Administration of Salubrinal Enhances Radiation-induced Cell Death of SW1353 Chondrosarcoma Cells

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Abstract. *Background: Chondrosarcoma is a common soft tissue malignancy. Although radiation induces DNA damage and integrated stress response (ISR), the sensitivity to ionizing radiation differs among tissues, and traditional radiotherapy for chondrosarcoma is not deemed effective. We examined whether administration of an ISR-inducing agent enhances radiosensitivity of chondrosarcoma. Materials and Methods: SW1353 chondrosarcoma cells and C28/I2 chondrocytes were irradiated with 1-10 Gy of X-rays and cultured with 1-20 μM salubrinal, which is known to induce ISR through inhibiting dephosphorylation of eukaryotic translation initiation factor 2α (eIF2α). Results: The numbers of cells were reduced after irradiation, and salubrinal further reduced them as well as their clonogenic survival. The levels of phosphorylated eIF2α were elevated by irradiation and administration of salubrinal. SW1353 cells treated with salubrinal after irradiation were more sensitive to radiation than those treated with salubrinal prior to irradiation. Conclusion: Salubrinal may serve as a potential chemotherapeutic agent for enhancing radiosensitivity, and its efficacy may depend upon the dose used and the timing of its administration.*

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Skeletal malignancies present a serious clinical problem, since they have a tendency to form tumors that are in general resistant to chemotherapy and radiotherapy (1). Approximately one-third of skeletal cancers are chondrosarcoma, making them the second most common form of tumors of bone and cartilage (2). To our knowledge, few studies have shown efficacy of traditional radiotherapy on chondrosarcoma, and surgical resection remains the main form of treatment (3). Although proton therapy has recently shown promise as a non-invasive treatment option (4), it would be desirable to develop a chemotherapeutic procedure that could enhance radiation sensitivity of chondrosarcoma.

To develop possible adjuvants in reducing radiation resistance of chondrosarcoma, many molecular mechanisms have been investigated (5, 6). One approach is to administer acridine orange and increase production of reactive oxygen species (ROS) to enhance apoptosis and elevate radiation sensitivity (7). Another approach is to facilitate apoptosis by modulating expression of apoptosis-linked genes such as B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma – extra large (Bcl-xL) (8). A mechanism involved in suppression of angiogenesis has been examined using selective inhibitors of growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (9). Although significant work has been conducted so far, alternate pathways and mechanisms that lead to enhanced radiation sensitivity could be explored in an effort to increase tumor cure in the clinic.

Salubrinal ($C_{21}H_{17}Cl_3N_4OS$; 479.8 Da) is a synthetic chemical agent known to elevate the levels of phosphorylated eukaryotic translation initiation factor 2α (eIF2α) (10), which is a prerequisite of the integrated stress response (ISR) (11). ISRs are induced by various stresses, including oxidation, nutrient deprivation, virus infection, and stress to the
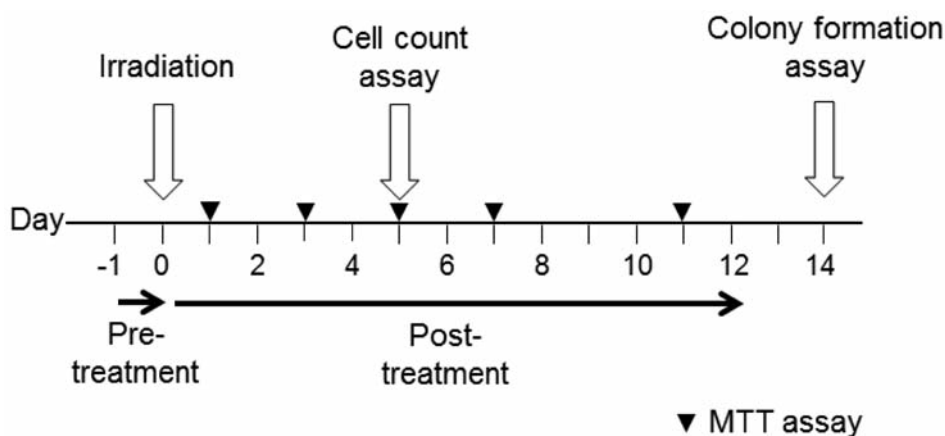
Figure 1. *Experimental timeline. Salubrinal was administered for 24 h prior to irradiation and removed before irradiation (pre-treatment), or 1 h after irradiation for 3-14 days (post-treatment).*

endoplasmic reticulum (12). Depending on the intensities of such stresses, cells may either initiate a repair mechanism that rescues damaged cells or activate the apoptotic pathway (13). Since radiation is reported to induce ISR by elevating the phosphorylated levels of eIF2α (14, 15), we addressed whether administration of salubrinal to irradiated cells would enhance the ISR and inhibit survival and proliferation of chondrosarcoma cells. Our hypothesis is that proliferation of chondrosarcoma cells is inhibited by treatment with salubrinal when combined with high doses of ionizing radiation.

In order to test this hypothesis, we conducted a series of *in vitro* experiments using SW1353 chondrosarcoma cells (16) together with C28/I2 chondrocyte cells as a control (17).

Materials and Methods

Cell culture. Human chondrosarcoma (SW1353; ATCC HTB-94) and C28/I2 chondrocyte cells were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin; Invitrogen, Grand Island, NY, USA). Cells were grown in T25 flasks at 37°C in a humidified atmosphere containing of 5% CO_2. All experiments were conducted three times, and the mean±s.e.m. was determined (Figure 1).

Irradiation. Cells were irradiated with a Faxitron X-ray machine (Faxitron Bioptics, Tucson, AZ, USA) or Precisions X-ray machine (160 kVp, North Branford, CT, USA). Cells were exposed to doses of 1, 5 or 10 Gy, at a dose rate of approximately 2.5 Gy/min, at room temperature.

Administration of salubrinal. Salubrinal (Tocris, Bristol, UK) was administered pre- or post-irradiation. The stock salubrinal solution of 10 mM was prepared using Dimethyl Sulfoxide (DMSO) as a solvent, and it was diluted in culture medium at a concentration of 0-20 μM. All cells were exposed to the same concentration of DMSO in the medium. For post-irradiation administration of

salubrinal, culture media were not changed, and no additional salubrinal was provided.

Proliferation, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and colony formation assays. The numbers of cells were determined using an optical microscope and a hemocytometer. Cells were trypsinized and stained with trypan blue (Sigma, St. Louis, MO, USA), 5 days after irradiation. In response to salubrinal administration, cell viability was determined using the MTT assay (18) on 1, 3, 5, 7, and 11 days after irradiation. Cells were incubated with MTT (0.05 mg/ml; Sigma) for 3 h at 37°C. The culture medium was then replaced with an equal volume of DMSO, and the concentration of formazan crystals dissolved in DMSO was determined by measuring the absorbance at 550 nm with a microplate reader (BioTek, Winooski, VT, USA).

For the colony formation (clonogenic survival) assay, irradiated cells were re-plated on day 1, followed by fixation and staining on day 14. During the two-week incubation period, culture media were not changed. Cells were fixed with a fixative solution (3:1 methanol/acetic acid) and stained with crystal violet (Sigma). The number of colonies consisting of 50 or more cells was counted. Colony formation was more identifiable in C28/I2 cells than in SW1353 chondrosarcoma cells.

Western blotting. Cells were harvested in a radio immune precipitation assay (RIPA) lysis buffer, containing inhibitors for proteases and phosphatases (Calbiochem, San Diego, CA, USA). Isolated proteins were fractionated using 10% sodium dodecyl sulfate (SDS) gels (BioRad, Hercules, CA, USA) and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Immunoblots were carried out using antibodies specific to eIF2α (Cell Signaling, Danvers, MA, USA) and phosphorylated eIF2α (Thermo Fisher Scientific, Waltham, MA, USA). After incubation with anti-rabbit IgG antibodies conjugated with horseradish peroxide (HRP) (Cell Signaling), signals were detected with ECL chemiluminescence. Images were captured using a Fujifilm Luminescent image analyzer (Fujifilm Co., Tokyo, Japan) and analyzed with Adobe Photoshop (CS5.5, Adobe Systems, San Jose, CA, USA).

Results

Altered cell viability by salubrinal administration is dose-dependent. Incubation with 0-20 μM salubrinal for 1-11 days reduced cell viability in C28/I2 cells and SW1353 cells. According to the MTT assay, viability of both C28/I2 and SW1353 cells was dependent upon the duration of incubation, as well as the dose of salubrinal (Figure 2). A higher salubrinal dose with a longer treatment time resulted in a greater reduction in cell viability. Compared to cells without salubrinal administration, for instance, salubrinal at 1, 2, 5, 10 and 20 μM reduced the absorbance reading on day 7 to 85, 75, 68, 56 and 39% (C28/I2 cells) and 94, 88, 80, 56 and 45% (SW1353 cells), respectively.

Reduced proliferation of C28/I2 and CW1353 cells after irradiation. Irradiation with 5 or 10 Gy significantly reduced the number of C28/I2 cells and CW1353 cells on day 5 (Figure 3). After irradiation with 5 and 10 Gy, the numbers of C28/I2 cells were reduced to 41±5% (mean±s.e.m.) and 19±1% of the non-irradiated numbers, respectively. The effect was more pronounced in the SW1353 cells, with a decrease in cell numbers to 30±9% (5 Gy) and 20±9% (10 Gy) of that of the non-irradiated cells after irradiation.

   In addition to irradiation, administration of salubrinal further reduced the number of live cells (Figure 3). Post-irradiation treatment with salubrinal was in general more effective as an enhancer of cell death than pre-treatment, and this trend was clearer in SW1353 than in C28/I2 cells. After irradiation with 10 Gy, for instance, treatment with 10 μM salubrinal reduced the normalized cell numbers to 17% (pre-treatment) and 13% (post-treatment) in C28/I2 cells, while these numbers were 12% (pre-treatment) and 6% (post-treatment) in SW1353 cells. The highest salubrinal concentration of 20 μM led to the lowest number of live C28/I2 and SW1353 cells.

Morphological changes in SW1353 colonies by radiation and salubrinal. We examined the effects of radiation and salubrinal administration on morphological changes of SW1353 cells and their colonies. Compared to non-treated cells that formed a dense cluster of spindle-shaped cells (Figure 4A), administration of 20 μM salubrinal reduced cell density as well as colony size (Figure 4B). Radiation with 5 Gy not only reduced the number of cells in each colony but also altered some cells from a regular spindle form to a thin irregular shape (Figure 4C). Addition of 20 μM salubrinal to irradiated cells with 5 Gy, further reduced the number of cells (Figure 4D). A higher radiation dose with 10 Gy, blocked formation of regular colonies in which most cells did not appear to be healthy and had a deformed cytoplasm (Figure 4E). In response to 10 Gy radiation and 20 μM salubrinal, the majority of cells died. Some live cells presented two nuclei, indicating mitotic arrest (Figure 4F).
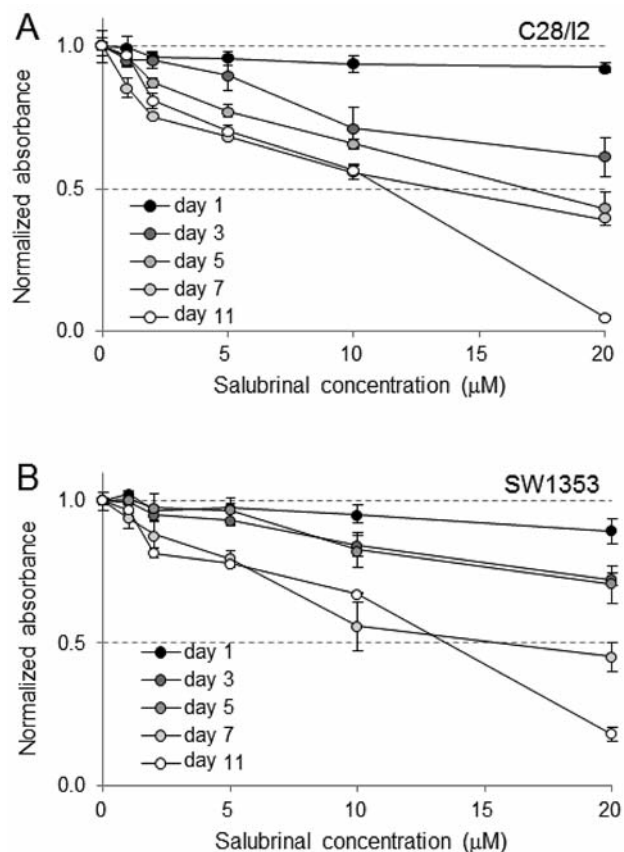

Figure 2. *Cell viability as measured by the MTT assay, after treatment with salubrinal administration for 1, 3, 5, 7 or 11 days. Viability of C28 (A) and SW1353 (B) cells.*

Reduced colony formation by simultaneous application of radiation and salubrinal. A colony formation (clonogenic survival) assay revealed that reproductive cell survival was reduced by application of radiation and salubrinal in a dose-dependent manner. These combined effects were observed both in C28/I2 and SW1353 cells. In C28/I2 cells, for instance, irradiation with 5 Gy resulted in surviving fractions of 0.113 (no salubrinal), 0.107 (2 μM), 0.035 (10 μM), and 0.009 (20 μM) (Figure 5A). In SW1353 cells, the normalized colony number was 0.059 (no radiation and 0 μM salubrinal) and 0.010 (5 Gy radiation and 0 μM salubrinal), and 0.012 (no radiation and 20 μM salubrinal) and 0.001 (10 Gy radiation and 20 μM salubrinal) (Figure 5B).

Elevation of phosphorylated (p-) eIF2α levels. The levels of phosphorylated eIF2α were elevated by irradiation and administration of salubrinal. In C28/I2 and SW1353 cells, irradiation with 10 Gy increased the levels of p-eIF2α at 24 h after irradiation (Figure 6A). Administration of 20 μM salubrinal increased the levels of p-eIF2α on day 1 (24 h) in
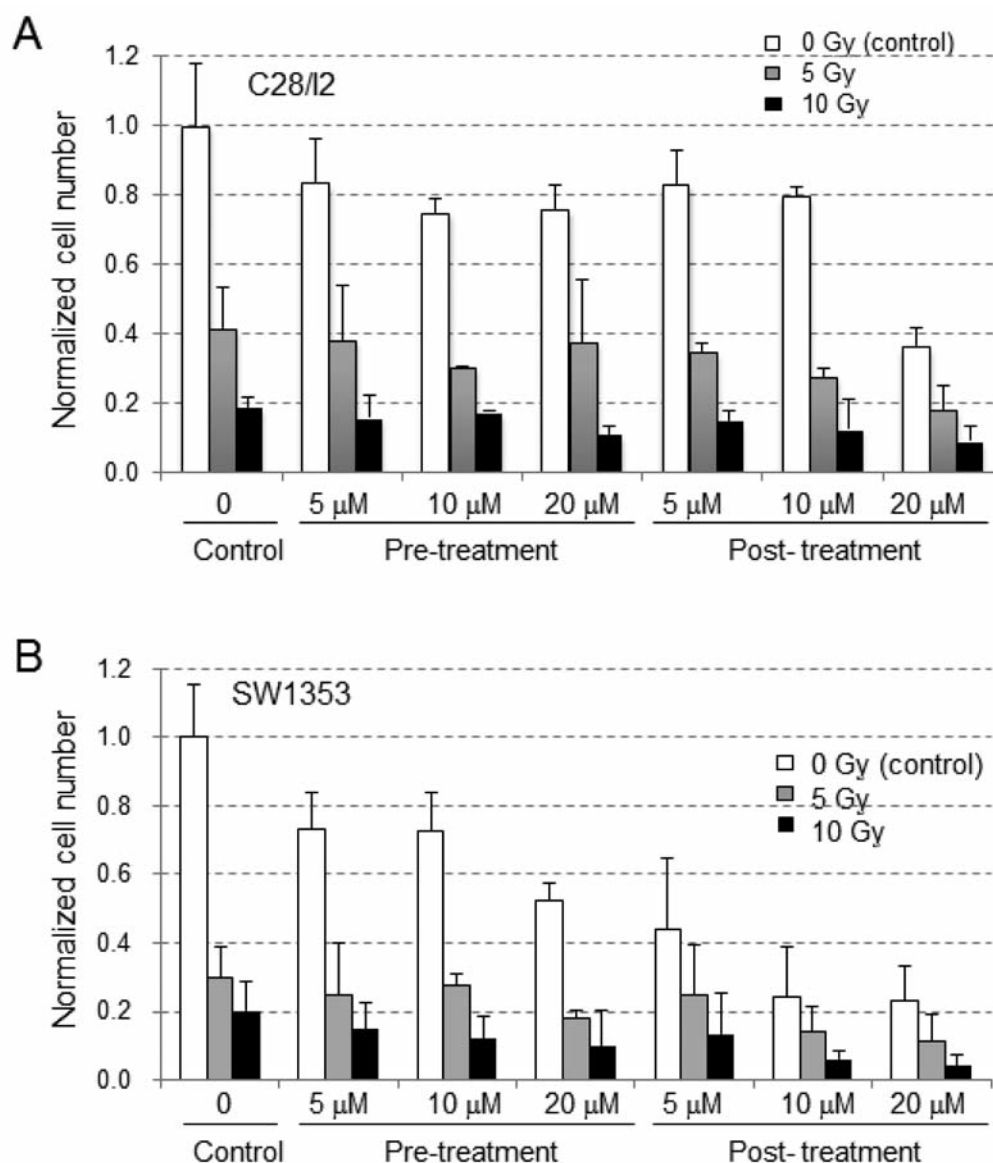
Figure 3. *Numbers of live cells after irradiation with 0, 5 or 10 Gy. Cells were pre-treated (pre-treatment) or post-treated (post-treatment) with 0-20 μM salubrinal. The control cells received no radiation or salubrinal. Number of C28 (A) and SW1353 (B) cells.*

SW1353 cells and on day 2 (48 h) in C28/I2 cells (Figure 6B). The levels of p-eIF2α were reduced on day 4 in both cell lines after up-regulation on day 1 or 2.

Discussion

This study demonstrates that SW1353 chondrosarcoma cells as well as C28/I2 chondrocyte cells are sensitized to ionizing radiation if treated with salubrinal; the compound reduces the proliferation and colony forming capability of these cells. SW1353 cells have been used to examine the efficacy of potential chemotherapy agents for growth inhibition (5, 8). To

our knowledge, however, few studies have been reported regarding their response to radiation. This is the first study that describes a combinatorial effect of radiation and administration of salubrinal, both of which elevate the levels of p-eIF2α.

To enhance radiation sensitivity, molecular signaling at various levels has been examined, including ROS generation, apoptosis in mitochondria, and angiogenesis (19). Radiation sensitivity differs among types of cancer cells, and a differential rate of ROS generation is hypothesized as a potential cause. To facilitate ROS-assisted cell death, agents that provide an additional oxygen source have been evaluated
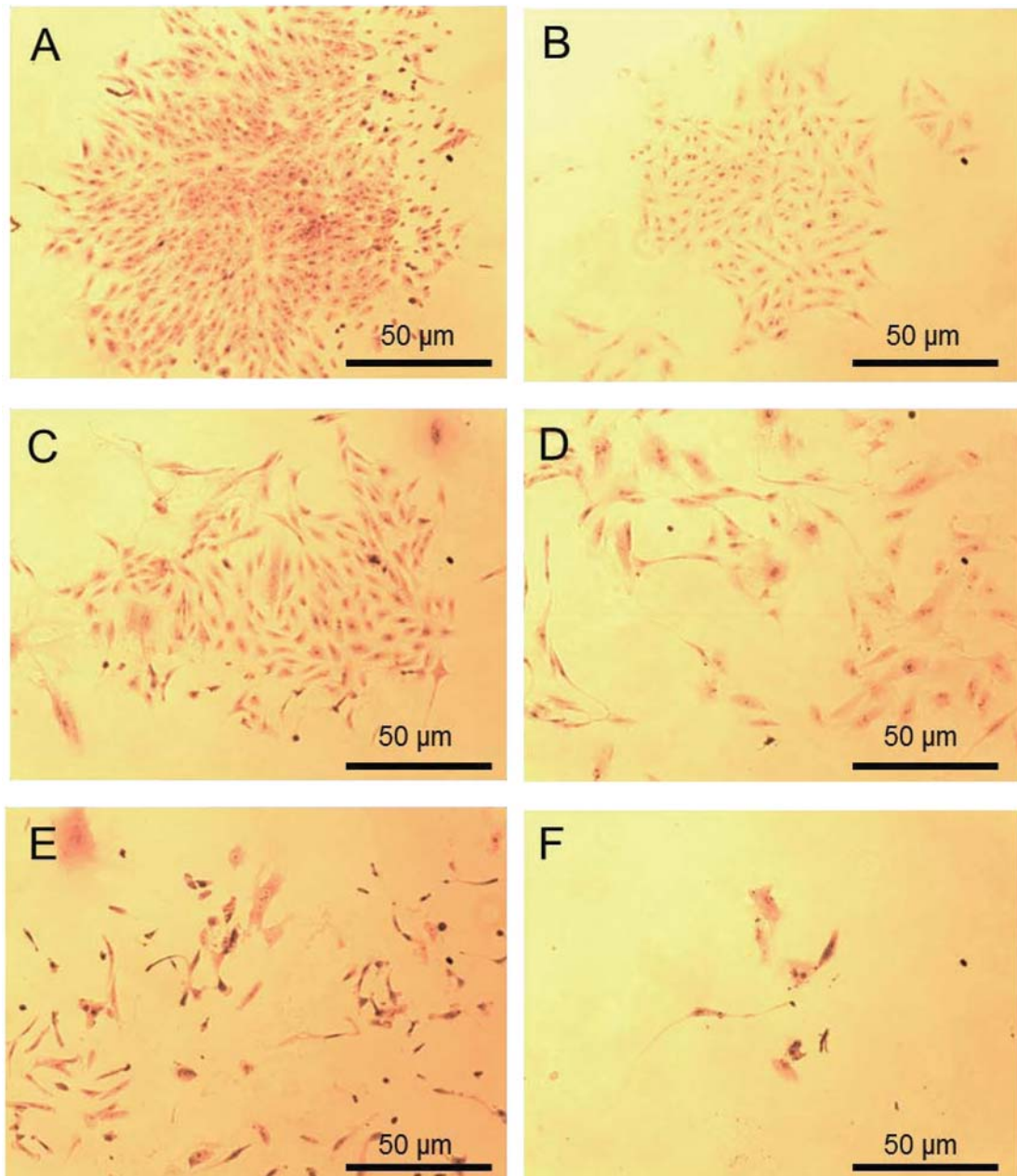
Figure 4. *Images of SW1353 cells in the colony formation assay stained with crystal violet. A: Control cells (no radiation, and no salubrinal administration). B: Cells post-treated with 20 μM salubrinal. C: Cells irradiated with 5 Gy. D: Cells irradiated with 5 Gy and post-treated with 20 μM salubrinal. E: Cells irradiated with 10 Gy. F: Cells irradiated with 10 Gy and post-treated with 20 μM salubrinal.*

(20). Other strategies include activation of apoptosis-linked genes in mitochondria and suppression of factors that stimulate vessel remodeling and blood circulation (21). In this study, we examined the effects of salubrinal as an agent that stimulates ISR through the elevation of p-eIF2α in a dose-dependent manner. ISRs are caused by varying stress sources including UV irradiation, nutrient deprivation, heat shock, and stress to the endoplasmic reticulum. ISRs can also be induced by oxidative stress, as well as X-ray irradiation. Western blot analysis revealed that the levels of p-eIF2α were elevated by irradiation with 10 Gy and administration of 20 μM salubrinal. By combinatorial application of two independent stimulators of p-eIF2α, survival of SW1353 chondrosarcoma cells was significantly reduced.
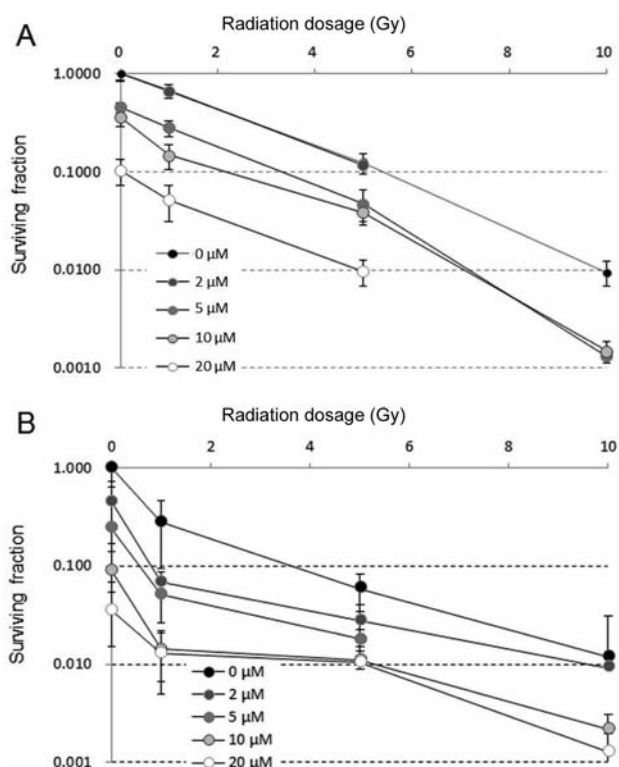
Figure 5. *Surviving fraction of cells after application of radiation and post-treatment with salubrinal. The normalized number of 1 corresponds to control cells without any treatment. Number of C28 (A) and SW1353 (B) colonies.*

Figure 6. *Alteration of the phosphorylated levels of eIF2α. A: Effects of 10 Gy irradiation. B: Effects of administration of 20 μM salubrinal.*

In the colony-formation assay, the slopes of survival curves for cells treated with different doses of salubrinal appear mostly parallel in a semi-logarithmic plot. Thus, a combinatorial effect of irradiation and salubrinal observed in this study was additive and not synergistic. However, the efficacy of salubrinal differed between pre- and post-treatments and salubrinal was not present during irradiation. A proper treatment window including a series of irradiations at a lower dosage might provide a further reduction in cell numbers and colony formation. Between the two lines of cells, we observed differences in their responses to salubrinal. Firstly, SW1353 cells were more sensitive to post-treatment than were C28/I2 cells. Secondly, the levels of p-eIF2α were elevated in one day in SW1353 cells, but in two days in C28 cells.

There are multiple strategies to prevent proliferation of sarcoma cells, including induction of mitotic arrest and apoptosis. Many irradiated SW1353 cells were not viable and unable to form colonies without inducing apoptosis. The decrease of cell density in the colonies of SW1353 cells is likely due to cell cycle inhibition, at least at lower doses. In this study, application of ionizing radiation and salubrinal was c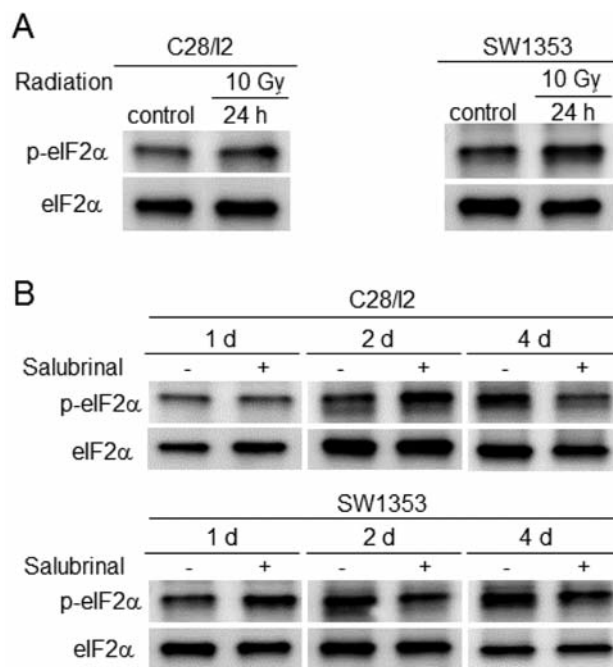ombinatorial but not simultaneous since their treatment windows did not overlap. Further studies are needed to characterize variations among cells and determine a proper schedule to sensitize chondrosarcoma cells without substantially affecting non-sarcoma cells. It is also necessary to directly examine expression of apoptosis-linked genes, such as caspase 3, in response to radiation and salubrinal. In summary, we demonstrated that administration of salubrinal enhanced radiation-induced inhibition of cell proliferation and reduced clonogenic cell survival. The results were consistent with the hypothesis that both radiation and salubrinal elevated the levels of p-eIF2α that led to ISR-linked apoptosis.

Acknowledgements

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