MEDICAL UNIVERSITY OF VIENNA

Integrative Logic of Combination Therapy and Drug Resistances in Cancer

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

Georg Winter

Supervisor:
Giulio Superti-Furga, PhD
CeMM, Research Center for Molecular Medicine
Lazarettgasse 14/ AKH BT 25.3 1090 Wien/AUSTRIA

Vienna, April 2013

Acknowledgements

In many perspectives, the time that I could spend in the laboratory of Giulio Superti-Furga and in general at the Center for Molecular Medicine was unique. To be able to interact and work in such a stimulating, creative and motivated environment was and is a privileged situation that I am very grateful for. In professional terms, this was the biggest adventure that I have undertaken so far and there are too many colleagues and friends to be mentioned here explicitly. However, there are people that were simply outstanding in the way they influenced my personal as well as professional development in these last almost five years.

Particularly, I want to mention my PhD supervisor Prof. Giulio Superti-Furga. His constant support, trust and the freedom he provided me with in order to be able to develop my personality as well as my scientific skills make him the biggest mentor I ever had outside of my private life. I am very grateful for having had the chance to meet Giulio and to have worked under his supervision. I always had and still have the impression that I can learn something new from him almost every day and the way how Giulio thinks about his profession have made me the scientist that I am today or at least would like to be one day. Thank you very much for everything you have done for me, I really hope that we will stay in touch throughout the next steps of my life. Whoever will follow you will have a hard time in keeping up the high standards that I got used to.

I would furthermore like to express my highest appreciations and a very special gratitude to Dr. Uwe Rix who has been working as a Postdoctoral Fellow in the lab of Giulio Superti-Furga until recently. He has taken care of me in a special way and I have learned tremendously from his experience as a scientist. A lot of the positive aspects of my studies would not have been possible without Uwe and in retrospect I think that he truly has been the perfect match for my character. He found a good balance in calming me down when my enthusiasm on new ideas or projects was too high and reinforced my trust and my belief in existing work. For me, Uwe developed from a well respected colleague to a good friend and I miss working with him a lot. Thanks for your mentorship and thanks for being a great friend.

Furthermore I want to express my gratitude and my deepest respect for Dr. Thijn Brummelkamp who has hosted me two times at his lab in Amsterdam and who has been one of the most inspiring and charismatic persons I met in my life as a scientist. For me, Thijn is one of the leading young investigators in Europe and I am very proud of the fact that we have an ongoing scientific exchange.

I also want to thank Florian Grebien, Kilian Huber, Markus Müllner and Stefan Kubicek as well as the entire “chemical proteomics” team for constant input and fruitful scientific discussions and advices. Similarly, I want to thank all members of the Superti-Furga lab for

being such mind- and respectful colleagues and everybody at CeMM for a countless number of favors.

Moreover, I would like to acknowledge my thesis committee members Prof. Veronika Sexl, Prof. Heinrich Kovar and Dr. Sebastian Nijman for their support and advice.

I want to thank my friends for understanding long hours in the lab and for the fun times that often enough served as welcomed distractions.

Finally I want to thank my mother who supported me from day zero on.

Declaration

The work of this thesis was accomplished at several academic institutions with the assistance of different collaborators. In the following, all contributions are listed in detail.

Chapter 3.1 has been published by Winter et al., 2011. The author of this thesis has designed and performed most of the experiments, analyzed and interpreted the data, made the figures and wrote the manuscript. U. Rix performed chemical proteomics experiments and wrote the manuscript. A. Lissat performed animal studies. A. Stukalov performed statistical analysis. M.K. Muellner contributed experimentally to small molecule drug screen. K.L. Bennett and J. Colinge analyzed proteomics data. S.M. Nijman gave experimental advice. S. Kubicek performed small molecule drug screen. H. Kovar gave experimental advice, U. Kontny contributed to the writing of the manuscript and supervised animal experiments, G. Superti-Furga had overall responsibility for the research and contributed to the writing of the manuscript.

Chapter 3.2 has been published by Winter et al., 2012. Detailed information on the contribution of all authors are listed under “Author contributions” on the last page of the manuscript.

Chapter 3.3 is intended for publication. The author of this thesis designed and performed most of the experiments, analyzed and interpreted the data, made the figures and wrote the manuscript. V. Blomen helped performing the haploid genetic screen and conducted statistical analysis. K. Huber and C. Trefzer helped to perform MRM measurements and gave experimental advice. T. Klampfl operated the next generation sequencer (Illumina HiSeq 2000) and helped with next-generation sequencing data handling. D. Chen created circos plot and graphical display of insertion sites. R. Kralovics gave experimental advice. S. Kubicek gave experimental advice. T.R. Brummelkamp codesigned study and gave experimental advice. G. Superti-Furga codesigned and supervised the study and wrote the manuscript.

All chapters of the thesis were written by the author. G. Superti-Furga provided input to the writing of the thesis.

Reprint permission for chapter 3.1 and 3.2 was obtained from Molecular Cancer Therapeutics/ AACR (chapter 3.1) and nature publishing group (chapter 3.2.).

Figure 1, 2 and 4 of this work were reprinted by permission of Elsevier. Figure 3 was reprinted by permission of nature publishing group. Figure 5 was reprinted by permission of RSC publishing.

Table of Contents

Abstract

Cancer is a genetic disease, the complexity of which started to be uncovered only recently by technological revolutions in the field of structural genomics. The resulting issues of functionally dissecting the “cancer genome”, assigning a biological meaning to each of the thousands of mutations that have been identified, is one of the biggest challenges of current cancer biology. However, the increasing information gained by this process opens up the door for a subsequent “pharmacologic revolution” where innovative chemistry can be used to pharmacologically exploit cancer-specific vulnerabilities that are linked to the underlying genomic aberrations. Collectively, this coalesces in the idea of “personalized medicine” where custom-designed therapeutic agents and combinations thereof are prescribed based on knowledge about the genetic wiring of a patient’s cancer molecular network.

Chemical biology is commonly referred to as the science at the interface of chemistry and biology and can participate in improving the understanding of aberrations driving cancer. Different approaches in chemical biology are applied in order to uncover unknown vulnerabilities in cancer subtypes or to understand the molecular mode of action of small molecule agents that are already used successfully in the clinics. Moreover, chemical biology has also been applied in order to dissect mechanisms of resistance to anti-cancer agents and to identify drug combinations that might prevent spontaneous acquisition of resistances.

Chemical genetics as well as chemical proteomics are among the most prominent subclasses of chemical biology and are, in an integrated form, the unifying topic of my thesis. We applied chemical biology centered approaches in order to uncover a novel vulnerability of a pediatric bone cancer, to identify a synergistic combination-treatment option for a drug-resistant form of chronic myeloid leukemia (CML) and finally to uncover a new carrier-mediated resistance mechanism to YM155, an anti-cancer compound currently in clinical trials for lung- cancer as well as other malignancies.

We screened several hundred small molecule kinase inhibitors in cell line models of Ewings sarcoma (ES), a pediatric cancer of the bone that is molecularly characterized by the expression of the oncogenic transcription factor fusion EWS-FLI1. We found that tozasertib, a promiscuous kinase inhibitor, displayed a remarkably high efficacy in killing ES cell line models and also featured selectivity for ES over other pediatric sarcomas. We pursued a chemical proteomics-centered target-deconvolution approach in order to identify protein targets of tozasertib relevant for the observed phenotype. We found that parallel inhibition of both aurora kinases A and B impairs the viability of ES cell lines in a cooperative manner

which we could validate via RNAi. We finally also proved efficacy of tozasertib in an *in-vivo* xenograft model.

In a second chapter of my thesis, we employed a focused drug combination screen in a CML model that featured resistance to the front-line treatment imatinib via a point-mutation in BCR-ABL, the oncogenic fusion kinase that hallmarks CML pathogenesis. We identified an exquisite synergy of the two small molecule kinase inhibitors danusertib and bosutinib in killing this imatinib-resistant cell line. We could show that the synergistic drug interaction was also preserved in primary mouse cells as well as in primary human patient cells. We uncovered the molecular logic underlying this strong synergy by a multi-level approach consisting of chemical proteomics, phosphoproteomics and transcriptomics. We intersected the target-profiles derived by chemical proteomics with alterations in the signaling networks after transient drug-exposure measured by phosphoproteomics and the resulting downstream change in the transcriptome assessed by microarray analysis. By doing so, we could identify a global downregulation of the gene-expression program maintained by the transcription factor c-Myc as point of convergence of the observed synergy. Importantly, both kinase inhibitors participate in the observed synergy via off-target effects that were non-obvious. This approach was, to the best of our knowledge, the first time that chemical- as well as phosphoproteomics were intersected with transcriptomics in order to decipher the molecular basis for a drug synergy.

The last chapter of my thesis is based on a recently developed genetic screening methodology dependent on a near-haploid CML cell line called KBM7. Via an insertional mutagenesis approach using a genetrap-virus, we could identify a novel resistance mechanism to the clinical survivin inhibitor YM155. We found that deletion of the solute carrier SLC35F2 renders KBM7, but also lung cancer cells, insensitive to otherwise toxic YM155 concentrations. We could show that downregulation of SLC35F2 is the major resistance mechanism to YM155 treatment in a genetrap-independent wildtype situation and that deletion of SLC35F2 prevents YM155 to enter the cells. Thus, using chemical genetics, we identified an unknown dependency of an anti-cancer compound on an entry route via a yet uncharacterized solute carrier. This makes SLC35F2 an important pharmaceutic parameter to consider.

Collectively, the present work shed light on unknown vulnerabilities or resistance mechanisms of Ewings sarcoma, chronic myeloid leukemia as well as Lung Cancer via innovative and integrative chemical biology-centered approaches and might thus aid to an improved understanding of those cancers.

Zusammenfassung

Krebs ist eine genetische Erkrankung deren Komplexität erst durch kürzlich errungene, immense Fortschritte in genetischen Analyseverfahren erkannt wurde. Damit einher geht die Problematik, das komplexe “Krebs-Genom” funktionell zu analysieren, sprich den tausenden neu entdeckten Mutationen eine biologische Funktion zu zuordnen. Diese funktionelle Annotierung stellt eine der größten Herausforderungen der modernen Krebsforschung dar. Gleichzeitig ebnet der damit verbundene Informationsgewinn den Weg für eine nachfolgende, “pharmakologische Revolution”. Konzeptionell können mithilfe von innovativen chemischen Ansätzen Therapien gefunden werden, deren Wirkungsweisen auf spezifische Krebs-Anomalien abzielen. Diese Theorie bildet die Basis der personalisierten Medizin, in der maßgeschneiderte Medikamente oder Medikamentenkombinationen auf den jeweiligen Patienten abgestimmt werden.

Chemische Biologie, der Wissenschaftszweig der traditionell an der Schnittfläche von Chemie und Biologie angesiedelt wird, kann einen Beitrag zum verbesserten Verständnis von krebsrelevanten Vorgängen liefern. Verschiedene Ansätze der Chemischen Biologie werden angewendet um bisher unbekannte therapeutische Angriffspunkte in diversen Krebsarten zu identifizieren oder um die molekulare Wirkungsweise von erfolgreich verabreichten Therapeutika besser zu verstehen. Experimentelle Strategien der Chemischen Biologie wurden außerdem erfolgreich angewendet um Resistenzmechanismen gegen Krebstherapeutika auf molekularer Ebene zu verstehen beziehungsweise um Kombinationen von Medikamenten zu identifizieren, die derartigen spontanen Resistenzmechanismen entgegenwirken können.

Zu den wichtigsten Subkategorien der Chemischen Biologie gehören die Chemische Genetik und die Chemische Proteomik die, in integrative Form, das Grundgerüst meiner Arbeit bilden. Im Verlauf dieser Arbeit wurden Studien mit einem chemisch-biologischen Fokus durchgeführt, mit deren Hilfe ein neuer molekularer Angriffspunkt in einem pädiatrischen Knochenkrebs, eine synergistische Medikamentenkombination in einer therapieresistenten Form der Chronisch Myeloischen Leukämie und ein neuer Resistenzmechanismus gegen ein Krebstherapeutikum in klinischen Studien identifiziert wurden.

Wir testeten hunderte Kinase-blocker in verschiedenen zellulären Modellsystemen des Ewings Sarkoms (ES), eines pädiatrischen Knochentumors. Das charakteristische molekulare Erkennungsmerkmal dieser Krebsart ist die Expression des natürlicherweise nicht vorkommenden onkogenen Fusionstranskriptionsfaktors EWS-FLI1. Es wurde gezeigt, dass der unspezifische Kinasen-blocker tozasertib hocheffektiv und hochselektiv das Wachstum von Ewings Sarkom- im Vergleich zu anderen pädiatrischen Krebsmodellen hemmt. Mittels einer chemisch-proteomischen Studie wurden alle Proteinkinasen identifiziert,

die durch tozasertib in ES Zellen inhibiert werden. Daraus wurde via RNA-Interferenz abgeleitet, dass die parallele Hemmung der beiden Aurora Kinasen A und B kooperativ die Viabilität von ES Zellmodellen beeinträchtigt. Schlussendlich wurde im Tierversuch gezeigt, dass tozasertib auch das Wachstum von ES Xenograft Modellen blockiert.

Im zweiten Kapitel meiner Arbeit führten wir fokussierte Kombinationsexperimente durch, um neue Behandlungsstrategien gegen eine therapieresistenten Form der Chronisch Myeloischen Leukämie (CML) zu finden. Es konnte gezeigt werden, dass die beiden Kinase-blocker danusertib und bosutinib stark synergistisch das Wachstum dieses therapieresistenten CML Modells hemmen. Dieser Effekt wurde weiters auch in primären Maus- und Patientenzellen gezeigt. Um ein molekulares Verständnis für diese Synergie zu entwickeln, wurde auf einen systembiologischen Ansatz zurückgegriffen. Dahingehend wurden Ansätze der Chemischen Proteomik, der Phosphoproteomik und der Transkriptomik integrativ mit einander vernetzt. Dadurch konnte gezeigt werden, dass die Medikamentenkombination einen systemweiten negativen Effekt auf das vom Transkriptionsfaktor Myc kontrollierte Genexpressionsmuster hat. Interessanterweise basiert dieser Effekt auf vorher nicht bekannten Nebenwirkungen („Off Target“ Effekten) von danusertib und bosutinib. In diesem ganzheitlichen Ansatz wurden erstmals drei orthogonale systembiologische Ansätze mit einander kombiniert um ein komplexes Verständnis für eine vielversprechende Medikamentenkombination zu entwickeln.

Das letzte Projekt, das Teil meiner Arbeit ist, basiert auf einer kürzlich veröffentlichten genetischen Technologie, welche auf einer annähernd haploiden CML Zelllinie namens KBM7 basiert. Durch retroviraler Insertionsmutagenese kann so in einem Pool an KBM7 Zellen jedes Gen deletiert werden. Mithilfe dieses Ansatzes konnte ein neuer Resistenzmechanismus gegen das klinische Krebstherapeutikum YM155 gefunden werden. Es wurde gezeigt, dass eine Deletion des „Solute Carrier“ Gens SLC35F2 in KBM7 Zellen aber auch in Lungenkrebszellen eine Resistenz gegenüber YM155 hervorruft. Weiters wurde gezeigt, dass auch in nicht retroviral transduzierten Zellen die verminderte Expression von SLC35F2 den hauptsächlichen Resistenzmechanismus gegen YM155 darstellt und dass die Deletion von SLC35F2 einer intrazellulären Akkumulation von YM155 entgegenwirkt. Zusammenfassend wurde eine neue genetische Technologie verwendet um die Abhängigkeit der Krebstherapeutikums YM155 von einem bis dato nicht charakterisierten Transporter zu beschreiben.

Die vorliegende Arbeit liefert neue Erkenntnisse hinsichtlich unbekannter molekularer Schwachstellen und neuer Resistenzmechanismen im Ewings Sarkom, in der Chronisch Myeloischen Leukämie und im Lungenkrebs. Diese Erkenntnisse wurden mit innovativen und integrativen Methoden der Chemischen Biologie gewonnen und können zu einem verbesserten Verständnis dieser Krebsarten beitragen.

1 Introduction

1.1 Biological Systems, Networks and Signal Transduction

"*It has been said: The whole is more than the sum of its parts. It is more correct to say that the whole is something else than the sum of its parts, because summing up is a meaningless procedure, whereas the whole-part relationship is meaningful*." (Koffka, 1935)

The modern view of cells and organisms is that of complex biological systems formed by molecular networks that shift in their wiring depending on perturbations linked to normal physiological challenges or pathology. (Ideker et al., 2001) Conceptually, molecular networks can be distinguished by the type of interactions that they depict. Among others, one can distinguish networks that describe large-scale interactions of proteins, signaling events, gene-regulatory mechanisms, genetic interactions or metabolic processes. The integration, transmission and interpretation of environmental signals are key features of biological systems which are fulfilled by signaling networks. Signaling networks consist of interconnections and links between signaling pathways that are caused by molecular junctions that receive information from multiple inputs. (Walhout et al., 2012) As opposed to individual (signaling) pathways that often delineate a rather linear conversion and transmission of a signal, networks aim at describing the global response of a system under investigation in response to an altered environmental condition or to any kind of perturbation. Due to complex interconnections and various forms of feedback loops, the behavior and properties of entire networks as multi-component units can often not be attributed to the behavior and wiring of individual components and subnetworks thereof. (Walhout et al., 2012) Post-genomic technologies like next generation sequencing and quantitative mass spectrometry allow the investigation of biological questions in a holistic manner which is of absolute necessity to understand complex systems behavior. This also relates to cancer as a paradigmatic pathophysiologic system in which particular molecular programs, such as cell-cycle, apoptosis and energetic homeostasis, are perturbed. (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011)

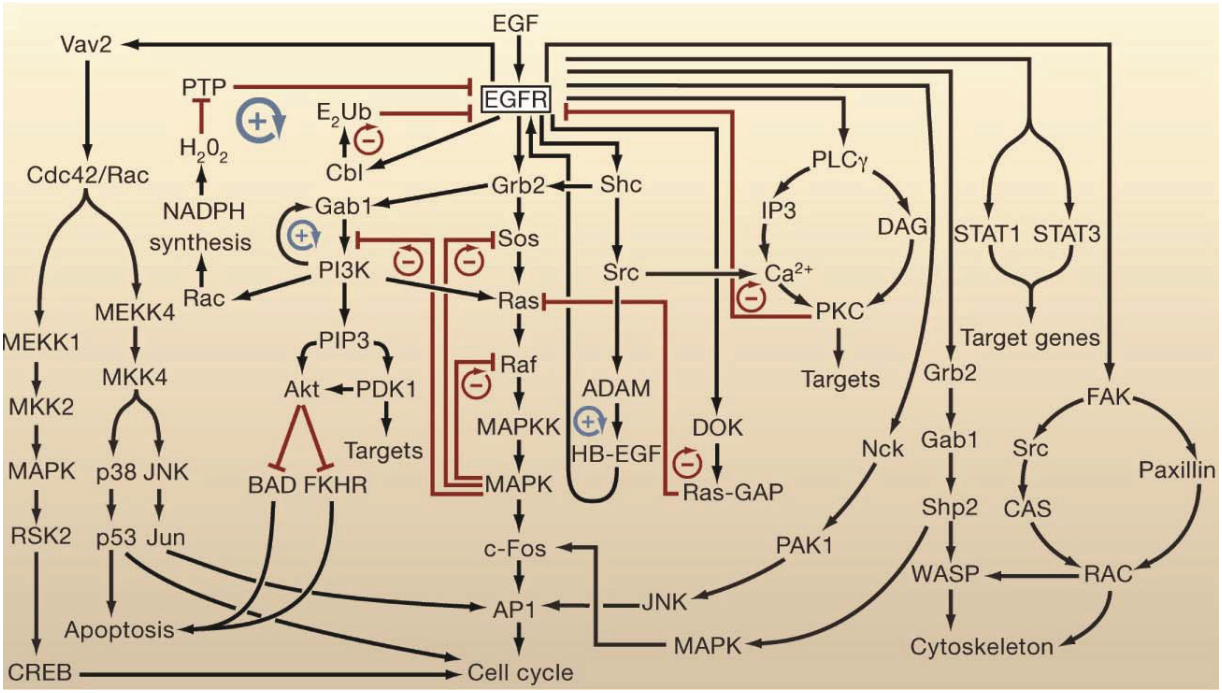


Figure 1 (taken and adapted from Lemmon, M. A. & Schlessinger J. 2010. Cell signaling by receptor tyrosine kinases. Cell, 141(7): 1117-1134). A schematic depiction of the EGF-receptor signaling network outlining the complex cellular responses after binding of EGF.

1.2 Cancer

Recent insights into the genomic complexity of a variety of cancers through large-scale sequencing efforts have shifted the perception of cancer. (Berger et al., 2012; Berger et al., 2011; Beroukhim et al., 2009; Campbell et al., 2010; Chapman et al., 2010; Hammerman et al., 2012; Stephens et al., 2012) Resulting from the ever increasing knowledge about genomic aberrations, the classification- and characterization strategies of cancers, which were primarily based on the affected organ or tissue, are now reconsidered in the light of the molecular and genetic lesions underlying and governing the malignancies. Today, cancer is regarded as a highly variable collection of diseases that are unified by a necessity to fulfill a common set of biological traits. These capabilities are thought to be essential for cancer initiation and progression. They are represented by sustaining proliferative- while downregulating growth suppressive signaling networks, inherent resistance to cell death mechanisms, inducing tumor vascularization by angiogenesis, activating an immortal replication potential and enabling invasive growth and metastasis. (Hanahan and Weinberg, 2000) More recently, reprogramming of energy metabolism, evading immune destruction, inflammation and genome instability have been added to the conceptional framework of the "hallmarks of cancer". (Hanahan and Weinberg, 2011) Moreover, different "stress phenotypes" have been proposed. (Luo et al., 2009b) Those are not necessarily thought to

be causative for tumorigenesis but are common features shared by most human cancers. These phenotypes are, for instance, metabolic stress, proteotoxic stress, mitotic stress, oxidative stress and stress caused by DNA-damage. (Luo et al., 2009b)

Collectively, these hallmarks also serve as conceptional intervention points for cancer therapy. As the underlying genomic alterations are a tumor- and even patient specific combination, a broad variety of therapeutics will be needed to effectively improve therapeutic options in a patient-specific manner.

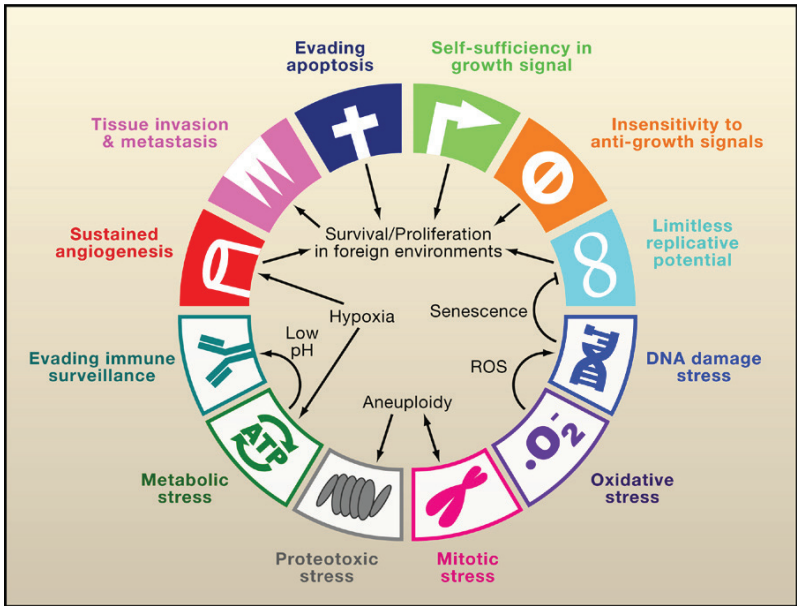


Figure 2:(taken from Luo J and Elledge SJ, Principles of cancer therapy: oncogene and non-oncogene addiction. Cell, 136(5): 823-837) depicts the first generation hallmarks of cancer as proposed by Hanahan and Weinberg supplemented with the cancer-specific intrinsic stress phenotypes.

1.3 Targeting cancer with small molecule drugs

Surgery followed by radiotherapy is for a variety of solid tumors still the chosen therapeutic intervention but, due to the focus of this work, will not be discussed in further detail here. (Delaney et al., 2005)

In terms of small molecule cancer drugs, one commonly distinguishes so called “targeted therapeutics” from classic chemotherapeutics and cytotoxic agents. Both classes take in principle advantage of the phenotypic traits of cancer cells mentioned above. While classic treatment regimens target hallmarks and stress phenotypes in an unselective way, targeted therapeutics are customized to cancer-specific alterations and are thus thought to have a greater window of opportunity and fewer side effects. (Martini et al., 2011) Vinblastine or Cisplatine may serve as examples of compounds commonly referred to as “classical

chemotherapeutics". (Siddik, 2003; Weaver and Cleveland, 2005) While the inhibition of microtubule-assembly (Vinblastine) or the cross linking of DNA (Cisplatine) is in principle toxic to every cell in the body, the selectivity that makes up the therapeutic window is gained by the fact that these compounds exploit the phenotypes of mitotic stress and DNA damage stress inherent to cancer cells. (Luo et al., 2009b) In contrast, most targeted therapeutics are designed to inhibit cancer cell vulnerabilities specifically caused by oncogenes and are thus thought to exploit the concept of "oncogene addiction" which describes a physiologic dependence of cancer cells on a malfunction of a specific gene or set of genes. (Weinstein, 2002) These dependencies are unique to the cancer phenotype and thus usually provide a good window of opportunity for therapeutic intervention. Imatinib (STI-571, Gleevec®) is a paradigmatic example of a targeted therapeutic intervention that exploits oncogene addiction directly by inhibiting the driving oncogene. (Druker, 2008) It is an ATP-competitive small molecule kinase inhibitor targeting the fusion kinase BCR-ABL, the driving oncogene of chronic myeloid leukemia (CML). However, not every targeted agent is designed to directly inhibit an oncogene product. For instance, small molecule agents targeting proteostasis show promising results in preclinical development as well as in the clinics. (Islam and Ambrus, 2008; Soucy et al., 2009) Although these agents are customized to target specific molecular machines like the 26s proteasome, they do not exploit the concept of oncogene addiction but benefit from elevated levels of proteotoxic stress observed in cancers. (Luo et al., 2009b) Other agents that are customized to target cancer-relevant phenotypes but rather exploit non-oncogene addiction are for instance heat shock protein 90 (HSP90) inhibitors like 17-AAG or histone-deacetylases (HDAC) inhibitors like Vorinostat. (Minucci and Pelicci, 2006; Trepel et al., 2010)

Of note, the initial promises and hopes into targeted therapies raised by the success of imatinib in the treatment of CML could only be fulfilled in a subset of cancers. (Druker et al., 2006; Joensuu, 2012) In other malignancies like lung cancer, efforts towards pharmacological inhibition of major oncogenes like the Endothelial Growth Factor Receptor (EGFR) with targeted small molecule kinase inhibitors erlotinib (Tarceva®) or gefitinib (Iressa®) have proven rather disappointing overall. (Cataldo et al., 2011) Targeted inhibitors against mutant B-RAF V600E (vemurafenib, Zelboraf®) or ALK (Crizotinib, Xalkori®) show promising results in patients suffering from melanoma and EML4-ALK-mutant non small cell lung carcinoma (NSCLC) respectively and resulted in market approval for both agents. (Chapman et al., 2011; Kwak et al., 2010) The long-term effects of either of these agents on the respective malignancies will be visible when data on long-term studies with large patient cohorts will become accessible in future.

1.4 Resistances to Targeted Cancer Drugs

The emergence of spontaneous drug resistance is a commonality that is shared by classical chemotherapeutic as well as targeted agents. This is often linked, as illustrated above for efforts targeting mutant EGFR in NSCLC, to moderate effects when new compounds are transferred from preclinical testing to clinical phase studies. (Maemondo et al., 2010) The benefits for patients are usually prolonged progression-free survival but not necessarily improved overall survival rates.

Thus, understanding the repertoire of possible adaptations that a cancerous cell harbors in order to acquire resistances to small molecule therapy is of utter importance for patient stratification, prediction of clinical efficacy of a drug and the development of proper biomarkers to monitor treatment success. Ultimately, the therapeutic goal would be to prevent emergence of drug resistance *a priori* by custom-designing combination treatments (conceptually, the importance of drug combinations is discussed in more detail in 1.5). In recent years, more and more effort has been put into uncovering resistance mechanisms to cancer drugs and the research field of chemical biology, especially its subcategory chemical genetics had a strong influence on many important discoveries that have been made. (Brummelkamp et al., 2006; Gregory et al., 2010; Muellner et al., 2011; Wacker et al., 2012)

Today, our understanding of the emergence of resistance mechanisms allows a first distinction between intrinsic and extrinsic resistance mechanisms. (Lamontanara et al., 2012) Intrinsic mechanisms describe mutations that occur at the level of the protein target of a small molecule. They have been widely observed in the field of targeted drugs, especially with kinase inhibitors where point mutations affecting the ATP binding pocket are among the most common resistance mechanisms for ATP-competitive inhibitors. Most commonly, the “gatekeeper” residue represents a prominent hotspot for resistance conferring mutations where an initially small amino acid residue like threonine is changed to a bulkier residue like isoleucine or methionine. Mutations in the gatekeeper residues have been observed, among others, in BCR-ABL (T315I), EGFR (T790M) and EML4-ALK (L1196M), thus conferring resistance to first-line inhibitors like imatinib, erlotinib and crizotinib. (Branford et al., 2002; Choi et al., 2010; Pao et al., 2005)

Of note, although mutations in the kinase domain are a very prominent resistance mechanism, they only represent a subset of potential point mutations that can affect the efficacy of kinase inhibitors. Detailed studies have been conducted in CML patients on imatinib treatment and have uncovered a broad variety of mutations also in regulatory domains of BCR-ABL. (Sherbenou et al., 2010) Similar observations have also been made with allosteric inhibitors that target the myristate pocket of BCR-ABL. (Zhang et al., 2010)

However, lead optimization and extensive small molecule screening on drug-resistant mutants have led to the discovery of small molecules capable of also inhibiting e.g. gatekeeper-mutant kinases for BCR-ABL, EGFR and ALK and first available clinical data are encouraging. (Cortes et al., 2012; O'Hare et al., 2009; Sakamoto et al., 2011; Zhou et al., 2009) Another intrinsic resistance mechanism is the upregulation of the protein levels of the drugged target itself; a mechanism that has also been seen as a response to pharmacological inhibition of BCR-ABL with imatinib and EGFR with erlotinib. (Gorre et al., 2001; Jimeno et al., 2005) Therapeutically, this resistance mechanism could partially be addressed by simple dose escalations, as it has for instance been shown in CML patients. (Marin et al., 2003)

Extrinsic resistance mechanisms usually describe alterations that cause the activation of downstream- or alternative signaling networks. These inductions of altered signaling events render the cancer cell insensitive to pharmacological inhibition of otherwise essential nodes, usually by turning on additional pro-survival and anti-apoptotic signaling events. Exciting research pursued in recent years has shed light on the mechanisms of extrinsic resistance mechanisms. In line with the focus of this thesis, the primary attention will again be put on extrinsic resistance mechanisms to kinase inhibitors.

Already for several years it has been known that overexpression of the tyrosine kinase LYN, a member of the family of SRC kinases, can lead to resistance to the BCR-ABL inhibitor imatinib. (Donato et al., 2003; Gamas et al., 2009) Therapeutically, LYN overexpression can be managed relatively easily by the use of later generation BCR-ABL inhibitors like dasatinib or bosutinib, both of which are highly potent inhibitors of LYN. (Bantscheff et al., 2007; Remsing Rix et al., 2009; Rix et al., 2007)

Recently, as already mentioned above, chemical genetics centered approaches have successfully been applied to explain a variety of mechanisms of acquired resistance to kinase inhibitors and to explain unexpectedly low efficacies in certain genetic backgrounds. Genetic screens using libraries of cDNA overexpression vectors representing the entire human kinome have been applied in order to dissect a resistance mechanism to pharmacologic inhibition of oncogenic B-RAF harboring the activating V600E mutation. Johannessen et al could show that overexpression of the MAPKK kinase COT1 can reverse the induction of cell death elicited by inhibition of B-RAF V600E. (Johannessen et al., 2010) Similar to melanoma, the B-RAF V600E mutation is also found in colon cancer, although at a significantly lower frequency with 70% of all primary melanomas versus only 10% of colon cancers presenting B-RAF V600E. (Davies et al., 2002; Di Nicolantonio et al., 2008) Nevertheless, already initial treatment success is severely attenuated in colon cancer as compared to melanoma. In a comprehensive study, Prahallad and colleagues used a

kinome-wide RNA interference (RNAi) strategy in order to unravel the resistance to- and the generally low efficacy of the B-RAF V600E inhibitor vemurafenib in colon cancer. They found that genetically silencing of EGFR synergized with vemurafenib inhibition and could furthermore show that this is due to upregulation of the activity of EGFR in response to vemurafenib treatment through a negative feedback loop. This resistance mechanism is specific for colon cancer and thus explains the disappointing results observed clinically. (Prahallad et al., 2012) Importantly, this finding also allowed assessing a follow-up therapeutic strategy of combining vemurafenib with EGFR kinase inhibitors to increase clinical efficacy. The fact that observations made in resistance screens and synergy-studies can be highly complementary is discussed in more detail in chapter 1.5 and 1.6. Another chemical genetics study addressed a resistance mechanism of lung cancers driven by EML4-ALK or mutant EGFR to crizotinib and erlotinib that involves upregulation of TGFβ signaling and consequently MAP kinase pathway activation via the MEDIATOR complex member MED12.(Huang et al., 2012)(Huang et al., 2012)

Conceptually similar observations of acquired resistance to kinase inhibitor treatment have been made with proteomics centered approaches. Important studies published in parallel by three labs have uncovered another resistance mechanism to inhibition of B-RAFV600E in an N-RAS mutant background. It was shown that in that specific genetic environment, vemurafenib treatment caused a dimerisation of C-RAF and (drug-inhibited) B-RAF V600E. This led to “paradoxical” MAP kinase pathway activation thus bypassing the inhibition of B-RAF V600E. (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010) In a similar scope, a recent study conducted by Britschgi et al. has shown the involvement of JAK2-STAT5 signaling in resistance to PI3K/mTOR blockade in breast cancer. (Britschgi et al., 2012) Finally, recent studies also suggest a close involvement of the tumor microenvironment in the resistance to anticancer kinase inhibitors via the secretion of growth factors. Signaling caused by the hepatocyte growth factor (HGF) has for instance been shown to cause resistance to RAF inhibitors. (Straussman et al., 2012; Wilson et al., 2012)

Importantly, not all extrinsic resistance mechanisms are signaling-mediated. Other extrinsic mechanisms can impinge on the pharmacokinetic properties of a compound. Usually, those mechanisms collaborate in downregulating the intracellular concentration of a drug in the tumor cell, thus leading to attenuated efficacy. Predominantly, these resistance mechanisms have been linked to an increased expression or elevated activity of the protein class of ATP binding cassette (ABC-) transporters. ABC transporters mediate an active, ATP dependent, efflux of a wide variety of xenobiotics including drugs across biological membranes. (Szakacs et al., 2006) However, out of the 48 known ABC transporters encoded in the human genome, only a smaller subset, represented predominantly by ABCB1 (P-GP, MDR1),

ABCG2 (BCRP1) and ABCC1 (MRP1), mediate the resistance mechanism called “multi-drug resistance” (MDR). (Allikmets et al., 1998; Dean et al., 2001; Doyle et al., 1998; Miyake et al., 1999) Multi-drug resistance is, as already indicated by the name, a relatively promiscuous resistance phenotype dependent on the chemical properties of a given compound. (Szakacs et al., 2006) ABC-transporter linked drug resistance has been observed for a multitude of different anti-cancer drugs of both the “classical” chemotherapeutics (irinotecan) as well as targeted agents like imatinib. (Ozvegy-Laczka et al., 2005; Sarkadi et al., 2006) However, initial hopes that were put on including MDR inhibitors in combinations of anti-cancer treatments were not confirmed by further clinical data that often showed unpredictable side effects caused by elevated compound plasma levels resulting from ABC transporter inhibition. (Daenen et al., 2004; Kolitz et al., 2004; Wattel et al., 1999)

Pharmacological resistance has also been linked to a decrease in drug uptake achieved by downregulation and inactivating mutations of drug transporters of the SLC (solute carrier family) members. (Dobson and Kell, 2008) SLC22A1 (OCT1) has for instance been shown to be necessary for uptake of a variety of anti-cancer agents, including imatinib. (Minematsu and Giacomini, 2011; White et al., 2006) Another recent study has been published by the laboratory of David Sabatini, identifying SLC16A1 (MCT1) as the major determinant of resistance to 3-bromopyruvate (3-BrPA). (Birsoy et al., 2012)

Finally, my thesis could contribute to this field as we performed a haploid genetic screen in order to find genes essential for the cytotoxicity of the clinical anti-cancer agent YM155. We could show the absolute necessity of the solute carrier family member 35 F2 (SLC35F2) for YM155 induced cell death by mediating its uptake. The study is presented in detail by the manuscript to be submitted in the chapter 3.3.

1.5 Drug combinations – a way to improve efficacy in cancer treatment?

In a systems-biology based perspective, cancer is, just like any other biological system, characterized by redundancy and multi-functionality that cooperatively make up the buffering capacity of a cancer cell and ultimately allow for adaption to therapeutic intervention over time. (Lehar et al., 2008a; Lehar et al., 2008b) These characteristics have been shown to limit single drug use in cancer therapy even for compounds that target cancer specific alterations and thus adhere to the concept of oncogene addiction. (Weinstein, 2002) In comparison to other biological systems, the potential of cancer to adapt to environmental factors like drug treatment is, as discussed in the previous chapter, accelerated by a high mutational rate and genetic flexibility. In some aspects this is comparable to features observed in HIV biology and related anti-viral therapeutic options. (Bock and Lengauer, 2012) Thus, in light of the fact that multi-component therapeutic approaches have revolutionized

the treatment of HIV infection, drug combinations appear a highly attractive option in the treatment of cancer.

However, the notion that higher selective pressure elicited by drug combinations can be beneficial for treatment is not new. As early as in 1939, C.I. Bliss proposed in his manuscript "the toxicity of poisons applied jointly" a conceptional framework for the calculation of expected drug additivity. (Bliss, 1939) Today, this method is still among the most frequently used models and is known as the "Bliss independency model". (Lehar et al., 2008a) Combination treatment also has a long-standing history in clinical cancer therapy and has significantly improved survival rates especially in different form of leukemias. (Dancey and Chen, 2006; Fullmer et al., 2010; Tam and Keating, 2010) Conceptually, the advantages of drug combinations over single drug treatments are many. First, a combination of two or more agents usually allows for reduced dosing of each individual drug and thus most often also leads to reduced adverse effects. (Dancey and Chen, 2006) Secondly, it has been shown that the efficacy of drug combinations is highly context-dependent and therefore usually yields better therapeutically relevant selectivity profiles. (Lehar et al., 2009) Finally, in the perspective of avoiding the acquisition of cancer drug resistance, the evolution of a drug resistant subclone should be hindered when two or more selective agents are applied simultaneously. (Bock and Lengauer, 2012)

Historically, the implementation of combination treatment was based on empirical testing of two agents with known individual activity that differed in their respective mode-of action. Given the ever increasing number of possible anti-cancer agents in preclinical or clinical trials and, connected to that, the rising number of possible combinations thereof, the throughput of empirical testing appears too low as an initial deconvolution step. To circumvent that problem, Borisy et al set out to develop a high throughput approach to test thousands of drug combinations for a given readout in preclinical cell culture models. (Borisy et al., 2003) This approach is based on the generation of two-dimensional dilution matrices for each compound pair which allows a first estimation of drug-cooperativity over a broader dose range (Figure 3). Different mathematical and statistical models as the already mentioned Bliss independence or the also frequently used Loewe additivity and Chou-Talalay method provide means to distinguish basically three different possible outcomes for a drug-drug interaction. (Loewe, 1953) (Chou and Talalay, 1984) The most unwanted interaction in a therapeutic perspective is an antagonistic interaction which is characterized by smaller effect of the drug combination as opposed to both single drug agents at the identical concentrations. An additive effect describes a drug-drug interaction where the net effect of the combination is in the range of the prediction based on Bliss or Lowe or other models like Chou-Talalay. This represents the most frequent interaction, especially when

both drugs do not show pronounced efficacy on their own. (Lehar et al., 2008a) Finally, the most desired outcome of a drug interaction study would be a synergistic interaction which is defined as yielding an effect that exceeds the expectations predicted based on the single drug efficacy. (Lehar et al., 2008a) Whereas for a purely additive interaction, the underlying mechanisms of action of the two single agents are most likely not interconnected, antagonistic as well as synergistic interactions reflect a functional connection of the targeted nodes or pathways.

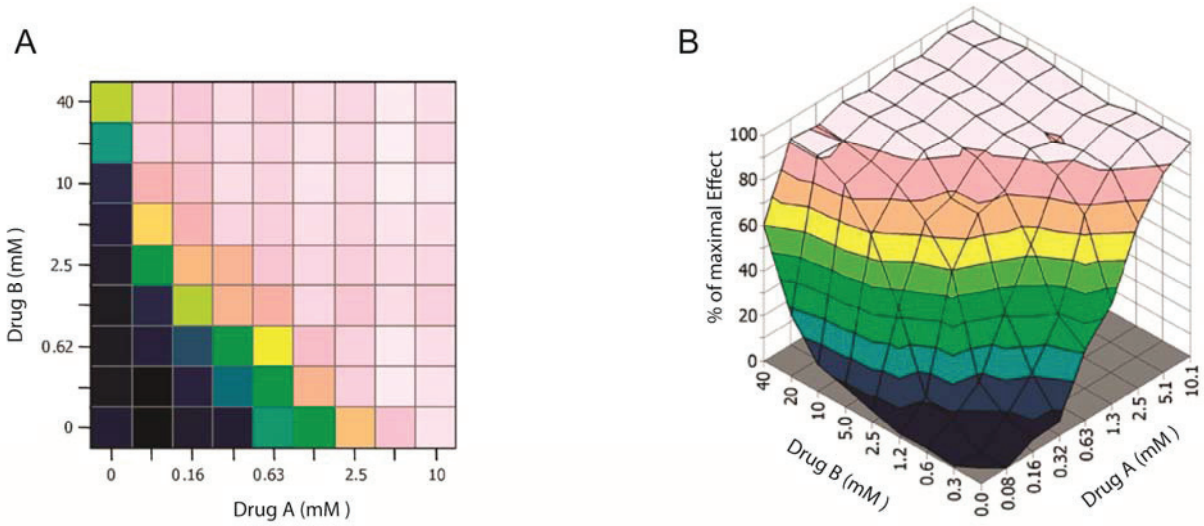


Figure 3: (taken and adapted from Lehar et al, Chemical combination effects predict connectivity in biological systems. Mol Syst Biol 2007, 3, 80.)
(A) Schematic representation of a 9x9 two-dimensional dilution matrix of two drugs (A and B respectively). Notably, the matrix is delimited by the two single drug dose response curves on the x-axis (drug A) and the y-axis (drug B). These values are used for computing the predicted additive value that is then compared to the experimentally derived value to assess for deviations thereof and thus a potentially synergistic drug interaction. **(B)** The matrix can be displayed also in a three-dimensional manner, resulting in a dose-response surface that features the measured values on its z-axis.

1.6 Approaches for the identification of synergistic drug combinations

The strategies to uncover synergistic interactions between anti-cancer drugs are diverse, ranging from experimental to theoretical approaches, but are certainly still biased to hypothesis-driven empirical testing. (Borisy et al., 2003; Cokol et al., 2011; Kalac et al., 2011; Nelander et al., 2008; Yang et al., 2008; Yeh et al., 2006) As already mentioned above, this empirical approach is based on the assessment of the combinatorial potential of two small molecules that are of sufficient single-drug toxicity but feature a diverse mechanism of action. Conceptionally, the likelihood to identify a synergistic drug interaction is comparable with the likelihood of identifying a synergistic interaction of two genetic perturbing agents, a so-called

"epistatic" interaction or "synthetic lethality". Initial considerations regarding the frequency of those epistatic interactions have been derived primarily from studies in yeast that suggest in general a relatively low frequency, thus potentially illustrating the bottleneck of empirical-based studies. (Costanzo et al., 2010; Roguev et al., 2008) Therefore, unbiased screens are a promising alternative as they provide means to interrogate hundreds or even thousands of drug-drug interactions for their synergistic potential in regard to a desired outcome (as for instance induction of apoptosis or, in more general terms, cell death as an initial step in therapeutic cancer applications). Important insights have been gained using drug-drug interaction screens for different cancer indications like imatinib-resistant CML, prostate cancer or multiple myeloma using the previously mentioned approach of Borisy et al. (Borisy et al., 2003; Ketola et al., 2012; Rickles et al., 2010; Winter et al., 2012) These approaches are all based exclusively on the use of small molecule compounds. This comes with certain possibilities but also challenges. For instance, it is not self-understood that the relevant protein target, inhibition of which underlies the observed phenotype, is a known. This is linked to the polypharmacologic features of many small molecules, that have been studied in detail for kinase inhibitors but also other agents like HDAC inhibitors. (Bradner et al., 2011; Knight et al., 2010) These issues are shared by virtually all phenotypic small molecule screens and are therefore, along with the resulting necessity of state-of the art drug target deconvolution approaches, discussed later in more detail (chapter 1.7). The second branch of unbiased screens for the identification of therapeutic combination strategies are chemical genetic approaches. This also illustrates the conceptually close interconnection to functional resistance screens that are discussed in 1.4. The major advantage of chemical genetic screens is the fact that they can be performed in pooled formats due to DNA-barcoding technologies. (Brummelkamp et al., 2006) The experimental setup of genetic drug-sensitivity studies and functional resistance studies usually only differs in the effector concentrations of the small molecule under investigation. Whereas resistance screens are predominantly performed as positive selection screens, synthetic lethal drug screens need to be assayed as negative selection or "drop out" screens which is technically more challenging. Important recent discoveries in the field of genetic drug sensitivity screens have for instance uncovered synthetic-lethal interactions of combined BCL-XL and MEK inhibition in Ras-mutant cancer models or a synthetic lethal interaction between BCR-ABL and the Wnt/$Ca^{2+/}$NFAT signaling pathway. (Corcoran et al., 2012; Gregory et al., 2010) Although chemical genetic screens have uncovered multiple therapeutic combination strategies that could be valuable in future, their direct applicability to cancer therapy is not always straightforward. To begin with, as the implementation of targeting techniques for therapeutic RNAi purposes into clinical practice is highly challenging, the process of genetic silencing via RNAi has to be transposed most often to pharmacological inhibition via a small molecule. (Pecot et al., 2011) This is

connected to two major problems. First, the inhibition of an enzymatic function of a protein or its interaction with other proteins or protein complexes is mechanistically not necessarily comparable with genetic silencing that leads to a general reduction of the concentration of a protein. For example, an inhibited enzyme can still fulfill scaffolding functions that are independent of its catalytic activity. A second, related issue is the notion that, in general, the chemically traceable space is limited to a subset of proteins encoded by those genes that make up the consequently called “druggable genome”. (Makley and Gestwicki, 2012; Russ and Lampel, 2005)

In general, drug-drug as well as drug-gene interaction screens feature technical advantages and disadvantages and harbor experimentally the largest benefit when used in an integrated manner.

1.6.1 Mechanistic subtypes of synergistic drug combinations

The molecular logic of identified synergistic drug-drug interactions can cover a very broad spectrum leading from altering pharmacokinetic properties to the exploitation of essential signaling functions e.g. by co-inhibition of two parallel and redundant pathways. Again, this illustrates the close connectivity to types of drug resistances discussed before. Examples that are representative for various types of synergies that have been observed are discussed briefly below.

First, synergies of two small molecules can converge on a single protein target that is affected by the compounds via an alternating mode of action. This has for instance been shown for combined treatment of CML cell line models with ATP-competitive- and allosteric inhibitors of BCR-ABL. (Zhang et al., 2010) Comparable synergies that converge (functionally) on one protein target have also been observed with combined inhibition of heat shock protein 90 (HSP90) and oncogenic kinases like c-kit in GIST or JAK2 in myeloproliferative neoplasms (MPNs). (Fiskus et al., 2011; George et al., 2004) There, the oncogenic kinase is pharmacologically impaired on two different levels. While the kinase inhibitor of the respective synergy inhibits the enzymatic function, the HSP90 inhibitor attenuates protein maturation and folding and thereby effectively reduces the protein abundance of the oncogenic client protein.

A combination strategy that exploits the cellular dependency on parallel signaling cascades has for instance been addressed for multiple myeloma (MM). MM patients treated with the proteasome inhibitor bortezomib showed promising response rates that resulted in the market approval for MM cases resistant to standard therapy in 2004. (Richardson et al.,

2003) Since 2008, bortezomib is part of the first line treatment of MM in combination with melphalan (a nitrogen mustard alkylating agent) and prednisone (a corticosteroid). (San Miguel et al., 2008) However, there is still a fraction of patients that do not benefit from bortezomib treatment. This has, at least partially, been attributed to an alternative degradation system of unfolded and ubiquitinated proteins termed the aggresome. (Bennett et al., 2005; Hideshima et al., 2005) This alternative degradation system depends on the function of HDAC6 in transporting aggregated proteins to the lysosome. Therefore, pharmacological inhibition of HDAC6 appeared an interesting therapeutic option to counteract alternative protein degradation. In that line, Hideshima and colleagues could show that the HDAC6 inhibitor tubacin synergizes with bortezomib in mediating antitumor activity in MM, leading to efforts in designing more potent and specific HDAC6 inhibitors for improving the treatment of MM. (Hideshima et al., 2005; Santo et al., 2012)

Another strategy of co-targeting redundancies is represented by efforts towards pharmacologic inhibition of members of the anti-apoptotic machinery in order to increase the efficacy of apoptosis-inducing small molecule therapeutic agents. In that regard, members of the BCL-2 family of anti-apoptotic proteins have successfully been targeted with small molecule agents like navitoclax (ABT-263) or obatoclax (GX15-070). (Perez-Galan et al., 2007; Tse et al., 2008) As expected, co-treatment with BCL-2 inhibitors and apoptosis inducing drugs like paclitaxel, bortezomib or a range of HDAC inhibitors resulted in pronounced synergistic induction of cell death. (Perez-Galan et al., 2007; Tan et al., 2011; Wei et al., 2010)

Finally, mechanisms of drug-synergies have also been associated with altered pharmacokinetics. For instance, it has been reported that the combination of imatinib and nilotinib acts beneficially in preclinical models of BCR-ABL positive leukemias. (Weisberg et al., 2007) This was relatively surprising as both agents have a highly similar target profile as assessed by chemical proteomics studies, although nilotinib is known to feature a higher potency for inhibition of BCR-ABL kinase activity. (Rix et al., 2007) However, it could be shown that the observed mechanism was not due to altered signaling properties of BCR-ABL or off-target effects of imatinib and nilotinib but rather due to the fact that imatinib treatment increases the intracellular concentration of nilotinib by inhibiting ABCB1 which mediates cellular efflux of nilotinib. (White et al., 2007) Similar observations have also been made with other kinase inhibitors. For instance, it has been reported that the synergistic potential of the dual EGFR/HER2 inhibitor lapatinib with chemotherapeutic agents is also due to the inhibitory effect of lapatinib on ABCG2 (BCRP) which limited the efflux of the chemotherapeutics. (Brozik et al., 2011; Perry et al., 2010)

Finally, another layer of complexity becomes evident when regarding the timing of the combination treatment. In some instances, “priming” of a cellular system by pre-treatment

with one small molecule of interest prior to the second compound might be beneficial compared to co-incubation of both agents simultaneously. In a recent study, Lee and colleagues observed a striking sensitization of triple negative breast cancer cell systems to drugs inducing genotoxic stress when the cancer cells are pretreated with EGFR inhibitors. Importantly, co-incubation with EGFR inhibitors and genotoxic stress did not result in a detectable drug synergy. Integrative network analysis led to the conclusion that pre-incubation with EGFR-inhibitors rewire the oncogenic state, thus rendering the cells more vulnerable to the induction of DNA damage. (Lee et al., 2012)

In summary, the efficacy of a small molecule drug can be influenced on a variety of different levels of its respective mode of action by another compound. The complexity inherent to the field of drug combinations renders it a highly challenging field of research with the potential to significantly impact therapeutic approaches in cancer in future. Systematic screens to uncover potential entry points for drug combinations in a pre-clinical setting are especially promising but require, like any other phenotypic screen, state-of the art tools for drug-target deconvolution. A review of currently used drug target deconvolution approaches is presented in 1.7.

1.7 Experimental Approaches to identify the cellular targets of small molecule agents

The identification of a protein target of a given small molecule with unknown molecular mechanism of action is a longstanding challenge in the field of chemical biology. The strong interest in a broad spectrum of target-deconvolution approaches stems from essentially two factors. First, high-content- or phenotypic screens became increasingly available and popular in recent years in industry as well as academia. (Young et al., 2008) High content screens allow inferring complex systems for a desired composite response elicited by exposure to small-molecules. The complexity of the readout as well as of the system is highly variable and multifaceted. While more facile readouts measure for instance the impact of small molecule agents on cellular viability and the induction of cell death, more complex readouts can for instance assess protein localization, viral replication or the impact of small molecules on changes in expression levels of a predefined set of genes (“gene expression high throughput screening”, GE-HTS). (Cohen et al., 2008; Huang et al., 2009; Minucci and Pelicci, 2006; Stegmaier et al., 2004) The complexity of the interrogated system is comparable variable. While in the majority of all phenotypic screens, cell line-models are inferred for a desired phenotype, recent advances allow even performing whole-organism screening in a high-throughput fashion, for instance using zebrafish as model organism. (Gut

et al., 2012) However, despite their heterogeneity, all high-content screening approaches share the necessity for a subsequent target deconvolution as the protein target(s) of most small molecule probes of high-throughput libraries are unknown.

The second related reason behind the interest and necessity of target-deconvolution approaches is due to the fact that a considerable proportion of all drugs that are used in the clinics today have been devised without a priori knowledge of their target or are basically used in patients based on an initially non-desired side effect. (Rotella, 2002) Also, serious adverse drug reactions (SADRs) were recently ranked as the fourth leading cause of death in the US. (Giacomini et al., 2007)

Some pharmaceutical agents are also used for multiple indications that are not necessarily functionally related to each other and are thus expected to have multiple underlying protein targets, inhibition of which contributes in a context-specific manner to the observed and desired clinical phenotype. One of the most prominent examples is methotrexate which is a compound featuring clinical efficacy in different human cancers as well as rheumatoid arthritis (RA). While the protein target that is responsible for the anti-cancer clinical efficacy of methotrexate is well established (dihydrofolate reductase, DHFR), a mechanistic understanding of its mode of action in RA remains elusive. (Chan and Cronstein, 2010) The complexity of the molecular logic of phenotypes elicited by small molecules can finally also result from parallel inhibition of two or more proteins simultaneously. These polypharmacologic features are inherent to various small molecule classes like for instance kinase inhibitors and represent an exquisite challenge in terms of deconvoluting their individual contribution. (Knight et al., 2010; Sourbier et al., 2013)

Currently available drug-target deconvolution strategies are predominantly employing either genetic or proteomic methodologies and are presumably most efficiently used when integrated with each other orthogonally. A recent example of integrative deconvolution of a phenotype caused by a combination of two polypharmacologic kinase inhibitors is part of this work and is presented in 3.2.

1.7.1 Genetic/Genomics approaches for target deconvolution of small molecules

Drug-Induced Haplo-Insufficiency Profiling (HIP) / Homozygous Profiling (HOP)

HIP profiling exploits the availability of genome-wide, barcoded deletion strains of *S. cerevisiae*. The assumption underlying HIP profiling in order to unravel the protein target of a small molecule of interest is that gene-dosage reduction of the respective target renders a yeast-cell more vulnerable to subsequent pharmacologic inhibition. In detail, genome-wide

deletion strains allow for assessing growth advantages or –disadvantages of a certain strain in response to an environmental pressure elicited by the compound of interest over time (Figure 4). If heterozygous deletion of a given yeast gene confers a disadvantage in surviving exposure to that drug, the respective strain will be depleted from the initial complex pool of heterozygous deletion clones over time. Given the fact that every yeast strain is "earmarked" by a DNA barcode in its genome, its relative abundance can be assayed using either hybridization based approaches like microarrays or next generation-sequencing based strategies. (Giaever et al., 1999; Smith et al., 2012)

Notably, this approach also features important disadvantages and limitations. First, the measured growth defect of a heterozygous gene deletion in response to drug treatment does not necessarily imply a physical interaction of the underyling geneproduct and the small molecule of interest. Thus, the protein encoded by the identified gene is not necessary the direct drug binding partner. This is a drawback of most if not all drug-target deconvolution approaches stemming from genetic methodologies. Secondly, given the evolutionary distance between yeast and humans, Haplo-Insufficiency Profiling is additionally limited to highly conserved genes and finally also to compounds that impact on cellular fitness. (Smith et al., 2010) An extension to HIP profiling has been established and termed Homozygous Profiling (HOP). The main difference is that HOP profiling relies on complete deletion strains of (non essential) genes in yeast. Thus, more complex chemical-genetics interaction profiles are established that are then compared to and intersected with genetic interaction profiles. (Lee et al., 2005; Parsons et al., 2006) Due to the high similarity to HIP profiling, HOP profiling suffers from the same disadvantages as discussed before.

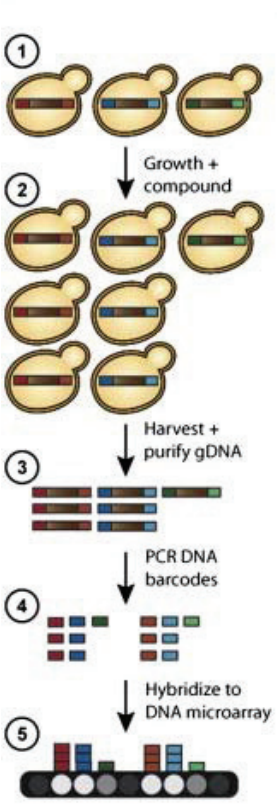


Figure 4 (taken from Smith AM et al, A survey of yeast genomic assays for drug and target discovery, *Pharmacol Ther* 2010, 127, (2), 156-64) displays schematically the workflow of an Haplo-Insufficiency Profiling experiment.

Comparative Gene Expression Profiling

Comparative gene expression profiling stems, like many systems-biology centered approaches, from initial systematic studies performed in yeast. Hughes and colleagues showed that a reference database of expression profiles representing the transcriptional response to 300 different genetic and chemical perturbations is capable of proposing a biological function to bioactive small molecule agents with unknown protein targets and to put largely uncharacterized genes into a novel biological context. (Hughes et al., 2000) Lamb and colleagues have subsequently expanded the logic that robust expression patterns elicited by perturbing agents of known mode of action can guide the classification of unknown agents to human cells. (Lamb et al., 2006) They established a publically available compendium of transcriptional responses of cultured human cell lines to a broad selection of bioactive small molecules. This approach called the “Connectivity Map” or “C-Map” is thus also based on the notion that small molecules impinging on the same cellular signaling network should elicit comparable changes in gene expression. In proof of concept studies, the C-Map has been used to validate two well known chemical agents (celastrol and gedunin) as inhibitors of heat shock protein 90 (HSP90). (Hieronymus et al., 2006) Since then, the Connectivity Map is a widely used tool in order to interpret and compare in an unbiased way the effect that genetic or small molecule- perturbing agents have on the transcriptional output of mammalian cells. Of note, comparative gene expression profiling rather assigns affected pathways and processes to a compound of interest. This is based on the “molecular footprints” it leaves on the transcriptome of a treated cell line. Not necessarily it elucidates the affected member of that pathway.

RNA-interference based Approaches

As already discussed previously, chemical genetics screens have been employed with great success in order to find modulator genes that, either upon stable or transient knockdown or overexpression, can alter the efficacy of a bioactive small molecule of interest in a given cellular system. However, these screens can not only be devised in order to identify novel synthetic lethalities or resistances but can conceptually also aid in target identification approaches. For instance, Jiang and colleagues could generate a shRNA signature that has proven successful in order to functionally classify chemotherapeutic agents of various different classes. (Jiang et al., 2011) In analogy to the previously mentioned HOP profiling, this functional-genetics based approach measures the impact of a collection of stable shRNA-based knockdowns on the cytotoxicity of a query-molecule of interest. It was shown that similar chemotherapeutics cluster together when they are grouped based on the composite impact of eight shRNAs on their respective efficacy. Based on that, this approach

could be used to assign a functional role to chemotherapeutic agents with unknown mechanism of action. However, to which degree this predictive signature can also be extended to non-chemotherapeutic agents has to be seen in future. Moreover, a standardization of that method in order to make it publically available has not yet been achieved. As all genetics-based target-deconvolution approaches, also RNA-interference based methods are not suited to provide evidence for a physical interaction or binding event between the small molecule of interest and the putative target protein. Again, also these assays are in general limited to cytotoxic compounds.

1.7.2 Proteomics approaches for target deconvolution of small molecules

Compound-Centered Chemical Proteomics and Activity Based Protein Profiling

Chemical proteomics is often referred to as a "post-genomic" version of drug-affinity chromatography which is enabled by high resolution mass spectrometry and sophisticated bioinformatics analysis. (Rix and Superti-Furga, 2009) In principle, chemical proteomics can be subdivided into activity based protein profiling (ABPP) and compound-centered chemical proteomics (CCCP). ABPP not necessarily has a focus on drug target deconvolution but is rather used to identify the enzymatically active subset of protein classes in a given cellular state. Among others, ABPP has for instance provided important insights into regulatory mechanisms and activity profiles of serine hydrolases and metalloproteases. (Bachovchin and Cravatt, 2012; Sieber et al., 2006) In brief, this approach is centered around a probe that usually consists of three parts: a reactive group that binds to and labels enzymatically active proteins of interest, a spacer providing flexibility and a reporter tag that is used for detection, enrichment and visualization. (Nomura et al., 2010) Given the focus of my thesis, concepts of ABPP will not be illustrated in further detail.

Compound-centered chemical proteomics (CCCP) is, in terms of target deconvolution, the better suited approach that harbors multiple advantages but also faces certain challenges. The first crucial step in a chemical proteomics experiment is to design and synthesize a version of the given small molecule compound under investigation that allows for immobilization. This is especially tricky for cases where no structure-activity data is available. Adding an otherwise not occurring chemical group which allows for the addition of a linker for immobilization can potentially interfere with the binding affinity to the one or more target(s). Thus, in an optimal scenario, the compound of interest should be immobilized using different chemical strategies. To ensure a retained activity, the coupleable analogue is usually assayed in recombinant (if a cognate target is known) and/or cellular or phenotypic assays (if the cognate target is unknown). Subsequently, after the analogue has been immobilized, the drug-affinity matrix can be incubated with a protein lysate of any desired source. This

illustrates one of the major advantages of chemical proteomics as it allows to probe, in an unbiased and proteome-wide fashion, for proteins in their naturally occurring, post-translationally modified version.(Rix and Superti-Furga, 2009) Dependent on the lysis conditions, chemical proteomics usually retrieves entire target complexes and pre-existing knowledge on protein-protein interaction data can add additional value. In fact, this feature has actively been exploited using HSP90-inhibitor centered chemoproteomic studies to derive novel insights into cancer-specific HSP-client networks. (Moulick et al., 2011) Studies focusing on this "direct" version of CCCP ("direct" as the query compound itself has to be modified) have, among others, elucidated the promiscuous nature of BCR-ABL inhibitors currently used in the clinics for the treatment of CML and GIST, have established Bromodomain containing proteins as a pharmacologically traceable protein class and have identified an axin-stabilizing small molecule (XAV939) as a novel way to pharmacologically antagonize Wnt signaling. (Huang et al., 2009; Nicodeme et al., 2010; Rix et al., 2007) As already mentioned above, the most labor-intensive part of a direct CCCP experiment is the derivatisation of the small molecule compound of interest in order to render it compatible for immobilization. Resulting from that, this direct approach is also not applicable to high- or even medium-throughput approaches. In order to circumvent that problem, Bantscheff and colleagues have immobilized a collection of highly unspecific kinase inhibitors, thus creating a diverse affinity matrix ("*kinobeads*") that allows for capturing a substantial part of the expressed kinome of the cell line or cell state of interest. (Bantscheff et al., 2007) Subsequently, any kinase inhibitor of interest can be profiled for competitive binding to this expressed and captured proteome in a dose-dependent way. In simple terms, proteins that are captured by the "*kinobeads*" are eluted using different concentrations of the kinase inhibitor of interest and the eluted fractions are subsequently investigated via quantitative proteomics. Thus, targets are expected to be competed away from the non-specific affinity matrix in a dose-dependent fashion. This methodology features the big advantage that the query compound does not have to be modified and this conceptual approach has been extended to other protein classes like HDACs and is compatible with medium-throughput application. (Bantscheff et al., 2011) Of note, these advantages also come at the price that the compound of interest is only exposed to a pre-selected subpart of the proteome. Thus, indirect CCCP introduces a bias relatively early on and might therefore rather be suited in order to unravel the promiscuity of a compound of interest with an already known target relative to other members of that target class. However, the concept of competing for binding to a given affinity matrix by spiking in non-modified, free compound has also been applied to direct CCCP experiments. (Ong et al., 2009) In fact, this experimental setup has facilitated the identification of "real" targets as opposed to "sticky" proteins that bind to the affinity matrix in a non-selective way which has been and still is one of the main problems

associated with chemical proteomics. Hence, a protein is only considered as a target of a small molecule of interest if it is not only enriched by an affinity matrix consisting of an immobilized but yet active form of that molecule but also if that binding event can be prevented by co- or pre-incubating the protein lysate with the original, non-modified version of the small molecule. Importantly, these properties would also be fulfilled by a protein that is strongly interacting and thus piggybacking on a protein actually physically binding to the small molecule. Therefore, even if competed away successfully, a protein cannot automatically be assigned as physically interacting with the query compound which has to be confirmed in validation experiments using for instance recombinant assays. As already briefly mentioned above, CCCP has been – in its direct as well as indirect form- further empowered using downstream quantitative proteomics. This has been performed with isobaric labeling (iTRAQ) as well as stable isotope labeling by amino acids (SILAC) and allows estimations of binding constants of the small molecule of interest to the collection of enriched and competed proteins. (Bantscheff et al., 2007; Daub et al., 2008) Both quantitation methods feature inherent advantages and drawbacks but will not be discussed here in further detail. Figure 5 depicts an overview of the approaches and methodologies of that chapter (taken from Target/s Identification Approaches – Experimental Biological Approaches by Giulio Superti-Furga, Kilian Huber and Georg Winter).

Importantly, proteomics-focused approaches for drug target deconvolution have also been extended to measuring the impact of drug treatments on the abundance of relevant post translational modifications. For instance, phosphoproteomics-centered approaches have revealed global alterations in signaling networks after transient exposure to kinase inhibitors. (Pan et al., 2009) Given both, the complexity of signaling networks, understanding of which is far from being complete, as well as the promiscuous nature of most if not all kinase inhibitors, it appears highly challenging to deduce the relevant affected kinase based on the alterations in the phospho-proteome. This holds true especially for cases without *a-priori* knowledge of target kinases. Conceptually, this approach has been not only applied for assessing the impact of kinase inhibitors on phosphorylation events but also to profile alterations of global acetylation in response to treatment with the HDAC inhibitors Vorinostat and MS-275. (Choudhary et al., 2009)

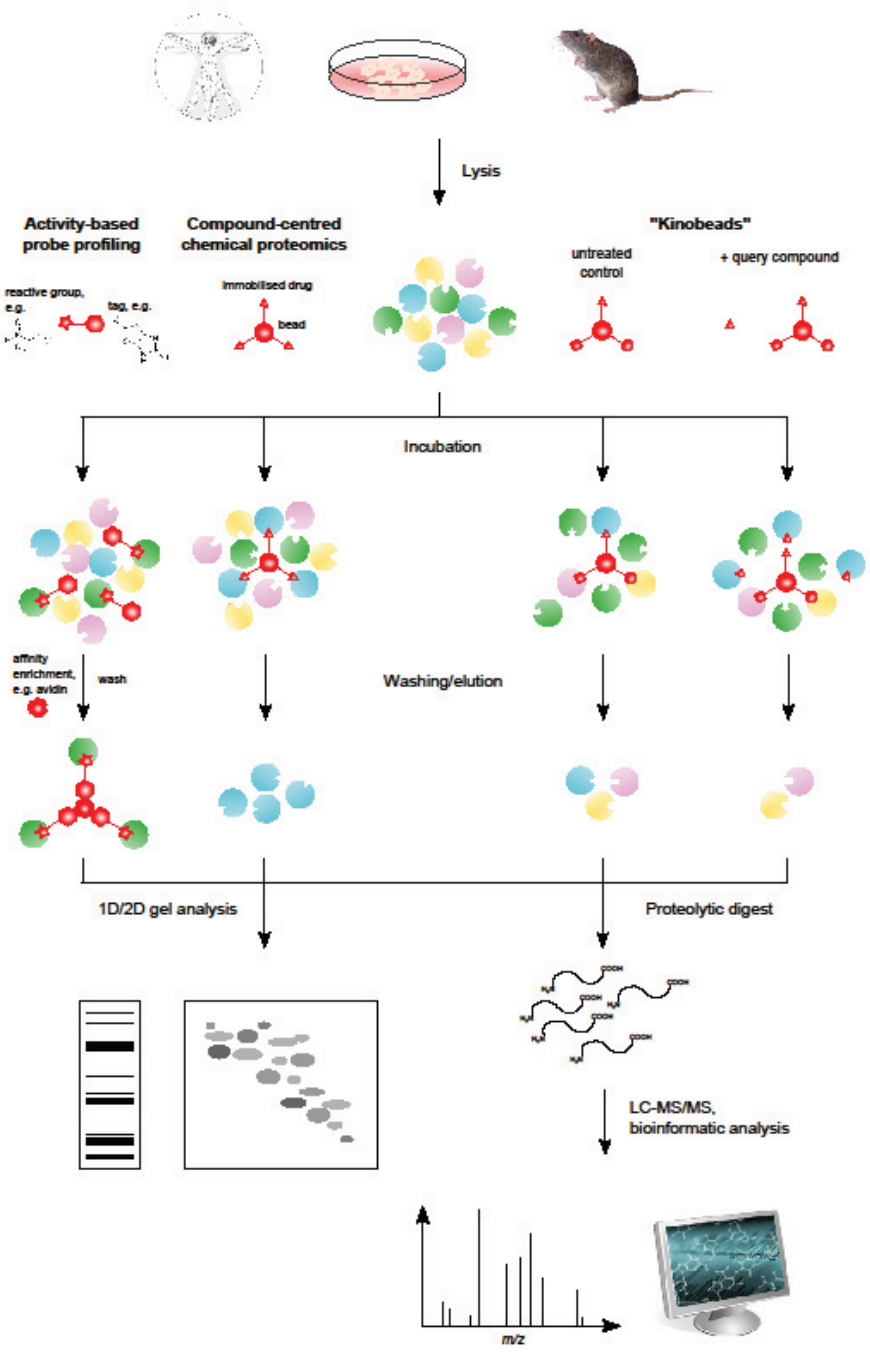


Figure 5 (taken from Superti-Furga, G., K. Huber, and G. Winter, *Designing Multi-Target Drugs*. RSC Drug Discovery, ed. R. Morphy and J. Harris. 2012, Cambridge: Royal Society of Chemistry. 256.) displays the various forms of proteomics approaches outlined in chapter 1.7.2 in a schematic workflow.

1.7.3 Other approaches for target deconvolution

Yeast-Three-Hybrid

The Yeast-three-Hybrid (Y3H) approach stems from the Yeast-two-Hybrid (Y2H) approach that is widely used in order to charter protein-protein interactions. (Chien et al., 1991)

Compared to the traditional Y2H approach, Y3H was modified in order to allow identification of protein binding partners of small molecules. In the Y2H setting, a known protein of interest is expressed as fusion to a DNA binding domain whereas the potentially interacting protein, usually part of a large cDNA expression vector collection, is fused to a transcriptional activator domain. Upon successful interaction of the candidate gene-product with the protein of interest, the DNA binding domain and the transcriptional activator domain are in close proximity, thus resulting in the expression of a marker gene of interest. (Chien et al., 1991) In order to map the interaction of a known small molecule to a candidate binding-protein, a third feature had to be added to the traditional Y2H methodology. Licitra and colleagues extended the approach by introducing well-defined and tight drug-protein interactions like methotrexate/DHFR that can be exploited as chemical anchors for investigating novel interactions. (Licitra and Liu, 1996) In detail, the small molecule under investigation has to be derivatized as a methotrexate fusion, thus linking it to DHFR that itself is, in analogy to the Y2H system, expressed as a DNA binding domain-fusion. Potential binding partners are again expressed as fusions to transcriptional activators. A binding event between the query (fusion-) molecule and a potential binding protein would thus again reconstitute the proximity between the DNA binding and the transcriptional activator domain and result in reporter-gene expression. Importantly, just as for the previously described chemical proteomics setup, the drug derivatization harbors the potential to interfere with the natural binding and activity of the non-modified compound. Therefore, the Y3H approach is also not compatible with higher throughput approaches and might explain the fact that, especially as compared to Y2H, Yeast-Three-Hybrid is a less frequently used approach for target deconvolution. Y3H has for instance been used to identify novel binding partners for cyclin dependent kinases or, more recently to identify that sulfasalazine inhibits tetrahydrobiopterin biosynthesis. (Becker et al., 2004; Chidley et al., 2011)

Small Molecule- and Protein Microarrays

Small Molecule Microarrays (SMM) have been established as a platform to probe, in a non-biased fashion, for binding events between small molecules that are immobilized on chemically reactive glass microscope slides and proteins of interest. (Falsey et al., 2001) One array consists of up to approximately ten thousand different features where each feature is represented by a spotted small molecule. The SMM would then subsequently be incubated with recombinant protein or a cell lysate of interest and a successful interaction between a protein of interest and one of the ten thousand features can be probed via a fluorescence signal stemming either from a labeled protein or from detecting the non-modified protein with a fluorescence-labeled antibody. Of note, this approach is rather suited

to screen for novel small molecule binders to a protein of interest rather than the previous approaches designed to deconvolute unknown binders to drugs of interest.. In that light, SMM have successfully been employed in ligand discovery for proteins like transcription factors that lack an obvious hydrophobic pocket that can be exploited for fashioning a small molecule. (Koehler, 2010) Moreover, SMM have also been used to identify an inhibitor of the challenging target class of extracellular proteins, namely the hedgehog protein ligand. (Stanton et al., 2009) Conceptually, SMM have also been “reversed” by devising Protein Microarrays that feature a library of proteins spotted on an array that are then probed for binding events with fluorescent or radioisotope-labeled derivatives of the small molecules under investigation. (MacBeath and Schreiber, 2000) Conceptually, this is closer to actual drug-deconvolution approaches. However, the approach has not yet been used widely in the scientific community, also due to the difficulty in obtaining purified full-length recombinant proteins for some target classes.

2 Aim of this study

The aim of this thesis was to explore the feasibility of the integration of several innovative post-genomic chemical biology approaches and systems-level evaluations to tackle fundamental problems of pharmacology. The focus has been on combinatorial drug action and on drug resistance. In short, I tried to uncover and explain novel therapeutic entry points for molecularly defined cancers like Ewings sarcoma and chronic myeloid leukemia (CML) using phenotypic drug and drug-combination screens. We also aimed to establish global gene-disruption screens in the haploid cell line KBM7 in order to unravel the genomic requirements of anti-cancer compounds like the clinical survivin inhibitor YM155.

The expected output of employing these single-drug, drug-drug or gene-drug perturbations was an improved understanding of the molecular wiring of Ewings sarcoma (ES). We aimed to discover putative synthetic dependencies on certain kinases created by the presence of the oncogenic transcription factor EWS-FLI11, the molecular lesion characteristic of ES. Moreover, we wanted to derive a systems-level perspective on synergistic drug interactions. Hence, we specifically screened for drug synergies in a CML cell line model that features a molecularly defined resistance mechanism against the CML frontline therapy Imatinib. We wanted to capture a holistic perspective of mechanisms elicited in these drug-resistant cells upon exposure to a novel synergistic drug interaction that we could identify. To do so, we employed a multi-level chemical biology approach focused on chemical proteomics as initial target deconvolution step.

Finally, we wanted to investigate the mode of action of the clinical anti-cancer compound YM155 that is in several phase II clinical trials and has, although being developed as an inhibitor of the anti-apoptotic protein survivin, a rather unclear mechanism of action. We employed a haploid global gene-disruption approach with the intention of elucidating genes that are required for the cytotoxicity of YM155 and might thus also play a role in resistance mechanisms or may functionally explain the clinically observed low response rate.

3 Results and Discussion

3.1 An Integrated Chemical Biology Approach Identifies specific vulnerability of Ewing's sarcoma to Combined Inhibition of Aurora kinases A and B

Winter GE, Rix U, Andrej Lissat, Alexey Stukalov, Markus K. Müllner, Keiryn L. Bennett, Jacques Colinge, Sebastian M. Nijman, Stefan Kubicek, Heinrich Kovar, Udo Kontny, and Giulio Superti-Furga

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

Therapeutic Discovery

Molecular Cancer Therapeutics

An Integrated Chemical Biology Approach Identifies Specific Vulnerability of Ewing's Sarcoma to Combined Inhibition of Aurora Kinases A and B

Georg E. Winter[1], Uwe Rix[1], Andrej Lissat[3], Alexey Stukalov[1], Markus K. Müllner[1], Keiryn L. Bennett[1], Jacques Colinge[1], Sebastian M. Nijman[1], Stefan Kubicek[1], Heinrich Kovar[2], Udo Kontny[3], and Giulio Superti-Furga[1]

Abstract

Ewing's sarcoma is a pediatric cancer of the bone that is characterized by the expression of the chimeric transcription factor EWS-FLI1 that confers a highly malignant phenotype and results from the chromosomal translocation t(11;22)(q24;q12). Poor overall survival and pronounced long-term side effects associated with traditional chemotherapy necessitate the development of novel, targeted, therapeutic strategies. We therefore conducted a focused viability screen with 200 small molecule kinase inhibitors in 2 different Ewing's sarcoma cell lines. This resulted in the identification of several potential molecular intervention points. Most notably, tozasertib (VX-680, MK-0457) displayed unique nanomolar efficacy, which extended to other cell lines, but was specific for Ewing's sarcoma. Furthermore, tozasertib showed strong synergies with the chemotherapeutic drugs etoposide and doxorubicin, the current standard agents for Ewing's sarcoma. To identify the relevant targets underlying the specific vulnerability toward tozasertib, we determined its cellular target profile by chemical proteomics. We identified 20 known and unknown serine/threonine and tyrosine protein kinase targets. Additional target deconvolution and functional validation by RNAi showed simultaneous inhibition of Aurora kinases A and B to be responsible for the observed tozasertib sensitivity, thereby revealing a new mechanism for targeting Ewing's sarcoma. We further corroborated our cellular observations with xenograft mouse models. In summary, the multilayered chemical biology approach presented here identified a specific vulnerability of Ewing's sarcoma to concomitant inhibition of Aurora kinases A and B by tozasertib and danusertib, which has the potential to become a new therapeutic option. *Mol Cancer Ther; 10(10); 1846–56. ©2011 AACR.*

Introduction

Ewing's sarcoma is the second most frequent bone cancer occurring in children and young adults (1). Even though many patients initially respond well to chemotherapy, 40% of these usually develop lethal recurrent disease, and 75% to 80% of patients with metastatic Ewing's sarcoma die within 5 years despite aggressive combinations of chemotherapy, radiation, and surgery (2, 3). In fact, metastasis is one of the most critical problems associated with Ewing's sarcoma, as approximately 15% to 20% of patients have overt metastasis upon diagnosis, and a yet undefined percentage of patients is believed to carry micrometastasis (4).

Thus, there is a profound need for novel therapies. Particularly desirable would be targeted therapies, which lack many of the acute and long-term side effects associated with classical chemotherapy, such as developmental impairment and elevated risk of cancer. At the molecular level, Ewing's sarcoma is defined by a chromosomal translocation, such as t(11;22)(q24;q12), which accounts for approximately 85% of all cases and which results in the translation of the aberrant gene product EWS-FLI1 (5). The causal role of EWS-FLI1 in the pathogenesis of Ewing's sarcoma results from the cooperativity of both fusion partners. Whereas EWS contributes a strong transcriptional activation domain, FLI1 features an ETS-type DNA-binding domain (5–7). Functionally, EWS-FLI1 acts as an aberrant transcription factor capable of deregulating more than 1,000 direct and indirect target genes (8). EWS-FLI1 has been also described to act as a

Authors' Affiliations: [1]Ce-M-M-Research Center for Molecular Medicine of the Austrian Academy of Sciences; [2]Children's Cancer Research Institute, St. Anna Kinderkrebsforschung, Vienna, Austria; and [3]Department of Pediatrics and Adolescent Medicine, Division of Pediatric Hematology and Oncology, University Medical Center, Freiburg, Baden-Württemberg, Germany

Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

G.E. Winter and U. Rix contributed equally to this work.

Corresponding Author: Giulio Superti-Furga, Ce-M-M-Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14, AKH BT 25.3, 1090 Vienna, Austria. Phone: 43-1-40160-70001; Fax: 43-1-40160-970000; E-mail: gsuperti@cemm.oeaw.ac.at

doi: 10.1158/1535-7163.MCT-11-0100

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

transcriptional repressor (9). This specific oncogenic lesion is responsible for the highly malignant phenotype and poor prognosis typical of Ewing's sarcoma. Despite the molecular understanding of the underlying pathology of the disease, development of targeted therapies has proven difficult. Promising steps have been made either by targeting the protein–protein interaction interface of EWS-FLI1 with its complex partner RNA helicase A or by inhibition of IGFR, CD99, or MGST1 (10–13). However, so far none of these approaches has received clinical approval.

Protein kinases have received significant attention over the past decade because many of these play important roles, for example, in cancer and can be readily engaged by small molecules. Being safe and effective against many molecularly defined malignancies, such as chronic myelogenous leukemia (CML; ref. 14), ErbB2-driven breast (15), or epidermal growth factor receptor–dependent lung cancer (16), kinase inhibitors are currently the most successful class of targeted drugs. Notably, some kinase inhibitors have also been combined with traditional chemotherapeutic agents for the treatment of particularly aggressive cancers such as Philadelphia chromosome–positive acute lymphoblastic leukemia (17). Therefore, combination of targeted therapy and chemotherapy could be a viable therapeutic option in Ewing's sarcoma.

Because Ewing's sarcoma features the defined molecular lesion EWS-FLI1, an attractive option is the investigation of possible synthetic lethal relationships (18). Globally, this concept has been exploited through genetic screens in yeast and RNAi screens in human cells (19, 20). Considering proven druggability, particularly kinome-wide RNAi screens are widely pursued (21). Alternatively, the kinome can be perturbed with small molecules. Both approaches display distinct advantages and a certain level of complementarity. However, there is a general difference in removing a protein by RNAi or inhibiting it by a small molecule. Although genomic approaches are not limited by the druggable chemical space, dose dependencies are easier to investigate using small molecules. Furthermore, kinase inhibitors are notoriously promiscuous (22), which offers the advantage of modulating multiple nodes at the same time and therefore provides a higher chance of uncovering complex mechanistic relationships. However, dissecting these relationships requires suitable downstream target identification and deconvolution approaches, such as chemical proteomics (23). Chemical proteomics is a postgenomic version of classical drug affinity chromatography enabled by high-resolution tandem mass spectrometry and downstream bioinformatics analysis, which can identify the cellular target spectra of screening hits (24–26).

Here, we apply such a multilayered approach that combines a chemical biology viability screen with a focused kinase inhibitor library and chemical proteomics–based target identification. Subsequent target deconvolution by RNAi identified parallel inhibition of Aurora kinases as a specific vulnerability of Ewing's sarcoma.

Materials and Methods

Cell culture and reagents

SK-ES-1, SK-N-MC, TC-71, A673, STA-7.2, RD, Rh30, U2OS, K562, and KU812 were obtained from the American Type Culture Collection; ASP14 was a gift of Javier Alonso (Instituto de Investigaciones Biomédicas). Nilotinib, dasatinib, bosutinib, and tozasertib were purchased from LC Laboratories; lapatinib, etoposide, doxorubicin, erlotinib, sorafenib, and sunitinib were purchased from Selleck Chemicals, and bafetinib was synthesized by WuXi AppTec. Furthermore, the customized kinase inhibitor library consisted of sublibraries derived from Tocris, Calbiochem, and Merck. All compounds were dissolved in dimethyl sulfoxide (DMSO) as 10 mmol/L stock solutions.

Western blotting

Western blotting was carried out as described in the manufacturer's manual for antibodies against Aurora kinases A (#3092; Cell Signaling) and B (611038; BD Transduction Laboratories) and actin (#AAN01; Cytoskeleton). Aurora A p-T288 (#3079; Cell Signaling) and pH3S10 (#05-806; Millipore) were detected after 12 hours nocodazole arrest. Drug treatment was carried out for 2 hours in the presence of 20 μmol/L MG-132.

Viability screen

Cells were plated at 40,000 cells/mL (RPMI 1640; 10% fetal calf serum). Drugs were added after 24 hours and incubated for 72 hours. Viability was measured by using the CellTiter-Glo Assay (Promega). Half-maximal effective concentration (EC_{50}) values were calculated by using Spotfire (TIBCO; duplicate analysis). All other viability measurements have been conducted with CellTiter-Glo in triplicate.

Chemical proteomics

Chemical proteomics experiments have been carried out as described previously (27).

Apoptosis and cell-cycle measurements

Cleaved caspase-3 was quantified by intracellular protein analysis, using flow cytometry. A total of 1×10^6 cells were fixed by using paraformaldehyde, washed with PBS, and stored overnight at −20°C in methanol. Antibodies against cleaved caspase-3 (Cell Signaling; #9661) and PE-conjugated goat anti-mouse (Southern Biotech; #4030_09) have been used. Cell-cycle analysis was carried out by staining DNA with propidium iodide after 36 hours exposure to tozasertib.

Synergy determination

Thirty-six–point dose–response matrices have been established as described elsewhere (28).

Knockdown assays

Knockdowns of Aurora kinases have been carried out by ON-TARGETplus Dharmacon SMARTpools in

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

triplicates (10 nmol/L, 24-well plate). Sequences of EWS-FLI1 RT primers were used for evaluation of knockdown efficiency in inducible ASP14 cell line upon request.

In vivo studies

A total of 2×10^6 TC-71 cells were injected into the gastrocnemius muscle of 5- to 7-week-old SCID/bg mice (Charles River). Single primary tumors developed in more than 90% of mice over 2 weeks. Mice were randomized into 3 groups with 11 animals each and intraperitoneally injected with tozasertib (50 or 100 mg/kg) or vehicle twice daily for 6 days. Tozasertib was first dissolved in DMSO at 100 mg/mL and then further diluted in 50% PEG 400 in 50 mmol/L phosphate buffer. Two diameters of the tumor sphere were measured every 2 days. Tumor volumes were approximated by using the formula: $V = (D \times d\,2/6) \times \pi$, where D is the longer and d is the shorter diameter. When tumors reached 2,000 mm^3, experiments were terminated. Mice studies were approved by the state regulatory board.

Statistical considerations

Two-way ANOVA test for the log of tumor growth ratio was used for statistical analysis of the pairwise differences between *in vivo* treatment groups. Tumor growth ratio was the volume of the tumor observed at a given day versus day 1. P values less than 0.05 were regarded as significant. For synergy determination, Bliss additivity was used to predict the combined response C for 2 single agents with their effects A and B ($C = A + B - A \times B$), where each effect is expressed as fractional inhibition between 0 and 1.

Results

Chemical biology screen reveals sensitivity of Ewing's sarcoma cell lines to several small molecule protein kinase inhibitors

To elucidate vulnerabilities of Ewing's sarcoma, we carried out a focused screen by probing 2 different Ewing's sarcoma cell lines (SK-ES-1 and SK-N-MC) with a library of 200 small molecule protein kinase inhibitors. Each compound was tested at 5 different concentrations ranging from 16 nmol/L to 10 μmol/L. Although the majority of compounds showed no or at best moderate effects, a subset of 16 (SK-ES-1) and 20 compounds (SK-N-MC), respectively, showed significant impairment of cellular viability, as indicated by EC_{50} values less than 1 μmol/L (Fig. 1; Supplementary Table S1). Among these were staurosporine and several of its derivates such as midostaurin (*N*-benzoyl-staurosporine) or UCN-01 (7-hydroxystaurosporine). Cumulatively, the known cognate targets of the identified screening hits, such as cyclin-dependent kinases (CDK), casein kinases (CK), and Aurora kinases, are predominantly implicated in cell cycle control. Tozasertib (VX-680, MK-0457) was prominently represented in both cell lines, as it was the most efficacious compound with an EC_{50} of 20 nmol/L in SK-N-MC and the second most efficacious compound with an EC_{50} of 30 nmol/L in SK-ES-1. Tozasertib and also danusertib (PHA-739358), another potent screening hit with EC_{50} values of 47.6 nmol/L (SK-M-MC) and 25.5 nmol/L (SK-ES-1), have initially been developed as Aurora kinase inhibitors but are known to target many other serine/threonine and tyrosine kinases as well (22, 29, 30). For instance, both compounds have been shown to potently inhibit ABL, its CML-relevant counterpart BCR-ABL, and some of the clinically relevant mutants, such as the gatekeeper mutant T315I, and are or have been in clinical trials for this indication (31, 32).

Effects of tozasertib and danusertib are specific for Ewing's sarcoma cells

To extend the initial observation made in SK-ES-1 and SK-N-MC, we determined individual drug effects of tozasertib and danusertib on cellular viability across a broader range of concentrations and Ewing's sarcoma cell lines (A673, TC-71, STA-ET-7.2). The derived EC_{50} values for these cell lines are consistent with previous observations, displaying a window of activity between 8.8 and 55 nmol/L for tozasertib and 22 and 37 nmol/L for danusertib (Fig. 2A; Supplementary Table S2). To further investigate the specificity and relative potency of these drugs for Ewing's sarcoma, we determined their effects on cell lines derived from the pediatric tumors rhabdomyosarcoma (RD, Rh30) and osteosarcoma (U2OS), as well as CML (K562, KU812). This comparison revealed specificity for Ewing's sarcoma cells, as they displayed EC_{50} values that are considerably lower than those in the other cell lines (Fig. 2A).

Finally, we compared the efficacy of tozasertib and danusertib in killing Ewing's sarcoma cell lines with a panel of kinase inhibitors (nilotinib, dasatinib, bosutinib, bafetinib, danusertib, tozasertib, lapatinib, erlotinib, sorafenib, and sunitinib) that are for the largest part already approved by the Food and Drug Administration or in later stages of clinical trials, but, most importantly, have well characterized target spectra across near kinome-wide kinase panels that were established previously (22, 24, 25, 33, 34). In fact, these studies show that collectively these drugs affect approximately 300 kinases, more than 200 of which are significantly inhibited at concentrations that are achievable in patients. We hypothesized that a combined comparison of the target spectra and the cellular efficacy of tozasertib with those well-characterized agents would facilitate subsequent target deconvolution. Tozasertib and danusertib were 60-fold more potent than the next best drug (bosutinib; SKI-606) and more than 100-fold more potent than the remaining drug panel (Fig. 2B). Therefore, we conjectured that the target spectrum of tozasertib must show significant differences to the less effective compounds and that differentially inhibited kinases would be prioritized for validation experiments.

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

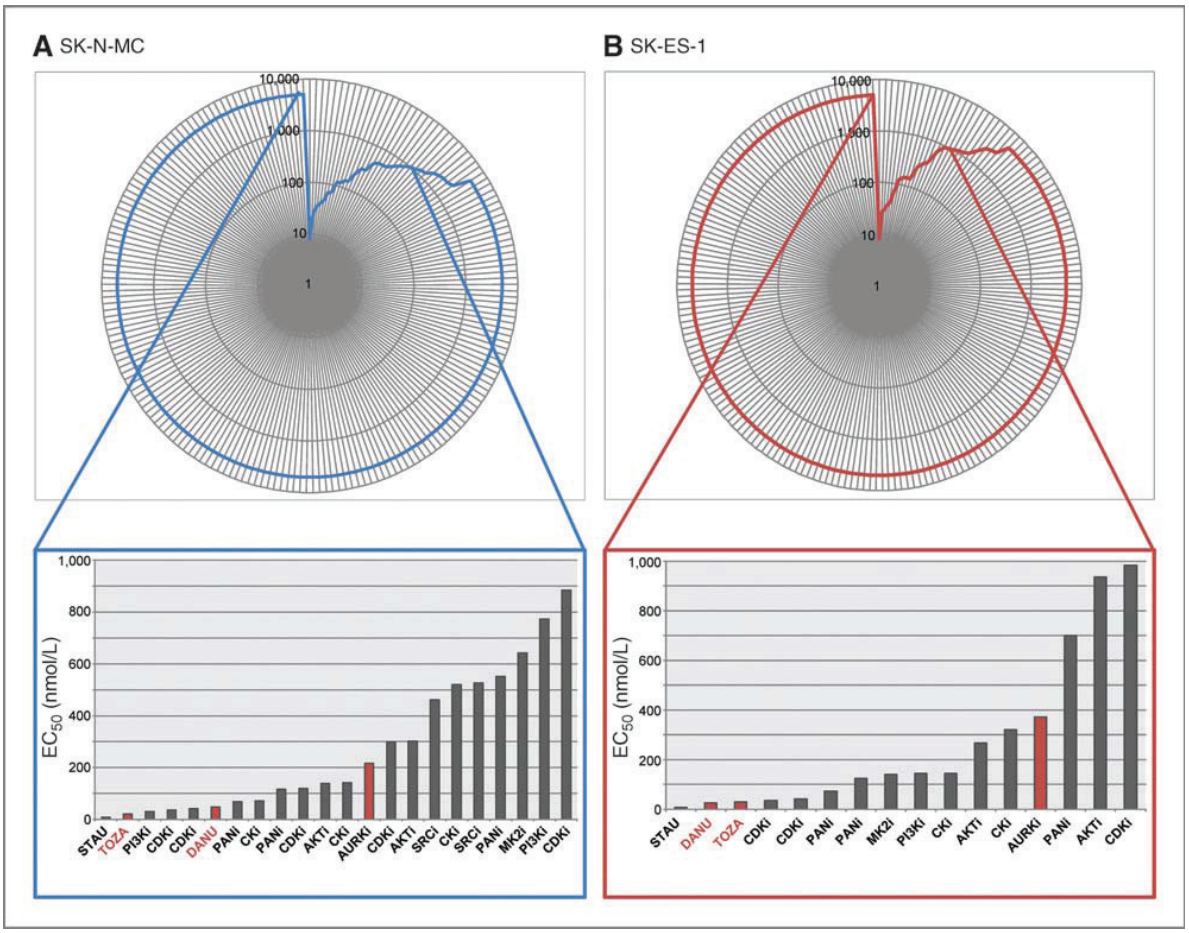


Figure 1. Focused kinase Inhibitor screen reveals sensitivity of Ewing's sarcoma cell lines to small molecule protein kinase inhibitors. A, EC_{50} values for inhibition of cell viability of all 200 protein kinase inhibitors in SK-N-MC cells. Compounds with EC_{50} values less than 1 μmol/L are displayed in the corresponding bar graph. Viability measurements were conducted by the CellTiter-Glo Viability Assay as described in Materials and Methods. B, same as for A, but using SK-ES-1 cells. For full data set see Supplementary Table S1. TOZA, tozasertib; DANU, danusertib; STAU, staurosporine.

Tozasertib interacts with 20 protein kinases in Ewing's sarcoma cells

To elucidate the mechanism of action of tozasertib in Ewing's sarcoma and furthermore compare its target ID with the drug panel mentioned earlier, we pursued a chemical proteomics approach as an initial step of target deconvolution (26). On the basis of the available cocrystal structure information, we designed an analogue of tozasertib (c-tozasertib) which features an *N*-aminobutyl linker (Fig. 3A). This modification allowed for immobilization on NHS-activated sepharose beads and subsequent affinity purification of interacting proteins without affecting kinase binding properties as confirmed by comparison of tozasertib and c-tozasertib for inhibition of ABL kinase activity (Fig. 3B). We identified a total of 20 and 16 kinases binding to c-tozasertib in lysates of the Ewing's sarcoma cell line SK-ES-1 and A673, respectively. Besides already known tozasertib targets such as Aurora kinase A, ABL, SRC, and FGFR1 (22), we also identified new potential targets, such as FAK, MAP2K5, MAP3K2, and TBK1 (Fig. 3C; Supplementary Table S3).

Comparison of the tozasertib target spectrum with the deconvolution panel drugs highlighted Aurora kinases A and B as unique targets, because they were approximately 50-fold more potently inhibited by tozasertib (and danusertib) than by any other drug (Supplementary Table S4). Any remaining target was at least as strongly affected by 1 or more other drugs within the panel and therefore was unlikely to account for the observed selectivity. To further validate the interaction between c-tozasertib and the endogenous Aurora kinases expressed in Ewing's sarcoma cells, we carried out competitive pull-down experiments with SK-ES-1 cell lysates by cotreatment with original tozasertib or DMSO. Interaction of Aurora kinases A and B with c-tozasertib was successfully competed for with tozasertib (Fig. 3D). Taken together, we

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

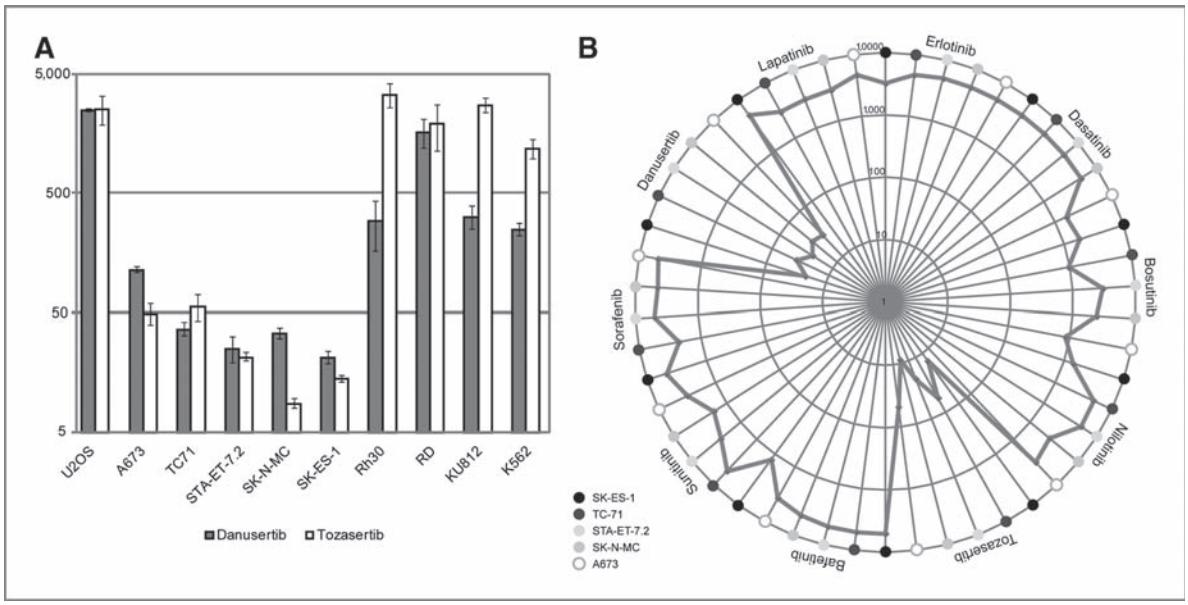


Figure 2. Tozasertib and danusertib selectively and potently impair viability of Ewing's sarcoma cell lines. A, tozasertib and danusertib show selectivity toward Ewing's sarcoma cell lines over other pediatric sarcoma (RD, Rh30, U20S) and leukemic (K562 and KU812) cell lines. B, EC_{50} values of danusertib and tozasertib compared with the drug deconvolution panel in the different Ewing's sarcoma cell lines. EC_{50} determination, based on measurements using the viability assay as described in Materials and Methods. Only tozasertib and danusertib treatments result in EC_{50} values in the low nanomolar range. EC_{50} values that exceeded 5 μmol/L are displayed as 5 μmol/L.

therefore focused on the effects of Aurora kinase inhibition in Ewing's sarcoma cells.

Combined inhibition of Aurora kinases A and B underlies tozasertib effect on Ewing's sarcoma cells

We asked the question, whether inhibition of Aurora kinases A and/or B represents the relevant mechanism of action of tozasertib in the killing of Ewing's sarcoma cells. First, we confirmed expression of Aurora kinases A and B for all Ewing's sarcoma cell lines used in this study via immunoblotting (Supplementary Fig. S1). RNAi knockdown experiments were carried out in SK-ES-1 (and SK-N-MC; Supplementary Fig. S2), as well as in the tozasertib-insensitive rhabdomyosarcoma control cell line RD, using siRNAs against Aurora kinases A and B. We observed similar impairment of viability for both cell lines when knocking down Aurora kinase A, but significantly reduced viability of SK-ES-1 compared with RD upon knockdown of Aurora kinase B (Fig. 4A). Whereas the effect of Aurora kinase B knockdown on viability of SK-ES-1 seems stronger than for Aurora kinase A, simultaneous knockdown of both Aurora kinases resulted in a dramatic reduction of viability in the Ewing's sarcoma cells, but only a mild effect in the control cell line, which cannot be attributed to knockdown of either kinase alone. This suggests a combined mode of action that may underlie the potent effect of the pan-Aurora kinase inhibitors tozasertib and danusertib.

Furthermore, in SK-ES-1 cells, tozasertib completely abrogated phosphorylation of T288 on Aurora kinase A, which is known to correlate with kinase activity, as well as phosphorylation of the validated Aurora kinase B downstream target histone 3 S10 in the mid-nanomolar range. These results validate Aurora kinases A and B as functional targets of tozasertib in Ewing's sarcoma cells and corroborate our results from the chemical proteomics binding assay (Fig. 4B).

Tozasertib treatment induces apoptosis and cell-cycle arrest

We carried out apoptosis measurements via intracellular fluorescence-activated cell sorting analysis of cleavage of caspase-3 as well as flow cytometric analysis of cell cycle with increasing concentrations of tozasertib. In both cell lines, SK-ES-1 and TC-71, tozasertib induced apoptosis as indicated by increasing levels of cells positive for cleaved caspase-3 (Fig. 4C). SK-ES-1 displays higher sensitivity toward tozasertib treatment than TC-71, thus recapitulating our findings of the viability assay. Moreover, flow cytometric cell-cycle analysis revealed tozasertib-induced arrest in G_2–M phase (Supplementary Fig. S3).

Induced knockdown of EWS-FLI1 causes downregulation of Aurora kinases A and B and confers resistance to tozasertib

It has been shown that EWS-FLI1 directly upregulates both Aurora kinases (35) and that transient transfection of EWS-FLI1 results in mislocalization of Aurora kinase B followed by mitotic defects (36). We investigated whether

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

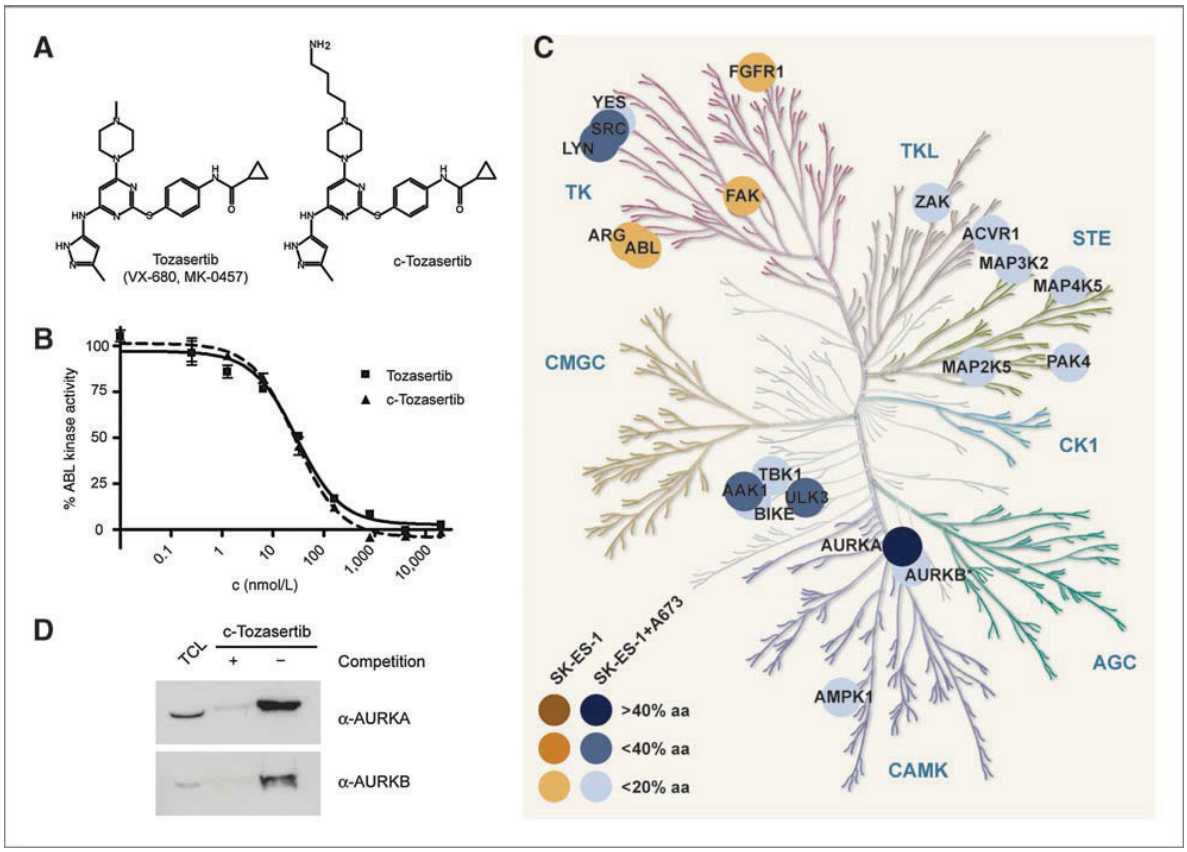


Figure 3. Characterization of the tozasertib target spectrum in Ewing's sarcoma cells (SK-ES-1, A673) by chemical proteomics. A, chemical structures of tozasertib and the coupleable analogue c-tozasertib. B, c-tozasertib (triangles, dashed line) retains c-ABL inhibitory potential compared with tozasertib (squares, solid line) as shown by *in vitro* kinase inhibition assays, which have been carried out as described previously (31). C, target profile of tozasertib in SK-ES-1 and A673 cells. Kinase targets identified in both cell lines are displayed in blue, and those only identified in SK-ES-1 in brown. Color intensities correlate with the observed amino acid sequence coverage of the respective targets thereby providing a semiquantitative surrogate parameter for interaction strength. All kinases that bound to c-tozasertib in A673 cells were also found in SK-ES-1 cells. Aurora kinase B is marked with an asterisk as it is being masked by Aurora kinase A peptides. Complete data are provided in the Supplementary Table S3. D, competitive binding of tozasertib at 10 μmol/L with immobilized c-tozasertib for Aurora kinases A and B in SK-ES-1 cell lysates as indicated by immunoblots.

the specific vulnerability of Ewing's sarcoma for tozasertib and danusertib is a consequence of the functional relationship between EWS-FLI1 and the Aurora kinases. Therefore, we used the A673-derived cell line ASP14 that allows for a doxycycline-inducible knockdown of EWS-FLI1 (37). As expected, we observed downregulation of Aurora kinases A and B at 24 and 48 hours after doxycycline-induced depletion of EWS-FLI1.

Knockdown of EWS-FLI1 has been shown to reduce viability of Ewing's sarcoma cells. Therefore, we examined whether the time window from doxycycline induction to reduction of both Aurora kinases on the protein level (48 hours) would allow for a subsequent 72-hour drug treatment. We observed a reduction of viability of 56% after 5 days of EWS-FLI1 knockdown, which was sufficient to discern differences elicited by tozasertib. We next tested whether the absence of EWS-FLI1 can confer resistance to pan-Aurora kinase inhibitors within this time window in contrast to an uninduced state. Forty-eight hours after induced EWS-FLI1 knockdown, cells were treated either with tozasertib or with etoposide, a topoisomerase II inhibitor, as a control drug. Doxycycline induction severely reduced the relative sensitivity toward tozasertib as compared with the noninduced state (Fig. 4B). This shift was seen only to a much lesser extent with etoposide showing that tozasertib sensitivity of Ewing's sarcoma cells is dependent on EWS-FLI1 expression (Supplementary Fig. S4).

Tozasertib synergizes with current chemotherapeutic options

To further investigate the therapeutic potential of pan-Aurora kinase inhibition in Ewing's sarcoma, we compared tozasertib with the chemotherapeutic agents, etoposide and doxorubicin, both of which are among the standard treatment options for Ewing's sarcoma. In both

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

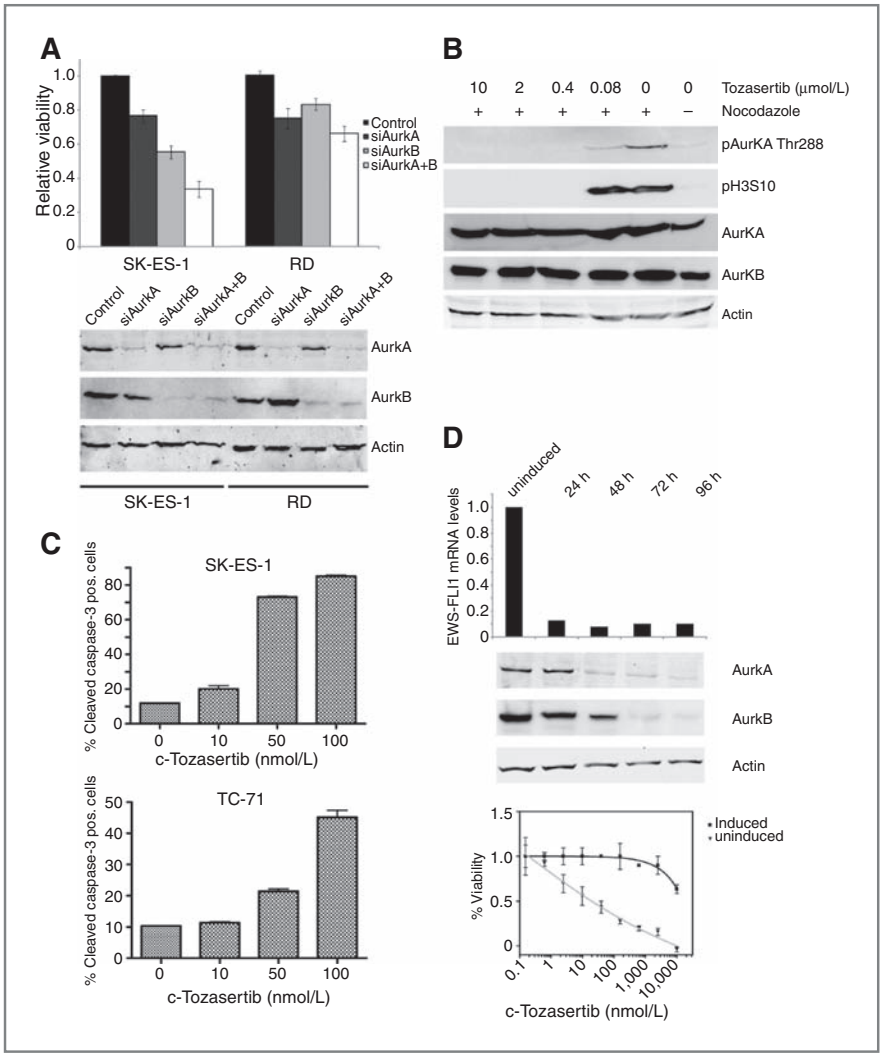


Figure 4. Combined inhibition of Aurora kinases A and B underlies tozasertib effect on Ewing's sarcoma cells. A, comparable knockdown levels in SK-ES-1 and RD result in similar impairment of viability regarding Aurora kinase A (AURKA), but substantially reduced viability regarding Aurora kinase B (AURKB) that is even more pronounced about the parallel knockdown of Aurora kinases A and B. B, phosphorylation events indicative of activity of Aurora kinase A (pAurkA Thr288) and B (pH3S10) are reduced in nocodazole-arrested cells upon increasing concentrations of tozasertib. C, induction of apoptosis of Ewing's sarcoma cells increases with escalating concentrations of tozasertib as specified by elevated levels of cleaved caspase-3–positive cells. D, doxycycline-inducible knockdown of EWS-FLI1 in ASP14 cells results in downregulation of Aurora kinases and causes resistance to tozasertib.

cell lines, SK-ES-1 and A673, tozasertib proved to be the most effective of the 3 agents (Fig. 5A). We were also interested in investigating possible synergistic or antagonistic effects resulting from the combination of pan-Aurora kinase inhibitors with these agents. Therefore, we carried out pairwise drug combination viability assays and constructed 3-dimensional dose–response surfaces delimited by the respective single dose–response curves. These experimentally derived dose–response surfaces were subsequently correlated to predicted values, based on the course of the single dose–response curves, that were generated using the Bliss additivity model (38). Calculating the differential volumes between predicted and measured inhibition allowed estimation of synergy over a broad concentration range and different ratios. This analysis revealed strong synergistic interactions between tozasertib and the 2 chemotherapeutic agents in both cell lines, particularly between tozasertib and etoposide in A673 cells. The largest positive deviation from predicted values and therefore the highest synergy was detected at low nanomolar concentrations of tozasertib and mid-nanomolar concentrations of etoposide, both of which are readily achievable in cancer patients (Fig. 5B; Supplementary Fig. S5; Supplementary Table S5).

Tozasertib causes reduction of tumor growth in a xenograft mouse model

To assess the potential of tozasertib to reduce Ewing's sarcoma tumor growth *in vivo*, mouse xenograft experiments were conducted. A total of 2×10^6 TC-71 Ewing's sarcoma cells were orthotopically injected in the gastrocnemius muscle of 5- to 7-week-old SCID/bg mice. Mice were examined on a daily basis for tumor formation.

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

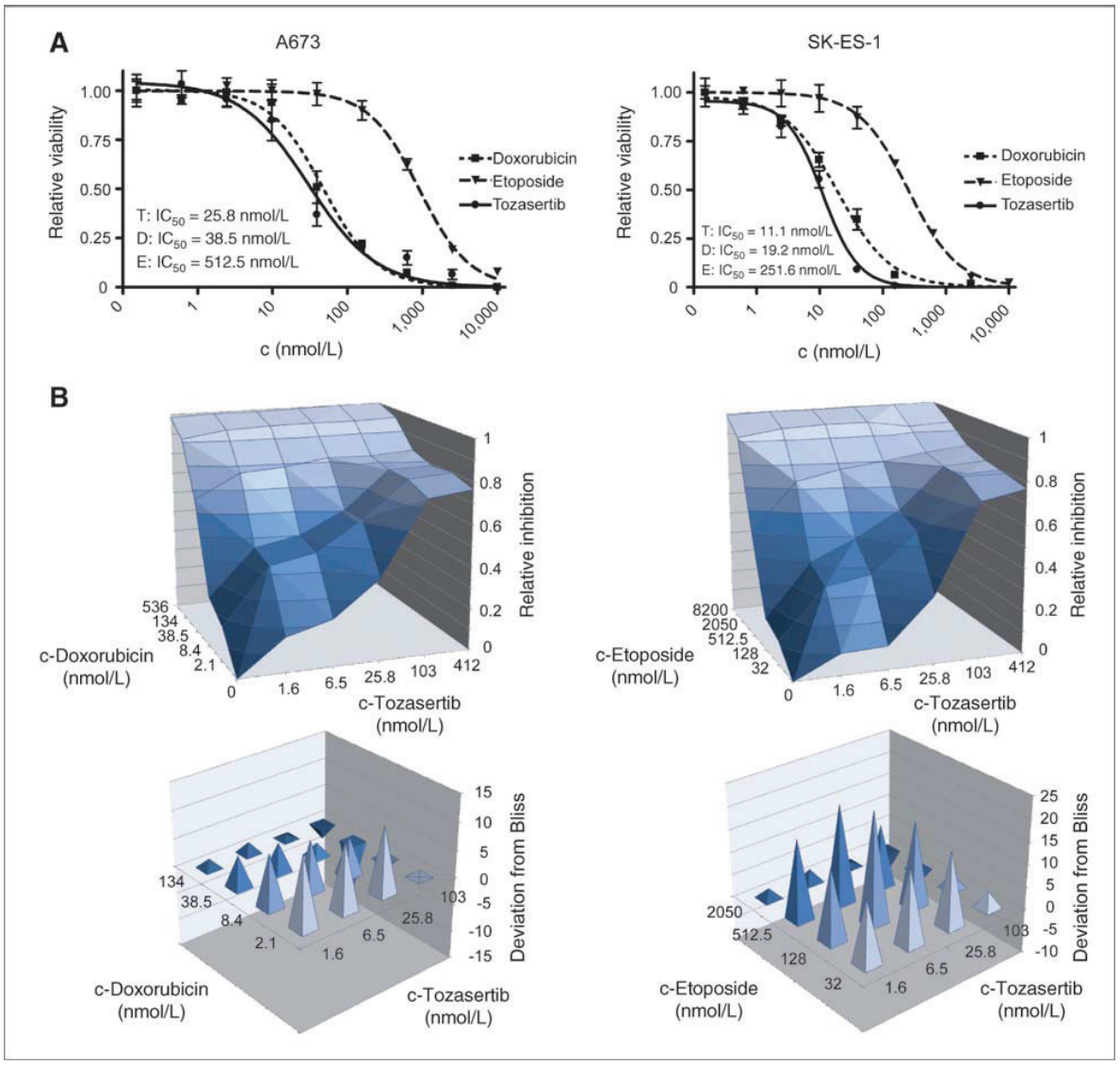


Figure 5. Tozasertib is more effective in comparison with etoposide and doxorubicin and shows synergistic potential with them. A, dose–response curves of tozasertib, doxorubicin, and etoposide in SK-ES-1 and A673. B, combined effect of tozasertib with etoposide or doxorubicin exceeded Bliss prediction, indicating synergy. Needle graphs indicate deviation from Bliss-predicted additivity. Dose–response surfaces are centered on the EC_{50} of each compound in the respective cell lines. Analysis was carried out in triplicates. Values depicted represent absolute deviations. Observed values were divided through SDs plus 15th percentile (Supplementary Table S5).

After tumors were palpable, mice were treated twice daily intraperitoneally with vehicle ($n = 11$), 50 mg/kg ($n = 11$), or 100 mg/kg ($n = 11$) tozasertib for 6 days. Subsequently, further tumor growth was monitored until day 11 when first tumors in the vehicle-treated group reached a volume exceeding 2,000 mm^3 initially set as termination criterion. Although treatment was stopped after day 6, a dose-dependent reduction in tumor growth was observed comparing the 100 mg/kg group with the vehicle-treated control group or the 50 mg/kg group that did not show an effect compared with vehicle control (Fig. 6). No toxic deaths occurred.

Discussion

We describe an integrated chemical biology approach based on small molecule screening combined with proteomics-assisted drug target identification and validation (23). This strategy allows for the discovery of novel agents with disease-relevant activity and the dissection of their

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

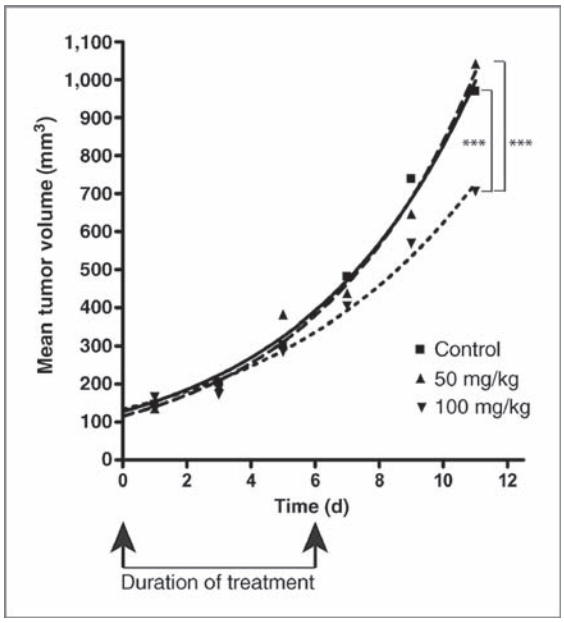


Figure 6. Tozasertib causes reduction of tumor growth *in vivo*. After orthotopical injection of 2×10^6 Ewing's sarcoma cells (TC-71) in the gastrocnemius muscle, mice were treated with vehicle control ($n = 11$), 50 mg/kg ($n = 11$), or 100 mg/kg ($n = 11$) tozasertib for 6 days. Furthermore, tumor growth was monitored until day 11. Two-way ANOVA test for the log of tumor growth ratio revealed significant differences between the 100 mg/kg treated group versus the vehicle-treated control group ($P = 9.876e\text{-}06$) and versus the 50 mg/kg treated group ($P = 3.591e\text{-}07$), whereas no significant difference was found between the control group and the 50 mg/kg treated group.

molecular mechanism of action. In turn, this provides for a better understanding of the underlying disease biology. We have applied this approach to Ewing's sarcoma, a pediatric bone cancer with high metastatic potential and unfavorable long-term prognosis in need for novel therapeutic options. Considering the significant side effects of high-dose chemotherapy it would be advantageous to implement not just improved but furthermore targeted therapies. Therefore, we have screened a library of 200 kinase inhibitors. Because of the promiscuous nature of drugs in general and kinase inhibitors in particular, this panel covers a wide range of the druggable kinome.

Our screen identified a number of kinase inhibitors potently killing Ewing's sarcoma cell lines. In light of the fact that many EWS-FLI1–upregulated target genes have been linked to cell cycle control and that knockdown of EWS-FLI1 results in growth arrest (8), it is notable that the cognate targets, such as CDKs, CKs, and Aurora kinases, of the majority of our screening hits were implicated in cell cycle regulation. Consistently, the CDK inhibitor roscovitine has been previously described as an effective inducer of apoptosis in Ewing's sarcoma cell lines (39). However, for CDKs and also for CKs we found a number of validated small molecule inhibitors among the ineffective compounds as well. A similar observation was made with inhibitors targeting phosphatidylinositol-3 kinases and AKT signaling, suggesting the possibility of off-target effects.

Kinase inhibitors are enriched for polypharmacologic features. Potentially, this can translate into initially unexpected side effects as predicted previously (40). However, targeting multiple gene products simultaneously can also be of significant benefit if several of these targets show disease relevance (41). In fact, as redundancy and buffering capacities are inherent features of many biological systems, several phenotypes of interest will only be revealed by such higher-order perturbations (42). These aspects are an advantage of multilayered chemical biology approaches and allowed the discovery that both Aurora kinases A and B contribute to the effect of tozasertib cooperatively. Moreover, additional tozasertib targets such as FAK and SRC were revealed by chemical proteomics and might indicate, due to their established role in promoting metastasis, a potential impact of tozasertib that extends beyond the scope of our initial screen (43).

Tozasertib and danusertib were, apart from staurosporine, the most potent screening hits with low- to mid-nanomolar activity in Ewing's sarcoma cell lines. Both compounds have been developed as pan-Aurora kinase inhibitors (29, 30). Of the approximately 20 tozasertib targets identified in these cells, Aurora kinases were highlighted by our deconvolution approach as potentially relevant targets. Aurora kinases A and B are serine/threonine kinases that play critical roles in mitosis (44). Among other functions, they are implicated in spindle assembly (Aurora kinase A), regulation of the mitotic checkpoint, and cytokinesis (Aurora kinase B; ref. 45). Aurora kinase A is located at the genomic locus *20q13.2*, often found to be amplified in several tumors and is a known oncogene capable of transforming fibroblasts. The genomic locus of Aurora kinase B (*17p13.1*) is not commonly amplified in human cancers. Nevertheless, increased mRNA and protein levels of Aurora kinase B have been reported in colorectal cancers (46). Consequently, Aurora kinases have gained significant attention as candidate targets in drug discovery, resulting in the development of various small molecule inhibitors that are currently in different stages of clinical trials, such as danusertib (29, 30, 44, 47). In the context of pediatric cancers, Aurora kinase A has been implicated in chemosensitivity of medulloblastoma cells (48). Furthermore, the Aurora kinase A–specific inhibitor MLN8237 has recently been shown to have efficacy in pediatric acute lymphoblastic leukemia and neuroblastoma, but interestingly to somewhat lesser extent also in Ewing's sarcoma (49). This report is consistent with our observations described here. Thus, knockdown of Aurora kinase A by RNAi reduces viability of SK-ES-1 cells to a minor extent, which is more pronounced over a longer period of time (Supplementary Fig. S2). Furthermore, tozasertib is approximately twice as potent as MLN8237 on a cellular level, which could be attributed to the additional

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

inhibitory effect on Aurora kinase B (Fig. 4A and B). However, reducing the levels of a protein by RNAi might not always be comparable with its pharmacologic inhibition. Therefore, an entirely unambiguous evaluation of individual contributions of Aurora kinases A and B to the observed phenotype is not possible.

It is noteworthy that Aurora kinases A and B have been described to be upregulated in Ewing's sarcoma by EWS-FLI1 (35). Our results furthermore show concomitant knockdown of Aurora kinases A and B in Ewing's sarcoma cells displays a potentiating effect as compared with single knockdown of either kinase. This effect seems to be specific for Ewing's sarcoma, as it is not observed in rhabdomyosarcoma cells. As tozasertib and danusertib are inhibiting Aurora kinases A and B with similar potencies (29, 30), this suggests a compound-intrinsic synergy, which might explain the specific vulnerability of Ewing's sarcoma cells toward these drugs. This is in line with our observation that downregulation of Aurora kinases A and B as a consequence of conditional EWS-FLI1 knockdown confers relative resistance toward tozasertib treatment as compared with an uninduced state or an unrelated control drug. Considering that EWS-FLI1 is the molecular lesion defining Ewing's sarcoma and would be, if not for lack of chemical tractability, a very attractive drug target, constitutes an interesting higher-order synthetic lethal relationship.

The mouse xenograft studies showed that this specific vulnerability of Ewing's sarcoma cells translates also into reduced tumor growth rates *in vivo*. Furthermore, it has been suggested that Aurora kinase inhibition might cooperate with chemotherapeutic drugs that induce DNA damage and cause cell-cycle arrest (44). Consistently, we observed strong synergy of tozasertib with the current standard chemotherapeutic drugs in Ewing's sarcoma, doxorubicin and etoposide, at low and therapeutically achievable dosages. Several Aurora kinase inhibitors, among them danusertib, are already in clinical trials for various cancers including some pediatric tumors, but not yet Ewing's sarcoma. Therefore, we believe that the specific vulnerability of Ewing's sarcoma cells toward pan-Aurora kinase inhibitors described here may represent an attractive and novel therapeutic option, the clinical evaluation of which could profit from ongoing similar trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Javier Alonso (Madrid) for providing ASP14 cells and Patrick Eyers (Sheffield) for advice on Aurora kinases. We also thank Manuela Gridling, Angelina Meier, Nora Fernbach, and Gunhild Jug.

Grant Support

This work was supported by the Austrian Federal Ministry for Science and Research (BMWF) under the GEN-AU program (GZ 200.142/1-VI/I/2006 and GZ BMWF-70.081/0018-II/1a/2008), the Austrian Academy of Sciences (ÖAW), and the European Commission (ASSET, HEALTH-F4-2010-259348).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 7, 2011; revised June 10, 2011; accepted July 6, 2011; published OnlineFirst July 18, 2011.

References

1. Bernstein M, Kovar H, Paulussen M, Randall RL, Schuck A, Teot LA, et al. Ewing's sarcoma family of tumors: current management. Oncologist 2006;11:503–19.
2. Grier HE, Krailo MD, Tarbell NJ, Link MP, Fryer CJ, Pritchard DJ, et al. Addition of ifosfamide and etoposide to standard chemotherapy for Ewing's sarcoma and primitive neuroectodermal tumor of bone. N Engl J Med 2003;348:694–701.
3. Linabery AM, Ross JA. Childhood and adolescent cancer survival in the US by race and ethnicity for the diagnostic period 1975–1999. Cancer 2008;113:2575–96.
4. Terrier P, Llombart-Bosch A, Contesso G. Small round blue cell tumors in bone: prognostic factors correlated to Ewing's sarcoma and neuroectodermal tumors. Semin Diagn Pathol 1996;13:250–7.
5. Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T, Peter M, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 1992;359:162–5.
6. May WA, Lessnick SL, Braun BS, Klemsz M, Lewis BC, Lunsford LB, et al. The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. Mol Cell Biol 1993;13:7393–98.
7. Lessnick SL, Braun BS, Denny CT, May WA. Multiple domains mediate transformation by the Ewing's sarcoma EWS/FLI-1 fusion gene. Oncogene 1995;10:423–31.
8. Prieur A, Tirode F, Cohen P, Delattre OEWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. Mol Cell Biol 2004;24:7275–83.
9. Hahm KB. Repression of the gene encoding the TGF-beta type II receptor is a major target of the EWS-FLI1 oncoprotein. Nat Genet 1999;23:481.
10. Erkizan HV, Kong Y, Merchant M, Schlottmann S, Barber-Rotenberg JS, Yuan L, et al. A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing's sarcoma. Nat Med 2009;15:750–6.
11. Scotlandi K, Benini S, Nanni P, Lollini PL, Nicoletti G, Landuzzi L, et al. Blockage of insulin-like growth factor-I receptor inhibits the growth of Ewing's sarcoma in athymic mice. Cancer Res 1998;58:4127–31.
12. Scotlandi K, Baldini N, Cerisano V, Manara MC, Benini S, Serra M, et al. CD99 engagement: an effective therapeutic strategy for Ewing tumors. Cancer Res 2000;60:5134–42.
13. Scotlandi K, Remondini D, Castellani G, Manara MC, Nardi F, Cantiani L, et al. Overcoming resistance to conventional drugs in Ewing sarcoma and identification of molecular predictors of outcome. J Clin Oncol 2009;27:2209–16.
14. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl–positive cells. Nat Med 1996;2:561–6.
15. Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med 2006;355:2733–43.
16. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353:123–32.

Published OnlineFirst July 18, 2011; DOI:10.1158/1535-7163.MCT-11-0100

17. Piccaluga PP, Paolini S, Martinelli G. Tyrosine kinase inhibitors for the treatment of Philadelphia chromosome-positive adult acute lymphoblastic leukemia. Cancer 2007;110:1178–86.
18. Hartwell LH, Szankasi P, Roberts CJ, Murray AW, Friend SH. Integrating genetic approaches into the discovery of anticancer drugs. Science 1997;278:1064–8.
19. Costanzo M, Baryshnikova A, Bellay J, Kim Y, Spear ED, Sevier CS, et al. The genetic landscape of a cell. Science 2010;327:425–31.
20. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. Nature 2009;462:108–12.
21. Potratz JC, Saunders DN, Wai DH, Ng TL, McKinney SE, Carboni JM, et al. Synthetic lethality screens reveal RPS6 and MST1R as modifiers of insulin-like growth factor-1 receptor inhibitor activity in childhood sarcomas. Cancer Res 2010;70:8770–81.
22. Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, et al. A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol 2008;26:127–32.
23. Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 2009;461:614–20.
24. Godl K, Gruss OJ, Eickhoff J, Wissing J, Blencke S, Weber M, et al. Proteomic characterization of the angiogenesis inhibitor SU6668 reveals multiple impacts on cellular kinase signaling. Cancer Res 2005;65:6919–26.
25. Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S, et al. Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. Nat Biotechnol 2007; 25:1035–44.
26. Rix U, Superti-Furga G. Target profiling of small molecules by chemical proteomics. Nat Chem Biol 2009;5:616–24.
27. Fernbach NV, Planyavsky M, Müller A, Breitwieser FP, Colinge J, Rix U, et al. Acid elution and one-dimensional shotgun analysis on an Orbitrap mass spectrometer: an application to drug affinity chromatography. J Proteome Res 2009;8:4753–65.
28. Borisy AA, Elliott PJ, Hurst NW, Lee MS, Lehar J, Price ER, et al. Systematic discovery of multicomponent therapeutics. Proc Natl Acad Sci U S A 2003;100:7977–82.
29. Harrington EA, Bebbington D, Moore J, Rasmussen RK, Ajose-Adeogun AO, Nakayama T, et al. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth *in vivo*. Nat Med 2004;10:262–7.
30. Carpinelli P, Ceruti R, Giorgini ML, Cappella P, Gianellini L, Croci V, et al. PHA-739358, a potent inhibitor of Aurora kinases with a selective target inhibition profile relevant to cancer. Mol Cancer Ther 2007;6: 3158–68.
31. Modugno M, Casale E, Soncini C, Rosettani P, Colombo R, Lupi R, et al. Crystal structure of the T315I Abl mutant in complex with the Aurora kinases inhibitor PHA-739358. Cancer Res 2007;67:7987–90.
32. Carter TA, Wodicka LM, Shah NP, Velasco AM, Fabian MA, Treiber DK, et al. Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. Proc Natl Acad Sci U S A 2005;102:11011–6.
33. Rix U, Hantschel O, Dürnberger G, Remsing Rix LL, Planyavsky M, Fernbach NV, et al. Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. Blood 2007;110:4055–63.
34. Remsing Rix LL, Rix U, Colinge J, Hantschel O, Bennett KL, Stranzl T, et al. Global target profile of the kinase inhibitor bosutinib in primary chronic myeloid leukemia cells. Leukemia 2009;23:477–85.
35. Wakahara K, Ohno T, Kimura M, Masuda T, Nozawa S, Dohjima T, et al. EWS-Fli1 upregulates expression of the Aurora A and Aurora B kinases. Mol Cancer Res 2008;6:1937–45.
36. Embree LJ, Azuma M, Hickstein DD. Ewing sarcoma fusion protein EWSR1/FLI1 interacts with EWSR1 leading to mitotic defects in zebrafish embryos and human cell lines. Cancer Res 2009;69: 4363–71.
37. Carrillo J, García-Aragoncillo E, Azorín D, Agra N, Sastre A, González-Mediero I, et al. Cholecystokinin downregulation by RNA interference impairs Ewing tumor growth. Clin Cancer Res 2007;13:2429–40.
38. Bliss CI. The toxicity of poisons applied jointly. Ann Appl Biol 1939; 26:585–615.
39. Tirado OM, Mateo-Lozano S, Notario V. Roscovitine is an effective inducer of apoptosis of Ewing's sarcoma family tumor cells *in vitro* and *in vivo*. Cancer Res 2005;65:9320–7.
40. Hantschel O, Rix U, Schmidt U, Bürckstümmer T, Kneidinger M, Schütze G, et al. The Btk tyrosine kinase is a major target of the Bcr-Abl inhibitor dasatinib. Proc Natl Acad Sci U S A 2007;104: 13283–8.
41. Li J, Rix U, Fang B, Bai Y, Edwards A, Colinge J, et al. A chemical and phosphoproteomic characterization of dasatinib action in lung cancer. Nat Chem Biol 2010;6:291–9.
42. Lehar J, Krueger A, Zimmermann G, Borisy A. High-order combination effects and biological robustness. Mol Syst Biol 2008;4:215.
43. Shor AC, Keschman EA, Lee FY, Muro-Cacho C, Letson GD, Trent JC, et al. Dasatinib inhibits migration and invasion in diverse human sarcoma cell lines and induces apoptosis in bone sarcoma cells dependent on SRC kinase for survival. Cancer Res 2007;67:2800–8.
44. Lens SM, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer 2010;10:825–41.
45. Lapenna S, Giordano A. Cell cycle kinases as therapeutic targets for cancer. Nat Rev Drug Discov 2009;8:547–66.
46. Marumoto T, Zhang D, Saya H. Aurora-A - a guardian of poles. Nat Rev Cancer 2005;5:42–50.
47. Dar AA, Goff LW, Majid S, Berlin J, El-Rifai WAurora kinase inhibitors–rising stars in cancer therapeutics? Mol Cancer Ther 2010;9: 268–78.
48. El-Sheikh A, Fan R, Birks D, Donson A, Foreman NK, Vibhakar R, et al. Inhibition of Aurora Kinase A enhances chemosensitivity of medulloblastoma cell lines. Pediatr Blood Cancer 2010;55:35–41.
49. Maris JM, Morton CL, Gorlick R, Kolb EA, Lock R, Carol H, et al. Initial testing of the Aurora kinase A inhibitor MLN8237 by the Pediatric Preclinical Testing Program (PPTP). Pediatr Blood Cancer 2010;55: 26–34.

3.2 Systems-pharmacology dissection of a drug synergy in imatinib-resistant CML

Georg E Winter, Uwe Rix, Scott M Carlson, Karoline V Gleixner, Florian Grebien, Manuela Gridling, André C Müller, Florian P Breitwieser, Martin Bilban, Jacques Colinge, Peter Valent, Keiryn L Bennett, Forest M White & Giulio Superti-Furga

nature chemical biology

ARTICLE

PUBLISHED ONLINE: 30 SEPTEMBER 2012 | DOI: 10.1038/NCHEMBIO.1085

Systems-pharmacology dissection of a drug synergy in imatinib-resistant CML

Georg E Winter[1,7], Uwe Rix[1,6,7], Scott M Carlson[2,6], Karoline V Gleixner[3], Florian Grebien[1], Manuela Gridling[1], André C Müller[1], Florian P Breitwieser[1], Martin Bilban[4], Jacques Colinge[1], Peter Valent[3,5], Keiryn L Bennett[1], Forest M White[2] & Giulio Superti-Furga[1]*

Occurrence of the BCR-ABL^{T315I} gatekeeper mutation is among the most pressing challenges in the therapy of chronic myeloid leukemia (CML). Several BCR-ABL inhibitors have multiple targets and pleiotropic effects that could be exploited for their synergistic potential. Testing combinations of such kinase inhibitors identified a strong synergy between danusertib and bosutinib that exclusively affected CML cells harboring BCR-ABL^{T315I}. To elucidate the underlying mechanisms, we applied a systems-level approach comprising phosphoproteomics, transcriptomics and chemical proteomics. Data integration revealed that both compounds targeted Mapk pathways downstream of BCR-ABL, resulting in impaired activity of c-Myc. Using pharmacological validation, we assessed that the relative contributions of danusertib and bosutinib could be mimicked individually by Mapk inhibitors and collectively by downregulation of c-Myc through Brd4 inhibition. Thus, integration of genome- and proteome-wide technologies enabled the elucidation of the mechanism by which a new drug synergy targets the dependency of BCR-ABL^{T315I} CML cells on c-Myc through nonobvious off targets.

Redundancy and multifunctionality are inherent characteristics of biological systems that limit the therapeutic opportunity of single-agent applications[1]. Combinations of drugs that yield a synergistic effect are thought to be the most effective way of countering biological buffering and also allow reduced dosing of each agent while increasing therapeutically relevant selectivity[2]. Recent advances in assaying the impact of small molecules on the transcriptome or the proteome in terms of drug binding or alterations in post-transcriptional modifications led to a complex picture of drug action that goes against the 'one drug, one target' paradigm[3–5]. Although each of the above-mentioned approaches generates a wealth of useful data, together they only allow for partial insight into the composite effects of small-molecule agents on complex cellular systems. These effects are a consequence of all on- and off-target drug effects and impairment of the related cellular processes, including changes in gene expression[6,7]. As a result of crosstalk at various levels, this complexity is markedly increased if two drugs are applied simultaneously. Deconvolution of the relevant cellular mechanism underlying a combined treatment with two drugs that yields a synergistic and therefore unpredictable effect is a particular challenge.

CML is a clonal hematopoietic disease hallmarked by the expression of the BCR-ABL fusion oncoprotein that results from a reciprocal translocation between chromosomes 9 and 22. BCR-ABL features a deregulated tyrosine kinase activity that drives a number of downstream signaling pathways, confers growth advantage and counteracts apoptosis[8]. The most prominent downstream pathways upregulated by BCR-ABL include the PI3K, STAT5 and MAPK pathways. Treatment of CML rapidly improved after the introduction of the first BCR-ABL inhibitor, imatinib (Gleevec, STI-571), which serves as a paradigmatic example for targeted therapies[9]. Imatinib causes complete remission and prolonged lifespan in the majority of patients with CML[9]. Nevertheless, it soon became apparent that a broad spectrum of possible resistance mechanisms toward imatinib treatment, for example, acquisition of point mutations in the ATP binding pocket or overexpression of LYN or BCR-ABL itself, necessitated the development of second- and third-generation BCR-ABL inhibitors such as nilotinib (Tasigna, AMN107) and dasatinib (Sprycel, BMS-354825)[10–14]. These later-generation agents have been successful in over-riding a broad variety of resistance mechanisms against imatinib. However, none of them is effective in patients with CML who harbor the so-called BCR-ABL 'gatekeeper mutations' at Thr315. Thus, these patients are in need of new therapeutic approaches, although promising experimental targeting strategies have been reported recently[15–18].

Here we describe a new synergistic interaction between the clinically tested multikinase inhibitors danusertib (PHA-739358) and bosutinib (SKI-606) that is specific for BCR-ABL gatekeeper mutation–transformed cells. We deciphered the molecular logic underlying the synergistic effect using a multilevel experimental approach that included proteome-wide measurements of drug-binding using chemical proteomics, global monitoring of alterations in phosphorylation states in response to drug treatment and genome-wide transcriptomics. Correlating the affected signaling pathways with drug-dependent transcription-factor signatures revealed reduced c-Myc activity as the key point of convergence.

To the best of our knowledge, this is the first description of a comprehensive dissection of a synergistic drug interaction using three different large-scale 'omics' data sets. In this study, we show that the systems-level cooperative effect obtained by applying danusertib and bosutinib in combination results from previously unappreciated features of both agents. We believe that this strategy

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria. [2]Department of Biological Engineering and Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. [3]Department of Internal Medicine I, Division of Hematology and Hemostaseology, Medical University of Vienna, Vienna, Austria. [4]Clinical Institute for Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Vienna, Austria. [5]Ludwig Boltzmann Cluster Oncology, Vienna, Austria. [6]Present addresses: Department of Drug Discovery, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA (U.R.) and Department of Biology, Stanford University, Stanford, California, USA (S.M.C.). [7]These authors contributed equally to this work. *e-mail: gsuperti@cemm.oeaw.ac.at

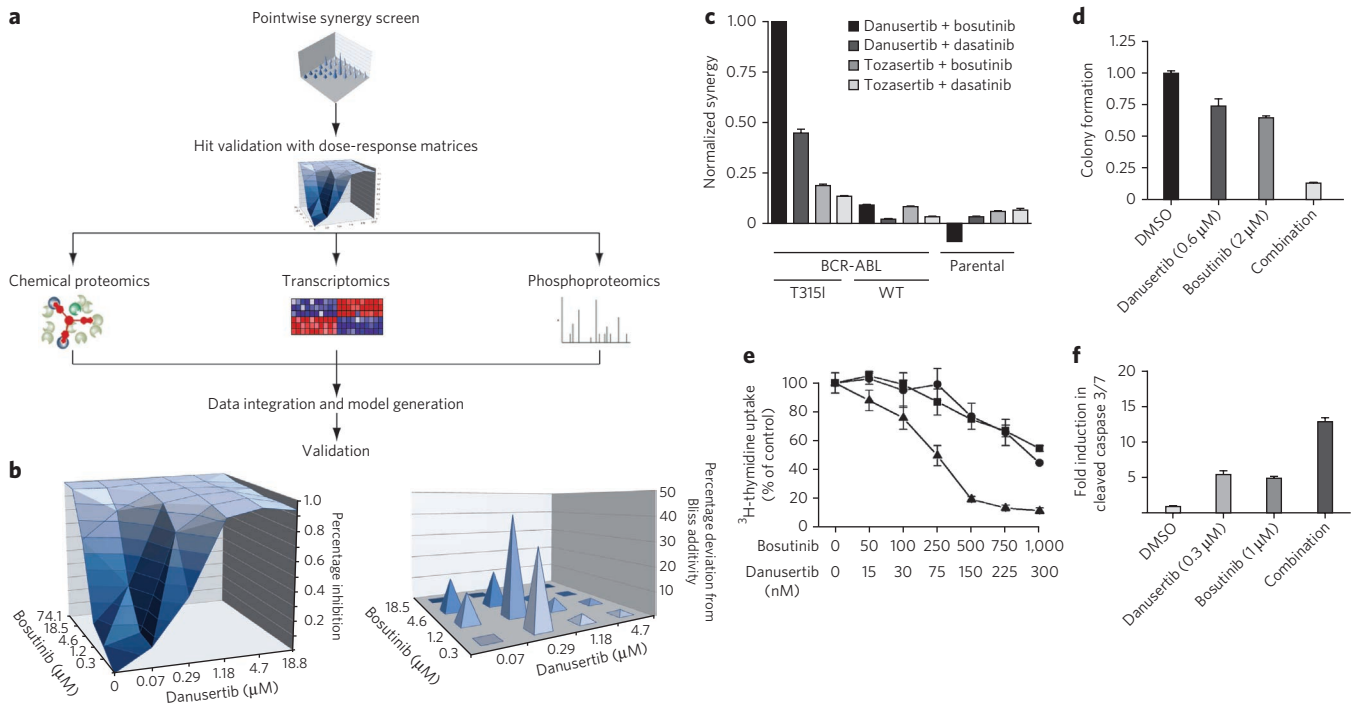


Figure 1 | Danusertib and bosutinib synergize specifically in BCR-ABL^{T315I} cells. (**a**) Schematic outline of the three-pronged approach for comprehensive capturing of the cellular response to the combined drug treatment. (**b**) The combined effect of danusertib and bosutinib in Ba/F3 BCR-ABL^{T315I} cells exceeds the Bliss prediction, indicating a synergistic interaction. Needle graphs depict deviation from the Bliss-predicted additivity and represent the mean of triplicates. (**c**) Comparison of the differential volumes between experimentally derived and Bliss-predicted values. Data are the mean ± s.d. of triplicates. WT, wild type. (**d**) Colony formation capability of primary cells retrovirally transduced with BCR-ABL^{T315I} after drug treatment. Data represent the mean ± s.d. of quadruplicates. (**e**) ^{3}H-thymidine uptake in primary cells from the peripheral blood of a patient with BCR-ABL^{T315I}–positive CML that were incubated with bosutinib (■-■) and danusertib (●-●) as single agents or in combination (at a 10:3 fixed ratio of drug concentrations; ▲-▲). (**f**) Induction of apoptosis as measured by cleavage of caspase 3 and caspase 7 (caspase 3/7). Data depict fold increases and are the mean ± s.d. of triplicates.

of gaining a functional understanding of a drug synergy may serve as a model for further mode-of-action studies.

RESULTS

Identification of synergy specific for BCR-ABL^{T315I} cells

The overall experimental strategy is outlined schematically in **Figure 1a**. It starts with point-wise synergy screens and their validation using three-dimensional dose-response surfaces, which is then followed by three parallel experimental lines of investigation: (i) determination of the cellular binding partners of the involved drugs, (ii) mapping the impact of the single agents as well as their combination on the transcriptome and (iii) charting the global changes in the phosphoproteome after drug exposure. Integration of the data sets generates hypotheses that are subsequently validated. We chose to investigate synergies that were specific for gatekeeper mutant–associated imatinib-resistant BCR-ABL. To identify such potential synergistic interactions, we used the Ba/F3 mouse pro-B cell line system that was retrovirally transduced with BCR-ABL^{T315I}, which conferred interleukin-3–independent growth properties. First, we generated dose-response curves for eight clinical BCR-ABL inhibitors known to be sufficiently safe in the relevant patient class (imatinib, nilotinib, dasatinib, bosutinib, bafetinib, tozasertib, danusertib and sorafenib). Most of the tested compounds were *per se* not effective against Ba/F3 BCR-ABL^{T315I} cells at clinically relevant concentrations (**Supplementary Results**, **Supplementary Table 1**). However, given that kinase inhibitors are generally known to share polypharmacologic features, we hypothesized that combining two agents that were initially not very efficacious could result in synergistic cell killing because of a cooperative effect of previously unappreciated off targets. Therefore, all possible pairwise combinations were tested in an effector concentration for a 20% maximal response (EC_{20}) by EC_{20} checkerboard design and evaluated by comparison of the experimentally derived impairment of cellular viability with the predicted combinatorial effect determined using the Bliss-additivity model (**Supplementary Table 2**)[19].

We observed a pronounced synergy between the pan–aurora kinase inhibitor danusertib and the dual ABL and SRC inhibitor bosutinib that we validated in detail by generating multifactorial dilutions of both agents, resulting in three-dimensional dose-response surfaces[20]. These were again correlated to the Bliss-predicted values. Calculating the differential volumes between the predicted and measured inhibitions allowed an estimation of synergy over a broad concentration range, where a positive interaction volume indicates synergy. We observed a strong synergism between danusertib and bosutinib in killing BCR-ABL^{T315I}–transformed cells in the lower, clinically relevant dose range, reaching up to 40% more inhibition than predicted by Bliss additivity (**Fig. 1b**). To further investigate the specificity of the observed synergy, we tested the combination of danusertib and bosutinib in a BCR-ABL wild-type background as well as in parental Ba/F3 cells. Danusertib and bosutinib only synergized in the gatekeeper-mutant background. Furthermore, using a combination of dasatinib and tozasertib, compounds with overlapping cognate targets, the detected synergy could be mimicked only to a much lesser extent, suggesting the possibility that off-target effects were responsible for the observed synergy (**Fig. 1c**).

Synergy is conserved in primary cells harboring BCR-ABL^{T315I}

We next asked whether the observed synergistic drug interaction is also conserved in a setting that is closer to the *in vivo* situation. Thus, we retrovirally transduced primary mouse bone-marrow cells

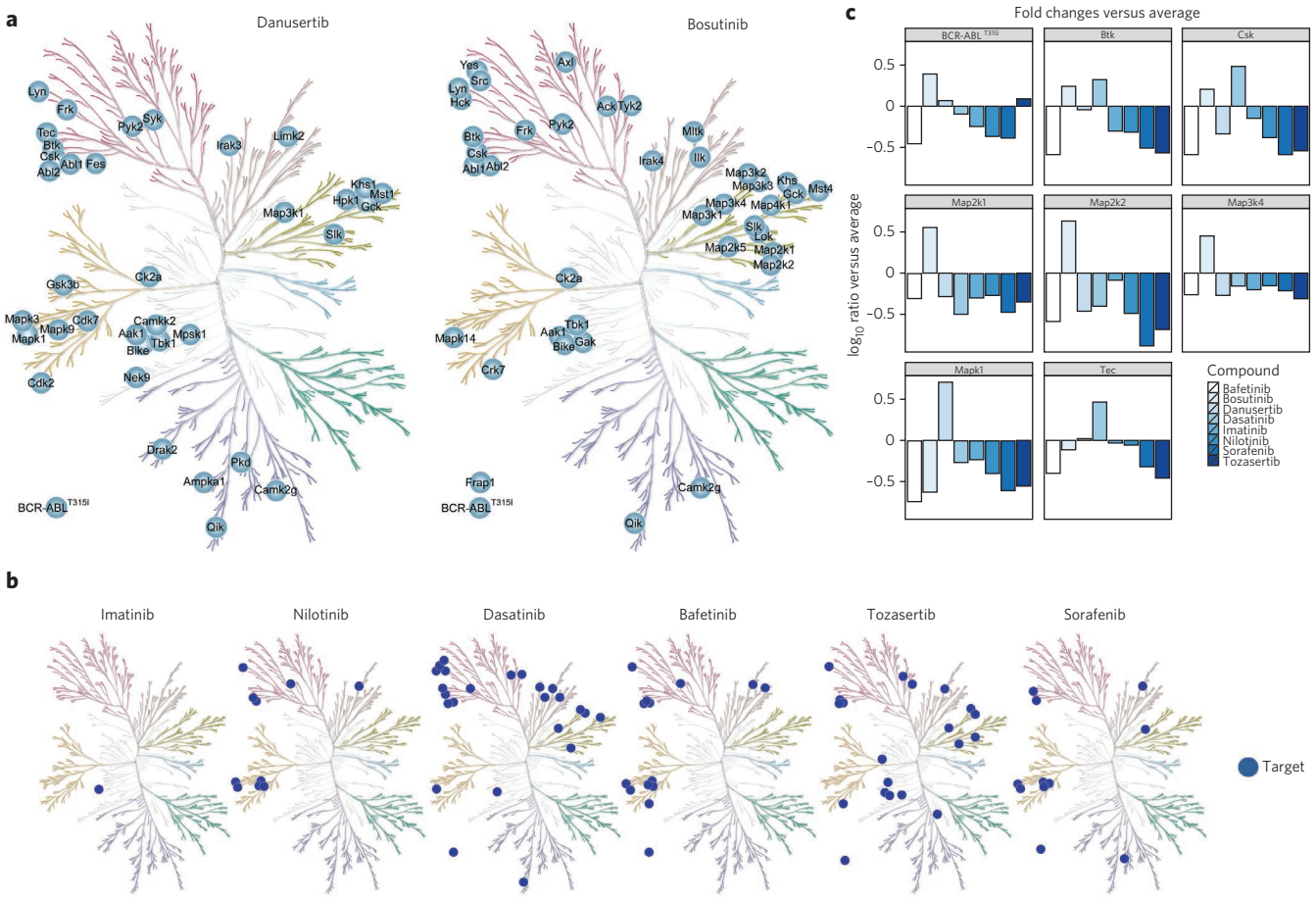


Figure 2 | Quantitative chemical proteomics reveals target spectra of eight clinical BCR-ABL kinase inhibitors and indicates impairment of MAPK signaling resulting from off-target effects of danusertib and bosutinib. (**a**) Comprehensive kinase target profiles of danusertib and bosutinib as determined by gel-free one-dimensional LC/MS analysis mapped onto the human protein kinome. The human kinome is reproduced courtesy of Cell Signaling Technology (http://www.cellsignal.com/). (**b**) Kinome-wide target spectra of the remaining six kinase inhibitors used in this study as determined by one-dimensional LC/MS. (**c**) Bar graph depiction of eight-plex iTRAQ ratios for selected kinases (for the full panel of identified kinases, see **Supplementary Fig. 7**). Intensities were quantified using the average reporter tag intensity as a reference.

with BCR-ABL^{T315I}, thereby rendering them growth-factor independent. A significant reduction ($P < 0.0001$) in colony-formation capability was observed in the presence of the drug combination as compared to the presence of either danusertib or bosutinib alone after 10 d of drug incubation (**Fig. 1d** and **Supplementary Fig. 1**). Moreover, the synergy between danusertib and bosutinib also translated into *ex vivo* proliferation assays using primary cells isolated from the peripheral blood of a patient suffering from advanced BCR-ABL^{T315I}–positive CML (**Fig. 1e**). In line with the cell line–derived data, the cooperative drug interaction was not observed using primary cells from a BCR-ABL^{WT}–positive patient (**Supplementary Fig. 2**). To rule out the possibility that the measured effect was caused solely by cooperative inhibitory effects on BCR-ABL^{T315I} activity, *in vitro* kinase assays were performed. These studies showed a buffering effect of the drug combination that did not exceed the single-drug efficacy of danusertib, which was the more effective individual compound (**Supplementary Fig. 3**). By characterizing the impairment of cellular viability on Ba/F3 BCR-ABL^{T315I} cells in more detail, we observed an increase in apoptosis that was specific in its collaborative nature for Ba/F3 BCR-ABL^{T315I} cells. In fact, we observed a 13-fold increase in cleaved caspase 3 and caspase 7 in cells treated with the drug combination compared to vehicle-treated cells (**Fig. 1f** and **Supplementary Fig. 4**). Assessing the impact on the cell cycle after treatment with a single drug and with the drug combination, we detected a G2 arrest that was observed specifically for the drug combination in the mutant BCR-ABL background (**Supplementary Fig. 5**).

Multiple off targets implicated in Mapk signaling

With the intention of uncovering the off-target effects of the two agents that may underlie the observed synergy, a chemical proteomics approach was pursued as an initial step in deconvoluting potential targets. Chemical proteomics is a post-genomic version of drug affinity chromatography and is enabled by high-resolution tandem mass spectrometry and downstream bioinformatics analysis[4]. Given that tozasertib and dasatinib, compounds that are related to danusertib and bosutinib, did not show a pronounced synergistic interaction, we hypothesized that the proteins underlying the cellular effect of the combination of danusertib and bosutinib would be specific binders of these drugs, thereby necessitating the identification of their cell-specific, proteome-wide target profiles. Analogs of all eight screened compounds were either available or designed for this study (**Supplementary Fig. 6**). For each compound, a modification that allowed immobilization on sepharose beads was introduced for the subsequent affinity purification of interacting proteins from lysates of the BCR-ABL^{T315I}–transformed Ba/F3 cells. Kinase-binding properties were not affected for any of the compounds, as confirmed by *in vitro* kinase assays for each analog compared to the parental small molecule (**Supplementary Table 3**). Using one-dimensional gel-free LC/MS chemical proteomics, a total of 68 protein kinase targets

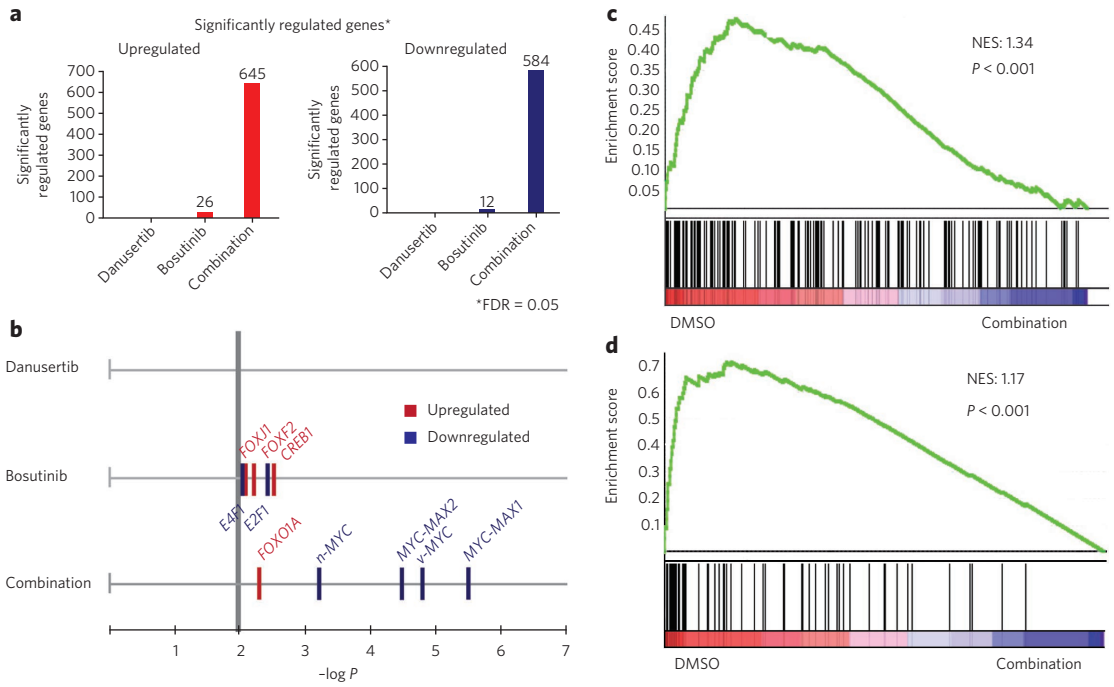


Figure 3 | Transcriptome-wide analysis indicates global downregulation of *c-Myc* target genes after combinatorial treatment with danusertib and bosutinib. (**a**) Bar graphs showing the number of significantly upregulated and downregulated genes in each comparative condition. (**b**) *P* values for the enrichment of motif gene sets (MSigDB) only taking significantly (FDR < 0.05) regulated genes into consideration. (**c**,**d**) Gene set enrichment analysis showing global downregulation of genes with *c-MYC* regulatory motifs (**c**) and experimentally validated *c-Myc* target genes (**d**). NES, normalized enrichment score.

were identified for all 8 kinase inhibitors and, more specifically, 40 kinases bound to bosutinib and 37 kinases bound to danusertib in Ba/F3 BCR-ABL^{T315I} cell lysates (**Fig. 2a**,**b**). To quantify the relative affinities of the identified kinase binders to the eight small molecules, the chemical proteomics experiments were extended using the iTRAQ methodology (isobaric tag for relative and absolute quantitation)[21,22]. These experiments were subsequently analyzed by two-dimensional gel-free LC/MS.

Intensity ratios were obtained for 43 kinases (**Fig. 2c**, **Supplementary Fig. 7** and **Supplementary Data Set 2**). In addition to reproducing known target-ligand interactions, such as identifying Tec kinase as a specific interactor of dasatinib and confirming the higher affinity of both bosutinib and dasatinib for Src or Csk, we also identified kinase targets that would almost exclusively bind to only one of the agents tested. In particular, we found various kinases that are implicated in Mapk signaling, such as Mapk1 (Erk2), Map2k1 (Mek1), Map2k2 (Mek2), Map3k3 and Pyk2 (Ptk2b), that have a strong propensity for binding to either danusertib or bosutinib but not to the other inhibitors tested (**Fig. 2c** and **Supplementary Fig. 7**). To further support the chemical proteomics–derived data, we performed additional *in vitro* enzymatic assays and competitive binding experiments (**Supplementary Figs. 8** and **9**). A subsequent pathway analysis using all the identified targets of either danusertib or bosutinib alone as well as their collective target spectrum as queries suggested that both agents had a substantial impact on the Mapk signaling cascade, both individually and cooperatively[23]. Using the DAVID bioinformatics interface, we observed a significant enrichment for the drug combination in the KEGG ($P = 8.98 \times 10^{-9}$, false discovery rate (FDR) = 9.2×10^{-6}) and BIOCARTA ($P = 2.49 \times 10^{-9}$, FDR = 2.8×10^{-6}) Mapk pathways that was not achieved to the same extent by querying the single target spectra of either danusertib or bosutinib and therefore indicated that the impact of both drugs on this pathway was not of a redundant nature but, rather, was of a cooperative nature (**Supplementary Table 4**)[23].

c-Myc targets are downregulated on a genome-wide scale

Post-translational modification of transcription factors, which causes transcriptional activation through nuclear translocation, homodimerization or heterodimerization, is a common mechanism by which signaling cascades can integrate environmental stimuli into altered transcriptional responses and, in turn, is an immediate answer by the cellular system to any given kind of intervention[24].

To determine the functional consequences of the simultaneous application of danusertib and bosutinib, we analyzed global transcriptional changes by generating differential gene expression profiles. Using the concentrations that resulted in the highest synergy, the impact of either of the drugs alone and their combination at an equal dose was compared to that of the vehicle control 6 h after drug exposure (**Supplementary Fig. 10**). At this time point, no signs of cell death or impaired cellular viability were detected (data not shown). Taking an FDR of 0.05 into consideration, there were no significantly regulated genes when Ba/F3 BCR-ABL^{T315I} cells were treated with 300 nM danusertib. Also, the number of significantly regulated genes resulting from treatment with 1 μM bosutinib was relatively low, with 26 upregulated and 12 downregulated genes. However, when both compounds were combined, severe alterations in the transcriptome were observed, with 645 genes significantly upregulated and 584 genes significantly downregulated (**Fig. 3a**, **Supplementary Fig. 11** and **Supplementary Data Set 1**). This observation supported the previous notion that the combined effect of both drugs is not explainable solely by the summation of the individual effects of the two agents alone and suggested that cooperative inactivation of a transcription factor might be linked to the observed cellular synergy. Therefore, we performed a database query with all significantly regulated genes in each measured condition using the Molecular Signature Database (MSigDB). Thus, the group of genes downregulated by the combined drug treatment was found to be specifically enriched for genes containing c-Myc regulatory motifs in their promoters (**Fig. 3b**).

To extend our findings, which were limited to the subset of significantly regulated genes, to the entire genome, we next conducted an unbiased gene set enrichment analysis[25] comparing all the conditions measured. This independent approach reproduced the finding that genes containing a c-Myc motif were strongly correlated with downregulation by combination drug treatment (**Fig. 3c**). Moreover, we evaluated the impact of the drug combination on an experimentally derived c-Myc transcriptional signature, which also revealed global downregulation of c-Myc–dependent target genes in the drug combination treatment as compared to DMSO treatment (**Fig. 3d**)[26].

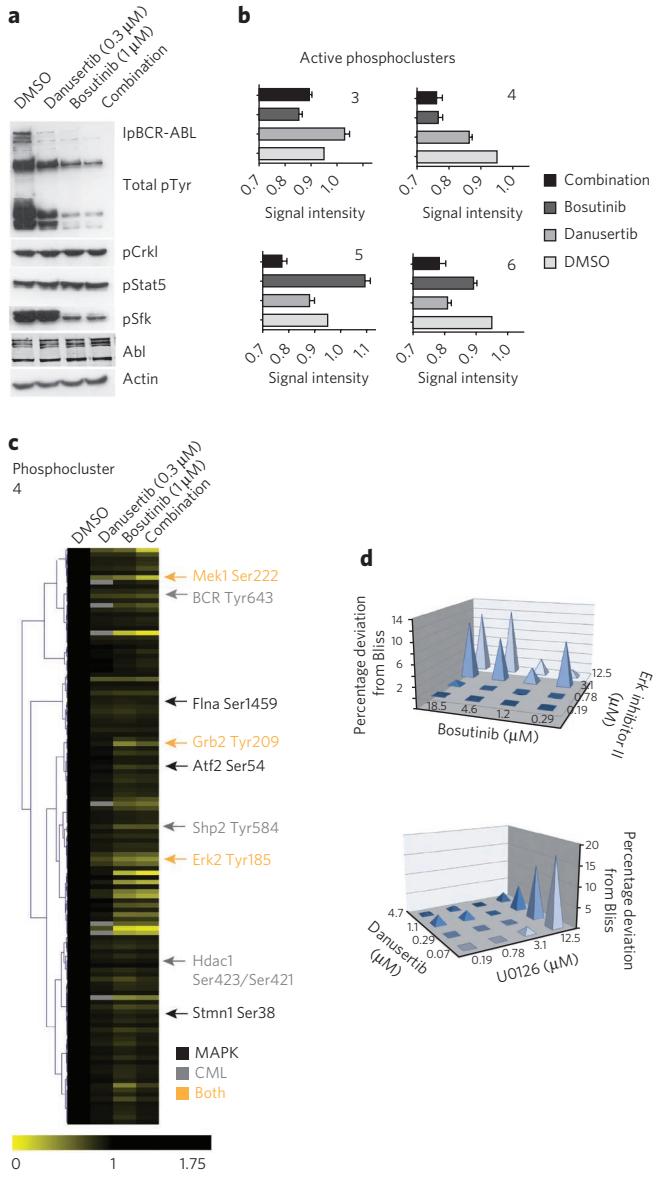


Figure 4 | Quantitative phosphoproteomics highlights the impact of drug combination on the MAPK signaling network and the functional relevance of inhibition of MEK and ERK. (**a**) Immunoblot of total phosphotyrosine (pTyr), pCrkl, pStat5, pSfk (Src family kinases), Abl and actin of Ba/F3 BCR-ABL^{T315I} cells treated as indicated for 2 h. (**b**) SOTA analysis yielding 11 clusters of differential drug response patterns for the observed phosphopeptides, four of which (clusters 3, 4, 5 and 6) are considered to be active clusters. All clusters are represented in **Supplementary Figure 13**. (**c**) Heatmap rendition of all phosphorylation sites comprising active SOTA cluster 4, which is enriched for members of the MAPK (black) and CML (gray) signaling pathways. Phosphopeptide signatures corresponding to proteins that participate in both pathways are highlighted in orange. (**d**) Synergy of danusertib and bosutinib can be mimicked by the replacement of danusertib with ERK inhibitor II and of bosutinib with the MEK inhibitor U0126, respectively. Needle graphs depict deviation from the Bliss-predicted additivity and represent the mean of triplicates. Raw immunoblot data for **a** are shown in **Supplementary Figure 24**.

Mapk signaling affected by drug action

We initially set out to investigate the consequence of single and combined drug treatment on the consensus BCR-ABL signaling network using immunoblotting (**Fig. 4a**). A gradual decrease in total phosphotyrosine levels, including BCR-ABL phosphorylation itself, was observed with both agents individually and in combination. Conversely, other well-known readouts for canonical BCR-ABL signaling, such as phosphorylation of Stat5 or Crkl, were not markedly impaired at the concentrations used and only decreased detectably when the concentrations of both compounds were increased fourfold (data not shown). As in a BCR-ABL wild-type signaling background, phosphorylated Stat5 (pStat5) and pCrkl are very sensitive readouts for BCR-ABL activity; this observation was unexpected and therefore necessitated a more unbiased, global phosphoproteomics approach (**Supplementary Fig. 12**). Using eight-plex iTRAQ, we assayed the four different treatment conditions (DMSO, danusertib, bosutinib and the combination of danusertib and bosutinib) at an early, 15-min time point and a late, 6-h time point. Approximately 700 phosphopeptides could be quantified at both time points, including 110 phosphotyrosine peptides and 563 phosphoserine or phosphothreonine peptides. Because of the dynamic nature of phosphorylation, the early time point was more informative. Further analysis was therefore conducted using the 15-min treatment time as a reference. To identify common patterns of response of each site to treatment with a single drug or the drug combination, an unsupervised, self-organizing phylogenetic clustering algorithm (SOTA) was applied that allocated each phosphopeptide to one unique cluster[27]. Thus, 11 distinct clusters of response patterns were identified that ranged from a completely unchanged phosphorylation status to cooperative downregulation of given phosphorylation sites and signaling cascades (**Fig. 4b**, **Supplementary Fig. 13** and **Supplementary Data Set 3**).

Subsequent pathway analysis of each separate cluster revealed enrichments for various pathways (**Supplementary Table 5**). Most notably, however, MAPK and CML pathways were specifically enriched ($P < 0.05$) in active response patterns, as represented in clusters 3, 4, 5 and 6. Notably, cluster 4 also contained phosphopeptides for Mek (Ser222) and Erk (Tyr185) (**Fig. 4c** and **Supplementary Methods**). This is in line with the results from the proteome-wide target survey using chemical proteomics that suggested Mek and Erk as specific binders of bosutinib and danusertib, respectively. Phosphorylation of these sites correlates with the active states of the respective kinases. In addition to Mek and Erk, the quantitative phosphoproteomics analysis also revealed differentially altered phosphorylation events on other proteins implicated in the BCR-ABL–MAPK signaling network (**Supplementary Fig. 14**). To gain more confidence in the functional relevance of the inhibition of Map kinases in the context of the observed synergy, we assessed whether Ba/F3 BCR-ABL^{T315I} cells would be more sensitive toward their inhibition as compared to Ba/F3 BCR-ABL^{WT} cells. Using various small-molecule inhibitors, we observed a trend toward a higher sensitivity for Mapk pathway inhibition in BCR-ABL^{T315I} cells (**Supplementary Figs. 15** and **16**).

Moreover, factorial dilutions were prepared in which either danusertib or bosutinib was replaced with a tool compound for the respective targets of interest. Replacing bosutinib with U0126, a widely used Mek inhibitor, and replacing danusertib with Erk inhibitor II or the Pyk2 kinase inhibitor PF431396 preserved the initially obtained synergy in the higher dose ranges of the tool

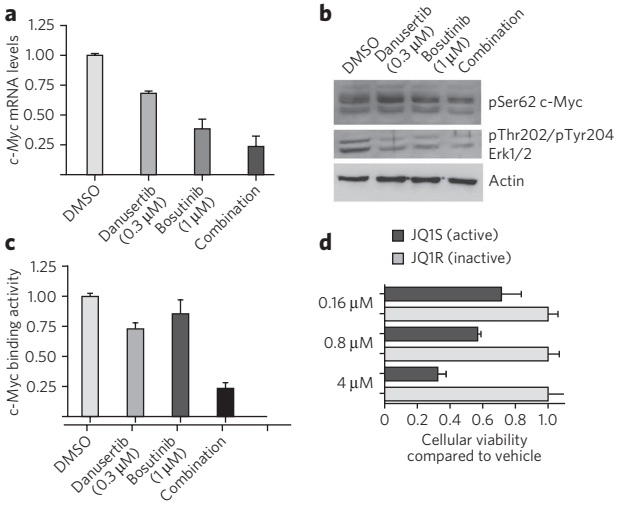


Figure 5 | Combination of danusertib and bosutinib interferes on a post-translational level with *c-Myc* transcriptional activity. (**a**) *c-Myc* mRNA levels after combined drug treatment for 6 h. Results represent the mean ± s.d. of triplicates. (**b**) Immunoblot of c-Myc phosphorylated at Ser62 (c-Myc pSer62), Erk1 and Erk2 phosphorylated at Thr202 and Tyr204, respectively (pThr202/pTyr204) and actin of Ba/F3 BCR-ABL^{T315I} cells treated as indicated for 2 h. (**c**) DNA binding activity of endogenous c-Myc determined by ELISA. Data are the mean ± s.d. of triplicates. (**d**) Viability of Ba/F3 BCR-ABL^{T315I} cells treated as indicated for 72 h. Results represent the mean ± s.d. of triplicates. Raw immunoblot data for **b** are shown in **Supplementary Figure 24**.

compounds. Pharmacological inhibition of Pyk2 resulted in reduced Erk phosphorylation (**Fig. 4d** and **Supplementary Figs. 17** and **18**).

Combined drug treatment impairs c-Myc activity

Following up on the transcriptome analysis, we investigated the molecular logic of the global downregulation of c-Myc target genes and the possible consequences of this downregulation on cellular fitness. *c-Myc* itself was among the most significantly downregulated genes, which was validated by quantitative RT-PCR. (**Fig. 5a** and **Supplementary Fig. 19**). Notably, this did not translate to downregulation of c-Myc protein until 48 h of drug treatment (**Supplementary Fig. 20a**). Thus, the downregulation at the mRNA level and the functional consequence of overall downregulation of c-Myc–dependent transcriptional networks seem to be independent events. To examine the possibility that the reduction in target gene expression was a result of altered cellular localization of c-Myc, nuclear extracts were generated after 48 h of combined drug treatment and were compared with vehicle-treated controls (**Supplementary Fig. 20b**). No obvious differences in nuclear localization were apparent, which is indicative of another mechanism of action. Intersecting the quantitative phosphoproteomics profiles with the transcriptomics data highlighted the Mapk signaling cascade, which was one of the most substantially affected pathways, as one of the most prominent upstream pathways of c-Myc. Erk is known to phosphorylate c-Myc at Ser62, which correlates with the transcriptional activity of c-Myc[28–31]. Hence, an immunoblot analysis of pErk (phosphorylated at Thr202 and Tyr204) was performed to validate the impact of the combined drug treatment on Mapk signaling (**Fig. 5b**). Both compounds showed relatively similar effects on their own and cooperativity when combined, thus validating the findings of the large-scale phosphoproteomics data set. In comparison, the dynamics of the phosphorylation of Ser62 were affected in a way that only the combined application of both kinase inhibitors had a measurable effect (**Fig. 5b** and **Supplementary Fig. 21**). To test whether this observation also translates into functional impairment of c-Myc activity, ELISA-based binding assays were performed using nuclear extracts from Ba/F3 BCR-ABL^{T315I} cells that were treated for 2 h with vehicle, single compounds or their combination to assess Myc binding to spotted double-stranded oligos that contain c-Myc consensus binding sites. Whereas danusertib and bosutinib alone had only a relatively mild impact on the binding of endogenous c-Myc to its consensus sequence, the combination treatment significantly ($P < 0.005$) reduced binding affinity at equal c-Myc loading (**Fig. 5c** and **Supplementary Fig. 22**). To further study the impact of targeting c-Myc in BCR-ABL^{T315I}–dependent cells, we used JQ1, a recently described BRD4 inhibitor that downregulates c-Myc on both the mRNA and protein levels[32,33]. Notably, only the active enantiomer JQ1S reduced c-Myc mRNA levels in Ba/F3 BCR-ABL^{T315I} cells, whereas treatment with the inactive enantiomer JQ1R did not (**Supplementary Fig. 23**). Consistently, only JQ1S reduced cellular viability, thus independently validating the dependence of Ba/F3 BCR-ABL^{T315I} cells on c-Myc (**Fig. 5d**).

DISCUSSION

We describe a systems-wide approach aimed at capturing and exploring the molecular mechanisms behind the synergistic drug-drug interaction of the clinical kinase inhibitors danusertib and bosutinib. This synergy was specific for CML cells featuring the BCR-ABL^{T315I} gatekeeper mutation and thus addresses an unmet medical need, as this mutation confers resistance to all currently approved kinase inhibitors for CML. The approach of integrating mass spectrometry–based target profiles with phosphoproteomic and transcriptional data sets should have broad applicability to other disease settings and biological questions.

The specificity of the observed synergistic drug interaction for the BCR-ABL^{T315I} gatekeeper-transformed cells over BCR-ABL wild-type cells was perhaps surprising. At least two explanations for this relationship seem feasible. One possible explanation might be that each drug is already highly potent against BCR-ABL wild-type–transformed cells, which may mask any other effect and make the detection of cooperative effects on downstream pathways technically difficult. Another explanation may be qualitative changes in the signaling properties of BCR-ABL^{T315I} as compared to wild-type BCR-ABL. Monitoring the cellular markers of wild-type BCR-ABL activity through careful titration of drug inhibition or stepwise induction of BCR-ABL expression has shown that phosphorylation of Stat5 is the most sensitive cellular readout for BCR-ABL kinase activity, followed by total phosphotyrosine levels and, finally, phosphorylation of Crkl (ref. 34). In BCR-ABL^{T315I} mutant cells, however, we found pStat5 and pCrkl levels to be relatively insensitive to BCR-ABL^{T315I} inhibition. Instead, BCR-ABL^{T315I} seemed to depend more strongly on Mapk signaling, as combined targeting of this pathway by bosutinib and danusertib conferred increased sensitivity. This seems consistent with qualitative changes in the signaling properties of BCR-ABL with mutations in the gatekeeper residue in addition to quantitative effects that may be cell-type and assay dependent[35,36]. Though we found a heightened dependence on, but not enhanced activity of, Map kinases in BCR-ABL^{T315I} cells, these may be related phenomena, and they highlight the particular synergy between the BCR-ABL and the Mapk pathways[18].

Both chemical proteomics and phosphoproteomics analyses suggested bosutinib as an inhibitor of the BCR-ABL gatekeeper kinase activity, which was subsequently confirmed by immunoblotting and *in vitro* kinase assays. It is, however, important to note that this enzymatic inhibition did not translate into substantial cellular efficacy of bosutinib in the context of BCR-ABL^{T315I}–positive CML, which is consistent with clinical reports[37]. This might indicate activation of an unknown downstream roadblock by bosutinib through one of its multiple off targets. It is also noteworthy that, although danusertib and bosutinib both inhibit BCR-ABL^{T315I}

kinase activity, combination *in vitro* kinase assays suggested that the observed synergy was not based on cooperativity in regard to the inhibition of BCR-ABL^{T315I} itself. Therefore, this effect must be predominantly attributed to inhibition of other targets.

Our observations converge on the Mapk signaling cascade as the predominantly affected pathway of the synergistic drug combination. Chemical proteomics identified several Map3ks, Map4ks and, particularly, Mek1 and Mek2 as specific targets of bosutinib, whereas Erk1 and Erk2 interacted exclusively with danusertib. In addition, Pyk2 (Ptk2b), one of the most prominent targets of danusertib (half-maximal inhibitory concentration (IC_{50}) = 79.9 nM; **Supplementary Fig. 8**), is known to also feed into Mapk signaling through Erk phosphorylation (**Supplementary Fig. 18**). Although BCR-ABL^{T315I} was bound by both drugs, the global phosphoproteome survey showed little combinatorial drug effects on canonical BCR-ABL downstream signals other than those culminating in the Mapk pathway, especially Gab2 Ser604, Mek1 Ser222 and Erk2 Tyr185. Querying the drug-dependent transcriptomic signature for significantly ($P < 0.01$) regulated transcription factor motifs highlighted c-Myc as a crucial factor in transmitting the cellular response to combined treatment with danusertib and bosutinib.

Myc has a well-established, but still not completely understood, role in a broad spectrum of human cancers because of its highly promiscuous features[38]. In the context of CML, c-Myc is required for BCR-ABL–mediated transformation[39]. It has also been reported as an essential gene in CML cell lines and has been linked to the clinical response to imatinib[40,41]. Because of the lack of a chemically tractable enzymatic function, pharmacological targeting of c-Myc is a formidable challenge for which promising steps have only recently been made[32,33]. The BRD4-targeting compound JQ1 seems to suppress mainly transcriptional expression of c-Myc. We have not been able to obtain reliable results in our attempts to downregulate c-Myc by alternative methods, such as inducible RNA expression, probably because c-Myc is essential for cellular survival. We found that combined drug treatment with bosutinib and danusertib led to reduced engagement of c-Myc DNA binding sites and downregulation of c-Myc target genes. Our results indicate regulation of c-Myc activity at the post-translational level. C-Myc is known to be phosphorylated on several N-terminal residues that are linked to protein stability and transcriptional activity[42]. Ser62 phosphorylation, which is known to be mediated by MAP kinase signaling[43], is among the most prominent post-translational modifications, and although it has been mainly described as a stabilizing event, there is evidence that it has a direct role in modulating transcriptional activity[30,43].

The ability to effectively integrate large omics data sets to elucidate the molecular effects of genetic or chemical perturbation of biological systems remains a challenge. This is particularly true for the application of multiple pharmacological agents[44]. The approach described here is empowered by the use of quantitative mass spectrometry–derived chemical proteomics profiling of the cellular targets of compounds. The obtained 'physical' link between the perturbing agent and the cellular repertoire of molecules is sufficiently direct and reliable to represent a strong vantage point for subsequent integration with other data sets through the use of network and pathway analysis. We have thus been able to elucidate the impact of a new synergistic drug interaction in a clinically relevant, highly drug-resistant disease setting. The study revealed a nonobvious synergistic mechanism of action that is elicited by several off targets of the two small molecules. Thus, the polypharmacology of kinase inhibitors with good safety profiles represents, in this case, an advantage that allows for their versatile and combinatorial use in the quest for stratified cancer therapy[6,7].

METHODS

Cell lines and reagents. The parental Ba/F3 cell line was obtained from the American Type Culture Collection and was cultured in RPMI and 10% fetal calf serum (FCS). Imatinib, nilotinib, dasatinib, bosutinib and tozasertib were purchased from LC Laboratories. Danusertib, CI-1040, AZD6244 and sorafenib were purchased from Selleck Chemicals. Bafetinib, c-bafetinib, c-dasatinib, c-nilotinib and c-tozasertib were synthesized by WuXi AppTec. C-imatinib, c-sorafenib, c-bosutinib and c-danusertib were synthesized by Siokem, Gateway Pharma, Vichem Chemie and AMRI, respectively. C-imatinib, c-nilotinib, c-bafetinib and c-sorafenib were esterified with *N*-Boc-glycine and deprotected with trifluoroacetic acid as part of the coupling procedure. U0126 and Erk inhibitor II were purchased from Sigma-Aldrich and Calbiochem, respectively. PF431396 was purchased from Tocris. JQ1S and JQ1R were kindly provided by J. Bradner (Dana-Farber Cancer Institute, Boston, Massachusetts, USA). The purity (>94%) of all compounds was confirmed using HPLC and MS analysis. All compounds were dissolved in DMSO as 10 mM stock solutions. Further chemical characterization data for c-imatinib, c-nilotinib, c-bafetinib, c-sorafenib and c-danusertib can be found in the **Supplementary Methods**.

Immunoblot analysis. Immunoblotting was performed according to the manufacturer's recommendations for antibodies to pStat5A and pStatB (1:1,000, Tyr694 and Tyr699 for pStat5A and pStat5B, respectively; 94-10C-9-10C-2, Millipore); pSrc family (1:1,000, Tyr416, 2101, Cell Signaling Technology); pCrkl (1:1,000, Tyr207, 3181, Cell Signaling Technology); actin (1:2,000, AAN01, Cytoskeleton); Abl (1:1,000, Ab-3, Calbiochem); total phosphotyrosine 4G10 (1:2,000, Upstate Biotechnology); total Myc (1:7,000, IR-Dye800 conjugated a-Myc epitope tag (Rb), 600-432-381, Rockland); pMyc (1:1,000, Ser62, ab51156, Abcam); Erk1 and Erk2 (1:2,500, M5670, Sigma-Aldrich); pErk1 and pErk2 (1:1,000, Thr202 and Tyr204 for isoforms 1 and 2, respectively, 9106, Cell Signaling Technology); and Rcc-1 (1:1,000, sc-55559, Santa Cruz).

Viability assays and synergy determination. Viability assays were performed in triplicates using the Cell Titer Glo assay (Promega) after 72 h of drug exposure to cells initially seeded at a density of 10^5 cells per ml. Point-wise synergy screening was performed by deriving EC_{20} values for each compound and combining them in every possible pairwise combination. Experimentally derived values were compared with values predicted by the Bliss additivity model[19]. Thirty-six–point dose-response matrices have been established and evaluated as described elsewhere[20].

Proteomics analysis and MS strategies. More detailed information on the immobilization of small molecules, affinity purification, tryptic digestion, peptide purification, iTRAQ labeling and liquid chromatography, as well as data extraction, database searching and relative quantification, is outlined in the **Supplementary Methods**.

Apoptosis and cell-cycle measurements. Induction of apoptosis was measured 16 h after drug exposure using the Caspase Glo assay (Promega) measuring the cleavage of caspase 3 and caspase 7. Experiments were performed in triplicate. The cell-cycle analysis was performed by staining DNA with propidium iodide after 36 h of exposure to the respective drugs.

Colony formation assay. After retroviral transduction of primary mouse bone marrow cells with BCR-ABL^{T315I} internal ribosomal entry site GFP, GFP-positive cells were isolated using fluorescence-activated cell sorting and seeded in cytokine-free methylcellulose. Colonies were scored after 10 d.

***Ex vivo* proliferation assays using primary patient material.** Primary cells were obtained from the bone marrow of two patients with advanced BCR-ABL^{T315I} CML and one patient with advanced CML and no detectable BCR-ABL mutation. Mononuclear cells were isolated using Ficoll. They were then incubated in triplicate in RPMI 1640 medium with FCS in the presence or absence of various concentrations of bosutinib or danusertib, either as single agents or in combination at a fixed ratio of drug concentrations at 37 °C for 48 h. After incubation, ^{3}H-thymidine (PerkinElmer; 0.5 µCi per well) was added for another 16 h. Cells were then harvested on filter membranes (Packard Bioscience) in a Filtermate 196 harvester (Packard Bioscience). Filters were then air dried, and the bound radioactivity was measured in a β-counter (Top-Count NXT, Packard Bioscience).

Kinase assays. Kinase assays for BCR-ABL^{T315I}, AURKA (c-danusertib) and PDGFRA (c-sorafenib) were performed using the KinaseProfiler (Millipore). PYK2 inhibition by danusertib was assessed using the SelectScreen ZLYTE platform (Life Technologies). MAP2K5 and MAP3K3 kinase assays were conducted with the STK-ELISA platform (Carna Biociences). CDK7 and NEK9 kinase assays were conducted using the off-chip Mobility Shift Assay (Carna Biosciences). C-imatinib and c-nilotinib were assayed *in vitro* for inhibition of recombinant full-length c-ABL (Upstate Biotechnology), as described previously[45]. All kinase assays were performed using ATP concentrations that approximately equaled the K_m of the respective kinase.

Microarray analysis. Information on treatment conditions and data handling can be found in the **Supplementary Methods**.

MYC binding assays. Binding of endogenous c-MYC to its consensus sites was assessed by TransAM DNA binding ELISA assays (Active Motif, 43396) according to the manufacturer's recommendations.

Statistics. Two-tailed *t* tests were used for statistical analyses, and a difference was considered significant when $P < 0.05$. Further statistical considerations of the data-analysis tools used in the manuscript are provided either in the **Supplementary Methods** (for the microarray analysis and SAM analysis tool) or in the respective original publications[23,25].

Received 29 June 2012; accepted 30 August 2012; published online 30 September 2012

References

1. Kitano, H. Cancer as a robust system: implications for anticancer therapy. *Nat. Rev. Cancer* **4**, 227–235 (2004).
2. Lehár, J. *et al.* Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nat. Biotechnol.* **27**, 659–666 (2009).
3. Lamb, J. *et al.* The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science* **313**, 1929–1935 (2006).
4. Rix, U. & Superti-Furga, G. Target profiling of small molecules by chemical proteomics. *Nat. Chem. Biol.* **5**, 616–624 (2009).
5. Pan, C., Olsen, J.V., Daub, H. & Mann, M. Global effects of kinase inhibitors on signaling networks revealed by quantitative phosphoproteomics. *Mol. Cell. Proteomics* **8**, 2796–2808 (2009).
6. Hopkins, A.L. Network pharmacology: the next paradigm in drug discovery. *Nat. Chem. Biol.* **4**, 682–690 (2008).
7. Knight, Z.A., Lin, H. & Shokat, K.M. Targeting the cancer kinome through polypharmacology. *Nat. Rev. Cancer* **10**, 130–137 (2010).
8. Quintás-Cardama, A. & Cortes, J. Molecular biology of BCR-ABL1–positive chronic myeloid leukemia. *Blood* **113**, 1619–1630 (2009).
9. Druker, B.J. Imatinib as a paradigm of targeted therapies. *Adv. Cancer Res.* **91**, 1–30 (2004).
10. Gorre, M.E. *et al.* Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* **293**, 876–880 (2001).
11. Donato, N.J. *et al.* Imatinib mesylate resistance through BCR-ABL independence in chronic myelogenous leukemia. *Cancer Res.* **64**, 672–677 (2004); erratum **64**, 2306 (2004).
12. Mahon, F.X. *et al.* Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. *Blood* **96**, 1070–1079 (2000).
13. Zhang, J., Yang, P.L. & Gray, N.S. Targeting cancer with small molecule kinase inhibitors. *Nat. Rev. Cancer* **9**, 28–39 (2009).
14. Bixby, D. & Talpaz, M. Seeking the causes and solutions to imatinib-resistance in chronic myeloid leukemia. *Leukemia* **25**, 7–22 (2011).
15. O'Hare, T. *et al.* AP24534, a pan–BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. *Cancer Cell* **16**, 401–412 (2009).
16. Eide, C.A. *et al.* The ABL switch control inhibitor DCC-2036 is active against the chronic myeloid leukemia mutant BCR-ABLT315I and exhibits a narrow resistance profile. *Cancer Res.* **71**, 3189–3195 (2011).
17. Grebien, F. *et al.* Targeting the SH2-kinase interface in Bcr-Abl inhibits leukemogenesis. *Cell* **147**, 306–319 (2011).
18. Packer, L.M. *et al.* Nilotinib and MEK inhibitors induce synthetic lethality through paradoxical activation of RAF in drug-resistant chronic myeloid leukemia. *Cancer Cell* **20**, 715–727 (2011).
19. Bliss, C.I. The toxicity of poisons applied jointly. *Ann. Appl. Biol.* **26**, 585–615 (1939).
20. Borisy, A.A. *et al.* Systematic discovery of multicomponent therapeutics. *Proc. Natl. Acad. Sci. USA* **100**, 7977–7982 (2003).
21. Breitwieser, F.P. *et al.* General statistical modeling of data from protein relative expression isobaric tags. *J. Proteome Res.* **10**, 2758–2766 (2011).
22. Ross, P.L. *et al.* Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* **3**, 1154–1169 (2004).
23. Dennis, G. Jr. *et al.* DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* **4**, P3 (2003).
24. Chen, X. *et al.* Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* **133**, 1106–1117 (2008).
25. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA* **102**, 15545–15550 (2005).
26. Schuhmacher, M. *et al.* The transcriptional program of a human B cell line in response to Myc. *Nucleic Acids Res.* **29**, 397–406 (2001).
27. Dopazo, J. & Carazo, J.M. Phylogenetic reconstruction using an unsupervised growing neural network that adopts the topology of a phylogenetic tree. *J. Mol. Evol.* **44**, 226–233 (1997).
28. Gupta, S., Seth, A. & Davis, R.J. Transactivation of gene expression by Myc is inhibited by mutation at the phosphorylation sites Thr-58 and Ser-62. *Proc. Natl. Acad. Sci. USA* **90**, 3216–3220 (1993).
29. Seth, A., Alvarez, E., Gupta, S. & Davis, R.J. A phosphorylation site located in the NH_2-terminal domain of c-Myc increases transactivation of gene expression. *J. Biol. Chem.* **266**, 23521–23524 (1991).
30. Hann, S.R. Role of post-translational modifications in regulating c-Myc proteolysis, transcriptional activity and biological function. *Semin. Cancer Biol.* **16**, 288–302 (2006).
31. Benassi, B. *et al.* c-Myc phosphorylation is required for cellular response to oxidative stress. *Mol. Cell* **21**, 509–519 (2006).
32. Delmore, J.E. *et al.* BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **146**, 904–917 (2011).
33. Filippakopoulos, P. *et al.* Selective inhibition of BET bromodomains. *Nature* **468**, 1067–1073 (2010).
34. Hantschel, O. *et al.* BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. *Nat. Chem. Biol.* **8**, 285–293 (2012).
35. Griswold, I.J. *et al.* Kinase domain mutants of Bcr-Abl exhibit altered transformation potency, kinase activity, and substrate utilization, irrespective of sensitivity to imatinib. *Mol. Cell. Biol.* **26**, 6082–6093 (2006).
36. Azam, M., Seeliger, M.A., Gray, N.S., Kuriyan, J. & Daley, G.Q. Activation of tyrosine kinases by mutation of the gatekeeper threonine. *Nat. Struct. Mol. Biol.* **15**, 1109–1118 (2008).
37. Cortes, J.E. *et al.* Safety and efficacy of bosutinib (SKI-606) in chronic phase Philadelphia chromosome-positive chronic myeloid leukemia patients with resistance or intolerance to imatinib. *Blood* **118**, 4567–4576 (2011).
38. Pelengaris, S., Khan, M. & Evan, G. c-MYC: more than just a matter of life and death. *Nat. Rev. Cancer* **2**, 764–776 (2002).
39. Sawyers, C.L., Callahan, W. & Witte, O.N. Dominant negative MYC blocks transformation by ABL oncogenes. *Cell* **70**, 901–910 (1992).
40. Luo, B. *et al.* Highly parallel identification of essential genes in cancer cells. *Proc. Natl. Acad. Sci. USA* **105**, 20380–20385 (2008).
41. Albajar, M. *et al.* MYC in chronic myeloid leukemia: induction of aberrant DNA synthesis and association with poor response to imatinib. *Mol. Cancer Res.* **9**, 564–576 (2011).
42. Adhikary, S. & Eilers, M. Transcriptional regulation and transformation by Myc proteins. *Nat. Rev. Mol. Cell Biol.* **6**, 635–645 (2005).
43. Meyer, N. & Penn, L.Z. Reflecting on 25 years with MYC. *Nat. Rev. Cancer* **8**, 976–990 (2008).
44. Palsson, B. & Zengler, K. The challenges of integrating multi-omic data sets. *Nat. Chem. Biol.* **6**, 787–789 (2010).
45. Rix, U. *et al.* A comprehensive target selectivity survey of the BCR-ABL kinase inhibitor INNO-406 by kinase profiling and chemical proteomics in chronic myeloid leukemia cells. *Leukemia* **24**, 44–50 (2010).

Acknowledgments

We thank J. Bradner (Dana Faber Cancer Institute–Harvard Medical School) for providing JQ1S and JQ1R and R. Giambruno, C. Tan, J. Bigenzahn and O. Hantschel for skillful advice and help. We also thank J. Lehar for inspiring discussions and S. Nijman and O. Hantschel for carefully reading this manuscript. We thank Cell Signaling Technology for allowing reproduction of the kinome map. We acknowledge L. Brecker for measuring NMR spectra. The present work was, in part, financed by the 'GEN-AU' initiative of the Austrian Federal Ministry for Science and Research (PLACEBO GZ BMWF-70.081/0018-II/1a/2008) as well as the Austrian Science Fund (P 24321-B21).

Author contributions

G.E.W. designed and performed the experiments, analyzed and interpreted the data, performed statistical analyses, made the figures and wrote the manuscript. U.R. designed and performed the experiments, analyzed and interpreted the data, performed statistical analyses, made the figures and wrote the manuscript. S.M.C. performed phospho-proteomics studies and analyzed and interpreted the resulting data. K.V.G. performed experiments in primary human samples and analyzed and interpreted the data. F.G. performed fluorescence-activated cell sorting analysis and helped perform colony formation assays. M.G. carried out immunoblot experiments. A.C.M. performed pre-phosphoproteomic screening studies and analyzed quantitative drug pulldowns by mass spectrometry. F.P.B. analyzed quantitative proteomics data and performed bioinformatic experiments. M.B. performed microarray experiments. J.C. analyzed chemical proteomics experimental data and performed bioinformatic analysis. P.V. planned experiments, contributed patient samples and gave advice on the research. K.L.B. planned experiments and analyzed chemical proteomics experiments. F.M.W. planned experiments and analyzed phosphoproteomics data. G.S.-F. conceived of the experimental strategy with G.E.W. and U.R., had overall responsibility for the research and wrote and edited the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to G.S.-F.

3.3 Global gene-disruption identifies novel resistance mechanism to the clinical survivin inhibitor YM155

Georg E Winter, Vincent Blomen, Kilian Huber, Claudia Trefzer, Doris Chen, Thorsten Klampfl, Robert Kralovics, Stefan Kubicek, Thijn R Brummelkamp and Giulio Superti-Furga

Global gene disruption identifies novel resistance mechanism to the clinical survivin inhibitor YM155

Georg E. Winter[1], Vincent Blomen[2], Kilian Huber[1], Claudia Trefzer[1], Manuela Gridling[1], Doris Chen[1], Kumaran Kandasamy[1], Thorsten Klampfl[1], Robert Kralovics[1], Stefan Kubicek[1], Thijn R. Brummelkamp[1,2] and Giulio Superti-Furga[1]

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14 1090 Vienna, Austria
[2]Division of Biochemistry, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands

Correspondence

Giulio Superti-Furga, Ph.D., CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14, AKH BT 25.3, 1090 Vienna, Austria, e-mail: gsuperti@cemm.oeaw.ac.at, phone: +43-1-40160-70001, fax: +43-1-40160-970000

Abstract

Understanding resistance mechanisms is important for improving therapeutic options in cancer. To determine the genomic requirements of the survivin-inhibitor YM155 being assessed in various cancer trials, we performed a global gene-disruption approach in haploid human cells and identified SLC35F2, an uncharacterised member of the solute carrier family, as the central mediator for cytotoxicity. Deletion of SLC35F2 induced a drug-tolerant cellular state by preventing intracellular drug accumulation. Our results establish SLC35F2 expression levels as a fundamental biomarker for an effort evaluating the clinical efficacy of YM155.

Introduction&Results

Genomic heterogeneity and a high mutational rate are inherent to human cancers and facilitate the adaption to environmental stimuli and selective pressures, thus conveying the acquisition of resistances against therapeutic intervention. Adoption of drug resistances is one of the most pressing challenges in treating cancer and is an issue shared by conventional chemotherapy as well as by targeted approaches exploiting cancer specific dependencies. Consequently, clinical benefits are often disappointing and short-lived.[1,2]
Classical models of drug resistances include secondary point mutations in the protein target of a drug, amplification of the target itself, hyperactivation of downstream- and parallel signalling networks or alterations in the tumor microenvironment.[3-6] However, drug resistances can also occur by alteration of pharmacokinetic parameters. This has been predominantly linked to increased activation or expression of efflux pumps of the ABC protein group.[7] Vice-versa, attenuating the efficacy of a small molecule drug via decreased intracellular concentrations can also happen due to a lower uptake levels if a compound depends on active or facilitated transport.[8,9] To what degree drug-like small molecules are subject to transporter dependencies is a very actively debated field of pharmacology.[10-13] While systematic assessments in yeast have shown that drug-carrier interactions are frequent, these dependencies are less well understood in humans which is attributable, among others, to increased genomic complexity and thus potential redundancies.[14]

YM155 (sepantronium bromide, **Figure 1A**) is a small molecule agent with *in vitro* and *in vivo* anti-tumor activity that is currently in several clinical trials for Non-Small Cell Lung Cancer (NSCLC), metastatic breast cancer and Non-Hodgkin's Lymphoma. Although used in patients, the precise mode of action of YM155 remains elusive. Downregulation of the anti-apoptotic protein survivin through binding to Interleukin Enhancer-binding Factor 3/NF110 transcription factor has been suggested as the primary mode of action of YM155 although recent studies propose induction of DNA damage as an alternative or additional

mode of action.[15-17] However, despite a remarkably potency *in vitro*, the first results of clinical trials with YM155 as single agent have proven rather disappointing.[18]

With the intention of elucidating the molecular logic of YM155-induced cytotoxicity as well as uncovering potential genetic roadblocks for its clinical efficacy, we devised a large scale insertional mutagenesis approach in the near-haploid human cell line KBM7.[19] This global gene-disruption approach provides means to create loss of function phenotypes and can thus be enabled to uncover the genomic requirements for the action of YM155.

In detail, we heavily mutagenized $1x10^8$ KBM7 cells with a retroviral gene-trap vector that inserts randomly into the genome. Insertions occur genome-wide, preferentially at actively transcribed genes, disrupt the genomic locus and confer truncations of the underlying transcript via a strong spice acceptor site coupled to a stop codon.[20] Subsequently, the complex mutagenized cell population was selected with 100 nM YM155 which resulted in the clonal outgrowth of approximately 1000 resistant colonies. To identify mutations in genes causally linked to drug resistance, genomic DNA was isolated from the entire $KBM7^{GT}$ (genetrapped) pool and sequenced using an inverse-PCR based protocol that was described previously.[20] We found a striking enrichment for retroviral insertions in a gene coding for the Solute Carrier Family Member 35 F2 (SLC35F2) with 122 independent insertion sites mapping to that locus (**Figure 1B, 1C**). In comparison to a large dataset of gene-trap insertions of a non selected KBM7 pool, this enrichment was highly significant (*p*-value = $7.58x10^{-299}$). Interestingly, there was no other locus in the genome that showed enrichment for retroviral insertions thus suggesting that SLC35F2 is, at the given drug concentrations, the dominant genetic determinant of drug sensitivity.

To validate the drug-gene interaction between YM155 and SLC35F2, two independent cell lines carrying gene-trap insertions in sense-orientation directly after the first exon of SLC35F2 were subcloned (henceforce referred to as $KBM7^{GT1}$ and $KBM7^{GT2}$). Successful disruption of the underlying genomic locus was shown via a nested PCR strategy using two primer pairs flanking the insertion site. A shift correlating with the size of the gene-trap cassette was specifically observed in both $KBM7^{GT}$ clones but was absent in $KBM7^{WT}$ cells (**Figure 1D**). We then assessed the impact of the disruption of the locus on SLC35F2 transcript levels. We used a RT PCR primer pair binding downstream of the gene-trap insertion site to measure SLC35F2 mRNA levels. In line with the successful disruption of the genomic locus, transcript levels of SLC35F2 were below the limit of detection for both $KBM7^{GT1}$ and $KBM7^{GT2}$ compared to $KBM7^{WT}$ cells (**Figure 1E**). Thus, the retroviral gene-trap approach generated two isogenic, c loss-of-function clones for SLC35F2.

This allowed for testing the influence of SLC35F2 gene deletion on YM155 efficacy in a dose-dependent manner. To do so, we assayed the impact of YM155 on $KBM7^{WT}$ or $KBM7^{GT}$ cells at various concentrations. After three days of drug incubation, we observed an

approximately 100-fold shift in the half-maximal effector concentration (EC_{50}) of YM155 in clones deficient of SLC35F2 as compared to wildtype cells. This shift was not observed when using the structurally unrelated control drug nilotinib (**Supplementary Figure 1**). To ultimately prove that the loss of SLC35F2 is causal for the observed resistance to YM155 treatment, we re-introduced C-terminal Flag-tagged SLC35F2 cDNA for stable expression in the $KBM7^{GT1}$ clone (**Figure 2A**). We observed a significant shift towards higher sensitivity to YM155 in the reconstituted clone ($KBM7^{recon}$) in three day drug-treatment assays, whereas sensitivity to nilotinib remained unaltered (**Supplementary Figure 1**). As YM155 is affecting the anti-apoptotic machinery, we went on to assay the potential of YM155 to induce apoptosis in this isogenic setup. We treated $KBM7^{WT}$, $KBM7^{GT1}$ and $KBM7^{recon}$ cells with increasing concentrations of YM155 for sixteen hours. Induction of apoptosis was assessed by AnnexinV/PI staining. We monitored a dose-dependent increase of apoptotic cells in $KBM7^{WT}$ that was, with the exception of the highest concentration of YM155, completely abrogated when compared to $KBM7^{GT1}$ although the basal level of apoptosis was marginally increased in $KBM7^{GT1}$ cells (**Figure 2B**). In contrast, re-introduction of SLC35F2 completely reverted the phenotype and restored apoptosis-induction to even elevated levels as compared in $KBM7^{WT}$ cells. (**Figure 2B**, **Supplementary Figure 2**).

We went on to study the effect of SLC35F2 deletion on the YM155 induced downregulation of survivin protein levels. Similar to the above described setting, we treated all three cell types with increasing concentrations of YM155 for 24 hours. Whereas in $KBM7^{WT}$ cells, survivin protein levels were severely reduced post drug treatment, we did not observe altered levels in $KBM7^{GT1}$ cells using the same assay conditions (**Figure 2C**). Again, the reconstituted clone showed a reversed phenotype with an extent of survivin downregulation comparable to wildtype cells (**Figure 2C**). In summary, these results indicate that YM155 is entirely dependent on SLC35F2 in its capability to cause apoptotic cell death and downregulation of the anti-apoptotic factor survivin.

In order to exclude that this dependency is restricted to KBM7 cells, we aimed to transfer the findings to another cell system. Given the clinical settings YM155 has been tested in, we have chosen to validate our findings in Ras-mutant A549 NSCLC cells. After introducing different lentiviral shRNA hairpins targeting SLC35F2, we found that stable knockdown of SLC35F2 caused resistance to YM155 treatment also in these lung cancer cells. (**Figure 2D**, **Supplementary Figure 3**). In order to investigate if SLC35F2 levels are rate-limiting for YM155 induced cytotoxicity also in A549 cells, we retrovirally overexpressed C-Flag tagged SLC35F2. Stable overexpression of SLC35F2 in A549 cells resulted in a hypersensitivity to YM155 treatment in stably overexpressing A549 cells in short- as well as long term treatment conditions (**Figure 2E**, **2F**). Thus, the significance of SLC35F2 for the toxicity of YM155 is not restricted to KBM7 cells but can be extended also to a clinically

relevant cell system. Also here, expression levels appear to be rate-limiting for the action of YM155.

To investigate whether the observed resistance to YM155 via loss of SLC35F2 protein function could also occur in a gene-trap independent situation, we treated non-mutagenized KBM7^{WT} cells over a period of three weeks with 200 nM YM155. Subsequently, we isolated 65 individual KBM7 cell clones (KBM7^{YM155R}) that were resistant to continuous drug exposure. We observed a frequency of monoclonal outgrowth of about 1 in 1x10^5 initially seeded cells. Notably, 12 out of 12 clones tested remained completely resistant to 200 nM YM155 also after drug withdrawal for additional 4 weeks (**Supplementary Figure 4**). Thus, the resistance mechanism is heritable and is not caused by a dynamic and reversible small subpopulation of resistant clones, a phenomenon recently described for lung cancer cell line models.[21] We sequenced the genomic locus and in parallel assessed the expression of SLC35F2 in all 65 isogenic subclones. SLC35F2 expression levels were below the limit of detection in the vast majority of the tested clones (**Figure 2G**). We performed Sanger Sequencing of the genomic locus of SLC35F2 in all 65 KBM7^{YM155R} cell lines but failed to identify any mutations that could potentially impact mRNA stability. Given the fact that SLC35F2 harbours a CpG island in its promoter-region, we went on to study the extent of DNA hypermethylation in seven drug-resistant clones compared to sensitive KBM7^{WT} cells. However, we did not observe augmented hypermethylation of the SLC35F2 CpG island in any of the tested KBM7^{YM155R} clones (data not shown). Therefore, the molecular mechanism behind the strong convergence on SLC35F2 downregulation in YM155-resistant and non-mutagenized settings requires further investigation.

Given that the genetic deletion of SLC35F2 completely abrogated the downregulation of survivin protein levels and therefore the most immediate known phenotype of YM155 action, we hypothesized a mechanism of resistance that is relatively upstream. SLC35F2 is annotated as part of a protein-family that facilitates the transport of nucleotide-sugars through biological membranes. As opposed to the majority of SLC35 family members which are predominantly characterized via intracellular localisation to the Golgi or the endoplasmic reticulum, SLC35F2 and its feline homologue have been reported to localise at the outer cell membrane.[22,23] In line with that, we assessed if SLC35F2 is facilitating the uptake of YM155 and if deletion of SLC35F2 would result in altered intracellular concentrations of YM155. In order to do so we devised Multiple Reaction Monitoring, a mass spectrometry-based method to assay the drug concentrations in KBM7^{WT}, KBM7^{GT1} and KBM7^{recon} cells after 90 minutes treatment with 2 µM YM155. We observed a ten-fold decrease in intracellular drug levels in KBM7^{GT1} cells as opposed to KBM7^{WT} cells. Moreover, we found that in the reconstituted clone, intracellular drug levels were restored to even higher levels as compared to KBM7^{WT} cells (**Figure 3A**). In line with these results, transient overexpression of C-terminal V5

tagged SLC35F2 in HEK293T cells yielded a fifteen-fold increase in intracellular drug levels compared to empty vector control (**Figure 3B**). Finally, we assessed the dependency of the SLC35F2 mediated transport on sodium gradient integrity by blocking the sodium/hydrogen exchanger 1 (SLC9A1) channel with amiloride in YM155 uptake experiments. We observed a dose-dependent reduction in YM155 uptake upon co-incubation with 0.1 and 0.5 mM amiloride, suggesting that SLC35F2 mediated transport through the plasmas-membrane depends on a functional Na/H^+ gradient (**Figure 3C**).

Discussion

Forward genetic screens have successfully been used to elucidate mechanisms of acquired resistance to targeted anti-cancer therapies and to gain insights into their respective mode of action.[5,24,25] Here, we have applied a recently developed global gene-disruption approach in order to identify genes that are functionally required for the survivin inhibitor YM155 which is currently under clinical investigation. To our surprise there was only a single gene (SLC35F2) that conferred resistance to 100 nM YM155 in two independent genetic screens performed on two different mutagenized cell batches. It could be that a novel screen performed under conditions of high ectopic expression of SLC35F2 may bypass the dominance of this genetic bottleneck and identify other genes required for YM155 action. Alternatively, the proposed DNA-damage phenotype elicited by YM155 is a process too pleiotropic to be dependent on the action of single gene-products in a non-redundant fashion and thus prevented the discovery of modulator genes downstream of compound-entry. Using isogenic knockout clones we provide evidence for an absolute dependency of YM155 on SLC35F2 in order to be able to accumulate in cells, downregulate survivin protein levels and induce apoptosis. Given the fact that YM155 is, as single agent as well as part of drug combinations, in clinical trials for various different malignancies like NSCLC and Non-Hodgkin's lymphoma, we believe that monitoring intratumoral expression levels may serve as an important and mechanistically understood biomarker for clinical response and could guide patient and sub-tumour type stratification. Interestingly, SLC35F2 has been found to be upregulated more than 20-fold (p-values:$1.28x10^{-12}$ and $3.65x10^{-5}$) in cassic- as well as desmoplastic medulloblastoma as compared to normal brain tissue and upregulation was also reported in glioblastomas.[26,27] Thus, using YM155 or derivatives thereof with a retained dependency on SLC35F2 could potentially serve as a novel therapeutic strategy for tumour specific targeting of these brain malignancies. Notably, a better understanding of the biological role and specificity of this upregulation in the context of tumour initiation or maintenance would require further investigation with a particular focus on identifying the endogenous ligand(s) transported by SLC35F2.

Performing clinical evaluation of a candidate drug oblivious of what appears to be an absolutely limiting factor of drug accessibility raises important questions on our current knowledge prioritization for investigational new drug applications.

Materials and Methods

Cell lines and reagents

A549 and HEK 293T cell lines were obtained from the American Type Culture Collection and were cultured in DMEM +10% FCS. YM155 and nilotinib were purchased from Selleck Chemicals (Houston, TX, USA) and LC Laboratories (Woburn, MA, USA). Lentiviral shRNA vectors targeting SLC35F2 were purchased from Open Biosystems and are part of the TRC collection.

Haploid genetic screen and sequence analysis

Haploid genetic screening was essentially performed as described recently.[20] In brief, virus was produced by transient transfection of the gene-trap plasmid along with packaging plasmids using Lipofectamine 2000 (invitrogen) in low-passage HEK 293T cells. Virus was concentrated via ultracentrifugation and used to mutagenize $1x10^8$ KBM7 cells via spinfection. The mutagenized pool was expanded for another week. Subsequently $1x10^8$ genetrapped cells were selected with 100 nM YM155 in 96 well plates ($1x10^5$ cells seeded/well). Drug resistant clones were pooled after 10 days of drug exposure, collected in a T175 flask and expanded to a total cell number of $3x10^7$ cells. Genomic DNA was isolated and retroviral insertion sites were detected via an inverse PCR protocol adopted to next generation sequencing.[20] The significance of the enrichment of insertions in a given gene was calculated by comparing the number of insertions of the YM155 selected population with an unselected, larger control dataset using the one-sided Fisher's exact test. The resulting p-values were false-discovery rate corrected.

Subcloning of SLC35F2 deficient KBM7 mutant cell line

Due to the high mutational burden of SLC35F2 in the YM155 selected pool (approximately 16% of all mapped insertions clustered in the SLC35F2 gene), no serial subcloning strategy was required. Instead, $KBM7^{GT}$ cells were seeded at a density of 0.1 cells/well in 384 well plates. Monoclonal colonies were then propagated to 96 well plates and DNA was isolated when 96 wells were near confluent from 60 $KBM7^{GT}$ clones. A nested PCR strategy (sequence of primers upon request) was conducted in order to identify clones harbouring a retroviral insertion directly after the first exon of SLC35F2. For final confirmation of genetrap-localization, the identity of the resulting PCR-product was confirmed by Sanger sequencing.

Determination of intracellular drug levels via multiple reaction monitoring

For YM155, $2x10^6$ cells of each assessed genotype ($KBM7^{WT}$, $KBM7^{GT1}$ and $KBM7^{recon}$) have been treated with 2µM YM155 for 2 hours at 37°C. Subsequently, cells have been washed

three times with ice-cold PBS and directly lysed in 300 µl 80% ice-cold methanol. Lysates were then cleared by centrifugation for 20 minutes at 4°C at 16000 g, supernatants were used for subsequent quantifications by MS. MRM settings were automatically generated using the IntelliStart software (Waters) and quantification was conducted based on the intensity of three daughter ions.

Viability assays

Cellular Viability was assayed using the Cell titer glo assay (Promega) according the manufacturer's recommendations. Standard assay setup consists of 72 hours drug exposure at various concentrations with 10000 or 3000 cells seeded initially (for 96 or 384 well plates). EC_{50} determination and curve fitting was conducted using Graphpad-Prism software.

Apoptosis assays

Induction of apoptosis was determined sixteen hours post drug exposure using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™) following the manufacturer's recommendations.

Lentiviral knockdowns

Lentiviral shRNA constructs from the TRC library have been ordered at Open Biosystems. Virus-production has been conducted using HEK293T producer cells in 6 wells cotransfecting lentiviral packaging plasmids. Successfully transduced target cells have been selected with 5 ng Puromycin/ml culture medium continuously for five days.

Author contributions

G.E.W. designed and performed most of the experiments, analyzed and interpreted the data, made the figures and wrote the manuscript. V.B. helped performing the haploid genetic screen and conducted statistical analysis. K.H. and C.T. helped to perform MRM measurements and gave experimental advice. M.G. assisted with immunoblot analysis. D.C. created circos plot and graphical display of insertion sites. K.K. analyzed data. T.K. operated the next generation sequencer (Illumina HiSeq 2000) and helped with next-generation sequencing data handling. R.K. gave experimental advice and supervised next generation sequencing. S.K. gave experimental advice. T.R.B. codesigned study and gave experimental advice. G.S.F. codesigned and supervised the study and wrote the manuscript.

Figure Legends

Figure 1 (A) The chemical structure of YM155. **(B)** Circos Plot depiction of sequencing results. Every gene that has been mapped with at least one insertion is depicted as a circle, the localization of which is based on its chromosomal position. The size of a circles correlates with the number of insertions that were mapped for that respective gene. P-values decrease from in-to outside and are depicted as log10(-log10) values. **(C)** All mapped insertions in the SLC35F2 locus. Red triangles mark insertions that are in sense orientation compared to SLC35F2, blue triangles mark antisense orientations. **(D)** SLC35F2 locus disruption PCR of KBM7-WT, SLC35F2^{GT1}, SLC35F2^{GT2}. **(E)** SLC35F2 mRNA levels in KBM7-WT, SLC35F2^{GT1}, SLC35F2^{GT2}.

Figure 2 (A) Flag immunoblot of total cell lysates of KBM7-WT, SLC35F2^{GT1} and SLC35F2^{recon} that is stably reconstituted with C-terminal Flag tagged SLC35F2. **(B)** Induction of apoptosis depicted by Annexin-V histograms after treatment of KBM7-WT, SLC35F2^{GT1} and SLC35F2^{recon} with different concentrations of YM155. **(C)** Immonoblot for survivin levels of KBM7-WT, SLC35F2^{GT1} and SLC35F2^{recon} cells exposed to various concentrations of YM155 **(D)** Colony formation assays of A549 cells stably transduced with a lentiviral shRNA hairpin targeting SLC35F2 or a non-targeting control hairpin in the presence of 40 nM and 80 nM YM155. **(E)** Cellular viability of A549 cells stably overexpressing SLC35F2 and an empty vector control after 72 hours of exposure to various concentrations of YM155. Results represent the mean ± s.d. of triplicates **(F)** Colony formation assays of A549 cells stably overexpressing SLC35F2 and an empty vector control in the presence of 20 nM YM155. **(G)** SLC35F2 mRNA expression levels in all 65 KBM7^{YM155R} resistant cells normalized to the expression in non-selected KBM7-WT cells.

Figure 3 (A) Intracellular YM155 levels as determined with multiple reaction monitoring (MRM) in KBM7-WT, SLC35F2^{GT1} and SLC35F2^{recon} cells exposed to 2μM YM155 for 90 minutes. Results represent the mean ± s.d. of triplicates **(B)** Intracellular YM155 levels as determined with MRM in HEK293T cells stably overexpressiong C-V5 tagged SLC35F2 or empty vector control. Results represent the mean ± s.d. of triplicates **(C)** Intracellular YM155 levels as determined with MRM in KBM7-WT cells upon co-incubation with 0.1 and 0.5 mM amiloride. Results represent the mean ± s.d. of triplicates

Supplementary Figure Legends

Supplementary Figure 1

Cellular viability of KBM7^{WT}, KBM7^{GT1} and KBM7^{recon} cells after 72 hours of exposure to various concentrations of YM155 **(A)** and nilotinib **(B)**. Results represent the mean ± s.d. of triplicates.

Supplementary Figure 2

FACS density plots of KBM7^{WT}, KBM7^{GT1} and KBM7^{recon} cells after 16 hours of exposure to various concentrations of YM155 depicting PI and annexin V signal intensities.

Supplementary Figure 3

(A) SLC35F2 mRNA levels of A549 cells stably transduced with shRNA hairpins targeting SLC35F2. Values are normalized to a non-targeting control sh-RNA and represent the mean ± s.d. of triplicates. **(B)** Colony formation assays of A549 cells stably transduced with a lentiviral shRNA hairpin targeting SLC35F2 or a non-targeting control hairpin in the presence of 20 nM, 40 nM and 80 nM YM155.

Supplementary Figure 4

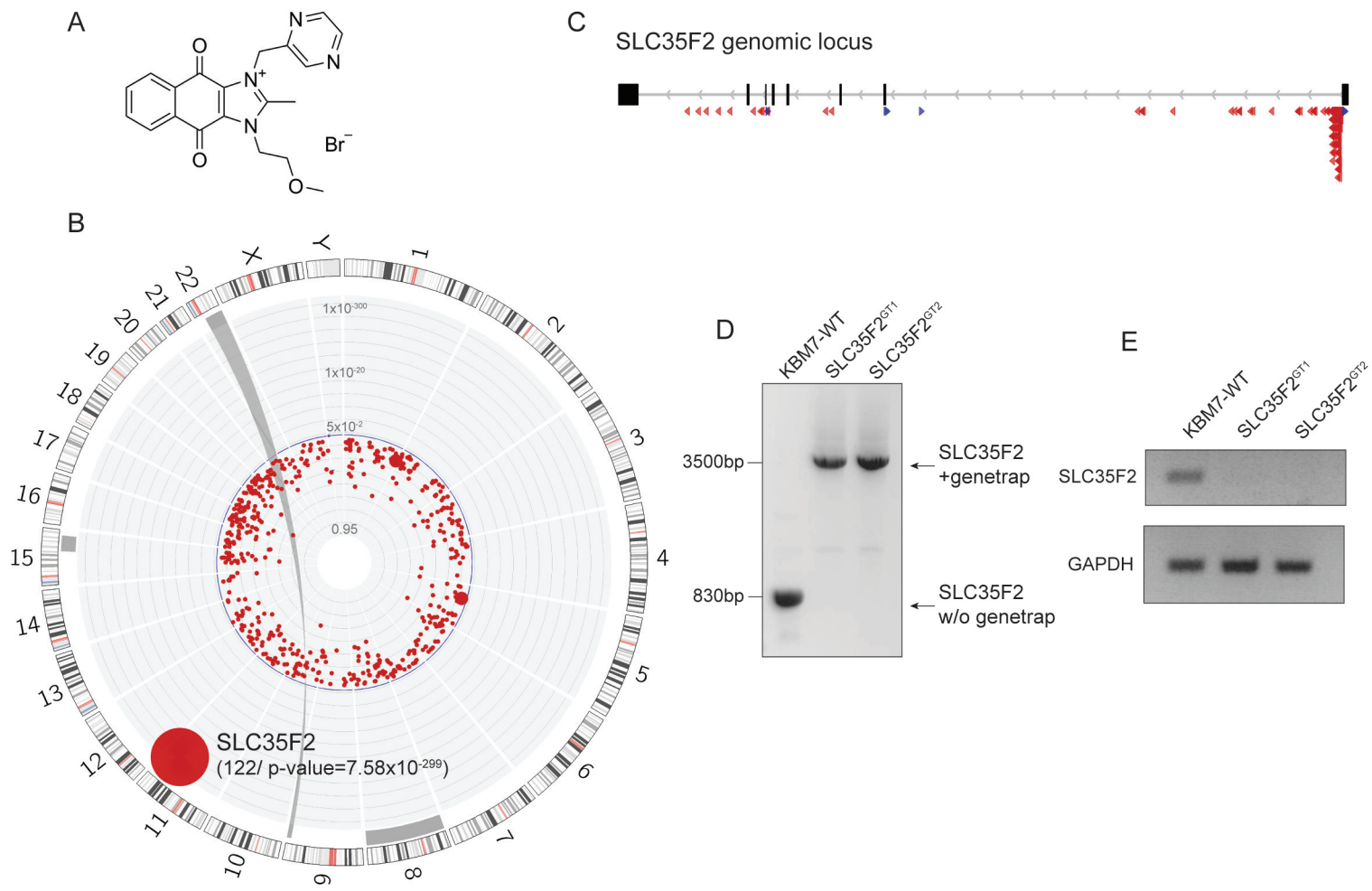
Cellular viability of 12 different KBM7^{YM155R} clones after 72 hours exposure to 200 nM YM155 after being expanded over a period of four weeks in drug-free media. Results represent the mean ± s.d. of triplicates.

References

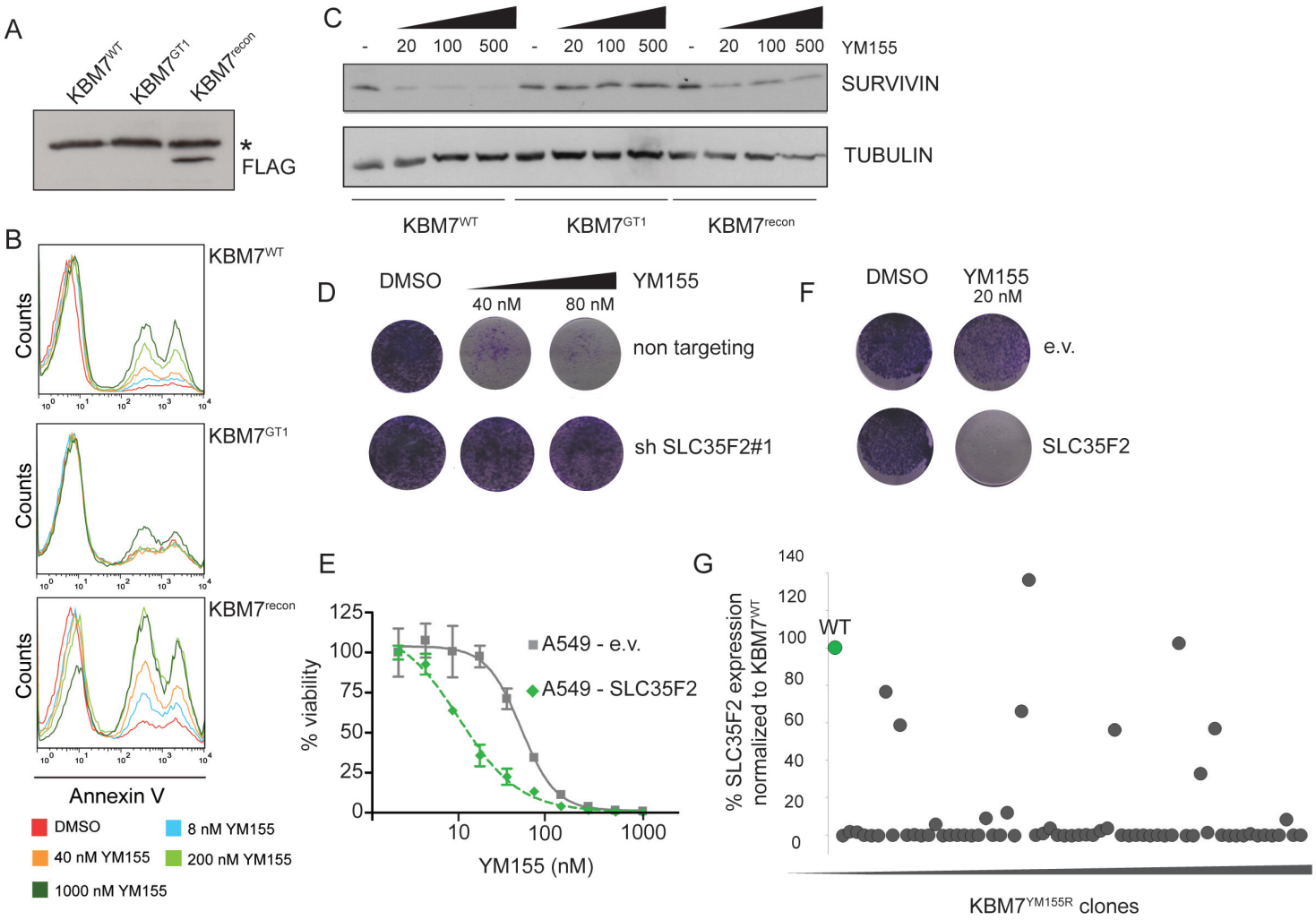
1. Maemondo, M. et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* **362**, 2380-8 (2010).
2. Cataldo, V.D., Gibbons, D.L., Perez-Soler, R. & Quintas-Cardama, A. Treatment of non-small-cell lung cancer with erlotinib or gefitinib. *N Engl J Med* **364**, 947-55 (2011).
3. Gorre, M.E. et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* **293**, 876-80 (2001).
4. Wagle, N. et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol* **29**, 3085-96 (2011).
5. Prahallad, A. et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature* **483**, 100-3 (2012).
6. Straussman, R. et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* **487**, 500-4 (2012).
7. Szakacs, G., Paterson, J.K., Ludwig, J.A., Booth-Genthe, C. & Gottesman, M.M. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* **5**, 219-34 (2006).
8. Gorlick, R. et al. Defective transport is a common mechanism of acquired methotrexate resistance in acute lymphocytic leukemia and is associated with decreased reduced folate carrier expression. *Blood* **89**, 1013-8 (1997).
9. Birsoy, K. et al. MCT1-mediated transport of a toxic molecule is an effective strategy for targeting glycolytic tumors. *Nat Genet* (2012).
10. Dobson, P.D. & Kell, D.B. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nat Rev Drug Discov* **7**, 205-20 (2008).
11. Kell, D.B., Dobson, P.D. & Oliver, S.G. Pharmaceutical drug transport: the issues and the implications that it is essentially carrier-mediated only. *Drug Discov Today* **16**, 704-14 (2011).
12. Sugano, K. et al. Coexistence of passive and carrier-mediated processes in drug transport. *Nat Rev Drug Discov* **9**, 597-614 (2010).
13. Giacomini, K.M. et al. Membrane transporters in drug development. *Nat Rev Drug Discov* **9**, 215-36 (2010).
14. Lanthaler, K. et al. Genome-wide assessment of the carriers involved in the cellular uptake of drugs: a model system in yeast. *BMC Biol* **9**, 70 (2011).
15. Nakahara, T. et al. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. *Cancer Res* **67**, 8014-21 (2007).
16. Glaros, T.G. et al. The "survivin suppressants" NSC 80467 and YM155 induce a DNA damage response. *Cancer Chemother Pharmacol* **70**, 207-12 (2012).
17. Nakamura, N. et al. Interleukin enhancer-binding factor 3/NF110 is a target of YM155, a suppressant of survivin. *Mol Cell Proteomics* **11**, M111 013243 (2012).
18. Giaccone, G. et al. Multicenter phase II trial of YM155, a small-molecule suppressor of survivin, in patients with advanced, refractory, non-small-cell lung cancer. *J Clin Oncol* **27**, 4481-6 (2009).
19. Carette, J.E. et al. Haploid genetic screens in human cells identify host factors used by pathogens. *Science* **326**, 1231-5 (2009).
20. Carette, J.E. et al. Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. *Nat Biotechnol* **29**, 542-6 (2011).
21. Sharma, S.V. et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* **141**, 69-80 (2010).
22. Sarangi, A., Bupp, K. & Roth, M.J. Identification of a retroviral receptor used by an envelope protein derived by peptide library screening. *Proc Natl Acad Sci U S A* **104**, 11032-7 (2007).

23. Hediger, M.A. et al. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction. *Pflugers Arch* **447**, 465-8 (2004).
24. Johannessen, C.M. et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* **468**, 968-72 (2010).
25. Brummelkamp, T.R. et al. An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. *Nat Chem Biol* **2**, 202-6 (2006).
26. Pomeroy, S.L. et al. Prediction of central nervous system embryonal tumour outcome based on gene expression. *Nature* **415**, 436-42 (2002).
27. Lee, J. et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell* **9**, 391-403 (2006).

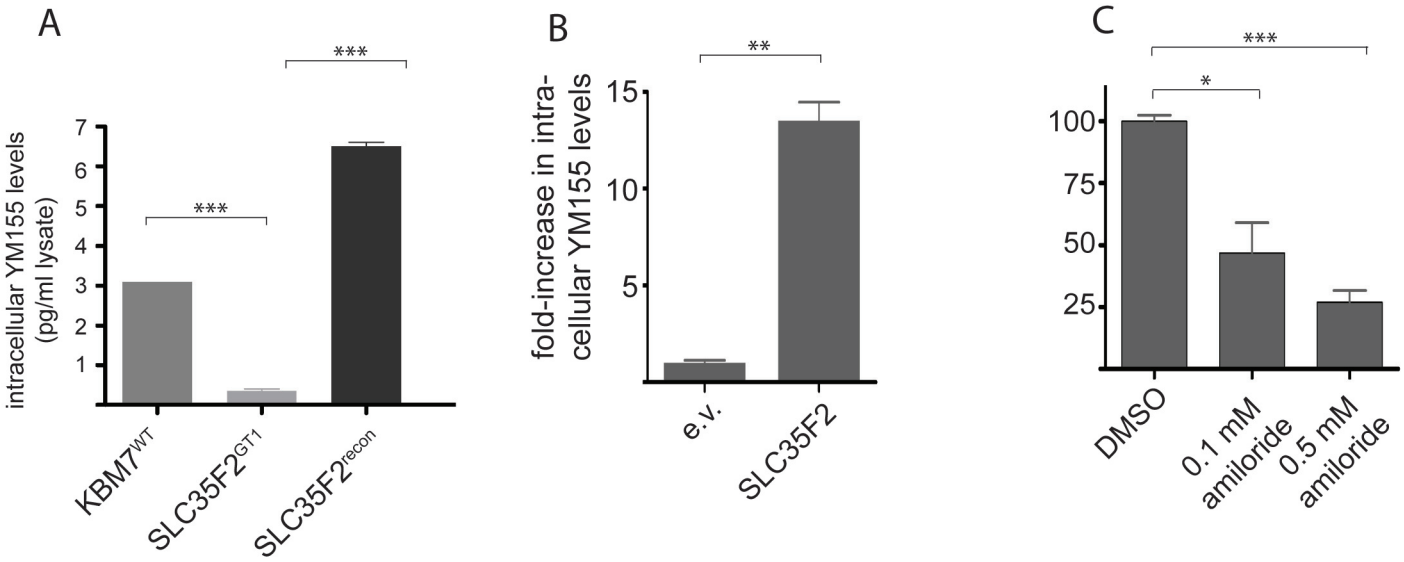
Winter et al Figure 1



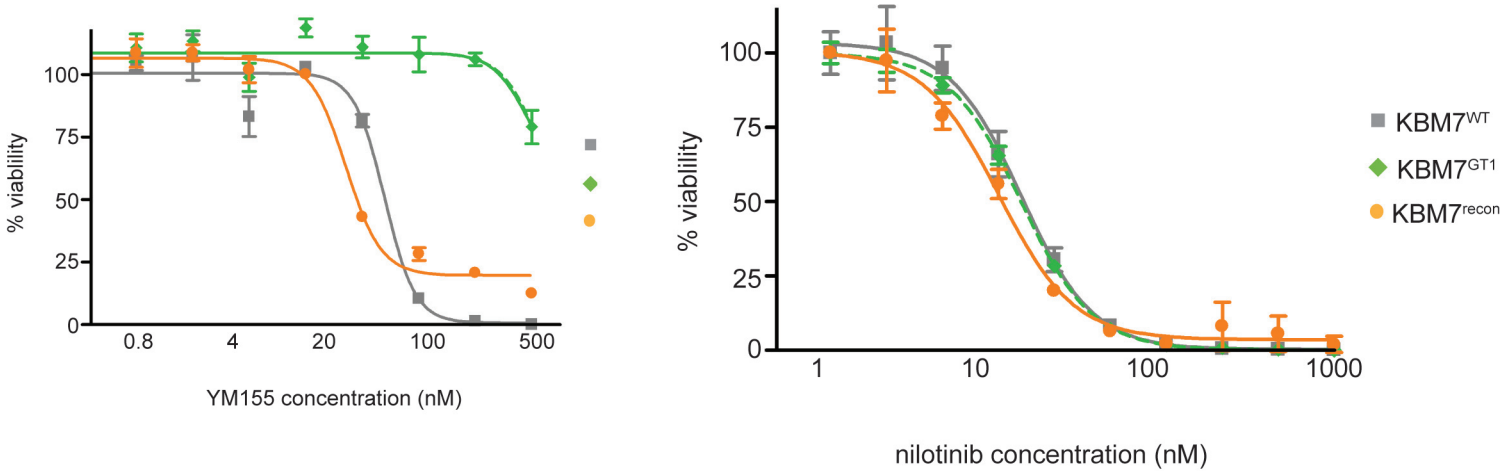
Winter et al Figure 2



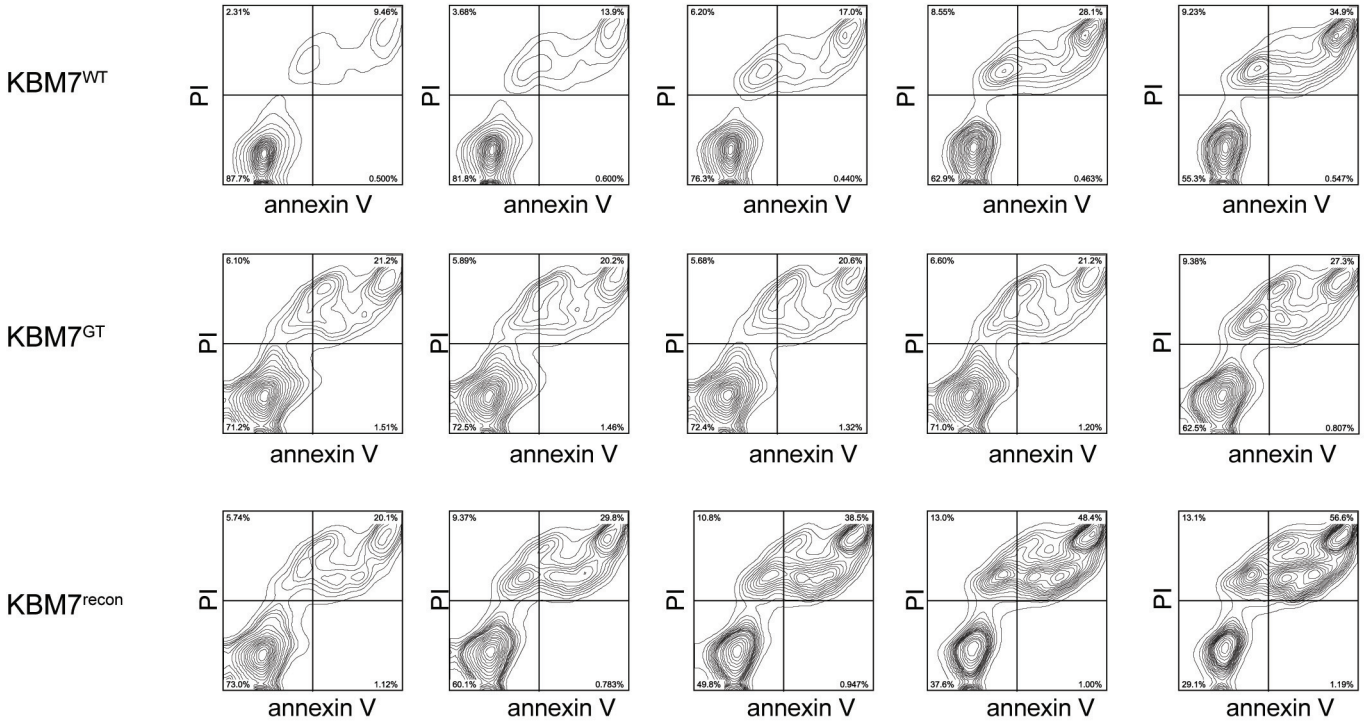
Winter et al Figure 3



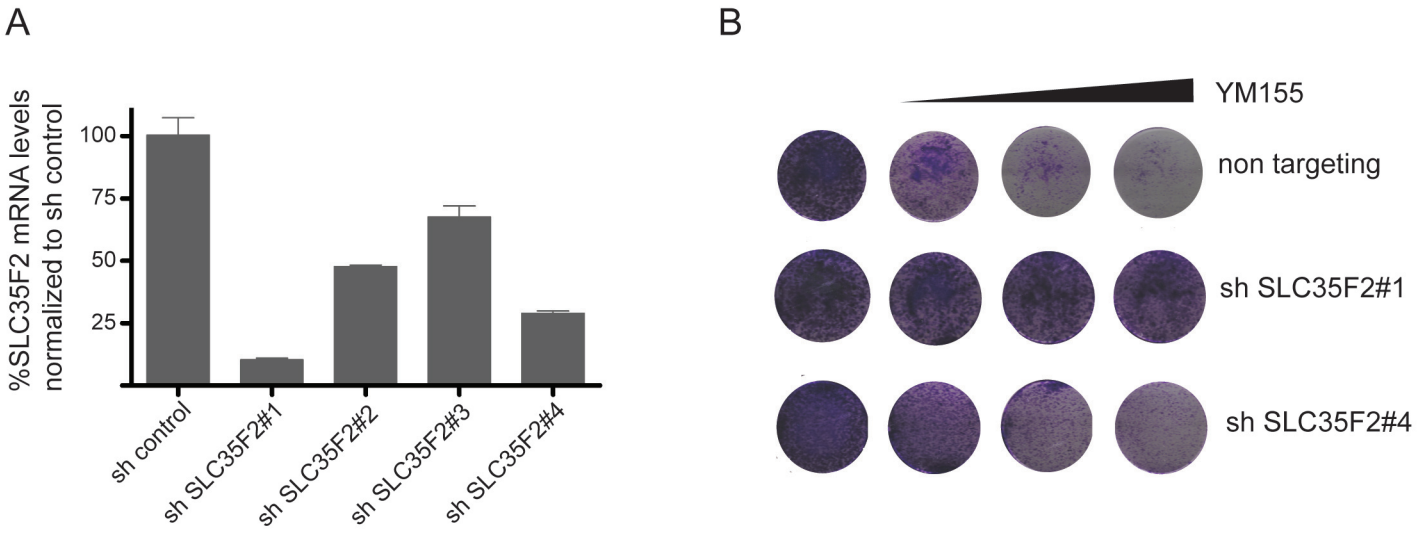
Winter et al
Supplementary Figure 1



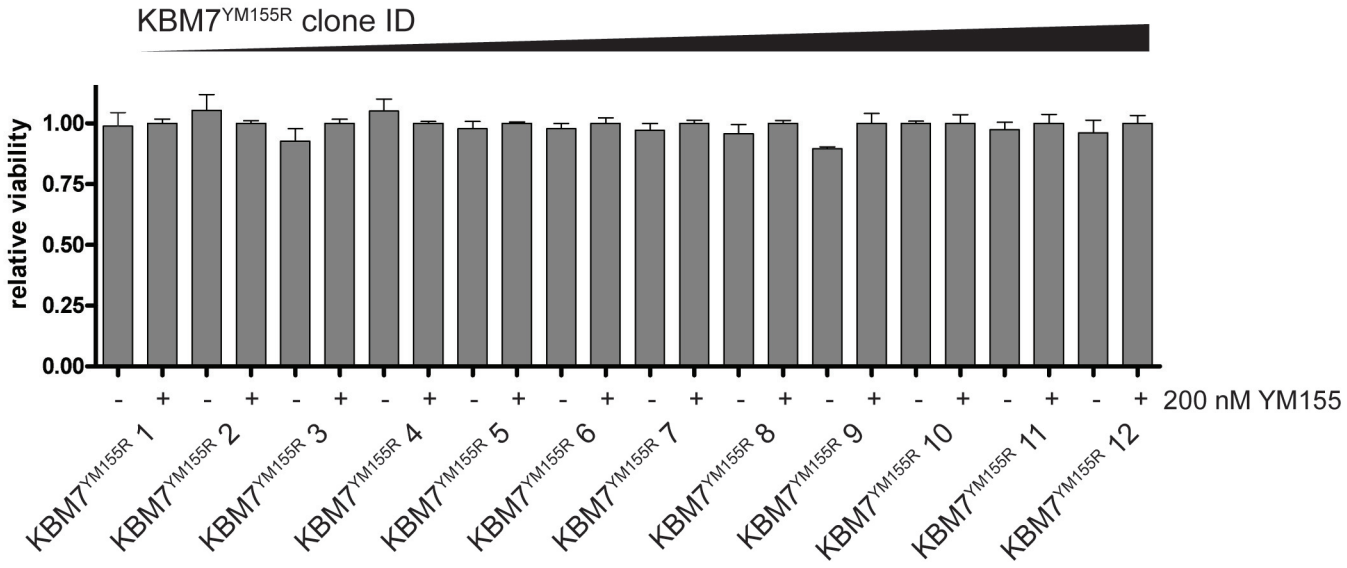
Winter et al
Supplementary Figure 2



Winter et al
Supplementary Figure 3



Winter et al
Supplementary Figure 4



4 Concluding Discussion

4.1 Contribution to the field of Cancer Chemical Biology

The highly complex and variable genetic make-up provides a cancer cell with a competitive advantage in growth and proliferation over other, non-malignant cells. These competitive advantages are conferred by a collection of phenotypic traits, collectively referred to as the "hallmarks of cancer". (Hanahan and Weinberg, 2011) However, these properties might also come at a certain price of a cancer-specific disadvantage and dependencies in distinct environmental conditions. (Kaelin, 2005) The idea of molecularly designed and personalized medicine is to uncover these disadvantages in a genotype-specific manner using, among others, phenotypic screens based on small molecule- or genetic perturbing agents. Knowledge derived thereof can subsequently be transposed into new therapeutic approaches.

Here, we employed experimentally different but conceptually unifying approaches to identify or further explore vulnerabilities of human malignancies of different origins like Ewings sarcoma or chronic myeloid leukemia. We used state-of the art target deconvolution approaches consisting of chemical proteomics, phosphoproteomics, transcriptomics and haploid genomics in order to explain efficacies of drugs or drug combinations. Collectively, we believe that the work presented here sheds light on the complex mechanisms that are caused by- or required for small molecule compounds in order to elicit a cellular response. Of note, we believe that our work highlights the even increased complexity if a cellular state is challenged with a combination of small molecule perturbing agents. As already mentioned in the introduction, there is a large conceptual overlap between performing chemical-genetics focused screens to identify resistance mechanisms to a given drug of interest and to systematic survey for compounds that show a non-additive effect when being combined with that same compound. Therefore we hope that in follow up studies, such complementary gene-drug and drug-drug interaction profiles will be integrated more frequently in order improve the understanding of mechanism of action of existing cancer drugs and thus also of the cancer-specific rewiring of essential signaling networks.

In the following subchapters 4.2 - 4.4, the projects that constitute the presented work are discussed in more detail.

4.2 A kinase inhibitor-focused small molecule screen to identify novel vulnerabilities in Ewings Sarcoma

Ewings sarcoma is a pediatric bone-cancer with poor long-term survival rates that is molecularly characterized by the expression of the naturally non-occurring fusion-transcription factor EWS-FLI11. (Delattre et al., 1992) Given the non-favorable side effects that are observed with the current chemotherapeutic treatment regimens, we aimed at finding novel therapeutic intervention points using a panel of two hundred kinase inhibitors, a large and well studied class of targeted small molecule agents. We identified, among others, a striking vulnerability of Ewings Sarcoma cell lines SK-N-ES and SK-N-MC to two small molecules danusertib and tozasertib that are known to be highly promiscuous and, among others, to target all three aurora kinases A, B and C. We extended our toxicity studies to other Ewings sarcoma cell line models as well as to cell lines model of other pediatric cancers like medulloblastoma or rhabdomyosarcoma and found out that the observed efficacy of danusertib and especially tozasertib is limited to Ewings sarcoma cell lines. In order to derive the specific target spectrum of tozasertib in these highly vulnerable cell lines, we employed a direct chemical proteomics approach. Thus, we devised a coupleable analogue of tozasertib that we used to generate a drug-affinity matrix suitable for enrichment of binding proteins. Our subsequent target deconvolution highlighted both aurora kinases A and B as relevant for the observed phenotypes as all other kinases that are bound by tozasertib were covered by other compounds of our initial screening library. Intriguingly, aurora kinases have been shown to be directly upregulated by EWS-FLI11. We thus assayed for a synthetic-lethal like interaction between EWS-FLI11 and aurora kinases. To do so, we made use of a cell line featuring an inducible knockdown of EWS-FLI11. As expected, both aurora kinases were downregulated on protein levels upon induction of EWS-FLI11 knockdown which interestingly was accompanied by a reduced sensitivity to treatment with tozasertib. Hence, it appears that the high sensitivity of ES cell lines to tozasertib and danusertib is actually directly connected to the genotype that is molecularly characterizing Ewings sarcoma. A similar drug-genotype interaction has recently also been established for Ewings sarcoma and PARP-inhibitors. (Barretina et al., 2012) Importantly, we could show that the polypharmacologic features of tozasertib, that we have extended further via our chemical proteomics experiments, is favorable for the observed efficacy in Ewings sarcoma. Using RNAi mediated knockdown experiments we observed that knockdown of aurora kinase A or B alone only marginally impairs cellular viability while parallel genetic perturbation increases the observed cytotoxicity. In line with that, another study that assessed the efficacy of the aurora kinase A specific inhibitor MLN8237 in pediatric cancers did not reveal a preference in killing Ewings sarcoma cell lines but rather an increased

sensitivity of neuroblastoma- and acute lymphoblastic leukemia models to MLN8327. (Maris et al., 2010) Collectively this suggests that the polypharmacologic features of tozasertib allowed for modulating of (at least) two different disease-relevant nodes simultaneously and that the observed exquisite vulnerability of Ewings sarcoma cell lines to tozasertib results from an intrinsic synergy elicited by inhibiting aurora kinases A and B simultaneously. Interestingly, we could further boost the efficacy of tozasertib in ES cell lines by combining it with genotoxic drugs like etoposide and doxorubicin that both are part of the current chemotherapeutic standard treatment. Hence, additional targeting of the genotoxic stress phenotype further increased the vulnerability of ES to pharmacologic inhibition of aurora kinases A and B. Finally, we could also prove efficacy of tozasertib in an *in-vivo* setting by conducting mouse xenograft experiments. We observed a dose-dependent, significant (p-value<0.05) impairment of tumor growth *in vivo*. However, as compared to the high efficacy that we observed *in vitro*, the effect observed in the xenograft experiments was rather disappointing. We believe that this is mostly attributable to the fact that we have chosen intraperitoneal injection of VX-680 as application method which might impair on the pharmacokinetic properties of VX-680 as compared to other application methods that have been used in the literature before. (Harrington et al., 2004) In light of more recent studies that were published after our approach and which suggested a significant impact of the timing when using kinase-inhibitors in combination with chemotherapeutic agents, it could be highly valuable to further extend our combination studies. (Lee et al., 2012) As already mentioned in the introduction, Lee and colleagues could show that priming of triple-negative breast cancer cells with inhibitors of EGFR renders them significantly more susceptible to pharmacological induction of DNA damage as compared to simultaneous application. As all of our combination studies have been conducted using a parallel treatment strategy, it appears tempting to check if switching to a sequential application strategy would alter the vulnerability of ES in either direction.

We believe that this approach illustrates the potential advantages of using small molecule chemical perturbing agents in phenotypic screens as opposed to genetic approaches. If coupled to state of the art downstream target deconvolution, this experimental setup also allows identifying phenotypes that are caused by targeting entire relevant networks if the employed small molecules feature a sufficient polypharmacology. Polypharmacology is an inherent feature of most small molecules but has been studied extensively for kinase inhibitors. (Knight et al., 2010; Rix and Superti-Furga, 2009) The elicited higher-order perturbation would unlikely be reflected in a comparable manner in genetic experiments. Genetic screens usually try to eliminate off-target effects by only focusing on groups of e.g. shRNA hairpins targeting the same gene if all or at least most of the hairpins yield the

phenotype of interest. (Jackson et al., 2003) As it is unexpected that independent hairpins that are designed to target the same gene also feature comparable unifying off target effects on the same secondary target, the complex effects of perturbing a cellular state with small molecules or even combinations thereof is hard to be phenocopied with RNAi. Thus, interrogating biological systems like cancer with small molecules harbors unique values that justify the challenges that are most often encountered in target deconvolution approaches following the initial phenotypic screens.

4.3 Deriving a systems-level understanding of a novel drug synergy in imatinib- resistant chronic myeloid leukemia

As already mentioned above, the acquisition of resistances to drugs is a major problem in the treatment of cancer. Given its paradigmatic role as a targeted treatment regimen, mechanisms that confer resistance against imatinib are among the best studied ones. (Lamontanara et al., 2012) Imatinib has yielded clinical excitement for its ability to target the fusion kinase BCR-ABL. BCR-ABL results from a reciprocal translocation between chromosomes 9 and 22 and its expression hallmarks the clonal hematopoietic disease chronic myeloid leukemia. Inhibition of BCR-ABL via imatinib blocks the aberrantly induced downstream signaling pathways (STAT5, MAPK, PI3K) and consequently induces death of the leukemic cells. Thus, imatinib also serves as a classical example for a drug exploiting the concept of oncogene-addiction.(Luo et al., 2009b; Weinstein, 2002) Clinically, it induces a complete remission and prolonged lifespan in the majority of patients. (Druker, 2004) One of the genomic aberrations causing resistance to imatinib with the most dismal prognosis is the so called gatekeeper mutation that changes the amino acid at position 315 from a threonine to a bulkier isoleucine residue. This single amino acid change prevents not only binding of imatinib but also of all other clinically approved CML drugs. The physical interaction between BCR-ABL^{T315I} and the respective drugs is sufficiently abrogated and neither of the compounds remains, at concentrations that are achievable in terms of plasma levels in patients, capable to inhibit BCR-ABL signaling. We thus set out in order to systematically probe for drug-drug interactions of BCR-ABL inhibitors that are either already approved for the treatment of CML or are in later stage clinical trials. Knowing that most kinase inhibitors are of promiscuous nature, we hypothesized that two compounds of an initially lower efficacy could nevertheless be exploited for their synergistic potential, given their polypharmacologic nature. As this drug-resistant form of CML also represents a high medical need we aimed to use compounds that have already been assessed and proven to be safe in humans, thus potentially shortening the distance between bench and bedside.

Using a combinatorial screening approach, we could identify as striking synergistic interaction between the two multi-kinase inhibitors danusertib and bosutinib. At the point of our initial discovery, both agents were in later stage clinical trials for potential second-line treatment option for patients that relapsed from imatinib treatment. Interestingly, bosutinib was meanwhile clinically approved for this indication. We validated our initial finding in primary mouse- and human patient cells. Surprisingly, the synergy was not observed in CML models harboring non-mutant BCR-ABL^{WT}. Potential reasoning for that can be found in the discussion of the manuscript (chapter 3.2.) In analogy to our finding that Ewings sarcoma was highly vulnerable to treatment with tozasertib (chapter 3.1) we were again in need to device a deconvolution strategy to molecularly understand the observed phenotype. However, in this case the combined perturbation was of increased complexity, thus we devised a multi-level target deconvolution approach. In detail we again set out to charter binding partners of bosutinib and danusertib in a proteome-wide fashion using direct chemical proteomics. To be able to capture and additional dimension, we also monitored global alterations in the phosphoproteome and the transcriptome upon single- or combined treatment with either of the two agents. Each of these holistic approaches alone provided valuable insights into the “molecular footprints” left by danusertib and/or bosutinib. However, we found that the, to some extend orthogonal approaches, significantly increase their value when being intersected with each other. Regarding the quality of the derived data, it is important to distinguish that chemical proteomics derives a direct and physical link between the perturbing agents and the molecular machines it interacts with. On the downside, this dataset lacks the functional downstream consequences that are caused by that interaction. Vice-versa, phosphoproteomics-signatures mirror the direct biochemical consequences after drug impact. Of note, when comparing the two time points we used to assess the alterations in the phosphoproteome, we found the early fifteen-minutes- much more informative than the late six hours time point. In order to integrate these two proteomics centered approaches, we have used protein network analysis to derive targeted complexes. We then correlated bound protein complexes with altered phosphorylation patterns using pathway analysis tools in order to uncover affected signaling pathways and networks. To our surprise, this analysis revealed that both compounds heavily impinge on the MAP kinase signaling network in a non-redundant manner. It turned out that this was due to previously not appreciated off-target effects of both agents. Finally, we integrated this data with changes in the transcriptome measured after six hours of drug treatment. As opposed to the relatively early time point that we used to assess affected phosphorylation signatures, this time point allowed for capturing the even more downstream cellular response. Again, we believe that in qualitative terms, there are strong differences between data that is derived from phosphoproteomics and transcriptomics analysis. This is partly due to the fact that the vast

majority of all phosphorylation sites actually lack a known function or biological meaning. Thus, a decrease in a given phosphorylation site does not necessarily imply that the function of the relevant protein or the relevant pathway is attenuated. In contrast, a change in the transcriptome depicts the impact of a perturbing agent as it is translated into a functional consequence by the cellular system. In that understanding, we hypothesized that the best way to integrate this transcriptome-derived “cellular interpretation” with our biochemical perturbation data would be to cluster the approximately 600 up- and downregulated genes based on the transcriptional regulators that govern their expression. In order to do so we made use of the Molecular Signature Database (MSigDB) as well as of a more unbiased approach, the Gene Set Enrichment Analysis (GSEA) that assesses changes on a transcriptome-wide scale without prior filtering for significantly altered genes. (Subramanian et al., 2005) Interestingly, c-MYC, one of the best-studies master regulators of gene expression, was in both assessments the most significantly affected transcription factor. Thus, using two complementary approaches, we could reduce the complexity from an initial transcriptome-wide scale down to essentially a single transcription factor molecularly governing these alterations. Notably, among a multitude of posttranslational modifications c-MYC is also phosphorylated via the MAP kinase pathway and this phosphorylation has been shown to play a role in protein stability as well as transcriptional activity. We could show that this phosphorylation on serine 62 is reduced upon combined treatment with danusertib and bosutinib. This goes along with a reduced binding affinity of c-MYC to consensus target sites. Finally, we also showed, using pharmacologic inhibition of BRD4, that downregulation of c-MYC also impacts on cellular viability. C-MYC has a prominent role in a variety of cancers and acts, as recently described, as a general amplifier of oncogenic signaling. (Lin et al., 2012) In line with our findings, it also has been shown that c-MYC is an essential gene in CML cell lines as well as that it is required for BCR-ABL mediated cellular transformation. (Luo et al., 2008; Sawyers et al., 1992)

We believe that our approach to functionally dissect the impact of a combined chemical perturbation on a cellular system harbors a multitude of advantages and is capable of capturing the systematic response in a comprehensive way. We used chemical proteomics as well as phosphoproteomics and transcriptomics in order to recapitulate the dynamics of the perturbation caused by combined treatment with danusertib and bosutinib. This lifecycle starts from the physical binding of both small molecules to their protein targets. For that purpose, chemical proteomics proved to be a formidable approach and can be, if coupled to bioinformatics analysis, used to charter entire molecular networks that are physically linked to the small molecules of interest. For our analysis, the unbiased nature of that approach was highly valuable as it allowed identifying the multiple off targets that impinge on the MAPK signaling network. Of note, bosutinib was previously known as a dual ABL/pan-SRC

inhibitor whereas danusertib was primarily appreciated as aurora kinase inhibitor. Thus, the multiple off target binding effects were neither known nor predictable. As already mentioned before, the binding of a protein to a drug-affinity matrix does not necessarily imply an enzymatic inhibition by that small molecule. Hence, we supported our chemical proteomics findings in a focused manner using *in vitro* kinase assays as well as in another unbiased fashion using quantitative, global phosphoproteomics. In order to deduce a comprehensive meaning from the approximately 700 phosphorylation sites that were mapped and quantified after single-or combined drug treatment, we applied a clustering algorithm that groups similarly altered sites together. Focusing on those groups that were, independent of the effect of either of the single compounds on that site, downregulated in the drug combination again highlighted the MAPK pathway as a hotspot for functional target inhibition. Therefore, in terms of the “lifecycle” of the chemical perturbation, we supported the binding affinities measured via chemical proteomics with a biochemical cellular response. The final intersection with the alterations measured in the transcriptional output of the affected system provided those biochemical measurements with a biological and functional meaning in terms of downregulation of c-Myc target genes. We think that our systems-biology focused approach outlined here can be a role-model for further target deconvolution strategies downstream of phenotypic small molecule screens or can be applied in order to derive a better understanding for drugs already used to treat patients with or without a known target. Phosphoproteomics was in our case specifically suited for supporting binding data with a biochemical impact as we investigated kinases as drug binding partners. However, also other posttranslational marks like acetylation or methylation can be assessed in an unbiased and quantitative manner and could therefore substitute for phosphoproteomics based on the nature of the captured protein target. (Choudhary et al., 2009; Uhlmann et al., 2012)

Moreover, our approach is not limited to cancer as a biological framework but can theoretically be applied to any biological question of interest. Additionally, it is not necessarily limited to cytotoxic compounds but can rather be coupled downstream of any phenotypic screen of interest.

A potential bottleneck might be the dependence on state of the art equipment where access to high-resolution mass spectrometry is key to successful chemical proteomics and phosphoproteomics results. Moreover, as described in 1.7.2, chemical proteomics also depends on a derivatisation of the compound of interest and thus also access to chemistry.

4.4 A haploid genetic screen identifies a novel genetic requirement of YM155 on the solute carrier SLC35F2

A functional understanding of resistance mechanisms to small molecule cancer therapy is of utter importance in order to device biomarkers for patient stratification. It should allow for the prediction of the clinical efficacy of a given compound based on the genetic wiring in a patient-specific manner. As already mentioned in the introduction, forward genetic screens have yielded important insights into various resistance mechanisms to targeted cancer therapies. Although some of these studies were based on stable overexpression of collections of cDNAs, functional genetics using transient or stable knockdown via RNAi have most extensively been used. (Huang et al., 2012; Johannessen et al., 2010; Prahallad et al., 2012) However, RNAi-based approaches also suffer from several drawbacks well known to the scientific community. To begin with, transient as well as stable knockdowns only yield a partial reduction in the expression level of the targeted gene product. For some applications, this is potentially not sufficient to elicit a given response or cellular output. Moreover, off target effects inherent to siRNA as well as shRNA are an additional source for the misinterpretation of an observed phenotype. (Jackson et al., 2003) At least to a certain degree, these off target effects are also causative for the generally low overlap and inter-lab reproducibility that have been linked to different RNA-interference based studies recently. For instance, these issues have been observed when three different labs employed genome-wide RNAi studies in order to find cellular host factors essential for HIV replication. Although each of these approaches yielded approximately around 300 hits, the datasets overlapped in as little as four candidate genes. (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008) A comparably low overlap of candidate genes was also observed when different groups used genome-wide RNAi strategies in order to molecularly define the synthetic-lethal dependencies of Ras-mutant cancers. (Barbie et al., 2009; Luo et al., 2009a; Scholl et al., 2009) Even more, STK33, the top hit emerging of the genome-wide screen published by Scholl et al was meanwhile confuted to feature a synthetic-lethal interaction as a custom-designed inhibitor with selectivity for STK33 (BRD-8899) failed in killing in a Ras-mutant genotype selective manner. (Luo et al., 2012)

Given these drawbacks, we decided to make use of an alternative genetic approach in order to find genes that are essential for the anti-cancer activity of a drug of interest. Retroviral insertional mutagenesis screens have proven to be extremely powerful when performed in a near haploid cell line called KBM7. This approach has been pioneered in order to find host factors required for various bacterial toxins and viruses. (Carette et al., 2009; Carette et al., 2011a; Carette et al., 2011b) The particular strength of this setup is explained by the fact that, given the haploid karyotype of the cells, an insertion of the genetrap vector, equipped

with a strong splice-acceptor site, confers a complete disruption of the genomic locus, thus yielding truncation of the underlying transcript. Therefore, this approach is capable of yielding loss-of function or “knockout” clones. This comes with several advantages. First, off-target effects that are, as described above, a major issue in RNAi based screening approaches are completely eliminated. The site of the retroviral insertion of thousands of clones can be precisely mapped in a pooled fashion using next generation sequencing. (Carette et al., 2011a) Moreover, whereas RNAi based approaches often suffer from incomplete target gene repression, haploid genetics usually confers a complete loss of function and thus creates a scenario of a complete gene-deletion. (Carette et al., 2009)

We decided to make use of this approach in order to find the genomic requirements of the clinical anti-cancer compound YM155. YM155 has initially been described as an inhibitor of the anti-apoptotic protein survivin as it downregulates survivin on transcript-and protein levels. (Nakahara et al., 2007) Consequently, it was shown to induce apoptotic cancer cell death in a variety of cell lines and *in-vivo* xenograft experiments, as single agent but also in combination experiments. (Nakahara et al., 2007; Yamanaka et al., 2011) Given the pronounced efficacy of YM155 in preclinical settings, it has been evaluated in several phase I and II clinical trials. (Giaccone et al., 2009; Satoh et al., 2009) Currently, it is investigated for its clinical efficacy in NSCLC, Melanoma and Non-Hodgkin’s Lymphoma (www.clinicaltrials.gov). We reasoned that a haploid genetic screen could potentially shed further light on the rather unclear molecular mode of action of YM155 as well improve the understanding of its rather modest clinical efficacy. (Giaccone et al., 2009; Glaros et al., 2012; Tolcher et al., 2012; Yamauchi et al., 2012)

Surprisingly, we could only identify a single gene that was highly significantly enriched for gene trap insertions. This gene (SLC35F2) belongs to the family of solute carriers, a large family of genes that evolved in order to transport metabolites and xenobiotics through biological membranes. (Hediger et al., 2004) We could show that the insertions which predominantly occurred directly after the first exon of SLC35F2 indeed conferred a complete knockout of SLC35F2. Deletion of SLC35F2 significantly abrogated the cytotoxicity and the potential to induce apoptosis of YM155 in a dose dependent manner. We extended our findings to other cell lines. The Ras mutant A549 NSCLC cell line proved to be resistant to YM155 upon stable knockdown of SLC35F2 whereas overexpression of SLC35F2 in the same cellular background confers a hypersensitivity to YM155. We could furthermore show that downregulation of SLC35F2 also happens in a genetrap independent manner in $KBM7^{WT}$ cells in response to treatment with YM155. In fact, as 57 out of 65 assayed YM155 resistant clones dropped in SLC35F2 expression levels below the limit of detection, we believe that acquired downregulation of SLC35F2 is, in the tested setting, the predominant mode of resistance also in non-mutagenized cells. However, the exact molecular mechanism

behind that downregulation remains elusive. Our initial efforts to sequence the genomic locus of SLC35F2 failed to identify any mutations and a subsequent bisulfate-sequencing strategy did not reveal increased DNA methylation in the CpG island located in the promoter region of SLC35F2.

There are several other options potentially explaining the observed downregulation of SLC35F2 in KBM7^{WT} cells that we did not assess in detail yet. First, we restricted our analysis to identify potential DNA hypermethylation in the drug resistant clones to the CpG island in the SLC35F2 promoter. However, DNA hypermethylation does not necessarily have to converge to that regulatory region alone. Other putative regulatory elements, especially enhancers of transcription, could have a distant localization that would not have been revealed by our focused sequencing strategy. In order to detect such aberrations in an unbiased manner, we would have to employ genome-wide bisulfate sequencing on each of the drug-resistant populations. However, SLC35F2 could also be silenced via other mechanisms of heterochromatin formation like an increase in repressive histone modifications (H3K9me3, H3K27me3, H3K20me3). Notably, the loss of SLC35F2 could also occur due to genetic or epigenetic aberrations in regulators governing the transcription or the mRNA stability of SLC35F2. However, given the fact that there was not a single other gene that showed enrichment for retroviral insertions, the non-redundant involvement of another factor in this process is rather unlikely.

Finally, given the annotation of SLC35F2 as solute carrier, we assessed if the intracellular concentrations of YM155 are altered as a consequence of deletion of SLC35F2. Interestingly, this was the case as we observed an approximately ten-fold decrease in intracellular YM155 levels in clones deficient of SLC35F2. We could rescue this effect upon stable reconstitution with SLC35F2 cDNA. This was somewhat surprising as the SLC family 35 is collectively annotated as nucleoside sugar transporter family. (Hediger et al., 2004) Intriguingly, while most of these family members feature a cytoplasmic localization at the Golgi or the endoplasmic reticulum, SLC35F2 and its feline homologue have been described to be localized at the cell surface. (Sarangi et al., 2007) Moreover, the structure of YM155, the now only known cargo of SLC35F2, does not feature any similarity to nucleoside-sugars. This opens up the possibility that the molecular function of SLC35F2 is potentially not correctly reflected by its annotation which itself is entirely based on sequence similarity. Further studies need to be conducted in order to derive a better understanding of the spectrum of metabolites and drugs that are transported by SLC35F2.

In my thesis, we have employed a genetic screen in order to dissect genes that are functionally required for the mechanism of action of the clinical anti-cancer compound YM155. Our genome-wide efforts converged on a single gene that facilitates the transport of YM155. The fact that genetic manipulation of only the uptake and thus the most upstream

component of the “lifecycle” of YM155 can rescue from its cytotoxicity tempts to hypothesize that it features a very broad and deleterious mechanism of action that cannot be attenuated by deletion of any other gene. Likewise, other compounds that impinge on the anti-apoptotic machinery of a cancer cell have yielded different results in haploid genetic screens. The BCL-2 inhibitor ABT737 has for instance been shown to critically depend on the pro-apoptotic genes BAX and NOXA. (Carette et al., 2011a) Thus, these results properly reflect the impairment of the anti-apoptotic machinery by Abt737. Given that, a genotoxic mode of action which has recently also been attributed to YM155 appears a feasible explanation for the almost digital outcome of the genetic screen. Because of the absolute dependency of YM155 on SLC35F2, we believe that monitoring the expression levels of the transporter could be highly valuable in terms of patient stratification for further clinical trials.

5 Abbreviations

3-BrPA	3-bromopyrovate
ABC	ATP binding cassette
ABCB1	ATP-binding cassette sub-family B member 1
ABCC1	ATP-binding cassette sub-family C member 1
ABCG2	ATP-binding cassette sub-family G member 2
ABL	Abelson murine leukemia viral oncogene homolog 1
ABPP	activity based protein profiling
ALK	anaplastic lymphoma receptor tyrosine kinase
ATP	Adenosine triphosphate
BAX	BCL2-associated X protein
BCL-2	B-cell CLL/lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCR	Breakpoint cluster region
BCRP1	breast cancer resistance protein 1
B-RAF	v-raf murine sarcoma viral oncogene homolog B1
BRD4	bromodomain containing 4
CCCP	compound centered chemical proteomics
CDK	cyclin dependent kinase
cDNA	complementary DNA
c-KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CML	Chronic Myeloid Leukemia
COT	Cancer Osaka thyroid oncogene
C-RAF	V-raf-1 murine leukemia viral oncogene homolog 1
DHFR	dihydrofolate reductase
DNA	Desoxyribonucleic acid
EGFR	endothelial growth factor receptor
EML4	echinoderm microtubule associated protein like 4
ES	Ewings Sarcoma
EWS	Ewing sarcoma breakpoint region 1
FLI1	Friend leukemia virus integration 1
GE-HTS	gene expression high throughput screening
GIST	gastrointestinal stromal tumor
GSEA	Gene Set Enrichment analysis
HDAC	histone deacetylase

HER2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 neuro/glioblastoma derived oncogene homolog (avian)
HGF	hepatocyte growth factor
HIP	haplo-insufficiency profiling
HIV	human immunodeficiency virus
HOP	homozygous profiling
HSP90	Heat shock protein 90
iTRAQ	isobaric tag for relative and absolute quantitation
JAK2	Janus Kinase 2
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MAP	mitogen activated protein
MCT1	monocarboxylic acid transporter 1
MDR	multidrug resistance
MDR1	multidrug resistance protein 1
MED12	mediator complex subunit 12
MEK	mitogen-activated protein kinase kinase
MM	multiple myeloma
MPN	myeloproliferative neoplasm
MRP1	multidrug resistance-associated protein 1
MSigDB	Molecular Signatures Database
mTOR	mammalian target of rapamycin
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NFAT	nuclear factor of activated T-cells
NOXA	horbol-12-myristate-13-acetate-induced protein 1 (PMAIP1)
N-RAS	neuroblastoma *RAS* viral (v-*ras*) oncogene homolog
NSCLC	non small cell lung cancer
OCT1	Organic cation transporter 1
PARP	Poly (ADP-ribose) polymerase
p-gp	p-glycoprotein 1
PI3K	phosphoinositide 3 kinase
RA	rheumatoid arthritis
RNA	Ribonucleic acid
RNA	RNA-interference
SADR	serious adverse drug reactions
shRNA	short hairpin RNA
SILAC	stable isotope labeling by amino acids
SLC	solute carrier

SLC16A1	solute carrier family 16, member 1
SLC22A1	solute carrier family 22 member 1
SLC35F2	Solute Carrier Family Member 35F2
SMM	small molecule microarrays
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
STAT5	signal transducer and activator of transcription 5
STK33	serine/threonine kinase 33
TGFb	transforming growth factor, beta
WNT	wingless type
Y2H	yeast two hybrid
Y3H	yeast three hybrid

6 References

Allikmets, R., Schriml, L.M., Hutchinson, A., Romano-Spica, V. and Dean, M. (1998) A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res*, **58**, 5337-5339.

Bachovchin, D.A. and Cravatt, B.F. (2012) The pharmacological landscape and therapeutic potential of serine hydrolases. *Nat Rev Drug Discov*, **11**, 52-68.

Bantscheff, M., Eberhard, D., Abraham, Y., Bastuck, S., Boesche, M., Hobson, S., Mathieson, T., Perrin, J., Raida, M., Rau, C., Reader, V., Sweetman, G., Bauer, A., Bouwmeester, T., Hopf, C., Kruse, U., Neubauer, G., Ramsden, N., Rick, J., Kuster, B. and Drewes, G. (2007) Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol*, **25**, 1035-1044.

Bantscheff, M., Hopf, C., Savitski, M.M., Dittmann, A., Grandi, P., Michon, A.M., Schlegl, J., Abraham, Y., Becher, I., Bergamini, G., Boesche, M., Delling, M., Dumpelfeld, B., Eberhard, D., Huthmacher, C., Mathieson, T., Poeckel, D., Reader, V., Strunk, K., Sweetman, G., Kruse, U., Neubauer, G., Ramsden, N.G. and Drewes, G. (2011) Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes. *Nat Biotechnol*, **29**, 255-265.

Barbie, D.A., Tamayo, P., Boehm, J.S., Kim, S.Y., Moody, S.E., Dunn, I.F., Schinzel, A.C., Sandy, P., Meylan, E., Scholl, C., Frohling, S., Chan, E.M., Sos, M.L., Michel, K., Mermel, C., Silver, S.J., Weir, B.A., Reiling, J.H., Sheng, Q., Gupta, P.B., Wadlow, R.C., Le, H., Hoersch, S., Wittner, B.S., Ramaswamy, S., Livingston, D.M., Sabatini, D.M., Meyerson, M., Thomas, R.K., Lander, E.S., Mesirov, J.P., Root, D.E., Gilliland, D.G., Jacks, T. and Hahn, W.C. (2009) Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature*, **462**, 108-112.

Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A.A., Kim, S., Wilson, C.J., Lehar, J., Kryukov, G.V., Sonkin, D., Reddy, A., Liu, M., Murray, L., Berger, M.F., Monahan, J.E., Morais, P., Meltzer, J., Korejwa, A., Jane-Valbuena, J., Mapa, F.A., Thibault, J., Bric-Furlong, E., Raman, P., Shipway, A., Engels, I.H., Cheng, J., Yu, G.K., Yu, J., Aspesi, P., Jr., de Silva, M., Jagtap, K., Jones, M.D., Wang, L., Hatton, C., Palescandolo, E., Gupta, S., Mahan, S., Sougnez, C., Onofrio, R.C., Liefeld, T., MacConaill, L., Winckler, W., Reich, M., Li, N., Mesirov, J.P., Gabriel, S.B., Getz, G., Ardlie, K., Chan, V., Myer, V.E., Weber, B.L., Porter, J., Warmuth, M., Finan, P., Harris, J.L., Meyerson, M., Golub, T.R., Morrissey, M.P., Sellers, W.R., Schlegel, R. and Garraway, L.A. (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*, **483**, 603-607.

Becker, F., Murthi, K., Smith, C., Come, J., Costa-Roldan, N., Kaufmann, C., Hanke, U., Degenhart, C., Baumann, S., Wallner, W., Huber, A., Dedier, S., Dill, S., Kinsman, D., Hediger, M., Bockovich, N., Meier-Ewert, S., Kluge, A.F. and Kley, N. (2004) A three-hybrid approach to scanning the proteome for targets of small molecule kinase inhibitors. *Chem Biol*, **11**, 211-223.

Bennett, E.J., Bence, N.F., Jayakumar, R. and Kopito, R.R. (2005) Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. *Mol Cell*, **17**, 351-365.

Berger, M.F., Hodis, E., Heffernan, T.P., Deribe, Y.L., Lawrence, M.S., Protopopov, A., Ivanova, E., Watson, I.R., Nickerson, E., Ghosh, P., Zhang, H., Zeid, R., Ren, X., Cibulskis, K., Sivachenko, A.Y., Wagle, N., Sucker, A., Sougnez, C., Onofrio, R., Ambrogio, L., Auclair, D., Fennell, T., Carter, S.L., Drier, Y., Stojanov, P., Singer, M.A., Voet, D., Jing, R., Saksena, G., Barretina, J., Ramos, A.H., Pugh, T.J., Stransky, N., Parkin, M., Winckler, W., Mahan, S., Ardlie, K., Baldwin, J., Wargo, J., Schadendorf, D., Meyerson, M., Gabriel, S.B., Golub, T.R., Wagner, S.N., Lander, E.S., Getz, G., Chin, L. and Garraway, L.A. (2012) Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature*, **485**, 502-506.

Berger, M.F., Lawrence, M.S., Demichelis, F., Drier, Y., Cibulskis, K., Sivachenko, A.Y., Sboner, A., Esgueva, R., Pflueger, D., Sougnez, C., Onofrio, R., Carter, S.L., Park, K., Habegger, L., Ambrogio, L., Fennell, T., Parkin, M., Saksena, G., Voet, D., Ramos, A.H., Pugh, T.J., Wilkinson, J., Fisher, S., Winckler, W., Mahan, S., Ardlie, K., Baldwin, J., Simons, J.W., Kitabayashi, N., MacDonald, T.Y., Kantoff, P.W., Chin, L., Gabriel, S.B., Gerstein, M.B., Golub, T.R., Meyerson, M., Tewari, A., Lander, E.S., Getz, G., Rubin, M.A. and Garraway, L.A. (2011) The genomic complexity of primary human prostate cancer. *Nature*, **470**, 214-220.

Beroukhim, R., Mermel, C.H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., Barretina, J., Boehm, J.S., Dobson, J., Urashima, M., Mc Henry, K.T., Pinchback, R.M., Ligon, A.H., Cho, Y.J., Haery, L., Greulich, H., Reich, M., Winckler, W., Lawrence, M.S., Weir, B.A., Tanaka, K.E., Chiang, D.Y., Bass, A.J., Loo, A., Hoffman, C., Prensner, J., Liefeld, T., Gao, Q., Yecies, D., Signoretti, S., Maher, E., Kaye, F.J., Sasaki, H., Tepper, J.E., Fletcher, J.A., Tabernero, J., Baselga, J., Tsao, M.S., Demichelis, F., Rubin, M.A., Janne, P.A., Daly, M.J., Nucera, C., Levine, R.L., Ebert, B.L., Gabriel, S., Rustgi, A.K., Antonescu, C.R., Ladanyi, M., Letai, A., Garraway, L.A., Loda, M., Beer, D.G., True, L.D., Okamoto, A., Pomeroy, S.L., Singer, S., Golub, T.R., Lander, E.S., Getz, G., Sellers, W.R. and Meyerson, M. (2009) The landscape of somatic copy-number alteration across human cancers. *Nature*, **463**, 899-905.

Birsoy, K., Wang, T., Possemato, R., Yilmaz, O.H., Koch, C.E., Chen, W.W., Hutchins, A.W., Gultekin, Y., Peterson, T.R., Carette, J.E., Brummelkamp, T.R., Clish, C.B. and Sabatini, D.M. (2012) MCT1-mediated transport of a toxic molecule is an effective strategy for targeting glycolytic tumors. *Nat Genet*.

Bliss, C.I. (1939) The toxicity of poisons applied jointly. *Ann. Appl. Biol.*, 585-615.

Bock, C. and Lengauer, T. (2012) Managing drug resistance in cancer: lessons from HIV therapy. *Nat Rev Cancer*, **12**, 494-501.

Borisy, A.A., Elliott, P.J., Hurst, N.W., Lee, M.S., Lehar, J., Price, E.R., Serbedzija, G., Zimmermann, G.R., Foley, M.A., Stockwell, B.R. and Keith, C.T. (2003) Systematic discovery of multicomponent therapeutics. *Proc Natl Acad Sci U S A*, **100**, 7977-7982.

Bradner, J.E., West, N., Grachan, M.L., Greenberg, E.F., Haggarty, S.J., Warnow, T. and Mazitschek, R. (2011) Chemical phylogenetics of histone deacetylases. *Nat Chem Biol*, **6**, 238-243.

Branford, S., Rudzki, Z., Walsh, S., Grigg, A., Arthur, C., Taylor, K., Herrmann, R., Lynch, K.P. and Hughes, T.P. (2002) High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood*, **99**, 3472-3475.

Brass, A.L., Dykxhoorn, D.M., Benita, Y., Yan, N., Engelman, A., Xavier, R.J., Lieberman, J. and Elledge, S.J. (2008) Identification of host proteins required for HIV infection through a functional genomic screen. *Science*, **319**, 921-926.

Britschgi, A., Andraos, R., Brinkhaus, H., Klebba, I., Romanet, V., Muller, U., Murakami, M., Radimerski, T. and Bentires-Alj, M. (2012) JAK2/STAT5 Inhibition Circumvents Resistance to PI3K/mTOR Blockade: A Rationale for Cotargeting These Pathways in Metastatic Breast Cancer. *Cancer Cell*, **22**, 796-811.

Brozik, A., Hegedus, C., Erdei, Z., Hegedus, T., Ozvegy-Laczka, C., Szakacs, G. and Sarkadi, B. (2011) Tyrosine kinase inhibitors as modulators of ATP binding cassette multidrug transporters: substrates, chemosensitizers or inducers of acquired multidrug resistance? *Expert Opin Drug Metab Toxicol*, **7**, 623-642.

Brummelkamp, T.R., Fabius, A.W., Mullenders, J., Madiredjo, M., Velds, A., Kerkhoven, R.M., Bernards, R. and Beijersbergen, R.L. (2006) An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. *Nat Chem Biol*, **2**, 202-206.

Campbell, P.J., Yachida, S., Mudie, L.J., Stephens, P.J., Pleasance, E.D., Stebbings, L.A., Morsberger, L.A., Latimer, C., McLaren, S., Lin, M.L., McBride, D.J., Varela, I., Nik-Zainal, S.A., Leroy, C., Jia, M., Menzies, A., Butler, A.P., Teague, J.W., Griffin, C.A.,

Burton, J., Swerdlow, H., Quail, M.A., Stratton, M.R., Iacobuzio-Donahue, C. and Futreal, P.A. (2010) The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature*, **467**, 1109-1113.

Carette, J.E., Guimaraes, C.P., Varadarajan, M., Park, A.S., Wuethrich, I., Godarova, A., Kotecki, M., Cochran, B.H., Spooner, E., Ploegh, H.L. and Brummelkamp, T.R. (2009) Haploid genetic screens in human cells identify host factors used by pathogens. *Science*, **326**, 1231-1235.

Carette, J.E., Guimaraes, C.P., Wuethrich, I., Blomen, V.A., Varadarajan, M., Sun, C., Bell, G., Yuan, B., Muellner, M.K., Nijman, S.M., Ploegh, H.L. and Brummelkamp, T.R. (2011a) Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. *Nat Biotechnol*, **29**, 542-546.

Carette, J.E., Raaben, M., Wong, A.C., Herbert, A.S., Obernosterer, G., Mulherkar, N., Kuehne, A.I., Kranzusch, P.J., Griffin, A.M., Ruthel, G., Dal Cin, P., Dye, J.M., Whelan, S.P., Chandran, K. and Brummelkamp, T.R. (2011b) Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature*, **477**, 340-343.

Cataldo, V.D., Gibbons, D.L., Perez-Soler, R. and Quintas-Cardama, A. (2011) Treatment of non-small-cell lung cancer with erlotinib or gefitinib. *N Engl J Med*, **364**, 947-955.

Chan, E.S. and Cronstein, B.N. (2010) Methotrexate--how does it really work? *Nat Rev Rheumatol*, **6**, 175-178.

Chapman, M.A., Lawrence, M.S., Keats, J.J., Cibulskis, K., Sougnez, C., Schinzel, A.C., Harview, C.L., Brunet, J.P., Ahmann, G.J., Adli, M., Anderson, K.C., Ardlie, K.G., Auclair, D., Baker, A., Bergsagel, P.L., Bernstein, B.E., Drier, Y., Fonseca, R., Gabriel, S.B., Hofmeister, C.C., Jagannath, S., Jakubowiak, A.J., Krishnan, A., Levy, J., Liefeld, T., Lonial, S., Mahan, S., Mfuko, B., Monti, S., Perkins, L.M., Onofrio, R., Pugh, T.J., Rajkumar, S.V., Ramos, A.H., Siegel, D.S., Sivachenko, A., Stewart, A.K., Trudel, S., Vij, R., Voet, D., Winckler, W., Zimmerman, T., Carpten, J., Trent, J., Hahn, W.C., Garraway, L.A., Meyerson, M., Lander, E.S., Getz, G. and Golub, T.R. (2010) Initial genome sequencing and analysis of multiple myeloma. *Nature*, **471**, 467-472.

Chapman, P.B., Hauschild, A., Robert, C., Haanen, J.B., Ascierto, P., Larkin, J., Dummer, R., Garbe, C., Testori, A., Maio, M., Hogg, D., Lorigan, P., Lebbe, C., Jouary, T., Schadendorf, D., Ribas, A., O'Day, S.J., Sosman, J.A., Kirkwood, J.M., Eggermont, A.M., Dreno, B., Nolop, K., Li, J., Nelson, B., Hou, J., Lee, R.J., Flaherty, K.T. and McArthur, G.A. (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*, **364**, 2507-2516.

Chidley, C., Haruki, H., Pedersen, M.G., Muller, E. and Johnsson, K. (2011) A yeast-based screen reveals that sulfasalazine inhibits tetrahydrobiopterin biosynthesis. *Nat Chem Biol*, **7**, 375-383.

Chien, C.T., Bartel, P.L., Sternglanz, R. and Fields, S. (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A*, **88**, 9578-9582.

Choi, Y.L., Soda, M., Yamashita, Y., Ueno, T., Takashima, J., Nakajima, T., Yatabe, Y., Takeuchi, K., Hamada, T., Haruta, H., Ishikawa, Y., Kimura, H., Mitsudomi, T., Tanio, Y. and Mano, H. (2010) EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med*, **363**, 1734-1739.

Chou, T.C. and Talalay, P. (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*, **22**, 27-55.

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M.L., Rehman, M., Walther, T.C., Olsen, J.V. and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*, **325**, 834-840.

Cohen, A.A., Geva-Zatorsky, N., Eden, E., Frenkel-Morgenstern, M., Issaeva, I., Sigal, A., Milo, R., Cohen-Saidon, C., Liron, Y., Kam, Z., Cohen, L., Danon, T., Perzov, N. and Alon, U. (2008) Dynamic proteomics of individual cancer cells in response to a drug. *Science*, **322**, 1511-1516.

Cokol, M., Chua, H.N., Tasan, M., Mutlu, B., Weinstein, Z.B., Suzuki, Y., Nergiz, M.E., Costanzo, M., Baryshnikova, A., Giaever, G., Nislow, C., Myers, C.L., Andrews, B.J., Boone, C. and Roth, F.P. (2011) Systematic exploration of synergistic drug pairs. *Mol Syst Biol*, **7**, 544.

Corcoran, R.B., Cheng, K.A., Hata, A.N., Faber, A.C., Ebi, H., Coffee, E.M., Greninger, P., Brown, R.D., Godfrey, J.T., Cohoon, T.J., Song, Y., Lifshits, E., Hung, K.E., Shioda, T., Dias-Santagata, D., Singh, A., Settleman, J., Benes, C.H., Mino-Kenudson, M., Wong, K.K. and Engelman, J.A. (2012) Synthetic Lethal Interaction of Combined BCL-XL and MEK Inhibition Promotes Tumor Regressions in KRAS Mutant Cancer Models. *Cancer Cell*.

Cortes, J.E., Kantarjian, H., Shah, N.P., Bixby, D., Mauro, M.J., Flinn, I., O'Hare, T., Hu, S., Narasimhan, N.I., Rivera, V.M., Clackson, T., Turner, C.D., Haluska, F.G., Druker, B.J., Deininger, M.W. and Talpaz, M. (2012) Ponatinib in refractory Philadelphia chromosome-positive leukemias. *N Engl J Med*, **367**, 2075-2088.

Costanzo, M., Baryshnikova, A., Bellay, J., Kim, Y., Spear, E.D., Sevier, C.S., Ding, H., Koh, J.L., Toufighi, K., Mostafavi, S., Prinz, J., St Onge, R.P., VanderSluis, B., Makhnevych, T., Vizeacoumar, F.J., Alizadeh, S., Bahr, S., Brost, R.L., Chen, Y., Cokol, M., Deshpande, R., Li, Z., Lin, Z.Y., Liang, W., Marback, M., Paw, J., San Luis, B.J., Shuteriqi, E., Tong, A.H., van Dyk, N., Wallace, I.M., Whitney, J.A., Weirauch, M.T., Zhong, G., Zhu, H., Houry, W.A., Brudno, M., Ragibizadeh, S., Papp, B., Pal, C., Roth, F.P., Giaever, G., Nislow, C., Troyanskaya, O.G., Bussey, H., Bader, G.D., Gingras, A.C., Morris, Q.D., Kim, P.M., Kaiser, C.A., Myers, C.L., Andrews, B.J. and Boone, C. (2010) The genetic landscape of a cell. *Science*, **327**, 425-431.

Daenen, S., van der Holt, B., Verhoef, G.E., Lowenberg, B., Wijermans, P.W., Huijgens, P.C., van Marwijk Kooy, R., Schouten, H.C., Kramer, M.H., Ferrant, A., van den Berg, E., Steijaert, M.M., Verdonck, L.F. and Sonneveld, P. (2004) Addition of cyclosporin A to the combination of mitoxantrone and etoposide to overcome resistance to chemotherapy in refractory or relapsing acute myeloid leukaemia: a randomised phase II trial from HOVON, the Dutch-Belgian Haemato-Oncology Working Group for adults. *Leuk Res*, **28**, 1057-1067.

Dancey, J.E. and Chen, H.X. (2006) Strategies for optimizing combinations of molecularly targeted anticancer agents. *Nat Rev Drug Discov*, **5**, 649-659.

Daub, H., Olsen, J.V., Bairlein, M., Gnad, F., Oppermann, F.S., Korner, R., Greff, Z., Keri, G., Stemmann, O. and Mann, M. (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol Cell*, **31**, 438-448.

Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B.A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G.J., Bigner, D.D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J.W., Leung, S.Y., Yuen, S.T., Weber, B.L., Seigler, H.F., Darrow, T.L., Paterson, H., Marais, R., Marshall, C.J., Wooster, R., Stratton, M.R. and Futreal, P.A. (2002) Mutations of the BRAF gene in human cancer. *Nature*, **417**, 949-954.

Dean, M., Rzhetsky, A. and Allikmets, R. (2001) The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res*, **11**, 1156-1166.

Delaney, G., Jacob, S., Featherstone, C. and Barton, M. (2005) The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. *Cancer*, **104**, 1129-1137.

Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G. and et al. (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature*, **359**, 162-165.

Di Nicolantonio, F., Martini, M., Molinari, F., Sartore-Bianchi, A., Arena, S., Saletti, P., De Dosso, S., Mazzucchelli, L., Frattini, M., Siena, S. and Bardelli, A. (2008) Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol*, **26**, 5705-5712.

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.

Donato, N.J., Wu, J.Y., Stapley, J., Gallick, G., Lin, H., Arlinghaus, R. and Talpaz, M. (2003) BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. *Blood*, **101**, 690-698.

Doyle, L.A., Yang, W., Abruzzo, L.V., Krogmann, T., Gao, Y., Rishi, A.K. and Ross, D.D. (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A*, **95**, 15665-15670.

Druker, B.J. (2004) Imatinib as a paradigm of targeted therapies. *Adv Cancer Res*, **91**, 1-30.

Druker, B.J. (2008) Translation of the Philadelphia chromosome into therapy for CML. *Blood*, **112**, 4808-4817.

Druker, B.J., Guilhot, F., O'Brien, S.G., Gathmann, I., Kantarjian, H., Gattermann, N., Deininger, M.W., Silver, R.T., Goldman, J.M., Stone, R.M., Cervantes, F., Hochhaus, A., Powell, B.L., Gabrilove, J.L., Rousselot, P., Reiffers, J., Cornelissen, J.J., Hughes, T., Agis, H., Fischer, T., Verhoef, G., Shepherd, J., Saglio, G., Gratwohl, A., Nielsen, J.L., Radich, J.P., Simonsson, B., Taylor, K., Baccarani, M., So, C., Letvak, L. and Larson, R.A. (2006) Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*, **355**, 2408-2417.

Falsey, J.R., Renil, M., Park, S., Li, S. and Lam, K.S. (2001) Peptide and small molecule microarray for high throughput cell adhesion and functional assays. *Bioconjug Chem*, **12**, 346-353.

Fiskus, W., Verstovsek, S., Manshouri, T., Rao, R., Balusu, R., Venkannagari, S., Rao, N.N., Ha, K., Smith, J.E., Hembruff, S.L., Abhyankar, S., McGuirk, J. and Bhalla, K.N. (2011) Heat shock protein 90 inhibitor is synergistic with JAK2 inhibitor and overcomes resistance to JAK2-TKI in human myeloproliferative neoplasm cells. *Clin Cancer Res*, **17**, 7347-7358.

Fullmer, A., O'Brien, S., Kantarjian, H. and Jabbour, E. (2010) Emerging therapy for the treatment of acute lymphoblastic leukemia. *Expert Opin Emerg Drugs*, **15**, 1-11.

Gamas, P., Marchetti, S., Puissant, A., Grosso, S., Jacquel, A., Colosetti, P., Pasquet, J.M., Mahon, F.X., Cassuto, J.P. and Auberger, P. (2009) Inhibition of imatinib-mediated apoptosis by the caspase-cleaved form of the tyrosine kinase Lyn in chronic myelogenous leukemia cells. *Leukemia*, **23**, 1500-1506.

George, P., Bali, P., Cohen, P., Tao, J., Guo, F., Sigua, C., Vishvanath, A., Fiskus, W., Scuto, A., Annavarapu, S., Moscinski, L. and Bhalla, K. (2004) Cotreatment with 17-allylamino-demethoxygeldanamycin and FLT-3 kinase inhibitor PKC412 is highly effective against human acute myelogenous leukemia cells with mutant FLT-3. *Cancer Res*, **64**, 3645-3652.

Giaccone, G., Zatloukal, P., Roubec, J., Floor, K., Musil, J., Kuta, M., van Klaveren, R.J., Chaudhary, S., Gunther, A. and Shamsili, S. (2009) Multicenter phase II trial of YM155, a small-molecule suppressor of survivin, in patients with advanced, refractory, non-small-cell lung cancer. *J Clin Oncol*, **27**, 4481-4486.

Giacomini, K.M., Krauss, R.M., Roden, D.M., Eichelbaum, M., Hayden, M.R. and Nakamura, Y. (2007) When good drugs go bad. *Nature*, **446**, 975-977.

Giaever, G., Shoemaker, D.D., Jones, T.W., Liang, H., Winzeler, E.A., Astromoff, A. and Davis, R.W. (1999) Genomic profiling of drug sensitivities via induced haploinsufficiency. *Nat Genet*, **21**, 278-283.

Glaros, T.G., Stockwin, L.H., Mullendore, M.E., Smith, B., Morrison, B.L. and Newton, D.L. (2012) The "survivin suppressants" NSC 80467 and YM155 induce a DNA damage response. *Cancer Chemother Pharmacol*, **70**, 207-212.

Gorre, M.E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P.N. and Sawyers, C.L. (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science*, **293**, 876-880.

Gregory, M.A., Phang, T.L., Neviani, P., Alvarez-Calderon, F., Eide, C.A., O'Hare, T., Zaberezhnyy, V., Williams, R.T., Druker, B.J., Perrotti, D. and Degregori, J. (2010) Wnt/Ca2+/NFAT signaling maintains survival of Ph+ leukemia cells upon inhibition of Bcr-Abl. *Cancer Cell*, **18**, 74-87.

Gut, P., Baeza-Raja, B., Andersson, O., Hasenkamp, L., Hsiao, J., Hesselson, D., Akassoglou, K., Verdin, E., Hirschey, M.D. and Stainier, D.Y. (2012) Whole-organism screening for gluconeogenesis identifies activators of fasting metabolism. *Nat Chem Biol*, **9**, 97-104.

Hammerman, P.S., Hayes, D.N., Wilkerson, M.D., Schultz, N., Bose, R., Chu, A., Collisson, E.A., Cope, L., Creighton, C.J., Getz, G., Herman, J.G., Johnson, B.E., Kucherlapati, R., Ladanyi, M., Maher, C.A., Robertson, G., Sander, C., Shen, R., Sinha, R., Sivachenko, A., Thomas, R.K., Travis, W.D., Tsao, M.S., Weinstein, J.N., Wigle, D.A., Baylin, S.B., Govindan, R. and Meyerson, M. (2012) Comprehensive genomic characterization of squamous cell lung cancers. *Nature*, **489**, 519-525.

Hanahan, D. and Weinberg, R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57-70.

Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell*, **144**, 646-674.

Harrington, E.A., Bebbington, D., Moore, J., Rasmussen, R.K., Ajose-Adeogun, A.O., Nakayama, T., Graham, J.A., Demur, C., Hercend, T., Diu-Hercend, A., Su, M., Golec, J.M. and Miller, K.M. (2004) VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. *Nat Med*, **10**, 262-267.

Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B.J., Anderson, D.J., Alvarado, R., Ludlam, M.J., Stokoe, D., Gloor, S.L., Vigers, G., Morales, T., Aliagas, I., Liu, B., Sideris, S., Hoeflich, K.P., Jaiswal, B.S., Seshagiri, S., Koeppen, H., Belvin, M., Friedman, L.S. and Malek, S. (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature*, **464**, 431-435.

Hediger, M.A., Romero, M.F., Peng, J.B., Rolfs, A., Takanaga, H. and Bruford, E.A. (2004) The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction. *Pflugers Arch*, **447**, 465-468.

Heidorn, S.J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvas, I., Dhomen, N., Hussain, J., Reis-Filho, J.S., Springer, C.J., Pritchard, C. and Marais, R. (2010) Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell*, **140**, 209-221.

Hideshima, T., Bradner, J.E., Wong, J., Chauhan, D., Richardson, P., Schreiber, S.L. and Anderson, K.C. (2005) Small-molecule inhibition of proteasome and aggresome function induces synergistic antitumor activity in multiple myeloma. *Proc Natl Acad Sci U S A*, **102**, 8567-8572.

Hieronymus, H., Lamb, J., Ross, K.N., Peng, X.P., Clement, C., Rodina, A., Nieto, M., Du, J., Stegmaier, K., Raj, S.M., Maloney, K.N., Clardy, J., Hahn, W.C., Chiosis, G. and Golub, T.R. (2006) Gene expression signature-based chemical genomic prediction identifies a novel class of HSP90 pathway modulators. *Cancer Cell*, **10**, 321-330.

Huang, S., Holzel, M., Knijnenburg, T., Schlicker, A., Roepman, P., McDermott, U., Garnett, M., Grernrum, W., Sun, C., Prahallad, A., Groenendijk, F.H., Mittempergher, L., Nijkamp, W., Neefjes, J., Salazar, R., Ten Dijke, P., Uramoto, H., Tanaka, F., Beijersbergen, R.L., Wessels, L.F. and Bernards, R. (2012) MED12 Controls the Response to Multiple Cancer Drugs through Regulation of TGF-beta Receptor Signaling. *Cell*, **151**, 937-950.

Huang, S.M., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., Hild, M., Shi, X., Wilson, C.J., Mickanin, C., Myer, V., Fazal, A., Tomlinson, R., Serluca, F., Shao, W., Cheng, H., Shultz, M., Rau, C., Schirle, M., Schlegl, J., Ghidelli, S., Fawell, S., Lu, C., Curtis, D., Kirschner, M.W.,

Lengauer, C., Finan, P.M., Tallarico, J.A., Bouwmeester, T., Porter, J.A., Bauer, A. and Cong, F. (2009) Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature*, **461**, 614-620.

Hughes, T.R., Marton, M.J., Jones, A.R., Roberts, C.J., Stoughton, R., Armour, C.D., Bennett, H.A., Coffey, E., Dai, H., He, Y.D., Kidd, M.J., King, A.M., Meyer, M.R., Slade, D., Lum, P.Y., Stepaniants, S.B., Shoemaker, D.D., Gachotte, D., Chakraburtty, K., Simon, J., Bard, M. and Friend, S.H. (2000) Functional discovery via a compendium of expression profiles. *Cell*, **102**, 109-126.

Ideker, T., Galitski, T. and Hood, L. (2001) A new approach to decoding life: systems biology. *Annu Rev Genomics Hum Genet*, **2**, 343-372.

Islam, A. and Ambrus, J.L. (2008) Bortezomib plus melphalan and prednisone for multiple myeloma. *N Engl J Med*, **359**, 2613; author reply 2613-2614.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P.S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol*, **21**, 635-637.

Jiang, H., Pritchard, J.R., Williams, R.T., Lauffenburger, D.A. and Hemann, M.T. (2011) A mammalian functional-genetic approach to characterizing cancer therapeutics. *Nat Chem Biol*, **7**, 92-100.

Jimeno, A., Rubio-Viqueira, B., Amador, M.L., Oppenheimer, D., Bouraoud, N., Kulesza, P., Sebastiani, V., Maitra, A. and Hidalgo, M. (2005) Epidermal growth factor receptor dynamics influences response to epidermal growth factor receptor targeted agents. *Cancer Res*, **65**, 3003-3010.

Joensuu, H. (2012) Adjuvant treatment of GIST: patient selection and treatment strategies. *Nat Rev Clin Oncol*, **9**, 351-358.

Johannessen, C.M., Boehm, J.S., Kim, S.Y., Thomas, S.R., Wardwell, L., Johnson, L.A., Emery, C.M., Stransky, N., Cogdill, A.P., Barretina, J., Caponigro, G., Hieronymus, H., Murray, R.R., Salehi-Ashtiani, K., Hill, D.E., Vidal, M., Zhao, J.J., Yang, X., Alkan, O., Kim, S., Harris, J.L., Wilson, C.J., Myer, V.E., Finan, P.M., Root, D.E., Roberts, T.M., Golub, T., Flaherty, K.T., Dummer, R., Weber, B.L., Sellers, W.R., Schlegel, R., Wargo, J.A., Hahn, W.C. and Garraway, L.A. (2010) COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature*, **468**, 968-972.

Kaelin, W.G., Jr. (2005) The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer*, **5**, 689-698.

Kalac, M., Scotto, L., Marchi, E., Amengual, J., Seshan, V.E., Bhagat, G., Ulahannan, N., Leshchenko, V.V., Temkin, A.M., Parekh, S., Tycko, B. and O'Connor, O.A. (2011) HDAC inhibitors and decitabine are highly synergistic and associated with unique gene-expression and epigenetic profiles in models of DLBCL. *Blood*, **118**, 5506-5516.

Ketola, K., Kallioniemi, O. and Iljin, K. (2012) Chemical biology drug sensitivity screen identifies sunitinib as synergistic agent with disulfiram in prostate cancer cells. *PLoS One*, **7**, e51470.

Knight, Z.A., Lin, H. and Shokat, K.M. (2010) Targeting the cancer kinome through polypharmacology. *Nat Rev Cancer*, **10**, 130-137.

Koehler, A.N. (2010) A complex task? Direct modulation of transcription factors with small molecules. *Curr Opin Chem Biol*, **14**, 331-340.

Koffka, K. (1935) *Principles of gestalt psychology*. Harcourt, Brace.

Kolitz, J.E., George, S.L., Dodge, R.K., Hurd, D.D., Powell, B.L., Allen, S.L., Velez-Garcia, E., Moore, J.O., Shea, T.C., Hoke, E., Caligiuri, M.A., Vardiman, J.W., Bloomfield, C.D. and Larson, R.A. (2004) Dose escalation studies of cytarabine, daunorubicin, and etoposide with and without multidrug resistance modulation with PSC-833 in untreated adults with acute myeloid leukemia younger than 60 years: final induction results of Cancer and Leukemia Group B Study 9621. *J Clin Oncol*, **22**, 4290-4301.

Konig, R., Zhou, Y., Elleder, D., Diamond, T.L., Bonamy, G.M., Irelan, J.T., Chiang, C.Y., Tu, B.P., De Jesus, P.D., Lilley, C.E., Seidel, S., Opaluch, A.M., Caldwell, J.S., Weitzman, M.D., Kuhen, K.L., Bandyopadhyay, S., Ideker, T., Orth, A.P., Miraglia,

L.J., Bushman, F.D., Young, J.A. and Chanda, S.K. (2008) Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. *Cell*, **135**, 49-60.
Kwak, E.L., Bang, Y.J., Camidge, D.R., Shaw, A.T., Solomon, B., Maki, R.G., Ou, S.H., Dezube, B.J., Janne, P.A., Costa, D.B., Varella-Garcia, M., Kim, W.H., Lynch, T.J., Fidias, P., Stubbs, H., Engelman, J.A., Sequist, L.V., Tan, W., Gandhi, L., Mino-Kenudson, M., Wei, G.C., Shreeve, S.M., Ratain, M.J., Settleman, J., Christensen, J.G., Haber, D.A., Wilner, K., Salgia, R., Shapiro, G.I., Clark, J.W. and Iafrate, A.J. (2010) Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*, **363**, 1693-1703.
Lamb, J., Crawford, E.D., Peck, D., Modell, J.W., Blat, I.C., Wrobel, M.J., Lerner, J., Brunet, J.P., Subramanian, A., Ross, K.N., Reich, M., Hieronymus, H., Wei, G., Armstrong, S.A., Haggarty, S.J., Clemons, P.A., Wei, R., Carr, S.A., Lander, E.S. and Golub, T.R. (2006) The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science*, **313**, 1929-1935.
Lamontanara, A.J., Gencer, E.B., Kuzyk, O. and Hantschel, O. (2012) Mechanisms of resistance to BCR-ABL and other kinase inhibitors. *Biochim Biophys Acta*.
Lee, M.J., Ye, A.S., Gardino, A.K., Heijink, A.M., Sorger, P.K., MacBeath, G. and Yaffe, M.B. (2012) Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell*, **149**, 780-794.
Lee, W., St Onge, R.P., Proctor, M., Flaherty, P., Jordan, M.I., Arkin, A.P., Davis, R.W., Nislow, C. and Giaever, G. (2005) Genome-wide requirements for resistance to functionally distinct DNA-damaging agents. *PLoS Genet*, **1**, e24.
Lehar, J., Krueger, A., Zimmermann, G. and Borisy, A. (2008a) High-order combination effects and biological robustness. *Mol Syst Biol*, **4**, 215.
Lehar, J., Krueger, A.S., Avery, W., Heilbut, A.M., Johansen, L.M., Price, E.R., Rickles, R.J., Short, G.F., 3rd, Staunton, J.E., Jin, X., Lee, M.S., Zimmermann, G.R. and Borisy, A.A. (2009) Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nat Biotechnol*, **27**, 659-666.
Lehar, J., Stockwell, B.R., Giaever, G. and Nislow, C. (2008b) Combination chemical genetics. *Nat Chem Biol*, **4**, 674-681.
Licitra, E.J. and Liu, J.O. (1996) A three-hybrid system for detecting small ligand-protein receptor interactions. *Proc Natl Acad Sci U S A*, **93**, 12817-12821.
Lin, C.Y., Loven, J., Rahl, P.B., Paranal, R.M., Burge, C.B., Bradner, J.E., Lee, T.I. and Young, R.A. (2012) Transcriptional amplification in tumor cells with elevated c-Myc. *Cell*, **151**, 56-67.
Loewe, S. (1953) The problem of synergism and antagonism of combined drugs. *Arzneimittelforschung*, **3**, 285-290.
Luo, B., Cheung, H.W., Subramanian, A., Sharifnia, T., Okamoto, M., Yang, X., Hinkle, G., Boehm, J.S., Beroukhim, R., Weir, B.A., Mermel, C., Barbie, D.A., Awad, T., Zhou, X., Nguyen, T., Piqani, B., Li, C., Golub, T.R., Meyerson, M., Hacohen, N., Hahn, W.C., Lander, E.S., Sabatini, D.M. and Root, D.E. (2008) Highly parallel identification of essential genes in cancer cells. *Proc Natl Acad Sci U S A*, **105**, 20380-20385.
Luo, J., Emanuele, M.J., Li, D., Creighton, C.J., Schlabach, M.R., Westbrook, T.F., Wong, K.K. and Elledge, S.J. (2009a) A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. *Cell*, **137**, 835-848.
Luo, J., Solimini, N.L. and Elledge, S.J. (2009b) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell*, **136**, 823-837.
Luo, T., Masson, K., Jaffe, J.D., Silkworth, W., Ross, N.T., Scherer, C.A., Scholl, C., Frohling, S., Carr, S.A., Stern, A.M., Schreiber, S.L. and Golub, T.R. (2012) STK33 kinase inhibitor BRD-8899 has no effect on KRAS-dependent cancer cell viability. *Proc Natl Acad Sci U S A*, **109**, 2860-2865.
MacBeath, G. and Schreiber, S.L. (2000) Printing proteins as microarrays for high-throughput function determination. *Science*, **289**, 1760-1763.
Maemondo, M., Inoue, A., Kobayashi, K., Sugawara, S., Oizumi, S., Isobe, H., Gemma, A., Harada, M., Yoshizawa, H., Kinoshita, I., Fujita, Y., Okinaga, S., Hirano, H.,

Yoshimori, K., Harada, T., Ogura, T., Ando, M., Miyazawa, H., Tanaka, T., Saijo, Y., Hagiwara, K., Morita, S. and Nukiwa, T. (2010) Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med*, **362**, 2380-2388.

Makley, L.N. and Gestwicki, J.E. (2012) Expanding the number of 'druggable' targets: non-enzymes and protein-protein interactions. *Chem Biol Drug Des*, **81**, 22-32.

Marin, D., Goldman, J.M., Olavarria, E. and Apperley, J.F. (2003) Transient benefit only from increasing the imatinib dose in CML patients who do not achieve complete cytogenetic remissions on conventional doses. *Blood*, **102**, 2702-2703; author reply 2703-2704.

Maris, J.M., Morton, C.L., Gorlick, R., Kolb, E.A., Lock, R., Carol, H., Keir, S.T., Reynolds, C.P., Kang, M.H., Wu, J., Smith, M.A. and Houghton, P.J. (2010) Initial testing of the aurora kinase A inhibitor MLN8237 by the Pediatric Preclinical Testing Program (PPTP). *Pediatr Blood Cancer*, **55**, 26-34.

Martini, M., Vecchione, L., Siena, S., Tejpar, S. and Bardelli, A. (2011) Targeted therapies: how personal should we go? *Nat Rev Clin Oncol*, **9**, 87-97.

Minematsu, T. and Giacomini, K.M. (2011) Interactions of tyrosine kinase inhibitors with organic cation transporters and multidrug and toxic compound extrusion proteins. *Mol Cancer Ther*, **10**, 531-539.

Minucci, S. and Pelicci, P.G. (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer*, **6**, 38-51.

Miyake, K., Mickley, L., Litman, T., Zhan, Z., Robey, R., Cristensen, B., Brangi, M., Greenberger, L., Dean, M., Fojo, T. and Bates, S.E. (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res*, **59**, 8-13.

Moulick, K., Ahn, J.H., Zong, H., Rodina, A., Cerchietti, L., Gomes DaGama, E.M., Caldas-Lopes, E., Beebe, K., Perna, F., Hatzi, K., Vu, L.P., Zhao, X., Zatorska, D., Taldone, T., Smith-Jones, P., Alpaugh, M., Gross, S.S., Pillarsetty, N., Ku, T., Lewis, J.S., Larson, S.M., Levine, R., Erdjument-Bromage, H., Guzman, M.L., Nimer, S.D., Melnick, A., Neckers, L. and Chiosis, G. (2011) Affinity-based proteomics reveal cancer-specific networks coordinated by Hsp90. *Nat Chem Biol*, **7**, 818-826.

Muellner, M.K., Uras, I.Z., Gapp, B.V., Kerzendorfer, C., Smida, M., Lechtermann, H., Craig-Mueller, N., Colinge, J., Duernberger, G. and Nijman, S.M. (2011) A chemical-genetic screen reveals a mechanism of resistance to PI3K inhibitors in cancer. *Nat Chem Biol*, **7**, 787-793.

Nakahara, T., Kita, A., Yamanaka, K., Mori, M., Amino, N., Takeuchi, M., Tominaga, F., Hatakeyama, S., Kinoyama, I., Matsuhisa, A., Kudoh, M. and Sasamata, M. (2007) YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. *Cancer Res*, **67**, 8014-8021.

Nelander, S., Wang, W., Nilsson, B., She, Q.B., Pratilas, C., Rosen, N., Gennemark, P. and Sander, C. (2008) Models from experiments: combinatorial drug perturbations of cancer cells. *Mol Syst Biol*, **4**, 216.

Nicodeme, E., Jeffrey, K.L., Schaefer, U., Beinke, S., Dewell, S., Chung, C.W., Chandwani, R., Marazzi, I., Wilson, P., Coste, H., White, J., Kirilovsky, J., Rice, C.M., Lora, J.M., Prinjha, R.K., Lee, K. and Tarakhovsky, A. (2010) Suppression of inflammation by a synthetic histone mimic. *Nature*, **468**, 1119-1123.

Nomura, D.K., Dix, M.M. and Cravatt, B.F. (2010) Activity-based protein profiling for biochemical pathway discovery in cancer. *Nat Rev Cancer*, **10**, 630-638.

O'Hare, T., Shakespeare, W.C., Zhu, X., Eide, C.A., Rivera, V.M., Wang, F., Adrian, L.T., Zhou, T., Huang, W.S., Xu, Q., Metcalf, C.A., 3rd, Tyner, J.W., Loriaux, M.M., Corbin, A.S., Wardwell, S., Ning, Y., Keats, J.A., Wang, Y., Sundaramoorthi, R., Thomas, M., Zhou, D., Snodgrass, J., Commodore, L., Sawyer, T.K., Dalgarno, D.C., Deininger, M.W., Druker, B.J. and Clackson, T. (2009) AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. *Cancer Cell*, **16**, 401-412.

Ong, S.E., Schenone, M., Margolin, A.A., Li, X., Do, K., Doud, M.K., Mani, D.R., Kuai, L., Wang, X., Wood, J.L., Tolliday, N.J., Koehler, A.N., Marcaurelle, L.A., Golub, T.R., Gould, R.J., Schreiber, S.L. and Carr, S.A. (2009) Identifying the proteins to which small-molecule probes and drugs bind in cells. *Proc Natl Acad Sci U S A*, **106**, 4617-4622.

Ozvegy-Laczka, C., Cserepes, J., Elkind, N.B. and Sarkadi, B. (2005) Tyrosine kinase inhibitor resistance in cancer: role of ABC multidrug transporters. *Drug Resist Updat*, **8**, 15-26.

Pan, C., Olsen, J.V., Daub, H. and Mann, M. (2009) Global effects of kinase inhibitors on signaling networks revealed by quantitative phosphoproteomics. *Mol Cell Proteomics*, **8**, 2796-2808.

Pao, W., Miller, V.A., Politi, K.A., Riely, G.J., Somwar, R., Zakowski, M.F., Kris, M.G. and Varmus, H. (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med*, **2**, e73.

Parsons, A.B., Lopez, A., Givoni, I.E., Williams, D.E., Gray, C.A., Porter, J., Chua, G., Sopko, R., Brost, R.L., Ho, C.H., Wang, J., Ketela, T., Brenner, C., Brill, J.A., Fernandez, G.E., Lorenz, T.C., Payne, G.S., Ishihara, S., Ohya, Y., Andrews, B., Hughes, T.R., Frey, B.J., Graham, T.R., Andersen, R.J. and Boone, C. (2006) Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. *Cell*, **126**, 611-625.

Pecot, C.V., Calin, G.A., Coleman, R.L., Lopez-Berestein, G. and Sood, A.K. (2011) RNA interference in the clinic: challenges and future directions. *Nat Rev Cancer*, **11**, 59-67.

Perez-Galan, P., Roue, G., Villamor, N., Campo, E. and Colomer, D. (2007) The BH3-mimetic GX15-070 synergizes with bortezomib in mantle cell lymphoma by enhancing Noxa-mediated activation of Bak. *Blood*, **109**, 4441-4449.

Perry, J., Ghazaly, E., Kitromilidou, C., McGrowder, E.H., Joel, S. and Powles, T. (2010) A synergistic interaction between lapatinib and chemotherapy agents in a panel of cell lines is due to the inhibition of the efflux pump BCRP. *Mol Cancer Ther*, **9**, 3322-3329.

Poulikakos, P.I., Zhang, C., Bollag, G., Shokat, K.M. and Rosen, N. (2010) RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature*, **464**, 427-430.

Prahallad, A., Sun, C., Huang, S., Di Nicolantonio, F., Salazar, R., Zecchin, D., Beijersbergen, R.L., Bardelli, A. and Bernards, R. (2012) Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature*, **483**, 100-103.

Remsing Rix, L.L., Rix, U., Colinge, J., Hantschel, O., Bennett, K.L., Stranzl, T., Muller, A., Baumgartner, C., Valent, P., Augustin, M., Till, J.H. and Superti-Furga, G. (2009) Global target profile of the kinase inhibitor bosutinib in primary chronic myeloid leukemia cells. *Leukemia*, **23**, 477-485.

Richardson, P.G., Barlogie, B., Berenson, J., Singhal, S., Jagannath, S., Irwin, D., Rajkumar, S.V., Srkalovic, G., Alsina, M., Alexanian, R., Siegel, D., Orlowski, R.Z., Kuter, D., Limentani, S.A., Lee, S., Hideshima, T., Esseltine, D.L., Kauffman, M., Adams, J., Schenkein, D.P. and Anderson, K.C. (2003) A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med*, **348**, 2609-2617.

Rickles, R.J., Pierce, L.T., Giordano, T.P., 3rd, Tam, W.F., McMillin, D.W., Delmore, J., Laubach, J.P., Borisy, A.A., Richardson, P.G. and Lee, M.S. (2010) Adenosine A2A receptor agonists and PDE inhibitors: a synergistic multitarget mechanism discovered through systematic combination screening in B-cell malignancies. *Blood*, **116**, 593-602.

Rix, U., Hantschel, O., Durnberger, G., Remsing Rix, L.L., Planyavsky, M., Fernbach, N.V., Kaupe, I., Bennett, K.L., Valent, P., Colinge, J., Kocher, T. and Superti-Furga, G. (2007) Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. *Blood*, **110**, 4055-4063.

Rix, U. and Superti-Furga, G. (2009) Target profiling of small molecules by chemical proteomics. *Nat Chem Biol*, **5**, 616-624.

Roguev, A., Bandyopadhyay, S., Zofall, M., Zhang, K., Fischer, T., Collins, S.R., Qu, H., Shales, M., Park, H.O., Hayles, J., Hoe, K.L., Kim, D.U., Ideker, T., Grewal, S.I., Weissman, J.S. and Krogan, N.J. (2008) Conservation and rewiring of functional modules revealed by an epistasis map in fission yeast. *Science*, **322**, 405-410.

Rotella, D.P. (2002) Phosphodiesterase 5 inhibitors: current status and potential applications. *Nat Rev Drug Discov*, **1**, 674-682.

Russ, A.P. and Lampel, S. (2005) The druggable genome: an update. *Drug Discov Today*, **10**, 1607-1610.

Sakamoto, H., Tsukaguchi, T., Hiroshima, S., Kodama, T., Kobayashi, T., Fukami, T.A., Oikawa, N., Tsukuda, T., Ishii, N. and Aoki, Y. (2011) CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. *Cancer Cell*, **19**, 679-690.

San Miguel, J.F., Schlag, R., Khuageva, N.K., Dimopoulos, M.A., Shpilberg, O., Kropff, M., Spicka, I., Petrucci, M.T., Palumbo, A., Samoilova, O.S., Dmoszynska, A., Abdulkadyrov, K.M., Schots, R., Jiang, B., Mateos, M.V., Anderson, K.C., Esseltine, D.L., Liu, K., Cakana, A., van de Velde, H. and Richardson, P.G. (2008) Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. *N Engl J Med*, **359**, 906-917.

Santo, L., Hideshima, T., Kung, A.L., Tseng, J.C., Tamang, D., Yang, M., Jarpe, M., van Duzer, J.H., Mazitschek, R., Ogier, W.C., Cirstea, D., Rodig, S., Eda, H., Scullen, T., Canavese, M., Bradner, J., Anderson, K.C., Jones, S.S. and Raje, N. (2012) Preclinical activity, pharmacodynamic, and pharmacokinetic properties of a selective HDAC6 inhibitor, ACY-1215, in combination with bortezomib in multiple myeloma. *Blood*, **119**, 2579-2589.

Sarangi, A., Bupp, K. and Roth, M.J. (2007) Identification of a retroviral receptor used by an envelope protein derived by peptide library screening. *Proc Natl Acad Sci U S A*, **104**, 11032-11037.

Sarkadi, B., Homolya, L., Szakacs, G. and Varadi, A. (2006) Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. *Physiol Rev*, **86**, 1179-1236.

Satoh, T., Okamoto, I., Miyazaki, M., Morinaga, R., Tsuya, A., Hasegawa, Y., Terashima, M., Ueda, S., Fukuoka, M., Ariyoshi, Y., Saito, T., Masuda, N., Watanabe, H., Taguchi, T., Kakihara, T., Aoyama, Y., Hashimoto, Y. and Nakagawa, K. (2009) Phase I study of YM155, a novel survivin suppressant, in patients with advanced solid tumors. *Clin Cancer Res*, **15**, 3872-3880.

Sawyers, C.L., Callahan, W. and Witte, O.N. (1992) Dominant negative MYC blocks transformation by ABL oncogenes. *Cell*, **70**, 901-910.

Scholl, C., Frohling, S., Dunn, I.F., Schinzel, A.C., Barbie, D.A., Kim, S.Y., Silver, S.J., Tamayo, P., Wadlow, R.C., Ramaswamy, S., Dohner, K., Bullinger, L., Sandy, P., Boehm, J.S., Root, D.E., Jacks, T., Hahn, W.C. and Gilliland, D.G. (2009) Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. *Cell*, **137**, 821-834.

Sherbenou, D.W., Hantschel, O., Kaupe, I., Willis, S., Bumm, T., Turaga, L.P., Lange, T., Dao, K.H., Press, R.D., Druker, B.J., Superti-Furga, G. and Deininger, M.W. (2010) BCR-ABL SH3-SH2 domain mutations in chronic myeloid leukemia patients on imatinib. *Blood*, **116**, 3278-3285.

Siddik, Z.H. (2003) Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*, **22**, 7265-7279.

Sieber, S.A., Niessen, S., Hoover, H.S. and Cravatt, B.F. (2006) Proteomic profiling of metalloprotease activities with cocktails of active-site probes. *Nat Chem Biol*, **2**, 274-281.

Smith, A.M., Ammar, R., Nislow, C. and Giaever, G. (2010) A survey of yeast genomic assays for drug and target discovery. *Pharmacol Ther*, **127**, 156-164.

Smith, A.M., Durbic, T., Kittanakom, S., Giaever, G. and Nislow, C. (2012) Barcode sequencing for understanding drug-gene interactions. *Methods Mol Biol*, **910**, 55-69.

Soucy, T.A., Smith, P.G., Milhollen, M.A., Berger, A.J., Gavin, J.M., Adhikari, S., Brownell, J.E., Burke, K.E., Cardin, D.P., Critchley, S., Cullis, C.A., Doucette, A., Garnsey, J.J., Gaulin, J.L., Gershman, R.E., Lublinsky, A.R., McDonald, A., Mizutani, H., Narayanan, U., Olhava, E.J., Peluso, S., Rezaei, M., Sintchak, M.D., Talreja, T., Thomas, M.P., Traore, T., Vyskocil, S., Weatherhead, G.S., Yu, J., Zhang, J., Dick, L.R., Claiborne, C.F., Rolfe, M., Bolen, J.B. and Langston, S.P. (2009) An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature*, **458**, 732-736.

Sourbier, C., Scroggins, B.T., Ratnayake, R., Prince, T.L., Lee, S., Lee, M.J., Nagy, P.L., Lee, Y.H., Trepel, J.B., Beutler, J.A., Linehan, W.M. and Neckers, L. (2013) Englerin A Stimulates PKCtheta to Inhibit Insulin Signaling and to Simultaneously Activate HSF1: Pharmacologically Induced Synthetic Lethality. *Cancer Cell*, **23**, 228-237.

Stanton, B.Z., Peng, L.F., Maloof, N., Nakai, K., Wang, X., Duffner, J.L., Taveras, K.M., Hyman, J.M., Lee, S.W., Koehler, A.N., Chen, J.K., Fox, J.L., Mandinova, A. and Schreiber, S.L. (2009) A small molecule that binds Hedgehog and blocks its signaling in human cells. *Nat Chem Biol*, **5**, 154-156.

Stegmaier, K., Ross, K.N., Colavito, S.A., O'Malley, S., Stockwell, B.R. and Golub, T.R. (2004) Gene expression-based high-throughput screening(GE-HTS) and application to leukemia differentiation. *Nat Genet*, **36**, 257-263.

Stephens, P.J., Tarpey, P.S., Davies, H., Van Loo, P., Greenman, C., Wedge, D.C., Nik-Zainal, S., Martin, S., Varela, I., Bignell, G.R., Yates, L.R., Papaemmanuil, E., Beare, D., Butler, A., Cheverton, A., Gamble, J., Hinton, J., Jia, M., Jayakumar, A., Jones, D., Latimer, C., Lau, K.W., McLaren, S., McBride, D.J., Menzies, A., Mudie, L., Raine, K., Rad, R., Chapman, M.S., Teague, J., Easton, D., Langerod, A., Lee, M.T., Shen, C.Y., Tee, B.T., Huimin, B.W., Broeks, A., Vargas, A.C., Turashvili, G., Martens, J., Fatima, A., Miron, P., Chin, S.F., Thomas, G., Boyault, S., Mariani, O., Lakhani, S.R., van de Vijver, M., van 't Veer, L., Foekens, J., Desmedt, C., Sotiriou, C., Tutt, A., Caldas, C., Reis-Filho, J.S., Aparicio, S.A., Salomon, A.V., Borresen-Dale, A.L., Richardson, A.L., Campbell, P.J., Futreal, P.A. and Stratton, M.R. (2012) The landscape of cancer genes and mutational processes in breast cancer. *Nature*, **486**, 400-404.

Straussman, R., Morikawa, T., Shee, K., Barzily-Rokni, M., Qian, Z.R., Du, J., Davis, A., Mongare, M.M., Gould, J., Frederick, D.T., Cooper, Z.A., Chapman, P.B., Solit, D.B., Ribas, A., Lo, R.S., Flaherty, K.T., Ogino, S., Wargo, J.A. and Golub, T.R. (2012) Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature*, **487**, 500-504.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S. and Mesirov, J.P. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*, **102**, 15545-15550.

Szakacs, G., Paterson, J.K., Ludwig, J.A., Booth-Genthe, C. and Gottesman, M.M. (2006) Targeting multidrug resistance in cancer. *Nat Rev Drug Discov*, **5**, 219-234.

Tam, C.S. and Keating, M.J. (2010) Chemoimmunotherapy of chronic lymphocytic leukemia. *Nat Rev Clin Oncol*, **7**, 521-532.

Tan, N., Malek, M., Zha, J., Yue, P., Kassees, R., Berry, L., Fairbrother, W.J., Sampath, D. and Belmont, L.D. (2011) Navitoclax enhances the efficacy of taxanes in non-small cell lung cancer models. *Clin Cancer Res*, **17**, 1394-1404.

Tolcher, A.W., Quinn, D.I., Ferrari, A., Ahmann, F., Giaccone, G., Drake, T., Keating, A. and de Bono, J.S. (2012) A phase II study of YM155, a novel small-molecule suppressor of survivin, in castration-resistant taxane-pretreated prostate cancer. *Ann Oncol*, **23**, 968-973.

Trepel, J., Mollapour, M., Giaccone, G. and Neckers, L. (2010) Targeting the dynamic HSP90 complex in cancer. *Nat Rev Cancer*, **10**, 537-549.

Tse, C., Shoemaker, A.R., Adickes, J., Anderson, M.G., Chen, J., Jin, S., Johnson, E.F., Marsh, K.C., Mitten, M.J., Nimmer, P., Roberts, L., Tahir, S.K., Xiao, Y., Yang, X., Zhang, H., Fesik, S., Rosenberg, S.H. and Elmore, S.W. (2008) ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res*, **68**, 3421-3428.

Uhlmann, T., Geoghegan, V.L., Thomas, B., Ridlova, G., Trudgian, D.C. and Acuto, O. (2012) A method for large-scale identification of protein arginine methylation. *Mol Cell Proteomics*, **11**, 1489-1499.

Wacker, S.A., Houghtaling, B.R., Elemento, O. and Kapoor, T.M. (2012) Using transcriptome sequencing to identify mechanisms of drug action and resistance. *Nat Chem Biol*, **8**, 235-237.

Walhout, A.J.M., Vidal, M. and Dekker, J. (2012) *Handbook of Systems Biology*. Academic Press.

Wattel, E., Solary, E., Hecquet, B., Caillot, D., Ifrah, N., Brion, A., Milpied, N., Janvier, M., Guerci, A., Rochant, H., Cordonnier, C., Dreyfus, F., Veil, A., Hoang-Ngoc, L., Stoppa, A.M., Gratecos, N., Sadoun, A., Tilly, H., Brice, P., Lioure, B., Desablens, B., Pignon, B., Abgrall, J.P., Leporrier, M., Fenaux, P. and et al. (1999) Quinine improves results of intensive chemotherapy (IC) in myelodysplastic syndromes (MDS) expressing P-glycoprotein (PGP). Updated results of a randomized study. Groupe Francais des Myelodysplasies (GFM) and Groupe GOELAMS. *Adv Exp Med Biol*, **457**, 35-46.

Weaver, B.A. and Cleveland, D.W. (2005) Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell*, **8**, 7-12.

Wei, Y., Kadia, T., Tong, W., Zhang, M., Jia, Y., Yang, H., Hu, Y., Tambaro, F.P., Viallet, J., O'Brien, S. and Garcia-Manero, G. (2010) The combination of a histone deacetylase inhibitor with the Bcl-2 homology domain-3 mimetic GX15-070 has synergistic antileukemia activity by activating both apoptosis and autophagy. *Clin Cancer Res*, **16**, 3923-3932.

Weinstein, I.B. (2002) Cancer. Addiction to oncogenes--the Achilles heal of cancer. *Science*, **297**, 63-64.

Weisberg, E., Catley, L., Wright, R.D., Moreno, D., Banerji, L., Ray, A., Manley, P.W., Mestan, J., Fabbro, D., Jiang, J., Hall-Meyers, E., Callahan, L., DellaGatta, J.L., Kung, A.L. and Griffin, J.D. (2007) Beneficial effects of combining nilotinib and imatinib in preclinical models of BCR-ABL+ leukemias. *Blood*, **109**, 2112-2120.

White, D.L., Saunders, V.A., Dang, P., Engler, J., Zannettino, A.C., Cambareri, A.C., Quinn, S.R., Manley, P.W. and Hughes, T.P. (2006) OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. *Blood*, **108**, 697-704.

White, D.L., Saunders, V.A., Quinn, S.R., Manley, P.W. and Hughes, T.P. (2007) Imatinib increases the intracellular concentration of nilotinib, which may explain the observed synergy between these drugs. *Blood*, **109**, 3609-3610.

Wilson, T.R., Fridlyand, J., Yan, Y., Penuel, E., Burton, L., Chan, E., Peng, J., Lin, E., Wang, Y., Sosman, J., Ribas, A., Li, J., Moffat, J., Sutherlin, D.P., Koeppen, H., Merchant, M., Neve, R. and Settleman, J. (2012) Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature*, **487**, 505-509.

Winter, G.E., Rix, U., Carlson, S.M., Gleixner, K.V., Grebien, F., Gridling, M., Muller, A.C., Breitwieser, F.P., Bilban, M., Colinge, J., Valent, P., Bennett, K.L., White, F.M. and Superti-Furga, G. (2012) Systems-pharmacology dissection of a drug synergy in imatinib-resistant CML. *Nat Chem Biol*, **8**, 905-912.

Yamanaka, K., Nakahara, T., Yamauchi, T., Kita, A., Takeuchi, M., Kiyonaga, F., Kaneko, N. and Sasamata, M. (2011) Antitumor activity of YM155, a selective small-molecule survivin suppressant, alone and in combination with docetaxel in human malignant melanoma models. *Clin Cancer Res*, **17**, 5423-5431.

Yamauchi, T., Nakamura, N., Hiramoto, M., Yuri, M., Yokota, H., Naitou, M., Takeuchi, M., Yamanaka, K., Kita, A., Nakahara, T., Kinoyama, I., Matsuhisa, A., Kaneko, N.,

Koutoku, H., Sasamata, M., Kobori, M., Katou, M., Tawara, S., Kawabata, S. and Furuichi, K. (2012) Sepantronium bromide (YM155) induces disruption of the ILF3/p54(nrb) complex, which is required for survivin expression. *Biochem Biophys Res Commun*, **425**, 711-716.

Yang, K., Bai, H., Ouyang, Q., Lai, L. and Tang, C. (2008) Finding multiple target optimal intervention in disease-related molecular network. *Mol Syst Biol*, **4**, 228.

Yeh, P., Tschumi, A.I. and Kishony, R. (2006) Functional classification of drugs by properties of their pairwise interactions. *Nat Genet*, **38**, 489-494.

Young, D.W., Bender, A., Hoyt, J., McWhinnie, E., Chirn, G.W., Tao, C.Y., Tallarico, J.A., Labow, M., Jenkins, J.L., Mitchison, T.J. and Feng, Y. (2008) Integrating high-content screening and ligand-target prediction to identify mechanism of action. *Nat Chem Biol*, **4**, 59-68.

Zhang, J., Adrian, F.J., Jahnke, W., Cowan-Jacob, S.W., Li, A.G., Iacob, R.E., Sim, T., Powers, J., Dierks, C., Sun, F., Guo, G.R., Ding, Q., Okram, B., Choi, Y., Wojciechowski, A., Deng, X., Liu, G., Fendrich, G., Strauss, A., Vajpai, N., Grzesiek, S., Tuntland, T., Liu, Y., Bursulaya, B., Azam, M., Manley, P.W., Engen, J.R., Daley, G.Q., Warmuth, M. and Gray, N.S. (2010) Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature*, **463**, 501-506.

Zhou, H., Xu, M., Huang, Q., Gates, A.T., Zhang, X.D., Castle, J.C., Stec, E., Ferrer, M., Strulovici, B., Hazuda, D.J. and Espeseth, A.S. (2008) Genome-scale RNAi screen for host factors required for HIV replication. *Cell Host Microbe*, **4**, 495-504.

Zhou, W., Ercan, D., Chen, L., Yun, C.H., Li, D., Capelletti, M., Cortot, A.B., Chirieac, L., Iacob, R.E., Padera, R., Engen, J.R., Wong, K.K., Eck, M.J., Gray, N.S. and Janne, P.A. (2009) Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature*, **462**, 1070-1074.

Curriculum Vitae

Personal Information

Name:	**Georg Winter**
Nationality:	Austrian
Day of Birth:	March 20th, 1985
Address:	Josefstädterstraße 47-49, 1080 Vienna, Austria
Phone:	+43 (0)6645286643 (m)
E-mail:	gwinter@cemm.oeaw.ac.at wintergeorg@hotmail.com
Skype:	georg.e.winter

Education

9/2008-present **Ph.D. in Applied Medical Sciences**
CeMM-Research Center for Molecular Medicine
Supervisor: Giulio Superti-Furga, Ph.D.
Title: "Integrative Approaches in Cancer Chemical Biology"

7/2007-9/2008 **M.S. in Molecular Biotechnology**
IMP-Institute for Molecular Pathology
Supervisor: Thomas Jenuwein, Ph.D.
(Grade: 'Very Good' [highest mark])
Title: "Generation of mutant mouse ES cells to study HMTase function"

Seminars and Poster Presentations

"An integrated chemical biology approach identifies specific vulnerability of Ewings sarcoma to combined inhibition of Aurora kinases A and B", Nature Chemical Biology meeting 2011, Boston, USA

"*Haploid genetic screens reveal genomic requirements of drugs and epistatic drug combinations*", CeMM SAB meeting 2011, Vienna, Austria

"Genomic requirements of drugs and drug combinations revealed by a global gene disruption approach" YSA meeting 2011, Vienna, Austria

"*Chemical proteomics and kinome profiling in pediatric sarcomas*" Molecular Signal Transduction summer school 2009, Spetses

Peer Review

Assisting *Nature Chemical Biology, Journal of Proteome Research, Blood, Molecular Cancer Therapeutics*

Languages

German (native), English (fluent),

Interests

Music, Arts, Soccer and Running

List of Publications

1. **Winter G.E.**, Rix U., Carlson S., Grebien F., Gridling M., Colinge J., Bennet K.L., White F. and Superti-Furga G.: Systems-pharmacology dissection of a drug synergy in imatinib resistant CML. *Nature Chemical Biology, 2012*

2. Superti-Furga, G., K. Huber, and **G. Winter**, *Designing Multi-Target Drugs*. RSC Drug Discovery, ed. R. Morphy and J. Harris. 2012, Cambridge: Royal Society of Chemistry. 256.

3. **Winter G.E.**, Rix U**.**, Lissat A., Stukalov A., Müllner M.K., Bennett K.L., Colinge J., Nijman S., Kubicek S., Kovar H., Kontny U. and Superti-Furga G.: An integrated chemical biology approach identifies specific vulnerability of Ewings sarcoma to combined inhibition of Aurora kinases A and B, *Mol.CancerTher*., 2011

4. Mohien C.U., Hartler J., Breitwieser F., Rix U., Remsing Rix L.L., **Winter G.E.**, Thallinger G.G., Bennett K.L., Superti-Furga G., Trajanowski Z. and Colinge J.: MASPECTRAS 2: An integration and analysis platform for proteomic data, *Proteomics,* 2010, 10(14), 2719-2722.

Statement

Parts of this thesis are taken from

Winter G.E., Rix U., Carlson S., Grebien F., Gridling M., Colinge J., Bennet K.L., White F. and Superti-Furga G.: Systems-pharmacology dissection of a drug synergy in imatinib resistant CML. *Nature Chemical Biology, 2012*

and

Winter G.E., Rix U**.**, Lissat A., Stukalov A., Müllner M.K., Bennett K.L., Colinge J., Nijman S., Kubicek S., Kovar H., Kontny U. and Superti-Furga G.: An integrated chemical biology approach identifies specific vulnerability of Ewings sarcoma to combined inhibition of Aurora kinases A and B, *Mol.CancerTher*., 2011

and

Superti-Furga, G., K. Huber, and **G. Winter**, *Designing Multi-Target Drugs*. RSC Drug Discovery, ed. R. Morphy and J. Harris. 2012, Cambridge: Royal Society of Chemistry. 256.