The Radiosensitizing Potential of Rigosertib and Dinaciclib in Ewing’s Sarcoma Cells

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ABSTRACT

Ewing’s sarcoma is the second most frequent bone cancer. However, it is still a relatively uncommon cancer, with only approximately 225 new cases diagnosed in patients under 20 years old each year in North America. Despite the low incidence of Ewing’s sarcoma, it is still deadly for a large proportion of metastatic cases. In addition, treatment can induce secondary cancers due to the high radiation dosage required for definitive radiotherapy. The purpose of this research is to test the possibility of chemotherapeutic agents to increase the radiosensitivity of Ewing’s sarcoma cells, as well as to compare the effects of each treatment type based on cell size, in order to improve the effectiveness and specificity of the treatment. The two inhibitors of interest for this study, Rigosertib and Dinaciclib, demonstrated increased radiosensitization for all cell types compared to the control. Additionally, there were no significant differences in radiosensitivity between cell types, indicating that both drugs function equally well in Ewing’s sarcoma cells regardless of differences in cell size. The mechanism of action for each inhibitor was examined by protein immunoblot analysis, which showed a decrease in PLK1 protein expression after treatment with Rigosertib, but no change in CDK1 expression following treatment with Dinaciclib. These results indicate the potential of using the inhibitors Rigosertib and Dinaciclib as effective radiosensitizing agents for the treatment of Ewing’s sarcoma.
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BACKGROUND

Incidence

Ewing’s sarcoma was first discovered by James Ewing in 1921, which he described as a “diffuse endothelioma of bone” (1). It occurs in a wide range of ages, although patients under 20 years old account for approximately 80% of new diagnoses (2). Additionally, it is more commonly diagnosed in males (male/female ratio, 1.3–1.5:1) (2). Ewing’s sarcoma is the second most frequent bone cancer (3-5). However, it is still a relatively uncommon cancer, with only approximately 225 new cases diagnosed in patients under 20 years old each year in North America (3).

Prognosis

Ewing's sarcoma typically progresses quickly. Skeletal lesions often form large tumors, which develop into soft tissue after a few weeks. The earliest symptom is pain, and may be associated with paresthesia, which is a tingling sensation of the skin. Tumor growth typically leads to swelling, and the affected site begins to feel tense, hard, elastic, tender, and warm to the touch (2). Minor fevers, along with other non-specific symptoms, are more commonly found in more advanced or metastatic stages, and affect approximately one-third of patients (3).

The prognosis for Ewing’s sarcoma patients with large primary tumors or metastases remains relatively poor despite aggressive therapy, such as combination treatments of high-dose chemotherapy, surgery, and radiotherapy. Patients with localized disease have approximately 70% survival rate (3). However, the survival rate for metastatic cases is much worse, with less than 30% chance of survival after the disease has spread (3).
Treatment

A majority of cases require a combination treatment involving chemotherapy, in addition to local disease control via surgery or radiation (3). Ewing’s sarcoma cells are relatively sensitive to radiation (1). In order to treat them, radiation doses of approximately 45-60 Gy are typically administered (4). However, radiation therapy can induce secondary cancers in a dose-related manner, with a significantly greater risk at doses greater than 40 Gy (3, 4). Therefore, there is likely a significant benefit in increasing the radiosensitivity of the cells in order to reduce the administered dose necessary for treatment.

Treatment of distant metastases in Ewing's sarcoma should include chemotherapy as well. Before the use of chemotherapeutic agents in cancer treatments, patients diagnosed with Ewing’s sarcoma had a long-term survival rate of less than 10% (2). However, most centers performing intensive chemotherapy treatments are now experiencing survival rates of 60-70% (2, 5), indicating the sensitivity of these cells to chemotherapy. Despite its role in increasing survival in Ewing’s sarcoma, chemotherapy has been shown to cause a number of late side effects, such as cardiotoxicity and infertility (4).

Local control of the disease through surgery is often performed as well. Previous reports have demonstrated a decrease in the local recurrence rate to less than 10%, as well as increases in the survival rate following wide resection of the primary tumor (2). Additionally, multiple retrospective analyses have concluded that, when possible, surgery is preferable to radiotherapy for local control (2, 6). However, there may be a selection bias favoring patients for whom surgery is an option (2, 7). Therefore, the decision between surgery and irradiation as a method of local treatment should be made on a case-by-case basis.
Characteristics

Although Ewing's sarcoma is typically classified as a bone tumor, it often exhibits characteristics of both mesodermal and ectodermal origin, making it difficult to classify (2). The exact point of origin of the growth is not clear, but the early rarefaction, or thinning of bony tissue, indicates that the disease begins in the blood vessels of the bone tissue (2).

Ewing's sarcoma is composed of a homogeneous population of small round cells with a high nuclear-to-cytoplasmic ratio that are arranged in sheets (2). However, James Ewing observed that in some sections, these cells were of increased size, while in others they were smaller and more compact (1). A similar observation was previously made within this laboratory for A4573 cells, and independent clones of these cells were generated based on size into “small” and “large” categories, while others were kept in the original, “mixed” state in order to compare their response to treatment. This was performed based on the assumption that the “small” cells may possibly require a shorter period of time to complete the cell cycle due to their size. If true, these cells would replicate faster than the “mixed” or “large” cells, and would likely be more aggressive. Additionally, since these cells are smaller in size, it is possible that they would be more likely to metastasize. If true, the relative effectiveness of the inhibitors on these cells would be of interest in developing an appropriate treatment for Ewing’s sarcoma.

Compared to osteosarcoma, flat bones of the axial skeleton are relatively more commonly affected in Ewing’s sarcoma, and malignancies tend to arise in the diaphysis rather than the metaphysis of long bones (3). Primary cancers typically arise in pelvic bones, the long bones of the lower extremities, and the bones of the chest wall. Metastases are also commonly found in the lungs, bone, and bone marrow, and are detectable in about 25% of patients (2).
Ewing's sarcoma typically appears ill-defined and permeative and is often accompanied by a periosteal reaction that affects the diaphyses of long bones (2). Several other types of periosteal reactions have been observed, including a ‘sunburst’ pattern indicating a perpendicular reaction, and ‘Codman's triangle’ indicating that the periosteum has lifted from the bone (2).

**Oncogenesis Determinants**

The EWS/FLI1 protein results from a t(11;22)(q24;q12) translocation that fuses the EWS gene on chromosome 22 to the FLI1 gene on chromosome 11 (8). Since the DNA-binding domain of FLI1 pairs with the transcription-regulating domain of EWS, an aberrant transcription factor is formed (9), leading to uncontrolled growth and proliferation of cells. This chromosomal abnormality is specific to Ewing’s sarcoma, and is detected in over 85% of cases (10). Thus, ESFTs contains a unique protein produced by a tumor-specific translocation with the potential for use as a molecular target, though no such treatments have been approved yet.

The chimeric EWS/FLI1 protein which results from this translocation is believed to affect the expression of cell cycle-regulatory molecules. It has recently been reported that Forkhead box M1 (FoxM1) expression is elevated in Ewing’s sarcoma (11). FoxM1 is associated with proliferation, cell cycle progression from G1/S and G2/M phases, as well as mitotic chromosome stability (12), and its overexpression has been shown to promote cell cycle progression (13). Polo-like kinase 1 (PLK1) is another critical cell-cycle mediator whose expression is believed to be affected by the EWS/FLI1 protein. It controls entry into the mitotic phase, spindle assembly, and centrosome maturation, and modulates the transition through the G2/M checkpoint (14).

Past studies have indicated that PLK1 regulates FoxM1 transcriptional activity by direct phosphorylation and therefore regulates mitotic progression (14). In late S and G2 phase, FoxM1
is initially phosphorylated by CDK1, creating docking sites for PLK1 (15). PLK1 then binds and directly phosphorylates FoxM1 (15). This activates FoxM1 transcriptional activity, resulting in the enhanced expression of key regulators of mitotic progression (15). As PLK1 is a target gene of FoxM1 (14), these interactions can generate a positive-feedback loop, leading to a further increase in PLK1 levels and FoxM1 activity. Since FoxM1 is the major transcription factor during G2/M transition, its activation is also necessary for progression into the M phase (14).

FoxM1 upregulation has been found in a majority of solid human cancers, and PLK1 overexpression is a poor prognostic feature in numerous types of cancer (16). Decreased expression of FoxM1 has also been previously shown to decrease the tumorigenicity of EWS cells (17), and to reduce expression of PLK1 (17). This suggest the potential role of these genes in the EWS/FLI1 signaling pathway, as well as their importance as potential targets of regulation in the treatment of Ewing’s sarcoma.

**Rigosertib**

Rigosertib (ON-01910) is a non-ATP-competitive inhibitor to PLK1, and is currently in Phase III clinical trials as a single agent and in combination with conventional chemotherapy in advanced and metastatic tumors (18). PLK1 is of significant interest in this study due to its role in the phosphorylation of FoxM1 in the EWS/FLI1 pathway (15). Rigosertib inhibits mitotic progression by arresting cells in the G2/M phase of the cell cycle and induces abnormalities in mitotic spindle formation, eventually leading to selective apoptosis in tumor cells, but not in normal cells (19). This drug has previously been shown to be an effective radiosensitizer in cervical carcinoma *in vitro* (20). However, there does not currently appear to be any published research on the radiosensitizing effects of Rigosertib on Ewing’s sarcoma cells.
**Dinaciclib**

Dinaciclib (SCH 727965) has reached phase III clinical trials for a range of cancer indications (21). This drug is of interest for this study due to its role in the inhibition of cyclin-dependent kinase (CDK) 1 (22), which serves as the priming kinases for PLK1 in the EWS/FLI1 pathway (15). However, Dinaciclib also acts through the inhibition of cyclin-dependent kinases (CDKs) 2, 5, and 9 (22). CDK1 and CDK2 allow for progression of cells through the S phase and into mitosis, respectively (23), and suppression of CDKs 1 and 2 has been shown to decrease the proportion of cells in the S phase while increasing those in the G2/M phase (24). Since cells are most radiosensitive in the G2/M phase of the cell cycle (25), Dinaciclib could also potentially serve as a radiosensitizing agent. In addition, CDK5 has been shown to play a role in cancer cell migration and anchorage-independent growth (26), and gene transcription requires the activity of CDK9 (23). Whether CDK inhibitors induce apoptosis by repressing transcription or by disrupting the cell cycle is unclear. There also does not currently appear to be any published research on the effectiveness of Dinaciclib in treating Ewing’s sarcoma.

**Purpose**

The purpose of this study is to evaluate whether Rigosertib could selectively synchronize Ewing’s sarcoma cells to G2/M phase and thus improve the efficacy of radiotherapy, as ionizing radiation is most effective in this phase (20). Additionally, this study will attempt to determine the efficacy of treating Ewing’s sarcoma cells with radiotherapy in combination with Dinaciclib in order to compare potential cancer treatment methods. These treatment conditions will also be applied to cells exhibiting “large,” “mixed,” and “small” morphologies in order to determine any potential differences in their replicative properties or sensitivity to treatment.
**Materials and Methods**

*Reagents*

DMEM and antibiotics were purchased from Corning (Manassas, VA, USA), Trypsin was purchased from Life Technologies (Frederick, MD, USA). Protease inhibitor cocktail was purchased from Roche Applied Science (Indianapolis, IN, USA), and 0.45µm MCE filter units were purchased from Fisher Scientific (Pittsburgh, PA, USA). Rigosertib (ON-01910) and Dinaciclib (SCH 727965) compounds were obtained from Selleckchem (Houston, TX, USA). The antibody against PLK1 was obtained from Cell Signaling Technologies (Beverly, MA, USA), and antibodies against FoxM1, FLI1, CDK1, and beta actin were obtained from Abcam (Cambridge, UK). Anti-mouse and anti-rabbit secondary antibodies were purchased from Cell Signaling Technologies. Pierce ECL Western Blotting substrate was purchased from Thermo Scientific (Rockford, IL, USA). Unless otherwise mentioned, most chemicals used in the study were molecular biology or cell culture grade and obtained either from Fisher Scientific or Corning.

*Cell Lines*

The Ewing’s sarcoma tumor cell lines, A4573, were obtained from the Notario laboratory collection. Independent clones of these cells were generated based on size into “small” and “large” categories, while the original cells were kept in their standard, “mixed” state. The cells were cultured on 10 cm tissue culture plates in Dulbecco's Modified Eagle Medium (DMEM, Corning) containing 100 U/mL penicillin, 100 µg/mL streptomycin (Corning), and 10% fetal bovine serum (FBS, Tissue Culture Shared Resource). Adherent monolayers were passaged every 3 to 5 days and grown at 37°C in a humidified atmosphere with 5% CO₂.
Cell Trypsinization, Inactivation, and Collection

After the cells reached ~80% confluence, the medium was aspirated, and the cells were washed with 1X phosphate buffered saline (PBS, Corning). Trypsin was then added to the plate to detach cells from the plates, once cells were released from plate, they were transferred to a tube containing PBS and FBS to inactivate the trypsin activity. The resulting cell suspension was centrifuged at 5000 rpm for 5 min, the supernatant was aspirated, and the cells in the pellet were then passaged by adding medium to the vial and re-plating them for further growth.

Inhibitor Exposure

Trypsinization, inactivation, and collection was performed for each cell type, and cell counting was performed using a hemocytometer (Fischer Scientific) to determine the cell numbers. These cells were then diluted with media so that 100,000 cells were plated into each well of 6-well polystyrene plates (Corning). After ~12 h, this medium was aspirated and new media containing a range of concentrations of Rigosertib and Dinaciclib was added to each plate. These cells were then permitted to grow for 72 h to allow sufficient time for the action of the inhibitor on growth and replication to be effective, due the ~28 h cell division time of Ewing’s sarcoma cells. Afterwards, these cells were lysed and BCA Protein Estimation was performed on the cell lysates from each well in order to determine the appropriate concentrations of each drug that produced ~80% survival in each cell line. This drug concentration was then used for later experimentation involving combination treatments with inhibitor followed by irradiation.
Radiation Exposure

Cell counting was performed for each cell type. These cells were then diluted with medium and plated to 10 cm collagen-coated plates (Corning), so that 1000 cells were placed on the plates to be exposed to 0 Gy and 2 Gy of radiation, and 2500 cells were placed on the plates to be exposed to 4 Gy and 8 Gy of radiation. This was done to ensure that the plates exposed to higher radiation levels would still exhibit a statistically significant number of surviving cell colonies. After ~12 h, these cells were exposed to the appropriate radiation dosage, and permitted to grow and replicate for two weeks before performing fixation, staining, and colony counting. The media was then aspirated and these cells were washed gently with PBS, and fixed to the plate with 100% methanol (Fischer Scientific) for 1 h. Once fixation was complete, methanol was aspirated, and cell clusters were stained with 100% ethanol and 1% Crystal Violet for 1 h. The plates were then gently rinsed with tap water and cell clusters were counted with a Manostat Colony Counter (PGC Scientifics).

Combined Inhibitor and Radiation Exposure

The inhibitor concentrations determined to produce 80% survival in each cell type was then used to study the effect of combination treatments. In this step, a similar procedure was used to that of radiation exposure alone. However, two hours prior to irradiation, the medium in each plate was aspirated, and new medium containing the appropriate inhibitor concentration was added to each plate. This allowed for the study of the radiosensitization potential of both inhibitors among each cell type.

For assays involving calculation of the sensitivity enhancement ratio (SER), a level of survival was chosen where the curves appeared to be log-linear (~10% survival), and the ratio of
the estimated radiation dose required to produce the same survival percentage for the untreated control was divided by the radiation dose required for the cells exposed to inhibitor.

**Western Blot Analysis**

Protein immunoblot analysis was performed on lysates of the “mixed” cell type in order to determine the effect each inhibitor type had, either alone or in combination with irradiation, on protein expression. To perform this analysis, the appropriate dosage of each inhibitor type plus or minus radiation was determined that would affect targets of protein expression while still allowing cells to retain confluence. These cells were then plated to 10 cm polystyrene plates and allowed to reach ~80% confluence. Each plate was then treated with the determined appropriate dosage, and permitted to grow and replicate for ~24 h.

The treated cells were lysed using a 1X RIPA buffer containing protease inhibitors (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA at pH 8). The collected lysates were kept on ice for 20 min, vortexing every 5 min. They were then centrifuged at 12,000 rpm at 4°C for 15 min. Total protein concentration in the cell lysates were then determined using the Pierce BCA Protein Assay Kit (Thermo Fisher) and recorded using a colorimetric plate reader.

Cell extracts were then subjected to SDS-Page, separated using Tris-Glycine SDS polyacrylamide gels (10-well of 4-20%, Invitrogen) and run at 150 V for 2 h. The resolved polypeptides were transferred to polyvinylidene difluoride (PVDF) membranes at 50V for another 2 h at 4°C. The membrane was then blocked with 2.5% skim milk in Tris-Buffered Saline and Tween 20 (TBST) and incubated overnight at 4°C with gentle rocking. The following morning, primary antibodies were added at room temperature (1:1000 dilution with 2.5% skim
milk in TBST) and incubated with gentle rocking for 1 h. The blots were then washed 3 times for 10 min each in 1X phosphate buffered saline supplemented with Tween-20 (PBST). The HRP-conjugated secondary antibodies were then added to PBST according to the primary antibody source (mouse 1:2000, rabbit 1:2000 with 2.5% skim milk in TBST) with gentle rocking for 1 h. The blots were then washed 3 times again for 10 min each in PBST. Pierce ECL western blotting substrate was added to the membranes for 3 min before being developed.

After developing each antibody, beta actin expression was determined in order to confirm that protein loading was equivalent for each sample. In order to do so, each membrane was washed for 7 min, stripped for reprobing with a mild stripping buffer mixture (0.2 M glycine, 3.5 mM SDS, and 1% Tween 20 at pH 2 and 37°C) for five min, and blocked for 45 min with 5% skim milk in TBST. The procedure described previously for adding primary antibodies and secondary antibodies was then repeated with beta actin (1:10000 dilution factor) and detected using Pierce ECL western blotting substrate. The expression of each antibody was then analyzed using ImageJ software, and compared with beta actin in order to determine differences in relative expression of proteins by treatment group.

Statistical Analysis

Unless otherwise indicated, all analyses were performed with two trials, each in triplicate. Data from quantification analyses were expressed as mean ± SD.
RESULTS

Inhibitor Survival

The ideal concentration range for each inhibitor type was determined by decreasing the concentrations by a factor of ten each time until the lowest concentration showed >80% survival for each cell type (not shown). The survival curve shown below includes the relative survival percentage for each cell type following treatment with the inhibitor Rigosertib (Figure 1). Representative photos of cells at certain inhibitor concentrations are shown as well in order to represent this effect visually (Figure 2).

Figure 1. Survival curve for A4573 “large,” “mixed,” and “small” cells following 72 h of exposure to Rigosertib concentrations of 0, 50, 100, 200, 500, and 1000 nM. Error bars represent ± SD.
These results shown in Figures 1 and 2 demonstrate ~80% survival for Rigosertib at a concentration of 200 nM, and that there is no significant difference in the effects of the inhibitors based on cell size.

The survival curve shown in Figure 3 includes the relative survival percentage for each cell following treatment with the inhibitor Dinaciclib. Representative photos of cells at certain inhibitor concentrations are shown as well in order to represent this effect visually (Figure 4).
Figure 3. Survival curve for A4573 “large,” “mixed,” and “small” cells following 72 h of exposure to Dinaciclib concentrations of 0, 0.5, 1, 2, 5, and 10 nM. Error bars represent ± SD.

Figure 4. Representative photos of cell growth for A4573 “large,” “mixed,” and “small” cells following 72 h of exposure to Dinaciclib concentrations of 0, 5, and 10 nM.
The results shown in Figures 3 and 4 demonstrate ~80% survival for Dinaciclib at a concentration of 6 nM, and that there is no significant difference in the effects of the inhibitor based on cell size.

**Determination of Radiosensitization Effect**

The plating efficiency for the “large” cells was determined to be ~51% (not shown). This was performed in order to establish the background levels of colony formation in the untreated controls. A radiation survival curve was then generated for the “large” cell type following inhibition by 200 nM Rigosertib or 6 nM Dinaciclib, followed by radiation exposures of 0, 2, 4, or 8 Gy, compared to the control (Figure 5).

!["Large" Cell Inhibitor + Radiation Survival Curve](image)

**Figure 5.** Radiation survival curve for A4573 “large” cell colonies following treatment with no inhibitor, 200 nM Rigosertib, or 6 nM Dinaciclib in combination with radiation exposures of 0, 2, 4, and 8 Gy. Both trials are shown for each treatment condition. Error bars represent ± SD, and are smaller than markers when not visible.

Representative photos of the counted plates used to produce the radiation survival curves are shown in Figure 6, below.
The results shown in Figures 5 and 6 indicate radiation exposure caused greater cell death in “large” cells when exposed to Rigosertib (SER: 1.5) or Dinaciclib (SER: 1.2) prior to and during irradiation. Rigosertib also appears to exhibit a slightly greater radiosensitization effect than does Dinaciclib.

The plating efficiency for the “mixed” cells was determined to be ~48% (not shown). A radiation survival curve was then generated for the “mixed” cell type following inhibition by 200 nM Rigosertib or 6 nM Dinaciclib, followed by radiation exposures of 0, 2, 4, or 8 Gy, compared to the control (Figure 7). Representative photos of the counted plates used to produce the radiation survival curve are shown in Figure 8, below.
Figure 7. Radiation survival curve for “mixed” cell colonies following treatment with no inhibitor, 200 nM Rigosertib, or 6 nM Dinaciclib in combination with radiation exposures of 0, 2, 4, and 8 Gy. Both trials are shown for each treatment condition. Error bars represent ± SD, and are smaller than markers when not visible.

Figure 8. Representative photos of colony survival for A4573 “mixed” cells following treatment with no inhibitor, 200 nM Rigosertib, or 6 nM Dinaciclib in combination with radiation exposures of 0, 2, 4, and 8 Gy.
The results shown in Figures 7 and 8 indicate radiation exposure caused greater cell death in the “mixed” cells when exposed to the inhibitors Rigosertib (SER: 2.0) or Dinaciclib (SER: 1.6) prior to and during irradiation. Additionally, Rigosertib appears to exhibit a slightly greater radiosensitization effect than does Dinaciclib.

The plating efficiency for the “small” cells was determined to be ~43% (not shown). A radiation survival curve was then generated for the “small” cell type following inhibition by 200 nM Rigosertib or 6 nM Dinaciclib, followed by radiation exposures of 0, 2, 4, or 8 Gy, compared to the control (Figure 9).

!["Small" Cell Inhibitor + Radiation Survival Curve](image)

**Figure 9.** Radiation survival curve for “small” cell colonies following treatment with no inhibitor, 200 nM Rigosertib, or 6 nM Dinaciclib in combination with radiation exposures of 0, 2, 4, and 8 Gy. Both trials are shown for each treatment condition. Error bars represent ± SD, and are smaller than markers when not visible.

Representative photos of the counted plates used to produce the radiation survival curve are shown in Figure 10, below.
The results shown in Figures 9 and 10 indicate radiation exposure caused substantially greater cell death in the “large” cells when exposed to the inhibitors Rigosertib (SER: 1.5) or Dinaciclib (SER: 1.4) prior to and during irradiation. Additionally, Rigosertib appears to exhibit a slightly greater radiosensitization effect than does Dinaciclib.

Some slight differences were found in the effects of radiation based on cell size as well. For example, Rigosertib exhibited a slightly greater radiosensitization effect for “mixed” cells (SER: 2.0) compared to that of “large” (SER: 1.5) and “small” (SER: 1.5) cells. Additionally, Dinaciclib exhibited a slightly greater radiosensitization effect for “mixed” cells (SER: 1.6) compared to “small” cells (SER: 1.4), which was also greater than for “large” cells (SER: 1.2).
While there are some differences in the survival percentages of the cell clusters based on morphological differences, these differences do not appear to be a significant. Therefore, it appears that the radiosensitization effect of Rigosertib and Dinaciclib on A4573 cells does not depend on cell morphology.

The SER values determined for the “large,” “mixed,” and “small” cells exposed to Rigosertib and Dinaciclib are compiled within Table 1, below.

<table>
<thead>
<tr>
<th>Sensitization Enhancement Ratio (SER)</th>
<th>Rigosertib</th>
<th>Dinaciclib</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Large&quot;</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>&quot;Mixed&quot;</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>&quot;Small&quot;</td>
<td>1.5</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 1. Calculated SER values for A4573 “large,” “mixed,” and “small” cells after exposure to 200 nM Rigosertib or 6 nM Dinaciclib.

This calculation was performed in order to determine the radiosensitization effect that these inhibitors had on Ewing’s sarcoma cells of each type.

**Western Immunoblot Detection**

Since there was no consistent trend demonstrating a difference in inhibitor or radiation effectiveness on cell survival based on cell morphology, it was assumed that the effects of each inhibitor would be consistent among each cell size. Therefore, the effects of each treatment in A4573 “mixed” cells were assumed to be representative of all cell types. Western blot detection was thus performed only on the “mixed” cell type in order to determine the possible effects of treatment on protein levels compared to the control. This was performed in order to determine the effect that inhibition or irradiation had on relative protein expression.

In this step, A4573 “mixed” cells were exposed to 200 nM Rigosertib or 6 nM Dinaciclib, followed by administration of either a 0 Gy or 2 Gy radiation dose ~2 h after
inhibition. After ~8 h, these cells were then lysed and protein expression was determined by western blot analysis. The protein expression for cells exposed to each treatment condition was compared to the loading control, beta actin. The protein detection data after inhibition is shown in Figure 11, below.

![Relative Protein Expression After Inhibition, With or Without Irradiation]

**Figure 11.** Relative protein expression of A4573 “mixed” cells treated with Rigosertib or Dinaciclib, with (+) or without (-) irradiation, compared to the untreated control. Beta actin was used as a loading control.

The protein detection data plotted in Figure 11 was analyzed from the developed films from each western blot, as represented in Figure 12, below.
The western blot results for Ewing’s sarcoma cells exposed to Rigosertib plus or minus radiation, showed decreased PLK1 protein expression, as well as decreased FoxM1 expression for non-irradiated cells, compared to the loading control, beta actin (Figure 11). However, no change was detected in EWS/FLI1 protein expression.

The western blot results for cells exposed to Dinaciclib, plus and minus radiation, showed no change in CDK1 expression compared to the loading control (Figure 11). Interestingly,
however, cells exposed Dinaciclib plus radiation showed a decrease in PLK1 expression, as well as decreased FoxM1 expression for non-irradiated cells.

Radiation also appeared to demonstrate a decrease in the expression of EWS/FLI1 and PLK1 proteins among all inhibitor treatment conditions (Figure 11), though no change in expression of FoxM1 or CDK1 was determined for these cells.

**DISCUSSION**

This study demonstrates that Rigosertib and Dinaciclib, inhibitors that are both currently undergoing clinical trials for other tumor types, function effectively in reducing the cell survival of Ewing’s sarcoma cells *in vitro* (Figures 2 and 4, respectively). Additionally, our results show that radiation is significantly more toxic following treatment with Rigosertib or Dinaciclib (Figures 8 and 10, respectively), suggesting that both drugs are effective radiosensitizers. This may be due to the fact that both Rigosertib and Dinaciclib likely play a role in halting cell cycle progression at the G2/M phase, which is the most radiosensitive portion of the cell cycle (20). This is of great interest because a radiation dose above 40 Gy is necessary in order to control Ewing’s sarcoma. For definitive radiotherapy, doses between 55 Gy and 60 Gy are usually given (3). Irradiation does have the potential to induce second cancers in a dose-related manner, with a significantly greater rate at administered doses above 40 Gy (3). Thus, the radiosensitizing potential of these drugs could be useful in reducing the radiation dose required for treatment of Ewing’s sarcoma patients.

Rigosertib appears to be a more effective radiosensitizer (Figure 7) than Dinaciclib (Figure 9), which indicates that this drug could be useful for further experimentation with potential combination treatments in Ewing’s sarcoma cells, though both treatment types are of
interest for future studies. Dinaciclib shows more effective cell killing at a lower concentration (Figure 3) than does Rigosertib (Figure 1), which might reduce the risk of other nonspecific effects taking place when administering treatments, though the effect of these inhibitors in normal cells would need to be determined by further experimentation. Additionally, inhibition of CDK5 could decrease the potential development of metastases (23), which are a significant indicator for decreased survival rates in patients (3). Previous studies have also shown that Dinaciclib exhibits superior activity with an improved therapeutic index in preclinical studies compared with flavopiridol, a CDK inhibitor in clinical trials as a cancer therapy agent (22). This has been proven by its ability to induce regression of established solid tumors in a range of mouse models following intermittent scheduling of doses below the Maximum Tolerable Dose (MTD) (22). This is of interest due to the fact that a lower inhibitor dosage may reduce the risk of adverse effects in patients receiving cancer treatment.

There were significant differences found in the effectiveness of some of the treatments based on size between the “small,” “mixed,” and “large” cell types (Figures 5, 7, and 9). However, there are a number of potential factors that may account for these differences. For example, the greater survival percentages for the “large” cells may possibly be due to the fact that their colonies are typically easier to visualize during counting due to their larger average size compared to that of the “mixed,” and especially the “small,” cell types. Additionally, the relatively low survival percentages for the “small” cell may possibly be due to their small colony size, which made it difficult to determine significant differences between groups based on their treatment conditions. Therefore, it appears that each combination of treatments could still be equally effective in treating Ewing’s sarcoma cells containing a range of morphological differences.
To determine whether the inhibitors Rigosertib and Dinaciclib regulated the protein expression levels of their molecular targets, western blots were performed on the A4573 “mixed” cells after treatment with each drug with or without irradiation. These results confirmed that PLK1 protein expression levels were reduced by Rigosertib (Figure 11). It also showed that FoxM1 expression was reduced for non-irradiated cells, but that EWS/FLI1 expression was unaffected (Figure 11). This suggests that Rigosertib, in addition to its known inhibitory effect on PLK1 kinase activity, may decrease PLK1 function by inducing a decrease in total PLK1 and FoxM1 protein levels. However, it does not appear to affect the translation of its putative upstream target, EWS/FLI1. This does not indicate that the activity of these proteins are unaffected, however, as PLK1 has been shown to phosphorylate FoxM1, a potential regulator of EWS/FLI1 activity (17). Therefore, Rigosertib may serve to decrease the activity of EWS/FLI1 without directly affecting their protein expression.

Additionally, these results showed that CDK1 protein expression was unaffected by Dinaciclib inhibition (Figure 11). However, Dinaciclib has been previously shown to function in CDK inhibition by directly interacting with the ATP site (27), thereby rendering the protein inactive. Therefore, the inhibitor likely functions by reducing CDK1 activity without affecting its protein expression. Additionally, cells exposed to a combination treatment of Dinaciclib and radiation exhibited decreased PLK1 expression (Figure 11), and non-irradiated cells treated with Dinaciclib demonstrated a decreased expression of FoxM1. This is not surprising, since CDK1 functions in an auto-amplification loop with PLK1, where phosphorylated CDK1 serves as a priming site for PLK1, which is then responsible for phosphorylation of FoxM1 (15). Since Dinaciclib appears to decrease the activity of CDK1 without affecting protein expression, it is
possible that it functions by indirectly affects the translation of PLK1 by means of a negative feedback loop. However, the role of irradiation in inducing this effect is unknown.

Radiation appears to have an effect on EWS/FLI1 and PLK1 protein expression as well, as exhibited by their reduced expression among all treatment types (Figure 11). However, further experimentation would be required in order to determine the physiological basis for this relationship.

**Suggestions for Future Studies**

- Perform western blot analysis on A4573 “small” and “large” cells to determine any potential differences in the effects of each inhibitor on protein expression based on cell size.
- Perform western blot analysis using antibodies against the other molecular targets of Dinaciclib, such as CDK2, CDK5, and CDK9 (21), in order to begin exploring its mechanism of inhibition.
- Test the effects of Dinaciclib and Rigosertib on the protein kinase activity of their target proteins in each cell type to further elucidate the mechanism of inhibition utilized by both inhibitors.
- Perform flow cytometry analysis on each cell type after treatment with Dinaciclib and Rigosertib, plus or minus radiation, in order to determine the effects of treatment on cell cycle progression.
- Treat each cell type with a combination of both inhibitors to determine if there is a synergistic effect of targeting multiple aspects of the putative signaling pathway.
• Perform these trials with Ewing’s sarcoma cell lines containing other types of translocations in order to determine the effect of these inhibitors on cells containing other potential aberrant aspects of their signaling pathways.

• Test the effects of both inhibitors on tumor growth \textit{in vivo} to further demonstrate their safety and effectiveness as potential treatments for Ewing’s sarcoma.

• Repeat these trials with other inhibitors of the putative Ewing’s sarcoma signaling pathway in order to determine the most effective therapy conditions that minimize the necessary dosage for treatment.
BIBLIOGRAPHY


