DEVELOPMENT OF TARGETED DRUG COMBINATIONS BLOCKING MULTI-DRIVER ONCOGENESIS

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MASTER OF SCIENCE IN BIOLOGICAL AND ENVIRONMENTAL SCIENCES

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ABSTRACT

Protein kinase inhibitors have been an effective treatment for cancers driven by an identifiable predominant protein kinase that drives cancer development. Most cancers, however, are supported by multiple independent drivers and cannot be effectively treated by targeted therapies that inhibits only a single driver. Instead, a combination targeted therapy with multiple targeted drugs to block all drivers is required. Developing combination targeted therapies for such cancers requires identification of the individual drivers and pharmacological understanding of the complex interactions between the drugs and the cancer targets. The current pharmacological models, based on the Hill equation, only describe the interaction between a drug and a single target in a biological system. Thereby, any observed effect is ascribed to the interaction with one target only. In practice, such drugs often inhibit multiple kinase targets, both on and off-target, and the resulting inhibition will be a compound of the effectiveness against all affected targets. Yet when such drugs are used for cancer therapy, only the target-specific inhibition is likely responsible for efficacy, while the off-target inhibition is likely the cause of toxicity. This perspective article discusses a recently developed biphasic pharmacological model for characterizing such complex interactions, assessing the contribution of individual drug targets, and predicting synergistic drug combinations for multi-driver cancers. This approach can produce mechanism-based and synergistic drug combinations against multi-driver cancers.
ACKNOWLEDGMENTS

To Dr. Sun and our lab group, Geng Chia, Dr. Ayrapetov, Dr. Wei and Abygail Chapdelanie

It has been an absolute pleasure working both in person and online with you all! From starting in the lab group with only Dr. Sun and Geng Chia to growing and with Dr. Wei, Dr. Ayrapetov, and Abby, I have learned so much and developed more skills than I ever thought possible. I look forward to reading of your experiments and research as you prepare your publications and future manuscripts, and will forever be available to assist where apt. I wish you all consistent cell culture, a clean incubator, and well replicated results!

To my Thesis Committee – Dr. Gregory, Dr. Hemme, and Dr. King

You have all be fantastic help over the past months, providing guidance, advice, direction, and expertise. It is my pleasure to present my research and this manuscript to you. Thank you for your kind words, consideration, and flexibility working through the rather unconventional process and development of my Master’s Thesis.

I look forward to reading your publications in the future, and hope for the good health of you and your families

To the CMB Department –

My time at URI has taught me of all things within and many beyond of the study of cellular life. Teaching and working under our faculty was an impactful experience, and the guidance proved keystone to my success. It is a great privilege to be able to graduate from under your care.
PREFACE

The following document is in Manuscript format, divided into three chapters.

Chapter 1 contains materials prepared for publication with a complete introduction to the materials, overview of the subject, and summary of conclusions from research.

Chapter 2 contains a complete presentation of the conclusions developed during research.

Chapter 3 contains the statistical analyses of the research.
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CHAPTER 1: DEVELOPMENT OF TARGETED DRUG COMBINATIONS

BLOCKING MULTI-DRIVER ONCOGENESIS

For publication in MDPI Cancers

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1. Targeted cancer therapy faces two main challenges

Targeted cancer therapies are most effective against cancers addicted to a predominant oncogenic driver(1,2). Successful examples include targeting BCR-Abl in chronic myeloid leukemia (CML)(3), ErbB2 or estrogen receptor in some breast cancers(4), mutated EGFR in non-small cell lung cancer (NSCLC)(5), and activated c-Kit in gastrointestinal stromal tumors (GIST)(6,7). Because cancer drivers are often protein kinases or activate protein kinases, and because protein kinases are relatively easy to develop inhibitors for, most targeted therapies are small molecule inhibitors or monoclonal antibodies that block the functions of protein kinases(8-11). They block specific molecular processes crucial in the proliferation of cancer cells and tend to cause much less toxicity than traditional cytotoxic chemotherapies.

Despite its dramatic success, targeted cancer therapy faces two critical challenges: acquired resistance and intrinsic resistance. Acquired resistance refers to patients who initially respond to a targeted therapy but invariably acquire resistance to it and relapse(12,13). Acquired resistance is due to mutations or amplifications that make the original targeted driver unresponsive to the drug(14-16) or activate additional pro-survival/proliferative pathways which are not affected by the current targeted therapy(17,18). Acquired resistance can be overcome by improved drugs targeting the mutated driver(19-23) or drug combinations that target the original and newly activated pathways(18,24).

Intrinsic resistance refers to the fact that most cancer patients are "intrinsically (or naturally) resistant" to targeted cancer therapies(12,25-27). Drug transport and efflux,
tumor microenvironment, physical barriers, tumor heterogeneity, and undruggable drivers (such as P53, KRAS)(13,27) are a few mechanisms potentially responsible for intrinsic resistance. In addition, the presence of multiple oncogenic drivers will support the development and proliferation of cancer and render it unresponsive to any targeted therapies which inhibition against any single driver. For these reasons, most cancers still have no approved targeted therapies. Only 8.33% of all US cancer patients are genomically eligible for targeted therapy, and only 4.9% benefited from such treatments in 2018(28). Developing a targeted therapy strategy against multi-driver cancers is the focus of this article.
2. Multi-driver oncogenesis

Despite a few well-established cancers driven by a predominant oncogene, most cancers are not driven by a predominant driver but rather by multiple drivers. Multi-driver cancer oncogenesis was proposed in the 1950's (29,30) and is well supported by modern genetic and molecular studies (31-36). Multi-driver proliferation is perhaps best established in colorectal cancers (CRC) (33,35,36). The initiation of CRC is often triggered by an oncogenic mutation in the adenomatous polyposis coli (APC) gene that transforms the harboring cell into a small adenoma. Some cells may acquire additional growth-stimulating mutations in KRAS, BRAF, PIK3CA, and others to gain additional proliferative advantages that lead to the full development of a metastatic tumor (32,33). It is estimated that CRC may contain three to more than 10 oncogenic drivers (33,36). A study (36) of 7664 tumors of 29 types revealed that a tumor carries ~four driver mutations on average, but the number varies widely (from 1 to >10) among cancer types. Another study found that 28% of cell response curves to drug inhibition are multiphasic (85). Thus, multi-driver oncogenesis is a broad and general mechanism underlying most cancers.

Two distinct scenarios can describe multi-driver oncogenesis. In the first scenario, a tumor contains multiple cancer cell populations, with each supported by a different predominant driver. This phenomenon is commonly referred to as tumor heterogeneity. In the second scenario, a cancer cell contains multiple oncogenic drivers, which collectively contribute to its growth and proliferation. Of course, a cancer may also contain multiple cell populations, with each cell also containing multiple drivers. With both scenarios and
their variations, the multi-driver cancers will be partially responsive to treatment and either relapse after treatment ends or continue growing throughout treatment with at a slower rate. This article focuses on the identification of effective drug combinations for cancer cell lines that contain multiple drivers.

When multiple drivers independently contribute to the proliferation and survival of a cancer, even when the function of any single drive is completely blocked, the other driver(s) would still be functionally intact, rendering mono-agent targeted therapy ineffective. For example, some ErbB2-positive breast cancers are intrinsically resistant to ErbB2-targeted therapy due to bypass signaling through other receptor or intracellular signaling pathways(37). Some epidermal growth factor receptor (EGFR)-mutated NSCLC patients are intrinsically resistant to EGFR-based therapies due to KRAS and BRAF mutations(38), or other receptor protein tyrosine kinases (rPTK), phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) signaling being activated(39). These examples exemplify a broad problem of how multi-driver oncogenesis complicates targeted therapy.

Multi-driver oncogenesis is also a plausible explanation for the failures of many initially highly promising targeted therapies. Src family kinases, IGF-1R and Akt are well-established cancer drivers in many cancer types(40-45), but mono-agent therapies targeting Src kinase(46-48), IGF-1R(49-52), and AKT(53-57) have failed in various cancers due to the lack of clinical efficacy. Although these kinases are known to be associated with a large number of cancers, they are rarely, if ever, demonstrated to be the sole drivers in any cancer or even in in vitro cancer models. Similarly, BRAF V600E is a
well-established cancer driver and an effective target in melanoma(58). However, BRAF inhibition is not effective against CRC and kidney cancers harboring the same mutation, suggesting that BRAF V600E is not a sole driver in these cancers(59-62).

Fully blocking multi-driver cancers would require a combination of targeted drugs, each blocking an individual driver. Growing evidence supports this conclusion. Combining BRAF inhibitors with drugs against EGFR, ErbB2, MEK, and/or PI3K-mTOR significantly improves the response rates in CRC(61,63-66) over BRAF inhibitor alone. Recently encorafenib (a BRAF inhibitor) in combination with cetuximab (EGFR antibody) was approved for metastatic CRC with BRAF (V600E) by the FDA(67). The effective drug combinations and the genetic evidence(31,33,36) strongly support that most cancers are likely multi-driver cancers.
3. Multi-driver oncogenesis complicates driver identification and combination drug discovery

Driver identification and formulation of drug combinations for multi-driver cancers are still primary challenges in cancer research. The wealth of genomic and transcriptomic information often suggests a long list of potential drivers in each cancer, and the functional significance of such putative drivers needs to be quantitatively evaluated by systems biology and pharmacology approaches. With the difficulty of identifying multiple drivers and quantifying their contribution to the cancer phenotype, preclinical efforts to identify effective drug combinations mostly rely on empirical combination screening (68-71). Due to the large number of targeted drugs and the exponentially larger number of potential drug combinations, most combination screens test pair-wise combinations of FDA-approved or experimental drugs at a limited number of concentrations. Many drug combinations have also been evaluated in clinical trials based on biological or pharmacological rationales supported by clinical and preclinical evidence (72), however a systematic preclinical platform for identifying mechanism-based drug combinations is still elusive.

A 2017 analysis (73) indicated that most of the benefits of approved combination therapies are derived from different patient subgroups benefiting from different components of the combination, rather than individual patients benefiting from synergy or additivity between the components of a combination. This finding indicated that developing truly synergistic combinations that can effectively treat a patient when the
individual components are far less effective is still a significant challenge in cancer drug
discovery.
4. Current pharmacological models for analyzing cancer cell inhibition by targeted drugs

One prerequisite for formulating rational drug combinations is a mathematical model with a mechanistic interpretation to provide understanding of how multi-driver cancer cells lines respond to individual drugs(74). Having such mathematical model allows for data to be both extrapolated and interpolated for the study of data and predictions of untested scenarios; and the model used have a direct link to the mechanisms tied to observed phenomenon. Currently, pharmacological analysis of how cancer cell lines respond to drugs is based on the Hill equation \( I = \frac{I_{\text{max}} \cdot D^n}{(IC_{50}^*)^n + D^n} \), which expresses the inhibition \( I \) as a function of the drug concentration \( D \) using three inhibitory parameters: the maximal inhibition at saturating concentration \( I_{\text{max}} \), the Hill slope or co-efficient \( n \), and the concentration of a drug that achieves 50% of \( I_{\text{max}} \) \( (IC_{50}^* ) \)(75-79). A simplified version assumes the \( I_{\text{max}} \) to be 100%. Thus, the equation becomes \( I = \frac{D^n}{(IC_{50}^*)^n + D^n} \), where the \( IC_{50} \) is the drug concentration that inhibits total cell viability by 50%(80). \( I_{\text{max}} \), \( IC_{50} \), \( IC_{50}^* \) and \( n \) can be derived from fitting dose-response data into these equations. The \( IC_{50} \) provided by the Hill function has been widely used to represent the potency with which a drug inhibits cancer cells, such as in the Genomics of Drug Sensitivity in Cancer (GDSC) database(80-82). However, an IC50 determined by a fitting of dose-response data to the Hill function only works well for drug/cell interactions that meet the assumptions of the Hill equation(76). As discussed below, the interaction between protein kinase inhibitors with multi-driver cancer cell lines do not follow the Hill equation nor align with the mechanistic interpretation of the Hill Equation.
The Hill equation was initially developed to describe the binding of \( \text{O}_2 \) and hemoglobin(77), and the Hill slope, \( n \), was introduced to account for the positive cooperative binding (\( n>1 \)). It has since become the primary pharmacological model widely used to describe the interaction between a drug and a biological system(76,78,79,83,84). The implicit assumption is that a ligand binds to a single target. If a drug affects a system by binding to more than one target with different affinities, the dose-response curve will be the sum of multiple responses. Such a multi-target composite response would not follow the Hill equation. Force fitting such multi-phasic cell response data into the Hill equation would lead to a mischaracterization and misunderstanding of the drug-cell interaction(78,79).

Strong evidence suggests that a considerable percentage of dose-response curves of cancer cells to targeted drugs do not conform to the Hill equation and the inhibition cannot be accurately represented by the Hill parameters(76). Many targeted drugs, such as those targeting the Akt/PI3K/mTOR pathway, have unusually shallow dose-response curves, and fitting such data into the Hill equation results in \( n \) values below 1, suggesting "negative cooperativity" in the inhibition. "Negative cooperativity" can be defined as the decreasing effectiveness of a drug as concentrations increase, a scenario which fits the shallow dose-response curves observed. However, there is still no plausible mechanistic explanation for negative cooperativity in modern pharmacology based on a single target interaction. On the other hand, two cases have been reported that a shallow response due to biphasic response, where a drug interacts with two types of targets, one with high affinity and another with low affinity. Such a biphasic response cannot be described by
the Hill equation because it is based on a single target interaction. Furthermore, a systematic analysis of published cancer cell dose-response curves revealed that a significant portion of such drug response curves are polyphasic, further suggesting a multi-target interaction for many cancer drugs. Further analysis revealed that the shallow and polyphasic response patterns are related to the multi-driver support in many cancer cells.
5. The response of only mono-driver cancer cells to targeted drugs follows the Hill equation.

To determine how multi-driver cancer cells respond to targeted drugs, we examined the responses of various cancer cell lines to a panel of protein kinase inhibitors(75,86-88) and identified inhibitors. The cell response to each inhibitor was assayed across 16 drug concentration, ranging from 0.61 nM to 20 μM in 2-fold intervals to provide a quantitative evaluation of inhibition.

Most cell lines responded to some kinase inhibitors, and the responses can be divided into two categories (Fig. 1). Category 1 is comprised of cells that are potently and fully inhibited by some inhibitors. HCC-827 is an NSCLC cell line driven by EGFR(89,90) activated by four mutations, gene amplification and overexpression(89,91). It is a mono-driver NSCLC cell line model. HCC-827 is highly sensitive to gefitinib (IC\textsubscript{50}=12.1±0.4 nM), erlotinib (IC\textsubscript{50}=15.4±0.5 nM) (Fig 1, red), and other EGFR inhibitors(75). Both gefitinib and erlotinib nearly fully blocked cell viability below 100 nM. This response pattern is consistent with HCC-827 being a mono-driver cancer cell driven by EGFR.

![Fig. 1. Responses of cancer cells to targeted drugs. Red: HCC-827, Black: CTV-1. Green: CRC or TNBC cells.](image-url)
CTV-1 is an M5-type acute myeloid leukemia (AML) cell line(92,93), with an undefined proliferative mechanism. Probing the cell line with the panel of protein kinase inhibitors revealed that Lck inhibitors potently and fully inhibit the cell line. Lck is a PTK in the Src family(87). Lck in CTV-1 cells is activated by over-expression and four activating mutations(87). Blocking Lck activity with dasatinib or bosutinib (Fig. 1, black) and other Lck inhibitors entirely blocks CTV-1 viability. The dose-response curves for both HCC-827 and CTV-1 fit the Hill equation well(88). The $I_{\text{max}}$'s were close to 100% against EGFR and Lck inhibitors, respectively, and the IC$_{50}$'s were at low nM levels, and the n values were between 1.7 and 2.7 (Table 1). The n values above 1 suggest positive cooperativity in inhibitor binding(88). Complete inhibition at low inhibitor concentrations indicated that blocking the intended targets, EGFR or Lck, is sufficient to entirely block the viability of these cells, a hallmark of mono-driver cancer cells.
6. Shallow inhibition curves as a result of multi-driver oncogenesis and lack of specificity of protein kinase inhibitors

However, most of the examined cells did not fit the profile of the mono-driver cancer cells, and fall in category 2. Fig. 1 (green) displays the responses of three CRC cell lines (HT-29, SK-CO-1, and NCI-H747) and two TNBC cell lines (MDA-MB-231 and MDA-MB-468) to their respective effective drugs. Two common features of category 2 cells are: 1) multiple inhibitors inhibited each cell line against different kinase targets, and 2) each dose-response curve is "shallow." Nearly all examined cases showed apparent inhibition at a low nM range, but failed to achieve complete inhibition even at 20 μM(Fig 1, Green). Fitted to the Hill equation, these responses resulted in varying $I_{max}$ and $IC_{50}$ values, but the $n$ values were consistently and significantly $<1$ (Table 1).

The $n$ values below 1 are a pharmacological enigma. They seem to suggest "negative cooperativity," but no mechanism for negative cooperativity has been demonstrated in any biological system.(76,78). Benzodiazepine clonazepam stimulating GABAA receptor in human neurons(94) and aniline binding to cytochrome CYP2E1(95) are the only two known examples where "negative cooperativity" was understood, and it was due to the ligands binding to the receptors on two sites with distinct affinities in both cases.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Drug</th>
<th>Hill analysis</th>
<th>Biphasic analysis</th>
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<tr>
<td></td>
<td>$I_{max}$ (%)</td>
<td>$IC_{50}$ (nM)</td>
<td>$n$</td>
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<tr>
<td>HT-29</td>
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<td>73 248 0.52</td>
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<td></td>
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<td>100 585 0.34</td>
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<tr>
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<td>Dasatinib</td>
<td>0.032</td>
<td>67 147 0.62</td>
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<td>CT-1</td>
<td>WH-4-023</td>
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<td>98 712 2.49</td>
</tr>
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</table>

Table 1. Results of Hill and biphasic analyses.
Considering the potential of multi-driver oncogenesis and the well-documented lack of specificity of protein kinase inhibitors, we thought such a biphasic inhibition a distinct possibility. The protein kinome is a large enzyme family that shares structural similarities. Thus, protein kinase inhibitors often lack specificity. A protein kinase inhibitor tends to bind to its intended target(s) with the best affinity but may also bind to many other protein kinases with gradually decreasing affinities (10, 96). For mono-driver cancers, a potent inhibitor would saturate and block the driver kinase to cause complete inhibition of cell viability, rendering the off-target interaction non-consequential. When multiple drivers sustain a cancer cell, even complete inhibition of any individual driver would only cause a partial inhibition of the cell viability. If the inhibitor also inhibits other kinases or causes additional toxicity with lower affinity, it could cause additional inhibition at higher concentrations. Thus, inhibition of a multi-driver cancer cell by any kinase inhibitor would likely result in a biphasic inhibition: a target-specific inhibition and an off-target inhibition. Such a biphasic inhibition may manifest itself as a shallow inhibition if the two phases are not well separated.

Examinations of the shallow dose responses indicated that some of them are indeed biphasic (Fig. 2, for...
example). To better quantify the biphasic inhibition, we developed a biphasic mathematical model \((I=F_1x[D]/([D]+K_{d1})+F_2x[D]/([D]+K_{d2}))\) that assumes that the inhibition \((I)\) by a drug has two phases: \(F_1\) and \(F_2\), as fractions of total cell viability, and each phase has an individual binding affinity \((K_{d1}\) and \(K_{d2}\)). We also assume that \(F_1\) and \(F_2\) add up to 1 (or 100%). When the 16-point dose-response data are fitted to the biphasic equation, it yields three inhibitory parameters, \(F_1/F_2\) ratio, \(K_{d1}\) and \(K_{d2}\). The biphasic parameters for some cells are shown in Table 1.

Each shallow inhibition curve is decomposed into two inhibitory phases: a high-affinity phase \((F_1\) and \(K_{d1}\)) and a low-affinity phase \((F_2\) and \(K_{d2}\)). For example, HT-29 is sensitive to BRAF inhibitor HG6-64-1, Mek inhibitor AZD-6244, IGF-1R inhibitor BMS-754807, and Src/Abl/PDGFR inhibitor dasatinib. These results and further Western blot analyses indicated that HT-29 uses BRAF-activated MAPK pathway, IGF-1R activated PI3K pathway and Src kinase as independent drivers(75). Similarly, we identified the KRAS-activated MAPK pathway and IGF-1R activated PI3K pathway as primary drivers in SK-CO-1 and NCI-H747. The same approach identified dasatinib (inhibiting Src) and AZD-6244 (inhibiting Mek) as biphasic inhibitors for TNBC cell line MDA-MB-231, suggesting that Mek and Src are two independent drivers for this cell line. The sensitivity to AZD-6244 is due to KRAS activation by a G13D mutation, thus activating the MAPK pathway(91,97). The same approach also identified EGFR inhibitor lapatinib and Akt inhibitor GSK690693 as two biphasic inhibitors for TNBC cell line MDA-MB-468 (Table 1). EGFR overexpression(91,98) and PTEN loss(99-103) are responsible for the sensitivity to lapatinib and GSK690693 for this cell line.
The results in Table 1 illustrate a critical comparison between the Hill and biphasic analyses and the comparison between mono-driver and multi-driver cancer cells. For the mono-driver cancer cells, CTV-1 and HCC-827, both approaches result in similar evaluations: \( I_{\text{max}} \approx F_1 \) and \( IC_{50} \approx K_{d1} \). For the multi-driver cancer cells, the difference is dependent on the off-target inhibition (indicated by \( K_{d2} \)). The more prominent the off-target inhibition is, the more discrepant the two analyses. For HG6-64-1 on HT-29, there is no off-target inhibition (\( K_{d2} > 100 \mu M \)), and the two analyses results in similar conclusions: \( I_{\text{max}} \) (51%) \( \approx \) \( F_1 \) (50%), and \( IC_{50} \) (16 nM) \( \approx \) \( K_{d1} \) (14 nM). In contrast, the off-target inhibition of lapatinib in MDA-MB-468 (\( K_{d2} = 3.1 \mu M \)) results in dramatically different evaluations by the two approaches. The Hill analysis suggests that lapatinib inhibits the cells fully (\( I_{\text{max}} = 100\% \)) with an \( IC_{50} \) of 190 nM, but the biphasic analysis indicates that lapatinib only inhibits the cell viability by 53% \( F_1 \) with \( K_{d1} \) of 17 nM by binding to its intended target EGFR. The remaining inhibition \( F_2 = 47\% \) is due to an off-target binding (\( K_{d2} = 3.1 \mu M \)). The Hill analysis suggests that lapatinib is a strong drug for MDA-MB-468, but the biphasic analysis concludes that lapatinib alone is a poor drug for MDA-MB-468 because it only inhibits 53% of cell viability by a target-specific mechanism. The off-target inhibition would likely contribute to toxicity rather than therapeutic efficacy.
7. Biphasic analysis helps predict effective drug combinations for multi-driver cancer cells

Identifying multiple kinase targets and their inhibitors for each multi-driver cell model provides a rational strategy for formulating effective drug combinations against these cancer cells, namely the combination of drugs, each blocking an independent target. Fig. 3 and Table 2 display the data for multiple cell models. For HT-29, combinations of AZD-6244/BMS-754807, HG6-64-1/dasatinib, dasatinib/BMS-754807 are all much more potent than the individual drugs, achieving dose reduction of >10-fold in all cases for 50% inhibition. CRC cell lines, SK-CO-1 and

---

**Table 2.** Drug IC\(_{50}\)'s (μM) for CRC and TNBC cells. AZD=AZD-6244; BMS=BMS-754807; HG6=HG6-64-1; Dasa=dasatinib; Lapa=lapatinib; GSK=GSK690693; ABD=AZD-6244/BMS-754807/dasatinib.
NCI-H747, are mildly inhibited by both AZD-6244 and BMS-754807, but the AZD-6244/BMS-754807 combination is much more potent. IC\textsubscript{70} was reduced by 1-2 orders of magnitude in all cases(75).

This approach is similarly applicable to triple negative breast cancer, another cancer type that still lacks effective targeted therapy. MDA-MB-231 is partially inhibited by AZD-6244 and dasatinib with shallow and biphasic characteristics, but fully inhibited by the AZD-6244 and dasatinib combination. The combination index for this drug combination is 0.029 at 70% inhibition, meaning a dose reduction of 34-fold. While lapatinib and GSK690693 mildly and biphasically inhibited MDA-MB-468, the drug combination is much more potent (dose reduction of 23-fold at 70% inhibition). The combinations for the two TNBC cells are also extremely cell-specific: the IC\textsubscript{50} of lapatinib/GSK690693 is 22 nM for MDA-MB-468, and 10 μM for MDA-MB-231, a 454-fold specificity. Similarly, the IC\textsubscript{50} of dasatinib/AZD-6244 is 73 nM for MDA-MB-231 and 15 μM for MDA-MB-468, a 200-fold difference\textsuperscript{88}. The striking cell specificity for the TNBC cells reflects the molecular heterogeneity and demonstrates that the identified drug combinations are rooted in the unique oncogenic mechanisms of these cells.
8. Shallow/biphasic inhibition is widespread among cancer cells

<table>
<thead>
<tr>
<th>Categories</th>
<th>Drug:</th>
<th>Doxorubicin</th>
<th>MK-2206</th>
<th>Gefitinib</th>
<th>BMS-754807</th>
<th>Dasatinib</th>
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<td></td>
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<td>29</td>
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<td>841</td>
<td>882</td>
<td>427</td>
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</tr>
</tbody>
</table>

Table 3 Summary table of GDSC analysis.
Contains the total number of cells / present in each of the 4 major & 2 minor categories from analysis. Cells for each drug in the Monophasic (MP) category were also categorized as being potent or late inhibition based on a the IC\textsubscript{50} distributions of the category as a whole.

Despite the strong evidence supporting the multi-driver nature of numerous cell lines analyzed so far, it is not clear how widespread multi-driver oncogenesis is in cancer development. To address this issue, we examined how a broad spectrum of over 800 cancer cell lines responded to inhibitors to some of the most promising targets, such as Src, EGFR, insulin/IGF-1R, and Akt. We analyzed how a broad spectrum of cancer cells respond to four targeted drugs, MK-2206 against Akt protein kinase, dasatinib against Abl and Src kinases, BMS-754807 against IGF-1R and insulin receptor and gefitinib against EGFR. These four kinase families have been shown to be widely involved in cancer development, yet drugs targeting these kinases have failed in clinical trials. The multi-driver oncogenesis hypothesis suggests one possible reason for the failure could be that despite their broad involvement in cancer development, these kinases may not be used as predominant drivers in cancers. The hypothesis predicts that: 1) If a cancer cell line is not dependent on a given kinase at all, then the cell line would not be inhibited by an inhibitor against the kinase; 2) if a cancer cell line is fully dependent on a given kinase for viability and proliferation, the cancer cell line would be potently inhibited with a
dose-response curve fitting the Hill equation; 3) If a cancer cell line is dependent on multiple driver kinases, a drug against one of the driver kinases would either cause a partial inhibition or biphasic inhibition. We also analyzed the dose response of a broad spectrum of cancer cells to doxorubicin. Doxorubicin is a broadly used chemotherapeutic agent against cancer. It intercalates into DNA and disrupts topoisomerase-II-mediated DNA repair; and 2) generates of free radicals and causes damage to cellular membranes, DNA and proteins (1). Thus, doxorubicin is a nonspecific regarding cell selectivity and target selectivity. Doxorubicin is analyzed as a benchmark for a non-targeted drug. Part 2 of this thesis details the analysis of these cancer cells to these drugs. The data are summarized in

The analysis supports the following observations. 1) Most cancer cells are potently inhibited by doxorubicin in a monophasic manner. Even though some cells displayed a relatively weak affinity, due to some undefined mechanism of drug resistance, most cancer cells still display monophasic characteristics. The ratio between monophasic and biphasic responses is about 3:1. This clearly reflects the non-selective nature of doxorubicin. 2) Very few cancer cells are potently inhibited by MK-2206, BMS-754807 and gefitinib, suggesting that the target kinases, Akt, IR/IGF-1R, and EGFR are rarely used as mono-drivers in cancer cells. Far more cancer cells respond to these targeted drugs in a biphasic manner than in a monophasic manner. The ratio between potent monophasic inhibition and biphasic inhibition is 4:246 for MK-2206, 10:51 for gefitinib, 10:265 for BMS-754807. This observation suggests that these targets are far more frequently used a one driver in a multi-driver cancer cell. 3) Dasatinib inhibits both
Abl and Src kinases, it has been approved for treatment of leukemia for its inhibition of Abl (Ref). Its ratio of potent monophasic inhibition versus biphasic inhibition is 39:94. The most potently inhibited cancer cells are all leukemia cell lines and most of them express BCR-Abl, a fusion between BCR and Abl genes that causes Abl activation. These BCR-Abl expressing cells are often mono-driver cancer cells fully dependent on the Abl activity. This observation also indicates that potent mono-phasic inhibition by a drug indeed correlated to the target being a mono-driver in the cancer cells. Taken together, the cell response data supports the hypothesis that multi-driver oncogenesis is a broad mechanism, and the development of targeted cancer therapy should incorporate this perspective into consideration.
**Future direction**

Targeted cancer therapy is at a critical juncture. In the last four decades, dramatic advances have been made in mono-agent cancer therapies. High-affinity inhibitors against most oncogenic kinases have been developed, some highly effective targeted therapies have become the standard of care for some cancers displaying appropriate biomarkers. However, these advances and broad clinical trials also made it abundantly clear that only a small percentage of cancers are responsive to mono-agent targeted therapy. An overwhelming majority of cancers rely on multiple oncogenic drivers and can only be effectively treated by combination targeted therapy. Identifying the oncogenic drivers and formulating drug combinations to block these drivers is the main obstacle preventing the reach of targeted therapy to a broad spectrum of cancers. Overcoming this obstacle could potentially broaden the reach of targeted therapy to most, if not all, cancers. Failing to do that will likely keep targeted cancer therapy as a niche option for treating a few exceptional cancer types caused by predominant mono-drivers. Multi-driver oncogenesis calls for a new pharmacological paradigm that combines drugs that block different drivers individually, but in combination cause full inhibition to a cancer. The biphasic analysis is capable of identifying oncogenic drivers and suggesting effective and synergistic drug combinations. At this point, this pharmacological approach has been applied only to a small number of cancer cell models. We anticipate that the broad application of this approach may lead to the identification of combination targeted therapy against a broad spectrum of multi-driver cancers.
References


22 Kishikawa, T. *et al.* Osimertinib, a third-generation EGFR tyrosine kinase inhibitor: A retrospective multicenter study of its real-world efficacy and safety in


28


Paller, C. J. et al. Design of phase I combination trials: recommendations of the Clinical Trial Design Task Force of the NCI Investigational Drug Steering


77 Hill, A. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. **40**, 4-7 (1910).


CHAPTER 2: Data Science and Drug Response Analysis

Summary:

The following document contains an expanded investigation into the data of the 5 tested drugs, doxorubicin, MK-2206, dasatinib, BMS-754807, and gefitinib. The data for each of the drugs were processed utilizing the same method, see section 1, and the resulting data assessed for patterns and trends in how cancer cells respond to these drugs. Each drug is discussed in separate sections which include a table of descriptive statistics, summary of the dose-response curve categorization, an overview of the results, graphs of relevant categories, and choice-picked additional content to highlight unique or otherwise important observations. Overall, these analyses support the hypothesis that more cancer cells rely on multiple oncogenic drivers than on single predominant drivers.
1. Methods of data analyses and comparisons

Data was acquired from the GDSC1 database (1) and separated by drug being tested into csv documents containing the dose-response curves for all cells tested against. Analysis was done in four major steps: Error Catching, Modeling, Categorizing, and Hand Sorting.

1. Error catching was looking for patterns of discrete “scattering” or inconsistent changes in the viability despite constant change to concentration of drug. We set a general threshold of 20%, where for a dose-response curve with any single point that was 20% higher than the point previous, meaning point with concentration one factor higher, then the data was considered error. Secondly, if a dose-response curve contained any two successive points where the sum changes equals a 20% increase in viability despite an increase in drug concentration, the data was considered error. Additional error checking was done by comparing the intensity reported for the NC-0, the 0 μM drug & 100% viability point to the following dose-response curve; if the NC-0 presented as greater than 10-fold higher than the intensities of the following points, the data was considered error. If a dose-response curve was found to be error, its data received an appended value stating “error”.

2. Modeling of the dose-response curves (dose-response curves) was performed through R script and the R package EmiR, a customizable minimizer created by Davide Pagano and Lorenzo Sostero (1). When provided with an equation that contains a defined input (x), an output (y) to be minimized, and a series of constants to be adjusted, typically (p1, p2, p3…), the minimizer will attempt different combinations of numbers as the
constants to achieve the lowest possible output. The minimizer chooses numbers for the constants based on a provided range, generally (0:10), and a chosen algorithm to adjust the constants based on the previously tested combinations and their outputs. For our work, we found that the IHS algorithm, Improved Harmony Search (2) to be most effective and efficient when provided 8000 iterations with population sizes of 200 and a retest based on the best combination if the output was an error greater than 0.3.

Utilizing the EmiR package with customized input and looping, we fit each dose-response curve to both the simplified Hill function (3,4) and the biphasic function (5,6) based on the tested concentrations as input (x) and the following constraints: Imax = 1, nH (0.01,10), IC_{50} (0.01,100), F_{1} (0,1), K_{d1} (0.01,100) and K_{d2} (0.1, 10000). The resulted minimization output the final error, via standard Root Mean Square Error (RMSE), and the combinations of constants. The output was appended to the initial database, providing a now complete CSV with the original data, the Biphasic constants, the Hill constants, and the dose-response curves generated for both the Biphasic and Hill constants.

3. Categorizing was done through a series of sorting and filtering the data. With each drug as its own csv file converted into a spread sheet, we removed the “error” labeled data into its own sheet, then sorted the no-inhibition (NI) data into its own sheet. NI was defined as observing no difference greater than 20% between the first and last three data points averaged together. With both the NI and error data removed, the remaining “Good Data” was sorted by nH value and separated depending if the nH value was above or below 0.8 into either the “monophasic” (MP) or “biphasic” (BP) group.
4. Hand sorting was the final step in organizing the data and moving datasets between categories. One common issue was a lower-than-expected initial data point, resulting in the average of the first 3 points to be disproportionately lower; or the initial data point was over 20% lower than the following, resulting in an error designation. In both cases, the data was assessed by hand as to whether it presented as erroneous scattering or a single outlier point at the lowest tested concentration, depending on the interpretation, the dose-response curve would be moved to the appropriate category. The next most common issue was an indiscreet scattering of data creating an almost sigmoidal wave, this error was identified by comparing all of the dose-response curves of a given category and moving through them one at a time. If a dose-response curve presented with a notable scattering that was higher in variation than the average change observed, the data was moved to error. Lastly, dose-response curves were shifted between MP, BP, and NI groups depending on their overall trend as compared with the rest of a categories pattern. Data from either the MP or BP groups would be moved into the NI group if no change of 20% or higher was observed, or if the scattering was variable between a value of 1.2 and 0.8. While data was shifted between MP and BP based on its proximity to the nH threshold of 0.8, due to situations where a nH value would be fit as either greater than 0.8 and an unreasonably high, or an nH value bellow 0.8 but a corresponding equal to the Kd1 and lower to the category over all. The final result of data processing was individual spreadsheets for each drug that separated dose-response curves into error, NI, MP, or BP for further analysis. The following sections will highlight the analysis for relevant results of five drugs: doxorubicin, BMS-754807, MK-2206, dasatinib, and gefitinib.
2. Doxorubicin

Doxorubicin is a broadly effective cancer drug in clinical applications (7-9). Two mechanisms are proposed to account for its effects on cancer cells: 1) intercalation into DNA and disruption of topoisomerase-II-mediated DNA repair, and 2) generation of free radicals and their damage to cellular membranes, DNA and proteins (8). Because of its broad effects through these mechanisms are not exerted by affecting a specific target molecule, it is not a targeted drug, but an effective drug for blocking cancer cell growth (9). For this study, it is chosen as an example of a non-targeted cancer drug, and a benchmark for how cancer cells respond to a non-targeted drug. The GDSC1 tested 865 cancer cell lines against doxorubicin (10). The categorization of cancer cell responses to doxorubicin is shown in Table 2.1.

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<tr>
<td>MP IC$_{50}$ &lt; 0.1 μM</td>
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<td>MP IC$_{50}$ &gt; 0.1 μM</td>
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<tr>
<td>Biphasic (BP)</td>
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**Table 2.1: Summary table of doxorubicin’s analysis.**
Shows the number of cells classified into each category. Additional differentiation was done to separate out the potent inhibition cases, where the IC$_{50}$ was below above 0.1 μM, from late inhibitions where the IC$_{50}$ was above 0.1 μM.

**Figure 2.1: Doxorubicin monophasic IC$_{50}$ plot.**
Plot of calculated IC$_{50}$’s for monophasic dose-response curves, shows a continuous spectrum of IC$_{50}$’s ranging from potent at nanomolar concentrations to requiring micromolar or higher.
Among all cells tested, the dose response data for 75 cells were judged to be erroneous. Among the 790 sets of good data, only 52 cells did not respond to doxorubicin.

Figure 2.2: Characteristics of doxorubicin inhibition of cancer cell lines. Figure 2.2.A shows the distribution of $F_1$ values from the biphasic category for doxorubicin, ranging from 0.25 to 0.89. Figure 2.2.B shows the distribution of the $K_{d1}$ values from the biphasic category, ranging from 0.01 μM to 7.65 μM. Figure 2.2.C is a correlation plot of the monophasic IC$_{50}$ and biphasic $K_{d1}$ for potent inhibitions where the IC$_{50}$ was below 0.11 μM, which generated an R value of 0.84. Figure 2.2.D is a correlation plot of the monophasic IC$_{50}$ and biphasic $K_{d1}$ for late inhibitions where the IC$_{50}$ above 0.11 μM, which generated an R value of -0.29. Figure 2.2.E is a correlation plot of the monophasic IC$_{50}$ and biphasic $F_1$ for potent inhibitions where the IC$_{50}$ below 0.11 μM, which generated an R value of -0.24. Figure 2.2.F is a correlation plot of the monophasic IC$_{50}$ and biphasic $F_1$ for late inhibitions where the IC$_{50}$ above 0.11 μM, which generated an R value of -0.66.
(NI category), confirming the broad and non-specific nature of doxorubicin effects on cancer cells. A total of 545 cells displayed monophasic inhibition patterns, and 193 cells displayed biphasic (shallow) responses to it. This ratio of 3:1 in monophasic to biphasic responses toward doxorubicin indicated that doxorubicin killed most cancer cells in each cell line by one mechanism. Among the monophasic responses to doxorubicin, data showed a near continuous spectrum of effectiveness, ranging from low nM to low μM (Figure 1). Over half of the monophasic responses have an IC\textsubscript{50} below 100 nM. This analysis is consistent with what we expected for a broad-spectrum cell toxicity agent. The non-cell selectivity is also consistent with the general toxicity observed with doxorubicin treatment, such as cardiotoxicity (7).

It is not clear if the biphasic responses by the 193 cell lines to doxorubicin are a reflection of the intrinsic mechanism of action or due to experimental error. As noted, 75 cell lines displayed dose-response patterns that are clearly erroneous due to data scattering. It can be expected that some cells may in theory have a monophasic response to doxorubicin, but data error could cause the inhibition patterns to appear to be biphasic or shallow. Biphasic analysis indicated that the \( K_{d1} \) and \( K_{d2} \) for the biphasic responses are scattered, further supporting the error-based interpretation. Figures 2.2 A and B show the distributions of \( K_{d1} \) and \( F_1 \) values for the biphasic data. Plot 2.2.A shows an \( F_1 \) value which ranges from 0.25 to 0.89 and have a higher population density approaching an \( F_1 \) value of 0.9, indicating a monophasic interactions. Additional differentiation was done to separate out the potent inhibition cases, where the IC\textsubscript{50} was below above 0.3 μM, from late inhibitions where the IC\textsubscript{50} was above 0.3 μM. The \( K_{d1} \) values shown in plot 2.2.B
span a range of values from the low threshold, 0.01μM to 7.65μM but the population is focused around a value of 0.1, which would be highly potent. Based on plots A and B together, the biphasic data favors a potency similar to the IC\textsubscript{50}s observed in the monophasic category and a curve more steep than shallow. Figures 2.2 C through F contain the correlation data of the F\textsubscript{1} and K\textsubscript{d1} values to their IC\textsubscript{50} counterpart, separated by whether the IC\textsubscript{50} value was above or below our potency threshold of 0.10μM (adjusted to 0.11μM due to non-regular clustering around 0.10μM and 0.20μM). Plots C and D compare the correlation between K\textsubscript{d1} and IC\textsubscript{50} of potent verses late dose-response curves in the biphasic category and returned R values of 0.84 for potent inhibition and clear scattering with R = -0.29 for the late inhibition; the K\textsubscript{d1} values correlate closely with IC\textsubscript{50} when inhibition is observed but wildly different when the inhibition is late or weak. Plots E and F show a similar story with correlation of biphasic F\textsubscript{1} and monophasic IC\textsubscript{50}, however the F\textsubscript{1} and IC\textsubscript{50} values operate as inversely related, where the higher the F\textsubscript{1} value correlates with lower IC\textsubscript{50} and vice-versa. Plot F provides a clear relationship matching expectations, where the late inhibitions, indicated by high IC\textsubscript{50}s have a negative correlation with F\textsubscript{1} of R=-0.66. Plot E contains more nuance, the plot generated an R value of -.024, which can be observed as an almost flat line of F\textsubscript{1} values around 0.8, regardless of the increasing IC\textsubscript{50}. The simplest observation from this relationship is that F\textsubscript{1} of the potent inhibitions favor higher values caused by a steeper and more monophasic slope. Comparing all of the plots together displays a pattern of decreasing effectiveness by doxorubicin within these dose-response curves that remains monophasic but with more gradual slopes. This conclusion matches well with the possible overlap of
doxorubicin’s two mechanisms and processes. As noted earlier, doxorubicin uses two mechanisms to block cell growth. Due to its complexity and the lack of more expansive data, this interpretation needs further research to validate.
3. MK-2206

The phosphatidylinositol 3 kinase pathway is a key signaling pathway that supports many processes related to cell growth and proliferation (11). A key signaling molecule of the pathway is Akt, a Ser protein kinase (12,13). Thus, Akt has long been considered an important target for anti-cancer therapy.

MK-2206 is a drug developed to block Akt activity and was considered as a promising drug for targeted therapy (14-17). However, clinical trials with MK-2206 has produced disappointing results (17,18).

We assessed if Akt is the sole driver in any cancer cells by examining the responses of a broad spectrum of cancer cells to MK-2206 (Table 3.1). In the GDSC1 database, out of 817 cells, 303 were not inhibited by MK-2206 to a statistically significant degree and 173 were categorized as erroneous. The fact the high portion of the cancer cells were not affected by MK-2206 suggests that the drug is not broadly toxic toward cancer cells, a hallmark of

| Table 3.1: Summary table of MK-2206’s analysis. Shows the number of cells classified into each category. Additional differentiation was done to separate out the potent inhibition cases, where the IC$_{50}$ was below above 0.3 μM, from late inhibitions where the IC$_{50}$ was above 0.3 μM. |

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<thead>
<tr>
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Figure 3.1: MK-2206 monophasic IC$_{50}$ plot. Plot of calculated IC$_{50}$’s for monophasic MK-2206 dose-response curves, shows a continuous spectrum of IC$_{50}$’s with a gradual decline in change as values peak above 1 μM. This pattern shows a distribution that favors values between 1 and 10 μM.
targeted drugs. Presumably the cancer cells that are responding to MK-2206 rely on the PI 3-kinase/Akt pathway at least to some degree.

Among the cancer cells that responded to MK-2206, 95 displayed a monophasic response pattern, while 246 response patterns were biphasic. Among the 95 cells displaying a monophasic responses to MK-2206, only four were potently inhibited with IC$_{50}$’s below 0.3 μM and 23 had IC$_{50}$’s below 1 μM. This result indicated that very low percentage 4/633 (0.63%) of 4. BMS-754807 cells are inhibited by MK-2206 with a reasonable potency. Thus, very few cancer cells are fully dependent on Akt for survival and proliferation. This conclusion is consistent with the clinical failures of this drug in numerous clinical trials. The cells displaying a monophasic response to the drug with
high IC\textsubscript{50}’s (>1 μM) are likely not dependent on Akt for survival as high concentration are required to inhibit cell viability.

As we have previously demonstrated the biphasic response can be interpreted as a drug binding two targets to cause cellular inhibition, one with high affinity and another with low affinity. The proportion of the phase 1 (F\textsubscript{1}) and phase 2 (F\textsubscript{2}) and their respective K\textsubscript{d}s (K\textsubscript{d1} and K\textsubscript{d2}) can be determined by the biphasic analysis. The 246 cells that displayed biphasic responses to MK-2206 were subjected to the biphasic analysis. The analysis revealed that the dose responses have a highly variable F\textsubscript{1}, ranging from 0.2 to near 1, with K\textsubscript{d1}s in the low nM range. This result indicated that MK-2206 can inhibit a target in these cells in low nM range, and this target plays a variable role in the viability of these cells. The remaining inhibition (F\textsubscript{2}) at a much higher K\textsubscript{d} (K\textsubscript{d2}) is caused by MK-2206 binding to a target of much lower affinity. As it is plausible to assign the high affinity target to be Akt, we can assign the low affinity target to be an off target other than Akt. This result suggests that in these cancer cells with biphasic responses, Akt plays a partial role in driving cell viability, and other pathways that are supporting the remaining portion of cell viability are co-drivers in these cancer cells.
4. BMS-754807

IGF-1R and insulin receptor have long been well established to be important contributors to cancer cell growth (19-23). It was anticipated that inhibitors for these receptor kinases would find a broad application in cancer therapy (19,20,24,25). BMS-754807 potently inhibits both receptors (26,27), displayed promising in vitro activity against numerous cancer cells and in animal models (28-31). However, it also failed in clinical trial for the lack of efficacy as a mono-agent therapy.

GDSC1 analyzed 882 cancer cell lines against BMS-754804, and the categories of their responses are summarized in Table 4.1. A large number of cell line responses (a total of 217) were considered to be erroneous due to obvious data scattering. Among the remaining 665 data sets, 179 displayed no inhibition by BMS-754807, 221 displayed monophasic responses, and 265 displayed biphasic responses. Analysis of the monophasic responses indicates

<table>
<thead>
<tr>
<th>BMS-754807</th>
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<tbody>
<tr>
<td>Monophasic (MP)</td>
</tr>
<tr>
<td>MP IC50 &lt; 0.3 μM</td>
</tr>
<tr>
<td>MP IC50 &gt; 0.3 μM</td>
</tr>
<tr>
<td>Biphasic (BP)</td>
</tr>
<tr>
<td>No Inhibition</td>
</tr>
<tr>
<td>Low Value</td>
</tr>
<tr>
<td>Error</td>
</tr>
<tr>
<td>Total Cells Analyzed:</td>
</tr>
</tbody>
</table>

Table 4.1: Summary table of BMS-754807’s analysis. Shows the number of cells classified into each category. Additional differentiation was done to separate out the potent inhibition cases, where the IC50 was below above 0.3 μM, from late inhibitions where the IC50 was above 0.3 μM.
that very few of them are potently inhibited. Fig 4.1 displays the IC$_{50}$’s of the monophasic responses. Only 10 dose-response curves had IC$_{50}$’s below 0.3 μM, and 56 had below 1 μM. Because BMS-754807 inhibits both IGF-1R and insulin receptor, and potent responses were achieved at 30 nM, it can be interpreted that only the 10 potently inhibited cells are likely dependent on IR or IGF-1R for cell viability. While the low affinity responses with IC$_{50}$’s approaching 1 μM would be due to BMS-754807 non-specifically blocking some other targets (off-target inhibition). This result is consistent with the failure of BMS-754807 as a mono-agent drug.

Figure 4.2: BMS-754807 biphasic dose-response curves. Figure 4.2.A shows the dose-response curves for BMS-754807 data demonstrating potent biphasic inhibition, defined by the an IC$_{50}$ value below 0.3 μM and an nH value below 0.8. Figure 4.2.B shows the dose-response curves for BMS-754807 data demonstrating late biphasic inhibition, defined by the an IC$_{50}$ value above 0.3 μM and an nH value below 0.8. The biphasic response patterns (a total of 265 cells) were analyzed by the biphasic analysis. The results showed that a significantly higher number
of cells (a total of 79) were inhibited by concentrations below 0.3 μM of BMS-754807. And 140 cells achieved an IC$_{50}$ of under 1 μM. These results indicated that a far larger number of cells are partially inhibited by BMS-754807, suggesting that IR or IGF-1R is partially responsible for the viability of these cells. A collection of the biphasic inhibition curves are presented in Figure 4.2. This analysis suggests that a large number of cancer cells (about 30% of those tested) are partially dependent on either insulin receptor or IGF-1R for viability. Furthermore, the biphasic analysis and differentiation of the $K_{d1}$ and $K_{d2}$ phases can be attributed to on-target effects by BMS-754807 causing a spectrum of inhibition from potent to late which are then ill-represented by the monophasic IC$_{50}$ due to the inhibition by off-target effects. Previous studies in colorectal cancer cells (6,32) and ongoing experimental analysis of cancer cell responses supports this interpretation.
5. Dasatinib

Dasatinib is a potent inhibitor of Abl and Src family kinases, with additional interactions with several other PTKs families albeit with less affinity (33-35). It is approved for therapy for chronic myeloid leukemia harboring BCR-Abl (36). Analysis was performed on data scale, spectrum of IC$_{50}$’s from the minimum to maximum thresholds from 427 cells and a summary of the categories is presented in Table 5.1. Dasatinib contained 52 cell data categorized as error, which left the remaining 375 cell response data as reliable. Of the reliable data, 225 cells were not inhibited by Dasatinib, and only 43 appeared as monophasic, with a total of 26 considered potent by an under 0.1 μM. A total of 11 cells had an of below 1 μM but above 0.1 μM. Based on these results, the most likely conclusion is that 26 cells were potently inhibited by on-target action of

<table>
<thead>
<tr>
<th>Dasatinib</th>
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<tbody>
<tr>
<td>Monophasic (MP)</td>
<td>43</td>
</tr>
<tr>
<td>MP IC$_{50}$ &lt; 0.1 μM</td>
<td>26</td>
</tr>
<tr>
<td>MP IC$_{50}$ &gt; 0.1 μM</td>
<td>17</td>
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<tr>
<td>Biphasic (BP)</td>
<td>94</td>
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<tr>
<td>No Inhibition</td>
<td>225</td>
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<tr>
<td>Low Value</td>
<td>13</td>
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<tr>
<td>Error</td>
<td>52</td>
</tr>
<tr>
<td>Total Cells Analyzed:</td>
<td>427</td>
</tr>
</tbody>
</table>

Table 5.1: Summary table of dasatinib’s analysis. Shows the number of cells classified into each category. Additional differentiation was done to separate out the potent inhibition cases, where the IC$_{50}$ was below above 0.1 μM, from late inhibitions where the IC$_{50}$ was above 0.1 μM.

Figure 5.1: Dasatinib monophasic IC$_{50}$ plot.
Plot of calculated IC$_{50}$’s for monophasic dasatinib dose-response curves showing a continuous, almost linear curve, logarithmic if converted to a standard.
dasatinib against the primary driver. While the remaining cells likely exhibited partial on-target inhibition or off-target inhibition, causing a late monophasic response. Plots of the monophasic potent responses compared to the late responses are present in Figure 5.2.

The biphasic category contained 94 cells, with 14 cells having an under 0.1 μM and a total of 49 cells under 1 μM. The results of this category follow suit with the monophasic, where there is a clear split between potent inhibition by dasatinib and late inhibition. The potent inhibition can be attributed to the on-target interactions causing inhibition of a primary driver, whereas the late inhibition are most likely off-target interactions. The difference in conclusions between the two categories comes in the shallow slopes observed in the biphasic category. With nH values ranging from 0.089 to 0.796, the

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**Figure 5.2: Dasatinib monophasic dose-response curves**

Figure 5.2.A shows the dose-response curves for dasatinib data demonstrating potent monophasic inhibition, defined by the an IC\(_{50}\) value below 0.1 μM and an nH value above 0.8.

Figure 5.2.B shows the dose-response curves for dasatinib data demonstrating late monophasic inhibition, defined by the an IC\(_{50}\) value above 0.1 μM and an nH value above 0.8.
biphasic category shows a spectrum of curves that are progressively less effective & require significantly higher concentrations to achieve inhibition. The consensus from this observed pattern is that dasatinib is capable of both on and off target effects even if the effects are not targeting a mono-driver cell. Combining the conclusions from both monophasic and biphasic sections, dasatinib is a potent targeted inhibitor and capable of preforming within cells utilizing multiple drivers at a similar scale to with single driver cells. Biphasic plots are presented in Figure 5.3.
Dasatinib presents on additional and unique trend as compared with all other observed drugs, the low value category. Low value is defined as curves where the initial point & lowest concentration is below 50% viability, and there is no observed decrease in viability after the first or second concentration. The category stands out with slope equal to no inhibition relationships, but still present a significant degree of inhibition. While there is no direct cause for this phenomenon, it can be attributed to the potential for dasatinib being a highly potent inhibitor even in cases where the

**Figure 5.3: Dasatinib biphasic dose-response curves.**

Figure 5.3.A shows the dose-response curves for dasatinib data demonstrating potent biphasic inhibition, defined by the an IC$_{50}$ value below 0.1 μM and an nH value 0.8. Figure 5.3.B shows the dose-response curves for dasatinib data demonstrating late biphasic inhibition, defined by the an IC$_{50}$ value above 0.1 μM and an nH value below 0.8.
interactions do not target all proliferation drivers, as previously discussed. In cases of low value curves, one hypothesis is that inhibition of dasatinib’s target causing a 50% or higher decrease in viability by removing its proliferation or survivability benefit, but does not affect the additional drivers at all. The result would be competitive inhibition of the target with high potency, but unable to completely inhibit viability. While unique and interesting, low value curves do not offer major insights into dasatinib’s functionality nor its effectiveness as a targeted therapy because the curves do not exhibit inhibition changes based on dosage, do not achieve complete inhibition of the cells, and account for only 13 datasets out of 427.

**Figure 5.4: Dasatinib plot of low value dose-response curve group.**
Plot of all data determined to fall into the low value category, with two accepted as lack of change after the second point. The low value category is defined as data that exhibits minimal change in viability between the lowest concentrations and the highest concentrations, but starting at a value below 0.4 μM.
6. Gefitinib

<table>
<thead>
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<th>Gefitinib</th>
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<tbody>
<tr>
<td>Monophasic (MP)</td>
<td>57</td>
</tr>
<tr>
<td>MP IC50 &lt; 0.3 μM</td>
<td>10</td>
</tr>
<tr>
<td>MP IC50 &gt; 0.3 μM</td>
<td>47</td>
</tr>
<tr>
<td>Biphasic (BP)</td>
<td>51</td>
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<tr>
<td>No Inhibition</td>
<td>413</td>
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<tr>
<td>Low Value</td>
<td>0</td>
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<tr>
<td>Error</td>
<td>320</td>
</tr>
<tr>
<td>Total Cells Analyzed</td>
<td>841</td>
</tr>
</tbody>
</table>

Table 6.1: Summary table of gefitinib's analysis. Shows the number of cells classified into each category. Additional differentiation was done to separate out the potent inhibition cases, where the IC$_{50}$ was below 0.3 μM, from late inhibitions where the IC$_{50}$ was above 0.3 μM.

EGFR mutation and activation leads to the development of certain problematic cancers, most notably in non-small cell lung cancer (37-39).

Because of the risk they pose, establishing anti-EGFR treatments has been a large undertaking, but resulted with only partial success. Anti-EGFR therapy has been effective for only a narrow range of cancers, such as non-small cell lung cancer (37,38,40), leaving many without viable targeted treatment options. We examined the EGFR inhibitor, gefitinib, to quantitatively assess the effectiveness of EGFR inhibition as a targeted therapy in the broad spectrum of cancer cell lines.
The GDSC1 contained 841 cells tested against gefitinib, 413 did not respond to gefitinib and another 320 cells presented with error by cell scattering. Inhibition was observed within 108 cells, split between 57 monophasic and 51 biphasic curves. The monophasic curves were fit to the Hill equation and the IC50’s were found to range between 0.035 μM and 2.18 μM. 10 cells responded with IC50’s below 0.3 μM, and a total of 42 cells responded below 1 μM. For the biphasic data, IC50’s were significantly more scattered, ranging from 0.01 μM to 6.97 μM. Despite the larger range, the biphasic curves contained 14 with IC50’s below 0.3 μM and 32 below 1 μM. Gefitinib presents with very direct conclusions as the ratio of potent biphasic to potent monophasic is approximately 1:1 and 49% of the cells were not inhibited; gefitinib is a capable inhibitor of
Figure 6.3: Gefitinib biphasic dose-response curves. Figure 6.3.A shows the dose-response curves for gefitinib data demonstrating potent biphasic inhibition, defined by an IC\(_{50}\) value below 0.1 μM and an nH value 0.8. Figure 6.3.B shows the dose-response curves for gefitinib data demonstrating late biphasic inhibition, defined by the an IC\(_{50}\) value above 0.1 μM and an nH value below 0.8.

For further consideration and validation of its conclusions, we examined the cell line HCC-827, a non-small cell lung cancer cell line containing several EGFR mutations and considered a model for the EGFR mutation-driven non-small cell lung cancer (6,41). HCC-827 displayed a potent inhibition with an IC\(_{50}\) value of 0.036 μM, indicating that gefitinib successfully inhibited EGFR. However, HCC-827 does not present as a perfect representation of Hill equation’s monophasic pattern and instead generated a negative cooperativity with an nH of 0.695. Examining HCC-827’s graph, Figure 6.3, shows a clear decrease in effectiveness as concentrations reach above the K\(_{d1}\) value and a shallow slope which does not reach complete inhibition at peak tested concentration, ending instead at a viability of...
22.6%. While the effect of gefitinib inhibition of EGFR is enough to severely impair the cell viability, it is not enough to fully inhibit the viability. Instead, considering HCC-827 as a biphasic interaction with a $K_d$ of 0.02 μM and an $F_1$ value of 0.818, we can interpret HCC-827 as a case-study for highly potent biphasic interactions. HCC-827 exhibits two definite regions, one on-target and high-affinity region and one off-target or low-affinity region. Hypotheses can be drawn from pattern to question how EGFR is being supplemented in the cells via innate immunity against gefitinib, or if the cells are manifesting an acquired immunity through mitigation of inhibition as EGFR is reduced. This topic would make for an interesting and possibly rewarding investigation. For the purpose of this paper, HCC-827’s impact is more important as supportive; comparing HCC-827 to others potently to gefitinib suggests that the pattern is consistent, varying only in whether or not complete inhibition is achieved and indeed, only a small portion of cancers utilize EGFR as an oncogenic driver, with an even smaller portion relying on it as the sole-driver.
7. Concluding remarks on cell inhibition analysis

The analysis of the responses of a broad spectrum of cancer cells to four once-promising targeted cancer drugs was conducted, and their response patterns were compared to their responses to doxorubicin. The analysis supports the following observations. 1) Far more cancer cells are potently inhibited by doxorubicin than the number of cells inhibited by the targeted drugs. This clearly is a reflection of the non-selective versus targeted nature of the two classes of drugs. 2) While majority of the cancer cells are potently and monophasically inhibited by doxorubicin, relatively small number of cancer cells are potently and monophasically inhibited by the targeted drugs. This is consistent with the fact that doxorubicin non-discriminately block DNS replication while the targeted drugs only block certain signaling steps in a given cancer cell. When that blocked signaling step is not important to a cancer cell, the cell would not be affected by that drug. 3) Far more cancer cells respond to the targeted drugs in a biphasic manner than in a monophasic manner. As we discussed previously the biphasic response is indicative of the targeted process only playing a partial role in a given cancer cell. This, in turn, suggests that there are additional signaling processes supporting cell viability other than the targeted process by a given drug. The presence of multiple mechanisms supporting cell viability is the essence of the multi-driver oncogenesis. Taken together, the cell response data supports the hypothesis that multi-driver oncogenesis is a broad mechanism, and the development of targeted cancer therapy should incorporate this perspective into consideration.
References:

1. Pagano D, Sostero L. EmiR: Evolutionary minimization for R. SoftwareX 2022;18


20. Ekyalongo RC, Yee D. Revisiting the IGF-1R as a breast cancer target. NPJ Precis Oncol 2017;1


32. Shen J, Li L, Yang T, Cheng N, Sun G. Drug Sensitivity Screening and Targeted Pathway Analysis Reveal a Multi-Driver Proliferative Mechanism and Suggest a Strategy of Combination Targeted Therapy for Colorectal Cancer Cells. Molecules 2019;24


The following pages contains the aggregation of correlation graphs for the data of drugs doxorubicin, dasatinib, MK-2206, BMS-754807, and gefitinib. Each collection consists of 9 plots and a brief summary of materials presented per plot.
Supplementary Figures 1.A-F: Correlation of Coefficients from BMS-754807 Data.

Figures A, D and G show the correlation between the $K_{d1}$ and $IC_{50}$ of the monophasic category in Log scale depending on documented potency, plot A shows all points in the data, plot B shows only the potently inhibited data, and plot G shows all of the late inhibition data. Figures A, D, and G report R values of 0.88, 0.91 and 0.83 respectively. The three figures indicate a strong and positive correlation between $K_{d1}$ and $IC_{50}$ which is stronger in cases of more potent inhibition.
Figures B, E, and H show the correlation of $K_{d1}$ and $IC_{50}$ of biphasic category data, separated by relative potency. Figures B, E, and H report $R$ values of 0.07, 0.60 and 0.01. While there is a positive correlation between the $K_{d1}$ and $IC_{50}$, it is only observed in data where inhibition was potent.

Figures C, F, and I show the correlation of $F_1$ and $IC_{50}$ of the biphasic category data, separated by relative potency. Due to the nature of $F_1$’s function, it is expected to negatively correlate with $IC_{50}$. Plots C, F, and I report $R$ values of -0.56, -0.44, and -0.53; all of the biphasic data shows a moderate degree of correlation between $IC_{50}$ and $F_1$, regardless of potency.
Supplementary Figures 2.A-F: Correlation of Coefficients from MK-2206 Data.

Figures A, D and G show the correlation between the $K_{d1}$ and $IC_{50}$ of the monophasic category in Log scale depending on documented potency. Figures A, D, and G report R values of 0.41, 0.99 and 0.39 respectively. Results indicate a strong and positive correlation only in the potently inhibited data, with lesser correlation in all other data.

Figures B, E, and H show the correlation of $K_{d1}$ and $IC_{50}$ of biphasic category data, separated by relative potency. Figures B, E, and H report R values of 0.13, 0.52 and.
0.12. There is a positive correlation between the $K_{d1}$ and $IC_{50}$ only with potently inhibited data, and almost no correlation otherwise. The lack of correlation in late inhibitions indicates a disjuncture in the modeling abilities of the Hill equation and biphasic equation, where it is more likely that partial inhibition that cannot be distinguished by the Hill equation is being generated.

Figures C, F, and I show the correlation of $F_1$ and $IC_{50}$ of the biphasic category data, separated by relative potency. Plots C, F, and I report R values of -0.71, -0.29, and -0.71; the data size for potent inhibition here is minimal at best, so its low correlation is not reliable. For the overall data, there is a consistent negative correlation that degrades as concentrations rise.
Supplementary Figures 3.A-F: Correlation of Coefficients from Gefitinib Data.

Figures A, D and G show the correlation between the \( K_{d1} \) and \( IC_{50} \) of the monophasic category in Log scale depending on documented potency. Figures A, D, and G report R values of 0.52, 0.81 and 0.40 respectively. Results indicate a strong and positive correlation only in the potently inhibited data, with partial correlation otherwise. Figures B, E, and H show the correlation of \( K_{d1} \) and \( IC_{50} \) of biphasic category data, separated by relative potency. Figures B, E, and H report R values of -0.21, 0.75 and -0.22. There is a positive correlation between the \( K_{d1} \) and \( IC_{50} \) only with potently inhibited
data, and an almost negative correlation otherwise due to the wide range of IC$_{50}$ values. The highly variable correlation observed is emblematic of gefitinib operating as a potent inhibitor when cells are driven by EGFR mutations alone, while being lacking in all other conditions. Figures C, F, and I show the correlation of F$_1$ and IC$_{50}$ of the biphasic category data, separated by relative potency. Plots C, F, and I report R values of -0.74, -0.75, and -0.68. All three plots provide moderate correlation that tracks as expected, where the larger the IC$_{50}$, indicating lower potency, causes smaller F$_1$ values, indicating a weaker on-target / high-affinity region.
**Supplementary Figures 4.A-F: Correlation of Coefficients from Dasatinib Data.**

Figures A, D and G show the correlation between the $K_{d1}$ and $IC_{50}$ of the monophasic category in Log scale depending on documented potency. Figures A, D, and G report R values of 0.76, 0.90 and 0.72 respectively. Results indicate a strong and positive correlation across data, with a slightly strong correlation in the potently inhibited data.

Figures B, E, and H show the correlation of $K_{d1}$ and $IC_{50}$ of biphasic category data, separated by relative potency. Figures B, E, and H report R values of 0.12, 0.62 and
0.08. There is a positive correlation between the $K_{d1}$ and IC$_{50}$ only with potently inhibited data, and no correlation otherwise, supporting the conclusion that dasatinib’s off-target effects are being summarized inside of the IC$_{50}$, decreasing its correlation with the $K_{d1}$.

Figures C, F, and I show the correlation of $F_1$ and IC$_{50}$ of the biphasic category data, separated by relative potency. Plots C, F, and I report R values of -0.54, -0.34, and -0.52. All three plots provide moderate correlation that tracks as expected, however the weaker correlation in potent data and its population dispersal closer to a value of 1, indicates that these data are more similar to a monophasic relationship than the rest of the biphasic category.
Supplementary Figures 5.A-F: Correlation of Coefficients from Doxorubicin Data.

Figures A, D and G show the correlation between the $K_{d1}$ and $IC_{50}$ of the monophasic category in Log scale depending on documented potency. Figures A, D, and G report R values of 0.86, 0.96 and 0.83 respectively. Results indicate a strong and positive correlation across data, with a slightly strong correlation in the potently inhibited data. The slight difference observed is mostly likely caused by the scattered outliers with lower ratios of $K_{d1}$ to $IC_{50}$.
Figures B, E, and H show the correlation of $K_{d1}$ and $IC_{50}$ of biphasic category data, separated by relative potency reporting R values of 0.47, 0.84 and -0.29. There is a strong positive correlation with potently inhibited data, and a weak negative correlation otherwise. The stark difference between the potent and late inhibitions can be observed through widespread scattering of $K_{d1}$ values which are notably smaller than their related $IC_{50}$s. With doxorubicin being a non-targeted chemotherapeutic drug, the wide scattering presented in the late inhibitions, and the presence of biphasic inhibitions in general, is likely due to a mixture of error in testing with cells presenting as resistant to doxorubicin.

Figures C, F, and I show the correlation of $F_1$ and $IC_{50}$ of the biphasic category data, separated by relative potency. Plots C, F, and I report R values of -0.62, -0.24, and -0.66. All three plots provide moderate correlation that tracks as expected with a weaker correlation in potent data that is matched by population dispersal closer to a value of 1, indicating a higher similarity in slope to monophasic.