*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 oftoxicity. 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.

*1.**Targeted cancer therapy faces two main challenges*

* Targeted cancer therapies are most effective against cancers addicted to a predominant oncogenic driver(1,2).
  + 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)
  + activated c-Kit in gastrointestinal stromal tumors (GIST)(6,7).
* 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) a
    - 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. F
    - 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).

*Multi-driver oncogenesis*

* Multi-driver cancer oncogenesis
  + proposed in the 1950's(29,30)
  + Supported by modern genetic and molecular studies(31-36).
  + Colorectal cancers (CRC)(33,35,36).
    - adenomatous polyposis coli (APC) gene that transforms the harboring cell into a small adenoma
    - 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.
    - tumor heterogeneity - a tumor contains multiple cancer cell populations, with each supported by a different predominant driver..
    - multiple oncogenic drivers, which collectively contribute to its growth and proliferation.
      * 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.
      * ErbB2-positive breast cancers are intrinsically resistant to ErbB2-targeted therapy due to bypass signaling through other receptor or intracellular signaling pathways(37).
      * 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).
  + Multi-driver oncogenesis is also a plausible explanation for the failures of many initially highly promising targeted therapie
    - Src family kinases, IGF-1R and Akt are well-established cancer drivers in many cancer types(40-45),
    - Src kinase(46-48)
    - IGF-1R(49-52)
    - AKT(53-57)
    - BRAF V600E (58) not effective against CRC and kidney cancers harboring the same mutation (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)
  + encorafenib (a BRAF inhibitor) in combination with cetuximab (EGFR antibody) was approved for metastatic CRC with BRAF (V600E) by the FDA(67) (31,33,36)

*3. Multi-driver oncogenesis complicates driver identification and combination drug discovery*

* preclinical efforts to identify effective drug combinations mostly rely on empirical combination screening(68-71).
* 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) - patients benefiting from synergy or additivity between the components of a combination.

*4. Current pharmacological models for analyzing cancer cell inhibition by targeted drugs*

* mathematical model with a mechanistic interpretation to provide understanding of how multi-driver cancer cells respond to individual drugs(74).
  + Hill equation (I = Imax \* Dn/((IC50\*)n+Dn)) that achieves 50% of Imax (IC50\*)(75-79).
  + A simplified version I = Dn/((IC50)n+Dn, where the IC50 (80).
  + IC50 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).
  + 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).
* The Hill equation
  + initially developed to describe the binding of O2 and hemoglobin(77), and the Hill slope, n, was introduced to account for the positive cooperative binding (n>1).
  + the primary pharmacological model widely used to describe the interaction between a drug and a biological system(76,78,79,83,84).
  + implicit assumption is that a ligand binds to a single target
    - dose-response curve will be the sum of multiple responses
    - 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).
  + Percentage of dose-response curves of cancer cells to targeted drugs do not conform to the Hill equation (76).
    - 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,
    - no plausible mechanistic explanation for negative cooperativity in modern pharmacology based on a single target interaction.
    - 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.
    - biphasic response cannot be described by the Hill equation because it is polyphasic

*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,
  + a panel of protein kinase inhibitors(75,86-88) and identified inhibitors.
  + assayed across 16 drug concentration, ranging from 0.61 nM to 20 uM in 2-fold intervals
  + HCC-827 is an NSCLC cell line driven by EGFR(89,90) activated by four mutations, gene amplification and overexpression(89,91).
    - sensitive to gefitinib (IC50=12.1+0.4 nM), erlotinib (IC50=15.4+0.5 nM) (Fig 1, red), and other EGFR inhibitors(75).
  + CTV-1 is an M5-type acute myeloid leukemia (AML) cell line(92,93), with an undefined proliferative mechanism.
    - 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. T
  + The dose-response curves for both HCC-827 and CTV-1 fit the Hill equation well(88).
    - The n values above 1 suggest positive cooperativity in inhibitor binding(88).

*6. Shallow inhibition curves as a result of multi-driver oncogenesis and lack of specificity of protein kinase inhibitors*

* Biphasic / shallow responses
  + CRC cell lines (HT-29, SK-CO-1, and NCI-H747)
  + two TNBC cell lines (MDA-MB-231 and MDA-MB-468)
  + Shared Features:
    - 1) multiple inhibitors inhibited each cell line against different kinase targets
    - 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 uM (Fig 1, Green).
    - n values were consistently and significantly <1 (Table 1).
      * + no mechanism for negative cooperativity has been demonstrated in any biological system(76,78).
        + Known Examples:

Benzodiazepine clonazepam stimulating GABAA receptor in human neurons(94)

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.

* 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).
  + Single potent inhibitor would saturate and block the driver kinase to cause complete inhibition of cell viability, rendering the off-target interaction non-consequential.
  + Complete inhibition of any individual driver would only cause a partial inhibition of the cell viability.
    - additional toxicity with lower affinity, it could cause additional inhibition at higher concentrations.
      * biphasic inhibition: a target-specific inhibition and an off-target inhibition.
      * shallow inhibition if the two phases are not well separated.
* biphasic mathematical model
  + (I=F1x[D]/([D]+Kd1)+F2x[D]/([D]+Kd2))
  + assumes that the inhibition (I) by a drug has two phases: F1 and F2, as fractions of total cell viability, and each phase has an individual binding affinity (Kd1 and Kd2). We also assume that F1 and F2 add up to 1 (or 100%).
  + yields three inhibitory parameters, F1/F2 ratio, Kd1 and Kd2
  + two inhibitory phases:
    - a high-affinity phase (F1 and Kd1)
    - a low-affinity phase (F2 and Kd2).
  + HT-29
    - sensitive to BRAF inhibitor HG6-64-1, Mek inhibitor AZD-6244, IGF-1R inhibitor BMS-754807, and Src/Abl/PDGFR inhibitor dasatinib.
    - HT-29 uses BRAF-activated MAPK pathway, IGF-1R activated PI3K pathway and Src kinase as independent drivers(75).
  + SK-CO-1 and NCI-H747.
    - the KRAS-activated MAPK pathway and IGF-1R activated PI3K
  + TNBC cell line MDA-MB-231
    - The same approach identified dasatinib (inhibiting Src) and AZD-6244 (inhibiting Mek)
      * 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, (91,97).
  + TNBC cell line MDA-MB-468
    - EGFR inhibitor lapatinib and Akt inhibitor GSK690693 as two biphasic inhibitors
    - EGFR overexpression(91,98) and PTEN loss(99-103) are responsible for the sensitivity to lapatinib and GSK690693
* mono-driver cancer cells
  + CTV-1 and HCC-827, both approaches result in similar evaluations: Imax≈F1 and IC50≈Kd1.
  + Not true for multi-drivers
* multi-driver cancer cells,
  + HG6-64-1 on HT-29, there is no off-target inhibition (Kd2>100 uM), and the two analyses results in similar conclusions: Imax (51%) ≈ F1 (50%), and IC50 (16 nM) ≈ Kd1 (14 nM).
  + lapatinib in MDA-MB-468 (Kd2=3.1 uM):
    - The Hill analysis suggests that lapatinib inhibits the cells fully (Imax=100%) with an IC50 of 190 nM,
    - biphasic analysis indicates that lapatinib only inhibits the cell viability by 53% (F1) with Kd1 of 17 nM by binding to its intended target EGFR
    - remaining inhibition (F2=47%) is due to an off-target binding (Kd2=3.1 uM).
    - 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*

* 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 NCI-H747,
  + mildly inhibited by both AZD-6244 and BMS-754807
  + AZD-6244/BMS-754807 combination is much more potent. IC70 was reduced by 1-2 orders of magnitude in all cases(75).
* MDA-MB-231
  + partially inhibited by AZD-6244 and dasatinib with shallow and biphasic characteristics
  + 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.
* MDA-MB-468,
  + lapatinib and GSK690693 mildly and biphasically inhibited
  + 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 IC50 of lapatinib/GSK690693 is 22 nM for MDA-MB-468, and 10 uM for MDA-MB-231,
  + a 454-fold specificity. Similarly, the IC50 of dasatinib/AZD-6244 is 73 nM for MDA-MB-231
  + 15 uM for MDA-MB-468, a 200-fold difference88.
  + 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*

* 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.

*9. 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.

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