HHS Public Access

Author manuscript

Expert Opin Ther Targets. Author manuscript; available in PMC 2020 November 14.

Published in final edited form as:

Expert Opin Ther Targets. 2019 November ; 23(11): 893–901. doi:10.1080/14728222.2019.1691526.

Not the comfy chair! Cancer drugs that act against multiple active sites

Laurence Booth[1], **Andrew Poklepovic**[2], **Paul Dent**[1]

[1]Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, VA 23298-0035, USA

[2]Department of Medicine, Virginia Commonwealth University, Richmond, VA 23298-0035, USA

Abstract

Introduction.—Signal transduction pathway discovery has led to the development of small molecule tyrosine kinase and serine/threonine kinase inhibitors which were based on catalytic site enzyme inhibition. Targeted kinases bind ATP and catalyze phosphate transfer, hence inhibitors that block ATP binding and its metabolism were predicted to have a known on-target specificity. However, they were also likely to have unknown or unrecognized targets because of similarities in ATP binding pockets. This on-target, off-target biology of kinase inhibitors means that many therapeutics act through unknown biological processes to mediate their anti-tumor effects. Some drugs or drug combinations stimulate autophagosome formation via their off-target effects which can reduce expression of histone deacetylase proteins and alter rates of transcription.

Areas Covered.—This perspective discusses drug therapies whose actions cannot be explained by their actions on the original targeted kinase; it concludes with a methodology to rapidly screen for changes in cell signaling via in-cell western immunoblotting.

Expert Opinion.—Most malignancies do not depend on survival signaling from one specific mutated proto-oncogene. This is particularly true for advanced, previously treated malignancies where multiple clonal variants of the primary tumor have evolved. Under these circumstances, the concept of a highly “personalized medicine” approach fails because it is unlikely that a specific therapy will kill all clonal variants of the tumor.

Keywords

autophagy; apoptosis; chaperone; drug; endoplasmic reticulum; ERK; histone deacetylase; kinase; MAP kinase; neratinib; off-target effect; pazopanib; pemetrexed; receptor tyrosine kinase; sorafenib; survival signaling

Corresponding Author: Paul Dent Ph.D., Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, VA 23298-0035. Phone: 804-628-0861; paul.dent@vcuhealth.org.

Declaration of interest

P Dent has received funding from Genzada Pharmaceuticals and Puma Pharmaceuticals. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

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

1. Introduction

1.1 ATP-binding site kinase inhibitors and multiple targets: Faulty theories; don't mention the off-target effects!

All small molecule inhibitors of kinase enzymes, simply by their docking with the ATP binding and catalytic site of the enzyme, are susceptible to both inhibition of the target kinase and of unknown kinases that have a similar active site conformation and amino acid docking moieties. This issue is important in several ways for drug development and for subsequent rational combination of any two drugs to achieve an enhanced anti-cancer effect. The first issue is in the early development stage of a compound, where a great deal of attention is focused on the compound – target interaction, with more modest assessments as to whether the compound interacts with other kinases via in vitro or more recently in silico assays. Once a compound has moved forward to the proto-drug stage, it is therefore most often assumed by industry-based biochemists that the agent inhibits its defined target and laboratory-based experiments are then focused at examining how the agent acts to inhibit its target, with a read-out of cell growth utilizing MTT-type assays [1–3]. Essentially, company-based drug development becomes a self-confirmatory process. Additional pre-clinical testing will determine the safety and anti-tumor efficacy of the agent in rodent models, followed by large animal models, and the eventual granting of an investigational new drug (IND) number by the FDA [4, 5]. The drug is then tested in cancer patients to determine its safety profile, and to observe if any anti-cancer efficacy / signal is present.

2. Rationally defining the correct drug concentration for in vitro studies; an African or a European swallow?

The key pieces of data for an *academic* developmental therapeutics biochemist to obtain prior to agnostically working with any drug after its initial phase I trial is the maximum safe plasma concentration of the agent (C max), the half-life and area under the curve (AUC) of the drug, and to a lesser extent its in vitro protein binding which is putatively indicative of how much 'free' drug is in the patient's plasma. Regardless of pre-clinical rodent data, the C max of any drug should be used to inform the scientist going forwards to rationally understand the biological actions of the drug. Thus hypothetically, if the C max of the drug is 10 μM and the half life is 8 hours, with 90% protein binding, in vitro cell-based assessments of the drug's biology in cells cultured in 10% serum cannot realistically use drug concentrations above 2 μM, if the researcher wishes to generate data that has translational relevance. A good example of this issue is with the renal and liver cancer FDA approved drug sorafenib tosylate. At the standard of care 400 mg twice daily (BID) ingestion, the C max has been observed between 5–15 μM [6–9]. However, based on its AUC and high levels of protein binding, yet obvious biological activity in patients, the levels of free active sorafenib in human plasma also cannot realistically be greater than ~ 2 μM. Despite this concept and the knowledge therein being freely available for many years, in 2019, multiple published manuscripts using 5 μM, 10 μM and greater sorafenib concentrations were still being reported [10–12]. This overarching concept then leads to a second idea which is that the label placed onto the bottle of pills by the drug company, claiming their drug inhibits a specific protein kinase, is very often only partially correct. i.e.

drug company scientists are fixated on developing their drug against the target they initially chose, and it is natural that a drug company scientist will not take a more agnostic approach towards development of their 'specific' marketable drug.

2.1 Approaching drug development in an agnostic fashion.

An agnostic approach towards developing specific drugs, and particularly drug combinations, is essential. Sorafenib was one of the first multi-kinase inhibitors approved by the FDA for the treatment of kidney and liver cancers [13–15]. It was followed by other multi-kinase inhibitors, e.g. imatinib, regorafenib, pazopanib, sunitinib, dasatinib and axitinib [16–21]. For all of these kinase inhibitors, in addition to their original target, e.g. sorafenib for RAF-1, other kinases are also inhibited which collectively all play key roles in the biological activity of each drug, e.g. sorafenib was also shown to inhibit Class III receptor tyrosine kinases [22, 23]. The agnostic in-depth approach to understanding sorafenib biology, however, demonstrated that the drug had an additional cohort of target enzymes; protein chaperones [24–27]. Sorafenib in its clinically relevant physiologic range is a low-affinity inhibitor of HSP90, HSP70 and GRP78. From the earliest reports, it was shown that sorafenib could rapidly induce an endoplasmic reticulum stress response, i.e. elevated phosphorylation of eIF2α S51, with PKR-like endoplasmic reticulum kinase (PERK) being responsible for S51 phosphorylation [28, 29]. The chaperone GRP78 acts as a rheostat for sensing ER stress; one the one hand GRP78 binds to PERK and keeps the kinase inactive; on the other hand, increased levels of denatured proteins cause GRP78 to dissociate from PERK, and for GRP78 to act as a chaperone which will attempt to renature the protein. Denuded PERK becomes activated, autophosphorylates, and phosphorylates and inactivates eIF2α S51. This reduces protein translation that in turn facilitates GRP78-mediated clearance of denatured proteins [30].

2.2 Well-described multi-kinase inhibitors: surprisingly found to regulate chaperone biology.

'There will be a rumor of things going astray!' The more studies you perform, apparently, the greater the number of drug-targets that are discovered. The most obvious question was, how and why does sorafenib so rapidly increase eIF2α S51 phosphorylation? As a kinase inhibitor of RAF-1 or of receptor tyrosine kinases, no obvious rapid signaling pathway could be hypothesized which would link those targets to activating PERK or increasing eIF2α phosphorylation. Purely by chance, from work in a different project using the celecoxib-derivative OSU-03012, we discovered that the key mechanism of action for this agent was dysregulation of GRP78, the chaperone which maintains PERK in its inactive state [27, 31]. By detecting GRP78 expression via in-cell immunoblotting we discovered that whilst OSU-03012 did not significantly reduce total GRP78 levels, at least not until many hours after exposure, the agent did rapidly cause epitopes at the NH2-terminus of GRP78 to become occluded [24–27, 31]. Nota bene; the GRP78 ATP binding site is located in the NH2-terminal portion of the protein. In vitro assays of GRP78 chaperone ATPase activity revealed that OSU-03012 was indeed an inhibitor of GRP78 with an IC50 of ~300 nM. OSU-03012 also had IC50 values against HSP90 and HSP70 were also in the ~300 nM range. Based on what appeared to be some structural similarities between OSU-03012, sorafenib and pazopanib, additional analyses were performed to define the dose-dependent

inhibition curve for the multi-kinase inhibitors against chaperone ATPase activities. For pazopanib, the IC50 for inhibition of purified GST-HSP90 was ~100 nM. For HSP70, the IC50 was ~500 nM. For sorafenib, the IC50 against HSP90 was ~400 nM and for HSP70 ~300 nM. All of these IC50 values for the chaperones are all significantly below the safe plasma C_{max} values for both drugs [32, 33]. Hence, the overall biological actions of both sorafenib and pazopanib represent a *swarming multitude* of altered protein – protein interactions and changed signaling by many signal transduction pathways. Kinases who are chaperoned via HSP90, and for whom neither sorafenib nor pazopanib are catalytic domain inhibitors can nevertheless be targets of these drugs via reduced chaperone functionality followed by proteolytic degradation of the kinase. Furthermore, as transformed cells express many more times the total level of proteins than non-transformed cells and are thus less reliant on chaperones for their survival, this mode of action provides for a therapeutic window for the treatment of cancer.

2.3 Neratinib and afatinib: if I said you had a beautiful structure; would you hold it against me?

Differences in chemical structure do not influence inhibition of the primary target but can profoundly influence drug actions on the other, 'unknown' targets. Neratinib and afatinib despite having quite different chemical structures both were developed as irreversible, i.e. suicide, inhibitors of ERBB1, ERBB2 and ERBB4 [34, 35]. They both block homo- and hetero-phosphorylation. Tyrosine phosphorylated ERBB family receptors act to causes downstream signaling through the ERK1/2, ERK5, JNK1/2, PI3K, STAT3 and NFκB pathways [36, 37]. Several publications using neratinib and afatinib have presented evidence that these kinase inhibitors not only prevent receptor tyrosine phosphorylation but can also act to promote receptor internalization and degradation [38–40]. Two groups demonstrated that whereas the ERBB1/2/4 inhibitor lapatinib can enhance ERBB2 expression, neratinib decreased ERBB2 levels [41–43]. In SKOV3 breast cancer cells low concentrations of neratinib could suppress endogenous and over-expressed wild type ERBB2 levels within four hours [44]. Others have suggested neratinib could decrease ERBB1 expression or down-regulate ERBB2 via ubiquitination and endocytic degradation [45–48]. Thus, over the past 10 years many groups have presented consistent evidence that suicide ERBB1/2/4 inhibitors can act as kinase inhibitors and also cause ERBB receptor degradation.

Our own initial experience with neratinib and afatinib arose in a project examining the interactions of ERBB1/2/4 inhibitors and the JAK1/2 inhibitor ruxolitinib [49]. Subsequently, by generating in vivo multiple afatinib resistant H1975 NSCLC tumors, our odyssey of studying the biology of neratinib began. The survival of afatinib resistant tumor clones was mediated by signaling from ERBB3/SCR, c-MET and c-KIT [50]. Surprisingly, afatinib resistant H1975 clones were still capable of being killed by neratinib [51]. During studies designed to explore this phenomenon, we observed in our loading controls that the residual ERBB1 levels in the resistant tumor cells rapidly declined. Furthermore, in a delayed fashion, the expression of our negative control receptors c-MET and c-KIT were also reduced. In contrast to neratinib, in our system afatinib could partially reduce ERBB receptor expression but was significantly less efficacious than neratinib at doing so. Collectively, we concluded that afatinib, but especially neratinib, had to be interacting with

other proteins, protein kinases (?), to promote the rapid internalization and degradation of receptors.

2.4 Further tales of the additional unexpected neratinib targets: 'Four shalt thou not count, neither count thou two, excepting that thou then proceed to three. Five is right out!'

In silico and in vitro studies can play a key role in discovering the potential multitude of targets for any small molecule kinase inhibitor. In 2011 Davis et al published a comprehensive study examining the kinase inhibitory properties of ~40 small molecule kinase inhibitors against the ~400 kinases within the human kinome [52]. These findings were subsequently confirmed by Klaeger et al. [1]. In the initial study, afatinib was shown to have a very restricted substrate specificity, essentially inhibiting only ERBB family receptor tyrosine kinases, whereas neratinib was shown to have many additional previously unknown targets. The majority of the newly discovered neratinib targets were serine / threonine kinases, rather than the targets of its initial development which were tyrosine kinases. Kinases inhibited by neratinib with IC50 values below 100 nM included; MST3, MST4, MAP4K5 and MAP4K3, GCN2, MAP4K1, MAP3K4, MST2 and YSK4. MST2/MST3 and MST4 all play key roles in regulating plasma membrane – Golgi trafficking as well as being part of the Hippo Pathway which in normal tissues regulates organ size and in oncology plays an influential role in promoting tumor growth, invasion and chemotherapy resistance [53, 54]. We demonstrated that neratinib could simultaneously reduce the protein levels of multiple growth factor receptors and plasma membrane-associated RAS proteins together with functionally inactivating the oncogenic Hippo Pathway; the downstream co-transcription factors Yes-associated protein (YAP) and TAZ left the nucleus and were degraded.

The kinase Sterile 20 (Ste20) is a component of the pheromone-response pathway in budding yeast. Several mammalian homologs to Ste20 have been identified including the PAKs, the GCN kinase family (MAP4Ks) and MST1/2/3/4 [55–57]. The Mammalian Sterile-Twenty-like kinases, MST1/2, are core signaling components of the Hippo Pathway [58, 59]. Simplistically, MST1/2 phosphorylate and activate the Large Tumor Suppressor, LATS1/2, as well as the Nuclear Dbf2-related, NDR1/2, kinases [60]. Essential in this process are the scaffold proteins of the *Mps one binder family*, MOB1, MOB4 and Salvador (SAV1) [61]. Other scaffold proteins such as RASSF1A and SAV1 can also activate MST1/2 by forming SARAH domain dependent complexes [62]. The amount of MST1:MOB1 complex versus the MST4:MOB4 complex can influence, for example, whether pathway signaling promotes growth or cell death [63]. Neratinib can act to inhibit MST1/2/3/4 but does not inhibit either LATS1/2 or NDR1/2; it in fact activates LATS1/2 [64]. Activated LATS1/2 or NDR1/2 phosphorylate and cause cytoplasmic sequestration of the transcriptional co-activator YAP and its paralog TAZ. The YAP/TAZ partners are TEAD transcription factors. Cytosolic YAP and TAZ can then be ubiquitinated and proteolytically degraded. YAP is an oncogene that enhances transcription of genes involved in cell proliferation by partnering with TEAD family of transcription factors; thus, inactivation of YAP promotes cell death [65]. More recently, and in addition to MST1/2, other members of the Ste20-like MAP4K kinase family have been shown to perform activating phosphorylation of LATS1/2. MST1/2 are not obligate for the regulation of YAP/TAZ.

Knock out of LATS1/2, but not of MST1/2, abolishes YAP/TAZ phosphorylation [66]. MAP4K1/2/3 and MAP4K4/6/7 are now known to be direct LATS1/2-activating kinases. Thus, combined deletion of the MAP4Ks and MST1/2, but neither alone, is required to completely suppress LATS1/2 and YAP/TAZ phosphorylation i.e. if MAP4Ks act as components of the expanded Hippo Pathway; an obvious question to ask is does neratinib cause a compensatory activation of any MAP4K that would promote YAP phosphorylation?

MAP4K family kinases function upstream of NDR1/2, and NDR1/2 as well as LATS1/2 kinases phosphorylate additional substrates besides YAP [67]. MST1/2/3 phosphorylate NDR1/2 on T444/T442, and the binding of the scaffold MOB1 to NDR1/2 is required to support the autophosphorylation of NDR1/2 on S281/S282. The activation of NDR1/2 can be mediated via different pathways: the inhibition of protein phosphatase 2A; the mutation of an autoinhibitory segment; and membrane targeting of NDR1/2 [67]. Hence, NDR1/2 can be regulated via different signaling mechanisms involving altered subcellular distribution and phosphorylation status. It has been argued that NDR1/2 kinases can also be acetylated, as proposed by the Cell Signaling database; neratinib can down-regulate HDAC expression via autophagy [68].

At present, definitive roles for the novel neratinib targets in the biology of the drug and anti-cancer efficacy are somewhat limited. Our own data demonstrated that the Hippo Pathway upstream kinases were inhibited by neratinib but that LATS1/2 were phosphorylated as were their downstream substrates YAP and TAZ [64]. MST3/4 control the apical brush border of epithelial cells, and the major dose limiting toxicity of neratinib in patients is diarrhea, arguing that neratinib is acting in an on-target MST3/4-dependent fashion to cause this event [69, 70]. MST3/4 also coordinate the phosphorylation of cytoskeletal proteins such as Ezrin/Radixin/Moesin (ERM) family to regulate plasma membrane ruffling [69, 70]. MAP4K5 is an apical kinase that interacts with GTP binding proteins downstream of G Protein Coupled Receptors, and links GPCR signaling into MAPK pathways [71–73]. MAP4K5 and MAP4K3 phosphorylate and activate the LATS1/2 kinases that in turn phosphorylate and inhibit YAP/TAZ, the main effectors of the Hippo Pathway [72]. MAP4K3 has also been shown to play an important role in amino acid signaling to mTOR/p70 S6K [73]. MST3/MST4 are expressed in both solid and liquid tumors. We demonstrated in both solid and liquid tumor cells that neratinib suppressed the autophosphorylation of MST3/4 and reduced phosphorylation of their direct substrate Ezrin [64]. Neratinib rapidly reduced the expression of K-RAS in both solid and liquid tumor cells, via LC3-associated phagocytosis (LAP) / autophagy [64].

2.5 Off-target effects of many anti-cancer drugs: pleiotropic cellular effects.

So far, we have discussed kinase inhibitors and their pleiotropic targets. However, some well-established chemotherapy agents also can have unknown off-target effects that play an essential role in their anti-tumor biology. For example, the non-small cell lung cancer therapeutic drug pemetrexed (Altima) was developed to inhibit thymidylate synthase (TS) in the pyrimidine synthetic pathway and has been a standard of care therapeutic in this disease, and subsequently mesothelioma, for nearly twenty years [74–76]. Approximately ten years ago, it was demonstrated that pemetrexed had at least one additional target, the enzyme

aminoimidazole carboxamide ribonucleotide formyl-transferase (AICART) which is a component in the de novo purine synthesis pathway [77, 78]. A required substrate of AICART is the compound 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP); hence, ZMP accumulates in cells treated with pemetrexed. ZMP is an allosteric activator of the AMP-dependent protein kinase (AMPK) [79]. The AMPK is a key sensor of AMP levels within a cell, particularly in response to ATP depletion. Reduced pyrimidine and purine biosynthesis also leads to a DNA damage stress response which activates the ataxia telangiectasia mutated (ATM) kinase [80]. One ATM substrate is the alpha subunit of the AMPK at position threonine 172, which causes AMPK catalytic activation [81]. Thus, via phosphorylation and allosteric mechanisms, pemetrexed can induce a large "ATP-deficiency" stress response via activation of the AMPK within a cancer cell. Downstream outputs of this stress response include inactivation of AKT, mTORC1 and mTORC2, in turn leading to complete activation of ULK1, increased phosphorylation of the autophagy gate-keeper protein ATG13 S318 which then acts to promote the formation of autophagosomes [82]. Thus, because of the focus on the primary mode of drug action the fully elucidated biologies of many long-term established cytotoxic anti-cancer agents have yet to be truly explored. More importantly, by understanding the intricate biologies of all anti-cancer drugs will permit biochemists to refine their approaches to rationally combine drugs to provide a greater tumor killing effect.

2.6 Developing a rapid through-put screening system: using multi-channel fluorescence HCS microscopes.

A major issue for all biochemists, pharmacologists and molecular biologists examining the interactions of drugs in a cancer therapeutics setting is being able to accrue large cohorts of data as rapidly as possible, thereby permitting more focused future experiments. Multi-channel fluorescence HCS microscopes are capable of combining high quality optics with a high-quality computer driven microscope stage, and with dedicated software, for example, to analyze the immunofluorescent staining intensity of individual cells, i.e. *true* in-cell western blotting. Although these machines can be used as a traditional microscope with glass slides, the majority of data sets obtained in our laboratory have used 96-well plates.

Three independent cultures derived from three thawed vials of cells of a tumor cell type are sub-cultured into individual 96-well plates. Twenty-four hours after plating, the cells are transfected with a control plasmid or a control siRNA, or with an empty vector plasmid or with plasmids to express various proteins. After another twenty four hours, the cells are ready for drug exposure(s). At various time-points after the initiation of drug exposure, cells are fixed in place using paraformaldehyde and using Triton X100 for permeabilization. Standard immunofluorescent blocking procedures are employed, followed by incubation of different wells with a variety of validated primary antibodies and subsequently fluorescent-tagged secondary antibodies are added to each well; in general, using more than two tagged antibodies in each well results in poorer data / image quality. Cells can be visualized at either 10X magnification for bulk assessments of immunofluorescent staining intensity or at 60X magnification for assessments of protein levels or protein-protein colocalization.

For studies at 10X magnification, the operator outlines for the computer controlling the microscope “what is a cell.” i.e. the operator manually inputs the criteria for each specific tumor cell line segregating away detection of what is obvious debris or a staining artefact. Routinely the machine assesses the fluorescent staining intensity of 100 cells. The microscope determines the background fluorescence in the well and in parallel randomly determines the mean fluorescent intensity of those 100 cells. Of note for scientific rigor is that the operator does not personally manipulate the microscope to examine specific cells; the entire fluorescent accrual method is independent of the operator. Once data from the first set of plated cells has been obtained, the second and third independent sets of plated cells can be processed through the machine. Thus, three independent sets of fluorescence data from the three individual cultures, with 300 cells / condition being assessed.

The total expression of a particular protein is assessed alongside additional staining to define the levels of different phosphorylation sites within the protein, e.g. total ULK1, Phospho-ULK1 S317, Phospho-ULK1 S757. Within these analyses it is also essential to include wells to define invariant protein loading controls, such as the total expression level of ERK2 or AKT. For phospho-proteins, data can be presented in two ways, either bar graphs where the total protein expression / loading is presented alongside changes in phospho-protein levels, or with just bars for the phospho-protein fluorescence data corrected for the amount of protein expression, i.e. the stoichiometry of protein phosphorylation. For proteins whose total expression changes after drug exposure, the use of invariant total ERK2 or total AKT expression is used instead as a loading control.

As with any methodology, there are potential pitfalls. Although our fixation procedure is relative mild using a weak paraformaldehyde solution, it is possible that some antigens could be masked or damaged by this approach. Even though we rigorously validate our antibodies using knock down and over-expression to assess on-target effects, there is still a possibility that off-target effects could be being measured. Our experience, however, has been the opposite to that of standard western blotting / SDS PAGE approaches. With the microscope, we have found that it consistently delivers better signal to noise data for antibodies that we have so far tested. For example, based on our own findings, generating ‘nice looking’ western blots for mTOR and for its two phosphorylation sites that we measure had proved very difficult by traditional methods – the data never looked ‘publication quality.’ Using siRNA approaches to confirm on-target antibody effects, we were subsequently able obtain a much stronger / signal to noise data using the microscope approach. Finally, cultures using microscope systems are only in two dimensions, and lack the additional rigor of studies in 3D. Multi-channel fluorescence HCS microscopes have also proved to be very useful at examining protein-protein interactions at 60X magnification. Three to four images of cells stained in the red and green fluorescence channels are taken for each treatment / transfection / condition. Images are approximately 4 MB sized files. Images are merged and the image intensity and contrast is then post-hoc altered in an identical fashion inclusive for each group of images / treatments / conditions, so that the image with the weakest intensity is still visible to the naked eye for publication purposes but also that the image with the highest intensity is still within the dynamic range, i.e. not over-saturated.

At present, many laboratories still utilize traditional western blotting with secondary antibodies conjugated to luciferase, with enhanced chemiluminescence and X-ray film as a read-out; this approach has a limited dynamic range and for the 21st Century lacks sufficient rigor. Other laboratories with access to fluorescent imagers use SDS PAGE with fluorescent tagged secondary antibodies, and these systems have a 5- to 6-log dynamic range. These systems can, at a gross level, also perform in-cell immunoblotting including co-staining in the red and green fluorescence channels. All of the above procedures require a considerable amount of operator input, including the isolation, lysis, clarification and loading of proteins onto an SDS PAGE gel, followed by transfer to immobilon. This creates inherent errors in defining small drug-induced alterations to expression and phosphorylation; these are all processing stages where the rigor of the experiment can be compromised. The use of multi-channel fluorescence HCS microscopes abolishes all of the intermediate steps as cells are fixed in situ. Furthermore, unlike traditional SDS PAGE, the proteins retain their native conformations which for a number of proteins, e.g. detecting changes in chaperone conformation cause by drug exposures, presented data that could not have been obtained using traditional SDS PAGE. Thus, the in-cell assessments of altered phosphorylation or expression using multi-channel fluorescence HCS microscopy provide data with more rigor and a much lower standard deviation difference, permitting changes of 20–30% intensity to be assessed for statistical significance.

An additional benefit of using a multi-channel fluorescence HCS microscope is that it promotes a greater level of rigorous non-manipulatable data. As mentioned earlier, the cells are fixed in place and stained, and then once the machine has been set to recognize the morphology of any specific tumor cell type, the role of the operator has ended. Data is obtained by the machine in a random fashion examining cell staining intensities wherever it detects cells; the operator cannot skew their data by plating more cells in one well compared to another, or by picking certain cells to scan, leaving other cells out. We believe that our approach using a multi-channel fluorescence HCS microscope together with its computer-controlled stage and imaging technology, is a valuable tool to assist research in developmental cancer therapeutics laboratories.

3. Conclusion: more targets per drug means more ways to kill a tumor cell.

As of 2020 the most exciting new cancer therapeutics are those using antibodies to enhance immuno-surveillance of tumors, causing T cell -mediated tumor cell destruction [83–87]. However, although some very impressive results have been observed in tumor types such as NSCLC and melanoma, many tumors are still considered “cold” and unresponsive. Thus, novel drugs and drug combinations require development to improve the bio-activity of checkpoint inhibitory antibodies. Understanding the “off-target” effects of kinase inhibitors, particularly as the relate to controlling the expression of histone deacetylase proteins, is an important component of this therapeutic approach.

One obvious way to enhance the efficacy of immunotherapy antibodies would be to rapidly decrease expression of cell surface proteins who act to prevent immune cell surveillance of

tumor cells, for example PD-L1, PD-L2, PD-1, or to increase the expression of proteins who would improve immune cell -mediated killing of tumor cells, e.g. Class I HLA proteins. One family of drugs, histone deacetylase inhibitors, are known to simultaneously reduce the levels of PD-L1 and increase expression of Class I MHCA [88]. Multiple clinical trials are underway exploring this possibility, for example and . What is less appreciated or understood is that the HDAC proteins themselves can be rapidly degraded via the actions of certain drugs or drug combinations [89–91]. Drugs or drug combinations which induce autophagosome formation can rapidly reduce the expression of HDACs1/2/3/6/8/10 and using molecular tools the altered expression of different HDAC proteins can be linked to the decline in PD-L1 expression and the enhanced levels of Class I MHCA. For example, a drug combination utilizing pemetrexed with the phosphodiesterase 5 inhibitor sildenafil (Viagra) results in autophagy-dependent tumor cell death and survival prolongation in mice whilst also causing the autophagic degradation of multiple HDAC proteins [83]. And it was the reduced expression of the HDAC proteins which was causal in the tumor cells expressing less PD-L1 and more Class I MHCA; in vivo, combined pemetrexed sildenafil exposure enhanced the efficacy of checkpoint inhibitory antibodies directed towards PD-1 or to CTLA4 against lung cancer tumors. The use of pazopanib and sorafenib in this fashion also can enhance the immunogenicity of tumor cells. Hence, just because a particular drug combination does not contain an HDAC inhibitor does not necessarily imply that HDAC expression and function has not been altered.

Thus, it should be clear to the reader that a simplistic acceptance of what a drug is claimed to inhibit cannot reflect the true likely pleiotropic actions of the agent. Not only can drugs have straightforward obvious additional targets, but also that drugs can have “unknown unknown” targets, e.g. sorafenib inhibited RAF-1, then Class III RTKs and finally and unexpectedly chaperone function. As noted above for HDACs, drugs or drug combinations that induce autophagy can reduce the levels not only of proteins such as HDACs, but also of proteins that play essential roles in maintaining protein stability, i.e. chaperones. Above all, when working with small molecule drugs, the essential modus operandi should always be to have an agnostic approach to one’s mechanistic analyses of any agent, regardless of what the drug manufacturer may state.

4. Expert Opinion

‘He’s not the Messiah’: Always be ready to discard data and change, moving your hypotheses where the data leads; do not cling to your favorite hypothesis and treat it like a God. Most malignancies are not addicted to survival signaling from one specific mutated proto-oncogene. Thus, to effectively treat these tumors requires the *rational* combination of multiple anti-tumor drugs. This is particularly true for advanced previously treated malignancies where multiple clonal variants of the primary tumor will have evolved [92]. Under these circumstances, the concept of a highly “personalized medicine” approach falls down as it is unlikely a specific therapy will be able to kill all clonal variants of the tumor in the same patient.

The problem for biochemists and pharmacologists designing novel therapies, based on combinations of various agents, is that what a drug company claims their compound inhibits

does not necessarily include all of the possible or probable actual targets of the agent. For example, the expected biology of sorafenib when first reported was that it would inhibit the kinase activities of RAF family serine / threonine kinases. For those of us who had been researching the biology of RAF-1 in the early 1990s, one of the few pieces of information we had was that the catalytic site of RAF-1, despite being a serine / threonine kinase, resembled more closely the catalytic sites of SRC family non-receptor tyrosine kinases [93]. Thus, it was not surprising when several groups reported sorafenib could also inhibit class III receptor tyrosine kinases.

When the senior author of this article was developing new therapeutic drug combinations for the treatment of liver, pancreatic and colorectal cancers in the 2000's, those two targets were the basis for the rational combination of the drug with histone deacetylase inhibitors, which attacked HSP90 function, and with pemetrexed, which increased autophagosome formation via a different pathway to sorafenib. This research led to several subsequent clinical trials , , and . was a phase I trial in liver cancer. was a phase I trial in all solid tumors and was so successful that a new trial phase II trial with drug company support, , was opened in TNBC. in pancreatic cancer is still on-going. At present, from 14 patients so far recruited to this trial, 10 were considered to have become eligible for Whipple surgery. Eight of the ten had R0 resections of their disease with one CR. Of the other two who at surgery exhibited metastatic disease, one is still alive three years after cessation of therapy. A stage IV pancreatic living for three years is quite rare.

What was not addressed in the pre-clinical studies was the conundrum previously discussed in this manuscript, namely, how was sorafenib activating PERK thereby enhancing eIF2α phosphorylation, suppressing expression of protective proteins such as MCL1 and BCL-XL, and increasing expression of the autophagy-regulatory proteins Beclin1 and ATG5? The answer came from work in another project that had demonstrated the celecoxib derivative OSU-03012 could modify the chaperone function of GRP78 / BiP / HSPA5. Subsequently, OSU-03012, sorafenib and another multi-kinase inhibitor pazopanib were shown in their safe physiologic range to inhibit the ATPase activities HSP90 family and HSP70 family chaperones, including GRP78. In other words, within 20 years of sorafenib's announcement to the world, it had gone from being a RAF kinase inhibitor, to a tyrosine kinase inhibitor, to a broad low affinity chaperone inhibitor. In a similar vein, the therapeutically useful CDK4/6 family of kinase inhibitors, e.g. palbociclib, have been shown to not only inhibit their on-target CDK4/6 targets, but also to induce autophagosome formation [94, 95]. So, yet again, a drug family designed to inhibit cell cycle progression via CDK4/6 inhibition can also enhance autophagosome formation which reduces the levels of multiple regulatory proteins. It is therefore apropos to ask; how can anyone rationally develop anti-cancer drug combinations when the biology of each drug is in all likelihood a moving target?

One way in which some of these drug-specificity issues could be forestalled in the future is at the level of drug discovery and drug development within a pharmaceutical company. Modern computer aided compound developmental strategies and in vitro assays with purified kinases will yield a "product" for further development. The next steps are usually cell-based assays in which the actions of the agent are tested, specifically examining the biology of the target to whom the agent has been designed. It is at this stage of drug

development where a new compound needs to be rigorously interrogated in an agnostic fashion to assess its biological actions in a diverse variety of tumor cell types, including those with defined oncogenic driver mutations. In multiple manuscripts, utilizing a multi-channel fluorescence HCS microscope, we have agnostically screened for drug effects on receptor tyrosine kinases, signaling intermediates and transcription factors and HDAC proteins. Hence, as the developers and patent owners of drugs that are brought to the clinic, it should fall directly upon the pharmaceutical companies themselves to thoroughly investigate and define the entire spectrum of each drug's biology and target enzymes, prior to their release into the scientific and clinical space.

Finally, consider the future directions of "cancer research." It remains a disconcerting fact that most 'card carrying' cancer researchers have never closely interacted with a practicing medical oncologist or pondered the possibilities of bench-to-bedside translation research. Many cancer researchers selected to serve on grant review committees who review translational research have themselves neither conceived nor published studies in which they performed bench-to-bedside research. As a collective, the cancer research community still stresses to the outside world the importance of "basic research." This claim was certainly true in the early 2000's, however today and onwards into the future, with the hundreds of phase I safely-tested drugs available for rational combinatorial studies, the over-emphasis on basic cancer research at the expense of true translational research is very probably holding back the development of new therapeutic modalities. It is hoped that the concepts and approaches described in this review article will encourage more cancer researchers to consider bench-to-bedside translational research and deliver a useful drug-combination "product" for the communities in which they live.

Funding

Support for the present study was funded from philanthropic funding from Massey Cancer Center and the Universal Inc. Chair in Signal Transduction Research. P Dent has received funding by the Commonwealth Health Research Board (CHRB) of Virginia and The US National Cancer Institute (R01 CA192613).

References

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

1. Klaeger S, Heinzlmeir S, Wilhelm M, Polzer H, et al. The target landscape of clinical kinase drugs. Science. 2017; 358: pii: eaan4368. doi: 10.1126/science.aan4368. [PubMed: 29191878]
2. Muttathukattil AN, Srinivasan S, Halder A, Reddy G. Role of Guanidinium-Carboxylate Ion Interaction in Enzyme Inhibition with Implication for Drug Design. J Phys Chem B. 2019 10 9. doi: 10.1021/acs.jpcb.9b06130.
3. Dobrydnev AV, Tkachuka TM, Atamaniuka VP, Popova MV. Quercetin-amino acid conjugates are promising anti-cancer agents in drug discovery projects. Mini Rev Med Chem. 2019 10 9. doi: 10.2174/1389557519666191009152007.
4. Deeks ED. Neratinib: First Global Approval. Drugs. 2017;77:1695–1704. [PubMed: 28884417]
5. Wilhelm S, Carter C, Lynch M, Lowinger T, et al. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. Nat Rev Drug Discov. 2006;5:835–44. [PubMed: 17016424]

6. Inaba H, Panetta JC, Pounds S, Wang L, et al. Sorafenib population pharmacokinetics and skin toxicities in children and adolescents with refractory/relapsed leukemia or solid tumor malignancies. Clin Cancer Res. 2019 8 27 pii: clincanres.0470.2019.
7. Strumberg D, Voliotis D, Moeller JG, Hilger RA, et al. Results of phase I pharmacokinetic and pharmacodynamic studies of the Raf kinase inhibitor BAY 43–9006 in patients with solid tumors. Int J Clin Pharmacol Ther. 2002;40:580–1. [PubMed: 12503822]
8. Awada A, Hendlisz A, Gil T, Bartholomeus S, et al. Phase I safety and pharmacokinetics of BAY 43–9006 administered for 21 days on/7 days off in patients with advanced, refractory solid tumours. Br J Cancer. 2005; 92:1855–61. [PubMed: 15870716]
9. Clark JW, Eder JP, Ryan D, Lathia C, et al. Safety and pharmacokinetics of the dual action Raf kinase and vascular endothelial growth factor receptor inhibitor, BAY 43–9006, in patients with advanced, refractory solid tumors. Clin Cancer Res. 2005;11:5472–80. [PubMed: 16061863]
10. Gavini J, Dommann N, Jakob MO, Keogh A, et al. Verteporfin-induced lysosomal compartment dysregulation potentiates the effect of sorafenib in hepatocellular carcinoma. Cell Death Dis. 2019; 10:749. [PubMed: 31582741]
11. Lin CH, Elkholy KH, Wani NA, Li D, et al. Ibrutinib potentiates anti-hepatocarcinogenic efficacy of sorafenib by targeting EGFR in tumor cells and BTK in immune cells in the stroma. Mol Cancer Ther. 2019 10 3 pii: molcanther.0135.2019.
12. Wang C, Wang H, Lieftink C, du Chatinier A, et al. CDK12 inhibition mediates DNA damage and is synergistic with sorafenib treatment in hepatocellular carcinoma. Gut. 2019 9 13 pii: gutjnl-2019–318506.
13. Takimoto CH, Awada A. Safety and anti-tumor activity of sorafenib (Nexavar) in combination with other anti-cancer agents: a review of clinical trials. Cancer Chemother Pharmacol. 2008;61:535–48. [PubMed: 18026728]
14. Strumberg D, Clark JW, Awada A, Moore MJ, et al. Safety, pharmacokinetics, and preliminary antitumor activity of sorafenib: a review of four phase I trials in patients with advanced refractory solid tumors. Oncologist. 2007;12:426–37. [PubMed: 17470685]
15. Flaherty KT, Schiller J, Schuchter LM, Liu G, et al. A phase I trial of the oral, multikinase inhibitor sorafenib in combination with carboplatin and paclitaxel. Clin Cancer Res. 2008;14:4836–42. [PubMed: 18676756]
16. Pan P, Wang L, Wang Y, Shen L, et al. Systematic Review and Meta-Analysis of -New-Generation Tyrosine Kinase Inhibitors versus Imatinib for Newly Diagnosed Chronic Myeloid Leukemia. Acta Haematol. 2019 9 12:1–13.
17. Fondevila F, Méndez-Blanco C, Fernández-Palanca P, González-Gallego J, et al. Anti-tumoral activity of single and combined regorafenib treatments in preclinical models of liver and gastrointestinal cancers. Exp Mol Med. 2019;51:109. [PubMed: 31551425]
18. Lee ATJ, Jones RL, Huang PH. Pazopanib in advanced soft tissue sarcomas. Signal Transduct Target Ther. 2019;4:16. [PubMed: 31123606]
19. Bæk Møller N, Budolfsen C, Grimm D, Krüger M, et al. Drug-Induced Hypertension Caused by Multikinase Inhibitors (Sorafenib, Sunitinib, Lenvatinib and Axitinib) in Renal Cell Carcinoma Treatment. Int J Mol Sci. 2019;20 pii: E4712. [PubMed: 31547602]
20. Brattås MK, Reikvam H, Tvedt THA, Bruserud Ø. Dasatinib as an investigational drug for the treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia in adults. Expert Opin Investig Drugs. 2019;28:411–420.
21. Gross-Goupil M, Kwon TG, Eto M, Ye D, et al. Axitinib versus placebo as an adjuvant treatment of renal cell carcinoma: results from the phase III, randomized ATLAS trial. Ann Oncol. 2018;29:2371–2378. [PubMed: 30346481]
22. Wilhelm SM, Carter C, Tang L, Wilkie D, et al. BAY 43–9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res. 2004;64:7099–109. [PubMed: 15466206]
23. Carlomagno F, Anaganti S, Guida T, Salvatore G, et al. BAY 43–9006 inhibition of oncogenic RET mutants. J Natl Cancer Inst. 2006;98:326–34. [PubMed: 16507829]

24. Chiou JF, Tai CJ, Huang MT, Wei PL, et al. Glucose-regulated protein 78 is a novel contributor to acquisition of resistance to sorafenib in hepatocellular carcinoma. Ann Surg Oncol. 2010;17:603–12. [PubMed: 19830497]

*25. Roberts JL, Tavallai M, Nourbakhsh A, Fidanza A, et al. GRP78/Dna K Is a Target for Nexavar/Stivarga/Votrient in the Treatment of Human Malignancies, Viral Infections and Bacterial Diseases. J Cell Physiol. 2015;230:2552–78. [PubMed: 25858032] Multi-kinase inhibitors, by inhibiting GRP78 function, prevent viruses from hijacking cellular machinery that is essential for virus replication. The same is true in cancer cells with endoplasmic reticulum stress and in bacteria that have evolutionary conserved chaperone domains.

*26. Booth L, Shuch B, Albers T, Roberts JL, et al. Multi-kinase inhibitors can associate with heat shock proteins through their NH2-termini by which they suppress chaperone function. Oncotarget. 2016; 7:12975–96. [PubMed: 26887051] Multi-kinase inhibitors such as pazopanib and sorafenib not only inhibit multiple kinase targets but also interact with the ATP binding sites of HSP90 family and HSP70 chaperone proteins. Thus, a complete understanding of how these drugs act as single agents or in combinations will be highly complex.

27. Booth L, Roberts JL, Tavallai M, Nourbakhsh A, et al. OSU-03012 and Viagra Treatment Inhibits the Activity of Multiple Chaperone Proteins and Disrupts the Blood-Brain Barrier: Implications for Anti-Cancer Therapies. J Cell Physiol. 2015;230:1982–98. [PubMed: 25736380]

28. Rahmani M, Davis EM, Crabtree TR, Habibi JR, et al. The kinase inhibitor sorafenib induces cell death through a process involving induction of endoplasmic reticulum stress. Mol Cell Biol. 2007;27:5499–513. [PubMed: 17548474]

29. Park MA, Zhang G, Martin AP, Hamed H, et al. Vorinostat and sorafenib increase ER stress, autophagy and apoptosis via ceramide-dependent CD95 and PERK activation. Cancer Biol Ther. 2008;7:1648–62. [PubMed: 18787411]

30. Zhu G, Lee AS. Role of the unfolded protein response, GRP78 and GRP94 in organ homeostasis. J Cell Physiol. 2015;230:1413–20. [PubMed: 25546813]

31. Booth L, Roberts JL, Ecroyd H, Tritsch SR, et al. AR-12 Inhibits Multiple Chaperones Concomitant With Stimulating Autophagosome Formation Collectively Preventing Virus Replication. J Cell Physiol. 2016;231:2286–302. [PubMed: 27187154]

32. NCT00978523.

33. Verheijen RB, Beijnen JH, Schellens JHM, Huitema ADR, et al. Clinical Pharmacokinetics and Pharmacodynamics of Pazopanib: Towards Optimized Dosing. Clin Pharmacokinet. 2017; 56: 987–997. [PubMed: 28185218]

34. Masood A, Kancha RK, Subramanian J. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in non-small cell lung cancer harboring uncommon EGFR mutations: Focus on afatinib. Semin Oncol. 2019 9 11 pii: S0093–7754(18)30191-X.

35. Paranjpe R, Basatneh D, Tao G, De Angelis C, et al. Neratinib in HER2-Positive Breast Cancer Patients. Ann Pharmacother. 2019; 53:612–620. [PubMed: 30607980]

36. Sooro MA, Zhang N, Zhang P. Targeting EGFR-mediated autophagy as a potential strategy for cancer therapy. Int J Cancer. 2018;143:2116–2125. [PubMed: 29574749]

37. Liu Q, Yu S, Zhao W, Qin S, Chu Q, Wu K. EGFR-TKIs resistance via EGFR-independent signaling pathways. Mol Cancer. 2018;17:53. [PubMed: 29455669]

38. Moll HP, Pranz K, Musteanu M, Grabner B, et al. Afatinib restrains K-RAS-driven lung tumorigenesis. Sci Transl Med 2018;10:eaao2301. [PubMed: 29925635]

39. Yamaoka T, Ohmori T, Ohba M, Arata S, et al. Distinct Afatinib Resistance Mechanisms Identified in Lung Adenocarcinoma Harboring an EGFR Mutation. Mol Cancer Res. 2017;15:915–928. [PubMed: 28289161]

40. Ebert K, Mattes J, Kunzke T, Zwingenberger G, et al. MET as resistance factor for afatinib therapy and motility driver in gastric cancer cells. PLoS One. 2019;14:e0223225. doi: 10.1371/journal.pone.0223225. [PubMed: 31557260]

41. Scaltriti M, Verma C, Guzman M, Jimenez J, et al. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene. 2009; 28:803–14. [PubMed: 19060928]

42. Collins DM, Gately K, Hughes C, Edwards C, et al. Tyrosine kinase inhibitors as modulators of trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity in breast cancer cell lines. Cell Immunol. 2017; 319:35–42. [PubMed: 28735814]
43. Collins DM, Conlon NT, Kannan S, Verma CS, et al. Preclinical Characteristics of the Irreversible Pan-HER Kinase Inhibitor Neratinib Compared with Lapatinib: Implications for the Treatment of HER2-Positive and HER2-Mutated Breast Cancer. Cancers (Basel). 2019;11 pii: E737. [PubMed: 31141894]
44. Cocco E, Javier Carmona F, Razavi P, et al. Neratinib is effective in breast tumors bearing both amplification and mutation of ERBB2 (HER2). PLoS One. 2018;13:e0200836. [PubMed: 30118499]
45. Kwak EL, Sordella R, Bell DW, Godin-Heymann N, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci U S A. 2005;102:7665–70. [PubMed: 15897464]
46. Zhang Y, Zhang J, Liu C, Du S, et al. Neratinib induces ErbB2 ubiquitylation and endocytic degradation via HSP90 dissociation in breast cancer cells. Sci Signal. 2018;11 pii: eaat9773. doi: 10.1126/scisignal.aat9773. [PubMed: 30301790]
47. Pal R, Wei N, Song N, Wu S, et al. Molecular subtypes of colorectal cancer in pre-clinical models show differential response to targeted therapies: Treatment implications beyond KRAS mutations. PLoS One. 2018;13:e0200836. [PubMed: 30118499]
48. Kruspig B, Monteverde T, Neidler S, Hock A, et al. The ERBB network facilitates KRAS-driven lung tumorigenesis. Sci Transl Med. 2018;10 pii: eaao2565. [PubMed: 29925636]
49. Tavallai M, Booth L, Roberts JL, Poklepovic A, et al. Rationally Repurposing Ruxolitinib (Jakafi (®)) as a Solid Tumor Therapeutic. Front Oncol. 2016;6:142. [PubMed: 27379204]
*50. Booth L, Roberts JL, Tavallai M, Webb T, et al. The afatinib resistance of in vivo generated H1975 lung cancer cell clones is mediated by SRC/ERBB3/c-KIT/c-MET compensatory survival signaling. Oncotarget. 2016;7:19620–30. [PubMed: 26934000]
*51. Booth L, Roberts JL, Poklepovic A, Avogadri-Connors F, et al. HDAC inhibitors enhance neratinib activity and when combined enhance the actions of an anti-PD-1 immunomodulatory antibody in vivo. Oncotarget. 2017;8:90262–90277. [PubMed: 29163826] Data in reference 50 demonstrated the mechanisms by which lung cancer cells became resistant to afatinib. Reference 51 demonstrated that afatinib-resistant cells could be killed by neratinib, and that neratinib could down-regulate expression of multiple growth factor receptors as well as RAS proteins.
**52. Davis MI, Hunt JP, Herrgard S, Ciceri P, et al. Comprehensive analysis of kinase inhibitor selectivity. Nat Biotechnol. 2011;29:1046–51. [PubMed: 22037378] The first demonstration that although afatinib and neratinib both inhibit their proposed targets, only neratinib had significant off-target activity against MAP4K ser/thr kinases.
53. Fuller SJ, McGuffin LJ, Marshall AK, Giraldo A, et al. A novel non-canonical mechanism of regulation of MST3 (mammalian Sterile20-related kinase 3). Biochem J. 2012;442:595–610. [PubMed: 22229648]
54. Klooster JP, Jansen M, Yuan J, Oorschot V, et al. Mst4 and Ezrin induce brush borders downstream of the Lkb1/Strad/Mo25 polarization complex. Dev Cell. 2009;16:551–62. [PubMed: 19386264]
55. Jang JW, Kim MK, Bae SC. Reciprocal regulation of YAP/TAZ by the Hippo pathway and the Small GTPase pathway. Small GTPases. 2018; 20:1–9.
56. Rawat SJ, Chernoff J. Regulation of mammalian Ste20 (Mst) kinases. Tr. Biochem Sci. 2015; 40:149–56.
57. Bae SJ, Luo X. Activation mechanisms of the Hippo kinase signaling cascade. Biosci Rep. 2018;38 pii: BSR20171469. doi: 10.1042/BSR20171469. [PubMed: 30038061]
58. Kong D, Zhao Y, Men T, Teng CB. Hippo signaling pathway in liver and pancreas: the potential drug target for tumor therapy. J Drug Target. 2015;23:125–33. [PubMed: 25470255]
59. Patel SH, Camargo FD, Yimlamai D. Hippo Signaling in the Liver Regulates Organ Size, Cell Fate, and Carcinogenesis. Gastroenterology. 2017;152:533–545. [PubMed: 28003097]
60. Hergovich A. The Roles of NDR Protein Kinases in Hippo Signalling. Genes (Basel). 2016;7 pii: E21. doi: 10.3390/genes7050021. [PubMed: 27213455]

61. Sharif AAD, Hergovich A. The NDR/LATS protein kinases in immunology and cancer biology. Semin Cancer Biol. 2018;48:104–114. [PubMed: 28579171]

62. Bitra A, Sistla S, Mariam J, Malvi H, et al. Rassf Proteins as Modulators of Mst1 Kinase Activity. Sci Rep. 2017;7:45020. doi: 10.1038/srep45020. [PubMed: 28327630]

63. Chen M, Zhang H, Shi Z, Li Y, et al. The MST4-MOB4 complex disrupts the MST1-MOB1 complex in the Hippo-YAP pathway and plays a pro-oncogenic role in pancreatic cancer. J Biol Chem. 2018; 293:14455–14469. [PubMed: 30072378]

**64. Dent P, Booth L, Roberts JL, Liu J, et al. Neratinib inhibits Hippo/YAP signaling, reduces mutant K-RAS expression, and kills pancreatic and blood cancer cells. Oncogene. 2019;38:5890–5904. [PubMed: 31253872] Neratinib blocks RTK and mutant K-RAS function, and through a compensatory mechanism, causes enhanced phosphorylation of LATS1/2, YAP and TAZ. This causes inactivation of the Hippo pathway.

65. Crawford JJ, Bronner SM, Zbieg JR. Hippo pathway inhibition by blocking the YAP/TAZ-TEAD interface: a patent review. Expert Opin Ther Pat. 2018. doi: 10.1080/13543776.2018.1549226.

66. Meng Z, Moroishi T, Mottier-Pavie V, Plouffe SW, et al. MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the Hippo pathway. Nat Commun. 2015;6:8357. doi: 10.1038/ncomms9357. [PubMed: 26437443]

67. Hergovich A. Regulation and functions of mammalian LATS/NDR kinases: looking beyond canonical Hippo signalling. Cell Biosci. 2013;3:32. doi: 10.1186/2045-3701-3-32. [PubMed: 23985307]

*68. Booth L, Roberts JL, Rais R, Cutler RE, et al. Neratinib augments the lethality of [regorafenib + sildenafil]. (2018) J. Cell. Physiol doi: 10.1002/jcp.27276.The first manuscript using molecular tools to conclusively demonstrate that neratinib caused RAS proteins to localize in intracellular vesicles.

69. Thompson BJ, Sahai E. MST kinases in development and disease. J Cell Biol. 2015;210:871–82. [PubMed: 26370497]

70. Chen S, Fang Y, Xu S, Reis C, et al. Mammalian Sterile20-like Kinases: Signalings and Roles in Central Nervous System. Aging Dis. 2018;9:537–552. [PubMed: 29896440]

71. Wang OH, Azizian N, Guo M, Capello M, et al. Prognostic and Functional Significance of MAP4K5 in Pancreatic Cancer. PLoS One. 2016;11:e0152300. doi: 10.1371/journal.pone.0152300. [PubMed: 27023625]

72. Meng Z, Moroishi T, Mottier-Pavie V, Plouffe SW, et al. MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the Hippo pathway. Nat Commun. 2015;6:8357. doi: 10.1038/ncomms9357. [PubMed: 26437443]

73. Hsu CL, Lee EX, Gordon KL, Paz EA, et al. MAP4K3 mediates amino acid-dependent regulation of autophagy via phosphorylation of TFEB. Nat Commun. 2018;9:942. doi: 10.1038/s41467-018-03340-7. [PubMed: 29507340]

74. Arbour KC, Riely GJ. Systemic Therapy for Locally Advanced and Metastatic Non-Small Cell Lung Cancer: A Review. JAMA. 2019;322:764–774. [PubMed: 31454018]

75. Kim RY, Sterman DH, Haas AR. Malignant Mesothelioma: Has Anything Changed? Semin Respir Crit Care Med. 2019;40:347–360. [PubMed: 31525810]

76. Frederickson AM, Arndorfer S, Zhang I, Lorenzi M, et al. Pembrolizumab plus chemotherapy for first-line treatment of metastatic nonsquamous non-small-cell lung cancer: a network meta-analysis. Immunotherapy. 2019;11:407–428. [PubMed: 30712477]

77. Racanelli AC, Rothbart SB, Heyer CL, Moran RG. Therapeutics by cytotoxic metabolite accumulation: pemetrexed causes ZMP accumulation, AMPK activation, and mammalian target of rapamycin inhibition. Cancer Res. 2009;69:5467–74. [PubMed: 19549896]

78. Rothbart SB, Racanelli AC, Moran RG. Pemetrexed indirectly activates the metabolic kinase AMPK in human carcinomas. Cancer Res. 2010;70:10299–309. [PubMed: 21159649]

79. Brooks HB, Meier TI, Geeganage S, Fales KR, et al. Characterization of a novel AICARFT inhibitor which potently elevates ZMP and has anti-tumor activity in murine models. Sci Rep. 2018;8:15458. [PubMed: 30337562]

80. Booth L, Roberts JL, Poklepovic A, Gordon S, et al. PDE5 inhibitors enhance the lethality of pemetrexed through inhibition of multiple chaperone proteins and via the actions of cyclic GMP and nitric oxide. Oncotarget. 2017;8:1449–1468. [PubMed: 27903966]
81. Tripathi DN, Chowdhury R, Trudel LJ, Tee AR, et al. Reactive nitrogen species regulate autophagy through ATM-AMPK-TSC2-mediated suppression of mTORC1. Proc Natl Acad Sci U S A. 2013;110:E2950–7. [PubMed: 23878245]
82. Alers S, Wesselborg S, Stork B. ATG13: just a companion, or an executor of the autophagic program? Autophagy. 2014;10:944–56. [PubMed: 24879146]
83. Booth L, Roberts JL, Poklepovic A, Dent P. [pemetrexed + sildenafil], via autophagy-dependent HDAC downregulation, enhances the immunotherapy response of NSCLC cells. Cancer Biol Ther. 2017;18:705–714. [PubMed: 28812434]
84. Booth L, Roberts JL, Poklepovic A, Kirkwood J, et al. HDAC inhibitors enhance the immunotherapy response of melanoma cells. Oncotarget. 2017;8:83155–83170. [PubMed: 29137331]
85. Booth L, Roberts JL, Poklepovic A, Dent P. Prior exposure of pancreatic tumors to [sorafenib + vorinostat] enhances the efficacy of an anti-PD-1 antibody. Cancer Biol Ther. 2019;20:109–121. [PubMed: 30142009]
86. Mohanty R, Chowdhury CR, Arega S, Sen P, et al. CAR T cell therapy: A new era for cancer treatment (Review). Oncol Rep. 2019. doi: 10.3892/or.2019.7335.
87. Leonetti A, Wever B, Mazzaschi G, Assaraf YG, et al. Molecular basis and rationale for combining immune checkpoint inhibitors with chemotherapy in non-small cell lung cancer. Drug Resist Updat. 2019;46:100644. [PubMed: 31585395]
88. Terranova-Barberio M, Thomas S, Ali N, Pawlowska N, et al. HDAC inhibition potentiates immunotherapy in triple negative breast cancer. Oncotarget. 2017;8:114156–114172. [PubMed: 29371976]
89. Dent P, Booth L, Poklepovic A, Hancock JF. Signaling alterations caused by drugs and autophagy. Cell Signal. 2019;64:109416. [PubMed: 31520735]
90. Booth L, Roberts JL, Kirkwood J, Poklepovic A, et al. Unconventional Approaches to Modulating the Immunogenicity of Tumor Cells. Adv Cancer Res. 2018;137:1–15. [PubMed: 29405973]
91. Booth L, Roberts JL, Sander C, Lee J, et al. The HDAC inhibitor AR42 interacts with pazopanib to kill trametinib/dabrafenib-resistant melanoma cells in vitro and in vivo. Oncotarget. 2017;8:16367–16386. [PubMed: 28146421]
92. Booth L, Roberts JL, Tavallai M, Chuckalovcak J, et al. [Pemetrexed + Sorafenib] lethality is increased by inhibition of ERBB1/2/3-PI3K-NFκB compensatory survival signaling. Oncotarget. 2016;7:23608–32. [PubMed: 27015562]
93. Heidecker G, Huleihel M, Cleveland JL, Kolch W, et al. Mutational activation of c-raf-1 and definition of the minimal transforming sequence. Mol Cell Biol. 1990;10:2503–12. [PubMed: 2188091]
94. Booth L, Roberts JL, Rais R, Cutler RE Jr, et al. Palbociclib augments Neratinib killing of tumor cells that is further enhanced by HDAC inhibition. Cancer Biol Ther. 2019;20:157–168. [PubMed: 30183517]

Article Highlights

- Targeted kinase inhibitors often have additional, unknown or unrecognized targets because of similarities in ATP binding pockets
- The concept of a highly “personalized medicine” approach to cancer can fail because it is unlikely that a specific therapy will kill all clonal variants of the tumor.
- Analyses of RNA levels can be helpful, but unless protein phosphorylation events are agnostically catalogued, the true biology of many anti-cancer agents cannot be fully understood.
- Multi-channel fluorescence microscopy, via in-cell immunoblotting, represents a state-of-the-art approach to define changes in protein expression, protein phosphorylation and protein localization in a 96-well plate format.
- The over-emphasis on basic research goals, rather than translational goals leading to new trials, is depriving stakeholders of novel rationally-developed drug combinations which would improve progression free and overall survival rates.