A multi-targeted probe-based strategy to identify signaling vulnerabilities in cancers
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ABSTRACT
Most cancer cells are dependent on a network of deregulated signaling pathways for survival and are insensitive, or rapidly evolve resistance, to selective inhibitors aimed at a single target. For these reasons, drugs that target more than one protein (polypharmacology) can be clinically advantageous. The discovery of useful polypharmacology remains serendipitous and is challenging to characterize and validate. In this paper we developed a non-genetic strategy for the identification of pathways that drive cancer cell proliferation and represent exploitable signaling vulnerabilities. Our approach is based on using a multi-targeted kinase inhibitor, SM1-71, as a tool compound to identify combinations of targets whose simultaneous inhibition elicits a potent cytotoxic effect. As a proof-of-concept, we applied this approach to a KRAS-dependent non-small cell lung cancer (NSCLC) cell line, H23-KRASG12C. Using a combination of phenotypic screens, signaling analyses and kinase inhibitors, we found that dual inhibition of MEK1/2 and insulin-like growth factor 1 receptor (IGF1R)/insulin receptor (INSR) is critical for blocking proliferation in cells. Our work supports the value of multi-targeted tool compounds with well-validated polypharmacology and target space as tools to discover kinase dependencies in cancer. We propose that the strategy described here is complementary to existing genetic-based approaches, generalizable to other systems, and enabling for future mechanistic and translational studies of polypharmacology in the context of signaling vulnerabilities in cancers.

INTRODUCTION
Over the last two decades cancer treatment has been revolutionized by a targeted approach to therapy in which a selective agent is developed to hit a single, specific target. Prominent examples of targeted therapies include selective kinase inhibitors that target BCR-ABL in Chronic Myelogenous Leukemia (CML) or mutant EGFR and EML4-ALK in non-small cell lung cancer (NSCLC) (1). Unfortunately, in some tumor types this approach is limited by the rapid emergence of drug resistance; in other cancers with multiple or conventionally undruggable driver mutations, targeted approaches can be hard to apply. Many tumors are genetically heterogeneous, harboring multiple genomic alterations in different combinations, which results in signaling plasticity and rapid evolvability. These properties of tumors have generated interest in developing drugs capable of simultaneously inhibiting multiple signaling pathways. Such inhibitors, often described as “polypharmacological agents” have long been utilized therapeutically in CNS diseases, infection, inflammatory diseases and psychiatric disorders, where selective inhibitors have failed (2,3). Examples of approved polypharmacological drugs include acetyl salicylic acid, paracetamol, clozapine, etc., that act by binding and interacting with several proteins, thereby exerting pharmacological effects that cannot be ascribed to a single molecular target (2).

In the field of kinase inhibitors, many approved drugs were developed with a particular target in mind but are now known to be multi-targeted. Their multi-targeting properties are a consequence of their binding mode. Kinase inhibitors have been developed to bind in conserved ATP-binding pockets and therefore have cross-reactivity towards other kinases with shared structural features in their active sites. The multi-targeted nature of most approved kinase inhibitors has now been confirmed and characterized through kinome-wide profiling technologies (4-10). The polypharmacology of kinase inhibitors has resulted in the discovery of new
indications for particular compounds. For example, imatinib was initially developed as a BCR-ABL inhibitor for the treatment of CML but its activity against c-KIT/PDGFR allowed it to be a successful drug for the treatment of gastrointestinal stromal tumors (GIST) (11,12). Similarly, crizotinib was developed as a MET inhibitor but its activity against ALK resulted in its approval for the treatment of EML4-ALK positive NSCLC. In several cases, kinase inhibitor polypharmacology has been shown to be important for anti-cancer activity. For example, the ability of ibrutinib to simultaneously inhibit BTK and HCK makes it a superior drug for treatment of Waldenstrom’s Macroglobulemia (WM) as compared to highly selective BTK inhibitors (13). Similarly, sorafenib, originally developed as a B-RAF inhibitor, is now known to target numerous other kinases including VEGFR, PDGFR, RET, DDR1/2 and FLT3 (4) leading to its later approval as a multikinase inhibitor for renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC) (14). Similarly, rationally designed inhibitors that simultaneously target bromodomains and kinases have shown superior potency compared to a single target inhibition (15). A recent report suggests that >60% of FDA-approved kinase inhibitors, including sorafenib, dasatinib, pazopanib, ponatinib, exert their mechanism in a multtargeted manner by targeting at least three or more kinases (16).

With a better understanding of molecular mechanisms governing tumor growth and progression, efforts are underway to rationally design drugs with precision polypharmacology. The most promising efforts combine structure-based analysis with medicinal chemistry campaigns to identify pharmacophores that potently inhibit two or more kinases. Using this approach, Apsel et al. (17) developed inhibitors that simultaneously target PI3K and tyrosine kinases to overcome resistance mediated by activation of one or the other signaling kinases. In a study that combined phenotypic- and target-based drug discovery approaches, Dar et al. identified inhibitors with polypharmacological profiles that exerted potent activity in a RET-kinase driven Drosophila model bearing multiple endocrine neoplasia 2 (18).

A major challenge in rationally designing cancer drugs with polypharmacology is to identify the subset of kinases that must be simultaneously inhibited to induce potent anti-proliferative effects in a particular tumor type. One way to address this is to conduct systematic phenotypic screens using drug combinations and/or gene knockout techniques (19-24). This approach is complicated by the difficulty of achieving simultaneous knock-down or knock-out of multiple targets in a single cell (such multi-gene knockouts are often lethal). In this study, we demonstrate an alternate strategy that uses a multi-targeted kinase inhibitor, SM1-71, with well-characterized polypharmacology as a chemical tool to investigate signaling vulnerabilities in cancer cells. As a proof-of-concept, we explored signaling vulnerabilities in a KRAS mutant NSCLC cell line, H23-KRASG12C, and demonstrated that dual inhibition of MEK1/2 and IGF1R/INSR is required for anti-proliferative activity in these cells. Our work provides a framework for leveraging a multi-targeted kinase inhibitor with known polypharmacology to identify key signaling pathways driving tumor cells. This further lays the path for development of active compounds with desired polypharmacology or effective combination therapies.

**RESULTS**

**Investigating the cytotoxic effect of SM1-71 across multiple cancer cell lines**
SM1-71 is a diaminopyrimidine kinase inhibitor that potently targets kinases both through reversible binding in the ATP-binding site and irreversible binding promoted by reaction of the SM1-71 acrylamide moiety with cysteine residues (25,26) (Fig. 1a). We synthesized the reversible analog of SM1-71, SM1-71-R, that lacks the acrylamide warhead and is thus incapable of forming covalent bonds as a control compound for our studies of cellular effects of SM1-71 (Fig. 1a). We previously used chemical proteomic approaches to elucidate ~54 kinase targets of SM1-71 (Rao et al., Cell Chem Biol, accepted, 2019) (Table S1) and identified 24 kinases as exhibiting an IC50 value < 10µM (Table 1). Thirteen of these kinases have well-annotated functions in promoting cell growth and proliferation including YES1, SRC, MAP2K2 (MEK2), AURKA, MAP2K1 (MEK1), MAP3K1, MAPK1 (ERK2), MAPK3 (ERK1), WEE1, IGF1R, INSR, DDR1 and MET. Based on the number of pro-proliferative targets in this list, we sought to determine which of these were important for the anti-proliferative activities of SM1-71 using multiple cancer cell lines.

We screened SM1-71 against a panel of cancer cell lines having diverse genotypes and tumor origin. These cells included five NSCLC lines (H23, H460, H1975, HCC827, H3122), three triple negative breast cancer (TNBC) lines (MDA-MB-453, MDA-MB-468, MDA-MB-231) as well as colon cancer (HCT116), pancreatic PDAC (MiaPaca2) and melanoma (A375) cell lines (see Table 2 for genotypes). For comparative purposes, we profiled investigational and clinically approved kinase inhibitors against their described nominal targets including PI3K, MEK1/2, ERK1/2, EGFR, B-RAF, ALK, MET and IGFR1 (Table 3). Cells were plated and 24h later treated with varying doses of compounds for a period of 72h. The CellTiter Glo reagent was added to the plates, which were then analyzed for cytostatic or cytotoxic effects potentially induced by the drugs. To overcome confounding effects of varying division rates between cell lines on estimates of drug potency and efficacy, we used our recently developed Growth Rate (GR) corrected values (27,28). We use GR50 as a measure of potency (analogous to IC50) and GRmax as a measure of maximal efficacy (analogous to Emax; Supplementary File-1). A GRmax value between 1 and 0 corresponds to partial growth inhibition, a value of 0 indicates complete cytostasis and negative value denotes cell killing (27). The GR values reported in Table S2 were computed using the online GR Calculator (http://www.grcalculator.org; see methods, 29).

In 8 of 11 cell lines tested SM1-71 induced potent cytotoxicity with nanomolar values for GR50 and negative GRmax values (Fig. 1b, Table S2). SM1-71 was significantly more potent (the GR50 value was lower) across all cell lines tested than highly optimized inhibitors of MEK1/2 (AZD6244), PI3K (BKM120), ALK (ceritinib), EGFR (osimertinib), EGFR & HER2 (lapatinib), ERK1/2 (SCH772984) and BRAF ( vemurafenib) (p < 0.01; Fig. 1c). In 3 of 11 lines (H3122, H460 and MDA-MB-453), SM1-71 was comparatively less potent with GR50 ranging from 0.1 to 5 µM (Fig. 1b). Based on these data, eight lines were classified as SM1-71 sensitive and three as resistant (p = 0.0005, difference in potency between sensitive and resistant cell lines) (Fig.1d). These results indicate that SM1-71 is broadly active on cancer cell lines, and suggest that multi-targeted agents exhibit improved cytotoxicity compared to highly optimized inhibitors.

Elucidating kinases responsible for mediating cytotoxic effects in KRAS mutant cells
From our growth inhibitory screen, we identified several cancer cell lines with different genetic backgrounds that were highly sensitive to SM1-71, while showing resistance to inhibitors designed to target single kinases. To demonstrate that SM1-71 serves as an effective multi-targeted chemical tool compound for revealing key signaling pathways driving growth and proliferation, we chose a single sensitive cell line to carry out further experimental analyses. We were especially interested in elucidating druggable targets in KRAS mutant cells, since RAS mutations are found across different tumors and lack effective targeted therapy (30,31). We carried out all our analyses in H23-KRASG12C NSCLC cells, which we recently employed to generate a global map of kinases that SM1-71 binds to (Rao et al., Cell Chem Biol, accepted, 2019). To identify kinase targets of SM1-71 responsible for mediating cytotoxic effects in H23-KRASG12C cells, we measured the phosphorylation status of kinases involved in MAPK and PI3K signaling pathways, two effectors downstream of KRAS, using Western blotting (Fig. 2a, see Fig. S1 for blots from two independent experiments). H23-KRASG12C cells were treated with 1 µM of SM1-71 or SM1-71-R (reversible analog), for a period of 2h, followed by drug washout and replacement with fresh media. Cells were collected and lysed 0h, 2h and 4h post-washout to distinguish between transient and reversible and prolonged and potentially irreversible inhibition by SM1-71 (SM1-71 can covalently bind to kinases such as MEK1/2, ERK1/2, SRC, etc. (Rao et al., Cell Chem Biol, accepted, 2019). To further distinguish between reversible and irreversible inhibition we performed treatment-washout experiments using SM1-71-R, which cannot form covalent adducts. We found that SM1-71 was potent as an inhibitor of phosphorylation on p-AKT^S473 and p-ERK1/2^T202/T204 prior to washout (at t = 0h) (Fig. 2a). Inhibition of p-ERK1/2^T202/T204 was sustained 2h post-washout. SM1-71-R resulted in inhibition of phosphorylation of only p-AKT^S473. We observed a concomitant increase in p-MEK1/2^S217/S221 levels after 2h and 4h post-washout, which is common following ERK1/2 inhibition as a consequence of disrupting negative feedback regulation (32). We observed p-AKT^S473 and p-ERK1/2^T202/T204 levels rising 2h and 4h post-washout with SM1-71 and SM1-71-R. Several reports have previously shown that kinases within the MAPK and PI3K pathways are reactivated in response to specific inhibitors that suppress negative feedback loops (33-35). We thus predict a similar phenomenon responsible for the reactivation of p-AKT^S473 and p-ERK1/2^T202/T204 signaling upon treatment with SM1-71 and SM1-71-R.

So far, signaling analysis using western blotting demonstrated direct cellular inhibition of MAPK and PI3K signaling pathways. Our data also revealed inhibition of p-AKT^S473 (PI3K pathway), which was likely affected through inhibition of an upstream receptor and not directly by targeting PI3K or AKT or mTOR (non-targets of SM1-71, Table S1). To test this possibility, we profiled a panel of 49 RTKs using an RTK array (R&D Systems) in which phosphorylation of RTKs (and their inhibition in the presence of SM1-71) was measured by exposing cell lysates to capture antibodies spotted in duplicates (per RTK) on a nitrocellulose membrane (see Fig. S2 for dot-blot results from the two independent experiments). H23-KRASG12C cells were treated with 1 µM SM1-71 or DMSO for 6h and lysed, following which the lysate was incubated with the RTK arrays. Phosphorylation signals were quantified for both the SM1-71 and DMSO-treated samples using the dot-blot analyzer (ImageJ software) and fold-change was calculated. These fold-
change values were averaged across two independent experiments to generate an average fold-change signal (± SEM) for each RTK. These average fold-change values for IGF1R (80-fold), INSR (12-fold) and MET (5-fold) have been plotted as bar graphs (Fig. 2b) (p < 0.0001, compared to INSR and MET fold-change). Our results indicate that among the 49 RTKs profiled, SM1-71 potently inhibited IGF1R, INSR and MET. We conclude that SM1-71 is active on at least three RTKs known to lie upstream of the PI3K signaling pathway. Furthermore, we identified each of these three RTKs, IGF1R, INSR and MET as direct targets of SM1-71 from our previous study (Table S1) (Rao et al., Cell Chem Biol, accepted, 2019).

Validation of key targets driving proliferation in H23-KRASG12C cells
To determine whether inhibition of IGF1R/INSR and/or MET is involved in downregulation of p-AKT\textsuperscript{S473} levels, we attempted to phenocopy the effects using combinations of kinase inhibitors. The effects of 1 µM SM1-71 was compared to that of an ALK/MET inhibitor (1 µM crizotinib), IGF1R inhibitor (AEW541), ERK1/2 inhibitor (SCH772984), pan-PI3K inhibitor (BKM120) or DMSO. H23-KRASG12C cells were incubated with the compound for 4h and phosphorylation of downstream kinases was assessed using Western blotting (Fig. 2c, see Fig. S3 for blots from two independent experiments). We found that crizotinib reduced pMET\textsuperscript{Y1234/1235} phosphorylation to background levels, partially reduced pAKT\textsuperscript{S473} levels but had no discernable effect on pERK1/2\textsuperscript{T202/Y204} levels. AEW541 reduced pIGF1R/p-INSR\textsuperscript{Y1135/1136} levels and caused complete inhibition of pAKT\textsuperscript{S473}, also with no effect on pERK1/2\textsuperscript{T202/Y204} (Fig. 2c). This inhibition of IGF1R/INSR and/or MET in H23-KRASG12C cells can downregulate the PI3K pathway without affecting the activity of the MAPK pathway. In contrast, SM1-71 reduced not only pIGF1R/p-INSR\textsuperscript{Y1135/1136}, pMET\textsuperscript{Y1234/1235} levels, but also pERK1/2\textsuperscript{T202/Y204}. We therefore, asked whether inhibition of MEK1/2 in combination with IGF1R/INSR or MET would recapitulate the cytotoxicity observed with SM1-71. We found that AZD6244 (MEK1/2 inhibitor) and AEW541 were weakly cytostatic on their own (GR\textsubscript{50} = 0.5 µM, GR\textsubscript{max} between 0 and 1) but when combined, were 5-fold more potent (GR\textsubscript{50} = 0.08 µM) and also cytotoxic, as indicated by a GR\textsubscript{max} value of -0.4 (Fig. 2d, supplementary file-1). As previously mentioned, a negative GR\textsubscript{max} value is indicative of cytotoxicity. Moreover, the MEK-IGFR1 inhibitor combination of AEW541 plus AZD6244, MEK-ERK-PI3K triple inhibitor combination of AZD6244 plus SCH772984 plus BKM120 and SM1-71 were all similar in potency and cytotoxicity. In contrast, the MEK-MET inhibitor combination of AZD6244 plus crizotinib was only weakly cytotoxic (Fig. 2e, supplementary file-1). Based on these data we propose that MEK1/2 and IGF1R/INSR are critical drivers of growth and proliferation in H23-KRASG12C cells. Furthermore, these are targeted by SM1-71, that results in inhibition of proliferation and induction of cell death.

Distinct molecular mechanisms drive different tumor types
The multi-targeted nature of SM1-71 makes it a valuable tool to interrogate cancer cell signaling across different cell lines and tumor types. Having investigated pathways responsible for driving growth in a sensitive cell line (H23-KRASG12C), we wished to further apply SM1-71 to understand what might be mediating resistance in some other cell lines. From our growth inhibitory screen, we identified H3122, H460 and MDA-MB-453 cells to be slightly more resistant (sub-micromolar/micromolar GR\textsubscript{50} values
compared to nanomolar values for sensitive cell lines) to the action of SM1-71. H3122 is a NSCLC line harboring an EML4-ALK translocation in which exposure to ceritinib, an ALK inhibitor, strongly inhibits proliferation (GR$_{50}$ = 0.05 μM, GR$_{max}$ = -0.78, Fig. 3a, Table S2). However, SM1-71 binds only weakly to the ALK oncogenic driver (data not shown). The H460 NSCLC cell line and the MDA-MB-453 TNBC cell line both harbor E545K and H1074R mutations in the PIK3CA gene. ([https://cancer.sanger.ac.uk/cosmic](https://cancer.sanger.ac.uk/cosmic)). This mutation introduces an oncogenic driver downstream of the RTKs such as IGF1R/INSR, which activates the PI3K pathway. We therefore predicted that a pan-PI3K inhibitor such as BKM120 would make these cell lines sensitive to SM1-71. We found that the combination of SM1-71 plus BKM120 reduced the GR$_{50}$ for SM1-71 > 3-fold in both cell lines suggesting that resistance is possibly a consequence of PIK3CA mutation (Fig. 3b, c). Of note, both HCT116 and H1975 harbor H1074R and G118D PIK3CA mutations, respectively and were sensitive to SM1-71. This suggests that the PIK3CA mutation is not a sufficient oncogenic driver to confer SM1-71 resistance. Such cell-line specific differences are observed with many kinase inhibitors and arise from the specific signaling biology of the lines (36,37). However, since SM1-71 has multiple targets, we cannot fully exclude other mechanisms that might be contributing towards drug sensitivity and resistance overall effects of the compound.

DISCUSSION

Several challenges including complex signaling networks, crosstalk with the tumor microenvironment, and onset of drug resistance are associated with treating cancers driven by multiple oncogenes. It is thus increasingly appreciated that only use of drugs that affect multiple signaling nodes will result in strong anti-proliferative effects and delay the onset of drug resistance. Such compounds, referred to as polypharmacological drugs, have been investigated in the past to treat polygenic diseases such as cancers, CNS disorders and inflammatory disorders. Our current work aims to promote the rational development of such inhibitors by developing means to unravel key signaling pathways that must be simultaneously inhibited to achieve maximum anti-tumor effects. By utilizing a multi-targeted kinase inhibitor, SM1-71, that serves as an effective tool compound, we were able to elucidate molecular mechanisms driving specific cancer cell types. In our previous study, we used SM1-71 to interrogate the human kinome to identify cysteines that can be targeted, owing to the covalent nature of the compound (Rao et al., Cell Chem Biol, accepted, 2019). In the present study, we extended the use of this compound to the investigation of signaling pathways that drive cellular proliferation in cancer cells. As a proof-of-concept, we demonstrated the utility of this chemical tool by using the KRAS mutant NSCLC cell line, H23-KRAS$^{G12C}$, and identified MEK1/2 and IGF1R/INSR as being key players in driving cellular proliferation. We corroborated our findings by using clinical and investigational kinase inhibitors to induce pharmacologic shutdown of kinases. Our results validated that dual-inhibition of MEK1/2 and IGF1R/INSR led to downregulation of the MAPK and PI3K pathways, which is responsible for inducing potent cytotoxic effects in H23-KRAS$^{G12C}$ cells. Our findings are further supported by the knowledge that mutant KRAS leads to constitutive activation of its downstream effector pathways, MAPK and PI3K, and thus, inhibiting both arms of the oncogene leads to potent anti-tumor effects (38-40). Furthermore, preclinical and clinical studies focusing on the dual-inhibition of MEK1/2 and IGF1R/INSR have
demonstrated beneficial effects across different types of cancers (41-44). Our study does not unequivocally prove the pharmacologically relevant targets of SM1-71; indeed, the functionally relevant targets of this compound are likely different in different cell types.

It is well established that for drugs that target a single oncogene (e.g. EGFR, BCR-ABL, BTK, etc.), gene knockout (e.g. RNAi or CRISPR-Cas9) or rescue experiments following site-directed mutagenesis that prevents the drug from binding the kinase, are powerful techniques to functionally validate the on-target effect of the drug. However, in the case of polypharmacological agents acting on polygenic tumors, similar methods pose many challenges (3). An alternative strategy is to use combinations of selective inhibitors targeting kinases that phenocopy the effects induced by a polypharmacological inhibitor. Given the multi-targeted properties of SM1-71, we adapted a similar strategy to pharmacologically validate potential targets.

Our study also draws attention to the benefits of using a multi-targeted chemical probe compared to a selective one. Traditionally, most chemical probes are designed to retain selectivity and specificity towards a single target, in order to characterize its role in a given cell type. However, in our current study, we demonstrate that chemical probes with multiple targets can serve as powerful tools to interrogate oncogenic drivers in cancer cells, in lieu of systematic combinatorial screens. By using a combination of cell-based assays and pharmacologic inhibitors, we outlined a framework for adopting multi-targeted kinase inhibitors with defined polypharmacology as effective chemical probes. As a proof-of-concept, we conducted all our analyses in the sensitive H23-KRAS\(^{G12C}\) cells; however, a similar strategy can be adopted to interrogate other cell types. In fact, we demonstrated this by extending our evaluation towards three cell lines that were relatively resistant to SM1-71 in the growth inhibitory screen. Further investigation is required to ascertain these mechanisms as causes for the lowered activity of SM1-71 in these less sensitive cell lines. Nonetheless, direct pharmacologic inhibition of targets provides compelling evidence towards the mechanism of cytotoxicity in both sensitive and resistant cells. Together, these illustrate the generalizability of this approach and implies that such probes can serve as effective means to elucidate signaling pathways driving tumor cells.

Polypharmacological agents are also associated with several limitations when it involves improving their properties through systematic medicinal chemistry efforts. It can be extremely difficult to hone potency and selectivity towards two or more desired targets whilst simultaneously achieving selectivity and the desired pharmacokinetic and toxicological profile. A further challenge relates to finding suitable experimental models that can faithfully predict response in the clinic. For example, a simple cell proliferation assay may be able to predict response to a BCR-ABL inhibitor but may be a poor predictor for a kinase inhibitor such as SM1-71 that inhibits through multiple targets. Specifically, in the case of SM1-71, the ability of the compound to block PI3K-pathway signaling through RTK inhibition may not be accurately modeled in simple cancer cell proliferation assays with unnatural levels of growth factors. Given that polypharmacological kinase inhibitors will continue to be discovered, we propose that these multi-targeted inhibitors can serve as effective research tools to help unravel pathways that must be targeted. Their
application for path-finding and vulnerability-identification will allow the development of rational combinations as well as potentially aide rational design of tailored polypharmacology agents.

METHODS

Cell culture

Cell lines used in the study included NSCLC cells H23 (KRAS\textsuperscript{G12C}), H460 (KRAS\textsuperscript{Q61H}, PIK3CA\textsuperscript{E545K}), H1975 (EGFR\textsuperscript{L858R/T790M}/CDKN2A/PIK3CA\textsuperscript{G118D/T P53}), HCC827 (EGFR\textsuperscript{deIE746-A750}), H3122 (EML4-ALK rearrangement), triple negative breast cancer (TNBC) cells MDA-MB-453 (PIK3CA\textsuperscript{H1047R}), MDA-MB-468 (PTEN/RB1/SMAD4/TP53), MDA-MB-231 (KRAS/BRAF/p53/CDKN2A), colorectal cells HCT116 (KRAS\textsuperscript{G13D}/CTNNB1/CDKN2A/PIK3CA\textsuperscript{H1047R}), pancreatic cells MiaPaca2 (KRAS\textsuperscript{G12C}/CDKN2A/TP53) and melanoma cells A375 (BRAF\textsuperscript{V600E}/CDKN2A). All the NSCLC cell lines were a generous gift from Dr. Pasi Janne [Dana Farber Cancer Institute (DFCI), Boston, MA]. Cell lines were tested for mycoplasma contamination (Lonza MycoAlert Kit Cat. # LT07-318) and authenticated using short tandem repeat (STR) profiling (Molecular Diagnostics Laboratory, DFCI, Boston, MA). NSCLC cell lines were maintained in RPMI-1640 media (ATCC, 30-2001), TNBC, MiaPaca2 and A375 cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Corning: 10-013-CV) media and HCT116 cells were maintained in McCoy’s 5A media (ATCC: 30-2007). All media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin (30-002-CI, Corning). Cells were grown in incubators maintained at 37°C and 5% CO₂.

Chemical Synthesis

Detailed methodology on the synthesis of compounds has been previously described (25).

Growth inhibition assay

Cells were plated in 384-well plates (3764, Corning) at a seeding density of 2000 cells/well using the Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific). Cells were treated with different doses of compounds for 72h using the automated D300 digital dispenser (HP-D300 Digital Dispenser) and normalized to 0.2% DMSO, 24h or 48h post-plating. Viability was measured 72h after treatment by adding 25 µL/well of CellTiter Glo (G7572, Promega) and reading plates using the Synergy H1 microplate reader (Biotek). Each experiment was carried out in technical triplicate and biological duplicate.

Growth inhibition analysis using the online growth rate (GR) calculator

Growth inhibition across cell lines was analyzed using the online GR calculator (http://www.grcalculator.org/grcalculator_de_vy/) developed by members of the LINCS-BD2K Data Coordination and Integration Center, Harvard Medical School (Clark et al., 2017), which is based on the method originally described by Hafner et al (27). The GR metrics (GR\textsubscript{50} and GR\textsubscript{max}) along with their corresponding IC\textsubscript{50} values were calculated using the online GR calculator based on cell division rates obtained for different cell lines. To measure cell doubling time, cells were seeded at an initial count of 500,000 in a 10-cm dish and final count was measured 3-4 days (72-96h) later using the TC20-automated cell counter (Bio-Rad). The doubling time was calculated using the formula: \[ DT = T \ln(2)/\ln(X_e/X_b) \], where T is the time of growth (hours), X\textsubscript{e} is the final cell count and X\textsubscript{b} is the initial cell count. The following doubling times were used: A375
(19.2h), H1975 (31.8h), H23 (40.6h), H3122 (32.5h), H460 (20.7h), HCC827 (29.7h), HCT116 (20.4h), MDA-MB-231 (44.5h), MDA-MB-453 (41.8h), MDA-MB-468 (35.3h) and MiaPaca2 (31.3h). Data from the CellTiter Glo assay along with cell division rates were uploaded on to the online GR calculator that generated dose-response curves, GR50, GRmax values along with all other statistical parameters that can be found in Supplementary File-1. Codes used in the analysis can be made available upon request.

**Western Blotting**

H23-KRAS\textsuperscript{G12C} cells were grown in 10 cm dishes and treated with given concentrations of compounds or DMSO for (2-6h). For the washout experiments, cells were washed twice with PBS followed by the addition of fresh media. Cells were then lysed at different time points post-washout. In case of non-washout experiments, cells were lysed right after treatment following quick rinses with cold PBS. Lysis was carried out using 300 µL/dish of cold M-PER Mammalian Protein Extraction Reagent (78505, Thermo Fisher Scientific) substituted with 100X HALT protease and phosphatase inhibitor (final concentration 1X) (78446, Thermo Fisher Scientific). After 30 min of incubation on ice (with rocking), lysate was centrifuged at 14,800 rpm for 15-20 mins at 4ºC. The supernatant (protein extract) was collected and quantified using the Micro BCA Protein Assay Kit (23235, Thermo Fisher Scientific). Samples were prepared by adding 2X-4X Laemmli Sample Buffer (161-0737, 161-0747 Bio-Rad) substituted with 2-mercaptoethanol (5% v/v) (Sigma Aldrich) and boiling at 95°C for at least 10 mins. Gel electrophoresis was performed using Precast Protein Gels (4569034, 4569036 Bio-Rad) loaded with 10-20 µg protein/well. Proteins were transferred on to PVDF membranes (0.45 µm pore size, Novex Life Technologies). 5% non-fat dry milk (9999S, Cell Signaling Technology) in Tris Buffered Saline (TBS) substituted with 0.1% Tween (TBST) solution was used as the blocking buffer and to prepare antibody solutions. Buffers used included 10X running (Tris/Glycine/SDS buffer, 161-0772, Biorad), 10X transfer (35040, Thermo Fisher Scientific) and 20X TBS (sc-362305, Santa Cruz Biotechnology). We purchased antibodies from Cell Signaling Technology for the following: pMEK1/2-S221 (2338S - rabbit), MEK1/2 (9126S - rabbit), pERK1/2-T202/Y204 (4370S - rabbit), ERK1/2 (4696S - mouse), pMET-Y1234/1235 (3077S - rabbit), MET (3148S - mouse), pIGF1R/pINSR-Y1135/1136 (3024S - rabbit), IGF1R (9750S - rabbit), pAKT-S473 (4060S - rabbit), AKT (4685S – rabbit). Primary antibodies were diluted to a final concentration of 1:1000 and we used a 1:10,000 concentration for secondary antibodies (Cell Signaling Technology). We purchased Beta-actin antibody from Santa Cruz (sc-47778 - mouse), which was used at a concentration of 1:5000. We incubated blots with SuperSignal West Dura Extended Duration Substrate (34076, Thermo Fisher Scientific) and visualized them using the myECL imager (Thermo Fisher Scientific). Each experiment was carried out twice and representative blots from a single experiment are shown in Fig. 2a, c and both blots are shown in Fig. S1, S3.

**Receptor Tyrosine Kinase (RTK) array**

H23-KRAS\textsuperscript{G12C} cells were grown in 10 cm dishes and treated with 1 µM SM1-71 or DMSO for 6h, washed, extracted and lysed as described in the Western blotting section. Following protein quantification, 500 µg of protein/sample was used to carry out the phospho-RTK array analysis according to the protocol described by the manufacturer (ARY001B, R&D Systems). Henceforth, all incubation and wash steps were accompanied by end-to-end rocking. Briefly, each array...
was incubated with 2 ml of Array Buffer 1 for 1h at room temperature. After removal of this blocking buffer, 500 µg of sample diluted in 1.5 mL Array Buffer 1 was added to each array and incubated overnight at 4 ºC. Following washes with 1X Wash Buffer, arrays were incubated with 2 ml of anti-phospho-tyrosine-HRP antibody diluted in Array Buffer 1 for 2h at room temperature (RT). Wash steps were repeated, and arrays were visualized by adding 1:1 ratio of the SuperSignal West Dura Extended Duration Substrate and scanning them using the myECL imager. Phosphorylation signals obtained were mapped to their respective RTKs using the reference RTK coordinates included in the kit (blots for both experiments are shown in Fig. S2). The experiment was repeated twice and the phosphorylation signals for each RTK was quantified using the dot-blot analyzer macro on the ImageJ 1.50i software (code and documentation available on http://image.bio.methods.free.fr/dotblot.html). Each phospho-RTK signal had two representative spots on a given array. Fold-change was calculated for SM1-71 and DMSO-treated samples for each RTK and averaged across the two independent experiments. These fold-change values ± SEM were plotted as bar graphs for IGF1R, INSR and MET. The GraphPad Prism 7.0 software was used to generate graphs and carry out statistical analysis.

Statistical analysis
All statistical analyses were carried out using the GraphPad Prism 7.0 software. In Fig. 1c and Fig. 2b, statistical analysis was carried out using one-way ANOVA. In Fig. 1d, statistical significance between the sensitive and resistant cell lines was calculated using the two-tailed unpaired t-test (p = 0.0005).

Conflict of Interest
Nathanael S. Gray is a founder, science advisory board member (SAB) and equity holder in Gatekeeper, Syros, Petra, C4, B2S and Soltego. The Gray lab receives or has received research funding from Novartis, Takeda, Astellas, Taiho, Janssen, Kinogen, Voronoi, Her2llc, Deerfield and Sanofi. Peter K. Sorger is a founder, SAB member and equity holder in Merrimack Pharmaceutical and Glencoe Software; he is on the Board of Directors of Applied Biomath and the SAB of RareCyte Inc. In the last five years the Sorger lab has received research funding from Novartis and Merck. Sorger declares that none of these relationships are directly or indirectly related to the content of this manuscript. Marc Hafner is currently an employee of Genentech, Inc and declares no conflict of interest.

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TABLES

Table 1: List of kinases inhibited by SM1-71 (IC_{50} value < 10\mu M) in the Multiplexed Inhibitor Beads (MIB) assay* and their role in promoting proliferation

| Kinase | Binding | Pro-proliferative? |
|--------|---------|--------------------|
| YES1   | Covalent| YES                |
| SRC    | Covalent| YES                |
| MAP2K2 | Covalent| YES                |
| AURKA  | Reversible| YES              |
| MAP2K1 | Covalent| YES                |
| MAP3K1 | Covalent| YES                |
| MAPK1  | Covalent| YES                |
| MAPK3  | Covalent| YES                |
| WEE1   | Reversible| YES             |
| IGF1R  | Reversible| YES             |
| INSR   | Reversible| YES             |
| DDR1   | Reversible| YES             |
| MET    | Reversible| YES             |
| GAK    | Covalent| NO                 |
| AAK1   | Covalent| NO                 |
| LIMK1  | Covalent| NO                 |
| BMP2K  | Covalent| NO                 |
| TEC    | Reversible| NO              |
| PTK2   | Reversible| NO              |
| MARK2  | Covalent| NO                 |
| PRKD3  | Reversible| NO              |
| LYN    | Reversible| NO              |
| TGFR2  | Covalent| NO                 |
| TNIK   | Reversible| NO              |

*Identified and reported in Rao S et al, Cell Chem Biol, accepted, 2019

Table 2: Cell lines used in the growth inhibitory screens

| Cell Line | Tumor Type | Mutation            |
|-----------|------------|---------------------|
| H23       | Lung       | KRAS-G12C/TP53      |
| H358      | Lung       | KRAS-G12C           |
| H460      | Lung       | KRAS-Q61K, PIK3CA-E545K, CDKN2A |
| Compound   | Nominal Targets                                                      |
|------------|---------------------------------------------------------------------|
| H1792      | Lung  
|            | KRAS-G12C, TP53                                                      |
| Calu-1     | Lung  
|            | KRAS-G12C                                                            |
| Calu-6     | Lung  
|            | KRAS-Q61K, TP53                                                      |
| H1975      | Lung  
|            | EGFR-L858R/T790M, CDKN2A, PIK3CA-G118D, TP53                          |
| HCC827     | Lung  
|            | EGFR-delE746-A750                                                    |
| H3122      | Lung  
|            | EML4-ALK rearrangement                                               |
| HCT116     | Colon  
|            | KRAS-G13D, CTNNB1, CDKN2A, PIK3CA-H1047R                              |
| Mia-Paca-2 | Pancreas  
|            | KRAS-G12C, CDKN2A, TP53                                              |
| MDA-MB-231 | Breast  
|            | KRAS-G13D, BRAF-G464V, TP53, CDKN2A, NF2                              |
| MDA-MB-468 | Breast  
|            | PTEN, RB1, SMAD4, TP53                                                |
| MDA-MB-453 | Breast  
|            | PIK3CA-H1047R                                                        |
| A375       | Melanoma  
|            | BRAF-V600E, CDKN2A                                                    |

**Table 3**: List of clinical and investigational kinase inhibitors used in the study

| Compound   | Nominal Targets |
|------------|-----------------|
| AZD6244    | MEK1/2          |
| SCH772984  | ERK1/2          |
| BKM120     | pan-PI3K        |
| Osimertinib| EGFR            |
| Lapatinib  | EGFR/HER2       |
| Ceritinib  | ALK             |
| Vemurafenib| BRAF-V600E      |
| AEW541     | IGF1R           |
| Crizotinib | MET/ALK         |
Figure 1: Growth inhibitory screen across multiple cancer cell lines. (a) Chemical scaffold of SM1-71 (covalent inhibitor) and its reversible analog, SM1-71-R. (b) GR$_{50}$ and GR$_{\text{max}}$ values from two independent experiments representing growth inhibitory potency of SM1-71 across eleven different cancer cell lines with mutations in KRAS, EGFR, BRAF, ALK and PIK3CA. A negative GR$_{\text{max}}$ value represents cytotoxicity. A GR$_{\text{max}}$ value between 0 and 1 indicates partial growth inhibition and a value equal to 0 represents complete growth arrest. (c) Average growth inhibitory potency (GR$_{50}$) from two independent experiments, across eleven cancer cell lines for SM1-71 and kinase inhibitors targeting MEK (AZD6244), PI3K (BKM120), ALK (ceritinib), EGFR/HER2 (Lapatinib), EGFR (osimertinib), ERK (SCH772984) and BRAF$^{V_{600E}}$ (Vemurafenib). Statistical analysis was carried out using one-way ANOVA. (d) Comparing the growth inhibitory potency of SM1-71 (logGR$_{50}$) averaged from two independent experiments across eight sensitive (H23, H1975, HCC827, A375, HCT116, MDA-MB-231, MDA-MB468 and MiaPaca2) and three resistant (H3122, H460 and MDA-MB-453) cell lines. Cells were treated 24h post-plating for 72h. Statistical analysis was carried out using the two-tailed unpaired t-test across the logGR$_{50}$ values (p = 0.0005). All statistical analyses were performed using the GraphPad Prism 7.0 software. All GR$_{50}$ and GR$_{\text{max}}$ values represent the average of two independent experiments, carried out in technical triplicate.
Figure 2: Characterizing the mechanism of SM1-71-associated cytotoxicity in H23-KRAS\textsuperscript{G12C} cells. (a) Western blot analysis of phosphorylation of kinases in cells treated with 1 \( \mu \text{M} \) SM1-71 or SM1-71-R or DMSO for 2h, followed by drug-washout, replacement with fresh drug-free media and subsequent lysis 0h, 2h and 4h post-washout. Blot shown is from one of two independent experiments performed (see Fig. S1 for both blots). (b) Phospho-RTK inhibition following treatment with 1 \( \mu \text{M} \) SM1-71 for 6h. RTK array blots from two independent experiments were quantified and average fold-inhibition (± SEM) for SM1-71 and DMSO treated samples have been represented in the bar graph. Statistical significance was carried out using one-way ANOVA on GraphPad Prism 7.0. Dot blots for each array are shown in Fig. S2, and for each independent experiment (p < 0.0001). (c) Western blot analysis of pMET\textsuperscript{Y1234/1235}, pIGF1R\textsuperscript{Y1135/1136}, pAKT\textsuperscript{S473} and pERK1/2\textsuperscript{T202/T204} treated with 1\( \mu \text{M} \) of SM1-71, SCH772984 (ERK), BKM120 (PI3K), crizotinib (MET), AEW541 (IGF1R) or DMSO for 4h. Blot shown is from one of two independent experiments performed (please refer to Fig. S3 to see blots from both experiments). (d, e) Growth inhibition assay in H23-KRAS\textsuperscript{G12C} cells treated with SM1-71 or AZD6244 (MEK), AEW541, crizotinib and their equimolar combinations (GR\textsubscript{50} values indicated within brackets). Cells were treated 24h post-plating for 72h. The GR\textsubscript{50} values represent the average of two independent experiments carried out in triplicate. Each point on the growth inhibition curve represents the average of two independent experiments carried out in technical triplicate ± SEM.
Figure 3: Evaluating cell line-dependent polypharmacology associated with SM1-71. (a) Growth inhibitory potency of SM1-71 and ceritinib in the EML4-ALK positive H3122 NSCLC cells. Growth inhibitory potency induced by SM1-71, BKM120 (PI3K inhibitor) or their equimolar combination in the PIK3CA mutant (b) H460 NSCLC and (c) MDA-MB-453 TNBC cell lines. Each point on the growth inhibition curves represent the average of two independent experiments carried out in technical triplicate ± SEM. Average GR50 values from two independent experiments have been shown for Fig. 3a-c. (d) Proposed mechanism of action of cytotoxicity in H23-KRASG12C cells by simultaneously inhibiting IGF1R/INSR and MEK1/2. (f) Proposed mechanisms to enhance potency in resistant cells by targeting the driver oncogene EML4-ALK (mechanism 1) or by blocking activating PI3K signaling (mechanism 2).
A multi-targeted probe-based strategy to identify signaling vulnerabilities in cancers
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