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Rigosertib is a more effective radiosensitizer than cisplatin in concurrent chemo-radiation treatment of cervical carcinoma, *in vitro* and *in vivo*

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Summary

Rigosertib is a novel anti-mitotic agent that induces selective G2/M arrest in cancer cells. Our study shows that rigosertib, when administered concurrently with radiotherapy, acts as a radiosensitizer with greater efficacy than cisplatin, the standard drug in concurrent chemo-radiotherapy for cervical malignancies. Rigosertib, combined with radiotherapy, appears to be a potential therapeutic agent for the clinical management of cervical carcinoma. Rigosertib as a radiosensitizer for cervical carcinoma

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Conflict of interests statement:

Dr. E.P. Reddy is the scientific founder, board member, stock owner, grant recipient and a paid consultant of Onconova Therapeutics, Inc. In addition, Dr. E. P. Reddy is a named inventor on pending and issued patents filed by Temple University and licensed to Onconova Therapeutics that are related to this work. The other authors declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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INTRODUCTION

Concurrent cisplatin-based chemo-radiotherapy constitutes the standard treatment for locally advanced cervical cancer. However, administration of cisplatin may result in hematological, gastrointestinal, renal toxicity and neuropathy. Moderate to severe toxicity often requires dose reduction of cisplatin, delay in the treatment or discontinuation of therapy. Thus, novel, less toxic and equally effective compounds are highly desirable.

Rigosertib is a synthetic benzyl styryl sulfone in clinical development that has been shown to have anti-tumor activity alone and in combination with other chemotherapeutic agents.¹⁻⁴ In laboratory studies, rigosertib has been shown to induce mitotic spindle abnormalities and abnormal centrosome localization. This leads to misalignment of chromosomes and activation of G₂/M checkpoint. These events finally result in mitotic catastrophe and cell death.^{1,5,6} One suggested mechanism of action includes the inhibition of one or more kinases regulators of G₂/M checkpoint, like Plk1, Chk-1 and Chk-2.⁵ Further, rigosertib has been shown to inhibit PI3K in mantle cell lymphoma cell lines⁵ and chronic lymphocytic leukemia cells.⁷ Since PI3K plays an important role in cellular processes essential for oncogenesis and tumor survival⁸, several PI3K inhibitors are undergoing clinical development.⁹ However, rigosertib is the only small molecule in development that inhibits both PI3K and Plk1 pathways. Rigosertib activates apoptosis by inducing pro-apoptotic factors from Bcl-2 family and at the same time inhibiting anti-apoptotic proteins such as Mcl-1.⁷

Based on its unique properties and mechanisms of action, rigosertib has been used in several Phase I-III clinical trials either as a single agent or in combination therapy in several types of solid tumors including breast, colon, pancreas, ovarian, lung, and head and neck.^{Reviewed in 6} Further, multiple studies have been conducted in patients with high risk myelodysplastic syndrome.^{Reviewed in 6} A wide range of outcome from stable disease to partial response was obtained in these clinical studies. In a xenograft mice study, rigosertib provided protective effect against doxorubicin-induced weight loss, cardiotoxicity and death.¹⁰ Further, *in vivo* studies show that rigosertib does not exhibit cytotoxicity in the blood, liver or brain.

Thus, rigosertib is shown to have anti-cancer activity with least toxicity in the non-neoplastic cells and it also shows strong synergy with several chemotherapeutic drugs. The purpose of this study was to evaluate whether rigosertib could selectively synchronize cancer cells to G₂/M phase and thus improve the efficacy of radiotherapy since ionizing radiation is most effective in this phase. The radiosensitization effect was further compared with that of cisplatin^{11,12}, an established radiosensitizer that is used as a standard chemotherapeutic agent for cervical cancer but has demonstrated a high level of toxicity.

RESULTS

Rigosertib is a potent and selective inhibitor of cancer cells proliferation

Rigosertib strongly inhibited the proliferation of cancer cell lines, with observed IC₅₀ values in the nanomolar range for both HeLa (115 nM) and C33A (45 nM) cells. In contrast,

rigosertib had a minimal effect on normal cell lines, BJ and Ect1/E6E7 ($IC_{50} > 0.1$ mM) (Fig. 1). IC_{50} values for cisplatin were 10-fold higher when compared to rigosertib in HeLa cells and about 50-fold higher in C33A cells (Fig. 1).

Rigosertib induces G₂/M arrest selectively in cancer cells

HeLa and C33A cells demonstrated a complete (>95%) G₂/M arrest at concentrations of rigosertib greater than 0.5 μ M (Fig. 2), while at less than 0.2 μ M, no clear perturbation of the cell cycle was evident (data not shown). Normal cells were less affected by rigosertib. BJ and Ect1/E6E7 cells increased in G₂/M phase by 30 and 60% respectively, after 24 hours of incubation with 1 μ M rigosertib (Fig. 2).

Rigosertib enhances the DNA damage caused by irradiation more efficiently than cisplatin

Expression of γ -H2AX 1 hour after irradiation, relative to basal expression in the same treatment group, constitutes a reliable index of DNA damage.¹³

To compare the radiosensitizing effect of rigosertib and cisplatin treatments, we calculated and compared the fractional increase of γ -H2AX intensity defined as the ratio between the γ -H2AX fluorescence mean intensity values for irradiated cells and un-irradiated cells for each treatment group (DMSO, rigosertib or cisplatin) using monovariant analysis (data not shown). In HeLa cells, rigosertib resulted in a 48% higher fractional increase value when compared to cisplatin, 1.71 (± 0.28 SD) and 1.15 (± 0.19 SD) respectively. In other words, a 6 Gy irradiation increased γ -H2AX fluorescence intensity of 15% for cisplatin pre-treatment, and 71% for rigosertib pre-treatment. Similar results were found with C33A cells, with fractional increase values of 2.08 (± 0.37 SD) and 1.25 (± 0.37 SD) respectively. A bivariate analysis, which considers DNA content (Fig. 3A), also showed that rigosertib was a more efficient radiosensitizer when compared to cisplatin. After γ -irradiation, rigosertib increased the number of γ -H2AX beyond-threshold events by 5 fold, while cisplatin treatment led to an increase of only about 2 fold in HeLa cells (Fig. 3B). This was also observed in C33A cells, in which rigosertib increased the index by 11-fold compared to 1.55-fold by cisplatin (Fig. 3B). Rigosertib also increased the index by about 2 fold when compared to IR alone while cisplatin did not show a significant increase in the index (Fig. 3B).

Rigosertib is a more potent radiosensitizer than cisplatin *in vitro*

Clonogenic survival dose-response assays were performed for both drugs with HeLa and C33A cells, and isoeffect doses were obtained (Fig. 4 insets). In HeLa cells, rigosertib 1 μ M corresponded to a surviving fraction of 10% ($sf_{10\%}$) and a comparable cisplatin isoeffect dose was obtained with 1.75 μ M (Fig. 4 inset). These $sf_{10\%}$ isoeffect doses were then used to show increases in the efficacy of irradiation (2–8 Gy) (Fig 4). To quantify the difference in radiosensitization properties between the two drugs, survival curves were interpolated at given fractions, and the Dose Modifying Factor (DMF), Dose Reduction Factor (DRF), Radiation Enhancement Ratios (RER), and Drug Enhancement Ratios (DER) were determined (Table 1). DMF was defined as the ratio between the radiation dose without and with the tested agent to achieve the same level of effect. For HeLa cells, $sf_{1\%}$ was obtained with 7.7 Gy radiation alone, with cisplatin 1.75 μ M followed by 6.1 Gy (DMF=1.26), and with rigosertib 1 μ M followed by 4.3 Gy (DMF=1.79). Similarly, $sf_{10\%}$ was obtained with a

5.2 Gy radiation alone, with cisplatin 1.75 μM followed by 4.3 Gy (DMF=1.21), and with rigosertib 1 μM followed by 2.4 Gy (DMF=2.17) (Table 1). A similar experiment was carried out using C33A cells with the $\text{sf}_{10\%}$ isoeffect doses for rigosertib and cisplatin, 0.2 μM for rigosertib and 1.5 μM for cisplatin (Fig. 4 insets). Results in C33A cells showed a higher radiosensitizing effect of rigosertib compared to cisplatin. The $\text{sf}_{1\%}$ was observed with 8.0 Gy irradiation, or with cisplatin 1.5 μM followed by 7.1 Gy (DMF=1.13), or rigosertib 0.2 μM followed by 5.7 Gy (DMF=1.40); $\text{sf}_{10\%}$ was observed with 5.1 Gy irradiation, or with cisplatin 1.5 μM followed by 4.5 Gy (DMF=1.13), or rigosertib 0.2 μM followed by 3.4 Gy (DMF=1.5) (Table 1). Dose Reduction Factor (DRF), defined as the ratio between the radiation dose with and without the tested agent to achieve the same level of effect, was also used to compare treatment groups. In cervical cancer cells, rigosertib showed a DRF between 0.46 and 0.71. By comparison, the DRF for cisplatin was measured between 0.79 and 0.88. Radiation enhancement ratios (RER), defined as the ratio of surviving fractions obtained at 6 Gy IR alone and drug+6 Gy IR and drug enhancement ratios, defined as the ratio of surviving fractions at drug concentration giving 10% survival (that is SF of 0.1) and drug+6 Gy IR (Table 1) also demonstrated that rigosertib is a more potent radiosensitizer than cisplatin for cervical cancer cells. Furthermore, the difference in clonogenic survival curves was statistically significant (ANOVA analysis; $p < 0.001$).

To assess the effects of rigosertib and cisplatin with or without radiation in the first two days following treatment, apoptosis was measured in both cancer and normal cells using annexin V and propidium iodide staining (Fig. e1; www.redjournal.org). As with clonogenic survival assays, rigosertib alone or in combination with radiation was more effective than cisplatin alone or in combination with radiation in inducing apoptosis in cancer cell lines. Normal fibroblast cells, BJ were resistant to any treatment while immortalized Ect1/E6E7 cells showed slight sensitivity to rigosertib but not to cisplatin (Fig. e1).

Rigosertib is a more potent radiosensitizer than cisplatin *in vivo*

Three independent animal experiments were performed. Six treatment conditions were utilized, and five animals were treated in each group. An experimental design schematic is shown in Fig. 5A. The dose of rigosertib was used according to Gumireddy et al¹, however, instead of 250 mg/kg dose every other day, 200 mg/kg was given 3 times in the first 36 hours and later two more dose were given during radiation therapy. A 3 mg/kg dose of cisplatin was selected based on the literature and also with 5 mg/kg cisplatin, a high toxicity was observed in our mouse model. Tumor volume was measured and plotted as a function of time (Fig. 5B). Number of days taken to reach a tumor volume of 500mm³ were determined. Activity of cisplatin was confirmed as single agent and in combination therapy with irradiation. Tumor growth was delayed by 41 days in animals treated with cisplatin alone. Combination therapy with cisplatin and irradiation was highly effective, showing radiosensitizing activity that delayed tumor growth by 64 days. Notably, this was longer than the sum of cisplatin or irradiation alone (respectively 41+16 = 57 days). Single agent rigosertib showed activity, but less than cisplatin, delaying tumor growth for 25 days, when compared to the vehicle treated group. However, when combined with irradiation, rigosertib treatment delayed tumor growth for 122 days. Moreover, 3 out of 15 animals from this combination group exhibited a cure, which lasted 50 days following last treatment. At 175

days follow-up, no tumor was visible (Fig. 5C). These findings were statistically significant ($p < 0.0001$). Further, body weight of the animals was obtained once every two weeks to assess the toxic effects of any of the treatments. As shown in Fig 5D, no significant differences in body weight were observed with any of the treatments groups compared to the vehicle group.

DISCUSSION

Our data show the activity of rigosertib in synchronizing cancer cells in G₂/M phase, the most radiation-sensitive phase of the cell cycle, and the consequent enhancement of DNA damage. Rigosertib treatment led to higher anti-tumor activity both *in vitro* and *in vivo*, when compared to irradiation alone or radiation combined with a cisplatin pre-treatment.

We first profiled the activity of rigosertib by dose-response assay using a panel of normal and cancer cells and confirmed that rigosertib is a potent cytotoxic agent (Fig. 1).¹⁻⁵ We also demonstrated that rigosertib is approximately 10-fold more active than cisplatin in this *in vitro* assay (Fig. 1).

To extend our findings, we verified by flow cytometry that a 24-hour incubation with rigosertib, 0.5–1 μ M, blocks cancer cells (HeLa and C33A cell lines) in G₂/M phase (Fig. 2). This activity was selective toward cancer cell lines. When normal cell lines (Ect1/E6E7 and BJ) were incubated at the same concentrations, the extent of the G₂/M peak was considerably smaller. Furthermore, Ect1/E6E7 cells showed a partial G₂/M increase while BJ cells seemed unaffected. This could be due to the fact that Ect1/E6E7 is a cell line obtained by immortalization through HPV E6 and E7 transfection thus, and although considered normal, it is indeed a G₁/S checkpoint compromised cell line. We believe that the action of rigosertib on these three cell lines can be explained by its anti-mitotic properties.¹⁻⁵ It is well described in the literature that a perturbation of spindle formation elicits the spindle checkpoint and causes cells to arrest in mitosis.¹⁴

It is well recognized that cells in G₂/M phase are more sensitive to the effects of ionizing radiation.¹⁵ Rigosertib acts to synchronize cells in the G₂/M phase following 24 hours of treatment and when irradiated show γ -H2AX expression greater than with irradiation alone or pre-treatment with cisplatin (Fig. 3A). Cisplatin causes DNA damage by irreversibly binding to DNA and forming a number of structurally different adducts with DNA such as intrastrand crosslinking of two nucleobases of single DNA strand and interstrand crosslinking of two different strands of one DNA molecule.¹⁶ Cisplatin works synergistically with irradiation by accumulating and intensifying DNA damage. In this way, cisplatin acts as a radiosensitizer. Since the S-phase is the most sensitive to the action of cisplatin, we expected an accumulation of DNA damage in that phase, and this was evident while using flow cytometry to detect γ -H2AX expression (Fig. 3A). In fact, γ -H2AX flow cytometry analysis showed that cisplatin treatment alone affected DNA integrity, while rigosertib did not (Fig. 3).

Both rigosertib and cisplatin were proven effective as radiosensitizers using clonogenic survival assay (Fig. 4 and table 1). Rigosertib proved to be a more potent radiosensitizer, achieving a 70% greater DMF when compared to the highest DMF obtained with cisplatin.

We realized that G₂/M synchronization might not be the exclusive mechanism involved in radiosensitization by rigosertib. The drug acted as an effective radiosensitizer in a clonogenic assay with C33A cells, for which the dose used (0.2 μ M) was too low to induce G₂/M block. The reason for this behavior could be due to the ability of rigosertib to inhibit the PI3K/Akt/mTOR/eIF4E-BP pathway⁵ which is required for the survival of cells exposed to radiation. In this study, Prasad et al have also shown that tumor cells treated with rigosertib exhibit rapid loss of pro-survival proteins BCL-XL and Mcl-1, which could further enhance the progression of tumor cells to apoptotic cell death. Additional studies aimed at defining the role of PI3K pathway in this activity are currently in progress which will further define the molecular basis for the observed radiosensitization activity seen with rigosertib.

In vivo experiments confirmed the activity of rigosertib as a radiosensitizer with potent anti-tumor activity and low toxicity profiles (Fig. 5D). In the animal group treated with rigosertib and irradiation, the mean tumor volume was the lowest among all 6 treatment groups and 3 out of 15 animals had no palpable tumor 175 days after tumor inoculation. No significant side effects appeared during the experiments. Further, the cisplatin radiosensitizing activity was verified, which appeared to be a simple cumulative effect between the drug and irradiation. Better cisplatin activity might be achieved with higher doses, but with much higher toxicity.

For future clinical application of rigosertib, it may need to be added to the standard of care therapy. Therefore, we also studied the effects of combining rigosertib with cisplatin and radiation therapy in HeLa cells (Table e1; www.redjournal.org). Exponentially growing HeLa cells were treated with rigosertib, cisplatin, and radiation and effects on cell survival were studied by clonogenic survival assay. The cytotoxicity of rigosertib was further enhanced by the addition of cisplatin and irradiation. At a clinically relevant dose of 2 Gy, rigosertib enhances the radiosensitivity and augments the combined cytotoxicity of cisplatin and radiation by one log kill. This data suggests that rigosertib can also be used in combination with cisplatin and radiation for cervical cancer therapy.

In conclusion, rigosertib appears to be more effective than cisplatin when combined with radiation. Although the mechanism is not fully characterized, we showed that rigosertib is capable of synchronizing cervical cancer cells in G₂/M, the most radiation-sensitive phase of the cell cycle, thus enhancing the efficacy of radiation treatment *in vitro* and *in vivo*. Thus, rigosertib in combination with irradiation could be a potential therapeutic agent for the clinical management of cervical carcinoma.

MATERIALS AND METHODS

Cell lines and culture conditions

Human cervical adenocarcinoma, HeLa, cervical squamous carcinoma, C33A, immortalized human ecto-cervical epithelial cell line, Ect1/E6E7 and a human fibroblast cell line, BJ were obtained from American Type Culture Collection (Manassas, VA). Cervical cancer cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and penicillin (100 IU/ml)-streptomycin (100 µg/ml). Ect1/E6E7 were grown in Keratinocyte Free Serum Medium (KFSM), supplemented with pre-qualified human recombinant Epidermal Growth Factor 1-53 (EGF 1-53), Bovine Pituitary Extract (BPE) and penicillin (100 IU/ml)-streptomycin (100 µg/ml). BJ cells were grown in Modified Eagle's Medium (MEM), supplemented with 10% FBS, MEM Non-Essential Amino Acids Solution (0.1 mM), sodium pyruvate (1 mM), and penicillin (100 IU/ml)-streptomycin (100 µg/ml). All growth media and supplements were purchased from Invitrogen Co. (Carlsbad, CA). Cells were maintained in a humidified incubator at 37 °C and 5% CO₂.

Drug formulations

Rigosertib, a synthetic benzyl styryl sulfone, was developed and kindly provided by Onconova Therapeutics, Inc. (Newtown, PA). The compound was dissolved in DMSO (Sigma, St. Louis, MO) and then diluted with culture medium or saline to a concentration of 0.1M. Cisplatin (Sigma, St. Louis, MO) was dissolved in saline to a stock solution of 3.3 mM and then diluted with medium to final concentration.

Growth inhibition and Viability Assays

Approximately 1×10^4 cells were plated per well in 96-well plates, followed by incubation with either rigosertib or cisplatin (1 nM to 0.1mM), 6h after plating for 5 continuous days. Cell viability was then assessed by Alamar Blue assay (Biotium Inc., Hayward, CA).

Clonogenic Survival Assay

HeLa and C33A cells were plated in appropriate number and treated with either rigosertib or cisplatin at different doses (0.1–10 µM) for 24 hours. After 10–12 days, colonies (>64 cells) were fixed with 10% formalin and stained with 0.5% crystal violet in absolute ethanol and counted for clonogenic survival. After a first drug dose-effect curve was obtained, cells from each treatment group were irradiated at different doses (2–8 Gy) using a γ -ray ¹³⁷Cs Irradiator in presence or absence of drugs (cisplatin- 1.75 µM in HeLa cells and 1.5 µM in C33A cells; Rigosertib- 1.0 µM in HeLa cells and 0.2 µM in C33A cells). Drugs were removed immediately after irradiation by changing the media.

Cell Cycle Analysis

Exponentially growing cells were incubated with rigosertib, at concentrations of 50 nM, 100 nM, 200 nM, 500 nM and 1 µM. Following incubation, at 24 hours, cells were harvested by detachment with 0.05% Trypsin-EDTA (Invitrogen Co. - Carlsbad, CA), centrifuged at 800 rpm for 10min, washed with PBS (Invitrogen Co. - Carlsbad, CA), and fixed with 70% ice

cold ethanol. Fixed cells were stained with propidium iodide (Sigma, St. Louis, MO). DNA content data were collected by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ). Cell cycle analysis was performed with FlowJo 8.6 software (Tree Star Inc., Stanford, CA).

DNA Damage/Repair Analysis

Exponentially growing cells were treated with rigosertib (1 μ M) or cisplatin (1.75 μ M) for 24 hours, prior to 6Gy γ -irradiation, followed by harvesting at 0 or 24h post-irradiation. Fixed cells were incubated with anti-phosphohistone H2AX (Ser139) antibody (Millipore Co., Billerica, MA) followed by Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen Co., Carlsbad, CA) and stained with propidium iodide (Sigma, St. Louis, MO) and FACScan (Becton Dickinson, Franklin Lakes, NJ) analysis was performed. In the monovariant analysis, only the γ -H2AX fluorescence intensity was considered for generating histograms and the relative γ -H2AX fluorescence intensity was then obtained by taking the ratio of irradiated cells to un-irradiated cells. For bivariate analysis, a cell cycle phase-specific γ -H2AX fluorescence intensity was obtained after setting a threshold on the γ -H2AX fluorescence intensity to discriminate positive events. The FlowJo 8.6 software (Tree Star Inc., Stanford, CA) was used to perform a cell cycle analysis with the Watson pragmatic method.

Tumor growth *in vivo*

Human cervical carcinoma xenografts were generated by subcutaneous injection of 5×10^5 exponentially growing HeLa cells into the dorsal aspect of the right foot of athymic nude mice (National Cancer Institute, Bethesda, MD). Tumor volume was measured by calipers and calculated by approximation to the volume of an ellipsoid ($H \times L \times W \times 0.523$). Animals with palpable tumors ($> 75 \text{ mm}^3$) were assigned to one out of the six groups of treatment: vehicle alone, vehicle + radiation treatment (RT), rigosertib alone, rigosertib + RT, cisplatin alone, and cisplatin + RT.

For RT, anesthetized animals (ketamine:xylazine mix 7:1 mg/ml for 100 μ L monodose) were confined to a lucite jig with a 0.5 cm lead cover shield to protect animals in individualized compartments from whole body exposure. Tumor bearing legs were projected through the center of the jig to an irradiation field measuring 7.5 cm in diameter. Palpable tumors received 21 Gy total dose, in 3 fractions of 7 Gy each, every 48 hours using a Phillips MGC orthovoltage unit operating at 320 kVp at 5.0 mA with 2.0 mm Cu filtration at a 31 cm source to surface distance operating at 2.2 Gy/min. Rigosertib, 200 mg/kg per dose, was injected intraperitoneally 3 times, 12 hours apart, with the last dose 6 hours before the first irradiation, then 24 hours before the second and the third irradiation doses. Cisplatin, 3 mg/kg per dose, was administered 30 minutes before each irradiation, as described elsewhere.¹⁷ The tumors were measured twice a week, along with assessment of health status and body weight was obtained once every two weeks.

Statistical analysis

IC₅₀, clonogenic survival and tumor growth were collected, recorded and analyzed for statistical significance with Prism 4.0 software (GraphPad Software Inc., San Diego, CA).

The ANOVA method was used to verify the statistical significance of the difference between the groups in each experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Alamar Blue viability assays for rigosertib in cancer and normal cell lines and cisplatin in cancer cell lines. Cells were incubated with the drugs for 5 continuous days before assessing cell viability and growth inhibition. Relative proliferation (percent of control) from 4 independent experiments for each cell line is shown with standard deviation. IC_{50} (concentration inhibiting 50% of proliferation) values were determined as shown.

Fig. 2.

FACS cell cycle analysis of cancer and normal cell lines after 24 hour incubation with rigosertib at different concentrations or DMSO control. Representative histograms are shown from 4 independent experiments performed for each cell line.

Fig. 3.

A. FACS analysis of DNA damage in cancer cells after 24 hour incubation with rigosertib, cisplatin or DMSO, with or without concurrent 6 Gy irradiation, detected by γ -H2AX expression (y-axis) and DNA content (x-axis). Mean percentage of events beyond threshold (from 4 independent experiments) is shown with standard deviation (SD). Cell cycle phases (G1, S, G2/M) and DNA content (2N, 4N) are marked. **B.** Ratios of mean percentage of events beyond threshold obtained with drug + IR and drug alone or IR alone derived from 3A are shown.

Fig. 4.

Clonogenic survival assays of cancer cells with rigosertib, cisplatin or DMSO, with or without concurrent irradiation at different doses (x-axis). Cells were incubated with the drugs at a concentration giving 10% cell survival for 24 hours. Immediately after irradiation at different doses, drugs were removed by changing the media and the colonies were allowed to form for 10–12 days. Surviving fraction (y-axis) of 10% (sf10%) and 1% (sf1%) is shown. **Insets.** Clonogenic survival assays of cancer cells with rigosertib and cisplatin. Cells were incubated with different concentrations of rigosertib or cisplatin for 24 hours and colonies were allowed to form for 10–12 days. Isoeffect concentrations of the drugs at 10% cell survival were determined.

Fig. 5.

A. A scheme of experimental design showing different doses and schedule of treatments used for *in vivo* studies.

Tumor growth curves from 3 independent experiments ($n=5$ per group each experiment, total $n=15$ per group) with HeLa xenografts treated with rigosertib, cisplatin or vehicle, with or without concurrent radiotherapy. In panel **B**, mean tumor volume per group is shown along with tumor growth delay at target volume. In panel **C**, single tumor volumes are shown, grouped by drug.

D. Animals were weighed once in every two weeks for 175 days and body weight is shown as a function of time.

Table 1

Summary of dose modifying and dose reduction factors and radiation and drug enhancement ratios following clonogenic survival assays in HeLa and C33A cells.

	DMF=IR dose at SF of 0.1 / IR dose with drug to get SF of 0.1		DRF=IR dose with drug to get SF of 0.1 / IR dose at SF of 0.1		RER= SF _{6 Gy} / SF _{drug+6 Gy}		DER= SF _{0.1} / SF _{drug+6 Gy}	
	Cisplatin	Rigosertib	Cisplatin	Rigosertib	Cisplatin	Rigosertib	Cisplatin	Rigosertib
HeLa	1.21	2.17	0.83	0.46	5	50	10	100
C33A	1.13	1.5	0.88	0.66	2.1	6.9	4.2	13.9