MEDICAL UNIVERSITY OF VIENNA

Chemical targeting of the membrane transporter for lactate SLC16A3

Doctoral thesis at the Medical University of Vienna

for obtaining the academic degree

Doctor of Philosophy

Submitted by

Vojtěch Dvořák, MSc.

Supervisor:

Prof. Dr. Giulio Superti-Furga

Center for Physiology and Pharmacology,

Medical University of Vienna

Währinger Straße 13A/HP, 1090 Vienna, Austria

CeMM Research Center for Molecular Medicine

of the Austrian Academy of Sciences

Lazarettgasse 14, AKH BT25.3, 1090 Vienna, Austria

Vienna, 08/2023

Declaration

This doctoral thesis is presented in a cumulative form and contains a research article and two review articles. The work presented in this thesis has been carried out in the laboratory of Giulio Superti-Furga at the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, unless stated otherwise. This thesis was written solely by Vojtech Dvorak with input from his supervisor Giulio Superti-Furga. The author of this thesis is a first author of all manuscript included in this thesis. Individual contributions of all co-authors are listed in prologue sections preceding each manuscript.

Review in the section 1.1.2 was published in Expert Opinion on Drug Discovery: V. Dvorak and G. Superti-Furga, **Structural and functional annotation of solute carrier transporters: implications for drug discovery,** *Expert Opinion on Drug Discovery (2023)*, doi: 10.1080/17460441.2023.2244760

Review in the section 1.2 was published in Frontiers in Pharmacology: V. Dvorak, T. Wiedmer, A. Ingles-Prieto, P. Altermatt, H. Batoulis, F. Bärenz, E. Bender, D. Digles, F. Dürrenberger, L. H. Heitman, A. P. IJzerman, D. B. Kell, S. Kickinger, D. Körzö, P. Leippe, T. Licher, V. Manolova, R. Rizzetto, F. Sassone, L. Scarabottolo, A. Schlessinger, V. Schneider, H. J. Sijben, A.-L. Steck, H. Sundström, S. Tremolada, M. Wilhelm, M. W. Muelas, D. Zindel, C. M. Steppan and G. Superti-Furga, **An overview of cell-based assay platforms for the solute carrier family of transporters**, *Frontiers in Pharmacology (2021)*, doi: 10.3389/fphar.2021.722889

The research article in the results section was published in Cell Chemical Biology: V. Dvorak, A. Casiraghi, C. Colas, A. Koren, T. Tomek, F. Offensperger, A. Rukavina, G. Tin, E. Hahn, S. Dobner, F. Frommelt, A. Boeszoermenyi, V. Bernada, J. T. Hannich, G. F. Ecker, G. E. Winter, S. Kubicek and G. Superti-Furga, **Paralog-dependent isogenic cell assay generates highly selective SLC16A3 inhibitors,** *Cell Chemical Biology (2023)*, doi: 10.1016/j.chembiol.2023.06.029

Table of content

Declaration ... **ii**

List of figures and tables ... **v**

Abstract ... **vi**

Zussamenfassung ... **vii**

Abbreviations ... **ix**

Acknowledgments ... **x**

1. Introduction ... **1**

1.1 Solute Carriers ... 1

1.1.1 Therapeutical targeting of SLCs ... 4

1.1.2 Review of impact of annotations of SLCs on drug discovery ... 8

1.2 Review of assays for SLCs. ... 26

1.3 SLC16 family of monocarboxylate transporters ... 57

1.4 Lactate ... 60

1.4.1 Lactate shuttle ... 62

1.4.2 Lactate as a signaling molecule ... 64

1.5 Lactate transporters ... 66

1.5.1. SLC16A1 (MCT1) ... 66

1.5.2. SLC16A3 (MCT4) ... 69

1.5.3. SLC16A7 (MCT2) ... 73

1.5.4. SLC16A8 (MCT3) ... 74

1.6 Aims of this thesis ... 75

2. Results ... **76**

3. Discussion 103

3.1 PARADISO is a versatile assay with potentially wide applicability. 103

3.2 Applying the PARADISO assay to SLC16A3. 105

3.3 SLC16A3 chemical probes & their applications 107

3.4 Conclusions & future prospects 112

References 113

Curriculum Vitae 131

List of figures and tables

Figure 1:	Variety of substrate across the SLC families	2
Figure 2:	The phylogenetic tree of monocarboxylate transporter family	58
Figure 3:	Glycolysis scheme	61
Figure 4:	Metabolic symbiosis between cells in TME	64
Figure 5:	Chemical structures of selected SLC16A1 inhibitors	69
Figure 6:	Chemical structures of selected SLC16A3 inhibitors	73

Table 1:	List of approved drugs targeting SLCs	7
Table 2:	Sequence identity among lactate transporters of SLC16 family	66

Abstract

The Solute Carrier (SLC) superfamily is a diverse group of more than 450 membrane transporters which in humans have a crucial role in chemical exchange between the cell and its environment as well as individual subcellular compartments. A multitude of studies connected the individual members of the SLC superfamily to a diverse spectrum of physiological processes and diseases. Moreover, SLCs are considered to be pharmacologically tractable ("druggable") and promising therapeutic targets in many and diverse diseases. Despite this, the SLC superfamily remains pharmacologically underexploited, mainly due to poor understanding of the biological functions of many SLCs and limited availability of tools such as biological assays or tool compounds that would be required to study them effectively.

The introductory section of this thesis briefly reviews some of the general characteristics of SLCs, with a particular focus on the SLC druggability and assay technologies. The recent progress, particularly in structural biology of SLCs, provided an opportunity to survey the mode of actions of existing SLC targeting drugs and thus re-fine the scope of SLC druggability. Subsequently, we provide an overview of the existing kind for SLC-focused assays and discuss their wider applicability as well as SLC characteristics that are important to assess SLC properties that can be monitored.

Ultimately, the focus of this thesis is developing a versatile assay strategy for SLC-oriented identification of cognate chemical compounds and consequent development of specific and selective probes targeting individual SLCs. To this end, we developed an assay system called Paralog-dependent isogenic cell assay, or PARADISO, which is based on exploiting the genetic interactions and functional overlap among paralog genes. The core principle relies on engineering a series of cell lines, each individually dependent on a particular paralog gene for its growth or survival fitness. These cell lines are then used in a logical cascade of screening steps that provide for high selectivity. We focused on lactate transporters of the SLC16 family and developed a highly selective and potent small molecule chemical inhibitor targeting SLC16A3. SL16A3, also known as MCT4 (monocarboxylate transporter 4) is a lactate transporter with an important and increasingly recognized role in several disease areas including cancer. The approach described in this thesis can in principle be used for other SLCs, but also other proteins, and can be especially useful when the access to other assays and tool compounds is limited.

Zussamenfassung

Die Solute Carrier (SLC)-Superfamilie ist eine vielfältige Gruppe von mehr als 450 Membrantransportern, die in Menschen eine entscheidende Rolle beim Austausch von Molekülen zwischen der Zelle und ihrer Umgebung sowie den einzelnen subzellulären Kompartimenten spielen. Eine Vielzahl von Studien hat die einzelnen Mitglieder der SLC-Superfamilie mit einem breiten Spektrum physiologischer Prozesse und Krankheiten in Verbindung gebracht. Darüber hinaus gelten SLCs als pharmakologisch modulierbare („druggable") und vielversprechende therapeutische Ziele in vielen verschiedenen Krankheiten. Trotzdem bleibt das therapeutische Potential der SLC-Superfamilie zu großen Teilen ungenutzt, hauptsächlich aufgrund mangelnden Verständnisses der biologischen Funktionen vieler SLCs und der begrenzten Verfügbarkeit von Werkzeugen wie biologischen Assays oder niedermolekularer Werkzeuge („tool compounds“), die für eine effektive Untersuchung erforderlich wären.

Der einführende Abschnitt dieser Arbeit beleuchtet kurz einige der allgemeinen Charakteristika von SLCs, wobei der Schwerpunkt insbesondere auf der SLC-Pharmakologie und den Assay-Technologien liegt. Jüngste Fortschritte - insbesondere im strukturbiologischen Verständnis von SLCs – ermöglichen die Untersuchung der Wirkungsweise bestehender SLC-Medikamente und somit ein präziseres Verständnis der SLC-Pharmakologie. Anschließend geben wir einen Überblick über die bestehenden SLC-spezifischen Assays und diskutieren ihre breitere Anwendbarkeit sowie SLC-Eigenschaften, die für die molekularbiologische Untersuchung von SLCs und entsprechende Assay-Entwicklung zentral sind.

Letztendlich konzentriert sich diese Arbeit auf die Entwicklung einer vielseitigen Assay-Strategie zur SLC-orientierten Identifizierung potentieller SLC-bindender Moleküle und zur anschließenden Entwicklung spezifischer und selektiver Moleküle zur gezielten Bindung und Inhibition einzelner SLCs zu entwickeln. Zu diesem Zweck haben wir ein Assay-System namens "Paralog-dependent isogenic cell assay" oder PARADISO entwickelt, das genetische Interaktionen und funktionaler Überschneidungen zwischen Paralog-Genen gezielt ausnutzt. Das Grundprinzip beruht auf der Konstruktion einer Reihe von Zelllinien, die jeweils individuell auf ein einzelnes Paralog-Gen der Familie für ihr Wachstum oder ihre Überlebensfähigkeit angewiesen sind. Diese Zelllinien werden dann in einer schrittweise Screening-Kampagne verwendet, um hoch selektive Inhibitoren zu identifizieren. Wir konzentrierten uns auf Laktat-Transporter der SLC16-Familie und entwickelten einen hochselektiven und potenten chemischen Inhibitor für SLC16A3. SLC16A3, auch als MCT4 (Monocarboxylat-Transporter 4) bekannt, ist ein Laktat-Transporter mit einer wichtigen und zunehmend anerkannten Rolle in verschiedenen Pathologien, einschließlich Krebs. Der in dieser Arbeit beschriebene Ansatz

kann grundsätzlich für andere SLCs, aber auch für andere Proteine verwendet werden und kann besonders nützlich sein, wenn der Zugang zu anderen Assays und molekularen Werkzeugen begrenzt ist.

Abbreviations

ABC	ATP-Binding cassette
CAF	Cancer associated fibroblast
ccRCC	clear cell renal cell carcinoma
CCLE	Cancer Cell Line Encyclopedia
CHC	α-cyano-4-hydroxycinnamate
DRIs	Dopamine reuptake inhibitors
ER	Endoplasmatic reticulum
GI	Genetic interaction
GWAS	Genome-wide association study
HTS	High-throughput screening
KO	Knock-out
LDH	Lactate dehydrogenase
MoA	Mode of action
MPC	Mitochondrial pyruvate carrier
NDRIs	Norepinephrine-dopamine reuptake inhibitors
NSCLC	Non-small cell lung cancer
NRIs	Norepinephrine reuptake inhibitors
OE	Overexpression
OXPHOS	Oxidative phosphorylation
RA	Rheumatoid arthritis
SAR	Structure-activity relationship
SGD	Structural Genomic Consortium
SL	Synthetic lethality
SLC	Solute carrier
SNRIs	Serotonin-norepinephrine reuptake inhibitors
SSRIs	Serotonin reuptake inhibitors
TCA	Tricarboxylic acid
TME	Tumour microenvironment
TRIs	Triple reuptake inhibitors
T2D	Type 2 diabetes
PK	Pharmacokinetic

Acknowledgments

As I am writing the last lines of this thesis, it is almost exactly to the day five years since I started my PhD journey at CeMM. During this time, I was lucky enough to work in the most stimulating environment I have ever experienced, surrounded by many brilliant and kind colleagues and friends. A lot has happened during this time, and it is impossible to mention everyone who supported me along the way, but there are several people I simply must thank here.

First and foremost, I would like to thank my supervisor and mentor Giulio Superti-Furga for giving me the opportunity and privilege to join his group and for all the support I received from him. His continuous enthusiasm, original thinking, and openness to explore new areas were truly inspiring. Working under Giulio's leadership provided me with countless opportunities to learn and helped me to grow both professionally and personally. The next person I wish to thank is Ariel Bensimon, whose mentoring during the first years of my PhD helped me tremendously, and whose advice I still keep on benefiting from up until today. Subsequently, I want to thank Andrea Casiraghi, whose guidance helped to really kick-off my chemical biology journey.

I would also like to thank my PhD committee, Stefan Kubicek, Gerhard Ecker and Elif Karagöz for finding the time to give me their valuable input. There are also many collaborators I hereby thank. While some of the collaborations materialized into manuscripts, there were a few others that did not. The latter includes collaborations with Emilio Casanova and Herwig Moll from Medical University of Vienna, Florian Grebien from University of Veterinary Medicine Vienna, Federica Benvenuti from ICGEB Tieste, and Thomas Licher from Sanofi. I also would like to thank Christopher Koth from Genentech, for providing me with an opportunity to do an internship in his group, which unfortunately did not happen due to the COVID-19 pandemic.

Next, I would like to thank the members of Giulio's laboratory. The impossibility of naming everyone here can be well illustrated by the fact that during my PhD I have had chance to work along in total with 72 members (past and present) of the GSF lab. Yet, those are the people who were the most important in creating the everyday atmosphere in the lab and from whom I learned the most. Similarly, I must collectively thank all CeMMies I had the chance to work with, who are too many to count. This includes all the scientists at CeMM, who are always willing to help and discuss the science, but also all the management and administrative personal who provided excellent support, making it possible to devote our maximum focus on science.

Of course, the past five years wouldn't have been possible without the continuous support of my family, my partner Mariem and my friends, so hereby I wish to thank them with all my heart. I know that I can always count on them.

"Few can foresee whither their road will lead them, till they come to its end."
J.R.R. Tolkien
Lord of the Rings: The two towers.

1. Introduction

1.1 Solute Carriers

Tight regulation of the internal chemical environment is essential for every cell. To control homeostasis, cells developed a variety of transporter systems that act as selective gates inside otherwise non-permeable membranes (Bar-Peled & Kory, 2022). According to some estimates, there are more than 1,500 genes in the human genome that encode proteins responsible for transport over biological membranes (Ye *et al*, 2014). These can be divided based on mechanistic and thermodynamic criteria into active, secondary active and passive transporters (Hediger *et al*, 2013). Active transporters require energy, mostly in form of ATP, to move their substrate and they are capable of transporting against a gradient over the membrane. This is the case for the ATP-binding cassette (ABC) transporters and different forms of ATPases. Secondary active transporters can shuttle their substrate against concentration gradient by coupling it with transport of another molecule down its gradient. This transport is characteristic for some families of the solute carrier (SLC) superfamily. Passive, or facilitative, transport follows the concentration gradient, moving the substrate from the side of the membrane with higher substrate concentration to the side with lower concentration. Facilitative transporters are common amongst many SLC transporter families and ion channels.

The SLC superfamily represents the largest group of membrane transporters and second largest group of membrane proteins (Höglund *et al*, 2011). Currently, there are over 450 members in the SLC superfamily, which are further divided into 65 canonical and several non-canonical families (Perland & Fredriksson, 2017). The members of the individual families are assigned based on sequence similarity (minimum 20 – 25% of amino acid identity with another member of the family) or based on functional similarity (f.e. the SLC25 family of mitochondrial transporters). This results in a substantial structural diversity within the SLC superfamily, spanning over 20 structurally distinct protein folds (Ferrada & Superti-Furga, 2022). SLCs are present not only at the plasma membrane of the cell, but also at the membranes of intracellular compartments (Pizzagalli *et al*, 2020). They play an important role not only in the exchange of solutes between cells, a cell and its environment, but also between intracellular compartments.

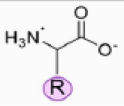
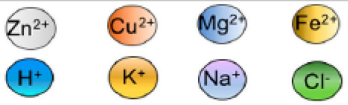
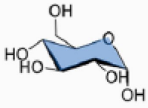
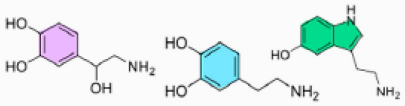
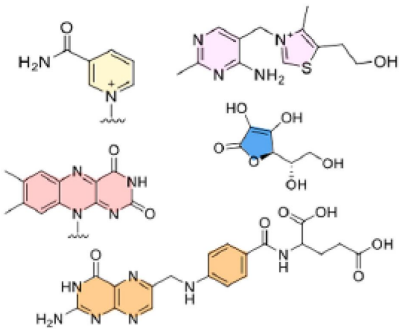
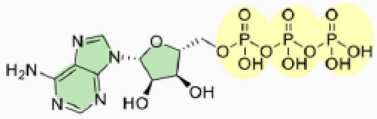
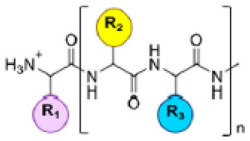
General/Representative Structure	Substrate	Solute Carrier Transporter
	Amino Acids	SLC1, SLC7, SLC17A6, SLC17A7, SLC17A8, SLC36, SLC38, SLC43A1, SLC43A2
	Inorganic cations and anions	SLC4, SLC8, SLC9, SLC11, SLC12, SLC20, SLC24, SLC30, SLC31, SLC34, SLC39, SLC40, SLC41
	Sugars	SLC2, SLC5A1, SLC5A2, SLC37A4, SLC45, SLC50, SLC60
	Lipids	SLC5A8, SLC5A12, SLC27, SLC51
	Neurotransmitters	SLC6A1, SLC6A2, SLC6A3, SLC6A4, SLC6A11, SLC6A12, SLC6A13, SLC18A1, SLC18A2, SLC18A3, SLC32
	Cofactors/Vitamins	SLC19, SLC23A1, SLC23A2, SLC25A17, SLC25A19, SLC25A32, SLC46, SLC52
	Nucleosides, Nucleotides	SLC28, SLC29, SLC35
	Peptides	SLC15

Figure 1: Variety of substrate across the SLC families. *Reprinted from* (Wang *et al*, 2019a)*, with permission from the publisher.*

SLCs transport a wide variety of substrates (**Figure 1**) and are involved in diverse biological processes. Foremost, SLCs are critical for the acquisition of nutrients from the cell's environment and for the export of metabolites ((Pizzagalli *et al*, 2020), some examples in Figure 1). Cells often alter expression level of transporters during growth or differentiation, ensuring that changing metabolic demands can be fulfilled (Palm & Thompson, 2017).

Many SLCs play a role in signal transduction. The endoplasmatic reticulum (ER) zinc transporter SLC39A7 (ZIP7), for example, is important for trafficking of the Notch (Nolin *et al*, 2019) and the TNF receptor (Fauster *et al*, 2018), and thereby playing a crucial role in both of these signaling cascades. Similarly, the MAP kinase pathway is modulated by copper which is in turn dependent on import through SLC31A1 (CTR1) (Brady *et al*, 2014). SLC38A9 is instrumental in the sensing of amino acids on lysosomes and is required for the activation of mTOR (Rebsamen *et al*, 2015; Wang *et al*, 2015). SLC15A4 acts as an adaptor protein that is necessary for TLR7/9 signaling (Heinz *et al*, 2020). SLC46A2 transports peptidoglycans that mediate immune reactions against bacteria in the skin (Bharadwaj *et al*, 2023). Several other SLCs have additional important roles in immune cells, such as the bicarbonate transporter SLC4A7 (NBCn1) that regulates the acidification of phagosomes in macrophages by balancing the cytoplasmic pH homeostasis (Sedlyarov *et al*, 2018). SLCs are also receptors for viruses. For instance, SLC10A1 (NTCP) is the receptor for the Hepatitis B and D virus (Yan *et al*, 2012) and SLC65A1 (NPC1) facilitates cellular entry of the Ebola virus (Carette *et al*, 2011; Côté *et al*, 2011). SLCs were also shown to play an important role during bacterial infections. For example, SLC11A1 (NRAMP1) plays an important role in host defense against *Salmonella* and other bacterial pathogens (Cunrath & Bumann, 2019).

SLCs are involved in essentially any physiological function. The absorption of nutrients in the gastrointestinal system would not be possible without transporters, including SLCs, and likewise the excretion of metabolites (Zhang *et al*, 2019b). SLCs transporting ions across plasma membrane, such as some members of the SLC12 family, are for example involved in regulation of blood pressure through renal salt reabsorption (Bazúa-Valenti *et al*, 2016). Similarly, transporters from several different families are expressed in the kidney, where they mediate the renal re-absorption of nutrients (Lewis *et al*, 2021). Members of the SLC9 family, the H^+/Na^+ exchangers, are important regulators of intracellular pH and cell volume (Casey *et al*, 2010). The erythrocytic bicarbonate transporter SLC4A1 (AE1), together with carbonic anhydrases, plays a crucial role in O_2/CO_2 exchange (Jennings, 2021). SLCs, in particular the SLC1 and SLC6 families, modulate neurotransmission by re-absorption of neurotransmitters at the synapses (Nguyen *et al*, 2022). Absorption of dietary iron and the maintenance of iron levels in plasma is mediated by SLC40A1 (Ferroportin) (Muckenthaler *et al*, 2017). Similar to these examples, numerous other physiological processes are influenced by SLC function.

Another important aspect of SLCs is their involvement in the transport of drugs. Several members of the SLCO and SLC22 families are particularly recognized for their role in the pharmacokinetics (PK) of several drugs (Nigam, 2014). Additionally, while it was widely believed that most of the drugs (and lipophilic compounds in general) cross the plasma membrane passively, an increasing amount of evidence suggests that transporters may have

a much broader role in these processes. In fact, an elegant study utilizing an unbiased genetic screening approach showed that the action of the steroid hormone ecdysone in *Drosophila* is fully dependent on a membrane transporter, suggesting that even highly lipophilic compounds require transporter (Okamoto *et al*, 2018). Similarly, a survey of 60 cytotoxic drugs, chosen to be representative of the chemical space of approved drugs, showed that the action of most drugs is at least partially affected upon knock-out (KO) of one or more SLC transporters, which indicates that the role of SLCs in the drug transport is broader than it was previously thought (Girardi *et al*, 2020b). This can have important consequences not only for the PK properties of a drug, but also for the development of drug resistances, especially in case of cancer therapeutics. In fact, the impact of transporters in cancer resistance occurs on several layers. First, cancer cells can down-regulate the transporter that is responsible for the uptake of a drug targeting an intracellular target, as exemplified by SLC35F2 and YM155 (Winter *et al*, 2014). Second, cancer cells can upregulate exporters. Even though ABC transporters, such as ABCC1, ABCB1 or ABCG2, are more frequently linked to this type of resistance, some evidence suggests that the SLC47 family may play a similar role (Nigam, 2014; Staud *et al*, 2013). Third, in case of competitive modulators, that target proteins interacting with metabolites, the expression levels of transporters can be crucial to the increase in the intracellular concentration of metabolites and thus outcompeting the binding of the drug. This mechanism was described for the acetyl-CoA competitive inhibitors of histone acetyl transferases and SLC5A6, a transporter important for the import of precursors for the coenzyme A biosynthesis (Bishop *et al*, 2023).

Importantly, a big portion of SLCs remain poorly understood. A review of the literature in the NCBI database in 2015 showed that SLCs displayed the highest publication asymmetry among all human gene families (César-Razquin *et al*, 2015). This means that the vast majority of publications focused only on a few SLCs, while most of the superfamily remains understudied. Furthermore, a report from 2020 indicates that as many as 30% of human SLCs lack any functional annotation (Meixner *et al*, 2020).

1.1.1 Therapeutical targeting of SLCs

Perturbation of the functions of SLCs can cause or contribute to variety of diseases. On one hand, it was estimated that mutations in at least a fourth of the SLC superfamily is linked to monogenic diseases (Schaller & Lauschke, 2019). On the other hand, SLCs are also linked to more complex diseases, ranging from neurological diseases to cancer. While in the first case the mutation in a gene is sufficient to cause a disease, in the latter, the involvement of an SLC

gene can be more complex. An increasing number of studies report on mechanisms on how SLCs are involved in complex diseases.

To date there are several SLC families that are targets of drugs approved by pharmaceutical regulatory agencies and organizations, across different indications (**Table 1**). Arguably, the monoamine transporters of the SLC6 family are the most prominent among these. Several members of the SLC6 family are responsible for the reuptake of neurotransmitters at synapses and are targets for monoamine reuptake inhibitors. This is a drug class that includes more than 40 FDA-approved molecules targeting the norepinephrine transporter SLC6A2 (NET), the dopamine transporter SLC6A3 (DAT) and the serotonin transporter SLC6A4 (SERT) (Wang *et al*, 2019a; Walter, 2005). These compounds can either be selective inhibitors of SLC6A4, known as serotonin reuptake inhibitors (SSRIs), selective inhibitors of SLC6A2, known as norepinephrine reuptake inhibitors (NRIs), selective inhibitors of SLC6A3, known as dopamine reuptake inhibitors (DRIs), or they could target multiple monoamine transporters. This includes serotonin-norepinephine reuptake inhibitors (SNRIs) targeting SLC6A2 and SLC6A4, norepinephrine-dopamine reuptake inhibitors (NDRIs) targeting SLC6A2 and SLC6A3 or triple reuptake inhibitors (TRIs) targeting all three transporters. Collectively these drugs are used for the treatment of variety of psychological disorders (Table 1) and their importance is reflected in the fact that monoamine reuptake inhibitors are one of the drug classes generating the most revenues (Oprea *et al*, 2018). Apart from monoamine reuptake inhibitors, of particular note, is tiagabine which targets the GABA transporter SLC6A1 (GAT1) for the treatment of epilepsy. Another important class of drugs are inhibitors of the sodium glucose co-transporter SLC5A2 (SGLT2), known as gliflozins. These drugs block glucose reabsorption in the kidney, causing glucosuria and were originally developed for the treatment of diabetes. Several clinical trials demonstrated that gliflozins show also benefits for patients suffering from kidney or heart diseases, further expanding the potential clinical utility of these drugs (Fonseca-Correa & Correa-Rotter, 2021). The case of gliflozins also illustrates another frequent feature of SLC-targeting drugs: serendipity of their discovery. Phlorizin – predecessor of gliflozins – originally isolated in 19th century from the bark of apple trees, was not only instrumental for research in renal physiology but was also shown to be an effective treatment in several animal models of diabetes in the second half of 20th century (White, 2010). Only later it was recognized that the effect of phlorizin is due to inhibition of glucose reabsorption in the kidney, and after characterization of SLC5A2, phlorizin was further developed into several members of the class now collectively called gliflozins (Fonseca-Correa & Correa-Rotter, 2021). Similarly, several other compounds were found to be SLC inhibitors only by characterization of the mode of action (MoA) of compounds originally discovered based on altering the phenotype in isolated tissue or *in vivo* models (Wang *et al*, 2019a). Other drugs shown to target SLCs are summarized in **Table 1**.

There are also several other chemical modulators currently undergoing clinical trials that can be first-in-class therapeutics. For example, an inhibitor of amino acid transporter SLC6A19 (B0AT1) is being tested in a phase I trial as a potential treatment of phenylketonuria (NCT05781399), while an inhibitor of the iron exporter SLC40A1 is being tested in a phase II trial as a potential treatment of sickle cell disease (NCT04817670). Additionally, several other second- and third-generation compounds, that target SLCs that are already targeted by approved drug, are also undergoing clinical trials. These include for example inhibitors of SLC22A12 (URAT1) that are being tested in a phase II trial as potential treatment of gout and kidney disorders (NCT03990363), conditions for which similar drugs are already approved (Wang *et al*, 2019a).

Importantly, since SLCs are in general considered to be highly druggable (described in detail in the next section), many more SLCs were proposed as promising therapeutical targets in different disease areas. These include autoimmune diseases, such as systemic lupus erythematosus (Heinz *et al*, 2020), rheumatoid arthritis (Fujii *et al*, 2015; Pucino *et al*, 2019) or psoriatic inflammation (Bharadwaj *et al*, 2023), cardiovascular diseases (Cluntun *et al*, 2021), neurological diseases (Qosa *et al*, 2016), cancer (Superti-Furga *et al*, 2017; Nwosu *et al*, 2023) and different metabolic diseases (Schumann *et al*, 2020).

SLC	Drug/ Drug class and example	Indications
SLC5A2 (SGLT2)	Gliflozins (canagliflozin, dapagliflozin, empagliflozin, ertugliflozin)	Type II diabetes (and more)
SLC6A1 (GAT-1)	Tiagabine	Epilepsy
SLC6A2 (NET)	SSRIs (citalopram, escitalopram, fluoxetine), SNRIs (desvenlafaxine, duloxetine, levomilnacipran), NRIs (atomoxetine), NDRIs (bupropion, dexmethylphenidate) DRIs (benztropine)	Major depression disorder, anxiety disorders, obsessive-compulsive disorder, attention-deficit hyperactivity disorder, narcolepsy
SLC6A3 (DAT)		
SLC6A4 (SERT)		
SLC9A3 (NHE3)	Tenapanor	Chronic kidney disease, hypertension
SLC10A1 (NTCP)	Bulevirtidine	Hepatitis D (blocks viral entry)
SLC10A2 (IBAT)	Elobixibat, odevixibat	Chronic constipation, pruritus (in progressive familiar intrahepatic cholestasis patients)
SLC12A1 (NKCC2)	Loop diuretics (furosemide, bumetanide)	Hypertension, edema
SLC12A2 (NKCC1)		
SLC12A3 (NCC)	Thiazide diuretics (Chlorothiazide, polythiazide)	
SLC18A1 (VMAT1)	Reserpine	Hypertension
SLC18A2 (VMAT2)		
	Tetrabenazine, deutetrabenazine, valbenazine	Huntington disease, tardive dyskinesia
SLC22A6 (OAT1)	Probenecid	Gout
SLC22A8 (OAT3)		
SLC22A11 (OAT4)		
SLC22A12 (URAT1)	Lesinurad	Gout
SLC25A4 (ANT1)	Clodronate	Osteoporosis
SLC25A5 (ANT2)		
SLC26A6 (ANT3)		
SLC29A1 (ENT1)	Adenosine reuptake inhibitors (dipyridamole, dilazep)	Vasodilator
SLC65A2 (NPC1L1)	Ezetimibe	High blood cholesterol

Table 1: List of approved drugs targeting SLCs. *Table reproduced from* (Wang *et al*, 2019a)*. Additional search for SLC-targeting drugs in Pharos database* (Kelleher *et al*, 2023) *uncovered modulators for SLC10A1, SLC10A2 (tested in clinical trials when review by Wang and colleagues was published).*

1.1.2 Review of impact of functional and structural annotations of SLCs on drug discovery

SLCs are considered to be a druggable, that is chemically and pharmacologically tractable, class of proteins, however despite ongoing efforts (summarized for example in an excellent review by Wang and colleagues (Wang *et al*, 2019a)), the basis of their druggability is still poorly formulated. Arguably one of the major factors in this could be the limited knowledge on structures and functions of many SLCs. At the same time, in recent years we have seen an increasing number of studies reporting SLC structures, some reporting several structures per SLC, capturing different stages of the transport process. We also witness an increasing number of studies assigning functions to previously uncharacterized SLCs. In addition, developments in algorithms for predicting the 3D protein structures with high precision provide structural insights into whole proteomes (Tunyasuvunakool *et al*, 2021; Jumper *et al*, 2021). These advancements present an opportunity to completely redefine the druggability of the SLC superfamily as a class.

The next section includes a review article that highlights some of the most important aspects of SLC druggability. In the article, we first describe the main transport mechanisms of SLCs and provide an overview of MoA for existing SLC chemical modulators. To do this, we have assembled all experimentally determined structures of human SLCs from the Uniprot and PDB databases, and we focused on structures determined in complex with inhibitors. Next, we discuss the functional features of SLCs that are the most important contributors to the SLC druggability, such as their expression and localization. Last but not least, we provide a perspective on the current trends, emerging technologies, and a brief outlook for SLC-oriented drug discovery.

The author of this thesis conceptualized and wrote the review together with his supervisor. The rights to include a full reprint of this review in this thesis are retained with the authors.

EXPERT OPINION ON DRUG DISCOVERY
https://doi.org/10.1080/17460441.2023.2244760

Taylor & Francis
Taylor & Francis Group

REVIEW

Structural and functional annotation of solute carrier transporters: implication for drug discovery

Vojtech Dvorak [a] and Giulio Superti-Furga [a,b]

[a]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [b]Center for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria

ABSTRACT

Introduction: Solute carriers (SLCs) represent the largest group of membrane transporters in the human genome. They play a central role in controlling the compartmentalization of metabolism and most of this superfamily is linked to human disease. Despite being in general considered druggable and attractive therapeutic targets, many SLCs remain poorly annotated, both functionally and structurally.

Areas covered: The aim of this review is to provide an overview of functional and structural parameters of SLCs that play important roles in their druggability. To do this, the authors provide an overview of experimentally solved structures of human SLCs, with emphasis on structures solved in complex with chemical modulators. From the functional annotations, the authors focus on SLC localization and SLC substrate annotations.

Expert opinion: Recent progress in the structural and functional annotations allows to refine the SLC druggability index. Particularly the increasing number of experimentally solved structures of SLCs provides insights into mode-of-action of a significant number of chemical modulators of SLCs.

ARTICLE HISTORY
Received 20 June 2023
Accepted 1 August 2023

KEYWORDS
Solute carriers; Slcs; transporters; drug discovery; druggability; structural annotation; functional annotation

1. Introduction

It is estimated that more than 30% of the human genome encodes proteins responsible for processing more than 200,000 metabolites that we encounter in ~3,000 possible chemical reactions [1]. Notably, individual steps in complex metabolic pathways frequently require specific environments, that are achieved only by strict compartmentalization across various tissues, between specific cell types, among the different organelles. Membrane transporters are crucial mediators of metabolic processes, controlling which molecules enter and leave different compartments, and thus play a key role in regulating metabolic, cellular and physiological processes. Around 10% of the human genome encodes transporter related proteins, which are divided into several classes [2]. Solute carriers (SLCs) represent the biggest and most diverse class of membrane transporters with currently more than 450 members divided into 66 canonical and several non-canonical families. SLCs are responsible for the transport of diverse substrates and many SLCs play a role in various physiological processes in health and disease, as well as the pharmacology of commonly used drugs [3]. Moreover, SLC transporters are considered to be a druggable class of proteins, albeit with only a handful being targeted by approved drugs so far, and several more being proposed as therapeutic targets in disease areas such as cancer, neurological disorders, metabolic diseases, immune disorders, or bacterial infections, to name a few [4]. Despite this, many of the SLC proteins remain understudied, and it is estimated that around a third of the human SLCs remain entirely orphan, that is without functional annotation [5,6]. In this review, we will focus on the structural and functional attributes of SLCs, particularly those that contribute to them being considered druggable (druggability is mainly defined by the evidence that other members, or enough members, of the same biochemical/structural class of proteins, have been amenable to chemical modulation [7]).

First, we will review transport mechanisms and the mode-of-action of chemical modulators, with focus on experimentally determined structures of SLCs in complex with inhibitors. Second, we will discuss functional features of SLCs, such as expression and localization, and their impact on drug discovery. Finally, we will provide a perspective on current trends and emerging technologies. Throughout this review, we will focus on human SLCs and provide examples for illustration.

2. Progress in structural annotation of SLCs

The SLC superfamily is an artificially pooled group of transporters with different evolutionary origins, divided into 66 canonical and several non-canonical families. Individual members are, in most instances, assigned into a family if they have > 20% amino acid sequence identity to other members of that family or, in some cases, based on functional similarity, which is resulting in a structurally diverse group of proteins [8]. The structural features of SLCs, such as multiple transmembrane domains, represent a significant challenge for studies that require protein purification and solubilization, hampering any

CONTACT Giulio Superti-Furga GSuperti@cemm.oeaw.ac.at CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria

Supplemental data for this article can be accessed online at https://doi.org/10.1080/17460441.2023.2244760

Article highlights

- SLCs represent a heterogenous group of membrane transporters that are considered druggable and attractive drug targets.
- Overview of experimentally determined SLC structures illustrates the advances in structural annotations of SLCs.
- Structures of SLCs in complex with chemical modulators provide an overview of mode-of-actions of SLC modulators and highlight the SLC druggability.
- Despite the progress in functional annotations, assigning functions to uncharacterized SLCs remains challenging.
- Information on the localization and substrate specificity of individual SLCs is particularly important for the SLC-oriented drug discovery.

downstream structural investigation. In fact, considerable amount of knowledge regarding the SLC structures and their mechanism-of-action was initially derived from prokaryotic homologs (that are frequently easier to study), as homology modeling of human SLCs on bacterial structures [9]. The studies utilizing bacterial homologues of human SLCs provided many insights that were instrumental for drug discovery. Only in recent years did we see an increase of available 3D structures of human SLCs, mostly due to progress in cryoEM techniques (Figure 1). Together with the advent of new algorithms for 3D structure prediction, such as AlphaFold [10,11], this represents a significant step forward in understanding the structural basis for SLC function. Before the beginning of 2022, only 25 experimentally determined structures of individual human SLCs were available, and accurate homology modeling was possible only for ~ 30% of human SLCs [12]. In contrast, as of March 2023, experimental structural data for more than 60 different human SLCs (in most cases several structures per individual SLC) are available from PDB (listed in the **supplementary table**), and highly accurate structural models for most SLCs, including homologs from different species, can be accessed from the AlphaFold database [10]. Moreover, classifications of 3D structure models empowered by AI-based algorithms expanded the number of folds present in human SLCs from 13 to over 20 [12,13], highlighting not only the progress in our understanding, but also the significant structural heterogeneity among SLC superfamily. In the next sections we will provide a brief introduction into SLC function, and then we will aim to provide an overview of the mechanisms-of-action of SLC chemical modulators. We argue that the advancements in the structural annotation of human SLCs, in particular due to increasing number of structures of SLCs in complex with ligands, now provide a solid basis to redefine the druggabbility of SLCs as a superfamily. In fact, we predict that SLC transporters will soon be as attractive as drug target classes as GPCRs, channels, kinases, and proteases once were.

2.1. SLC function

In general, SLCs mediate their function by alternating between the outward-open, inward-open, and occluded conformation (the latter representing a conformation in which the substrate binding site is not accessible from either side), all of which prohibit the simultaneous access from both sides of the membrane. This also defines a fundamental difference to channels, with which transporters are sometimes mistaken: when channels are 'open,' there is direct contact between solvent from both side of the membrane. With transporters, proteins with a much lower capacity than channels, this is never the case. The conformational alternate states of transporters allow changes in accessibility to the substrate binding site and transport of substrate with a fixed stoichiometry per transport cycle. SLCs are in general facilitative uniporters, or secondary active co-transporters (although exceptions exits). While facilitative transporters move their substrate across the membrane based on the concentration gradient, the secondary active transport couples the transport of multiple molecules or ions. This, in principle, allows to transport molecules against their concentration gradient by coupling it to transport of a second molecule/ion down a gradient and could be achieved either as symport or antiport, based on the directionality of the co-transport [9]. In the case of symporters, the transporter can typically undergo a conformational change upon binding of the molecules that are being co-transported and the sequence of binding of these may vary. For instance, in lactate/H^+ symporters, the lactate binding site becomes accessible due to protonation of a conserved aspartic acid residue, that loosens the salt bridge between this residue and a conserved arginine that is then essential for the recognition of the substrate, thereby opening access to the binding site [14,15]. Similar binding of substrate and co-transported ions was described in many SLCs, and in some cases also applies to binding of SLC modulators [16]. In case of antiporters, the coupling can happen as two independent events, such as in the case of the cysteine-glutamine exchanger SLC7A11 (xCT). In this transporter the binding of extracellular cysteine triggers the conformational change from the outward-open conformation to the inward-open conformation, resulting in cysteine uptake [17]. The substrate binding site becomes accessible for glutamine, for which most cells face a high intracellular concentration, and its binding results in another conformational switch, followed by extracellular glutamine release, and thus completing the antiport cycle and regenerating again the outward-open conformation. Importantly, while the main substrates of SLCs may vary, the molecules coupled to the transport are frequently similar across different SLCs, particularly in case of ions [6]. This could be important for drug discovery as it provides a possibility to develop assays based on similar principles for different SLC families. For example, assays based on assessing the pH changes connected to H^+ (co-)transport are applicable to SLC9 (Na^+/H^+ exchangers) and SLC16 (Monocarboxylate transporters) families. Similarly, assays based on assessment of changes in membrane potential could be applied to SLC1 (high-affinity glutamate and neutral amino acid transporters), SLC6 (Na^+ and Cl^- dependent neurotransmitter transporters), SLC8 (Na^+/Ca^{2+} exchangers) and SLC15 (H^+ oligopeptide co-transporters) families, to name just the most prominent [18].

In general three main types of transport mechanisms are considered: (1) rocker-switch, (2) gated-pore (or rocking bundle), and (3) elevator [9]. For rocker-switch transporters, the binding of the substrate triggers conformational change during which the transporter transits through several conformations, including the occluded state, into opening to the opposite conformation and substrate release, with the transporter moving essentially around the substrate [16] (Figure 2a). Binding of substrate for transporters utilizing the gated-pore mechanism occurs between structurally

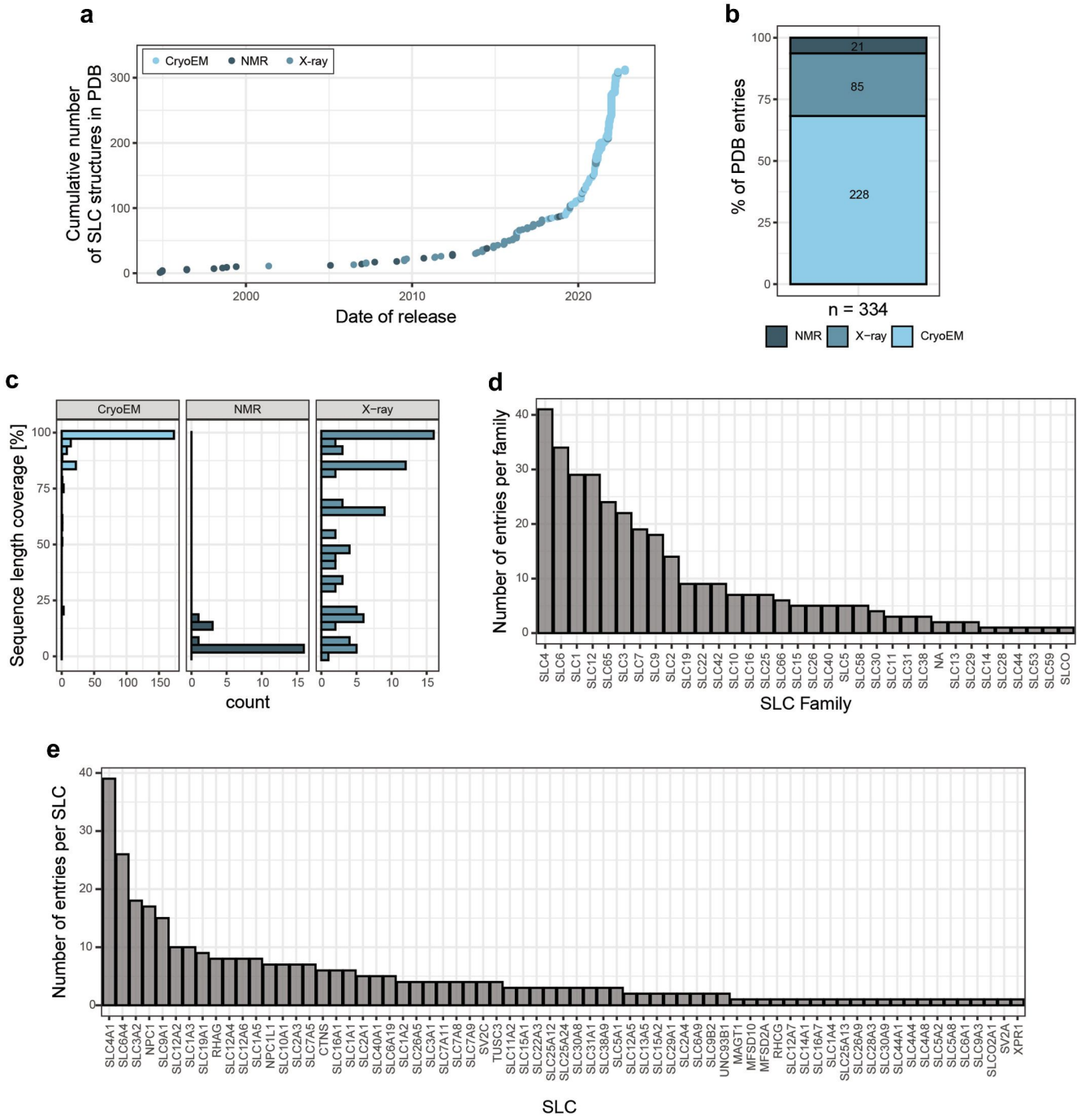


Figure 1. Survey of available SLC structures at PDB (as of end of March 2023). (a) Cumulative number of SLC structures available at PDB over time. The biggest increase is visible in recent years with advancements in cryoEM. (b) Distribution of SLC structures according to used methodology. Numbers inside the bar plot correspond to the numbers of entries per method. (c) The length of constructs used for structural determination per methodology as reported in PDBe. Note: This number reflects the length of the construct, some parts of the structure may still not be resolved (f.e. less organized termini or loop regions). (d) Number of entries per SLC family. (e) Number of entries per SLC.

distinct mobile and scaffold domains. This engagement initiates a bending of a mobile domain around a substrate binding site, resulting in movement of gating segments, a conformational switch, and release of substrate to the opposite site of the membrane [19] (Figure 2b). Lastly, transporters employing the elevator mechanism consist similarly of a static scaffolding domain and a mobile domain, however, the substrate binding site is largely or entirely within the mobile domain that slides up and down on the scaffolding domain between the inward-open, occluded, and outward-open conformations in an 'elevator-like' fashion [20] (Figure 2c). The transport mechanism is tightly connected to the fold, with the two most common folds MFS and LeuT utilizing typically the rocker-switch and gated-pore mechanisms and elevator mechanism being employed by for example Glt, UraA or

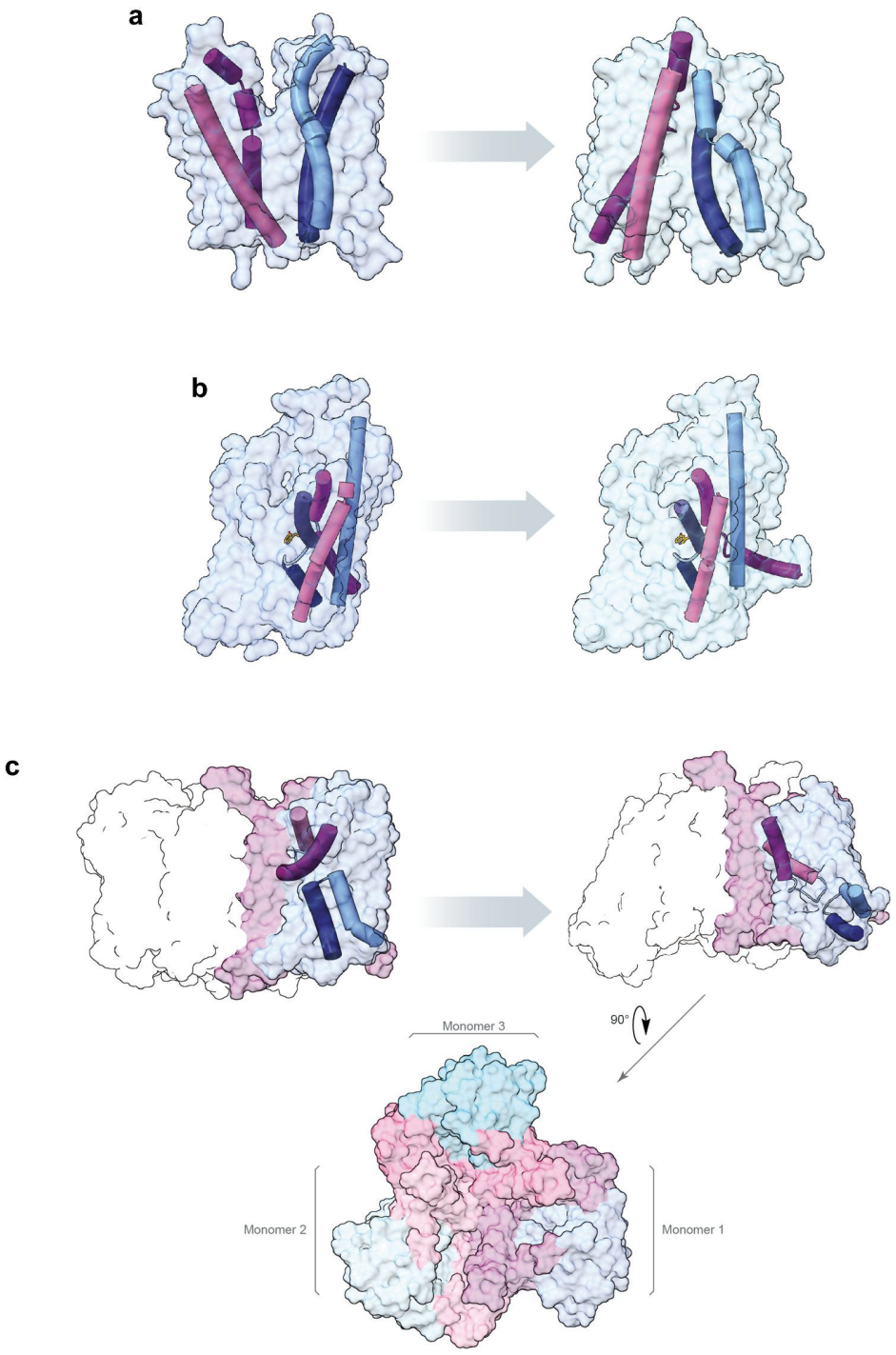


Figure 2. Transition from outward- to inward-open conformation for the major transport mechanisms of SLCs. (a) The MFS fold is typically connected with a rocker-switch mechanism, as exemplified by SLC16A1. Rocker-switch transporters typically move around the orthosteric site localized in the center. Helices crucial for gating are shown. (Outward-open PDB 6LZ0; Inward-open PDB 7DA5). (b) The LeuT fold is a typical gated-pore transporter, as exemplified by SLC6A4. Transporters utilizing gated-pore mechanism consist of scaffold domain and mobile domain that bend around the orthosteric site. Only helices responsible for gating of the substrate binding site shown, substrate bound in orthosteric site (Outward-open PDB 7LIA; Inward-open PDB 7LI9). (c) Elevator mechanism illustrated by SLC1A5 (Glt fold). During the transport cycle, the transport domain (blue) moves while the scaffold domain (pink) stays static. Helices mediating gating of the transport domain are shown. Protein is a trimer, only one monomer colored in the upper section (Outward-open PDB 7BCQ; Inward-open PDB 6GCT).

NhaA folds. Interestingly, while many transporters can clearly function in both directions, that is mediating both influxes, as well as effluxes depending on the concentration gradients, others seem to transport in one direction only. The ability of SLCs to mediate the bi-directional transport is likely resulting from combined characteristics of the SLC substrate as well as SLC structure, and thus should be carefully evaluated for each SLC individually.

The fact that structures of SLCs are mobile may well contribute to their druggability, by providing multiple conformations that could be targeted by drugs in alternate modes.

Moreover, SLCs can form homo- or hetero-mers, further expanding on their structural heterogeneity, and therefore expanding potential druggable interfaces and modes. To further explore general druggability, we will focus in the next sections on ways how SLC function can be chemically modulated.

2.2. Inhibiting the SLC function

Small molecules typically inhibit SLCs by disrupting some of the steps of the alternating access mechanism. Table 1 provides an overview of structures of human SLCs solved in complex with a chemical modulator, together with brief information on the mechanism-of-action. These kinds of mechanism-of-action studies on chemical modulators of SLC proteins, greatly facilitate the drug discovery process, in several ways. First, by providing the mechanistic understanding how SLCs transport their substrates and how to explore this for development of chemical modulators. Second, since most of the SLC structures became available only recently, many of the existing modulators can be further improved by structure-based drug design (SBDD). Third, similarly, the ligand binding site can be used as a blueprint for virtual screening campaigns and subsequently SBDD to develop novel chemotypes, perhaps with more favorable drug-like properties [23,33]. Fourth, to build homology models of transporters with limited structural information that can be used similarly for virtual screening and SBDD campaigns or to study transport mechanism [51]. Fifth and not least, these studies are crucial in describing the mode-of-action of existing inhibitors, such as at which conformation of the transporter the inhibitor binds (we will return to this topic in Section 3.1). Of course, inhibitors also help understanding the relationship between the transporters and their natural cargos, often presenting structural similarity, and allow to test function in a variety of settings, including animal studies and human *ex vivo* systems.

Of note, to describe the precise molecular interactions and to unlock the full potential of SBDD, it is generally considered that the resolution should be better than ~ 2.5 Å [52]. Even though many structures mentioned in this review do not reach this resolution, they are still highly informative for a general overview on how drugs bind to their targets. Which different mechanisms of SLC modulators exist and how they bind we will address in the next section based on several examples.

2.2.1. Orthosteric modulators

Given the alternating-access model of transport, all SLCs have a substrate binding 'orthosteric' site, that, in principle, is amenable for binding of small molecules. This represents the physical core of the SLC druggability landscape. Indeed, the SLC structures solved in complex with inhibitors show that most of the inhibitors bind to the orthosteric site and sterically prevent the transporter from switching conformations (Table 1). All major transport mechanisms are susceptible to this type of inhibition. The rocker-switch mechanism of the adenosine uniporter SLC29A1 (ENT1) is inhibited by bridging the orthosteric site with 'opportunistic' sites by adenosine reuptake inhibitors, preventing the closing of gating residues [46] (Figure 3a). Likewise, the gated-pore mechanism of the glucose transporter SLC5A2 (SGLT2) is inhibited by a glucoside moiety of glifozin binding to the sugar-binding site, while the hydrophobic tail extending into the external vestibule and thus similarly blocking the conformation switch [30] (Figure 3b). Similar mechanisms are utilized by inhibitors of transporters using the elevator mode. For example, citrate transporter SLC13A5 (NaCT) inhibitor PF-06649298 binds the transporter at the substrate binding site in the inward-open conformation with the citrate-mimicking moiety, while bulkier benzyl and tert-butyl groups interact with the scaffolding domain [44] (Figure 3c). This prevents the sliding movement of the transport domain, that would bring the transporter into outward-open conformation. In comparison, WAY213613 inhibits the elevator transport of the amino acid transporter SLC1A2 (EAAT2) by binding the orthosteric site with its L-asparagine moiety and preventing the closure of those residues responsible for gating the transport domain by a phenyl 2-bromo-4,5-difluorophenoxy moiety (BDP) [21] (Figure 3d). These examples illustrate the general properties utilized by many of the SLC inhibitors, that may be generally applicable.

In general, binding to the orthosteric site should result in a competition between inhibitor and substrate, however this does not have to apply to all SLC orthosteric inhibitors. This phenomenon is nicely illustrated by the example of ibogaine binding to the serotonin transporter SLC6A4 (SERT). Since ibogaine did not show a competition with the substrate, it was initially suspected that it binds at an alternative binding site. However the structure of SLC6A4 in complex with ibogaine showed that the transporter is stabilized in an inward-open/occluded conformation, and hence is not accessible for competition from the extracellular site [53]. Similarly, the cryoEM structure of the GABA transporter SLC6A1 (GAT1) in complex with tiagabine, a clinically approved anti-epileptic drug, suggests a two-step mode-of-action [31]. First, the drug binds the outward-open conformation of the transporter and is accessible for competition with the substrate. Second, the transporter changes the conformation into an inward-open state, in which the drug remains bound due to its large steric group that prevents the dissociation. Importantly, for most of the drugs that bind the inward-open/occluded conformations, it is not clear if they are previously transported by their target, or if they get into the cell (or organelle) through other means. The possibility of competition between transporter substrate and the inhibitor in cells thus depends mainly on two factors: In which direction the transporter is (mainly) transporting and which conformation is stabilized by the inhibitor. The survey of structures in Table 1 uncovered that most of the orthosteric inhibitors stabilize their targets in outward-open/outward-occluded conformation. While this can be advantageous from the medicinal chemistry perspective (e.g. less issues with cell penetration or the possibility to exploit conjugation of the drug), it also brings the minor disadvantage of substrate competition, in case that the targeted transporter is an importer. Even though efforts to develop conformation-specific inhibitors are limited, some are already reported. An elegant example is a study focusing

Table 1. Structure of human SLCs in complex with chemical modulators.

SLC	Fold	Drug	Drug MoA	Ref
SLC1A2 (EAAT2)	Glt	WAY213613	Binds orthosteric site, locks the inward-open conformation by preventing transporter gating and conformation switch.	CryoEM [21]
SLC1A3 (EAAT1)	Glt	UCPH101	Allosterically blocks elevator-like movement of transport domain in outward-open conformation.	X-ray [22]
SLC1A5 (ASCT2)	Glt	L*c*-BPE	Binds substrate binding site, stabilizes the outward-open conformation.	CryoEM [23]
SLC2A1 (GLUT1)	MFS	GLUT-i1	Locks the inward-open conformation, binds very similarly to glucose (substrate).	X-ray [24]
		GLUT-i2		
		Cytochalasin B		
SLC2A3 (GLUT3)	MFS	SA47	Outward-open conformation, blocking substrate binding.	X-ray [25]
SLC2A4 (GLUT4)	MFS	Cytochalasin B	Inward-open conformation, glucose binding site, similarly, to binding in SLC2A1.	Cryo-EM [26]
SLC4A1 (AE1)	UraA	H_2DIDS/DIDS	Blocking the access channel from extracellular site into substrate binding site, locking outward-open conformation. DIDS and DEPC can bind covalently.	CryoEM [27], X-ray [28]
		DEPC		CryoEM [27]
		Dipyridamole		
		Niflumic Acid	Binds between gate and core domains and prevents conformation change.	
SLC5A1 (SGLT1)	LeuT	LX2671	Binds the substrate binding site, stabilizing the outward-open conformation.	CryoEM [29]
SLC5A2 (SGLT2)	LeuT	Empaglifozin	Locks the outward-open conformation by binding to substrate binding site and extending to the external vestibule.	CryoEM [30]
SLC6A1 (GAT1)	LeuT	Tiagabine	Binds orthosteric site two-step binding inhibiton: competitive outward-open and noncompetitive inward-open.	CyoEM [31]
SLC6A4 (SERT)	LeuT	Ibogaine	Binding in (central) substrate binding site. Locks inward-open/occluded conformation. Non-competitive.	cryoEM [32]
		'8090		cryoEM [33]
		S-citalopram	Locks outward-open conformation by two binding events – in orthosteric (higher affinity) and allosteric sites (lower affinity).	X-ray [34]
		Setraline	Binds (central) substrate binding site in outward-open conformation.	X-ray [35]
		Fluvoxamine		
		Paroxetine		
		Vilazodone	Binds in allosteric site in outward-open conformation.	CryoEM [36]
SLC6A9 (GlyT1)	LeuT	Cmpd1 (Bitopertin analogue)	Binds orthosteric site in inward-open conformation, noncompetitive.	X-ray [37]
SLC7A5 (LAT1)	LeuT	JX-078	Inhibitor stabilizes the outward-occluded conformation.	CryoEM [38]
		Diiodo-Tyrosine	Binds outward-open conformation, sterically prevents conformational switch.	
SLC7A11 (xCT)	LeuT	Erastin	Inward-open conformation, binds intracellular vestibule.	CryoEM [39]
SLC9A1 (NHE1)	NhaA	Caripore	Binding in Na^+ binding site blocks sliding of core and dimerization domains, locking outward-open conformation.	CryoEM [40]
SLC12A2 (NKCC1)	LeuT	Bumetanide	Binds orthosteric site in outward-open conformation.	CryoEM [41]
SLC12A3 (NCC)	LeuT	Polythiazide	Binds substrate binding site. Locks the outward-open conformation.	CryoEM [42]
SLC12A4 (KCC1)	LeuT	VU0463271	Binds orthosteric site, outward-open conformation.	CryoEM [43]
SLC13A5 (NaCT)	Glt	PF-06649298	Substrate-mimicking moiety binds orthosteric site, while bulkier groups interact with scaffold domain, locking inward-open conformation.	CryoEM [44]
SLC16A1 (MCT1)	MFS	AZD3965	Binds central substrate binding site, locks in outward-open conformation.	CryoEM [15]
		BAY8002		
		7ACC2	Binds substrate binding site, inward-open conformation.	
SLC22A3 (OCT3)	MFS	Decynium-22	Orthosteric site, outward-open, sterically blocks.	CryoEM [45]
		Corticosterone		
SLC29A1 (ENT1)	MFS	Dilazep	Inhibitors bridge orthosteric site with distinct 'opportunistic' sites, locking outward-open conformation.	X-ray [46]
		NBMPR		
SLC40A1 (Ferroportin)	MFS	Hepcidin	Binds substrate loaded transporter, stabilizing the outward-open conformation, and preventing the substrate release.	CryoEM [47]
		Vamifeport	Binds in hepcidin binding site, locking the transporter in outward-open/occluded state.	CryoEM [48]
SLC65A1 (NPC1)	NPC1	Itraconazole	Binding sterically blocks the substrate access to the sterol binding domain.	CryoEM [49]
SLC65A2 (NPC1L1)	NPC1	Ezetimide	Binding in the central cavity causes deformation of sterol-sensing domain, resulting in low endocytosis, hence low transport function.	CryoEM [50]

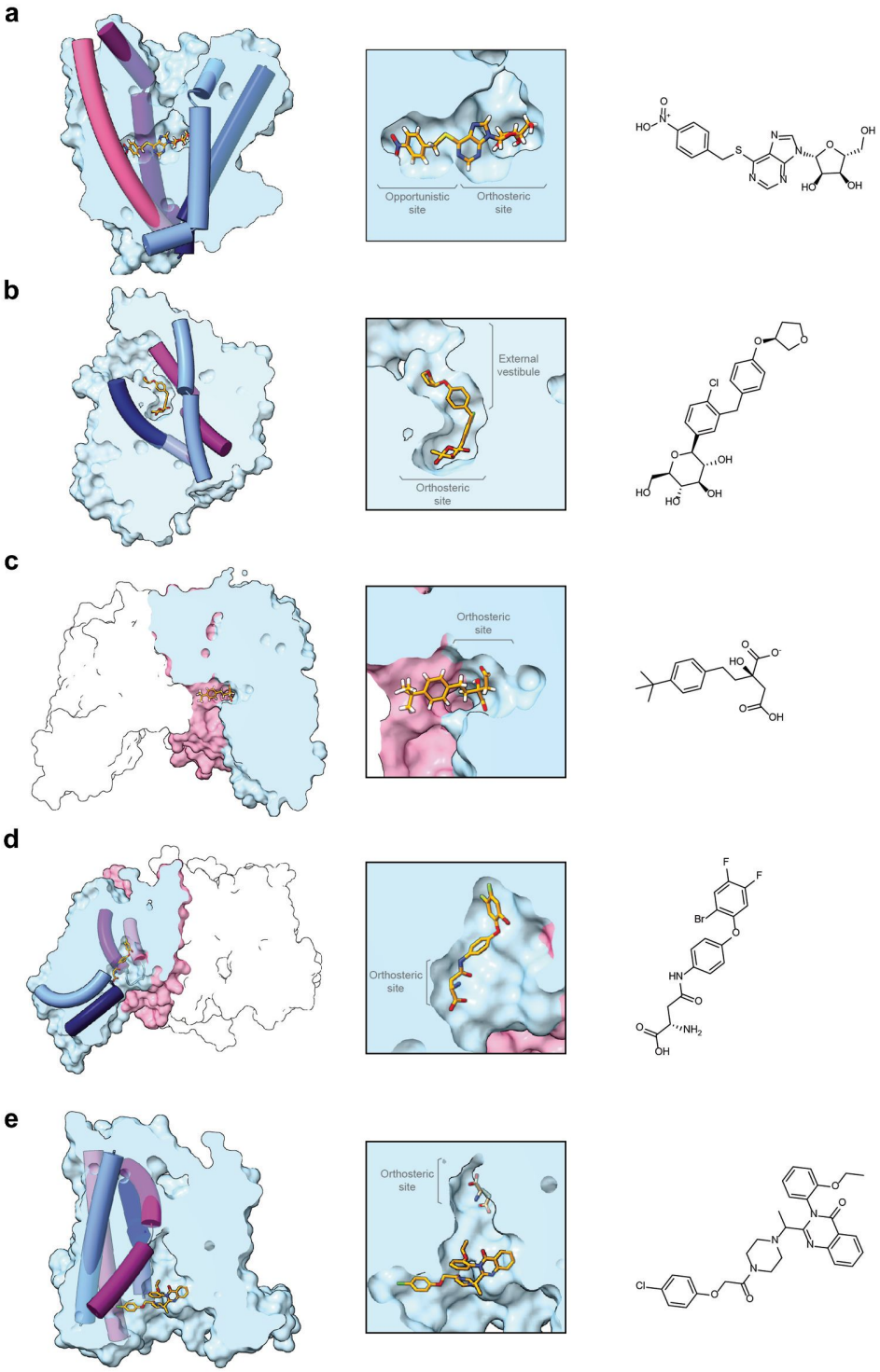


Figure 3. Orthosteric inhibitors of SLCs. (a) SLC29A1 (rocker-switch mechanism) in complex with NBMPR (PDB 6OB6). The Inhibitor extends to the opportunistic site, where it sterically prevents conformational switch. (b) SLC5A2 (gated-pore mechanism) in complex with empagliflozin (PDB 7VSI). Inhibitor partially occupies the external vestibule thus limiting the movement of gating helices. (c) SLC13A5 (elevator mechanism) in complex with PF-06649298 (PDB 7JSJ). The inhibitor blocks sliding of transport domain (blue) by extending to the scaffold domain (pink). Protein is dimer, only one monomer colored. (d) SLC1A2 (elevator mechanism) in complex with WAY213613 (PDB 7XR6). Inhibitor binds in the orthosteric site and surrounded cavity transport domain (blue) and prevents closing of gating helices. Protein is trimer, only one monomer colored. (E) SLC7A11 (gated-pore mechanism) in complex with erastin (PDB 7EPZ). Erastin binds to intracellular vestibule rather than orthosteric site. Glutamine added to orthosteric site in the detail section from structure of glutamine bound inward-open SLC7A11 (PDB 7P9U). Color coding of helices corresponds to Figure 1 (in B light pink (TM2) removed to keep the ligand is visible). Chemical structures of corresponding inhibitors are provided.

on the development of inhibitors binding SLC2A1 (GLUT1) and SLC2A3 (GLUT3) in outward-open conformation [25]. In this study, the authors utilized several screening methods, in one of them using SLC2A3 with mutations stabilizing the outward-open conformation, and were able to develop SA47, conformation-specific inhibitor of SLC2A3 and SLC2A1. A second

interesting example is a recent campaign to develop SLC6A4 inhibitors mimicking the inward-open stabilization of ibogaine [33]. This study started from virtual screening against the SLC6A4-ibogaine complex structure, followed by structure-based optimization and rigorous validation of compounds, including cryoEM and *in vivo* models.

Another possible mechanism of inhibition that can be observed with several inhibitors is the binding in the cavities near the orthosteric site, leading to blocking the access to the substrate-binding site. For example, the comparison of structures of the cysteine/glutamine exchanger SLC7A11 solved in complex with glutamine [17] and the inhibitor erastin [39] (both inward-open), suggest that erastin binds in the intracellular vestibule, in a shallower pocket, instead of the substrate binding site that is deeper in the structure (Figure 3e). Similarly, binding of H_2DIDS (4,4′-Diisothiocyanatodihydrostilbene-2,2′-Disulfonic Acid) or DEPC (Diethyl Pyrocarbonate) in SLC4A1 (AE1) occurs in the channel leading from the extracellular space to the buried substrate-binding site, thus preventing the substrate from binding [27]. Interestingly, these cases also show one of the rare covalent bindings, as both inhibitors can covalently modify lysines in SLC4A1. Finally, a similar mechanism of inhibition was proposed for lysosomal cholesterol transporter Niemann-Pick C1 (NPC1, member of SLC65 family) and the anti-fungal drug itraconazole [49]. Although this type of inhibition does not seem to be frequent, these studies illustrate that in principle, cavities surrounding the substrate binding site could be druggable similarly to the substrate-binding sites.

2.2.2. Allosteric modulators

Next to the modulators that are binding to orthosteric site, some SLCs have also allosteric sites, that can be targeted by chemical modulators. In general, allosteric modulators were suggested to provide several unique advantages over molecules targeting the orthosteric site [54]. For example, they can have a structure very different from the substrate, thus potentially providing a better selectivity and minimizing the risk of potential side-effects. Also, the presence of allosteric sites is considered to be targetable not only by inhibitors, but also potentially by 'positive allosteric modulators' (PAM), or agonists, compounds enhancing the function [55].

Allosteric modulators are in particularly well described for the SLC1 family of transporters of glutamate and neutral amino acids. $UCPH_{101}$ inhibits SLC1A3 (EAAT1) allosterically, by connecting the transporter domain with the scaffolding domain, outside the ligand binding site, toward the intracellular part of the transporter [22] (Figure 4a). This leads to the blockage of the elevator movement, resulting in an arrest of the transporter in the outward facing state. A similar allosteric site was also suggested in SLC1A5 (ASCT2) [56]. In contrast, SLC1A2 (EAAT2) is targeted by a PAM, and based on homology models, the binding site was proposed to reside toward the outer opening part of the transporter [57,58]. Even though the allosteric sites proposed by these studies for SLC1A2 and SLC1A3 are slightly different, they share an interface between the transporter domain and the scaffolding domain, suggesting commonality and potentially broader applicability of this mechanism to other transporters with elevator mechanism [51].

Another family of SLCs with well-defined allosteric regulatory sites is the sodium- and chloride-dependent neurotransmitter family, SLC6. Here, the allosteric site (called S2, compared to substrate binding site S1) was identified in the structure of serotonin transporter SLC6A4 [34], an SLC with particularly rich pharmacology and availability of experimentally solved structures. The S2 site resides in the extracellular vestibule in the outward-open conformation. It was proposed that several ligands bind there and modulate transporter activity by altering the kinetics of the ligand dissociation from the substrate binding site [59]. This binding modality was first described in the structure of SLC6A4 in complex with (S)-citalopram [34]. Interestingly, (S)-citalopram inhibits the transporter by two binding events, one at the allosteric site and another one at the central substrate binding site, with higher affinity to the substrate binding site (Figure 4b). The allosteric site was then further characterized, and it was shown that it can bind serotonin, but also chemical modulators such as Lu AF60027 and vilazodone with high affinity [36,59,60]. In fact, Lu AF60027 was specifically developed to bind the allosteric site, based on the binding of (S)-citalopram. Interestingly, it was proposed that a similar secondary substrate binding site should be present in many other transporters with LeuT fold, further expanding the druggability of these transporters [9].

Several further studies proposed allosteric modulators for other SLCs, albeit some without support of experimentally solved structures and in many cases also without direct target engagement evidence. Examples include the already mentioned PAM for SLC1A2 [57,58], and compounds targeting SLC5A7 (CHT1) [61,62] and SLC6A3 (DAT) [63]. Even though the evidence for chemically targetable allosteric sites on these additional SLCs is limited as for now, it is tempting to speculate that these sites may exist in many SLCs, particularly in those with gated-pore or elevator mechanisms. In addition, analysis of allosteric sites in known allosteric proteins suggested that allostery is more common in multi-meric and multi-domain proteins and that allosteric sites are frequently located at the oligomeric interfaces [64]. Indeed, many SLCs have been proposed to act as multimers, particularly those with elevator transport mechanism [9]. Some evidence suggests that multimerization of SLCs may enhance the transport function [14,65]. Thus, it should be possible to imagine molecules that enhance transport function by promoting the multimerization state of the transporter [54]. Moreover, it is possible that the elucidation of the mode-of-action of existing SLC inhibitors will uncover additional modulators acting in an allosteric fashion. Importantly, as already described in the previous section, the lack of competition between the inhibitor and substrate should not be taken alone as evidence for allosteric binding of the modulator [66].

3. Functional annotation of SLCs

Similarly, to their heterogenous structures, SLCs are very heterogenous in their function. The substrates of SLCs vary greatly, from ions, through nutrients and metabolites,

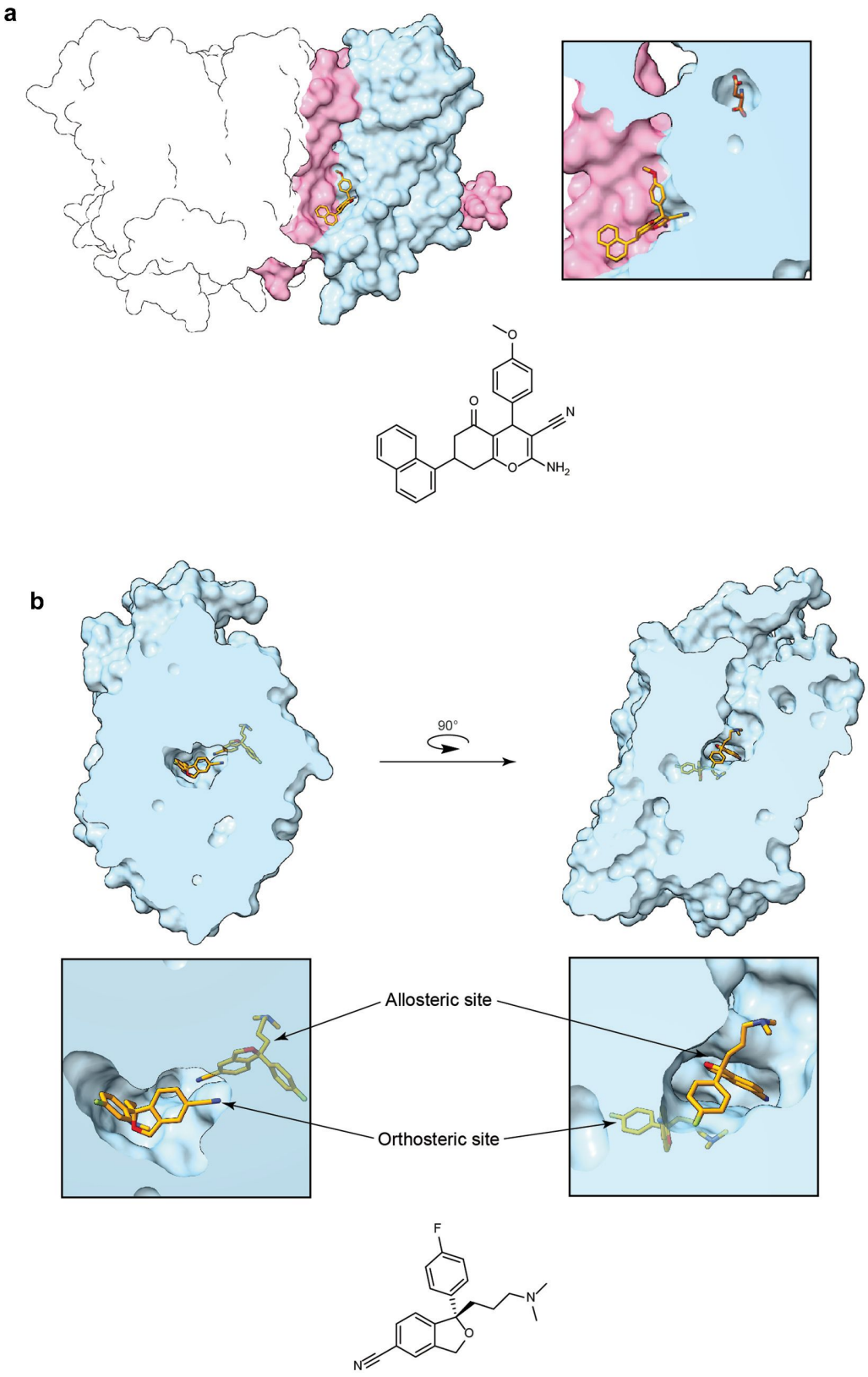


Figure 4. Allosteric inhibitors of SLCs. (a) UCPH101 inhibits SLC1A3 by binding between transport (blue) and scaffold (pink) domains outside orthosteric site. In detail section the orthosteric site is shown with aspartic acid (PDB 7AWM). (b) (S)-citalopram binds in both orthosteric and allosteric sites in SLC6A4 (PDB 5I73). Chemical structures of corresponding inhibitors are provided.

signaling molecules and xenobiotics and drugs. Even considering only the more than 200'000 human metabolites reported, the numbers are too large to imagine a 1:1 relationship between transporters and their cargo [67]. It follows, that, unlike GPCRs and nuclear hormones, transporters cannot be simply 'deorphanized.' Rather, we should speak of functional assignments of transporters. The task of assigning function to transporters is further made more complex by the greatly varying expression of different SLCs, with some transporters being expressed ubiquitously and the expression of others confined to specific cell types, or even individual cell states. At the same time, SLCs are responsible not only for the exchange of solutes between cells and their environment, but also between the intracellular compartments. Together with a high degree of functional overlap and redundancy, this makes assignment of biochemical and biological functions to individual SLC genes a difficult undertaking.

For long time, most of the research efforts have concentrated only on a subset of SLCs, leaving the majority of the SLC superfamily understudied [5]. Consequently, many SLCs remained with no significant functional annotation. In recent years we have seen an increase in number of studies proposing a function for an 'uncharacterized' SLC for the first time. In many cases the functional assignment is enabled by new technologies. For example, the utilization of technologies enabling an organelle pulldown and rapid metabolomic profiling led to the discovery of a role in pigmentation of the lysosomal cystine transporter MFSD12 [68]. Similarly, substrate or function of several SLCs was described for the first time in studies using genetic screening technologies. This was the case for a mitochondrial serine transporter with a key role in one-carbon metabolism SFXN1 [69] or for the mitochondrial NAD transporter SLC25A51 [70–72]. Additionally, for several SLCs, the biological functions were described by investigating the mechanism-of-action of drugs discovered in phenotypic screening. For example, identification of targets of compounds that interfere with the NOTCH pathway showed that endoplasmic reticulum (ER) zinc transporter SLC39A7 (ZIP7) has an essential role in trafficking of NOTCH [73], while the role of the same transporter in trafficking of TNF receptor was uncovered from genetic screen [74]. Finally, functions for some SLCs were proposed based on genome-wide association studies (GWAS) and mechanistic follow-ups. In this way, SLC22A24 was found to be involved in re-absorption of anionic steroids in the kidney [75], and FLVCR1 was shown as an important mediator of choline transport [76]. Studies that lead to functional annotation of SLCs, such as examples described above, have a strong potential impact on drug discovery through two main effects. First, by describing the biological role of the transporter, they can provide a rationale for therapeutical targeting of the particular transporter in a certain disease. Second, since many SLCs are involved in the transport of drugs themselves [77], understanding the substrate specificity and expression patterns of individual SLCs could provide a better understanding of drug distribution, or could be even leveraged into designing drugs or probes targeted to specific compartments through specific SLC-mediated transport. Nutrients or their metabolites likely affect the uptake and distribution of specific drugs and *vice versa* [78,79]. This may be particularly important in the intestine, the kidney, the liver, at the blood-brain barrier, at the blood-cerebrospinal fluid barrier of the choroid plexus, and the placenta. Despite pioneering work on individual drugs and transporters, the pharmacogenomic aspects of membrane transporters, taking into account the genomic variants of the human transportome-genome, is still at the beginning [78,80–84].

In the next sections, we will focus on two aspects of the functional annotations of SLCs that play a particularly important role in drug discovery and overall druggability of SLCs, and that is their localization and their substrate annotations.

3.1. SLC localization

An important aspect contributing to the druggability of SLC is their localization. The majority of the SLCs are expressed on the plasma membrane [85,86], hence, to target these, the requirement to cross the membrane in order to modulate these could be simpler compared to intracellular targets. This is the case especially for modulators that bind the SLC in the outward-open conformation (which is the case for the majority of the modulators in Table 1). While most orally available modulators still need to be able to cross membranes in order to be bioavailable, there are also interesting exceptions among SLC drugs. Because the bile acid transporter SLC10A2 (IBAT) is expressed on the luminal side of the intestine, some of the drug candidates targeting this transporter were deliberately modified to decrease the absorption, thus limiting the systemic exposure [4]. While this strategy could be limited only for targeting the SLCs expressed on luminal surfaces of the gastrointestinal tract, other SLCs expressed on plasma membrane could be targeted by molecules with generally limited capacities to cross the membranes, such as antibodies, macrocycles, or peptides.

The usage of antibodies to block SLC function is mainly investigational and reports of developing antibodies to modulate SLC function are currently limited. SLC40A1 (Ferroportin) is worth mentioning in this context. SLC40A1 is the only known iron exporter in mammals and is essential for iron absorption as well as maintaining the plasma iron concentration and iron distribution between different cell types [87]. One of the mechanisms of control of these processes is via the peptidic hormone hepcidin [87]. Binding of hepcidin to SLC40A1 leads to internalization and subsequent degradation of the transporter, and hence reduced iron efflux. Efforts have been made to downregulate SLC40A1 via hepcidin-inspired peptides, as well as via small molecule inhibitors developed for the treatment of iron overload conditions, such as vamifeport [88,89]. Conversely, a monoclonal antibody developed for a treatment of anemia in patients with chronic kidney disease that binds SLC40A1, while preserving the transport function, prevents binding of hepcidin and thus stabilizes the transporter [90]. Even though this is a very special case, it illustrates the variety of possible modulations of plasma membrane SLCs as well as opportunities in using negative or positive modulators of the same SLC, depending on a context.

Similarly to the antibodies, reports on SLC-targeting macrocycles are currently limited. Screening campaigns of

rapamycin-inspired macrocycles (rapafucins) yielded inhibitors of SLC2A1, SLC2A3 and SLC2A4 (GLUT4) rapaglutin A [91] as well as the SLC29A1 inhibitor rapadocin [92]. The effect of the latter is, to some extent, dependent on FKBP12. Rapafucins are designed to preserve the FKBP binding of rapamycin, while exchanging the mTOR binding domain of rapamycin to allow for binding of other ligands, suggesting that the macrocycle probably engages the transporter from the intracellular site [92]. Additionally, since many SLCs can act as receptors for viruses, it has recently been reported that a macrocyclic peptide prevents cellular entry of hepatitis B and D by binding to SLC10A1 (NTCP), while preserving the transport function [93]. Since several other SLCs were shown to be receptors of viruses, or toxins, similar approach could be utilized also for these SLCs.

Another important aspect that is influenced by the localization of SLCs regards the amenability to targeted protein degradation. Previous work from our laboratory showed that SLCs are amenable for targeted protein degradation (TPD) [94]. This study showed degradability of SLCs localized to different subcellular compartments, with some limitations in case of SLCs localized to Golgi and the inner mitochondria membrane, due to inaccessibility to degradation machinery. To some extent, this is a limitation raising from the degradation tag (dTAG) system, that relies on the localization of protein termini, and could be probably at least partially overcame by designing proteolysis targeting chimeras (PROTACs). Since the design of novel PROTACs is usually based on existing inhibitors as warheads, it is crucial to know which conformation is being targeted. This is given by the subcellular localization of E3 ligases that are currently available for PROTAC design, as well as the potentially different degradation efficacy dictated by the subcellular context [95]. For example, if an SLC is localized to the plasma membrane, it is crucial to use a warhead that binds the inward-open conformation, since E3 ligases are localized intracellularly. Conversely, to design PROTACs targeting SLCs localized to the membrane of the Golgi, it may be advantageous to use molecules that bind the outward-open (that is cytoplasm-facing) conformation of the transporter. Of note, binders targeting the outward-open conformation could be still utilized for technologies such as lysosome-targeting chimaeras (LYTACs) [96].

3.2. SLC substrates

One of the crucial pieces of information for determining the SLC function is, which substrate an SLC transports. A survey from 2020 estimated that approximately 30% of all human SLCs lack any reliable information about their substrates or ligands [6]. This represents one of the major bottlenecks in SLC-oriented drug discovery, since the understanding of the substrate specificity remains an important requirement for setting up most of the screening assays [18]. In addition, since many SLC-targeted chemical modulators are analogues to substrates, the prior knowledge of SLC substrate(s) can help pre-selecting chemical libraries/strategies for screening. Campaigns that start with screening of molecules that are structurally similar to SLC substrates are relatively frequent and can yield high-quality inhibitors. The discovery of PF-06649298, a highly potent inhibitor of the citrate transporter SLC13A5, started from screening of molecules structurally similar to citrate [97]. The discovery of AZ1422, an inhibitor of the lactate transporter SLC16A3 (MCT4), started from screening of compounds containing carboxylic acids and hence mimicking lactate [98]. This strategy can be valuable especially in cases when access to assays that would be suitable for large high-throughput screening (HTS) campaign is limited.

Numerous chemical modulators derived from substrate analogues exhibit limited selectivity, but then there also others that display a high degree of selectivity. Some of these differences in selectivity could be explained by examining the structures of transporters bound with substrate-mimicking inhibitors. While the substrate binding sites may be highly conserved across similar transporters, the cavities that are frequently near these sites may display large structural differences. This can be illustrated with the already mentioned examples of orthosteric inhibitors. The selectivity of citrate-mimicking inhibitor of SLC13A5 is achieved by the presence of a bulky group that prevents binding to the products of the paralog genes SLC13A2 (NaDC1) and SLC13A3 (NaDC3) [44] (Figure 5(a,b)). Similarly, while the substrate binding sites of SLC1A2 and SLC1A3 are highly similar, the substrate-mimicking inhibitor WAY213613 is selective to SLC1A2. This selectivity is mediated by residues in the site binding the BDP moiety that is near the substrate binding site, and less conserved [21]. The selectivity of SLC29A1 inhibitors over closely related SLC29A2 (ENT2) and SLC29A3 (ENT3) is mediated mainly by different amino acid residues in 'opportunistic sites' rather than in the proper substrate binding site [46] (Figure 5c,d). Similar to this is also the basis for selectivity of gliflozins to SLC5A2 over SLC5A1, but it can be also the case for many other orthosteric inhibitors, that are not analogues to the substrates. Of note, the residues involved in the gating of transporter can be similarly important for substrate specificity as residues in the orthosteric site [16], as exemplified by an elegant comparison of glucose transporters homologues from *Plasmodium falciparum* and mammals [99]. This further highlights the possibilities for enhancing the selectivity of orthosteric inhibitors by engaging residues outside the substrate binding site.

Last but not least, since most inhibitors bind the substrate binding site, the functional annotation can be used to counter-screen SLCs even from different families. This can be particularly important for substrate-inspired inhibitors. For example, 7ACC2, a compound originally developed as an inhibitor of the plasma membrane transporters of lactate and pyruvate [100], was later shown to inhibit the mitochondrial pyruvate carrier (MPC, SLC54) [101]. This 'off-target' would not have been expected based on protein homology considerations. It is important to note that since substrates of many SLCs overlap with the substrates of enzymes, it is also possible that substrate-inspired inhibitors may inhibit other intracellular proteins that normally bind the same substrate.

4. Expert opinion

Despite notable progress in recent years in our understanding of functions and structures of the SLC superfamily of

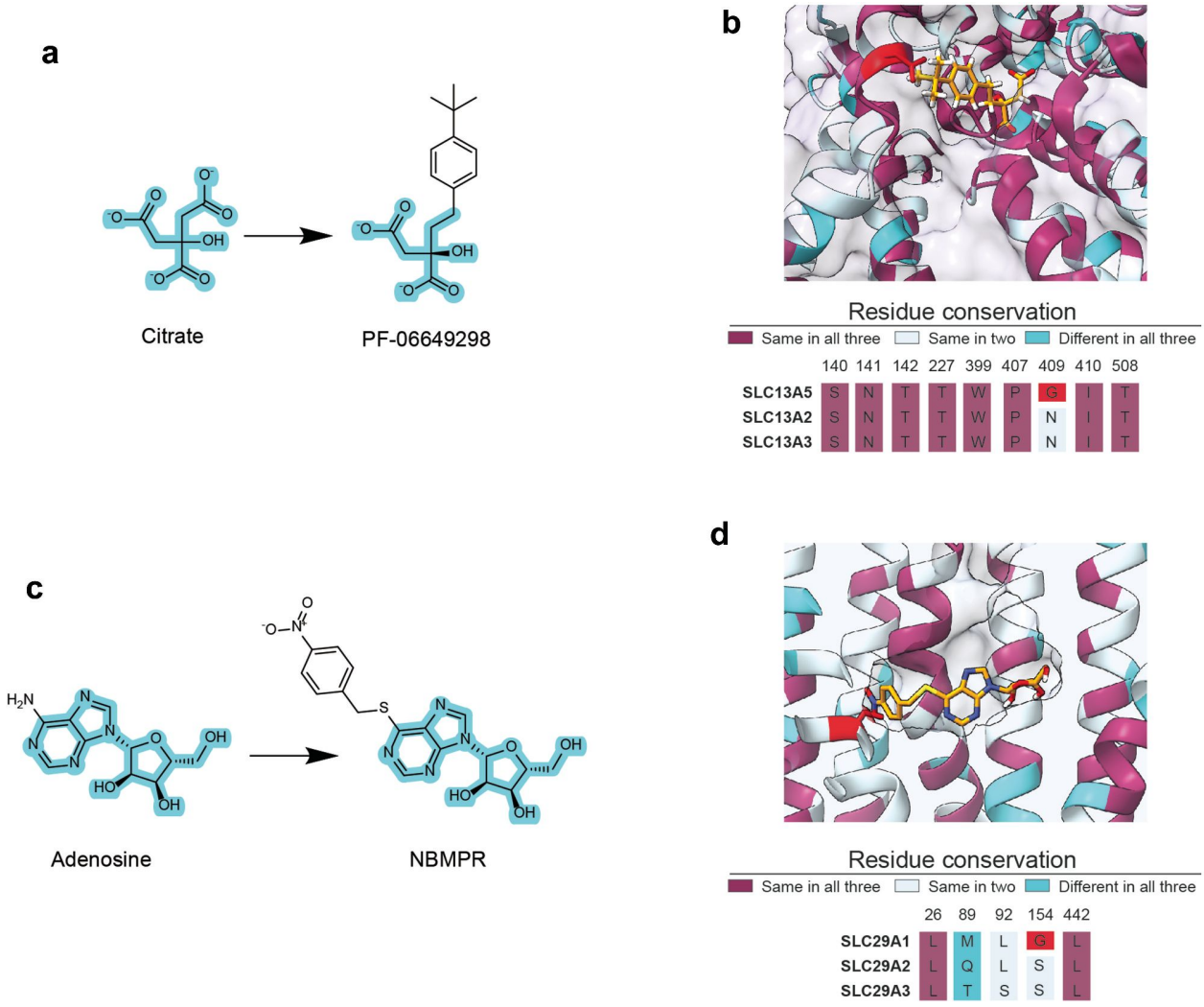


Figure 5. Selectivity of substrate-mimicking inhibitors. (A) Chemical structures of SLC13A5 substrate (citrate) and substrate-mimicking inhibitor PF-06649298. (B) PF-06649298 binding site, colored by conservation across SLC13A5, SLC13A2 and SLC13A3. The selectivity to SLC13A5 is mainly mediated by G409, and subsequent steric clash by corresponding asparagine in SLC13A2 and SLC13A3 (red) [44]. Numbering reflects the residue position in SLC13A5 (PDB 7JSJ). (C) Chemical structures of SLC29A1 substrate (adenosine) and substrate-mimicking inhibitor NBMPR. (D) NBMPR binding site, colored by conservation across SLC29A1, SLC29A2 and SLC29A3. The selectivity to SLC29A1 is mainly mediated by G154, and subsequent steric clash by corresponding serine in SLC29A2 and SLC29A3 [46]. Numbering reflects residue position in SLC29A1 (PDB 6OB6).

membrane transporters, the biochemical and biological function for many SLC remains elusive. The rapidly growing number of SLC structures, empowered by the progress in cryoEM, is contributing not only to the understanding of the transport cycles, but also to the understanding of the mode-of-action of chemical modulators targeting SLCs. Also, the number of SLC targeting drugs is constantly increasing, even though it still lags behind other classes of similar relevance [102,103]. Moreover, the majority of new compounds targeting SLCs is usually against SLCs that are already targeted [104]. We believe that this trend illustrates well the gaps in the functional and structural understanding of the SLC superfamily, as well as in the availability of tools such as chemical modulators, screening assays or high-quality antibodies. Moreover, the typical inertia that can be ascribed to the necessity to de-risk drug discovery campaigns with 'mee-toos' targets is at play also with SLC targets [105].

Both structural, but in particular functional annotation will benefit from advancements in drug discovery. With increasing number of high-quality chemical modulators, it will be easier to set up screening assays and to investigate the biological functions of SLCs but also to get additional insights into structural conformations. At the same time, increasing understanding in SLC function, transport mechanism, as well as structure will enhance the opportunities to design screening assays along with providing the rationale for targeting SLCs in different disease areas and thus empower drug discovery efforts broadly and effectively. Ultimately, the most important pre-clinical validation of therapeutic targets occurs by using high-quality chemical probes in complex disease models. Only by expanding the number of chemical probes targeting SLCs we will be able to fully test their potential as drug targets as well as druggability [106].

In our opinion, the poor functional annotation of the SLC superfamily may lead to underestimations of the numbers of

druggable, as well as 'PROTACable' SLCs [107]. Given that virtually all SLCs have orthosteric sites that evolved to bind small molecules, we would argue that all SLCs should be inherently druggable. One possible caveat with the orthosteric modulators could be their selectivity. Indeed, there are more than 60 SLCs transporting amino acids, belonging to 11 different SLC families [108]. While the orthosteric sites in these transporters may display similarities, surrounding cavities are frequently more diverse, and hence their engagement could increase the selectivity, as we discussed in Section 3.2. Moreover, while our survey of SLC-modulator complex structures showed perhaps unsurprisingly that most of the ligands bind the orthosteric site, it also showed that some SLCs could be modulated by binding other pores and cavities, or by allosteric compounds often at the interface between multimers. In the cases in which the orthosteric site and surrounding sites may be too conserved, targeting some of these additional sites could provide selectivity. Additionally, if an SLC on the plasma membrane is being targeted, utilization of monoclonal antibodies, or complex macrocycles that would bind outside the orthosteric site, can further expand possible SLC-targeting strategies.

Moreover, we believe that some of the possible issues with selective inhibition could be overcome by targeted protein degradation. In this case, the selectivity is not driven solely by the binding, but also by the formation of the ternary complexes between the targeted protein and the ubiquitin ligase. It is well established that for example promiscuous inhibitors of kinases could be turned into highly selective PROTACs [109]. We would expect that the same could be true for SLC transporters. Similarly, since even warheads with lower binding affinity and/or only transient interactions could be sufficient for PROTAC development, this could be an interesting path for modifying ligands with low affinity, or even for substrates. In addition, at least some SLCs display surfaces that can likely bind small molecules without impacting the transport function. Such molecules could also be developed into bivalent molecules such as PROTACs, and further expand the possibilities of chemical modulation of SLCs. While currently there are not many compounds like this reported, we think that their number will grow in the future. Additional compounds may induce or prevent proximity and alter equilibria among many proteins that directly or indirectly affect SLC proteostatic regulation or activity. These could be detected by precisely focused phenotypic screens with chemical libraries covering enough diversity, by screens employing proteomics or by design [110–113].

While in many cases the degradation of SLC probably does not provide significant advantages over selective inhibition, there are areas where degradation could be superior. Such examples could be cases of SLCs that are receptors for viruses, or in SLCs that play a role as scaffolding proteins. At the time of the writing, to the best of our knowledge, the only PROTACs targeting SLCs reported in the literature is the d9A-series reported by Bensimon and colleagues [94]. We are expecting that the number of SLC targeting degraders, PROTACs in particular, will grow in the future. This will not only expand the ways of pharmacological modulations but will also allow to compare all benefits between the inhibitors and degraders in SLC superfamily.

While historically, most of the assays used in the SLC-oriented drug discovery were focused on measuring substrate uptake, in recent years there is an increasing interest in binding assays. For example, thermal shift assays have been increasingly applied to SLCs [114,115]. Indeed, there are also beautiful examples of chemical modulators of SLC discovered through chemoproteomic approaches [116]. Approaches like these can be used for many SLCs at the same time, circumventing the need for SLC-specific assays [113]. Although these assays are not sufficient to drive a whole drug discovery campaign, they provide a promising basis to find hit compounds that can be used to set up orthogonal assays and to be further improved. This is particularly useful in cases of SLCs for which no modulating chemical exists, in which even a relatively poor-quality modulator can be used as control in an assay development.

Many SLCs are connected to diseases through loss-of-function mutations, that can lead to transport deficiency, protein misfolding or limited ability to localize correctly [81]. The development of molecules that can address these aspects, such as PAMs, correctors, potentiators or pharmaco-chaperones, is recently getting more attention [117–120]. It is still not entirely clear which SLCs could be targeted by these approaches, however as we briefly discussed in Section 2.2.2 (Allosteric modulators), some ideas are already emerging. We are expecting to see an increasing number of reports on positive pharmacological modulators in the future, that will provide clearer definition of druggability of SLCs with these types of modulators. Finally, increasing the understanding of expression patterns in combination with substrate specificities among individual transporters hold promise to lead to the development of drugs and probes that would be transported by specific SLCs and thus affect only specific cell types. This principle is used for positron emission tomography (PET) scans; however we are starting to see this entering also drug design [104].

In summary we are convinced that despite some already existing cases of SLC-targeting blockbusters, we are experiencing the dawn of SLC transporter pharmacology. As major modulators of the interface between chemistry and biology at biological membranes, by their sheer number and versatility, and by their gatekeeping role in cellular and organismic metabolism, SLC transporters are likely to be become the most important target of new drugs of the coming decade. We hope that the evidence offered in this review should persuade or at least intrigue even the more skeptical readers.

Acknowledgments

The authors thank Ulrich Goldmann for help with assembling the SLC structures, Evandro Ferrada for discussions during the writing of the review and S. Andreas Angermayr for critical reading, proofreading and administrative support.

Funding

The authors acknowledge support by the Austrian Academy of Sciences and the Innovative Medicines Initiative 2 Joint Undertaking (grant agreement No 777372, ReSOLUTE).

Declaration of interest

G Superti-Furga is a co-founder of the companies Proxygen and Solgate and a coauthor on patents related to SLCs. He is also the Academic Project Coordinator of the IMI grant Resolute in partnership with Pfizer Inc, Novartis, Bayer Healthcare, Sanofi, Boehringer Ingelheim and Vifor Pharma. The G Superti-Furga laboratory receives funds from Pfizer. V Dvorak is a coauthor on patent applications related to SLCs. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

ORCID

Vojtech Dvorak http://orcid.org/0000-0002-9496-370X
Giulio Superti-Furga http://orcid.org/0000-0002-0570-1768

References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Bar-Peled L, Kory N. Principles and functions of metabolic compartmentalization. Nat Metab. 2022;4(10):1232–1244. doi: 10.1038/s42255-022-00645-2
2. Hediger MA, Clémençon B, Burrier RE, et al. The ABCs of membrane transporters in health and disease (SLC series): Introduction. Mol Aspects Med Internet. 2013;34:95–107. doi: 10.1016/j.mam.2012.12.009
3. Lin L, Yee SW, Kim RB, et al. SLC transporters as therapeutic targets: Emerging opportunities. Nat Rev Drug Discov Internet. 2015;14:543–560. doi: 10.1038/nrd4626
4. Wang W, Gallo L, Jadhav A, et al. The druggability of solute carriers. J Med Chem Internetacs.jmedchem.9b01237. 2019; https://pubs.acs.org/doi/abs/10.1021/acs.jmedchem.9b01237
•• **Comprehensive review on SLC druggability.**
5. César-Razquin A, Snijder B, Frappier-Brinton T, et al. A call for systematic research on solute carriers. Cell. 2015;162(3):478–487. doi: 10.1016/j.cell.2015.07.022
6. Meixner E, Goldmann U, Sedlyarov V, et al. A substrate-based ontology for human solute carriers. Mol Syst Biol. 2020;16(7):1–9. doi: 10.15252/msb.20209652
•• **Excelent resource for functional annotations of SLCs.**
7. Hopkins AL, Groom CR The druggable genome. Nat Rev Drug Discov Internet. 2002;1(9):727–730. doi: 10.1038/nrd892
8. Perland E, Fredriksson R. Classification systems of secondary active transporters. Trends Pharmacol Sci. 2017;38(3):305–315. doi: 10.1016/j.tips.2016.11.008
9. Drew D, Boudker O. Shared molecular mechanisms of membrane transporters. Annu Rev Biochem. 2016;85(1):543–572. doi: 10.1146/annurev-biochem-060815-014520
• **Excelent review describing the main transport mechanisms that are shared among SLCs.**
10. Tunyasuvunakool K, Adler J, Wu Z, et al. Highly accurate protein structure prediction for the human proteome. Nature InternetAvailable from. 2021;596(7873):590–596. doi: 10.1038/s41586-021-03828-1
•• **Highly accurate predictions of protein structures for the majority of proteomes from multiple species.**
11. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. Nature Internet. 2021;596:583–589. doi: 10.1038/s41586-021-03819-2
12. Ferrada E, Superti-Furga G. A structure and evolutionary-based classification of solute carriers. iScience Internet. 2022;25:105096. doi: 10.1016/j.isci.2022.105096
13. Xie T, Chi X, Huang B, et al. Rational exploration of fold atlas for human solute carrier proteins. Structure Internet. 2022;30(9):1321–1330.e5. doi: 10.1016/j.str.2022.05.015
14. Zhang B, Jin Q, Xu L, et al. Cooperative transport mechanism of human monocarboxylate transporter 2. Nat Commun Internet. 2020;11:1–10. doi: 10.1038/s41467-020-16334-1
15. Wang N, Jiang X, Zhang S, et al. Structural basis of human monocarboxylate transporter 1 inhibition by anti-cancer drug candidates. Cell Internet. 2021;184(2):370–383.e13. doi: 10.1016/j.cell.2020.11.043
16. Drew D, North RA, Nagarathinam K, et al. Structures and general transport mechanisms by the major facilitator superfamily (MFS). Chem Rev Internet. 2021;121(9):5289–5335. doi: 10.1021/acs.chemrev.0c00983
17. Parker JL, Deme JC, Kolokouris D, et al. Molecular basis for redox control by the human cystine/glutamate antiporter system xc−. Nat Commun Internet. 2021;12(1):7147. http://www.ncbi.nlm.nih.gov/pubmed/34880232
18. Dvorak V, Wiedmer T, Ingles-Prieto A, et al. An overview of cell-based assay platforms for the solute carrier family of transporters. Front Pharmacol [Internet]. 2021;12:1–31. doi: 10.3389/fphar.2021.722889
19. Del Alamo D, Meiler J, Mchaourab HS. Principles of alternating access in LeuT-fold transporters: commonalities and divergences. J Mol Biol Internet. 2022;434:167746. doi: 10.1016/j.jmb.2022.167746
20. Garaeva AA, Slotboom DJ Elevator-type mechanisms of membrane transport. Biochem Soc Trans Internet. 2020;48(3):1227–1241. doi: 10.1042/BST20200290
21. Kato T, Kusakizako T, Jin C, et al. Structural insights into inhibitory mechanism of human excitatory amino acid transporter EAAT2. Nat Commun Internet. 2022;13(1):4714. doi: 10.1038/s41467-022-32442-6
22. Canul-Tec JC, Assal R, Cirri E, et al. Structure and allosteric inhibition of excitatory amino acid transporter 1. Nature Internet. 2017;544(7651):446–451. doi: 10.1038/nature22064
• **Landmark study describing the allosteric inhibition of SLC1A3.**
23. Garibsingh RAA, Ndaru E, Garaeva AA, et al. Rational design of ASCT2 inhibitors using an integrated experimental-computational approach. Proc Natl Acad Sci U S A. 2021;118(37):118. doi: 10.1073/pnas.2104093118
• **Interesting study combining rational drug design based on homology model and cryoEM structure.**
24. Kapoor K, Finer-Moore JS, Pedersen BP, et al. Mechanism of inhibition of human glucose transporter GLUT1 is conserved between cytochalasin B and phenylalanine amides. Proc Natl Acad Sci U S A. 2016;113(17):4711–4716. doi: 10.1073/pnas.1603735113
25. Wang N, Zhang S, Yuan Y, et al. Molecular basis for inhibiting human glucose transporters by exofacial inhibitors. Nat Commun Internet. 2022;13(1):2632. doi: 10.1038/s41467-022-30326-3
• **Study describing development of conformation specific inhibitor.**
26. Yuan Y, Kong F, Xu H, et al. Cryo-EM structure of human glucose transporter GLUT4. Nat Commun Internet. 2022;13(1):2671. doi: 10.1038/s41467-022-30235-5
27. Capper MJ, Yang S, Stone AC, et al. Substrate binding and inhibition of the anion exchanger 1 transporter. bioRxiv [Internet]. 2022;2022 Feb 11p. 480130. http://biorxiv.org/content/early/2022/02/12/2022.02.11.480130.abstract
28. Arakawa T, Kobayashi-Yurugi T, Alguel Y, et al. Crystal structure of the anion exchanger domain of human erythrocyte band 3. Science Internet. 2015;350(6261):680–684. doi: 10.1126/science.aaa4335
29. Niu Y, Cui W, Liu R, et al. Structural mechanism of SGLT1 inhibitors. Nat Commun Internet. 2022;13(1):6440. doi: 10.1038/s41467-022-33421-7
30. Niu Y, Liu R, Guan C, et al. Structural basis of inhibition of the human SGLT2–MAP17 glucose transporter. Nature Internet. 2022;601(7892):280–284. http://www.ncbi.nlm.nih.gov/pubmed/34880493

31. Motiwala Z, Aduri NG, Shaye H, et al. Structural basis of GABA reuptake inhibition. Nature Internet. 2022;606(7915):820–826. doi: 10.1038/s41586-022-04814-x
32. Coleman JA, Yang D, Zhao Z, et al. Serotonin transporter–ibogaine complexes illuminate mechanisms of inhibition and transport. Nature Internet. 2019;569(7754):141–145. doi: 10.1038/s41586-019-1135-1
    - **Structure of SLC6A4 in complex with ibogaine, showing atypical othosteric inhibition.**
33. Singh I, Seth A, Billesbølle CB, et al. Structure-based discovery of conformationally selective inhibitors of the serotonin transporter. Cell Internet. 2023;186(10):2160–2175.e17. doi: 10.1016/j.cell.2023.04.010
34. Coleman JA, Green EM, Gouaux E. X-ray structures and mechanism of the human serotonin transporter. Nature. 2016;532 (7599):334–339. doi: 10.1038/nature17629
    - **Study reporting the first SLC6A4 structure and structural evidence of the drug bounding in the allosteric site of SLC6A4.**
35. Coleman JA, Gouaux E. Structural basis for recognition of diverse antidepressants by the human serotonin transporter. Nat Struct Mol Biol Internet. 2018;25:170–175. doi: 10.1038/s41594-018-0026-8
36. Plenge P, Yang D, Salomon K, et al. The antidepressant drug vilazodone is an allosteric inhibitor of the serotonin transporter. Nat Commun Internet. 2021;12(1):5063. doi: 10.1038/s41467-021-25363-3
37. Shahsavar A, Stohler P, Bourenkov G, et al. Structural insights into the inhibition of glycine reuptake. Nature Internet. 2021;591:677–681. doi: 10.1038/s41586-021-03274-z
38. Yan R, Li Y, Müller J, et al. Mechanism of substrate transport and inhibition of the human LAT1-4F2hc amino acid transporter. Cell Discov Internet. 2021;7:0–7. doi: 10.1038/s41421-021-00247-4
39. Yan R, Xie E, Li Y, et al. The structure of erastin-bound Xct–4F2hc complex reveals molecular mechanisms underlying erastin-induced ferroptosis. Cell Res Internet. 2022;32(7):687–690. http://www.ncbi.nlm.nih.gov/pubmed/35352032
40. Dong Y, Gao Y, Ilie A, et al. Structure and mechanism of the human NHE1-CHP1 complex. Nat Commun Internet. 2021;12. doi: 10.1038/s41467-021-23496-z
41. Zhao Y, Roy K, Vidossich P, et al. Structural basis for inhibition of the Cation-chloride cotransporter NKCC1 by the diuretic drug bumetanide. Nat Commun. 2022;13(1). doi: 10.1038/s41467-022-30407-3
42. Fan M, Zhang J, Lee C-L, et al. Structure and thiazide inhibition mechanism of the human Na–Cl cotransporter. Nature Internet. 2023;614(7949):788–793. doi: 10.1038/s41586-023-05718-0
43. Zhao Y, Shen J, Wang Q, et al. Structure of the human cation–chloride cotransport KCC1 in an outward-open state. Proc Natl Acad Sci Internet. 2022;119(27): doi: 10.1073/pnas.2109083119
44. Sauer DB, Song J, Wang B, et al. Structure and inhibition mechanism of the human citrate transporter NaCT. Nature Internet. 2021;591:157–161. doi: 10.1038/s41586-021-03230-x
45. Khanppnavar B, Maier J, Herborg F, et al. Structural basis of organic cation transporter-3 inhibition. Nat Commun Internet. 2022;13 (1):6714. doi: 10.1038/s41467-022-34284-8
46. Wright NJ, Lee S-Y. Structures of human ENT1 in complex with adenosine reuptake inhibitors. Nat Struct Mol Biol Internet. 2019;26:599–606. doi: 10.1038/s41594-019-0245-7
47. Billesbølle CB, Azumaya CM, Kretsch RC, et al. Structure of hepcidin-bound ferroportin reveals iron homeostatic mechanisms. Nature Internet. 2020;586:807–811. doi: 10.1038/s41586-020-2668-z
48. Lehmann EF, Liziczai M, Drożdżyk K, et al. Structures of ferroportin in complex with its specific inhibitor vamifeport. bioRxiv [Internet]. 2022;2022 Aug 29p. 505642. http://biorxiv.org/content/early/2022/08/29/2022.08.29.505642.abstract.
49. Long T, Qi X, Hassan A, et al. Structural basis for itraconazole-mediated NPC1 inhibition. Nat Commun Internet. 2020;11:1–11. doi: 10.1038/s41467-019-13917-5
50. Hu M, Yang F, Huang Y, et al. Structural insights into the mechanism of human NPC1L1-mediated cholesterol uptake. Sci Adv Internet. 2021;7(29):1–16. doi: 10.1126/sciadv.abg3188
51. Garibsingh RA, Schlessinger A. Advances and challenges in rational drug design for SLCs. Trends Pharmacol Sci Internet. 2019;1–11. doi: 10.1016/j.tips.2019.08.006
52. Renaud JP, Chari A, Ciferri C, et al. Cryo-EM in drug discovery: achievements, limitations and prospects. Nat Rev Drug Discov Internet. 2018;17:471–492. doi: 10.1038/nrd.2018.77
53. Niello M, Gradisch R, Loland CJ, et al. Allosteric modulation of neurotransmitter transporters as a therapeutic strategy. Trends Pharmacol Sci Internet. 2020;41:446–463. doi: 10.1016/j.tips.2020.04.006
54. Kremer DM, Lyssiotis CA. Targeting allosteric regulation of cancer metabolism. Nat Chem Biol. 2022;18(5):441–450. doi: 10.1038/s41589-022-00997-6
55. Changeux JP, Christopoulos A. Allosteric modulation as a unifying mechanism for receptor function and regulation. Cell Internet. 2016;166:1084–1102. doi: 10.1016/j.cell.2016.08.015
56. Dong Y, Wang J, Garibsingh RA, et al. Conserved allosteric inhibition mechanism in SLC1 transporters. bioRxiv [Internet]. 2022;2022 Sep 21p. 508810. http://biorxiv.org/content/early/2022/09/22/2022.09.21.508810.abstract
57. Kortagere S, Mortensen OV, Xia J, et al. Identification of novel allosteric modulators of glutamate transporter EAAT2. ACS Chem Neurosci. 2018;9(3):522–534. doi: 10.1021/acschemneuro.7b00308
58. Abram M, Jakubiec M, Reeb K, et al. Discovery of (R)- N-Benzyl-2-(2,5-dioxopyrrolidin-1-yl)propanamide [(R)-AS-1], a novel orally bioavailable EAAT2 modulator with drug-like properties and potent antiseizure activity in vivo. J Med Chem. 2022;65(65):11703–11725. doi: 10.1021/acs.jmedchem.2c00534
59. Yang D, Gouaux E Illumination of serotonin transporter mechanism and role of the allosteric site. Sci Adv Internet. 2021;7(49):eabl3857. doi: 10.1126/sciadv.abl3857
60. Plenge P, Abramyan AM, Sørensen G, et al. The mechanism of a high-affinity allosteric inhibitor of the serotonin transporter. Nat Commun Internet. 2020;11:1–12. doi: 10.1038/s41467-020-15292-y
61. Ruggiero AM, Wright J, Ferguson SM, et al. Nonoisotopic assay for the presynaptic choline transporter reveals capacity for allosteric modulation of choline uptake. ACS Chem Neurosci Internet. 2012;3 (10):767–781. doi: 10.1021/cn3000718
62. Choudhary P, Armstrong EJ, Jorgensen CC, et al. Discovery of compounds that positively modulate the high affinity choline transporter. Front Mol Neurosci. 2017;10:1–17. doi: 10.3389/fnmol.2017.00040
63. Aggarwal S, Liu X, Rice C, et al. Identification of a novel allosteric modulator of the human dopamine transporter. ACS Chem Neurosci. 2019;10(8):3718–3730. doi: 10.1021/acschemneuro.9b00262
64. Xie J, Lai L. Protein topology and allostery. Curr Opin Struct Biol Internet. 2020;62:158–165. doi: 10.1016/j.sbi.2020.01.011
65. Yan Q, Lu Y, Zhou L, et al. Mechanistic insights into GLUT1 activation and clustering revealed by super-resolution imaging. Proc Natl Acad Sci Internet. 2018;115(27):7033–7038. doi: 10.1073/pnas.1803859115
66. Blat Y Non-competitive inhibition by active site binders. Chem Biol Drug Des Internet. 2010;75(6):535–540. doi: 10.1111/j.1747-0285.2010.00972.x
67. Wishart DS, Guo A, Oler E, et al. HMDB 5.0: the human metabolome database for 2022. Nucleic Acids Res Internet. 2022;50(D1):D622–D631. doi: 10.1093/nar/gkab1062
68. Adelmann CH, Traunbauer AK, Chen B, et al. MFSD12 mediates the import of cysteine into melanosomes and lysosomes. Nature Internet. 2020;588:699–704. doi: 10.1038/s41586-020-2937-x
69. Kory N, Wyant GA, Prakash G, et al. SFXN1 is a mitochondrial serine transporter required for one-carbon metabolism. Science. 2018;362 (6416):80–. doi: 10.1126/science.aat9528
70. Girardi E, Agrimi G, Goldmann U, et al. Epistasis-driven identification of SLC25A51 as a regulator of human mitochondrial NAD import. Nat Commun Internet. 2020;11:6145. doi: 10.1038/s41467-020-19871-x
71. Luongo TS, Eller JM, Lu M-J, et al. SLC25A51 is a mammalian mitochondrial NAD+ transporter. Nature Internet. 2020;588:174–179. doi: 10.1038/s41586-020-2741-7
72. Kory N, Uit de Bos J, van der Rijt S, et al. MCART1/SLC25A51 is required for mitochondrial NAD transport. Sci Adv Internet. 2020;6 (43): doi: 10.1126/sciadv.abe5310

73. Nolin E, Gans S, Llamas L, et al. Discovery of a ZIP7 inhibitor from a Notch pathway screen. Nat Chem Biol Internet. 2019;15(2):179–188. doi: 10.1038/s41589-018-0200-7
- **This study utilizes chemical biology to uncover a hit from phenotypic screen, finding the role of SLC39A7 as a Notch pathway modulation.**
74. Fauster A, Rebsamen M, Willmann KL, et al. Systematic genetic mapping of necroptosis identifies SLC39A7 as modulator of death receptor trafficking. Cell Death Differ Internet. 2018;26(6):1138–1155. http://www.nature.com/articles/s41418-018-0192-6
75. Yee SW, Stecula A, Chien HC, et al. Unraveling the functional role of the orphan solute carrier, SLC22A24 in the transport of steroid conjugates through metabolomic and genome-wide association studies [Internet]. PLoS Genet. 2019;15(9):e1008208. doi: 10.1371/journal.pgen.1008208
76. Kenny TC, Khan A, Son Y, et al. Integrative genetic analysis identifies FLVCR1 as a plasma-membrane choline transporter in mammals. Cell Metab [Internet]. 2023;p. 1–15. https://linkinghub.elsevier.com/retrieve/pii/S1550413123001304
77. Girardi E, César-Razquin A, Lindinger S, et al. A widespread role for SLC transmembrane transporters in resistance to cytotoxic drugs. Nat Chem Biol Internet. 2020;16(4):469–478. doi: 10.1038/s41589-020-0483-3
78. Schlessinger A, Welch MA, van Vlijmen H, et al. Molecular modeling of drug–transporter interactions—an international transporter consortium perspective. Clin Pharmacol Ther. 2018;104(5):818–835. doi: 10.1002/cpt.1174
79. Zhang Y, Zhang Y, Sun K, et al. The SLC transporter in nutrient and metabolic sensing, regulation, and drug development. J Mol Cell Biol. 2018;11(1):1–13. doi: 10.1093/jmcb/mjy052
80. Yee SW, Chen L, Giacomini KM Pharmacogenomics of membrane transporters: past, present and future. Pharmacogenomics Internet. 2010;11(4):475–479. doi: 10.2217/pgs.10.22
81. Schaller L, Lauschke VM. The genetic landscape of the human solute carrier (SLC) transporter superfamily. Hum Genet Internet. 2019;138:1359–1377. doi: 10.1007/s00439-019-02081-x
82. DeGorter MK, Xia CQ, Yang JJ, et al. Drug transporters in drug efficacy and toxicity. Annu Rev Pharmacol Toxicol Internet. 2012;52(1):249–273. doi: 10.1146/annurev-pharmtox-010611-134529
83. Franke RM, Gardner ER, Sparreboom A Pharmacogenetics of drug transporters. Curr Pharm Des Internet. 2010;16(2):220–230. doi: 10.2174/138161210790112683
84. Nies AT, Schaeffeler E, Schwab M. Hepatic solute carrier transporters and drug therapy: Regulation of expression and impact of genetic variation. Pharmacol Ther Internet. 2022;238:108268. doi: 10.1016/j.pharmthera.2022.108268
85. Pizzagalli MD, Bensimon A, Superti-Furga G. A guide to plasma membrane solute carrier proteins. FEBS J. 2020;288(9):2784–2835. doi: 10.1111/febs.15531
86. Giacomini KM, Yee SW, Koleske ML, et al. New and emerging research on solute carrier and ATP binding cassette transporters in drug discovery and development: outlook from the international transporter consortium. Clin Pharmacol Ther. 2022;112(3):540–561. doi: 10.1002/cpt.2627
87. Muckenthaler MU, Rivella S, Hentze MW, et al. A red carpet for iron metabolism. Cell [Internet]. 2017;168:344–361. doi: 10.1016/j.cell.2016.12.034
88. Manolova V, Nyffenegger N, Flace A, et al. Oral ferroportin inhibitor ameliorates ineffective erythropoiesis in a model of β-thalassemia. Journal Of Clinical Investigation Internet. 2019;130(1):491–506. http://www.ncbi.nlm.nih.gov/pubmed/31638596
89. Ramos E, Ruchala P, Goodnough JB, et al. Minihepcidins prevent iron overload in a hepcidin-deficient mouse model of severe hemochromatosis. Blood Internet. 2012;120(18):3829–3836. doi: 10.1182/blood-2012-07-440743
90. Sheetz M, Barrington P, Callies S, et al. Targeting the hepcidin–ferroportin pathway in anaemia of chronic kidney disease. Br J Clin Pharmacol. 2019;85(5):935–948. doi: 10.1111/bcp.13877
91. Guo Z, Cheng Z, Wang J, et al. Discovery of a potent GLUT inhibitor from a library of rapafucins by using 3D microarrays. Angew Chem Int Ed Engl Internet. 2019;58(48):17158–17162. doi: 10.1002/anie.201905578
92. Guo Z, Hong SY, Wang J, et al. Rapamycin-inspired macrocycles with new target specificity. Nat Chem Internet. 2019;11:254–263. doi: 10.1038/s41557-018-0187-4
93. Passioura T, Watashi K, Fukano K, et al. De Novo macrocyclic peptide inhibitors of hepatitis b virus cellular entry. Cell Chem Biol Internet. 2018;25(7):906–915.e5. doi: 10.1016/j.chembiol.2018.04.011
- **Development of macrocylic compounds that prevent interactions between SLC and a viral protein, reducing the intracellular entry of the virus, while not affecting the SLC transport function.**
94. Bensimon A, Pizzagalli MD, Kartnig F, et al. Targeted degradation of SLC transporters reveals amenability of multi-pass transmembrane proteins to ligand-induced proteolysis. Cell Chem Biol Internet. 2020;27(6):728–739.e9. doi: 10.1016/j.chembiol.2020.04.003
- **This study shows for a first time that SLCs are amenable for targeted protein degradation and introduces first SLC-targeted PROTAC.**
95. Simpson LM, Glennie L, Brewer A, et al. Target protein localization and its impact on PROTAC-mediated degradation. Cell Chemical Biology Internet. 2022;29(10):1482–1504.e7. doi: 10.1016/j.chembiol.2022.08.004
96. Banik SM, Pedram K, Wisnovsky S, et al. Lysosome-targeting chimaeras for degradation of extracellular proteins. Nature Internet. 2020;584:291–297. doi: 10.1038/s41586-020-2545-9
97. Huard K, Brown J, Jones JC, et al. Discovery and characterization of novel inhibitors of the sodium-coupled citrate transporter (NaCT or SLC13A5). Sci Rep. 2015;5(1):1–13. doi: 10.1038/srep17391
98. Kawatkar A, Clark RA, Hopcroft L, et al. Chemical biology approaches confirm MCT4 as the therapeutic target of a cellular optimized hit. ACS Chem Biol [Internet]. 2023; http://www.ncbi.nlm.nih.gov/pubmed/36602435.
99. Qureshi AA, Suades A, Matsuoka R, et al. The molecular basis for sugar import in malaria parasites. Nature Internet. 2020;578:321–325. doi: 10.1038/s41586-020-1963-z
100. Draoui N, Schicke O, Fernandes A, et al. Synthesis and pharmacological evaluation of carboxycoumarins as a new antitumor treatment targeting lactate transport in cancer cells. Bioorganic Med Chem Internet. 2013;21:7107–7117. doi: 10.1016/j.bmc.2013.09.010
101. Corbet C, Bastien E, Draoui N, et al. Interruption of lactate uptake by inhibiting mitochondrial pyruvate transport unravels direct antitumor and radiosensitizing effects. Nat Commun Internet. 2018;9:1208. doi: 10.1038/s41467-018-03525-0
102. Santos R, Ursu O, Gaulton A, et al. A comprehensive map of molecular drug targets. Nat Rev Drug Discov Internet. 2017;16:19–34. doi: 10.1038/nrd.2016.230
103. Avram S, Halip L, Curpan R, et al. Novel drug targets in 2021. Nat Rev Drug Discov Internet. 2022;21(5):328. doi: 10.1038/d41573-022-00057-7
104. Casiraghi A, Bensimon A, Superti-Furga G. Recent developments in ligands and chemical probes targeting solute carrier transporters. Curr Opin Chem Biol Internet. 2021;62:53–63. doi: 10.1016/j.cbpa.2021.01.012
105. Aronson JK, Green AR Me-too pharmaceutical products: History, definitions, examples, and relevance to drug shortages and essential medicines lists. Br J Clin Pharmacol Internet. 2020;86(11):2114–2122. doi: 10.1111/bcp.14327
106. Müller S, Ackloo S, Al Chawaf A, et al. Target 2035 – update on the quest for a probe for every protein. RSC Med Chem Internet. 2022;13(1):13–21. http://xlink.rsc.org/?DOI=D1MD00228G
107. Schneider M, Radoux CJ, Hercules A, et al. The PROTACtable genome. Nat Rev Drug Discov Internet. 2021;20:789–797. doi: 10.1038/s41573-021-00245-x
108. Kandasamy P, Gyimesi G, Kanai Y, et al. Amino acid transporters revisited: new views in health and disease. Trends Biochem Sci Internet. 2018. https://linkinghub.elsevier.com/retrieve/pii/S0968000418301014
109. Bondeson DP, Smith BE, Burslem GM, et al. Lessons in PROTAC design from selective degradation with a promiscuous warhead.

Cell Chemical Biology Internet. 2018;25(1):78–87.e5. doi: 10.1016/j.chembiol.2017.09.010
110. Toriki ES, Papatzimas JW, Nishikawa K, et al. Rational chemical design of molecular glue degraders. ACS Cent Sci Internet. 2023;9(5):915–926. doi: 10.1021/acscentsci.2c01317
111. Lazear MR, Remsberg JR, Jaeger MG, et al. Proteomic discovery of chemical probes that perturb protein complexes in human cells. Molecular Cell Internet. 2023;83(10):1725–1742.e12. doi: 10.1016/j.molcel.2023.03.026
112. Gerry CJ, Schreiber SL. Unifying principles of bifunctional, proximity-inducing small molecules. Nat Chem Biol Internet. 2020;16:369–378. doi: 10.1038/s41589-020-0469-1
113. Conway LP, Li W, Parker CG. Chemoproteomic-enabled phenotypic screening. Cell Chem Biol Internet. 2021;28:371–393. doi: 10.1016/j.chembiol.2021.01.012
114. Hashimoto M, Girardi E, Eichner R, et al. Detection of chemical engagement of solute carrier proteins by a cellular thermal shift assay. ACS Chem Biol Internet. 2018;13(6):1480–1486. doi: 10.1021/acschembio.8b00270
115. Chatzikyriakidou Y, Ahn D-H, Nji E, et al. The GFP thermal shift assay for screening ligand and lipid interactions to solute carrier transporters. Nat Protoc Internet. 2021;16(12):5357–5376. doi: 10.1038/s41596-021-00619-w
116. Parker CG, Galmozzi A, Wang Y, et al. Ligand and target discovery by fragment-based screening in human cells. Cell. 2017;168(3):527–541.e29. doi: 10.1016/j.cell.2016.12.029
117. Van Goor F, Hadida S, Grootenhuis PDJ, et al. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. Proc Natl Acad Sci U S A. 2009;106(44):18825–18830. doi: 10.1073/pnas.0904709106
118. Bhat S, El-Kasaby A, Freissmuth M, et al. Functional and biochemical consequences of disease variants in neurotransmitter transporters: a special emphasis on folding and trafficking deficits. Pharmacol Ther [Internet]. 2021;222:107785. doi: 10.1016/j.pharmthera.2020.107785
119. Bhat S, Newman AH, Freissmuth M How to rescue misfolded SERT, DAT and NET: targeting conformational intermediates with atypical inhibitors and partial releasers. Biochem Soc Trans Internet. 2019;47(3):861–874. doi: 10.1042/BST20180512
120. Rowe SM, Verkman AS. Cystic fibrosis transmembrane regulator correctors and potentiators. Cold Spring Harb Perspect Biol. 2013;3(7):a009761–a009761. doi: 10.1101/cshperspect.a009761

1.2 Review of assays for SLCs.

One of the major bottlenecks to study SLCs is the general lack of appropriate tools (César-Razquin *et al*, 2015; Superti-Furga *et al*, 2020). This considers antibodies, tool compounds, datasets, but also biological assays. Yet, the availability of assays is critical for the development of chemical modulators and for drug discovery. Moreover, the adoption of existing technologies that could be utilized for SLCs is frequently hampered by lack of information on which SLCs are amenable to which assays. The aim of the review included in this section is to provide an overview of technologies and screening platforms available for SLC-oriented research and to help rationalize the suitability of individual SLCs or families to individual technologies. In this review we discuss what the general characteristics of assays are, the features of individual SLCs that are important for choosing an assay, and some of the most important assay technologies. The assays are divided based on their principles into transport, functional, binding, and phenotypic assays.

The author of this thesis conceptualized and wrote most of this review together with his supervisor and with input from Tabea Wiedmer and Alvaro Ingles-Prieto. The rest of the co-authors contributed by writing subsections about individual assay technologies, that were then assembled and edited by the author of this thesis with input from Tabea Wiedmer and Alvaro Ingles-Prieto. Daniela Digles performed the data mining in figure 12, contributed to writing of the discussion and provided feedback throughout the writing. Data for figure 12D were provided by Claire M. Steppan. Figures 1 and 12 were made by the author of this thesis, all the other figures were made by Alvaro Ingles-Prieto. The rights to include a full reprint of this review in this thesis are retained with the authors.

frontiers in Pharmacology

REVIEW
published: 10 August 2021
doi: 10.3389/fphar.2021.722889

An Overview of Cell-Based Assay Platforms for the Solute Carrier Family of Transporters

*Vojtech Dvorak[1], Tabea Wiedmer[1], Alvaro Ingles-Prieto[1], Patrick Altermatt[2], Helena Batoulis[3], Felix Bärenz[4], Eckhard Bender[3], Daniela Digles[5], Franz Dürrenberger[2], Laura H. Heitman[6], Adriaan P. IJzerman[6], Douglas B. Kell[7,8], Stefanie Kickinger[5], Daniel Körzö[5], Philipp Leippe[9], Thomas Licher[4], Vania Manolova[2], Riccardo Rizzetto[10], Francesca Sassone[10], Lia Scarabottolo[10], Avner Schlessinger[11], Vanessa Schneider[5], Hubert J. Sijben[6], Anna-Lena Steck[2], Hanna Sundström[2], Sara Tremolada[10], Maria Wilhelm[2], Marina Wright Muelas[7], Diana Zindel[3], Claire M. Steppan[12] and Giulio Superti-Furga[1,13]**

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria, [2]Vifor (International), St. Gallen, Switzerland, [3]Drug Discovery Sciences–Lead Discovery, Bayer Pharmaceuticals, Wuppertal, Germany, [4]Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany, [5]Department of Pharmaceutical Sciences, University of Vienna, Vienna, Austria, [6]Division of Drug Discovery and Safety, LACDR, Leiden University, Leiden, Netherlands, [7]Department of Biochemistry and Systems Biology, Institute of Systems, Molecular and Integrative Biology, University of Liverpool, Liverpool, United Kingdom, [8]Novo Nordisk Foundation Centre for Biosustainability, Technical University of Denmark, Kgs. Lyngby, Denmark, [9]Department of Chemical Biology, Max Planck Institute for Medical Research, Heidelberg, Germany, [10]Axxam SpA, Bresso (Milano), Italy, [11]Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [12]Pfizer Worldwide Research, Development and Medical, Groton, MA, United States, [13]Center for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria

OPEN ACCESS

Edited by:
Heike Wulff,
University of California, Davis,
United States

Reviewed by:
Yurong Lai,
Gilead, United States
William M. Pardridge,
University of California, Los Angeles,
United States

***Correspondence:**
Giulio Superti-Furga
gsuperti@cemm.oeaw.ac.at

Specialty section:
This article was submitted to Drug Metabolism and Transport, a section of the journal Frontiers in Pharmacology

Received: 10 June 2021
Accepted: 19 July 2021
Published: 10 August 2021

Citation:
Dvorak V, Wiedmer T, Ingles-Prieto A, Altermatt P, Batoulis H, Bärenz F, Bender E, Digles D, Dürrenberger F, Heitman LH, IJzerman AP, Kell DB, Kickinger S, Körzö D, Leippe P, Licher T, Manolova V, Rizzetto R, Sassone F, Scarabottolo L, Schlessinger A, Schneider V, Sijben HJ, Steck A-L, Sundström H, Tremolada S, Wilhelm M, Wright Muelas M, Zindel D, Steppan CM and Superti-Furga G (2021) An Overview of Cell-Based Assay Platforms for the Solute Carrier Family of Transporters. Front. Pharmacol. 12:722889. doi: 10.3389/fphar.2021.722889

The solute carrier (SLC) superfamily represents the biggest family of transporters with important roles in health and disease. Despite being attractive and druggable targets, the majority of SLCs remains understudied. One major hurdle in research on SLCs is the lack of tools, such as cell-based assays to investigate their biological role and for drug discovery. Another challenge is the disperse and anecdotal information on assay strategies that are suitable for SLCs. This review provides a comprehensive overview of state-of-the-art cellular assay technologies for SLC research and discusses relevant SLC characteristics enabling the choice of an optimal assay technology. The Innovative Medicines Initiative consortium RESOLUTE intends to accelerate research on SLCs by providing the scientific community with high-quality reagents, assay technologies and data sets, and to ultimately unlock SLCs for drug discovery.

Keywords: solute carrier, cell-based assay, drug discovery, chemical screening, transporters, SLC

INTRODUCTION

Cells need to tightly control the chemical exchange between the intracellular and extracellular environment to maintain homeostasis, cellular integrity and safeguarding identity. Around 10% of the human genome encodes for proteins dedicated to the transport of molecules across cellular membranes, such as ATP-binding cassette transporters (ABC), ATPases, ion channels and solute carriers (SLCs) (Hediger et al., 2013). SLCs represent the second biggest group of membrane proteins and the biggest group of transporters (Höglund et al., 2011). Currently the SLC group or, better, supergroup or superfamily, as it includes proteins with different folds and phylogenetic origin, counts more than 450 members. Membership is based on either sequence or functional similarity

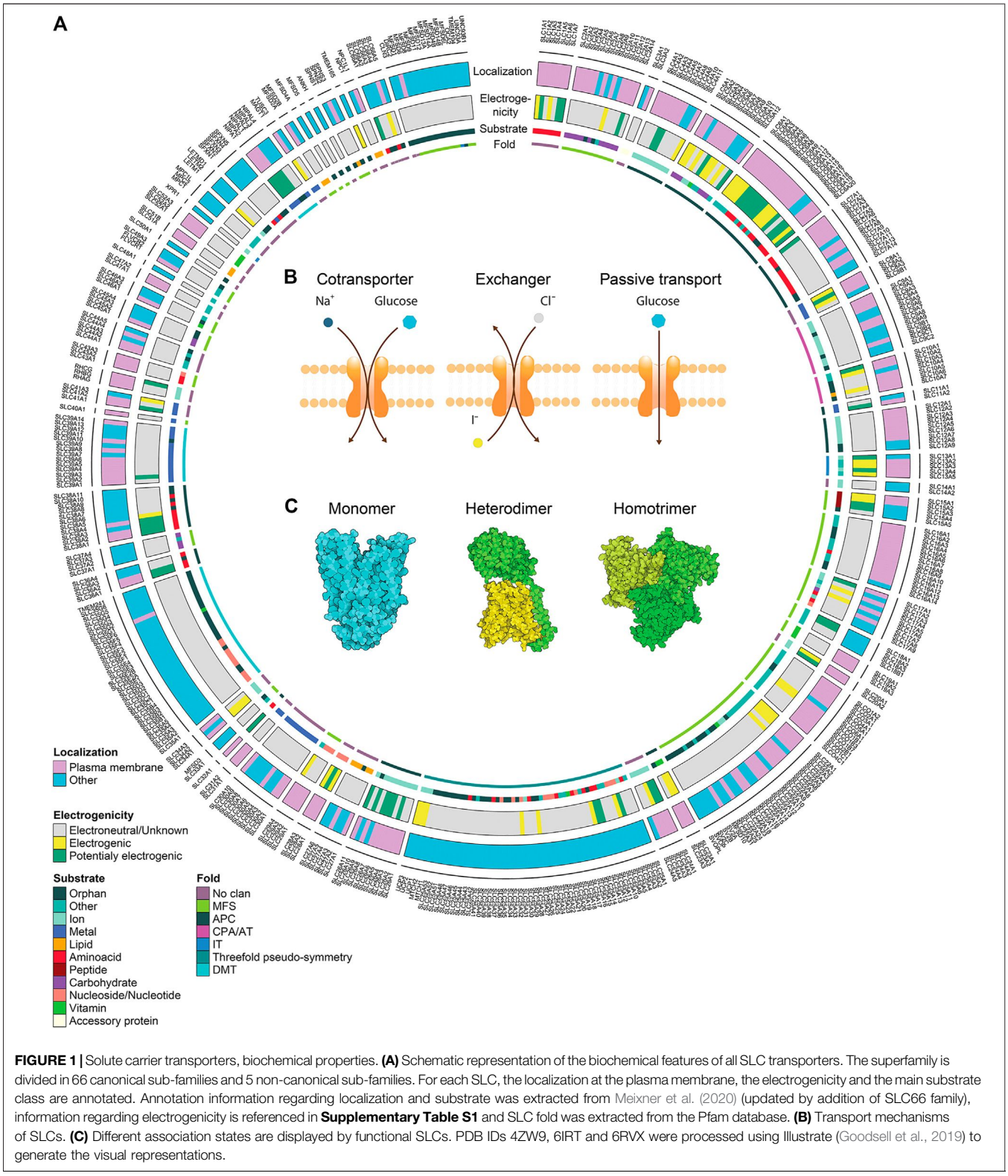


FIGURE 1 | Solute carrier transporters, biochemical properties. **(A)** Schematic representation of the biochemical features of all SLC transporters. The superfamily is divided in 66 canonical sub-families and 5 non-canonical sub-families. For each SLC, the localization at the plasma membrane, the electrogenicity and the main substrate class are annotated. Annotation information regarding localization and substrate was extracted from Meixner et al. (2020) (updated by addition of SLC66 family), information regarding electrogenicity is referenced in **Supplementary Table S1** and SLC fold was extracted from the Pfam database. **(B)** Transport mechanisms of SLCs. **(C)** Different association states are displayed by functional SLCs. PDB IDs 4ZW9, 6IRT and 6RVX were processed using Illustrate (Goodsell et al., 2019) to generate the visual representations.

(**Figure 1A**). SLCs are divided into 66 "classical" or canonical families and five new families or non-canonical families (Perland and Fredriksson, 2017; Gyimesi, 2020).

SLCs are responsible for the transport of a large spectrum of molecules including nutrients, metabolites, xenobiotics (such as phytochemicals), small molecule drugs and metal ions (Pizzagalli

et al., 2020). Given the character and breadth of their substrate spectrum, it is not surprising that SLCs vary in their structure, regulation and tissue expression which is tightly coupled to the metabolic state of cells (Zhu and Thompson, 2019). This entails that SLCs are not only involved in key physiological processes, such as absorption of nutrients in the gut or ion reabsorption in kidney, but also in specialized cellular tasks, like the acidification of cytoplasm (Sedlyarov et al., 2018), amino acids sensing (Rebsamen et al., 2015; Wang et al., 2015), metal sensing (Zhang C. et al., 2020), efferocytosis (Morioka et al., 2018) or regulation of cell mass (Demian et al., 2019).

In addition, increasing amount of evidence suggests that most drugs and steroid hormones may require transporters to enter cells (Dobson and Kell, 2008; Okamoto et al., 2018; Girardi et al., 2020a). Since the expression of some SLCs is restricted only to certain tissues and cell types (O'Hagan et al., 2018), it should be possible to tailor compounds to target specific populations of cells through SLC affinity. This principle is well known from PET imaging, based on the fact that cancer cells tend to upregulate glucose transporters and glycolysis and can therefore be visualized with labelled glucose. The same principle was recently used to develop a fluorescent probe for activated macrophages (Park et al., 2019). Tailoring compounds for specific SLC-mediated drug delivery is also a promising strategy for enabling drugs to cross the blood-brain barrier (Puris et al., 2020). In addition, membrane transporters may influence the pharmacokinetic profile of a drug, and mutation or downregulation of a transporter may lead to development of resistance and treatment failure (Winter et al., 2014). Since SLCs play a role in drug-drug interactions and single nucleotide polymorphisms could affect both drug pharmacokinetics and pharmacodynamics, FDA guidelines recommend to consider these factors when evaluating drug efficiency (Giacomini et al., 2010; FDA, 2020).

At least half of the SLCs are linked to human diseases, including diabetes, gout, high blood pressure, asthma, inflammatory bowel disease, chronic kidney disease, mental disorders, cancer, and a plethora of inborn errors of metabolism, highlighting their medical relevance and therapeutic significance (Giacomini et al., 2010). In addition, recent studies reported that SLCs may be involved in the regulation of different signaling pathways involved in cancer and other diseases, such as copper transporters and MAPK pathway, or zinc transport as a modulator of Notch pathway activity (Brady et al., 2014; Nolin et al., 2019). Moreover, SLCs are acting as cellular receptors for the entry of viruses (Côté et al., 2011; Sainz et al., 2012), which can be impeded with high-affinity protein binders (Passioura et al., 2018).

Due to all the mentioned reasons and the fact that SLCs are increasingly considered amenable drug targets, the interest in SLC-oriented drug discovery is rapidly increasing (Garibsingh and Schlessinger, 2019; Avram et al., 2020, 2021; Superti-Furga et al., 2020). For instance, SLCs offer diverse structural features that favor interactions with drug-like molecules as well as appropriate accessibility to drug interactions, as more than half of SLCs are localized to the plasma membrane (Meixner et al., 2020; Pizzagalli et al., 2020). This also allows targeting SLCs with larger molecules, such as high-affinity binders, antibodies and macrocycles (Wang W. W. et al., 2019).

Despite all facts mentioned above, only a small proportion of SLCs are so far targeted by drugs or chemical probes. There are three main factors hampering the development of new chemical entities able to modulate SLC activity. First, the majority of this supergroup is relatively understudied and biological functions or substrates of many SLCs remain elusive (César-Razquin et al., 2015; Meixner et al., 2020). Second, there is a lack of high-quality biological tools, specific and reliable reagents and dedicated databases. Lastly, the number of functional assays required to study such a diverse group of targets is still limited.

To address the state of the art regarding this last point, we here provide an overview of the cell-based assay technologies currently available for SLC-focused research.

Why focus on cell-based assays and not include *in vitro* assays? For both practical and discovery strategy reasons. Practical as this overview is already sizeable as is. Strategic as we are convinced that cellular assays are better suited primary assays for proteins that are difficult to express recombinantly and purify. We consider the proper folding, natural embedding in a lipid bilayer of physiological complexity, proper cellular glycosylation pattern together with other post-translational modifications, and, most importantly, the natural repertoire and concentration of protein interaction partners, all as parameters of great importance for assessing the chemical engagement of SLC transporters. It is reasonable to assume that these parameters critically contribute to the specificity of action of individual SLCs. It is only recently that it has become possible to engineer human cells with an ease, precision and scale that has not been hitherto considered feasible (Xie and Fussenegger, 2018). Many of the assays considered in this review have been empowered by cell engineering technologies. Therefore, this review does not include assays involving recombinant, purified proteins and that are, essentially, biophysical. The review should rather serve as a guide and a starting point for choosing assay systems for anybody considering a chemical screen on SLCs or interested in studying SLC function in intact cells or at least with SLCs embedded in a cell-derived natural environment.

Assay technologies presented here are applied and developed further in the RESOLUTE consortium, a public-private partnership funded by the Innovative Medicines Initiative (IMI) of the European Union. RESOLUTE aims at empowering the research community with open-access reagents and data to unlock the SLC family for drug discovery. One of the main goals of RESOLUTE is to systematically assess the suitability of transport assay technologies for individual SLCs and develop them further (Superti-Furga et al., 2020). We are expecting to update this review with experience gained throughout the project.

Choose Wisely: Biophysical and Biochemical Profile of Solute Carriers

SLC family members are diverse in many aspects and it is important to carefully consider features of both the SLC under study and the assay platform. This brief overview of general SLC features should act as a rationale for choosing the best-suited assay platform.

Transport Type

In contrast to active transporters using ATP as a source of energy, such as ABC transporters or P-Type ATPases, SLCs are transporting their substrates either in 1) facilitative mode, or 2) secondary active mode (Hediger et al., 2013) (**Figure 1B**). Facilitative transport is moving compounds along their own gradient, similar to ion channels. Compared to ion channels, SLCs are working in an alternating access mechanism, meaning that the SLC is actively moving its gate with a fixed stoichiometry per transport cycle, and thus SLCs have a transport rate that is several orders of magnitude smaller (Hediger et al., 2013).

Secondary active transport typically couples the movement of two different molecules. Since concentration gradients across membranes are a vital feature of cells, many SLCs take advantage of such gradients to couple the transport of different molecules. While one molecule moves along its gradient, the energy can be used to power the transport of another molecule against its gradient. Depending on the transport direction of both molecules, the SLC is either a symporter, i.e. molecules follow the same direction, or antiporter, i.e. molecules move in the opposite direction (**Figure 1B**). The transport rate may be proportional to the gradient of the coupled molecule. Secondary active transport is most frequently coupled to ions, mainly Na^+, Cl^-, K^+ or H^+ (Bai et al., 2018; Meixner et al., 2020), but other molecules may be coupled as well, for example SLC7A11 is exchanging glutamate for cysteine. This gives the possibility to assess changes in concentrations of coupled molecules as a surrogate of transport.

Solute Carrier Structural Features

Visualization of the SLCs' structure is critical for describing their transport mechanisms and molecular function. Over the past years, multiple structures of human SLCs and their homologs have been determined (Garibsingh and Schlessinger, 2019). Some SLC families have unique structures that are unrelated in evolution to structures from other SLC families (e.g. SLC1), while some SLC families are related in structure and fold (Schlessinger et al., 2010) (**Figure 1A**). For example, the members of the SLC7 (e.g. SLC7A5/LAT1 (Yan et al., 2019)) and SLC6 (SERT (Coleman et al., 2019)) families adopt a LeuT fold, while members of SLC2 (SLC2A1/GLUT1 (Deng et al., 2014)) and SLC16 (SLC16A7/MCT2 (Zhang B. et al., 2020)) display the MFS fold. SLCs are dynamic proteins that adopt different conformations during transport. Structural description of the transport mechanism experimentally or computationally is critical for the rational design of small molecule ligands (i.e., inhibitors, substrates, and activators). Many SLC members use an "alternating transport" mechanism, in which substrates are transported across the membrane as the protein alternates between inward-facing, occluded, and outward-facing conformations (Jardetzky, 1966). Different folds utilize different variations of this mechanisms, where commonly observed mechanisms are the "rocker switch" (e.g. SLC2), "rocking bundle" (SLC6), and "elevator" (SLC1 (Boudker and Verdon, 2010)).

Electrogenicity

Given the fact that many molecules transported by SLCs are charged, the transport cycle may result in charge displacement across the membrane. For example, SLC4A4 cotransports Na^+ and HCO_3^-, typically in stoichiometry 1:2, and each transport cycle results in an additional intracellular negative charge. Similar observations with many other transporters open the possibility to use functional assays based on changes in membrane potential, such as electrophysiology or voltage-sensitive dyes. To the best of our knowledge, no systematic collection of the electrogenic properties of SLCs is available. We therefore collected this specific property from the literature focusing on literature for human SLCs, but also reporting data from other mammalian studies if no evidence for human SLCs was found. The literature research was based on the reviews collected in the Bioparadigm SLC tables (www.bioparadigms.org) and the original literature referenced in there. For the remaining SLCs, additional literature was collected with a special focus on SLCs that were potentially electrogenic according to their transport reaction as described in the IUPHAR/BPS Guide to Pharmacology (Armstrong et al., 2020) or the Gene Ontology (Ashburner et al., 2000; Carbon et al., 2021), or according to the description in Uniprot (Bateman et al., 2021). In total we found evidence of electrogenicity for 115 mammalian SLCs from 35 families (**Figure 1A**, **Supplementary Table S1**), corresponding to around 25% of all human SLCs. While for some SLCs the evidence of electrogenic transport – and amenability to assays based on this principle – is sufficient, for many SLCs there are no studies investigating this property, and thus the number of electrogenic SLCs is rather underestimated.

Redundancy

As already indicated, many SLCs are widely expressed throughout the body, while expression of other SLCs is restricted to only a few cell types (O'Hagan et al., 2018). Additionally, SLCs may have multiple isoforms, which may associate with specific cell types. These isoforms normally differ in their C- or N- termini, which may result in different protein-protein interactions (PPIs), transport efficiency, transport stoichiometry, or localization (McAlear et al., 2006; Shirakabe et al., 2006; Mazurek et al., 2010; Yoo et al., 2020).

Conversely, redundancy is also found among the substrates, as many substrates are transported by more than one SLC. For example, some 60 SLCs are thought to be competent for the transport of the 21 proteogenic amino acids (Kandasamy et al., 2018), of which approximately half is capable of shuttling glutamine (Meixner et al., 2020). As typical cell lines express around 200 different SLCs (César-Razquin et al., 2018; O'Hagan et al., 2018), more than one SLC may be potentially able to transport a particular substrate in any given cell, irrespectively of the actual subcellular localization, state of activity or actual transport rate. At the same time, while some SLCs can transport a wide range of substrates, other SLCs are specific only for one substrate. Redundancy can thus be a very challenging aspect when assaying SLCs in cellular systems. However, the cellular system can be skewed to reduce the redundancy, either by comparing several cell lines with different SLC expression profiles or by genetically alternating the levels of expression. Genetic manipulations may however introduce transcriptional and metabolic adaptations and thus potentially muddle cause and consequence when assessing individual SLCs.

Such effects may be larger, the longer the cell can adapt to the genetic perturbation. Hence short-term perturbations, such as inducible systems, selective inhibitors or targeted protein degradation, may be advantageous (Bensimon et al., 2020; Wu et al., 2020). Short term perturbations may be also used as a control to set up the assay since the availability of selective inhibitors for SLCs is limited. Alternatively, the wild-type SLC can be compared with the SLC bearing a transport-deficient mutation.

Localization

Cellular localization of a particular SLC is a crucial consideration for assay choice. Some SLCs transport molecules only across the membranes of intracellular compartments, like the SLC25 family expressed on the mitochondrial membranes, while others are not restricted to only one organelle, such as SLCs with multiple isoforms, which are expressed in different organelles (Mazurek et al., 2010; Yoo et al., 2020). Annotation of SLC localization based on literature search revealed that around half of the SLCs are localized at least partially to the plasma membrane (Meixner et al., 2020) (**Figure 1A**). Since many assay technologies measure changes in substrates at the whole-cell level, special attention should be devoted to the choice of an assay for an intracellularly localized SLC. This limitation can be overcome by artificially redirecting intracellular SLCs to the plasma membrane (Lisinski et al., 2001; Forbes and Gros, 2003; Wang Y. et al., 2019). However, redirection will also alter parameters, such as local ion gradients. Thus, assays that are compatible with the intracellular localization, such as for example assays based on genetically encoded sensors, or fluorescent substrates, are generally preferable. Alternatively, some of the intracellular SLCs can be assayed in permeabilized cells (Kuznetsov et al., 2008), or organelles isolated using techniques such as LysoIP (Abu-Remaileh et al., 2017) or MitoIP (Chen et al., 2017). This approach was recently used to characterize SLC localized in melanosomes (Adelmann et al., 2020).

Regulators/Modulators of Solute Carrier Function and Localization

To function properly, many SLCs require chaperones, oligomerization or interaction with other proteins, which may regulate their function in several ways. Protein-protein interactions (PPIs) play an important role in subcellular localization. While localization of some SLCs is determined by a signal peptide, other SLCs require more extensive interactions for trafficking. For instance, members of SLC16 family require chaperone proteins basigin (CD147) or embigin (gp70) for translocation to the plasma membrane (Felmlee et al., 2020). Other SLCs may be restricted to vesicles, and only after a secondary signal will be translocated to the plasma membrane, such as the insulin responsive glucose transporter SLC2A4 (Jaldin-Fincati et al., 2017).

Some SLCs necessitate oligomerization for functioning (**Figure 1C**). Heteromerization is required, for example, for SLC families 3 and 7 (Fotiadis et al., 2013), 51 (Ballatori et al., 2013) and 54 (Herzig et al., 2012). Other SLCs form homomers, such as SLC4A4 forming homodimers (Huynh et al., 2018), or SLC1A3 forming homotrimers (Canul-Tec et al., 2017). However, the importance of homomerization for transporter function may vary. A recent study on SLC2A1 employing super-resolution microscopy suggested that SLCs may form dynamic clusters of different size with distinct transport activity (Yan et al., 2018). A similar phenomenon was observed with SLC16A7, where homodimerization increased transport activity, suggesting cooperativity between two subunits (Zhang B. et al., 2020).

PPIs are also important for modulation of SLC function. Known positive regulators include IRBIT, a regulator of SLC9A3, SLC4A4 and SLC26A6 function (Ando et al., 2014), or MAP17 regulating SLC5A2 (Coady et al., 2017). Among known negative regulators are PASCIN1 for SLC12A5 function and expression (Mahadevan et al., 2017), the ubiquitin ligase RNF5 for SLC1A5 and SLC38A2 (Jeon et al., 2015), and OS9 for ER-associated degradation of SLC12A1 (Seaayfan et al., 2016). As these interactions typically do not happen in isolation, but in the complex cellular environment where many SLCs exist in large multi-protein complexes, different interactors may affect transport functions in different ways (Haase et al., 2017; Mahadevan et al., 2017) and they may influence assay settings or production of recombinant protein for *in vitro* assays (Kost et al., 2005). At the same time, interactions can be explored for indirect pharmacological modulation of SLC function.

Post-translational modifications (PTMs), such as glycosylation, SUMOylation, phosphorylation or acetylation, regulate function and trafficking of certain SLCs (Pedersen et al., 2016; Czuba et al., 2018). Importantly, glycosylation can affect drug binding (Hoover et al., 2003). Other factors that may modulate the transport function are for example pH (Webb et al., 2016), membrane potential or binding of small molecules to intracellular non-substrate binding sites (Scalise et al., 2015; Windler et al., 2018). Additionally, SLC mediated transport can be slowed down by decreasing the temperature, which can be exploited in assay development.

Select Carefully: Assay Throughput and Chemical Space

A key factor for a novel drug discovery campaign is the selection of compounds for screening, which determines the throughput capacity required from an assay. While large chemical libraries can be successfully screened only in high throughput (HTP) assays, capable of testing millions of compounds, focused chemical libraries can be screened effectively with lower throughput (LTP). HTP assays typically implement simple protocols and their quality is primarily determined based on the Z' factor, which quantifies the assay window (Zhang et al., 1999). Importantly, HTP assays require often special instrumentation, rigorous assay optimization and follow-up secondary screening campaigns to validate the results (Walters and Namchuk, 2003). Assays with LTP may require less optimization and sometimes also provide more information (e.g. kinetics). LTP assays can be sufficient as a secondary screening assay in chemical screening campaigns, or if a transporter for a selected substrate or a drug is investigated (Yee et al., 2019). Importantly, many LTP assays can be adapted to HTP mode. An interesting compromise between

library size and the chemical space bias are fragment-based approaches, shown to be applicable to SLCs (Parker et al., 2017a). Alternatively, to reduce the number of compounds for experimental validation, large compound libraries can be pre-screened using virtual screening approaches.

Virtual screening, a computational approach, is an efficient approach to evaluate the activity of large compound libraries against a specific protein. Virtual screening can be grouped into ligand-based approaches where an algorithm is developed based on a known set of small molecule ligands, and structure-based virtual screening or molecular docking that evaluates the complementarity between small molecules and an experimentally determined SLC structure or a computational model. Ligand-based approaches have been used to identify small molecules for a range of SLC targets (reviewed in (Türková and Zdrazil, 2019)). One limitation of ligand-based approaches is the availability of known active compounds to develop predictive models.

Alternatively, molecular docking on a 3D molecular structure is commonly used to predict activity of relatively unbiased, and often massive, compound libraries, which is critical for identifying novel chemical scaffolds (Irwin and Shoichet, 2016). A combination of virtual screening and focused chemical libraries employing LTP assays might be a powerful approach to reveal promising drug candidates (Geier et al., 2013; Huard et al., 2015). However, this approach is limited to SLCs with sufficient structural information to warrant meaningful docking models. Recently it was shown that combining both ligand- and structure-based approaches can be a powerful approach to identify SLC drug interactions (Schlessinger et al., 2018) such as the case of SLC22A24 deorphanization (Yee et al., 2019).

Importantly, the choice of the chemical library, as well as screening technology depends on the availability of resources, including budget, platforms, instruments, and chemistry.

CELL-BASED ASSAY TECHNOLOGIES FOR SOLUTE CARRIER ORIENTED SCREENING

The choice of the most suitable assay is not only dictated by the characteristics of a particular SLC, but also by the goal of the screening. While many biological questions related to the role of individual transporters in biological processes can be answered only in animal models (Jiménez-Valerio et al., 2016; Pisarsky et al., 2016; Nakata et al., 2020), or with different approaches, such as genetic screens (Fauster et al., 2018; Kory et al., 2018; Sedlyarov et al., 2018; Girardi et al., 2020a), this review will focus on target-based cellular technologies for chemical screening in drug discovery campaigns or mechanistic transport studies. Most presented assays are best suited for *in vitro* applications, which may limit physiological relevance. However many assays can be applied, for example, with *ex vivo* isolated tissues or perfused organs. Since SLC function is determined also by concentration gradients, the assay system can be brought closer to physiological conditions by for example using physiological medium (Cantor, 2019; Rossiter et al., 2021).

In the next section, we outline key considerations and provide an overview of a range of assay technologies that we have successfully adopted for SLCs. Without going into experimental details, we summarize information on the principles operating within the assays, some parameters to consider and some SLC families for which the assay may be particularly suitable. The reader is referred to the literature for further information.

Assays are divided based on their assay principle (**Figure 2**, **Table 1**) (Wang W. W. et al., 2019). Cell-based substrate transport assays are more suitable to screen for SLC inhibitors or to connect the SLC to its substrate; while binding assays can identify molecules that bind to the SLC but not necessarily alter transport. These could be further developed as chemical modulators of function, as corrector, potentiator, stabilizer or degrader depending on the target SLC (Gerry and Schreiber, 2020; Lopes-Pacheco, 2020). Functional assays can uncover SLC inhibitors, as well as modulators of transporter function, and thus can be advantageous when screening for SLC activators.

Substrate Uptake Assays

The most commonly used strategy to assess SLC transport function are substrate uptake assays (Wang W. W. et al., 2019). This approach directly assesses the transport function by measuring the changing concentrations of a transported molecule extra- and intracellularly (**Figure 2**). Cellular systems are most widely used, but uptake assays can also be performed in vesicles, such as liposomes or in microinjected oocytes from *Xenopus laevis* (Nimigean, 2006).

Radioligand Uptake Assay

Radioligand uptake assays are vastly employed to study the structure and function of transporters. In general, a radiolabeled substrate is used to quantitatively study the substrate uptake across the plasma membrane into a closed compartment (e.g. whole cells, perfused organs, tissue pieces, synaptosomes, vesicles) (Sucic and Bönisch, 2016). The inhibitory potency of a ligand is probed through the competition with the radiolabeled substrate. The transporter of interest can either be endogenously expressed in a native system or heterogeneously expressed. Transient expression in diverse cell lines has become increasingly popular because different cloned transporters can be probed under the same assay conditions. Additionally, site-directed mutagenesis studies can be performed. This makes radioligand uptake assays an excellent tool to study the molecular determinants governing activity and selectivity of a compound. It is noteworthy that radioligand uptake assays measure a functional effect and the obtained activity values, typically IC_{50} values, do not directly reflect the affinity of the tested compounds.

Technical Requirements and Level of Throughput

A radiolabeled substrate, typically ^{3}H labeled, is required to perform the assays. Such radioligands are either commercially available (e.g. for the monoamine transporters SLC6A2-4

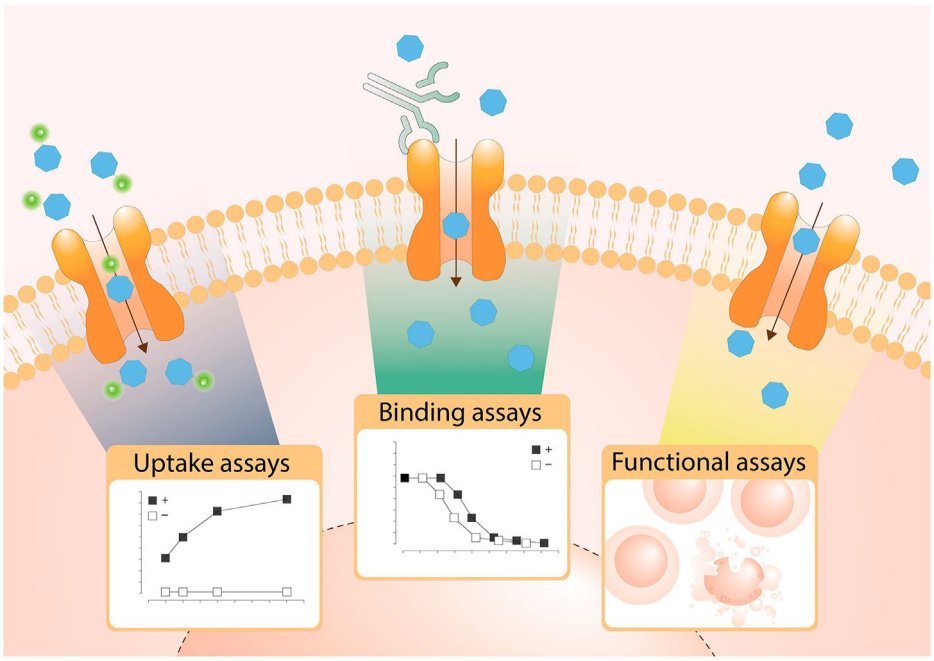


FIGURE 2 | Overview of the types of cell-based transport assays described in this review. Uptake assays directly measure the changes in the transported substrate across a cellular membrane. Binding assays report on protein stabilization upon binding of a molecule to the SLC in a cellular environment. Functional assays assess secondary effects in cells as a consequence of substrate transport.

transporters ^{3}H-norepinephrine, ^{3}H-dopamine, ^{3}H-imipramine, respectively) (Sucic and Bönisch, 2016) or they can be synthesized as demonstrated for the GABA transporter SLC6A12 and the creatine transporter SLC6A8 (Al-Khawaja et al., 2018). In order to measure the amount of radioactive substrate, which was transported inside the cells, the cells are lysed, a scintillation cocktail is added, and the plates are analyzed with a scintillation counter. Performing radioligand uptake assays requires multiple washing steps which results in LTP.

Experimental Setup

Sucic and Bönisch have described in detail how to perform radioligand uptake assays with special focus on neurotransmitter transporters (Sucic and Bönisch, 2016). On the day prior to the uptake experiment, the cells are plated into multiple well plates, which were precoated with Poly-D-Lysine, to ensure attachment of the cells to the plate. On the day of the uptake assay, the wells are washed multiple times with buffer and are then incubated with the radiolabeled substrate together with different concentrations of inhibitors. Additionally, positive and negative controls are performed by incubating wells with a high concentration of the radioligand to measure maximum inhibition as well as with buffer to measure nonspecific inhibition. The uptake is stopped by multiple washing steps with ice-cold buffer. Finally, the cells are lysed, a scintillation cocktail is added, and the plate is analyzed with a scintillation counter. Data analysis is typically performed by fitting the data to a sigmoidal-dose response model by applying nonlinear-regression in order to obtain IC_{50} values. K_i values can be calculated for competitive inhibitors according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

The amount of radiolabeled substrate, the number of plated cells and the incubation time highly depend on the nature of the transporter and need to be optimized accordingly.

Suitable Solute Carrier Families

Radioligand uptake assays have been widely employed to study diverse SLC families, including: SLC1 (Garibsingh et al., 2018), SLC2 (Tripp et al., 2017), SLC6 (Borden, 1996; Núñez et al., 2000; Al-Khawaja et al., 2014, 2018; Hofmaier et al., 2014; Richter et al., 2019), SLC7 (Chien et al., 2018), SLC10 (De Bruyn et al., 2011), SLCO (De Bruyn et al., 2011), SLC13 (Colas et al., 2017), or SLC22 (Erdman et al., 2006).

Assay Advantages, Limitations and Approximate Costs

The advantage of radioligand uptake assays is that different transporters as well as mutants can be measured in the same assay set-up under the same conditions. A disadvantage of the assay is that only a functional effect is measured and not the actual binding affinity. For measuring binding affinities other assays such as Surface Plasmon Resonance (SPR), isothermal titration (ITC) (Rajarathnam and Rösgen, 2014) or radioligand binding assays (Sucic and Bönisch, 2016) can be utilized. Another profound shortcoming of the radioligand uptake assay is that it cannot distinguish between inhibitors and substrates. The actual costs for performing the assay depend highly on the cost of the radioligand.

TABLE 1 | Overview of assays presented in this review. Examples of intracellular SLCs are highlighted in bold.

	Assay	Special technical requirements	SLC suitability	Level of throughput	Advantages	Limitations
Substrate uptake assays	Radioligand uptake assay	Radiolabeled SLC substrate	Widely suitable (e.g. SLC1, SLC2, SLC6, SLC7, SLC10, SLCO, SLC13, SLC22)	Low	1) Versatility	1) Radioactive readout 2) Cannot distinguish inhibitors from substrates
	Fluorescent substrate uptake assay	Fluorescent SLC substrate	Widely suitable (e.g. SLC6, SLC10, SLC18, SLC27, SLCO, SLC22, SLC47, **intracellular SLCs with microscopy readout**)	High	1) Simple setup 2) Kinetics	1) Not suitable for testing of compounds with fluorescent or quenching properties
	Genetically encoded biosensors	GE biosensor	Widely suitable (e.g. SLC1, SLC2, SLC5, SLC26, SLC12, SLC16, SLC42, **SLC54**)	Medium to high (sensor and readout dependent)	1) Possibility to target the sensor to a specific subcellular compartments 2) Dynamic range and sensitivity 3) No need of cell loading with dyes 4) Temporal resolution	1) Robust expression of the sensor is required
	MS-based transport assays for metabolites or ion trace elements	Mass spectrometer (ICP-MS for ion trace elements)	Applicable to most SLC families **(intracellular SLCs upon organelle isolation)**	Low	1) Detection of multiple analytes 2) Specificity and direct measurement of substrates	1) Specialist knowledge required
Binding assays	Thermal shift assay		Widely suitable (e.g. SLC2, SLC16)	Low to medium	1) Direct protein-ligand interaction 2) Label free 3) Versatility	1) Not all ligands will shift Tm 2) Possible loss of interaction due to high T 3) Prone to false negative results
Functional assays	Fluorescent dyes	FLIPR/Hamamatsu FDSS (or similar) plate reader	Widely suitable (e.g. SLC1, SLC4, SLC6, SLC9, SLC12 SLC16)	High	1) Simple protocols 2) Flexibility 3) Good dynamic range 4) Temporal resolution	1) Loading of cells with dyes 2) High costs
	Electrophysiology	Patch clamp experimental setup	Electrogenic SLCs in plasma membrane (e.g. SLC8)	Low	1) High accuracy 2) Real-time measurement 3) Single-cell analysis	1) Limited to electrogenic SLCs 2) Small signal window
	SSM-based electrophysiology	SURFE2R	Electrogenic SLCs (e.g. SLC1, SLC8, SLC15, SLCO, **intracellular SLCs upon organellar membrane isolation**)	Low to medium	1) High accuracy 2) Real-time measurement 3) High signal amplification	1) Membrane potential cannot be applied 2) Limited usability if transporter function depends on PPI
	SLC-GPCR coupling		Limited to SLCs transporting GPCR ligands (e.g. SLC63, SLC59)	High	1) Specificity and sensitivity	1) Many steps requiring optimization and posing confounding factors 2) Risk of false positive/negative hits
	Label-free impedance-based assay	xCELLigence real-time cell analyser	Limited to SLCs transporting GPCR ligands (e.g. SLC6, SLC29)	High	1) Label-free and non-invasive 2) Real-time measurement	1) Prone to false positive/negative hits
	SLC coupling to nuclear hormone receptor		Limited to SLC transporting nuclear hormone ligands (e.g. SLC10, SLC16, SLCO, SLC22)	High	1) Unmodified SLC substrate 2) Real-time measurement	1) Redundant SLC expression may limit usability
	Phenotypic assay		Widely suitable (e.g. SLC16, **SLC25**)	High	1) Viability readout 2) High specificity in case of reciprocal interaction	1) Prior knowledge of a strong genotype-phenotype connection required

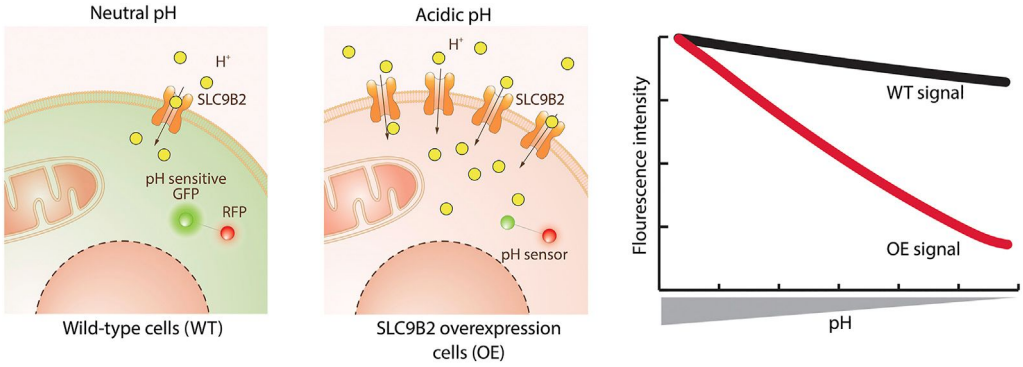


FIGURE 3 | Transport assay using a genetically encoded biosensor. The exemplified assay uses a protein sensor to detect changes in cellular pH caused by the substrate transported by the SLC. The sensor encodes a pH sensitive green fluorescent protein (GFP) linked to a red fluorescent protein (RFP – used for normalization). Wild-type (WT) cells have a neutral cytoplasmic pH where GFP is active. Upon overexpression of SLC9B2 (a proton importer) and addition of its substrate, the increased concentration of protons lowers the cytoplasmic pH. This causes the quenching of the GFP and therefore a decrease in fluorescence intensity compared to WT cells.

Fluorescent Substrate Uptake Assay

Fluorescent surrogate substrate assays rely on transport of a fluorescently labeled analogue of an SLC's natural substrate (e.g. a BODIPY-labeled fatty acid, an Alexa-labeled peptide) or a fluorescent drug or dye which acts as an alternative substrate of the SLC (Fardel et al., 2015; O'Hagan and Kell, 2020). This approach allows to monitor the activity of SLCs in cells in real time.

Technical Requirements and Level of Throughput

This assay strategy requires a fluorescently labeled substrate and a conventional fluorometric microplate reader, ideally allowing for real time monitoring of fluorescence changes within the cell. For HTP screening, a reader compatible with 384 or 1,536 well plates and with integrated robotic handling systems is advantageous. Alternatively, cells can be analyzed by microscopy on single slides or in multi titer plates by high content imaging to visualize the distribution of the fluorescent substrate within intracellular compartments.

Experimental Setup

A cell line overexpressing the SLC of interest in an inducible or constitutive manner is generated for comparing SLC-mediated and unspecific substrate uptake in the same cellular background. To run the assay, the growth medium is removed, and cells are incubated with transport buffer. In case of sym- or antiporters, this buffer should contain relevant ions which are co-transported along with the fluorescent substrate. In addition, a cell-impermeable quenching agent can be added to the buffer to eliminate extracellular fluorescence and thus enhance the signal-to-noise ratio (Wemhöner et al., 2006; Zhou et al., 2010). Finally, the fluorescent substrate is added, and its uptake is monitored for several minutes. In presence of a quencher, the assay can be run in a homogenous format and uptake can be monitored continuously. Without addition of a quencher, washing steps with transport buffer need to be included after substrate addition to remove the remaining fluorescent substrate from the extracellular space.

For assay optimization, cell clones are selected based on the signal-to-background ratio of fluorescent substrate uptake and – if available – the observed activity of known potent and selective tool compounds. Further clone selection criteria include qPCR and western blotting to quantitate protein expression levels. To find optimal assay conditions, the K_m of the fluorescent surrogate substrate is estimated (Wittwer et al., 2013), and competition experiments with the unlabeled physiological substrate can be performed.

Suitable Solute Carrier Families

Fluorescent surrogate substrate uptake assays are used broadly and have successfully been applied to various SLC families such as SLC6 (Zwartsen et al., 2017), SLC10 (Mita et al., 2006), SLC18 (Hu et al., 2013), SLC27 (Sandoval et al., 2010; Zhou et al., 2010), SLCO and SLC22 (Fardel et al., 2015), and SLC47 (Yasujima et al., 2010; Fardel et al., 2015)

Assay Advantages, Limitations and Approximate Costs

Fluorescent surrogate substrates offer the advantage of performing a rapid, simple and homogenous assay without washing steps, if performed in presence of a quenching agent. Thus, fluorescent surrogate substrate assays are amenable for HTP screening aiming for rapid characterization of lead compounds and can replace more laborious and cost intensive approaches like using radiolabeled substrates or isolation and fractionation of natural substrates. Furthermore, the activity of SLCs can be monitored in real time and cellular process such as trafficking, sequestration or compartmentalization of fluorescent solutes can be visualized. The limitations of this strategy include the need to identify a fluorescent surrogate substrate, which is likely not feasible for every SLC. Also, compounds which are autofluorescent or fluorescence quenchers can interfere with the readout. The costs of the assay largely depend on the costs of the surrogate substrate.

Genetically Encoded Biosensors

Genetically encoded fluorescent biosensors are proteins that bind an analyte or sense a physical property and translate its concentration into a change in fluorescence, either intensiometric or ratiometric. Beyond the well-known calcium- and voltage-indicators such as the GCaMP (Dana et al., 2019) and ASAP (Villette et al., 2019) series, biosensors for a diverse array of cellular analytes now exist (Greenwald et al., 2018). Since biosensors can be specifically targeted to cellular compartments by appropriate targeting motifs, they hold promise to measure intracellular transport. Biosensors measuring ions such as Ca^{2+}, Cl^- or H^+, as for example GCaMP (Nakai et al., 2001), SuperClomeleon (Kuner and Augustine, 2000; Zhong et al., 2014) and pHluorins (Miesenböck et al., 1998), are especially suitable for the assessment of SLC transport, either by detection of the primary substrate or coupled ions (**Figure 3**).

Technical Requirements and Level of Throughput

A cell line co-expressing the SLC of interest together with a biosensor for the transported substrate is required. The change in fluorescence can be detected by microscopy, flow cytometry or using a plate reader. Ideally, instruments are equipped with perfusion or injection modules to enable a time-resolved study of transport. Plate-based measurements of biosensors are generally applicable to HTP screening by the use of plate readers such as FLIPR or Hamamatsu FDSS that can accommodate 384-well plates format.

Experimental Setup

For a successful assay, the cell line should be optimized for homogenous and stable expression levels of both SLC and biosensor, as both will influence the dynamic range. The gene coding for the biosensor is always introduced exogenously and its expression should be examined for correct subcellular targeting and absence of overexpression or folding artifacts. Before the experiment, cells can be starved or treated with drugs to deplete intracellular levels of transporter substrate. Next, cells are incubated in an appropriate assay buffer containing test compounds (e.g. drug candidates). In the case of intensiometric biosensors, a first measurement needs to be performed for normalization. Then, the substrate is added, and the resulting fluorescence change is either recorded immediately to measure kinetics of the transport reaction or with a time-delay to measure the steady-state level. Fitting the concentration of the externally supplied substrate against the fluorescence change results in an apparent $K_{0.5}$, or IC_{50}, representing the combination of biosensor affinity, transporter properties and metabolic conversion of the substrate. Alternatively, the substrate concentration can be held constant while varying the test compound concentration for IC_{50} determinations. As an additional benefit of ratiometric biosensors, the fluorescence change can be converted into absolute intracellular concentrations with the requirements of careful calibration and ratio-processing (Hou et al., 2011; Pomorski et al., 2013).

Suitable Solute Carrier Families

Biosensors can be widely applied to study SLCs which transport ions or metabolites detectable by a biosensor (Sanford and Palmer, 2017). Biosensors were successfully employed in a number of assays for glucose transporters from families SLC2 and SLC5 (Takanaga and Frommer, 2010; Keller et al., 2019), chloride transport mediated by the SLC26 family (Galietta et al., 2001; Zhong et al., 2014), iodide transported by the SLC12 family (Valdez-Flores et al., 2016), glutamine transported by the SLC1 family (Gruenwald et al., 2012), pyruvate transported by the SLC54 family (Arce-Molina et al., 2020), pyruvate and lactate transported by the SLC16 family (Contreras-Baeza et al., 2019) and ammonium ions transported by yeast homologues of SLC42 family (Ast et al., 2017).

Assay Advantages, Limitations and Approximate Costs

Biosensors can directly measure the concentration of the substrate, offer temporal resolution and can be targeted to cellular compartments. Biosensors overcome the need of cell loading with chemical organic dyes, potentially affecting cell physiology. Biosensor-based assays are inexpensive and essentially have the costs of running a cell culture. A practical disadvantage can be the requirement of the expression of two genes, transporter and biosensor. The main limitation is the availability of biosensors, most of which have been developed to study signaling events and not for transport measurements. However, these can be optimized or repurposed for transporter assays. For instance, a popular class of biosensors is expressed on the plasma membrane for measuring release of neurotransmitters, such as glutamate (Marvin et al., 2013), GABA (Marvin et al., 2019), glycine (Zhang et al., 2018), dopamine (Patriarchi et al., 2018; Sun et al., 2018), norepinephrine (Feng et al., 2019), acetylcholine (Jing et al., 2018), and serotonin (Wan et al., 2020). These biosensors could be co-expressed with SLCs transporting their ligand, similar to coupling of SLC transport to G protein coupled receptor (GPCR) downstream signaling (Vlachodimou et al., 2019). After ligand application and SLC-dependent transport into cells, the reduction of extracellular ligand concentration will decrease the biosensor's apparent affinity. In fact, pharmacological inhibition of SLCs involved in the clearance of glutamate were measurable with biosensors and support this assay strategy (Armbruster et al., 2016, 2019; Pinky et al., 2018; Wan et al., 2020).

Mass Spectrometry-Based Transport Assay

Mass spectrometry (MS) is an analytical technique that measures the mass to charge ratio (m/z) of molecules present in samples. These measurements are used to calculate the exact molecular weight of components and thus identify and quantify the compounds in the sample. This technique is widely used in metabolomics, the study of low molecular weight molecules that take part in metabolic reactions required for maintenance, growth and function of cells (Oliver et al., 1998). Metabolomics analysis by MS is a powerful tool to determine transporter substrates by measuring the uptake or excretion of small molecule compounds by cells (**Figure 4**).

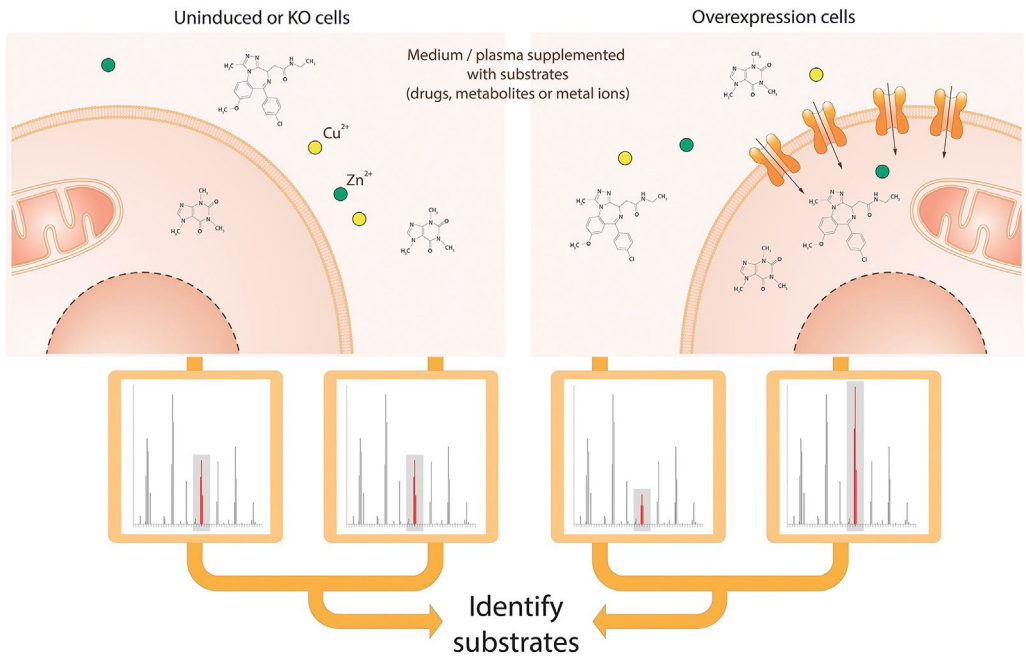


FIGURE 4 | Schematic view of the MS-based transport assay for SLCs. Cells are incubated in medium or plasma containing a mix of metabolites, drugs and ions. After incubation, medium and/or intracellular fractions are extracted and prepared for MS analysis, followed by alignment and identification of molecules or ions. Both the comparison of identified molecules or ions in cellular extracts and medium as well as the comparison of cells with the SLC of interest knocked-out and overexpressed enable the identification of the metabolites, drugs or ions that are transported by the SLC of interest.

Technical Requirements and Level of Throughput

The assay requires incubation of cells or organelles in an appropriate medium for a short period of time. Comparison of the uptake of compounds by genetically engineered cell lines with SLC knock-out or overexpression (Gründemann et al., 2005), as well as the manipulation of uptake medium conditions (e.g. pH, addition of inhibitors or other compounds for competition (Dickens et al., 2018)) can facilitate the identification of transporter substrates. Subsequently samples must be appropriately prepared for mass spectrometric analysis (Dunn et al., 2011; Vuckovic, 2012).

Cajka and Fiehn provide an excellent review of the various MS technologies available for metabolomics, along with advantages and limitations (Cajka and Fiehn, 2016). Untargeted MS following methodologies and guidelines in (Brown et al., 2005; Broadhurst and Kell, 2007; Dunn et al., 2011; Mullard et al., 2015; Broadhurst et al., 2018) enable the measurement of differences in the uptake of a broad range and number (thousands) of compounds by cell lines (Wright Muelas et al., 2020). Targeted MS can alternatively be used, limiting the number of compounds measured (typically <200) but at the same time enabling absolute quantification. Throughput depends on the approach and instrumentation used.

Experimental Setup

The following steps describe the preparation of intra- and extracellular samples for MS analysis over a time course (Wright Muelas et al., 2020). Following incubation of cells in uptake medium, spent medium is collected after centrifugation, followed by extraction using methanol. The remaining cell pellet is washed, followed by quenching and extraction of intracellular metabolites using methanol. The spent medium and intracellular extracts are subsequently lyophilised, and reconstituted in water ready for analysis by LC-HRMS/MS.

Suitable Solute Carrier Families

Mass spectrometry analysis of transporter substrates is applicable to most SLC families.

Assay Advantages, Limitations and Approximate Costs

Advantages of the assay are that sampling over a period of time enables transport kinetics to be measured. Untargeted metabolomics allows measurement of relative changes in a wide range of compounds, known and unknown, potentially leading to novel substrate identification. Whilst fewer compounds can be reliably measured using targeted techniques, these enable quantification of the changes in specific compounds to be measured. A disadvantage with both methods is the requirement for expensive instrumentation with specialist knowledge and skills required to run and maintain, along with complex data processing and analysis. However, these disadvantages are outweighed by the wealth of information provided by these assays. This experimental set up is particularly well suited to SLCs expressed at the plasma membrane. Similar approaches have been reported for SLCs localized in cellular organelles such as lysosomes and mitochondria, but require additional cell line engineering to

enable the pulldown approach ensuring a quick and efficient organelle isolation (Abu-Remaileh et al., 2017; Chen et al., 2017).

Mass Spectrometry-Based Analysis of Intracellular Ionic Trace Elements

Trace elements in their ionic form mediate biochemical reactions in human cells by acting as enzyme cofactors or centers for stabilizing protein structures. Deficit or accumulation of these substances lead to cell toxicity and severe diseases in humans and therefore, intracellular trace ion concentrations (i.e. the "ionome") must be tightly controlled. Inductively coupled plasma mass spectrometry (ICP-MS) is the most sensitive method able to determine and quantify the human "ionome" by detecting isotopes at a very low concentration. The analytical technique is widely used in the pharmaceutical industry to detect and quantify elemental impurities. However, in recent studies, ICP-MS also enables to profile trace elements in mammalian cells (Malinouski et al., 2014; Tsai et al., 2014; Konz et al., 2017). Using cell lines with a genetically deleted or artificially overexpressed SLC allows a systematic identification of SLCs involved in the transport of ions (**Figure 4**).

Technical Requirements and Level of Throughput

Ionomics assays use mammalian cells overexpressing or bearing knock-out genes encoding particular transporters to quantify the change of inorganic ions by ICP-MS upon cell lysis. ICP-MS-based ionomics is rather a LTP assay, as a significant volume of sample is required.

Experimental Setup

To analyze the amount of intracellular ions present in the sample (i.e. ^{23}Na, ^{24}Mg, ^{31}P, ^{32}S, ^{39}K, ^{44}Ca, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{97}Mo), HEK293 cells stably expressing SLC transporters under the control of a doxycycline inducible promoter are grown in standard cell culture medium, naturally containing a selection of ions and metals. Transporter expression is induced by overnight addition of doxycycline. The next day cells are thoroughly washed with an isotonic Tris/choline-chloride based wash buffer, to completely remove all extracellular ions and subsequently lysed with a Tris/choline-chloride/Triton X-100 based lysis buffer, not containing any of the measured ions. The sample is then ionized by the inductively coupled plasma and the ions are transferred to the mass spectrometer, where they are separated based on their mass-to-charge ratio (m/z). The detector receives a signal proportional to the quantity of ions present in the sample. The ion intensities are normalized to either cell lysate protein concentration or intensity of ^{31}P, which were shown to change linearly with the number of cells harvested. To evaluate the contribution of a particular SLCs in the transport of inorganic ions, the normalized intensity ratios of the ions are compared between HEK293 cells with or without induced overexpression of a particular SLC.

Due to the sensitivity of ICP-MS, an exhaustive optimization of washing steps, cell lysis, cell count normalization, ion detection and statistical analysis is required to precisely detect intracellular ion levels (Malinouski et al., 2014; Konz et al., 2017).

Suitable Solute Carrier Families

This assay format was shown to be suitable for both efflux and influx transporters of metal ions and metalloids including, but not limited to, the aforementioned ions (Malinouski et al., 2014; Konz et al., 2017). Examples of these transporters are from families SLC11, SLC30, SLC31, SLC39, and SLC40. Furthermore, we speculate that SLCs for which the main substrate transport is driven by metal ions detectable with ICP-MS may also be amenable to this assay technology.

Assay Advantages, Limitations and Approximate Costs

Determination of the intensities of the monoisotopic ion ensures specificity and allows direct measurement of inorganic SLC substrates. The method also allows normalization based on either protein concentration of cell lysates or amount of ^{31}P with the limitation in case of studies with phosphate transporters. Furthermore, the assay was demonstrated to be suited for SLCs located on the plasma membrane or endoplasmic reticulum. The use of this approach for smaller subcellular compartments (mitochondria, lysosomes, vesicles, etc.) has not been evaluated systematically so far, and it may require isolation of organelles to obtain ion intensities above the limit of detection. The described ionomics assay is applicable in a LTP mode (6-well plate). Another limitation of the assay is that the ICP-MS is a relatively expensive equipment and therefore not available in the vast majority of labs.

Binding Assays

Binding assays are based on assessing direct interactions between the compound and the target. These assays can be useful to find binders of SLCs, not necessarily only compounds acting as inhibitors. Such binders can function as pharmacological chaperones, potentiate or prevent PPIs (Passioura et al., 2018), or can be modified into chemical chimeras such as PROTACs (Schreiber, 2019).

Using approaches of chemical proteomics, binding assays can be focused on compound (chemical-centric) or protein (target-centric), depending on the nature of the bait. Chemical-centric methods, recently reviewed in (Robers et al., 2020), have been used for many years to deconvolute targets from phenotypic screens, or to profile off-target effects of compounds on a proteome level. Methods such as photoaffinity labelling and *Click* chemistry, or thermal proteome profiling (TPP), are capable of reporting low affinity and less abundant interactions – in principle including a drug and its transporter (Parker and Pratt, 2020). Thus, these methods may be suitable starting points to screen for the SLC responsible for transport of an investigated compound. However, MS-based proteome-wide profiling frequently exhibits a bias towards soluble proteins, and thus interactions with SLCs may be underrepresented, though examples of their use to deconvolute a SLC as a direct target of drugs exist (Parker et al., 2017b).

Target-centric binding assays are in general suitable for HTP chemical screening (Alexandrov et al., 2008; Hall et al., 2016) and especially in combination with technologies such as DNA-encoded libraries can screen very large chemical libraries (Passioura et al., 2018). However, these assays frequently require purified protein. Since protein purification for membrane proteins with several transmembrane domains is in general considered challenging (Wang W. W. et al., 2019), we focus on the cellular thermal shift assay, which has been recently optimized for SLCs and does not require purified protein (Hashimoto et al., 2018).

Thermal Shift Assay

The thermal shift assay (TSA) using cells is based on the behavior of a protein exposed to increasing temperatures (Martinez Molina et al., 2013). Upon reaching a certain temperature (melting temperature – Tm), the thermodynamic stability of the protein fold is disrupted, resulting in protein unfolding and aggregation with other unfolded proteins. Interaction of the protein with a small molecule can result in partial thermal stabilization, and thus in a shift in Tm (**Figure 5**). In this way, direct protein-ligand engagement can be assayed. In comparison to the *in vitro* TSA (Alexandrov et al., 2008; King et al., 2016; Tavoulari et al., 2019), the cellular TSA assesses protein-ligand interactions in a cellular environment and does not require purified protein. While the TSA was originally established as a method to determine a drug-target engagement, in recent years the cellular TSA is emerging also as an assay for primary screening (Shaw et al., 2019).

Technical Requirements and Level of Throughput

For performing a cellular TSA experiment, a source of heating (such as thermoblock or PCR cycler) and a readout discriminating native from aggregated protein are necessary. The choice of readout determines the assay throughput. The most widely used method with Western blotting (WB) as a readout (Martinez Molina et al., 2013) can test only limited compound-target combinations but technologies such as AlphaScreen (Almqvist et al., 2016) or split reporters can enable screening in HTP (reviewed in Henderson et al., 2019). Using MS as a readout, the method can be applied to study target engagement of a single compound on proteome level (Huber et al., 2015; Reinhard et al., 2015).

Experimental Setup

Since the method was originally introduced for cytoplasmic proteins (Jafari et al., 2014), modifications were necessary for membrane proteins (Hashimoto et al., 2018). Typically, compounds are incubated either with intact cells or cell lysates. The use of intact cells accounts for membrane crossing or metabolic modifications of the compound. Next, samples are aliquoted and exposed to heating. Lysing the cells prior to a heating step could facilitate easier aggregation of membrane proteins after melting, however some studies with membrane protein lysed the cells only after the heating step (Huber et al., 2015; McMillan et al., 2018; Kawatkar et al., 2019). To avoid resolubilization of aggregates, a mild detergent should be used (Reinhard et al., 2015). Finally, the remaining protein in native conformation is quantified in each sample. The most commonly used technique is to remove aggregates with centrifugation and quantify the native protein by WB, but readouts employing reporters can specifically distinguish native protein, and thus the removal of aggregates is not necessary (Martinez et al., 2018). Dose dependency can be confirmed via an isothermal dose-response fingerprint ($ITDRF_{CETSA}$) experiment where the sample is treated with several ligand concentrations at constant temperature (Martinez Molina et al., 2013).

Length of compound incubation, compound concentration, sample volume, cell density, heating duration, and the efficiency of native–aggregated protein discrimination should all be optimized first. If available, a potent and selective ligand, such as a specific inhibitor, can be used to determine the possible degree of Tm shift. However, similarly potent inhibitors targeting the same protein can have a different degree of Tm shift (Kawatkar et al., 2019). Although the most widely used heating duration is 3 min, longer heating duration could result in a better Z' factor and thus be beneficial for HTP screening (Martinez et al., 2018).

Suitable Solute Carrier Families

A proof-of-principle study showed thermal stabilization of members of SLC1 and SLC16 families upon treatment with available inhibitors, and in the case of SLC16 also stabilization with substrate (Hashimoto et al., 2018). Although to our knowledge only few other studies use the cellular TSA to target SLCs, namely SLC2 family (McMillan et al., 2018; Reckzeh et al., 2019), a number of studies have applied the cellular TSA for transmembrane proteins (Huber et al., 2015; Reinhard et al., 2015; Kawatkar et al., 2019) and SLCs were also detected in TPP studies (Reinhard et al., 2015), demonstrating the potential to apply the method more broadly.

Assay Advantages, Limitations and Approximate Costs

Advantages are that the TSA is probing the direct interaction of a target with a compound, its versatility and that it can use lysate, intact cells, and even whole tissue (Martinez Molina et al., 2013). However, the major limitation is that not all compounds binding the protein will shift the Tm for reasons like insufficient stabilization of the structure or loss of the interaction between protein and compound due to high, non-physiological temperatures. Thus, while the method is relatively resistant to false-positive results, false-negative results can occur. The Drug Affinity Responsive Target Stability (DARTS) assay represents an alternative assay technology similar to the TSA that can assess drug-target engagement for SLC inhibitors (Lomenick et al., 2009; Benjamin et al., 2018). The costs of running a LTP cellular TSA are basically equal to the costs of running a WB experiment, while costs of a HTP TSA depend on the readout and thus on reagents.

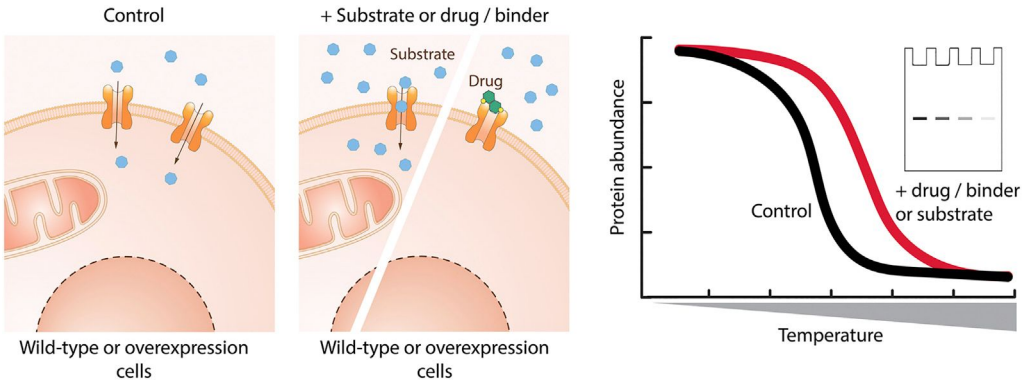


FIGURE 5 | Cellular binding assay based on thermal shift. Cells are incubated with the molecule of interest, lysed, and exposed to increasing temperature. The remaining protein in native conformation is quantified by western blotting or using reporters. Binding of a small molecule stabilizes the protein of interest and leads to a shift in the melting temperature of the protein of interest.

Functional Assays

In contrast to substrate uptake assays, functional assays do not assess the transporter activity directly, but are measuring the secondary effects caused by SLC driven transport, such as changes in membrane potential or intracellular pH. While employing functional assays to poorly characterized SLCs may be challenging, implementation for SLCs which are sufficiently characterized may be relatively easy, and many of these assays can be also easily optimized for HTP. Functional assays should be followed-up with a counter-screening campaign, to confirm that the primary screening hits are truly connected to SLC mediated transport.

Fluorescent Dyes

A number of functional assays is based on fluorescent dyes, which are either sensitive to changes in membrane potential or in ion concentrations (Yu et al., 2015). Membrane potential sensitive dyes measure changes of charges across the cell membrane. FLIPR membrane potential dye (Molecular Devices) is a lipophilic, anionic, bis-oxonol dye able to cross the plasma membrane and to measure voltage changes by its potential-dependent accumulation and redistribution (Wolff et al., 2003) (**Figure 6**). When the cells are depolarized, the dye enters the cells, causing an increase in fluorescent signal, conversely, cell hyperpolarization results in dye exit and decreased fluorescence. Ion sensitive dyes measure changes in the concentration of a specific ion, such as calcium, sodium, potassium or changes in pH. Several calcium-sensitive dyes are available, with different calcium affinities and different excitation/emission spectra. Among these, Fluo-8 dyes were developed to improve other dyes (e.g. Fluo-3, Fluo-4) in terms of loading and brightness. Among pH sensitive dyes, the most used is 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM), a non-charged indicator that rapidly diffuses inside the cell, where intracellular esterases cleave the ester bond releasing BCECF, which fluoresces according to the intracellular pH (Ozkan and Mutharasan, 2002; Benjamin et al., 2005). Sodium sensitive dyes are used to detect changes in Na^+ concentrations. Two of the most frequently used are Asante Natrium Green and CoroNa (Iamshanova et al., 2016). In contrast to Ca^{2+} and pH sensitive dyes, Na^+ sensitive dyes are not well-suited for HTP screening due to low sensitivity and a poor signal-to-background ratio for SLC targets (Yu et al., 2015). Potassium transport is frequently studied though exploiting thallium as a surrogate ion for potassium. Some examples are the FLIPR Potassium Assay kit (Molecular Devices) and the FluxOR Potassium Ion Channel Assay (Thermo Fisher). The increase in cytosolic thallium is detected using the thallium-sensitive dye indicator (Weaver et al., 2004).

Technical Requirements and Level of Throughput

Detection of fluorescent indicator dyes is achieved by means of a fluorescence plate reader (such as Fluorescent Imaging Plate Reader (FLIPR) or Hamamatsu FDSS) able to excite the probe and to read its emission. This instrumentation together with the characteristics of the dyes enable the HTP of the assay. Alternatively, fluorescence can be assessed by microscopy or flow cytometry.

Experimental Setup

The cell line with the expression of the SLC of interest together with an adequate control cell line (mock control that does not express the target, not-induced if the target is overexpressed with an inducible system or knock-out cell line) is loaded with the fluorescent dye in a suitable buffer for a period that typically ranges from 10 min to 1 h. Then, the solution is exchanged to an appropriate assay buffer containing test compounds (e.g. drug candidates). Next the buffer containing a transporter substrate is added and changes in fluorescence are measured. By fitting the concentration of the tested compound against the changes in fluorescence, the EC_{50} value is obtained.

Suitable Solute Carrier Families

The membrane potential dye has been used to study electrogenic transporters from families SLC1 (Jensen and Bräuner-Osborne, 2004) and SLC6 (Benjamin et al., 2005;

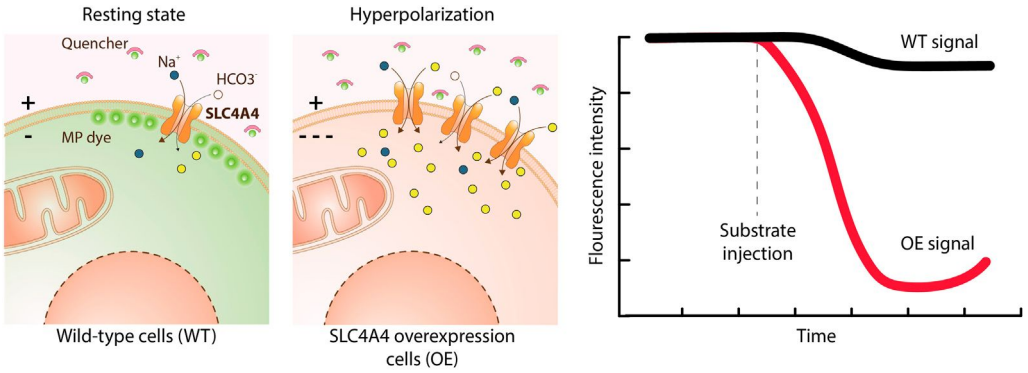


FIGURE 6 | Transport assay using a membrane potential dye. This assay uses a chemical dye to detect changes in the membrane potential (MP) caused by the ions transported by an electrogenic SLC. The dye coupled to a quencher is added to the medium. In the resting state, some dye enters the cell causing a fluorescent intensity that serves as a reference. Upon membrane hyperpolarization the dye does not penetrate in the cells and remains attached to the quencher, resulting in a fluorescence decrease. Upon depolarization of the membrane the dye detaches from the quencher and penetrates into the cells, eliciting a signal increase. Overexpression of SLC4A4 (a 1:Na^+/3:HCO^{3-} co-transporter) and addition of its substrates leads to hyperpolarization and a decrease in fluorescence intensity over time compared to wild-type cells.

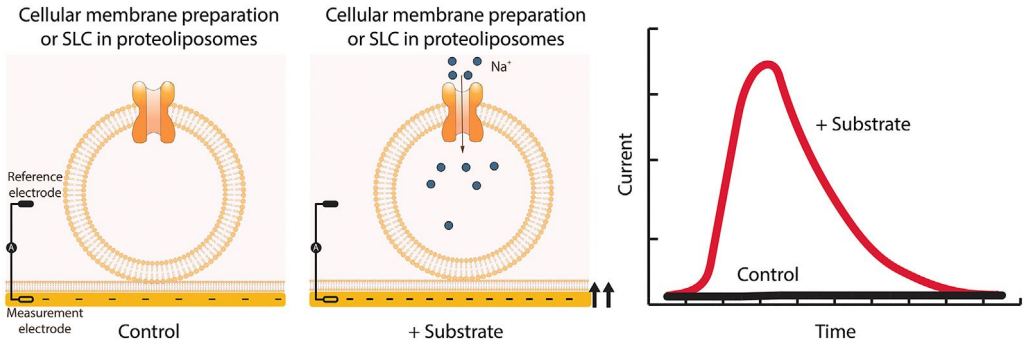


FIGURE 7 | SSM-based electrophysiology applied to SLCs. Membrane preparations from cells overexpressing the SLC of interest are applied to the sensor and together form a capacitively coupled membrane system. Therefore, charge translocation at the protein containing membrane can be detected via the SSM. After addition of the SLC substrate, changes in membrane potential are recorded. Only transient currents are measured, and the peak current represents the maximum speed of the transport.

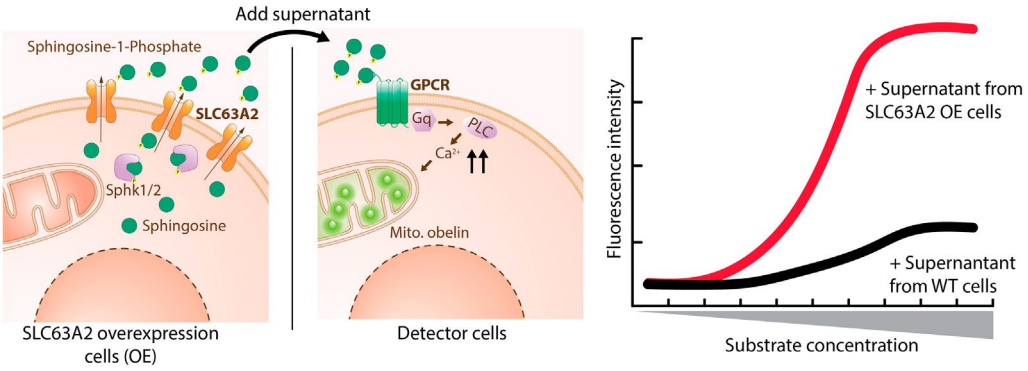


FIGURE 8 | SLC-GPCR coupling assay applied to SLC63A2. Sphingosine is phosphorylated by Sphk1/2 and exported by SLC63A2 OE cells through SLC63A2 into medium. Supernatant from these cells is then applied to detector cells, which stably express a S1P specific GPCR and the Ca^{2+} reporter Obelin. Activation of the GPCR as a surrogate readout for SLC63A2 transport of S1P is quantified by the increase of reporter fluorescence.

Danthi et al., 2019). BCECF dye has been used to study SLCs from SLC9 (Windler et al., 2018), SLC16 (Contreras-Baeza et al., 2019) and SLC12 families (Reynolds et al., 2007). Potassium transport was measured with the thallium dye (as a surrogate) for SLC12 family (Carmosino et al., 2013) and with the sodium indicator Asante Natrium Green for SLC4 and SLC12 families (Noor et al., 2018).

Assay Advantages, Limitations and Approximate Costs

The main limitation in the use of dyes is that a cell loading step is required, with the consequent risk of affecting cell physiology. Nevertheless, most of the dyes are very easy to load and require a single incubation step without washing the cell monolayer (Wolff et al., 2003), which results in a rapid and HTP assay. In addition, these assays are flexible and have low temporal resolution, given the dyes' fast responses. Finally, the use of probes brings along with it some elevated costs.

Electrophysiology

Since its discovery by Neher and Sakmann (Neher and Sakmann, 1976), the patch clamp technique has been widely used to study membrane electrical activity and the underlying ion currents in excitable cells. Today patch clamp is still recognized as the golden standard technique to study voltage- and ligand-gated ion channels, as well as mechanosensitive, transient receptor potential (TRP) channels and electrogenic proteins, such as pumps or transporters (Brown and Greenberg, 2016). Compared to surrogate techniques, such as fluorescence or luminescence assays, patch clamp allows not only to identify active molecules on the target of interest, but it also provides information about the mechanism of action of a compound. Due to its direct measurement of net charge fluxes across the membrane, patch clamp is a very powerful tool for mechanistic studies.

Technical Requirements and Level of Throughput

Patch clamp requires one skilled person to run a so-called "electrophysiology setup". The basic version is composed of an inverted microscope, an operational amplifier and a digital-analogic transducer coupled with a computer for data collection and analysis. Usually, a Faraday cage is included for electrical isolation and a fluidic perfusion system is in place to apply compounds diluted in physiological saline solutions (Rubaiy, 2017). Since the technique is versatile, any laboratory currently equipped with the setup can extend this approach to study SLCs without major changes to the protocols already in place. The high informativity is given at the cost of the intrinsic LTP.

Experimental Setup

The day before the experiment SLC-expressing cells are seeded as single isolated cells on coated glass coverslips. On the day of experiment the coverslips with cells are placed in the recording chamber of the inverted microscope equipped with the headstage of the operational amplifier to run the patch clamp experiment. A glass micropipette is filled with the solution mimicking the cytosolic environment, and firmly stabilized on the headstage of a micromanipulator. The tip of the micropipette is carefully attached to the cell membrane. To form an electrical seal between the micropipette and cell membrane, a constant negative pressure is applied. The membrane is ruptured by a sudden pulse of negative pressure or by brief applications of currents. Afterwards, the voltage clamp configuration is used to modulate the cell membrane potential by applying voltage waveforms specifically designed to favor the activation of the SLC under investigation.

Typically, a perfusion system is integrated in the manual patch clamp setup to apply inhibitors, activators or substrates directly on the patched cell. If such a system is not available, compounds can be directly applied by pipetting small amounts of solution in the recording chamber.

The changes in the membrane currents upon application of substrates/inhibitors are recorded in real-time and analyzed offline, with the major advantage being the internal normalization control for each application of a given compound since transmembrane current is measured before and after.

Suitable Solute Carrier Families

The SLC of interest needs to be electrogenic (**Supplementary Table S1**) and expressed in sufficient amount at the plasma membrane. Patch clamp was used for example to validate the effects of two molecules (SEA0400 and KB-R9743) now recognized as reference SLC8A1 inhibitors (Elias et al., 2001; Lee et al., 2004).

Assay Advantages, Limitations and Approximate Costs

The main advantage is the high time resolution and the accuracy of the readout signal, allowing direct monitoring of electrogenic protein activity and their modulation by compounds in real-time. Single cell analysis of a stable clone provides information about homogeneity of the cell line, i.e. the percentage of cells functionally expressing the SLC of interest. A disadvantage for its use for SLCs may be that the net charge caused by electrogenic transport is much lower compared to the opening of an ion channel. The amplitude of recorded signals may not be high enough to allow a dose-response experiment and different techniques are required for a full pharmacological characterization, unless the SLC is expressed at very high levels.

Solid-Supported Membrane Based Electrophysiology and Surface Electrogenic Event Reader Technology

Solid-supported membrane (SSM)-based electrophysiology was especially developed for the measurement of transporters such as SLCs, which are difficult to investigate using conventional electrophysiology (Nanion Technologies Munich, 2021; Schulz et al., 2008; Bazzone et al., 2013, 2017). The methodology differs from conventional electrophysiology. Instead of living cells, the methodology uses diverse native or artificial membrane vesicles, such as reconstituted protein in proteoliposomes or membrane preparations from organelles, cells or tissue samples (Nanion Technologies Munich, 2021; Geibel et al., 2006; Bazzone et al., 2013, 2017). The membrane sample is added to a SSM, which consists of a lipid monolayer on top of a thiolated gold coated

sensor chip. This leads to the stable adsorption of the added membranes to the SSM and the formation of a capacitively coupled membrane. The experiment starts in the presence of buffer lacking the SLC substrate. During the experiment the buffer is exchanged for a solution containing the SLC substrate. The substrate gradient established by fast solution exchange is the main driving force and the transport of charged substrates or ions into the liposomes or vesicles generates a membrane potential. The potential is detected via capacitive coupling between the membrane and the SSM on the gold layer of the sensor. As soon as the membrane potential equals the chemical driving force, the transport process comes to a halt. The *surface electrogenic event reader* technology (*SURFE^{2}R*) (Nanion Technologies Munich, 2021) employs SSM-based electrophysiology and allows the measurement of up to 10^9 transporters at the same time to yield the best signal to noise ratio (**Figure 7**).

Technical Requirements and Level of Throughput

SSM-based electrophysiology requires a *SURFE^{2}R* instrumentation, which is available as a *SURFE^{2}R N1* for LTP assays, or as a *SURFE^{2}R 96 SE* enabling HTP and automatization in a 96-well plate like format (96 sensors in parallel) (Nanion Technologies Munich, 2021). Each membrane containing the protein of interest is suitable for sample preparation and for measurements using SSM-based electrophysiology, but nevertheless a high protein density and purity can compensate for low turnover and low electrogenicity (Nanion Technologies Munich, 2021; Bazzone et al., 2013, 2017). Normally, the transporter of interest is either recombinantly overexpressed or used from native tissue including different organisms. Commonly used expression systems range from bacteria to eukaryotic cell lines. Also, cell-free expression systems have been used to assay transporter function with SSM-based electrophysiology, where protein is purified, followed by reconstitution into liposomes of ~100 nm in diameter at high protein densities (Barthmes et al., 2016; Bazzone et al., 2017). An advantage of reconstituted samples is the possibility to vary the membrane composition of the sample which can affect the protein function or ion gradient stabilities (Bazzone et al., 2013, 2017). Due to its mechanical robustness, SSM-based electrophysiology has a high potential for screening applications, allowing determination of the dose dependence of 100 compounds in less than 30 min (Nanion Technologies Munich, 2021; Bazzone et al., 2013, 2017). A *SURFE^{2}R 96SE* system (Nanion Technologies Munich, 2021) allows the recording of 96 wells in parallel and the automatization of experimental workflows including sensor preparation, data analysis and export, and practically results in the measurement of six 96-well plates per day (Nanion Technologies Munich, 2021).

Experimental Setup

For the laboratory setup, a detailed protocol performing experiments was published by Bazzone et al. in 2013 (Bazzone et al., 2013). Sensor preparation includes the thiolization of the sensor surface, the assembly of the lipid layer, and finally the application of membranes to the sensor (Nanion Technologies Munich, 2021). The protein containing membrane and the SSM will form a capacitively coupled membrane system and therefore, charge translocation at the protein containing membrane can be detected by capacitive coupling via the SSM. Upon substrate addition, transient currents are recorded, whereas the peak current represents the maximum speed of the transport. Due to the high stability of the SSM, up to one hundred sequential measurements can be performed on the same sample and allow the determination of parameters such as EC_{50} or IC_{50} (Nanion Technologies Munich, 2021). SSM-based electrophysiological experiments only require 0.1 – 1 µg protein per sensor (Nanion Technologies Munich, 2021; Bazzone et al., 2013, 2017).

Suitable Solute Carrier Families

The method is suitable for the detection of any kind of reaction associated with a charge displacement or with the change in water accessibility close to a charge (electrogenic transporters listed in **Supplementary Table S1**). The SLC families which were measured using this method are for example SLC1, SLC6, SLC8, SLC15 or SLCO (Geibel et al., 2006; Bazzone et al., 2017; Gerbeth-Kreul et al., 2021; Nanion Technologies Munich, 2021). In addition, since SSM-based electrophysiology has been used to assess the function of mitochondrial proteins, this technique may be applied to intracellular SLCs (Watzke et al., 2010).

Assay Advantages, Limitations and Approximate Costs

The technology allows real-time data acquisition with a high time resolution and a high signal amplification compared to conventional patch-clamp (Nanion Technologies Munich, 2021). SSM-based electrophysiology additionally allows to resolve fast binding kinetics and EC_{50} or IC_{50} determination in a HTP manner (Nanion Technologies Munich, 2021).

In contrast to patch clamp and voltage clamp techniques, SSM-based electrophysiology cannot be used to apply a membrane potential (Bazzone et al., 2013). Transporter characterization is therefore restricted to transport modes which do not rely on a membrane potential (Bazzone et al., 2013). In general, SSM-based electrophysiology has no limitations concerning the type of the transporter, but voltage clamp or patch clamp methods can have advantages, if intracellular components like binding proteins are required for protein functionality (Bazzone et al., 2013, 2017). Limitations can arise, if solution exchange creates large artifact currents which happens when the substrate interacts strongly with the SSM like in the case of lipophilic compounds (Bazzone et al., 2013).

Costs per data point are dependent on the assay protocol, especially how many activations, concentrations of compounds or conditions are tested in one well/sensor.

Overall, SSM-based electrophysiology is an ideal methodology in cases where conventional electrophysiology cannot be applied and is also attractive for screening applications in drug discovery especially because of its robustness and its potential for automation (Geibel et al., 2006; Nanion Technologies Munich, 2021).

Solute Carrier-G Protein Coupled Receptor Coupling

An SLC-GPCR coupling assay is based on detecting the SLC substrate via GPCR engagement. The assay consists of two parts: first, the SLC is stimulated to export its substrate, and second, a GPCR which recognizes the substrate as a ligand is used for detection (**Figure 8**).

Technical Requirements and Level of Throughput

The hardware requirements for an SLC-GPCR coupling assay are a fluorescence or luminescence microtiter plate reader which ideally allows for kinetic measurements. GPCR activation typically leads to a change in the intracellular calcium or cyclic adenosine monophosphate (cAMP) concentrations, or to altered gene expression. These events can be easily detected by using a typical "GPCR toolbox" consisting mainly of fluorescent dyes and genetically encoded luminescent biosensors (Thomsen et al., 2005; Zhang and Xie, 2012). In brief, calcium responsive fluorescent dyes (e.g. Fluo-8) or photoproteins (e.g. aequorin) can be used to detect increases in intracellular calcium (Ma et al., 2017). Changes in cAMP levels can be detected e.g. via a cAMP-responsive luciferase (Fan et al., 2008), or proximity-based homogenous assays relying on cAMP-antibodies (Williams, 2004). Alterations in gene expression are typically monitored by inserting a reporter, e.g. a luciferase, into the respective genomic locus. All these assays can be run in 384-well-plates which allows for HTP.

Experimental Setup

For assay development, two cell lines are generated: one expressing the SLC and the other cell line expressing the corresponding GPCR. In a first step, the GPCR assay is independently optimized using the GPCR ligand (i.e. the SLC substrate) from an external source, as well as known inhibitors of the GPCR to verify sensitive detection of GPCR activation, inhibition, and desensitization. In a second step, the supernatant of the SLC-expressing cell line can be used for GPCR activation. At this stage, different SLC stimulation parameters can be evaluated, and clonal selection can be performed. It is also conceivable to express both the SLC and the GPCR in a single cell line. Specifically, for assaying SLC59A2 (MFSD2B) and SLC63A2 (Spinster2, SPNS2), we generated cell lines which stably express SLC59A2 or SLC63A2 + SPHK1 (sphingosine kinase 1) and feed these cells with (unlabeled) sphingosine for several hours. Intracellular sphingosine kinases phosphorylate the pre-substrate to S1P, which is exported by the SLC. Transferring the SLC cells' supernatant to cells co-expressing S1P3 (a Gq coupled S1P receptor) as well as the calcium photoprotein obelin results in a luminescent calcium response.

In order to make the assay more amenable to HTP, we adapted it to a desensitized format: Continuous agonist stimulation eventually leads to GPCR desensitization, inactivation or internalization (Siehler and Guerini, 2006; Rajagopal and Shenoy, 2018). Thus, instead of detecting the SLC substrate (S1P) as an agonist, it can also be detected as a functional antagonist when applying a second agonist challenge using the substrate (S1P) from an external source. A previously desensitized GPCR will remain "silent", while not previously stimulated GPCRs will show an agonistic signal. Once optimized, we adapted the desensitized format to a co-culture set-up where S1P3 and SLC cells are seeded into the same well, to eliminate the need for supernatant transfer.

Suitable Solute Carrier Families

We have successfully applied this strategy to set up assays for the two sphingosine-1-phosphate (S1P) transporters SLC63A2 and SLC59A2. Previously described assays for these transporters were low in throughput because they involved laborious sample preparation followed by TLC (thin layer chromatography) or HPLC (high performance liquid chromatography) detection of radiolabeled or fluorescent S1P (Kawahara et al., 2009; Hisano et al., 2012; Vu et al., 2017; Kobayashi et al., 2018). This approach is applicable to any other SLC which can export a GPCR ligand into the extracellular medium.

Assay Advantages, Limitations and Approximate Costs

SLC-GPCR coupling involves several steps which need optimization and pose confounding factors that may lead to false positive/negative hits. For HTP screening, this approach is therefore most suitable for SLCs which cannot be assayed with more straightforward options such as membrane potential or pH-sensitive dyes, a fluorescent surrogate substrate, or a substrate-specific biosensor. On the other hand, GPCR coupling poses a highly specific and sensitive tool for substrate detection. Therefore, SLC-GPCR coupling can be a valuable downstream or orthogonal assay for hit validation. The cost of an SLC-GPCR coupling assay heavily depends on the GPCR readout strategy. In the S1P transporter assay described above, the cost is low with coelenterazine (obelin's luminophore) being the most expensive reagent per well.

Label-free Impedance-Based Assay

Label-free cell-based assays have emerged in recent years as a versatile platform to monitor changes in cellular properties such as adhesion, proliferation, growth and morphology (Xi et al., 2008). Several platforms, e.g. optical and impedance-based technologies, have been used to develop such assays as drug discovery tools for protein classes like GPCRs (Yu et al., 2006). In principle, activation of a GPCR on living adherent cells generates a whole-cell response dependent on coupling to intracellular signaling pathways and cellular background, leading to temporal changes in cell morphology which are detected in real-time (Scott and Peters, 2010). Impedance-based assays, e.g. xCELLigence (Doornbos and Heitman, 2019), have been extensively used for functional characterization of GPCR agonists, antagonists, and allosteric modulators (Doornbos et al., 2018). Since SLC substrates can act as GPCR ligands, this technology is suitable for assaying SLCs. The resulting assay, in which SLC activity or inhibition can be measured via GPCR activation, was termed the "transport activity through transport activation" (TRACT) assay (Sijben et al., 2021).

Technical Requirements and Level of Throughput

Requirements to run xCELLigence experiments are a general cell culture facility, temperature and CO_2 controlled environment (e.g. cell culture incubator), an xCELLigence real-time cell analysis (RTCA) instrument and E-plates, which are the main consumable of this application. The xCELLigence RTCA system has multiple plate configurations ranging from 16 to 96 wells up to 384 wells which are amenable to HTP screening (Halai and Cooper, 2012). To allow detection of SLC activity, a (preferably adherent) cell line is required with heterologous or endogenous expression of both the SLC and a concomitant GPCR.

Experimental Setup

Reports on label-free cell-based transporter assays have been limited (Wong et al., 2012). Recently, a label-free impedance-based assay was developed using xCELLigence to assess functional activity of SLC29A1 (equilibrative nucleoside transporter 1 or ENT1) in living cells (Vlachodimou et al., 2019). Here, activation of adenosine receptors (ARs) by adenosine, a SLC29A1 substrate/AR agonist, is used as a readout. The assay is based on the hypothesis that active SLC29A1 mediates influx of adenosine when extracellular concentrations are higher than cytosolic adenosine, thereby controlling the tone and magnitude of adenosine-mediated signaling events. Upon addition of exogenous adenosine to cells that endogenously express both SLC29A1 and ARs, adenosine is partially taken up via SLC29A1, while the remaining extracellular adenosine is able to activate ARs expressed on the cell membrane. When SLC29A1 transport is blocked by an inhibitor, the extracellular concentration of exogenous adenosine is increased which leads to augmented activation of ARs resulting in an enhanced cell response. This provides an assay window which has been used to characterize inhibitory potency (Vlachodimou et al., 2019) and binding kinetics (Vlachodimou et al., 2020) of SLC29A1 inhibitors. More recently, this assay principle was validated for the human dopamine transporter (DAT, SLC6A3) in two cell lines with heterologous expression of DAT (**Figure 9**) (Sijben et al., 2021).

Suitable Solute Carrier Families

SLCs that are suitable for assessment with a TRACT assay should have a known substrate ascribed to them, meaning that orphan transporters are not amenable. So far, TRACT assays have been developed for SLC29A1 and SLC6A3 using the xCELLigence technology. In theory, any SLC that transports a substrate which at the same time is an agonist for a membrane-bound GPCR is admissible for this assay. Examples of these are SLCs for monoamine neurotransmitters (e.g. SLC6A2, SLC6A4), glutamate (SLC1) (Doornbos et al., 2018), carboxylic acids (SLC16), and fatty acids (SLC27). To further widen the scope, any SLC that is involved in or influences a process that leads to detectable changes in cell morphology could potentially be assessed (Wang W. W. et al., 2019). However, this remains to be demonstrated experimentally.

Assay Advantages, Limitations and Approximate Costs

Label-free cell-based assays detect whole-cell responses that are essentially an accumulation of all intracellular signaling events resulting from a perturbation. This allows the researcher to capture comprehensive information in real-time without the use of non-physiological labels and invasive methods. The cumulative signal may also be perceived as a disadvantage of this approach as it produces a "black box" readout, which warrants thorough signal validation during assay development. Additionally, compounds inducing off-target effects that mask or amplify observed cell responses can result in false positives or false negatives, which are mitigated with appropriate counter screens. Main costs for running xCELLigence assays come down to the E-plate consumables. Some protocols describe the reuse of E-plates which provides perspective to reduce overall assay costs (Stefanowicz-Hajduk et al., 2016).

Solute Carrier Coupling to Nuclear Hormone Receptor

Human nuclear hormone receptors (Maglich et al., 2001) are ligand dependent transcription factors, which activate or repress the transcription of genes after binding the corresponding hormone. 48 members of this protein family are known. While the majority are still orphans, the associated ligands for some of those or their precursors are SLC substrates.

An example are steroid sulfates, transported by SLC10A6 (sodium dependent organic anion transporter or SOAT), but also by members of the SLCO (OATP) and SLC22 (OAT) families (Pizzagalli et al., 2003; Ugele et al., 2003). As a result of transporter activity, the intracellular hormone precursor concentration is increased. After the sulfate has been cleaved off by steroid sulfatase (Reed et al., 2005), the agonism of the active steroid is measured at the corresponding nuclear receptor.

There are two established methods to quantify nuclear receptor activity in cellular assays (Schulman and Heyman, 2004; Pinne and Raucy, 2014). The first protocol is a transactivation assay based on the transient or stable expression of the nuclear hormone receptor combined with a plasmid carrying a response element for this receptor in front of a reporter gene like β-galactosidase or luciferase. The second method employs a fusion between the ligand binding domain of the nuclear receptor and the yeast GAL4 protein (yeast galactose metabolic genes inducing transcription factor 4), in combination with a vector carrying the GAL4 UAS (upstream activating sequence) in front of a reporter enzyme (**Figure 10**).

Technical Requirements and Level of Throughput

The assay needs to be performed in a cell culture lab and requires a suitable microtiter plate reader for luminescence or absorbance measurements. Both assay systems, the protocol using the full-length nuclear receptor as well as the GAL4 fusion method can be adapted to a HTP format. The GAL4 fusion protocol shows in general higher signal windows.

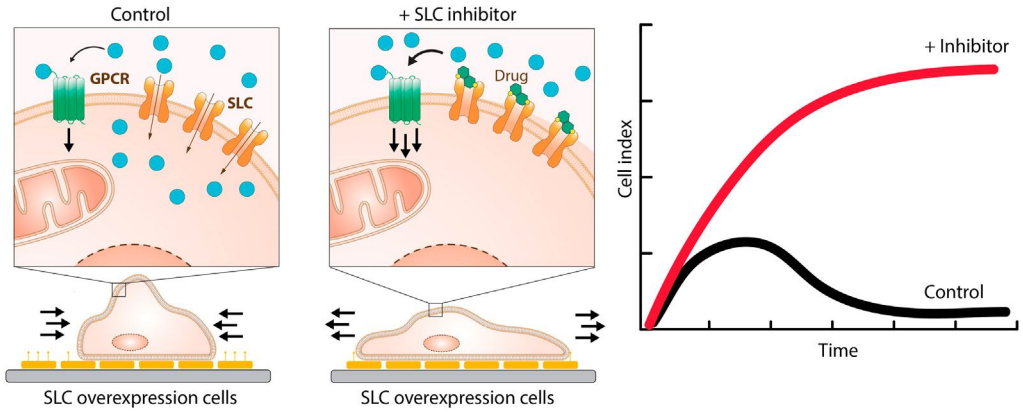


FIGURE 9 | TRACT assay. Activation of a GPCR leads to changes in cellular morphology which can be quantified using the xCELLigence system. Exogenous addition of a SLC substrate which is at the same time a GPCR ligand to cells expressing both the SLC and the GPCR will lead to partial uptake and activation of the GPCR, measured by morphological changes with the xCELLigence real-time cell analysis (RTCA) instrument. Overexpression of the SLC leads to increased uptake of the substrate, which attenuates the GPCR-mediated cell response. When SLC transport is blocked by an inhibitor, the extracellular concentration of the SLC substrate/GPCR ligand is increased which leads to augmented activation of the GPCR and an enhanced cell response.

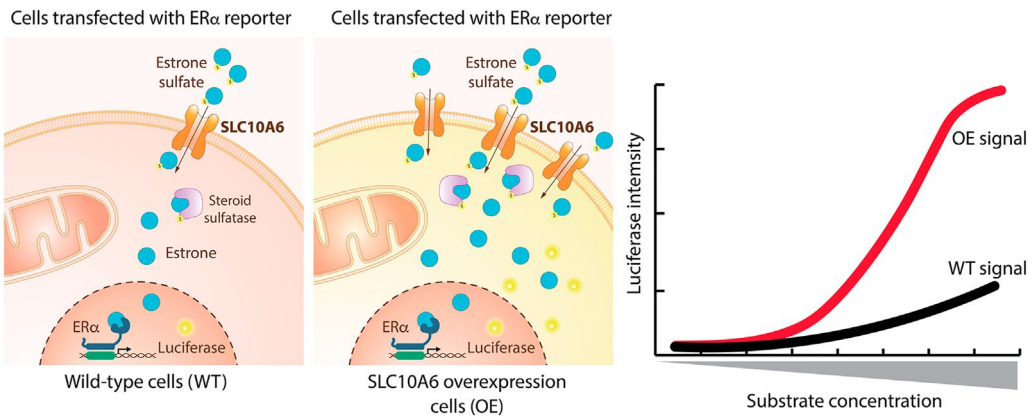


FIGURE 10 | SLC coupling to nuclear hormone receptor applied to SLC10A6. A cell line expressing a reporter plasmid combining a response element of the estrogen receptor alpha (ERα) and a luciferase encoding gene is treated with estrone sulfate, which is imported to the cytoplasm by SLC10A6 and cleaved by the steroid sulfatase. The product estrone then binds to the estrone-responsive element and activates luciferase expression from the reporter. Inducible overexpression of SLC10A6 leads to increased uptake of estrone sulfate and therefore increased luciferase intensity.

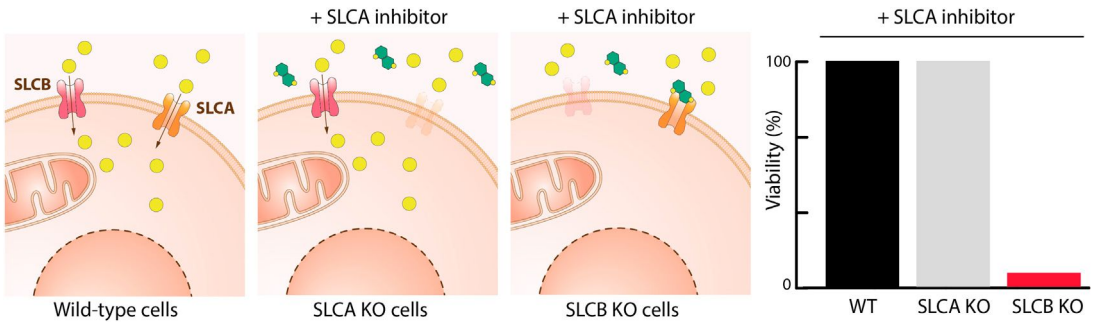


FIGURE 11 | Phenotypic assay based on synthetic lethality. WT cells are expressing SLCA and SLCB which are two transporters with a strong negative genetic interaction. KO of SLCA results in cells dependent on SLCB and *vice versa*. Therefore, selective inhibitors of SLCA kill only SLCB KO cells.

Experimental Setup

In order to establish a transporter assay cell line, the expression constructs for the SLC, the corresponding nuclear hormone receptor and its reporter plasmid are transfected into a suitable mammalian host cell line. An inducible expression of the target SLC will enable the analysis of the contribution from the endogenous SLCs in the host cell line to the total hormone uptake. The assay starts by incubating the test compounds with the cells, then the SLC substrate is added for a given time, followed by its removal and an additional incubation time for the cells to express the reporter. Optimization of SLC substrate concentration and incubation time can improve assay performance.

Suitable Solute Carrier Families

In principle all SLCs for which nuclear receptor agonists or their precursors are substrates can be assayed in this format based on steroid sulfates and corresponding nuclear hormone receptors, such as SLC10A6, members of the SLCO and SLC22A families. Thyroid hormone transporters like SLC16A2 and SLC16A10 might also be amenable for this assay type (Visser et al., 2011).

Assay Advantages, Limitations and Approximate Costs

An advantage of this assay method is the use of unmodified, natural SLC substrates and its independence of electrogenicity or cotransport of certain ions. An important limitation for some substrates is redundancy, especially when respective SLCs are endogenously expressed in the host cell line. The costs of running the assay are low.

Phenotypic Assays

Phenotypic assays (PAs) rely on a "visible" or observable read-out as a proxy for a biological/biochemical function. PAs useful for transporter research typically involve cell lines, although employing a variety of models, from yeast to fish, is feasible in principle. Among the cell line-based assays one can distinguish phenotypes that are based on inherent cellular properties, such as fitness/survival, cellular differentiation, or adhesion. The key principle relies on the ability of genetics to demonstrate that a particular cellular feature is dependent on the function of a particular transporter, no matter how indirect the phenotype. While there are many possible kinds of this assay, the simplest measures viability of human cells engineered for a dependency on a particular SLC. The best approach to circumvent redundancy and establish a robust genotype-phenotype relationship is to conduct a genetic screen in a cell line with inactivation of the targeted SLC. The simplest readout is growth/survival, hijacking the principle of synthetic lethality: while cell viability upon inactivation of either transporter A or transporter B is minimally affected, inactivation of both transporters leads to severly reduced fitness (Girardi et al., 2020b; Huang et al., 2020). In this setting, the fitness of the cell line with inactivated transporter B becomes dependent on transporter A, which provides a platform for screening for chemical modulators of the function of transporter A (**Figure 11**).

Technical Requirements and Level of Throughput

The assay requires the ability to genetically engineer a cell line of choice and the necessary tools are vectors allowing to express Cas9 and transporter-specific guides or chemical probes for the SLCs under investigation. If there is no prior knowledge on synthetic lethality to other SLCs, a prior genetic screen may be required. A cell-based viability assay is amenable to HTP screening.

Experimental Setup

Key requirement is establishing the unambiguous SLC-phenotype relationship. This includes the ability to engineer human cell lines to depend on a particular SLC gene and at least a second control cell line that is isogenic.

The first round of chemical screening is started with the engineered cell line dependent on the SLC of interest. All compounds which exhibit toxicity in the first round are screened in the secondary chemical screen, which includes additional isogenic control cell lines (e.g. WT parental cell line, and cell line dependent on the reciprocal SLC). Afterwards, all compounds which reduce viability only in the cell line dependent on the targeted SLCs are considered as a hit. Since this screening campaign enriches compounds targeting all genes with a synthetic lethal relationship to the targeted SLC, hits should be followed up for example with a binding assay to further filter compounds which interact physically with the targeted SLC.

Suitable Solute Carrier Families

The great advantage of this approach is that it is amenable to all SLC families, irrespective of their subcellular localization, topology or abundance, provided there is evidence of a genetic interaction between the SLC of interest and another protein and both are expressed in the cell line of choice. For example, strong negative genetic interactions were confirmed between SLC16A1 (MCT1) and SLC16A3 (MCT4), or between SLC25A28 (Mitoferrin-2) and SLC25A37 (Mitoferrin-1) (Girardi et al., 2020b). Using the parental cell line and the SLC16A1 KO as controls, differential activity of compounds against the SLC16A1 KO cell line yielded *bona fide* SLC16A3 inhibitors (Dvorak and Superti-Furga, unpublished).

Assay Advantages, Limitations and Approximate Costs

The main advantage of this assay is the straightforward readout based on cell viability, which is suitable for HTP screening. Since many of the genetic interactions are of reciprocal character, it is possible to counter screen for specificity by screening hits from the primary screen in reciprocal knock-out cells and WT cells. This approach should filter all compounds which affect viability of cells independently of the targeted SLC, and thus provide a fast path to compound specificity. The main limitation is the requirement of a strong genotype – phenotype association, which may require a prior dedicated genetic screen. Phenotypic assays can be applied also for screening for transporters of cytotoxic compounds (Girardi et al., 2020a).

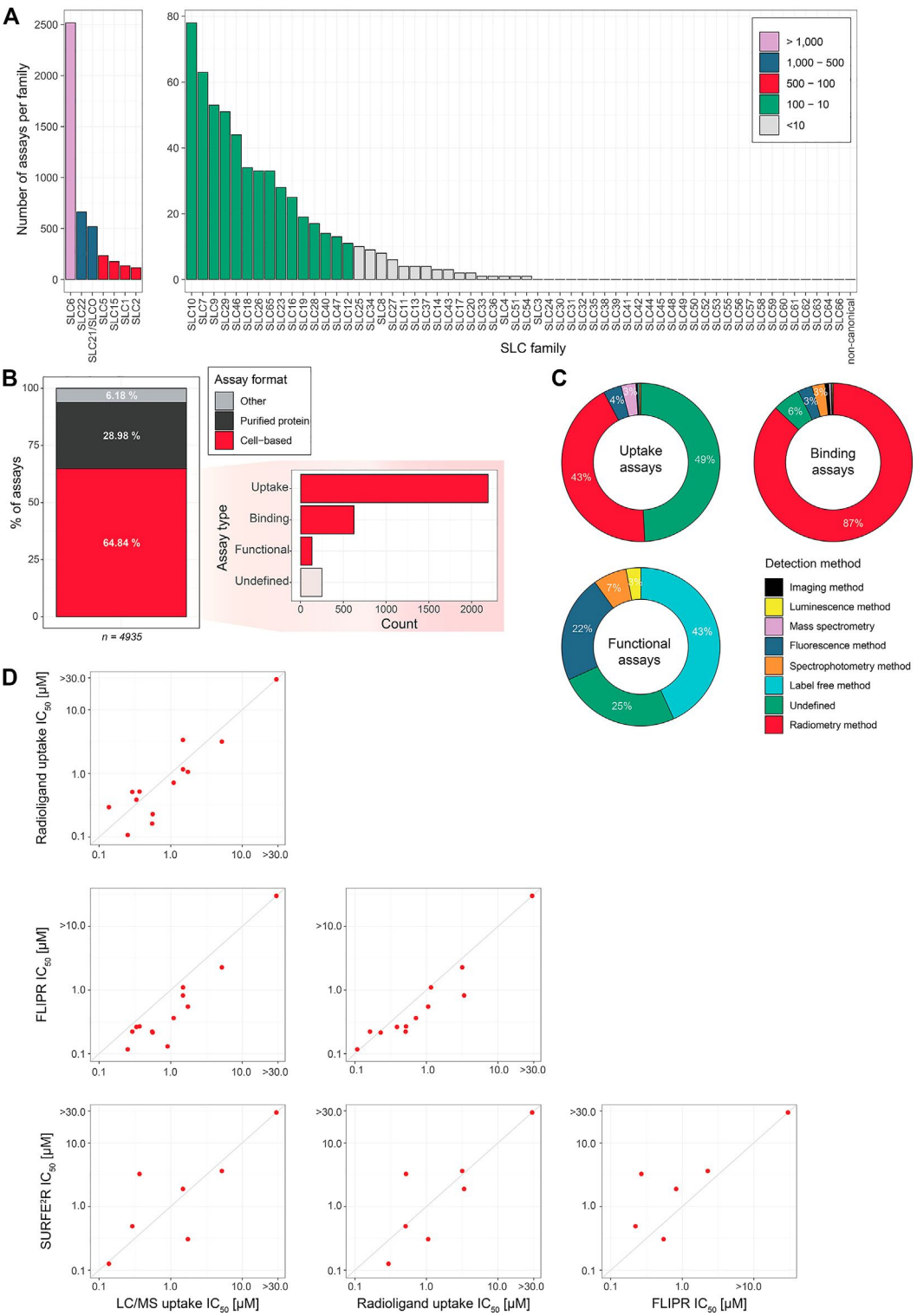


FIGURE 12 | Overview and comparison of assay techniques in use. **(A)** Number of assays reported in ChEMBL per SLC family. **(B)** Distribution of assays based on assay format and assay type (cell-based assays only). Assay format was determined from the BAO label reported in the ChEMBL database (e.g. cell-based format and single protein format). Assay type was assigned according to manually created rules (see supplementary material) **(C)** Detection methods employed by each assay type (cell-based assays only). The detection method was assigned to the different categories based on manually created rules (see Supplementary table 2) **(D)** Comparison of IC_{50} values of SLC13A5 inhibitors obtained by different assays (Data retrieved from Huard et al. (2016); Pajor et al. (2016)); IC_{50} of >10, or >30 µM respectively, refers to the detection limit).

DISCUSSION

The experimental methods and approaches that can be used to interrogate the function of SLC transporters are as diverse as this class of integral membrane proteins, with different phylogenetic origin, fold and transport mechanism. To enable drug discovery efforts, an extensive toolbox of technologies is required to study the diverse transport mechanisms utilized by SLC transporters as well as the broad nature of substrates transported.

For an overview on the different assay techniques in use, we analyzed the assays for SLCs available in the ChEMBL database Version 28 (Davies et al., 2015; Gaulton et al., 2017) using KNIME (Berthold et al., 2009). In total, 4,935 assays (where each publication counts as one assay) were reported for 120 different SLCs (see **Supplementary Table S2**). These data are highly asymmetric, with the vast majority of assays performed only on a handful of SLC families, while there are less than 10 assays reported for more than a half of SLC families (**Figure 12A**). While this can be to some extent biased from the scope of the database, these observations are in line with a previously reported publication asymmetry in the SLC superfamily, in which a small fraction of SLCs are the object of a large proportion of literature on SLCs (César-Razquin et al., 2015). Our overview also showed that the majority of assays in ChEMBL were based on cell-based formats (**Figure 12B**). This is in line with the subject of the current review and reflects the challenges connected to purification and handling of complex membrane proteins.

We further characterized the assays according to the text of the assay description by ChEMBL. Text based rules were created manually to distinguish different assay types (e.g. uptake or binding) and detection methods (e.g. radiometry or label free methods). The full list of rules as well as the list of ChEMBL assays with assigned labels are given in the **Supplementary Material**. As cell-based assays are the focus of this review we concentrated on these assays for the overview given in **Figures 12B,C**. The majority of the cell-based assays are uptake assays, accounting for nearly 70%. When investigating the detection methods, we see that radiometry is heavily used both for uptake and binding assays. For the uptake assays we could not assign any detection method to almost 50% according to the assay description ("undefined"), but one can assume that radiometry was the method of choice in a large proportion of these cases as well. Only in the functional assays we do not see measurements of radioactivity. Instead, label free and fluorescence detection methods are used for measuring the outcome in functional cell-based assays. The total number of these assays is however still small, as functional assays make up only four percent of all cell-based assays (**Figure 12C**). We expect the frequency of functional assays to increase in the future due to more knowledge on less-studied SLCs, which should open the possibilities to study these SLCs particularly using functional assays. A better understanding of the SLC superfamily and its individual members will also facilitate the decision on which SLCs are amenable to which assay technologies.

The choice of an appropriate assay format is expected to change as a SLC drug discovery program progresses from its initial efforts to identify chemical matter that modulates the SLC transporter in the HTP screening phase where tens of thousands of compounds are screened, to file mining and virtual screening, and finally to SAR and lead development support. Multiple assay formats are often employed throughout the life of a program not only to address practical issues such as throughput and cost concerns but also to consider assay specific liabilities such as compound interference, cytotoxicity, off target interferences etc. Like with other target classes, as a hit matures to lead and preclinical and then clinical candidate, it will transit through several of these assays in different combinations.

To address the question if pharmacology of small molecules is influenced by choice of assay technology, we inspected previously obtained data on the potency of several SLC13A5 inhibitor chemotypes across different technologies in a cellular system in which SLC13A5 was overexpressed (Huard et al., 2016; Pajor et al., 2016): uptake of radiolabeled citrate, uptake of citrate monitored by LC/MS, uptake of co-transported sodium using FLIPR to monitor membrane potential and solid supported based electrophysiology using SURFE^{2}R (**Figure 12D**). In sum, the potency of the SLC13A5 inhibitors correlated across all the technologies tested. This suggests that data obtained with one assay should represent a reliable starting base for another assay. It also suggests that modern assay technology can be calibrated in such a way to be robust. While we consider this observation rewarding and promising, this does not necessarily imply that the discovery efficiency should be equal, as sensitivity may differ, and certain molecules may have properties that manifest differentially in the different assays. All in all, based on the variety and robustness of available assays, SLCs should be considered an attractive and amenable target class.

FUTURE PERSPECTIVES

From our perspective the future of SLCs as major target class in drug discovery seems certain. The number of reagents, tools, assays and publications are increasing constantly. Only in the last two years we counted three new biotech start-ups focused entirely on SLCs, the first of what surely is likely to be a whole generation of new businesses. The majority of pharmaceutical companies either already include SLC targets in their project portfolio or are rapidly evaluating the opportunity. Among the novel drugs with new mechanism of action reported for 2020, SLCs feature prominently (Avram et al., 2021). Though the dataset is small, it heralds the beginning of a new phase in SLC pharmacology. The RESOLUTE consortium intends to empower the scientific community with reagents, datasets and assays and invites it to contribute to exploit transporters more broadly for drug discovery. Through their action, transporters link metabolism to a variety of cellular and organismal processes, all relevant to disease. At a yet more futuristic and ecological level, transporters of roughly the same kind, across the animal, plant, fungal and microbial kingdom, ensure flow and integration of chemical matter in a global way. Thus, what we are learning about the assays

required in the field of SLC transporter pharmacology for curing human diseases, may turn out to be useful also for nutrition, animal and crop production, managing of microbial communities and more.

AUTHOR CONTRIBUTIONS

The manuscript was written through the contributions of all authors. VD, TW, AIP and GSF contributed to the conceptualization of the review and editing. GSF provided supervision.

FUNDING

This work is part of the RESOLUTE project that has received funding from the Innovative Medicines Initiative 2 Joint Undertaking under Grant Agreement No. 777372. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA. In addition, we acknowledge support by the Austrian Academy of Sciences, the Vienna Science and Technology Fund (WWTF, Grant No. LS17-051), Austrian Science Fund (FWF, Grant No. W1232), Biotechnology and Biological Sciences Research Council (BBSRC, Grant No. BB/P009042/1), Novo Nordisk Foundation (grant NNF20CC0035580), National Institutes of Health (NIH, Grant No. R01 GM108911), Bayer AG, Vifor Pharma, Sanofi and Pfizer Inc. The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. All authors declare no other competing interests.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.722889/full#supplementary-material

REFERENCES

Abu-Remaileh, M., Wyant, G. A., Kim, C., Laqtom, N. N., Abbasi, M., Chan, S. H., et al. (2017). Lysosomal Metabolomics Reveals V-ATPase- and mTOR-dependent Regulation of Amino Acid Efflux from Lysosomes. *Science* 358, 807–813. doi:10.1126/science.aan6298

Adelmann, C. H., Traunbauer, A. K., Chen, B., Condon, K. J., Chan, S. H., Kunchok, T., et al. (2020). MFSD12 Mediates the Import of Cysteine into Melanosomes and Lysosomes. *Nature* 588, 699–704. doi:10.1038/s41586-020-2937-x

Al-Khawaja, A., Haugaard, A. S., Marek, A., Löffler, R., Thiesen, L., Santiveri, M., et al. (2018). Pharmacological Characterization of [3H] ATPCA as a Substrate for Studying the Functional Role of the Betaine/GABA Transporter 1 and the Creatine Transporter. *ACS Chem. Neurosci.* 9, 545–554. doi:10.1021/acschemneuro.7b00351

Al-Khawaja, A., Petersen, J. G., Damgaard, M., Jensen, M. H., Vogensen, S. B., Lie, M. E. K., et al. (2014). Pharmacological Identification of a Guanidine-Containing β-Alanine Analogue with Low Micromolar Potency and Selectivity for the Betaine/GABA Transporter 1 (BGT1). *Neurochem. Res.* 39, 1988–1996. doi:10.1007/s11064-014-1336-9

Alexandrov, A. I., Mileni, M., Chien, E. Y. T., Hanson, M. A., and Stevens, R. C. (2008). Microscale Fluorescent Thermal Stability Assay for Membrane Proteins. *Structure* 16, 351–359. doi:10.1016/j.str.2008.02.004

Almqvist, H., Axelsson, H., Jafari, R., Dan, C., Mateus, A., Haraldsson, M., et al. (2016). CETSA Screening Identifies Known and Novel Thymidylate Synthase Inhibitors and Slow Intracellular Activation of 5-fluorouracil. *Nat. Commun.* 7, 1–11. doi:10.1038/ncomms11040

Ando, H., Kawaai, K., and Mikoshiba, K. (2014). IRBIT: A Regulator of Ion Channels and Ion Transporters. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1843, 2195–2204. doi:10.1016/j.bbamcr.2014.01.031

Arce-Molina, R., Cortés-Molina, F., Sandoval, P. Y., Galaz, A., Alegría, K., Schirmeier, S., et al. (2020). A Highly Responsive Pyruvate Sensor Reveals Pathway-Regulatory Role of the Mitochondrial Pyruvate Carrier MPC. *Elife* 9, 1–18. doi:10.7554/eLife.53917

Armbruster, M., Dulla, C. G., and Diamond, J. S. (2019). Effects of Fluorescent Glutamate Indicators on Neurotransmitter Diffusion and Uptake. bioRxiv, Cold Spring Harbor Laboratories New York. doi:10.1101/2019.12.13.875724

Armbruster, M., Hanson, E., and Dulla, C. G. (2016). Glutamate Clearance Is Locally Modulated by Presynaptic Neuronal Activity in the Cerebral Cortex. *J. Neurosci.* 36, 10404–10415. doi:10.1523/JNEUROSCI.2066-16.2016

Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Sharman, J. L., et al. (2020). The IUPHAR/BPS Guide to PHARMACOLOGY in 2020: Extending Immunopharmacology Content and Introducing the IUPHAR/MMV Guide to MALARIA PHARMACOLOGY. *Nucleic Acids Res.* 48, D1006–D1021. doi:10.1093/nar/gkz951

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene Ontology: Tool for the Unification of Biology. *Nat. Genet.* 25, 25–29. doi:10.1038/75556

Ast, C., Foret, J., Oltrogge, L. M., De Michele, R., Kleist, T. J., Ho, C.-H., et al. (2017). Ratiometric Matryoshka Biosensors from a Nested Cassette of green- and orange-emitting Fluorescent Proteins. *Nat. Commun.* 8. doi:10.1038/s41467-017-00400-2

Avram, S., Halip, L., Curpan, R., and Oprea, T. I. (2020). Novel Drug Targets in 2019. *Nat. Rev. Drug Discov.* 19, 300. doi:10.1038/d41573-020-00052-w

Avram, S., Halip, L., Curpan, R., and Oprea, T. I. (2021). Novel Drug Targets in 2020. *Nat. Rev. Drug Discov.* 20, 333. doi:10.1038/d41573-021-00057-z

Bai, X., Moraes, T. F., and Reithmeier, R. A. F. (2018). Structural Biology of Solute Carrier (SLC) Membrane Transport Proteins. *Mol. Membr. Biol.* 34, 1–32. doi:10.1080/09687688.2018.1448123

Ballatori, N., Christian, W. V., Wheeler, S. G., and Hammond, C. L. (2013). The Heteromeric Organic Solute Transporter, OSTα-Ostβ/slc51: A Transporter for Steroid-Derived Molecules. *Mol. Aspects Med.* 34, 683–692. doi:10.1016/j.mam.2012.11.005

Barthmes, M., Liao, J., Jiang, Y., Brüggemann, A., and Wahl-Schott, C. (2016). Electrophysiological Characterization of the Archaeal Transporter NCX_Mj Using Solid Supported Membrane Technology. *J. Gen. Physiol.* 147, 485–496. doi:10.1085/jgp.201611587

Bateman, A., Martin, M. J., Orchard, S., Magrane, M., Agivetova, R., Ahmad, S., et al. (2021). UniProt: The Universal Protein Knowledgebase in 2021. *Nucleic Acids Res.* 49, D480–D489. doi:10.1093/nar/gkaa1100

Bazzone, A., Barthmes, M., and Fendler, K. (2017). *SSM-based Electrophysiology for Transporter Research*. 1st ed. Elsevier, 31–83. doi:10.1016/bs.mie.2017.05.008

Bazzone, A., Costa, W. S., Braner, M., Călinescu, O., Hatahet, L., and Fendler, K. (2013). Introduction to Solid Supported Membrane Based Electrophysiology. *JoVE* 1, 1–11. doi:10.3791/50230

Benjamin, D., Robay, D., Hindupur, S. K., Pohlmann, J., Colombi, M., El-Shemerly, M. Y., et al. (2018). Dual Inhibition of the Lactate Transporters MCT1 and MCT4 Is Synthetic Lethal with Metformin Due to NAD+ Depletion in Cancer Cells. *Cel Rep.* 25, 3047–3058.e4. doi:10.1016/j.celrep.2018.11.043

Benjamin, E. R., Skelton, J., Hanway, D., Olanrewaju, S., Pruthi, F., Ilyin, V. I., et al. (2005). Validation of a Fluorescent Imaging Plate Reader Membrane Potential Assay for High-Throughput Screening of glycine Transporter Modulators. *J. Biomol. Screen.* 10, 365–373. doi:10.1177/1087057104274090

Bensimon, A., Pizzagalli, M. D., Kartnig, F., Dvorak, V., Essletzbichler, P., Winter, G. E., et al. (2020). Targeted Degradation of SLC Transporters Reveals Amenability of Multi-Pass Transmembrane Proteins to Ligand-Induced Proteolysis. *Cel Chem. Biol.* 27, 728–739.e9. doi:10.1016/j.chembiol.2020.04.003

Berthold, M. R., Cebron, N., Dill, F., Gabriel, T. R., Kötter, T., Meinl, T., et al. (2009). KNIME - the Konstanz Information Miner. *SIGKDD Explor. Newsl.* 11, 26–31. doi:10.1145/1656274.1656280

Borden, L. (1996). GABA Transporter Heterogeneity: Pharmacology and Cellular Localization. *Neurochem. Int.* 29, 335–356. doi:10.1016/0197-0186(95)00158-1

Boudker, O., and Verdon, G. (2010). Structural Perspectives on Secondary Active Transporters. *Trends Pharmacol. Sci.* 31, 418–426. doi:10.1016/j.tips.2010.06.004

Brady, D. C., Crowe, M. S., Turski, M. L., Hobbs, G. A., Yao, X., Chaikuad, A., et al. (2014). Copper Is Required for Oncogenic BRAF Signalling and Tumorigenesis. *Nature* 509, 492–496. doi:10.1038/nature13180

Broadhurst, D., Goodacre, R., Reinke, S. N., Kuligowski, J., Wilson, I. D., Lewis, M. R., et al. (2018). Guidelines and Considerations for the Use of System Suitability and Quality Control Samples in Mass Spectrometry Assays Applied in Untargeted Clinical Metabolomic Studies. *Metabolomics* 14, 72. doi:10.1007/s11306-018-1367-3

Broadhurst, D. I., and Kell, D. B. (2007). Statistical Strategies for Avoiding False Discoveries in Metabolomics and Related Experiments. *Metabolomics* 2, 171–196. doi:10.1007/s11306-006-0037-z

Brown, A. P., and Greenberg, H. Z. (2016). Patch Clamp. *Br. J. Hosp. Med.* 77, C74–C77. doi:10.12968/hmed.2016.77.5.C74

Brown, M., Dunn, W. B., Ellis, D. I., Goodacre, R., Handl, J., Knowles, J. D., et al. (2005). A Metabolome Pipeline: From Concept to Data to Knowledge. *Metabolomics* 1, 39–51. doi:10.1007/s11306-005-1106-4

Cajka, T., and Fiehn, O. (2016). Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics. *Anal. Chem.* 88, 524–545. doi:10.1021/acs.analchem.5b04491

Cantor, J. R. (2019). The Rise of Physiologic Media. *Trends Cel Biol.* 29, 854–861. doi:10.1016/j.tcb.2019.08.009

Canul-Tec, J. C., Assal, R., Cirri, E., Legrand, P., Brier, S., Chamot-Rooke, J., et al. (2017). Structure and Allosteric Inhibition of Excitatory Amino Acid Transporter 1. *Nature* 544, 446–451. doi:10.1038/nature22064

Carbon, S., Douglass, E., Good, B. M., Unni, D. R., Harris, N. L., Mungall, C. J., et al. (2021). The Gene Ontology Resource: Enriching a GOld Mine. *Nucleic Acids Res.* 49, D325–D334. doi:10.1093/nar/gkaa1113

Carmosino, M., Rizzo, F., Torretta, S., Procino, G., and Svelto, M. (2013). High-Throughput Fluorescent-Based NKCC Functional Assay in Adherent Epithelial Cells. *BMC Cell Biol.* 14, 16. doi:10.1186/1471-2121-14-16

César-Razquin, A., Girardi, E., Yang, M., Brehme, M., Saez-Rodriguez, J., and Superti-Furga, G. (2018). In Silico prioritization of Transporter-Drug Relationships from Drug Sensitivity Screens. *Front. Pharmacol.* 9, 1–10. doi:10.3389/fphar.2018.01011

César-Razquin, A., Snijder, B., Frappier-Brinton, T., Isserlin, R., Gyimesi, G., Bai, X., et al. (2015). A Call for Systematic Research on Solute Carriers. *Cell* 162, 478–487. doi:10.1016/j.cell.2015.07.022

Chen, W. W., Freinkman, E., and Sabatini, D. M. (2017). Rapid Immunopurification of Mitochondria for Metabolite Profiling and Absolute Quantification of Matrix Metabolites. *Nat. Protoc.* 12, 2215–2231. doi:10.1038/nprot.2017.104

Cheng, Y., and Prusoff, W. H. (1973). Relationship between the Inhibition Constant (K1) and the Concentration of Inhibitor Which Causes 50 Per Cent Inhibition (I50) of an Enzymatic Reaction. *Biochem. Pharmacol.* 22, 3099–3108. doi:10.1016/0006-2952(73)90196-2

Chien, H.-C., Colas, C., Finke, K., Springer, S., Stoner, L., Zur, A. A., et al. (2018). Reevaluating the Substrate Specificity of the L-type Amino Acid Transporter (LAT1). *J. Med. Chem.* 61, 7358–7373. doi:10.1021/acs.jmedchem.8b01007

Coady, M. J., El Tarazi, A., Santer, R., Bissonnette, P., Sasseville, L. J., Calado, J., et al. (2017). MAP17 Is a Necessary Activator of Renal Na+/Glucose Cotransporter SGLT2. *Jasn* 28, 85–93. doi:10.1681/ASN.2015111282

Colas, C., Schlessinger, A., and Pajor, A. M. (2017). Mapping Functionally Important Residues in the Na+/Dicarboxylate Cotransporter, NaDC1. *Biochemistry* 56, 4432–4441. doi:10.1021/acs.biochem.7b00503

Coleman, J. A., Yang, D., Zhao, Z., Wen, P.-C., Yoshioka, C., Tajkhorshid, E., et al. (2019). Serotonin Transporter-Ibogaine Complexes Illuminate Mechanisms of Inhibition and Transport. *Nature* 569, 141–145. doi:10.1038/s41586-019-1135-1

Contreras-Baeza, Y., Sandoval, P. Y., Alarcón, R., Galaz, A., Cortés-Molina, F., Alegría, K., et al. (2019). Monocarboxylate Transporter 4 (MCT4) Is a High Affinity Transporter Capable of Exporting Lactate in High-Lactate Microenvironments. *J. Biol. Chem.* 294, 20135–20147. doi:10.1074/jbc.RA119.009093

Côté, M., Misasi, J., Ren, T., Bruchez, A., Lee, K., Filone, C. M., et al. (2011). Small Molecule Inhibitors Reveal Niemann-Pick C1 Is Essential for Ebola Virus Infection. *Nature* 477, 344–348. doi:10.1038/nature10380

Czuba, L. C., Hillgren, K. M., and Swaan, P. W. (2018). Post-translational Modifications of Transporters. *Pharmacol. Ther.* 192, 88–99. doi:10.1016/j.pharmthera.2018.06.013

Dana, H., Sun, Y., Mohar, B., Hulse, B. K., Kerlin, A. M., Hasseman, J. P., et al. (2019). High-performance Calcium Sensors for Imaging Activity in Neuronal Populations and Microcompartments. *Nat. Methods* 16, 649–657. doi:10.1038/s41592-019-0435-6

Danthi, S. J., Liang, B., Smicker, O., Coupland, B., Gregory, J., Gefteas, E., et al. (2019). Identification and Characterization of Inhibitors of a Neutral Amino Acid Transporter, SLC6A19, Using Two Functional Cell-Based Assays. *SLAS DISCOVERY: Advancing Sci. Drug Discov.* 24, 111–120. doi:10.1177/2472555218794627

Davies, M., Nowotka, M., Papadatos, G., Dedman, N., Gaulton, A., Atkinson, F., et al. (2015). ChEMBL Web Services: Streamlining Access to Drug Discovery Data and Utilities. *Nucleic Acids Res.* 43, W612–W620. doi:10.1093/nar/gkv352

De Bruyn, T., Ye, Z.-W., Peeters, A., Sahi, J., Baes, M., Augustijns, P. F., et al. (2011). Determination of OATP-, NTCP- and OCT-Mediated Substrate Uptake Activities in Individual and Pooled Batches of Cryopreserved Human Hepatocytes. *Eur. J. Pharm. Sci.* 43, 297–307. doi:10.1016/j.ejps.2011.05.002

Demian, W. L., Persaud, A., Jiang, C., Coyaud, É., Liu, S., Kapus, A., et al. (2019). The Ion Transporter NKCC1 Links Cell Volume to Cell Mass Regulation by Suppressing mTORC1. *Cel Rep.* 27, 1886–1896.e6. doi:10.1016/j.celrep.2019.04.034

Deng, D., Xu, C., Sun, P., Wu, J., Yan, C., Hu, M., et al. (2014). Crystal Structure of the Human Glucose Transporter GLUT1. *Nature* 510, 121–125. doi:10.1038/nature13306

Dickens, D., Rädisch, S., Chiduza, G. N., Giannoudis, A., Cross, M. J., Malik, H., et al. (2018). Cellular Uptake of the Atypical Antipsychotic Clozapine Is a Carrier-Mediated Process. *Mol. Pharmaceutics* 15, 3557–3572. doi:10.1021/acs.molpharmaceut.8b00547

Dobson, P. D., and Kell, D. B. (2008). Carrier-mediated Cellular Uptake of Pharmaceutical Drugs: An Exception or the Rule?. *Nat. Rev. Drug Discov.* 7, 205–220. doi:10.1038/nrd2438

Doornbos, M. L. J., and Heitman, L. H. (2019). *Label-free Impedance-Based Whole Cell Assay to Study GPCR Pharmacology*. 2nd ed.. Elsevier, 179–194. doi:10.1016/bs.mcb.2018.08.003

Doornbos, M. L. J., Van der Linden, I., Vereyken, L., Tresadern, G., IJzerman, A. P., Lavreysen, H., et al. (2018). Constitutive Activity of the Metabotropic Glutamate Receptor 2 Explored with a Whole-Cell Label-free Biosensor. *Biochem. Pharmacol.* 152, 201–210. doi:10.1016/j.bcp.2018.03.026

Dunn, W. B., Broadhurst, D., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., et al. (2011). Procedures for Large-Scale Metabolic Profiling of Serum and Plasma Using Gas Chromatography and Liquid Chromatography Coupled to Mass Spectrometry. *Nat. Protoc.* 6, 1060–1083. doi:10.1038/nprot.2011.335

Elias, C. L., Lukas, A., Shurraw, S., Scott, J., Omelchenko, A., Gross, G. J., et al. (2001). Inhibition of Na+/Ca2+ Exchange by KB-R7943: Transport Mode Selectivity and Antiarrhythmic Consequences. *Am. J. Physiology-Heart Circulatory Physiol.* 281, H1334–H1345. doi:10.1152/ajpheart.2001.281.3.h1334

Erdman, A. R., Mangravite, L. M., Urban, T. J., Lagpacan, L. L., Castro, R. A., de la Cruz, M., et al. (2006). The Human Organic Anion Transporter 3 (OAT3; SLC22A8): Genetic Variation and Functional Genomics. *Am. J. Physiology-Renal Physiol.* 290, F905–F912. doi:10.1152/ajprenal.00272.2005

Fan, F., Binkowski, B. F., Butler, B. L., Stecha, P. F., Lewis, M. K., and Wood, K. V. (2008). Novel Genetically Encoded Biosensors Using Firefly Luciferase. *ACS Chem. Biol.* 3, 346–351. doi:10.1021/cb8000414

Fardel, O., Le Vee, M., Jouan, E., Denizot, C., and Parmentier, Y. (2015). Nature and Uses of Fluorescent Dyes for Drug Transporter Studies. *Expert Opin. Drug Metab. Toxicol.* 11, 1233–1251. doi:10.1517/17425255.2015.1053462

Fauster, A., Rebsamen, M., Willmann, K. L., César-Razquin, A., Girardi, E., Bigenzahn, J. W., et al. (2018). Systematic Genetic Mapping of Necroptosis Identifies SLC39A7 as Modulator of Death Receptor Trafficking. *Cell Death Differ* 26, 1138–1155. doi:10.1038/s41418-018-0192-6

FDA (2020). Clinical Drug Interaction Studies — Cytochrome P450 Enzyme- and Drug Interactions Guidance for Industry. *FDA Guid. Doc.* 1, 1–27.

Felmlee, M. A., Jones, R. S., Rodriguez-Cruz, V., Follman, K. E., and Morris, M. E. (2020). Monocarboxylate Transporters (SLC16): Function, Regulation, and Role in Health and Disease. *Pharmacol. Rev.* 72, 466–485. doi:10.1124/pr.119.018762

Feng, J., Zhang, C., Lischinsky, J. E., Jing, M., Zhou, J., Wang, H., et al. (2019). A Genetically Encoded Fluorescent Sensor for Rapid and Specific *In Vivo* Detection of Norepinephrine. *Neuron* 102, 745–761.e8. doi:10.1016/j.neuron.2019.02.037

Forbes, J. R., and Gros, P. (2003). Iron, Manganese, and Cobalt Transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) Expressed at the Plasma Membrane. *Blood* 102, 1884–1892. doi:10.1182/blood-2003-02-0425

Fotiadis, D., Kanai, Y., and Palacín, M. (2013). The SLC3 and SLC7 Families of Amino Acid Transporters. *Mol. Aspects Med.* 34, 139–158. doi:10.1016/j.mam.2012.10.007

Galietta, L. J. V., Haggie, P. M., and Verkman, A. S. (2001). Green Fluorescent Protein-Based Halide Indicators with Improved Chloride and Iodide Affinities. *FEBS Lett.* 499, 220–224. doi:10.1016/S0014-5793(01)02561-3

Garibsingh, R.-A. A., Otte, N. J., Ndaru, E., Colas, C., Grewer, C., Holst, J., et al. (2018). Homology Modeling Informs Ligand Discovery for the Glutamine Transporter ASCT2. *Front. Chem.* 6, 1–12. doi:10.3389/fchem.2018.00279

Garibsingh, R.-A. A., and Schlessinger, A. (2019). Advances and Challenges in Rational Drug Design for SLCs. *Trends Pharmacol. Sci.* 40, 790–800. doi:10.1016/j.tips.2019.08.006

Gaulton, A., Hersey, A., Nowotka, M., Bento, A. P., Chambers, J., Mendez, D., et al. (2017). The ChEMBL Database in 2017. *Nucleic Acids Res.* 45, D945–D954. doi:10.1093/nar/gkw1074

Geibel, S., Flores-Herr, N., Licher, T., and Vollert, H. (2006). Establishment of Cell-free Electrophysiology for Ion Transporters: Application for Pharmacological Profiling. *J. Biomol. Screen.* 11, 262–268. doi:10.1177/1087057105285110

Geier, E. G., Schlessinger, A., Fan, H., Gable, J. E., Irwin, J. J., Sali, A., et al. (2013). Structure-based Ligand Discovery for the Large-Neutral Amino Acid Transporter 1, LAT-1. *Proc. Natl. Acad. Sci.* 110, 5480–5485. doi:10.1073/pnas.1218165110

Gerbeth-Kreul, C., Pommereau, A., Ruf, S., Kane, J. L., Kuntzweiler, T., Hessler, G., et al. (2021). A Solid Supported Membrane-Based Technology for Electrophysical Screening of B0AT1-Modulating Compounds. *SLAS DISCOVERY: Advancing Sci. Drug Discov.* 26, 783–797. doi:10.1177/24725552211011180

Gerry, C. J., and Schreiber, S. L. (2020). Unifying Principles of Bifunctional, Proximity-Inducing Small Molecules. *Nat. Chem. Biol.* 16, 369–378. doi:10.1038/s41589-020-0469-1

Giacomini, K. M., Giacomini, K. M., Huang, S. M., Tweedie, D. J., Benet, L. Z., Brouwer, K. L., et al. (2010). Membrane Transporters in Drug Development. *Nat. Rev. Drug Discov.* 9, 215–236. doi:10.1038/nrd3028

Girardi, E., César-Razquin, A., Lindinger, S., Papakostas, K., Konecka, J., Hemmerich, J., et al. (2020a). A Widespread Role for SLC Transmembrane Transporters in Resistance to Cytotoxic Drugs. *Nat. Chem. Biol.* 16, 469–478. doi:10.1038/s41589-020-0483-3

Girardi, E., Fiume, G., Goldmann, U., Sin, C., Müller, F., Sedlyarov, V., et al. (2020b). *A Systematic Genetic Interaction Map of Human Solute Carriers Assigns a Role to SLC25A51/MCART1 in Mitochondrial NAD Uptake*. bioRxiv, Cold Spring Harbor Laboratories New York, 275818. doi:10.1101/2020.08.31.275818

Goodsell, D. S., Autin, L., and Olson, A. J. (2019). Illustrate: Software for Biomolecular Illustration. *Structure* 27, 1716–1720.e1. doi:10.1016/j.str.2019.08.011

Greenwald, E. C., Mehta, S., and Zhang, J. (2018). Genetically Encoded Fluorescent Biosensors Illuminate the Spatiotemporal Regulation of Signaling Networks. *Chem. Rev.* 118, 11707–11794. doi:10.1021/acs.chemrev.8b00333

Gruenwald, K., Holland, J. T., Stromberg, V., Ahmad, A., Watcharakichkorn, D., and Okumoto, S. (2012). Visualization of Glutamine Transporter Activities in Living Cells Using Genetically Encoded Glutamine Sensors. *PLoS One* 7, e38591–13. doi:10.1371/journal.pone.0038591

Gründemann, D., Harlfinger, S., Golz, S., Geerts, A., Lazar, A., Berkels, R., et al. (2005). Discovery of the Ergothioneine Transporter. *Proc. Natl. Acad. Sci.* 102, 5256–5261. doi:10.1073/pnas.0408624102

Gyimesi, G. (2020). SLC66 Lysosomal Amino Acid Transporters (Version 2020.5) in the IUPHAR/BPS Guide to Pharmacology Database. *GtoPdb CITE* 2020. doi:10.2218/gtopdb/F1048/2020.5

Haase, J., Grudzinska-Goebel, J., Müller, H. K., Münster-Wandowski, A., Chow, E., Wynne, K., et al. (2017). Serotonin Transporter Associated Protein Complexes Are Enriched in Synaptic Vesicle Proteins and Proteins Involved in Energy Metabolism and Ion Homeostasis. *ACS Chem. Neurosci.* 8, 1101–1116. doi:10.1021/acschemneuro.6b00437

Halai, R., and Cooper, M. A. (2012). Using Label-free Screening Technology to Improve Efficiency in Drug Discovery. *Expert Opin. Drug Discov.* 7, 123–131. doi:10.1517/17460441.2012.651121

Hall, M. D., Yasgar, A., Peryea, T., Braisted, J. C., Jadhav, A., Simeonov, A., et al. (2016). Fluorescence Polarization Assays in High-Throughput Screening and Drug Discovery: A Review. *Methods Appl. Fluoresc.* 4, 022001. doi:10.1088/2050-6120/4/2/022001

Hashimoto, M., Girardi, E., Eichner, R., and Superti-Furga, G. (2018). Detection of Chemical Engagement of Solute Carrier Proteins by a Cellular Thermal Shift Assay. *ACS Chem. Biol.* 13, 1480–1486. doi:10.1021/acschembio.8b00270

Hediger, M. A., Clémençon, B., Burrier, R. E., and Bruford, E. A. (2013). The ABCs of Membrane Transporters in Health and Disease (SLC Series): Introduction. *Mol. Aspects Med.* 34, 95–107. doi:10.1016/j.mam.2012.12.009

Henderson, M. J., Holbert, M. A., Simeonov, A., and Kallal, L. A. (2019). High-Throughput Cellular Thermal Shift Assays in Research and Drug Discovery. *SLAS DISCOVERY: Advancing Sci. Drug Discov.* 25, 137–147. doi:10.1177/2472555219877183

Herzig, S., Raemy, E., Montessuit, S., Veuthey, J.-L., Zamboni, N., Westermann, B., et al. (2012). Identification and Functional Expression of the Mitochondrial Pyruvate Carrier. *Science* 337, 93–96. doi:10.1126/science.1218530

Hisano, Y., Kobayashi, N., Yamaguchi, A., and Nishi, T. (2012). Mouse SPNS2 Functions as a Sphingosine-1-Phosphate Transporter in Vascular Endothelial Cells. *PLoS One* 7, e38941. doi:10.1371/journal.pone.0038941

Hofmaier, T., Luf, A., Seddik, A., Stockner, T., Holy, M., Freissmuth, M., et al. (2014). Aminorex, a Metabolite of the Cocaine Adulterant Levamisole, Exerts Amphetamine like Actions at Monoamine Transporters. *Neurochem. Int.* 73, 32–41. doi:10.1016/j.neuint.2013.11.010

Höglund, P. J., Nordström, K. J. V., Schiöth, H. B., and Fredriksson, R. (2011). The Solute Carrier Families Have a Remarkably Long Evolutionary History with the Majority of the Human Families Present before Divergence of Bilaterian Species. *Mol. Biol. Evol.* 28, 1531–1541. doi:10.1093/molbev/msq350

Hoover, R. S., Poch, E., Monroy, A., Vázquez, N., Nishio, T., Gamba, G., et al. (2003). N-glycosylation at Two Sites Critically Alters Thiazide Binding and Activity of the Rat Thiazide-Sensitive Na+:Cl− Cotransporter. *Jasn* 14, 271–282. doi:10.1097/01.ASN.0000043903.93452.D0

Hou, B.-H., Takanaga, H., Grossmann, G., Chen, L.-Q., Qu, X.-Q., Jones, A. M., et al. (2011). Optical Sensors for Monitoring Dynamic Changes of Intracellular Metabolite Levels in Mammalian Cells. *Nat. Protoc.* 6, 1818–1833. doi:10.1038/nprot.2011.392

Hu, G., Henke, A., Karpowicz, R. J., Sonders, M. S., Farrimond, F., Edwards, R., et al. (2013). New Fluorescent Substrate Enables Quantitative and High-Throughput Examination of Vesicular Monoamine Transporter 2 (VMAT2). *ACS Chem. Biol.* 8, 1947–1954. doi:10.1021/cb400259n

Huang, A., Garraway, L. A., Ashworth, A., and Weber, B. (2020). Synthetic Lethality as an Engine for Cancer Drug Target Discovery. *Nat. Rev. Drug Discov.* 19, 23–38. doi:10.1038/s41573-019-0046-z

Huard, K., Brown, J., Jones, J. C., Cabral, S., Futatsugi, K., Gorgoglione, M., et al. (2015). Discovery and Characterization of Novel Inhibitors of the Sodium-Coupled Citrate Transporter (NaCT or SLC13A5). *Sci. Rep.* 5, 1–13. doi:10.1038/srep17391

Huard, K., Gosset, J. R., Montgomery, J. I., Gilbert, A., Hayward, M. M., Magee, T. V., et al. (2016). Optimization of a Dicarboxylic Series for *In Vivo* Inhibition of Citrate Transport by the Solute Carrier 13 (SLC13) Family. *J. Med. Chem.* 59, 1165–1175. doi:10.1021/acs.jmedchem.5b01752

Huber, K. V. M., Olek, K. M., Müller, A. C., Tan, C. S. H., Bennett, K. L., Colinge, J., et al. (2015). Proteome-wide Drug and Metabolite Interaction Mapping by thermal-stability Profiling. *Nat. Methods* 12, 1055–1057. doi:10.1038/nmeth.3590

Huynh, K. W., Jiang, J., Abuladze, N., Tsirulnikov, K., Kao, L., Shao, X., et al. (2018). CryoEM Structure of the Human SLC4A4 Sodium-Coupled Acid-Base Transporter NBCe1. *Nat. Commun.* 9. doi:10.1038/s41467-018-03271-3

Iamshanova, O., Mariot, P., Lehen'kyi, V. Y., and Prevarskaya, N. (2016). Comparison of Fluorescence Probes for Intracellular Sodium Imaging in Prostate Cancer Cell Lines. *Eur. Biophys. J.* 45, 765–777. doi:10.1007/s00249-016-1173-7

Irwin, J. J., and Shoichet, B. K. (2016). Docking Screens for Novel Ligands Conferring New Biology. *J. Med. Chem.* 59, 4103–4120. doi:10.1021/acs.jmedchem.5b02008

Jafari, R., Almqvist, H., Axelsson, H., Ignatushchenko, M., Lundbäck, T., Nordlund, P., et al. (2014). The Cellular thermal Shift Assay for Evaluating Drug Target Interactions in Cells. *Nat. Protoc.* 9, 2100–2122. doi:10.1038/nprot.2014.138

Jaldin-Fincati, J. R., Pavarotti, M., Frendo-Cumbo, S., Bilan, P. J., and Klip, A. (2017). Update on GLUT4 Vesicle Traffic: A Cornerstone of Insulin Action. *Trends Endocrinol. Metab.* 28, 597–611. doi:10.1016/j.tem.2017.05.002

Jardetzky, O. (1966). Simple Allosteric Model for Membrane Pumps. *Nature* 211, 969–970. doi:10.1038/211969a0

Jensen, A. A., and Bräuner-Osborne, H. (2004). Pharmacological Characterization of Human Excitatory Amino Acid Transporters EAAT1, EAAT2 and EAAT3 in a Fluorescence-Based Membrane Potential Assay. *Biochem. Pharmacol.* 67, 2115–2127. doi:10.1016/j.bcp.2004.02.013

Jeon, Y. J., Khelifa, S., Ratnikov, B., Scott, D. A., Feng, Y., Parisi, F., et al. (2015). Regulation of Glutamine Carrier Proteins by RNF5 Determines Breast Cancer Response to ER Stress-Inducing Chemotherapies. *Cancer Cell* 27, 354–369. doi:10.1016/j.ccell.2015.02.006

Jiménez-Valerio, G., Martínez-Lozano, M., Bassani, N., Vidal, A., Ochoa-de-Olza, M., Suárez, C., et al. (2016). Resistance to Antiangiogenic Therapies by Metabolic Symbiosis in Renal Cell Carcinoma PDX Models and Patients. *Cel Rep.* 15, 1134–1143. doi:10.1016/j.celrep.2016.04.015

Jing, M., Zhang, P., Wang, G., Feng, J., Mesik, L., Zeng, J., et al. (2018). A Genetically Encoded Fluorescent Acetylcholine Indicator for *In Vitro* and *In Vivo* Studies. *Nat. Biotechnol.* 36, 726–737. doi:10.1038/nbt.4184

Kandasamy, P., Gyimesi, G., Kanai, Y., and Hediger, M. A. (2018). Amino Acid Transporters Revisited: New Views in Health and Disease. *Trends Biochem. Sci.* 43, 752–789. doi:10.1016/j.tibs.2018.05.003

Kawahara, A., Nishi, T., Hisano, Y., Fukui, H., Yamaguchi, A., and Mochizuki, N. (2009). The Sphingolipid Transporter Spns2 Functions in Migration of Zebrafish Myocardial Precursors. *Science* 323, 524–527. doi:10.1126/science.1167449

Kawatkar, A., Schefter, M., Hermansson, N.-O., Snijder, A., Dekker, N., Brown, D. G., et al. (2019). CETSA beyond Soluble Targets: a Broad Application to Multipass Transmembrane Proteins. *ACS Chem. Biol.* 14, 1913–1920. doi:10.1021/acschembio.9b00399

Keller, J. P., Marvin, J. S., Lacin, H., Lemon, W. C., Shea, J., Kim, S., et al. (2019). *In Vivoglucose Imaging in Multiple Model Organisms with an Engineered Single-Wavelength Sensor*. bioRxiv, Cold Spring Harbor Laboratories New York, 571422. doi:10.1101/571422

King, M. S., Kerr, M., Crichton, P. G., Springett, R., and Kunji, E. R. S. (2016). Formation of a Cytoplasmic Salt Bridge Network in the Matrix State Is a Fundamental Step in the Transport Mechanism of the Mitochondrial ADP/ATP Carrier. *Biochim. Biophys. Acta (Bba) - Bioenerg.* 1857, 14–22. doi:10.1016/j.bbabio.2015.09.013

Kobayashi, N., Kawasaki-Nishi, S., Otsuka, M., Hisano, Y., Yamaguchi, A., and Nishi, T. (2018). MFSD2B Is a Sphingosine 1-phosphate Transporter in Erythroid Cells. *Sci. Rep.* 8, 1–11. doi:10.1038/s41598-018-23300-x

Konz, T., Migliavacca, E., Dayon, L., Bowman, G., Oikonomidi, A., Popp, J., et al. (2017). ICP-MS/MS-Based Ionomics: A Validated Methodology to Investigate the Biological Variability of the Human Ionome. *J. Proteome Res.* 16, 2080–2090. doi:10.1021/acs.jproteome.7b00055

Kory, N., Wyant, G. A., Prakash, G., uit de Bos, J., Bottanelli, F., Pacold, M. E., et al. (2018). SFXN1 Is a Mitochondrial Serine Transporter Required for One-Carbon Metabolism. *Science* 362, eaat9528. doi:10.1126/science.aat9528

Kost, T. A., Condreay, J. P., and Jarvis, D. L. (2005). Baculovirus as Versatile Vectors for Protein Expression in Insect and Mammalian Cells. *Nat. Biotechnol.* 23, 567–575. doi:10.1038/nbt1095

Kuner, T., and Augustine, G. J. (2000). A Genetically Encoded Ratiometric Indicator for Chloride. *Neuron* 27, 447–459. doi:10.1016/S0896-6273(00)00056-8

Kuznetsov, A. V., Veksler, V., Gellerich, F. N., Saks, V., Margreiter, R., and Kunz, W. S. (2008). Analysis of Mitochondrial Function *In Situ* in Permeabilized Muscle Fibers, Tissues and Cells. *Nat. Protoc.* 3, 965–976. doi:10.1038/nprot.2008.61

Lee, C., Visen, N. S., Dhalla, N. S., Le, H. D., Isaac, M., Choptiany, P., et al. (2004). Inhibitory Profile of SEA0400 [2-[4-[(2,5-Difluorophenyl) methoxy]phenoxy]-5-Ethoxyaniline] Assessed on the Cardiac Na+-Ca2+Exchanger, NCX1.1. *J. Pharmacol. Exp. Ther.* 311, 748–757. doi:10.1124/jpet.104.070805

Lisinski, I., Schürmann, A., Joost, H.-G., Cushman, S. W., and Al-Hasani, H. (2001). Targeting of GLUT6 (Formerly GLUT9) and GLUT8 in Rat Adipose Cells. *Biochem. J.* 358, 517–522. doi:10.1042/0264-6021:358051710.1042/bj3580517

Lomenick, B., Hao, R., Jonai, N., Chin, R. M., Aghajan, M., Warburton, S., et al. (2009). Target Identification Using Drug Affinity Responsive Target Stability (DARTS). *Proc. Natl. Acad. Sci.* 106, 21984–21989. doi:10.1073/pnas.0910040106

Lopes-Pacheco, M. (2020). CFTR Modulators: The Changing Face of Cystic Fibrosis in the Era of Precision Medicine. *Front. Pharmacol.* 10, 1–29. doi:10.3389/fphar.2019.01662

Ma, Q., Ye, L., Liu, H., Shi, Y., and Zhou, N. (2017). An Overview of Ca2+mobilization Assays in GPCR Drug Discovery. *Expert Opin. Drug Discov.* 12, 511–523. doi:10.1080/17460441.2017.1303473

Maglich, J. M., Sluder, A., Guan, X., Shi, Y., McKee, D. D., Carrick, K., et al. (2001). Comparison of Complete Nuclear Receptor Sets from the Human, *Caenorhabditis elegans* and Drosophila Genomes. *Genome Biol.* 2, 1–7. doi:10.1186/gb-2001-2-8-research0029

Mahadevan, V., Khademullah, C. S., Dargaei, Z., Chevrier, J., Uvarov, P., Kwan, J., et al. (2017). Native KCC2 Interactome Reveals PACSIN1 as a Critical Regulator of Synaptic Inhibition. *Elife* 6, 1–34. doi:10.7554/eLife.28270.001

Malinouski, M., Hasan, N. M., Zhang, Y., Seravalli, J., Lin, J., Avanesov, A., et al. (2014). Genome-wide RNAi Ionomics Screen Reveals New Genes and Regulation of Human Trace Element Metabolism. *Nat. Commun.* 5, 3301. doi:10.1038/ncomms4301

Martinez, N. J., Asawa, R. R., Cyr, M. G., Zakharov, A., Urban, D. J., Roth, J. S., et al. (2018). A Widely-Applicable High-Throughput Cellular thermal Shift Assay (CETSA) Using Split Nano Luciferase. *Sci. Rep.* 8, 9472. doi:10.1038/s41598-018-27834-y

Marvin, J. S., Borghuis, B. G., Tian, L., Cichon, J., Harnett, M. T., Akerboom, J., et al. (2013). An Optimized Fluorescent Probe for Visualizing Glutamate Neurotransmission. *Nat. Methods* 10, 162–170. doi:10.1038/nmeth.2333

Marvin, J. S., Shimoda, Y., Magloire, V., Leite, M., Kawashima, T., Jensen, T. P., et al. (2019). A Genetically Encoded Fluorescent Sensor for *In Vivo* Imaging of GABA. *Nat. Methods* 16, 763–770. doi:10.1038/s41592-019-0471-2

Mazurek, M. P., Prasad, P. D., Gopal, E., Fraser, S. P., Bolt, L., Rizaner, N., et al. (2010). Molecular Origin of Plasma Membrane Citrate Transporter in Human Prostate Epithelial Cells. *EMBO Rep.* 11, 431–437. doi:10.1038/embor.2010.51

McAlear, S. D., Liu, X., Williams, J. B., McNicholas-Bevensee, C. M., and Bevensee, M. O. (2006). Electrogenic Na/HCO3 Cotransporter (NBCe1) Variants Expressed in Xenopus Oocytes: Functional Comparison and Roles of the Amino and Carboxy Termini. *J. Gen. Physiol.* 127, 639–658. doi:10.1085/jgp.200609520

McMillan, E. A., Ryu, M.-J., Diep, C. H., Mendiratta, S., Clemenceau, J. R., Vaden, R. M., et al. (2018). Chemistry-First Approach for Nomination of Personalized Treatment in Lung Cancer. *Cell* 173, 864–878.e29. doi:10.1016/j.cell.2018.03.028

Meixner, E., Goldmann, U., Sedlyarov, V., Scorzoni, S., Rebsamen, M., Girardi, E., et al. (2020). A Substrate-based Ontology for Human Solute Carriers. *Mol. Syst. Biol.* 16, 1–9. doi:10.15252/msb.20209652

Miesenböck, G., De Angelis, D. A., and Rothman, J. E. (1998). Visualizing Secretion and Synaptic Transmission with pH-Sensitive green Fluorescent Proteins. *Nature* 394, 192–195. doi:10.1038/28190

Mita, S., Suzuki, H., Akita, H., Hayashi, H., Onuki, R., Hofmann, A. F., et al. (2006). Inhibition of Bile Acid Transport across Na+/taurocholate Cotransporting Polypeptide (SLC10A1) and Bile Salt export Pump (ABCB 11)-coexpressing LLC-PK1 Cells by Cholestasis-Inducing Drugs. *Drug Metab. Dispos.* 34, 1575–1581. doi:10.1124/dmd.105.008748

Molina, D. M., Jafari, R., Ignatushchenko, M., Seki, T., Larsson, E. A., Dan, C., et al. (2013). Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular thermal Shift Assay. *Science* 341, 84–87. doi:10.1126/science.1233606

Morioka, S., Perry, J. S. A., Raymond, M. H., Medina, C. B., Zhu, Y., Zhao, L., et al. (2018). Efferocytosis Induces a Novel SLC Program to Promote Glucose Uptake and Lactate Release. *Nature* 563, 714–718. doi:10.1038/s41586-018-0735-5

Mullard, G., Allwood, J. W., Weber, R., Brown, M., Begley, P., Hollywood, K. A., et al. (2015). A New Strategy for MS/MS Data Acquisition Applying Multiple Data Dependent Experiments on Orbitrap Mass Spectrometers in Non-targeted Metabolomic Applications. *Metabolomics* 11, 1068–1080. doi:10.1007/s11306-014-0763-6

Nakai, J., Ohkura, M., and Imoto, K. (2001). A High Signal-To-Noise Ca2+ Probe Composed of a Single green Fluorescent Protein. *Nat. Biotechnol.* 19, 137–141. doi:10.1038/84397

Nakata, T., Creasey, E. A., Kadoki, M., Lin, H., Selig, M. K., Yao, J., et al. (2020). A Missense Variant inSLC39A8confers Risk for Crohn's Disease by Disrupting Manganese Homeostasis and Intestinal Barrier Integrity. *Proc. Natl. Acad. Sci. USA* 117, 28930–28938. doi:10.1073/pnas.2014742117

Nanion Technologies Munich (2021). The Solid Supported Membrane (SSM): "Measuring Transporter Currents" and "SURFE^{2}R 96SE - High Throughput Transporter Research". Available at: https://www.nanion.de/en/products/surfe2r-96se.html.

Neher, E., and Sakmann, B. (1976). Single-channel Currents Recorded from Membrane of Denervated Frog Muscle Fibres. *Nature* 260, 799–802. doi:10.1038/260799a0

Nimigean, C. M. (2006). A Radioactive Uptake Assay to Measure Ion Transport across Ion Channel-Containing Liposomes. *Nat. Protoc.* 1, 1207–1212. doi:10.1038/nprot.2006.166

Nolin, E., Gans, S., Llamas, L., Bandyopadhyay, S., Brittain, S. M., Bernasconi-Elias, P., et al. (2019). Discovery of a ZIP7 Inhibitor from a Notch Pathway Screen. *Nat. Chem. Biol.* 15, 179–188. doi:10.1038/s41589-018-0200-7

Noor, Z. N., Deitmer, J. W., and Theparambil, S. M. (2018). Cytosolic Sodium Regulation in Mouse Cortical Astrocytes and Its Dependence on Potassium and Bicarbonate. *J. Cellular Physiol.* 234 (1), 89–99. doi:10.1002/jcp.26824

Núñez, E., López-Corcuera, B., Vázquez, J., Giménez, C., and Aragón, C. (2000). Differential Effects of the Tricyclic Antidepressant Amoxapine on glycine Uptake Mediated by the Recombinant GLYT1 and GLYT2 glycine Transporters. *Br. J. Pharmacol.* 129, 200–206. doi:10.1038/sj.bjp.0703049

O'Hagan, S., Wright Muelas, M., Day, P. J., Lundberg, E., and Kell, D. B. (2018). GeneGini: Assessment via the Gini Coefficient of Reference "Housekeeping" Genes and Diverse Human Transporter Expression Profiles. *Cell Syst* 6, 230–244.e1. doi:10.1016/j.cels.2018.01.003

O'Hagan, S., and Kell, D. B. (2020). Structural Similarities between Some Common Fluorophores Used in Biology, Marketed Drugs, Endogenous Metabolites, and Natural Products. *Mar. Drugs* 18. doi:10.3390/md18110582

Okamoto, N., Viswanatha, R., Bittar, R., Li, Z., Haga-Yamanaka, S., Perrimon, N., et al. (2018). A Membrane Transporter Is Required for Steroid Hormone Uptake in Drosophila. *Dev. Cel* 47, 294–305.e7. doi:10.1016/j.devcel.2018.09.012

Oliver, S., Winson, M. K., Kell, D. B., and Baganz, F. (1998). Systematic Functional Analysis of the Yeast Genome. *Trends Biotechnol.* 16, 373–378. doi:10.1016/s0167-7799(98)01214-1

Ozkan, P., and Mutharasan, R. (2002). A Rapid Method for Measuring Intracellular pH Using BCECF-AM. *Biochim. Biophys. Acta (Bba) - Gen. Subjects* 1572, 143–148. doi:10.1016/S0304-4165(02)00303-3

Pajor, A. M., de Oliveira, C. A., Song, K., Huard, K., Shanmugasundaram, V., and Erion, D. M. (2016). Molecular Basis for Inhibition of the Na+/Citrate Transporter NaCT (SLC13A5) by Dicarboxylate Inhibitors. *Mol. Pharmacol.* 90, 755–765. doi:10.1124/mol.116.105049

Park, S.-J., Kim, B., Choi, S., Balasubramaniam, S., Lee, S.-C., Lee, J. Y., et al. (2019). Imaging Inflammation Using an Activated Macrophage Probe with Slc18b1 as the Activation-Selective Gating Target. *Nat. Commun.* 10, 1–7. doi:10.1038/s41467-019-08990-9

Parker, C. G., Galmozzi, A., Wang, Y., Correia, B. E., Sasaki, K., Joslyn, C. M., et al. (2017a). Ligand and Target Discovery by Fragment-Based Screening in Human Cells. *Cell* 168, 527–541.e29. doi:10.1016/j.cell.2016.12.029

Parker, C. G., Kuttruff, C. A., Galmozzi, A., Jørgensen, L., Yeh, C.-H., Hermanson, D. J., et al. (2017b). Chemical Proteomics Identifies SLC25A20 as a Functional Target of the Ingenol Class of Actinic Keratosis Drugs. *ACS Cent. Sci.* 3, 1276–1285. doi:10.1021/acscentsci.7b00420

Parker, C. G., and Pratt, M. R. (2020). Click Chemistry in Proteomic Investigations. *Cell* 180, 605–632. doi:10.1016/j.cell.2020.01.025

Passioura, T., Watashi, K., Fukano, K., Shimura, S., Saso, W., Morishita, R., et al. (2018). De Novo Macrocyclic Peptide Inhibitors of Hepatitis B Virus Cellular Entry. *Cel Chem. Biol.* 25, 906–915.e5. doi:10.1016/j.chembiol.2018.04.011

Patriarchi, T., Cho, J. R., Merten, K., Howe, M. W., Marley, A., Xiong, W.-H., et al. (2018). Ultrafast Neuronal Imaging of Dopamine Dynamics with Designed Genetically Encoded Sensors. *Science* 360, eaat4422. doi:10.1126/science.aat4422

Pedersen, N. B., Carlsson, M. C., and Pedersen, S. F. (2016). Glycosylation of Solute Carriers: Mechanisms and Functional Consequences. *Pflugers Arch. - Eur. J. Physiol.* 468, 159–176. doi:10.1007/s00424-015-1730-4

Perland, E., and Fredriksson, R. (2017). Classification Systems of Secondary Active Transporters. *Trends Pharmacol. Sci.* 38, 305–315. doi:10.1016/j.tips.2016.11.008

Pinky, N. F., Wilkie, C. M., Barnes, J. R., and Parsons, M. P. (2018). Region- and Activity-dependent Regulation of Extracellular Glutamate. *J. Neurosci.* 38, 5351–5366. doi:10.1523/JNEUROSCI.3213-17.2018

Pinne, M., and Raucy, J. L. (2014). Advantages of Cell-Based High-Volume Screening Assays to Assess Nuclear Receptor Activation during Drug Discovery. *Expert Opin. Drug Discov.* 9, 669–686. doi:10.1517/17460441.2014.913019

Pisarsky, L., Bill, R., Fagiani, E., Dimeloe, S., Goosen, R. W., Hagmann, J., et al. (2016). Targeting Metabolic Symbiosis to Overcome Resistance to Anti-angiogenic Therapy. *Cel Rep.* 15, 1161–1174. doi:10.1016/j.celrep.2016.04.028

Pizzagalli, F., Varga, Z., Huber, R. D., Folkers, G., Meier, P. J., and St.-Pierre, M. V. (2003). Identification of Steroid Sulfate Transport Processes in the Human Mammary Gland. *J. Clin. Endocrinol. Metab.* 88, 3902–3912. doi:10.1210/jc.2003-030174

Pizzagalli, M. D., Bensimon, A., and Superti-Furga, G. (2020). A Guide to Plasma Membrane Solute Carrier Proteins. *FEBS J.* 288, 2784–2835. doi:10.1111/febs.15531

Pomorski, A., Kochańczyk, T., Miłoch, A., and Krężel, A. (2013). Method for Accurate Determination of Dissociation Constants of Optical Ratiometric Systems: Chemical Probes, Genetically Encoded Sensors, and Interacting Molecules. *Anal. Chem.* 85, 11479–11486. doi:10.1021/ac402637h

Puris, E., Gynther, M., Auriola, S., and Huttunen, K. M. (2020). L-type Amino Acid Transporter 1 as a Target for Drug Delivery. *Pharm. Res.* 37. doi:10.1007/s11095-020-02826-8

Rajagopal, S., and Shenoy, S. K. (2018). GPCR Desensitization: Acute and Prolonged Phases. *Cell Signal.* 41, 9–16. doi:10.1016/j.cellsig.2017.01.024

Rajarathnam, K., and Rösgen, J. (2014). Isothermal Titration Calorimetry of Membrane Proteins - Progress and Challenges. *Biochim. Biophys. Acta (Bba) - Biomembranes* 1838, 69–77. doi:10.1016/j.bbamem.2013.05.023

Rebsamen, M., Pochini, L., Stasyk, T., de Araújo, M. E. G., Galluccio, M., Kandasamy, R. K., et al. (2015). SLC38A9 Is a Component of the Lysosomal Amino Acid Sensing Machinery that Controls mTORC1. *Nature* 519, 477–481. doi:10.1038/nature14107

Reckzeh, E. S., Karageorgis, G., Schwalfenberg, M., Ceballos, J., Nowacki, J., Stroet, M. C. M., et al. (2019). Inhibition of Glucose Transporters and Glutaminase Synergistically Impairs Tumor Cell Growth. *Cel Chem. Biol.* 26, 1214–1228.e25. doi:10.1016/j.chembiol.2019.06.005

Reed, M. J., Purohit, A., Woo, L. W. L., Newman, S. P., and Potter, B. V. L. (2005). Steroid Sulfatase: Molecular Biology, Regulation, and Inhibition. *Endocr. Rev.* 26, 171–202. doi:10.1210/er.2004-0003

Reinhard, F. B. M., Eberhard, D., Werner, T., Franken, H., Childs, D., Doce, C., et al. (2015). Thermal Proteome Profiling Monitors Ligand Interactions with Cellular Membrane Proteins. *Nat. Methods* 12, 1129–1131. doi:10.1038/nmeth.3652

Reynolds, A., Parris, A., Evans, L. A., Lindqvist, S., Sharp, P., Lewis, M., et al. (2007). Dynamic and Differential Regulation of NKCC1 by Calcium and cAMP in the Native Human Colonic Epithelium. *J. Physiol.* 582, 507–524. doi:10.1113/jphysiol.2007.129718

Richter, M., Moroniak, S. J., and Michel, H. (2019). Identification of Competitive Inhibitors of the Human Taurine Transporter TauT in a Human Kidney Cell Line. *Pharmacol. Rep.* 71, 121–129. doi:10.1016/j.pharep.2018.10.005

Robers, M. B., Friedman-Ohana, R., Huber, K. V. M., Kilpatrick, L., Vasta, J. D., Berger, B.-T., et al. (2020). Quantifying Target Occupancy of Small Molecules within Living Cells. *Annu. Rev. Biochem.* 89, 557–581. doi:10.1146/annurev-biochem-011420-092302

Rossiter, N. J., Huggler, K. S., Adelmann, C. H., Keys, H. R., Soens, R. W., Sabatini, D. M., et al. (2021). CRISPR Screens in Physiologic Medium Reveal Conditionally Essential Genes in Human Cells. *Cel Metab.* 33, 1248–1263.e9. doi:10.1016/j.cmet.2021.02.005

Rubaiy, H. N. (2017). A Short Guide to Electrophysiology and Ion Channels. *J. Pharm. Pharm. Sci.* 20, 48–67. doi:10.18433/J32P6R

Sainz, B., Barretto, N., Martin, D. N., Hiraga, N., Imamura, M., Hussain, S., et al. (2012). Identification of the Niemann-Pick C1-like 1 Cholesterol Absorption Receptor as a New Hepatitis C Virus Entry Factor. *Nat. Med.* 18, 281–285. doi:10.1038/nm.2581

Sandoval, A., Chokshi, A., Jesch, E. D., Black, P. N., and Dirusso, C. C. (2010). Identification and Characterization of Small Compound Inhibitors of Human FATP2. *Biochem. Pharmacol.* 79, 990–999. doi:10.1016/j.bcp.2009.11.008

Sanford, L., and Palmer, A. (2017). *Recent Advances in Development of Genetically Encoded Fluorescent Sensors*. 1st ed. Elsevier, 1–49. doi:10.1016/bs.mie.2017.01.019

Scalise, M., Pochini, L., Pingitore, P., Hedfalk, K., and Indiveri, C. (2015). Cysteine Is Not a Substrate but a Specific Modulator of Human ASCT2 (SLC1A5) Transporter. *FEBS Lett.* 589, 3617–3623. doi:10.1016/j.febslet.2015.10.011

Schlessinger, A., Matsson, P., Shima, J. E., Pieper, U., Yee, S. W., Kelly, L., et al. (2010). Comparison of Human Solute Carriers. *Protein Sci.* 19, 412–428. doi:10.1002/pro.320

Schlessinger, A., Welch, M. A., van Vlijmen, H., Korzekwa, K., Swaan, P. W., and Matsson, P. (2018). Molecular Modeling of Drug-Transporter Interactions-An International Transporter Consortium Perspective. *Clin. Pharmacol. Ther.* 104, 818–835. doi:10.1002/cpt.1174

Schreiber, S. L. (2019). A Chemical Biology View of Bioactive Small Molecules and a Binder-Based Approach to Connect Biology to Precision Medicines. *Isr. J. Chem.* 59, 52–59. doi:10.1002/ijch.201800113

Schulman, I. G., and Heyman, R. A. (2004). The Flip Side. *Chem. Biol.* 11, 639–646. doi:10.1016/j.chembiol.2003.12.021

Schulz, P., Garcia-Celma, J. J., and Fendler, K. (2008). SSM-based Electrophysiology. *Methods* 46, 97–103. doi:10.1016/j.ymeth.2008.07.002

Scott, C. W., and Peters, M. F. (2010). Label-free Whole-Cell Assays: Expanding the Scope of GPCR Screening. *Drug Discov. Today* 15, 704–716. doi:10.1016/j.drudis.2010.06.008

Seaayfan, E., Defontaine, N., Demaretz, S., Zaarour, N., and Laghmani, K. (2016). OS9 Protein Interacts with Na-K-2Cl Co-transporter (NKCC2) and Targets its Immature Form for the Endoplasmic Reticulum-Associated Degradation Pathway. *J. Biol. Chem.* 291, 4487–4502. doi:10.1074/jbc.M115.702514

Sedlyarov, V., Eichner, R., Girardi, E., Essletzbichler, P., Goldmann, U., Nunes-Hasler, P., et al. (2018). The Bicarbonate Transporter SLC4A7 Plays a Key Role in Macrophage Phagosome Acidification. *Cell Host & Microbe* 23, 766–774.e5. doi:10.1016/j.chom.2018.04.013

Shaw, J., Dale, I., Hemsley, P., Leach, L., Dekki, N., Orme, J. P., et al. (2019). Positioning High-Throughput CETSA in Early Drug Discovery through Screening against B-Raf and PARP1. *SLAS DISCOVERY: Advancing Sci. Drug Discov.* 24, 121–132. doi:10.1177/2472555218813332

Shirakabe, K., Priori, G., Yamada, H., Ando, H., Horita, S., Fujita, T., et al. (2006). IRBIT, an Inositol 1,4,5-trisphosphate Receptor-Binding Protein, Specifically Binds to and Activates Pancreas-type Na+/HCO3- Cotransporter 1 (pNBC1). *Proc. Natl. Acad. Sci.* 103, 9542–9547. doi:10.1073/pnas.0602250103

Siehler, S., and Guerini, D. (2006). Novel GPCR Screening Approach: Indirect Identification of S1P Receptor Agonists in Antagonist Screening Using a Calcium Assay. *J. Receptors Signal Transduction* 26, 549–575. doi:10.1080/10799890600932246

Sijben, H. J., van den Berg, J. J. E., Broekhuis, J. D., IJzerman, A. P., and Heitman, L. H. (2021). A Study of the Dopamine Transporter Using the TRACT Assay, a Novel *In Vitro* Tool for Solute Carrier Drug Discovery. *Sci. Rep.* 11, 1–14. doi:10.1038/s41598-020-79218-w

Stefanowicz-Hajduk, J., Adamska, A., Bartoszewski, R., and Ochocka, J. R. (2016). Reuse of E-Plate Cell Sensor Arrays in the xCELLigence Real-Time Cell Analyzer. *Biotechniques* 61, 117–122. doi:10.2144/000114450

Sucic, S., and Bönisch, H. (2016). "Classical Radioligand Uptake and Binding Methods in Transporter Research: An Emphasis on the Monoamine Neurotransmitter Transporters," in *Neurotransmitter Transporters: Investigative Methods*. Editors H. Bönisch and H. H. Sitte (New York, NY: Springer New York), 1–21. doi:10.1007/978-1-4939-3765-3_1

Sun, F., Zeng, J., Jing, M., Zhou, J., Feng, J., Owen, S. F., et al. (2018). A Genetically Encoded Fluorescent Sensor Enables Rapid and Specific Detection of Dopamine in Flies, Fish, and Mice. *Cell* 174, 481–496.e19. doi:10.1016/j.cell.2018.06.042

Superti-Furga, G., Lackner, D., Wiedmer, T., Ingles-Prieto, A., Barbosa, B., Girardi, E., et al. (2020). The RESOLUTE Consortium: Unlocking SLC Transporters for Drug Discovery. *Nat. Rev. Drug Discov.* 19, 429–430. doi:10.1038/d41573-020-00056-6

Takanaga, H., and Frommer, W. B. (2010). Facilitative Plasma Membrane Transporters Function during ER Transit. *FASEB j.* 24, 2849–2858. doi:10.1096/fj.09-146472

Tavoulari, S., Thangaratnarajah, C., Mavridou, V., Harbour, M. E., Martinou, J. C., and Kunji, E. R. (2019). The Yeast Mitochondrial Pyruvate Carrier Is a Hetero-dimer in its Functional State. *EMBO J.* 38, 1–13. doi:10.15252/embj.2018100785

Thomsen, W., Frazer, J., and Unett, D. (2005). Functional Assays for Screening GPCR Targets. *Curr. Opin. Biotechnol.* 16, 655–665. doi:10.1016/j.copbio.2005.10.008

Tripp, J., Essl, C., Iancu, C. V., Boles, E., Choe, J.-y., and Oreb, M. (2017). Establishing a Yeast-Based Screening System for Discovery of Human GLUT5 Inhibitors and Activators. *Sci. Rep.* 7, 6197. doi:10.1038/s41598-017-06262-4

Tsai, C.-Y., Larson, C. A., Safaei, R., and Howell, S. B. (2014). Molecular Modulation of the Copper and Cisplatin Transport Function of CTR1 and its Interaction with IRS-4. *Biochem. Pharmacol.* 90, 379–387. doi:10.1016/j.bcp.2014.06.019

Türková, A., and Zdrazil, B. (2019). Current Advances in Studying Clinically Relevant Transporters of the Solute Carrier (SLC) Family by Connecting Computational Modeling and Data Science. *Comput. Struct. Biotechnol. J.* 17, 390–405. doi:10.1016/j.csbj.2019.03.002

Ugele, B., St-Pierre, M. V., Pihusch, M., Bahn, A., and Hantschmann, P. (2003). Characterization and Identification of Steroid Sulfate Transporters of Human Placenta. *Am. J. Physiology-Endocrinology Metab.* 284, E390–E398. doi:10.1152/ajpendo.00257.2002

Valdez-Flores, M. A., Vargas-Poussou, R., Verkaart, S., Tutakhel, O. A. Z., Valdez-Ortiz, A., Blanchard, A., et al. (2016). Functionomics of NCC Mutations in Gitelman Syndrome Using a Novel Mammalian Cell-Based Activity Assay. *Am. J. Physiology-Renal Physiol.* 311, F1159–F1167. doi:10.1152/ajprenal.00124.2016

Villette, V., Chavarha, M., Dimov, I. K., Bradley, J., Pradhan, L., Mathieu, B., et al. (2019). Ultrafast Two-Photon Imaging of a High-Gain Voltage Indicator in Awake Behaving Mice. *Cell* 179, 1590–1608.e23. doi:10.1016/j.cell.2019.11.004

Visser, W. E., Friesema, E. C. H., and Visser, T. J. (2011). Minireview: Thyroid Hormone Transporters: The Knowns and the Unknowns. *Mol. Endocrinol.* 25, 1–14. doi:10.1210/me.2010-0095

Vlachodimou, A., IJzerman, A. P., and Heitman, L. H. (2019). Label-free Detection of Transporter Activity via GPCR Signalling in Living Cells: A Case for SLC29A1, the Equilibrative Nucleoside Transporter 1. *Sci. Rep.* 9, 1–10. doi:10.1038/s41598-019-48829-3

Vlachodimou, A., Konstantinopoulou, K., IJzerman, A. P., and Heitman, L. H. (2020). Affinity, Binding Kinetics and Functional Characterization of Draflazine Analogues for Human Equilibrative Nucleoside Transporter 1 (SLC29A1). *Biochem. Pharmacol.* 172, 113747. doi:10.1016/j.bcp.2019.113747

Vu, T. M., Ishizu, A.-N., Foo, J. C., Toh, X. R., Zhang, F., Whee, D. M., et al. (2017). Mfsd2b Is Essential for the Sphingosine-1-Phosphate export in Erythrocytes and Platelets. *Nature* 550, 524–528. doi:10.1038/nature24053

Vuckovic, D. (2012). Current Trends and Challenges in Sample Preparation for Global Metabolomics Using Liquid Chromatography-Mass Spectrometry. *Anal. Bioanal. Chem.* 403, 1523–1548. doi:10.1007/s00216-012-6039-y

Walters, W. P., and Namchuk, M. (2003). Designing Screens: How to Make Your Hits a Hit. *Nat. Rev. Drug Discov.* 2, 259–266. doi:10.1038/nrd1063

Wan, J., Peng, W., Li, X., Qian, T., Song, K., Zeng, J., et al. (2020). A Genetically Encoded GRAB Sensor for Measuring Serotonin Dynamics in Vivo. bioRxiv, Cold Spring Harbor Laboratories New York. doi:10.1101/2020.02.24.962282

Wang, S., Tsun, Z.-Y., Wolfson, R. L., Shen, K., Wyant, G. A., Plovanich, M. E., et al. (2015). Lysosomal Amino Acid Transporter SLC38A9 Signals Arginine Sufficiency to mTORC1. *Science* 347, 188–194. doi:10.1126/science.1257132

Wang, W. W., Gallo, L., Jadhav, A., Hawkins, R., and Parker, C. G. (2019a). The Druggability of Solute Carriers. *J. Med. Chem.* 63, 3834–3867. doi:10.1021/acs.jmedchem.9b01237

Wang, Y., Li, P., Song, F., Yang, X., Weng, Y., Ma, Z., et al. (2019b). Substrate Transport Properties of the Human Peptide/Histidine Transporter PHT2 in Transfected MDCK Cells. *J. Pharm. Sci.* 108, 3416–3424. doi:10.1016/j.xphs.2019.06.016

Watzke, N., Diekert, K., and Obrdlik, P. (2010). Electrophysiology of Respiratory Chain Complexes and the ADP–ATP Exchanger in Native Mitochondrial Membranes. *Biochemistry* 49, 10308–10318. doi:10.1021/bi1011755

Weaver, C. D., Harden, D., Dworetzky, S. I., Robertson, B., and Knox, R. J. (2004). A Thallium-Sensitive, Fluorescence-Based Assay for Detecting and Characterizing Potassium Channel Modulators in Mammalian Cells. *J. Biomol. Screen.* 9, 671–677. doi:10.1177/1087057104268749

Webb, B. A., White, K. A., Grillo-Hill, B. K., Schönichen, A., Choi, C., and Barber, D. L. (2016). A Histidine Cluster in the Cytoplasmic Domain of the Na-H Exchanger NHE1 Confers pH-Sensitive Phospholipid Binding and Regulates Transporter Activity. *J. Biol. Chem.* 291, 24096–24104. doi:10.1074/jbc.M116.736215

Wemhöner, A., Frick, M., Dietl, P., Jennings, P., and Haller, T. (2006). A Fluorescent Microplate Assay for Exocytosis in Alveolar Type II Cells. *J. Biomol. Screen.* 11, 286–295. doi:10.1177/1087057105285284

Williams, C. (2004). cAMP Detection Methods in HTS: Selecting the Best from the Rest. *Nat. Rev. Drug Discov.* 3, 125–135. doi:10.1038/nrd1306

Windler, F., Bönigk, W., Körschen, H. G., Grahn, E., Strünker, T., Seifert, R., et al. (2018). The Solute Carrier SLC9C1 Is a Na+/H+-exchanger Gated by an S4-type Voltage-Sensor and Cyclic-Nucleotide Binding. *Nat. Commun.* 9, 1–13. doi:10.1038/s41467-018-05253-x

Winter, G. E., Radic, B., Mayor-Ruiz, C., Blomen, V. A., Trefzer, C., Kandasamy, R. K., et al. (2014). The Solute Carrier SLC35F2 Enables YM155-Mediated DNA Damage Toxicity. *Nat. Chem. Biol.* 10, 768–773. doi:10.1038/nchembio.1590

Wittwer, M. B., Zur, A. A., Khuri, N., Kido, Y., Kosaka, A., Zhang, X., et al. (2013). Discovery of Potent, Selective Multidrug and Toxin Extrusion Transporter 1 (MATE1, SLC47A1) Inhibitors through Prescription Drug Profiling and Computational Modeling. *J. Med. Chem.* 56, 781–795. doi:10.1021/jm301302s

Wolff, C., Fuks, B., and Chatelain, P. (2003). Comparative Study of Membrane Potential-Sensitive Fluorescent Probes and Their Use in Ion Channel Screening Assays. *J. Biomol. Screen.* 8, 533–543. doi:10.1177/1087057103257806

Wong, S.-H., Gao, A., Ward, S., Henley, C., and Lee, P. H. (2012). Development of a Label-free Assay for Sodium-dependent Phosphate Transporter NaPi-IIb. *J. Biomol. Screen.* 17, 829–834. doi:10.1177/1087057112442961

Wright Muelas, M., Roberts, I., Mughal, F., O'Hagan, S., Day, P. J., and Kell, D. B. (2020). An Untargeted Metabolomics Strategy to Measure Differences in Metabolite Uptake and Excretion by Mammalian Cell Lines. *Metabolomics* 16, 1–12. doi:10.1007/s11306-020-01725-8

Wu, T., Yoon, H., Xiong, Y., Dixon-Clarke, S. E., Nowak, R. P., and Fischer, E. S. (2020). Targeted Protein Degradation as a Powerful Research Tool in Basic Biology and Drug Target Discovery. *Nat. Struct. Mol. Biol.* 27, 605–614. doi:10.1038/s41594-020-0438-0

Xi, B., Yu, N., Wang, X., Xu, X., and Abassi, Y. (2008). The Application of Cell-Based Label-free Technology in Drug Discovery. *Biotechnol. J.* 3, 484–495. doi:10.1002/biot.200800020

Xie, M., and Fussenegger, M. (2018). Designing Cell Function: Assembly of Synthetic Gene Circuits for Cell Biology Applications. *Nat. Rev. Mol. Cel Biol.* 19, 507–525. doi:10.1038/s41580-018-0024-z

Yan, Q., Lu, Y., Zhou, L., Chen, J., Xu, H., Cai, M., et al. (2018). Mechanistic Insights into GLUT1 Activation and Clustering Revealed by Super-resolution Imaging. *Proc. Natl. Acad. Sci. USA* 115, 7033–7038. doi:10.1073/pnas.1803859115

Yan, R., Zhao, X., Lei, J., and Zhou, Q. (2019). Structure of the Human LAT1-4F2hc Heteromeric Amino Acid Transporter Complex. *Nature* 568, 127–130. doi:10.1038/s41586-019-1011-z

Yasujima, T., Ohta, K.-y., Inoue, K., Ishimaru, M., and Yuasa, H. (2010). Evaluation of 4′,6-Diamidino-2-Phenylindole as a Fluorescent Probe Substrate for Rapid Assays of the Functionality of Human Multidrug and Toxin Extrusion Proteins. *Drug Metab. Dispos.* 38, 715–721. doi:10.1124/dmd.109.030221

Yee, S. W., Stecula, A., Chien, H.-C., Zou, L., Feofanova, E. V., van Borselen, M., et al. (2019). Unraveling the Functional Role of the Orphan Solute Carrier, SLC22A24 in the Transport of Steroid Conjugates through Metabolomic and Genome-wide Association Studies. *Plos Genet.* 15, e1008208. doi:10.1371/journal.pgen.1008208

Yoo, H. C., Park, S. J., Nam, M., Kang, J., Kim, K., Yeo, J. H., et al. (2020). A Variant of SLC1A5 Is a Mitochondrial Glutamine Transporter for Metabolic Reprogramming in Cancer Cells. *Cel Metab.* 31, 267–283.e12. doi:10.1016/j.cmet.2019.11.020

Yu, H.-b., Li, M., Wang, W.-p., and Wang, X.-l. (2015). High Throughput Screening Technologies for Ion Channels. *Acta Pharmacol. Sin* 37, 34–43. doi:10.1038/aps.2015.108

Yu, N., Atienza, J. M., Bernard, J., Blanc, S., Zhu, J., Wang, X., et al. (2006). Real-time Monitoring of Morphological Changes in Living Cells by Electronic Cell Sensor Arrays: An Approach to Study G Protein-Coupled Receptors. *Anal. Chem.* 78, 35–43. doi:10.1021/ac051695v

Zhang, B., Jin, Q., Xu, L., Li, N., Meng, Y., Chang, S., et al. (2020a). Cooperative Transport Mechanism of Human Monocarboxylate Transporter 2. *Nat. Commun.* 11, 1–10. doi:10.1038/s41467-020-16334-1

Zhang, C., Sui, D., Zhang, T., and Hu, J. (2020b). Molecular Basis of Zinc-dependent Endocytosis of Human ZIP4 Transceptor. *Cel Rep.* 31, 107582. doi:10.1016/j.celrep.2020.107582

Zhang, J.-H., Chung, T. D., and Oldenburg, K. R. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.* 4, 67–73. doi:10.1177/108705719900400206

Zhang, R., and Xie, X. (2012). Tools for GPCR Drug Discovery. *Acta Pharmacol. Sin.* 33, 372–384. doi:10.1038/aps.2011.173

Zhang, W. H., Herde, M. K., Mitchell, J. A., Whitfield, J. H., Wulff, A. B., Vongsouthi, V., et al. (2018). Monitoring Hippocampal glycine with the Computationally Designed Optical Sensor GlyFS. *Nat. Chem. Biol.* 14, 861–869. doi:10.1038/s41589-018-0108-2

Zhong, S., Navaratnam, D., and Santos-Sacchi, J. (2014). A Genetically-Encoded YFP Sensor with Enhanced Chloride Sensitivity, Photostability and Reduced pH Interference Demonstrates Augmented Transmembrane Chloride Movement by Gerbil Prestin (SLC26a5). *PLoS One* 9, e99095. doi:10.1371/journal.pone.0099095

Zhou, W., Madrid, P., Fluitt, A., Stahl, A., and Xie, X. (2010). Development and Validation of a High-Throughput Screening Assay for Human Long-Chain Fatty Acid Transport Proteins 4 and 5. *J. Biomol. Screen.* 15, 488–497. doi:10.1177/1087057110369700

Zhu, J., and Thompson, C. B. (2019). Metabolic Regulation of Cell Growth and Proliferation. *Nat. Rev. Mol. Cel Biol.* 20, 436–450. doi:10.1038/s41580-019-0123-5

Zwartsen, A., Verboven, A. H. A., van Kleef, R. G. D. M., Wijnolts, F. M. J., Westerink, R. H. S., and Hondebrink, L. (2017). Measuring Inhibition of Monoamine Reuptake Transporters by New Psychoactive Substances (NPS)

in Real-Time Using a High-Throughput, Fluorescence-Based Assay. *Toxicol. Vitro* 45, 60–71. doi:10.1016/j.tiv.2017.05.010

Conflict of Interest: Research of the RESOLUTE consortium is in the precompetitive space. Authors PA, FD, AS, HS, MW are employed by Vifor. HB and EB are employed by Bayer. DZ was employed by Bayer during the manuscript preparation. TL and FB are employed by Sanofi. RR, FS, LS and ST are employed by Axxam. CS is employed by Pfizer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

1.3 SLC16 family of monocarboxylate transporters

The monocarboxylate transporter family, SLC16, has 14 members responsible for transport of nutrients and metabolites, such as short-chain fatty acids, pyruvate, lactate, or amino acids, hormones, or drugs (Bosshart *et al*, 2020; Felmlee *et al*, 2020). The SLC16 family is part of the major facilitator superfamily (MFS), and hence transporters of SLC16 family operate with a rocker-switch mechanism typical of that protein fold (Bosshart *et al*, 2020). The transport of several members of the SLC16 family is coupled to the co-transport of H^+ and therefore these members are also implicated in the cellular pH homeostasis. A subset of the SLC16 family members form complexes with the ancillary proteins basigin (CD147) or embigin (GP70), an interaction that is important for their trafficking and proper function (Halestrap, 2013). Based on the structural similarities, and the substrate specificities, the SLC16 family can be divided into several subgroups (**Figure 2**). Of note, the mismatching MCT and SLC16 numbering can be confusing and requires special attention. There are several examples of changing MCT numbering, for example SLC16A5 was initially called MCT5, but later, upon the characterization of *SLC16A4* cDNA, was renamed to MCT6 (Felmlee *et al*, 2020). For this reason and throughout this thesis, I am using the unambiguous HUGO SLC nomenclature reflecting gene names. For clarity, the MCT names are added in the Figure 2.

The first subgroup can be defined around SLC16A1, SLC16A3, SLC16A7 and SLC16A8. These transporters are mainly responsible for transport of lactate, pyruvate and ketone bodies (Draoui & Feron, 2011). Collectively this subgroup is the most studied among SLC16 family and will be discussed in more details in the next section.

SLC16A2 and SLC16A10 form a second subgroup based on similarities in the structure and the substrate specificity. SLC16A2 is one of the major transporters of thyroid hormones (Bernal *et al*, 2015). Mutations reducing the SLC16A2 function are causing the Allan-Herndon-Dudley syndrome, a rare neurological disorder linked to the X chromosome manifested by a developmental delay, intellectual disabilities, and impairment of muscle activity (Remerand *et al*, 2019). SLC16A10 was also described as a transporter of thyroid hormones, but compared to SLC16A2, SLC16A10 shows lower affinity for thyroid hormones, but is instead also capable of transporting aromatic amino acids (Bernal *et al*, 2015).

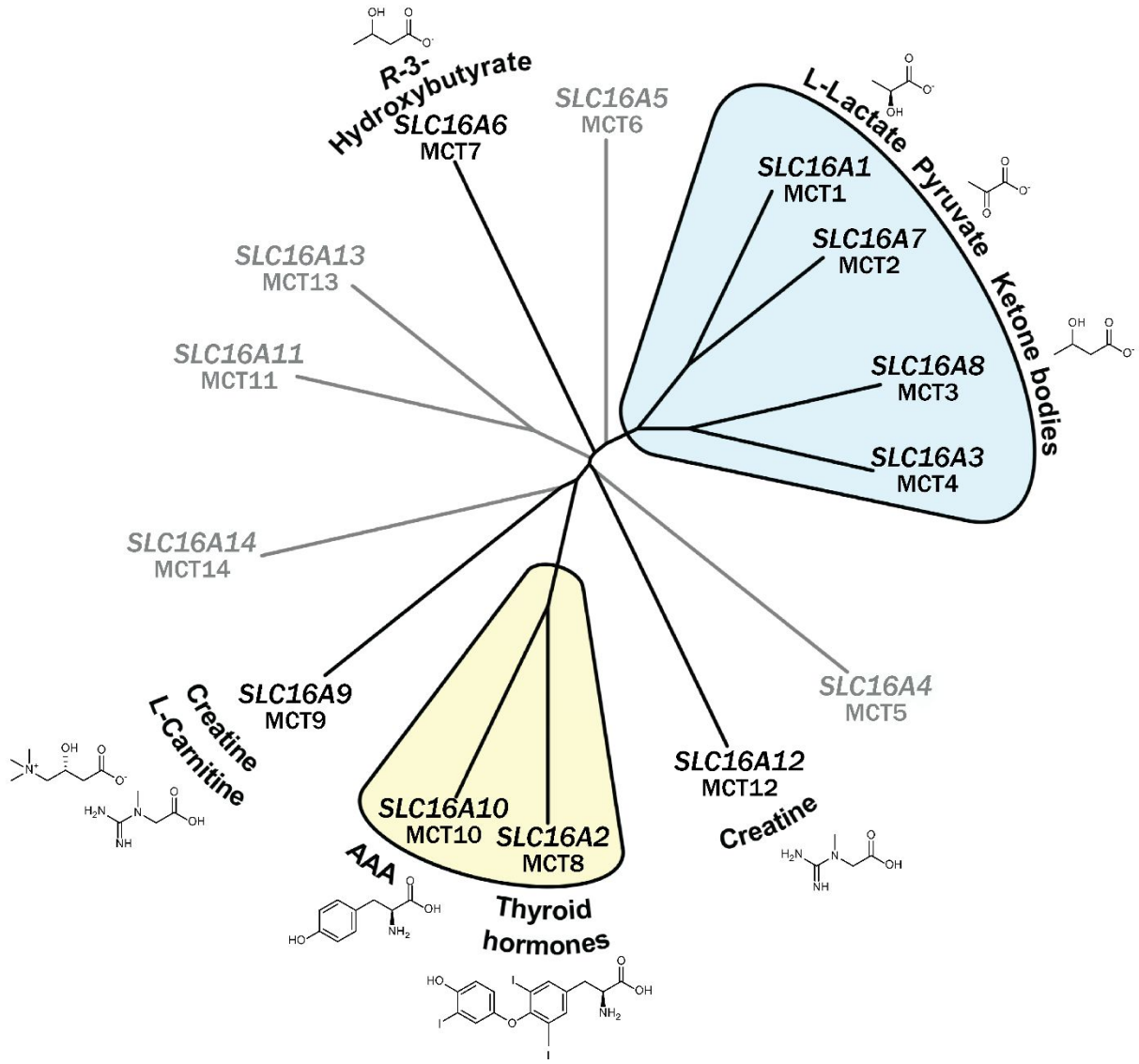


Figure 2: The phylogenetic tree of monocarboxylate transporter family. *Substrates of the individual transporters are indicated. Transporters with insufficient substrate annotation are in grey. Chemical structures of the exemplary substrates added for illustrator purposes. Illustration adapted based on* (Bosshart *et al*, 2020) *with permission from the publisher.*

SLC16A9 and SLC16A12 can be considered as a third subgroup in the SLC16 family, based on similarity in their substrate specificity. SLC16A9 is thought to be a transporter of carnitine and creatine (Futagi *et al*, 2020; Suhre *et al*, 2011). Moreover, polymorphisms in *SLC16A9* were linked to higher plasma levels of uric acid and higher risk of gout development (Nakayama *et al*, 2013; Kolz *et al*, 2009). SLC16A12 is a creatine transporter expressed mainly in retina and kidney (Abplanalp *et al*, 2013; Takahashi *et al*, 2020). Variants in *SLC16A12* were reported in patients suffering from eye diseases, such as cataracts (Kloeckener-Gruissem *et al*, 2008).

The functions of the rest of the members of the SLC16 family are currently not clear. Gene variants of *SLC16A11* and *SLC16A13* were linked to type 2 diabetes (T2D) in genome-wide association studies (GWAS; (Williams Amy *et al*, 2014; Hara *et al*, 2014)). An additional follow-up study found that the haplotypes associating with T2D cause lower expression of *SLC16A11*, reduced localization in the plasma membrane due to reduced interaction with the ancillary protein basigin as well as reduced transport activity (Rusu *et al*, 2017). The same study also showed that SLC16A11 can transport pyruvate with H^+ as a coupled ion. Another follow up study focusing on the role of SLC16A11 based on mouse models (Zhao *et al*, 2019) however, showed inconsistency with the human data and was later disputed by the authors of the original study (Hoch *et al*, 2019). Hence to fully explain the physiological roles of SLC16A11 more research is needed. The function of SLC16A13 is also not well understood. A recent study utilizing a *slc16a13*$^{-/-}$ murine model suggested that this transporter is capable of lactate transport and therapeutical targeting could be beneficial in T2D and similar diseases, however these data still need to be validated with the human ortholog (Schumann *et al*, 2021). SLC16A6 was proposed to be a transporter of ketone bodies (Hugo *et al*, 2012) and taurine (Higuchi *et al*, 2022), based on studies conducted with zebrafish and rat orthologs. However, further studies are needed to show that this transporter has a same function in humans. Studies reporting roles for SLC16A4, SLC16A5 and SLC16A14 are currently limited. SLC16A5 is expressed in several organs including intestine, kidney and liver, and was shown to be capable of transporting several xenobiotics, including bumetanide, nateglinide, probenecid or some of the prostaglandins (Murakami *et al*, 2005). The same study showed that SLC16A5 is not capable of transporting other substrates typical for the SLC16 family, such as lactate and tryptophan. Studies utilizing the *slc16a5*$^{-/-}$ mouse model pointed to a role of this transporter in lipid and glucose homeostasis (Jones *et al*, 2019; Ren *et al*, 2022), but were not able to pinpoint the exact substrate specificity. To the best of the author's knowledge, there are currently no studies demonstrating possible physiological roles or substrate specificities for SLC16A4 and SLC16A14.

1.4 Lactate

Lactate was for a long time considered to be a waste product of anaerobic metabolism, but in recent years it is becoming increasingly clear that the physiological roles of lactate are much more complex. The following section will describe some of the recent studies on lactate with the purpose of providing a brief overview on possible roles of lactate in human physiology. The comprehensive overview of functions of lactate is beyond the scope of this thesis and can be found in the excellent reviews by George Brooks (Brooks, 2018) and by Li and colleagues (Li *et al*, 2022).

Lactate is produced during glycolysis, the metabolic process of converting a glucose molecule into two molecules of pyruvate (**Figure 3**) (Chandel, 2015). Pyruvate can then either be imported into mitochondria where it enters the tricarboxylic acid (TCA) cycle or it can be converted by lactate dehydrogenase (LDH) to lactate, which is then secreted into the extracellular space typically by SLC16A3. The conversion of pyruvate to lactate is necessary to regenerate NAD^+ that can be reduced to NADH during glycolysis, allowing glycolysis to persist. Importantly, apart from being a fast source of ATP, glycolysis can also supply molecules for other metabolic pathways, and hence can be an important source of intermediates for biosynthetic pathways. This includes: 1) the pentose-phosphate pathway, the source of NADPH, a co-factor in reductive biosynthesis, and ribose, a building block for synthesis of nucleotides; 2) the hexosamine pathway, required for glycosylation; 3) glycerol synthesis, a building block for synthesis of complex lipids; and 4) serine-glycine-one carbon metabolism; a pathway important for the synthesis of glutathione, nucleotides, and methylation reactions. Importantly, the metabolic conversion of pyruvate in the mitochondria powers the oxidative phosphorylation (OXPHOS) that produces higher ATP yields (per molecule of glucose) compared to glycolysis and conversion of pyruvate to lactate but required the presence of oxygen. It was proposed that the reason for the Warburg effect, i.e. the observation that tumors prefer glycolysis over OXPHOS even in the presence of oxygen, is to obtain a large pools of glycolytic intermediates that can fuel the biosynthetic mentioned previously (DeBerardinis & Chandel, 2020). Of note, it is now clear that the Warburg effect does not prevent OXPHOS in tumors, as it was previously believed.

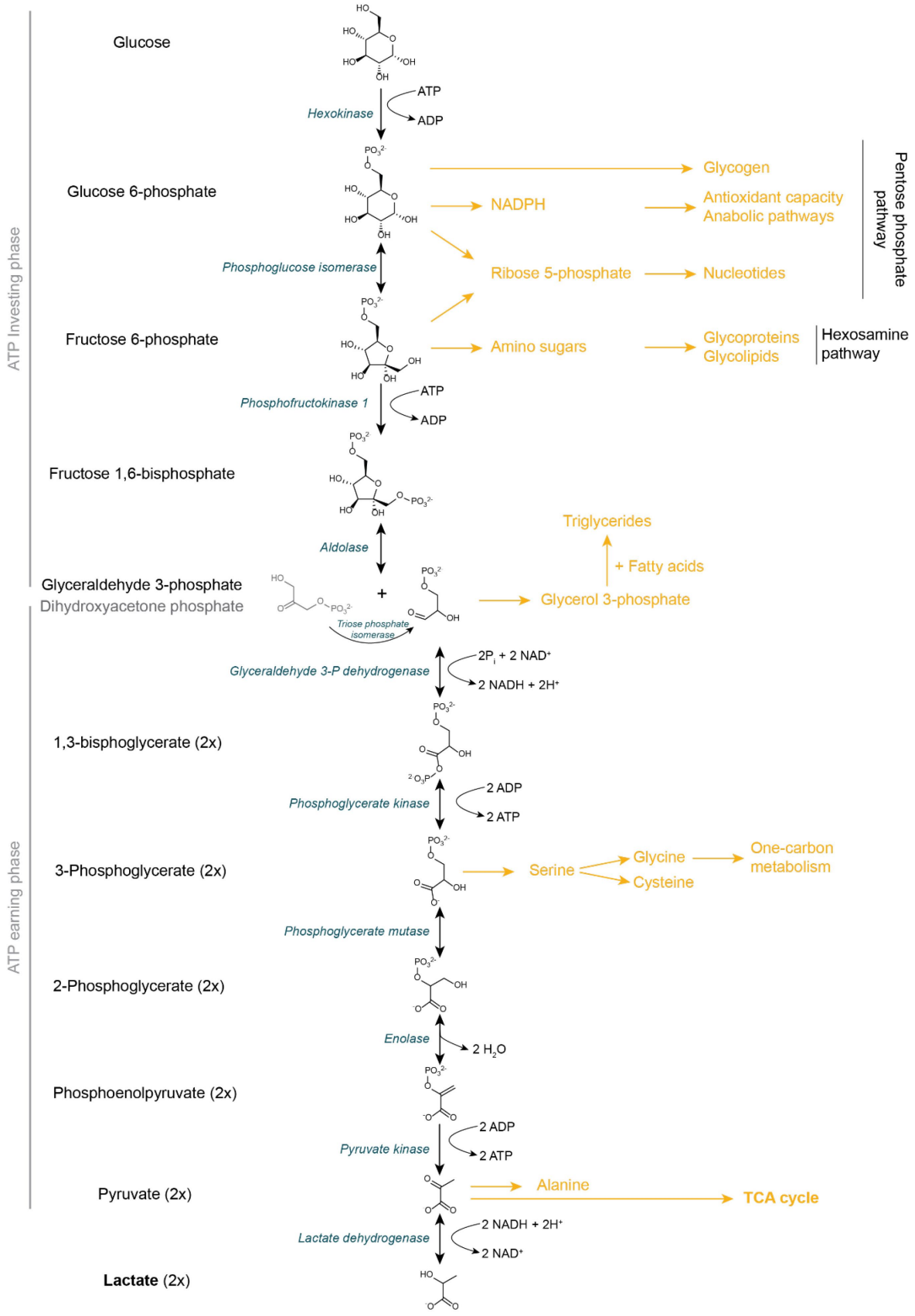


Figure 3: Glycolysis scheme. *Enzymes responsible for induvial steps in blue; alternative metabolic pathways in yellow. Figure based in* (Chandel, 2015)*, with permission from the publisher.*

1.4.1 Lactate shuttle

Lactate can be generated by a plethora of cell types under fully aerobic conditions, similarly to what Warburg observed in tumor samples (Brooks, 2018). Furthermore, lactate can be rapidly exchanged by different tissues or cell types within one tissue, and it can serve as an energy source in the so-called lactate shuttle. Importantly, it was shown that lactate is used as one of the major carbon sources for the TCA cycle, and that on molar basis the turnover flux of lactate is the fastest compared to other carbon sources (Hui *et al*, 2017).

Moreover, by using ^{13}C-labelled lactate, it has been shown that the lactate carbons contribute to the TCA cycle in all tissues in mouse (Hui *et al*, 2017). Similarly, lactate can be a predominant carbon source for the TCA cycle in human lung tumours *in vivo* (Faubert *et al*, 2017), tumours in genetically engineered mouse models (GEMMs) of lung and pancreatic[1] cancers (Hui *et al*, 2017), or for metastasizing cells, as shown in melanoma patient-derived xenograft (PDX) models (Tasdogan *et al*, 2020). Lactate can be used as a carbon source for fatty acid synthesis (Chen *et al*, 2016), a phenomenon that seems to be particularly important in cancer cells (Zhao *et al*, 2020), but also for example in some types of immune cells (Pucino *et al*, 2019).

It was proposed that pyruvate generated from glycolysis rarely flows directly into the TCA cycle, but is rather converted to lactate, released into the blood stream and from there enters again (different) cells, where it gets converted to pyruvate and feeds into the TCA cycle (Rabinowitz & Enerbäck, 2020). This allows de-coupling of glycolysis and TCA cycle, processes that are otherwise linked because NADH and pyruvate produced by glycolysis need to be cleared in the mitochondria (Brooks, 2018). Using lactate as a primary energy source in most tissues also allows to reserve glucose for energy production in tissues that are glucose dependent (such as erythrocytes, or under certain circumstances the brain) and for biochemical reactions that use glucose as substrate, such as the pathways mentioned previously (Rabinowitz & Enerbäck, 2020). Moreover, it was shown that several tissues, including heart, brain, or skeletal muscles (during excise) prefer lactate over glucose (Rabinowitz & Enerbäck, 2020; Brooks, 2018).

The relationship between the organs with high lactate production and high lactate consumption can be considered an inter-organ lactate shuttle. On the level of same tissue (intra-organ), the lactate shuttle was described in several organs. A typical example of an organ with a well described lactate shuttle is the skeletal muscle (Fishbein *et al*, 2002). The fast-twitch fibres tend to be glycolytic and produce high amounts of lactate. Lactate, together with H^+ ions, gets

[1] In the pancreatic cancer model was the predominant source glutamine, lactate was second.

exported into the environment and eventually a part of it is imported into slow-twitch muscle fibres, where it serves as a metabolic fuel. Similarly, in the brain, astrocytes produce lactate that is then transported to neurons where it gets oxidized (Magistretti & Allaman, 2018). The intra-organ lactate shuttle received most attention in the context of tumour microenvironment (TME), where it is known also to be key to metabolic symbiosis (**Figure 4**) (Draoui & Feron, 2011; Lyssiotis & Kimmelman, 2017). It was shown in several different types of cancers that the tumour mass can be compartmentalized into regions with different metabolic phenotypes (reviewed in (Ippolito *et al*, 2019)). Specifically, cancer cells in the tumour regions with limited oxygen access express high levels of LDHA, an isoform of LDH that is primary catalyzing conversion of pyruvate to lactate. They also, produce high levels of lactate that is then exported through SLC16A3 into the environment. There, it is available to cells that are closer to the vasculature. These cells import lactate through SLC16A1, convert it to pyruvate by LDHB (LDH isoform that is primary converting lactate to pyruvate), and then utilize it to meet their metabolic needs. Similar relationships were described also between cancer cells and cancer-associated fibroblasts (CAFs) and were shown to be important mediators of resistance to therapies targeted at angiogenesis or to tyrosine kinase inhibitors (Apicella *et al*, 2018; Pisarsky *et al*, 2016; Allen *et al*, 2016; Jiménez-Valerio *et al*, 2016). Importantly, the high levels of lactate in the TME have an impact on the immune system and reducing lactate levels in the TME was shown to induce the immune response against cancer cells (Wang *et al*, 2021c).

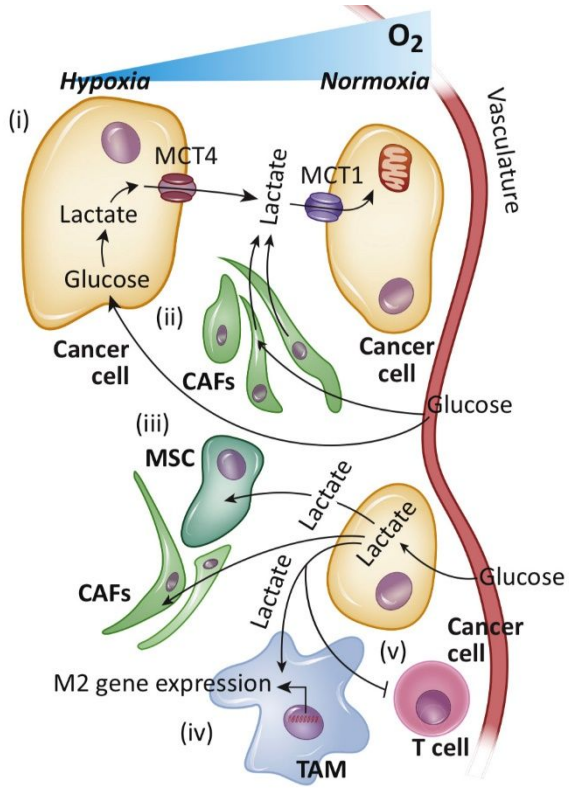


Figure 4: Metabolic symbiosis between cells in TME*. Cancer cells in more hypoxic regions of the tumour (i) or CAFs (ii) tend to consume high levels of glucose and produce lactate, that gets exported by SLC16A3 (MCT4). Lactate in the TME can feed (i) cancer cells in less hypoxic regions of the tumour or (iii) mesenchymal stem cells (MSC) or CAFs, (iv) drive polarization of macrophages towards the tumour associated macrophage (TAM; pro-tumorigenic) phenotype, or (v) inhibit immune cells, such as T cells. The cells consuming the lactate use typically SLC16A1 (MCT1) to import the lactate from the environment. Reprinted from* (Lyssiotis & Kimmelman, 2017)*, with permission from the publisher.*

1.4.2 Lactate as a signaling molecule

While the levels of glucose are kept relatively steady in the blood (around 5 mM), the concentrations of lactate can transiently rise from ~1 mM to ~5 mM in the circulation (as seen during hard excise), and local concentrations of lactate, for example in the inflamed tissues or TME, could reach levels up to 40 mM (Brooks, 2018). This is important because lactate has also a role as a signaling molecule, particularly in the context of immune system. Lactate can for example influence the migration of immune cells, production of cytokines and differentiation of immune cells (Certo *et al*, 2021). This could be either through, for example, activation of G-protein coupled receptor 81 (GPR81), a recently described receptor for lactate, or through effects on metabolism (Certo *et al*, 2022). A landmark study published in 2019 showed that lactate can be also used as a substrate for a kind of post-translational modification called lactylation, and that lactylation of histones is involved in the polarization of macrophages

(Zhang *et al*, 2019a). A study focused on the global lactylome in samples collected from hepatocellular carcinoma patients, showed that lactylation can affect also other proteins, especially metabolic enzymes, and that lactylation can modulate the enzymatic activity of these proteins (Yang *et al*, 2023). Importantly, lactate can also modulate the function of proteins by non-covalent interactions. A recent study utilizing thermal proteome profilling, a technique described in more detail in the review in section 1.3, showed that lactate forms complexes with zinc that then bind and inhibit the active site of SENP1, a negative regulator of APC/C complex that is required for mitotic exit during cell cycle (Liu *et al*, 2023). Interestingly the study shows that lactate accumulates upon the mitotic entry and further suggests that elevated intracellular lactate levels are required for the mitotic exit, further expanding the importance of lactate in cell physiology.

Last but not least, circulating lactate can be converted by the liver back to glucose in the Cori cycle (Brooks, 2018). Hence, lactate fulfills at least three roles: (1) it represents an energy source, (2) it is a molecule involved in signaling and regulation of protein function, and (3) it serves as a carbon source for gluconeogenesis and lipid synthesis. Moreover, de-regulation of lactate homeostasis was observed in a variety of different diseases and has been proposed as a promising therapeutic target. Importantly, the transport of lactate plays a crucial role in most of the biological functions of lactate. At physiological pH the vast majority of the lactic acid molecules can be found in form of lactate anion and H^+, both substrates for H^+ coupled monocarboxylate transporters (Draoui & Feron, 2011).

1.5 Lactate transporters

To date, several transporters of lactate have been described: SLC16A1, SLC16A3, SLC16A7 and SLC16A8. These are all H^+-coupled co-transporters capable of shuttling lactate, pyruvate and ketone bodies such as butyrate (Draoui & Feron, 2011). All these transporters depend on the interaction with the ancillary proteins basigin and/or embigin for proper localization and function (Kirk *et al*, 2000). SLC16A1, SLC16A3 and SLC16A8 preferably form complexes with basigin, while SLC16A7 prefers embigin (Halestrap, 2013). Despite their similarities in the amino acid sequence and substrate specificity, the function of these transporters differs, mainly due to different substrate affinities and their expression patterns. Even though all four proteins are highly homologous, SLC16A1 shares most structural similarity with SLC16A7 and the closest homologue of SLC16A3 is SLC16A8, as illustrated in **Table 2** based on the amino acid identity. Importantly, the SLC16 family lactate transporters are considered to be bi-directional, depending on the concentration of their substrates.

As already mentioned, SLC16A11 and SLC16A13 were proposed as transporters with similar substrate specificity similar to SLC16A1/3/7/8, however these studies still need to be confirmed (Rusu *et al*, 2017; Schumann *et al*, 2021). Additionally, lactate was shown to be transported by the sodium dependent transporters SLC5A8 (considered as high affinity) and SLC5A12 (considered as low-affinity) (Gopal *et al*, 2007; Ganapathy *et al*, 2008).

Structural identity [%]	**SLC16A1**	**SLC16A3**	**SLC16A7**	**SLC16A8**
SLC16A1	100	44.57	59.21	39.43
SLC16A3	44.57	100	46.74	56.93
SLC16A7	59.21	46.74	100	42.77
SLC16A8	39.43	42.77	56.93	100

Table 2: Sequence identity among lactate transporters of SLC16 family. *Table reproduced from* (Felmlee *et al*, 2020).

1.5.1. SLC16A1 (MCT1)

SLC16A1 is ubiquitously expressed and is in general considered to function as a major lactate importer (Fishbein *et al*, 2002). Its role in lactate transport was described in for example erythrocytes, heart, skeletal muscles, and liver, where it feeds the Cori cycle. In the mouse the KO of *slc16a1* is embryonically lethal (Lengacher *et al*, 2013), while loss-of-function mutations in the human are connected to severe ketoacidosis (van Hasselt *et al*, 2014). In cancer cells that co-express SLC16A1 and SLC16A3 the blockage of SLC16A1 led to reduced pyruvate export, while the export of lactate was not affected, suggesting that SLC16A1 could be a pyruvate exporter in this setting (Hong *et al*, 2016). Interestingly, SLC16A1 was also proposed as a transporter of succinate, a dicarboxylate that can be converted to monocarboxylate and

then, in this form, recognized by SLC16A1 (Reddy *et al*, 2020). This conversion was observed in exercising muscles, due to an increase in intracellular pH which, by getting close to the pKa of succinate, leads to the conversion to the monocarboxylic state, followed by transport through SLC16A1. Upon export, succinate recovers the dicarboxylic form, binds its receptors on surrounding cells and mediates signaling that has a critical role in muscle adaptation and remodeling upon excise. The role of SLC16A1 in succinate transport was confirmed also in the heart, where succinate accumulates in cytoplasm during ischemia (Prag *et al*, 2021).

SLC16A1 in cancer

High levels of SLC16A1 expression were reported in many types of cancers (Ippolito *et al*, 2019), and therefore SLC16A1 was proposed as a therapeutic target in oncology. Indeed, several studies suggested that cancer cells may be sensitive to SLC16A1 knock-down or inhibition (Doherty *et al*, 2014; Curtis *et al*, 2017). In the melanoma models, the inhibition of SLC16A1 led to the reduction in distant metastasis, while the growth of subcutaneous tumors was not affected (Tasdogan *et al*, 2020). The authors of this study uncovered that lactate utilization is important for the survival of circulating tumor cells. This is due to preferential utilization of lactate in the TCA cycle, thus preserving glucose to fuel the pentose-phosphate pathway, generating NADPH and subsequent protection from ROS. This agrees with a previous study that showed that inhibition of SLC16A1 leads to lactate accumulation, subsequent inhibition of glycolysis, and reduction in NADPH and glutathione levels (Doherty *et al*, 2014). Along the same line, SLC16A1 was suggested as a potential therapeutic target to overcome resistance to ferroptosis, a form of cell death dependent on the accumulation of reactive lipid radicals, in liver cancer (Zhao *et al*, 2020). Moreover, SLC16A1 is a transcriptional target of Myc, a proto-oncogene, and is frequently upregulated in tumors (Doherty *et al*, 2014). Indeed expression of SLC16A1 correlates with prognosis in for example breast or lung cancer patients (Hong *et al*, 2016; Doherty *et al*, 2014). Interestingly, it was also proposed that SLC16A1 could facilitate the mitochondrial import of lactate, representing an intracellular lactate shuttle (Brooks, 2018). While this theory remains to a certain extend controversial, several studies convincingly showed presence and metabolism of lactate in mitochondria (Chen *et al*, 2016; Li *et al*, 2023).

Chemical modulators of SLC16A1

One of the first compounds reported as a SLC16A1 inhibitor was α-cyano-4-hydroxycinnamate (CHC; **Figure 5A**), however this inhibitor displays low potency and low specificity (CHC inhibits

multiple SLC16s and displays higher potency to f.e. mitochondrial pyruvate carrier compared to SLC16s (Halestrap & Denton, 1975)) (Puri & Juvale, 2020). Efforts to optimize this scaffold led to series of dual SLC16A1 and SLC16A3 inhibitors with potency improved more than a thousand-fold (Jonnalagadda *et al*, 2019) (**Figure 5B**). The series of the arguably most prominent SLC16A1 inhibitors originates in 2005, when SLC16A1 was found as a target of a drug candidate originally developed for immunosuppression (**Figure 5C**) (Murray *et al*, 2005). In this study, the authors followed up on compounds originally discovered from a phenotypic screen that measured compounds that reduced proliferation of T cells. By applying chemical proteomics, the study identified SLC16A1 as a target. This series was further progressed to the molecule AR-C155858 (**Figure 5D**). This compound displayed a good selectivity over SLC16A3, but only mildly higher potency to SLC16A1 over SLC16A7 (Ovens *et al*, 2010). Moreover, AR-C155858 showed also improved PK properties, compared to its predecessors (Påhlman *et al*, 2013), and became an tool in investigating biological roles of SLC16A1. Additional development of this compound series yielded the clinical candidate AZD3965 (**Figure 5E**). Similarly to AR-C15585, AZD3965 does not affect SLC16A3, but the selectivity to SLC16A1 is only 6-fold higher compared to SLC16A7 (Curtis *et al*, 2017). Screening of cancer cell lines showed that cell lines derived from lymphomas are particularly sensitive to AZD3965 (Curtis *et al*, 2017). In 2013 AZD3965 entered a phase I clinical trial conducted in patients with advanced solid tumors, diffuse large B cell lymphoma, and Burkitt lymphoma (clinical trial registered under NCT01791595). Recently published data from this trial showed that AZD3965 was tolerated in a dose that produces target engagement, side effects were mainly related to temporal changes in the retina, and importantly, established a dosing and markers for further clinical studies (Halford *et al*, 2023). At the time of writing of this thesis, AZD3965 is ready for phase II, and offered for out-licensing by Cancer Research Horizons (part of Cancer Research UK). It is expected that the drug will be used either in cancers with high *SLC16A1* and low *SLC16A3* expression and/or in combinations with other therapies.

BAY-8002 (**Figure 5F**) is another inhibitor of SLC16A1 with excellent selectivity over SLC16A3, but only mild selectivity over SLC16A7 (Quanz *et al*, 2018). The properties of this compound are very similar to AZD3965, including a comparable binding to SLC16A1 (Wang *et al*, 2021b). Since the publication of the study by Quanz and colleagues there has been no report of BAY-8002 being progressed into clinical trials yet, despite promising pre-clinical data. Apart from inhibitors of SLC16A1 mentioned above, there are several other compounds that were developed (reviewed in (Puri & Juvale, 2020).

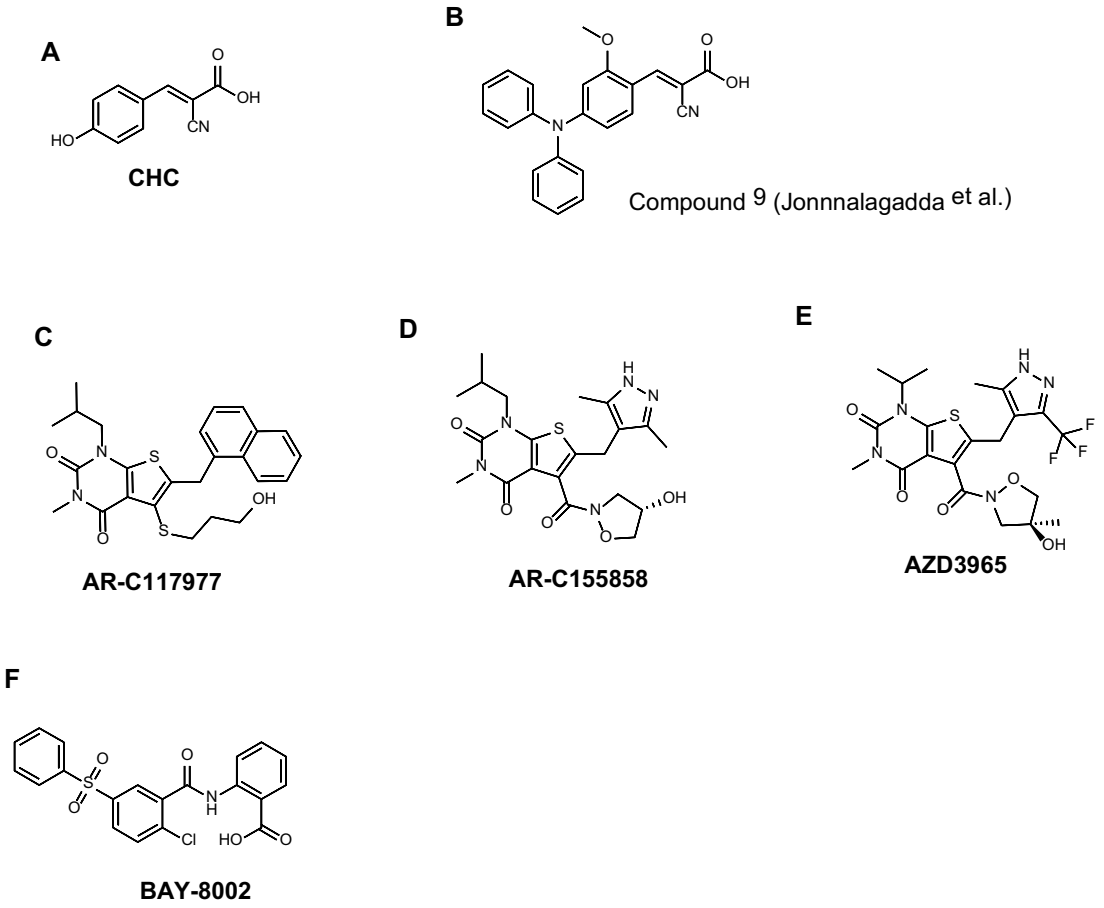


Figure 5: Chemical structures of selected SLC16A1 inhibitors.

1.5.2. SLC16A3 (MCT4)

SLC16A3 is a low affinity transporter that is in general considered to be a major lactate exporter (Contreras-Baeza *et al*, 2019). It plays its main role in highly glycolytic cells, including white skeletal muscle fibres (particularly during excise), astrocytes, immune cells, or chondrocytes (Halestrap, 2013). Moreover, as one of the targets of HIF-1α, the expression of SLC16A3 can rapidly increase during hypoxia (Ullah *et al*, 2006).

SLC16A3 in cancer

A multitude of reports showed that SLC16A3 plays an important role in cancer. It was shown that breast cancer cell lines, in particular the $HER2^+$ subset, are dependent on SLC16A3 (Baenke *et al*, 2015). Moreover, this study reports that *SLC16A3* expression is regulated by the PI3K-Akt signalling pathway. In context of breast cancer, expression of *SLC16A3* was particularly elevated in invasive tumours, and high levels of *SLC16A3* were predictive of poor survival (Baenke *et al*, 2015; Doyen *et al*, 2014). A study focused on non-small cell lung cancer

(NSCLC) found that basigin is frequently di-methylated on lysine 234 in tumours, accompanied to increased interaction with SLC16A3 and subsequent higher levels of SLC16A3 at the plasma membrane and increased lactate export (Wang *et al*, 2021a). This also correlated with tumour progression, pathological grading, and poor survival. SLC16A3 was also shown to be an important mediator of therapy resistance in NSCLC mouse xenograft model (Apicella *et al*, 2018). In that study authors showed that resistance to kinase inhibitors (EGRF and MET) can be developed due to increased lactate levels in the TME and resulting enhancement of metabolic symbiosis between the cancer cells and CAFs. In that case, the lactate produced by tumour cells and exported through SLC16A3 was taken up by CAFs, which further stimulated CAFs to produce cytokines mediating the adaptive treatment resistance. Moreover, silencing of *SLC16A3* in the tumour cells led to re-sensitization of tumour cells to therapy. Similarly, metabolic symbiosis plays a role also in resistance to treatments targeting tumour angiogenesis. Studies utilizing mouse models of pancreatic neuroendocrine cancer, breast cancer and renal cancer showed that resistance to angiogenesis inhibitors can develop through metabolic compartmentalization and enhanced metabolic symbiosis, that can be overcome by silencing of *SLC16A3* (Allen *et al*, 2016; Jiménez-Valerio *et al*, 2016; Pisarsky *et al*, 2016). A genome-wide RNA interference screen found that SLC16A3 is essential in clear cell renal cell carcinoma (ccRCC) cell lines (Gerlinger *et al*, 2012). The same study also analysed samples from ccRCC patients and found that SLC16A3 was significantly upregulated in primary tumours compared to normal kidney tissue and at the same time the expression was even higher in metastatic lesions. High levels of SLC16A3 were also correlating with significantly worse prognosis. Similarly, SLC16A3 was shown to be elevated in bladder cancer were higher expression was associated with inferior survival and silencing of *SLC16A3* led to reduced growth of bladder cancer cells *in vitro* as well as in orthotopic xenograft mouse model (Todenhofer *et al*, 2018). Elevated levels of SLC16A3 in cancer cells compared to normal tissue were found also in pancreatic cancer (Baek *et al*, 2014). High expression of *SLC16A3* correlated with poor prognosis and silencing of *SLC16A3* in pancreatic cancer cell lines with high endogenous expression resulted in cell death (Baek *et al*, 2014). Additionally, this study showed that experimental reduction of *SLC16A3* expression in pancreatic cancer cell lines resulted in a metabolic crisis, that could be overcome by upregulation of mitochondrial metabolism, increase utilization of glutamine, and by induction of micropinocytosis or autophagy. Inhibition of any of these processes created synergistic effects with silencing of *SLC16A3*. Results were additionally confirmed in mouse xenograft models, in which silencing of *SLC16A3* expression slowed tumour growth.

SLC16A3 in other diseases

SLC16A3 also plays a role in heart hypertrophy and hearth failure. It is estimated that cardiomyocytes under normal conditions produce ~60-90% of their ATP from fatty acid oxidation (Fernandez-Caggiano *et al*, 2020). The rest of the ATP comes predominantly from oxidation of pyruvate that is produced equally by glycolysis and conversion from lactate. However, during pathological hearth hypertrophy and heart failure the cardiomyocyte metabolism shifts towards glycolysis that is uncoupled from mitochondrial metabolism (Ritterhoff & Tian, 2023). Several studies showed that this could be caused by decreased expression of the mitochondrial pyruvate carrier (MPC) (Cluntun *et al*, 2021; Fernandez-Caggiano *et al*, 2020; Zhang *et al*, 2020b; McCommis *et al*, 2020). Increased glycolysis associated with the failing heart is connected to increased production of lactate that is exported by SLC16A3 (Cluntun *et al*, 2021). Importantly, Cluntun and colleagues showed that inhibition of SLC16A3 in mouse models of heart hypertrophy leads to accumulation of lactate in cardiomyocytes and subsequent conversion of lactate back to pyruvate, restoration of MPC expression and finally mitochondrial pyruvate oxidation, restoring the physiological state.

As already mentioned, lactate accumulates in the tissue microenvironment during inflammation, which may lead to further amplification of the inflammation (Certo *et al*, 2021). Given that SLC16A3 is a major known lactate exporter, it was proposed that therapeutic targeting of SLC16A3 could be beneficial in the context of inflammatory diseases. It was reported that expression of *SLC16A3* is increased in rheumatoid arthritis (RA) (Fujii *et al*, 2015; Pucino *et al*, 2023). Moreover, Fujii and colleagues have shown that silencing of *slc16a3* in joints of mice with collagen-induced arthritis reduced the disease severity. Future studies will show whether SLC16A3 could indeed become an interesting target in RA and other autoimmune/inflammatory conditions.

Chemical modulators of SLC16A3

In comparison to SLC16A1, the availability of chemical modulators for SLC16A3 was poor until very recently. Several serendipitous discoveries lead to the identification of compounds originally developed for different indications able to also inhibit SLC16A3 as an off-target effect. Syrosingopine (**Figure 6A**), a compound originally developed in the 1970s for treatment of hypertension, was highlighted in a drug repurposing screen focused on the identification of drugs that would enhance the anti-proliferative effects of metformin in cancer cells (Benjamin *et al*, 2016). A follow-up study found that the MoA of syrosingopine in this setting, was due to inhibition of SLC16A1 and SLC16A3, with higher affinity towards SLC16A3 (Benjamin *et al*, 2018). Similarly, diclofenac (**Figure 6B**), well known as an non-steroidal anti-inflammatory

drug, was found to inhibit SLC16A3 and SLC16A1 as an off-target effect (Sasaki *et al*, 2016; Renner *et al*, 2019). Of note, as already mentioned, CHC and it's derivates are also inhibitors of SLC16A3.

The first selective and potent SLC16A3 inhibitor reported in the literature was VB124 (**Figure 6C**; (Cluntun *et al*, 2021)). Cluntun and colleagues used VB-124 in a mouse model of heart hypertrophy, to show that SLC16A3 inhibition could revert heart hypertrophy and subsequent heart failure. To characterize this compound, the authors also performed a genome-wide CRISPR/Cas9 resistance screen, identifying genes involved in OXPHOS as synthetically lethal with the compound. Given the known interplay of lactate metabolism and OXPHOS, this suggested an on-target effect of the agent. The second compound reported in the literature was MSC-4381 (**Figure 6D**; (Heinrich *et al*, 2021)). This compound showed a good potency and selectivity for SLC16A3 over SLC16A1, similarly to VB124. Moreover, MSC-4381 showed good PK properties and favourable oral bioavailability in mouse. Upon co-administration with AZD3965 in the MC38 murine colorectal cancer syngeneic model, the authors showed intracellular lactate accumulation in tumour cells. Based on experiments conducted with fluorescent analogues, the authors also suggested that the compound bound the inward-open conformation of the transporter. MSC-4381 also became a part of the chemical probe collection of the Structural Genomic Consortium (SGC) and is currently undergoing deeper selectivity profiling and characterization (Tredup *et al*, 2023). Two further selective SLC16A3 inhibitors were reported by AstraZeneca. Firstly, AZ1422 (**Figure 6E**) was discovered in a screening campaign of compounds selected based on the presence of carboxylate in a lactate uptake assay and subsequent optimization of the hits (Kawatkar *et al*, 2023). Even though this series of compounds showed good potency and selectivity, their PK properties were not optimal. Throughout the project, however, the scientists developed a functional screening assay that allowed to screen a bigger collection of compounds, leading to the discovery of a second series of compounds. This lead to the development of AZD0095 (**Figure 6F**) (Goldberg *et al*, 2022). This compound showed better PK properties, with potential for oral administration in the clinic and very good selectivity for SLC16A3 over SLC16A1. The compound showed good selectivity also in unbiased chemoproteomics experiment and secondary pharmacology panel of almost 200 targets (Goldberg *et al*, 2022). Several other compounds targeting SLC16A3 were recently described in the patent literature.

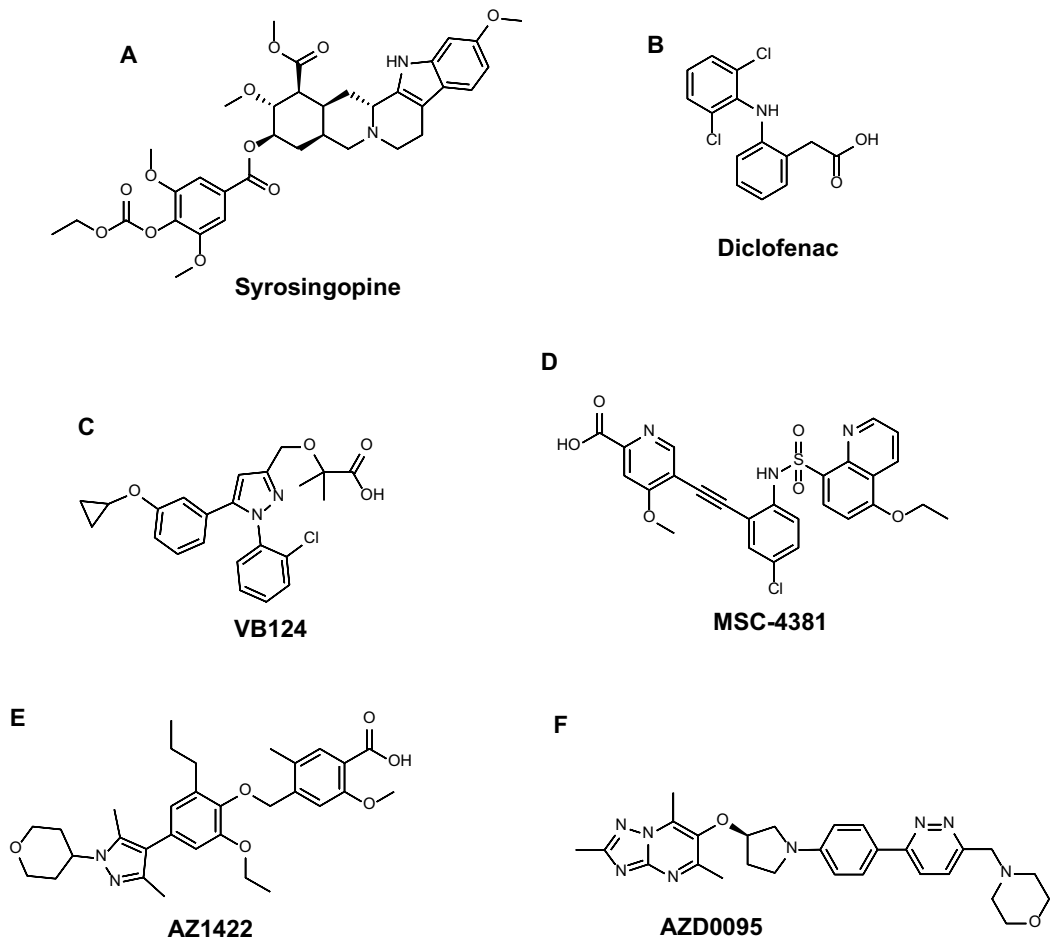


Figure 6: Chemical structures of selected SLC16A3 inhibitors

1.5.3. SLC16A7 (MCT2)

Among all lactate transporters, SLC16A7 shows the highest affinity for pyruvate and lactate among all lactate transporters (Draoui & Feron, 2011). The expression of SLC16A7 is more restricted compared to SLC16A1 and SLC16A3, mainly to tissues or cell types that rely on OXPHOS, or perform gluconeogenesis or lipogenesis, such as neurons, testis, kidney or adipose tissues (Fisel *et al*, 2018; Fishbein *et al*, 2002). The cryoEM structure of human SLC16A7 suggests that the protein can dimerize, and that the dimerization can increase the transport function of the individual subunits (Zhang *et al*, 2020a). SLC16A7 is less studied than SLC16A1 and SLC16A3, and most of the research focuses on its role in the brain, where SLC16A7 mediates the lactate/pyruvate uptake in neurons (Magistretti & Allaman, 2018). In the context of cancer, SLC16A7 was found to be upregulated in prostate cancer (Pertega-Gomes *et al*, 2015). Additionally, a study utilizing a mouse model of breast cancer showed than import of pyruvate through SLC16A7 is required for formation of a metastatic niche in the lung (Elia *et al*, 2019). Mechanistically, the pyruvate imported by SLC16A7 induces α-ketoglutarate production and subsequent activation of collagen hydroxylation. This leads to

remodelling of the extracellular matrix in the lung that allows the formation of metastasis. Moreover, the same study showed that inhibition of pyruvate metabolism in several mouse models is sufficient to reduce the growth of lung metastasis. To date, there are no selective inhibitors of SLC16A7, however, due to the high structural similarity to SLC16A1, SLC16A7 is an off-target of many SLC16A1 inhibitors, such as AZD3965 and BAY-8002.

1.5.4. SLC16A8 (MCT3)

SLC16A8 is exclusively expressed in the retinal pigment epithelium (RPE) and it was shown that genetic variants associate with age-related macular degeneration (Fritsche *et al*, 2013, 2016). A follow-up mechanistic study based on RPE cells differentiated from induced pluripotent stem cells (iPSCs) obtained from patient carrying two copies of *SLC16A8* allele identified in the GWAS studies showed that this variant results in an absence of SLC16A8 and subsequent deficit in transepithelial lactate transport (Klipfel *et al*, 2021). Moreover, a *slc16a8* $^{-/-}$ mouse showed a reduction in visual function while preserving an otherwise normal phenotype (Daniele *et al*, 2008). These studies suggest that SLC16A8 plays an important role in vision.

In summary, several members of the SLC16 family have already shown to be interesting drug targets. With time, it will be interesting to see which will become successful in clinical trial and also what the other SLC16 family members, with less known function, may bring for pharmacology.

1.6 Aims of this thesis

The motivation for this thesis stems from the large pharmacological opportunity represented by the SLC16 family combined with the historical difficulty with reliable and focused screening assay. Recent developments in understanding functional genetic redundancy in human cells accompanied with high-precision and efficient genomic engineering prompted the ambition to use the SLC16 family as prototypic target group for the development of a new strategy in phenotypic assay-based drug discovery. In this thesis I aim to:

1) Develop a highly specific assay strategy that can be used for SLCs and could be, in principle, transferable to different SLCs and other proteins.
2) Use the assay to develop a chemical probe that can be used as a valuable tool to dissect the biological roles of its target, and for the development of a pre-clinical drug candidate.

2. Results

One of the major factors hampering SLC-oriented research and SLC-oriented drug discovery is the lack of biological assays. Purification of proteins for biophysical assays is challenging due to the complex SLC structures that include many transmembrane spanning regions. Furthermore, the development of cell-based systems is impaired by frequent co-expression of multiple SLCs with redundant function, resulting in low assay specificity.

In the following manuscript we set out to develop a cell-based assay system utilizing genetic interactions (GIs) and using this system to develop a chemical probe. As target we chose the lactate transporter SLC16A3. We started by developing a series of cell lines individually dependent on different lactate transporters from the SLC16 family for their fitness. Next, we used these cell lines for a chemical screening campaign. In the first step, we screened a collection of approximately 90,000 compounds in cells dependent on SLC16A3, followed by counter-screening steps in cell lines dependent on other paralogs. This led to identification of two compound series selectively killing only the SLC16A3 dependent cells. Compounds from both series were then tested in orthogonal assays that confirmed binding and inhibition of SLC16A3. Further development of the screening hits yielded slCeMM1, an inhibitor of SLC16A3 displaying excellent potency and selectivity. In order to explain the selectivity and binding mode of slCeMM1, we build a homology model of SLC16A3 and generated several SLC16A3 mutants that resulted in reduced effect of the inhibitor. Finally, by employing a photoaffinity probe and chemical proteomics we demonstrated selectivity of slCeMM1 on proteome level.

The author of this thesis conceptualized this work together with his supervisor and performed most of the experiments. The homology model of SLC16A3 was generated by Claire Colas, chemical screening was performed by Anna Koren and Tatjana Tomek and the chemoinformatic analysis of the results was performed by the author and Andrea Casiraghi. The chemoproteomics experiment was performed by the author with assistance from Fabian Offensperger, Andrea Rukavina, Gary Tin, Elisa Hahn and Sarah Dobner and the analysis was done by Andrea Rukavina and Fabian Frommelt. Andras Boeszoermenyi provided feedback, discussions and contributed to design of several experiments. Viktoriia Bernada helped with tissue culture. Stefan Kubicek, Gerhard F. Ecker, Georg E. Winter and J. Thomas Hannich provided supervision for members of their groups that contributed to this project. Throughout the project, the data were discussed with all co-authors.

Cell Chemical Biology

CellPress
OPEN ACCESS

Brief Communication

Paralog-dependent isogenic cell assay cascade generates highly selective SLC16A3 inhibitors

Vojtech Dvorak,[1] Andrea Casiraghi,[1] Claire Colas,[2] Anna Koren,[1] Tatjana Tomek,[1] Fabian Offensperger,[1] Andrea Rukavina,[1] Gary Tin,[1] Elisa Hahn,[1] Sarah Dobner,[1] Fabian Frommelt,[1] Andras Boeszoermenyi,[1] Viktoriia Bernada,[1] J. Thomas Hannich,[1] Gerhard F. Ecker,[2] Georg E. Winter,[1] Stefan Kubicek,[1] and Giulio Superti-Furga[1,3,4,*]

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, 1090 Vienna, Austria
[2]Department of Pharmaceutical Sciences, University of Vienna, 1090 Vienna, Austria
[3]Center for Physiology and Pharmacology, Medical University of Vienna, 1090 Vienna, Austria
[4]Lead contact
*Correspondence: GSuperti@cemm.oeaw.ac.at
https://doi.org/10.1016/j.chembiol.2023.06.029

SUMMARY

Despite being considered druggable and attractive therapeutic targets, most of the solute carrier (SLC) membrane transporters remain pharmacologically underexploited. One of the reasons for this is a lack of reliable chemical screening assays, made difficult by functional redundancies among SLCs. In this study we leveraged synthetic lethality between the lactate transporters SLC16A1 and SLC16A3 in a screening strategy that we call paralog-dependent isogenic cell assay (PARADISO). The system involves five isogenic cell lines, each dependent on various paralog genes for survival/fitness, arranged in a screening cascade tuned for the identification of SLC16A3 inhibitors. We screened a diversity-oriented library of ~90,000 compounds and further developed our hits into slCeMM1, a paralog-selective and potent SLC16A3 inhibitor. By implementing chemoproteomics, we showed that slCeMM1 is selective also at the proteome-wide level, thus fulfilling an important criterion for chemical probes. This study represents a framework for the development of specific cell-based drug discovery assays.

INTRODUCTION

The solute carrier (SLC) superfamily of membrane transporters plays an important role in cellular homeostasis and metabolism. An increasing number of SLCs is being linked to different human diseases, which is reflected in a growing interest in SLC-oriented drug discovery.[1,2] Yet if compared with other protein families, the SLC superfamily remains pharmacologically underexploited.[3] This originates on one hand from the fact that most SLCs are relatively understudied and on the other hand from a lack of tools, such as chemicals targeting SLC function or appropriate biological assays.[4]

Development of biophysical assays using purified proteins is challenging due to the complexity of SLC structures, containing multiple transmembrane-passing helices and hence complicated protein purification. Development of cell-based assays, on the other hand, is hampered by frequent redundancies in the SLC function, and expression of multiple SLCs capable of transporting same substrates by the same cell, representing a challenge for assay specificity.[1,5] Ideally, a chemical screening assay would combine the advantages of the physiological, cellular context of the target with the precision of assays employing purified protein. This could be attempted by engineering a cell-based system with reduced functional genetic redundancies. One possibility how to identify such redundancies is to exploit genetic interactions, in particular synthetic lethality.[6] This concept was originally derived from fly and yeast genetics and later rationalized for finding cancer specific drug targets. Unbiased genetic interaction screens showed that genetic dependencies are very powerful in uncovering functional overlap and relatedness between genes involved in similar cellular processes.[7,8] While the exploitation of genetic interactions for drug discovery has been so far mostly oriented toward synthetic lethality as a vulnerability of cancer cells,[9] in principle this concept could be leveraged to uncover functional redundancies and to increase the functional resolution of cell-based systems. This could be particularly useful in protein groups like SLCs, where a large portion of the superfamily remains orphan, and thus the identification of functional overlaps is challenging.

Although lactate is sometimes viewed simply as a metabolic waste product, it is becoming clear that it has a role as a circulating nutrient as well as signaling molecule.[10,11] In fact, lactate can efficiently shuttle carbon atoms between different tissues, as well as different cell types within tissues, and is a major substrate for the TCA cycle.[12] Monocarboxylate transporters SLC16A1 (MCT1), SLC16A3 (MCT4), SLC16A7 (MCT2), and

SLC16A8 (MCT3) are responsible for the transport of lactate across the plasma membrane and play a critical role in this process.[13] Even though the functions of individual SLC16 lactate transporters can be considered redundant to some extent, SLC16A1 is typically considered the major lactate importer while SLC16A3 is a major lactate exporter, mainly due to the differences in affinities to lactate and pyruvate.[13] Similarly, all four paralogs vary in their expression pattern across tissues, as well as cell lines, with, at one extreme, SLC16A1 being expressed ubiquitously, and at the other extreme, SLC16A8 being exclusively expressed in retinal tissue.[14] Although all four paralogs are highly similar in their structures, the degree of similarity between them varies. While SLC16A1 and SLC16A3 share 39% of amino acid identity, the identity between SLC16A1 and SLC16A7 is 59% and the identity between SLC16A3 and SLC16A8 is 57%.[15] The structural similarity represents a challenge for development of selective inhibitors. This is illustrated by the existing SLC16A1 inhibitors, such as AZD3965 or BAY-8002, that display decent selectivity over SLC16A3, but lack selectivity over SLC16A7.[16] Modulation of the levels of lactate through targeting lactate transport was proposed as a target in several therapeutic areas, including cancer, inflammatory diseases, and heart hypertrophy (recently reviewed in[17]). While SLC16A1 inhibitors exist already for many years, and the most clinically advanced SLC16A1 inhibitor recently completed a phase I trial,[18] inhibitors for SLC16A3 have been emerging only recently.[19–23]

In this study, we have implemented the synthetic lethality concept to focus a phenotypic screening assay on SLC16A3. By manipulating the expression of lactate transporters in HAP1 cells, we created a paralog-dependent isogenic cell line (PARADISO) assay strategy, employing a series of perfectly isogenic cell lines, individually dependent on four different paralog genes for their fitness/survival, or the original wild-type cell line as a control. PARADISO is arranged in a cascade logic that allowed us to screen a diversity-oriented library of ~90,000 compounds. Two chemotypes inhibiting SLC16A3 in a highly selective manner were discovered through the PARADISO screen. We have further validated both chemotypes with orthogonal assays and derived slCeMM1, an SLC16A3 inhibitor with potency <100 nM and great selectivity over the other three additional paralogs. Finally, by implementing chemical proteomics, we showed that slCeMM1 is selective also at the proteome-wide level, fulfilling a chemical probe criterion.

RESULTS

Development of a paralog specific screening assay

To develop a paralog-specific screening system, we started from an SLC genetic interaction network recently reported for the HAP1 haploid human cell line.[24] This is a very well characterized cell line, for which the genome and proteome have been described, and in which we have been able to identify the so-called essentialome, all genes functionally required for fitness under standard cell culture condition.[25] By virtue of being haploid and otherwise preserve all salient features and pathways of other tissue culture cell lines, it allows for genomic engineering without interference of a second (or often multiple, such as in HEK293 and HeLa) allele.[26,27] As a proof-of-concept we focused on SLC16A1 and SLC16A3, lactate transporters with a strong and reciprocal genetic interaction (Figure S1A). Multiple studies previously noted a functional redundancy between SLC16A1 and SLC16A3 and reported frequent sensitivity to inhibition of SLC16A1 in cell lines not expressing *SLC16A3*.[16,28–31] To date, four transporters of lactate in the SLC16 family have been described,[13] so we hypothesized that it should be possible to manipulate expression of all four paralog genes, to generate cell lines that are each dependent on individual transporters (Figure 1A). In this system, we expect cell line expressing only *SLC16A1* to be sensitive to SLC16A1 inhibitors, while not being affected by molecules selectively inhibiting SLC16A3, SLC16A7, or SLC16A8. Similarly, cell lines expressing only individual other paralog genes would be sensitive only to compounds specifically inhibiting the corresponding protein products, while compounds acting as dual inhibitors would affect cells lines expressing the cognate paralogs while having no effect on cell lines expressing other paralogs.

First, we compared the sensitivity of HAP1 WT, HAP1 *SLC16A1*$^{-/-}$, and HAP1 *SLC16A3*$^{-/-}$ cells to SLC16A1/SLC16A7 inhibitors AZD3965 and to BAY-8002 as well as the SLC16A3/SLC16A1 inhibitor Syrosingopine. We found that only *SLC16A3*$^{-/-}$ cells were sensitive to AZD3965 and BAY-8002 (Figures 1B and S1B), while *SLC16A1*$^{-/-}$ cells were more sensitive to Syrosingopine, in agreement with previously reported higher affinity of Syrosingopine to SLC16A3 compared to SLC16A1.[30] To further confirm that this dependency is mediated by transport function, we re-expressed the transporters either as WT, or as a transport-deficient mutant, bearing a mutation in a conserved arginine residue critical for the function (SLC16A3^{R278K}[32] and SLC16A1^{R313Q}[33]) and re-tested the inhibitors (Figures S1D and S1E). While WT transporters rescued the previously observed effect, the transport-deficient version did not, confirming that the synthetic lethality was dependent on the transport function (Figures 1C, 1D, and S1C). Interestingly, RNA-Seq showed that HAP1 cells endogenously do not express *SLC16A7* or *SLC16A8* (Figures 1E and S1F), further suggesting that fitness of HAP1 cells was dependent on expression of at least one functional lactate transporter. To create isogenic cell lines with dependencies on these two paralogs, we overexpressed (OE) individually *SLC16A7* and *SLC16A8* in HAP1 *SLC16A3*$^{-/-}$ cell line and subsequently knocked-out *SLC16A1* (Figures 1A and S1G). No visible effect on cell fitness was observed upon the *SLC16A1* knockout, indicating that essential functions, and synthetic lethality, was rescued by exogenously introduced paralogs. Moreover, to test the dependency, we compared the sensitivity of these cells to AZD3965 and BAY-8002 and found that HAP1 *SLC16A3*$^{-/-}$, *SLC16A1*$^{-/-}$, *SLC16A7* OE were sensitive to AZD3965 and BAY-8002, while HAP1 *SLC16A3*$^{-/-}$, *SLC16A1*$^{-/-}$, *SLC16A8* OE cells were not (Figures 1F and S1H). These results are in agreement with previous studies reporting that both AZD3965 and BAY-8002 inhibit SLC16A7, albeit with lower affinity compared to SLC16A1,[16,29] suggesting that these cell lines are dependent on the newly introduced SLC. These data showed that by manipulating expression of lactate transporters in HAP1 isogenic cell lines, survival/fitness dependency on individual paralogs can be generated.

Chemical screening identified selective SLC16A3 inhibitors

Next, we used the PARADISO assay logic to convey a chemical screening campaign targeting SLC16A3 (Figure 2A). First, we

CellPress
OPEN ACCESS

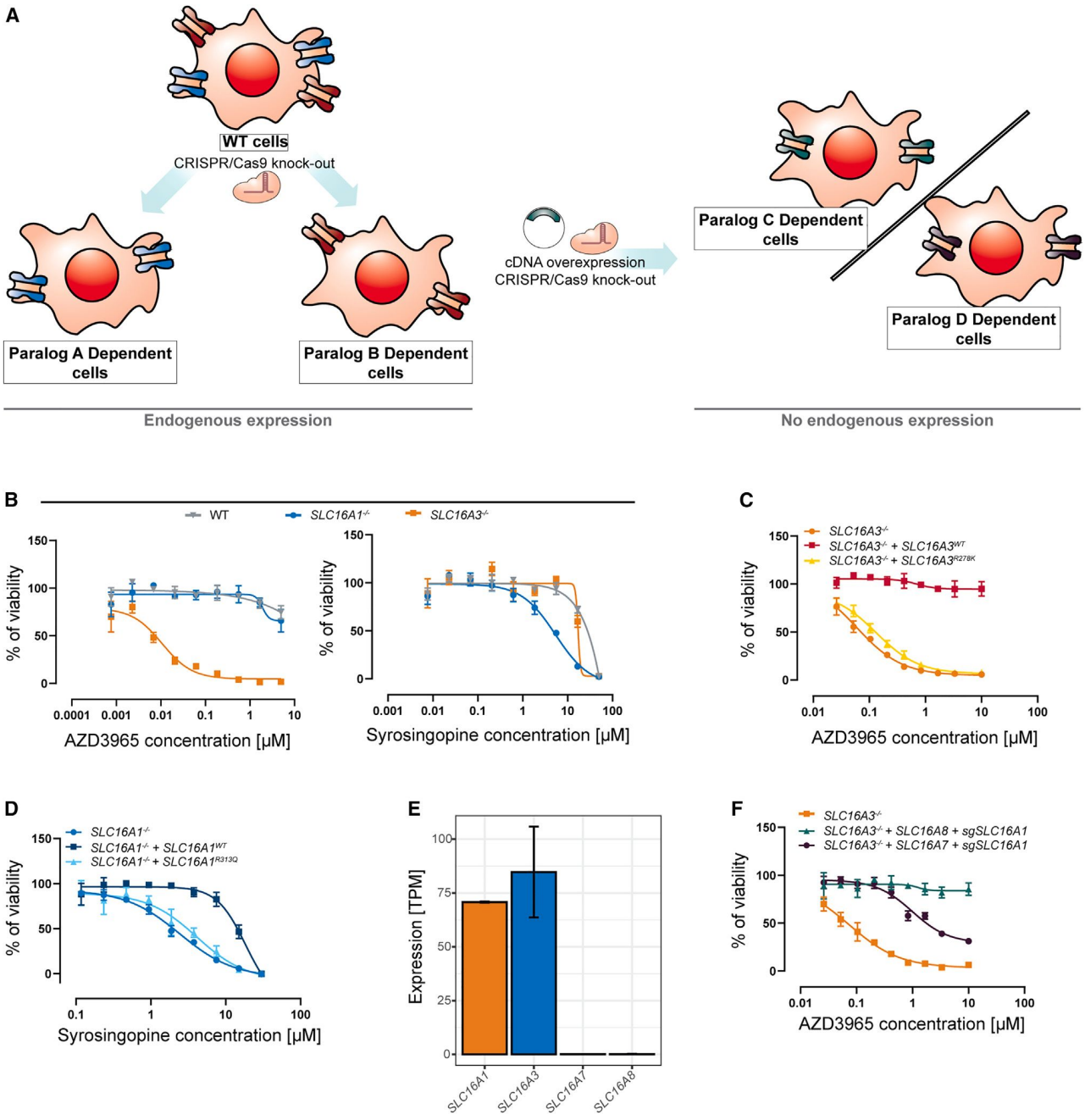


Figure 1. PARADISO assay validation for SLC16 family lactate transporters

(A) Paralog-dependence isogenic cell assay logic.

(B) Sensitivity of HAP1 WT, HAP1 *SLC16A1*$^{-/-}$, and HAP1 *SLC16A3*$^{-/-}$ cell lines to AZD3965 (SLC16A1/SLC16A7 inhibitor) and Syrosingopine (SLC16A3/SLC16A1 inhibitor).

(C) Sensitivity of HAP1 *SLC16A3*$^{-/-}$ and HAP1 *SLC16A3*$^{-/-}$ with *SLC16A3*^{WT} or *SLC16A3*^{R278K} overexpression to AZD3965.

(D) Sensitivity of HAP1 *SLC16A1*$^{-/-}$ and HAP1 *SLC16A1*$^{-/-}$ with *SLC16A1*^{WT} or *SLC16A1*^{R313Q} overexpression to Syrosingopine.

(E) RNA seq data showing expression of lactate transporters in HAP1 WT cells.

(F) Sensitivity of SLC16A7 (*SLC16A3*$^{-/-}$ + *SLC16A7* + *sgSLC16A1*) and SLC16A8 (*SLC16A3*$^{-/-}$ + *SLC16A8* + *sgSLC16A1*) dependent cells to AZD3965. All data are reported as mean ± SD.

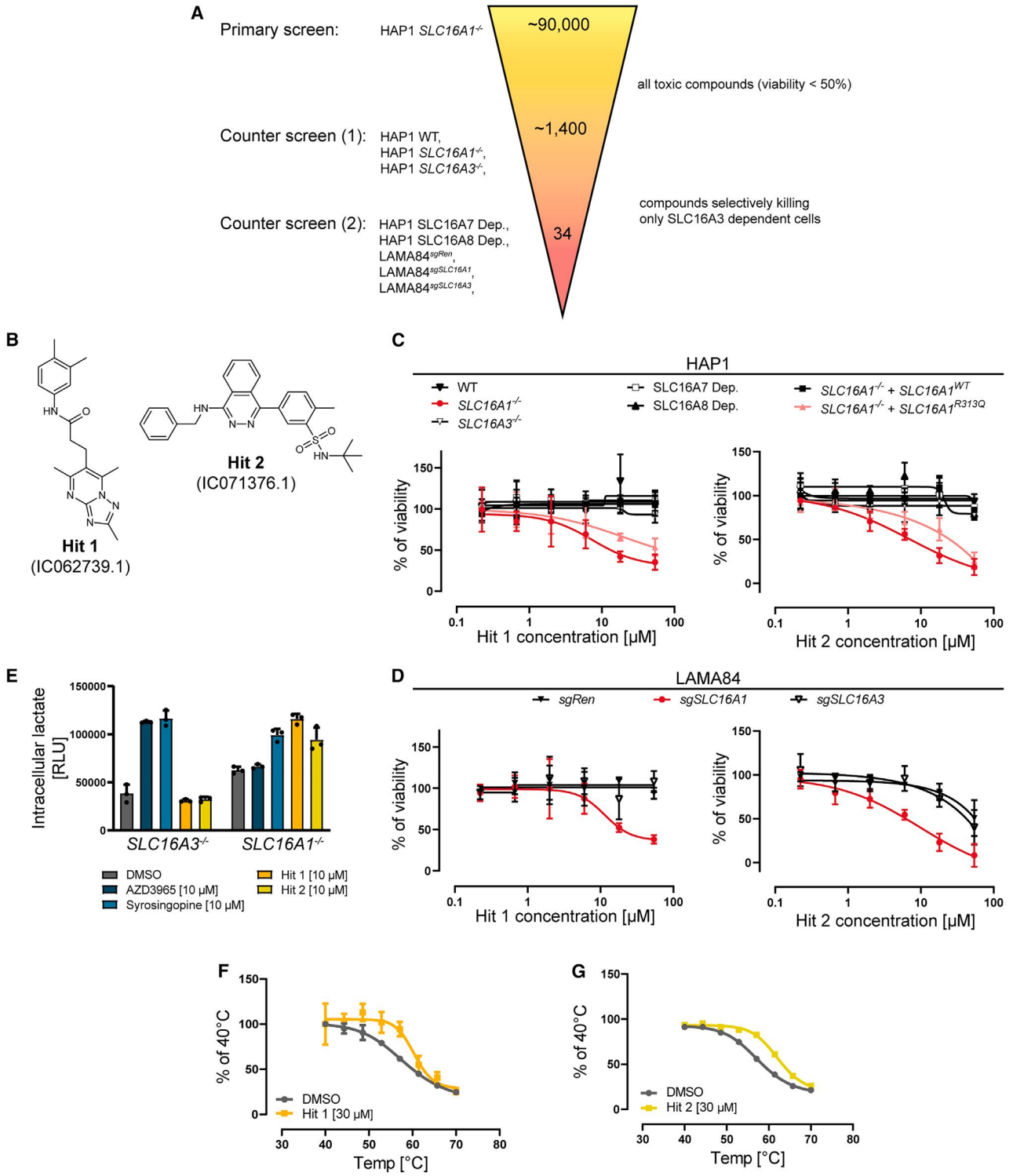


Figure 2. Chemical screening using PARADISO assay uncovered selective SLC16A3 (MCT4) inhibitors

(A) Schematic representation of the SLC16A3 targeted chemical screening, indicating cell lines number of compounds, and hit criteria used in individual screening steps.

(B) Chemical structures of representative hits based on triazolopyrimidine and phthalazine scaffolds.

(C) Data from the chemical screen for HAP1 cells lines.

(D) Data from the chemical screen for LAMA84 cell lines.

(legend continued on next page)

used our in-house diversity-oriented library of approximately 90,000 compounds to screen for compounds reducing viability in HAP1 *SLC16A1*$^{-/-}$ cells. We identified 1,410 compounds reducing the viability >50% compared to DMSO. To separate compounds acting on SLC16A3 from non-specific ones, we counter-screened the toxic compounds in dose response settings using not only HAP1 WT, but also *SLC16A1*$^{-/-}$ and *SLC16A3*$^{-/-}$ cell lines. This yielded 34 compounds able to selectively kill only the HAP1 *SLC16A1*$^{-/-}$ cells, dependent on SLC16A3 function, but not any of the other two isogenic cell lines. To profile the selectivity further and to increase the confidence in our hits, we expanded the dose-ranging validations to additional cell lines including HAP1 with engineered SLC16A7 or SLC16A8 dependency, HAP1 *SLC16A1*$^{-/-}$ overexpressing *SLC16A1*^{WT} or *SLC16A1*^{R313Q}, and similarly to HAP1, we used LAMA84^{sgRen}, LAMA84^{sgSLC16A1}, and LAMA84^{sgSLC16A3} (SLC16A1-SLC16A3 synthetic lethality reported previously in,[31] Figure S1I). We found seven compounds sharing a triazolopyrimidine scaffold and six compounds with shared phtalazine core (Table S1, examples in Figure 2B), which killed selectively only cell lines dependent on SLC16A3 (Figures 2C and 2D). Next, we confirmed that the treatment with compounds from both series resulted in intracellular lactate accumulation in HAP *SLC16A1*$^{-/-}$, but not in HAP1 *SLC16A3*$^{-/-}$ cells, suggesting that compounds inhibited SLC16A3 mediated lactate export (Figure 2E). To validate the binding of compounds to SLC16A3, we used the thermal shift assay using cell lysates and split nano-luciferase as readout. We observed a shift in the melting temperature of SLC16A3 upon treatment with both, triazolopyrimidine (Figure 2F) as well as phthalazine (Figure 2G) based compounds, suggesting binding to SLC16A3.

slCeMM1 as a selective inhibitor of SLC16A3

We further tested several analogs from both series using the PARADISO assay (Table S1). In addition, for a selection of compounds, the metabolic stability in liver microsomes was assessed (Figure S2A). These results led us to selection of slCeMM1 for further studies (Figure 3A). Further characterization showed that slCeMM1 inhibited growth of HAP1 cells dependent on SLC16A3 (IC_{50} = 0.88 μM), but not on SLC16A1, SLC16A7 or SLC16A8, and this effect was rescued by re-introduction of *SLC16A1*^{WT} but not transport-deficient mutant *SLC16A1*^{R313Q} (Figure 3B). The functional assay showed intracellular lactate accumulation upon treatment with slCeMM1 in SLC16A3 dependent cells (IC_{50} = 91 nM), but not in cells dependent on SLC16A1 (Figure 3C). Finally, binding to SLC16A3 was confirmed using the thermal shift assay (Figure 3D).

To investigate the mode of action of slCeMM1, as well as the possible structural basis for the selectivity toward SLC16A3, we built a homology model based on recently published structures of the paralog proteins[34–36] and our structure-activity relationship (SAR) data. Docking of slCeMM1 suggested binding of the compound to the outward-open conformation of the transporter (Figure 3E). For the initial validation of the docking pose, we grouped residues important for the binding based on location in the protein structure (Figure S3A), mutated them to alanine and tested binding of slCeMM1 using the thermal shift assay. Loss of slCeMM1-mediated stabilization was observed in: (1) residues important exclusively for binding to the outward-open conformation (L68, F243, and P246; Figure S3B); (2) residues involved in lactate/H^+ recognition and transport (K40, D274, and R278; Figure S3C); (3) residues localized in the central part of the structure (Y34, Y72, S156, Y332, and G336; Figure S3D). This suggested that at least a subset of residues in each group is critical for slCeMM1 binding. Comparing our model with structure of SLC16A1 bound to AZD3965[34] suggested similarities between the binding sites (Figure S3E). Since our preliminary results showed that alanine mutant groups had reduced function (data not shown), we decided to focus on residues mediating selectivity between SLC16A3 and SLC16A1 using similar approach as Wang and colleagues.[34] To do this, we selected several residues surrounding slCeMM1-binding pocket that are not conserved between SLC16A1 and SLC16A3 and mutated them individually for corresponding residues from SLC16A1 (F130L, A153M, V239F, P246L, and Y332F, Figure 3F). Immunofluorescence showed that staining pattern between the mutants and WT SLC16A3 is similar and that all mutants are capable of trafficking to the plasma membrane (Figure 3G). To determine the functionality, we overexpressed the mutants in HAP1 *SLC16A3*$^{-/-}$ cells and tested the sensitivity to AZD3965. All mutants showed rescue phenotype comparable with WT SLC16A3, except V239F, which displayed weaker rescue (Figure S3F). Taken together, these data show that most of our mutants were functional; however, the function of V239F may be reduced. Next, we tested overexpression of SLC16A3 mutants on the effect of slCeMM1 in HAP1 *SLC16A1*$^{-/-}$ cells. We observed that all tested mutations rescued the slCeMM1 effect partially (Figures 3H and 3I), indicating that tested residues are involved in slCeMM1 binding.

slCeMM1-PAP uncovered selectivity at the proteome level

To determine the selectivity of slCeMM1 using an unbiased chemoproteomic approach, we synthesized a photoaffinity probe based on the slCeMM1 scaffold (slCeMM1-PAP; Figure 4A). The diazirine moiety enables covalent crosslinking upon photo activation and a terminal alkyne handle allows for conjugation to azide-reporter tags via click chemistry.[37] First, we tested the biological activity of slCeMM1-PAP by comparing sensitivity of HAP1 *SLC16A1*$^{-/-}$ and HAP1 WT with the parental compound. Both compounds showed similar effects, however the PAP was slightly less effective in *SLC16A1*$^{-/-}$ cells and showed increased toxicity in WT cells at high concentrations (Figure 4B). This suggests that the compound is biologically active but may have off-target effects at high concentrations (Figure 4B).

We then assessed photoaffinity labeling of SLC16A3 in a HAP1 *SLC16A3-StrepHA* overexpressing cell line. Cells were treated with slCeMM1-PAP in the presence of different concentrations

(E) Effect of indicated molecules on intracellular lactate accumulation in HAP1 *SLC16A1*$^{-/-}$ and HAP1 *SLC16A3*$^{-/-}$ cell lines 6 h after treatment.
(F) Thermal shift assay in lysates of HEK293T cells stably expressing *HiBiT-SLC16A3* upon treatment with Hit 1 or DMSO. Modified HiBiT lytic assay used as readout.
(G) as in (F) but for Hit 2. All data are reported as mean ± SD.

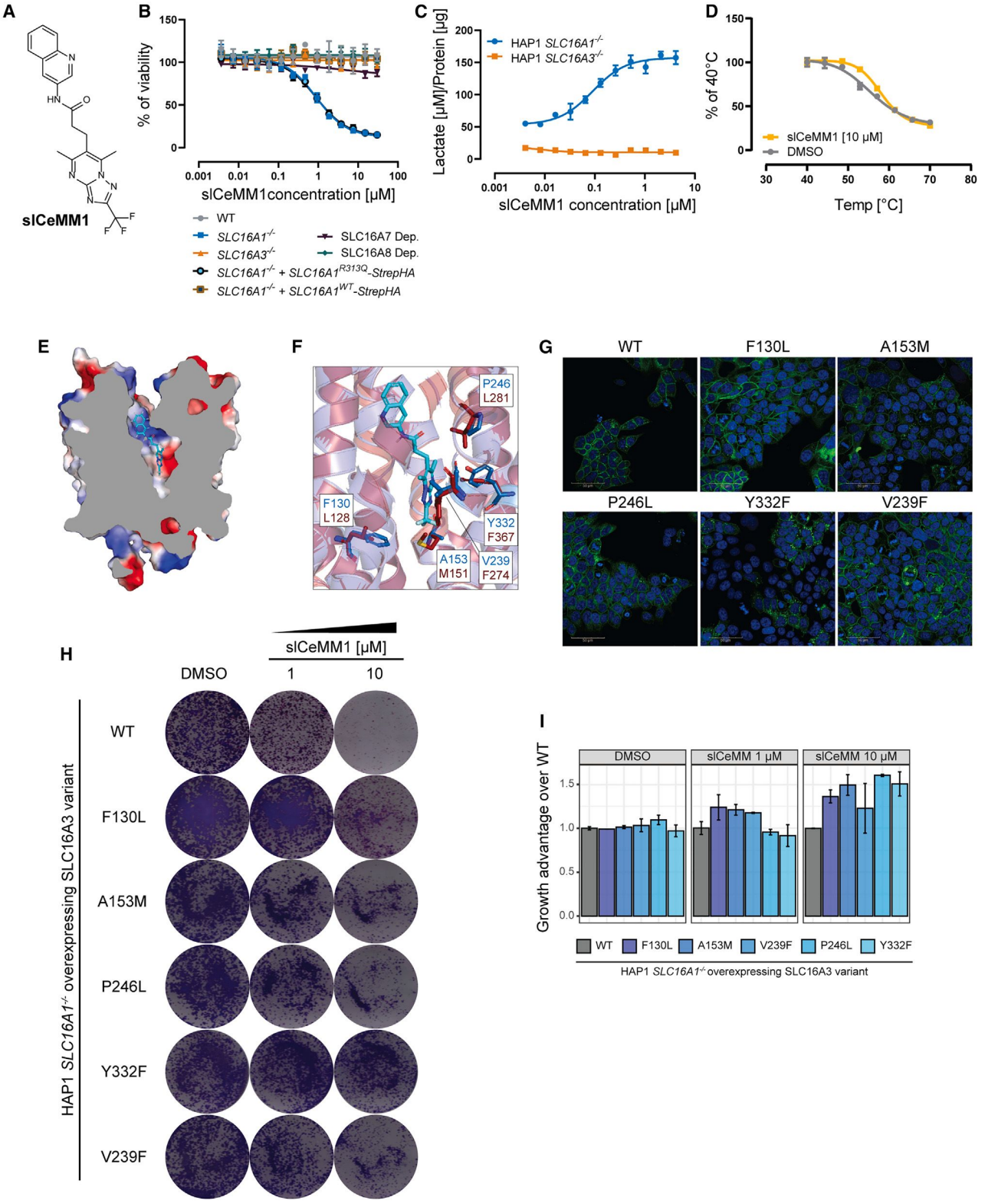


Figure 3. slCeMM1 is a selective and potent SLC16A3 (MCT4) inhibitor

(A) Chemical structure of slCeMM1.

(B) Viability of HAP1 cell lines upon treatment with slCeMM1 (SLC16A7 Dep. refers to HAP1 *SLC16A3*$^{-/-}$ + *SLC16A7* OE + *sgSLC16A1* cells and SLC16A8 Dep. refers to HAP1 *SLC16A3*$^{-/-}$ + *SLC16A8* OE + *sgSLC16A1* cells).

(legend continued on next page)

of slCeMM1 or its inactive, structurally related analog, S1_007 (Figure S2B, Table S1) as free competitors. The diazirine moiety of slCeMM1-PAP was then crosslinked with UV; cells were lysed followed by protein extraction and pulldown pre-enrichment of SLC16A3 using StrepHA tag. The alkyne handle of slCeMM1-PAP was then conjugated to the TAMRA-N_3 reporter and visualized by SDS-PAGE and in-gel fluorescence scanning. The slCeMM1-PAP showed good labeling that was reduced by slCeMM1 competition, but not by S1_007 (Figure S2C).

To identify binding targets of slCeMM1, we performed quantitative mass-spectrometry (MS) based proteomics experiments using HAP1 and MDA-MB-231 cell lines. We compared the enrichment of proteins by slCeMM1-PAP and conditions where slCeMM1-PAP was competed with 5X concentration of slCeMM1 or S1_007 as control. Proteins that bind slCeMM1 should be enriched by slCeMM1-PAP, and their abundance should be reduced upon competition with slCeMM1, but not S1_007.[38] To do this, we pre-treated cells with slCeMM1 or S1_007, followed by addition of slCeMM1-PAP, UV crosslinking, protein extraction, click-chemistry conjugation of a biotin-N_3, pulldown enrichment, and MS analysis of interacting proteins (Figure 4C). In total, slCeMM1-PAP enriched 15 proteins in HAP1 and 9 proteins in MDA-MB-231 (log2 fold change over constant region fragment (CRF) > 2, Figures 4D and 4E). In both models, SLC16A3 showed the most prominent reduction in labeling upon competition with slCeMM1, suggesting overall good selectivity of the compound for this target. Moreover, the inactive structurally related compound S1_007 did not change the abundance of SLC16A3 (Figures S2D and 4E). A similar behavior was observed only in the case of the DCTPP1, however, this protein showed relatively low overall enrichment (slCeMM1-PAP/CRF log2 fold change of 0.98 in HAP1 and 1.02 in MDAMB231, respectively), suggesting that DCTPP1 could be a highly abundant off-target at high concentrations of slCeMM1. SLC16A1, endogenously expressed only in HAP1, was detected, but not enriched, supporting the paralog selectivity of slCeMM1. These data provide strong evidence for SLC16A3 engagement of slCeMM1. At the whole proteome level, slCeMM1 displays an overall good selectivity.

DISCUSSION

Development of highly specific cell-based assays for drug discovery targeting SLCs remains challenging mainly due to frequent functional redundancies. In this study, we established a strategy, the PARADISO assay that reduces redundancies, thus establishing an unambiguous SLC-phenotype relationship. We used the SLC16 family lactate transporters to establish a proof-of-concept. Previous studies focused on discoveries of inhibitors for SLC16A1/SLC16A3 relied on the use of cell lines with differential expression of lactate transporters. For example Quanz and colleagues used primarily DLD-1 to measure SLC16A1 and EVSA-T or 786-O for SLC16A3-mediated transport.[16] Heinrich and colleagues used MDA-MB-231 for SLC16A3 and SNU-398 for SLC16A1.[20] In our study we used a series of isogenic cell lines arranged in a logical cascade allowing the efficient identification of chemical agents acting in a highly selective manner in a cellular setting, in which the target is folded, modified, and localized naturally. Importantly, one of the strengths of the PARADISO system is that we could assess selectivity regarding the SLC16A7 and SLC16A8 proteins, products of gene paralogs that are usually not expressed individually in cell lines. Because of this counter-screens using cell lines with differential expression levels of SLC16 paralogs has been the only, suboptimal, option until now. This is particularly important because the closest related paralog of SLC16A3 is indeed SLC16A8.[15] Additionally, the isogenic background of the PARADISO system provides confidence that differences in phenotype across the cell lines can be confidently attributed to the paralog that is assessed, rather than confounding dependencies arising from different genetic backgrounds. Using the PARADISO assay, we found two chemotypes selectively inhibiting SLC16A3 and we optimized the initial hits into slCeMM1. Furthermore, using an unbiased chemoproteomic approach we were able to confirm selectivity on a whole proteome level, suggesting further versatility of the PARADISO approach.

Several highly potent inhibitors of SLC16A3 were recently reported,[19,20] and several less potent/selective SLC16A3 inhibitors were described previously.[30,39–41] When finalizing this manuscript, Kawatkar and colleagues and Goldberg and colleagues reported another SLC16A3 inhibitors with nM potency, the latter based on a triazolopyrimidine scaffold.[22,23] To the best of our judgment, slCeMM1 may well be the compound with the most rigorously defined selectivity among SLC16A3 paralogs, and with an experimentally assessed selectivity among cellular proteins, as defined by a proteomic approach. We therefore believe that our SLC16A3 inhibitor fulfills the criteria for a chemical probe[42] and will thus become instrumental for the scientific community to assess the specific role of SLC16A3/MCT4 more broadly. Despite the possibly lower potency compared to the recently discovered SLC16A3 inhibitors,[19,20,22,23] this puts slCeMM1 into a unique position for dissecting the specific biological role of SLC16A3 or validating it as a therapeutic target.[43] In particular, slCeMM1 should be a valuable additional tool that helps to chemically validate SLC16A3 as a target based on experiments with gene knock-out/knock-down strategies. This includes cases where SLC16A3 has been suggested as possible target in disease areas such as cancer[44–48] or rheumatoid

(C) Effect of slCeMM1 treatment on intracellular lactate accumulation HAP1 *SLC16A1*$^{-/-}$ or HAP1 *SLC16A3*$^{-/-}$ after 6 h.
(D) Thermal shift assay in lysates of HEK293T cells stably expressing *HiBiT-SLC16A3* upon treatment with Hit 1 or DMSO.
(E) SLC16A3 homology model in outward-open conformation with docked slCeMM1.
(F) Overlap of SLC16A3 homology model (blue) with docked slCeMM1 and CryoEM structure of SLC16A1 (red; PDB 6LYY).
(G) Immunofluorescence staining of HiBiT-tag in HAP1 *SLC16A1*$^{-/-}$ cells with overexpression of *SLC16A3* variants (all constructs expressed with HiBiT tag). Scale bars 50 μm.
(H) Crystal violet staining of HAP1 *SLC16A1*$^{-/-}$ cells, stably overexpressing indicated *SLC16A3* variants, treated with slCeMM1.
(I) Growth advantage calculate from doubling times of HAP1 *SLC16A1*$^{-/-}$ cells, stably overexpressing indicated *SLC16A3* variants, treated with slCeMM1. Data in (B-D) and (I) are presented as mean ± SD.

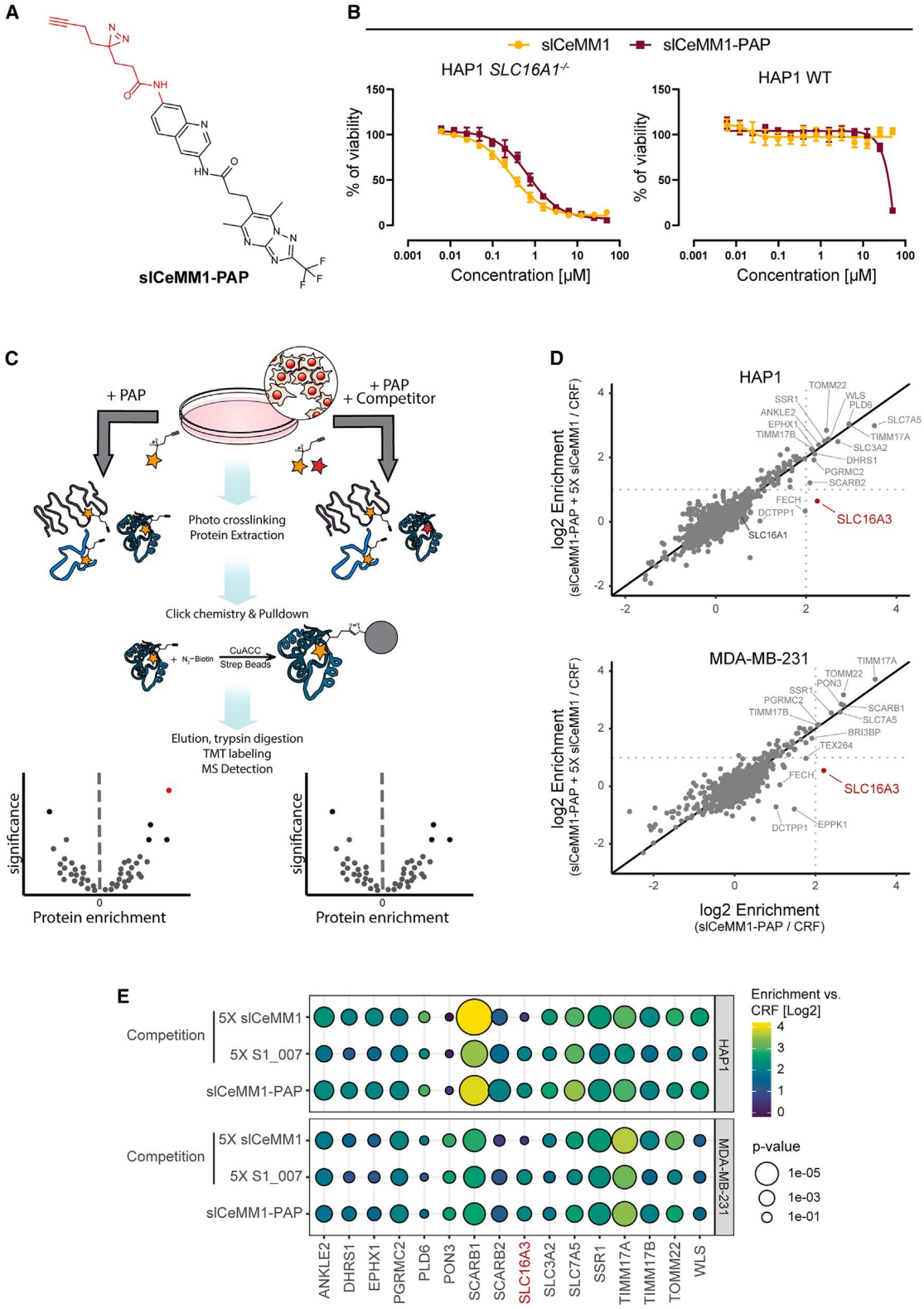


Figure 4. Chemoproteomic profiling of slCeMM1 selectivity

(A) Chemical structure of slCeMM1-PAP.

(B) Viability of HAP1 *SLC16A1*$^{-/-}$ and HAP1 WT upon treatment with slCeMM1 or slCeMM1-PAP. Data reported as mean ± SD.

(legend continued on next page)

arthritis.[49] In addition, our study reports a homology model of SLC16A3 based on recently published structures as well as our SAR data. Our outward-open model complements the SLC16A3 model in the AlphaFold database, which is in inward-facing conformation, further expanding the possibilities for optimizations of drugs targeting SLC16A3.[50]

In summary, the presented study provides a framework for the development of highly specific cell-based assays for drug discovery, which may be widely applicable not only to SLCs, but also to other challenging targets possessing similar functional redundancies. In our view, the main limitation of this approach is the requirement for strong genotype-phenotype connections that may require a prior genetic screen for their identification. While genetic interactions solely among SLCs may be limited,[24,51] an increasing number of studies are reporting unique SLC dependencies characterized by expression of metabolic genes. For example AML cells depend on myo-inositol import through SLC5A3 due to silencing of *ISYNA1*, an enzyme involved in myo-inositol biosynthesis,[52] or ovarian cancer cells depend on phosphate exporter SLC53A1 (XPR1) due to overexpression of phosphate importer *SLC34A2*.[53] In principle, the PARADISO assay design would also be applicable in these cases, expanding its potential to additional opportunities beyond SLC-SLC genetic interactions. In addition, using the PARADISO approach with cell viability as readout combines the advantages of target oriented and phenotypic screening, and thus is able to also capture compounds that modulate the target indirectly, yet in a highly specific manner.

Limitations of the study

The PARADISO approach is not suitable, as designed, for genes that do not have paralogs or are essential to start with. Another limitation of our study is the sparse SAR information for the slCeMM1, as well as for the phthalazine series of compounds due to the limited access to medicinal chemistry. Similarly, while the liver microsome stability assay suggests overall good metabolic stability of slCeMM1, additional experiments to determine the *in vivo* usability of slCeMM1 are required. In addition, even though we were able to validate the homology model and binding of slCeMM1 with mutagenesis, our study implements a homology model instead of an experimentally determined structure.

SIGNIFICANCE

The manuscript describes a chemical screening strategy allowing the straightforward identification of compounds acting selectively on an individual paralog among its peers. It is based on a cascade of assays using perfectly isogenic human cell lines engineered to be dependent on individual paralog genes. The approach is in principle applicable to any target expressed in a human cell line and thus has general validity. Here it is used for the identification and characterization of potent, specific, and selective inhibitors of the *SLC16A3* gene encoded membrane transporter of lactate and other monocarboxylates, an increasingly attractive target in a variety of diseases. Elucidation of the molecular basis for specificity compared to paralogs at the structural level and demonstration of selectivity among cellular proteins render the new compounds attractive chemical probes for the scientific community, empowering the precise assessment of its role in a variety of experimental settings. Several features make the two series of identified compounds attractive points of departure for drug discovery campaigns.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Cell lines
- METHOD DETAILS
  - Plasmids and stable cell line generation
  - Viability assay
  - Chemical screening
  - Thermal shift assay in lysates
  - Intracellular lactate measurement
  - Chemoproteomics
  - Immunofluorescence staining and imaging
  - Crystal violet proliferation assay
  - Doubling-time assay
  - qPCR
  - Immunoblotting
  - Homology modeling and docking
  - Liver microsome stability assay
  - Synthesis of slCeMM1 and slCeMM1-PAP
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Chemoproteomics
  - Liver microsome stability assay

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chembiol.2023.06.029.

ACKNOWLEDGMENTS

We thank the members of Superti-Furga laboratory, members of the RESOLUTE consortium and members of Solgate GmbH, in particular Ariel

(C) Schematic representation of the chemoproteomic experiment with slCeMM1-PAP.
(D) Proteins enriched by 1 μM slCeMM1-PAP compared to enriched proteins in competition with 5 μM slCeMM1. Enrichment calculated over 50 μM CRF (structure corresponding to red part of slCeMM1-PAP in (A)). Dotted lines represent thresholds for protein enrichment (slCeMM1-PAP enrichment (log2) > 2) and competition (slCeMM1-PAP + 5X slCeMM1 enrichment (log2) < 1).
(E) Comparison of abundances of proteins across tested conditions. Only proteins enriched by slCeMM1-PAP with Log2 enrichment >2 for at least one of the cell lines are shown. Color corresponds to Log2 enrichment over CRF and size corresponds to p value (ANOVA hypothesis test).

Bensimon, for critical discussions and suggestions. CeMM is supported by the Austrian Academy of Sciences. This work was further supported by Vienna Science and Technology Fund (WWTF LS17-051 Cellular Color Chart) and the European Research Council (ERC AdG 695214 GameofGates).

AUTHOR CONTRIBUTIONS

Conceptualization, V.D. and G.S.-F.; Methodology V.D., A.C., C.C., F.O., A.R., G.T., F.F., A.B., J.T.H., G.E.W., S.K., and G.S.-F.; Formal analysis V.D., A.C., A.K., T.T., A.R., and F.F.; Investigation, V.D., A.C., C.C., A.K., T.T., F.O., A.R., G.T., E.H., S.D., F.F., and V.B.; Data Curation, A.K., A.R., and F.F.; Writing – Original Draft, V.D., C.C., A.R., and G.S.-F.; Visualization, V.D. and C.C.; Supervision, J.T.H., G.F.E., G.E.W., S.K., and G.S.-F.; Project Administration, V.D. and G.S.-F., Funding Acquisition, G.S.-F.

DECLARATION OF INTERESTS

V.D., A.C., and G.S.-F. are co-authors of patent applications related to presented study. S.K., G.E.W., and G.S.-F. are co-founders of company related to SLCs. G.S.-F. is the Academic Project Coordinator of the IMI RESOLUTE/Resolution consortium in partnership with Pfizer, Novartis, Bayer, Sanofi, Boehringer Ingelheim and Vifor Pharma. G.S.-F., G.E.W., and S.K. laboratories receive funds from Pfizer.

Received: January 27, 2023
Revised: May 2, 2023
Accepted: June 30, 2023
Published: July 28, 2023

REFERENCES

1. Wang, W.W., Gallo, L., Jadhav, A., Hawkins, R., and Parker, C.G. (2020). The Druggability of Solute Carriers. J. Med. Chem. *63*, 3834–3867. https://doi.org/10.1021/acs.jmedchem.9b01237.
2. Lin, L., Yee, S.W., Kim, R.B., and Giacomini, K.M. (2015). SLC transporters as therapeutic targets: Emerging opportunities. Nat. Rev. Drug Discov. *14*, 543–560. https://doi.org/10.1038/nrd4626.
3. César-Razquin, A., Snijder, B., Frappier-Brinton, T., Isserlin, R., Gyimesi, G., Bai, X., Reithmeier, R.A., Hepworth, D., Hediger, M.A., Edwards, A.M., et al. (2015). A Call for Systematic Research on Solute Carriers. Cell. https://doi.org/10.1016/j.cell.2015.07.022.
4. Superti-Furga, G., Lackner, D., Wiedmer, T., Ingles-Prieto, A., Barbosa, B., Girardi, E., Goldmann, U., Gürtl, B., Klavins, K., Klimek, C., et al. (2020). The RESOLUTE consortium: unlocking SLC transporters for drug discovery. Nat. Rev. Drug Discov. *19*, 429–430. https://doi.org/10.1038/d41573-020-00056-6.
5. Dvorak, V., Wiedmer, T., Ingles-Prieto, A., Altermatt, P., Batoulis, H., Bärenz, F., Bender, E., Digles, D., Dürrenberger, F., Heitman, L.H., et al. (2021). An Overview of Cell-Based Assay Platforms for the Solute Carrier Family of Transporters. Front. Pharmacol. *12*, 722889–722931. https://doi.org/10.3389/fphar.2021.722889.
6. Hartwell, L.H., Szankasi, P., Roberts, C.J., Murray, A.W., and Friend, S.H. (1997). Integrating genetic approaches into the discovery of anticancer drugs. Science *278*, 1064–1068. https://doi.org/10.1126/science.278.5340.1064.
7. Costanzo, M., VanderSluis, B., Koch, E.N., Baryshnikova, A., Pons, C., Tan, G., Wang, W., Usaj, M., Hanchard, J., Lee, S.D., et al. (2016). A global genetic interaction network maps a wiring diagram of cellular function. Science *353*, aaf1420. https://doi.org/10.1126/science.aaf1420.
8. Costanzo, M., Hou, J., Messier, V., Nelson, J., Rahman, M., VanderSluis, B., Wang, W., Pons, C., Ross, C., Ušaj, M., et al. (2021). Environmental robustness of the global yeast genetic interaction network. Science *372*, eabf8424. https://doi.org/10.1126/science.abf8424.
9. Huang, A., Garraway, L.A., Ashworth, A., and Weber, B. (2020). Synthetic lethality as an engine for cancer drug target discovery. Nat. Rev. Drug Discov. *19*, 23–38. https://doi.org/10.1038/s41573-019-0046-z.
10. Rabinowitz, J.D., and Enerbäck, S. (2020). Lactate: the ugly duckling of energy metabolism. Nat. Metab. *2*, 566–571. https://doi.org/10.1038/s42255-020-0243-4.
11. Brooks, G.A. (2018). The Science and Translation of Lactate Shuttle Theory. Cell Metab. *27*, 757–785. https://doi.org/10.1016/j.cmet.2018.03.008.
12. Hui, S., Ghergurovich, J.M., Morscher, R.J., Jang, C., Teng, X., Lu, W., Esparza, L.A., Reya, T., Zhan, L., Yanxiang Guo, J., et al. (2017). Glucose feeds the TCA cycle via circulating lactate. Nature *551*, 115–118. https://doi.org/10.1038/nature24057.
13. Draoui, N., and Feron, O. (2011). Lactate shuttles at a glance: from physiological paradigms to anti-cancer treatments. Dis. Model. Mech. *4*, 727–732. https://doi.org/10.1242/dmm.007724.
14. Bosshart, P.D., Charles, R.P., Garibsingh, R.A.A., Schlessinger, A., and Fotiadis, D. (2021). SLC16 Family: From Atomic Structure to Human Disease. Trends Biochem. Sci. *46*, 28–40. https://doi.org/10.1016/j.tibs.2020.07.005.
15. Felmlee, M.A., Jones, R.S., Rodriguez-Cruz, V., Follman, K.E., and Morris, M.E. (2020). Monocarboxylate Transporters (SLC16): Function, Regulation, and Role in Health and Disease. Pharmacol. Rev. *72*, 466–485. https://doi.org/10.1124/pr.119.018762.
16. Quanz, M., Bender, E., Kopitz, C., Grünewald, S., Schlicker, A., Schwede, W., Eheim, A., Toschi, L., Neuhaus, R., Richter, C., et al. (2018). Preclinical efficacy of the novel monocarboxylate transporter 1 inhibitor BAY-8002 and associated markers of resistance. Mol. Cancer Ther. *17*, 2285–2296. https://doi.org/10.1158/1535-7163.MCT-17-1253.
17. Li, X., Yang, Y., Zhang, B., Lin, X., Fu, X., An, Y., Zou, Y., Wang, J.X., Wang, Z., and Yu, T. (2022). Lactate metabolism in human health and disease. Signal Transduct. Target. Ther. *7*, 305. https://doi.org/10.1038/s41392-022-01151-3.
18. Plummer, R., Halford, S., Jones, P., Wedge, S., Hirschberg, S., Veal, G., Payne, G., Chenard-Poirier, M., Keun, H., and Banerji, U. (2018). A first-in-human first-in-class (FIC) trial of the monocarboxylate transporter 1 (MCT1) inhibitor AZD3965 in patients with advanced solid tumours. Ann. Oncol. *29*, iii9. https://doi.org/10.1093/annonc/mdy048.008.
19. Cluntun, A.A., Badolia, R., Lettlova, S., Parnell, K.M., Shankar, T.S., Diakos, N.A., Olson, K.A., Taleb, I., Tatum, S.M., Berg, J.A., et al. (2021). The pyruvate-lactate axis modulates cardiac hypertrophy and heart failure. Cell Metab. *33*, 629–648.e10. https://doi.org/10.1016/j.cmet.2020.12.003.
20. Heinrich, T., Sala-Hojman, A., Ferretti, R., Petersson, C., Minguzzi, S., Gondela, A., Ramaswamy, S., Bartosik, A., Czauderna, F., Crowley, L., et al. (2021). Discovery of 5-{2-[5-Chloro-2-(5-ethoxyquinoline-8-sulfonamido)phenyl]ethynyl}-4-methoxypyridine-2-carboxylic Acid, a Highly Selective in Vivo Useable Chemical Probe to Dissect MCT4 Biology. J. Med. Chem. *64*, 11904–11933. https://doi.org/10.1021/acs.jmedchem.1c00448.
21. Puri, S., and Juvale, K. (2020). Monocarboxylate transporter 1 and 4 inhibitors as potential therapeutics for treating solid tumours: A review with structure-activity relationship insights. Eur. J. Med. Chem. *199*, 112393. https://doi.org/10.1016/j.ejmech.2020.112393.
22. Goldberg, F.W., Kettle, J.G., Lamont, G.M., Buttar, D., Ting, A.K.T., McGuire, T.M., Cook, C.R., Beattie, D., Morentin Gutierrez, P., Kavanagh, S.L., et al. (2023). Discovery of Clinical Candidate AZD0095, a Selective Inhibitor of Monocarboxylate Transporter 4 (MCT4) for Oncology. J. Med. Chem. *66*, 384–397. https://doi.org/10.1021/acs.jmedchem.2c01342.
23. Kawatkar, A., Clark, R.A., Hopcroft, L., Roaquin, D.A., Tomlinson, R., Zuhl, A.M., Lamont, G.M., Kettle, J.G., Critchlow, S.E., Castaldi, M.P., et al. (2023). Chemical Biology Approaches Confirm MCT4 as the Therapeutic Target of a Cellular Optimized Hit. ACS Chem. Biol. *18*, 296–303. https://doi.org/10.1021/acschembio.2c00666.
24. Girardi, E., Fiume, G., Goldmann, U., Sin, C., Müller, F., Sedlyarov, V., Srndic, I., Agerer, B., Kartnig, F., Meixner, E., et al. (2020). A systematic genetic interaction map of human solute carriers assigns a role to

SLC25A51/MCART1 in mitochondrial NAD uptake. Preprint at bioRxiv. https://doi.org/10.1101/2020.08.31.275818.

25. Blomen, V.A., Májek, P., Jae, L.T., Bigenzahn, J.W., Nieuwenhuis, J., Staring, J., Sacco, R., van Diemen, F.R., Olk, N., Stukalov, A., et al. (2015). Gene essentiality and synthetic lethality in haploid human cells. Science *350*, 1092–1096. https://doi.org/10.1126/science.aac7557.

26. Bürckstümmer, T., Banning, C., Hainzl, P., Schobesberger, R., Kerzendorfer, C., Pauler, F.M., Chen, D., Them, N., Schischlik, F., Rebsamen, M., et al. (2013). A reversible gene trap collection empowers haploid genetics in human cells. Nat. Methods *10*, 965–971. https://doi.org/10.1038/nmeth.2609.

27. Essletzbichler, P., Konopka, T., Santoro, F., Chen, D., Gapp, B.V., Kralovics, R., Brummelkamp, T.R., Nijman, S.M.B., and Bürckstümmer, T. (2014). Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. Genome Res. *24*, 2059–2065. https://doi.org/10.1101/gr.177220.114.

28. Marchiq, I., Le Floch, R., Roux, D., Simon, M.P., and Pouyssegur, J. (2015). Genetic disruption of lactate/H+ symporters (MCTs) and their subunit CD147/BASIGIN sensitizes glycolytic tumor cells to phenformin. Cancer Res. *75*, 171–180. https://doi.org/10.1158/0008-5472.CAN-14-2260.

29. Curtis, N.J., Mooney, L., Hopcroft, L., Michopoulos, F., Whalley, N., Zhong, H., Murray, C., Logie, A., Revill, M., Byth, K.F., et al. (2017). Pre-clinical pharmacology of AZD3965, a selective inhibitor of MCT1: DLBCL, NHL and Burkitt's lymphoma anti-tumor activity. Oncotarget *8*, 69219–69236. https://doi.org/10.18632/oncotarget.18215.

30. Benjamin, D., Robay, D., Hindupur, S.K., Pohlmann, J., Colombi, M., El-Shemerly, M.Y., Maira, S.M., Moroni, C., Lane, H.A., and Hall, M.N. (2018). Dual Inhibition of the Lactate Transporters MCT1 and MCT4 Is Synthetic Lethal with Metformin due to NAD+ Depletion in Cancer Cells. Cell Rep. *25*, 3047–3058.e4. https://doi.org/10.1016/j.celrep.2018.11.043.

31. Pemovska, T., Bigenzahn, J.W., Srndic, I., Lercher, A., Bergthaler, A., César-Razquin, A., Kartnig, F., Kornauth, C., Valent, P., Staber, P.B., and Superti-Furga, G. (2021). Metabolic drug survey highlights cancer cell dependencies and vulnerabilities. Nat. Commun. *12*, 7190. https://doi.org/10.1038/s41467-021-27329-x.

32. Sasaki, S., Kobayashi, M., Futagi, Y., Ogura, J., Yamaguchi, H., Takahashi, N., and Iseki, K. (2013). Crucial Residue Involved in L-Lactate Recognition by Human Monocarboxylate Transporter 4 (hMCT4). PLoS One *8*, e67690. https://doi.org/10.1371/journal.pone.0067690.

33. van Hasselt, P.M., Ferdinandusse, S., Monroe, G.R., Ruiter, J.P.N., Turkenburg, M., Geerlings, M.J., Duran, K., Harakalova, M., van der Zwaag, B., Monavari, A.A., et al. (2014). Monocarboxylate Transporter 1 Deficiency and Ketone Utilization. N. Engl. J. Med. *371*, 1900–1907. https://doi.org/10.1056/nejmoa1407778.

34. Wang, N., Jiang, X., Zhang, S., Zhu, A., Yuan, Y., Xu, H., Lei, J., and Yan, C. (2021). Structural basis of human monocarboxylate transporter 1 inhibition by anti-cancer drug candidates. Cell *184*, 370–383.e13. https://doi.org/10.1016/j.cell.2020.11.043.

35. Zhang, B., Jin, Q., Xu, L., Li, N., Meng, Y., Chang, S., Zheng, X., Wang, J., Chen, Y., Neculai, D., et al. (2020). Cooperative transport mechanism of human monocarboxylate transporter 2. Nat. Commun. *11*, 2429. https://doi.org/10.1038/s41467-020-16334-1.

36. Bosshart, P.D., Kalbermatter, D., Bonetti, S., and Fotiadis, D. (2019). Mechanistic basis of L-lactate transport in the SLC16 solute carrier family. Nat. Commun. *10*, 2649. https://doi.org/10.1038/s41467-019-10566-6.

37. Parker, C.G., and Pratt, M.R. (2020). Click Chemistry in Proteomic Investigations. Cell *180*, 605–632. https://doi.org/10.1016/j.cell.2020.01.025.

38. Robers, M.B., Friedman-Ohana, R., Kilpatrick, L., Huber, K.V.M., Vasta, J.D., Berger, B.T., Chaudhry, C., Hill, S., Müller, S., Knapp, S., and Wood, K.V. (2020). Quantifying Target Occupancy of Small Molecules within Living Cells. Annu. Rev. Biochem. *89*, 557–581. https://doi.org/10.1146/annurev-biochem-011420-092302.

39. Futagi, Y., Kobayashi, M., Narumi, K., Furugen, A., and Iseki, K. (2018). Identification of a selective inhibitor of human monocarboxylate transporter 4. Biochem. Biophys. Res. Commun. *495*, 427–432. https://doi.org/10.1016/j.bbrc.2017.10.025.

40. Renner, K., Bruss, C., Schnell, A., Koehl, G., Becker, H.M., Fante, M., Menevse, A.N., Kauer, N., Blazquez, R., Hacker, L., et al. (2019). Restricting Glycolysis Preserves T Cell Effector Functions and Augments Checkpoint Therapy. Cell Rep. *29*, 135–150.e9. https://doi.org/10.1016/j.celrep.2019.08.068.

41. Jonnalagadda, S., Jonnalagadda, S.K., Ronayne, C.T., Nelson, G.L., Solano, L.N., Rumbley, J., Holy, J., Mereddy, V.R., and Drewes, L.R. (2019). Novel N,N-dialkyl cyanocinnamic acids as monocarboxylate transporter 1 and 4 inhibitors. Oncotarget *10*, 2355–2368. https://doi.org/10.18632/oncotarget.26760.

42. Arrowsmith, C.H., Audia, J.E., Austin, C., Baell, J., Bennett, J., Blagg, J., Bountra, C., Brennan, P.E., Brown, P.J., Bunnage, M.E., et al. (2015). The promise and peril of chemical probes. Nat. Chem. Biol. *11*, 536–541. https://doi.org/10.1038/nchembio.1867.

43. Garbaccio, R.M., and Parmee, E.R. (2016). The Impact of Chemical Probes in Drug Discovery: A Pharmaceutical Industry Perspective. Cell Chem. Biol. *23*, 10–17. https://doi.org/10.1016/j.chembiol.2015.11.011.

44. Baenke, F., Dubuis, S., Brault, C., Weigelt, B., Dankworth, B., Griffiths, B., Jiang, M., MacKay, A., Saunders, B., Spencer-Dene, B., et al. (2015). Functional screening identifies MCT4 as a key regulator of breast cancer cell metabolism and survival. J. Pathol. *237*, 152–165. https://doi.org/10.1002/path.4562.

45. Gerlinger, M., Santos, C.R., Spencer-Dene, B., Martinez, P., Endesfelder, D., Burrell, R.A., Vetter, M., Jiang, M., Saunders, R.E., Kelly, G., et al. (2012). Genome-wide RNA interference analysis of renal carcinoma survival regulators identifies MCT4 as a Warburg effect metabolic target. J. Pathol. *227*, 146–156. https://doi.org/10.1002/path.4006.

46. Baek, G., Tse, Y.F., Hu, Z., Cox, D., Buboltz, N., McCue, P., Yeo, C.J., White, M.A., DeBerardinis, R.J., Knudsen, E.S., and Witkiewicz, A.K. (2014). MCT4 Defines a Glycolytic Subtype of Pancreatic Cancer with Poor Prognosis and Unique Metabolic Dependencies. Cell Rep. *9*, 2233–2249. https://doi.org/10.1016/j.celrep.2014.11.025.

47. Todenhöfer, T., Seiler, R., Stewart, C., Moskalev, I., Gao, J., Ladhar, S., Kamjabi, A., Al Nakouzi, N., Hayashi, T., Choi, S., et al. (2018). Selective inhibition of the lactate transporter MCT4 reduces growth of invasive bladder cancer. Mol. Cancer Ther. *17*, 2746–2755. https://doi.org/10.1158/1535-7163.MCT-18-0107.

48. Pisarsky, L., Bill, R., Fagiani, E., Dimeloe, S., Goosen, R.W., Hagmann, J., Hess, C., and Christofori, G. (2016). Targeting Metabolic Symbiosis to Overcome Resistance to Anti-angiogenic Therapy. Cell Rep. *15*, 1161–1174. https://doi.org/10.1016/j.celrep.2016.04.028.

49. Fujii, W., Kawahito, Y., Nagahara, H., Kukida, Y., Seno, T., Yamamoto, A., Kohno, M., Oda, R., Taniguchi, D., Fujiwara, H., et al. (2015). Monocarboxylate transporter 4, associated with the acidification of synovial fluid, is a novel therapeutic target for inflammatory arthritis. Arthritis Rheumatol. *67*, 2888–2896. https://doi.org/10.1002/art.39270.

50. Tunyasuvunakool, K., Adler, J., Wu, Z., Green, T., Zielinski, M., Žídek, A., Bridgland, A., Cowie, A., Meyer, C., Laydon, A., et al. (2021). Highly accurate protein structure prediction for the human proteome. Nature *596*, 590–596. https://doi.org/10.1038/s41586-021-03828-1.

51. Shi, X., Reinstadler, B., Shah, H., To, T.L., Byrne, K., Summer, L., Calvo, S.E., Goldberger, O., Doench, J.G., Mootha, V.K., and Shen, H. (2022). Combinatorial GxGxE CRISPR screen identifies SLC25A39 in mitochondrial glutathione transport linking iron homeostasis to OXPHOS. Nat. Commun. *13*, 2483. https://doi.org/10.1038/s41467-022-30126-9.

52. Wei, Y., Huang, Y.H., Skopelitis, D.S., Iyer, S.V., Costa, A.S.H., Yang, Z., Kramer, M., Adelman, E.R., Klingbeil, O., Demerdash, O.E., et al. (2022). SLC5A3-Dependent Myo-inositol Auxotrophy in Acute Myeloid

Leukemia. Cancer Discov. *12*, 450–467. https://doi.org/10.1158/2159-8290.CD-20-1849.

53. Bondeson, D.P., Paolella, B.R., Asfaw, A., Rothberg, M.V., Skipper, T.A., Langan, C., Mesa, G., Gonzalez, A., Surface, L.E., Ito, K., et al. (2022). Phosphate dysregulation via the XPR1–KIDINS220 protein complex is a therapeutic vulnerability in ovarian cancer. Nat. Cancer *3*, 681–695. https://doi.org/10.1038/s43018-022-00360-7.

54. Bigenzahn, J.W., Collu, G.M., Kartnig, F., Pieraks, M., Vladimer, G.I., Heinz, L.X., Sedlyarov, V., Schischlik, F., Fauster, A., Rebsamen, M., et al. (2018). LZTR1 is a regulator of RAS ubiquitination and signaling. Science *362*, 1171–1177. https://doi.org/10.1126/science.aap8210.

55. Gabler, F., Nam, S.Z., Till, S., Mirdita, M., Steinegger, M., Söding, J., Lupas, A.N., and Alva, V. (2020). Protein Sequence Analysis Using the MPI Bioinformatics Toolkit. Curr. Protoc. Bioinforma. *72*, e108. https://doi.org/10.1002/cpbi.108.

56. Pei, J., Kim, B.H., and Grishin, N.V. (2008). PROMALS3D: A tool for multiple protein sequence and structure alignments. Nucleic Acids Res. *36*, 2295–2300. https://doi.org/10.1093/nar/gkn072.

57. Eswar, N., Webb, B., Marti-Renom, M.A., Madhusudhan, M., Eramian, D., Shen, M., Pieper, U., and Sali, A. (2006). Comparative Protein Structure Modeling Using Modeller Modeling Structure from Sequence 5.6.1. Curr. Protoc. Bioinforma. 1–30.

58. Shen, M.Y., and Sali, A. (2006). Statistical potential for assessment and prediction of protein structures. Protein Sci. *15*, 2507–2524. https://doi.org/10.1110/ps.062416606.

59. Laskowski, R.A., Rullmannn, J.A., MacArthur, M.W., Kaptein, R., and Thornton, J.M. (1996). AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR. J. Biomol. NMR *8*, 477–486. https://doi.org/10.1007/BF00228148.

60. Sherman, W., Beard, H.S., and Farid, R. (2006). Use of an induced fit receptor structure in virtual screening. Chem. Biol. Drug Des. *67*, 83–84. https://doi.org/10.1111/j.1747-0285.2005.00327.x.

61. Sastry, G.M., Adzhigirey, M., Day, T., Annabhimoju, R., and Sherman, W. (2013). Protein and ligand preparation: Parameters, protocols, and influence on virtual screening enrichments. J. Comput. Aided Mol. Des. *27*, 221–234. https://doi.org/10.1007/s10822-013-9644-8.

62. Perez-Riverol, Y., Bai, J., Bandla, C., García-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., Kundu, D.J., Prakash, A., Frericks-Zipper, A., Eisenacher, M., et al. (2022). The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. Nucleic Acids Res. *50*, D543–D552. https://doi.org/10.1093/nar/gkab1038.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HA High Affinity Rat mAb	Roche	Cat# 11867423001; RRID: AB_390918
Anti-pan ACTIN Rabbit polyclonal	Cytoskeleton, Inc.	Cat# AANO1; RRID: AB_10708070
MCT1 Antibody (H-1)	Santa Cruz Biotechnology	Cat# sc-365501; RRID: AB_10841766
Peroxidase-AffiniPure Goat anti-Rabbit IgG (H+L) antibody	Jackson ImmunoResearch Labs	Cat# 111-035-003; RRID: AB_2313567
Mouse Anti-HiBiT mAb (Clone 30E5)	Promega	Cat# CS2006A01; RRID: AB_2924793
Goat anti-Mouse IgG (H+L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor Plus 488	Thermo Fisher Scientific	Cat# A-11001; RRID: AB_2534069
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-11006; RRID: AB_2534074
Bacterial and virus strains		
One Shot™ Stbl3™ Chemically Competent E. coli	Invitrogen	Cat# C737303
Chemicals, peptides, and recombinant proteins		
AZD3965	MedChemExpress	Cat# HY-12750
BAY-8002	Selleckchem	Cat# S8747
Bortezomib	Selleckchem	Cat# S1013
Syrosingopine	Sigma-Aldrich	Cat# SML1908
slCeMM1	This study	N/A
slCeMM1-PAP	This study	N/A
S1_007	Chemspace	CSMS00334283126
Azide-PEG3-biotin	Sigma-Aldrich	Cat# 762024-25MG
5-TAMRA-Azide	Jena Bioscience	Cat# CLK-FA008-1
3-[3-(but-3-yn-1-yl)-3H-diazirin-3-yl]-N-methylpropanamide (CRF)	Enamine	Cat# EN300-7355163
Critical commercial assays		
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	Cat# G7572
Nano-Glo® HiBiT Lytic Detection System	Promega	Cat# N3040
Lactate-Glo™ Assay	Promega	Cat# J5022
Q5® Site-Directed Mutagenesis Kit	New England Biolabs	Cat# E0554S
Deposited data		
HAP1 RNA sequencing data	RESOLUTE consortium	GEO: GSE234043
slCeMM1 Chemoproteomics	This study	https://doi.org/10.6019/PXD040089
Experimental models: Cell lines		
HAP1	Horizon Genomics	N/A
HAP1 SLC16A1-/-	Horizon Genomics	HZGHC002882c010
HAP1 SC16A3-/-	Horizon Genomics	HZGHC001844c001
MDA-MB-231	Gift from W. Berger's lab	N/A
LAMA84 sgRen	Pemovska et al.[31]	N/A
LAMA84 sgSLC16A1	Pemovska et al.[31]	N/A
LAMA84 sgSLC16A3	Pemovska et al.[31]	N/A
HEK293T	ATCC	CRL-3216

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
listed in the Table S2		
Recombinant DNA		
pDONR221_SLC16A1	RESOLUTE consortium	Addgene #131934
pDONR221_SLC16A3	RESOLUTE consortium	Addgene #131899
pDONR221_SLC16A7	RESOLUTE consortium	Addgene #131947
pDONR221_SLC16A8	RESOLUTE consortium	Addgene #131959
pLentiCRISPRv2	Gift from F. Zhang lab	Addgene #52961
psPAX2	Gift from D. Trono lab	Addgene #12260
pMD2.G	Gift from D. Trono lab	Addgene #12259
pRRL	Bigenzahn et al.[54]	N/A
pRRL strepHA	Bigenzahn et al.[54]	N/A
pJTI R4 CMV-TO pA Vector	Thermo Fisher Scientific	Cat# A15006
Software and algorithms		
Prism v9	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
R	The R Foundation	https://www.R-project.org/
Proteome Discoverer	Thermo Fisher Scientific	https://www.thermofisher.com/order/catalog/product/OPTON-31014
Other		
Pierce Streptavidin Agarose	Thermo Fisher Scientific	Cat# 20353
TMTpro 16plex Label Reagent Set	Thermo Fisher Scientific	Cat# A44521
StrepTactin Sepharose (50% suspension)	IBA	Cat# 2-1201-010

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Giulio Superti-Furga (GSuperti@cemm.oeaw.ac.at).

Materials availability

Materials and reagents generated in this study are available upon a reasonable request from lead contact (GSuperti@cemm.oeaw.ac.at) and may require completed Material Transfer Agreement. Synthesis of slCeMM1 and slCeMM1-PAP is reported in the Synthesis section of methods.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE database (http://www.proteomexchange.org) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. RNA seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines

All HAP1 (donor sex: male) cell lines were cultivated in IMDM medium. HEK293T (donor sex: female) were cultivated in DMEM medium. LAMA84 (donor sex: female) and MDA-MB-231 (donor sex: female) cell lines were cultivated in RPMI medium. All media were supplemented with 10% FBS (v/v) and P/S (100U/ml penicillin and 100 mg/ml streptomycin).

CellPress
OPEN ACCESS

METHOD DETAILS

Plasmids and stable cell line generation

pDONR221 vectors containing *SLC16A1*, *SLC16A3*, *SLC16A7* and *SLC16A8* cDNAs were gift from Resolute consortium. HiBiT tag and/or mutations were introduced using site directed mutagenesis (New England Biolabs; oligonucleotides listed in Table S2) and pDONR221 vectors. cDNAs were cloned into gateway-compatible vectors pRRL (for lentiviral expression, EF1a promotor driven expression, described previously[54]) or pJTI (for transient transfection, Thermo Fisher Scientific) using LR recombinase (Thermo Fisher Scientific). For CRISPR/Cas9 mediated knock-out generation, gRNA oligonucleotides were annealed and cloned into pLentiCRISPRv2 vector using golden gate assembly (SLC16A1 gRNA sequence: CACCGACAGACGTATAGTTGCTGTA). All vectors were confirmed by Sanger sequencing. Plasmids were chemically transformed into Stbl3 E.coli, plated on agar plates containing selection antibiotics and grew overnight. Single colonies were picked and further grow overnight in a liquid culture containing selection antibiotics. Plasmids were subsequently purified using QIAprep spin miniprep kit (Qiagen).

For the lentiviral transduction, HEK293T cells were transfected with psPAX2, pMDG2.G and expression vector using PEI. Approximately 12 hours post transfection, medium was replaced and after 48 – 72 hours the medium containing the lentiviral particles was harvested, filtered through 0.45 μm filter, mixed with Polybrene (5 μg/ml, Hexadimethrine bromide, Sigma Aldrich) and added to sub-confluent target cells. 24 hours after the transduction, medium was exchanged and 24 hours later selection antibiotics were added for 3 – 7 days to select infected cells. Knock-out and overexpression efficiency was evaluated by immunoblot and/or immunofluorescence.

Viability assay

Viability assays were run in 96 well plates, or 384 well plates, depending on number of tested conditions in experiment. Drugs were diluted in 2-fold or 3-fold serial dilution in complete growing medium to obtain 2X of final concentration (100 μl for 96 well plate, 25 μl for 384 well plate). Cells were then harvested, counted, diluted in complete growing medium and seeded in additional 100 μl per well for 96 well plate of 25 μl for 384 well plate respectively.

For 96 well plates, 2,500 cells per well were used and for 384 well plates, 1,000 cells per well were used respectively. Viability was measured after 72 hours using CellTiter-Glo assay (Promega) with a plate reader (SpectraMax i3x, Molecular Probes). Data were normalized to DMSO-treated controls (100% viability) and 5 μM Bortezomib controls (0% viability) and four-parameter dose response fitting curves were fitted using GraphPad Prism. All assays were run in at least technical triplicates and data is presented as mean +/- SD.

Testing of analogues of hits compounds was done first in 3-fold dilutions ranging from 30 μM to 4.6 nM. Compounds that were inactive were discarded after this step. Active compounds were re-tested in 2-fold dilutions with the concentration range adapted based on the first result.

Chemical screening

Prior to cell seeding, compounds were transferred into 384 well plates using acoustic liquid handler (Echo, Labcyte). Next, 1,000 cells (HAP1) or 1,500 cells (LAMA-84) per well were seeded using a dispenser (Thermo Fisher Scientific) in 50 μl of complete growth media per well. After 72 hours, viability was measured using CellTiter-Glo assay (Promega) with a plate reader (EnVision, PerkinElmer). Signal was then normalized to DMSO (100%) and Bortezomib (0%) controls.

In primary screen, 89,607 compounds were tested in concentration 10 μM (0.1% DMSO), in a single technical replicate using HAP1 *SLC16A1*$^{-/-}$ cell line. For the counter screening only compounds reducing viability below 50% were progressed. During the first counter screening, concentrations 13, 4.3, 1.4 and 0.5 μM were tested in technical duplicate in HAP1 WT, HAP1 *SLC16A1*$^{-/-}$ and HAP1 *SLC16A3*$^{-/-}$ cell lines. Resulting dose response curves were inspected manually. For second round of counter screening, compounds selectively killing only HAP1 *SLC16A1*$^{-/-}$ and structurally related compounds were selected. In this step compounds were tested in final concentrations 27, 9, 3, 1, 0.33 and 0.11 μM in technical triplicate in HAP1 WT, HAP1 *SLC16A1*$^{-/-}$, HAP1 *SLC16A3*$^{-/-}$, HAP1 SLC16A7 dependent, HAP1 SLC16A8 dependent, HAP1 *SLC16A1*$^{-/-}$ + *SLC16A1*^{WT} OE, HAP1 *SLC16A1*$^{-/-}$ + *SLC16A1*^{R313Q} OE, LAMA84^{sgRen}, LAMA84^{sgSLC16A1} and LAMA84^{sgSLC16A3} cell lines.

Thermal shift assay in lysates

Cells were grown on 10 cm dishes until reaching confluency ~80 – 90%. Cells were then washed with ice cold PBS and lysed with 3 ml of a lysis buffer (100 mM ammonium sulfate, 400 mM NaCl, 10% glycerol (v/v), 0.5% DDM (Sigma-Aldrich; w/v) and EDTA-free protease inhibitor cocktail (Roche) for 20 min on ice. Dishes were harvested by scraping, and lysates were cleared by centrifugation 14,000*g* for 20 min at 4°C. Supernatants were transferred to new tube, mix thoroughly, aliquoted and incubated with tested compounds for 60 min on ice. After that, individual aliquots were divided into PCR strips (50 μl per tube, 2 or 3 technical replicates per condition) and heated in gradient PCR thermocycler for 6 min (40°C to 70°C gradient), followed by 3 min incubation in 25°C. Next, samples were transfer on ice, technical replicates were pooled into 1.5 ml tubes, and protein aggregates were removed by centrifugation at 14,000*g* for 40 min at 4°C. For the detection of remaining protein, 25 μl of the supernatant was mixed

with 5 μl of HiBiT detection mastermix (0.3 μl LgBIT Protein Nano-Glo + 0.6 μl HiBiT Lytic Substrate + 4.1 μl lysis buffer per sample) in black 384 well plate, incubated for 10 min at dark, and luminescence was measured on a plate reader (SpectraMax i3x, Molecular Probes).

All experiments were performed using cells expressing *SLC16A3* tagged with HiBiT. For experiment comparing SLC16A3 as a WT and as mutant groups, 293T cells were transiently transfected using PEI and pJTI constructs two days before the experiments. For thermal shift assays comparing DMSO and compounds, HAP1 cells with stable expression were used.

Intracellular lactate measurement

To assess the intracellular lactate accumulation, 30,000 cells per well were seeded in 96 well plate. Next day, cells were incubated with tested compounds for 6 hours at 37°C and 5% CO_2 atmosphere, washed twice with 200 μl PBS, lysed with 22.5 μl 0.2 N HCl for 5 min while shaking, neutralized with 7.5 μl 1 M Tris-base buffer and mixed. Next, 30 μl of lactate detection reagent (Lactate-Glo, Promega) was added and plates were incubated for 60 min at RT and luminescence was measured with a plate reader (SpectraMax i3x, Molecular Probes). Absolute lactate concentrations were extrapolated from standard curve. To normalize the lactate levels to protein content, mirror plates were used. Here cells were lysed with RIPA buffer supplemented with Benzonase (15 min at room temperature (RT)) and protein concentration was measured with BCA assay (Thermo Fisher Scientific). All measurements were performed in technical triplicates, data are presented as mean +/- SD.

Chemoproteomics

Photoaffinity probe labelling of living cells

Cells were incubated with pre-treatment compounds (or DMSO) in serum free medium for 30 min in the incubator. Next slCeMM1-PAP (final concentration 1 μM) or CRF (final concentration 50 μM) were added, and cells were incubated for additional 5 min in the incubator. Cells were then irradiated with UV (365 nm, 5 min, 4°C), harvested, washed twice with ice cold PBS and cell pellets were stored at -80°C.

In-gel fluorescence analysis

Labelled pellets were lysed in buffer containing HEPES (50 mM, pH 8.0), NaCl (150 mM), NP-40 (0.5% v/v), PMSF (1 mM), EDTA-free protease inhibitor cocktail (Roche), Avidin (IBA, 2-0204-015; 1 μg/ml) and phosphatase inhibitor cocktail 2+3 (Sigma-Aldrich) for 15 min on RT. During the lysis samples were sonicated. Lysates were then cleared by centrifugation (14,000*g*, 20 min at 4°C). Protein concentration was measured with BCA assay (Thermo Fisher Scientific) and normalized across the samples. Streptactin Sepharose beads (IBA, 2-1201-010) were washed and equilibrated in the lysis buffer and incubated with the normalized samples for 1 hour at 4°C on a rotator (the amount of beads per sample corresponded to 40 μl of slurry). Next, samples were loaded to equilibrated Biospin columns (BioRad, 7326207) washed once with buffer containing HEPES (50 mM, pH 8.0), NaCl (150 mM), NP-40 (0.5% v/v), PMSF (1 mM) and EDTA-free protease inhibitor cocktail (Roche) and twice with buffer containing HEPES (50 mM, pH 8.0) and NaCl (150 mM). Captured protein was then eluted by incubating washed beads on thermoshaker (37°C, 300 rpm, 15 min) with 45 μl of PBS buffer containing 1% SDS, 2.5 mM D-biotin (Alfa Aeser, A14207), 2 mM $MgCl_2$ and protease inhibitor cocktail. Eluates were then collected to separate tube by centrifugating the columns at 3,000*g* for 1 min. Volume of eluates was measured and normalized across the samples. Next, to facilitate copper catalyzed azide-alkyne cycloaddition (CuAAC) between the PAP and TAMRA reporter, 50 μl of the sample was mixed with 6 μl of freshly prepared master mix containing 25 μM 5-TAMRA-azide (Jena Bioscience, CLK-FA008), 0.1 mM Tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)-methyl]-amin (TBTA, Sigma-Aldrich 678937-50MG), 1 mM $CuSO_4$ (Sigma Aldrich, C1297-100G; freshly prepared) and 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich, 75259-1G), mixed and while protected from the light incubated at thermoshaker (25°C, 300 RPM) for 1 hour. Reaction was then stopped by adding 18.6 μl of 4X concentrated Laemmli sample buffer and 25 μl of samples were used for SDS-PAGE. After the separation, gels were de-stain (40% Methanol and 10% acetic acid) and fluorescent signal was visualized with BioRad ChemiDoc. As a loading control, gels were stained with Coomassie blue.

Sample preparation for MS chemoproteomic analysis

Labelled cell pellets were thaw on ice and resuspended in PBS buffer containing 1% SDS, 2 mM $MgCl_2$, EDTA-free protease inhibitor cocktail (Roche) and Benzonase. Samples were incubated on thermoshaker (37°C, 300 rpm) for 30 min. Next, lysates were cleared by centrifugation (14,000*g*, 4°C, 30 min), supernatants were transferred to low protein binding tubes and protein was quantified with 660 nm Protein Assay Reagent (Thermo Fisher Scientific, 22660). Next, samples were normalized (based on the sample with the lowest protein concentration 545 μg per sample was used) and mixed with reagents to facilitate the CuAAC reaction (per sample: 450 μl of phosphate buffer; 25 μl of 5 mM Azide-PEG3-biotin (Sigma-Aldrich, 762024-25MG); 25 μl of 1:2 mixture of 20 mM $CuSO_4$ (Sigma Aldrich, C1297-100G; freshly prepared) and 50 mM THPTA (Sigma Aldrich, 762342-500MG); 35 μl of 100 mM Aminoguanidine hydrochloride (Sigma Aldrich, 396494-25G); 35 μl of 100 mM L(+)-ascorbic acid sodium salt (Carl Roth, 3149.1; freshly prepared). Samples were then vortexed and incubated on a rotator for 1 hour at 25°C. Next, 3 ml of ice-cold acetone (Carl Roth, 7328.1) was added, samples were vortexed and left at -80°C for 30 to 60 min, to facilitate protein precipitation, followed by centrifugation (18,000*g*, 4°C, 30 min). Acetone was then carefully discarded, tubes with remaining protein pellets were left under fume hood to dry and stored at -20°C until samples were processed further.

Next, pellets were resuspended in 300 μl of 1% SDS by sonication (90 sec ON, 30 sec OFF, 5 cycles in total, using 4°C cooled Bioruptor Pico, Diagenode). Samples were then reduced by incubation of samples with 30 μl of 50 mM TCEP (Sigma-Aldrich,

75259-1G; final concentration 4.5 mM) for 1 hour at 56°C. After that, pH was adjusted by adding 80 μl HEPES (AppliChem, A6916,0125; 1 M, pH 7.5) and samples were alkylated by incubation with 45 μl of freshly prepared 200 mM iodoacetamide (Sigma Aldrich, I1149-5G; final concentration 20 mM) at 25°C for 30 min. Alkylated samples were then mixed with 100 μl streptavidin agarose beads (Thermo Scientific, 20353) washed and equilibrated with PBS. 1.35 ml PBS was added, and samples were incubated at 25°C for 1 hour on a rotator. Next, beads were separated from flow through by centrifugation, flow through was discarded, beads were resuspended in PBS buffer containing 0.2% SDS and transferred to equilibrated Minispin columns (Bio-Rad, 7326207). Beads were then washed 16-times with 0.5 ml PBS buffer containing 8 M Urea (Sigma-Aldrich, 51456-500G) and 4-times with 0.5 ml of PBS buffer. Columns were then closed with bottom caps, beads were resuspended in 1 ml digestion buffer (50 mM ammonium bicarbonate, 0.2 M guanidine hydrochloride and 1 mM $CaCl_2$ in HPLC grade H_2O (VWR, 83645.320P)), transferred to new low-protein binding tube, centrifuged for 30 sec, supernatant was discarded and beads were resuspended in 250 μl digestion buffer, 10 μl trypsin (Promega, V5117) was added (1 μg in total) and samples were left rotating at 37°C incubator overnight (~14 hours).

Next day beads were separated by centrifugation (1,000*g*, 30 sec), supernatants were transferred to a new low-protein binding tube, beads were resuspended again with 200 μl of HPLC grade H_2O, centrifuged and supernatants combined with the digest. For SPE clean-up, 200 μl tips were plunged with Empore C18 disk material (Sigma-Aldrich, 66883-U), 24 μl oligo R3 solution (Thermo Fisher Scientific, 1133903; 15 mg/ml in acetonitrile (ACN)) was added, tips were placed to collection tubes, centrifuged at 1,000*g* for 1 min, C18 was activated by washing twice with 100 μl 100% ACN (VWR, 83640.320) and subsequently equilibrated with 200 μl 0.1% trifluoroacetic acid (TFA; Sigma Aldrich, 1082620100). Samples were then acidified with 16 μl 30% TFA (~1% final) and subsequently loaded on equilibrated tips, washed with 0.2% TFA and eluted by applying twice 50 μl of elution buffer (90% ACN + 0.01% TFA in H_2O), each followed by centrifugation (1,000*g*, 3 min). Eluates were then dried in vacuum centrifuge (45°C, 1-2 hours until completely dry). Dried pellets were stored at -20°C until TMT-labelling.

For the TMTpro 16plex (Thermo Fisher Scientific, A44521) labelling, dried pellets were reconstituted in 15 μl HEPES (100 mM in HPLC grade H_2O, pH 8.5). Respective TMTpro labels were added to the samples (4 μl of 0.01 mg/μl stocks dissolved in acetonitrile) and samples were vortexed and incubated at thermoshaker (25°C, 300 RPM) for 1 hour. Reaction was then stopped by adding 1.5 μl of freshly prepared 5% hydroxylamine solution in H_2O for HPLC (Sigma Aldrich, 467804-10ML) and incubating samples on thermoshaker (25°C, 300 RPM) for 15 min. After that all samples were pooled into a single tube and 1 ml of freshly prepared ammonium formate ((AF; Sigma-Aldrich, 70221-25G-F), pH 10 adjusted with 25% ammonia solution) was added. Next, similarly as in previous step, 200 μl tip plunged with Empore C18 disk material and oligo R3 solution was prepared: tip was plunged with C18, 24 μl of oligo R3 solution was added, tip was placed to collection tubes, centrifuged at 1,000 x *g* for 1 min, C18 was activated by washing twice with 100 μl 100% ACN and washed again twice with 200 μl 20 mM AF solution. After that, pooled TMTpro labeled sample was applied to the tip in several steps, each followed by centrifugation and discarding of flow through. After whole sample volume was loaded on the tip, sample was washed with 200 μl 20 mM AF. Next elution was performed into 5 separate fractions, by on-tip high pH fractionation each with different ACN dilution in 20 mM FA: 1st fraction with 16% ACN, 2nd fraction with 20% ACN, 3rd fraction with 24% ACN, 4th fraction with 28% ACN, 5th fraction with 80% ACN. Each elution step was done by applying 50 μl of appropriate elution solution, followed by centrifugation with 1,000*g* for 2 min and then applying additional 20 μl of appropriate elution buffer followed by 1,000*g* centrifugation for 1 min. All fractions were then dried in vacuum centrifuge set to 45°C. Dried pellets were then stored in -20°C until the acquisition, for which samples were reconstituted in 20 μl of 0.1 TFA.

2D-RP/RP liquid chromatography – Tandem mass spectrometry analysis

Mass spectrometry analysis was performed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) via a Nanospray Flex Ion Source (Thermo Fisher Scientific) interface. Peptides were loaded onto a trap column (PepMap 100 C18, 5 μm, 5 × 0.3 mm, Thermo Fisher Scientific) at a flow rate of 10 μL/min using 0.1% TFA as loading buffer. After loading, the trap column was switched in-line with an Acclaim PepMap nanoHPLC C18 analytical column (2.0 μm particle size, 75 μm IDx500mm, catalog number 164942, Thermo Fisher Scientific). The column temperature was maintained at 50°C. Mobile phase A consisted of 0.4% formic acid in water, and mobile phase B consisted of 0.4% formic acid in a mixture of 90% acetonitrile and 10% water. Separation was achieved using a four-step gradient over 151 min at a flow rate of 230 nL/min (increase of initial gradient from 6% to 9% solvent B within 1 min, 9% to 30% solvent B within 146 min, 30% to 65% solvent B within 8 min, 65% to 100% solvent B within of 1 minute and 100% solvent B for 6 minutes before equilibrating to 6% solvent B for 23 minutes prior to the next injection). In the liquid junction setup, electrospray ionization was enabled by applying a voltage of 1.8 kV directly to the liquid being sprayed, and non-coated silica emitter was used.

The mass spectrometer was operated in a data-dependent acquisition mode (DDA) and used a synchronous precursor selection (SPS) approach. For both MS2 and MS3 levels, we collected a 400–1600 m/z survey scan in the Orbitrap at 120 000 resolution (FTMS1), the AGC target was set to 'standard' and a maximum injection time (IT) of 50 ms was applied. Precursor ions were filtered by charge state (2-6), dynamic exclusion (60 s with a ±10 ppm window), and monoisotopic precursor selection. Precursor ions for data-dependent MSn (ddMSn) analysis were selected using 10 dependent scans (TopN approach). A charge-state filter was used to select precursors for data-dependent scanning. In ddMS2 analysis, spectra were obtained using one charge state per branch (from z=2 to z=5) in a dual-pressure linear ion trap (ITMS2). The quadrupole isolation window was set to 0.7 Da and the collision-induced dissociation (CID) fragmentation technique was used at a normalized collision energy of 35%. The normalized AGC target was set to 200% with a maximum IT of 35 ms. During the ddMS3 analyses, precursors were isolated using SPS waveform and

different MS1 isolation windows (1.3 m/z for z=2, 1.2 m/z for z=3, 0.8 m/z for z= 4 and 0.7 m/z for z = 5). Target MS2 fragment ions were further fragmented by high-energy collision induced dissociation (HCD) followed by orbitrap analysis (FTMS3). The normalized HCD collision energy was set to 45% and the normalized AGC target was set to 300% with a maximum IT of 100 ms. The resolution was set to 50 000 with a defined scanning range of 100 to 500 m/z. Xcalibur Version 4.3.73.11 and Tune 3.4.3072.18 were used to operate the instrument.

Immunofluorescence staining and imaging

Cells were seeded onto poly-L-lysine hydrobromide (Sigma-Aldrich, P6282) coated black 96 well plates (PerkinElmer). Upon reaching sub-confluency, cells were fixed with formaldehyde (4% in PBS) for 15 min on RT, permeabilized in blocking solution (0.3% Triton X-100, 10% FBS in PBS) for 1 hour at RT. Staining with primary antibodies was performed overnight at 4°C in blocking solution, cells were subsequently washed with blocking buffer (3x) and fluorophore conjugated secondary antibody diluted in blocking buffer was applied for 1 hour at RT. Next, cells were washed with blocking buffer (3x), stained with DAPI (1:1,000 in PBS) for 15 min at RT and imaged using Opera Phenix High Content Screening System (PerkinElmer). A full list of used antibodies is described in key resources table.

Crystal violet proliferation assay

For the proliferation assay, 5,000 cells per well were seeded into 6 well plate and treated immediately. Medium with drugs was refreshed every 3 days. After 7 days, cells were washed with PBS, fixed (4% formaldehyde in PBS) for 15 min at RT, washed with water and stained with crystal violet (0.1% in water). Plates were then washed again with water and left to dry.

Doubling-time assay

15,000 cells per well were seeded and treated immediately in 6 well plates. Cells were harvested and counted with CASY counter (Roche) after 1, 2 and 3 days. The doubling time was estimated using exponential growth equation in Graphpad Prism, and the growth advantage was calculated by comparing the doubling time of WT and variants in each condition. All experiments were performed in technical duplicates.

qPCR

RNA was isolated using RNeasy Mini kit (Qiagen). Genomic DNA was digested using DNase I (Fermentas, #EN0525) and the cDNA was synthesized using RevertAid reverse transcriptase (Thermo Fisher Scientific, EP0441) and oligo dT primers according to manufacturer's protocols. qPCR was performed on LightCycler 480 (Roche) using Luna Universal qPCR Master Mix (New England Biolabs, M3003L) according to manufacturer's protocol. Expression changes were calculated using ΔΔCt method. All samples were run in biological triplicate and technical duplicates.

Immunoblotting

Cells for immunoblotting were grown to sub-confluency in 6 well plates, washed with ice-cold PBS, harvested by scrapping, washed again and pellets were stored at -80°C until further processing. Next, pellets were thaw and lysed with RIPA buffer (25 mM Tris/HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitors 2+3 (Sigma-Aldrich) for 15 min on ice. Lysates were cleared by centrifugation (14,000*g*, 20 min, 4°C), protein concentration was determined with BCA assay (Thermo Fisher Scientific) and protein levels were normalized across all samples. Laemmli sample buffer was added to protein extracts without boiling. Samples were resolved with SDS-PAGE and transferred to nitrocellulose membranes Amesham Protran 0.45 μm (GE Healthcare). Membranes were stained with ponceau red, washed, blocked in 5% milk for 1 hour at RT and incubated in primary antibodies overnight at 4°C. Next day, membranes were washed (3x, 5 min) and incubated with horseradish peroxidase conjugated secondary antibodies for 1 hour at RT. Membranes were then washed (3x 5 min), and signal was detected using ECL Western blotting system (Thermo Fisher Scientific) on BioRad ChemiDoc.

Homology modeling and docking

Homology models of SLC16A3 in inward-open and outward-open conformations were built. First, the most reliable templates to generate models were identified using the HHPred server,[55] as well as visually inspecting available structures of SLC16A3 close homologs, i.e. structures of SLC16A1 (PDB IDs: 6LZ0, 6LYY, 7CKR, 7KO[34]), SLC16A7 (PDB ID: 7BP3[35]) and sfMCT (PDB ID: 6HCL[36]). The best templates were prioritized based on their sequence similarity to the target, their conformational state, the resolution of their structures, as well as whether or not they were bound to a ligand. SLC16A1 was selected as the most suitable template for homology modeling, as it presents a high sequence similarity to SLC16A3 (49%) and has been experimentally determined in multiple conformational states bound to substrates and inhibitors.[34]

A sequence alignment of all the 14 members of the human SLC16 family was generated using PROMALS 3D.[56] For each conformation (outward-open, using 6LYY as template and inward-open, using 7CKO as template) of SLC16A3, 100 models were generated using MODELLER 9.22.[57] Models were ranked according to their Z-DOPE score.[58] The quality of the best scoring models were further evaluated with PROCHECK[59]: the outward-open and inward-open models presented 96.2 and 97.4 % of residues in the most favored regions of the Ramachandran plot, respectively.

Induced-Fit docking calculations were performed with the Schrödinger suite.[60] The models were first prepared using the Protein Preparation Wizard[61] within Maestro with default options. During this step, the proteins are protonated and energy-minimized. The docked ligands were prepared with LigPrep, with default options, with the exception of the pH range set at 7±0.5.

The binding sites were defined around the centroid ligand bound to the structure used as template for homology modelling (i.e. AZD3965 in the outward-facing state, and 7ACC2 in the inward-facing state).

Induced fit docking of slCeMM1 was performed using OPLS3e force field, a ligand conformational sampling within 2.5 kcal/mol, a van der Waals scaling of 0.5, 20 maximum number of poses, Prime refinement of residues within 5 Å of ligand poses. Interaction fingerprint clustering was then performed on the resulting poses. Finally, the best-scored poses within the most populated cluster were selected for visual inspection.

Liver microsome stability assay

Metabolic stability in human, mouse and rat liver microsomes was tested by WuXi DMPK Nanjing as follow:

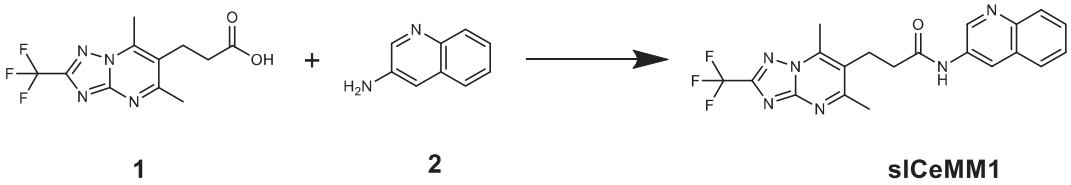
Working solutions of tested compounds were prepared by diluting 5 μl of compound (10 mM DMSO stock) with 495 μl acetonitrile (ACN). NADPH cofactors were prepared by diluting β-Nicotinamide adenine dinucleotide phosphate reduced form, tetrasodium salt (NADPH·4Na (Vendor: BONTAC, Cat. No. BT04) into 10 mM $MgCl_2$ to obtain 10 mM working solution concentration. Liver microsomes were prepared by dilution of Human (Corning, Cat No. 452117, Lot No. 38297), CD-1 Mouse (Xenotech, Cat no. M1000, Lot no. 2110092) or SD Rat (Xenotech Cat No. R1000, Lot No. 2110178) in 100 mM potassium phosphate buffer to final concentration 0.56 mg/ml. As a stop solution was used cold ACN (4°C) containing 200 ng/ml tolbutamide and 200 ng/ml labetalol as internal standards.

Blank plate was prepared by combining 54 μl liver microsomes with 6 μl of NADPH cofactor, followed by addition of 180 μl quenching solution. Incubation plates (T60 and NCF60 (no cofactor)) were prepared by combining 445 μl of microsome working solution with 5 μl of compound working solution in pre-warmed plate, followed by mixing. For the NCF60 sample, 50 μl of buffer was added and plate was incubated for 60 min at 37°C while shaking. Quenching plate T0 was prepared by mixing 180 μl of quenching solution and 6 μl of NADPH cofactor followed by chilling to prevent evaporation. 54 μl of the mixture from T60 plate was transferred to quenching plate, followed by addition of 44 μl NADPH to T60 incubation plate and 60 min incubation at 37°C while shaking. At 5, 15, 30, 45 and 60 min, 180 μl quenching solution was added to quenching plate, mixed, and 60 μl sample was serially transfer from T60 plate per time point to quenching plate. For NCF60, 60 μl sample was transferred from NCF60 incubation plate to quenching plate containing quenching solution at 60 min time point. Next to tested compounds, Testosterone, Diclofenac and Propafenone was measured as controls. Final concentration of each component in the incubation medium was 0.5 mg protein/ml for microsomes, 1 μM tested compound, 0.99% ACN, 0.01% DMSO.

All sampling plates were shaken for 10 min, centrifuged at 4,000 rpm for 20 min at 4C, 80 μl supernatant transferred to 240 μl HPLC water, mixed and shaken for 10 min. Each bioanalysis plate was sealed and shaken for 10 min prior to LC-MS/MS analysis.

Synthesis of slCeMM1 and slCeMM1-PAP

slCeMM1

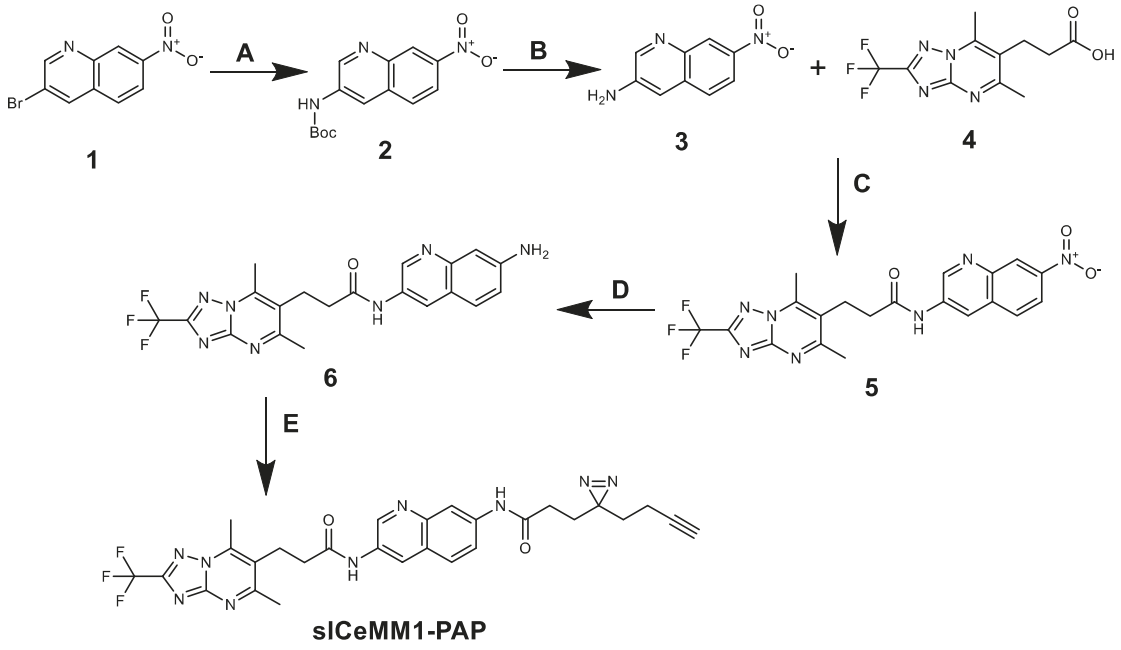


To a stirred solution of compound 1 (2 g, 6.94 mmol, 1 eq) in DCM (20 mL), 1,1'-Carbonyldiimidazole (1.46 g, 9.03 mmol, 1.3 eq) was added. The reaction mixture was stirred at RT for 1 h. Then, compound 2 (1 g, 6.94 mmol, 1 eq) was added, and the resulting solution was stirred at RT overnight. After that, the reaction mixture was washed with H_2O (20 mL x 2). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure to afford 2.5 g of crude target material, which was purified by FC ($CHCl_3$:CH_3CN) to obtain pure product slCeMM1 (1 g, 35% yield).

1H NMR (400 MHz, dmso) δ 10.43 (s, 1H), 8.87 (d, J = 2.5 Hz, 1H), 8.70 (d, J = 2.5 Hz, 1H), 7.93 (t, J = 7.4, 7.4 Hz, 2H), 7.69 – 7.60 (m, 1H), 7.57 (t, J = 7.5, 7.5 Hz, 1H), 3.19 (t, J = 7.8, 7.8 Hz, 2H), 2.85 (s, 3H), 2.76 (s, 3H), 2.72 (dd, J = 8.8, 6.9 Hz, 2H).

LC-MS m/z 415.0 [M+1]

sICeMM1-PAP



Step A: 3-bromo-7-nitroquinoline (compound **1**) (2.81 g, 11.1 mmol), Cs_2CO_3 (10.85 g, 33.31 mmol), $Pd_2(dba)_3$ (0.25 g, 0.27 mmol), Xantphos (0.32 g, 0.55 mmol), tert-butyl carbamate (1.43 g, 12.21 mmol) and dioxane (40 mL) were placed into the Schlenk flask (100 mL). The resulting solution was stirred at 100°C for 16 h. After completion of the reaction, the mixture was filtered through SiO_2, the pad was washed with EtOAc (100 mL), and the combined filtrates were evaporated in a vacuo to obtain tert-butyl (7-nitroquinolin-3-yl) carbamate (compound **2**) (1.53 g, 47.7 % yield).

Step B: To a stirred solution of tert-butyl (7-nitroquinolin-3-yl) carbamate (1.53 g, 5.29 mmol) in MeOH (10 mL), HCl (4 M solution in dioxane, 20 mL) was added. The resulting solution was stirred at RT for 16 h. The solvent was evaporated to obtain 7-nitroquinolin-3-aminium chloride (compound **3**) (1.01 g, 85% yield) as a brown solid.

Step C: 7-nitroquinolin-3-aminium chloride (compound **3**) (1.01 g, 4.5 mmol) was dissolved in DMF (10 mL), and 3-(5,7-dimethyl-2-(trifluoromethyl)-[1,2,4]triazolo[1,5-a]pyrimidin-6-yl)propanoic acid (compound **4**) (1.18 g, 4.09 mmol), HATU (2.02 g, 5.31 mmol) were added thereto followed by addition of N, N-Diisopropylethylamine (2.11 g, 16.36 mmol, 2.85 mL). The resulting mixture was stirred at 50 °C for 16 h. The solvent was evaporated in a vacuum. EtOAc was added to the residue, and a brown precipitate was observed. The insoluble solids were collected by filtration and washed with EtOAc. After drying under vacuum, the desired product 3-(5,7-dimethyl-2-(trifluoromethyl)-[1,2,4] triazolo [1,5-a]pyrimidin-6-yl)-N-(7-nitroquinolin-3-yl)propenamide (compound **5**) was obtained as a brown powder (1.26 g, 61.1% yield).

Step D: 3-(5,7-dimethyl-2-(trifluoromethyl)- [1,2,4] triazolo [1,5-a]pyrimidin-6-yl)-N-(7-nitroquinolin-3-yl) propenamide (compound **5**) (1.26 g, 2.74 mmol) was dissolved in AcOH (5 mL), and 10% Pd/C (0.3 g) was added. The flask was evacuated, backfilled with hydrogen gas from a balloon, and left to stir overnight at RT. The reaction mixture was filtered, and the solvent was rotary evaporated to obtain N-(7-aminoquinolin-3-yl)-3-(5,7-dimethyl-2-(trifluoromethyl)- [1,2,4] triazolo [1,5-a] pyrimidin-6-yl) propenamide (compound **6**) (1 g, 84.67% yield).

Step E: N-(7-aminoquinolin-3-yl)-3-(5,7-dimethyl-2 -(trifluoromethyl)- [1,2,4] triazolo[1,5-a] pyrimidin-6-yl)propenamide (compound **6**) (1 g, 2.32 mmol) was dissolved in DMF (7 mL), and 3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propanoic acid (0.38 g, 2.32 mmol), HATU (1.14 g, 3.01 mmol) were added thereto followed by addition of N, N-Diisopropylethylamine (1.2 g, 9.28 mmol, 1.62 mL). The resulting mixture was stirred at RT for 48 h. The solvent was evaporated to obtain a crude product. After HPLC (Column: SunFire 100*19 mm, 5 μM, Mobile Phase: system 0.5-6.5 min 63% water- 37% ACN 30 ml/min (loading pump 4 ml ACN) target mass 578) was obtained 3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(3-(3-(5,7-dimethyl-2-(trifluoromethyl)-[1,2,4]triazolo[1,5-a] pyrimidin-6-yl)propanamido)quinolin-7-yl)propenamide (**sICeMM1-PAP**) (0.239 g, 17.8% yield).

1H NMR (400 MHz, dmso) δ 10.38 (s, 1H), 10.22 (s, 1H), 8.79 (d, J = 2.5 Hz, 1H), 8.60 (d, J = 2.5 Hz, 1H), 8.33 (d, J = 1.9 Hz, 1H), 7.85 (d, J = 8.9 Hz, 1H), 7.64 (dd, J = 8.8, 2.1 Hz, 1H), 3.18 (t, J = 7.9, 7.9 Hz, 2H), 2.90 – 2.81 (m, 4H), 2.76 (s, 3H), 2.72 – 2.67 (m, 2H), 2.21 (t, J = 7.6, 7.6 Hz, 2H), 2.03 (td, J = 7.5, 7.4, 2.7 Hz, 2H), 1.80 (t, J = 7.6, 7.6 Hz, 2H), 1.63 (t, J = 7.4, 7.4 Hz, 2H).

LC-MS m/z 578.4 [M+1]

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters are reported in the figure legend of the paper.

Chemoproteomics

Following data acquisition, the acquired raw data files were processed using the Proteome Discoverer v.2.4.1.15 platform, with a TMT16plex quantification method selected. In the processing step, we used the Sequest HT database search engine and the Percolator validation software node to remove false positives with a false discovery rate (FDR) of 1% at the peptide and protein level under stringent conditions. The search was performed with full tryptic digestion against the human proteome (Canonical, reviewed, 20 304 sequences) and appended known contaminants and streptavidin, with a maximum of two allowable miscleavage sites. Methionine oxidation (+15.994 Da) and protein N-terminal acetylation (+42.011 Da), as well as methionine loss (-131.040 Da) and protein N-terminal acetylation with methionine loss (-89.030 Da) were set as variable modifications, while carbamidomethylation (+57.021 Da) of cysteine residues and tandem mass tag (TMT) 16-plex labeling of peptide N termini and lysine residues (+304.207 Da) were set as fixed modifications. Data were searched with mass tolerances of ±10 ppm and ±0.6 Da for the precursor and fragment ions, respectively. Results were filtered to include peptide spectrum matches with Sequest HT cross-correlation factor (Xcorr) scores of $\geq$1 and high peptide confidence assigned by Percolator. MS3 signal-to-noise (S/N) values of TMTpro reporter ions were used to calculate peptide/protein abundance values. Peptide spectrum matches with precursor isolation interference values of $\geq$70%, SPS mass matches $\leq$65%, and average TMTpro reporter ion S/N $\leq$10 were excluded from quantification. Both unique and razor peptides were used for TMT quantification. Correction of isotopic impurities was applied. Data were normalized to total peptide abundance to correct for experimental bias and scaled "to all average". Protein ratios are directly calculated from the grouped protein abundances using an ANOVA hypothesis test. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[62] partner repository with the dataset identifier PXD040089 and 10.6019/PXD040089.

Liver microsome stability assay

The equation of first order kinetics was used to calculate $T_{1/2}$ and $Cl_{int(mic)}$(µl/min/mg):

$$C_t = C_0 \cdot e^{-k_e \cdot t}$$

$$when\ C_t = \frac{1}{2}C_0,$$

$$T_{\frac{1}{2}} = \frac{Ln\ 2}{k_e} = \frac{0.693}{k_e}$$

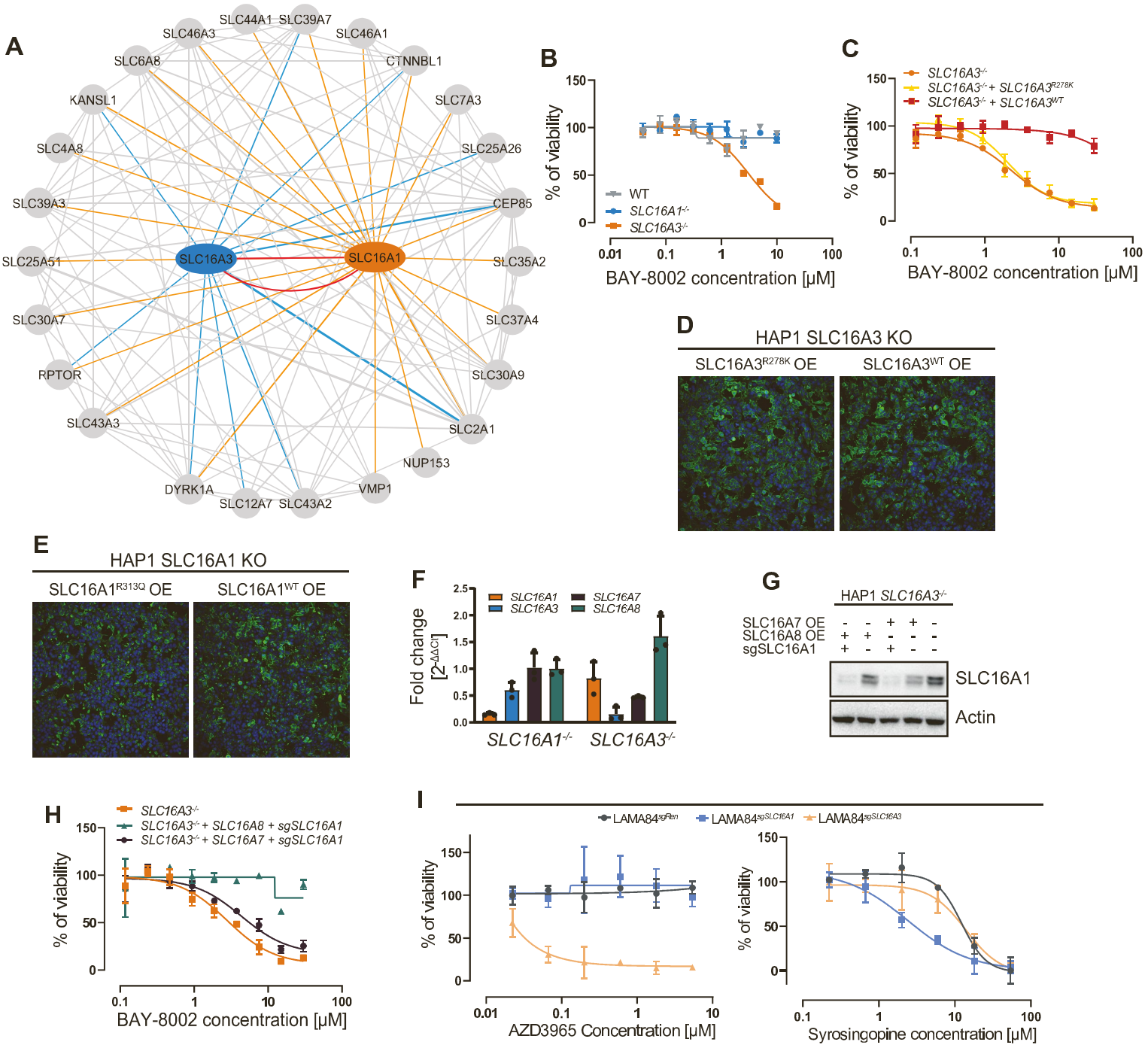
$$CL_{int(mic)} = \frac{0.693}{In\ vitro\ T_{\frac{1}{2}}} \cdot \frac{1}{\frac{mg}{ml} microsomal protein\ in\ reaction}$$

Cell Chemical Biology, Volume *30*

Supplemental information

Paralog-dependent isogenic cell assay cascade generates highly selective SLC16A3 inhibitors

Vojtech Dvorak, Andrea Casiraghi, Claire Colas, Anna Koren, Tatjana Tomek, Fabian Offensperger, Andrea Rukavina, Gary Tin, Elisa Hahn, Sarah Dobner, Fabian Frommelt, Andras Boeszoermenyi, Viktoriia Bernada, J. Thomas Hannich, Gerhard F. Ecker, Georg E. Winter, Stefan Kubicek, and Giulio Superti-Furga



Supplementary Figure 1: Synthetic lethality between *SLC16A1* (MCT1) and *SLC16A3* (MCT4) can be explored chemically, related to Figure 1.

(A) Genetic interaction network of *SLC16A1* and *SLC16A3* (1st degree neighborhood, adapted from Girargi et al.[1], negative interactions with GI score < -5 shown).

(B) Sensitivity of HAP1 WT, HAP1 *SLC16A1*$^{-/-}$ and HAP1 *SLC16A3*$^{-/-}$ cell lines to BAY-8002 (SLC16A1/SLC16A7 inhibitor).

(C) Sensitivity of HAP1 *SLC16A3*$^{-/-}$, HAP1 *SLC16A3*$^{-/-}$ with *SLC16A3*^{WT} or HAP1 *SLC16A3*$^{-/-}$ with *SLC16A3*^{R278K} overexpression to BAY-8002.

(D) Immunofluorescence staining of HA-tag in HAP1 *SLC16A3*$^{-/-}$ with *SLC16A3*^{WT} or *SLC16A3*^{R278K} (both constructs expressed with StrepHA tag).

(E) Same as (D) but for with *SLC16A1*^{WT} or *SLC16A1*^{R313Q} in HAP1 *SLC16A1*$^{-/-}$ cells.

(F) qPCR showing relative change in expression of lactate transporters in HAP1 *SLC16A1*$^{-/-}$ and HAP1 *SLC16A3*$^{-/-}$ cell lines compared to WT.

(G) Western blotting confirmation of SLC16A1 protein level redustion in SLC16A7 (HAP1 *SLC16A3*$^{-/-}$ + *SLC16A7* OE + *sgSLC16A1*) and SLC16A8 (HAP1 *SLC16A3*$^{-/-}$ + *SLC16A8* OE + *sgSLC16A1*) dependent cell.

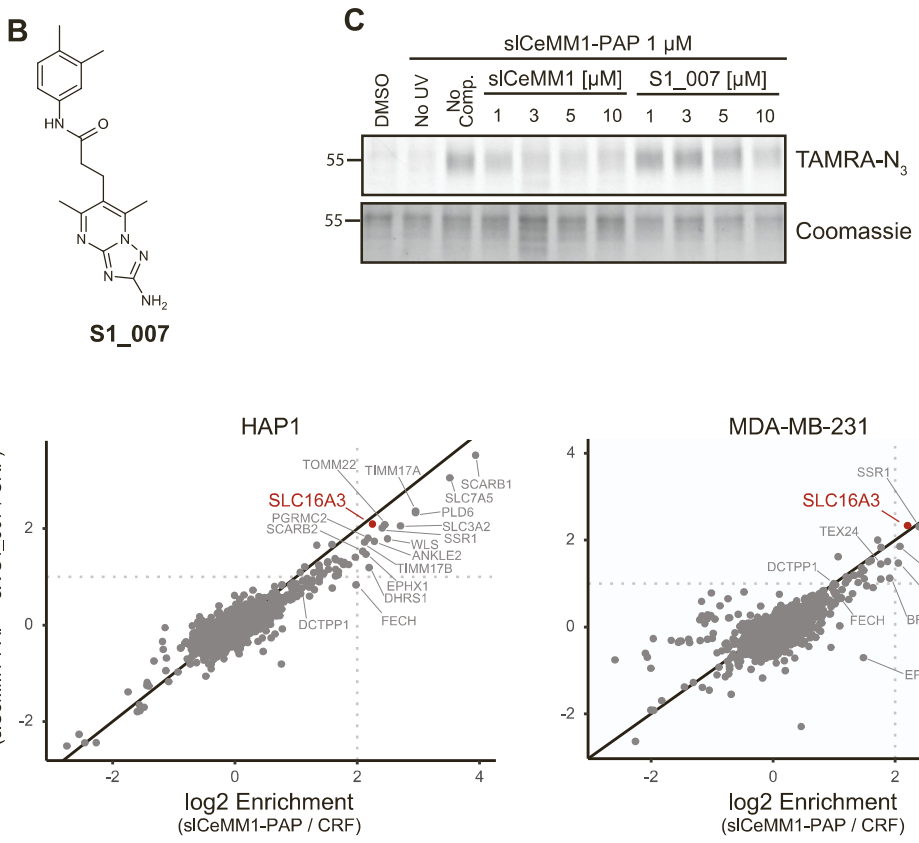
(H) Sensitivity of SLC16A7 and SLC16A8 dependent cells to BAY-8002.

(I) Sensitivity of LAMA84^{sgRen}, LAMA84^{sgSLC16A1} and LAMA84^{sgSLC16A3} to AZD3965 and Syrosingopine.

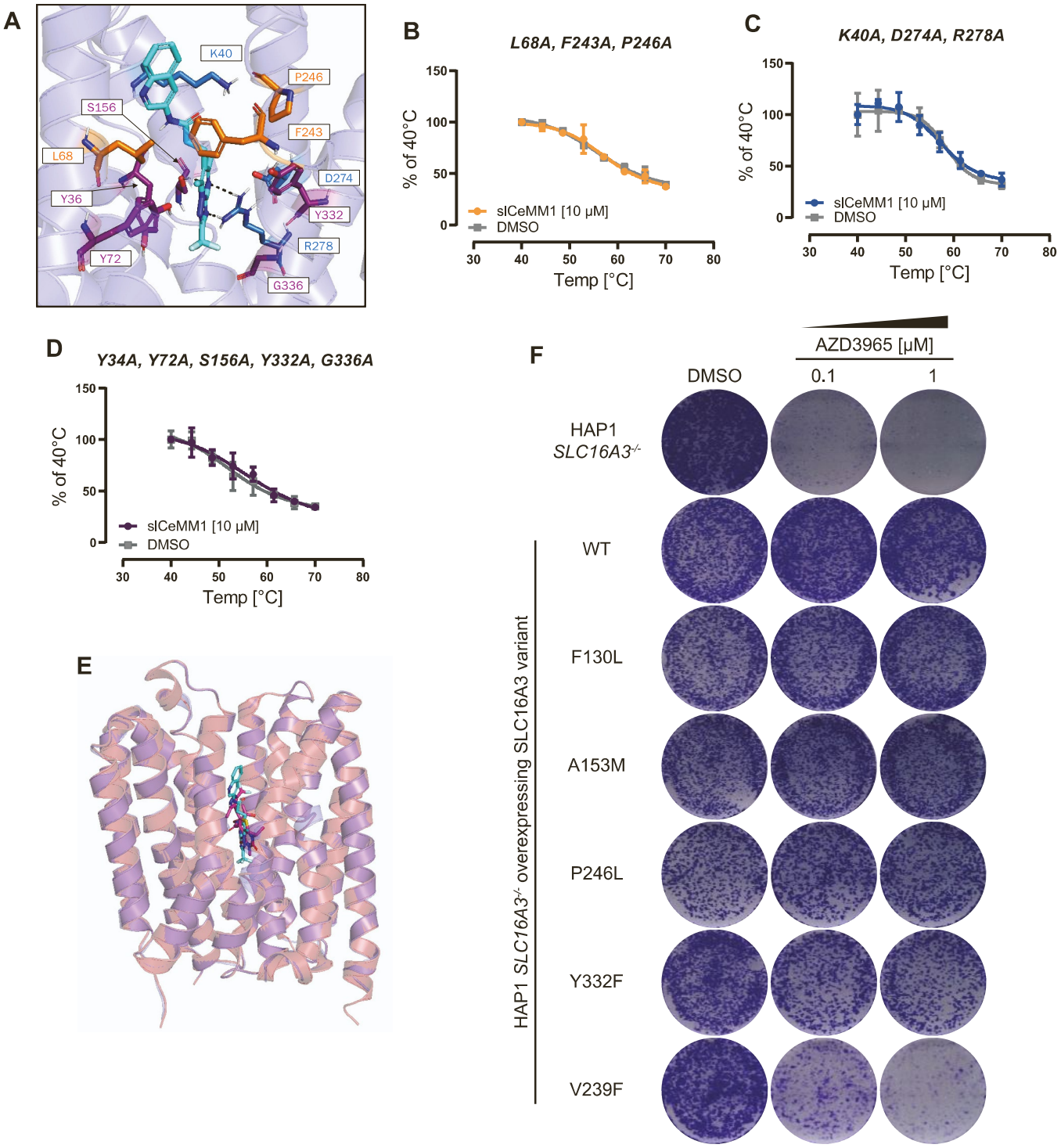
Data presented as mean +/- SD.

A

Compound	Human Liver Microsomes		Rat or Mouse Liver Microsomes	
	$T_{1/2}$ [min]	$CL_{int(mic)}$ (µl/min/mg)	$T_{1/2}$ [min]	$CL_{int(mic)}$ (µl/min/mg)
sICeMM1	**>145**	**<9.6**	**84.8**	**16.3**
S1_002	49.1	28.3	4.7	296.3
S1_005	9.9	139.9	3.5	309
S1_003	6.4	215.5	3.8	366.7
S1_015	2.2	591.6	1.6	881.5
S2_002	46.2	30	37	37.4
S2_003	8.2	169.3	2.2	627
S2_004	2.3	599.3	0.7	1899.2
S2_005	0.6	2159.7	0.6	2474



Supplementary Figure 2: Additional sICeMM1 characteristics (related to Figure 3) and sICeMM1-PAP chemoproteomics (related to Figure 4).
(A) Liver microsome stability assay data summary. sICeMM1 and S2_003 tested in mouse liver microsomes, rest of the compounds tested in liver microsomes from rat.
(B) Chemical structure of S1_007.
(C) In-gel fluorescence of SLC16A3-StrepHA pulldown enriched samples. Coomassie staining shown as a loading control.
(D) Proteins enriched by 1 µM sICeMM1-PAP compared to enriched proteins in competition with 5 µM S1_007. Enrichment calculated over 50 µM CRF (structure corresponding to red part of sICeMM1-PAP in Fig. 4A). Dotted lines represent thresholds for protein enrichment (sICeMM1-PAP enrichmenr (log2) > 2) and competition (sICeMM1-PAP + 5X S1_007 enrichment (log2) < 1).



Supplementary Figure 3: Additional SLC16A3 (MCT4) homology model data, related to Figure 3.
(A) SLC16A3 homology model in outward-open conformation with docked sICeMM1, highlighted residues involved in sICeMM1 binding, color coded based on position in the structure (yellow - "outward group", purple - "central group") or function (blue - residues involved in lactate/H^+ recognition).
(B) Thermal shift assay in lysates of HEK293T transiently transfected to express *HiBiT-SLC16A3* with mutations in residues involved in sICeMM1 binding, localized close to extracellular opening of the transporter cavity ("outward group" - L68A, F243A, P246A).
(C) Same as in (B), but with mutated residues involved in lactate/H^+ recognition ("functional group" - K40A, D274A, R278A).
(D) Same as in (B), but with mutated residues localized closer to the center of the structure ("central group" - Y34A, Y72A, S156A, Y332A, G336A).
(E) Overlap of SLC16A3 homology model (blue) with docked sICeMM1 (cyan) and CryoEM structure of AZD3965 (magenta) bound to SLC16A1 (red)[2].
(F) Crystal violet staining of HAP1 *SLC16A3*$^{-/-}$ cells, stably overexpressing indicated SLC16A3 variants, treated with sICeMM1.

Supplementary References

1. Girardi, E., Fiume, G., Goldmann, U., Sin, C., Müller, F., Sedlyarov, V., Srndic, I., Agerer, B., Kartnig, F., Meixner, E., et al. (2020). A systematic genetic interaction map of human solute carriers assigns a role to SLC25A51/MCART1 in mitochondrial NAD uptake. bioRxiv, 2020.08.31.275818.

2. Wang, N., Jiang, X., Zhang, S., Zhu, A., Yuan, Y., Xu, H., Lei, J., and Yan, C. (2021). Structural basis of human monocarboxylate transporter 1 inhibition by anti-cancer drug candidates. Cell 184, 370-383.e13. 10.1016/j.cell.2020.11.043.

3. Discussion

3.1 PARADISO is a versatile assay with potentially wide applicability.

Despite the relatively understudied status, the SLC superfamily is clearly getting more and more attention in recent years, particularly in the drug discovery. As I argued in the review in the section 1.1.2, we believe that it is mainly due to structural features of SLCs that favor interactions with small molecules. Nonetheless, the development of chemical modulators targeting SLCs remains challenging, mainly due to the lack of assays and other tools, which is connected to the overall poor understanding of the SLC superfamily.

The impact of lack of assays on the SLC-focused drug discovery can be illustrated in several ways. For instance, most of the new compounds targeting SLCs that were introduced in recent years are inhibitors of SLCs that were already targeted (Casiraghi *et al*, 2021). Moreover, many of these SLCs are also the ones that are best studied. For instance, in the last few years we witnessed the introduction of several new inhibitors targeting SLC2A1 (GLUT1), a glucose transporter that is among the most extensively studied SLCs (Casiraghi *et al*, 2021; César-Razquin *et al*, 2015), and is targeted by many existing inhibitors, including a chemical probe (Siebeneicher *et al*, 2016). At the same time, as argued by Aled Edwards and colleagues, there is a trend suggesting that proteins for which we have available tools (chemical modulators in particular) tend to be studied more, creating perpetuation of bias (Edwards *et al*, 2011). This trend is certainly visible among SLCs (Schlessinger *et al*, 2023). The lack of tool compounds can be detrimental for an assay development, since for most of the assays it is crucial to have control-compounds. One such example was already mentioned in the section 1.5.2: The discovery of AZD0095 was possible due to an inhibitor developed previously that was used in development of HTS assay (Goldberg *et al*, 2022).

How to start developing compounds targeting SLCs for which we do not have an assay, or a tool compound? One possible approach could be using approaches based on chemical proteomics. Approaches like this typically rely on covalent interactions between the targeted protein and chemical compounds (briefly discussed in the review in the section 1.3). There are several different solutions for these assays in terms of chemistry, ranging from compound libraries based on electrophilic warheads (reviewed, for example, in (Keeley *et al*, 2020; Boike *et al*, 2022)), to functionalization of fragments with diazirine groups (Parker *et al*, 2017; Wang *et al*, 2019b). These approaches can be very powerful but have limitations. For instance, they are inherently “chemistry-first” and “target-second”, which can be a disadvantage for the “target-first” applications. On the other hand, as I discuss in the review in the section 1.1.2, the chemical structure of the transporter substrate can be a reasonable starting point for the development of inhibitors. However, this is possible only for transporters with prior knowledge

of the substrate. Of note, some of the existing datasets that were generated with these methods can be mined in order to generate starting points for the development of chemical modulators (Boatner *et al*, 2023).

Another approach would be a setup based on phenotypic assays that are skewed towards the protein of interest. As we argue in the review in the section 1.3, the essential aspect of the target-centric phenotypic assay is to demonstrate that the measured phenotype is dependent on the function of the particular transporter. To this end, this thesis proposes to streamline the genetic complexity in a cell by focusing on GIs, and exploiting synthetic lethality (SL). SL is frequently observed among paralog genes, and thus the genetic screens comparing phenotypes of KO versus WT cells could be very powerful to uncover functional overlaps (Costanzo *et al*, 2016, 2021; Xin & Zhang, 2023). One of the potential drawbacks of this approach is that complex buffering interaction between more than two paralogs can be hard to identify and may require a combinatorial screen, rapidly increasing the complexity of the experiment (Dede *et al*, 2020). In many cases, potential GIs have been uncovered from some of the many resources that are available. This includes for example the DepMap and the Cancer Cell Line Encyclopedia (CCLE) projects (Ghandi *et al*, 2019), or also published datasets (Girardi *et al*, 2020c; Horlbeck *et al*, 2018). As we have shown in our study, GIs are a starting point for the development of highly specific screening assays using the PARADISO approach. Since the specificity of the PARADISO approach relies on counter-screening, it may be necessary to compare the effect of the tested molecules in several cell lines, preferentially dependent on different paralog genes. According to some estimates, around 70% of the protein-coding genes in the human genome have one of more paralogs (Ibn-Salem *et al*, 2017), indicating that the full potential of the PARADISO approach could be used widely, not only for SLCs, but also for other gene families. Of note, not all paralogs are capable of functional compensation due to possible functional divergence (Xin & Zhang, 2023). Consequently, if possible, all cell lines used in the PARADISO cascade should be first validated. How about proteins without paralogs? One way around this would be to use homologs from other species. This strategy is sometimes used in transporter research, and was for example instrumental in the identification of human mitochondrial NAD^+ transporter (Luongo *et al*, 2020; Girardi *et al*, 2020a). Finally, the PARADISO approach should be suitable in cases of GIs/SLs that are not exclusive between paralog genes, but also between a specific paralog and other factor, or even in the cases where the GI/SL is dependent on the environment. These cases, however, require very good understanding of the basis of the GI/SL in order to set up meaningful controls for the counter-screening.

3.2 Applying the PARADISO assay to SLC16A3.

To showcase our assay system, we chose to target the lactate transporter SLC16A3. The reasons are four-fold. First of all, the data previously obtained in the Superti-Furga laboratory (Girardi *et al*, 2020c; Pemovska *et al*, 2021), as well as other groups (Marchiq *et al*, 2015; Curtis *et al*, 2017; Benjamin *et al*, 2018; Quanz *et al*, 2018) provided a strong evidence for the existence of SL between the human *SLC16A1* and *SLC16A3* genes. Second, at the beginning of this project, there were no selective inhibitors for SLC16A3. Yet SLC16A3 was emerging as a very promising therapeutic target, providing for a third reason. The fourth and the last reason was practicality. There were several high-quality molecules targeting SLC16A1 available (AZD3965 and BAY-8002), and there was also a tool compound, syrosingopine, targeting SLC16A3. We reasoned that since the SL between *SLC16A1* and *SLC16A3* is reciprocal, we could take advantage of the SLC16A1 targeting compounds, and start setting up the screening system targeting SLC16A1, using these compounds for validation, and then flip this assay to target SLC16A3. Moreover, the promiscuity of SLC16A1 inhibitors towards SLC16A7 also helped us in validating the *de novo* dependencies on SLC16A7 and SLC16A8. Subsequently, despite its suboptimal selectivity, using syrosingopine as a tool compound we could test the reciprocity of the system, increasing the confidence in the approach. Before proceeding with the HTS, we benchmarked the assay using smaller drug libraries, such as CLIMET, a library consisting of metabolism targeted drugs, including AZD3965 (Pemovska *et al*, 2021). We were able to confirm the suitable assay window for the HTS (measured as Z' factor (Zhang *et al*, 1999)), and by confirming that the only drug in the CLIMET library selectively killing the *SLC16A3* KO cells among the PARADISO cell line panel was AZD3965, confirmed the validity of the strategy (data not shown).

From the HTS we found two scaffolds showing excellent selectivity to SLC16A3. Importantly, we were able to validate both scaffolds with a binding assay (cell-based thermal shift) as well as a functional assay (lactate accumulation), providing high confidence that both scaffolds are indeed SLC16A3 inhibitors. Next, we tested several analogues from both series. Mainly due to easier access to analogues of the triazolopyrimidine series, we favored this series. We were able to establish basic structure-activity relationship (SAR) rules, and subsequently generate slCeMM1, our lead compound. Next, we derived a photo-affinity probe, and by chemoproteomics showed in two independent cell lines that slCeMM1 is selective proteome-wide.

Next, we wanted to describe the molecular basis of slCeMM1 selectivity. Since there are no experimentally solved 3D structures of SLC16A3, we turned to homology modelling. As templates for the modelling we initially used: (i) crystal structure of the prokaryotic homologue

from *Syntrophobacter fumaroxidans* (sfMCT), solved in outward-open conformations in complex with lactate or thiosalycate (sfMCT ligand, produced during the crystallization) (Bosshart *et al*, 2019), (ii) cryoEM structure of human SLC16A7, solved in the inward-open apo-state and (iii) cryoEM structures of human SLC16A1 solved in complexes with lactate (outward-open), AZD3965 (outward-open), BAY-8002 (outward-open), 7ACC2 (inward-open) and the apo-state (inward-open) (Wang *et al*, 2021b). Importantly, to rank individual models, we used our SAR data. Initially, we built models in inward- as well as outward-open conformations and used both for docking of slCeMM1 and its analogues. The docking results favored the binding of slCeMM1 to the outward-open conformation. By mutating SLC16A3 residues selected based on the docking results and testing the binding of slCeMM1 using two types of assays, we were able to validate the results of the docking.

We also tried an alternative approach involving a gain-of-function experiment. Through exchanging the residues that were predicted to mediate the selectivity and binding of slCeMM1 between SLC16A3 and SLC16A1, we wanted to create a mutant of SLC16A1 with the capacity to bind slCeMM1. To this end we mutated seven residues in total, and despite the individual mutations had no, or only a mild, effect on the transport function, the mutant harboring all seven mutations showed loss of transport activity (data not shown). Similarly, by swapping multiple residues from SLC16A1 onto SLC16A3, we tried to generate a variant of SLC16A3 with complete resistance to slCeMM1. However also in this case we obtained transporters with altered transport function. Despite this, the effects of OE of single amino acid mutants of SLC16A3 showed sufficient difference in rescue of slCeMM1 effect, validating the homology model and docking results. The homology model can now serve as a template for further structure-based optimization of slCeMM1 and other compounds targeting SLC16A3.

One of the classic ways to show the on-target effect is to demonstrate that the effect of perturbation is rescued by introducing mutations in the target protein (Kaelin, 2017; Bunnage *et al*, 2015). Hence, by the identification of point mutations in *SLC16A3* that result in decreased effect of slCeMM1, we were able to provide an additional line of evidence for the SLC16A3 engagement. To this end, we tested also a complementary approach based on the occurrence of spontaneous resistance in sensitive cells that are deficient in the DNA mismatch repair pathway, resulting in higher accumulation of missense mutations. This approach was used successfully for example in (Mayor-Ruiz *et al*, 2020). To do this, we knocked out *MSH6* in HAP1 *SLC16A1*$^{-/-}$ cells and exposed these cells to various concentrations of the SLC16A3 inhibitors for several weeks. We were able to isolate several independent cell lines resistant to the SLC16A3 inhibitors (all cell lines were cross-resistant to inhibitors from both series that we identified in the HTS, including slCeMM1). Next, we performed a deep sequencing targeted to *SLC16A1* and *SLC16A3*, but we did not find any missense mutations that were significantly

enriched in the resistant cell lines compared to the parental cells (data not shown). Our results obtained from validations of the homology model showed that single mutations in *SLC16A3* result only in a mild rescue of the slCeMM1 binding, suggesting that even through a spontaneous resistance possibly several independent mutations would be needed to rescue the effect of SLC16A3 inhibitors sufficiently. On the other hand, as already mentioned, the resistance to SLC16A3 perturbation, as well as SLC16A1 perturbation, could be mediated by metabolic adaptations, such as an increase in OXPHOS, and decrease in glycolysis (will be discusses further below). The lack of enrichment in missense mutations in this experiment could be explained by the more straight-forward development of resistance mediated by metabolic adaptations, or even by upregulation of other *SLC16A3* paralogs, such as *SLC16A8,* rather than accumulation of mutations in *SLC16A3*. We were not able to follow-up further on this experiment due to time constraints, however future profiling of cell lines generated in this experiment can bring additional insights into resistance mechanisms to SLC16A3 inhibition in cancer cells.

3.3 SLC16A3 chemical probes & their applications

Our results show that slCeMM1 fulfils criteria for chemical probes, as stated by SGC. These are: (1) *in* vitro potency < 100 nM (IC_{50} or Kd); (2) > 30-fold selectivity over proteins in the same family; (3) cellular on-target activity < 1 µM; (4) extensive off-target profiling outside the target family; (5) structurally related negative control; (6) no pan-assay interference (commonly known as PAINs (Baell & Walters, 2014)) element. slCeMM1 fulfils all criteria, and hence should be considered the first SLC16A3 chemical probe. In addition, slCeMM1 has an advantage over other SLC16A3 inhibitors, because we were able to show the selectivity over all four paralogs, not only SLC16A1, as was the case in other studies (Cluntun *et al*, 2021; Heinrich *et al*, 2021; Kawatkar *et al*, 2023; Goldberg *et al*, 2022). This can be particularly important in the SLC16 family, as exemplified by SLC16A1 inhibitors that frequently inhibit SLC16A7 as their off-target. Importantly, the lactate transporters from the SLC16 family can be divided into two sub-groups based on their amino acid sequence identity: one being SLC16A1 and SLC16A7 and second being SLC16A3 and SLC16A8 (Figure 2 and Table 2). Therefore, the selectivity of SLC16A3 inhibitors should be compared against SLC16A8 rather than SLC16A1. To the best of my knowledge, the only compound that currently fulfils this criterion is slCeMM1. This can be important not only from the perspective of chemical probes, but also for potential drug candidates, since SLC16A8 plays an important role in vision and its inhibition could lead to potential defects in the retina. Of note, results of phase I trial of

AZD3965 reported temporal changes in retina as relatively frequent side-effect, suggesting that retina may be particularly sensitive to changes in lactate levels (Halford *et al*, 2023).

One potential temporal disadvantage of the slCeMM1 compared to other SLC16A3 inhibitors is the lack of PK characterization. Even though slCeMM1 showed very good stability in the liver microsomes, we have not yet been able to further characterize the metabolic stability and other PK properties in more detail. Of note, such PK experiments can be costly and time-consuming and are typically not covered by the financial framework of academic research. AZD0095 (Goldberg *et al*, 2022) and MSC-4381 (Heinrich *et al*, 2021) showed overall good PK properties, and were used successfully in *in vivo* models. Reports on VB124 did not provide details about the PK properties of the compound, but also showed activity in a murine model (Cluntun *et al*, 2021). Another potential shortcoming of slCeMM1 might be the lower potency compared to the three compounds AZD0095, MSC-4381 or VB124. Potency, however, may be hard to compare, as the IC_{50} for the four compounds were each obtained in different assays and experimental setting. It is well known that IC_{50} values depend on assays and experimental conditions and hence the direct comparison between different experimental setups can be misleading.

Nevertheless, we are convinced that slCeMM1 will be an important tool for exploring biological roles of SLC16A3 in different systems, particularly due to its selectivity and overall chemical probe characteristics. This is especially important for validating SLC16A3 as a therapeutic target (Hu *et al*, 2013). While chemical probes can be complementary to genetic approaches for the target validation, they also provide unique advantages. Their implementation can be easy and straightforward across many different models, they act rapidly comparted to genetic inactivation, and often in reversible fashion. Importantly, by using chemical probes, it is possible to distinguish between direct target inhibition and effects that would result from disruption of scaffolding functions upon gene knock-out (Arrowsmith *et al*, 2015). All these parameters are important, because they can provide clear knowledge on the tractability and translatability of their target, especially if multiple structurally unrelated probes are used (Garbaccio & Parmee, 2016).

Despite their potential importance, only very few chemical probes targeting SLCs are currently available. The chemical probe catalogue of the SGC, a highly curated collection of chemical probes ((Müller *et al*, 2018); https://www.thesgc.org/chemical-probes), currently lists only three probes, targeting SLC2A1, SLC9A1 (NHE1) and SLC13A5 (NaCT), with MSC-4381 currently being evaluated as potential SLC16A3 chemical probe (Tredup *et al*, 2023). This is very poor, given the fact that there are more than 400 SLCs. Importantly, most of the inhibitors used in SLC-research are poor quality tool compounds, usually manifested by the lack of selectivity.

While careful use of these compounds, for example to test the assay window during assay development can be tolerated, their usage to answer biological questions in complex model systems, should be avoided. This issue is summarized in more detail in an excellent perspective by Blagg and Workman (Blagg & Workman, 2017). Of note, there are different criteria for chemical probes than for drugs. The main differences are that chemical probes need to be highly selective with a defined MoA, while drugs are not always selective and their MoA is frequently not understood, as exemplified by aspirin, metformin or acetomiphen. On the other hand, drugs need to be safe, effective and bioavailable (Arrowsmith *et al*, 2015).

In which areas could slCeMM1 be utilized? As already indicated in the introduction, one of the big disease areas in which SLC16A3 was proposed as a therapeutic target is cancer. Within this context, slCeMM1 should be used to validate some of the studies that proposed SLC16A3 as therapeutic target using genetics. These include several studies focused on SLC16A3 as target for potential monotherapy, for example in renal cancer (Gerlinger *et al*, 2012), or breast cancer (Baenke *et al*, 2015). Initially, the effect of slCeMM1 should be tested in a wide panel of cell lines, similarly to BAY-8002 (Quanz *et al*, 2018) or AZD3965 (Curtis *et al*, 2017). One potential obstacle in this could be that, at least in our hands, slCeMM1 seemed to inhibit proliferation, rather than causing a cytotoxic effect, which would be easier to screen. Nevertheless, the original studies which are reporting compounds targeting SLC16A1 showed a similar behavior (Murray *et al*, 2005), and yet they were successfully tested in hundreds of cell lines (Quanz *et al*, 2018; Curtis *et al*, 2017). An efficient way how to test slCeMM1, and potentially other SLC16A3 inhibitors, would be by screening it as a part of the PRISM platform, a part of the DepMap project. This platform provides a highly optimized HTS of drug sensitivity in hundreds of DNA barcoded cell lines in a multiplexed fashion (Yu *et al*, 2016). This approach allows for highly precise quantification of individual cells based on their barcodes, and therefore should be well suited for identification of cytostatic effects in high throughput. Moreover, the results can be directly correlated to results obtained from testing of thousands other compounds, as well as other datasets in CCLE and DepMap, providing additional potential to understand the underlying mechanisms of sensitivity to tested drugs (Corsello *et al*, 2020). A combination of this platform and the selectivity of slCeMM1 should be very powerful to get novel insights into role of SLC16A3 in the context of cancer.

The SL relationship between *SLC16A1* and *SLC16A3* also received a lot of attention in cancer, and targeting both transporters was suggested as a potential avenue how to explore the Warburg effect associated with cancer metabolism therapeutically (Benjamin *et al*, 2018; Marchiq *et al*, 2015; Pemovska *et al*, 2021). Indeed, many studies showed that one of the mechanisms of resistance which inhibits lactate export in the tumours expressing only one paralog, is the upregulation of other paralogs (as already discussed elsewhere in this thesis).

Beyond this there were several other mechanisms of resistance reported, mostly due to metabolic adaptations. For example, Doherty and colleagues showed that one of the possible mechanisms of resistance for inhibition of lactate export is a shift from high glycolytic fluxes toward OXPHOS (Doherty *et al*, 2014). Similar findings were reported also by Baenke and colleagues, showing that silencing of *SLC16A3* leads to increased OXPHOS and glutamine consumption and corresponding enhanced sensitivity to glutaminase inhibition and metformin (Baenke *et al*, 2015). These findings are further supported by multiple studies showing the synergistic effects between drugs like metformin and inhibition of the lactate export (Benjamin *et al*, 2016, 2018; Marchiq *et al*, 2015). Similar studies could potentially be important for defining biomarkers of slCeMM1 action, but also to show that testing synergies between inhibition of lactate export and other metabolism targeting drugs could be a valid therapeutic strategy exploiting cancer metabolism.

Another big area in which slCeMM1 could be used in the context of cancer, is to investigate the role of SLC16A3 in the TME and in metabolic symbiosis. Also here, the most straightforward option would be to use slCeMM1 to validate findings based on knock-out or knock-down of *SLC16A3*. This includes for example the role of metabolic symbiosis in resistance to therapies targeted at angiogenesis (Allen *et al*, 2016; Jiménez-Valerio *et al*, 2016; Pisarsky *et al*, 2016). Indeed, these studies suggested SLC16A3 as a potential target to overcome the resistance and re-sensitize the tumors to angiogenesis inhibitors. Goldberg and colleagues showed that AZD0095 has a synergistic effect with angiogenesis-targeted therapy in xenograft models, validating the notion with SLC16A3 inhibitor (Goldberg *et al*, 2022). However, the cell line used in this study expressed only very low levels of *SLC16A1*, and hence it remains to be seen if SLC16A3 is a good therapeutical target also in other models, such as those used in the original studies. Other resistance mechanisms implicating the metabolic symbiosis in the TME involved tyrosine kinase inhibitors (Apicella *et al*, 2018). In this case, the lactate, secreted by cancer cells through SLC16A3, prompted CAFs to secrete growth factors that mediated the resistance. The disruption of the metabolic symbiosis by silencing of *SLC16A3* specifically in the tumor cells, or by administration of AZD3965, resulted in the re-sensitization of the tumours to the treatment. Using slCeMM1 in this context could validate the SLC16A3 as a therapeutic target in this context. Moreover, these studies indicate that use of SLC16A1 and SLC16A3 inhibitors more broadly in models of drug resistant cancer could uncover additional examples of resistance mediated by metabolic symbiosis.

Numerous studies demonstrated that accumulation of lactate in the TME negatively affects the immune response directed at cancer cells (reviewed in (Wang *et al*, 2021c)). Several studies suggested that inhibition of lactate transporters can decrease the concentration of lactate in the TME, enhancing the immune response, and acting synergistically with immunotherapy

(Renner *et al*, 2019; Qian *et al*, 2023; Kumagai *et al*, 2022). Moreover, since SLC16A1 inhibitors were shown to cause immunosuppression (Murray *et al*, 2005) targeting metabolic symbiosis by inhibiting the SLC16A3 may be superior in this context. Testing the synergy between slCeMM1 and immunotherapy in different models could provide insight into the role of SLC16A3 in the modulation of immunotherapy response, and potentially pave the way for introducing the immunotherapy into more cancer types.

Despite most research focusing on SLC16A3 has been performed in the context of cancer, there are several reports rationalizing the SLC16A3 targeting in other disease areas. This include already for example already mentioned inflammatory diseases or heart diseases (Fujii *et al*, 2015; Cluntun *et al*, 2021). In this context slCeMM1 should be used for validating SLC16A3 as a therapeutic target. Importantly, slCeMM1 could be tested in additional models to find new roles for SLC16A3 in other diseases. Especially in inflammatory diseases inhibition of SLC16A3 could be an interesting therapeutic avenue, mainly due to high local lactate concentrations and its impact on the immune cells (Certo *et al*, 2021).

Thus, there we expect the compound identified and characterized in this thesis, slCeMM1, to be useful in establishing the potential role of its target in a variety of pathophysiological settings and hence, derive the attractivity of developing other highly selective SLC16A3 inhibitors.

3.4 Conclusions & future prospects

In summary, this thesis provides an overview of SLC-oriented drug discovery with focus on their druggability and available assay technologies and offers a novel approach for targeting SLCs and a new chemical modulator targeting SLC16A3. Importantly, the PARADISO assay was able to generate compounds with properties of chemical probe, demonstrating its potential. Moreover, one of the strengths of the PARADISO approach is its potential universality and applicability to poorly characterized targets or targets that are impossible to configures in biochemical assays. One of the follow-up projects resulting from this thesis is aiming at using the PARADISO approach to target mitochondrial iron transporters SLC25A28 and SLC25A37, also known as mitoferrins. These transporters are emerging as therapeutical targets in metastatic cancers (Cuadros *et al*, 2022), but are currently considered highly challenging therapeutical targets due to limited availability of assays, and virtually no tool compounds that can be used for assay development. At the same time, both transporters show SL interactions which is ideal for the PARADISO assay (Girardi *et al*, 2020c). The application of the PARASIDO assay in cases like this will verify the potential of this approach.

The chemical probe generated in this assay provides a valuable tool to explore the biological roles of SLC16A3 and can be utilized to validate the potential of this transporter as a therapeutic target. Moreover, the compounds identified in this thesis could be a first step for the development of SLC16A3 targeted drugs, with potential usage in cancer, inflammation, or heart diseases.

References

Abplanalp J, Laczko E, Philp NJ, Neidhardt J, Zuercher J, Braun P, Schorderet DF, Munier FL, Verrey F, Berger W, *et al* (2013) The cataract and glucosuria associated monocarboxylate transporter MCT12 is a new creatine transporter. *Hum Mol Genet* 22: 3218–26

Allen E, Miéville P, Warren CM, Saghafinia S, Li L, Peng MW & Hanahan D (2016) Metabolic Symbiosis Enables Adaptive Resistance to Anti-angiogenic Therapy that Is Dependent on mTOR Signaling. *Cell Rep* 15: 1144–1160

Apicella M, Giannoni E, Fiore S, Ferrari KJ, Fernández-Pérez D, Isella C, Granchi C, Minutolo F, Sottile A, Comoglio PM, *et al* (2018) Increased Lactate Secretion by Cancer Cells Sustains Non-cell-autonomous Adaptive Resistance to MET and EGFR Targeted Therapies. *Cell Metab* 28: 848-865.e6

Arrowsmith CH, Audia JE, Austin C, Baell J, Bennett J, Blagg J, Bountra C, Brennan PE, Brown PJ, Bunnage ME, *et al* (2015) The promise and peril of chemical probes. *Nat Chem Biol* 11: 536–41

Baek GH, Tse YF, Hu Z, Cox D, Buboltz N, McCue P, Yeo CJ, White MA, DeBerardinis RJ, Knudsen ES, *et al* (2014) MCT4 Defines a Glycolytic Subtype of Pancreatic Cancer with Poor Prognosis and Unique Metabolic Dependencies. *Cell Rep* 9: 2233–2249

Baell J & Walters MA (2014) Chemistry: Chemical con artists foil drug discovery. *Nature* 513: 481–483

Baenke F, Dubuis S, Brault C, Weigelt B, Dankworth B, Griffiths B, Jiang M, MacKay A, Saunders B, Spencer-Dene B, *et al* (2015) Functional screening identifies MCT4 as a key regulator of breast cancer cell metabolism and survival. *J Pathol* 237: 152–165

Bar-Peled L & Kory N (2022) Principles and functions of metabolic compartmentalization. *Nat Metab* 4: 1232–1244

Bazúa-Valenti S, Castañeda-Bueno M & Gamba G (2016) Physiological role of SLC12 family members in the kidney. *Am J Physiol - Ren Physiol* 311: F131–F144

Benjamin D, Colombi M, Hindupur SK, Betz C, Lane HA, El-Shemerly MYM, Lu M, Quagliata L, Terracciano L, Moes S, *et al* (2016) Syrosingopine sensitizes cancer cells to killing by metformin. *Sci Adv* 2: 1–12

Benjamin D, Robay D, Hindupur SK, Pohlmann J, Colombi M, El-Shemerly MY, Maira SM, Moroni C, Lane HA & Hall MN (2018) Dual Inhibition of the Lactate Transporters MCT1 and MCT4 Is Synthetic Lethal with Metformin due to NAD+ Depletion in Cancer Cells. *Cell Rep* 25: 3047-3058.e4

Bernal J, Guadaño-Ferraz A & Morte B (2015) Thyroid hormone transporters--functions and clinical implications. *Nat Rev Endocrinol* 11: 406–17

Bharadwaj R, Lusi CF, Mashayekh S, Nagar A, Subbarao M, Kane GI, Wodzanowski KA, Brown AR, Okuda K, Monahan A, *et al* (2023) Methotrexate suppresses psoriatic skin inflammation by inhibiting muropeptide transporter SLC46A2 activity. *Immunity* 56: 998-1012.e8

Bishop TR, Subramanian C, Bilotta EM, Garnar-Wortzel L, Ramos AR, Zhang Y, Asiaban JN, Ott CJ, Rock CO & Erb MA (2023) Acetyl-CoA biosynthesis drives resistance to histone acetyltransferase inhibition. *Nat Chem Biol*

Blagg J & Workman P (2017) Choose and Use Your Chemical Probe Wisely to Explore Cancer Biology. *Cancer Cell* 32: 9–25

Boatner LM, Palafox MF, Schweppe DK & Backus KM (2023) CysDB: a human cysteine database based on experimental quantitative chemoproteomics. *Cell Chem Biol* 30: 683-698.e3

Boike L, Henning NJ & Nomura DK (2022) Advances in covalent drug discovery. *Nat Rev Drug Discov* 21: 881–898

Bosshart PD, Charles RP, Garibsingh RAA, Schlessinger A & Fotiadis D (2020) SLC16 Family: From Atomic Structure to Human Disease. *Trends Biochem Sci*: 1–13

Bosshart PD, Kalbermatter D, Bonetti S & Fotiadis D (2019) Mechanistic basis of L-lactate transport in the SLC16 solute carrier family. *Nat Commun* 10: 2649

Brady DC, Crowe MS, Turski ML, Hobbs GA, Yao X, Chaikuad A, Knapp S, Xiao K, Campbell SL, Thiele DJ, *et al* (2014) Copper is required for oncogenic BRAF signalling and tumorigenesis. *Nature* 509: 492–496

Brooks GA (2018) The Science and Translation of Lactate Shuttle Theory. *Cell Metab* 27: 757–785

Bunnage ME, Gilbert AM, Jones LH & Hett EC (2015) Know your target, know your molecule. *Nat Chem Biol* 11: 368–372

Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N, Kuehne AI, Kranzusch PJ, Griffin AM, Ruthel G, *et al* (2011) Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* 477: 340–3

Casey JR, Grinstein S & Orlowski J (2010) Sensors and regulators of intracellular pH. *Nat Rev Mol Cell Biol* 11: 50–61

Casiraghi A, Bensimon A & Superti-Furga G (2021) Recent developments in ligands and chemical probes targeting solute carrier transporters. *Curr Opin Chem Biol* 62: 53–63

Certo M, Llibre A, Lee W & Mauro C (2022) Understanding lactate sensing and signalling. *Trends Endocrinol Metab* 33: 722–735

Certo M, Tsai C-H, Pucino V, Ho P-C & Mauro C (2021) Lactate modulation of immune responses in inflammatory versus tumour microenvironments. *Nat Rev Immunol* 21: 151–161

César-Razquin A, Snijder B, Frappier-Brinton T, Isserlin R, Gyimesi G, Bai X, Reithmeier RA, Hepworth D, Hediger MA, Edwards AM, *et al* (2015) A Call for Systematic Research on Solute Carriers. *Cell* 162: 478–87

Chandel NS (2015) Navigating Metabolism Cold Spring Harbor Laboratory Press

Chen Y-J, Mahieu NG, Huang X, Singh M, Crawford PA, Johnson SL, Gross RW, Schaefer J & Patti GJ (2016) Lactate metabolism is associated with mammalian mitochondria. *Nat Chem Biol* 12: 937–943

Cluntun AA, Badolia R, Lettlova S, Parnell KM, Shankar TS, Diakos NA, Olson KA, Taleb I, Tatum SM, Berg JA, *et al* (2021) The pyruvate-lactate axis modulates cardiac hypertrophy and heart failure. *Cell Metab* 33: 629-648.e10

Contreras-Baeza Y, Sandoval PY, Alarcón R, Galaz A, Cortés-Molina F, Alegriá K, Baeza-Lehnert F, Arce-Molina R, Guequén A, Flores CA, *et al* (2019) Monocarboxylate transporter 4 (MCT4) is a high affinity transporter capable of exporting lactate in high-lactate microenvironments. *J Biol Chem* 294: 20135–20147

Corsello SM, Nagari RT, Spangler RD, Rossen J, Kocak M, Bryan JG, Humeidi R, Peck D, Wu

X, Tang AA, *et al* (2020) Discovering the anti-cancer potential of non-oncology drugs by systematic viability profiling. *Nat cancer* 1: 235–248

Costanzo M, Hou J, Messier V, Nelson J, Rahman M, VanderSluis B, Wang W, Pons C, Ross C, Ušaj M, *et al* (2021) Environmental robustness of the global yeast genetic interaction network. *Science* 372

Costanzo M, VanderSluis B, Koch EN, Baryshnikova A, Pons C, Tan G, Wang W, Usaj M, Hanchard J, Lee SD, *et al* (2016) A global genetic interaction network maps a wiring diagram of cellular function. *Science (80-)* 353

Côté M, Misasi J, Ren T, Bruchez A, Lee K, Filone CM, Hensley L, Li Q, Ory D, Chandran K, *et al* (2011) Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. *Nature* 477: 344–348

Cuadros AM, Fernández-Garcia J, Planque M, Altea-Manzano P, Schalley T, Vermeire I, Broekaert D, Wu Q, Van Brussel T, Leduc S, *et al* (2022) In vivo CRISPR screen defines Slc25a37 as an organ-specific regulator of antioxidant metabolism in metastasis. *bioRxiv*: 2022.09.03.506468

Cunrath O & Bumann D (2019) Host resistance factor SLC11A1 restricts Salmonella growth through magnesium deprivation. *Science (80-)* 366: 995–999

Curtis NJ, Mooney L, Hopcroft L, Michopoulos F, Whalley N, Zhong H, Murray C, Logie A, Revill M, Byth KF, *et al* (2017) Pre-clinical pharmacology of AZD3965, a selective inhibitor of MCT1: DLBCL, NHL and Burkitt's lymphoma anti-tumor activity. *Oncotarget* 8: 69219–69236

Daniele LL, Sauer B, Gallagher SM, Pugh EN & Philp NJ (2008) Altered visual function in monocarboxylate transporter 3 (Slc16a8) knockout mice. *Am J Physiol Cell Physiol* 295: C451-7

DeBerardinis RJ & Chandel NS (2020) We need to talk about the Warburg effect. *Nat Metab* 2: 127–129

Dede M, McLaughlin M, Kim E & Hart T (2020) Multiplex enCas12a screens detect functional buffering among paralogs otherwise masked in monogenic Cas9 knockout screens. *Genome Biol* 21: 262

Doherty JR, Yang C, Scott KEN, Cameron MD, Fallahi M, Li W, Hall MA, Amelio AL, Mishra

JK, Li F, *et al* (2014) Blocking lactate export by inhibiting the Myc target MCT1 Disables glycolysis and glutathione synthesis. *Cancer Res* 74: 908–20

Doyen J, Trastour C, Ettore F, Peyrottes I, Toussant N, Gal J, Ilc K, Roux D, Parks SK, Ferrero JM, *et al* (2014) Expression of the hypoxia-inducible monocarboxylate transporter MCT4 is increased in triple negative breast cancer and correlates independently with clinical outcome. *Biochem Biophys Res Commun* 451: 54–61

Draoui N & Feron O (2011) Lactate shuttles at a glance: from physiological paradigms to anti-cancer treatments. *Dis Model Mech* 4: 727–32

Edwards AM, Isserlin R, Bader GD, Frye S V., Willson TM & Yu FH (2011) Too many roads not taken. *Nature* 470: 163–5

Elia I, Rossi M, Stegen S, Broekaert D, Doglioni G, van Gorsel M, Boon R, Escalona-Noguero C, Torrekens S, Verfaillie C, *et al* (2019) Breast cancer cells rely on environmental pyruvate to shape the metastatic niche. *Nature* 568: 117–121

Faubert B, Li KY, Cai L, Hensley CT, Kim J, Zacharias LG, Yang C, Do QN, Doucette S, Burguete D, *et al* (2017) Lactate Metabolism in Human Lung Tumors. *Cell* 171: 358-371.e9

Fauster A, Rebsamen M, Willmann KL, César-Razquin A, Girardi E, Bigenzahn JW, Schischlik F, Scorzoni S, Bruckner M, Konecka J, *et al* (2018) Systematic genetic mapping of necroptosis identifies SLC39A7 as modulator of death receptor trafficking. *Cell Death Differ* 26: 1138–1155

Felmlee MA, Jones RS, Rodriguez-Cruz V, Follman KE & Morris ME (2020) Monocarboxylate Transporters (SLC16): Function, Regulation, and Role in Health and Disease. *Pharmacol Rev* 72: 466–485

Fernandez-Caggiano M, Kamynina A, Francois AA, Prysyazhna O, Eykyn TR, Krasemann S, Crespo-Leiro MG, Vieites MG, Bianchi K, Morales V, *et al* (2020) Mitochondrial pyruvate carrier abundance mediates pathological cardiac hypertrophy. *Nat Metab* 2: 1223–1231

Ferrada E & Superti-Furga G (2022) A structure and evolutionary-based classification of solute carriers. *iScience* 25: 105096

Fisel P, Schaeffeler E & Schwab M (2018) Clinical and Functional Relevance of the Monocarboxylate Transporter Family in Disease Pathophysiology and Drug Therapy. *Clin*

Transl Sci 11: 352–364

Fishbein WN, Merezhinskaya N & Foellmer JW (2002) Relative distribution of three major lactate transporters in frozen human tissues and their localization in unfixed skeletal muscle. *Muscle Nerve* 26: 101–12

Fonseca-Correa JI & Correa-Rotter R (2021) Sodium-Glucose Cotransporter 2 Inhibitors Mechanisms of Action: A Review. *Front Med* 8: 1–7

Fritsche LG, Chen W, Schu M, Yaspan BL, Yu Y, Thorleifsson G, Zack DJ, Arakawa S, Cipriani V, Ripke S, *et al* (2013) Seven new loci associated with age-related macular degeneration. *Nat Genet* 45: 433–9, 439e1-2

Fritsche LG, Igl W, Bailey JNC, Grassmann F, Sengupta S, Bragg-Gresham JL, Burdon KP, Hebbring SJ, Wen C, Gorski M, *et al* (2016) A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat Genet* 48: 134–43

Fujii W, Kawahito Y, Nagahara H, Kukida Y, Seno T, Yamamoto A, Kohno M, Oda R, Taniguchi D, Fujiwara H, *et al* (2015) Monocarboxylate transporter 4, associated with the acidification of synovial fluid, is a novel therapeutic target for inflammatory arthritis. *Arthritis Rheumatol* 67: 2888–2896

Futagi Y, Narumi K, Furugen A, Kobayashi M & Iseki K (2020) Molecular characterization of the orphan transporter SLC16A9, an extracellular pH- and Na+-sensitive creatine transporter. *Biochem Biophys Res Commun* 522: 539–544

Ganapathy V, Thangaraju M, Gopal E, Martin PM, Itagaki S, Miyauchi S & Prasad PD (2008) Sodium-coupled monocarboxylate transporters in normal tissues and in cancer. *AAPS J* 10: 193–199

Garbaccio RM & Parmee ER (2016) The Impact of Chemical Probes in Drug Discovery: A Pharmaceutical Industry Perspective. *Cell Chem Biol* 23: 10–17

Gerlinger M, Santos CR, Spencer-Dene B, Martinez P, Endesfelder D, Burrell RA, Vetter M, Jiang M, Saunders RE, Kelly G, *et al* (2012) Genome-wide RNA interference analysis of renal carcinoma survival regulators identifies MCT4 as a Warburg effect metabolic target. *J Pathol* 227: 146–156

Ghandi M, Huang FW, Jané-Valbuena J, Kryukov G V., Lo CC, McDonald ER, Barretina J,

Gelfand ET, Bielski CM, Li H, *et al* (2019) Next-generation characterization of the Cancer Cell Line Encyclopedia. *Nature* 569: 503–508

Girardi E, Agrimi G, Goldmann U, Fiume G, Lindinger S, Sedlyarov V, Srndic I, Gürtl B, Agerer B, Kartnig F, *et al* (2020a) Epistasis-driven identification of SLC25A51 as a regulator of human mitochondrial NAD import. *Nat Commun* 11: 6145

Girardi E, César-Razquin A, Lindinger S, Papakostas K, Konecka J, Hemmerich J, Kickinger S, Kartnig F, Gürtl B, Klavins K, *et al* (2020b) A widespread role for SLC transmembrane transporters in resistance to cytotoxic drugs. *Nat Chem Biol* 16: 469–478

Girardi E, Fiume G, Goldmann U, Sin C, Müller F, Sedlyarov V, Srndic I, Agerer B, Kartnig F, Meixner E, *et al* (2020c) A systematic genetic interaction map of human solute carriers assigns a role to SLC25A51/MCART1 in mitochondrial NAD uptake. *bioRxiv*: 2020.08.31.275818

Goldberg FW, Kettle JG, Lamont GM, Buttar D, Ting AKT, McGuire TM, Cook CR, Beattie D, Morentin Gutierrez P, Kavanagh SL, *et al* (2022) Discovery of Clinical Candidate AZD0095, a Selective Inhibitor of Monocarboxylate Transporter 4 (MCT4) for Oncology. *J Med Chem* 4

Gopal E, Umapathy NS, Martin PM, Ananth S, Gnana-Prakasam JP, Becker H, Wagner CA, Ganapathy V & Prasad PD (2007) Cloning and functional characterization of human SMCT2 (SLC5A12) and expression pattern of the transporter in kidney. *Biochim Biophys Acta* 1768: 2690–7

Halestrap AP (2013) The SLC16 gene family-Structure, role and regulation in health and disease. *Mol Aspects Med* 34: 337–349

Halestrap AP & Denton RM (1975) The specificity and metabolic implications of the inhibition of pyruvate transport in isolated mitochondria and intact tissue preparations by alpha-Cyano-4-hydroxycinnamate and related compounds. *Biochem J* 148: 97–106

Halford S, Veal GJ, Wedge SR, Payne GS, Bacon CM, Sloan P, Dragoni I, Heinzmann K, Potter S, Salisbury BM, *et al* (2023) A Phase I Dose-escalation Study of AZD3965, an Oral Monocarboxylate Transporter 1 Inhibitor, in Patients with Advanced Cancer. *Clin Cancer Res* 29: 1429–1439

Hara K, Fujita H, Johnson TA, Yamauchi T, Yasuda K, Horikoshi M, Peng C, Hu C, Ma RCW, Imamura M, *et al* (2014) Genome-wide association study identifies three novel loci for

type 2 diabetes. *Hum Mol Genet* 23: 239–246

van Hasselt PM, Ferdinandusse S, Monroe GR, Ruiter JPN, Turkenburg M, Geerlings MJ, Duran K, Harakalova M, van der Zwaag B, Monavari AA, *et al* (2014) Monocarboxylate Transporter 1 Deficiency and Ketone Utilization. *N Engl J Med* 371: 1900–1907

Hediger MA, Clémençon B, Burrier RE & Bruford EA (2013) The ABCs of membrane transporters in health and disease (SLC series): Introduction. *Mol Aspects Med* 34: 95–107

Heinrich T, Sala-Hojman A, Ferretti R, Petersson C, Minguzzi S, Gondela A, Ramaswamy S, Bartosik A, Czauderna F, Crowley L, *et al* (2021) Discovery of 5-{2-[5-Chloro-2-(5-ethoxyquinoline-8-sulfonamido)phenyl]ethynyl}-4-methoxypyridine-2-carboxylic Acid, a Highly Selective in Vivo Useable Chemical Probe to Dissect MCT4 Biology. *J Med Chem* 64: 11904–11933

Heinz LX, Lee J, Kapoor U, Kartnig F, Sedlyarov V, Papakostas K, César-Razquin A, Essletzbichler P, Goldmann U, Stefanovic A, *et al* (2020) TASL is the SLC15A4-associated adaptor for IRF5 activation by TLR7-9. *Nature* 581: 316–322

Higuchi K, Sugiyama K, Tomabechi R, Kishimoto H & Inoue K (2022) Mammalian monocarboxylate transporter 7 (MCT7/Slc16a6) is a novel facilitative taurine transporter. *J Biol Chem* 298: 101800

Hoch E, Florez JC, Lander ES & Jacobs SBR (2019) Gain-of-Function Claims for Type-2-Diabetes-Associated Coding Variants in SLC16A11 Are Not Supported by the Experimental Data. *Cell Rep* 29: 778–780

Höglund PJ, Nordström KJV, Schiöth HB & Fredriksson R (2011) The solute carrier families have a remarkably long evolutionary history with the majority of the human families present before divergence of Bilaterian species. *Mol Biol Evol* 28: 1531–1541

Hong CS, Graham NA, Gu W, Espindola Camacho C, Mah V, Maresh EL, Alavi M, Bagryanova L, Krotee PAL, Gardner BK, *et al* (2016) MCT1 Modulates Cancer Cell Pyruvate Export and Growth of Tumors that Co-express MCT1 and MCT4. *Cell Rep* 14: 1590–1601

Horlbeck MA, Xu A, Wang M, Bennett NK, Park CY, Bogdanoff D, Adamson B, Chow ED, Kampmann M, Peterson TR, *et al* (2018) Mapping the Genetic Landscape of Human Cells. *Cell* 174: 953-967.e22

Hu G, Henke A, Karpowicz RJ, Sonders MS, Farrimond F, Edwards R, Sulzer D & Sames D (2013) New fluorescent substrate enables quantitative and high-throughput examination of vesicular monoamine transporter 2 (VMAT2). *ACS Chem Biol* 8: 1947–1954

Hugo SE, Cruz-Garcia L, Karanth S, Anderson RM, Stainier DYR & Schlegel A (2012) A monocarboxylate transporter required for hepatocyte secretion of ketone bodies during fasting. *Genes Dev* 26: 282–93

Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, Esparza LA, Reya T, Zhan L, Yanxiang Guo J, *et al* (2017) Glucose feeds the TCA cycle via circulating lactate. *Nature* 551: 115–118

Ibn-Salem J, Muro EM & Andrade-Navarro MA (2017) Co-regulation of paralog genes in the three-dimensional chromatin architecture. *Nucleic Acids Res* 45: 81–91

Ippolito L, Morandi A, Giannoni E & Chiarugi P (2019) Lactate: A Metabolic Driver in the Tumour Landscape. *Trends Biochem Sci* 44: 153–166

Jennings ML (2021) Cell physiology and molecular mechanism of anion transport by erythrocyte band 3/AE1. *Am J Physiol - Cell Physiol* 321: C1028–C1059

Jiménez-Valerio G, Martínez-Lozano M, Bassani N, Vidal A, Ochoa-de-Olza M, Suárez C, García-del-Muro X, Carles J, Viñals F, Graupera M, *et al* (2016) Resistance to Antiangiogenic Therapies by Metabolic Symbiosis in Renal Cell Carcinoma PDX Models and Patients. *Cell Rep* 15: 1134–1143

Jones RS, Tu C, Zhang M, Qu J & Morris ME (2019) Characterization and Proteomic-Transcriptomic Investigation of Monocarboxylate Transporter 6 Knockout Mice: Evidence of a Potential Role in Glucose and Lipid Metabolism. *Mol Pharmacol* 96: 364–376

Jonnalagadda S, Jonnalagadda SK, Ronayne CT, Nelson GL, Solano LN, Rumbley J, Holy J, Mereddy VR & Drewes LR (2019) Novel N,N-dialkyl cyanocinnamic acids as monocarboxylate transporter 1 and 4 inhibitors. *Oncotarget* 10: 2355–2368

Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, *et al* (2021) Highly accurate protein structure prediction with AlphaFold. *Nature* 596: 583–589

Kaelin WG (2017) Common pitfalls in preclinical cancer target validation. *Nat Rev Cancer* 17: 441–450

Kawatkar A, Clark RA, Hopcroft L, Roaquin DA, Tomlinson R, Zuhl AM, Lamont GM, Kettle JG, Critchlow SE, Castaldi MP, *et al* (2023) Chemical Biology Approaches Confirm MCT4 as the Therapeutic Target of a Cellular Optimized Hit. *ACS Chem Biol*

Keeley A, Petri L, Ábrányi-Balogh P & Keserű GM (2020) Covalent fragment libraries in drug discovery. *Drug Discov Today* 25: 983–996

Kelleher KJ, Sheils TK, Mathias SL, Yang JJ, Metzger VT, Siramshetty VB, Nguyen D-T, Jensen LJ, Vidović D, Schürer SC, *et al* (2023) Pharos 2023: an integrated resource for the understudied human proteome. *Nucleic Acids Res* 51: D1405–D1416

Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN & Halestrap AP (2000) CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *EMBO J* 19: 3896–904

Klipfel L, Cordonnier M, Thiébault L, Clérin E, Blond F, Millet-puel G, Mohand-saïd S, Goureau O, Sahel JA, Nandrot EF, *et al* (2021) A splice variant in slc16a8 gene leads to lactate transport deficit in human ips cell-derived retinal pigment epithelial cells. *Cells* 10: 1–18

Kloeckener-Gruissem B, Vandekerckhove K, Nürnberg G, Neidhardt J, Zeitz C, Nürnberg P, Schipper I & Berger W (2008) Mutation of solute carrier SLC16A12 associates with a syndrome combining juvenile cataract with microcornea and renal glucosuria. *Am J Hum Genet* 82: 772–9

Kolz M, Johnson T, Sanna S, Teumer A, Vitart V, Perola M, Mangino M, Albrecht E, Wallace C, Farrall M, *et al* (2009) Meta-analysis of 28,141 individuals identifies common variants within five new loci that influence uric acid concentrations. *PLoS Genet* 5: e1000504

Kumagai S, Koyama S, Itahashi K, Tanegashima T, Lin Y tzu, Togashi Y, Kamada T, Irie T, Okumura G, Kono H, *et al* (2022) Lactic acid promotes PD-1 expression in regulatory T cells in highly glycolytic tumor microenvironments. *Cancer Cell* 40: 201-218.e9

Lengacher S, Nehiri-Sitayeb T, Steiner N, Carneiro L, Favrod C, Preitner F, Thorens B, Stehle J-C, Dix L, Pralong F, *et al* (2013) Resistance to diet-induced obesity and associated metabolic perturbations in haploinsufficient monocarboxylate transporter 1 mice. *PLoS One* 8: e82505

Lewis S, Chen L, Raghuram V, Khundmiri SJ, Chou CL, Yang CR & Knepper MA (2021) "SLC-omics" of the kidney: Solute transporters along the nephron. *Am J Physiol - Cell Physiol* 321: C507–C518

Li X, Yang Y, Zhang B, Lin X, Fu X, An Y, Zou Y, Wang JX, Wang Z & Yu T (2022) Lactate metabolism in human health and disease. *Signal Transduct Target Ther* 7

Li X, Zhang Y, Xu L, Wang A, Zou Y, Li T, Huang L, Chen W, Liu S, Jiang K, *et al* (2023) Ultrasensitive sensors reveal the spatiotemporal landscape of lactate metabolism in physiology and disease. *Cell Metab* 35: 200-211.e9

Liu W, Wang Y, Bozi LHM, Fischer PD, Jedrychowski MP, Xiao H, Wu T, Darabedian N, He X, Mills EL, *et al* (2023) Lactate regulates cell cycle by remodelling the anaphase promoting complex. *Nature* 616: 790–797

Luongo TS, Eller JM, Lu M-J, Niere M, Raith F, Perry C, Bornstein MR, Oliphint P, Wang L, McReynolds MR, *et al* (2020) SLC25A51 is a mammalian mitochondrial NAD+ transporter. *Nature* 588: 174–179

Lyssiotis CA & Kimmelman AC (2017) Metabolic Interactions in the Tumor Microenvironment. *Trends Cell Biol* 27: 863–875

Magistretti PJ & Allaman I (2018) Lactate in the brain: from metabolic end-product to signalling molecule. *Nat Rev Neurosci* 19: 235–249

Marchiq I, Le Floch R, Roux D, Simon MP & Pouyssegur J (2015) Genetic disruption of lactate/H+ symporters (MCTs) and their subunit CD147/BASIGIN sensitizes glycolytic tumor cells to phenformin. *Cancer Res* 75: 171–180

Mayor-Ruiz C, Bauer S, Brand M, Kozicka Z, Siklos M, Imrichova H, Kaltheuner IH, Hahn E, Seiler K, Koren A, *et al* (2020) Rational discovery of molecular glue degraders via scalable chemical profiling. *Nat Chem Biol* 16: 1199–1207

McCommis KS, Kovacs A, Weinheimer CJ, Shew TM, Koves TR, Ilkayeva OR, Kamm DR, Pyles KD, King MT, Veech RL, *et al* (2020) Nutritional modulation of heart failure in mitochondrial pyruvate carrier–deficient mice. *Nat Metab* 2: 1232–1247

Meixner E, Goldmann U, Sedlyarov V, Scorzoni S, Rebsamen M, Girardi E & Superti-Furga G (2020) A substrate-based ontology for human solute carriers. *Mol Syst Biol* 16: 1–9

Muckenthaler MU, Rivella S, Hentze MW & Galy B (2017) A Red Carpet for Iron Metabolism. *Cell* 168: 344–361

Müller S, Ackloo S, Arrowsmith CH, Bauser M, Baryza JL, Blagg J, Böttcher J, Bountra C,

Brown PJ, Bunnage ME, *et al* (2018) Donated chemical probes for open science. *Elife* 7: 1–15

Murakami Y, Kohyama N, Kobayashi Y, Ohbayashi M, Ohtani H, Sawada Y & Yamamoto T (2005) Functional characterization of human monocarboxylate transporter 6 (SLC16A5). *Drug Metab Dispos* 33: 1845–51

Murray CM, Hutchinson R, Bantick JR, Belfield GP, Benjamin AD, Brazma D, Bundick R V., Cook ID, Craggs RI, Edwards S, *et al* (2005) Monocarboxylate transporter MCT1 is a target for immunosuppression. *Nat Chem Biol* 1: 371–376

Nakayama A, Matsuo H, Shimizu T, Ogata H, Takada Y, Nakashima H, Nakamura T, Shimizu S, Chiba T, Sakiyama M, *et al* (2013) Common missense variant of monocarboxylate transporter 9 (MCT9/SLC16A9) gene is associated with renal overload gout, but not with all gout susceptibility. *Hum Cell* 26: 133–6

Nguyen YTK, Ha HTT, Nguyen TH & Nguyen LN (2022) The role of SLC transporters for brain health and disease. *Cell Mol Life Sci* 79: 1–21

Nigam SK (2014) What do drug transporters really do? *Nat Rev Drug Discov* 14: 29–44

Nolin E, Gans S, Llamas L, Bandyopadhyay S, Brittain SM, Bernasconi-Elias P, Carter KP, Loureiro JJ, Thomas JR, Schirle M, *et al* (2019) Discovery of a ZIP7 inhibitor from a Notch pathway screen. *Nat Chem Biol* 15: 179–188

Nwosu ZC, Song MG, di Magliano MP, Lyssiotis CA & Kim SE (2023) Nutrient transporters: connecting cancer metabolism to therapeutic opportunities. *Oncogene* 42: 711–724

Okamoto N, Viswanatha R, Bittar R, Li Z, Haga-Yamanaka S, Perrimon N & Yamanaka N (2018) A Membrane Transporter Is Required for Steroid Hormone Uptake in Drosophila. *Dev Cell* 47: 294-305.e7

Oprea TI, Bologa CG, Brunak S, Campbell A, Gan GN, Gaulton A, Gomez SM, Guha R, Hersey A, Holmes J, *et al* (2018) Unexplored therapeutic opportunities in the human genome. *Nat Rev Drug Discov* 17: 317–332

Ovens MJ, Davies AJ, Wilson MC, Murray CM & Halestrap AP (2010) AR-C155858 is a potent inhibitor of monocarboxylate transporters MCT1 and MCT2 that binds to an intracellular site involving transmembrane helices 7-10. *Biochem J* 425: 523–30

Påhlman C, Qi Z, Murray CM, Ferguson D, Bundick R V., Donald DK & Ekberg H (2013) Immunosuppressive properties of a series of novel inhibitors of the monocarboxylate transporter MCT-1. *Transpl Int* 26: 22–9

Palm W & Thompson CB (2017) Nutrient acquisition strategies of mammalian cells. *Nature* 546: 234–242

Parker CG, Galmozzi A, Wang Y, Correia BE, Sasaki K, Joslyn CM, Kim AS, Cavallaro CL, Lawrence RM, Johnson SR, *et al* (2017) Ligand and Target Discovery by Fragment-Based Screening in Human Cells. *Cell* 168: 527-541.e29

Pemovska T, Bigenzahn JW, Srndic I, Lercher A, Bergthaler A, César-Razquin A, Kartnig F, Kornauth C, Valent P, Staber PB, *et al* (2021) Metabolic drug survey highlights cancer cell dependencies and vulnerabilities. *Nat Commun* 12: 7190

Perland E & Fredriksson R (2017) Classification Systems of Secondary Active Transporters. *Trends Pharmacol Sci* 38: 305–315

Pertega-Gomes N, Vizcaino JR, Felisbino S, Warren AY, Shaw G, Kay J, Whitaker H, Lynch AG, Fryer L, Neal DE, *et al* (2015) Epigenetic and oncogenic regulation of SLC16A7 (MCT2) results in protein over-expression, impacting on signalling and cellular phenotypes in prostate cancer. *Oncotarget* 6: 21675–84

Pisarsky L, Bill R, Fagiani E, Dimeloe S, Goosen RW, Hagmann J, Hess C & Christofori G (2016) Targeting Metabolic Symbiosis to Overcome Resistance to Anti-angiogenic Therapy. *Cell Rep* 15: 1161–1174

Pizzagalli MD, Bensimon A & Superti-Furga G (2020) A guide to plasma membrane solute carrier proteins. *FEBS J*

Prag HA, Gruszczyk A V., Huang MM, Beach TE, Young T, Tronci L, Nikitopoulou E, Mulvey JF, Ascione R, Hadjihambi A, *et al* (2021) Mechanism of succinate efflux upon reperfusion of the ischaemic heart. *Cardiovasc Res* 117: 1188–1201

Pucino V, Certo M, Bulusu V, Cucchi D, Goldmann K, Pontarini E, Haas R, Smith J, Headland SE, Blighe K, *et al* (2019) Lactate Buildup at the Site of Chronic Inflammation Promotes Disease by Inducing CD4+ T Cell Metabolic Rewiring. *Cell Metab* 30: 1055-1074.e8

Pucino V, Nefla M, Gauthier V, Alsaleh G, Clayton SA, Marshall J, Filer A, Clark AR, Raza K & Buckley CD (2023) Differential effect of lactate on synovial fibroblast and macrophage

effector functions. *Front Immunol* 14: 1183825

Puri S & Juvale K (2020) Monocarboxylate transporter 1 and 4 inhibitors as potential therapeutics for treating solid tumours: A review with structure-activity relationship insights. *Eur J Med Chem* 199: 112393

Qian Y, Galan-Cobo A, Guijarro I, Dang M, Molkentine D, Poteete A, Zhang F, Wang Q, Wang J, Parra E, *et al* (2023) MCT4-dependent lactate secretion suppresses antitumor immunity in LKB1-deficient lung adenocarcinoma. *Cancer Cell* 41: 1363-1380.e7

Qosa H, Mohamed LA, Alqahtani S, Abuasal BS, Hill RA & Kaddoumi A (2016) Transporters as Drug Targets in Neurological Diseases. *Clin Pharmacol Ther* 100: 441–453

Quanz M, Bender E, Kopitz C, Grünewald S, Schlicker A, Schwede W, Eheim A, Toschi L, Neuhaus R, Richter C, *et al* (2018) Preclinical efficacy of the novel monocarboxylate transporter 1 inhibitor BAY-8002 and associated markers of resistance. *Mol Cancer Ther* 17: 2285–2296

Rabinowitz JD & Enerbäck S (2020) Lactate: the ugly duckling of energy metabolism. *Nat Metab* 2: 566–571

Rebsamen M, Pochini L, Stasyk T, De Araújo MEG, Galluccio M, Kandasamy RK, Snijder B, Fauster A, Rudashevskaya EL, Bruckner M, *et al* (2015) SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature* 519: 477–481

Reddy A, Bozi LHM, Yaghi OK, Mills EL, Xiao H, Nicholson HE, Paschini M, Paulo JA, Garrity R, Laznik-Bogoslavski D, *et al* (2020) pH-Gated Succinate Secretion Regulates Muscle Remodeling in Response to Exercise. *Cell* 183: 62-75.e17

Remerand G, Boespflug-Tanguy O, Tonduti D, Touraine R, Rodriguez D, Curie A, Perreton N, Des Portes V, Sarret C & RMLX/AHDS Study Group (2019) Expanding the phenotypic spectrum of Allan-Herndon-Dudley syndrome in patients with SLC16A2 mutations. *Dev Med Child Neurol* 61: 1439–1447

Ren T, Jones RS & Morris ME (2022) Untargeted metabolomics identifies the potential role of monocarboxylate transporter 6 (MCT6/SLC16A5) in lipid and amino acid metabolism pathways. *Pharmacol Res Perspect* 10: e00944

Renner K, Bruss C, Schnell A, Koehl G, Becker HM, Fante M, Menevse AN, Kauer N, Blazquez R, Hacker L, *et al* (2019) Restricting Glycolysis Preserves T Cell Effector Functions and

Augments Checkpoint Therapy. *Cell Rep* 29: 135-150.e9

Ritterhoff J & Tian R (2023) Metabolic mechanisms in physiological and pathological cardiac hypertrophy: new paradigms and challenges. *Nat Rev Cardiol*

Rusu V, Hoch E, Mercader JM, Gymrek M, von Grotthuss M, Fontanillas P, Spooner A, Altshuler DM, Florez JC, Jacobs SBR, *et al* (2017) Type 2 Diabetes Variants Disrupt Function of SLC16A11 through Two Distinct Mechanisms. *Cell* 170: 199-212.e20

Sasaki S, Futagi Y, Ideno M, Kobayashi M, Narumi K, Furugen A & Iseki K (2016) Effect of diclofenac on SLC16A3/MCT4 by the Caco-2 cell line. *Drug Metab Pharmacokinet* 31: 218–223

Schaller L & Lauschke VM (2019) The genetic landscape of the human solute carrier (SLC) transporter superfamily. *Hum Genet* 138: 1359–1377

Schlessinger A, Zatorski N, Hutchinson K & Colas C (2023) Targeting SLC transporters: small molecules as modulators and therapeutic opportunities. *Trends Biochem Sci*: 1–14

Schumann T, König J, Henke C, Willmes DM, Bornstein SR, Jordan J, Fromm MF & Birkenfeld AL (2020) Solute carrier transporters as potential targets for the treatment of metabolic disease. *Pharmacol Rev* 72: 343–379

Schumann T, König J, von Loeffelholz C, Vatner DF, Zhang D, Perry RJ, Bernier M, Chami J, Henke C, Kurzbach A, *et al* (2021) Deletion of the diabetes candidate gene Slc16a13 in mice attenuates diet-induced ectopic lipid accumulation and insulin resistance. *Commun Biol* 4: 826

Sedlyarov V, Eichner R, Girardi E, Essletzbichler P, Goldmann U, Nunes-Hasler P, Srndic I, Moskovskich A, Heinz LX, Kartnig F, *et al* (2018) The Bicarbonate Transporter SLC4A7 Plays a Key Role in Macrophage Phagosome Acidification. *Cell Host Microbe* 23: 766-774.e5

Siebeneicher H, Cleve A, Rehwinkel H, Neuhaus R, Heisler I, Müller T, Bauser M & Buchmann B (2016) Identification and Optimization of the First Highly Selective GLUT1 Inhibitor BAY-876. *ChemMedChem* 11: 2261–2271

Staud F, Cerveny L, Ahmadimoghaddam D & Ceckova M (2013) Multidrug and toxin extrusion proteins (MATE/SLC47); role in pharmacokinetics. *Int J Biochem Cell Biol* 45: 2007–11

Suhre K, Shin S-Y, Petersen A-K, Mohney RP, Meredith D, Wägele B, Altmaier E, CARDIoGRAM, Deloukas P, Erdmann J, *et al* (2011) Human metabolic individuality in biomedical and pharmaceutical research. *Nature* 477: 54–60

Superti-Furga G, Crews CM & Cochran J (2017) Where is the Future of Drug Discovery for Cancer? *Cell* 168: 564–565

Superti-Furga G, Lackner D, Wiedmer T, Ingles-Prieto A, Barbosa B, Girardi E, Goldmann U, Gürtl B, Klavins K, Klimek C, *et al* (2020) The RESOLUTE consortium: unlocking SLC transporters for drug discovery. *Nat Rev Drug Discov* 19: 429–430

Takahashi M, Kishimoto H, Shirasaka Y & Inoue K (2020) Functional characterization of monocarboxylate transporter 12 (SLC16A12/MCT12) as a facilitative creatine transporter. *Drug Metab Pharmacokinet* 35: 281–287

Tasdogan A, Faubert B, Ramesh V, Ubellacker JM, Shen B, Solmonson A, Murphy MM, Gu Z, Gu W, Martin M, *et al* (2020) Metabolic heterogeneity confers differences in melanoma metastatic potential. *Nature* 577: 115–120

Todenhofer T, Seiler R, Stewart C, Moskalev I, Gao J, Ladhar S, Kamjabi A, Nakouzi N Al, Hayashi T, Choi S, *et al* (2018) Selective inhibition of the lactate transporter MCT4 reduces growth of invasive bladder cancer. *Mol Cancer Ther* 17: 2746–2755

Tredup C, Ndreshkjana B, Schneider NS, Tjaden A, Kemas AM, Youhanna S, Lauschke VM, Berger B-T, Krämer A, Berger LM, *et al* (2023) Deep Annotation of Donated Chemical Probes (DCP) in Organotypic Human Liver Cultures and Patient-Derived Organoids from Tumor and Normal Colorectum. *ACS Chem Biol* 18: 822–836

Tunyasuvunakool K, Adler J, Wu Z, Green T, Zielinski M, Žídek A, Bridgland A, Cowie A, Meyer C, Laydon A, *et al* (2021) Highly accurate protein structure prediction for the human proteome. *Nature* 596: 590–596

Ullah MS, Davies AJ & Halestrap AP (2006) The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. *J Biol Chem* 281: 9030–7

Walter MW (2005) Monoamine reuptake inhibitors: highlights of recent research developments. *Drug Dev Res* 65: 97–118

Wang K, Huang W, Chen R, Lin P, Zhang T, Ni YF, Li H, Wu J, Sun XX, Geng JJ, *et al* (2021a)

Di-methylation of CD147-K234 Promotes the Progression of NSCLC by Enhancing Lactate Export. *Cell Metab* 33: 160-173.e6

Wang N, Jiang X, Zhang S, Zhu A, Yuan Y, Xu H, Lei J & Yan C (2021b) Structural basis of human monocarboxylate transporter 1 inhibition by anti-cancer drug candidates. *Cell* 184: 370-383.e13

Wang S, Tsun ZY, Wolfson RL, Shen K, Wyant GA, Plovanich ME, Yuan ED, Jones TD, Chantranupong L, Comb W, *et al* (2015) Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science (80-)* 347: 188–194

Wang W, Gallo L, Jadhav A, Hawkins R & Parker CG (2019a) The Druggability of Solute Carriers. *J Med Chem*: acs.jmedchem.9b01237

Wang Y, Dix MM, Bianco G, Remsberg JR, Lee H-Y, Kalocsay M, Gygi SP, Forli S, Vite G, Lawrence RM, *et al* (2019b) Expedited mapping of the ligandable proteome using fully functionalized enantiomeric probe pairs. *Nat Chem* 11: 1113–1123

Wang Z-H, Peng W-B, Zhang P, Yang X-P & Zhou Q (2021c) Lactate in the tumour microenvironment: From immune modulation to therapy. *EBioMedicine* 73: 103627

White JR (2010) Apple Trees to Sodium Glucose Co-Transporter Inhibitors: A Review of SGLT2 Inhibition. *Clin Diabetes* 28: 5–10

Williams Amy AL, Jacobs Suzanne SBR, Moreno-Macías H, Huerta-Chagoya A, Churchhouse C, Márquez-Luna C, Gómez-Vázquez MJ, Burtt Noël NP, Aguilar-Salinas CA, González-Villalpando C, *et al* (2014) Sequence variants in SLC16A11 are a common risk factor for type 2 diabetes in Mexico. *Nature* 506: 97–101

Winter GE, Radic B, Mayor-Ruiz C, Blomen VA, Trefzer C, Kandasamy RK, Huber KVM, Gridling M, Chen D, Klampfl T, *et al* (2014) The solute carrier SLC35F2 enables YM155-mediated DNA damage toxicity. *Nat Chem Biol* 10: 768–773

Xin Y & Zhang Y (2023) Paralog-based synthetic lethality: rationales and applications. *Front Oncol* 13: 1–12

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, *et al* (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 2012: 1–28

Yang Z, Yan C, Ma J, Peng P, Ren X, Cai S, Shen X, Wu Y, Zhang S, Wang X, *et al* (2023) Lactylome analysis suggests lactylation-dependent mechanisms of metabolic adaptation in hepatocellular carcinoma. *Nat Metab* 5: 61–79

Ye AY, Liu Q-R, Li C-Y, Zhao M & Qu H (2014) Human transporter database: comprehensive knowledge and discovery tools in the human transporter genes. *PLoS One* 9: e88883

Yu C, Mannan AM, Yvone GM, Ross KN, Zhang Y-L, Marton MA, Taylor BR, Crenshaw A, Gould JZ, Tamayo P, *et al* (2016) High-throughput identification of genotype-specific cancer vulnerabilities in mixtures of barcoded tumor cell lines. *Nat Biotechnol* 34: 419–23

Zhang B, Jin Q, Xu L, Li N, Meng Y, Chang S, Zheng X, Wang J, Chen Y, Neculai D, *et al* (2020a) Cooperative transport mechanism of human monocarboxylate transporter 2. *Nat Commun* 11: 1–10

Zhang, Chung & Oldenburg (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* 4: 67–73

Zhang D, Tang Z, Huang H, Zhou G, Cui C, Weng Y, Liu W, Kim S, Lee S, Perez-Neut M, *et al* (2019a) Metabolic regulation of gene expression by histone lactylation. *Nature* 574: 575–580

Zhang Y, Taufalele P V., Cochran JD, Robillard-Frayne I, Marx JM, Soto J, Rauckhorst AJ, Tayyari F, Pewa AD, Gray LR, *et al* (2020b) Mitochondrial pyruvate carriers are required for myocardial stress adaptation. *Nat Metab* 2: 1248–1264

Zhang Y, Zhang Y, Sun K, Meng Z & Chen L (2019b) The SLC transporter in nutrient and metabolic sensing, regulation, and drug development. *J Mol Cell Biol* 11: 1–13

Zhao Y, Feng Z, Zhang Y, Sun Y, Chen Y, Liu X, Li S, Zhou T, Chen L, Wei Y, *et al* (2019) Gain-of-Function Mutations of SLC16A11 Contribute to the Pathogenesis of Type 2 Diabetes. *Cell Rep* 26: 884-892.e4

Zhao Y, Li M, Yao X, Fei Y, Lin Z, Li Z, Cai K, Zhao Y & Luo Z (2020) HCAR1/MCT1 Regulates Tumor Ferroptosis through the Lactate-Mediated AMPK-SCD1 Activity and Its Therapeutic Implications. *Cell Rep* 33: 108487

Curriculum Vitae

Name:	Vojtěch Dvořák
Date of birth:	November 29, 1993
Nationality:	Czech
Email:	vojtech.d.dvorak@gmail.com
ORCID:	0000-0002-9496-370X

Education

2018 - present	**PhD**, Medical University, Vienna, Austria Molecular signal transduction
2016 – 2018	**Mgr (MSc)**, Masaryk University Brno, Czech Republic Experimental biology - Animal physiology and immunology
2013 – 2016	**Bc (BSc)**, Masaryk University Brno, Czech Republic Experimental biology – Animal physiology and immunology

Research and work experience

2018 – present	**CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences**, Vienna, Austria Giulio Superti-Furga laboratory PhD thesis: "*Chemical targeting of the membrane transporter for lactate SLC16A3"*
2015 – 2018	**Institute of Biophysics of the Czech Academy of Sciences, Department of Cytokinetics**, Brno, Czech Republic Karel Souček laboratory Diploma thesis: *"Plasticity and heterogeneity of circulating tumour cells"* Bachelor thesis: *"Circulating tumour cells as a window to biology of metastasis"*
2014, fall	**Well Consulting**, Brno Czech Republic

Conferences and Meetings

EACR Cancer Metabolism, 11 – 13 October 2022, Bilbao, Spain

SLC13A5 International Research Roundtable 2022, Oral presentation, *7 – 8 September 2022, ISTA, Austria*

EMBO Workshop: Membrane transporters as essential elements of cellular function and homeostasis, Oral presentation, *23 – 27 August 2022, Chania, Greece*

EMBO Virtual Workshop: Lactate: Unconventional roles of a nutrient along tumor landscape, Poster presentation, *21 – 23 June 2021, Online*

16th YSA PhD Symposium, Poster presentation, *17 – 18 June 2021, Online*

6th Prague-Weizmann Summer School Advances in Drug Discovery, Poster presentation, *2 – 6 September 2019, Prague, Czech Republic*

EACR-FEBS Advanced Lecture Course: Molecular Mechanisms in Signal Transduction and Cancer, Poster presentation, *16 – 24 August 2019, Spetses, Greece*

11th BioMedical Transporters Conference, Poster presentation, *4 – 8 August 2019, Lucerne, Switzerland*

15th YSA PhD Symposium, Poster presentation, *13 – 14 June 2019, Vienna, Austria*

International Conference Analytical Cytometry IX, Poster presentation, *14 – 17 October 2017, Prague, Czech Republic*

List of publications

Structural and functional annotation of solute carrier transporters: implications for drug discovery, V. Dvorak and G. Superti-Furga, *Expert Opinion on Drug Discovery (2023)*, doi: 10.1080/17460441.2023.2244760

Paralog-dependent isogenic cell assay generates highly selective SLC16A3 inhibitors, V. Dvorak, A. Casiraghi, C. Colas, A. Koren, T. Tomek, F. Offensperger, A. Rukavina, G. Tin, E. Hahn, S. Dobner, F. Frommelt, A. Boeszoermenyi, V. Bernada, J. T. Hannich, G. F. Ecker, G. E. Winter, S. Kubicek and G. Superti-Furga, *Cell Chemical Biology (2023)*, doi: 10.1016/j.chembiol.2023.06.029

An overview of cell-based assay platforms for the solute carrier family of transporters, V. Dvorak, T. Wiedmer, A. Ingles-Prieto, P. Altermatt, H. Batoulis, F. Bärenz, E. Bender, D. Digles, F. Dürrenberger, L. H. Heitman, A. P. IJzerman, D. B. Kell, S. Kickinger, D. Körzö, P. Leippe, T. Licher, V. Manolova, R. Rizzetto, F. Sassone, L. Scarabottolo, A. Schlessinger, V. Schneider, H. J. Sijben, A.-L. Steck, H. Sundström, S. Tremolada, M. Wilhelm, M. W. Muelas, D. Zindel, C. M. Steppan and G. Superti-Furga, *Frontiers in Pharmacology (2021)*, doi: 10.3389/fphar.2021.722889

Targeted degradation of SLC transporters reveals amenability of multi-pass transmembrane proteins to ligand-induced proteolysis, A. Bensimon, M. D. Pizzagalli, F. Kartnig, V. Dvorak, P. Essletzbichler, G. E. Winter and G. Superti-Furga, *Cell Chemical Biology (2020)*, doi: 10.1016/j.chembiol.2020.04.003