

## 8-Aminoadenosine Enhances Radiation-induced Cell Death in Human Lung Carcinoma A549 Cells

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### Radiosensitization/8-Aminoadenosine (8-NH<sub>2</sub>-Ado)/Apoptosis.

The combination of a chemotherapeutic agent and radiation is widely applied to enhance cell death in solid tumor cells in cancer treatment. The purine analogue 8-aminoadenosine (8-NH<sub>2</sub>-Ado) is known to be a transcription inhibitor that has proved very effective in multiple myeloma cell lines and primary indolent leukemia cells. In this report, to examine whether 8-NH<sub>2</sub>-Ado had the ability to enhance the radiation-induced cell killing in solid tumor cells, human lung adenocarcinoma A549 cells were irradiated in the presence and absence of 8-NH<sub>2</sub>-Ado. 8-NH<sub>2</sub>-Ado significantly increased reproductive cell death and apoptosis in A549 cells exposed to X-rays. When peptide inhibitors against caspase-3, -8, and -9 were utilized to evaluate the involvement of caspases, all inhibitors suppressed the enhancement of radiation-induced apoptosis, suggesting that not only mitochondria-mediated apoptotic signal transduction pathways but also death receptor-mediated pathways were involved in this enhancement of apoptosis. In addition, in the cells exposed to the treatment combining X-irradiation and 8-NH<sub>2</sub>-Ado, reduction of the intracellular ATP concentration was essential for survival, and down-regulation of the expression of antiapoptotic proteins such as survivin and XIAP was observed. These results indicate that 8-NH<sub>2</sub>-Ado has potential not only as an anti-tumor drug for leukemia and lymphoma but also as a radiosensitizing agent for solid tumors.

### INTRODUCTION

Anticancer drugs that affect the metabolism of nucleic acids are widely used to sensitize tumor cells to radiation treatment.<sup>1)</sup> For example, gemcitabine and 5-fluorouracil, which have inhibitory effects on DNA and RNA synthesis through their metabolites, have been shown to have radiosensitizing effects on tumor cell lines.<sup>1–3)</sup> Recently, we have reported the development of a nucleoside analogue, 1-(3-C-ethynyl-β-D-ribo-pentofuranosyl) cytosine (ECyd, TAS106), that strongly inhibits RNA synthesis, and combination treatment using this drug and radiation synergistically enhances not only reproductive cell death but also apoptosis in various solid tumors, i.e., gastric tumor cell lines MKN45, MKN28 and murine rectum adenocarcinoma cell line Colon26. In

addition, this combination treatment has been demonstrated to induce significant apoptotic cell death and growth delay of transplanted tumors in mice.<sup>4–7)</sup> This novel nucleoside antitumor drug was developed to be phosphorylated by uridine/cytidine kinase II, which was reported to be highly expressed in tumor cells,<sup>8)</sup> and it was demonstrated that treatment with ECyd attenuated the expression of anti-apoptotic proteins, proliferation-associated proteins and checkpoint-associated proteins, i.e., survivin, cIAP, HIF-1α, VEGF, Bcl-2, Bcl-X<sub>L</sub>, ERK, Akt, Cdk2, Wee1 and Cyclin B, by inhibition of RNA synthesis, resulting in radiosensitization *in vitro* and *in vivo*.<sup>4,7)</sup> Furthermore, a purine derivative, purvalanol A, which is not only a cyclin-dependent kinase inhibitor but also an RNA polymerase II inhibitor, enhances radiation-induced cell death through induction of apoptosis.<sup>9)</sup> Kazuno *et al.* reported that the combination of ECyd with cisplatin (CDDP) synergistically enhanced apoptosis in cell lines OCC1 and LX-1 and delayed the growth rates of their xenograft tumors.<sup>10)</sup> These results suggest that RNA synthesis inhibiting agents efficiently potentiate cell death and growth delay when the combination with a genotoxic agent such as X-irradiation or CDDP is utilized.

In previous studies, 8-aminoadenosine (8-NH<sub>2</sub>-Ado), shown in Fig. 1, was demonstrated to have strong hemato-

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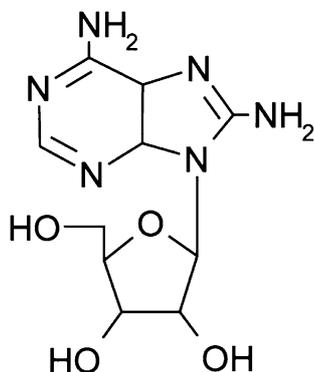


Fig. 1. The chemical structure of 8-aminoadenosine.

logical toxicity through its ability to inhibit RNA synthesis.<sup>11–15</sup> This inhibition of RNA synthesis occurred through multiple mechanisms such as exhaustion of the ATP pool needed for RNA constituent and polyadenylation reactions, inhibition of phosphorylation of RNA polymerase II and transcription termination through incorporation of 8-NH<sub>2</sub>-ATP into RNA at the 3'-terminal position.<sup>15</sup> Furthermore, in multiple myeloma cells exposed to 8-NH<sub>2</sub>-Ado, it was reported that accumulation of 8-NH<sub>2</sub>-ATP and depletion of intracellular ATP occurred and apoptotic cell death was induced by inhibition of not only RNA synthesis but also DNA synthesis.<sup>11</sup> Another report also showed that 8-NH<sub>2</sub>-Ado induced apoptotic cell death by inhibiting the expression of anti-apoptotic proteins Mcl-1 and XIAP in chronic lymphocytic cells.<sup>12</sup> These observations imply that 8-NH<sub>2</sub>-Ado has the ability to induce radiosensitization for solid tumors.

Since non-small cell lung cancer (NSCLC) cells are relatively resistant to ionizing radiation, combined treatment with radiotherapy and chemotherapy has been extensively used in the management of many types of solid malignancies including NSCLC.<sup>16–18</sup> This approach in NSCLC has shown increased response rate, survival, and local control rate.<sup>16</sup> Although *cis*-diamminedichloroplatinum (cisplatin) is one of the most widely used chemotherapeutic agents for combination treatment with radiotherapy,<sup>17,18</sup> more effective sensitizing reagent was needed. Thus, in this study, we investigated the effect of a novel antitumor drug, 8-NH<sub>2</sub>-Ado, on radiation-induced reproductive cell death and apoptosis in NSCLC cell line A549.

## MATERIALS AND METHODS

### Reagents

8-NH<sub>2</sub>-Ado was synthesized according to the method described by Holmes and Robins.<sup>19</sup> Carbobenzoyl-Val-Ala-Asp-fluoromethane (Z-VAD-fmk), acetyl-Leu-Glu-His-Asp-aldehyde (Ac-LEHD-CHO), acetyl-Ile-Glu-His-Asp-aldehyde (Ac-IETD-CHO) and acetyl-Asp-Glu-Val-

Asp-aldehyde (Ac-DEVD-CHO) were purchased from Peptide Institute (Osaka, Japan). Propidium iodide (PI) and adenosine were obtained from Sigma-Aldrich (St. Louis, MO). The following antibodies were used for Western blotting: anti-cytochrome c (BD Pharmingen, Erebodgem, Belgium), anti-XIAP (BD Transduction Laboratories, San Jose, CA), anti-survivin, anti-caspase-3, anti-caspase-8 (Cell Signaling Technology, Beverly, MA), anti-Bcl-2, anti-Bcl-X<sub>L</sub> (WAKO, Osaka, Japan), and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The chemiluminescence detection kit, Western Lightning Plus-ECL, was purchased from Perkin Elmer (Boston, MA).

### Cell culture, X-irradiation and drug treatment

Human lung carcinoma A549 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>. X-irradiation was performed with a Shimadzu PANTAK HF-350 X-ray generator (1.0-mm Al filter, 200 kVp, 20 mA, Shimadzu, Kyoto, Japan). Fifty micromolar of caspase inhibitors Z-VAD-fmk, Ac-LEHD-CHO, Ac-IETD-CHO and Ac-DEVD-CHO, were added 1 h before irradiation.

### Clonogenic survival assay

Cells were seeded on 6-cm dishes and treated with 8-NH<sub>2</sub>-Ado 6 h after seeding. Immediately after treatment, cells were exposed to X-rays and incubated for 12 h. They were then washed with PBS prior to the addition of fresh medium, and incubated for 10 days. Then they were fixed with methanol and stained with Giemsa solution (Sigma-Aldrich). Colonies containing more than 50 cells were scored as surviving cells. The surviving fraction at each dose was calculated with respect to the plating efficiency of the non-irradiated control and dose-response curves were plotted. The survival curves were fitted to a linear-quadratic model by data analysis software Origin 7 (OriginLab Co. Northampton, MA).

### Detection of apoptotic cells by fluorescence microscopy with propidium iodide staining.

Cells were collected at the indicated times after treatment with irradiation and fixed with 1% glutaraldehyde/PBS solution. The fixed cells were washed and resuspended in 20 μl of PBS containing 40 μg/ml propidium iodide (PI) for 15 min. At least 200 cells were scored using an Olympus BX61 microscope (Olympus Optical Co., Ltd., TOKYO, Japan) with reflected-light fluorescence and cells with chromatin condensation and fragmentation were counted as apoptotic cells.

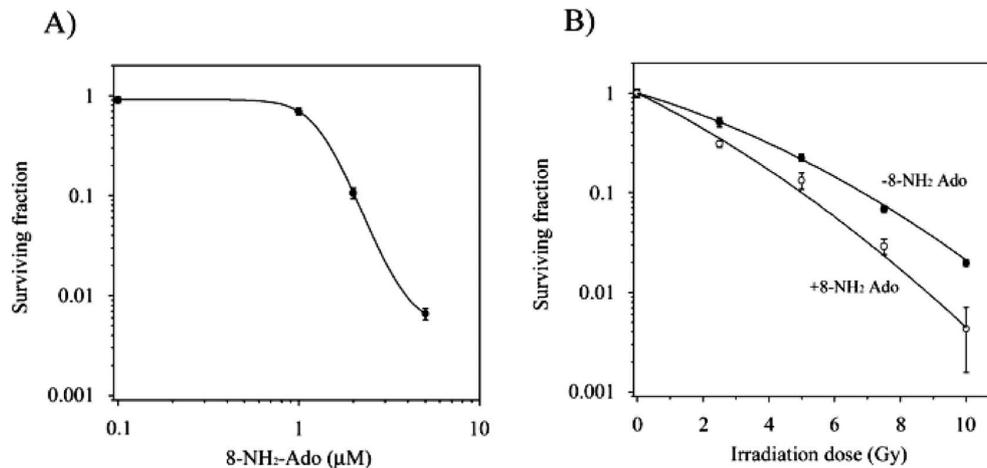
### Measurement of intracellular ATP

The amount of ATP was measured using a luciferin-luciferase ATP assay system (TOYO B-Net CO., Ltd., Tokyo, Japan). Briefly, 5 × 10<sup>3</sup> cells in 96-well plates were

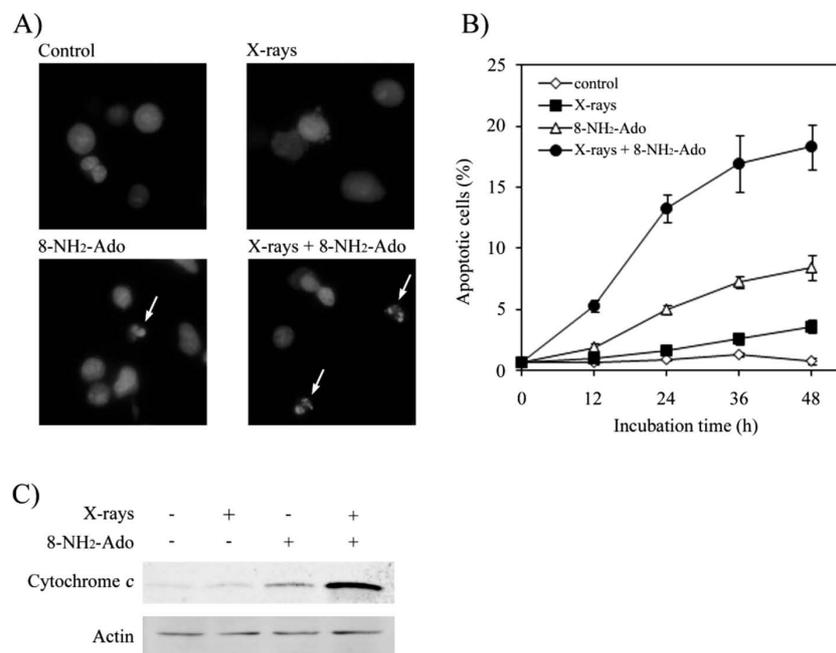
treated with the indicated reagents and incubated at room temperature for 30 min in the dark. The relative light intensity was recorded with a Luminescencer-JNR (AB-2100, ATTO Corporation, Tokyo, Japan).

#### SDS-PAGE and Western blotting

Cells were collected and lysed in lysis buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 µg/ml leu-



**Fig. 2.** Effects of 8-NH<sub>2</sub>-Ado on clonogenic ability in A549 cells. (A) Concentration dependence of the cytotoxicity of 8-NH<sub>2</sub>-Ado to A549 cells. Cells were cultured for 12 h with 0.1–5 µM 8-NH<sub>2</sub>-Ado and incubated for 10 days for colony formation. (B) Dose response curves of A549 cells exposed to X-rays with (+8-NH<sub>2</sub>-Ado) and without 1 µM 8-NH<sub>2</sub>-Ado (-8-NH<sub>2</sub>-Ado). Data are expressed as mean ± SE for three independent experiments.



**Fig. 3.** Effects of 8-NH<sub>2</sub>-Ado on radiation-induced apoptosis in A549 cells. (A) Typical photographs of nuclei stained with propidium iodide (PI). Cells were treated with 20 Gy of X-irradiation alone, 10 µM 8-NH<sub>2</sub>-Ado alone and the combination of 8-NH<sub>2</sub>-Ado and radiation. After incubation for 48 h, the cells were stained with PI and the morphological changes of nuclei were detected by fluorescence microscopy. Arrows indicate cells with typical apoptotic morphological changes. (B) Radiation-induced apoptosis induction (% of total cells) in A549 cells. Cells were incubated for the indicated time after treatment. After PI staining, at least 200 cells were scored by fluorescence microscopy. Data are expressed as means ± SE for three independent experiments. (C) Western blot analysis of cytochrome c release from mitochondria to cytosol at 12 h after treatment.

peptin, 10 µg/ml aprotinin and 10 µg/ml pepstatin) and kept on ice for 30 min. After centrifugation at 15,000 rpm for 15 min at 4°C, supernatants were collected. Three-fold concentrated Laemmli's sample buffer (0.1875 M Tris-HCl [pH 6.8], 15% β-mercaptoethanol, 6% SDS, 30% glycerol and 0.006% bromophenol blue) was added to these supernatants, and samples were boiled for 5 min. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (ADVANTEC Toyo, Tokyo, Japan). The membranes were probed with anti-survivin, anti-caspase-3, anti-caspase-8, anti-Bcl-2, anti-Bcl-X<sub>L</sub> or anti-actin in TBST buffer (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl, 0.1% Tween-20) containing 3% or 5% nonfat skim milk overnight at 4°C. After being probed with HRP-conjugated secondary antibodies, bound antibodies were detected with Western Lightning Plus-ECL.

#### Analysis of the release of cytochrome c from mitochondria

Cells were collected and resuspended in permeabilization buffer (75 mM NaCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 250 mM sucrose, 0.03% digitonin, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 10 µg/ml pepstatin) and kept on ice for 5 min. After centrifugation at 800 g for 10 min at 4°C, the supernatants were further centrifuged at 13,000 g for 10 min at 4°C. The cytochrome c in the resulting supernatants was detected using SDS-PAGE and Western blotting as described above.

## RESULTS

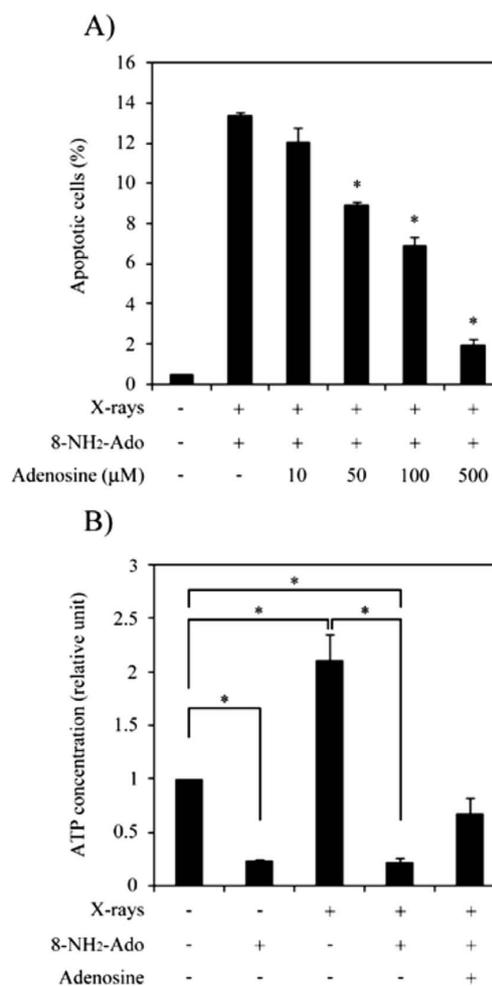
#### 8-NH<sub>2</sub>-Ado enhanced radiosensitivity in A549 cells in clonogenic survival assay

First, to determine whether 8-NH<sub>2</sub>-Ado had radiosensitization effects, we measured the survival fractions of A549 cells exposed to X-rays with 8-NH<sub>2</sub>-Ado. Figure 2A shows the cytotoxicity when A549 cells were treated with 8-NH<sub>2</sub>-Ado for 12 h. At the concentration of 1 µM, more than 65% of cells were alive and this concentration was chosen for the clonogenic survival assay. The survival curves in A549 cells exposed to X-rays with or without 1 µM 8-NH<sub>2</sub>-Ado for 12 h are shown in Fig. 2B. The 10% lethal dose (D<sub>10</sub>) of the survival fraction was reduced from 6.8 Gy in the control to 4.9 Gy by the treatment with 8-NH<sub>2</sub>-Ado. The sensitizing enhancement ratio (SER), which was estimated by the D<sub>10</sub>, was 1.39. This observation indicated that the treatment with 8-NH<sub>2</sub>-Ado significantly enhanced the loss of clonogenic ability.

#### 8-NH<sub>2</sub>-Ado enhanced radiation-induced apoptosis in A549 cells

Next, we investigated the effects of 8-NH<sub>2</sub>-Ado (10 µM) on radiation-induced apoptosis in A549 cells. Figure 3A shows fluorescent microscopic observations of morpho-

logical changes of cell nuclei, DNA fragmentation and chromatin condensation. There were very few apoptotic cells induced by X-irradiation alone. However, the combination treatment significantly induced typical apoptotic changes. The time course of quantitative measurements of apoptotic cells is shown in Fig. 3B. When A549 cells were treated with 8-NH<sub>2</sub>-Ado and X-irradiation, apoptotic cells were increased at 12 h after treatment. After 48 h, many apoptotic cells induced by combination treatment (18.3 ± 1.8%) were observed, whereas a marginal increase of apoptosis was



**Fig. 4.** Relationship between ATP depletion and enhancement of radiation-induced apoptosis by 8-NH<sub>2</sub>-Ado. (A) Effect of adenosine on enhancement of radiation-induced apoptosis by 8-NH<sub>2</sub>-Ado. Adenosine was added to the medium at the same time as the combination treatment, followed by incubation for 24 h. Data are expressed as means ± SE for three independent experiments. \*p < 0.05, vs. X-rays + 8-NH<sub>2</sub>-Ado by Student's *t*-test. (B) Ratio of the intracellular ATP level to that of non-treated cells. Cells treated with 20 Gy of X-irradiation, 10 µM 8-NH<sub>2</sub>-Ado and radiation, 8-NH<sub>2</sub>-Ado, radiation and 500 µM adenosine were incubated for 24 h and amount of ATP was measured using a luciferin-luciferase ATP assay system.

induced by irradiation alone ( $3.6 \pm 0.6\%$ ) or 8-NH<sub>2</sub>-Ado alone ( $8.4 \pm 1.0\%$ ). Moreover, we tested whether 8-NH<sub>2</sub>-Ado enhanced the radiation-induced cytochrome c release from mitochondria 12 h after treatment. The treatment with irradiation alone did not induce any release of cytochrome c from mitochondria, whereas the treatment with 8-NH<sub>2</sub>-Ado hardly induced release of cytochrome c from mitochondria in unirradiated A549 cells, but synergistically enhanced it in irradiated A549 cells (Fig. 3C).

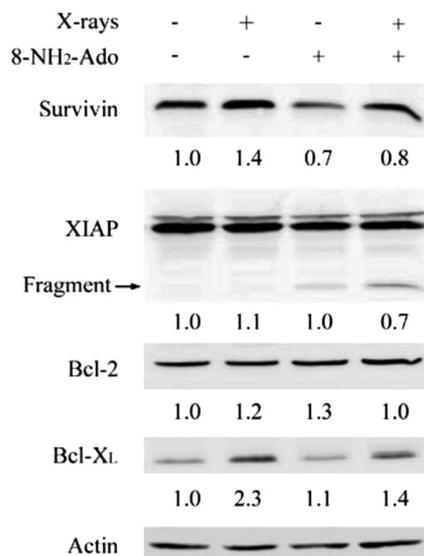
#### Adenosine suppressed enhancement of radiation-induced apoptosis by 8-NH<sub>2</sub>-Ado

Adenosine and 8-NH<sub>2</sub>-Ado are known to be converted to ATP and 8-NH<sub>2</sub>-ATP by adenosine kinase and other enzymes.<sup>11</sup> If phosphorylation of 8-NH<sub>2</sub>-Ado is essential to lead the radiosensitization and this phosphorylation reaction is a competitive reaction to the same enzyme for adenosine, addition of exogenous adenosine to the culture medium seems to ameliorate the killing effect of 8-NH<sub>2</sub>-Ado. Thus, to examine the effect of adenosine on 8-NH<sub>2</sub>-Ado-induced apoptosis and intracellular ATP level, A549 cells were co-treated with the large amounts (10  $\mu$ M–500  $\mu$ M) of adenosine and 10  $\mu$ M 8-NH<sub>2</sub>-Ado for 24 h. As shown in Fig. 4A, radiation-induced apoptosis in the presence of 10  $\mu$ M 8-NH<sub>2</sub>-Ado was attenuated by adenosine in a dose-dependent manner. Addition of more than 50  $\mu$ M adenosine significantly inhibited, and addition of a high concentration of adenosine (500  $\mu$ M) remarkably abolished, the apoptosis induced by irradiation and 8-NH<sub>2</sub>-Ado. Figure 4B shows the intracellular ATP level of A549 cells exposed to 8-NH<sub>2</sub>-Ado

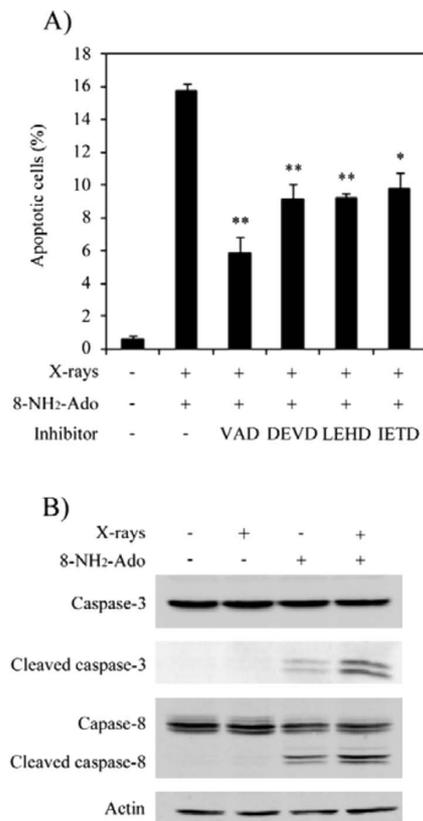
alone, irradiation alone, the combination treatment with 8-NH<sub>2</sub>-Ado and irradiation, and this combination treatment in the presence of adenosine. The treatment with 8-NH<sub>2</sub>-Ado alone reduced the basal ATP level. Irradiation alone induced an approximately 2-fold increase of the control ATP level, but the treatment with 8-NH<sub>2</sub>-Ado reduced this radiation-induced ATP increase (to one-fifth of the control ATP level). This reduction of the ATP level of A549 cells treated with 8-NH<sub>2</sub>-Ado and X-rays was restored to the control level by the addition of 500  $\mu$ M adenosine.

#### 8-NH<sub>2</sub>-Ado inhibited the expression of anti-apoptotic proteins

To determine the molecular mechanism for enhancement of apoptosis by 8-NH<sub>2</sub>-Ado, we examined the effect of 8-



**Fig. 5.** Western blot analysis of expression of anti-apoptotic proteins. Cells treated with 20 Gy of ionizing radiation, 10  $\mu$ M 8-NH<sub>2</sub>-Ado and the combination of 8-NH<sub>2</sub>-Ado and radiation were incubated for 24 h. Actin was used as a loading control. Fold changes in protein levels were quantified and normalized to Actin.



**Fig. 6.** Caspase dependence on enhancement of radiation-induced apoptosis by 8-NH<sub>2</sub>-Ado. (A) Effects of various caspase inhibitors on the enhancement of radiation-induced apoptosis by 8-NH<sub>2</sub>-Ado. Various caspase inhibitors, Z-VAD-fmk, Ac-DEVD-CHO, Ac-LEHD-CHO and Ac-IETD-CHO, were added to the medium 1 h before combination treatment and then cells were incubated for 24 h. Data are expressed as means  $\pm$  SE for three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 vs. X-rays + 8-NH<sub>2</sub>-Ado by Student's *t*-test. (B) Western blot analysis of active fragments of caspase-3 and caspase-8. Cells treated with 20 Gy of X-irradiation, 10  $\mu$ M 8-NH<sub>2</sub>-Ado and combination of 8-NH<sub>2</sub>-Ado and irradiation were incubated for 24 h. Actin was used as a loading control.

NH<sub>2</sub>-Ado on the expression of anti-apoptotic proteins. Figure 5 shows Western blotting for survivin, XIAP, Bcl-2 and Bcl-X<sub>L</sub>. The irradiation alone increased the expression of survivin and Bcl-X<sub>L</sub>, but not XIAP or Bcl-2 (second lane of Fig. 5), and the treatment with 8-NH<sub>2</sub>-Ado reduced this irradiation-induced enhancement of survivin and Bcl-X<sub>L</sub> expression as shown in the last lane of Fig. 5.

#### *8-NH<sub>2</sub>-Ado enhanced radiation-induced apoptosis in a caspase-dependent manner*

To examine the involvement of caspase in apoptosis induced by the combination of 8-NH<sub>2</sub>-Ado and radiation, the broad spectrum caspase inhibitor Z-VAD-fmk, caspase-3 inhibitor Ac-DEVD-CHO, caspase-9 inhibitor Ac-LEHD-CHO or caspase-8 inhibitor Ac-IETD-CHO, was added to culture 1 h before irradiation. As shown in Fig. 6A, apoptosis induced by the combination treatment was significantly suppressed by addition of the broad-spectrum caspase, caspase-9 and caspase-3 inhibitors. Furthermore, the caspase-8 inhibitor also inhibited the enhancement of radiation-induced apoptosis. Activation of caspase-3 and caspase-8 was also confirmed by Western blotting as shown in Fig. 6B. Active (cleaved) fragments of caspase-3 were not observed after the treatment with irradiation alone, whereas a faint band was detected after the treatment with 8-NH<sub>2</sub>-Ado alone. Large amounts of the active fragments were observed in the cells treated with 8-NH<sub>2</sub>-Ado and irradiation. A similar blotting pattern was observed for caspase-8 activation in A549 cells. These results indicated that the apoptosis induced by combination treatment was associated with not only activation of caspase-9 and -3 but also activation of caspase-8.

## DISCUSSION

Several studies have shown that 8-chloroadenosine (8-Cl-Ado) and 8-NH<sub>2</sub>-Ado have strong hematological toxicity through their ability to inhibit RNA synthesis, and 8-Cl-Ado is currently in a Phase I clinical study for patients with chronic lymphocytic leukemia.<sup>11-15</sup> 8-NH<sub>2</sub>-Ado was reported to be more potent as shown by induction of apoptosis-related cleavage of poly (ADP-ribose) polymerase compared with 8-Cl-Ado. Furthermore, the intracellular accumulation of 8-NH<sub>2</sub>-ATP is 38-fold higher than that of 8-Cl-ATP in leukemia cells and leads to greater analogue-mediated declines in the ATP pool and mRNA synthesis.<sup>15</sup> To confirm this characteristic of 8-NH<sub>2</sub>-Ado in solid tumor A549 cells, we assessed the accumulation of 8-NH<sub>2</sub>-ATP and depletion of intracellular ATP by high performance liquid chromatography (HPLC). There was a concentration-dependent increase in 8-NH<sub>2</sub>-ATP and decrease in the intracellular ATP (data not shown), indicating that 8-NH<sub>2</sub>-Ado also reduced the level of intracellular ATP in A549 cells. Furthermore, as shown in Fig. 4B, luciferin-luciferase ATP assay demonstrated that X-

irradiation enhanced intracellular ATP level and 8-NH<sub>2</sub>-Ado treatment removed intracellular ATP from A549 cells exposed to X-rays. In the present study, treatment with sub-lethal doses of 8-NH<sub>2</sub>-Ado was demonstrated to synergistically enhance loss of clonogenicity in the solid tumor cell line A549 exposed to X-rays, as shown in Fig. 2B, indicating that 8-NH<sub>2</sub>-Ado has the potential to be used as a therapeutic agent in not only hematopoietic tumor cells but also solid tumor cells. If the X-ray-induced increase of intracellular ATP level is essential for cell growth to maintain the clonogenic ability in A549 cells, a mechanism for sensitization may be removal of intracellular ATP by 8-NH<sub>2</sub>-Ado as shown in Fig. 4B. Another possible mechanism for radiosensitization of reproductive cell death by 8-NH<sub>2</sub>-Ado is inhibition of survival signaling molecules, i.e., phosphorylation of p38, ERK and Akt, leading to autophagy, since 8-NH<sub>2</sub>-Ado was reported to be able to disrupt these survival signal transduction pathways.<sup>13,14</sup> Further studies using solid tumor cells are required to answer this question.

As shown in Fig. 3, 8-NH<sub>2</sub>-Ado was demonstrated to enhance radiosensitization in not only reproductive cell death as revealed by clonogenic assay, but also apoptotic cell death as estimated by morphological changes (Fig. 3A and 3B). It is reported that 8-NH<sub>2</sub>-Ado inhibits mRNA transcription.<sup>15</sup> Therefore, to clarify the molecular mechanisms of enhancement of radiation-induced apoptosis by 8-NH<sub>2</sub>-Ado, we tested the effects of 8-NH<sub>2</sub>-Ado on the expression levels of anti-apoptotic proteins using Western blot analysis. As shown in Fig. 5, treatment with 8-NH<sub>2</sub>-Ado slightly suppressed the expression of survivin and XIAP, which are important members of the inhibitor of apoptosis (IAP) family. We previously reported that ECyd, which RNA synthesis in tumor cells, suppressed not only survivin but also Bcl-2, Bcl-X<sub>L</sub>, cell cycle-related proteins and proliferation-related proteins in gastric tumor cell lines MKN45, MKN28 and murine rectum adenocarcinoma cell line Colon26.<sup>4,7</sup> Treatment with purvalnol A, which acts as not only a CDK inhibitor but also an RNA polymerase inhibitor, induces radiosensitization and apoptosis in MKN45 and MKN28.<sup>9</sup> Recently, it was reported that survivin strongly inhibited the signal transduction of apoptosis after cytochrome c release by irradiation<sup>20</sup> and that overexpression of wild-type survivin attenuated ECyd-induced radiosensitization of apoptosis.<sup>4</sup> Wang *et al.* reported that inhibition of XIAP expression by RNAi inhibited the proliferation and enhanced the radiosensitivity of laryngeal carcinoma HEp-2 cells.<sup>21</sup> Furthermore, when A549 cells were exposed to X-rays alone, Bcl-X<sub>L</sub> expression was enhanced 2.3-fold in comparison with the control level and 8-NH<sub>2</sub>-Ado reduced this radiation-induced increase response (Fig. 5). These facts indicate that treatment with 8-NH<sub>2</sub>-Ado induced a proapoptotic condition in A549 cells, and this condition might be contributable, at least partially, to the enhancement of radiation-induced apoptosis.

Interestingly, when peptide inhibitors against caspase-3, -8, -9 were utilized to evaluate the involvement of caspases in this increase of apoptosis, all the inhibitors suppressed the enhancement of radiation-induced apoptosis (Fig. 6). These results suggested that not only mitochondria-mediated apoptotic pathways but also death receptor-mediated pathways were involved in this radiation-induced apoptosis in the presence of 8-NH<sub>2</sub>-Ado. In previous reports,<sup>22,23)</sup> we showed that X-irradiation induced the expression of Fas and death receptor DR5 and that treatment with Fas or TNF-related apoptosis-inducing ligand (TRAIL) enhanced caspase-8-dependent apoptosis in gastric cancer cell lines MKN45 and MKN28, lung cancer cell line A549 and prostate cancer cell line DU145. This indicated that the death-receptor-related apoptotic pathway involved in Fas and DR5 and caspase-8 was intact in many solid tumor cells, including A549 cells. Furthermore, Yang *et al.* showed that doxorubicin-induced activation of caspase-8 and caspase-9 was enhanced by reconstitution of caspase-3 in MCF-7 (a caspase-3 deficient cell line), suggesting that there was caspase-3 mediated-feedback, and that caspase-3 played an important role in maximizing the activation of apical caspases and cross talk between two major apoptotic pathways (the mitochondrial pathway and death receptor pathway).<sup>24)</sup> This feedback may be involved in the enhancement of radiation-induced apoptosis by 8-NH<sub>2</sub>-Ado.

In summary, we demonstrated that 8-NH<sub>2</sub>-Ado has multiple functions to influence cell survival and apoptosis signal transduction molecules, i.e., the intracellular ATP levels and expression level of survivin and XIAP, and that 8-NH<sub>2</sub>-Ado enhances the radiation-induced cell death in A549 cells. These results indicate that 8-NH<sub>2</sub>-Ado has potential not only as an anti-tumor drug for leukemia and lymphoma but also as a radiosensitizing agent for solid tumors. Furthermore, these results suggested that combination therapy using 8-NH<sub>2</sub>-Ado and radiation is useful for increase of response rate, survival, and local control rate in NSCLC.

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