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Pharmacology Discovery Services

WHITE PAPER

Profiling Oncology Drug Candidates Through Complementary *in vitro* Assays and *in vivo* Models to Better Understand Their Potential Clinical Applications

INTRODUCTION

In the lead optimization and candidate selection stages of drug discovery, diverse yet complementary investigations can provide a comprehensive profile of a compound's activity, efficacy, and safety. To obtain as complete a profile as possible, orthogonal approaches–including *in vitro* binding and biochemical assays, cell-based assays, and *in vivo* safety and efficacy models–can collectively provide insights into the potential clinical utility of a compound. When thoroughly interrogating a compound, no single data set, analysis, or interpretation should be taken in isolation. It is the comprehensive profile of a potential candidate, that enables a more rigorous and relevant assessment of its clinical potential.

To obtain as complete a profile as possible of a potential candidate no single piece of data should be taken in isolation.

In this white paper, we describe a profiling strategy that includes both *in vitro* and *in vivo* approaches to characterize sorafenib, an ATP-competitive multi-kinase inhibitor with clinical applications in multiple cancer types. Data for vemurafenib, an anti-neoplastic B-Raf kinase inhibitor, is also included to highlight how profiling compounds can shed light onto potentially different applications in the clinic.

KINASES AS DRUG TARGETS

With the discovery of the proto-oncogene, c-Src, in 1978 by J. Michael Bishop and Harold E. Varmus (Levinson *et al.* (1978) **Cell** 15, 561-72), the therapeutic potential for kinases as drug targets in cancer was established. In the following three decades many independent discoveries have collectively confirmed the notion that kinases are relevant drug targets. In 1986, staurosporine was identified as an effective inhibitor of the protein kinase C family of enzymes. However, it is a broad spectrum inhibitor that has a diverse profile leading to safety issues that prevented it from becoming a marketed drug. These early discoveries led to concerns that targeting the ATP binding site of kinases would not allow for sufficient selectivity to permit identification of a safe and selective drug. In the 1990s, the concept of kinase profiling became widely adopted which led to the discovery of safe and well-tolerated kinase inhibitors. The seminal approval of imatinib, now commercially known as Gleevec®, is a prime example of this drug class. Subsequently, numerous kinase drug discovery programs have resulted in a large number of drug approvals covering a wide variety of therapeutic applications.

B-RAF AS A DRUG TARGET

In 2002, a landmark paper was published (Davies *et al.* (2002) **Nature** 417, 949-54) that reported the association of activating mutations in the BRAF gene with human cancer, including what is now termed the V600E mutation. The paper provided a robust validation of the target, or at least a correlation of the mutation of a genetic target, with human disease. While melanoma was reported as having a particularly high incidence of the B-Raf V600E mutation, the presence of activating B-Raf mutations was confirmed in other cancers as well, including, but not limited to: colon, papillary thyroid, ovarian, and non-small-cell lung cancer. Onyx Pharmaceuticals identified compounds subsequently licensed by Bayer that targeted mutated B-Raf. One such compound, sorafenib, is an ATP-competitive multi-kinase inhibitor that displays activity against several different kinases of therapeutic relevance. It consequently inhibits a variety of cellular processes as a relatively broad spectrum kinase inhibitor with dose-dependent anti-tumor activity that induces autophagy and apoptosis in tumors. In addition to its desirable anti-cancer efficacy, sorafenib has suitable drug metabolism and pharmacokinetic properties that provide a favorable bioavailability profile.

IN VITRO PHARMACOLOGY PROFILING OF SORAFENIB

Concerning kinase inhibitors, such as sorafenib, complementary approaches can generate a more informative *in vitro* profile. Two such options include competition binding assays and functional biochemical assays. The goal of both methods is to assess the selectivity of a compound and the specific targeted activity. These data can assist medicinal chemistry efforts in achieving the desired activity and safety characteristics of a compound. Gaining knowledge about the broader *in vitro* profile of a compound, outside of that shown against the immediate drug target(s), can also assist efforts concerning asset repurposing.

The advent of *in vitro* pharmacology profiling was a key advance in the successful application of kinase inhibitors in the clinic. Binding and functional biochemical assays are two technologies that may be employed to generate an *in vitro* profile.

IN VITRO PROFILE OF SORAFENIB WITH KINOME*scan*

KINOME*scan*® is a service that provides site-directed target competition binding information to enable the quantification of interactions between a test compound and more than 450 kinases, and disease-relevant mutants, through the use of qPCR technology. "Hits" are identified by measuring the amount of captured test compound versus control samples. Dissociation constants for test compound-kinase interactions may also be calculated by measuring the amount of captured test compound on the solid support as a function of the test compound concentration.

Figure 1 shows a graphical representation of hits identified with sorafenib where each red circle indicates a kinase to which sorafenib was bound. The size of the circle is indicative of the affinity of the drug-target interaction; interactions with Kd values less than 3 µM are shown. These data show that sorafenib primarily binds with kinases in the TK, TKL, and CMGC families and has a relatively high degree of selectivity (Davis *et al.* (2011) **Nature** Biotechnology 29, 1046-51).

The binding affinities of sorafenib and another Raf inhibitor, vemurafenib, for B-Raf, B-Raf(V600E), and c-Raf were determined using the KINOME*scan*® platform (see Table 1). While sorafenib showed a modest affinity for the wild type B-Raf kinase, it has marginally greater affinity for the B-Raf(V600E) and c-Raf kinases, by ~2-fold. In contrast, vemurafenib showed more potent affinity for all three mitogenic kinases, with an approximately 16-fold greater affinity for wild type B-Raf than sorafenib, and ~5-fold greater affinity for B-Raf(V600E) and c-Raf. This difference in potency may be a significant factor in explaining why vemurafenib demonstrated superior clinical utility to sorafenib in tumors bearing B-Raf(V600E).

Interestingly, both drugs displayed an affinity for several kinases that are known to be associated with angiogenesis and vascular remodeling, namely CSF1R, Flt3, PDGFRA, PDGFRB, and VEGFR/Kdr. However, with respect to these targets, sorafenib showed a markedly tighter binding affinity than vemurafenib. In fact, vemurafenib only showed a modest, measurable affinity for Flt3, PDGFRA, and PDGFRB, in ascending order of potency. However, sorafenib demonstrated potent, double digit nanomolar Kd values for all five kinases evaluated, suggesting that it has greater anti-angiogenic potential than vemurafenib. Collectively, these observations are in alignment with biological data derived from evaluation of both drugs in cellular assays (see subsequent sections of this white paper), as well as the clinical approval of each for markedly different tumor types.

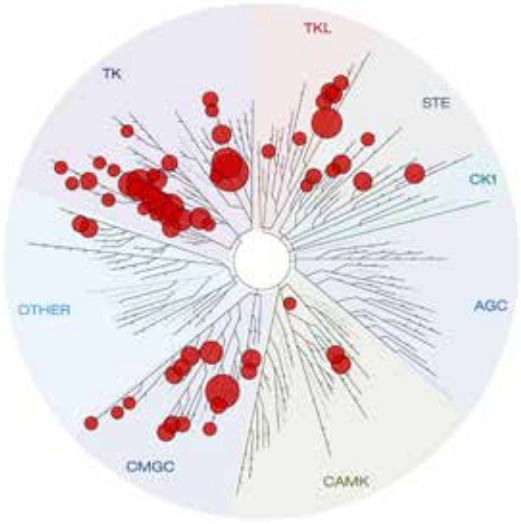

Figure 1. Sorafenib profile with KINOME*scan*

Kinase		Sorafenib KINOME*scan* Kd (nM)	Vemurafenib KINOME*scan* Kd (nM)
RAF Kinases	B-Raf	540	33
	B-Raf(V600E)	260	50
	c-Raf/Raf-1	230	43
Angiogenesis-Associated Kinases	CSF1R	28	>30,000
	Flt3	13	2200
	PDGFRA	62	830
	PDGFRB	37	100
	VEGFR2/Kdr	59	>30,000

Table 1. Binding affinities for Sorafenib and Vemurafenib.

KinaseProfiler™ is a radiometric activity-based assay platform that directly measures the kinase's catalytic activity to enable the detection of ATP-competitive, substrate-competitive and allosteric kinase inhibitors. The KinaseProfiler service offers over 420 kinases, comprised primarily of wild-type kinases, but it also contains several mutant kinases of key therapeutic interest allowing determination of a thorough understanding of a kinase inhibitor's profile.

Concerning sorafenib, Figure 2 shows a complete analysis of this drug using the KinaseProfiler service. This analysis demonstrates that sorafenib has a somewhat broad profile against a large number of kinases. Interestingly, and in line with the original therapeutic direction for sorafenib, it is capable of inhibiting all of the Raf protein kinase family members: A-Raf, B-Raf (wild-type and V600E mutant), and c-Raf. This fulfills the original objective for which sorafenib was originally developed. However, further analysis of the data shows that sorafenib targets several other kinases of interest to oncology drug discovery, including Flt1, Flt3, and KDR (also known as VEGFR2), as well as PDGFRβ. These additional kinases are of interest as they are all targets that are relevant to the process of angiogenesis, on which many tumor types show a critical dependence, with some cancers showing greater sensitivity to anti-angiogenic agents than others.

In comparing the kinase profile of sorafenib with vemurafenib, a slightly more selective profile is illustrated by the latter (Figure 3). As with sorafenib, vemurafenib also targets the Raf family of protein kinases, A-Raf, B-Raf (wild-type and V600E mutant) and c-Raf, as well as a few other kinases, but it is seen to be more selective than sorafenib when the two kinase profiles are compared.

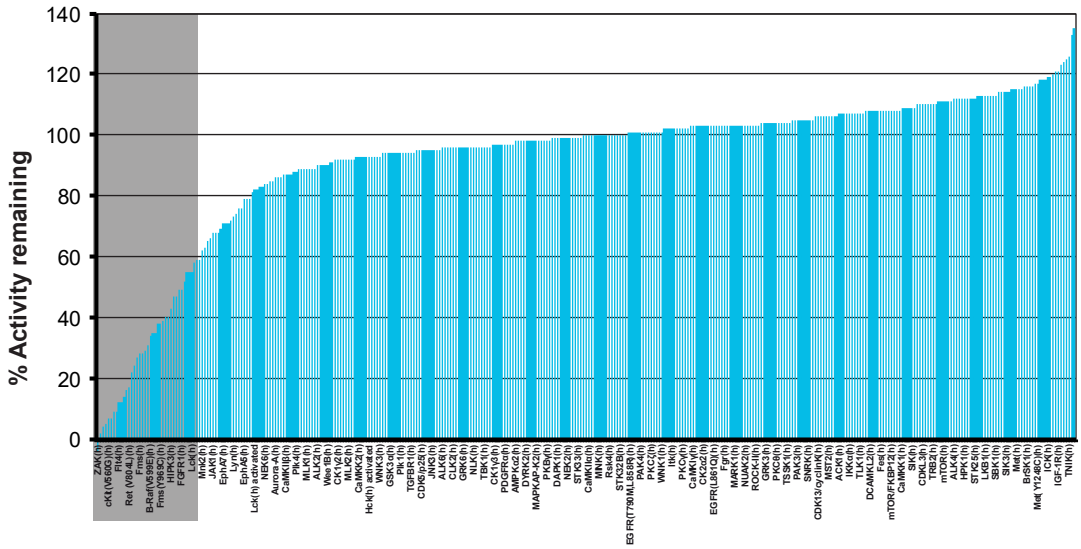

Figure 2. Sorafenib profile with KinaseProfiler (tested at 300 nM and K_m for ATP).

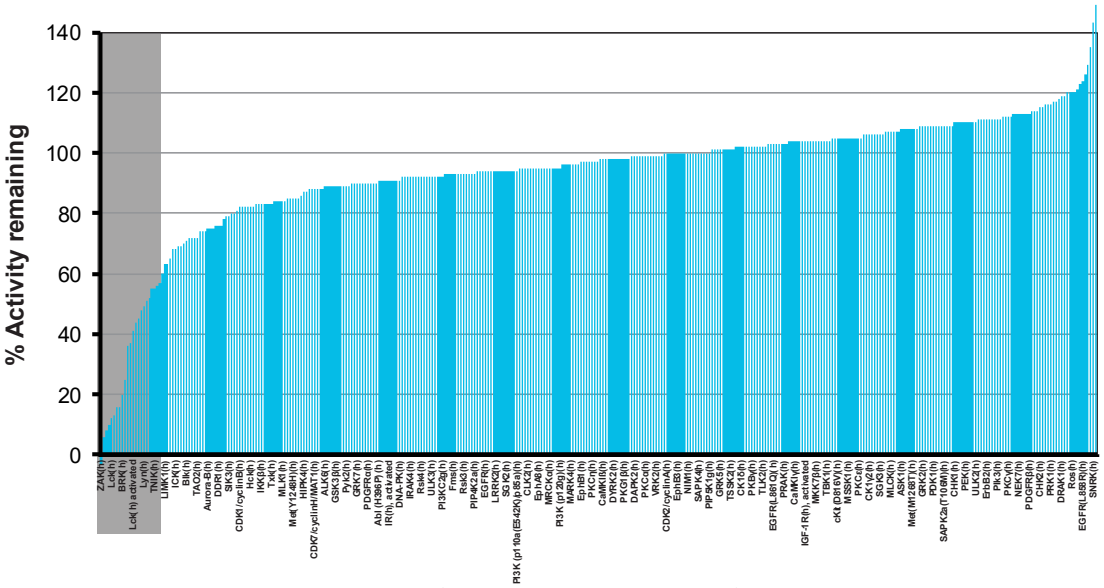

Figure 3. Vemurafenib profile with KinaseProfiler (tested at 300 nM and K_m for ATP).

PHENOTYPIC PROFILING OF ANTI-CANCER COMPOUNDS

Human cell-based phenotypic assays provide the opportunity to test anti-cancer compounds in a cellular context, for physiological insights into potency and selectivity across multiple cancer types. These assays can often validate, refute, or even predict results that are generated through *in vitro* pharmacological methods. Cell-based phenotypic assays can also provide information to help guide the design of *in vivo* pharmacology efficacy studies.

THE ONCOPANEL CELL-BASED PROFILING SERVICE

The OncoPanel™ service incorporates 300+ genomically diverse human tumor cell lines covering more than 25 different tissue types and sub-types. Data analysis options with OncoPanel include cell proliferation, induction of apoptosis, cell cycle status or the addition of other custom markers. A univariate genomic analysis may also be conducted on primary OncoPanel data that enables the identification of predictive genomic biomarkers of response to test agents. The identification of genomic biomarkers potentially allows the stratification of patient populations in the clinic by identifying tumor types that are most likely to be responsive to a test agent.

Figure 4 shows proliferation assay data obtained for sorafenib using the OncoPanel service. The figure shows a plot of the log of the observed IC_{50} value (in nM) for each cell line tested with each vertical bar representing an individual tumor cell line. The far left-hand side of the figure shows the cell lines that are most sensitive in terms of reduced cellular proliferation, with significant activity highlighted by the gray box. Sorafenib was developed as a B-Raf kinase inhibitor yet there does not appear to be a robust correlation between the presence of a B-Raf activating mutation in cell lines (shown in red) and sensitivity to sorafenib. Other cell lines of potential interest, from a B-Raf biology perspective, are those that contain activating mutations of the NRAS gene (shown in black) and those displaying over-expression of the gene for c-Kit (shown in yellow). If these cell lines are dependent upon signaling via N-Ras or c-Kit, and B-Raf kinase activity is being significantly inhibited, the notion of oncogene addiction might suggest it plausible to expect that a B-Raf inhibitor could inhibit the proliferation of these cell lines. However, that isn't observed with sorafenib.

In comparison, Figure 5 contains OncoPanel profile data for vemurafenib. When the data are examined, it is clear that the most sensitive cell lines, in terms of reduced cellular proliferation, cluster tightly with the presence of B-Raf activating mutations (shown in red). This is such a strong correlation that the data set has an associated p-value of as much as 10^{-22} (depending upon the specific statistical test performed). These data are highly significant, and this observation is borne out by the successful use of vemurafenib to treat tumors containing activated mutant B-Raf in the clinic. Vemurafenib is marketed as the drug Zelboraf™ and

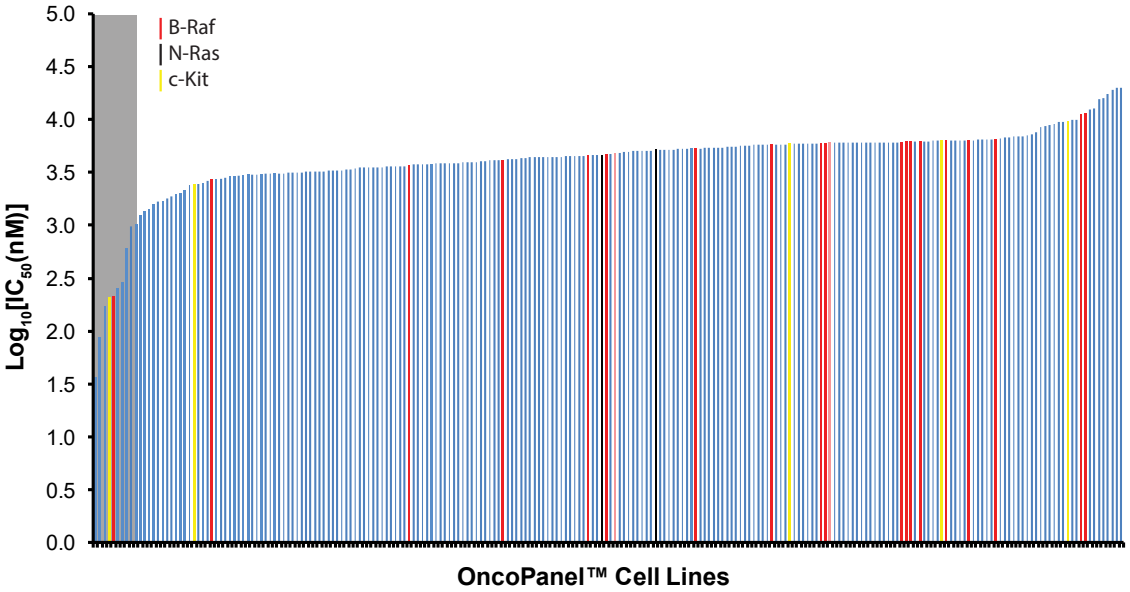

Figure 4. Sorafenib profile with the OncoPanel Cell-based Profiling Service (tested from 0.64 nM to 20 µM).

is approved for the treatment of metastatic melanoma-bearing B-Raf activating mutations, with ongoing clinical trials to address its utility in treating other tumors bearing B-Raf activating mutations. Cell lines bearing mutationally activated N-Ras or over-expressing c-Kit are also seen to be somewhat sensitive to this drug, this is in contrast to the profile seen with sorafenib. Looking carefully at the genomic composition of the affected cell lines one can begin to appreciate that there may be genomic characteristics that correlate with sensitivity and those that can also potentially correlate with resistance.

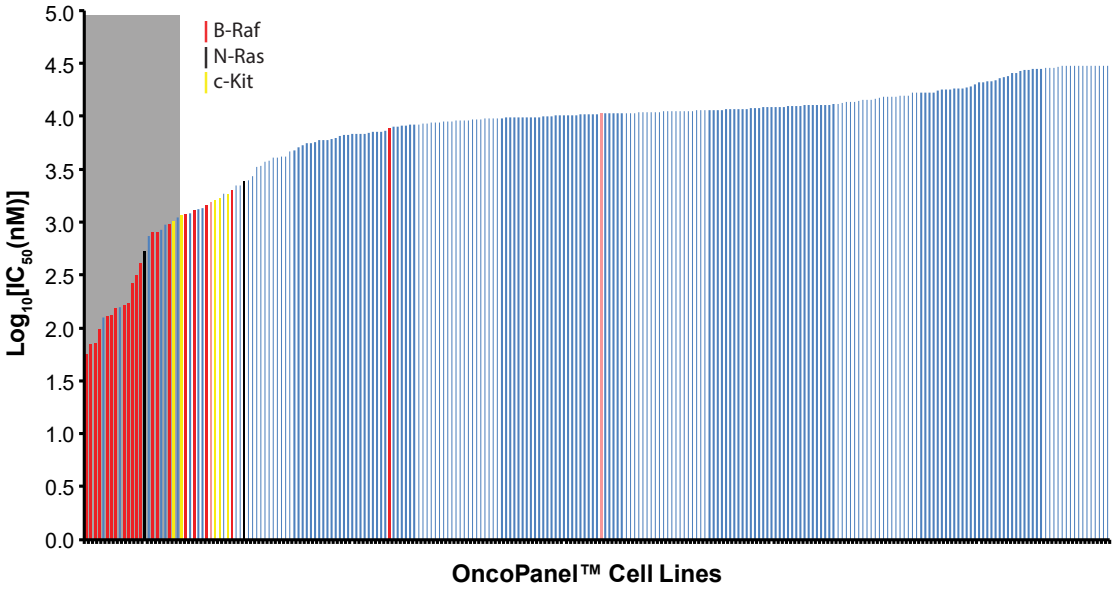

Figure 5. Vemurafenib profile with the OncoPanel Cell-based Profiling Service (tested from 0.95 nM to 30 µM).

UNIVARIATE GENOMIC ANALYSIS OF ONCOPANEL PROLIFERATION ASSAY DATA

Predictive genomic biomarker identification for sorafenib with proliferation assay data from the OncoPanel™ service can identify what, if any, genomic markers are statistically significant. This is done by orthogonal evaluation of drug response in two different statistical tests to help minimize any inherent bias exerted by either test on the analysis outcome. In order to be called as truly significant, only features showing significance in both statistical tests are considered. Using the Fisher's Exact Test and the Student's T-test, a variety of genomic features were deemed significant (data not shown) and further evaluated to identify whether the presence of such features correlates with either sensitivity or resistance to the tested drug. This further evaluation compares the odds ratio with the observed effect to identify whether specific genomic features correlate with resistance or sensitivity.

Figure 6 shows plots of the odds ratio (y-axis) versus the effect (x-axis) for both sorafenib (left panel) and vemurafenib (right panel). Genomic features with a high positive odds ratio and a positive effect are associated with resistance to the test agent while those features with a high negative odds ratio and a negative effect are associated with sensitivity. These features are shown in Figure 6 in the top-right and bottom-left quadrants, respectively. As was observed visually in the OncoPanel drug response profiles in Figures 4 and 5, the presence of activating mutations in the BRAF gene are strongly associated with sensitivity to vemurafenib in this statistical analysis, while this correlation is not seen with sorafenib. It is thus no great surprise that vemurafenib has been approved to treat cancers in which the BRAF gene has been mutated resulting in an activated form of the B-Raf protein kinase.

In addition to the evaluation of mutations, differential gene expression can also be evaluated for potential correlation with sensitivity or resistance to a tested agent. Figure 7 shows plots of the log of the calculated p-value for each gene versus the fold change for sorafenib (left panel) and vemurafenib (right panel). Genes with a positive fold change (highlighted in blue) are associated with resistance and those with a negative fold change (highlighted in red) are associated with sensitivity to the agent tested. Only the top twenty genes with the highest statistical significance are annotated for each drug.

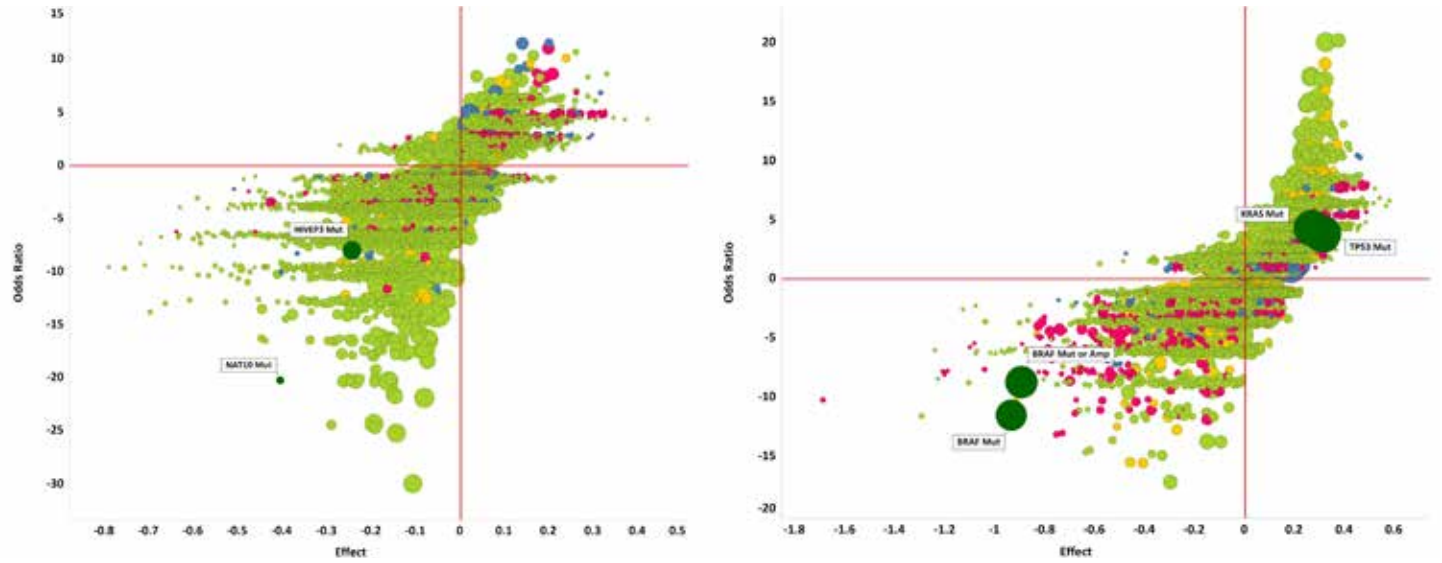

Figure 6. Mutation analysis odds ratio versus effect plots for sorafenib (left) and vemurafenib (right).

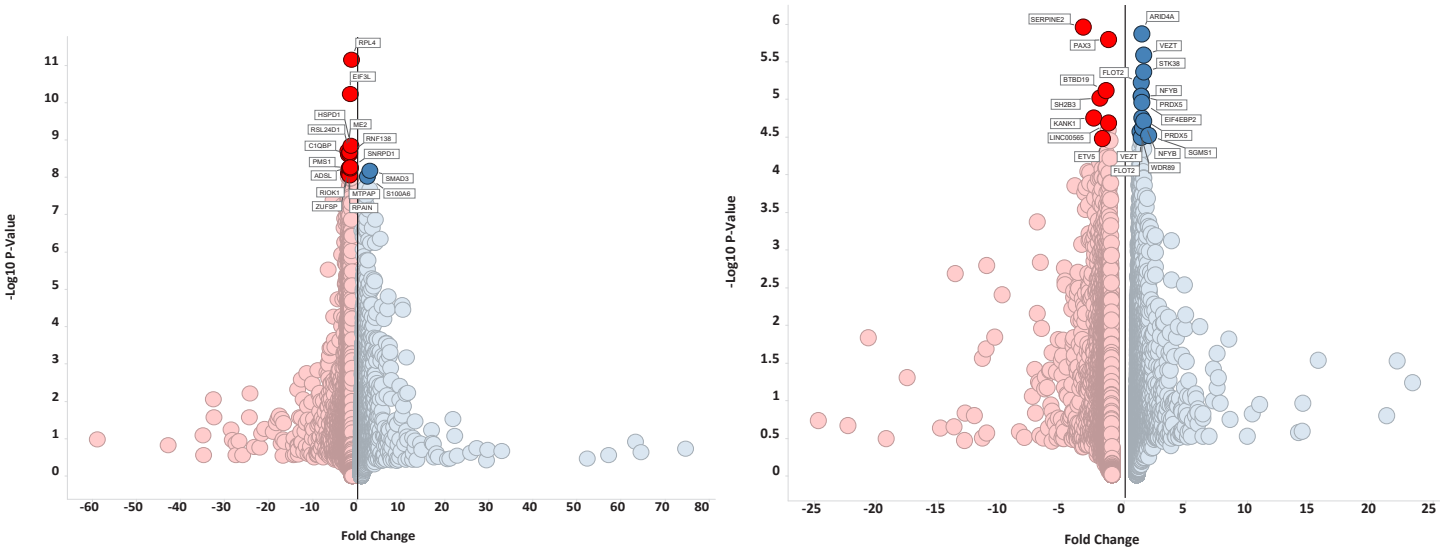

Figure 7. Differential gene expression analysis plots for sorafenib (left) and vemurafenib (right).

Based on the differential gene expression analysis, differences in profiles between sorafenib and vemurafenib are again notable. Sensitivity to vemurafenib is associated with the differential expression of genes associated with such functions as signaling and transcriptional regulation, as well as some of the less well-understood functions. However, resistance to vemurafenib is associated with genes such as ARID4A (AT-rich interaction domain 4A), STK38 (serine/threonine kinase 38), FLOT2 (flotillin 2), and PRDX5 (peroxiredoxin 5). The products of these genes modulate Rb function, control cell morphogenesis and proliferation, stabilize growth factor receptor signaling and protect against certain forms of stress and induced cell death. It is easy to see why overexpression of these genes may result in resistance to vemurafenib.

In contrast, sensitivity to sorafenib correlated with the differential expression of genes involved in a range of cellular functions, including a large number of genes that regulate protein synthesis and stability. Of note is the association of resistance to sorafenib with differential expression of the genes SMAD3 and S100A6. Interestingly, SMAD3 expression has been linked with vascular remodeling, and is associated with familial thoracic aortic aneurism and dissection. S100A6 encodes a calcium-sensing protein that has been linked with several cancers, including melanoma.

While the OncoPanel™ univariate genomic analysis profiles are quite different between sorafenib and vemurafenib, it is clear that different cellular functions and processes are associated with sensitivity or resistance to these two drugs. Consequently, it is no great surprise that the two drugs have been approved for the treatment of different types of cancer and different functional reasons.

The BioMAP® Phenotypic Profiling Platform encompasses multiple human primary cell-based assay systems designed to recapitulate key aspects of human tissue and disease biology, together with translational biomarker readouts. The BioMAP Diversity PLUS® Panel consists of 12 individual human *in vitro* disease model systems with 148 biologically and clinically relevant protein-based biomarker endpoints. Diversity PLUS was designed to serve as a comprehensive and unbiased assessment of the impact of a drug candidate on a broad scope of human biology that can inform on both primary and secondary pharmacology, as well as indication selection. The detection of biological activities of compounds in this validated panel, coupled to comprehensive analytical tools and a comprehensive reference database can provide actionable insights on efficacy, safety, and mechanism of action.

Figure 8 shows the BioMAP Diversity PLUS profile of sorafenib tested at four concentrations. The 12 BioMAP systems are represented by icons and divided by vertical lines. The biomarkers are listed on the X-axis and the activity of the test agents are displayed as a log ratio compared to a relevant vehicle control (Y-axis). Gray arrows indicate the antiproliferative impact of the drug at one or more concentrations; black arrows (not present) indicate cytotoxic impact at one or more concentrations. The gray shaded area in the middle represents a historical vehicle control range and is a component of the criteria used to determine key activities that meet established significance criteria, in order to be annotated on the profile. Overall, sorafenib moderately impacts patient-related biology including modulation of inflammation-related and immunomodulatory biomarkers.

Figure 9 compares the profile of sorafenib at the 1.1 µM concentration with a similar concentration of vemurafenib tested in the BioMAP Diversity PLUS Panel; differentiating activities are shown. The agents differ in their impact on B cell response in the BT system, a model of T cell-dependent B cell activation. Vemurafenib, but not sorafenib, decreases IgG production; an effect that is likely related to its ability to inhibit the BCR signaling mediators Lck and Lyn, as revealed by KinaseProfiler™ analysis. Additionally, vemurafenib modulates the expression levels of extracellular matrix-related proteins, including MMPs, Collagen-III, PAI-1, and uPAR while sorafenib does not. KinaseProfiler analysis also revealed that vemurafenib inhibits TRAF2 and NCK Interacting Kinase (TNIK). The latter kinase is an activator of the Wnt signaling pathway, which in turn regulates the expression of extracellular matrix components. Aberrant Wnt signaling is tightly associated with many types of cancers and may represent a novel mode of action for vemurafenib in oncology indications. These two drugs also share three common activities (data not shown), potentially indicating a phenotypic signature for their common target(s).

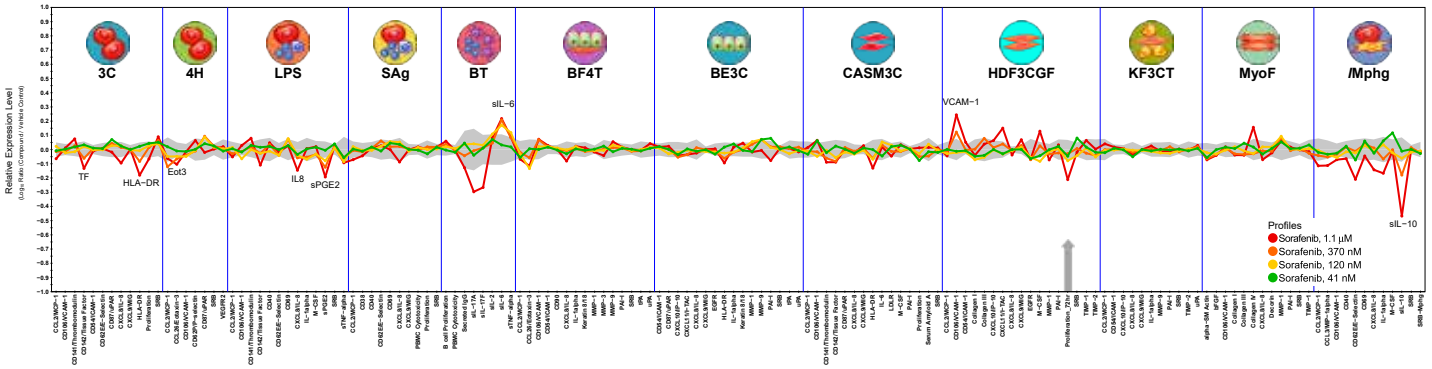

Figure 8. BioMAP Diversity PLUS profile of Sorafenib at four concentrations.

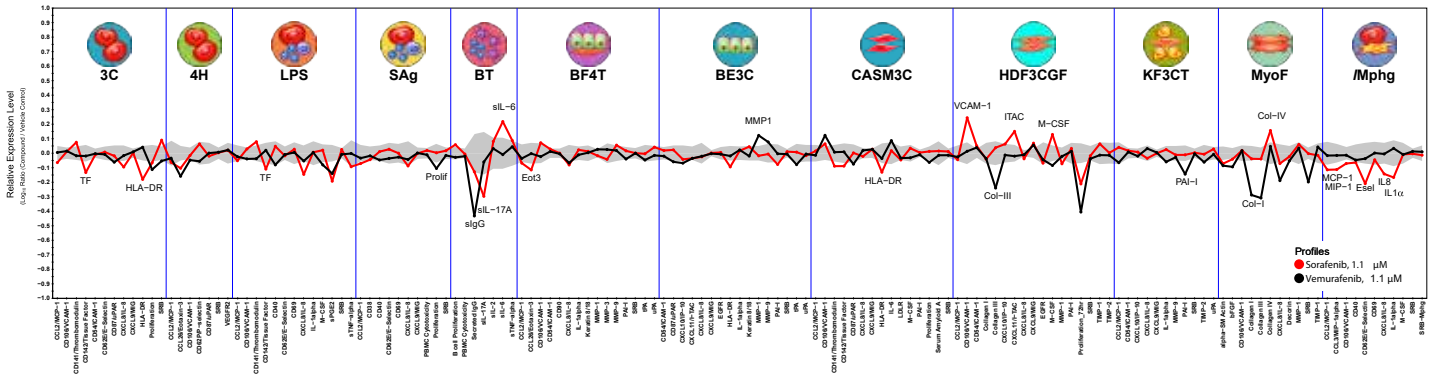

Figure 9. Comparative overlay analysis of the BioMAP Diversity PLUS profiles of sorafenib (red) and vemurafenib (black) at 1.1 µM. Differentiating biomarkers are annotated.

IN VIVO EFFICACY OF SORAFENIB – STUDY CONDUCTED BY PHARMACOLOGY DISCOVERY SERVICES

In addition to *in vitro* enzymatic and cell-based assays, *in vivo* efficacy models are a key component of the drug discovery process to assess clinical potential. Figure 10 shows *in vivo* efficacy data for sorafenib and three immuno-oncology therapeutics (targeting CTLA-4, PD-L1, and PD-1) in the Renca kidney cancer syngeneic mouse model. The immuno-oncology therapeutics do not display spectacular activity at the doses administered although perhaps the anti-PD-L1 agent does show some activity. Sorafenib does demonstrate excellent anti-tumor activity in this model. With sorafenib, the growth of tumors is essentially halted and the drug effect is tumoristatic, or the growth rate of the tumors are significantly diminished when compared with the no treatment control. What is significant here is that the response of Renca, a renal cancer cell line, ultimately correlates very well with the clinical utility of sorafenib which is approved for the treatment of renal cell carcinoma. Given the more vascularized nature of many renal cancers, this observation is in line with some of the *in vitro* findings from the OncoPanel™ genomic analysis.

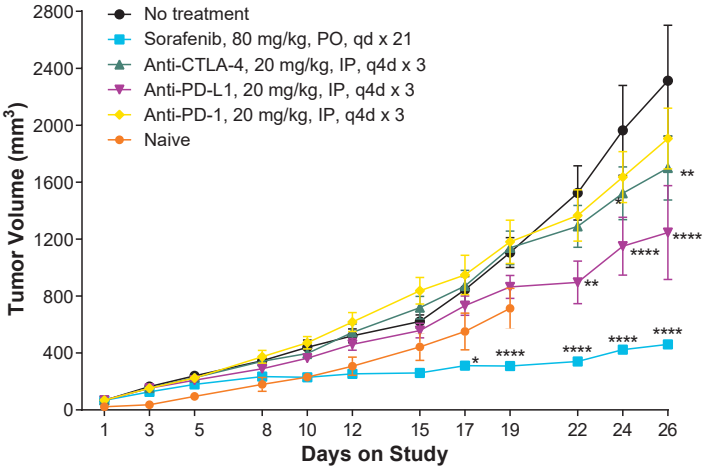

Figure 10. Sorafenib tested in a Renca syngeneic mouse model sorafenib (red) and vemurafenib (black) at 1.1 µM. Differentiating biomarkers are annotated. Two-way ANOVA followed by Bonferroni post-tests were applied for comparison between the "No treatment" and test substance-treated groups (*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001).

SORAFENIB IN THE CLINIC

Sorafenib was evaluated in a number of clinical trials, with an initial therapeutic indication for melanoma due to the high incidence of activating B-Raf mutations in that cancer type and the documented *in vitro* activity of sorafenib against the Raf family of protein kinases. Ultimately sorafenib did not display sufficient clinical utility for that indication. Interestingly, there was a subset of melanoma patients responsive to sorafenib, so it did have some benefit in these patients. However, as supported by the previously discussed *in vitro* biochemical, cell-based and genomic biomarker data, none of the patients that benefited from sorafenib had the presence of a B-Raf mutation in their tumors.

Based on the potential for efficacy resulting from other kinase inhibitory activities, sorafenib was evaluated in kidney cancer clinical trials. It showed great benefit in treating clear cell renal carcinoma and ultimately received marketing approval in 2005 as Nexavar™. It was also identified that sorafenib has utility in liver cancer and marketing approval for that indication was granted in 2007. Subsequently, sorafenib has also received marketing approval for thyroid carcinomas that are resistant to radioiodine (I-131) treatment.

CONCLUSION

The collection of data presented here, obtained from *in vitro* biochemical and cell-based assays and *in vivo* studies, provides insight into what ultimately became sorafenib's clinical utility. The *in vitro* binding and biochemical profiles of sorafenib with KINOME*scan*® and KinaseProfiler™, respectively, demonstrate that while the drug has activity against the Raf kinases, it also shows activity against a broader number of angiogenesis-relevant targets, primarily in the TK, TKL, and CMGC kinase families. These data go some way towards explaining sorafenib's clinical utility in both renal cell carcinomas and hepatocellular carcinomas, as they are more heavily vascularized tumors.

Cell-based assays can help to validate, predict, or in some cases refute, aspects of a biochemical profile. Data for sorafenib from the OncoPanel™ Cell-based Profiling Service demonstrated that anti-cancer activity was not dependent upon the Raf family of kinases especially when compared with the same data set for vemurafenib. For sorafenib, the OncoPanel results correlate with the *in vitro* data generated with KINOME*scan* and KinaseProfiler services. A univariate genomic analysis with those OncoPanel data further supports the notion that sorafenib does not specifically target the Raf kinase pathway, but rather other cellular pathways and related processes.

BioMAP® Phenotypic Profiling revealed that sorafenib had relatively limited impact on biomarkers covering a broad scope of human tissue biology outside the tumor cells themselves. In BioMAP systems containing endothelial cells, the few biomarkers impacted by sorafenib included those associated with angiogenesis such as tissue factor and prostaglandin E2, further supporting the observed clinical utility of sorafenib in treating vascularized tumors. BioMAP profiling also indicated that in contrast, vemurafenib was more broadly active, with antiproliferative effects on angiogenesis-related endothelial cells, as well as on T cells and fibroblasts, consistent with this drug having more of an anti-mitogenic profile. Vemurafenib impacted inflammation and immune and tissue remodeling activities, including modulation of extracellular matrix-related biomarkers such as VCAM-1. Taken together these data indicate that sorafenib may have a more restricted biological effect and is less potent against its biological target, thus restricting its efficacy to select cancer types.

In vivo evaluation of sorafenib in the Renca kidney cancer mouse model did display quite promising efficacy; these data support how sorafenib ultimately demonstrated therapeutic utility in the clinic. Importantly, renal cancer is a different therapeutic indication than that originally intended for sorafenib, so these results demonstrate how an agent can be repurposed based on informed use of both *in vitro* and *in vivo* data.

When a pharmacological profile of a compound is generated no one piece of data should be taken in isolation. *In vitro*, cellular phenotypic and *in vivo* data can each provide insights into potential clinical utility.

These collective data demonstrate the value of generating as comprehensive a compound profile as possible to identify appropriate therapeutic indications for a drug. Comprehensive profiles of a compound generated from complimentary approaches can also help to improve medicinal chemistry efforts for lead optimization, allowing more successful progression along the drug discovery pipeline. Collectively, it is most important to be aware that no single data set, *in vitro* or *in vivo*, should be taken in isolation. Knowing the complete compound profile, and making sense of all of the different aspects of that profile, is what creates the highest probability of success in the clinic.

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