Rigosertib Induces Mitotic Arrest and Apoptosis in RAS-Mutated Rhabdomyosarcoma and Neuroblastoma

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ABSTRACT

Relapsed pediatric rhabdomyosarcomas (RMS) and neuroblastomas (NBs) have a poor prognosis despite multimodality therapy. In addition, the current standard of care for these cancers includes vinca alkaloids that have severe toxicity profiles, further underscoring the need for novel therapies for these malignancies. Here, we show that the small-molecule rigosertib inhibits the growth of RMS and NB cell lines by arresting cells in mitosis, which leads to cell death. Our data indicate that rigosertib, like the vinca alkaloids, exerts its effects mainly by interfering with mitotic spindle assembly. Although rigosertib has the ability to inhibit binding to the RAS-binding domains of RAS effectors, such as the Raf family members (ARAF, BRAF, and RAF1) and PI3 kinase isoforms (p110α and β), thus blocking the interaction with active RAS (2). In this way, rigosertib decreases signaling through the RAS/RAF/MEK/ERK MAPK and the PI3 kinase/AKT/mTOR pathways. Rigosertib also induces mitotic and oxidative stress, which activates the stress MAPKs, p38 and JNK. The stress MAPKs phosphorylate components of the RAS pathway, including Raf family members and SOS1, an RAS guanine nucleotide exchange factor, which further decreases signaling through the RAS/RAF/MEK/ERK MAPK pathway (3). The mitotic stress induced by rigosertib may be due to the fact that rigosertib, or a degradation product of rigosertib, binds to an intradimer site between α- and β-tubulin in a manner similar to colchicine, which prevents microtubule growth (4–6).

Functionally, rigosertib has been shown to have anticancer effects across a range of malignancies both preclinically and clinically. Rigosertib is a potent inhibitor of tumor growth in HCT116 and A549 xenograft models of RAS-mutated colorectal and lung adenocarcinoma, respectively, and represses the growth of RAS-mutated pancreatic intraepithelial neoplasia in a genetically engineered mouse model (2). Rigosertib also suppresses extramedullary hematopoiesis in a KRASG12D-driven model of the pediatric myeloproliferative neoplasm, juvenile myelomonocytic leukemia (7). Clinically, rigosertib has shown efficacy in trials for adults with a variety of solid tumors, with complete or partial responses noted in subjects with thymic carcinoma, pancreatic ductal adenocarcinoma, and head and neck squamous cell carcinoma (8–10). Rigosertib has also been efficacious in hematologic malignancies, particularly Hodgkin lymphoma and high-risk myelodysplastic syndrome (11–13). No studies evaluating the efficacy of rigosertib in patients with RAS-mutated cancers have been conducted to date. However, in patients with metastatic pancreatic adenocarcinoma, tumors primarily driven by mutations in KRAS, the combination of rigosertib and gemcitabine failed to show an...
RAS is a common driver of pediatric cancers, including solid tumors, such as PAX-fusion negative rhabdomyosarcoma (EN-RMS; ref. 15), malignant peripheral nerve sheath tumor (16), relapsed neuroblastoma (NB; ref. 17), and malignant ectomesenchymoma (18), as well as hematologic malignancies such as juvenile myelomonocytic leukemia (19). In the current study, we aimed to investigate the activity of rigosertib in models of FN-RMS and NB that harbor mutations in one of the RAS isoforms, HRA5, KRAS, or NRAS. FN-RMS and NB are both embryonal tumors, with cells of origin being skeletal muscle (20) and sympathoadrenal precursors (21), respectively. We also aimed to identify the mechanism of action of rigosertib in the context of pediatric solid tumors to identify appropriate biomarkers for pharmacodynamic studies in future clinical trials of rigosertib for pediatric patients.

Materials and Methods

Cell lines and reagents

The RMS cell lines RD, SMS-CTR, BIRCH, RH4, RH30, RH18 and RMS-YM were obtained from J. Khan (National Cancer Institute, Bethesda, MD). The NB cell lines SKNAS, NBEB, SHEP, SY5Y, and IMR5 were obtained from C. Thiele (National Cancer Institute, Bethesda, MD). CHP212 was obtained from the Childhood Cancer Repository. The RASSles MEFs were obtained through collaboration with the NCI RAS initiative. All cell lines were confirmed to be Mycoplasma negative using the MycoAlert kit (Lonza), and their identity was confirmed by STR fingerprinting before experimental use. BIRCH, RMS-YM, RH18, SKNAS, NBEB, SHEP, SY5Y, and IMR5 were grown in RPMI with 10% FBS. The other cell lines were grown in DMEM with 10% FBS. Trametinib was obtained from the NIH Developmental Therapeutics Program (RRID:SCR_003057). Rigosertib was obtained through a Cooperative Research and Development Agreement with Onconova Therapeutics. Biotinylated rigosertib and ON0911 were a gift of P. Reddy (Icahn School of Medicine at Mount Sinai, New York, NY). DAPI (D1306) was obtained from Thermo. N-acyctlycisteine (A9165), biotin (B4501), and menadione (M2518) were obtained from Sigma. SB-203580 (S1076; ref. 22), nocodazole (S2775), albendazole (S1640), and crombretastatin A4 (S7783; ref. 23) were obtained from SelleckChem. Vincristine was obtained from the NIH Division of Veterinary Resources veterinary pharmacy.

Immunofluorescence

Cells plated on fibronectin coated coverslips (10 μg/mL) were treated as indicated and fixed with 4% paraformaldehyde in PBS. Cells were permeabilized with PBS containing 0.5% Triton X-100 for 10 minutes and then washed three times with PBS containing 0.1% Triton X-100. The cells were then incubated in PBS with 0.1% Triton X-100, 2% BSA and 0.1% sodium azide (blocking buffer) for 10 minutes at 4°C. Cells were incubated at room temperature for 1 hour with primary antibodies (Supplementary Table S1) diluted in blocking buffer. Cells were washed four times with blocking buffer and then incubated for 1 hour at room temperature with secondary antibody (Supplementary Table S1) diluted 1:200 in blocking buffer. Cells were washed four times with PBS containing 0.1% Triton X-100 and once with PBS. The cells were then incubated with 300 nmol/L DAPI for 5 to 10 minutes and rinsed three times with PBS. The coverslips were mounted with Dako Fluorescence mounting medium. Microscope images were captured using a Leica SP8 laser-scanning confocal microscope using a ×63, 1.4 numerical aperture objective (Leica Microsystems Inc.). Stacks of 0.3-μm slices were collected. Maximum projection images are presented. Image analysis was performed in ImageJ (RRID:SCR_003070).

Rigosertib pull down and mass spectrometry

RD cells in the logarithmic growth phase were lysed in PBS with added 0.15% Tween 20, 1 mmol/L DTT and HALT protease/ phosphatase inhibitors. Nuclei were lysed by passing the cell lysate through a 20G syringe five times. The resulting whole-cell lysates were clarified by centrifugation at 16,000 rpm at 4°C for 10 minutes. Approximately 1 μg of total lysate was used per condition. 50 μmol/L of free biotin, biotinylated rigosertib, or biotinylated ON01911 was added to the cell lysate. The samples were incubated at 4°C for 2 hours before the addition of 50-μL NeutrAvidin beads (Thermo). The bead lysate slurries were incubated on an end-over-end rotator overnight at 4°C. Beads were washed three times in PBS before resuspension in LDS sample buffer (Thermo). Denatured samples were separated by SDSPAGE for in-gel trypsin digestion as described previously (24). For mass spectrometry analysis, peptides were trapped on a trapping column and separated on a 75-μm × 15-cm, 2-μm Acclaim PepMap reverse phase column (Thermo) using an UltiMate 3000 RSLCnano HPLC (Thermo). Peptides were separated at a flow rate of 300 nL/min followed by online analysis by tandem mass spectrometry using a Thermo Orbitrap Fusion mass spectrometer. Parent full-scan mass spectra were collected in the Orbitrap mass analyzer set to acquire data at 120,000 FWHM resolution; ions were then isolated in the quadrupole mass filter, fragmented within the HCD cell (HCD normalized energy 32%, stepped ±3%), and the product ions analyzed in the ion trap. Proteome Discoverer 2.2 (Thermo, RRID:SCR_014477) was used to search the data against human proteins from the UniProt database (RRID:SCR_004426) using SequestHT. The search was limited to tryptic peptides, with maximally two missed cleavages allowed. Cysteine carbamidomethylation was set as a fixed modification, and methionine oxidation set as a variable modification. The precursor mass tolerance was 10 ppm, and the fragment mass tolerance was 0.6 Da. The Percolator node (RRID:SCR_00287) was used to score and rank peptide matches using a 1% FDR.

Cell growth assay

Dose–response, matrix, and time-course viability curves were generated by quantifying percentage of cell confluence from phase contrast images of cells in 384-well plates. Images were taken every 4 hours using an Incucyte ZOOM (Essen Bioscience). Cells were plated to achieve 20% confluence at the time of drug dosing. Each condition was assayed in triplicate. The 50% inhibitory concentration (IC50) values were calculated using GraphPad Prism version 7 (RRID:SCR_002798). Synergy was calculated according to the Bliss Independence model (25).

Clonogenic assay

RD, SMS-CTR, RH30, SKNAS, or CHP212 cells were plated at a density of 100 cells per well in 6-well tissue culture plates. The plates were incubated for 2 weeks before fixing in 10% formaldehyde and staining with 0.01% crystal violet.

Annexin V assay

RD or SMS-CTR cells were treated with vehicle (DMSO) or 2 μmol/L rigosertib for 48 hours before harvesting and incubating...
with APC-labeled human recombinant annexin V (BioLegend) and Sytox Green (Thermo) according to the manufacturers’ instructions. Samples were read on a FACS(Cantoflow cytometer (BD Biosciences) and percent annexin positive cells were calculated in FlowJo (RRID:SCR_008530).

**Xenograft experiments**

Xenograft studies were approved by the Animal Care and Use Committee (ACUC) of the NCI-Bethesda or the Frederick National Laboratory for Cancer Research (FNLCR). FNLCR is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals. SCID beige mice were purchased from Charles River laboratories. All animals were female, and all were injected at 4–8 weeks of age. For the RD rigosertib experiment, 2 million cells were injected orthotopically into the gastrocnemius muscle in the left hind leg of 13 SCID-Beige mice. After 3 weeks, the mice were randomized into vehicle (n = 6) and rigosertib (n = 7) groups (equivalent tumor size in each group). Rigosertib was prepared daily in PBS and stored at 4°C until use. The mice in the treatment group received rigosertib at a dose of 150 mg/kg (100 μL of a 30 mg/mL solution) by intraperitoneal injection twice daily 5 days per week; the vehicle group received the same volume of vehicle by intraperitoneal injection twice daily 5 days per week. Mice received rigosertib until they reached study endpoint. For the RD vincristine experiment, 2 million cells were injected as above. The mice were randomized into vehicle (n = 5) and vincristine groups with 10 mice per group. The vincristine group received 1 mg/kg weekly via tail vein injection. Vincristine was diluted in PBS immediately before injection. Mice received vincristine or vehicle for a period of 28 days at which time the treatment was stopped, and the mice were observed for tumor development. For the SKNAS experiment, 2 million cells were injected subcutaneously in the left flank of 9 SCID beige mice. After 2 weeks, the mice were randomized into vehicle (n = 4) and rigosertib (n = 5) groups and treated as described for mice bearing RD tumors.

In all experiments, the tumor dimensions were measured twice a week with digital calipers to obtain two diameters of the tumor sphere, from which the tumor volume was determined using the equation (D × d)³/6 × 3.14 (where D = the maximum diameter and d = the minimum diameter; ref. 26). For the intramuscular injections, the whole hindlimb was measured, whereas for the subcutaneous injections, the tumor itself was measured. Animals were euthanized when they reached tumor endpoint, which was defined as when the tumor measured greater than 18 mm in any direction for the tumors injected intramuscularly and 20 mm in any direction for the tumors injected subcutaneously. Animals were also euthanized if the tumors showed signs of ulceration or caused significant discomfort to the animal, in accordance with the humane endpoints recommended by our institutional ACUC.

**Capillary immunoassays**

Cell lysates were prepared in MPER (Thermo). Fresh-frozen tumor samples were prepared in TPER (Thermo) using a TissueRuptor. Cell or tumor lysates were mixed with 1× sodium dodecyl sulfate master mix containing sample buffer (ProteinSimple) dithiothreitol, and fluorescently labeled standards (ProteinSimple) and were heated at 70°C for 10 minutes before being loaded into Peggy Sue instrument (ProteinSimple) for analysis. During electrophoresis, proteins were separated by molecular weight while migrating through the separation matrix (ProteinSimple). Separated proteins were immobilized on the capillary wall using UV light, and incubated with a blocking reagent (ProteinSimple), followed by immunoprobeing with respective primary antibodies and horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse secondary antibodies (ProteinSimple, Jackson Immunoresearch). A 1:1 mixture of luminol and peroxide (ProteinSimple) was added to generate chemiluminescence, which was captured by a CCD camera. The digital image was analyzed by Compass software (ProteinSimple, RRID:SCR_018574). Target protein quantities were determined by quantifying the signal strength (peak area). The Simple Western total protein assay was used as a loading control. In the total protein analysis module, proteins were separated by MW and immobilized in the capillary, before incubation with biotinylating reagent (ProteinSimple), followed by HRP–streptavidin (ProteinSimple) for chemiluminescent detection.

**DNA content analysis**

Cells were fixed with 70% ethanol before staining with propidium iodide/Triton-X-100 (Thermo). Stained cells were analyzed using a Sony SA3800 instrument at the CCR Flow Cytometry Core facility. Cell cycle analysis was performed in ModFit LT version 5 using default parameters.

**Immunoblot experiments**

Antibodies used in this study are listed in Supplementary Table S1. Cells in culture were washed twice with ice-cold PBS before lysis in lysis buffer (Cell Signaling Technology) with added HALT protease and phosphatase inhibitors (Thermo). Cells were lysed at 4°C for 10 minutes and the resulting lysates were clarified by centrifugation at 16,000 rpm at 4°C for 10 minutes. Protein concentration of the resulting supernatant was estimated by BCA assay (Thermo). Ten to 40 micrograms of sample were run on NuPage 4%-12% BisTris minigels (Novex) and transferred to polyvinylidene difluoride membranes (Amersham). Membranes were blocked in 5% nonfat dried milk in TBST for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C. HRP-conjugated anti-rabbit or mouse antibodies (Cell Signaling Technology) were used as secondary antibodies, as indicated in Supplementary Table S1. Protein was visualized using SuperSignal West Femto maximum sensitivity substrate (Thermo) on a ChemiDoc imager (Bio-Rad).

**Caspase-Glo 3/7 assay**

Cells were plated at a density of 10–20,000 cells/well in 96-well plates. The next day, cells were treated with DMSO, 2 μmol/L rigosertib, 10 mmol/L NAC, and 20 mmol/L SB-203580 both individually and in combination for 24 hours. The caspase-Glo 3/7 assay (Promega) protocol was followed per manufacturer’s recommendations. Luminescence was read on a SpectraMax M5 (Molecular Devices).

**ROS-Glo assay**

RD or SMC-CTR cells were plated at a density of 10,000 cells/well in 96-well plates. Media alone (no cells) were also plated as a negative control. The next day, cells or media were treated with DMSO, 2 μmol/L rigosertib, or 50 μmol/L menadione for 24 hours. The ROS-glo protocol (Promega) was followed per the manufacturer’s instructions. Luminescence was read on a SpectraMax M5 (Molecular Devices).

**Intracellular tubulin polymerization assay**

RD and SKNAS cells were plated at a density of 500,000 cells per well in a 6-well dish 24 hours before treatment with increasing
concentrations of rigosertib. After 4 hours of rigosertib treatment, the cells were lysed in a hypotonic lysis buffer (1 mmol/L MgCl₂, 2 mmol/L EGTA, 20 mmol/L Tris HCl pH 6.8, 0.13% IGEPA, 5 mmol/L taxol) for 10 minutes at 37°C as previously described (27). The cell lysates were centrifuged at 15,000 rpm for 10 minutes at room temperature. The resulting supernatants (S), containing soluble tubulin, were removed and the resulting pellets (P), containing polymerized tubulin, were resuspended in a volume of hypotonic lysis buffer equivalent to the removed supernatant. Equal volumes of S and P for each treatment condition were subjected to SDS-PAGE followed by immunoblotting for α-tubulin. Band densities were quantified using ImageJ.

High-throughput cell viability assays

A total of 130 cancer cell lines were previously screened against rigosertib, which is a component of various NCATS compound libraries (28, 29). The majority (87) of these cell lines express wild-type RAS, whereas a subset (42) express either an HRAS, KRAS, or NRAS mutant. Potency was determined in 1,536-well tissue culture plates pre-plated with 11 concentrations of rigosertib. Cells were grown in the presence of rigosertib for 48 hours before the addition of CellTiter Glo (Promega) to assess cell viability. Luminescent signal was measured on a ViewLux instrument and data were normalized to DMSO-treated wells as 100% viability and no cells controls as 0% viability. The area under the dose–response curve (AUC) from the resultant 11-point dose–response curves was calculated using a standard trapezoidal method. The AUCs were z-transformed to compare the activity of rigosertib with that of the other compounds within that library for each cell line, such that a negative z-score denotes greater potency. This integration and harmonization of screening data were carried out in Palantir Foundry, through the NIH Integrated Data Analysis Platform (NIDAP).

Results

Rigosertib potently decreases the proliferation of RMS and NB cell lines

To determine the efficacy of rigosertib in RMS and NB cell lines, we investigated the effects of increasing concentrations of rigosertib on cell confluence using live cell imaging. Cell lines with and without RAS mutations were used in this analysis (Supplementary Table S2). Importantly, in the cell lines used, the presence of a mutation in a RAS isoform confers a functional dependency on that RAS isoform (30). The IC₅₀ value of rigosertib in RMS (Fig. 1A) and NB (Fig. 1B) cell lines was determined to be in the submicromolar range using this assay. However, no statistically significant difference in IC₅₀ value was observed between cell lines harboring a mutation in an RAS isoform (HRAS, KRAS, or NRAS) and those expressing wild-type (WT) RAS. To confirm that rigosertib efficacy is independent of RAS mutation status, we also investigated the efficacy of rigosertib in a panel of isogenic RAS-dependent MEFs obtained from the NCI RAS Initiative (Fig. 1C). These MEFs were derived from NRAS- and HRAS-null mice, and the endogenous KRAS allele was subsequently removed via Cre–lox-mediated recombination. These RASSL MEFs were then transduced with WT KRAS4b, KRAS4bG12V or BrafV600E, with the resulting MEF lines being dependent upon their respective transgene for proliferation (31). Rigosertib was equally potent at inhibiting cell viability in MEFs expressing either WT KRAS4b, KRAS4bG12V, or importantly BrafV600E, which lacks expression of any of the RAS isoforms, validating that the activity of rigosertib does not depend on RAS status. In addition to the efficacy observed in a short-term assay of cell viability, rigosertib treatment also inhibited cell growth in a 14-day clonogenic assay in RAS-mutated RMS (RD, SMS-CTR), RMS cells expressing WT RAS (RH30) and RAS-mutated NB (CHP212, SKNAS) cell lines (Fig. 1D). In the clonogenic experiments, the cell lines appeared to be more sensitive to rigosertib than the RMS cells. To extend our analysis, we compared the potency of rigosertib in additional pan-cancer RMS WT and RAS mutant cell lines that have been subjected to a high-throughput drug screen at the National Center for Advancing Translational Sciences. Potency in this screen is represented as the cell viability AUC. In this dataset, there was no difference in potency for rigosertib in RMS WT or RAS-mutant cell lines; however, the MEK inhibitor, trametinib, was more potent (had a lower AUC) in RAS-mutant as compared with RAS WT cells (Fig. 1E). Taken together, the above results suggest that the effects of rigosertib on cell viability are independent of mutant RAS expression.

Rigosertib induces apoptosis and mitotic arrest in RMS and NB cell lines

To determine the mechanism by which rigosertib affects RMS and NB cell line viability, we tested whether rigosertib was able to induce apoptosis in these cells. Rigosertib induced caspase 3/7 activity (Fig. 2A) and phosphatidylserine externalization as detected by annexin V staining (Fig. 2B) in RMS-mutant RMS cells lines, RD and SMS-CTR, suggesting apoptosis was induced in these cells. Caspase 3/7 activity was also induced by rigosertib in RMS WT FN-RMS cell lines, RMS-YM and RH18 (Supplementary Fig. S1A), as well as RAS mutant (SKNAS and NREB) and RAS WT (SHEP and SYSY) NB cell lines (Supplementary Fig. S1B). However, rigosertib also induced G₂-M arrest in RMS and NB cells, as determined by DNA content analysis (Fig. 2C), suggesting that the rigosertib effects in these cell lines are both cytotoxic and cytostatic. To further characterize the cell-cycle block induced by rigosertib, we determined the effect of rigosertib on histone H3 S10 phosphorylation, which is a marker specific for cells in mitosis. Rigosertib increased phospho-histone H3 in both RMS and NB cells as determined by immunoblot (Fig. 2D) and immunofluorescence (Fig. 2E) experiments, suggesting that rigosertib arrests pediatric solid tumor cells in mitosis. The cellular effects of rigosertib were observed in SMS-CTR cells in as few as 4 hours (Supplementary Fig. S1C) and were irreversible after removal of drug from the cell culture media (Supplementary Fig. S1D).

Rigosertib does not inhibit signaling through RAS effectors in RMS and NB cells

To determine whether rigosertib inhibits signaling through the RAS effector pathways RAF/MEK/ERK and PI3 kinase/AKT/mTOR, we serum-starved RD and SMS-CTR RMS cells, treated with vehicle (DMSO) or rigosertib, and then stimulated with either vehicle (PBS) or IGF1 (Fig. 3A). IGF1 stimulation did not increase ERK phosphorylation within each treatment group because each of these cell lines harbors an RAS isoform mutation, which confers constitutive signaling through the RAF/MEK/ERK pathway. IGF1 stimulation, however, did increase AKT phosphorylation in serum-starved RD and SMS-CTR, suggesting that the serum starvation conditions were sufficient. Rigosertib treatment increased ERK phosphorylation but did not affect AKT phosphorylation compared with DMSO-treated cells in the serum-starved and IGF1-stimulated conditions in both cell lines. In contrast with previous studies in MDA-MB-231 cells in which rigosertib treatment induced the phosphorylation of sites that suppress MEK1 activity (T286, T292) but not those that result in MEK activation (S217,S221; ref. 32), we found that rigosertib treatment in RD cells increased both positive and negative phosphorylation on MEK1 (Fig. 3B). Cotreatment with the allosteric MEK inhibitor,
trametinib, decreased MEK1 phosphorylation at T286 relative to rigosertib treatment alone. Trametinib did not impact rigosertib-induced T292 phosphorylation. Importantly, however, trametinib prevented rigosertib-induced ERK phosphorylation. Results in SKNAS were similar to those obtained in RMS lines in that IGF1 stimulation in SKNAS did not increase ERK phosphorylation but did increase AKT phosphorylation. In contrast with the results in RMS cells, rigosertib treatment did not impact ERK phosphorylation compared with DMSO-treated cells in the serum-starved or IGF1-stimulated conditions in SKNAS (Fig. 3C). Rigosertib induced
Figure 2.
Rigosertib induces apoptosis and G2–M-phase arrest in RAS-mutant RMS and NB cell lines. **A**, Caspase 3/7 activity, determined by Caspase-Glo, 18 hours after rigosertib treatment of the indicated RMS (RD or SMS-CTR) cell lines. *, P < 0.05; ***, P < 0.001 for comparison with DMSO, as determined by two-way ANOVA with Tukey’s multiple comparison test. **B**, RD or SMS-CTR cells were treated with DMSO or 2 µmol/L rigosertib for 48 hours, after which cells were stained with Annexin/Sytox green and analyzed by flow cytometry. Percentage of apoptotic cells were defined as cells that were annexin positive and Sytox positive or negative. ***, P < 0.001 and ****, P < 0.0001 as determined by two-way ANOVA with Sidak’s multiple comparison test. **C**, Treatment with 2 µmol/L rigosertib for 24 hours induces G2–M arrest in RMS (top) or NB (bottom) cells as determined by DNA content analysis. **D**, Treatment with 2 µmol/L rigosertib for 24 hours induces mitotic arrest in RMS (RD and SMS-CTR, top) or NB (SKNAS and NBEB, bottom) cells as determined by phosphorylated histone H3 immunoblot. **E**, Treatment with 250 nmol/L rigosertib for 24 hours induces mitotic arrest in RD (top) and SKNAS (bottom) cells as determined by phosphorylated histone H3 immunofluorescence. Representative images are shown. Quantification of seven independent fields shown at right. *, P < 0.05; ***, P < 0.001 as determined by two-way ANOVA with Sidak’s multiple comparison test.
positive and negative phosphorylation events on MEK1 in SKNAS, similar to the effects in the RD cell line (Fig. 3D). These results suggest that rigosertib does not decrease signaling through RAS effector pathways in RMS or NB cell lines.

Because trametinib decreases ERK phosphorylation induced by rigosertib, we hypothesized that trametinib and rigosertib might synergistically inhibit RMS cell viability. To test this hypothesis, we performed a matrix viability experiment in RD cells (Fig. 3E). Using the Bliss independence model, we were able to identify several conditions in which rigosertib and trametinib were synergistic. For example, the addition of 30 nmol/L trametinib to 1 μmol/L rigosertib prevents RD cell growth (Fig. 3F). These results support the conclusion that inhibitors of the RAF/MEK/ERK pathway can synergize with rigosertib in RMS.
Rigosertib activates stress MAPK pathways in RMS and NB cells

Rigosertib treatment of CLL cells (33) and HeLa cells (3) activates the JNK stress MAPK pathway following release of mitochondrial reactive oxygen species (ROS). In HeLa cells, activated JNK phosphorylates and negatively regulates components of the RAS/RAF/MEK/ERK MAPK pathway. To test the hypothesis that rigosertib treatment stimulates ROS production in RMS cells, we performed a ROS-glo assay (Supplementary Fig. S2A). In this experiment, rigosertib stimulated ROS production in RD to the same extent as menadione, a known stimulator of ROS production (34). The ability of rigosertib to stimulate ROS was inhibited by cotreatment with the ROS scavenger, N-acetylcysteine (NAC). Compared with RD cells, SMS-CTR cells produced less ROS as a result of either rigosertib or menadione treatment. The ability of NB cells to produce ROS as a result of rigosertib treatment was unable to be determined because of the high degree of spontaneous oxidation of the RPMI media components in which these cells are cultured.

Importantly, rigosertib treatment of RMS cells induced phosphorylation, and thus activation, of both the p38 stress MAPK and MKK4, the activator of the stress MAPK, JNK (Fig. 4A). Rigosertib-induced phosphorylation of p38 and MKK4 was inhibited by cotreatment with NAC. To determine whether the cytotoxic and cytostatic effects of rigosertib in RMS cells were dependent upon activation of p38, we used the pharmacologic inhibitor of p38, SB-203580. SB-203580 inhibits the kinase activity of p38, but its effect on p38 phosphorylation is cell-type dependent (35, 36). Cotreatment with SB-203580 inhibited rigosertib-induced p38 phosphorylation in SMS-CTR but not RD cells. The cotreatment did inhibit rigosertib-induced phosphorylation of MAPKAPK2, a p38 substrate, in RD cells, indicating inhibition of p38 catalytic activity (Fig. 4B). These data suggest that SB-203580 inhibits p38 activity in RD and SMS-CTR cells. Consistent with previous studies (3), neither NAC (Fig. 4C) nor SB-203580 (Fig. 4D) treatment had a significant effect on rigosertib-induced G2–M arrest, suggesting that ROS release occurs independent of the arrest in the cell cycle. In addition, neither NAC nor SB-203580 treatment prevented rigosertib-induced caspase 3/7 activity (Fig. 4E and F) or PARP cleavage (Fig. 4A and B).

Unlike the results in RMS cells, rigosertib did not induce phosphorylation of p38 in SKNAS or NBEB, and rigosertib induced MKK4 phosphorylation in NBEB but not SKNAS (Supplementary Fig. S2B). NAC treatment had very little effect on p38, MAPKAPK2, and MKK4 phosphorylation in the presence and absence of rigosertib in both SKNAS and NBEB cells; however, SB-203580 treatment did decrease MAPKAPK2 phosphorylation in SKNAS and p38 phosphorylation in NBEB, indicating inhibition of p38 catalytic activity (Supplementary Fig. S2B). NAC neither inhibited G2–M arrest due to rigosertib in SKNAS, nor inhibited rigosertib-induced caspase 3 activation in SKNAS or NBEB (Supplementary Fig. S2C and S2D). SB-203580 similarly neither affected rigosertib-induced G2–M arrest nor caspase 3 activation (Supplementary Fig. S2C and S2E), consistent with the results in RMS cells. Taken together, these results suggest that rigosertib induces apoptosis in RMS and NB cells via a mechanism that is independent of the rigosertib-induced activation of the stress MAPK pathways.

Rigosertib interacts with tubulin in RMS and NB cells to induce mitotic spindle defects

To identify the mechanism by which rigosertib induces mitotic arrest in RMS cells, we affinity purified rigosertib-binding proteins from RD cell lysates using a biotin–rigosertib conjugate and identified the interacting proteins using mass spectrometry. Free biotin as well as a biotin conjugate of an inactive isomer of rigosertib, ON 01911, were used as negative controls in this experiment, as previously described (2). Mass spectrometry identified TUBB2A/B (β-tubulin) as a specific interactor with rigosertib but not free biotin or biotinylated ON 01911 (Supplementary Table S3). To determine whether the interaction between rigosertib and tubulin has functional relevance, we assessed levels of acetylated tubulin, a marker of microtubule stability, in RD and SKNAS cells. Acetylated tubulin was decreased in RD and SKNAS cells treated with rigosertib, as determined by quantitative capillary immunofluorescence (Fig. 5A), indicating decreased microtubule stability in cells treated with rigosertib. Acetylated tubulin, then, represents an appropriate pharmacodynamic marker for on-target rigosertib activity in RMS and NB. In a complementary experiment, treatment of RD or SKNAS cells with increasing concentrations of rigosertib resulted in a dose-dependent shift of tubulin from the polymerized form in the pellet fraction (P) to the soluble form (S), consistent with a destabilization of microtubules (Fig. 5B). Importantly, immunofluorescence experiments in RD and SKNAS cells revealed that rigosertib treatment induces mitotic spindle defects, including a reduction of spindle length and an increase in tripolar and tetrapolar spindles (Fig. 5C). These results confirm that rigosertib cytotoxicity in pediatric cancer cell lines is due, at least in part, to induction of mitotic spindle defects. Previous studies showed that rigosertib binds to the colchicine-binding site at the β-tubulin intradimer interface (5). We therefore hypothesized that other tubulin-binding drugs might have efficacy in pediatric cancer cells. We screened a panel of drugs, including nocodazole, albendazole, and combretastatin A4, which also bind to tubulin dimers in the colchicine-binding site, for their ability to decrease proliferation in the RMS cell line, SMS-CTR (Supplementary Fig. S3A). Like rigosertib, combretastatin A4 showed no selectivity for RAS-mutated cells in the NCATS screening dataset (Supplementary Fig. S3B). Low concentrations of combretastatin A4 also induced mitotic spindle defects in RD and SKNAS cells (Supplementary Fig. S3C), indicating a similar mechanism of action to rigosertib.

Rigosertib has limited efficacy in mouse models of RMS and NB

The vinca alkaloids, which are inhibitors of tubulin polymerization, are commonly used chemotherapeutics in the treatment of pediatric RMS and NB. We hypothesized that rigosertib, functioning as an inhibitor of tubulin polymerization, might also be effective in the treatment of these pediatric cancers. To test this hypothesis, we evaluated the effect of rigosertib on in vivo tumor growth. In orthotopic RD xenograft models, rigosertib treatment delayed tumor growth (Supplementary Fig. S4A) such that resulting tumors were smaller compared with tumors from mice treated with vehicle when measured at the end of the study (Fig. 6A). This delay in tumor growth resulted in a modest survival advantage for the rigosertib-treated mice (Fig. 6B). In contrast, in a separate experiment, treatment with the vinca alkaloid vincristine that has an IC50 value of approximately 10 nmol/L in RD cells in vitro (37), prevented tumor growth in an orthotopic RD xenograft model (Supplementary Fig. S4B). In addition, rigosertib did not delay tumor growth in a heterotopic SKNAS xenograft model (Supplementary Fig. S4C). Importantly, no toxicity was observed in mice receiving rigosertib, including no significant body weight loss over the treatment period (Fig. 6C). Pharmacodynamic assessment of tumors harvested at the study endpoint revealed that acetylated tubulin did not decrease in the tumors from mice treated with rigosertib as compared with vehicle control tumors (Fig. 6D). These results suggest that the limited efficacy of rigosertib in this model is due to early acquisition of resistance to rigosertib or poor target
Figure 4.
The cell-cycle block induced by rigosertib treatment in RMS cells promotes generation of reactive oxygen species and activation of the stress MAPK cascades in RMS cells. 

A, Immunoblot analysis of phosphorylated p38 (p-p38), phosphorylated MKK4, and cleaved PARP of RD (left) or SMS-CTR (right) cells treated with 2 μmol/L rigosertib with or without 10 mmol/L N-acetylcysteine (NAC) for 18 hours.

B, Immunoblot analysis of p-p38, p-MAPKAPK2 (a p38 substrate), total MAPKAPK2, and cleaved PARP of RD (left) or SMS-CTR (right) cells treated with 2 μmol/L rigosertib with or without cotreatment with 20 μmol/L SB-203580 (a p38 inhibitor) for 18 hours.

C, NAC cotreatment does not prevent rigosertib-induced G2–M arrest in RD (top) or SMS-CTR (bottom) as determined by DNA content analysis after 24 hours of treatment.

D, SB-203580 cotreatment does not prevent rigosertib-induced G2–M arrest in RD (top) or SMS-CTR (bottom) as determined by DNA content analysis after 24 hours of treatment.

E, NAC cotreatment does not prevent rigosertib-induced caspase 3/7 activity in RD (left) or SMS-CTR (right) as determined by Caspase-glo after 18 hours of treatment.

F, SB-203580 cotreatment does not prevent rigosertib-induced caspase 3/7 activity in RD (left) or SMS-CTR (right) as determined by Caspase-glo after 18 hours of treatment.
engagement. The cell line xenografts used in this study were grossly encapsulated and poorly vascularized, both of which could contribute to poor drug penetration.

Discussion
In this study, we show that rigosertib treatment decreases viability in RMS and NB cells. This decrease in cell viability occurs through the binding of rigosertib to tubulin, an interaction that destabilizes microtubules, and results in the induction of apoptosis and/or mitotic arrest. Mitotic arrest then stimulates production of ROS, as it does in other cellular contexts (38). ROS production stimulates the stress MAPK pathways; however, these pathways do not contribute to further apoptosis or inhibition of the RAS/RAF/MEK/ERK MAPK pathway in the RAS-mutated, RMS and NB cells used in this study. The lack of apoptosis induced by ROS production in RMS cells is not unexpected, because although skeletal muscle cells have high antioxidant capacity (39), extreme levels of ROS induce necrosis, not apoptosis, in skeletal muscle myoblasts, which have a similar gene expression pattern to FN-RMS cells (29). RMS is sensitive to other therapeutics that induce ROS in vitro and in vivo; however, the precise mechanism by which these agents inhibit RMS growth is not completely described (40, 41). Increased levels of ROS stimulate ERK phosphorylation in myoblasts, which could explain why rigosertib

Figure 5. Rigosertib interacts with tubulin in RMS cells, which destabilizes microtubules and induces mitotic spindle defects. A, Treatment of RD or SKNAS with 2 μmol/L rigosertib for 24 hours decreases acetylation of α-tubulin as determined by quantitative capillary immunoassay. B, RD (top) or SKNAS (bottom) were treated with increasing concentrations of rigosertib for 4 hours before lysis and separation of polymerized (P) and soluble (S) tubulin by centrifugation. The pellets were resuspended in a volume equal to that of the soluble fraction. Equal volumes of P and S for each condition were subjected to SDS-PAGE and immunoblot for α-tubulin. C, Treatment of RD (top) or SKNAS (bottom) with 250 nmol/L rigosertib for 24 hours induces mitotic spindle defects in unsynchronized cells, as determined by immunofluorescence staining of α-tubulin (mitotic spindles) and pericentrin (centrosomes). Representative images are shown. Boxed insets are shown as zoomed images at right and display multipolar mitotic spindles and other spindle defects.
Rigosertib delays time to tumor progression in an RD xenograft model. A, Tumor volume on day 19 after initiation of vehicle or rigosertib (150 mg/kg i.p. twice daily) treatment of SCID beige mice bearing orthotopic RD xenografts. Tumors are significantly smaller in the rigosertib treated group compared with the vehicle group ($P = 0.0397$, student $t$ test). B, Rigosertib treatment significantly prolongs survival, denoted as time to develop a tumor of greater than 2 cm$^3$ ($P = 0.0111$, Mantel–Cox test). C, Rigosertib treatment did not decrease mouse body weight by more than 20% in RD xenograft models (dashed line). D, Rigosertib treatment did not decrease α-tubulin acetylation in RD xenografts, a pharmacodynamic indicator of response to the drug, indicating poor tumor penetration of the drug.

Several lines of evidence support the fact that the mechanism of action of rigosertib in pediatric cancers is not primarily RAS-dependent. First, rigosertib demonstrates no increased cytotoxicity in pediatric cancer cell lines harboring mutant RAS alleles as compared with those expressing WT RAS, and in fact, rigosertib was cytotoxic to MEFs devoid of RAS expression. Second, although inhibitors of components of the RAS/RAF/MEK/ERK MAPK pathway induce cell-cycle arrest in the G1 phase in FN-RMS (29) and NB cell lines (43), rigosertib induces a mitotic phase arrest. Third, rigosertib treatment does not decrease ERK phosphorylation in these pediatric cancer cell lines.

We do not observe an interaction between biotinylated rigosertib and RAS effectors by mass spectrometry; however, this does not rule out that such an interaction is occurring. In contrast, we did observe an interaction between biotinylated rigosertib and β-tubulin in RMS cells. Several additional experiments support the hypothesis that rigosertib is functioning as a microtubule-stabilizing agent in RMS and NB cells. First, rigosertib, like other microtubule-stabilizing agents, induces mitotic arrest in these cells. In addition, rigosertib treatment induces mitotic spindle defects, decreases tubulin acetylation, and decreases intracellular polymerized tubulin. Therefore, although rigosertib may have a role as an RAS-mimetic in RMS and NB cell lines, RAS/RAF/MEK/ERK MAPK antagonism is not the primary mechanism by which rigosertib exerts cytotoxic effects in this context. Interestingly, many kinase inhibitors, including the MET inhibitor tivantinib, the PI3 kinase inhibitor buparlisib, and the SRC inhibitor KX2-361, also affect microtubule function (44–46). Rigosertib, then, may function as both a microtubule-stabilizing agent and an RAS mimic in some cellular contexts.

Inhibitors of microtubule polymerization, particularly the vinca alkaloids vincristine and vinorelbine, are commonly used in the treatment of pediatric RMS and NB. Vincristine in combination with other agents comprises the standard-of-care chemotherapeutic regimen for the upfront treatment of low- (47), intermediate- (48), and high-risk RMS (44). Vincristine is also one of the chemotherapeutics used in the induction of remission in patients newly diagnosed with high-risk NB (49) and in the treatment of primary refractory NB (50). Vinorelbine is part of the maintenance chemotherapy regimens used in RMS treatment (51) and is combined with temsirolimus in the treatment of RMS patients in first relapse (52). The clinical use of the vinca alkaloids is complicated by several debilitating adverse effects, including peripheral neuropathy (53). In addition, other chemotherapeutics that interact with tubulin, such as nab-paclitaxel (54) and eribulin (55), have promising preclinical activity in RMS models. Although no pediatric clinical trials of rigosertib have currently been conducted, the side-effect profile of rigosertib in adult trials has been favorable compared with that of the vinca alkaloids. Peripheral neuropathy has not been noted as an adverse event associated with rigosertib treatment. In fact, the most commonly reported adverse events reported in trials of oral rigosertib were dysuria, hematuria, nausea, fatigue and diarrhea, all of which were reversible with discontinuation of the drug (8). Thus, rigosertib might represent a better-tolerated alternative to vinca alkaloids in the treatment of RMS and NB. The efficacy of rigosertib could be augmented by combination with conventional chemotherapeutics or molecularly targeted agents, such as trastuzumab, which we investigated in this study.

In conclusion, rigosertib potently decreases viability of pediatric solid tumor cancer cells in vitro through its ability to interact with tubulin. Alternate methods of administration that might improve the intratumoral penetration of rigosertib and combining rigosertib with other agents such as trastuzumab merit further investigation.

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