Inhibition of activin signaling in lung adenocarcinoma increases the therapeutic index of platinum chemotherapy

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Resistance to platinum chemotherapy is a long-standing problem in the management of lung adenocarcinoma. Using a whole-genome synthetic lethal RNA interference screen, we identified activin signaling as a critical mediator of innate platinum resistance. The transforming growth factor–β (TGFβ) superfamily ligands activin A and growth differentiation factor 11 (GDF11) mediated resistance via their cognate receptors through TGFβ-activated kinase 1 (TAK1), rather than through the SMAD family of transcription factors. Inhibition of activin receptor signaling or blockade of activin A and GDF11 by the endogenous protein follistatin overcame this resistance. Consistent with the role of activin signaling in acute renal injury, both therapeutic interventions attenuated acute cisplatin-induced nephrotoxicity, its major dose-limiting side effect. This cancer-specific enhancement of platinum-induced cell death has the potential to dramatically improve the safety and efficacy of chemotherapy in lung cancer patients.

INTRODUCTION

Over one-third of lung cancers are adenocarcinomas, an aggressive malignancy that is frequently inoperable at diagnosis. Despite the introduction of targeted therapy and immunotherapy, most patients still receive platinum chemotherapy. Although platinum agents remain the cornerstone of treatment, responses remain below 30% (1). Therefore, strategies to improve the efficacy of chemotherapy could improve outcomes for many lung cancer patients. More than 147 potential mechanisms of platinum resistance have been proposed (2), thus presenting a far more complex problem than the expression of multidrug resistance proteins (2). Here, we report the results of a whole-genome synthetic lethal small interfering RNA (siRNA) screen for innate platinum resistance genes. Although the screen identified several genes that encode therapeutically targetable proteins, a network analysis revealed that activin signaling, part of the transforming growth factor–β (TGFβ) superfamily (3), is an important mediator of resistance. We further show that activin A and the related ligand growth differentiation factor 11 (GDF11) activate TGFβ-activated kinase 1 (TAK1), an SMAD-independent component of TGFβ signaling known to be a critical regulator of cell death (4). Finally, we show that inactivation of activin signaling with a small-molecule inhibitor or with the endogenous activin/GDF11-binding protein follistatin (FST) can overcome innate platinum resistance in vivo.

RESULTS

Optimization of an in vitro model of platinum-resistant lung adenocarcinoma

On the basis of the work of Hall et al. (5), we concluded that the instability of cisplatin in dimethyl sulfoxide (DMSO) made it unsuitable for a screen of this size, whereas carboplatin is stable in aqueous solution. We therefore assessed a series of human lung cancer cell lines for sensitivity to carboplatin in vitro (Fig. 1A). Taking into account that the peak plasma concentration of carboplatin in humans is reported as 30 to 50 μg/ml, and the lack of evidence that platinum compounds are concentrated within tumors (6), we classified A549, NCI-H358, NCI-H2009, and NCI-H727 as platinum-resistant adenocarcinoma cell lines (Fig. 1A).

We further assessed the well-characterized KRAS-mutant lung adenocarcinoma cell line A549, derived from a treatment-naïve patient,
Fig. 1. An in vitro siRNA screen for carboplatin synthetic lethality in lung adenocarcinoma cells. (A) Carboplatin median inhibitory concentration (IC50) values in lung cancer cell lines, classified as platinum-resistant or sensitive in vitro. n = 4, mean ± SEM. Dotted line indicates peak plasma concentration for carboplatin in humans. (B) Viability of A549 and LX22CL cells treated with a range of carboplatin concentrations in vitro. n = 4, mean ± SEM. Red dashed line indicates peak plasma concentrations for carboplatin in humans; black dashed line, concentration chosen for screen. (C) γH2AX immunofluorescence images in A549 cells treated with carboplatin for 24 hours, counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 5 μm. (D) Overview of the synthetic lethal screen methodology. (E) Results of a whole-genome siRNA screen for carboplatin synthetic lethality for each gene in order of sensitization index. The top 5% of hits are shown in red. Potentially targetable genes are listed. (F) Secondary screening of the top 230 hits, depicted as a sensitization index heat map. Deconvolution of the primary screen was performed using individual siRNAs (1 to 4) and pooled siRNAs targeting the same genes in HEK293 cells. (G) Western blot analysis of knockdown efficiency of ACVR1B, FKBP3, and MCL1 siRNAs orthogonal to the siRNAs used in the screen. (H) Viability of A549 cells treated with nontargeting (NT) or specific siRNAs in combination with a range of carboplatin concentrations in vitro. IC50 carboplatin concentrations are shown. n = 4, mean ± SEM.
as a model of innate chemoresistance. As shown in Fig. 1B, the platinum response curve from plotting cell viability as log_{10} on the y axis according to the method of Stewart (2) indicates that A549 cells exhibit active saturable resistance. By contrast, the small cell lung cancer (SCLC) cell line LX22CL (7) was highly sensitive (Fig. 1B). Detection of DNA damage in A549 cells occurred at carboplatin concentrations well below the I_{50} (Fig. 1C), suggesting that carboplatin export is not the predominant mechanism of innate resistance. On the basis of these data, we chose A549 cells as the model for our synthetic lethal screen.

A screen for carboplatin synthetic lethality in A549 cells

The screen was based on the method of Whitehurst et al. (8), using cell viability as an endpoint. Optimization was performed with siRNA targeting polo-like kinase 1 (siPLK1), a positive control inducing platinum-independent cell death, or mammalian target of rapamycin (siMTOR) as a control known to induce sensitization to platinum (fig. S1A) (2). A sublethal concentration of carboplatin (25 μg/ml) was chosen empirically (fig. S1A). These controls revealed that the screen performed within acceptable limits of variability, quality, and reproducibility determined by Z' factor analysis (fig. S1B) (8). The screen was then performed using the Dharmacon whole-genome siRNA library along with vehicle or carboplatin (Fig. 1D) generating a sensitization index, a measure of the capacity for a given siRNA increase in response to carboplatin, for all 18,178 genes (Fig. 1E). High-confidence hits were defined by the rank order of the sensitization index and the false discovery rate (FDR)–corrected P value (table S1).

The top 230 hits underwent deconvolution screening in A549 cells, and as pooled siRNAs in the immortalized human renal epithelial cell line human embryonic kidney (HEK) 293 cells, representing a noncancer control. Of these, 79.1% passed the deconvolution screening, defined as sensitization in response to three of the four individual siRNAs targeting each under the same conditions as the primary screen (Fig. 1F). By contrast, only 24.6% of the pooled siRNAs sensitized HEK293 cells to carboplatin. Orthogonal siRNA validation with siRNAs of unrelated sequence was then performed on three targets chosen arbitrarily, activin receptor 1B (ACVR1B), FK506-binding protein 3 (FKBP3), and myeloid cell leukemia 1 (MCL1). This demonstrated that knockdown of the target protein was associated with sensitization to carboplatin in vitro (Fig. 1, G and H). These data show that the screening methodology could reliably identify gene targets commonly expressed in lung adenocarcinoma.

Identification of candidate therapeutic targets

The Dharmacon library contains 4795 genes arbitrarily classified as “druggable” (9). Searching of online databases revealed that 36 of these genes coded for proteins that could be inhibited by large or small molecules, and 15 of these genes were previously implicated in mediating platinum resistance (table S2). Several candidate genes encode proteins that can be inhibited by compounds or antibodies in clinical use (table S2). Although these therapies may improve the response to platinum, combinatorial toxicity may represent a barrier to effective translation. We therefore pursued an alternative approach by looking for signaling pathways that coordinate innate platinum resistance.

Network analysis of platinum resistance genes

The top 5% of high-confidence hits were submitted for a network analysis using ClueGo/Cytoscape (Fig. 2) (10). Upstream, drivers of the platinum response were dominated by cytokines and mitochondrion-activated protein kinase (MAPK) signaling. Downstream, the predominance of DNA repair genes in the analysis is consistent with previous reports that removal of platinum adducts is a major factor in resistance (11). As shown in Fig. 2, signaling through TAK1, a member of the MAP3K family, appeared to be a critical signaling node strongly linked downstream to pathways mediating cell cycle arrest and DNA repair. Because TAK1 has been linked to the response to cellular stress, including the generation of reactive oxygen species, necrosis, and apoptosis (4), we explored potential strategies to disable this signaling node as a therapeutic approach.

TGFβ superfamily genes as mediators of innate platinum resistance

To further explore TAK1 as a mediator of innate resistance, we reanalyzed the results of the primary RNA interference screen to identify candidate genes related to the TGFβ signaling pathway. Figure 3A shows that knockdown of the TGFβ orthologs GDF11 and inhibin βA (INHBA), and their receptor heterodimers, enhanced the efficacy of carboplatin in vitro. GDF11 can activate both TGFβ and activin receptors (12) and has been implicated in the pathogenesis of hematopoietic failure (13) and sarcopenia (14). In the context of inflammation, INHBA homodimerizes to form activin A, a TGFβ superfamily ligand implicated in tissue injury, fibrosis, and cachexia (15). Although canonical TGFβ signaling has been implicated in epithelial cancers as a mediator of drug resistance, our screen did not identify the SMAD family transcription factors, which mediate the related phenotypes of stemness and epithelial-mesenchymal transition (16).

Western blot analysis of A549 cells treated with carboplatin in vitro revealed up-regulation of phospho-TAK1 (p-TAK1), as well as the 55-kDa activin A precursor and its active 25-kDa form, and an increase in the expression of the activin receptor ACVR1B (Fig. 3, B and C). Using an antibody that recognizes both GDF8 and GDF11 (14), we also saw increased expression of the 45-kDa precursor form. Although we observed a modest increase in the expression of the 67-kDa TGFβ precursor protein, we could not detect expression of the 12-kDa active form in response to carboplatin (Fig. 3B). In keeping with the data shown in Fig. 3A, carboplatin did not induce phosphorylation of SMAD2/3 (Fig. 3B and fig. S2A), thus confirming the results of the primary screen suggesting that the transcriptional signaling arm of the TGFβ superfamily does not play a role in mediating resistance in A549 cells.

To further validate TAK1 signaling as a mediator of platinum treatment resistance, we first showed that the TAK1 inhibitor LLZ-1640-2 (17) sensitized A549 cells to carboplatin in vitro (Fig. 3D). Interrogation of events downstream of TAK1 in this model supported the results of the primary screen by showing that LLZ-1640-2 blocked the phosphorylation of MAPK kinase 4 (MKK4) and P38 (Fig. 3E), as well as phosphorylation of MCL1 threonine 184/187, a P38 target site that mediates MCL1 stabilization (18). These data are supported by orthogonal siRNA validation of MCL1 (Fig. 1, G and H) and are consistent with a model in which TAK1 signaling mediates platinum resistance through MAPK signaling and stabilization of MCL1 (Fig. 3A). The importance of TAK1 as a mediator of platinum resistance was further supported in A549 cells by orthogonal siRNA directed at MAP3K7, the gene encoding TAK1 (Fig. 3F), and a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) knockout.
Fig. 2. Network analysis of the top 5% of hits from the primary screen in A549 cells. Each node represents an enriched pathway, and the size of each node reflects the statistical significance of the term. The thickness of the interconnecting lines reflects the number of interactions between each term. Colors represent broader categories, where all nodes that share the same color represent interrelated terms. IFN, interferon; TCR, T cell receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; JNK, c-Jun N-terminal kinase; TRAF1, TNF receptor–associated factor 1; NFκB, nuclear factor κB; cGMP, guanosine 3′,5′-monophosphate; DCC, dicyclohexylcarbodiimide; LDL, low-density lipoprotein; GPCR, G protein–coupled receptor; RIG-I, retinoic acid–inducible gene 1; MDAS, melanoma differentiation–associated gene 5; ISG15, interferon-stimulated protein, 15 KDa; FGFR, fibroblast growth factor receptor; CaM, calmodulin; IRS, insulin receptor substrate; ATM, ataxia telangiectasia–mutated.
Combined inhibition of ACVR1B and TGFBR1, TAK1 signaling, and platinum resistance

To precisely define the mechanism of TAK1 activation in response to carboplatin in lung adenocarcinoma cells, we used the small-molecule SB-505124, a potent and specific inhibitor of ACVR1B...
and TGFβR1 (19). Treatment with SB-505124 overcome resistance at clinically relevant concentrations of carboplatin without single-agent activity (Fig. 4A). In each case, robust up-regulation of either activin A or GDF8/11 was seen, as well as phosphorylation of TAK1 (p-TAK1), consistent with TAK1 activation in response to carboplatin (Fig. 4B). Consistent with the data presented in Fig. 3B, we did not observe phosphorylation of SMAD2/3 in response to carboplatin in these cell lines; however, in each case, the induction of MCL1 expression was inhibited or abolished (Fig. 4B and fig. S3A). By contrast, SB-505124 treatment did not enhance the effect of carboplatin in platinum-sensitive cell lines (fig. S4A). NCI-H1299 cells did not up-regulate p-TAK1 or MCL1 in response to carboplatin, whereas in NCI-H1975 cells, the modest TAK1 response was not blocked by SB-505124 (fig. S4B). In all three platinum-sensitive lines, we did not detect SMAD2/3 phosphorylation (fig. S3B).

Sensitization to cisplatin over a range of clinically relevant concentrations was also observed in A549 cells, demonstrating that this effect is seen with the two platinum agents most commonly used in the treatment of lung adenocarcinoma (fig. S4C). Similar results were seen with the alkylating agent busulfan, but not with doxorubicin, docetaxel, pemetrexed, or gemcitabine (fig. S4C). These results suggest that adduct-forming cytotoxic drugs such as cisplatin and busulfan specifically activate TAK1 downstream of ACVR1B/TGFBR1.

**Expression of TGFβ superfamily components in lung adenocarcinoma**

Analysis of The Cancer Genome Atlas (TCGA) lung adenocarcinoma data set (20) revealed that genes represented in our primary screen including the ligands INHBA, INHBB, GDF11, the receptors ACVR1B and TGFBR1, and their relevant heterodimerization partners were all highly expressed (Fig. 5A). Analysis of the KMPlot lung adenocarcinoma data set (21) showed that expression of GDF11 and its receptors TGFBR1 and ACVR1B was associated with a reduction in overall survival (Fig. 5B). In keeping with published data (22), expression of activin components INHBA and INHBB was also associated with poor outcome. (Fig. 5B). By contrast, expression of TGFβ1 was strongly associated with a favorable outcome, once again in keeping with published studies (23). By integrating these data with the results of our primary screen, we postulated that GDF11, acting through both TGFβ and activin receptors, and activin A, acting through activin receptors, might mediate innate platinum resistance in lung adenocarcinoma.

To further address the relationship of activin A and GDF8/11 expression with platinum resistance, we performed an immunohistochemical analysis of diagnostic core biopsy samples from 59 patients with stage IV lung adenocarcinoma without activating mutations in either EGFR or ALK treated with platinum-based chemotherapy (table S3). Expression of activin A, or coexpression of both activin A and GDF8/11, was associated with significant (P < 0.05) reduction in progression-free survival (Fig. 5, C and D, and fig. S5). These results suggest that activin signaling, driven by activin A and/or GDF11, plays an important role in mediating innate platinum resistance in lung adenocarcinoma.

**Inhibition of ACVR1B/TGFBR1 signaling, platinum resistance, and nephrotoxicity in vivo**

We next asked whether the ACVR1B/TGFBR1 inhibitor SB-505124 could increase the efficacy of carboplatin in an athymic nude mouse model. We chose the A549 cell line based on its engraftment efficiency, highly reproducible rate of growth in vivo, and resistance to a single dose of carboplatin (60 mg/kg) (fig. S6A), a dose that reliably induces a complete response in the LX22 SCLC xenograft model (fig. S6A). In keeping with our in vitro data, we also observed robust expression of both ACVR1B and TGFBR1 in A549 xenografts (Fig. 6A).

We administered SB-505124 to mice with A549 flank xenografts in combination with carboplatin according to the regimen depicted in Fig. 6B. As expected from our in vitro data, SB-505124 alone had no effect, but administration of SB-505124 with a single dose of carboplatin resulted in durable responses without the need for maintenance therapy in five animals (Fig. 6, C and D).

Nephrotoxicity is the major dose-limiting toxicity of cisplatin (24), and multiple lines of evidence implicate activation of TAK1 (25) and TGFβ (26) signaling in the pathogenesis of acute kidney injury. We therefore treated mice with SB-505124 in combination with a single nephrotoxic dose of cisplatin, as described in Fig. 6E. Endpoint measurements of serum creatinine and urea demonstrated that SB-505124 was highly effective at reducing cisplatin-mediated kidney injury (Fig. 6, F and G). Periodic PAS staining of kidney sections from cisplatin-treated animals (Fig. 6G) showed changes characteristic of acute tubular injury, with staining in the medulla of the affected kidneys. In contrast, kidneys from the animals treated with cisplatin in combination with SB-505124 resembled controls (Fig. 6G). High-magnification images (fig. S6B) demonstrated that the typical features of acute tubular necrosis, including loss of the epithelial brush border, tubular necrosis, tubular dilation, and cast formation, were attenuated by the SB-505124 treatment.

**Inhibition of activin A/GDF11 signaling, platinum resistance, and nephrotoxicity in vivo**

One potential disadvantage of small-molecule inhibitors in combination with chemotherapy is unexpected toxicity due to on-target or off-target effects. We therefore considered whether FST might serve as a more tractable therapeutic approach. FST is an endogenous protein that regulates several developmental, reproductive, and inflammatory components of the TGFβ signaling pathway by binding to and inhibiting the activity of activin A, GDF8, and GDF11 (27). FST does not bind to or inhibit TGFβ1 and TGFβ2, therefore providing an experimental tool to support our data suggesting that ACVR1B and TGFBR1 are activated by activin A and/or GDF11.

Before testing the efficacy of FST for platinum sensitization in vivo, we determined the expression of its molecular targets, activin A and GDF8/11, in A549, NCI-H358, and NCI-H727 xenografts, as well as PDX1.1, a treatment-naïve, chemoresistant patient-derived xenograft. In addition, we performed the same assessment in the immunocompetent murine Lewis lung adenocarcinoma (LLC) model (Fig. 7A). Quantitative analysis showed that expression of both activin A and GDF8/11 was variable, and in the case of NCI-H727 cells, lower than expected based on our in vitro results (Fig. 7B). Tumors generated in the KRas/Tp53 conditional mouse model of lung adenocarcinoma (28) did not express either activin A or GDF8/11 (fig. S7A).

Purified recombinant human FST was administered along with carboplatin according to the regimen outlined in Fig. 7C. Figure 7D shows that FST had no effect on the growth of A549 flank xenografts as a single agent. However, administration of FST enhanced the effect of carboplatin, with 7 of 10 mice achieving a complete response...
Administration of carboplatin with FST also resulted in a dramatic improvement in survival consistent with durable and biologically meaningful therapeutic response (Fig. 7E). Similar results were seen in the NCI-H358 and PDX1.1 models, both of which overexpressed GDF8/11 (Fig. 7, A, B, and F, and fig. S7B). Combination carboplatin/FST treatment also had a significant effect on survival in the fast-growing LLC model; however, no effect was seen in the NCI-H727 xenografts, which did not overexpress activin A or GDF8/11 in vivo (Fig. 7, A, B, and F, and fig. S7B). Using the experimental protocol shown in Fig. 7G, administration of FST also prevented renal failure (Fig. 7H) and kidney injury (Fig. 7I and fig. S7C) in response to a single nephrotoxic dose of cisplatin. These data provide strong support for the development of FST as an inhibitor of activin/GDF11 signaling to overcome innate platinum resistance in lung adenocarcinoma.

DISCUSSION

To increase efficacy, platinum agents are typically given in combination with a taxane, pemetrexed, or gemcitabine (29). These regimens are largely based on historical clinical trial data rather than on mechanism and result in additional toxicity when compared to platinum.
given as a single agent (29). Moreover, pharmacologic strategies aimed at improving platinum responses have the potential to increase toxicity if they target pathways that repair platinum DNA adducts in normal cells. Our results suggest that a mechanism-based approach to reversing platinum resistance could be used to identify more specific and less toxic combination therapies.

A wealth of evidence supports a role for TGFβ signaling through the SMAD transcription factors as an important mediator of chemoresistance in many different tumor types (30). Our screen found that the MAP3K TAK1 is an important mediator of platinum resistance in lung adenocarcinoma and that its activation is driven by signaling through activin and TGFβ in response to the ligands activin A and GDF8/11 staining. Statistical significance was calculated using log-rank analysis. CI, confidence interval; HR, hazard ratio.

Fig. 5. Expression of TGFβ superfamily components in lung adenocarcinoma. (A) Heat map depicting expression of genes belonging to the TGFβ superfamily in the TCGA lung adenocarcinoma data set. (B) Association between GDF11, TGFβ1, INHBA, INHBB, ACVR1B, and TGFBR1 expression and overall survival in the KMPlot lung adenocarcinoma data set. (C) Activin A and GDF8/11 expression detected by immunohistochemistry (IHC) in stage IV lung adenocarcinoma. Immunoperoxidase signal is shown in brown, counterstained with hematoxylin. Scale bars, 50 μm. (D) Kaplan-Meier analysis of progression-free survival (PFS) in a cohort of patients with stage IV lung adenocarcinoma (n = 59) treated with platinum-based chemotherapy. Survival outcomes are shown with respect to quantitative immunohistochemical analysis of activin A and GDF8/11 staining. Statistical significance was calculated using log-rank analysis. CI, confidence interval; HR, hazard ratio.
Fig. 6. Inhibition of ACVR1B/TGFR1 signaling in vivo. (A) ACVR1B and TGFR1 expression detected by immunohistochemistry in A549 xenografts. Immunoperoxidase signal is shown in brown, counterstained with hematoxylin. Scale bars, 100 μm. (B) Schema describing an experiment to determine the effect of combination carboplatin and SB-505124 treatment on the growth of A549 flank xenograft tumors. (C) Growth of A549 xenografts in mice treated with vehicle control, SB-505124, and/or Carbo. Data are presented as the growth of tumors in independent mice. (D) Kaplan-Meier analysis of survival to ethical endpoint of mice from the same experiment. n for each treatment group is shown. ***P < 0.001 compared to mice treated with vehicle + cisplatin (CisPt), log-rank test. (E) Schema describing an experiment to determine the effect of treatment with SB-505124 on acute kidney injury induced by CisPt. (F) Serum urea and creatinine concentrations in mice treated with vehicle, SB-505124, and/or CisPt. Blood samples were taken on day 5. n = 7, mean ± SEM. **P < 0.01, ***P < 0.001, one-way ANOVA with Bonferroni correction. (G) Top: Macroscopic appearance of kidneys from mice from the experiment in (E) and (F). Scale bar, 2 mm. Bottom: Photomicrographs of periodic acid-Schiff (PAS)–stained sections of whole kidneys. Scale bar, 2 mm.

and GDF11. In contrast to SMAD signaling, the TAK1 component of the TGFβ pathway is more amenable to small-molecule targeting (30). Notably, our data suggest that ACVR1B/TGFR1 signaling mediates protection against chemotherapy in cancer cells and promotes kidney injury. Up-regulation of activin signaling is also a feature of distal airway epithelial cells in response to inflammation (3). On the basis of the putative cell of origin of lung adenocarcinoma, the up-regulation of activin signaling in the face of chemotherapeutic stress may be a consequence of cell lineage rather than genotype.

There are several points that limit the interpretation of our results. First, we have focused on innate platinum resistance by performing our screen on the treatment-naïve cell line A549, and thus, we cannot make definitive conclusions regarding acquired resistance. Second, we concentrated on KRAS/NRAS mutant models as a clinical priority, and so we cannot generalize our results to lung adenocarcinomas without a defined oncogenic mutation. Third, we have not identified a mechanism to explain why TAK1 activation rather than SMAD-mediated transcription is favored by lung adenocarcinoma cells in response to platinum chemotherapy. Finally, the differences in pharmacodynamics between cisplatin and carboplatin (11) will need to be considered when considering the potential efficacy of FST in humans.

Recombinant FST as a potential platinum-sensitization agent has several advantages over small molecule–targeting strategies, because it is an endogenous protein that does not require humanization. Our in vivo data show that FST can dramatically enhance the efficacy of FST in humans.
Fig. 7. Inhibition of activin/GDF11 signaling in vivo. (A) Activin A and GDF8/11 expression detected by immunohistochemistry in A549, NCI-H358, NCI-H727, and PDX1.1 xenografts, and LLC allografts. Immunoperoxidase signal is shown in brown, counterstained with hematoxylin. Scale bars, 100 μm. (B) Quantification of the data shown in (A). n = 4, mean ± SEM. (C) Schema describing an experiment to determine the effect of combination carboplatin and FST treatment on the growth of A549 flank xenograft tumors. (D) Growth of A549 flank xenografts in nude mice treated with vehicle control, FST, and/or Carbo. Data are presented as the growth of tumors in independent mice. (E) Kaplan-Meier analysis of survival to ethical endpoint of mice from the same experiment, n for each experimental group is shown. # P < 0.0001 compared to mice treated with vehicle + cisplatin (Pt), log-rank test. (F) Kaplan-Meier analysis of survival to ethical endpoint of nude mice bearing NCI-H358, NCI-H727, PDX1.1, and LLC flank tumors treated with phosphate-buffered saline (PBS; control), FST, and/or Carbo as shown in (C). n for each group is shown. ***P < 0.001, *P < 0.05 compared to mice treated with vehicle + Pt, log-rank test. (G) Schema describing an experiment to determine the effect of treatment with FST on acute kidney injury induced by CisPt. (H) Serum urea and creatinine concentrations in mice treated with PBS, FST, and/or CisPt. Blood samples were taken on day 5. n = 7, mean ± SEM. **P < 0.01, one-way ANOVA with Bonferroni correction. (I) Top: Macroscopic appearance of kidneys from mice from the experiment in (G) and (H). Scale bar, 2 mm. Bottom: Photomicrographs of PAS-stained sections of whole kidneys from the same experiment. Scale bar, 2 mm.
therefore may hold promise as a treatment for cancer cachexia and sarcopenia (15). We conclude that FST could have potential to improve the efficacy of chemotherapy, while at the same time attenuating a major dose-limiting toxicity with the potential to reverse some of the debilitating systemic effects of cancer.

MATERIALS AND METHODS

Study design

In vitro experiments were performed as quadruplicate technical replicates, with the mean value taken as one data point. Each experiment was then repeated as four biological replicates to generate the final data and statistical analysis. In vivo tumor experiments were randomized and blinded. Each mouse cohort is reported as the combination of two separate experiments with 3 to 5 mice in each group, resulting in a total of 6 to 10 animals per treatment arm.

Reagents

Sources and details of all reagents and antibodies are listed in table S4. Recombinant FST 288 protein (PB01) was supplied by Paranta Biosciences Limited.

Carboplatin response in vitro

Initial determination of cell line responses was performed in a 96-well plate format. Cells were seeded at 5000 cells per well in a 96-well plate on day 0, treated with carboplatin on day 2, and viability was determined using AlamarBlue after 72 hours according to the manufacturer’s instructions.

Cell culture

Cancer cell lines were grown in advanced RPMI with 1% newborn calf serum and penicillin (100 U/ml) and streptomycin (100 µg/ml). HEK293 cells were grown in Dulbecco’s minimum essential medium with 10% fetal bovine serum and penicillin-streptomycin at the same concentration. Cell culture experiments were performed without antibiotics. Cell line ID and mycoplasma testing was performed every 6 months. All cell lines were purchased from the American Type Culture Collection except for LX22CL (7).

CRISPR/Cas9 genome editing

Targeting of MAP3K7 in A549 cells was performed using the GeneScript lentiviral system, with the guide RNA sequence AGAGCCTGAT-GACTCGTTGT. Lentivirus was generated from HEK293T cells transfected with CRISPR/Cas9 plasmids and proprietary packaging plasmids using Lipofectamine3000 according to the manufacturer’s instructions. Cells were infected with lentivirus with polybrene for 1 hour. Cells were then probed with anti-H2AX for 2 hours at room temperature, then washed with PBS before addition of anti-mouse IgG-568 and DAPI, and then washed and mounted. Images were taken using the Nikon C1 microscope.

Gene expression analysis

RNA sequencing data were downloaded from the TCGA lung adenocarcinoma study through cBioPortal (31). Heat maps were generated using R/Bioconductor.

Immunohistochemistry

This study was performed as an anonymized, retrospective cohort analysis of patients with stage IV lung adenocarcinoma in accordance with the Declaration of Helsinki, Australian law, and the National Health and Medical Research Council guidelines and with the approval of the Monash Health Human Research Ethics Committee. Biopsies were taken from patients at Monash Medical Centre from 2009 to 2015. Formalin-fixed, paraffin-embedded sections were stained as described (32) with the antibodies listed in table S4.

In vivo experiments

All experiments were conducted in accordance with Australian government guidelines and with the approval of the Monash University Animal Ethics Committee. PDX1.1 was generated in-house from an untreated patient with a KRAS mutant lung adenocarcinoma using previously described methods (7). Cells in 50 µl of RPMI were added to 50 µl of Matrigel and injected into the flanks of Balb/c nude mice as follows: 1 × 10^6 cells per flank for A549, NCI-H727, or PDX1.1; and 1 × 10^7 per flank for NCI-H358. C57Bl6 mice were injected with 1 × 10^6 LLC cells per flank. Measurements of tumor volume were calculated using the following formula: V = (xs^2 × xl)/2, where xs is the measurement of the shorter axis and xl is the measurement of the longer axis.

For treatment with SB-505124, mice with A549 xenografts measuring 200 mm^3 were randomly assigned to receive vehicle (10% DMSO + 90% saline) only, SB-505124 (5 mg/kg) only, carboplatin (60 mg/kg) + vehicle, or carboplatin (60 mg/kg) + SB-505124 (5 mg/kg) i.p. as described in Fig. 6B. Mouse weight (fig. S8) and tumor volume were measured daily. Mice were sacrificed when tumors reached 500 mm^3. For FST experiments with A549, NCI-H358, NCI-H727, and PDX1.1 models, mice with tumors at 200 mm^3 were randomly assigned to vehicle (PBS) only, FST (2 µg, Paranta Biosciences Limited), carboplatin (60 mg/kg) + vehicle, or FST + carboplatin, intraperitoneally (ip), as described in Fig. 7C. Mouse weight (fig. S8) and tumor volume were measured daily. Mice were sacrificed when tumors reached 500 mm^3 or skin ulceration was observed with A549 xenografts, or at 800 mm^3 with NCI-H358, NCI-H727, and PDX1.1 xenografts.

Nephrotoxicity experiments were conducted with male C57Bl6 mice randomly assigned to receive cisplatin (15 mg/kg), PBS, vehicle (10% DMSO + 90% saline), 2 µg of FST, or SB-505124 (5 mg/kg) by intraperitoneal injection as shown in Figs. 6E and 7G. On day 6, mice were sacrificed using CO2 inhalation, and blood was collected. Kidneys were dissected and fixed in neutral buffered formalin for 24 hours, bisected, and then fixed for an additional 24 hours. Serum analysis of urea and creatinine concentrations was performed using a clinical Beckman Coulter analyzer.

Orthogonal siRNA validation

This was performed in a 96-well plate format using Dharmacon On-Target siRNAs. Two days after siRNA treatment, increasing concentrations...
concentrations of carboplatin were added to wells in quadruplicate. Cells (5000 per well) were seeded in a 96-well plate and left to attach for 6 to 8 hours. Cells were then transfected with siRNA using Lipofectamine RNAiMax (Thermo Fisher Scientific) in OptiMEM overnight. Medium was then replaced, and after 48 hours, cells were treated with vehicle (PBS) or carboplatin. After 72 hours, viability was determined using AlamarBlue fluorescence assay.

Pharmacological validation of hits generated by siRNA screening

Cells (5000 per well) were plated into a 96-well plate and left to attach for 6 to 8 hours. Medium was then replaced with fresh medium containing LLZ-1640-2 (10 μM), SB-505125 (5 μM), or vehicle. After 48 hours, the medium was replaced with medium containing increasing concentrations of carboplatin. Plates were then left to incubate for 3 days, and the cell viability was assayed using AlamarBlue.

Primary and secondary siRNA screens

A549 cells were used in a parallel 384-well plate format, with or without the addition of carboplatin (25 μg/ml) using the Dharmacon siGenome Smartpool library. Library plates were obtained lyophilized, and daughter plates were generated, where four 96-well plates were combined to make 384-well plates in batches of four. Cells (1000 per well) were reverse-transfected using Lipofectamine RNAiMax and OptiMEM overnight. Medium was then replaced. After a further 24 hours, cells were treated with vehicle (PBS) or carboplatin. After 5 days, viability was determined using AlamarBlue.

Using the death and sensitization controls, we generated quality assessment data using the Z’ factor, where \( Z' = 1 - \left[ 3 \times (\sigma_{\text{high control}} + \sigma_{\text{low control}}) \right] / [\mu_{\text{high control}} - \mu_{\text{low control}}] \), \( \sigma = SD \), and \( \mu = mean \). Current screening guidelines suggest that \( Z' \geq 0.5 \) is excellent (±12 SD from mean), \( Z' \geq 0 \) is acceptable (±6 SD from mean), and \( Z' < 0 \) is unacceptable and the plate cannot be analyzed (8). The sensitization index is calculated after normalization and averaging and is a function of the viability of cells treated with siRNA and vehicle over the viability of cells treated with siRNA and platinum (8).

Western blot analysis

Western blotting was performed on cell lysates as previously described (33). Details of Western blot antibodies are listed in table S4. Examples of full-length blots for each antibody and the image cropping used are shown in fig. S9.

Statistical analysis

For the whole-genome siRNA screen, we used a four-part statistical pipeline for data analysis: (i) data triage using visual plate inspection, heat mapping, and linear correlation plotting; (ii) normalization using the percentage of negative control method, where each value on the plate is divided by the mean of the negative controls on the plate; (iii) quality metrics generated by incorporating positive, negative, death, and sensitization controls in each plate; and (iv) hit identification using a two-step method (8). Genes were ranked by the sensitization index. These data were then analyzed using multiple t tests and raw P values subjected to FDR adjustment (34). Each experiment used four plates, where two of these plates received carboplatin treatment and the other two plates received the vehicle control treatment. Statistical methods for the remainder of the paper are described in the legends.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/10/451/eaat3504/DC1

Fig. S1. Controls used in the siRNA screen.

Fig. S2. Controls for SMAD2/3 expression and carboplatin responses in HEK293 cells in vitro.

Fig. S3. Controls for FST on the platinum response in vivo.

Fig. S4. Examples of full-length Western blots with image cropping.

Table S1. Complete data set from the primary siRNA screen (provided as an Excel file).

Table S2. High-confidence hits targetable with existing small or large molecules.

Table S3. Characteristics of the patient cohort used to generate immunohistochemical data.

Table S4. Details of reagents used (provided as an Excel file).

REFERENCES AND NOTES


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Inhibition of activin signaling in lung adenocarcinoma increases the therapeutic index of platinum chemotherapy


Sci Transl Med 10, eaat3504.
DOI: 10.1126/scitranslmed.aat3504

Blocking activin actively treats cancer
Platinum-based chemotherapy is a mainstay of treatment for lung cancer, but resistance to this therapy is a common problem, as are dose-limiting side effects, particularly kidney toxicity. To search for mechanisms that may contribute to treatment resistance, Marini et al. performed a whole-genome RNA interference screen and identified the activin pathway, which can be targeted. The authors demonstrated that inhibition of this pathway using a small molecule or a protein called follistatin can offer a dual benefit in that it potentiates the effects of platinum drugs in mouse models of cancer and also protects the animals from kidney damage. These findings suggest that activin inhibitors could be a valuable addition to platinum chemotherapy, enhancing the efficacy of treatment while also allowing the use of higher doses or longer periods of drug exposure.