Ibrutinib blocks YAP1 activation and reverses BRAFi resistance in melanoma cells

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Non-standard Abbreviation List:

BRAF - B-Raf Proto-Oncogene
MEK - Mitogen-Activated Protein Kinase Kinase 1
BTK - Bruton Tyrosine Kinase
SRC - SRC Proto-Oncogene
YAP1 - Yes1 Associated Transcriptional Regulator
PDGFRA - Platelet Derived Growth Factor Receptor Alpha
KIT - KIT Proto-Oncogene
LATS - Large Tumor Suppressor Kinase
TAZ - WW Domain Containing Transcription Regular 1
DAPI - 4′,6-diamidino-2-phenylindole
CRISPR - clustered regularly interspaced short palindromic repeats
STR - Short Tandem Repeat
TIDE - Tracking of Indels by Decomposition
LISA - landscape in silico subtraction analysis
CMap - Connectivity Map
GTEx - Genotype-Tissue Expression
LINCS - Library of Integrated Network-Based Cellular Signatures
sRGES - Summarized Reverse Gene Expression Score
OCTAD - Open Cancer TherApeutic Discovery
CTRPv2 - Cancer Therapeutics Response Portal
TEAD - TEA Domain Transcription Factor 1
SFK - Src Family Kinase
Abstract:

Most BRAF-mutant melanoma tumors respond initially to BRAFi/MEKi therapy, although few patients have durable long-term responses to these agents. The goal of this study was to utilize an unbiased computational approach to identify inhibitors which reverse an experimentally derived BRAFi resistance gene expression signature. Using this approach, we found that ibrutinib effectively reverses this signature and we demonstrate experimentally that ibrutinib re-sensitizes a subset of BRAFi-resistant melanoma cells to vemurafenib. Ibrutinib is used clinically as an inhibitor of the Src-family kinase BTK; however, neither BTK deletion nor treatment with acalabrutinib, another BTK inhibitor with reduced off-target activity, re-sensitized cells to vemurafenib. These data suggest that ibrutinib acts through a BTK-independent mechanism in vemurafenib re-sensitization. To better understand this mechanism, we analyzed the transcriptional profile of ibrutinib-treated BRAFi-resistant melanoma cells and found that the transcriptional profile of ibrutinib was highly similar to that of multiple SRC kinase inhibitors. Since ibrutinib, but not acalabrutinib, has appreciable off-target activity against multiple SRC family kinases, it suggests that ibrutinib may be acting through this mechanism. Furthermore, genes that are differentially expressed in ibrutinib-treated cells are enriched in YAP1 target genes and we showed that ibrutinib, but not acalabrutinib, reduces YAP1 activity in BRAFi-resistant melanoma cells. Taken together, these data suggest that ibrutinib, or other SRC family kinase inhibitors, may be useful for treating some BRAFi/MEKi-refractory melanoma tumors.

Significance Statement: MAPK-targeted therapies provide dramatic initial responses, but resistance develops rapidly; a subset of these tumors may be rendered sensitive again by treatment with an approved src-family kinase inhibitor – ibrutinib – potentially providing improved clinical outcomes.

Introduction:

Approximately 90% of melanoma tumors harbor activating mutations in the MAPK pathway and most of these tumors have BRAFV600 mutations (Hodis, Watson et al. 2012). Most BRAF-mutant melanoma tumors initially respond to BRAF inhibitors (BRAFi), however, this response is often short-lived and most tumors develop resistance (Larkin, Ascierto et al. 2014, Robert, Karaszewska et al. 2015). Mechanisms of resistance to BRAFi/MEKi therapy most commonly occur through re-activation of the mitogen activated protein kinase (MAPK) pathway (Nazarian, Shi et al. 2010, Poulikakos, Persaud et al. 2011, Shi, Moriceau et al. 2012, Shi, Hugo et al. 2014, Van Allen, Wagle et al. 2014, Hugo, Shi et al. 2015, Johnson, Menzies et al. 2015, Moriceau, Hugo et al. 2015, Shaffer, Dunagin et al. 2017, Song, Piva et al. 2017). However, there are few if any effective clinical interventions that overcome BRAFi resistance after it develops. In this study, we sought to identify compounds which reverse a BRAFi resistance gene signature. This systems-based approach has been widely explored in cancer drug discovery (Lamb, Crawford et al. 2006, Jahchan, Dudley et al. 2013, Chen, Ma et al. 2017, Chen, Wei et al. 2017, Subramanian, Narayan et al. 2017), yet few studies have investigated resistance in melanoma. Ultimately, the goal is to identify drugs which could be combined with BRAFi/MEKi therapy to prevent or reverse drug resistance.

One advantage to using this approach is that it allows for the identification of compounds whose effects may result from complex polypharmacology. There are several examples of the clinical utility of drugs that exhibit polypharmacology, including crizotinib, afatinib, ceritinib, dasatinib, erlotinib, nilotinib, ponatinib, and imatinib (Antolin, Workman et al. 2016). In the case of imatinib, it was first developed to inhibit a BCR-ABL fusion protein in Chronic Myeloid Leukemia (Buchdunger, Zimmermann et al. 1996, Druker, Tamura et al. 1996, Deininger, Goldman et al. 1997). But later imatinib was used to target dermatofibrosarcoma protuberans tumors harboring gene fusions which result in aberrant PDGFR activation. It is also clinically used to treat gastrointestinal stromal tumors which have activating PDGFR or KIT mutations since imatinib has off-target activity against PDGFR and KIT (Shimizu, O’Brien et al. 1999, Greco, Roccato et al. 2001, Joensuu, Roberts et al. 2001, Sjoblom, Shimizu et al. 2001, Tuveson, Willis et al. 2001, Rubin, Schuetze et al. 2002). Several molecules, many of which are not kinase inhibitors, are currently under clinical investigation and have a
mechanism of action linked to previously unappreciated off-target effects (Lin, Giuliano et al. 2019). These examples likely represent only a fraction of circumstances in which kinase inhibitor polypharmacology is clinically relevant. Because of this, there have been recent large-scale efforts to profile kinase inhibitor polypharmacology (Klaeger, Heinzlmeir et al. 2017). Defining the entire polypharmacology network will likely result in a sizeable increase in the number of clinically actionable applications.

The goal of this exploratory study is to perform an unbiased computational screen to identify chemical compounds that reverse BRAFi resistance in melanoma cells. In this study we identify a new role for ibrutinib, an FDA-approved BTK inhibitor, in reversing BRAFi resistance in melanoma in silico and in vitro. Our studies suggest that ibrutinib may modulate Yes1-associated transcriptional regulator (YAP1) activation in at least some BRAFi resistant melanoma cells. YAP1 is a transcriptional regulator that can function as a co-activator or a co-repressor for different genes (Kim, Kim et al. 2015). YAP1 activity is regulated by the actin cytoskeleton, as well as through changes in the phosphorylation state of YAP1 (Zhao, Wei et al. 2007, Oka, Mazack et al. 2008, Wada, Itoga et al. 2011, Zhao, Li et al. 2012). Some phosphorylation events on YAP1 by LATS1/2 lead to inactivation and subsequent proteasomal degradation (Zhao, Li et al. 2010) whereas phosphorylation at other sites, targeted by YES1 and other kinases, is critical for YAP1 nuclear translocation and activation (Rosenbluh, Nijhawan et al. 2012). YAP1 is activated in BRAFi-resistant melanoma cells; and silencing or deletion of YAP1 reverses BRAFi resistance (Hugo, Shi et al. 2015, Lin, Sabnis et al. 2015, Kim, Kim et al. 2016, Fisher, Grun et al. 2017, Misek, Appleton et al. 2020). In addition to melanoma, YAP1 has been implicated in many other cancer types including breast cancer (Chen, Sun et al. 2012), glioblastoma (Orr, Bai et al. 2011), pancreatic cancer (Zhang, Nandakumar et al. 2014), hepatocellular carcinoma (Tschaharganeh, Chen et al. 2013), and non-small-cell lung cancer (Chaib, Karachaliou et al. 2017). Despite the importance of YAP1 in cancer, it is still difficult to pharmacologically target YAP1. Verteporfin, a drug used to treat macular degeneration, blocks YAP1-TEAD activity in vitro, but in some models has limited efficacy in vivo (Lui, Xiao et al. 2019). Since YAP1 activity is regulated by its phosphorylation state, it may be possible to utilize the polypharmacology of FDA-approved kinase inhibitors to indirectly block YAP1 activation. Our findings suggest that ibrutinib can block the nuclear accumulation of YAP1 and also alter the expression of YAP1 target genes.

Materials and Methods:

Cell lines, reagents, and antibodies: Parental (denoted by a P suffix in the cell line name) and matched isogenic BRAFi-resistant cells (denoted by an R suffix in the cell line name) were either a gift (M229P/R, M238P/R) from Dr. Roger Lo (UCLA) or generated in our laboratory (UACC62P/R). These cells were generated and cultured as described below (Misek, Appleton et al. 2020).

Luteolin (#10004161), BVT-948 (#16615), ketoprofen (#10006661), lestaurtinib (#12094), L-NMMA (#10005031), ibrutinib (#16274), acalabrutinib (#19899), fadrozole (#24272), letrozole (#11568), exemestane (#15008), and vemurafenib (#10618) were purchased from Cayman Chemical (Ann Arbor, USA). Pyrvinium pamoate (#HY-A0293) was purchased from MedChemExpress (Monmouth Junction, USA). Clofilium tosylate (#C2365) was purchased from Sigma Aldrich (St. Louis, USA). All compounds (except L-NMMA) were diluted in DMSO to a stock concentration of 10 mM. L-NMMA was diluted in H2O to a stock concentration of 0.5 mM. All compounds were aliquoted and stored at -20°C.

Antibodies against YAP1 (#14074) and TAZ (#83669) were purchased from Cell Signaling (Danvers, USA). An antibody against Actin (#sc1616) was purchased from Santa Cruz Biotechnology (Dallas, USA). Donkey anti-Mouse800 (#926-3221), Donkey anti-Goat680 (#926-68074), and Donkey anti-Rabbit680 (#926-68073) immunoblotting secondary antibodies were purchased from LI-COR (Lincoln, USA). Anti-rabbit-HRP (#7074) immunoblotting secondary was purchased from Cell Signaling Technology. Alexa Fluor goat anti-rabbit488 (#A11034) and donkey anti-goat488 (A11055) were purchased from Invitrogen (Carlsbad, USA).
Cell culture: Cells were cultured in DMEM (ThermoFisher, Waltham, USA #11995-065) supplemented with 10% FBS (ThermoFisher, #10437-028) and 1% Antibiotic-Antimycotic (ThermoFisher, #15240062) and were passaged at approximately 75% confluence. The BRAFi-resistant cell line variants were maintained in culture medium supplemented with 2 µM vemurafenib. Vemurafenib was removed from the culture medium when cells were seeded for experiments, except where otherwise indicated. Cells were routinely tested for mycoplasma contamination by DAPI staining. Short Tandem Repeat (STR) profiling on all cell lines was performed at the MSU genomics core. In all cases, isogenic pairs of cell lines had identical STR profiles. After thawing cells were used for either 2 months or 20 passages, whichever came first. In all experiments the cell concentration, but not density, was normalized. For all experiments the cells were seeded and analyzed at sub-confluent cell densities.

Cloning/CRISPR: For CRISPR experiments the sgRNA were: sgControl (5’-TCCCCGAGACCATTAGGG-3’), sgBTK#1 (5’-ATGAGTATGACTTTGAACGT-3’), and sgBTK#2 (5’-CCCTTCATCATATAACCT-3’). These guide sequences were cloned into pLentiCRISPRv2-Puro (from Feng Zhang, Addgene plasmid #52961). Successful cloning was confirmed by Sanger sequencing. To measure knockout efficiency, amplicons containing the CRISPR cut sites were amplified from the genomic DNA with PCR and the ratio of frameshifted/functional DNA species was measured with Sanger sequencing using the Tracking of Indels by Decomposition (TIDE) algorithm (Brinkman, Chen et al. 2014). The primers for gDNA amplification and Sanger sequencing are listed in (Supplemental Data 1).

Virus preparation and infection: HEK-293T cells were seeded into 10-cm plates at a density of 4x10⁶ cells/plate and the cells were allowed to attach overnight. The next day the cells were transfected with a plasmid cocktail containing 5000 ng of the pLentiCRISPRv2 plasmid, 5000 ng of psPAX2 (Addgene plasmid #12260), 500 ng of pMD2.G (Addgene plasmid #12259), and 20 µL of Lipofectamine 2000 (ThermoFisher, #11668019) in 400 µL of OptiMEM (ThermoFisher, #31985070). The next morning the medium was changed to 10 mL of fresh complete culture medium, and the following day each plate was supplemented with an additional 5 mL of culture medium. After 24 h, the culture medium was harvested and filtered through a 0.45-µm syringe filter. Virus was stored at 4ºC and was used within 2 weeks.

Melanoma cells were seeded into 10-cm plates at a density of 5x10⁵ cells/plate in 10 mL of complete culture medium. While the cells were still in suspension, 3 mL of viral supernatant was added to each plate. The cells were incubated with virus overnight, then the medium was changed to 10 mL of fresh medium. After 24 h, the medium was changed to 10 mL of fresh medium supplemented with puromycin (2 µM). The cells were cultured in the presence of selection antibiotic until all the cells on the kill control plate died (approximately 3 days). Individual clones for the CRISPR cell lines were not selected, but instead we used a pooled infection approach. Validation of CRISPR knockout efficiency was performed by Sanger sequencing as described above.

Viability experiments: Cells were seeded into 384-well tissue culture plates (PerkinElmer, Waltham, USA, #6007689) at a density of 1000 cells/well in 20 µL of growth medium. The next day, compounds were pre-diluted in growth medium then added to the 384-well plates so that the final volume of each well was 40 µL. A PBS or growth medium barrier was added to the outer wells of the plate to limit evaporation. Cells were cultured under these conditions for 72 h. To assess viability, 8 µL of CellTiter-Glo (Promega, Madison, USA, #G7573) was added to each well. Plates were incubated on orbital shaker for 5 min at room temperature, then briefly centrifuged (4000 rpm, 60 s) before being read on a Bio-Tek Synergy Neo plate reader with the #11 and #41 Ex/Em filter cubes. Viability signal is plotted versus log (Vemurafenib concentration) for each treatment condition using a four-parameter logistic curve model.

Flow cytometry:
Cell cycle: Cells were rinsed once in PBS before being trypsinized, washed once in PBS and immediately fixed in 70% ethanol for 20 min at room temperature. The cells were washed once and were re-suspended in PBS supplemented with 20 µg/mL propidium iodide (#P1304MP, ThermoFisher) and 200 µg/mL RNaseA. The cells were briefly mixed and were incubated on ice for 20 min. Following incubation, the cells were filtered through a 70 µm filter and were run on an
Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, USA). Data were analyzed with the FCS Express flow cytometry analysis software package.

**Annexin V/Propidium Iodide**: Both floating and adherent cells were collected by trypsinization. The cells were pelleted, washed once in PBS, and then re-suspended in 200 µL of Annexin V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl\(_2\)) and 1 µL of APC-conjugated Annexin V (ThermoFisher, #A35110) on ice in the dark for 20 min. The cells were pelleted and re-suspended in 500 µL Annexin V binding buffer with 2 µg/mL propidium iodide. After 20 min the cells were filtered through a 70 µM filter and were run on an Accuri C6 flow cytometer. Data were analyzed with the FCS Express flow cytometry analysis software package.

**DEVD Assay**: Both the floating and attached cells were collected, rinsed as described above and then lysed in 200 µL of Triton-X100 lysis buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with protease/phosphatase inhibitors. The lysates were centrifuged at 20,000g for 15 min. In a 384-well plate 10 µL of 2x Cytobuffer (100 mM PIPES pH 7.4, 20% glycerol, 2 mM EDTA, 1 mM DTT, 40 µM DEVD-AFC (Arango, Parihar et al. 2012) (Enzo Biochem, Farmingdale, USA, #ALX260032M005), 5 µL of lysis buffer, and 5 µL of cellular lysate was added to each well. In control wells an extra 5 µL of lysis buffer was added in place of the cellular lysate. The plates were prepared on ice to limit enzymatic activity. The plates were read on a Bio-Tek Synergy Neo plate reader at an excitation wavelength of 400 nm and an emission wavelength of 500 nm. Reads were taken every 60 sec for 1 h and caspase3/7 activity is expressed as fold change in nM/AFC/mg/min.

**Colonies formation**: Cells were seeded into 6-well plates at a density of 1000 cells/well and were allowed to attach overnight. The next day the medium was changed, and the cells were treated as described in the figure legends. The growth medium was changed every 3 days. After 14 days the cells were fixed in 3.7% formaldehyde and the cells were stained with crystal violet. Images of the plates were acquired on a flat-bed scanner.

**Immunofluorescence staining**: Cells were seeded into 8-well chamber slides and were treated as indicated in the figure legends. Cells were fixed with 3.7% formaldehyde for 15 min, and then blocked in 2% BSA PBS-Triton X-100 (0.1%) for 1 h at room temperature. Cells were incubated overnight at 4°C in primary antibody at a (1:1,000) dilution in blocking buffer. Cells were washed 3x in PBS then were incubated in the appropriate secondary antibody at a (1:1,000) dilution for 1 h at room temperature. Cells were washed 3x in PBS then were mounted in ProLong Gold Antifade + DAPI (ThermoFisher, #P36935). Slides were cured overnight at room temperature, and then transferred to 4°C. Slides were imaged on a Nikon TE2000-U fluorescence microscope at 20x magnification.

For all immunofluorescence experiments, images were blinded with an R script before quantification. We repeated all immunofluorescence experiments at least three times and typically analyzed 5-10 fields per biological replicate. In total we analyzed at least 200 cells per experimental group, but in most cases over 1000 cells per experimental group. For subcellular localization experiments, data are represented as stacked bar graph wherein the fraction of cells that have predominantly nuclear, pan-cellular, or cytosolic localization is plotted as a fraction of the total cells. A cell was considered to have “cytosolic” localization if there was a clear nuclear exclusion. Inversely a cell was described as having “nuclear” localization if the staining intensity was appreciably higher than in the cytosol. If there was no apparent difference between the nuclear and cytosolic staining, then the cell was described as having “pan-cellular” distribution.

**RNA-Seq sample/data processing**: Total cellular RNA was extracted from drug-treated M229R cells using the Qiagen (Hilden, Germany) RNeasy kit (#74104) with three biological replicates per cell line. All RNA samples had a RIN score > 8. Libraries were prepared using the Illumina TruSeq Stranded mRNA Library Preparation Kit, prepared libraries were quality controlled and quantified using a Qubit and Labchip Bioanalyzer. Libraries were pooled and run on a NovaSeq6000 instrument. Sequencing was performed by 2 x 150 bp paired-end read format. Base calling was done by Illumina RTA and converted to FASTQ using bcl2fastq software. Sequencing was performed at a depth of approximately 30 M reads/sample. Quality control was performed on the FASTQ files using FastQC v0.11.5, and reads were trimmed.
using Trimmomatic v0.33. Reads were mapped using HISAT2 v2.1.0 and analyzed using HTSeq v0.6.1. Differential gene expression was calculated using edgeR. Raw RNA-Seq reads and processed HTSeq read counts are available on GEO under GSE145990. When appropriate RNA-Seq data was upper quintile normalized prior to analysis.

Datasets: Sources for the previously published RNA-Seq data used in this study are as follows. M229P/R and M238P/R RNA-Seq data was downloaded from GSE75313 (Song, Piva et al. 2017). UACC62P/R RNA-Seq data was previously generated by our group and was deposited under GSE115938 (Misek, Appleton et al. 2020). The PRISM drug response dataset was downloaded from the Cancer Dependency Map (DepMap) data download portal (depmap.org/portal/download).

LISA: Epigenetic landscape in silico subtraction analysis (LISA) was run on lisa.cistrome.org (Qin, Fan et al. 2020). Gene lists were filtered to include only significantly differentially expressed genes with a false discovery rate (FDR) < 0.01. Gene set 1 was filtered to include only upregulated genes, and gene set 2 was filtered to include only downregulated genes. Only the top 500 genes were used in each list. In cases where there were fewer than 500 differentially expressed genes, only the genes which had an FDR < 0.01 were included in the analysis. The ChIP-Seq output data was plotted as a scatter plot of enrichments in the upregulated vs downregulated gene sets.

Connectivity map analysis: The top 200 upregulated/downregulated genes with a false discovery rate (FDR) < 0.01 were analyzed to identify Connectivity Map (CMap) Classes which have similar gene expression perturbation signatures on the online clue.io portal. In cases where there were fewer than 200 upregulated or downregulated genes with an FDR < 0.01, only genes which passed the FDR cutoff were included in the analysis.

OCTAD Datasets and RNA-Sequence processing: We used the same pipeline to process RNA-Seq samples from public databases such as TCGA, TARGET, GTEx, and SRA and compiled them into one single dataset called OCTAD (Zeng, Glicksberg et al. 2019). Whenever possible, RNA-Seq samples used in this study were processed using the same pipeline to mitigate batch effects. In addition, RUVg (Risso, Ngai et al. 2014) was used to remove unwanted variation, and weakly expressed genes were removed while computing differentially expressed genes. Normalized raw counts were used for differential expression (DE) analysis and TPM was used for other analyses. The clustering of these samples with melanoma samples compared to non-melanoma primary tumor samples demonstrates the feasibility of performing differential expression analysis between cell lines and tissue samples (Fig. S1).

Disease signature creation: Gene expression data from BRAFi-resistant melanoma cell lines was compared with either 50 healthy normal skin samples from the GTEx database, or to BRAF/V600E-mutant melanoma tumor samples to generate BRAFi-resistance gene expression signatures. We used edgeR to perform differential expression (DE) analysis (log2 fold change > 1, adjusted p-value < 0.001) (Robinson, McCarthy et al. 2010). The detailed data processing and parameter selection were detailed in the OCTAD study (Zeng, Glicksberg et al. 2019). The enrichment of the genes in the BRAFi-resistance gene signatures was computed with ssGSEA (Hanzelmann, Castelo et al. 2013). The association of enrichment scores for both of the signatures with patient survival was computed and visualized using the survminer package. Patient mutation status and survival data were retrieved from cBioPortal (Gao, Aksoy et al. 2013). EnrichR was used for pathway enrichment analysis (Kuleshov, Jones et al. 2016).

Drug prediction: The Library of Integrated Network-Based Cellular Signatures (LINCS) database containing gene expression profiles for compound-treated cells has been widely used for candidate drug prediction in our previous studies (Chen, Greenside et al. 2015, Chen, Ma et al. 2017). The LINCS library is comprised of 476,251 signatures and 22,268 genes including 978 landmark genes. The 1,974 mapped drugs listed in the Repurposing Hub were considered in this study (Corsello, Bittker et al. 2017). To compute RGES scores, which are a quantitative metric of how well a compound reverses a gene signature, we first ranked genes based on their expression values in each drug signature. An enrichment score for each set of up- and down-regulated disease genes was computed separately using a Kolmogorov–Smirnov-like
statistic, followed by the combination of scores from both sides. The score is based on the number of the genes (up or down-regulated) at either the top or bottom of a drug-gene list ranked by expression change after drug treatment. One compound might have multiple available expression profiles because they were tested in various cell lines, drug concentrations, treatment durations, or even different replicates, resulting in multiple RGES for one drug-disease prediction. We termed this score Summarized Reverse Gene Expression Score (sRGES). The computation of RGES and the summarization RGES were detailed elsewhere and recently implemented as a standalone R package (Zeng, Glicksberg et al. 2019). Compounds were filtered to include only compounds that had a sample size greater than 1 in the LINCS L1000 dataset and were filtered to exclude compounds that were anti-neoplastic or were previously studied in melanoma. A sRGES threshold of -0.3 was the cutoff for compounds which effectively reversed the BRAFi resistance signature.

**Statistical Analysis:** For all analyses, the sample sizes were not pre-specified prior to performing the experiment. When appropriate, statistical analysis was performed with one-way ANOVA with Bonferroni correction to correct for multiple hypothesis testing. Owing to the exploratory nature of this study, p-values can not be interpreted as hypothesis testing and can only be interpreted as a descriptive metric. Error bars indicate the standard deviation of independent biological replicates.

**Results:**

**Identification of compounds which reverse a BRAFi resistance signature.**

We employed a systems-based approach to identify compounds that reverse an experimentally derived BRAFi resistance signature. This approach was originally proposed in the Connectivity Map project (Lamb, Crawford et al. 2006), and was extended in other studies (Sirota, Dudley et al. 2011, Chen, Wei et al. 2017), including a recent study from the Chen lab (Chen, Ma et al. 2017) which discusses the computational methodology used in this study in detail. Sample collection, signature creation, sRGES computation, and in silico validation were streamlined in the Open Cancer TherApeutic Discovery (OCTAD) pipeline which was described in the Materials and Methods section. This approach has been applied to identify potential therapeutic compounds for primary cancers, but this study is our first attempt to apply this method to study drug resistance.

To identify compounds that killed BRAFi-resistant melanoma cell lines we first leveraged RNA-seq data from three isogenic cell line pairs (M229P/R, M238P/R, and UACC62P/R) to generate a BRAFi resistance signature. However, the small reference population (parental cells) in this analysis made it difficult to generate a robust resistance signature for this analysis. As such, we took an alternative approach to compare the resistant cell lines with a reference population of healthy skin samples in the OCTAD database, with the idea being that differences between melanoma cells and healthy skin may reflect biologically meaningful differences in signaling pathway activation, and thus, compound sensitivity. Out of the 558 healthy skin samples in the OCTAD database we identified the 50 samples whose gene expression profiles correlated best with BRAFi resistant samples. Then by comparing these two groups of samples 191 differentially expressed genes that are included in the LINCS 978 landmark genes were identified (log2 fold-change >1 and adjusted p-value < 0.001). We subsequently used this set of genes as a resistance signature and used the sRGES method as described in the Materials and Methods section to identify compounds which reverse this expression signature. To this end, we identified 245 compounds with a sRGES score lower than -0.3, the cutoff for meaningful reversal of the resistance signature.

We next wanted to benchmark our computational predictions against known compound sensitivity data (Fig. S2). One of the cell lines used in our analysis, UACC62P, was included in the CTRPv2 compound screening dataset. For the compounds in the CTRPv2 dataset that overlapped with the compounds included in our analysis we found a strong correlation (Spearman: -0.47, p-value: 1.6e-9) between the known drug sensitivity available in the CTRPv2 dataset and the predicted reversal score (Fig. S3).
The goal of the computational screen (Fig. 1A) in this study was to identify compounds that suppress BRAFi-resistant melanoma cells. After filtering the compound list (Supplemental Data 2) to only include compounds that have an sRGES score < -0.3, which indicates reversal of the transcriptional signature of BRAFi-resistant cells, we applied several additional filtering metrics to further refine the compound list. First, we removed all compounds that did not have a well-annotated mechanism of action or are broadly cytotoxic chemotherapeutic agents. In total, 71 of these compounds passed these filtering criteria. Compounds with a promiscuous mechanism of action, such as sertindole, or compounds with molecular targets is not expressed at an appreciable level, such as CGP-71683, were then removed. Finally, compounds where the molecular target was known to be important in BRAFi resistance (18 of the top 40) were also excluded from further study, since our goal was to identify new potential targets and learn new biology about BRAFi resistance mechanisms (Supplemental Data 2). Our approach did, in fact, identify numerous compounds previously implicated in overcoming BRAFi resistance, including latrunculin-b and dasatinib, highlighting the effectiveness of this computational strategy. Ultimately, we selected nine compounds, not previously implicated in BRAFi resistance, for further experimental validation. These compounds include: targinine, pyrvinium, GR-127935, lestaurtinib, luteolin, BVT-948, ketoprofen, clofilium, and ibrutinib.

The nine selected compounds that reversed the BRAFi resistance gene expression signature in silico (Fig. 1B/Supplemental Data 2) were examined for their ability to inhibit growth of matched parental and BRAFi-resistant melanoma cell lines. Four of these compounds, Pyrvinium, BVT-948, Clofilium, and GR-127935, reduced cell viability in both M229P and M229R cells, with no apparent selectivity for one over the other (Fig. S3). This lack of selectivity is likely because both the parental and resistant cells were compared to normal tissue, instead of being directly compared against each other. Next, we created a gene expression resistance signature consisting of 87 genes by comparing the gene expression data from the resistant cell lines with BRAF\textsupscript{V600E}-mutant primary melanoma tumor samples in the OCTAD database. The expression signature is associated with poor overall survival in melanoma patients with BRAF\textsupscript{V600E} mutations (p = 0.006, Cox model), but not with BRAF\textsuperscript{WT} melanoma patients (p = 0.028), suggesting that this gene expression signature may be clinically relevant (Fig. S4). Using the improved BRAFi resistance signature, 3 of the 9 selected compounds (ibrutinib, pyrvinium, and lestaurtinib) were among the top 5% of compounds identified, with ibrutinib being the most effective in reversing the BRAFi resistance signature (Fig. S5). These findings solidified our prioritization of ibrutinib in further studies.

Ibrutinib re-sensitizes BRAFi-resistant cells to vemurafenib.

We reasoned that compounds which reverse a BRAFi resistance gene expression signature should also reverse BRAFi resistance in melanoma cells in an experimental setting. To test this hypothesis, we profiled the synergy between vemurafenib and the top 9 hits from the computational screen in a 14x7 concentration response matrix with vemurafenib to identify compounds that can potentiate vemurafenib response. Of the 9 selected compounds, many had single agent activity (Fig. S6), but only ibrutinib reversed BRAFi resistance (Fig. 1C, red curves). This low target validation rate (1/9) may be because our filtering criteria excluded compounds whose annotated mechanisms were already known to be associated with BRAFi resistance.

Even though our computational screen was performed using RNA-Seq data from all three isogenic parental and resistant cell line pairs, only M229R showed synergistic re-sensitization to vemurafenib by ibrutinib in our 72h cell viability assay. Importantly, synergistic growth inhibition was also observed in a long-term colony formation assay (Fig. S7). Since BRAF inhibitors are known to arrest melanoma cells at the G1 checkpoint, we reasoned that if ibrutinib is truly re-sensitizing the resistant cells to vemurafenib it should also re-sensitize the cells to vemurafenib-induced G1 arrest. As expected, we found by cell cycle analysis, that M229P cells accumulate in G0/G1 state during vemurafenib treatment whereas M229R cells do not. Consistent with re-sensitization, when ibrutinib is combined with vemurafenib, M229R cells now accumulate in G0/G1 (Fig. 1D). There was also an increased level of Annexin V-positive cells in the combination-
treated group, although there was no change in Caspase 3/7 activity or PARP cleavage (Fig. S8). Taken together, these data suggest that ibrutinib re-sensitizes a subset of BRAFi-resistant cell lines to vemurafenib.

BTK deletion or inhibition does not re-sensitize BRAFi-resistant cells to vemurafenib.

Since ibrutinib is known to have targets other than BTK (Herman, Montraveta et al. 2017, Klaeger, Heinzlmeir et al. 2017, Patel, Balakrishnan et al. 2017) we wanted to know whether BTK was responsible for BRAFi resistance. To test this hypothesis experimentally, we generated BTK knock out cell pools using CRISPR. BTK mRNA expression is low in both M229P and M229R (Fig. S9) cells and protein is undetectable by immunoblotting, so we determined knockout efficiency by Sanger sequencing of gDNA amplicons which contain the region of the CRISPR cut site. The Sanger sequencing traces were subsequently de-convoluted with the TIDE algorithm (Brinkman, Chen et al. 2014) to identify the fraction of cells that had functional knockout (Fig. 2A /S10). Using this approach, we found that the functional knockout efficiency was approximately 70%. Even though ibrutinib is used clinically as a BTK inhibitor, deletion of BTK did not alter the vemurafenib response in either the parental or resistant cells (Fig. 2B). This suggested to us that ibrutinib may be re-sensitizing the cells through off-target inhibition of other kinases instead of by on-target inhibition of BTK. Since acalabrutinib is a BTK inhibitor analog of ibrutinib with reduced off-target activity (Herman, Montraveta et al. 2017, Patel, Balakrishnan et al. 2017), we asked whether acalabrutinib is capable of reversing BRAFi resistance. Acalabrutinib failed to re-sensitize BRAFi-resistant cells to vemurafenib (Fig. 2C), consistent with the lack of effect BTK deletion on BRAFi re-sensitization. Taken together, these data indicate that ibrutinib re-sensitizes BRAFi-resistant cells to vemurafenib independently from on-target BTK inhibition.

Transcriptional response to ibrutinib treatment.

To better understand how ibrutinib re-sensitizes BRAFi-resistant cells to vemurafenib we performed RNA-seq on M229R cells after treatment with vemurafenib, ibrutinib, acalabrutinib, or combinations. Consistent with the observation that ibrutinib, but not acalabrutinib, re-sensitizes BRAFi-resistant cells to vemurafenib we found that 101 genes were differentially expressed (FDR < 0.01) with ibrutinib treatment while there were no differentially expressed genes with acalabrutinib treatment (Fig. 3A) compared with untreated M229R cells. Compared to single agent treatment, there was a synergistic induction of differential gene expression with vemurafenib combined with ibrutinib. Also, the combination of vemurafenib and ibrutinib significantly reversed the BRAFi resistance signature used in our compound sensitivity predictions (Spearman correlation = -0.25, p-value = 0.0007) (Fig. 3B). We then identified networks of differentially expressed genes in cells cultured in the presence of ibrutinib or the combination of vemurafenib and ibrutinib. With either single agent ibrutinib or the combination of ibrutinib and vemurafenib, the gene networks were primarily associated with development of various organs (Fig. S11). To understand the effect of ibrutinib on melanoma cells in greater detail, we profiled transcriptional regulators that are predicted to be altered in cells cultured with ibrutinib or the combination of ibrutinib and vemurafenib using Landscape In Silico deletion Analysis (LISA) (Qin, Fan et al. 2020) to identify transcription factors which may contribute to the differential gene expression in compound-treated cells. Among the top transcription regulators identified were YAP1 and two transcription factors, TEAD1 and TEAD4, which are bound by YAP1 (Fig. 3C). This enrichment was observed in genes that are both downregulated and upregulated by ibrutinib treatment. YAP1, in addition to its canonical role as a transcriptional co-activator, can also function as a transcriptional repressor(Kim, Kim et al. 2015). Thus, this is consistent with the idea that ibrutinib targets both YAP1-mediated gene induction and repression. (Kim, Kim et al. 2015).

We reasoned that inhibitors with the same functional target as ibrutinib should have a similar transcriptional signature to ibrutinib. To address this, we compared the gene expression signatures of ibrutinib- and vemurafenib-treated cells to the signatures of other compounds in the Connectivity Map (Cmap) dataset. SRC inhibitors had a highly similar transcriptional signature to that of ibrutinib (Fig. 3D). This observation is interesting since ibrutinib, but not acalabrutinib, has off-target activity against multiple SRC family kinases (SFKs) We compared the kinase gene expression based upon our RNASEq data in M229P/R with the experimental Kd values of ibrutinib and identified YES1 and SRC as potential off targets of ibrutinib. (Fig. S12) (Herman, Montraveta et al. 2017, Patel, Balakrishnan et al. 2017). As further support that the method that we employed, we also performed the same comparison with vemurafenib-treated cells and found high
similarity with BRAF and MEK inhibitors in the Cmap dataset, which is consistent with the pharmacology of vemurafenib. Together, these results suggested to us that ibrutinib may alter YAP1 activity through targeting of SRC and/or other SRC-family kinases.

**Ibrutinib reduces the nuclear accumulation of YAP1.**

YAP1 has been previously implicated in BRAFi resistance (Hugo, Shi et al. 2015, Lin, Sabnis et al. 2015, Kim, Kim et al. 2016, Fisher, Grun et al. 2017, Misek, Appleton et al. 2020), so it is critical to determine whether ibrutinib acts through altering YAP1 activity. Transcriptionally inactive YAP1 is sequestered in the cytosol and upon various stimuli YAP1 can translocate into the nucleus where it modulates gene transcription, often in partnership with TEAD transcription factors. As we previously demonstrated (Misek, Appleton et al. 2020), M229R cells have an increased nuclear/cytosolic ratio of YAP1 localization. The precise mechanism by which YAP1 is activated in these cells is still unclear. Consistent with our computational predictions in M229R cells which are resensitized to vemurafenib by ibrutinib, ibrutinib reduced the proportion of cells with nuclear YAP1 localization, whereas acalabrutinib had no effect on YAP1 localization (Fig. 4A/B). These data further support that ibrutinib mediates resensitization through a BTK-independent mechanism, involving YAP1.

Our data showed some efficacy of ibrutinib as a monotherapy in both UACC62P and UACC62R cells, but little effect on sensitization to vemurafenib, suggesting that ibrutinib is unlikely to be targeting vemurafenib resistance mechanisms, like YAP1 in those cells. Accordingly, ibrutinib, as well as acalabrutinib, had no effect on YAP1 localization in M238R or UACC62R cells (Fig. 4C/D). It is possible that YAP1 could be regulated through other mechanisms in these cells, such as crosstalk with the actin cytoskeleton. It is also possible that SRC-mediated phosphorylation of the YAP suppressor LATS1/2 could be important in M229R cells, but not UACC62R or M238R cells (Oka, Mazack et al. 2008). We also observed an increase in the fraction of cells with predominantly nuclear localization of TAZ, another TEAD coregulator, in all three resistant cell lines but neither ibrutinib nor acalabrutinib altered TAZ localization (Fig. S13). While there are likely additional targets of ibrutinib in melanoma cells, our data provide evidence that in a subset of BRAFi-resistant cells, off target activity of ibrutinib, independent of BTK, can alter YAP, which may contribute to resensitization to BRAFi treatment.

**Discussion:**

In this study we used an unbiased computational approach to identify compounds that reverse a gene expression signature associated with melanoma BRAFi resistance. Through this approach, we have identified a role for ibrutinib in resensitizing a subset of melanoma cells with acquired BRAFi resistance to vemurafenib.

Ibrutinib is an oral BTK inhibitor approved for use in chronic lymphocytic leukemia and mantle cell lymphoma. The lack of efficacy of acalabrutinib, a recently approved, more selective BTK inhibitor, or genetic ablation of BTK, on vemurafenib resensitization demonstrates that off target inhibition of another kinase(s) underlies the effect. Further investigation supports the notion that ibrutinib-mediated resensitization to vemurafenib, may occur through inhibition of Src family kinases. The transcriptomic signature of ibrutinib-treated cells is similar to that of cells treated with SRC family kinase inhibitors. Other studies have also identified a role for SFKs in BRAFi resistance (Girotti, Pedersen et al. 2013, Fallahi-Sichani, Becker et al. 2017, Feddersen, Schillo et al. 2019), further supporting the idea that off-target anti-SFK activity of potential melanoma therapeutics may be mechanistically important. One study in particular characterized a novel dual RAP/SRC inhibitor which retains activity against melanoma tumors which had previously developed resistance to dabrafenib/trametinib therapy (Girotti, Lopes et al. 2015). It is also conceivable that ibrutinib off-targets, other than SFKs, may also be important in melanoma.

In our previous work we found that a subset of BRAFi-resistant melanoma cells an accumulation of YAP1 in the nucleus. The precise mechanism by which YAP1 is activated in these cells is still unclear but may be the result of autocrine/paracrine signaling, cell-intrinsic signaling/epigenetic alterations, or differences in cell-to-cell contact or cell density. Consistent with the idea that changes in YAP1 localization control activity, we found that nuclear
accumulation, which renders YAP1 transcriptionally active, is prevented by ibrutinib, but not acalabrutinib (Basu, Totty et al. 2003). Several lines of evidence point to a critical role for SFKs in modulation of YAP1 activation. For example, YES1, a SFK that can be inhibited by ibrutinib (Klaeger, Heinzlmeir et al. 2017), phosphorylates and activates YAP1 (Rosenbluh, Nijhawan et al. 2012). Other SFKs including LCK, as well as SRC itself, have also been demonstrated to modulate YAP1 activation (Lamar, Xiao et al. 2018, Sugihara, Werneburg et al. 2018), suggesting that modulation of YAP1 activity could be a general feature of SFKs. Consideration of the effect of ibrutinib on YAP1 localization, as well as the established role of YAP1 in BRAFi resistance, leads us to propose a model in which ibrutinib reverses resistance by reducing the nuclear accumulation of YAP1. It is certainly possible that other mechanisms contribute to ibrutinib’s reversal of BRAFi resistance. For example, while we saw no effect on TAZ localization, modulation of other gene regulatory factors could be important. In (Fig. 3C) RELA activity was predicted to be altered by ibrutinib; in (Supplemental Data 2) multiple RELA/NF-κB inhibitors were predicted to reverse a BRAFi resistance signature. So, it is possible that ibrutinib could reverse BRAFi resistance by modulating NF-κB.

Understanding the polypharmacology of ibrutinib is crucial effectively re-purposing ibrutinib, an FDA approved drug, or related SFK inhibitors for the treatment of BRAFi-resistant melanoma. A clinical trial testing ibrutinib as a single agent therapy in patients with treatment-refractory metastatic melanoma (NCT02581930) is currently ongoing. Based on our findings that ibrutinib treatment alone is ineffective in BRAFi resistant or BRAFi-naïve (Fig. S14) melanoma cell lines, we would not expect strong efficacy of ibrutinib as a single agent in the clinic, at least through cancer cell autonomous mechanism. However, our data do suggest that ibrutinib may re-sensitize a subset of resistant melanoma to BRAF inhibitors, so combining ibrutinib with BRAF inhibitors may be more efficacious than monotherapy. Beyond melanoma, ibrutinib is used clinically to treat mantle cell lymphoma and chronic lymphocytic leukemia, and YAP1 has been implicated in both diseases (Byrd, Furman et al. 2013, Wang, Rule et al. 2013). Suggesting that the findings from this study may also be important in understanding differences in treatment response in these patients as well. It would be important to identify biomarkers, perhaps related to YAP1 activity or nuclear localization that would predict activity of SFK inhibition in BRAFi-resistant melanomas.

In this study we demonstrate that ibrutinib re-sensitizes a subset of BRAFi-resistant melanoma cells to vemurafenib. Mechanistically, we propose a model in which off-target SFK inhibition results in decreased YAP1 activity. Although, this model is likely incomplete and more experimental validation will need to be performed to fully understand how ibrutinib reverses BRAFi resistance. The translational potential of this research is increased by the fact that ibrutinib is already FDA-approved, and thus can be used off-label for other indications. These data would suggest that ibrutinib or other SFK inhibitors, many of which are already FDA-approved, could have utility in BRAFi/MEKi-resistant melanoma tumors, as well as other YAP1-driven cancers.

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Authorship Contributions:

Participated in research design: Misek, Newbury, Chen, Gallo, Neubig

Conducted experiments: Misek,

Contributed new reagents or analytical tools: Doseff, Chen,

Performed data analysis: Misek, Newbury, Chekalin, Paithankar,

Wrote or contributed to the writing of the manuscript: Misek, Newbury, Chekalin, Chen, Gallo, Neubig
References:


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Figure Legends:
Figure 1. Ibrutinib re-sensitizes BRAFi-resistant cells to vemurafenib. A. The unfiltered in silico compound library contains 12,441 compounds that span a diversity of chemotypes and molecular targets; many of these compounds are FDA approved or at one point entered clinical trials. Compounds were first filtered to only include compounds that significantly reverse a BRAFi resistance signature (sRGES < -0.3), resulting in 214 compounds that passed this cutoff. These compounds were further filtered to remove compounds that are broadly cytotoxic and to remove compounds lacking a well-annotated mechanism of action. From the resulting ranked list of 71 compounds we selected 9 compounds whose primary target had not been previously implicated in BRAFi resistance for further experimental validation. These compounds were selected primarily based upon the mechanism of action, with the goal of identifying new biology underlying BRAFi resistance. B. The BRAFi-resistance signature was computed by comparing BRAFi-resistant cell lines and normal tissue samples. Red boxes indicate upregulated genes, and blue boxes indicate downregulated genes. Loxoprofen was included as a control since this compound was not predicted to reverse the BRAFi-resistance signature. For compounds with multiple gene expression profiles, the profile with the median RGES was chosen for visualization. The sRGES values for the BRAFi-resistance signature and the compound-treated signatures are listed above the heatmap. A negative sRGES score indicates reversal of the BRAFi resistance signature by the indicated compound. C. M229P/R, UACC62P/R, and M238P/R cells were treated in a dose response matrix of ibrutinib (top concentration 10 µM, ½ dilution series) and vemurafenib (top concentration 10 µM, ½ dilution series). After 72 h, viability was measured with CellTiter-Glo. (n = 3 biological replicates) D. M229P/R cells were treated with -/+ 2 µM vemurafenib and -/+ 1 or 5 µM ibrutinib for 72 h. The cells were stained and analyzed by flow cytometry as described in materials and methods (n = 3 biological replicates). Significant differences of G0/G1 for compound treated samples vs the relevant DMSO control are indicated (One-way ANOVA, * p < 0.01 vs M229P-DMSO, # p < 0.01 vs M229R-DMSO).

Figure 2. BTK deletion or inhibition does not alter vemurafenib sensitivity. A. M229P/R BTK\textsuperscript{K0} cells were generated as described in Materials and Methods. Sanger sequencing was performed to measure the extent of BTK deletion in M229P/R cell pools. The fraction of cells with functional BTK deletion was quantified with TIDE (n = 3 biological replicates).
replicates)/ B. M229P/R sgControl and sgBTK cells were treated with 14 concentrations of vemurafenib (10 µM top concentration, ½ dilution series) and, after 72 h, viability was measured with CellTiter-Glo as described in Materials and Methods. (n = 3 biological replicates) C. M229P/R cells were treated with 7 different concentrations of acalabrutinib (10 µM top concentration, ½ dilution series) and 14 different concentrations of vemurafenib (10 µM top concentration, ½ dilution series). After 72 h, viability was measured with CellTiter-Glo (n = 3 biological replicates).

Figure 3. Transcriptional response to ibrutinib and vemurafenib treatment in BRAFi resistant cells. A. M229R cells were treated with DMSO, vemurafenib (Vem) (2 µM), ibrutinib (Ibrut) (5 µM), acalabrutinib (Acala) (5 µM), or the combinations as indicated. After 24 h RNA was extracted and RNA-Seq was performed as described in the materials and methods. The number of differentially expressed genes compared with DMSO control treated cells is shown for each treatment condition. B. A heatmap of the BRAFi resistance signature is shown in leftmost column and impact of compound treatments on reversal of BRAFi signature gene expression. For each treatment condition (A=Acala; I=Ibrut, V=Vem), the fold change in gene expression was compared to the DMSO control. The median expression value for each gene from three biological replicates was used. For each treatment group the fold change in gene expression was compared to the DMSO control. Red boxes indicate that the gene is upregulated, and blue boxes indicate that the gene is downregulated. Of all treatments, vemurafenib + ibrutinib significantly reversed the BRAFi resistance signature (Spearman correlation = -0.25, p-value = 0.0007). C. LISA analysis of differentially expressed genes in the ibrutinib and vemurafenib + ibrutinib treatment groups for prediction of transcriptional regulators. Data analysis was performed as described in Materials and Methods. X- and Y-axis values are enrichment p-values. Highly predicted transcription regulators are indicated with YAP1 and its transcriptional partners, TEAD1 and TEAD4, indicated as red dots. D. Similarity scores for CMap class analysis was performed as described in Materials and Methods. Transcriptional signatures of ibrutinib, vemurafenib, or vemurafenib + ibrutinib were compared to transcriptional signatures in the Cmap dataset.

Figure 4. Ibrutinib blocks YAP1 nuclear localization. All cells were treated with Ibrutinib or Acalabrutinib at 5 µM or vehicle control for 24 has indicated prior to being fixed and stained. A. M229P/R cells were stained with an anti-YAP1 antibody as described in the materials and methods section. The percentage of cells with nuclear, cytosolic, or pan-cellular YAP1 localization was quantified as described in the materials and methods section. B. Representative images from the experiment in Fig 4A. C. M238P/R or D. UACC62P/R cells were stained with an anti-YAP1 antibody as described in the materials and methods section. The percentage of cells with nuclear, cytosolic, or pan-cellular YAP1 localization was quantified as described in the materials and methods section. Statistical analysis (one-way ANOVA) was performed on % of cells with nuclear YAP1 localization where p < 0.01 was considered statistically significant. Bars marked with # indicate a statistically significant difference when compared with DMSO-treated parental cells and bars marked with * indicate a statistically significant difference when compared with DMSO-treated resistant cells (n = 3 biological replicates for all imaging experiments).
Figure 2

(A) Knockout Efficiency (%)

(B) Relative Viability vs. [Vemurafenib], M

(C) M299P and M299R with different Acalabrutinib concentrations.