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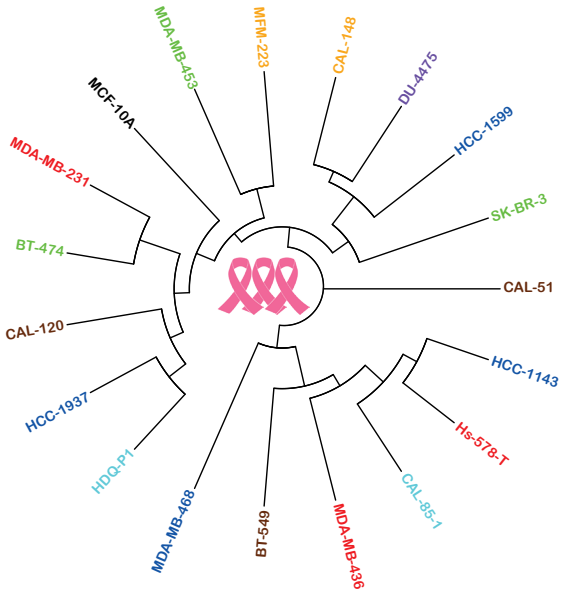
PRSON GAUTAM

Chemical Systems Biology Studies of Triple Negative Breast Cancer Cell Lines



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The Doctoral Programme in Biomedicine (DPBM)

CHEMICAL SYSTEMS BIOLOGY STUDIES OF TRIPLE NEGATIVE BREAST CANCER CELL LINES

Prson Gautam

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine, University of Helsinki, for public examination in lecture hall 3, Biomedicum Helsinki, Haartmaninkatu 8, on Thursday 14th of December 2017, at 12 noon.

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It always seems impossible until it's done.

-Nelson Mandela

Success is a journey, not a destination. The doing is often more important than the outcome.

-Arthur Ashe

To you mom
without you I would not have had my presence .....

Table of contents

Abbreviations

AA	activity area
AR	androgen receptor
ATP	adenosine triphosphate
AUC	area under curve
Bcl-2	B-cell lymphoma 2
BL1	basal-like 1
BL2	basal-like 2
BRCA1	breast cancer 1
CCLE	Cancer Cell Line Encyclopedia
CDK	cyclin-dependent kinase
CGP	Cancer Genome project
CTLA4	cytotoxic T lymphocyte antigen 4
DIP	drug induced proliferation
DMSO	dimethyl sulfoxide
DSRT	drug sensitivity and resistance testing
DSS	drug sensitivity score
EC_{50}	half maximal effective concentration
EGFR	epidermal growth factor receptor
Emax	maximum effect
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
GI_{50}	half growth inhibition
GR	growth rate
GSK	GlaxoSmithKline
HDAC	histone deacetylase
HER2	human epidermal growth factor receptor 2
HR	hormonal receptor
HSP90	heat shock protein 90
IC_{50}	half maximal inhibitory concentration
IM	immunomodulator
IMPDH	Inosine-5'-monophosphate dehydrogenase
KEGG	Kyoto Encyclopedia of Genes and Genomes

KISS	kinase inhibition sensitivity score
LAR	luminal androgen receptor
LC_{50}	half lethal concentration
LHRH	luteinizing hormone-releasing hormone
M	mesenchymal-like
MAPK	mitogen-activated protein kinase kinase
MEK	mitogen-activated protein kinase
MRI	magnetic resonance imaging
MSL	mesenchymal stem cell-like
mTOR	mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAMPT	nicotinamide phosphoribosyltransferase
NCI60	National Cancer Institute-60 tumor cell line screen
NDR	normalized drug response
Notch-3	neurogenic locus notch homolog protein 3
PAI-1	plasminogen activator inhibitor-1
PARP	Poly-ADP-ribose polymerase
PD-1	programmed cell death
PI	percent inhibition
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PKIS	published kinase inhibitor set
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
qDT	quantitative drug target
RPPA	reverse phase protein array
RTK	receptor tyrosine kinase
SD	standard deviation
sDSS	selective DSS
SERD	selective estrogen receptor degrader
SERM	selective estrogen receptor modulator
SMKI	small molecule kinase inhibitor
STAT3	signal transducer and activator of transcription3
TGI	total growth inhibition
TK	tyrosine kinase
TNBC	triple negative breast cancer
uDT	unary drug target
UNC	unclassified

List of original publications

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I. **Prson Gautam***, Leena Karhinen*, Agnieszka Szwajda, Sawan Kumar Jha, Bhagwan Yadav, Tero Aittokallio, Krister Wennerberg. "Identification of selective cytotoxic and synthetic lethal drug responses in triple negative breast cancer cells." *Mol. Cancer*, 2016. **15**(1), 1-16.

II. Abhishekh Gupta*, **Prson Gautam***, Krister Wennerberg & Tero Aittokallio. "A normalized drug response metric improves accuracy and consistency of drug sensitivity quantification in cell-based screening." *Nat. Commun*, 2017, under review

III. Agnieszka Szwajda, **Prson Gautam**, Leena Karhinen, Jing Tang, Bhagwan Yadav, Sawan Kumar Jha, Jani Saarela, Laura Turunen, Tea Pemovska, Krister Wennerberg & Tero Aittokallio. "Systematic mapping of kinase addiction combinations in breast cancer cells by integrating drug sensitivity and selectivity profiles." *Chem. Biology*, 2015. **22**(8): p 1144-55

* Equal contribution

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Publications related to the study but not included in thesis

Mpindi JP, Yadav B, Östling P, **Gautam P**, Malani D, Murumägi A, Hirasawa A, Kangaspeska S, Wennerberg K, Kallioniemi O & Aittokallio T. "Consistency in drug response profiling." Nature, 2016. 540 (7631): E5-E6.

Al-Ali H, Lee DH, Danzi MC, Nassif H, **Gautam P**, Wennerberg K, Zuercher B, Drewery DH, Lee JK, Lemmon VP, Bixby JL. "Rational polypharmacology: systematically identification and engaging multiple drug targets to promote axon growth." ACS Chem Biol.,2015. 10(8):1939-51.

Duellman S, Zhou W, Meisenheimer P, Vidugiris G, Cali J, **Gautam P**, Wennerberg K, and Vidugiriene J. "Bioluminescent, non-lytic, real-time cell viability assay and use in inhibitor screening." Assay Drug Dev Technol., 2015. 13(8):456-465.

Vidugiriene J, Leippe D, Sobol M, Vidugiris G, Zhou W, Meisenheimer P, **Gautam P**, Wennerberg K, Cali J. "Bioluminescent Cell-Based NAD(P)/NAD(P)H assays for rapid dinucleotide measurement and inhibitor screening." Assay Drug Dev Technol., 2014. 12(9-10):514-526.

Abstract

Triple negative breast cancer (TNBC) is a highly aggressive type of breast cancer that accounts for 15-20% of breast cancer cases. Targeted therapy remains to be established for TNBC that lacks estrogen receptors (ERs), progesterone receptors (PRs) and human epidermal growth factor receptor HER2, and therefore fails to respond to hormonal and anti-HER2 treatment. This limits the therapy to traditional chemotherapy, radiation and surgery, which is only beneficial to a fraction of TNBC patients. Transcriptomics-based subtyping of TNBC into six classes highlights the heterogeneity within the TNBC diseases, but it is unclear how the transcriptomics-based subtypes link to effective therapeutic strategies, resulting in a poor clinical prognosis in comparison to other breast cancer subtypes. Hence, there is an imminent need for identifying molecular markers and druggable targets against TNBC.

This study is focused on the establishment of functional profiling of TNBC cell lines based on their drug vulnerabilities, and to identify novel druggable signaling nodes. We studied a panel of 16 TNBC cell lines using a functional profiling approach in which we measured the responses of TNBC cells to 304 oncology compounds and 355 GSK published kinase inhibitors. The clustering analysis based on overall drug-responses did not match the transcriptomics-based subtypes, suggesting the presence of extensive heterogeneity in TNBC and that the genomic or transcriptomic profiles do not always reflect the functional behavior of these cells.

First, to go beyond standard anti-proliferative drug effects, we established a multiplexed readout for both cell viability and cytotoxicity. We identified many drug classes (such as anti-mitotics, anti-metabolites, mTOR inhibitors), which generally are assumed to have cytotoxic effects, mostly exhibited strong effects on cell viability but failed to kill the cells. However, in a subset of the cell lines, they induced a selective cell death. In those cases, we identified differential levels of protein markers linked to the cytotoxic responses (e.g. high level PAI-1

linked to anti-mitotics), suggesting their potential use in clinics for therapeutic decision. These results highlighted that simple multiplexed cell viability and cytotoxicity measurements provide more insight in cellular responses towards the treatment and thereby may help in providing better translationally predictive readouts.

Second, we devised a novel drug response metric, called normalized drug response (NDR), which accounts for many kinds of screening artifacts such as signal growth rate differences in positive and negative control, as well as in drug-treated conditions. We found that the NDR metric is a time-independent method and it significantly improved the drug response curve fitting. In addition, the NDR metric allowed us to differentiate the drug biology from a single viability readout, and enabled classifying the drug effects into four classes: lethal, effective, non-effective and growth-stimulatory. Our NDR will be of great value in cell-based high throughput drug screening approaches as it cuts down the cost and time for the replicate experiments and further validation with cytotoxicity assay.

Lastly, we used computational approach to decipher the kinase signal addiction of breast cancer cell lines by integrating vulnerabilities to kinase inhibitors and their polypharmacology data. We developed the kinase inhibition sensitivity score (KISS) to predict single and combinatorial signal addictions. For this study, we used 40 approved or investigational kinase inhibitors with well-defined target selectivities. With this approach, we predicted and validated novel synergistic inhibitor combinations against TNBC cells, such as dasatinib with axitinib, bosutinib with foretinib or pazopanib, and nintedanib with enzastaurin combinations for HCC1937 cells. This study suggests that drug sensitivity profiling is a powerful strategy for de-convolving cancer cell specific target addictions.

Introduction

J. Michael Bishop and Harold E. Varmus's concept of genetic alterations of oncogenes (activation) and tumor suppressor genes (loss) leading to cancer serves as a milestone in cancer biology [1, 2]. The phenomenon known as "oncogene addiction" led to the concept of targeted therapy [3, 4]. The establishment of the concept that cancer cells cannot survive without key oncogenic activities (addictions) led to a huge effort in thorough molecular characterization of cancers. Such molecular profiling enabled rational design of targeted therapeutics against oncogenic addictions (mutations) selective to cancer cells while sparing healthy cells. During the past decades, cancer drug discovery has to a large extent been focused on developing agents that either inhibit oncogenic targets or restore the normal function of tumor suppressors. However, cancers often carry multiple genomic alterations that lead to rewiring of signaling networks or compensatory pathway activation, therefore making it difficult to pinpoint particular oncogenic addictions. This calls for the need of alternate strategies to de-convolve cancer biology and hence to identify the specific oncogenic addictions/their combinations. Moreover, far from all known oncogenic drivers can currently be pharmacologically targeted, highlighting the complexity in translating oncogenes to effective targeted drugs. For instance, *RAS* mutations are prevalent in 20-25% of all human cancers, yet anti-RAS treatments remain as an unmet need.

Cancers harboring undruggable genetic aberrations increases the complexity in cancer drug discovery. Extensive 'omics' (genomics, epigenomics, transcriptomics, proteomics, metabolomics) studies of TNBC have already highlighted the heterogeneity and complexity of the disease at both the molecular and clinical levels. The primary therapy is limited to surgery, radiotherapy and cytotoxic chemotherapy and there is therefore an unmet demand for effective targeted therapy against TNBC. However, the heterogeneity of the disease argues that no single solution will be found. This highlights a need for a precision/personalized therapy stratification based on additional functional profiling of cancer. The accessibility of a broad range of

molecularly targeted and selectivity-profiled drugs creates an unprecedented opportunity to establish a drug sensitivity-based functional profiling of cancers, with the aim to establish functional disease stratification and novel therapeutic strategies. Furthermore, the availability of an increasing number of approved and investigational oncological drugs offers a unique possibility for rapid clinical translation of findings generated from the functional studies.

Most solid tumors are therapeutically challenging not only due to the lack of targeted therapies but also because of the development of resistance to targeted monotherapies. Monotherapies are proven to generally be inadequate because of rapid development of *de novo* or acquired drug resistance arguing for the establishment of precision drug combination strategies to efficiently target the cancer cells [5, 6]. However, defining effective drug combinations upfront is often challenging, and due to increasing number of targeted compounds, testing all potential combinations is experimentally challenging [7] and expensive even in a high throughput setting. Therefore, computational systems approaches are required to recognize the potential drug combinations. An integrated systems biology approach, which incorporates drug sensitivity data, drug activity profiles and cancer signaling networks could establish a more profound understanding of cancer biology and novel stratified therapeutic strategies.

In cell-based chemical screening approaches, quantification of drug responses is crucial to define drug vulnerabilities, in terms of both efficacy and selectivity. Traditionally, end-point viability or toxicity measurement assays are used to characterize the drug-induced effects in cell line screens. Percent inhibition (PI) is a normalization metric, which is used to delineate the effect of individual dose response data by normalizing the values between positive and negative controls. However, such an end point normalization metric suffers heavily from experimental limitations, because it disregards the experimental aspects such as cell growth rate, seeding discrepancies, and cell-health measurement assay artifacts both in control conditions and under drug treatments. This has raised a debate about inconsistency in drug

screening results and the question of how to enhance the reproducibility in cancer cell drug screening.

Review of the literature

1. Breast cancer

The rate of breast cancer mortality has fallen dramatically since the 1980s because of refined drug regimens (hormonal and targeted therapies) along with technological advancement in breast cancer screening (mammography, MRI, among others) making it possible to both diagnose and effectively treat at an early stage of disease[8]. Still, breast cancer is the second leading cause of cancer death in women worldwide, only outnumbered by lung cancer. According to GLOBOCAN2012, breast cancer accounts for 25% of newly diagnosed cases of all cancer in women, claiming the lives of ~522,000 women worldwide each year [9]. Almost 5% of breast cancers are considered as familial, where *BRCA1* and *BRCA2* genes mutations are the most commonly mutated inherited genes linking to the disease [10-12]. Women with germline mutations in *BRCA1* have a lifetime breast cancer development risk of 55-65% whereas the same number for germline with *BRCA2* mutations is 45% [13]. Still, somatic mutations are perceived as the most common causes of breast cancer. Alterations in *TP53*, *RB1*, *GATA3*, *PTEN*, *PI3CA*, *CCND1*, *FGFR1*, *MYC, MAP3K*1, and *ERBB2* are the ten most prevalent somatic genetic events driving breast cancer [14].

Even though breast cancer by its name is a single disease, it is highly heterogeneous and can be histologically differentiated into 21 subtypes and molecularly into at least four subtypes, which vary in tissue morphology, treatment susceptibility, prognosis and risk assessment [15-20].

1.1. Breast cancer subtypes

The routinely used subtypes of breast cancer in clinical practice are categorized based on the profiles of three immunohistochemistry markers: hormonal receptors (estrogen and progesterone receptors),

human epidermal growth factor receptor 2 (HER2/ERBB2) and a cell proliferation marker Ki67 [18, 21]. Based on receptor levels, breast cancer is divided into 4 molecular subtypes: luminal A, luminal B, HER2 enriched and triple negative (figure 1).

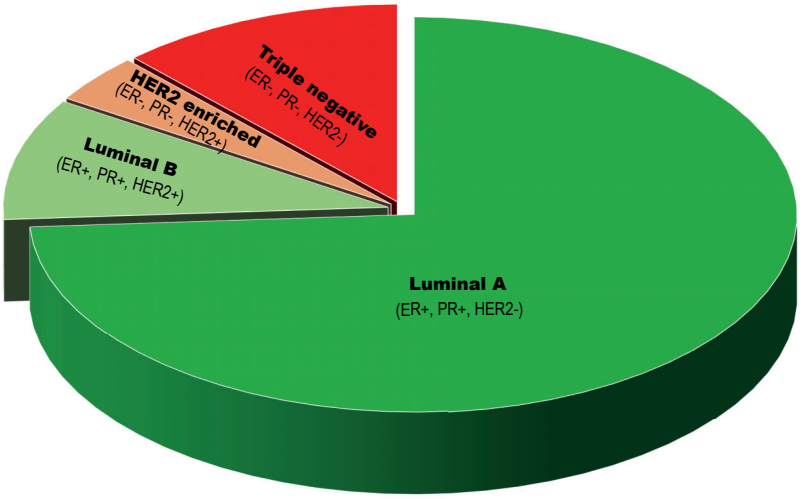

Figure 1: Classification of breast cancer based on receptor level [22]. Pie slice sizes represent fractions of total numbers of breast cancer cases.

1.1.1. Luminal A

Luminal A type of breast cancers express estrogen receptor (ER) and/or progesterone receptor (PR) but lack HER2 amplification (HER2-). They account for about 70% of total breast cancer. They have the most favorable prognosis among the major breast cancer subtypes since they are relatively slow growing and less aggressive.

1.1.2. Luminal B

In addition to estrogen and progesterone receptors, luminal B type breast cancers also overexpress HER2 (HER2+) and their prevalence is about 10% of the total cases. The luminal B subtype is regarded as more aggressive than luminal A. Luminal B breast cancers also express high levels of Ki67 protein, indicating a large population of actively proliferating cells [23].

1.1.3. HER2 enriched

This is a subtype that accounts for about 4% of breast cancers. HER2 enriched breast cancers have amplified HER2 expression but lack the expression of both hormonal receptors (HR). They tend to be more aggressive than HR+ breast cancers and HER2 enriched breast cancer patients therefore have poorer prognosis than patients with luminal subtypes [24].

1.1.4. Triple negative

As the name indicates, tripe negative breast cancers (TNBC) lack both estrogen and progesterone hormonal receptor expression and HER2 amplification. They are the most aggressive and recurring type of breast cancers with the poorest prognosis of all [25-28]. TNBC is often confused or restricted to basal subtype, because of high rates of overlap in molecular signature between TNBC and basal breast cancer subtype (~70%)[29]. In addition to lack of ER, PR and HER2 as in TNBC, basal breast cancers also often exhibit enhanced expression of EGFR and cytokeratin 5/6 [30]. TNBC is described in more detail in section 1.3.

1.2. Breast cancer therapy options

Breast cancer treatment options depends on several factors: stage, grade, type, menopausal state, among others. The stage of cancer is defined by the size and extent of its metastasis, where higher stage numbers indicate greater extent of metastasis and poorer prognosis. The grade, unlike the stage, only defines the extent of cell differentiation, where higher grades indicate gradually poorer differentiation. Most patients go through surgery (stage I-III); tumor excision (lumpectomy) or whole breast removal (mastectomy). Surgery is mostly followed by adjuvant therapy to avoid relapse of the disease. The adjuvant therapies given to the patients are usually radiotherapy, cytotoxic chemicals (e.g. doxorubicin, cyclophosphamide, docetaxel), hormonal therapy (e.g. tamoxifen and raloxifene), and growth factor targeted therapy (e.g. trastuzumab and lapatinib). The outcome of the treatment depends on the disease stage. The 5-year survival rate of

breast cancer patients drops dramatically from almost 100% for stage I patients to 22% for stage IV patients. Stage II patients have a 5-year survival of 93% and stage III have 72% [31, 32].

As a first line of therapy, hormone receptor positive (HR+) breast cancer patients are given hormonal therapies, which include selective estrogen receptor modulators (SERMs), such as tamoxifen; selective estrogen receptor degrader (SERDs) like fulvestrant; aromatase inhibitors, like anastrozole, which blocks the production of estrogen. Alternatively, luteinizing hormone-releasing hormone (LHRH) antagonist goserelin is also efficacious in treating HR+ breast cancer [33]. Administration of tamoxifen to ER+ breast cancer patients has resulted in a 39% reduction in recurrence rate averaged over all time periods and around 45-50% reduction in mortality rate throughout the first 15 years [34, 35]. For HER2 amplified breast cancer patients, humanized monoclonal antibodies against HER2 such as trastuzumab and pertuzumab form the first-line of therapy [36]. Enhanced disease-free survival and overall survival are observed with anti-HER2 therapies in HER2 amplified breast cancer patients [37].

Table 1: Approved targeted therapies against different breast cancers.

Drug	Brand name	Therapy type	Drug class	Administration
Anastrozole	Arimidex	Hormonal therapy	Aromatase inhibitor	Oral
Exemestane	Aromasin	Hormonal therapy	Aromatase inhibitor	Oral
Letrozole	Femara	Hormonal therapy	Aromatase inhibitor	Oral
Fulvestrant	Faslodex	Hormonal therapy	SERD	Injection
Goserelin	Zoladex	Hormonal therapy	LHRH antagonist	Injection
Leuprolide	Lupron	Hormonal therapy	LHRH antagonist	Injection
Megestrol acetate	Megace	Hormonal therapy	Progestin	Oral
Tamoxifen	Nolvadex	Hormonal therapy	SERM	Oral
Toremifene	Fareston	Hormonal therapy	SERM	Oral
Raloxifene	Evista	Hormonal therapy	SERM	Oral
Trastuzumab	Herceptin	Anti-HER2 therapy	Monoclonal antibody	Injection
Pertuzumab	Perjeta	Anti-HER2 therapy	Monoclonal antibody	Injection
Ado-trastuzumab emtansine	Kadcyla	Anti-HER2 therapy	Antibody drug conjugate	Injection
Lapatinib	Tykerb	Anti-HER2 therapy	Kinase inhibitor	Oral
Neratinib	Nerlynx	Anti-HER2 therapy	Kinase inhibitor	Oral
Everolimus	Afinitor	mTOR-targeted therapy	Kinase inhibitor	Oral
Palbociclib	Ibrance	CDK4/6-targeted therapy	Kinase inhibitor	Oral
Ribociclib	Kisqali	CDK4/6-targeted therapy	Kinase inhibitor	Oral
Abemaciclib	Verzenio	CDK4/6-targeted therapy	Kinase inhibitor	Oral

Devoid of hormonal receptors and HER2 amplification, TNBC still remains a therapeutic challenge as neither hormonal nor anti-HER2 therapy have any effect on it. To date, the therapy against TNBC is limited to conventional cytotoxic chemotherapies, which highlights the imminent need to establish targeted therapy for TNBC management.

1.3. Triple Negative Breast Cancer

TNBC is a highly aggressive subtype of breast cancer, which accounts for 15-20% of breast cancer cases and exhibits the poorest prognosis among all the breast cancer subtypes [38]. The 5-year survival rate of TNBC patients is significantly low, 77% as compared to 93% in non-TNBC patients [39]. TNBC tumors are larger in size, usually grade II-III and lymph node positive [27]. They also have higher rate of distant recurrence/metastasis to brain and lungs, but less likely to bones [27, 40]. At molecular level, most TNBC tumors exhibit myoepithelial/basal markers such as increased expression of EGFR, cytokeratins (CK5, CK6, CK14, CK17) and KIT [41]. The most recurrent genetic aberrations in TNBC are loss of *TP53*, *RB1* and *BRCA1/2*, along with activating mutations in the PI3K pathway, loss of *PTEN* or *INPP4B* and amplification of *MCL1* and *MYC* [17, 42]. Around 20% of TNBC patients also harbor *BRCA1/2* germline mutations, whereas additional downregulation of BRCA1/2 in TNBC is also associated with epigenetic alterations and overexpression of negative regulators of the BRCA1 promoter (like Id4) [43-46].

TNBC is a highly heterogeneous breast cancer subtype. Lack of the identification of common oncogenic driver alterations has made it highly challenging for the development of targeted therapy. To address this challenge, several studies have been carried out to classify TNBC cases into subtypes based on their molecular profiles. While comparing the overall gene expression profile (7,770 genes) of 97 TNBC samples, Kreike *et al.* divided them into five subgroups [25]. Similarly, Lehmann *et al.* classified TNBC into seven subgroups based on genome-wide gene expression profiling (six defined classes and one undefined), which exhibit distinct transcriptomic signatures: two basal subtypes (BL1 and BL2), two mesenchymal subtypes (M and MSL), an

immunomodulatory (IM) subtype, and a luminal androgen receptor positive (LAR) subtype [47]. In a recent study, Burstein *et al.* also established a similar classification schema of TNBC by analyzing the genomic and transcriptomic profiles of 198 tumors, in which they defined four subtypes; basal-like/immune-suppressed (BLIS), basal-like/immune activated (BLIA), mesenchymal (MES), and luminal/androgen receptor (LAR) [48]. Elsawaf *et al.* assigned four subtypes based on expression of 13 biomarkers (BCL2, CK7, CK14, CK18, CK19, CK56, CD117, EGFR, Ki67, p16, p53, vimentin, WT1); two basal (A and B), a basoluminal and luminal [49].

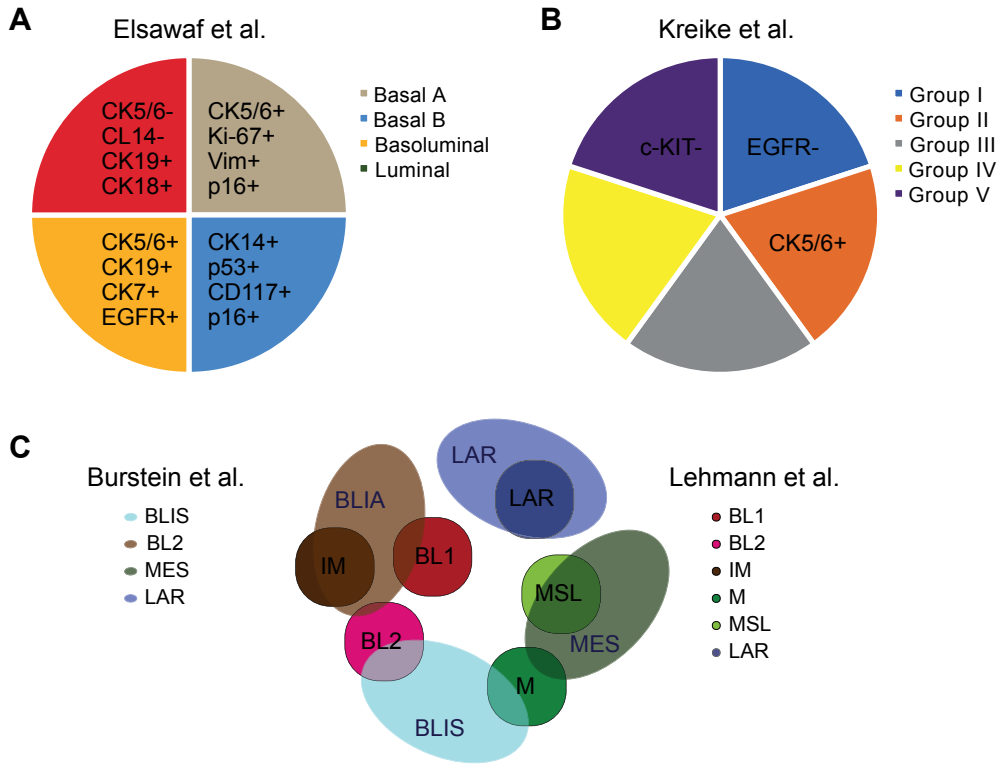

Figure 2: Molecular classification of TNBC. A) Subgrouping of TNBC into 4 groups based on expression level of 13 biomarkers [49], highlighted are expression level of respective biomarkers. B) Subtyping of TNBC into 5 groups based on the Kreike *et al.* study [25]. Group IV and V do not have any unique markers. C) Overlap between TNBC subtypes based on the Burstein et al. and Lehmann *et al.* studies [47, 48].

Several other studies have reported clustering of TNBC based on the expression of specific markers. For example, a subgroup lacking cell-cell tight junction protein claudin was defined as "claudin-low" and a subgroup enriched in immune marker interferon as "interferon-rich" [50, 51]. However, the classification schema defined by Lehmann *et al.* is widely accepted since the subgroups exhibit particular gene expression signatures, and *in vitro* studies in TNBC cell lines hint that these subtypes also differ in their response towards drugs targeting specific driver signaling pathways [47].

1.3.1. Basal like 1 (BL1) subtype

The BL1 subtype has an enriched gene profile related to cell cycle and cell division pathways evident with high expression of proliferation markers such as *AURKA*, *AURKB*, *TTK*, *MYC*, *PLK1*, *BIRC5*, *CENPA*, *CENPF*, *BUB1*, *CCNA2*, *PRC1*, *NRAS*, *MKI67*. They are also heavily enriched in the expression of DNA damage response pathways/components including *CHEK1*, *MDC1, RAD*, *FANCA*, *FANCG*, *RAD51*, *RAD21*, *RAD54BP*, *NBN*, *EXO1*, *MSH2*, *MCM10*.

1.3.2. Basal like 2 (BL2) subtype

The transcriptomic profile of the BL2 subtype is enriched for growth factor signaling components such as *EGFR*, *IGFR*, *NGF*, *MET* and Wnt/β-catenin pathways. Additionally, BL2 is also enriched for glycolysis and gluconeogenesis pathways. As the BL2 subtype TNBCs express *TP63* and *MME* (CD10), they are regarded to be of myoepithelial tissue origin.

1.3.3. Immuno-modulators (IM) subtype

The IM subtype has enriched immune cell response gene signatures. These include *IRF1*, *IRF7*, *IRF8*, *ITK*, *JAK1*, *JAK2*, *STAT1*, *STAT4*, *STAT5A*, *LCK*, *LYN*, *NFKB1*, *NFKBIA*, *NFKBIE*, *ITK*, *RELB*, *BTK*, and *ZAP70*.

1.3.4. Mesenchymal (M) subtype

The M subtype has enriched gene expression related to cell motility (Rho pathway), cell-differentiation (TGF-β/SMAD, Wnt/β-catenin and ALK pathways) and extracellular matrix (ECM) receptor interactions.

1.3.5. Mesenchymal stem like (MSL) subtype

The MSL subtype share gene ontologies for several biological processes with the M subtype, such as cell motility, growth and differentiation pathways. However, the MSL subtype is different from M since it also includes stem cell markers such as *ABCB1*, *ABCA8*, *ALDHA1*, *BCL2*, *BMP2*, *PROCR*, *HOXA5*, *HOXA10*, *MEIS1*, *MEIS2*, *MSX*, *MEOX1*, *MEOX2*, *BMP2*, *ENG*, *KDR*, *NGFR*, *VCAM1*. The MSL subtype is also highly enriched for genes associated with growth factor signaling such as *EGFR*, *PDGFR*, calcium signaling, inositol phosphate metabolism, ERK1/2 signaling, G-protein coupled receptor signaling, ABC transporter, adipocytokine signaling and angiogenesis (*KDR*, *TEK*, *TIE*, *EPSA1*).

1.3.6. Luminal Androgen Receptor (LAR) subtype

The LAR subtype has a gene expression profile enriched in hormonally regulated pathways including steroid synthesis and estrogen metabolism, which include *AR*, *DHCR24*, *ALCAM*, *FASN*, *FGFR4*, *FKBP5*, *SPEDF*, *APOD*, *PIP*, *CLON8*.

1.4. TNBC therapy: current status and future trends

Despite the extensive molecular profiling, no single targeted TNBC therapy is yet established. The lack of targeted therapies limits the treatment options to traditional cytotoxic chemotherapy, radiation and surgery, which are curative only to a fraction of TNBC patients. The first-line therapy includes anthracyclines (doxorubicin, epirubicin), microtubule inhibitors (paclitaxel, docetaxel, vinorelbine, eribulin, ixabepilone), antimetabolites (capecitabine), cyclophosphamide and platinum compounds (carboplatin, cisplatin), mostly given in

combinations [52, 53]. Masuda et. al. compared pathologic complete response (pCR) rate among 132 patients treated with neoadjuvant chemotherapies (paclitaxel, doxorubicin and cyclophosphamide) [54] and observed that BL1 patients demonstrate highest pCR rate (52%) followed by MSL (23%) and LAR (10%). BL2 had the lowest response rate (0%) [54]. The mitotic inhibitors seem to be effective only in the subgroup of patients exhibiting high proliferation rates and impaired DNA repair machinery. Such different pCR rates in different TNBC subgroups strongly argue for the need of further patient stratification and precision medicine for TNBC.

Several targeted therapies are being investigated in the clinic, exploiting the potential molecular targets incurred from TNBC expression signature [55]. Few of the clinical trials are run with molecular inclusion criteria (precision approach), where only those patients that exhibit the defined molecular signature (or markers) qualify for the treatment. For example, several Poly-ADP ribose polymerase (PARP) inhibitors (e.g. olaparib, (ClinicalTrials.gov identifiers: NCT026815562, NCT03205761); veliparib, (NCT02849496); talazoparib, (NCT02401347); and rucaparib (NCT01074970)) are currently active in late phase clinical studies, mostly against *BRCA* mutation carriers as PARP inhibitors appeared to be highly effective against BRCA mutant TNBC [55, 56]. Similarly, the checkpoint kinase 1 (CHK1) inhibitor prexasertib is being investigated (phase II, NTC02203513) against *BRCA1/2* mutant metastatic TNBC patients. A phase II basket trial is being carried out with the CDK4/6 inhibitor ribociclib against CDK4/6 pathway activated tumors, including TNBCs (NCT02187783).

Anti-androgen agents, such as bicalutamide, enzalutamide, enobosarm, seviteronel, are in early clinical studies against AR+ TNBCs [55]. The androgen receptor (AR) signaling enriched subtype LAR also exhibit high rate of *PIK3CA* (the gene encoding the PI3Kα protein) activating mutations, which make both AR and PI3K potential targets against AR+ TNBCs. The combination of androgen antagonists and PI3K inhibitors has been reported to yield additive inhibitory effect on tumor growth against TNBC [57]. Currently, the combination of the PI3K inhibitor

taselisib and the anti-androgen enzalutamide is being evaluated in a phase II clinical trial against AR+ TNBCs (NCT02457910).

An interim analysis of the on-going I-SPY2 (novel personalized clinical trial) phase II trial (NCT01042379, expected to be completed by December 2018) has estimated that the combination of veliparib and carboplatin will enhance pCR in TNBC patients (51%) compared to the control arm treated with standard neoadjuvant therapy only (26%), and based in an interim analysis, the combination has been estimated 88% likely to be successful in a phase III trial [58].

On the other hand, most of the targeted therapies that are currently active in clinical studies against TNBC are without any molecular inclusion criteria. For example, another cyclin dependent kinase 4/6 (CDK4/6) inhibitor trilaciclib, is being investigated in phase II clinical trials against TNBCs (NCT02978716). Apoptosis inducing agent selinexor is being assessed in a phase II trial for metastatic TNBC (NCT02402764). DNA methyltransferase and histone deacetylase (HDAC) inhibitors have been reported to reverse the epithelial to mesenchymal transition (EMT) process and hence inhibit the tumor initiating cells in TNBC. Therefore, the HDAC inhibitor entinostat is currently being explored in clinical trials (e.g. NCT02708680) [59]. HSP90, an important molecular chaperone known to stabilize various growth factor receptors (EGFR, VEGFR) and mutant oncoproteins (p53, PI3K, AKT), is regarded as a potential target for TNBC [60, 61]. The HSP90 inhibitor onalespib is currently under clinical evaluation for TNBC (NCT02474173).

Receptor tyrosine kinases (RTK) and signal transduction pathway components play a vital role in cell proliferation, differentiation, metabolism, cell survival and apoptosis, making them important targets for cancer treatment. There are several RTK inhibitors and other signal transduction kinase inhibitors explored in different level of clinical trials against TNBC [55]. Even though these protein kinase inhibitors have shown great results in *in vitro* or preclinical models, they have not been very successful in clinic as single agents due to cellular reprogramming and rapid development of resistance. One of the best

examples is the failure of EGFR inhibitor (cetuximab) in the clinic against TNBC in spite of the high prevalence (~70%) of overexpressed EGFR in TNBC. It was later discovered that inhibition of EGFR leads to the activation of compensatory AKT and ERBB3 signaling pathways, which ultimately mediates resistance [62]. A phase I study of pan-EGFR family inhibitor neratinib combined with mTORC1 inhibitor temsirolimus resulted in synergistic anti-tumor activity [63]. The observed anti-tumor activity may be due to polypharmacology effect of neratinib as it is expected to not only block EGFR signaling but also the compensatory ERBB3 signaling pathways. This rather urges for the use of drugs with multi-targets (or combination therapy), which lead to concurrent blockage of the compensatory signaling pathways. The current clinical trials of protein kinase inhibitors are mostly assessed along with conventional chemotherapies or compensatory pathway inhibitors.

A number of agents targeting angiogenesis, one of the hallmarks of cancer, (e.g. bevacizumab, sunitinib, sorafenib, axitinib, pazopanib) are already approved for clinical use against various cancers, but not TNBC. Several anti-angiogenic therapies targeting VEGFR (bevacizumab, NCT01898177; sorafenib, NCT02624700; apatinib, NCT03243838; cediranib, NCT01116648) are currently being explored against TNBC in clinical settings [55]. However, the effects of anti-angiogenic therapies against TNBC are debatable as they have failed to show clinical significance in the past [64-66]. It is rather shown that anti-angiogenic treatment enhances invasion and vasculogenic mimicry in TNBC [67].

Tumor infiltrating lymphocytes (TILs) and immunotherapy against cancer (immune oncology) have gained tremendous attention in recent years [68-70]. The subtype of TNBC defined by enhanced expression of genes involved in different immune pathways like T-cell signaling, antigen processing, interleukin pathways, cytotoxic T-lymphocyte antigen 4 (CTLA4) pathways, among others advocate the potential of using immune oncology agents against TNBCs [47, 71]. TNBC is reported to have high level of TILs, suggesting the potential of immune oncology therapy against it [72]. Several immune checkpoint inhibitors are currently being clinically investigated against TNBCs, specifically

CTL4A inhibitors (ipilimumab), and programmed cell death 1 (PD-1)/PD-1 ligand (PD-L1) inhibitors (atezolizumab, pembrolizumab, nivolumab, durvalumab, PDR001). For example, in a phase I study of atezolizumab for metastatic TNBC patients, responders (10-13%) had a long-lasting benefit [73]. Currently atezolizumab in combination paclitaxel is being tested in a phase III clinical trial against metastatic TNBC (NCT03125902).

In summary, even today, most clinical trials against TNBCs are carried out in randomized fashion, treating them as a single disease. Since TNBC is such a heterogeneous subtype of breast cancer, treating it as a single disease may not be the best approach. It is already alarming as we have witnessed a plethora of targeted agents showing high potency in preclinical studies but fail in the randomized trials. Therefore, this calls for further patient stratification, umbrella trials (trials with molecular inclusion criteria) and tailored treatment strategies to establish effective therapeutics against TNBC.

2. Precision medicine approach for TNBC

Unlike the general "one drug – one disease" philosophy for cancer therapeutic development, precision medicine refers to tailored or stratified treatment based on different molecular and pathological traits exhibited by a particular individual or group of patients suffering from a particular disease. Cancer is one of the most heterogeneous types of disease marked by both molecular and clinical differences. Even patients suffering from same type of cancer exhibit different genetic and epigenetic makeups and molecular characteristics. This makes it highly unlikely to discover a golden bullet that cures all the patients in most cancer forms. Therefore, there is a need for developing stratified therapeutic options. For this, sub-classification of the disease based on its unique molecular characteristics and subsequent tailored therapeutics against the respective subgroup is expected to aid to accurately diagnose the disease and make effective personalized treatment decisions.

The main advantages of precision/personalized medicine can be summarized in two points:

1. Establishing effective targeted therapy directed towards specific tumor pathology or specific signaling pathways along with patient stratification.
2. Avoiding unnecessary treatments, hence minimizing the risks of side-effects as well as the cost of ineffective or adverse treatments.

Recently, precision medicine has gained enormous attention boosted by readily assessable sequencing technologies and existing success stories of stratified therapy against different driver mutations; for example, targeting the fusion gene *BCR-ABL1* in chronic myeloid leukemia, the *EML4-ALK* fusion or mutant *EGFR* in non-small-cell lung cancer, and mutant *BRAF* in melanoma, which significantly extended survival the respective disease patients [74-76]. Breast cancer therapy in general is one of the best examples of precision medicine, which changed the paradigm for treatment of cancer. In fact, the history of precision medicine in breast cancer dates back to sixties, far before the human genome project, which gave rise to anti-hormonal therapy (anti-estrogen) directed against the HR-positive patients. This was established solely based on tissue histopathology (receptor level). Even now the anti-estrogen treatment is guided by molecular markers rather than genetic markers since genomics fails to capture this driver abnormality. Patient stratification and receptor status based therapy has significantly improved the prognosis of receptor positive breast cancers. The best example is estrogen receptor antagonists (e.g. tamoxifen, the first successful precision therapy in cancer), which significantly reduced the mortality rate and reoccurrence of the disease in hormone receptor positive breast cancer patients [34, 77, 78]. Similarly, anti-HER2 therapies (e.g. trastuzumab), increased both progress-free survival and overall survival rate of HER2-positive breast cancer patients [79-81]. Another example of phenotypic-based precision medicine exhibiting considerable impact in clinics is the use of anti-androgen therapy against prostate cancer. These clinical outcomes revolutionized the field of modern precision medicine.

Large studies on cancer cell lines (covering different tissue types, some specific to TNBC) using genomics, transcriptomics, proteomics, epigenomics and extensive functional profiling based on drug sensitivity and loss-of-function phenotypes have been carried out in an attempt to understand the disease in detail and to establish tailored treatment strategies [82-92]. Even though it has become possible to favorably treat receptor-positive breast cancers, TNBC still poses a challenge for clinical treatment and management. Despite extensive molecular profiling of TNBC, the key genetic events driving the disease are still unclear. Different molecular subtyping approaches have not yet yielded any targeted therapy against TNBC and also, the drug response prediction power of such molecular subtypes is relatively poor except for immune enriched or LAR subtypes [25, 93]. This argues for the need of drug sensitivity based phenotypic profiling of TNBC to establish actionable subtypes. Further phenotypic profiles can be matched with molecular/genetic signatures to establish phenotypic subgroup-specific biomarkers for patient stratification against the particular treatment option. Rather than putting patients in a single group while performing clinical trials, which is mostly the case in TNBC, biomarker based patient stratification and biomarker-driven trials should be implemented.

3. Drug sensitivity testing for precision medicine

The availability of approved and investigational compounds active against a wide range of biological targets provides an unprecedented opportunity in drug repositioning studies [94, 95]. Chemical perturbation screens employing broadly targeted compound libraries could allow us to identify individualized novel drug vulnerabilities that go beyond the prediction of current genomic analysis and that can readily be translated to clinics [94, 96]. In addition, it may also help to identify and establish novel drug-target interactions with clinical application [95].

As we have realized that any given single anticancer therapy is likely to be effective only in a subset of patients, *in vitro*/*ex vivo* drug vulnerability testing holds promises in predicting drug efficacy

addressing the heterogeneity and genomic complexity of human cancers [95, 97, 98]. The establishment of the first of its kind, the National Cancer Institute 60 cell line (NCI60) platform [82] revolutionized the high-throughput cancer cell line screening approach in drug discovery. In the seminal study, 60 distinct cell lines representing 9 different tumor types were screened against a panel of anticancer compounds. Later on, it was realized that use of only 60 cell lines was not enough for capturing genetic and phenotypic diversity, heterogeneity and responders to low-frequency targeted therapies [98]. Thus, more recent studies like the Cancer Genome Project (CGP) [99] and Cancer Cell Line Encyclopedia [13] [83] have expanded the cell line panel to several hundreds. Specifically, CGP included 727 cell lines representing 16 tumor types screened against 138 drugs, whereas CCLE included 947 cell lines representing 36 tumor types screened against 24 drugs. Along with the drug vulnerability data, they also compared the genomic profiles of the cell lines for the establishment of biomarkers as predictors of therapeutic intervention. However, with such pan-cancer approaches there has been minimal success in establishing novel predictive biomarkers, which are limited to well-known oncogenic drivers. This therefore calls for cancer type/subtype specific precision medicine approaches rather than pan-cancer studies.

Studies on well-characterized HER2-positive breast cancer cell lines with anti-HER2 compounds have revealed the potential of drug screening approaches in establishing the association between drug sensitivity and activated signaling pathway or molecular subtypes [100, 101]. Similarly, in a larger scale study led by Gray and Spellman, where 45 different breast cancer cell lines representing different transcriptional subtype of breast cancer were tested against 77 different approved/investigational drugs, they were able to establish subtype specific drug responses [102]. For example, they found that basal cell lines expressing upregulated DNA-damage response proteins were selectively sensitive to cisplatin, whereas ERBB2 amplified cell lines had increased HSP90 expression and responded to geldanamycin, an HSP90 inhibitor. This advocates the usefulness of screening larger panels of tumor cell lines that incorporates different subtypes of the specific tumor. This will help to further decipher the subtype-related

molecular pattern and establish precision therapeutics against the disease.

In general, four factors play a major role in successful use of drug sensitivity screening to identify phenotypic cancer vulnerabilities:

1. selection of cell line panel
2. selection of compound library
3. selection of cell health measurement system and
4. drug response quantification technique

3.1. Cell line panel

It is already evident that cancer is a heterogeneous group of diseases exhibiting diverse tissues of origin, morphology, phenotype and clinical responses. Hence, the cell line screen-based drug positioning approach should try to include a larger panel of cell lines, which incorporates all representative subtypes of a particular cancer. Incorporating various kinds of morphologically, physiologically and genetically divergent cell lines in the analysis will provide a power in predicting therapeutic outcome in relation to its molecular pattern. However, the challenge here is to come up with optimal/critical number of cell lines upfront. For example, even if we take large-scale screening studies like CCLE and CGP, the number of cell lines are generally modest when it comes to individual cancer types. Rather than such pan-cancer approaches, analysis on collections of as highly diverse cell lines as possible from an individual cancer type is expected to give greater insight into the disease.

3.2. Compound library

It is crucial to have an unbiased compound collection, representing myriad of drug mechanisms of action, to identify and establish cell line selective signaling node vulnerabilities as well as non-specific cytotoxics. One of the limitations realized later for the seminal NCI60 study was the use of generic cytotoxics in the screens as they have higher rate of response, making it difficult to differentiate tissue/sub-type selective response or tumor heterogeneity [98]. Later, the

compound collection was expanded, currently it consists of an array of chemical compounds and natural products ranging from nonspecific cytotoxic agents to targeted therapies. With the promises of targeted therapy to establish clinically applicable context specific treatments, all subsequent big drug screening studies has focused more on the targeted therapies. Barretina et. al. studied the effect of 24 targeted oncology compounds across 479 cell lines, where the tested compounds were mostly tyrosine kinase inhibitors [83].

The increasing number of well-characterized pharmacologically active approved and investigational compounds allows the identification and establishment of new readily translatable drug repurposing paradigms. Therefore, the use of approved and investigational anticancer compounds in the screens is common nowadays. Daemen et al profiled 70 breast cancer cell lines with 90 approved or experimental compounds to predict the drug sensitivities based on the molecular profiles of the cell lines [85]. Small molecule probes with selective molecular targets can serve as tool compounds to find novel druggable molecular target addictions (dependencies for cell survival) as well as to cross validate the selective molecular mechanisms for cancer cell vulnerabilities [103]. Inactive pro-drugs, which only get activated (metabolized) within the body (specific to tissue, organ or systemic fluids), allow us to target specific tissue and reduce adverse effects [104]. However, since these pro-drugs are limited to specific body metabolism, active metabolites that mimic the pro-drugs in *in vitro* settings are required. The big drug screening approaches like Genomic of Drug Sensitivity in Cancer (GDSC) [89, 105] and Therapeutic Response Portal (CTRP v2) [106] compiled their compound library with anticancer approved, experimental and tool compounds incorporating both cytotoxic and targeted agents.

In a recent study carried out by Licciardello et al, a collection of FDA approved clinical compounds (308 biochemically diverse compounds), which also includes non-oncology therapeutic agents, called the CeMM Library of Unique Drugs (CLOUD) was used to screen prostate cancer cell lines [107]. Combination screens of CLOUD compounds were performed to identify the synergistic drug combinations that result in

reduction of prostate cancer cell viability. They were able to establish a novel synergistic combination (the antiandrogen flutamide and the vitamin K antagonist phenprocoumon) that is effective against androgen receptor (AR) mutant prostate cancer [107]. This highlights the need of widening the target space beyond known oncology targets. Thus, a prudent approach to build a comprehensive compound library collection for anti-cancer activity screens should be to include pharmacologically active compounds with known bioactivities (target inhibition profile) and well-characterized target space. Moreover, compounds with similar mode of actions should also ideally cover structural diversity to cross-validate the mechanisms of action.

3.2.1. Kinase inhibitors

Due to their pivotal role in cellular functioning, kinases are one of the most attractive oncology therapeutic target classes [108]. Protein kinases are the enzymes that modify other proteins by transferring a phosphate group from ATP (mostly) to amino acids with a free hydroxyl group (most often serine, threonine or tyrosine). They play key roles in signal transduction and regulate a vast range of cellular activities including metabolism, immune regulation, and survival. Besides, many protein kinases can act as oncogenes [109, 110], most common are hyperactivation of protein tyrosine kinases (EGFR, ERBB2, FGFR, JAK, ABL, ALK, RET, KIT, etc.) and the PI3K lipid kinases and their downstream protein kinases like AKT, mTOR, and p70S6K. Therefore, due to their tremendous therapeutic potential and “druggability”, protein kinases are the most heavily targeted target proteins in oncology drug discovery. Most kinase inhibitors target the highly conserved ATP-binding pocket of the catalytic domain which can result in inhibitor promiscuity [111]. Such promiscuity leads to a polypharmacology effect, which often can lead to a detrimental clinical outcome when inhibition of unwanted (off-target) kinases occurs, resulting in reduced efficacy or loss of therapeutic windows, but can in some cases also improve clinical efficacy if they target compensatory pathways [112].

Over the past two decades, protein kinases have been intensively investigated as targets to treat neoplastic diseases. The potential of

kinase inhibitors was first evident when trastuzumab (Herceptin), a monoclonal antibody against HER2 was approved for HER2-positive breast cancer in 1998. In 2001, first small molecule kinase inhibitor (SMKI) was approved for use in cancer; the BCR-ABL inhibitor imatinib (Gleevec) against chronic myeloid leukemia (CML), which directed the investigation massively towards SMKIs. To date, 45 drugs targeting kinases have been approved as cancer therapeutics; 39 SMKIs and 6 monoclonal antibodies (Figure 3), along with hundreds of inhibitors that are currently being explored in clinical trials [113-116]. Five SMKIs have been approved for treatment of advanced stage breast cancer; the two HER2 inhibitors lapatinib (Tykerb) and neratinib (Nerlynx), the three CDK4/6 inhibitors palbociclib (Ibrance), ribociclib (Kisqali) and abemaciclib (Verzenio) and the allosteric mTORC1 inhibitor everolimus (Afinitor). Similarly, two monoclonal antibodies targeting HER2 have been approved for breast cancer treatment, trastuzumab (Herceptin) and pertuzumab (Perjeta). An antibody-drug conjugate ado-trastuzumab emtansine (Kadcyla) is approved for treatment of trastuzumab resistance breast cancer patients. All these were approved for HER2-positive breast cancer except everolimus, palbociclib, ribociclib and abemaciclib, which have been approved for hormone-receptor positive, HER2-negative breast cancer [117-122].

The protein kinases can be broadly divided into seven major kinase groups based on sequence similarity (highlighted in bold) as follows: **TK**, tyrosine-kinase; **TKL**, tyrosine kinase-like; **STE**, homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinase; **CK1**, casein kinase ; **AGC**, protein kinase A, protein kinase G, and protein kinase C containing families; **CAMK**, calcium/calmodulin-dependent protein kinase; **CMGC**, cyclin dependent kinase, mitogen-act protein kinase, glycogen synthase kinase 3, and dual specificity protein kinase CLK containing families. Beside these, there are three other kinase classes, which exhibit structural similarity to protein kinases, including lipid kinases PI3K; atypical, ABC1, Alpha, Brd, PDHK, PIKK, RIO, TIF1 containing families; and others containing families that do not fall in any of the mentioned families. Out of 518 enzymatically active human protein kinases, 299 have been proposed as potential therapeutic targets and more than 70 000 structurally unique compounds targeting them are currently

described [113, 123]. Even though extensive studies are being held to target the kinome, clinically active drugs are limited to only a small subset of human kinases, mostly TK, CMGC and PI3K kinases, neglecting a large set of kinome (Figure 4). This emphasizes the necessity of exploring kinases of other classes, which might hold equal potential as anti-cancer targets.

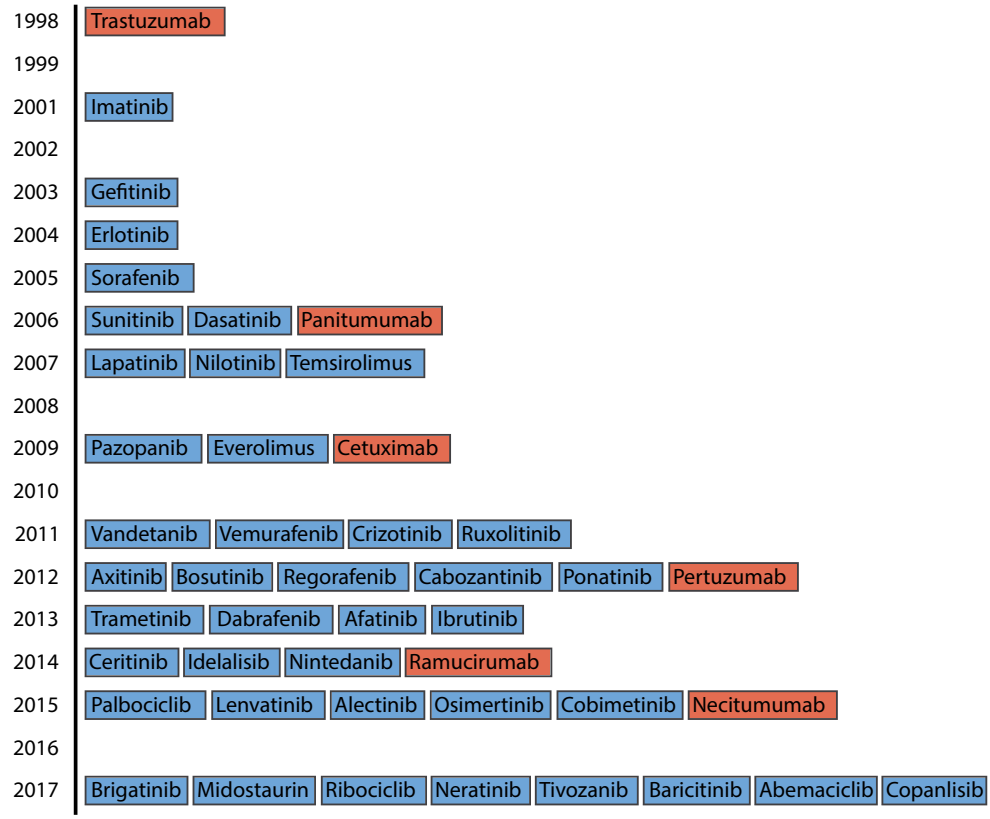

Figure 3: Summary of clinically approved kinase inhibitors, by their year of initial approval. Small molecule inhibitors are shown in blue and kinase-targeted monoclonal antibodies are shown in red. (fda.gov, ema.europa.eu/ema/)

To explore the neglected kinases targets, researchers at GlaxoSmithKline (GSK) generated a compound collection, which has a broad kinome coverage and are published as the Published Kinase Inhibitor Set (PKIS). Compound collections such as PKIS can help to mitigate the gap in kinome target coverage beyond TK [124, 125]. The compound collection was made available for research use with structure

and bioactivity data. The kinome activity of the compounds have been assayed (NANOSYN) against 220 kinases, which includes 89 TKs and 131 non-TKs. Among non-TKs, it covers 34 AGC, 34 CAMK, 25 CMGC, 13 STE, 6 TKL, 4 CK1, 5 lipid, 2 atypical and 12 other kinases.

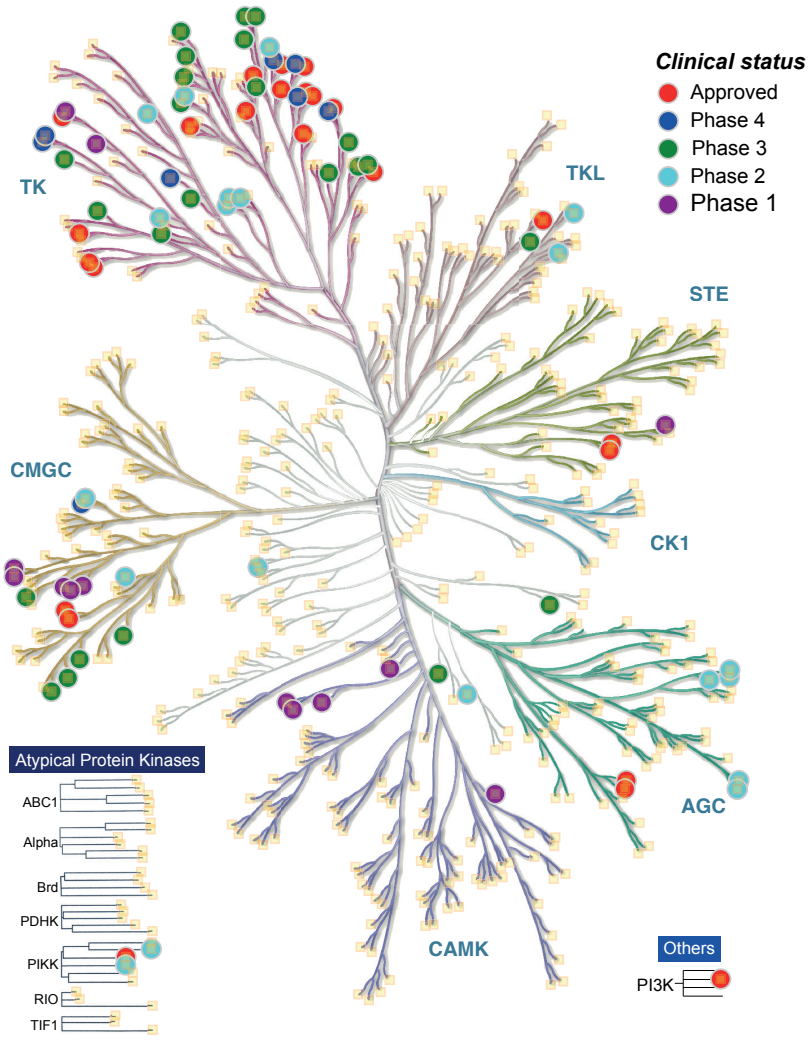

***Figure 4*:** Current kinome coverage of kinase inhibitors in clinical evaluation. The highest clinical status for the kinase inhibitor is considered. Illustration produced using KinMap from BioMed X (kinhub.org/kinmap/).

3.2.2. Oncogenic target deconvolution

Recent development in omics profiling has allowed extensive exploration of the genomic alterations in patient tumors, leading to a better understanding of the genes playing a role in cancer [126-128]. Profiling of mutational and histological variations in tumors from various cancer types have revealed that the genomic landscape of tumors is very heterogeneous and complex, making it difficult to pin-point the driver genes [129]. Moreover, it has been difficult to translate this knowledge into clinical practice as the functional consequences of many genetic alterations are not known, and often the altered gene products are difficult to target with pharmacologically agents [130]. This calls for alternative ways to identify the signaling addictions of cancer cells.

Phenotypic high-throughput chemical screenings provide complementary (omics) information about the drug sensitivity phenotypes of cancer cells, and have made it possible to identify promising drugs for anticancer treatment. By integrating the information of drug response of cancer cells with their known mechanism of actions or primary targets, it is possible to identify the driver addictions or biological processes or pathways that are important for the survival of cancer cells [131, 132]. However, most drugs are promiscuous and exhibit polypharmacology effect [133, 134], therefore making it difficult to attribute the effect of the drug to the direct inhibition of its primary on-targets. Several approaches have been taken previously to integrate the off-target effects of drug with drug response profiles to identify cell-line specific driver addictions. For example, Tang et al utilized a systems pharmacology approach to predict the cell line specific dependencies by mapping the drug target inhibition network with drug screen data [135]. In other study, Al-Ali et al used a machine learning approach to de-convolve cell line specific kinase targets utilizing the kinase selectivity profiles and drug response profiles [136]. Similarly, other molecular profiles can also be integrated with drug response profiles and drug target binding profiles to identify novel target addictions. For instance, Rees et al correlated the drug sensitivity profile in a panel of cell lines with their gene expression levels to

identify novel mechanism of action of drugs as well as novel target addictions [137].

3.3. Cell health measurement system

Most commonly, cell proliferation, cell viability and cytotoxicity assays have been used to test the effect of certain compounds on the cells [138]. Cell proliferation/ cell growth assays are the assays to measure the change in proportion of dividing cells in a given condition. Two major ways to quantify cell proliferation are measurement of DNA synthesis and amount of cell proliferation markers (antigens). Cell viability assays, also regarded as indirect cell proliferation assays, assess the number of viable cells by quantifying metabolic activity or amount of ATP. Highly sensitive and robust assays compatible with a multi-well plate reader formats are preferred in high throughput screening (HTS) approaches. As HTS mostly is performed as an end point assay, highly robust and reproducible detection methods are favored. The first HTS compatible homogenous cell viability assay developed was the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) salt colorimetric assay [139]. The MTT assay measures the reducing capability of viable cells. The colorless MTT is reduced to a purple formazan dye by the mitochondrial NAD(P)H-dependent oxidoreductase enzyme [140] (Figure 5A).

Later on, it was realized that the MTT assay was not robust enough for HTS because it required additional step to dissolve the water insoluble, precipitating formazan product before reading the absorbance. It was also found to be toxic to cells and interfere with different compounds, hence giving false hits [141-143]. Other refined tetrazolium reagents like MTS, XTT, WST were subsequently developed, which unlike MTT generate water soluble formazan dyes [144-146] and therefore are easier to use in HTS assays. The amount of generated reduced dye is proportional to number of viable cells. Similar to the concept of tetrazolium reagents, cell permeable, fluorescence based redox indicator ‘resazurin reduction assay’ was developed later [147]. It was highly appreciated in the HTS application as it is more robust and sensitive assay [148]. It measures the redox capability of viable cells

(mitochondrial activity) to transform resazurin (Alamar blue) to fluorescent resorufin (Figure 5B).

Technological advancements in establishing stable versions of firefly derived luciferase led to the development of robust cell viability measurement assays detecting the amount of cellular-derived ATP (a well validated cell viability marker), which were rapidly adapted to HTS [149] (Figure 5C). For example, the CellTiter-Glo (Promega) reagent works in a "add-mix-measure" format, eliminating all the multiple steps like media removal, cell washing and reagent dilution. The reagent lyses the cells and the ATP is distributed homogenously in the reaction mixture making it a robust measurement system. It was widely accepted in HTS settings since it was significantly more sensitive than previous assays (higher signal window as well allowing detection of as few as 10 cells/well) and had faster detection time compared to previous fluorescence or absorbance systems.

Further development of engineered luciferases (NanoLuc, derived from a marine shrimp luciferase) and a small molecule pro-substrate has allowed 'real-time' monitoring of cell health or quantifying viable cell number with a sensitive luminescence readout [150]. The pro-substrate is cell permeable, hence viable/ metabolically active cells reduce the pro-substrate to substrate, which diffuses back to culture medium where it reacts with the luciferase producing a luminescent signal (Figure 5D). NanoLuc is small (19 kDa) relative to firefly luciferase (60 kDa) or *Renilla* luciferase (36 kDa) and also have broader thermal and pH stability [151]. NanoLuc produces a signal that is 150-fold brighter than firefly and *Renilla* luciferases, has fastest response, and has exceptional stability *in vitro*, making it an ideal detection system in high throughput settings [151]. The real-time measurement provides advantages such as monitoring cell proliferation over time, which cannot easily be incurred from endpoint viability readouts and allows the user to follow compound effects over time.

As the basic principle of viability assays is to estimate the number of viable cells left after treatment, their readouts lack the crucial information about the extent of dead cells. Dye exclusion assays, which

utilize cell membrane non-permeable dyes to stain dead cells, have also been adopted in drug screening approaches [152] (Figure 5E). The most commonly used dyes are propidium iodide, ethidium bromide and cyanine dyes which bind to DNA of the membrane compromised cells. Cyanine dyes are more preferred for real-time cytotoxicity measurement in HTS settings as they have better sensitivity and reduced toxicity [153]. Some examples of cyanine-based dyes are CellTox Green (Promega) and SyTox Green (ThermoFisher). Cyanine dyes have two or more positive charges making them totally cell impermeable as compared to ethidium bromide which has single positive charge resulting in slow entry of dye even in viable cells [154]. Unlike propidium iodide, the cyanine dyes are non-toxic to the cells, allowing it to be used as an end point as well as kinetic measurement of cytotoxicity. With a multiplexed cyanine dye and RealTime-Glo assay, one can follow both the viability and cytotoxicity kinetics from the same experiment.

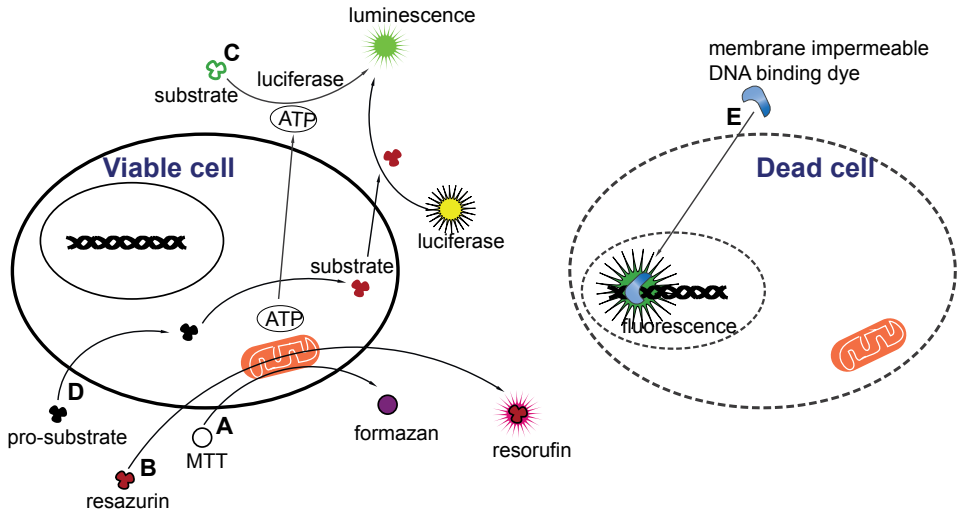

Figure 5: Illustration of different cell viability and cytotoxicity assays. A) MTT assay. B) Resazurin reduction assay. C) CellTiter-Glo assay. D) RealTime-Glo assay. E) Dye exclusion toxicity assays.

Other types of assays that have been used are apoptotic assays, which detect different stages of programmed cell death (like DNA fragmentation, caspase activity, loss of membrane polarization). Some

new apoptotic assays like dynamic BH3 profiling give more mechanistic insight into cell death [155]. However, all apoptotic assays share a common drawback that elevated level of apoptotic signaling does not necessarily result in cell death [156, 157]. This holds especially true for cancer cells, which can survive high levels of caspase activity or severe aneuploidy [158]. Besides, the temporary nature of apoptosis makes it challenging to capture the apoptotic signal at the right time and once the cells are dead, the apoptotic signal is typically lost. Therefore, apoptotic assays should preferably be complemented with cytotoxicity readouts. Even when the apoptotic and cytotoxicity responses correlate with each other, the dynamics of apoptosis varies a lot between the drugs and cell lines. This complicates the comparative analysis between the models and treatments.

The technical advances in microscopy and flow cytometry techniques has allowed the complementing of the multi-well plate reader assays with high content analysis. These systems provide much added information on cellular feature/phenotypes, like assessing multiple cellular markers and differentiating distinct phenotypic subpopulation [159-161]. These additional data points become especially relevant when addressing drug sensitivities in highly heterogenic primary samples. The major drawbacks with these technologies however are that they are quite laborious, technically challenging and expensive, therefore often limiting their use in dedicated facilities. In addition, data analysis and integration for these techniques remain a challenge.

Reduction in cell viability is often assumed to be correlated with enhanced cytotoxicity, which is not always the case. Moreover, simple viability or cytotoxicity assays do not always provide sufficient data to address mechanistic complexity in inhibition of many targets. Therefore, it is important to understand what cellular event we want to monitor in response to certain compounds and then choose the detection assay and interpret the data correctly.

3.4. Drug response quantification

Different parameters of dose-response curves are used to quantitate the effect of compounds in high throughput drug screening studies. The

most commonly used parameters are half maximum effects such as the half maximal effect concentrations (EC_{50}, IC_{50}), or summary metrics, such as area under the dose-response curve (AUC), activity area (AA) or multi-parametric drug sensitivity score (DSS) (illustrated in Figure 6) [83, 94, 96, 106, 162-164]. The whole concept emerged from the seminal NCI-60 tumor cell line screening effort where drug sensitivity and resistance was quantified by different parameters, which also incorporated initial cell density at initiation time T_0 and therefore allowed for estimation of the concentration at which 50% growth inhibition occurs (GI_{50}, concentration at which increase in cell density in treated condition is half of control condition), total growth inhibition (TGI, concentration at which cell density at treated condition is equal to initial seeded cell density) and 50% lethal concentration (LC_{50}, concentration at which cell density in treated condition reaches half the initial seeded cell density) [82, 165].

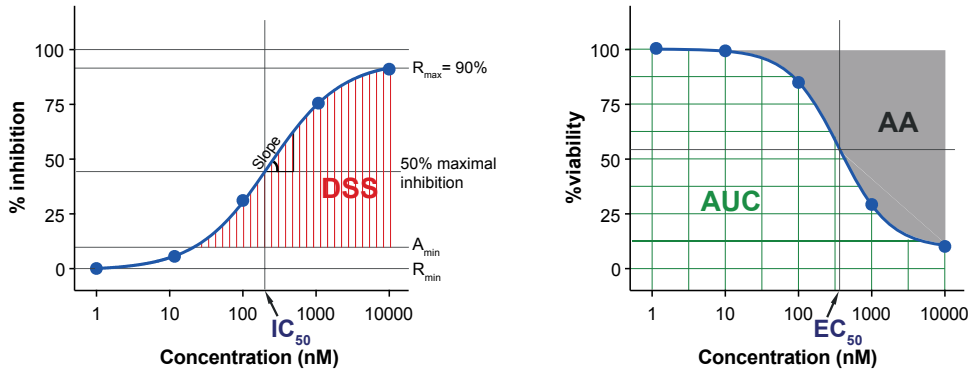

Figure 6: Illustration of different parameters of dose response curves applied for quantification of drug effects. IC_{50}: half maximal inhibitory concentration, EC_{50}: half maximal effective concentration, DSS: drug sensitivity score, AUC: area under the dose-response curve, AA: activity area.

Traditionally, in high throughput drug screening approaches, the end point drug effects are recorded and presented as percent inhibition (PI), calculated by normalizing the estimated viable cell counts with negative (vehicle, no effect) and positive (known killing agent) controls [83, 94, 99]. However, such kind of end point assays disregard the dynamic variability that could occur due to experimental artifacts such as cell

seeding discrepancy, differing growth rate of the cells, changes in background noise over time or signal bleed-through, among others (Figure 7). This highlights that noise-prone data are being collected and could partly explain the ongoing debate on inconsistencies observed in large-scale drug response profiling data [163, 166-168].

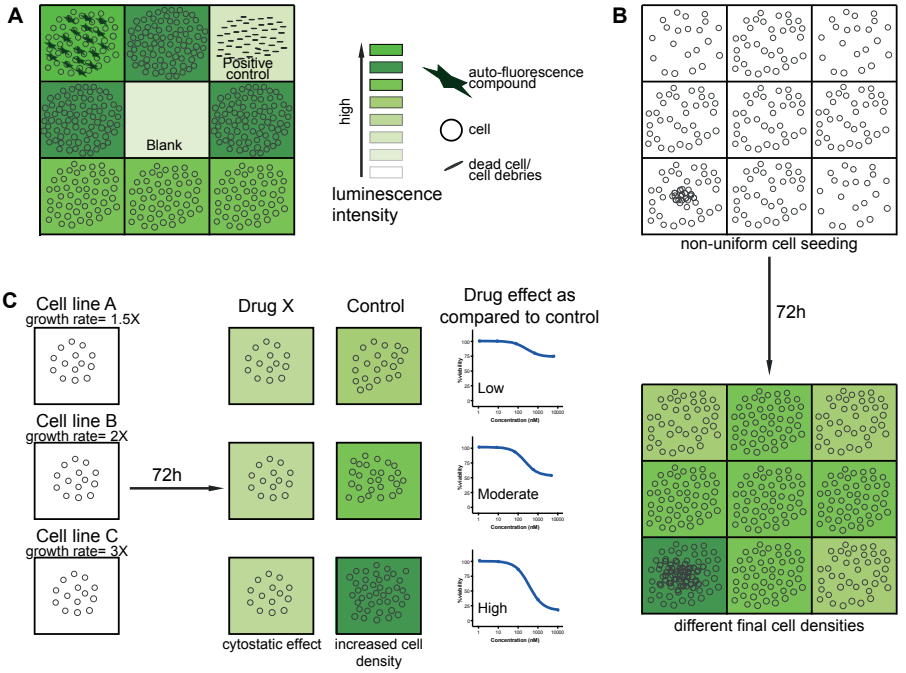

***Figure 7*:** Schematic diagram illustrating different time dependent experimental artifacts encountered in the end point high throughput drug screening assays. A) Figure representing different background noises, signal bleed through and false hit from auto-fluorescence drug. The signal bleed through is illustrated in the figure as follow: 'Blank' well is not supposed to give any luminescence signal but due to highly luminescent neighboring wells blank well also exhibits some luminescence. Most studies ignore positive control or background noise [82, 169, 170], which can grow over time and skew the drug effect analysis. B) Representation of differential luminescence signals at an end point because of cell seeding discrepancies. A skewness of single point in dose response curve due to non-uniform cell seeding can lead to a false over/under estimation of drug effect. C) Effect of growth rate of cells results in over/under estimation of drug response quantification. Figure describes an example where the effect of cytostatic drug X (completely blocks the cell division) in three different cell lines with differing growth rate. Initially equal number of cells are seeded for all three A, B, C cell lines, the drug exhibit same effect in all the cell lines limiting the cell number to the initial count. But when normalized/compared to negative control where the final cell densities are different corresponding their growth rates, the effect of drug X appears different, making it seem less effective against cell line A, moderately effective against cell line B and highly effective against cell line C.

Along with the seminal NCI60 metrics (GI50, LC50), two independent drug response metrics GI50 and TGI were recently devised to limit the time-dependent biases produced by the end-point measurement metrics, namely the growth rate (GR) metric and drug induced proliferation (DIP) rate [169, 170]. Both of these metrics take into account cell-line specific growth rates as well as drug-induced growth rates, and experimental artifacts including cell seeding discrepancy. However, both of them as well as the seminal NCI60 metrics ignore the positive control and/or background noise. Both GR and DIP are based on real-time imaging data. Hence, even though they seem like very promising metrics, GR and DIP cannot be readily be implemented in high throughput drug screening.

Technological advancement in the high content live imaging methods would resolve most of these artifacts and help in better understanding drug effects. However, the lack of such techniques compatible with HTS still normally limits drug effect assessment to endpoint measurements. Recent developments of live viability measurement assays has provided an opportunity to address experimental artifacts inherent to HTS to a certain extent.

Aims of the study

The main aim of the thesis was to explore drug vulnerabilities based on functional profiling of triple negative breast cancer (TNBC) cell lines to better define the molecular background of TNBC and to identify novel druggable targets. The aims can be briefly summarized as follows:

1. Functionally classify TNBC cell lines, directly connecting them to druggable vulnerabilities
2. Kinome-wide exploration of TNBC cell lines to identify and establish novel and putative drug targets.
3. Identify selective synergistic drug combinations against TNBCs.

Materials and methods

Material and methods used in this thesis are described in detail in the original publications (I-III). Below is the summary of materials and methods in brief.

4. Cell lines

Twenty-three different breast cancer cell lines along with one non-cancerous breast epithelial cell line and one pancreatic cancer cell line were used for this thesis work. The use of different cell lines in respective studies are highlighted in Table 2. The cell lines were obtained from ATCC or DSMZ except MIA-PaCa-2, which was a generous gift from Prof. Channing Der, University of North Carolina, NC, USA. All the cells were maintained in their respective media (Table 2) at 37°C with 5% CO_2 in humidified incubator as per provider's instruction. DMEM with 2.2 g/l sodium bicarbonate (adapted for 5% CO_2 incubation), RPMI-1640 and McCoy media were purchased from Life Technologies, Lonza and Sigma-Aldrich respectively.

5. Inhibitors

A compound collection consisting of 351 different oncology compounds covering wide range of molecular targets were used in the study (Appendix 1). The collection consisted of commercially available approved (43%), investigational (45%) and pre-clinical experimental (12%) anti-cancer compounds. The compounds were obtained from the National Cancer Institute Drug Testing Program and the Structural Genomics Consortium or purchased from Active Biochem, Axon Medchem, Cayman Chemical Company, ChemieTek, Enzo Life Science, LC Laboratories, MedChemExpress, Merck, Santa Cruz Biotechnology, Selleck, Sequoia Research Products, Sigma-Aldrich and Tocris Biosciences. The compounds were dissolved in DMSO or water and stored in pressurized Storage Pods (Roylan Developments Ltd.) filled with inert nitrogen gas according to the manufacturer's instructions.

Table 2: The list of cell lines used in the studies.

Cell line	Cell Origin	Source	Growth Medium	cell seeding /well (384-well plate)	Study
MDA-MB-468	breast adenocarcinoma	ATCC	RPMI 1640+ 5% FBS+ 2 mM glutamine	1000	I, III
CAL-85-1	breast adenocarcinoma	DSMZ	DMEM + 10% FBS+ 2 mM Glutamine+ 1 mM sodium pyruvate	1200	I, III
CAL-120	breast adenocarcinoma	DSMZ	DMEM + 10% FBS	1000	I, III
MDA-MB-231	breast adenocarcinoma	ATCC	DMEM + 10% FBS + 10 μg/ml insulin	1500	I, II, III
MDA-MB-436	breast adenocarcinoma	ATCC	DMEM + 10%FBS + 10 μg/ml insulin	1000	I, III
CAL-148	breast adenocarcinoma	DSMZ	DMEM+ 20% FBS+ 2 mM glutamine+ 0.01 μg/ml EGF	2000	I, IIII
SK_BR-3	breast adenocarcinoma	ATCC	McCoy's + 10% FBS	2000	I, III
MDA-MB-361	breast adenocarcinoma	ATCC	DMEM + 10% FBS	1500	II
HCC-1143	breast carcinoma	DSMZ	RPMI 1640+20% FBS+ 2 mM glutamine	1000	I, III
HCC-1599	breast carcinoma	DSMZ	RPMI 1640+ 10% FBS	8000	I, III
HCC-1937	breast carcinoma	ATCC	RPMI 1640+10% FBS+ 2 mM glutamine	1000	I, III
HDQ-P1	breast carcinoma	DSMZ	DMEM + 10% FBS	1000	I, II, III
BT-549	breast carcinoma	ATCC	RPMI 1640+ 10% FBS+ 2 mM glutamine	1000	I, III
CAL-51	breast carcinoma	DSMZ	DMEM + 20% FBS	2000	I, III
Hs-578T	breast carcinoma	ATCC	RPMI 1640+ 10% FBS+ 2 mM glutamine	1000	I, III
DU-4475	breast carcinoma	DSMZ	RPMI 1640+ 20% FBS	1500	I, III
MFM-223	breast carcinoma	DSMZ	EMEM + 10% FBS + 2 mM glutamine	2000	I, III
MDA-MB-453	breast carcinoma	DSMZ	DMEM/F12 + 10% FBS	2000	I, III
BT-474	breast carcinoma	ATCC	DMEM + 10% FBS + 10 μg/ml insulin	2000	I, II, III
T-47D	breast ductal carcinoma	ATCC	RPMI 1640+ 10% FBS	2000	III
ZR-75-1	breast ductal carcinoma	ATCC	RPMI 1640+ 10%FBS+ 2 mM glutamine	1000	III
MCF-7	breast ductal carcinoma	ATCC	EMEM + 10%FBS + 10 μg/ml insulin	1500	II, III
MCF-10A	noncancerous	ATCC	MEMB/F12+ supplement vials + 100 ng/ml Cholera toxin	1000	I, III
MIA-PaCa-2	pancreas carcinoma	Der lab	DMEM + 10% FBS	750	II

6. Drug Sensitivity and Resistance Testing (DSRT)

The drug sensitivity and resistance testing (DSRT) platform that has been described previously [94] was adopted for breast cancer cell lines. The compounds were plated to white clear bottom 384-well plates (Corning #3712) using an Echo 550 Liquid Handler (Labcyte) in 5 increasing concentrations in 10-fold dilutions covering a 10,000-fold concentration range of (e.g. 1-10 000 nM). 100 µM benzethonium chloride ($BzCl_2$) and 0.1% dimethyl sulfoxide **(**DMSO) were used as positive and negative controls respectively. All subsequent liquid handling was performed using a MultiDrop dispenser (Thermo Scientific). The pre-dispensed chemicals were dissolved in 5 µl of culture media, containing cytotoxicity measurement reagent (CellTox Green, Promega) and in study II viability measuring reagent (RealTime-Glo, Promega) was additionally supplemented. Two viability reagents CellTiter-Glo (Promega) and CellTiter-Blue (Promega) along with cytotoxicity measurement dye CellTox-Green were explored in study I. Multiplexed RealTime-Glo and CellTox Green was explored in study II and CellTiter-Blue was used in study III. Twenty microliters cell suspension containing required number of cells (defined in Table 2) per well were seeded in the drugged plates. After 72 h incubation, the cell viability (luminescence for CellTiter-Glo and RealTime-Glo and fluorescence in case of CellTiter-Blue) and cytotoxicity (fluorescence) was recorded using a PHERAstar FS plate reader (BMG Labtech).

7. DSRT Data Analysis

The raw luminescence/fluorescence data were analyzed in Breeze software, an in house developed data analysis and management software to generate the dose response curves. Utilizing multiple parameters from the dose response curve, drug sensitivity score (DSS) was calculated for each single drug treatment [162]. For drug combinations, synergy score was calculated using Bliss independence model and ZIP model [171, 172]. The drug combination matrices were analyzed with the web application 'SynergyFinder' [173] (https://synergyfinder.fimm.fi).

8. siRNA Screening

The siRNAs were purchased from Qiagen or Ambion. Kinase siRNAs along with scrambled non-targeting siRNAs and death control siRNAs (8-10 nM final concentration depending on the experiment) were added to 384 well plates (Corning #3712) with the Echo 550 Liquid Handler (Labcyte). 5 µl of Opti-MEM (Life Technologies) containing 50 nl of Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) was added to each well of pre-siRNA-added plates using a MultiDrop Combi nl dispenser (Thermo Scientific) and incubated at room temperature for 20 min on an orbital shaker. 20 µl of cell suspension (25000 cell per 1 ml) were seeded on the siRNA plate and the plates were maintained in culture for 96 h. In case of pooled screening, three different siRNAs against a particular target were mixed at equal concentration to obtain a final combined concentration of 10 nM. The viability of cells after siRNA treatments was assessed with CellTiter-Glo (Promega). The luminescence measurements were converted to % inhibition in respect to positive and negative controls.

9. Statistical analyses

Statistical analyses were performed using GraphPad Prism software or R programming. Pearson correlation coefficient was used to evaluate the correlation between screening data (DSS). Root mean squared distance (RMSD) and baseline distance were used to evaluate the improvement in dose response curve fits. Wilcoxon rank sum test was used to assess the difference in mean RMSD, and F-test was used to assess the variance of baseline distance.

Results and discussion

10. Identification of TNBC selective drug sensitivities

10.1. Viability inhibition does not directly correlate with cytotoxicity

Typically, multi-well plate compatible cell viability measurement assays are preferred in high throughput chemo-sensitivity screenings. The metabolic activity of the cells is measured as an estimate for number of viable cells. Some commonly used methods involve quantifying the amount of energy molecules like ATP, NADH/NADPH or the redox capability of the cells (as described in Figure 5)[138, 174]. Generally, a reduction in viability is assumed to be directly correlated to cytotoxicity. Therefore, to test whether viability inhibition complements cytotoxicity, along with cell viability measurement assays (CellTiter-Glo, measures amount of ATP; CellTiter-Blue, measures redox potential of cells), we also applied a cytotoxicity measurement assay (CellTox Green, a membrane impermeable DNA binding dye) in study **I**. We collected a panel of breast cancer cell lines including 16 TNBC, 2 HER+ and one non-cancerous triple negative breast epithelial cell line (details in table 1) that we screened against 301 oncology compounds (listed in Appendix 1). The drug effect was recorded as drug sensitivity score (DSS) [162]. Average DSS for all compounds screened across 19 cell lines were computed, both for viability and cytotoxicity data.

Upon comparing average DSS of tested compounds, the two independent cell viability assays (CellTiter-Glo and CellTiter-Blue) yielded high correlation (rcor= 0.89, Figure 8A). On the other hand, a comparison of the multiplexed cell viability (CellTiter-Glo) and cytotoxicity (CellTox Green) readouts revealed lower correlation (rcor= 0.67, Figure 8B). Most single agent anti-cancer compounds showing activity for the viability readout had no or little cytotoxic effects, rather only caused a cytostatic effect. A relatively low number of drugs exhibited cytotoxic effect (only 20 drugs had average DSS more than 5). The classes of compounds that displayed high viability inhibition but

failed to kill the cells included PI3K/mTOR inhibitors (such as dactolisib, GSK2126458, PF-04691502), CDK inhibitors (alvocidib, SNS-032, UCN-01), HSP90 inhibitors (tanespimycin, BIIB021), NAMPT inhibitor (daporinad), tubulin stabilizer (taxanes), protein synthesis inhibitor (omacetaxine), and RNA and DNA synthesis inhibitor (dactinomycin). The compounds showing such effects are highlighted with green color in Figure 8B.

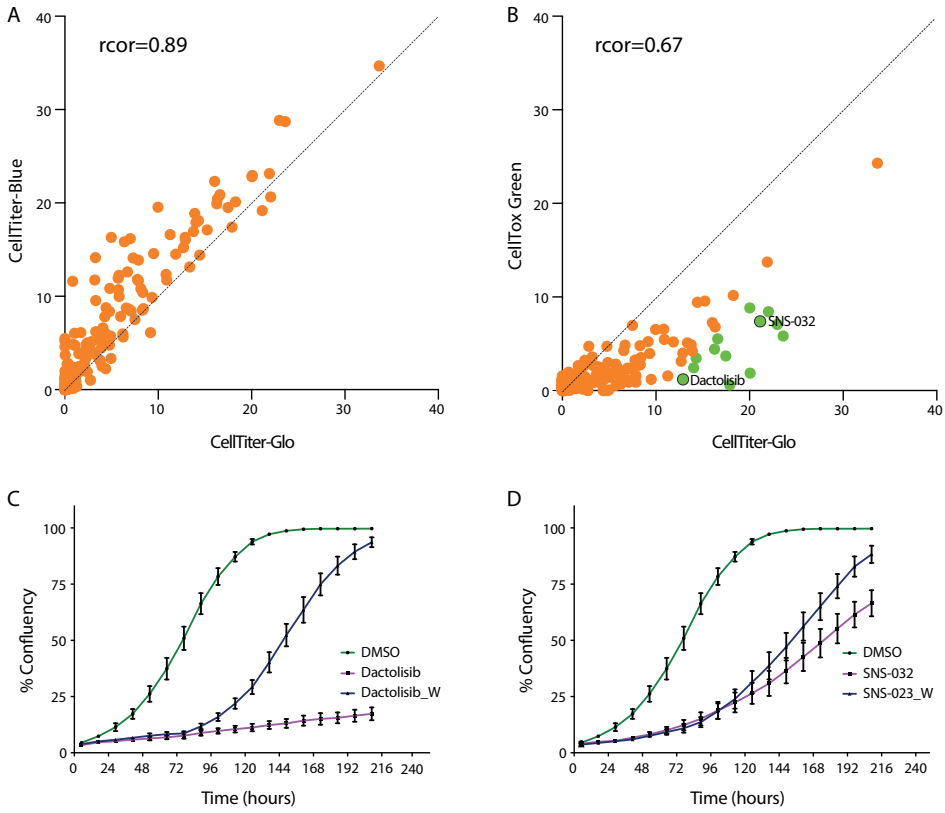

Figure 8: Difference between the cytotoxic and cytostatic effect. Upper panel shows scatter plots comparing DSS computed using two different viability readouts (A) and viability and cytotoxicity readouts (B). The drugs exhibiting strong viability inhibition but comparatively less toxicity are highlighted in green. Case examples of such drugs exhibiting high viability inhibition and low toxicity; (C) mTOR/PI3K inhibitor dactolisib; (D) CDK inhibitor SNS-032. Error bar represents SEM (n=3); "W" represents a condition where drug is washed away after 72 h.

There could be two reasons behind drug responses displaying high viability inhibition but no corresponding toxicity. The drug effect might

either be a reversible static effect or transient metabolic shutdown or a non-reversible/permanent metabolic shutdown such as senescence. Therefore, to unravel the nature of such drug classes we collected 8 drugs from the different drug classes that exhibited no apparent toxic effect but dramatic viability inhibition in most of the cell lines, including the mTOR/PI3K inhibitor dactolisib, the PI3K inhibitor pictilisib, the CDK inhibitor SNS-032, the NAMPT inhibitor daporinad, the IMPDH inhibitor AVN-944, and the survivin inhibitor YM155. A drug effect reversibility test was performed with these drugs against a representative TNBC cell line (CAL-51) to explore their mechanism of action, where after the drug (used at 10 times its IC50) treatment for 72 h, either was replenished or washed away and the cells were maintained in culture for 6 more days while their growth was followed in a live cell microscope. The cell growth (confluence) was monitored/recorded for the whole period of time. Detailed experimental results can be found in Study **I**, Figure 3.

Strikingly, we found that the cytostatic effects of all the drugs were reversible and in some instances the cells overcame the inhibitory effect even in the presence of the drug after a few days. The reversible effects were well-pronounced for dactolisib, pictilisib, daporinad and AVN-944. In the presence of drugs the cell growth was ceased/inhibited but as soon as the drugs were washed away the cells started dividing again (dactolisib behavior shown in Figure 8C as a representative effect). Furthermore, everolimus, methotrexate, SNS-032, and YM155 only caused a transient growth inhibitory effect and in a matter of few days their effect was lost; the cells began to grow normally even in the presence of the compounds (SNS-032 behavior shown in Figure 8D as representative effect). The resistance developed by the cells against these compounds is most likely because of cellular reprogramming triggered in response to the drugs. This implies that these agents alone might not be efficacious in the clinic, rather they should be combined with other agents that will inhibit the activated compensatory pathway(s).

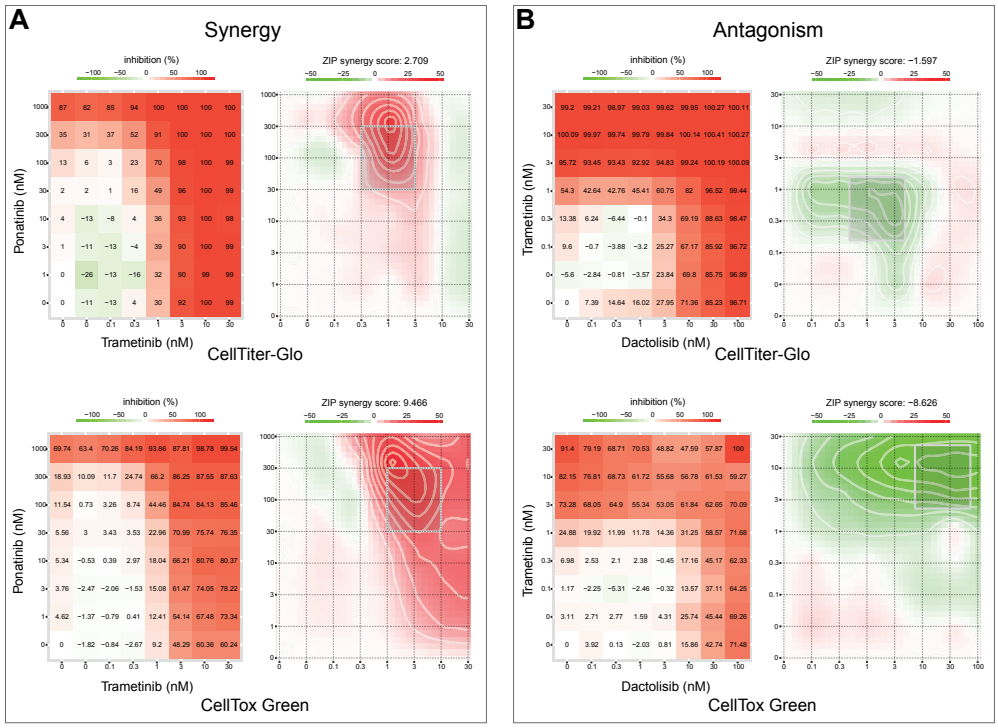

Figure 9: Assessing cytotoxicity is important to unravel synergistic and antagonistic effects in drug combination screening. A) In DU4475, a combination of ponatinib and trametinib exhibits synergistic effect that is more pronounced in cytotoxicity readout. B) In DU4475, a combination of dactolisib and trametinib displays an antagonistic effect, which is only pronounced in the cytotoxicity readout.

We found that assessing cytotoxicity along with viability drug combination studies provide much more information than mere viability readouts, for example in testing concentration combination matrices. The real synergistic (lethal) effect might be obscured in the viability readout for those kinds of drugs posing high viability inhibition but no toxicity, as single drug already showed the maximum viability inhibition effect masking the actual combination effect. Such examples are illustrated in Figure 9A, where synergy between trametinib (MEK inhibitor) and ponatinib (pan-selective TK inhibitor) in DU4475 cells was found to be more pronounced in cytotoxicity readout. Additionally, toxicity readout also captured counterproductive antagonistic effects, which were missed by viability measurements. For example, Figure 9B illustrates the antagonism between trametinib and dactolisib in DU4475, which was only observed with cytotoxicity readout. Here,

trametinib almost completely inhibited the metabolic activity of DU4475 above 1 nM already as a single agent obscuring the effect of added dactolisib, whereas the magnitude of cytotoxicity does not correspond to the metabolic inhibition, allowing exhibition of antagonistic effect.

These results highlighted the common misconception that reduced viability directly correlates to the induction of cytotoxicity. Strong viability/metabolic inhibition does not necessarily represent cellular toxicity, rather it may just represent metabolic shutdown/cell-cycle arrest or quiescent phenotype of the cells. Cells can ultimately reverse these kinds of effects. This highlights that drugs/compounds with reversible cytostatic responses may not be the ideal therapeutic in clinical setting. Hence, we need to thoroughly comprehend drug mechanisms beyond mere viability inhibition.

10.2. Drug sensitivity based functional profiling of TNBC

With an aim to functionally classify TNBC, a panel of TNBC cell lines representing all 6 transcriptomic subtypes [47] (Figure 10A) were clustered based on the selective drug sensitivities of 301 oncology compounds, which included a broad array of drugs including conventional cytotoxic chemotherapies, metabolic modifiers, apoptotic modulators, kinase inhibitors, hormonal therapies, immune-modulators, epigenetic modifiers, and others (Appendix 1). To determine the selective drug sensitivities towards TNBC (or a particular cell line), selective DSS (sDSS) was calculated for each compound by subtracting the average DSS of that compound over a large panel of in-house cell line screening data (150 diverse cell lines comprising of 5 different tissue types, based on CellTiter-Glo assay) from the cell line-specific response.

A

Cell line	Subtype
HCC-1143 HCC-1599 HCC-1937 MDA-MB-468	Basal-like 1 (BL1)
CAL-85-1 HDQ-P1	Basal-like 2 (BL2)
DU-4475	Immunomodulatory (IM)
BT-549 CAL-120 CAL-51	Mesenchymal-like (M)
MDA-MB-231 MDA-MB-436 Hs-578-T	Mesenchymal Stem-like (MSL)
CAL-148 MFM-223 MDA-MB-453	Luminal androgen receptor (LAR)

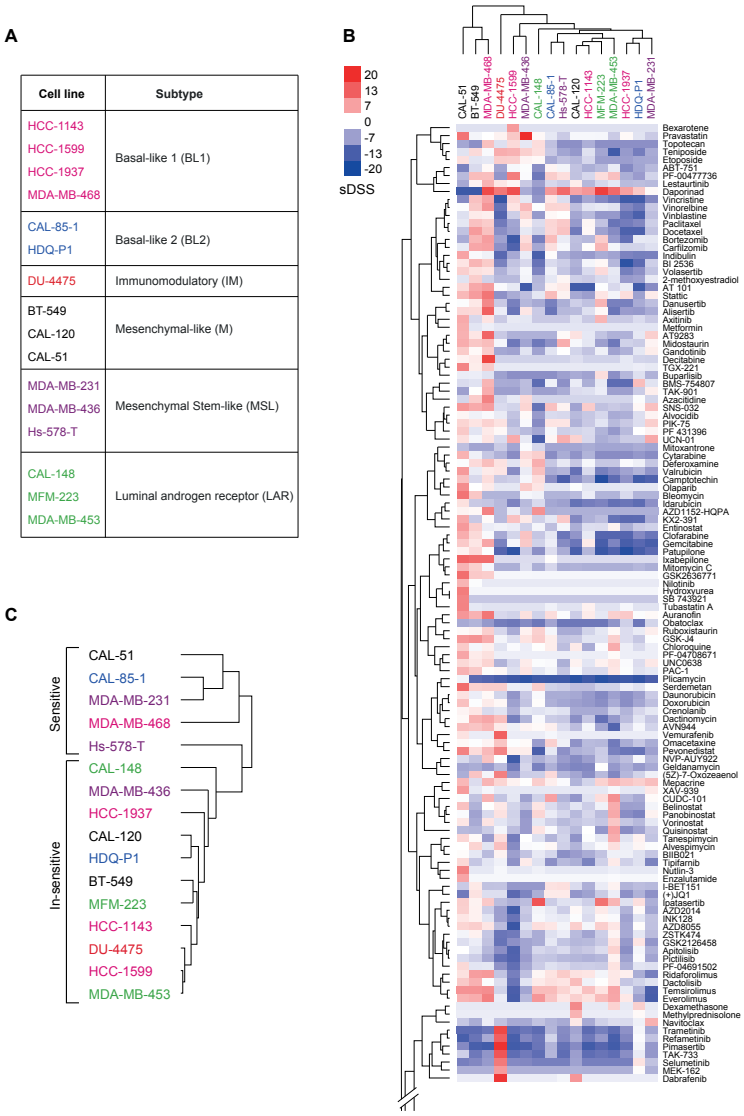

Figure 10: Variability between drug sensitivity and transcriptomics based TNBC subtyping. A) The tested cell lines grouped and colored based on transcriptomics based TNBC subtypes [47]. B) A clustered heat map based on sDSS of TNBC compared to the average response of a panel of 150 different cell lines from 7 different tissue types (viability readout). This only represents a portion of heat map where drugs are clustered vertically and cell lines horizontally (cell lines are color coded according to their transcriptomic subtypes as highlighted in A). Red (positive sDSS)) represent selective responses whereas blue (negative sDSS) represents below average responses. C) Cell line clustering based on selective drug sensitivity (sDSS) based on cytotoxicity data (sDSS calculated by subtracting average DSS of 30 cell lines originating from 3 tissue types).

While comparing sDSS across the TNBC cell lines, we found that the cell lines exhibited highly heterogeneous response patterns towards the compound library and not a single compound showed selective responses in all the TNBC cell lines tested. We further tried to cluster/subgroup the TNBC cell lines based on their drug vulnerabilities and match the clustering with published transcriptomics based TNBC subtypes [47]. Strikingly, drug sensitivity driven clustering was not directly linked to the transcriptomic subtyping of the cell lines (Figure 10B). In addition, we also performed the cell line clustering based on individual compound or classes of compounds sensitivities, but as opposed to previously reported studies [47-49], we could not link specific vulnerabilities to any specific transcriptomic-subtype.

Further, clustering analysis on cytotoxicity measurement based drug sensitivity data (sDSS calculated as difference against an average DSS over 30 cell lines screened in house) yielded a different pattern than those of both viability-based and transcriptomics-based subgroupings. In spite of heterogeneity in drug responses, cell lines could be broadly divided into two groups, sensitive and insensitive based on cytotoxicity data. The cell lines in the sensitive group included MDA-MB-231, MDA-MB-468, CAL-51, CAL-85-1 and Hs-578-T as they showed sensitivity (cytotoxicity) towards mitotic inhibitors (including taxanes, CDK inhibitors, *Vinca* alkaloids, topoisomerase I inhibitors) and HDAC inhibitors (Figure 10C) along with a few other inhibitors.

We further looked for molecular markers that could be linked with cytotoxic effect of the sensitive cell lines to respective drugs/classes. First, we explored recurrent genetic alterations prevalent in TNBC. Mutations in DNA repair genes (*BRCA2* and *ATM*) appeared to possibly be enriched in sensitive cell lines but no striking link between mutations and the cytotoxic responses stood out (Figure 11).

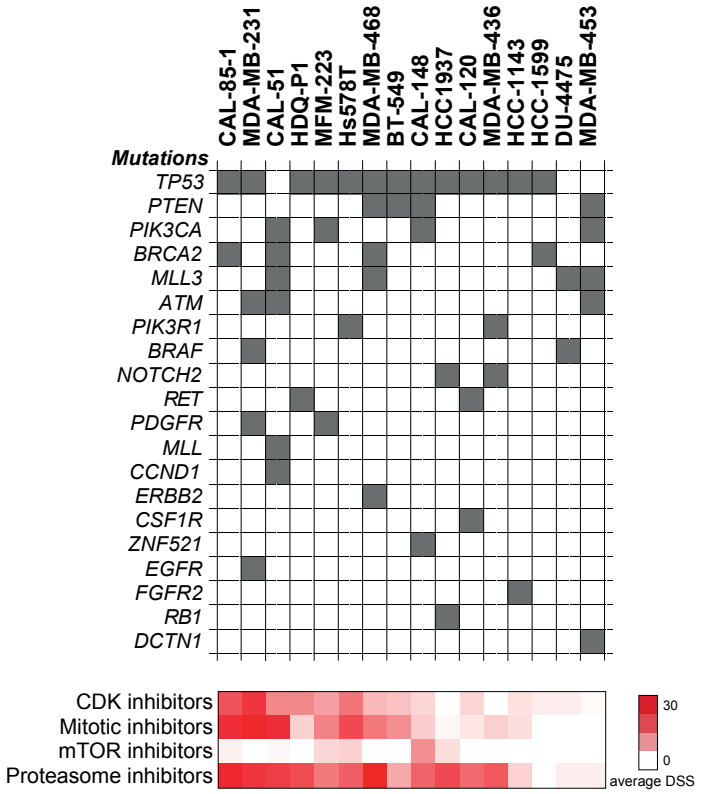

Figure 11: Association analysis between the recurrent genetic alterations in TNBC with the observed cytotoxic effects of different classes of drugs.

Next, we analyzed published protein marker expression profiles (reverse phase protein array data, Daemen et al. 2013) of the tested cell lines [85]. By comparing proteomic profiles of mitotic inhibitors (taxanes, CDK- and CHK- inhibitors) sensitive and insensitive cell lines, we identified differential levels of protein markers linked to the cytotoxic responses to mitotic inhibitors. For example, higher protein levels of PKCα, FGFR1, c-Jun, Caveolin-1 and low levels of RAB25, pSTAT3 (Y705), NOTCH3, Bcl-2 and HER2 were observed in antimitotics-sensitive cell lines than in insensitive cell lines (Figure 12). Supporting our discoveries, all these proteins (and their respective levels) has been linked to TNBC drug responses in other studies. For example, high levels of pSTAT3, Bcl-2 and HER2 have been reported to correlate with resistance to chemotherapy [175-177]; high level of c-Jun, RAB25 and low level of caveolin-1 have been linked to poor prognosis [178-180];

low levels of NOTCH3 has been linked to apoptosis induction [181]; and high levels of PKCα and FGFR1 have been linked to growth of TNBC cell lines [182, 183].

Similarly, differential levels of protein markers were found between proteasome inhibitor-sensitive and -insensitive cell lines. Proteasome inhibitor-sensitive cell lines exhibited elevated protein expression levels of PAI1, MKP-1 and pAKT (T308), phospho-p38 (T180/182) and low levels of PTEN, NOTCH3 and CCND1. Our results suggest that patient stratification based on these protein markers could be explored in clinics for the treatment of TNBC patients with mitotic inhibitors (such as taxanes) as well as proteasome inhibitors.

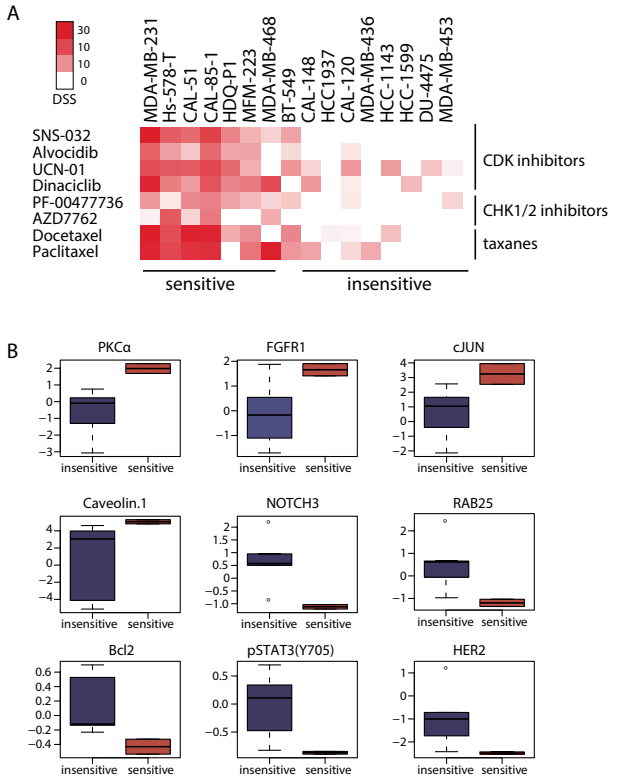

***Figure 12*:** Potential predictive biomarkers for lethal effect of antimitotics. A) DSS based heat map illustrating discrete effects of different mitotic inhibitors, broadly dividing cell lines to sensitive and insensitive groups. B) Differential expression of different protein markers between mitotic inhibitor sensitive and insensitive cell lines. (Protein expression data retrieved from Daemen et al. 2013 [85]).

All in all, the results advocate that precision/personalized therapeutic strategies-based proteomic biomarker stratification may be a more effective way to target TNBCs rather than therapies based on transcriptomics subtyping, or that a more refined analysis of TNBC transcriptomics is needed for linking it to broad drug sensitivities.

10.3. mTOR inhibitors antagonize the effect of diverse classes of drugs

mTOR is an extensively studied kinase and regarded as a potential cancer therapeutic target [184]. Recently, the rapamycin analogue everolimus was approved for HR+, HER2- breast cancer and numerous other mTOR inhibitors are being currently evaluated in clinical settings. However, we found that at the cellular level PI3K/AKT/mTOR pathway inhibitors were non-cytotoxic to almost all cell lines screened except CAL148. To achieve further insight, we performed combination testing with mTOR inhibitors with our oncology drug collection (301 compounds) with an aim to identify compounds that enhance the cytotoxic effect of mTOR inhibitor. We profiled the drug responses of the mTOR sensitive cell line CAL-51 (viability data) in the absence and presence of single concentrations (ten times IC_{50}) of mTOR inhibitors: the rapalog everolimus (10 nM) (Figure 13A) and the ATP competitive mTOR kinase inhibitor dactolisib (100 nM) (Figure 13B).

Surprisingly, we found that the mTOR inhibitors had an almost exclusive antagonistic effect on the cell viability and cytotoxic activity of other anti-cancer drugs, which includes anti-metabolites, anti-mitotics, topoisomerase, HDAC, PARP, and proteasome inhibitors along with few kinase inhibitors, among others (a detailed list can be found in Study **I**, Figure 6 and supplementary Figure 4). One plausible reason for this is that mTOR inhibition shuts down the cell metabolism and growth thereby limiting/blocking the effect of other anti-cancer drugs. Daporinad, a NAMPT inhibitor, exhibited synergism in combination with everolimus but only as a cytostatic response (Figure 13A). Our results suggest that a cautious consideration should be taken while combining mTOR inhibitors with conventional chemotherapies including standard first line of therapies like gemcitabine and

doxorubicin, which might be counterproductive in the clinical setting. Even though our results are limited to *in vitro* level and might not fully imitate the responses in a complex *in vivo* setting, it urges for caution in combining mTOR inhibitors with other anti-cancer drugs.

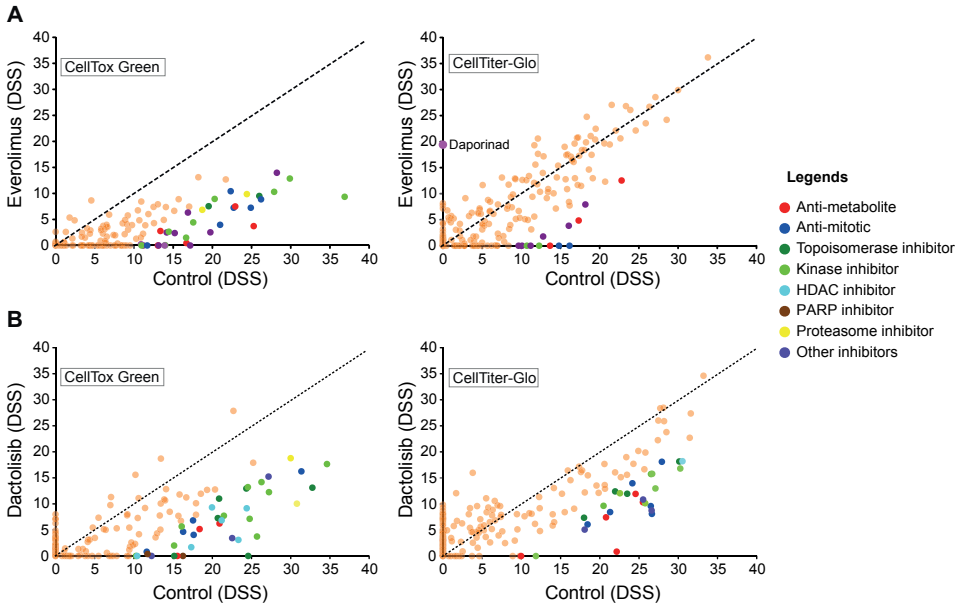

Figure 13: mTOR inhibitors exhibit antagonistic effect when combined to various classes of drugs in CAL-51. Scatter plot comparing DSS for mono-treatment (x-axis) with DSS for combination effect (y-axis) of 10 nM everolimus (A) and 100 nM dactolisib (B). The drugs with DSS difference higher than 10 are highlighted in the plots, different colors represent respective drug classes as listed in the color legend.

11. Normalized Drug Response (NDR)

11.1. Formulation of NDR metric

Typically, *in vitro* and *ex vivo* drug screening experiments are typically carried out as an end point assay (most often 72 h), where the effects of the drugs are assessed utilizing different viability or cytotoxicity measurement systems, covering a given concentration range of the drug. A normalized drug response is often generated as % inhibition (PI) values, which are calculated by normalizing the estimated viable

cell counts with negative (non-effective agent, vehicle) and positive (total killing agent) controls. Alternatively, growth inhibition (GI) values are used in some studies [82, 102], which considers growth rate of cell line (calculated as cell count at 72 h minus cell count at 0 h in non-treated cells). In these cases, the 72 h and 0 h data are extracted from two different replicate plates.

The end point PI-based drug effect quantification disregards the dynamic variability that occurs due to experimental limitation/artifacts, including uneven cell seeding, variable growth rates of cells, and background noise. This leads to the problem related to data reproducibility or consistency. To address such limitations, we devised a novel response metric, normalized drug response (NDR) that accounts for all time-dependent assay artifacts including signal escalation rate in positive and negative control as well as in drug-treated conditions. NDR is calculated utilizing two-point screening readouts, first at the start of the experiment and second at the end-point. This was possible due to recently developed real-time measurement systems [150].

The normalized drug response (NDR) was calculated as:

$$NDR = \max\left(-1, \frac{1 - 2^{(log_2(foldChange_{drug})/log_2(foldChange_{posCtrl}))}}{1 - 2^{(log_2(foldChange_{negCtrl})/log_2(foldChange_{posCtrl}))}}\right)$$

where the fold change between the readouts at start and end-point of the measurement was given as:

$$\text{foldChange}_{\text{condn}} = \frac{\text{End}_{\text{Readout}_{\text{condn}}}}{\text{Start}_{\text{Readout}_{\text{condn}}}}$$

In other words, NDR was computed by two-step normalizations, where each data point was first normalized to its initial readings and then to the positive and negative controls.

NDR not only scored the drug effect in single value but also defined the drug effects/behaviors (Figure 14). Grounded on the single NDR values, the drug effects could be characterized as:

$$NDR = \begin{cases} > 1 & \textit{growth promoting effect} \\ = 1 & \textit{normal growth as in negative control} \\ = 0 & \textit{complete growth inhibition} \\ = -1 & \textit{complete killing} \end{cases}$$

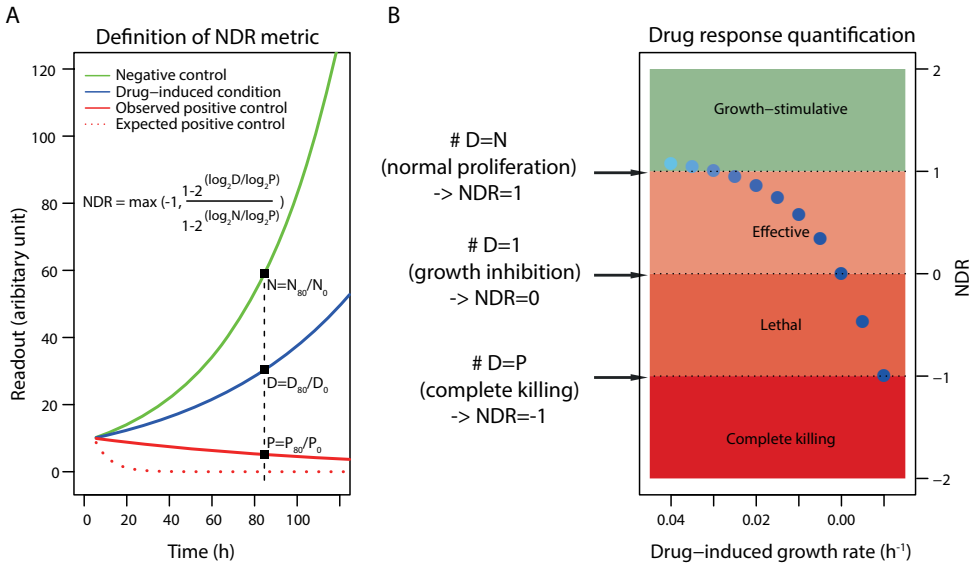

Figure 14: Illustration of NDR formulation. A) Simulation of NDR calculation at a specific time point (here 80 h of treatment) considering the dynamic change of different treatment conditions (blue line represents drug treatment, green represents negative control and red represents positive control. B) Illustration of range of drug effects respective to NDR based drug response quantification.

11.2. NDR improved reproducibility and drug response curve fits

The consistency in large-scale drug screening approaches are highly debated [163, 166-168]. Thus, we investigated if the NDR metric could improve the reproducibility in cell-based chemo-sensitivity screens. One of the major factors affecting the reproducibility is discrepant cell seeding. To explore this, we screened the breast cancer cell line MCF-7 in duplicate experiments with 131 oncology drugs. MCF-7 grows as cell aggregates and is technically challenging to obtain as single cell suspension, and cell seeding uniformity is therefore often compromised. Based on a PI normalized readout, the two replicate experiments were

highly discrepant, whereas NDR normalization markedly improved the consistency/reproducibility (Figure 15A). Z'-factor, which is one commonly used quality control measures for screening (should be ≥0.5) [185], was significantly improved after employing NDR compared to PI (Figure 15B).

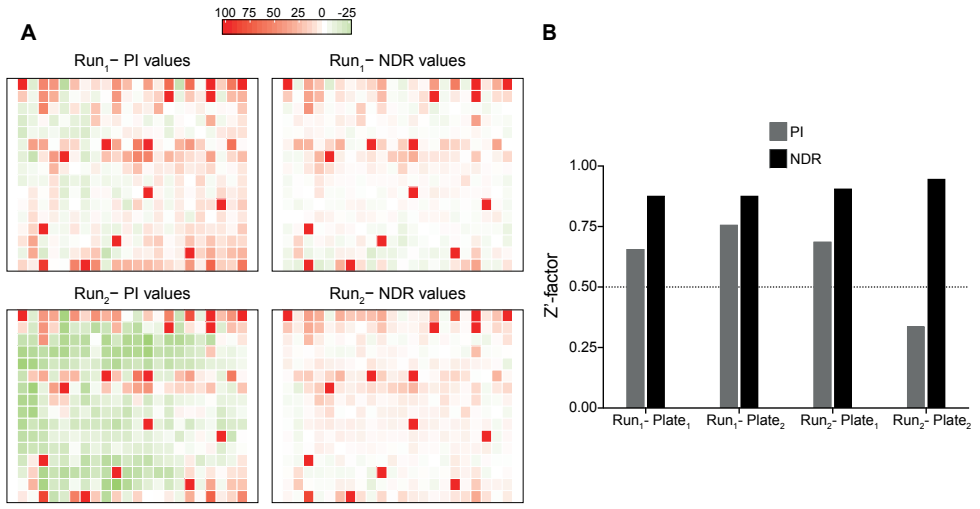

Figure 15: NDR improved reproducibility and quality of high throughput drug screening. A) Comparisons of replicate plates where the drug effects are assessed with PI and NDR. NDR cleaned up the noisy PI data. B) Bar diagram showing the improved Z'-factor of using NDR over PI.

Thereafter, we evaluated whether NDR improved drug response curve fitting, which is critical in drug response quantification. For this, we evaluated two dose response quality measures, root mean squared distance (RMSD) and baseline distance from dose response curves (Figure 16A). Ideally, both measures are zero. We found that NDR significantly improved both RMSD and baseline distance values of dose response curves (Figure 16). Further, we used NDR method to analyze existing datasets and observed significant improvement in RMSD and baseline distance values for already existing drug screening datasets, including those from the Genomics of Drug Sensitivity in Cancer (GDSC1000) [89, 105] and Cancer Therapeutics Response Portal (CTRPv2) [106, 164] (Figure 16D,E) projects. Since the pre-existing datasets lacked the initial time point data, estimates based on cell-

specific growth rate were used (growth rate incurred from in-house MDA-MB-231 screening.

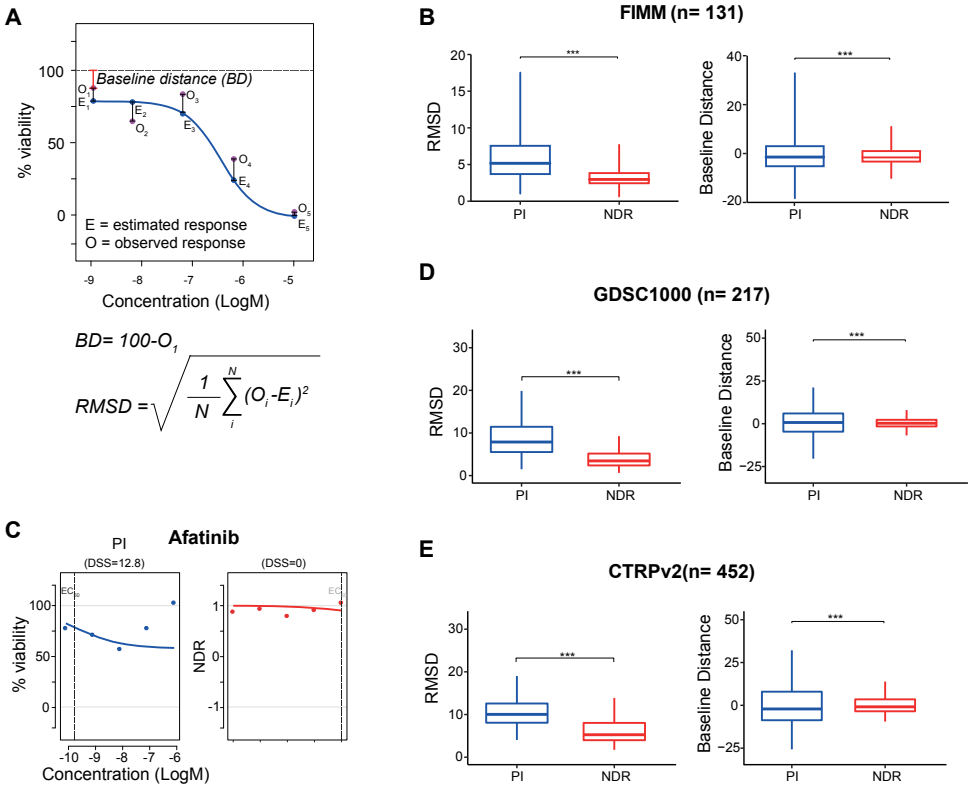

Figure 16: NDR significantly improves curve fitting. A) Schematic representation (definition) of RMSD and baseline distance (BD). B) Box plot comparing the average RMSD and baseline distance computed for dose response curves in MDA-MB-231 cell line between PI and NDR screened in house at FIMM. C) An example illustrating the improved curve fits of EGFR/HER2 inhibitor afatinib in MDA-MB-231 from FIMM dataset. D), E) RMSD and baseline distance comparisons in GDSC1000 and CTRPv2 respectively. **p<0.05, ***p<0.005.

Because of the limited number of screening data points, even an error in an individual data point of the dose response curve can lead to over/under assessment of drug effects. Our results demonstrated that NDR not only reduced the experimental artifacts and improved curve fitting but also alleviated the experimental artifacts inherent to high throughput drug screening. The curve fitting improvements in pre-existing GDSC and CCLE (CTRPv2) datasets emphasized the wide

applicability of NDR. Altogether, these results advocate that the application of NDR metric might partly solve the existing problems with inconsistency in large-scale drug screening studies.

11.3. NDR reflected both viability and cytotoxicity

Along with the real-time cell viability assay, we also utilized an independent cytotoxicity measurement system (CellTox Green) to cross-validate the reliability of cell viability readout-based NDR drug response. Viability and cytotoxicity assays were multiplexed in the same experiment, screening five different cancer cell lines with varying growth rates, cellular phenotypes and tissue of origin. When comparing NDR-based dose responses of drugs to their cytotoxicity counterparts, good agreement was observed, while this was not the case with PI based data (Figure 17).

First, in MDA-MB-361 and MIA-PACA-2 the magnitude of filanesib (a kinesin spindle protein inhibitor) effect based on NDR corresponds to the induced cytotoxicity (Figure 17A). In contrast, filanesib had no to little effect on other three cell lines, evident both in NDR-based and toxicity response curves. On the other hand, from the PI response metric, it was not possible to identify the response pattern seen with toxicity readout, and it did not allow us to distinguish the magnitude of the drug effect exhibited in the different cell lines.

Second, unlike the PI metric, NDR can differentiate a cytostatic effect from a cytotoxic effect. For example, omacetaxine (a protein synthesis inhibitor) was toxic towards all cell lines tested except MCF-7, where it exhibited a cytostatic effect (Figure 17B). This behavior of omacetaxine towards MCF-7 was captured by the NDR but not the PI metric.

Third, NDR eliminated the false hit calling, including over or under estimation of drug effect. For instance, based on PI, tipifarnib (a farnesyltransferase inhibitor) had a strong inhibition effect on MDA-MB-231, which did not correspond to the cytotoxicity readout (Figure 17C). NDR response, on the other hand, corresponded with the cytotoxicity and showed that tipifarnib had no inhibition effect in MDA-

MB-231. The false positive effect seen with PI was due to uneven cell seeding; fewer cells had been dispensed in the wells with the highest two concentrations.

Finally, NDR revealed growth-stimulatory effects of drugs that might be counterproductive in clinics. For example, in HDQ-P1 cells, pevonedistat (a NEDD8 activating enzyme inhibitor) exhibited growth-stimulating effect (Figure 17D).

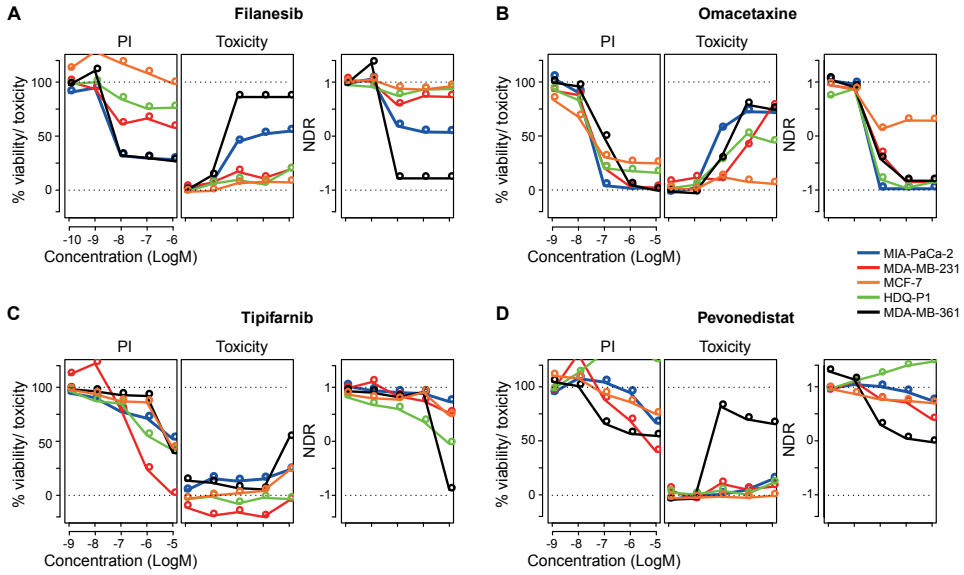

Figure 17: NDR illustrates both cytostatic and cytotoxic behavior of drugs. Dose response curves of five cell lines based on %viability (PI), %toxicity and NDR-metric for four representative drugs. A) Differing magnitude of viability inhibition and cytotoxicity of filanesib on the cell lines. B) Cytostatic nature of omacetaxine on MCF-7. C) Non-effective behavior of tipifarnib on MDA-MB-231. D) Growth-stimulatory effect of pevonedistat on HDQ-P1.

These results highlight that even with a single viability readout, a wide spectrum of drug behaviors can be evaluated employing the NDR-metric. This is beneficial in high throughput drug screening approaches as it eliminates the time and cost for further cytotoxicity assessments.

12. Network pharmacology enables prediction of selective kinase combination addictions

Kinases have gathered great attention as potential therapeutic targets against cancer due to their pivotal role in signal transduction and regulation of vast range of cellular activities including growth, survival, immune regulation and metabolism. In addition, they are often oncogenically mutated in cancers [109]. Even though kinases have been explored extensively, only a small number of kinase inhibitors have shown significant clinical efficacy so far. The availability of a large number of molecularly targeted compounds as well as drug vulnerabilities has created the opportunity to use their drug vulnerability data to predict the pathways/signaling nodes those are essential for cancer progression but are not necessarily mutated. However, most of the targeted compounds are promiscuous resulting in polypharmacology or 'off target' effects, which might play both ways as having counterproductive or enhancing therapeutic effects. To address the challenge of predicting key targetable vulnerabilities of cancer cells as well as efficacy of kinase inhibitors, we developed a phenotype-based systems pharmacology approach that integrates comprehensive drug sensitivity profiling with systems-level drug-target networks to provide functional insights into both single and multi-target models of the underlying oncogenic signaling cascades in individual breast cancer cell lines.

To identify signal addictions, we used the observed drug responses (DSS values) of 21 breast cancer cell lines (19 from study **I** as well as the 2 HR+ cell lines T-47-D and ZR-75-1) against 40 kinase inhibitors with extensive biochemical target profiling information (i.e. where kinome-wide profiling had been performed using purified proteins *in vitro*). The drug target data was obtained from biochemical assay data (K_d values) against 205 kinases from the study of Davis et al [186], which were targeted by the screened 40 kinase inhibitors. A kinase was considered as a target of a particular inhibitor if the K_d value was below 100 nM or at max 50-fold higher than the most potent K_d level of the given compound, whichever was lower. Analyzing cell line-specific inhibitor sensitivity profiles (DSS) together with drug-target network (kinase

targets of the respective inhibitor), we devised a kinase addiction quantification metric, which we termed kinase inhibitor sensitivity score (KISS). The analysis was based on the assumption that inhibitors of cell line specific essential kinases should effectively inhibit viability of that particular cell line. In other words, KISS for an individual kinase target is calculated as an average of drug responses over those drugs targeting the particular kinase (study **III**, Figure 1). For example, if an imaginary kinase X (Kin_X) is inhibited by three different drugs A, B and C with respective DSSs of 5, 10, 20 in a particular cell line, then the KISS for Kin_X in the given cell line is calculated as (5+10+20)/3=11.66.

KISS was computed separately for each kinase target for each cell line. Our KISS approach successfully predicted known kinase addictions in respective breast cancer cell lines, including ERBB2 addiction in the HER2+ cell lines SK-BR-3 and BT-474; BRAF addiction in DU-4475, a cell line carrying a *BRAF*(V600E) mutation and PI3K in the *PIK3CA* mutant cell lines MCF-7 (carrying an E545K mutation) and T47D (H1047R mutation) (study **III**, Figure 2D). In addition, KISS predicted several novel cell line specific kinase addictions, which were further validated with independent siRNA and/or compound assessment. For example, ARK5, CAMK2a, MST1, SNARK1 in CAL51 and DYRK1A, DYRK1B, PKCδ in Hs-578T (study **III**, Figure 4, Table S5). Strikingly, KISS-based clustering of TNBC cell lines displayed better agreement with transcriptomics based subtyping than the basic DSS-based subgrouping described in study I (study **III**, Figure 3). This could be because of the power of KISS to extract specific addictions that correspond to the subtype specific transcriptomic signatures, which are obscured in DSS data due to polypharmacology effect of drugs.

Inhibiting a single node or pathway may not always be effective in treating cancer due to network rewiring and activation of compensatory signaling pathways leading to rapid development of drug resistance [187, 188] (also evident in study I). Hence, concurrent selective inhibition of multiple signaling pathways/nodes might provide a more effective way to treat cancers. However, identifying an effective drug combination *a priori* is quite challenging and it is currently impossible to experimentally test every possible drug combination and/or

treatment regime. Therefore, systems biology approaches that may predict effective combinations are important to explore.

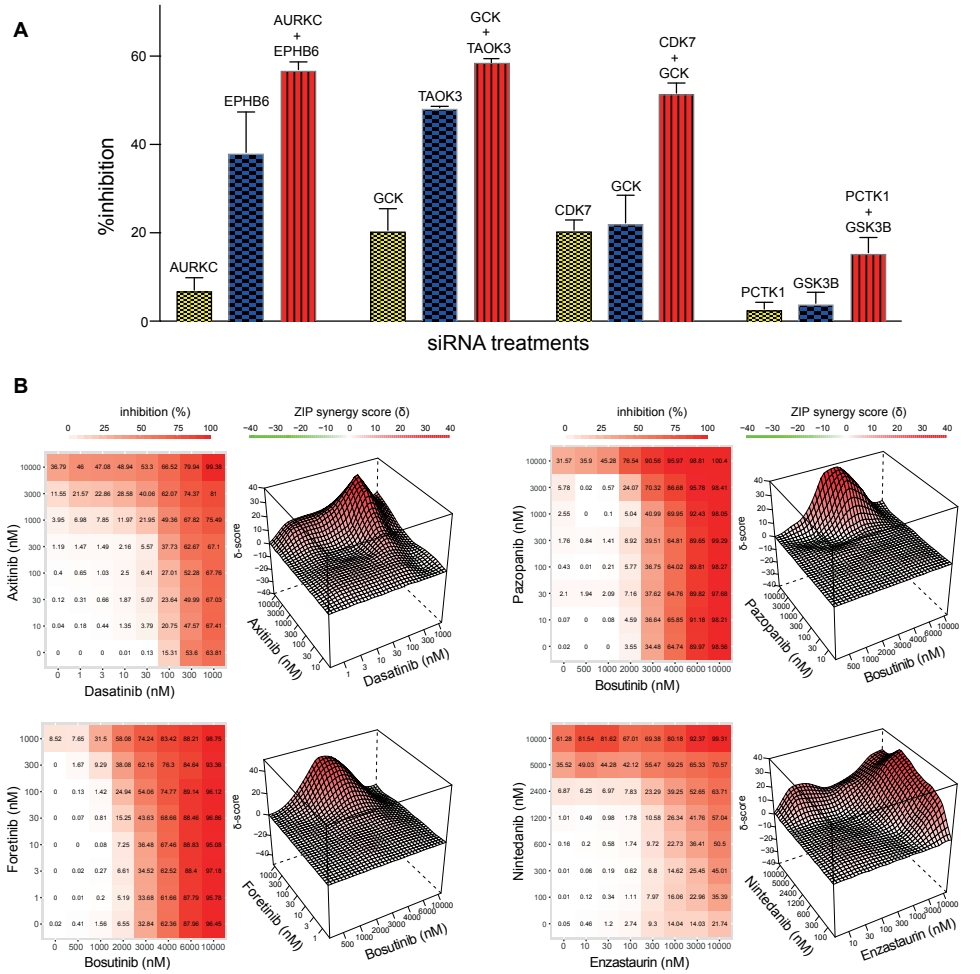

Figure 18: Synergistic compound combinations and kinase pairs co-essentiality in HCC1937. A) Validation of the combinatorial KISS-predicted kinase co-inhibition using siRNA knockdowns. B) Drug combination matrices and their corresponding 3D synergy landscape visualizations illustrating synergy between the combined drug pairs, including axitinib (AURKC inhibitor) with dasatinib (EPHB6 inhibitor), pazopanib (TAOK3 inhibitor) with bosutinib (GCK inhibitor), foretinib (CDK7 inhibitor) with bosutinib (GCK inhibitor) and nintedanib (PCTK1 inhibitor) with enzastaurin (GSK3b inhibitor).

In-line with the concept, we further extended the KISS approach to predict kinase pairs whose co-inhibition leads to enhanced cellular

toxicity (synergy), which we called combinatorial KISS. Synthetic lethal kinase pairs were selected in way that the effect of co-inhibition is higher than the single kinase selective inhibitors. Combinatorial KISS aided in predicting synergistic drug combinations in an unbiased fashion. Combinatorial KISS successfully predicted co-inhibition of EPHB6 with AURKC, GCK with TAOK3, GCK with CDK7 and GSK3b with PCTK1 in HCC1937 cells. Combinatorial siRNA-mediated knockdown of these kinase pairs exhibited markedly enhanced viability inhibition as compared to the individual kinase inhibition (Figure 18). By mapping the kinases back to their respective inhibitors, these predictions ultimately provided synergistic drug combinations effective against HCC1937, including axitinib (AURKC inhibitor) with dasatinib (EPHB6 inhibitor), pazopanib (TAOK3 inhibitor) with bosutinib (GCK inhibitor), foretinib (CDK7 inhibitor) with bosutinib (GCK inhibitor) and nintedanib (PCTK1 inhibitor) with enzastaurin (GSK3b inhibitor) (Figure 18).

These results suggest that the systems biology approach, which utilizes polypharmacology and functional screening data, helps to identify cell-line specific vulnerabilities or pathway addictions. In contrast to the limited power of genomic approaches in predicting druggable targets, phenotypic-profiling based approach provided an unprecedented opportunity to de-convolve druggable driver alterations. The KISS approach can be implemented in future precision therapy based drug development since it allows to dissect the unintended target specificities from the phenotypic screening data and provide mono and multi-target addictions in an individual cancer cell model.

Conclusions

This study highlights the need to go beyond the mere cell viability readouts for drug sensitivity assessment of the cell lines or primary cancer cells. Since cell viability does not directly correlate to proliferation or cell death, a simple multiplexed cell viability and cytotoxicity readout can provide more insights into the drug mode-of-action, hence aiding the design of rational treatment options. With this simple approach, we found several investigational oncology compounds that failed to induce cell line specific cytotoxicity, but rather only caused a reversible cytostatic or anti-metabolic effect against TNBC cell lines. As the effect of such cytostatic drugs can be reversed, these drugs should perhaps be used cautiously in the clinic.

Currently available high throughput screening-compatible assays for measuring cell viability and toxicity still have many limitations. They do not provide much information about the mechanisms of drug treatment-derived cytopathological changes. With such assays, the mechanism of cell toxicity cannot be addressed. For example, they cannot distinguish between apoptosis and necrosis or immunogenic cell death. The intra-tumor heterogeneity, which is a crucial aspect in primary samples, cannot be captured with the current toxicity measurements assays. These assays do not differentiate between the responder and non-responder cell populations. Such end-point toxicity assays do not tell much about physiological drug responses as well, like drug kinetics, metabolism, and its mechanism of action. This limits the detection of sensitivities to all classes of cancer drugs. To some extent, most of these limitations in high throughput chemical screening approach can be addressed with high content microscopy. Technological advancements in high throughput live imaging and establishment of optimal molecular markers are likely to enable deep understanding of drug effects.

Real-time measurement systems provide us an opportunity to de-convolute various types of drug responses including cytostatic, cytotoxic

and growth stimulatory effects only from cell viability measurements. A simple two-step normalization technique we called NDR, significantly reduced the false hit calling and increased the consistency and reproducibility in high throughput drug screening approaches. This approach will be a significant asset for future cell based high throughput drug screenings as it saves time, effort and cost by eliminating the need to perform cytotoxicity assays or replicate screens. The NDR approach is widely applicable not only for established cell lines but also against technically challenging and slow growing patient derived samples. With the technological advancements in multi-time point high-content imaging adapted for high throughput setting, the application of NDR could also be easily expanded to imaging data for more accurate evaluation of the effect of drugs on cells. Moreover, the effect of drugs could be accurately assessed at subpopulation level (eg. persister cells) and in co-culture system.

In this work, we categorized TNBC cell lines into sensitive and insensitive groups based on whether mitotic inhibitors induced cytotoxic vs. cytostatic responses. Selective cytotoxic responses to mitotic inhibitors among the TNBC cell lines could be linked to differential expression of protein markers, such as high levels of PKCα, FGFR1, c-Jun, Caveolin-1 expression and low levels of RAB25, pSTAT3(Y705), NOTCH3, Bcl-2. This highlights the possibility of exploring the expression of these molecules for patient stratification and therapy against mitotic inhibitors like taxanes. This also suggests that protein biomarkers may better predict the therapeutic responses to many drugs than genomic biomarkers.

Kinase inhibitors possess great potential in cancer therapeutics as they regulate pathways responsible for cell survival and proliferation [108]. However, because most of the kinase inhibitors target the highly conserved ATP binding site of kinases [189], they are known to be promiscuous, making it tough to pinpoint the selective inhibition of an individual kinase. Most of the clinically effective kinase inhibitors are relatively promiscuous and their polypharmacology effect resulting in the concurrent blocking of complementary pathways may be the reason behind their clinical significance [131, 134]. To address these challenges,

we developed a systems network pharmacology approach, which integrated drug sensitivity profiles and kinase target space of the respective drugs to identify and establish cell line specific kinase addictions or co-addictions. With the KISS approach, we predicted and validated selective kinase dependencies and co-dependencies in TNBC cell lines, for instance co-dependencies like CDK7 & GSK3B and MEK5 & AURKC in HCC1937 cell line. Our approach can be easily expanded or applied to patient derived samples to identify the molecular addictions driving cancer progression and relapse, thereby stratifying tailored drug or drug combination approaches for individual patients.

The absence of highly recurrent targetable genomic driver mutations and molecular alterations in TNBCs limits the possibilities of therapeutic design directly targeting a particular driver oncogene. In addition, TNBC is not only a heterogeneous disease with respect to its molecular characteristics, but it also exhibits marked heterogeneity in its phenotypic responses to drugs. We and others found that the overall drug sensitivity correlates poorly with the transcriptomics based subtyping of TNBC [22, 90]. Therefore, I belive that drug sensitivity-based functional profiling of large and diverse panels of TNBC samples (cell lines or patient-derived primary samples) along with their putative biomarkers can provide better ways to stratify TNBCs for the development of effective therapeutics.

The observations in this study were based on drug sensitivity assays of conventional TNBC cell lines. Therefore, further studies on alternative cancer models that more closely recapitulate the primary tumor features, such as patient tumor derived cells (PDC) [190] or xenograft (PDX) cell lines [191] or conditionally reprogrammed progenitor-like cells (CRC) [192, 193] may provide better ways to stratify and find promising therapeutic targets. Although these approaches are in preliminary stages of development, they allow the possibility for testing *ex vivo* drug responses in high-throughput settings. Further, emerging techniques like patient tumor *ex vivo* 3D- organoid cultures or organotypic tissue slice systems provide a possibility to profile more realistic and complex tumor models [194-197]. These models better capture the tumor characteristics such as intra-tumor heterogeneity and

tumor microenvironment which play a major role in determining drug efficacy [197-199]. However, it has been difficult to adapt these techniques to high throughput settings. In summary, functional profiling allows unbiased determination of new druggable signaling nodes and may aid in establishment of new precision targeted therapeutic regimes.

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Appendix 1: List of compounds used in the studies.

Drug.Name	Mechanism/Targets	High.phase/Approval status	High conc. (nM)	Study
Pilocarpine	Non-selective muscarinic receptor agonist	Approved	40000	I
Gefitinib	EGFR inhibitor	Approved	10000	I, III
Metformin	AMPK activator	Approved (non-oncology)	100000	II
Anagrelide	PDE-3, PLA2 inhibitor	Approved	10000	I, II
Imatinib	Abl, Kit, PDGFRB inhibitor	Approved	10000	I, II, III
Idarubicin	Topoisomerase II inhibitor	Approved	1000	I, II
Vatalanib	VEGFR-1 & -2 inhibitor	Investigational (Ph 3)	10000	I, III
Erlotinib	EGFR inhibitor	Approved	10000	I, II, III
Dexamethasone	Glucocorticoid, immunomodulatory agent	Approved	10000	I, II
Auranofin	Antirheumatic agent	Approved	2500	I
Tretinoin	Retinoic acid receptor agonist	Approved	10000	I, II
Simvastatin	HMG CoA reductase inhibitor	Approved (non-oncology)	10000	I
Goserelin	Gonadotropin releasing hormone superagonist	Approved	10000	I
Raloxifene	Selective estrogen receptor modulator	Approved	10000	I
Camptothecin	Topoisomerase I inhibitor	Probe	5000	I
Plicamycin	RNA synthesis inhibitor	Approved	10000	I
Rofecoxib	COX-2 inhibitor	Approved	10000	I
Lapatinib	HER2, EGFR inhibitor	Approved	1000	I, III
Bortezomib	Proteasome inhibitor (26S subunit)	Approved	1000	I, II
Letrozole	Aromatase inhibitor	Approved	10000	I
Bexarotene	Antineoplastic agent	Approved	10000	I
Celecoxib	Selective COX-2 inhibitor	Approved	10000	I, II
Anastrozole	Aromatase inhibitor	Approved	10000	I
Bicalutamide	Nonsteriodal antiandrogen	Approved	10000	I
Clofarabine	Antimetabolite; Purine analog	Approved	10000	I, II
Lomustine	Alkylating nitrosourea compound	Approved	10000	I
Vincristine	Microtubule depolymerizer	Approved	1000	I, II
Vinorelbine	Microtubule depolymerizer	Approved	10000	I
Altretamine	Formaldehyde release, alkylating agent	Approved	10000	I
Aminoglutethimide	Anti-steroid, aromatase inhibitor	Approved	10000	I
Vinblastine	Microtubule depolymerizer	Approved	1000	I

Chlorambucil	Nitrogen mustard alkylating agent	Approved	10000	I
Thalidomide	Immunosuppresant	Approved	10000	I
Clomifene	Selective oestrogen receptor modulator	Approved	10000	I
Dacarbazine	Alkylating agent	Approved	10000	I
Cyclophosphamide	Alkylating agent	Approved	40000	I
Cytarabine	Antimetabolite, interferes with DNA synthesis	Approved	10000	I, II
Finasteride	type II 5-α reductase inhibitor	Approved	10000	I
Fluorouracil	Antimetabolite	Approved	10000	I
Flutamide	Nonsteroidal antiandrogen	Approved	10000	I
Ifosfamide	Nitrogen mustard alkylating agent	Approved	10000	I
Imiquimod	Immunomodulatory agent, TLR7 agonist	Approved	2500	I
Levamisole	Immunomodulatory agent	Approved	10000	I
Melphalan	Nitrogen mustard alkylating agent	Approved	12500	I
Methylprednisolone	Glucocorticoid, immunomodulatory agent	Approved	10000	I, II
Mitoxantrone	Topoisomerase II inhibitor	Approved	1000	I, II
Paclitaxel	Mitotic inhibitor, taxane microtubule stabilizer	Approved	1000	I, II
Prednisolone	Glucocorticoid, immunomodulatory agent	Approved	10000	I
Procarbazine	Alkylating agent	Approved	10000	I
Prednisone	Immunomodulatory agent	Approved	10000	I
Mepacrine	Unclear. PLA2 inhibitor. NF- $\varkappa$B inhibitor, p53 activator	Approved	50000	I, II
Topotecan	Topoisomerase I inhibitor. Camptothecin analog	Approved	10000	I, II
Temozolomide	Alkylating agent	Approved	100000	I
Fulvestrant	Estrogen receptor antagonist	Approved	1000	I
Megestrol acetate	Progestogen	Approved	10000	I
Tamoxifen	Estrogen receptor antagonist	Approved	10000	I
Mechlorethamine	Nitrogen mustard alkylating agent	Approved	1e+05	I
Methotrexate	Antimetabolite; Anti-folate agent	Approved	5000	I, II
Nilutamide	Nonsteroidal antiandrogen	Approved	10000	I
Mitotane	Antineoplastic agent	Approved	10000	I
Allopurinol	Xanthine oxidase inhibitor	Approved	10000	I
Busulfan	Alkylating antineoplastic agent	Approved	1e+05	I
Deferoxamine	Iron chelator	Approved (non-oncology)	10000	I
Hydroxyurea	Antineoplastic agent	Approved	1000000	I, II
Mercaptopurine	Antimetabolite	Approved	10000	I
Thioguanine	Antimetabolite; Purine analog	Approved	10000	I
Carmustine	Alkylating agent	Approved	10000	I
Thio-TEPA	Alkylating agent	Approved	50000	I

Pipobroman	Alkylating agent	Approved	10000	I
Tacrolimus	Binds FKBP12, causes inhibition of calcineurin	Approved	10000	I
AT 101	Bcl-2 family inhibitor	Investigational (Ph 2)	100000	I
BI 2536	PLK1 inhibitor	Investigational (Ph 2)	1000	I, III
Pravastatin	HMG CoA reductase inhibitor	Approved (non-oncology)	10000	I, II
Exemestane	Aromatase inhibitor	Approved	10000	I
Irinotecan	Topoisomerase I inhibitor	Approved	10000	I
Palbociclib	CDK4/6 inhibitor	Approved	10000	I, II
AT9283	Aurora A & B, Jak2, Flt, Abl inhibitor	Investigational (Ph 2)	1000	I
Navitoclax	Bcl-2/Bcl-xL inhibitor	Investigational (Ph 2)	10000	I, II
Selumetinib	MEK1/2 inhibitor	Investigational (Ph 3)	10000	I, III
Veliparib	PARP inhibitor	Investigational (Ph 3)	10000	I, II
Afatinib	EGFR inhibitor	Approved	1000	I, II, III
Dovitinib	FGFR inhibitor	Investigational (Ph 3)	10000	I, III
Obatoclax	Bcl-2 family inhibitor	Investigational (Ph 3)	10000	I
Crizotinib	ALK, c-Met inhibitor	Approved	1000	I, II, III
Danusertib	Aurora, Ret, TrkA, FGFR-1 inhibitor	Investigational (Ph 2)	10000	I
Foretinib	MET, VEGFR2 inhibitor	Investigational (Ph 2)	1000	I, III
Abiraterone	P450 17α-hydroxylase-17,20-lyase inhibitor	Approved	5000	I
Tanespimycin	HSP90 inhibitor	Investigational (Ph 2)	10000	I
Alvespimycin	HSP90 inhibitor	Investigational (Ph 2)	1000	I
SNS-032	CDK inhibitor	Investigational (Ph 2)	10000	I, III
ABT-751	Microtubule depolymerizer.	Investigational (Ph 2)	10000	I
Serdemetan	HDM2-p53 antagonist	Investigational (Ph 1)	10000	I
BIIB021	HSP90 inhibitor	Investigational (Ph 2)	10000	I
CUDC-101	HDAC & EGFR, Her2 inhibitor	Investigational (Ph 1)	10000	I
Decitabine	Nucleoside analog DNA methyl transferase inhibitor	Approved	10000	I
Alvocidib	CDK inhibitor	Investigational (Ph 2)	10000	I, II, III
Amonafide	Topoisomerase II inhibitor / DNA intercalator	Investigational (Ph 3)	10000	I
Tipifarnib	Farnesyltransferase inhibitor	Investigational (Ph 3)	10000	I, II
Pimasertib	MEK1/2 inhibitor	Investigational (Ph 2)	10000	I
Ponatinib	Broad TK inhibitor	Approved	1000	I, II
Perifosine	AKT/PI3K inhibitor	Investigational (Ph 3)	2500	I
Motesanib	VEGFR, PDGFR, Ret, Kit inhibitor	Investigational (Ph 2)	10000	I, III
Cediranib	KDR/Flt/VEGFR inhibitor	Investigational (Ph 3)	1000	I, III
PF-04691502	PI3K/mTOR inhibitor	Investigational (Ph 2)	10000	I
Rucaparib	PARP inhibitor	Approved	10000	I

Tarenflurbil	γ-secretase inhibitor	Investigational (Ph 3)	10000	I
Tivozanib	VEGFR1, 2, 3, c-Kit, PDGFRB inhibitor	Investigational (Ph 3)	10000	I
AZD1775	Wee1 inhibitor	Investigational (Ph 2)	10000	I, II
PF-00477736	Chk1 inhibitor	Investigational (Ph 1)	10000	I
AZD7762	Chk1 inhibitor	Investigational (Ph 1)	1000	I
Barasertib	Aurora B inhibitor	Investigational (Ph 3)	1000	I, II, III
AZD8055	mTOR inhibitor	Investigational (Ph 1)	10000	I
Belinostat	HDAC inhibitor	Approved (US)	10000	I, II
Bimatoprost	Prostaglandin analog	Approved	5500	I
Doramapimod	p38MAPK inhibitor	Investigational (Ph 1)	10000	I, III
Bryostatin 1	PKC activator	Investigational (Ph 1)	100	I
Tacedinaline	HDAC inhibitor	Investigational (Ph 3)	1000	I
BMS-754807	IGF1R inhibitor	Investigational (Ph 2)	10000	I, II
Idelalisib	PI3K inhibitor, p110δ-selective	Approved	10000	I, II
Trametinib	MEK1/2 inhibitor	Approved	250	I, II
Sonidegib	Smothened (Hh) inhibitor	Approved	10000	I
OSI-027	mTOR inhibitor	Investigational (Ph 2)	10000	I
Refametinib	MEK1/2 inhibitor	Investigational (Ph 2)	10000	I
Everolimus	binds FKBP12, causes inhibition of mTORC1	Approved	100	I, II
Ruxolitinib	JAK1&2 inhibitor	Approved	10000	I, II, III
Fasudil	ROCK, PKA, PKG, PRK inhibitor, prodrug	Approved [200]	50000	I
Indibulin	Microtubule depolymerizer	Investigational (Ph 2)	10000	I
Iniparib	PARP inhibitor	Investigational (Ph 3)	10000	I
MK-2206	AKT inhibitor	Investigational (Ph 2)	1000	I
Alisertib	Aurora A inhibitor	Investigational (Ph 3)	10000	I, II
Nelarabine	Nucleoside analog, DNA, RNA synth inhibitor	Approved	10000	I
Nilotinib	Abl inhibitor	Approved	10000	I, II, III
Luminespib	HSP90 inhibitor	Investigational (Ph 2)	1000	I, II
2-methoxyestradiol	Angiogenesis inhibitor	Investigational (Ph 2)	10000	I
Plerixafor	CXCR4 antagonist	Approved	10000	I
Vemurafenib	B-Raf(V600E) inhibitor	Approved	10000	I, II, III
Vandetanib	VEGFR, EGFR, RET inhibitor	Approved	1000	I, III
Sepantronium bromide	Survivin inhibitor	Investigational (Ph 2)	10000	I
Linsitinib	IGF1R, IR inhibitor	Investigational (Ph 2)	10000	I
Tepotinib	c-Met inhibitor	Investigational (Ph 1)	1000	I
Lestaurtinib	FLT3, JAK2, TrkA, TrkB, TrkC inhibitor	Investigational (Ph 3)	1000	I
Dasatinib	Abl, Src, Kit, EphR... Inhibitor	Approved	1000	I, II, III

Tofacitinib	JAK3, JAK2(V617F) inhibitor	Approved	5000	I, III
Fingolimod	S1PR antagonist	Approved	10000	I
Axitinib	VEGFR, PDGFR, KIT inhibitor	Approved	10000	I, II, III
Saracatinib	Src, Abl inhibitor	Investigational (Ph 3)	10000	I
Bosutinib	Abl, Src inhibitor	Approved	10000	I, II
Canertinib	pan-HER inhibitor	Investigational (Ph 3)	10000	I, III
Lenalidomide	Immunomodulatory	Approved	100000	I, II
Panobinostat	HDAC inhibitor	Approved	1000	I, II
Pazopanib	VEGFR inhibitor	Approved	10000	I, III
Tandutinib	FLT3, PDGFR, KIT inhibitor	Investigational (Ph 2)	1000	I, III
Temsirolimus	binds FKBP12, causes inhibition of mTORC1	Approved	100	I
Vorinostat	HDAC inhibitor	Approved	10000	I, II
Entinostat	HDAC inhibitor	Investigational (Ph 2)	10000	I
Enzastaurin	PKCβ inhibitor	Investigational (Ph 3)	10000	I, III
Olaparib	PARP inhibitor	Approved	10000	I, II
Masitinib	KIT inhibitor	Investigational (Ph 3)	10000	I, III
Pictilisib	PI3K inhibitor, pan-class I	Investigational (Ph 2)	10000	I, III
Vismodegib	Smothened (Hh) inhibitor	Approved	10000	I, II
Sorafenib	B-Raf, FGFR-1, VEGFR-2 & -3, PDGFR-β, KIT, and FLT3 inhibitor	Approved	1000	I, II, III
Seliciclib	CDK2/7/9 inhibitor	Investigational (Ph 2)	10000	I
Patupilone	Epothilone microtubule stabilizer	Investigational (Ph 3)	1000	I
Docetaxel	Taxane microtubule stabilizer	Approved	1000	I
Pentostatin	Antimetabolite; Purine analog	Approved	10000	I
Sirolimus	binds FKBP12, causes inhibition of mTORC1	Approved	100	I
Estramustine	Alkylating agent	Approved	10000	I
Floxuridine	Antimetabolite; Analog of 5-fluorouracil	Approved	10000	I
Gemcitabine	Antimetabolite; Nucleoside analog	Approved	1000	I, II
Teniposide	Topoisomerase II inhibitor	Approved	10000	I
Dactinomycin	RNA and DNA synthesis inhibitor	Approved	1000	I
Streptozocin	Alkylating glucosamine-nitrosourea agent	Approved	10000	I
Cladribine	Antimetabolite; Purine analog	Approved	1000	I
Mitomycin C	Antineoplastic antibiotic; DNA crosslinker	Approved	10000	I
Carboplatin	Platinum-based antineoplastic agent	Approved	100000	I
Cisplatin	Platinum-based antineoplastic agent	Approved	100000	I
Pemetrexed	Dihydrofolate reductase inhibitor	Approved	10000	I
Oxaliplatin	Platinum-based antineoplastic agent	Approved	100000	I
Uramustine	Alkylating agent	Approved	10000	I

Daunorubicin	Topoisomerase II inhibitor	Approved	1000	I
Etoposide	Topoisomerase II inhibitor	Approved	10000	I, II
Arsenic(III) oxide	Thioredoxin reductase inhibitor	Approved	2500	I
Doxorubicin	Topoisomerase II inhibitor	Approved	1000	I
Valrubicin	Topoisomerase II inhibitor	Approved	5000	I
Sunitinib	Broad TK inhibitor	Approved	1000	I, II, III
Ixabepilone	Epothilone microtubule stabilizer.	Approved	1000	I
Azacitidine	Nucleoside analogue DNA methyl transferase inhibitor	Approved	10000	I, II
Fludarabine	Antimetabolite; Purine analog	Approved	10000	I, II
Bleomycin	Glycopeptide antibiotic; causes DNA breaks	Approved	10000	I
Dactolisib	mTOR/(PI3K) inhibitor	Investigational (Ph 2)	1000	I
Quizartinib	FLT3 inhibitor	Investigational (Ph 3)	1000	I, II, III
APR-246	p53 activator, thioredoxin reductase 1 inhibitor	Investigational (Ph 1)	10000	I
Carfilzomib	Proteasome inhibitor (20S subunit)	Approved	1000	I, II
Gandotinib	JAK2 inhibitor	Investigational (Ph 2)	10000	I
AZ 3146	Mps1 kinase (TTK) inhibitor	Probe	10000	I
S-trityl-L-cysteine	Eg5 inhibitor	Probe	40000	I
Sotrastaurin	PKC inhibitor	Investigational (Ph 2)	10000	I
Geldanamycin	HSP90 inhibitor	Probe	10000	I
Bendamustine	Nitrogen mustard alkylating agent	Approved	10000	I
Cyclopamine	Smothened inhibitor	Probe	10000	I
Midostaurin	Broad TK (FLT3, KIT, RET, JAK, EGFR...) inhibitor	Approved (US)	10000	I, II, III
Regorafenib	B-Raf, c-Kit, VEGFR2 inhibitor	Approved	10000	I, II
XAV-939	Tankyrase-1 and -2	Probe	10000	I
UCN-01	PKCβ, PDK1, Chk, Cdk2 inhibitor	Investigational (Ph 2)	10000	I
Ruboxistaurin	PKCβ inhibitor	Investigational (Ph 3)	10000	I, III
Capecitabine	5-FU prodrug	Approved	10000	I
Chloroquine	Antimalaria agent; chemo/radio sensitizer	Approved	100000	I, II
Valproic acid	HDAC inhibitor	Approved	1000000	I, II
Tivantinib	MET inhibitor	Investigational (Ph 2)	1000	I
Niraparib	PARP inhibitor	Approved (US)	10000	I
Volasertib	PLK1 inhibitor	Investigational (Ph 3)	1000	I, II
Toremifene	selective estrogen receptor modulator	Approved	10000	I
Lasofoxifene	Selective estrogen receptor modulator	Approved	1000	I
Galiellalactone	STAT3-DNA interaction inhibitor	Probe	25000	I
Omacetaxine	Protein synthesis inhib (80 S ribosome)	Approved	10000	I, II
NVP-RAF265	"C-Raf" inhibitor, unclear MoA	Investigational (Ph 2)	1000	I, III

Nutlin-3	MDM2 inhibitor	Probe	10000	I
Neratinib	EGFR inhibitor	Approved (US)	1000	I
Mocetinostat	HDAC inhibitor (HDAC1 & 2-selective)	Investigational (Ph 2)	10000	I
Rabusertib	Chk1 inhibitor	Investigational (Ph 2)	1000	I
Galunisertib	TGF-B/Smad inhibitor	Investigational (Ph 2)	1000	I, II
Linifanib	VEGFR, PDGFR, CSF-1R, FLT3 inhibitor	Investigational (Ph 3)	1000	I, III
Brivanib	VEGFR inhibitor	Investigational (Ph 3)	1000	I, III
Buparlisib	PI3K inhibitor, pan-class I	Investigational (Ph 2)	10000	I
Nintedanib	VEGFR, PDGFR, FGFR inhibitor	Approved	10000	I, II, III
Apitolisib	PI3K/mTOR inhibitor	Investigational (Ph 2)	10000	I
AZD4547	FGFR inhibitor	Investigational (Ph 2)	1000	I, II
VER 155008	HSP70 inhibitor	Probe	10000	I
Daporinad	NAMPT inhibitor	Investigational (Ph 2)	1000	I
Cabozantinib	VEGFR2, Met, FLT3, Tie2, Kit and Ret inhibitor	Approved	1000	I
AZD1480	JAK1/2, FGFR inhibitor	Investigational (Ph 1)	1000	I
Ridaforolimus	binds FKBP12, causes inhibition of mTORC1	Investigational (Ph 3)	100	I
Sonolisib	PI3K inhibitor, pan-class I. Irreversible	Investigational (Ph 2)	10000	I
Quisinostat	HDAC inhibitor	Investigational (Ph 2)	1000	I
Tosedostat	Aminopeptidase inhibitor	Investigational (Ph 3)	10000	I, II
PF-04708671	p70S6K inhibitor	Probe	10000	I
Binimetinib	MEK1/2 inhibitor	Investigational (Ph 2)	1000	I
Tamatinib	Syk inhibitor	Investigational (Ph 1)	10000	I
KX2-391	non-ATP competitive Src inhibitor	Investigational (Ph 2)	10000	I
PF-3845	FAAH inhibitor	Probe	10000	I
MK-0752	γ-secretase/notch inhibitor	Investigational (Ph 2)	1000	I, II
TAK-733	MEK1/2 inhibitor	Investigational (Ph 1)	1000	I
SB 743921	Mitotic inhibitor. Eg5/KSP inhibitor	Investigational (Ph 2)	100	I
Omipalisib	PI3K/mTOR inhibitor	Investigational (Ph 1)	1000	I
Ibrutinib	Btk inhibitor	Approved	1000	I, II
TAK-901	Aurora, Src family, JAK3, RTK inhibitor	Investigational (Ph 1)	1000	I
Fostamatinib	Syk inhibitor	Investigational (Ph 2)	2500	I, III
JQ1	BET family inhibitor	Probe	10000	I
(-) JQ1	Inactive stereoisomer of (+) JQ1	Probe	10000	I
Infigratinib	FGFR inhibitor	Investigational (Ph 1)	1000	I, II
Momelotinib	JAK1 & 2 inhibitor	Investigational (Ph 2)	10000	I, II
Sapanisertib	mTOR inhibitor	Investigational (Ph 1)	1000	I, II
PIK-75	PI3Kα selective inhibitor	Probe	10000	I

TGX-221	PI3Kβ selective inhibitor	Probe	10000	I
Tubacin	HDAC6 inhibitor	Probe	10000	I
Tubastatin A	HDAC6 inhibitor	Probe	10000	I
Atorvastatin	HMG CoA reductase inhibitor	Approved	10000	I
Varespladib	Secretory phospholipase A2 inhibitor	Investigational (Ph 2)	10000	I
StemRegenin 1	AHR antagonist, stem cell regenerating	Probe	10000	I
VX-11E	ERK1 & 2 inhibitor	Probe	2500	I
PFI-1	BET family inhibitor	Probe	30000	I
4-hydroxytamoxifen	Selective estrogen receptor modulator	Investigational (Ph 2)	10000	I
15D-PGJ2	Endogenous PPARγ ligand, prostaglandin, NFκB signaling inhibitor	Probe	3000	I
RD162	AR antagonist	Probe	10000	I
Enzalutamide	AR antagonist	Approved	10000	I
1-methyl-D-tryptophan	Indolamine 2,3-dioxygenase 1 and 2 inhibitor	Investigational (Ph 2)	5000	I
Dacomitinib	pan-HER inhibitor	Investigational (Ph 3)	1000	I
Dinaciclib	CDK inhibitor	Investigational (Ph 3)	1000	I, II
Dabrafenib	B-Raf(V600E) inhibitor	Approved	2500	I
I-BET151	BET family inhibitor	Probe	10000	I
Ralimetinib	p38MAPK inhibitor	Investigational (Ph 2)	10000	I, II
Crenolanib	PDGFRA and PDGFRB inhibitor	Investigational (Ph 2)	10000	I, II
PF-4800567	CK1epsilon inhibitor	Probe	10000	I
PF-670462	CK1epsilon and CK1δ inhibitor	Probe	10000	I
ZSTK474	PI3Kγ selective inhibitor	Investigational (Ph 1)	10000	I, II
PAC-1	procaspase-3 activator	Investigational (Ph 1)	10000	I
Vistusertib	mTOR inhibitor, ATP-competitive	Investigational (Ph 2)	10000	I, II
CPI-613	pyruvate dehydrogenase, α-ketoglutarate dehydrogenase inhibitor	Investigational (Ph 2)	10000	I, II
Roxadustat	HIF prolyl hydroxylase inhibitor	Investigational (Ph 2)	10000	I, II
Pevonedistat	NAE inhibitor	Investigational (Ph 1)	10000	I, II
GSK2636771	PI3Kβ selective inhibitor	Investigational (Ph 1)	10000	I
AT-406	XIAP, cIAP1, cIAP2 inhibitor	Investigational (Ph 1)	10000	I
(5Z)-7-Oxozeaenol	TAK1 inhibitor	Probe	10000	I
Rebastinib	Allosteric ABL, FLT3, TIE2, TRKA inhibitor	Investigational (Ph 1)	1000	I
Lomeguatrib	O6-methylguanine-DNA methyltransferase inhibitor	Investigational (Ph 2)	10000	I
GSK269962	ROCK1 and ROCK2 inhibitor	Probe	10000	I
PF 431396	FAK/PYK2 inhibitor	Probe	10000	I
GSK650394	SGK1 & 2 inhibitor	Probe	10000	I
AVN944	IMPDH inhibitor	Investigational (Ph 2)	10000	I

Venetoclax	Bcl-2-selective inhibitor	Approved	1000	I, II
BMS-911543	JAK2 inhibitor	Investigational (Ph 1)	10000	I
Ipatasertib	AKT inhibitor	Investigational (Ph 2)	10000	I, II
IOX-2	PHD2 inhibitor	Probe	50000	I
GSK-J4	JMJD3 (histone demethylase) inhibitor	Probe	100000	I
UNC1215	L3MBTL3 inhibitor	Probe	10000	I
SGC0946	DOT1L inhibitor	Probe	10000	I
UNC0642	G9a/GLP inhibitor	Probe	10000	I
GSK343	EZH2 inhibitor	Probe	1000	I
UNC0638	G9a/GLP inhibitor	Probe	10000	I
C646	p300/CREB-binding protein (CBP) inhibitor	Probe	25000	I
Stattic	STAT3 SH2 domain inhibitor	Probe	50000	I
Rocilinostat	HDAC-6 selective inhibitor	Investigational (Ph 1)	10000	II
Lonafarnib	Farnesyl transferase inhibitor	Investigational (Ph 3)	100000	II
Alpelisib	PI3Kα inhibitor	Investigational (Ph 2)	2500	II
Pomalidomide	Immunomodulatory agent, anti-angiogenic	Approved	10000	II
SGI-1776	PIM kinase inhibitor	Investigational (Ph 1)	10000	II
Rigosertib	Ras-Raf interaction inhibitor	Investigational (Ph 3)	10000	II
Duvelisib	PI3K inhibitor	Investigational (Ph 3)	500	II
SCH772984	ERK1 & 2 inhibitor	Probe	10000	II
Abemaciclib	CDK4/6 inhibitor	Investigational (Ph 3)	2500	II
Ceritinib	ALK inhibitor	Approved	2500	II
Hydroxyfasudil	ROCK, PKA, PKG, PRK inhibitor	(Approved Japan)	19000	II
Gedatolisib	PI3K/mTOR inhibitor	Investigational (Ph 2))	1000	II
AZD-1080	GSK3 inhibitor	Investigational (Ph 1)	10000	II
Silmitasertib	CSNK2A1 inhibitor	Investigational (Ph 2)	10000	II
AZD-8186	PI3Kβ inhibitor	Investigational (Ph 1)	1000	II
NVP-LCL161	IAPs, SMAC mimetic	Investigational (Ph 2)	25000	II
Tazemetostat	EZH2 inhibitor	Investigational (Ph 2)	10000	II
Pinometostat	DOT1L inhibitor	Investigational (Ph 1)	1000	II
Selinexor	CRM1 inhibitor	Investigational (Ph 2)	10000	II
Birabresib	BET family inhibitor	Investigational (Ph 2)	10000	II
NVP-LGK974	PORCN inhibitor	Investigational (Ph 1)	10000	II
Pacritinib	FLT3/JAK2	Investigational (Ph 3)	10000	II
Idasanutlin	MDM2 inhibitor	Investigational (Ph 3)	10000	II
Filanesib	KSP/Eg5 inhibitor	Investigational (Ph 2)	1000	II
Cobimetinib	MEK1/2 inhibitor	Approved (US)	1000	II

AZD1208	PIM1, 2, 3 kinase inhibitor	Investigational (Ph 1)	10000	II
BGB324	Axl inhibitor	Investigational (Ph 1)	10000	II
Entospletinib	SYK inhibitor	Investigational (Ph 2)	5000	II
AMG-232	MDM2 inhibitor	Investigational (Ph 2)	10000	II
GSK525762	BET family inhibitor	Investigational (Ph 1)	10000	II
Taselisib	PI3K α, δ, (γ) selective inhibitor	Investigational (Ph 3)	1000	II
Glasdegib	Smo inhibitor	Investigational (Ph 2)	1000	II
A-1210477	MCL-1 inhibitor	probe	50000	II
VS-4718	FAK inhibitor	Investigational (Ph 1)	10000	II
Ulixertinib	ERK inhibitor	Investigational (Ph 2)	10000	II
LY3009120	pan-RAF inhibitor	Investigational (Ph 1)	10000	II
Prexasertib	Chk1 inhibitor	Investigational (Ph 2)	10000	II
Epacadostat	IDO inhibitor	Investigational (Ph 3)	10000	II
Napabucasin	CSC inhibitor, STAT3 mediated	Investigational (Ph 3)	20000	II
Enasidenib	IDH2-R140Q inhibitor	Approved (US)	10000	II
CCT196969	pan-RAF/Src inhibitor	Probe	25000	II
Gilteritinib	FLT3/AXL inhibitor	Investigational (Ph 3)	1000	II
A-1331852	Bcl-XL inhibitor	Probe	1000	II
Ivosidenib	IDH1 R132H/R132C inhibitor	Investigational (Ph 2)	10000	II
TIC10	ERK & AKT inhibitor, TRAIL inducer	Investigational (Ph 2)	25000	II
ABC294640	Sphingosine kinase 2 inhibitor	Investigational (Ph 2)	50000	II
RO5126766	dual RAF/MEK inhibitor	Investigational (Ph 1)	1000	II
AZD6738	ATR inhibitor	Investigational (Ph 1)	25000	II
Entrectinib	TRK, ROS1, ALK inhibitor	Investigational (Ph 2)	1000	II
Motolimod	TLR8 agonist	Investigational (Ph 2)	10000	II

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