Enhancement of Radiosensitivity by DNA Hypomethylating Drugs through Apoptosis and Autophagy in Human Sarcoma Cells

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Abstract
The targeting of DNA methylation in cancer using DNA hypomethylating drugs has been well known to sensitize cancer cells to chemotherapy and immunotherapy by affecting multiple pathways. Herein, we investigated the combinational effects of DNA hypomethylating drugs and ionizing radiation (IR) in human sarcoma cell lines both in vitro and in vivo. Clonogenic assays were performed to determine the radiosensitizing properties of two DNA hypomethylating drugs on sarcoma cell lines we tested in this study with multiple doses of IR. We analyzed the effects of 5-aza-dC or SGI-110, as DNA hypomethylating drugs, in combination with IR in vitro on the proliferation, apoptosis, caspase-3/7 activity, migration/invasion, and Western blotting using apoptosis- or autophagy-related factors. To confirm the combined effect of DNA hypomethylating drugs and IR in our in vitro experiment, we generated the sarcoma cells in nude mouse xenograft models. Here, we found that the combination of DNA hypomethylating drugs and IR improved anticancer effects by inhibiting cell proliferation and by promoting synergistic cell death that is associated with both apoptosis and autophagy in vitro and in vivo. Our data demonstrated that the combination effects of DNA hypomethylating drugs with radiation exhibited greater cellular effects than the use of a single agent treatment, thus suggesting that the combination of DNA hypomethylating drugs and radiation may become a new radiotherapy to improve therapeutic efficacy for cancer treatment.

Key Words: DNA hypomethylating drugs, 5-aza-dC, SGI-110, Ionizing radiation, Apoptosis, Autophagy

INTRODUCTION
Sarcoma is a heterogeneous group comprised of over 160 different bone and soft tissue neoplasms and categorized more than 60 malignancies in terms of diverse biological and clinical characteristics (Gage et al., 2019). Therefore, the variability of all the subtypes of sarcomas is not well defined due to the heterogeneity of the disease, with various subtypes in treatment responses, behavior, and biology (Toro et al., 2006; Brennan et al., 2014; Kneisl et al., 2014; Trans-Atlantic Retroperitoneal Sarcoma Working Group, 2018). Although the etiology associated with the pathogenesis of sarcomas is largely unknown, recent decades have expanded on the understanding of the underlying biological and genetic alterations of many sarcoma subtypes and found that the genetics of sarcoma are highly variable. Some types of sarcoma exhibit simple genetic characteristics driven by chromosomal translocations that lead to fusion oncoproteins, including Ewing sarcoma and synovial sarcoma. Other types of sarcomas have complex genomic alterations, such as osteosarcoma, which is driven by copy number alterations or gene mutations (Schaefer et al., 2017). Recently, there has been increasing evidence that many types of sarcoma are associated with widespread epigenetic dysregulation (Jain et al., 2010; Schaefer et al., 2017).

The transcriptional gene silencing is regulated by epigenetic mechanisms such as the DNA methylation, the histone modification, and alterations of nucleosome positions at the DNA level. Unlike genetic events, epigenetic events are reversible, which makes epigenetic regulation extremely interesting from the point of view in developing new therapeutic technologies.

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for cancer treatment. In recent decades, DNA hypomethylating drugs (decitabine and 5-aza-2’-deoxycytidine, also known as 5-aza-dC) have been reported to have anticancer activities in patients with leukemia, myelodysplastic syndrome (MDS), and some other solid tumors (Kaminskas et al., 2005; Yoo et al., 2006). Most recently, SGI-110 (Guadecitabine), which is a second-generation hypomethylating prodrug and a dinucleotide that combines 5-aza-dC and deoxyguanosine (Astex Pharmaceuticals, Inc., Cambridge, UK) has been shown to be less susceptible to deamination by cytidine deaminase and more stable in water solutions (Yoo, 2007), thus convincing it an alternative to 5-aza-dC so far. Many reports have indicated that hypomethylating drugs are a critical chemotherapy that plays through epigenetic mechanisms to target cancer instead of inducing general toxicity, as has been seen with other chemotherapies (Sato et al., 2017). In addition, a few studies have revealed that the combination of these epigenetic regulation with traditional chemotherapy or radiotherapy could improve the therapeutic technique (Kristensen et al., 2009; Kelly et al., 2010).

Ionizing radiation is one of the conventional approaches for local control, and the effects of preoperative radiotherapy in sarcomas include reducing tumor size before surgery and decreasing the risk of local recurrence (Ta et al., 2009). However, sarcoma patients always present with radio-resistance, which consequently prevents successful treatment. Therefore, there has not been progress in the radiotherapy of human sarcoma in terms of increasing the tumor response to irradiation with targeted sensitizers.

In the present study, we investigated the combined effect of radiation with epigenetic inhibitors (particularly, DNA hypomethylating drugs) in sarcoma cell lines and explored the biological mechanisms of radiosensitization that is mediated by epigenetic regulation in human sarcoma cells.

**MATERIALS AND METHODS**

**Cell culture, drug treatment, and irradiation**

Human sarcoma cell lines (SK-LMS-1, SK-UT-1, SK-UT-1B, and SK-ES-1) were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in MEM (Welgene, Daegu, Korea) containing 10% fetal bovine serum (HyClone Laboratories Inc, Logan, UT, USA) and 1% antibiotic-antimycotic (Thermo Fisher, Waltham, MA, USA) and maintained at 37°C in an atmosphere of 20% O₂ and 5% CO₂ incubator. Cells were treated with 5-aza-dC (0.1, 0.5 or 1 µM) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) or SGI-110 (0.5 or 1 µM) (APEXBio, Houston, TX, USA) once daily for 3 days. Cells were incubated with 5-aza-dC or SGI-110 for 3 days before and 24 h after irradiation. Cells that were treated with 5-aza-dC or SGI-110 for 3 days without exposure to radiation or those cells that were solely exposed to irradiation were used as controls. Irradiation (gamma-rays) of cells was performed with a 137Cs ray source (Eckert & Ziegler, Berlin, Germany) at a dose rate of 2.6 Gy/min.

**Clonogenic assays**

Cells were seeded in 6-well plates (5,000 cells/well) and treated with 5-aza-dC or SGI-110. After drug treatment, cells were irradiated at the specific doses. Twelve to fourteen days after seeding, colonies were fixed and stained with 1.25% crystal violet. The number of colonies containing at least 50 cells was determined.

**Cell proliferation assay and cell viability assay**

Cell proliferation was determined by using a CCK-8 assay (Dojindo Molecular Technologies, Rockville, MD, USA). Cells (2×10³ cells/well) were seeded in 6-well plates and incubated at 37°C. After 48 h, the cells were washed twice with PBS, and CCK-8 reagent and culture media (1:1 ratio) were added to each well for 1 h. After the reaction, the absorbance range from 450 nm was measured by using a Spectra Max (Molecular Devices, San Jose, CA, USA).

**Apoptosis assay**

The evaluation of cell apoptosis was performed by using flow cytometry. DNA hypomethylating drugs or irradiated cells were assessed by using an Annexin V/FITC Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). The cells were stained with FITC, Annexin V, and 7-amino-actinomycin (7-AAD), after which they were incubated for 15 min in the dark.

**Western blot analysis**

Cells were lysed with cell lysis buffer. Equal amounts of total proteins were loaded onto 4-12% SDS–PAGE gels and transferred to PVDF membranes (GE Health care Life Sciences, Marlborough, MA, USA). The membranes were blocked with 5% skim milk in TBST buffer and incubated with primary antibodies - anti-DNMT1 (Abcam, Cambridge, UK), anti-cleaved caspase 3, anti-E-cadherin, anti-cleaved caspase 9, anti-cleaved PARP1, anti-ATG3, anti-ATG5, anti-ATG7, anti-ATG12, anti-ATG16L1 (Cell Signaling Technology, Denver, MA, USA), anti-LC3A (Novus Biologicals, Littleton, CO, USA), anti-Beclin (BD Biosciences), and anti-ß-actin (Protein Tech Group, Rosemont, IL, USA) overnight at 4°C. The membranes were subsequently incubated with specific horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized by using a Al600 system (GE Healthcare, Marlborough, MA, USA).

**Caspase 3/7 activity assay**

Measurements of caspase 3/7 activities in cells were performed using the commercially available Caspase-Glo 3/7 Assay Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

**Migration and invasion assay**

Cell migration was determined by using transwell plates (8 µm pore size, Corning Costar, Merck, Darmstadt, Germany), and invasion assays were performed by using Matrigel-coated invasion chambers (8 µm pore size, Corning Costar, Merck). The upper chamber contained osteosarcoma cells in serum-free medium, and the lower chamber contained MEM supplemented with 10% FBS. Both migration and invasion assays were performed following the manufacturer’s instructions. Photographs were taken using a Qicam image camera system mounted on a Nikon ECLIPSE 80i microscope (Nikon, Minato, Tokyo, Japan).

**In vivo subcutaneous xenograft models**

Six-week-old male athymic nude mice obtained from OrientBio (Seongnam, Korea) were maintained with freely pro-
vided sterile food and water under specific pathogen-free conditions. After a week-long adaptation period to the new environment, the mice were randomized into nine different groups before tumor implementation (n=3 mice per group). All of the animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Dongnam Institute of Radiological and Medical Sciences (DI-RAMS) (DI-2019–009). To establish the tumor xenograft models, SK-UT-1 or SK-LMS-1 cancer cells (which were treated as indicated) were subcutaneously injected (2×10^6 cells in 200 µL PBS) into the flank of each mouse. Tumor growths were measured 2 times a week. Tumor volume (mm^3) was calculated with the "(W^2×L)/2" formula (Faustino-Rocha et al., 2013). Two-way ANOVA was used to compare the tumor volume in mice treated with the combination therapies versus those treated with either agent alone. At the termination of the experiment, tumors were retrieved from the euthanized mice to measure the tumor weights. Volumes and weights of the tumors are expressed as the mean ± SEM.

Statistical analysis
The results in this study are presented as the mean ± standard deviation. The significance of data was estimated using either two tailed paired or unpaired student's t test and ANOVA. p values of <0.05 were considered to be statistically significant. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Prism software Inc., San Diego, CA, USA).

RESULTS
Low-dose DNA hypomethylating drugs can change the level of DNMT1 in human sarcoma cells
First, we determined whether sarcoma cell lines affected the protein expression of well-known regulators of DNA methylation, such as DNA-methyltransferase 1 (DNMT1), via the classical DNA hypomethylating drug (5-aza-dC) or second-generation DNA hypomethylating prodrug (SGI-110). By using several doses (0.1, 0.5, and 1 µM) of these drugs, Western blot analyses showed that the protein levels of DNMT1 were significantly decreased in sarcoma cells after treatment with these two DNA hypomethylating drugs. Interestingly, we found that a low dose (0.1 µM) of these drugs was sufficient to induce enough epigenetic inhibition in sarcoma cell lines; accordingly, a low dose (0.1 µM) was chosen for subsequent experiments to examine the combined effects of DNA hypomethylating drugs (5-aza-dC or SGI-110) and IR (Fig. 1).

DNA hypomethylating drugs and sarcoma cell radiosensitivity
To characterize whether 5-aza-dC or SGI-110 increased the radiosensitivity of sarcoma cell lines to IR, we performed conventional clonogenic assays to compare the cytotoxic effects of 5-aza-dC or SGI-110, IR at different doses by itself, and combination treatments of each DNA hypomethylating drug and IR in four different sarcoma cell lines (SK-LMS-1, SK-UT-1, SK-UT-1B, and SK-ES-1). Sarcoma cells were pretreated with 5-aza-dC (0.1 µM) or SGI-110 (0.1 µM) for 72 h before being exposed to various doses of IR (0.1, 0.5, 2, 4, and 6 Gy), including a lower dose, without knowing the cytotoxicity level that was induced by IR, and the cells were plated to assess the colony-forming efficiency. In response to IR, there was a dose-dependent reduction in cell survival with or without DNA hypomethylating drugs (5-aza-dC or SGI-110). After pretreatment with 5-aza-dC or SGI-110, radiation-induced cell death was significantly increased, accompanied by the formation of few and small colonies in only SK-LMS-1 and SK-UT-1 [(Fig. 2A) but not in SK-UT-1B and SK-ES-1 (data not shown, Supplementary Fig. 1)] cell lines, compared to those in the IR control groups (p<0.01). For the SK-LMS-1 cells, there was a 24% decrease in the number of colonies formed after treatment with 5-aza-dC (0.1 µM) alone, a 54% decrease after treatment with SGI-110 (0.1 µM) alone, and 1% (0.1 Gy), 5% (0.5 Gy), 20% (2 Gy), 53% (4 Gy), and 83% (6 Gy) decreases after exposure to IR alone; in addition, a 40-94% or 59-94% decrease in the number of colonies was elicited after the combination treatment of 5-aza-dC or SGI-110 with IR, respectively (p<0.01). For the SK-UT-1 cells, there was no decrease in the number of colonies that was formed after treatment with 5-aza-dC alone, as well as a 20% decrease after treatment with SGI-110 alone, a 1-84% decrease after exposure to IR alone, and a 31-97% or 47-95% decrease in the number of colonies that was formed after the combination treatment of 5-aza-dC or SGI-110 with IR, respectively (p<0.001). These observations indicated that the radiosensitization that was caused by 5-aza-dC or SGI-110 in sarcoma cell lines was statistically significant at radiation doses higher than 4 Gy (p<0.01) (Fig. 2A).

The combination of DNA hypomethylating drugs and IR inhibits cell proliferation in vitro and in vivo
To evaluate whether 5-aza-dC or SGI-110 in combination with IR affects cell proliferation, we performed a CCK-8 assays in two sarcoma cell lines (SK-LMS-1 and SK-UT-1) because there was no significant change in the number of colonies that was formed after combination treatment with 5-aza-dC, SGI-110, and IR in the other two cell lines. In addition, low doses (0.1 and 0.5 Gy) of sole IR exposure to both sarcoma cell lines indicated no significant changes in cell growth compared to untreated controls. When the 5-aza-dC or SGI-110 were added to this system, the number of colonies was reduced by 54% (0.1 µM) and 54% (0.5 µM) of the control, respectively.

Fig. 1. Low-dose 5-aza-dC and SGI-110 treatments can induce demethylation in human sarcoma cell lines. Western blots show the protein expression levels of DNMT1 (180 kD) in human sarcoma cell lines (SK-LMS-1, SK-UT-1, SK-UT-1B, and SK-ES-1) treated with different doses (0.1/0.5/1 µM) of 5-aza-dC or SGI-110. The blots were probed by using anti-DNMT1 (upper panel) and anti-β-actin antibodies.

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control cells, thus we excluded the combination treatment of 5-aza-dC or SGI-110 in subsequent experiments. For the SK-LMS-1 cells, there was a 1-39% decrease in proliferation after treatment with 5-aza-dC (0.1 µM) or SGI-110 (0.1 µM) and/or IR (2, 4, and 6 Gy). Cells were seeded into 6-well plates (5,000 cells/well) and treated with 5-aza-dC or SGI-110, either alone or in combination with IR, and clonogenic survival was determined. After 2 weeks, cultures were fixed with ethanol and stained with 1.25% crystal violet. Representative photographs of the single colonies are also shown. (B) CCK-8 assays in SK-LMS-1 (left) and SK-UT-1 (right) cell lines that were treated with 5-aza-dC (0.1 µM) or SGI-110 (0.1 µM) and/or irradiation (2, 4, and 6 Gy). Data are expressed as the mean ± standard deviation of the triplicate experiments. Statistical analysis data are presented as the means ± SD, n=3. *p<0.05, **p<0.01, ***p<0.001 compared with control or single treatments.

Based on in vitro data indicating significant growth inhibition of cells treated with the combination of DNA hypomethylating drugs (5-aza-dC or SGI-110) and IR, we investigated whether tumor growth inhibition could be observed in vivo. To investigate the anticancer efficacy of 5-aza-dC or SGI-110 in vivo, xenograft studies were performed in athymic nude mice. Both SK-LMS-1 and SK-UT-1 cells that were treated with either 5-aza-dC or SGI-110 and IR were subcutaneously injected into athymic nude mice. In this experiment, only 2 and 4 Gy IR doses were chosen because there was a similar combination effect between 4 and 6 Gy from in vitro data, thus demonstrating that a relatively lower dose of IR could induce a sufficient combination effect involving radiation and epigenetic inhibitors. Fig. 3A shows the significant tumor growth delay in response to the combination treatment with 5-aza-dC or SGI-110 and IR (2 and 4 Gy) compared with tumor growth of single treatment with either 5-aza-dC, SGI-110, or IR. In addition, a significantly different tumor size from both SK-LMS-1 and SK-UT-1 cells that were treated with both 5-aza-dC and IR was observed compared to that of tumors that were established from both cells that were treated with either 5-aza-dC or IR alone (Fig. 3B). Strikingly, similar effects were observed after
Fig. 3. The anticancer effect of the combination of DNA hypomethylating drugs and IR in the SK-LMS-1 and SK-UT-1 xenograft tumor models. Tumor growth was measured following 5-aza-dC or SGI-110 treatments either alone and in combination with irradiation (2 and 4 Gy) in nude mice. SK-LMS-1 and SK-UT-1 cells (2×10⁶ cells) that had been treated with 5-aza-dC or SGI-110 and irradiated (2 and 4 Gy) were subcutaneously injected into nude mice (n=3 for each sample group), and the average tumor size was measured two or three times weekly for 7 weeks. Tumor volume and body weight were measured every three days. At the termination of the experiments, all of the mice were sacrificed. (A) Tumor volume of SK-LMS-1 cells (left) and SK-UT-1 cells (right). (B) Representative images of tumor tissues (left) and tumor weight (right). Data are expressed as the mean ± standard deviation of the three independent experiments. No significant (NS) was not indicated but statistically significant p value was indicated in the graph. *p<0.05, **p<0.01, ***p<0.001 compared with control or single treatments.
SGI-110 treatment (Fig. 3). Therefore, these results strongly suggested that the combination treatment of 5-aza-dC or SGI-110 with IR affects synergistic tumor growth inhibition both in vitro and in vivo.

To further determine the combined effect of DNA hypomethylating drugs and IR on the metastasis of sarcoma cell lines, the migratory and invasive potential of SK-LMS-1 and SK-UT-1 cells were assessed using migration and invasion assays. As shown in Fig. 4, the migrating and invading cells in the combination of DNA hypomethylating drugs and IR group were significantly decreased compared with those in the control, 5-aza-dC, SGI-110, or IR alone in both sarcoma cell lines. Our data indicated that the combination of DNA hypomethylating drugs and IR affects sarcoma cell migration and invasion.

**Fig. 4.** Combination treatment of 5-aza-dC or SGI-110 with IR contributes to suppressing invasive traits. Migration and invasion assays in (A) SK-LMS-1 and (B) SK-UT-1 cells. Data are means ± SE from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared with control or single treatments.
The combination of DNA hypomethylating drugs and IR contributes to inducing apoptosis in sarcoma cell lines

To determine the mechanisms that are associated with DNA hypomethylating drug (5-aza-dC and SGI-110)-induced radiosensitivity of sarcoma cells, as well as the inhibition of cell growth both in vitro and in vivo, we employed three different methods to determine the level of cell death due to apoptosis after treatment with either the single agents or the combination treatments. Specifically, we used Annexin V/FITC staining and caspase 3/7 assays (which are known as being key effectors of apoptosis in mammalian cells), in order to determine whether this effect was mediated by caspases; additionally, we used immunoblot analysis to measure apoptotic factors (Fig. 5).

Annexin V analysis (Fig. 5) showed a significant increase in apoptotic cells (3.9 fold), as well as an increase in caspase 3/7 activities (3.1-fold), in SK-UT-1 cells after treatment with either DNA hypomethylating drugs, IR (2 and 4 Gy), or their combination, compared with control cells (p<0.01). In comparison, there was a significant increase in apoptotic cells (1.9-fold) and caspase 3/7 activities (2-fold) in SK-LMS-1 cells after treatment with either DNA hypomethylating drugs, IR (2 and 4 Gy), or their combination, compared with control cells (p<0.05). Studies from SK-LMS-1 cells (Fig. 5A) showed that combination treatment resulted in greater cell death than either the uses of DNA hypomethylating drugs or IR alone; the
fact that apoptosis was not significantly increased suggests that apoptosis is not involved in inducing cell death.

Interestingly, our Western blot analysis demonstrated that the protein levels of caspases 3 and 9 were also higher in cells treated with the combination of 5-aza-dC and IR than in cells treated with either 5-aza-dC or IR alone, as well as in control cells. Cleaved PARP1, a well known molecule inducing apoptosis (Kondo and Kondo, 2006; Faustino-Rocha et al., 2013), was also increased in cells treated with 5-aza-dC or IR alone, compared with control cells (Fig. 5C, Supplementary Fig. 2). Strikingly, the combination of SGI-110 treatment with IR led to a similar result, in that the protein levels of the key factors of apoptosis were significantly increased in human sarcoma cells. Taken together, as shown in Fig. 5, based on the synergistic cytotoxicity that was observed with the combined DNA hypomethylating drug treatment and IR exposure in SK-LMS-1 cells (compared to SK-UT-1 cells), it was clear that cell death was present, but not through the mechanism of significant apoptosis. These findings suggest that other mechanisms, such as autophagy, may be associated with promoting cell death.

The combination of DNA hypomethylating drugs and IR contributes to inducing autophagy in sarcoma cell lines

To examine whether the combination treatment of DNA hypomethylating drugs and IR induced autophagy in SK-LMS-1 cells, we analyzed the levels of autophagic characteristic markers, including ATG3, ATG5, ATG7, ATG12, ATG16L1, LC3, and Beclin by using Western blotting. As shown in Fig. 6, the expression levels of most of the ATG3, ATG5, ATG7, ATG12, ATG16L1, and Beclin markers were increased by the combination of both DNA hypomethylating drugs and IR, rather than by either treatment with DNA hypomethylating drugs or IR alone. More interestingly, although similar effects were observed at the LC3 protein level, the combination of SGI-110 and IR induced a significant increase in LC3 protein expression, compared with the combination of 5-aza-dC and IR, thus suggesting that SGI-110 is more associated with inducing the autophagy pathway to cancer cell death. LC3-II expression levels were significantly increased in the combination treatment of SGI-110 and IR, compared to the control or single agent treatments with either DNA hypomethylating drugs or IR alone (Fig. 6). It has been known that LC3-I converted into LC3-II via lipidation by an ubiquitin-like system during the autophagy process. Afterward, LC3-II stays with autophagosomes until the fusion with lysosomes is completed; therefore, this phenomenon can be used as an autophagy marker (Kondo and Kondo, 2006). Consequently, these results suggested that the combination treatment of DNA hypomethylating drugs and IR in SK-LMS-1 cells can also contribute to autophagic death.

DISCUSSION

IR is a well-known genotoxic agent that cause to key molecular damage through both direct and indirect biological mechanisms. Exposure to IR leads to both activation or inactivation of multiple signaling pathways that play key roles in cell survival or death (Goodhead, 1994). In the clinical setting, radiotherapy is most frequently used as the primary or adjuvant therapy in combination with surgery, chemotherapy, or both treatments and widely used as a standard treatment for many types of cancer. Most recently, there has been growing evidence that epigenetic mechanisms, such as DNA methylation and histone modification, are associated with transcriptional gene silencing and may be involved in control of radiosensitivity in cancer cells (De Schutter et al., 2009; Flotho et al., 2009). However, little information is available regarding the influences of DNA hypomethylating drugs on radiosensitization (Dote et al., 2005; Hofstetter et al., 2010). In this context, the combined uses of epigenetic drugs with radiotherapy are particularly interesting and have revealed to improve efficacy in several solid tumors (Dote et al., 2005; De Schutter et al., 2009; Qiu et al., 2009; Wang et al., 2012).

Regarding the reversibility of epigenetic modification changes occurring in cancer, it was hypothesized that DNA hypermethylation at promoter CpG islands was reversed to re-express silenced genes and to reprogram cancer cells to a more normal-like state. Decitabine (5-aza-dC) is a well-known

![Graphs](images)

**Fig. 6.** The combination of 5-aza-dC or SGI-110 and IR induced autophagic death in the SK-LMS-1 cell line. The combination of 5-aza-dC or SGI-110 and IR contributed to inducing autophagy. ATG3, ATG5, ATG7, ATG12, ATG16L1, Beclin, and LC3 expression levels in SK-LMS-1 cells treated with 5-aza-dC, SGI-110, or IR alone and in combination was determined by using Western blotting. Representative images of three individual experiments are shown. The statistical data analysis are presented as the means ± SD, n=3. *p<0.05, **p<0.01, ***p<0.001 compared with control and single treatments.
that is associated with both apoptosis and autophagy in sar-
DNA hypomethylating drugs and IR enhanced the cell death.
In addition, we found that a lower dose of 5-aza-
In several studies, guadecitabine demonstrated improved bio-
availability and increased half-life acting as a hypomethylating
drug (Griffiths et al., 2013).
In the present study, a low dose of 5-aza-dC or SGI-110
(0.1 µM) was used in combination with irradiation, although
numerous reports have indicated that 5-aza-dC (5 µM) was
required to reactivate the expression of most silenced genes
(Schuebel et al., 2007). As shown in Fig. 1, we tested the
DNMT1 expression level with different doses (0.1, 0.5, and
1 µM) of 5-aza-dC or SGI-110 in sarcoma cell lines and ob-
served that a lower dose (0.1 µM) of DNA hypomethylating
drugs was sufficient to inhibit global DNMT1 levels in sarcoma
cell lines. In addition, we found that a lower dose of 5-aza-
dC or SGI-110 (0.1 µM) and a nontoxic level of IR (2 Gy or 4
Gy) were sufficient to induce anticancer effects in sarcoma
cell lines. In this regard, our data strongly support the hypothesis
that giving lower doses of the drug would lead to more effective
for therapeutic approach and improved clinical trials (Issa
and Kantarjian, 2009).
Here, we used two different DNA hypomethylating drugs.
Specifically, 5-aza-dC (decitabine) is a well-known demethyl-
ating drug and FDA approved agent for MDS, and the other
drug is a next generation DNA hypomethylating drug that has
been recently developed and is currently being investigated in
clinical trials. Unlike previous studies, although we used low
doses (0.1 µM) of both DNA hypomethylating drugs in sar-
coma cell lines, the global level of DNMT1 protein was signifi-
cantly decreased (Fig. 1). The data presented in this study indi-
cicates that 5-aza-dC and SGI-110 increased radiosensitivity
in two sarcoma cell lines (SK-LMS-1 and SK-UT-1), which is
consistent with reports on zebularine, another DNA hypometh-
ylating drug (Dote et al., 2005). We also found that both DNA
inhibitors increased radiation-induced cell death, as has been
evaluated in in vitro and in vivo xenograft models. In addition,
it was interesting to observe that the combination treatment of
DNA hypomethylating drugs and IR enhanced the cell death
that is associated with both apoptosis and autophagy in sar-
coma cell lines.
Autophagy has been classified as a second form of pro-
grammed cell death. First, it was observed in cells that were
exposed to starvation, which implicating that it presumably a
cell protective mechanism to survive during starvation or when
they were exposed to environmental toxicity. Therefore, it
thought to be that autophagy enhances both cancer cell death
and survival (Levine, 2007; Dalby et al., 2010). Kroemer and
Levine (2008) suggest two possible explanations of the bio-
logical role for autophagy in cell death. One is that autophagy
only coexists with cell death, and the other is that autophagy
could be a cell death mechanism (Kroemer and Levine, 2008).
Collectively, the cellular function of autophagy in human can-
cer still remains controversial.
Little is known about the cellular mechanisms of the radio-
sensitivity that is induced by epigenetic inhibitors. Recently,
we and other researchers have reported that the combinatio-
ns of epigenetic inhibitors with radiotherapy are capable of in-
creasing apoptosis in several solid tumors and is regarded as
a potential mechanism for radiosensitization (Qiu et al., 2009;
Kim et al., 2012, 2014; Kwon et al., 2017). Autophagy has
been suggested as a novel mechanism of eliciting synergistic
cytotoxicity via several chemotherapeutic agents in sarcomas
in vitro or in vivo (Lambert et al., 2008; Li et al., 2013). Here we
present evidence that the combination treatment of DNA hy-
pomethylating drugs and radiation is also capable of increas-
ing autophagic cell death in sarcoma cell lines.
In the beginning of this study, we started analyzing the radi-
ation response in four different sarcomas that originated from
different tissues, such as bone, uterine, and vulva tissues; how-
ever, we ended up studying the use of two different cell
lines, including uterine leiomyosarcomas (ULMSs). ULMS is a
rare gynecologic malignancy with a low survival rate. Although
it is a rare tumor that accounts for less than 1% of all uterine
malignancies, over two thirds of patients with ULMS that has
extended among the uterus experience tumor recurrence af-
iter initial chemotherapy (Dinh et al., 2004). Human ULMS oc-
curs infrequently and exhibit obstacles for understanding the
etiology of the disease making it difficult to develop efficient
therapies. Therefore, our experimental findings suggest that
the combined effect of DNA hypomethylating drugs and radia-
tion might provide a new approach for the development of a
new therapeutic strategy.

CONFLICT OF INTEREST
The author declares no conflict of interest.

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