

AT-101, a Pan-Bcl-2 Inhibitor, Leads to Radiosensitization of Non-small Cell Lung Cancer

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Introduction: Radiotherapy has a central role in the treatment of lung cancer. However, its effectiveness is often limited, in part, because of the defects in key apoptosis regulators, such as Bcl-2 family members, that contribute to cancer ability to evade apoptosis. In this study, we tested AT-101, a pan-Bcl-2 inhibitor, as a potential radiosensitizer in lung cancer.

Methods and Results: Clonogenic assays were used to determine the radiosensitivity of multiple lung cancer cell lines. On the basis of their relative response to radiotherapy, lung cancer cells were stratified into two groups, and a representative cell line of each group was selected for more in-depth study: A549 (resistant) and HCC2429 (sensitive). The expression levels of antiapoptotic (Bcl-2, Bcl-X_L, and Mcl-1) and proapoptotic (Bax, Bak, and Bid) Bcl-2 proteins were determined for each group. Although the levels of Bcl-2 and Mcl-1 were low for both groups, Bcl-X_L expression was dramatically higher in A549, whereas almost not detected in HCC2429. The levels of Bax/Bak were 40% higher in HCC2429 compared with A549. When administered alone, AT-101 resulted in increased apoptosis in concentration-dependent manner against both groups, with enhanced activity in HCC2429 even at lower concentration. Furthermore, AT-101 promoted radiosensitivity of A549 and HCC2429 cells ($p < 0.005$). Consistent with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay findings, A549 cells required increased AT-101 dose to achieve a similar cytotoxicity to HCC2429 cells.

Conclusions: These investigations suggest that the Bcl-2 family may serve as effective therapeutic targets in lung cancer. Further clinical studies are warranted to assess the potential of AT-101 as an agent that enhances the therapeutic ratio of radiotherapy in lung cancer.

Key Words: Apoptosis, Bcl-2, Lung cancer, Radiotherapy.

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Lung cancer remained the leading cause of cancer mortality in 2008,¹ despite multimodality therapy. The majority of these cases (~80%) are non-small cell lung cancer (NSCLC), and the global 5-year overall survival is only 15%.¹ Although radiotherapy has a central role in the treatment of locally advanced NSCLC, the effectiveness of this modality, however, is often limited, in part, because of the presence of defects in cell death mechanisms. Therefore, the identification of novel therapeutic strategies is critical for improving outcome. Manipulation of radiation-induced cell death is one such strategy,² and it has been proposed to enhance therapeutic ratio in lung cancer. Of the different radiation-induced cell death processes, apoptosis has been a promising target.³ Apoptosis molecularly represents a cascade of caspase proteases released from the mitochondria and regulated by Bcl-2 proteins family. Bcl-2 was originally identified in the lymphoma as a result of a *t*(14;18) chromosome translocation prevalent in B cells.⁴ The Bcl-2 family comprises antiapoptotic members, such as Bcl-2, Mcl-1, and Bcl-X_L, and proapoptotic members, such as Bax, Bak, and Bid.⁵ The members of the Bcl-2 family control the integrity of the outer mitochondrial membrane and, thus, are critical in determining the susceptibility of cells to apoptosis induced by the intrinsic pathway. The balance between cell survival and cell death is modulated by the ratios and interactions of antiapoptotic and proapoptotic Bcl-2 family proteins. Therefore, in cancer cells, alterations in the amounts of these antiapoptotic Bcl-2 proteins allow survival to promote cell survival, likely generated by phenotypic abnormalities acquired during tumorigenesis. These defects also contribute to the ability of cancer cells to evade treatment-induced apoptosis and thus to confer resistance to various therapeutic agents, including radiotherapy.

In fact, overexpression of Bcl-2 or Bcl-X_L is observed in several cancers, including hematologic malignancies, colorectal, prostate, and breast cancers,^{6–11} and it has been shown to confer resistance to various anticancer agents.^{12–14} Understanding how radiation affects Bcl-2-dependent lung cancer cell death may enable the development of strategies to restore apoptotic sensitivity in lung tumors and ultimately improve locoregional control and prognosis.

In this study, we focused on the investigation of whether NSCLC cells are dependent on Bcl-2 proteins. We tested the potential of AT-101 (*R*(-)-gossypol acetic acid), a natural BH3 mimetic small molecule that binds to the BH3 domain of Bcl-2, Bcl-X_L, and Mcl-1, which has been reported recently to induce apoptosis in multiple cancer cell lines with high levels of Bcl-2 and/or Bcl-X_L,^{15–17} as a radiosensitizer in

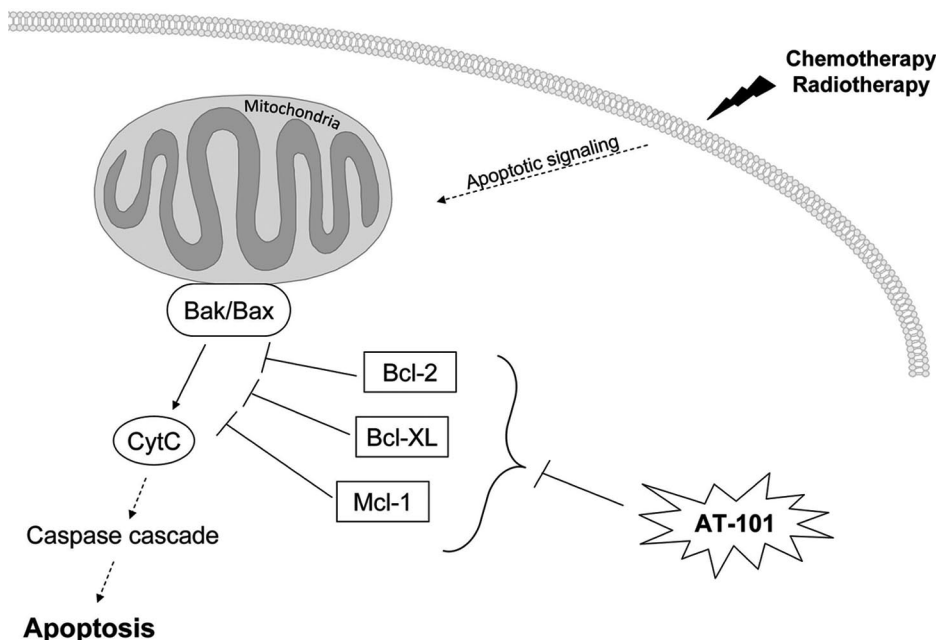


FIGURE 1. Schematic overview showing the action of AT-101 in the apoptotic signaling cascade. Antiapoptotic members of the Bcl-2 family (Bcl-2, Mcl-1, and Bcl-X_L) are targeted by AT-101 to allow proapoptotic members (Bax/Bak) to mediate apoptosis.

lung cancer. Gossypol is a product isolated from cottonseeds and roots, which has been studied previously for several therapeutic uses. More recently, it was found that AT-101, the racemic (–) enantiomer of gossypol (*R*-(–)-gossypol acetic acid), binds with nanomolar affinity to Bcl-2 (320 nmol/liter), Bcl-X_L (480 nmol/liter), and Mcl-1 (180 nmol/liter).¹⁸ AT-101 inhibits Bcl-2 by acting as a BH3 mimetic and disrupts the heterodimerization of Bcl-2 with proapoptotic family members,¹⁸ and it acts directly on the mitochondria to overcome Bcl-2- and Bcl-X_L-mediated apoptosis resistance. A schematic overview of AT-101 action is shown in Figure 1. Single-agent activity of (–)-gossypol has been shown in a variety of cancer cell lines, including chronic lymphocytic leukemia, prostate, and colon carcinoma.^{19–21} Furthermore, synergy was seen with docetaxel in prostate cancer cell lines.²⁰ However, the effects of AT-101 in NSCLC were not reported yet. By using Bcl-2 family proteins profiling and in vitro response to AT-101, we found that lung cancer cell lines harbor different sensitivity to radiation associated with their Bcl-2 status. Our findings also provide rationale for the clinical testing of AT-101 and Bcl-2 inhibition in patients with NSCLC.

MATERIALS AND METHODS

Cell Lines and Chemical

Human NSCLC cells were obtained from the following sources: A549 from American Type Culture Collection (Manassas, VA) and HCC2429 was a gift from Dr. Tao Dang (Vanderbilt University, Nashville, TN). All the cells were cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen, Grand Island, NY), both supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and humidified 5% CO₂. AT-101 was provided by Ascenta Therapeutics (San Diego, CA). Stock solution was prepared

in 100% dimethyl sulfoxide (DMSO) and stored at –20°C. The drug was diluted in fresh media before each experiment. Anti-Bcl-2, anti-Mcl-1, anti-Bcl-X_L, anti-Bak, anti-Bid, anti-Bax, and anticaspase-3 antibodies were purchased from Cell Signaling Technology (Danvers, MA) and anti-β-actin from Sigma (St. Louis, MO).

Clonogenic Assay

A549 and HCC2429 lung cancer cells were treated with DMSO or AT-101 (5 μM, for 14 hours). The cells were irradiated with 0 to 6 Gy as indicated at a dose rate of 1.8 Gy/min, using a ¹³⁷Cs irradiator (J.L. Shepherd and Associates, Glendale, CA). After irradiation, cells were incubated at 37°C for 8 to 10 days. Cells were fixed for 15 minutes with 3:1 methanol:acetic acid and stained for 15 minutes with 0.5% crystal violet (Sigma) in methanol. After staining, colonies were counted using a cutoff of 50 viable cells. Surviving fraction was calculated as (mean colony counts)/(cells inoculated) × (plating efficiency), where plating efficiency was defined as (mean colony counts)/(cells inoculated for nonirradiated controls). The radiation dose enhancement ratio (DER) was calculated as the dose (Gy) for radiation alone divided by the dose (Gy) for radiation plus AT-101 (normalized for AT-101 toxicity) necessary for a surviving fraction of 0.25. Experiments were conducted in triplicate, and mean, SD, and *p* values (using a Student *t* test) were calculated. Error bars were calculated as ± standard error by pooling of the results of three independent experiments.

Cell Proliferation Assay

Logarithmically growing lung cancer cell lines were cultured, washed, counted, and plated at 5000 to 15,000 cells per well in duplicate wells of 96-well plates and were incubated overnight. All the experimental conditions were performed with three replicates. The sample plates were incubated for 14 hours

in 300 μ l of Dulbecco's modified eagle medium containing AT-101 or solvent controls. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were then performed according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). The MTT assay measures the cell survival based on mitochondrial conversion of MTT from a soluble tetrazolium salt into an insoluble colored formazan precipitate, which is dissolved in DMSO, and quantitated using spectrophotometry.²² Percent absorbance relative to control was plotted as a linear function of drug concentration.

Apoptosis Assay

A549 and HCC2429 cells (5×10^5) were plated in 10-mm dishes for each data point. After overnight incubation at 37°C, cells were treated with 10 μ M AT-101 and irradiated with 6 Gy. Then, cells were treated with 1 ml Accutase (Innovative Cell Technologies, San Diego, CA) for 4 minutes (keeping all floating cells) and counted for each sample. The cells were centrifuged and resuspended in $1 \times$ binding buffer at a concentration of 1×10^6 /ml, and 100 μ l of the solution (5×10^5 cells) were transferred to a 5-ml fluorescence-activated cell sorter tube and combined with 1 μ l of Annexin V/fluorescein isothiocyanate and 1 μ l of propidium iodide. After incubation for 30 minutes at room temperature in the dark, 300 μ l of $1 \times$ binding buffer were added to each tube. The rate of apoptosis was measured using the Annexin V/fluorescein isothiocyanate apoptosis detection kit I (Pharmingen, San Diego, CA) with flow cytometry.

Immunoblotting

After the treatment with 10 μ M AT-101 or not, A549 and HCC2429 lung cancer cells (5×10^5) were treated with various doses of radiation and collected at various time points. The cells were harvested and then washed with ice-cold phosphate-buffered saline (PBS) twice before the addition of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM ethylene diaminetetraacetic acid, 1% NP40, 50 mM NaF, 1 mM Na_3VO_4 , 1 mM NaMoO_4 , and cocktail inhibitor [5 μ l/ml; Sigma]). Protein concentration was quantified by the Bio-Rad method. Equal amounts of protein were loaded into each well and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, followed by transfer onto polyvinylidene fluoride membranes (BIO-RAD, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in phosphate buffered saline-Tween 20 for 1 hour at room temperature. The blots were then incubated overnight at 4°C with Actin (Sigma) antibodies. After washing with PBS, the membranes were incubated with goat antirabbit IgG secondary (1:1000, Santa Cruz Biotechnologies, Santa Cruz, CA) antibody for 45 minutes at room temperature. Immunoblots were developed by using the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) and autoradiography.

Statistical Analysis

To generate isobolograms, data from the MTT assays were analyzed using CalcuSyn software (Biosoft, Cambridge, UK). The software analyzes drug interactions based on the method of Chou and Talalay.²³ The data presented represent mean \pm SD. All tests of significance were made with a

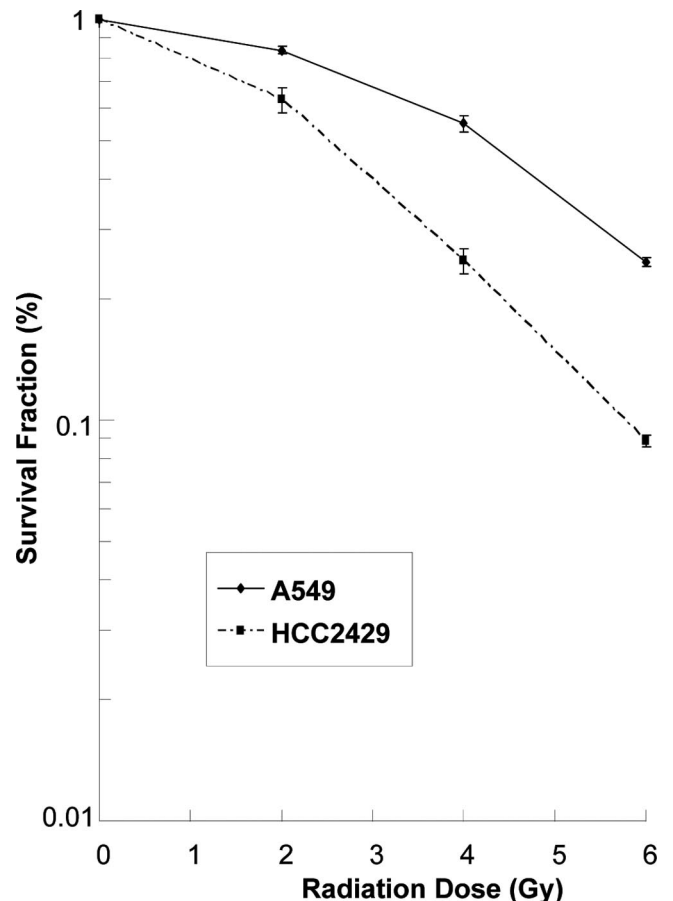


FIGURE 2. Clonogenic lung cancer survival after radiotherapy. Clonogenic assay showing sensitivity of A549 and HCC2429 lung cancer cells. Cells were irradiated with the indicated doses of radiation. After 8 days, colonies were stained and scored for colony formation, and the surviving fractions were plotted in a semi-log format. The mean \pm standard deviation of three separate repeated experiments are shown.

two-sided Student *t* test, and the differences were considered statistically significant when *p* value was <0.05 . Asterisks (*) represent statistical significance (**p* <0.05 and ***p* <0.01). A statistical package, SAS v8.2, was used for all analyses.

RESULTS

Clonogenic Lung Cancer Cells Survival After Radiotherapy

To investigate whether lung cancer is resistant to radiotherapy, we used clonogenic assays to study intrinsic radiosensitivity of several lung cancer cell lines, which were dichotomized in two groups: the A549 cell line was a representative of the radioresistant phenotype, whereas the HCC2429 cell line was a representative of the more radio-sensitive phenotype (Figure 2). Clonogenic survival assay demonstrated an 80% decreased survival in the HCC2429 group when compared with the A549 group (DER = 1.80, *p* <0.04), suggesting a dramatic difference in sensitivity to radiation between the two groups.

Radiation-Induced Apoptosis in Lung Cancer Cells Lines

To determine whether radiotherapy leads to apoptosis in lung cancer cells, Western Blot analysis was used to detect caspase-3 activation (pro-caspase-3 cleavage) at different time points (0–72 hours) after treatment with 6 Gy. As shown in Figure 3A, the expression of activated caspase-3 was not detected in A549 lung cancer cell line even at late time points, such as 72 hours. In contrast, HCC2429 cell group exhibited significant apoptosis induction, particularly after 48 and 72 hours. These results suggest that A549 lung cancer cells are more resistant to radiation-induced apoptosis than HCC2429 cells.

Bcl-2 Protein Family Expression in Lung Cancer Cell Lines

Protein expression levels of the Bcl-2 family in lung cancer cell lines were determined by Western blot analysis, as shown in Figure 4A. Quantification of relative density of Bcl-2 proteins after normalization to actin expression is represented in Figure 4B. Bak, Bax, and Bid proteins were highly expressed in both cell groups, whereas Mcl-1 expression was slightly lower. In addition, Bcl-X_L was robustly

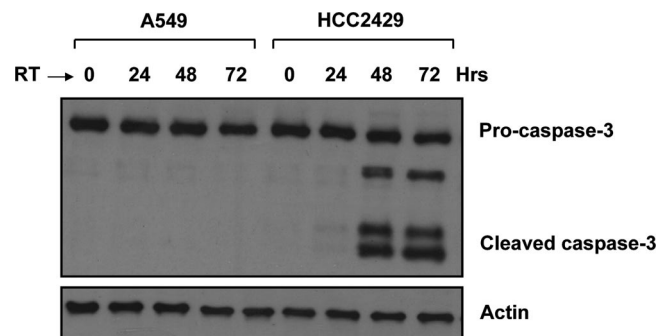


FIGURE 3. Radiation-induced apoptosis in lung cancer cell lines. Caspase-3 activation during lung cancer cells apoptosis after radiation treatment. Representative caspase-3 cleavage pattern analyzed using Western blot analysis. Actin was probed to demonstrate equal loading.

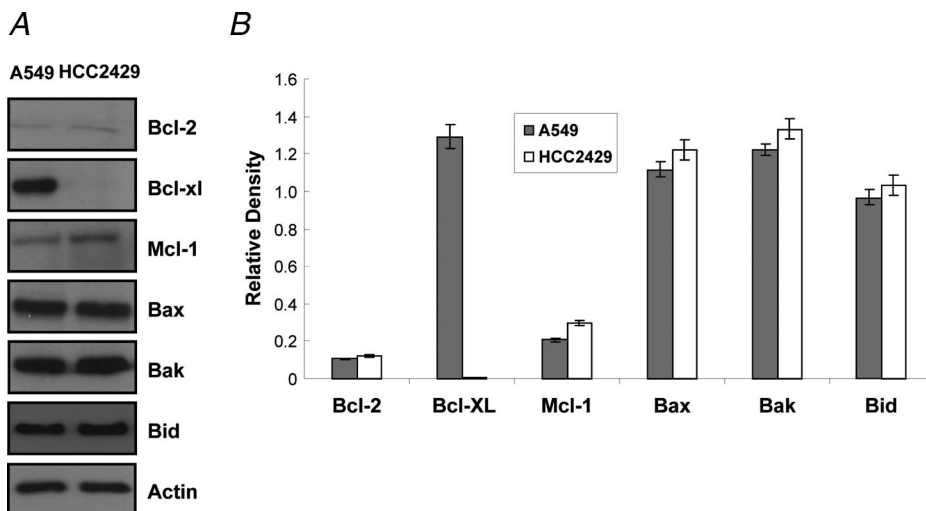


FIGURE 4. Bcl-2 family protein expression in lung cancer cell lines. *A*, Representative Western blot analysis showing relative levels of expression of Bcl-2 family proteins in A549 and HCC2429 lung cancer cell lines. Actin was probed to demonstrate equal loading. *B*, Densitometry data present relative expression of Bcl-2 family proteins after normalization to actin expression. Data are shown as mean (columns) with standard deviation (bars).

expressed in A549, with HCC2429 lacking detectable expression. Finally, both cell lines expressed Bcl-2 at some low level. These results suggest that resistant and sensitive lung cancer cell lines harbor a different Bcl-2 proteins profile, with higher levels of Bcl-X_L antiapoptotic proteins in A549 cells and relatively higher levels of Mcl-1 in HCC2429 cells. These findings suggest differential regulation in the expression of Mcl-1 and Bcl-X_L proteins in the lung cancer cells.

Differential Activity of AT-101 for Apoptosis Induction in Lung Cancer Cells

We next investigated the efficacy of AT-101, a pan-Bcl-2 inhibitor, in the treatment of lung cancer cells by determining the induction of apoptosis. For this study, Western blot analysis was used to detect caspase-3 activation (procaspase-3 cleavage) at different time points (14 and 26 hours) after treatment with different doses of AT-101 (1 and 10 μ M). As shown in Figure 5A, the level of apoptosis was progressively elevated with increased doses of AT-101 in both lung cancer cell groups. Although 1 μ M of AT-101 did not induce apoptosis, a 10 μ M was able to induce dramatic levels of apoptosis in HCC2429 cells at both 14 and 26 hours, when compared with DMSO control. In A549 cells, similar dose of AT-101 was also able to significantly induce apoptosis compared with control, particularly after 26 hours although to a much lower extent compared with HCC2429 cells. Taken together, these results suggest that the effect of AT-101 on apoptosis is dose dependent in both cell groups and that HCC2429 cells are more sensitive to AT-101 than A549.

AT-101 Promotes Radiation-Induced Apoptosis in Lung Cancer Cells

We next tested whether AT-101 would enhance apoptosis in response to radiotherapy in lung cancer cells. By using immunoblotting, we examined the level of activated caspase-3 after treatment with DMSO (control), AT-101 alone (10 μ M), radiation alone (6 Gy), and combination of both AT-101 and radiation (Figure 5B). Consistent with our previous findings, AT-101 alone was able to induce apoptosis in HCC2429 cells

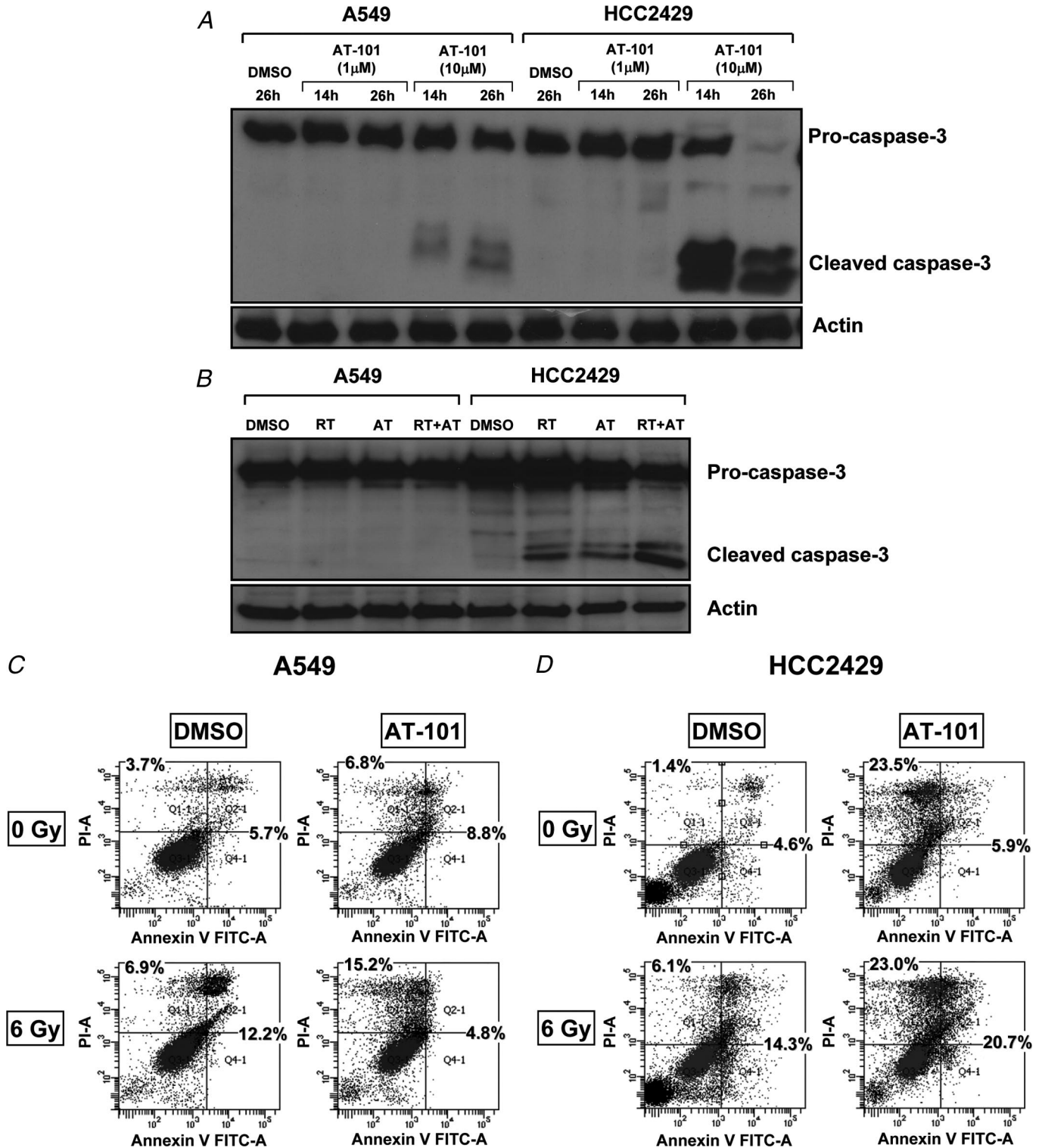


FIGURE 5. Apoptosis after AT-101 treatment with or without radiation in lung cancer cell lines. A549 and HCC2429 cells were pretreated with AT-101 for 2 hours followed by radiation (0 or 6 Gy). *A*, Caspase-3 activation during A549 and HCC2429 lung cancer cells apoptosis after dimethyl sulfoxide (DMSO), 1 or 10 μM AT-101, at 14 and 26 hours. Actin was probed to demonstrate equal loading. *B*, Caspase-3 activation during A549 and HCC2429 lung cancer cells apoptosis after DMSO, AT-101 (10 μM for 14 hours), radiation (6 Gy), or combination of both AT-101 and radiation (6 Gy) at 36 hours. Actin was probed to demonstrate equal loading. A549 (*C*) and HCC2429 (*D*) cells were stained with Annexin V and propidium iodide and analyzed using flow cytometry. This experiment was performed in triplicate and representative diagrams of Annexin V assays are shown.

and not in A549 cells after 36 hours. Similarly, radiation alone was able to induce apoptosis in HCC24229 cells and not in A549-resistant cells. After AT-101 treatment, enhanced radiation-induced apoptosis was observed in HCC2429 cells and not in A549 cells compared with radiation alone.

Annexin V flow cytometric analysis was used to quantify the levels of apoptosis in both cell lines. As shown in Figure 5C, D, radiation resulted in increased Annexin V-positivity in both cell lines, with a twofold increase in A549 cells (12.2% for 6 Gy versus 5.7% for 0 Gy) and a threefold increase in HCC2429 cells (14.3% for 6 Gy versus 4.6% for 0 Gy). In contrast, addition of AT-101 to radiation in A549 cells failed to further increase apoptosis compared with radiation alone, whereas it increased apoptosis in HCC2429 cells in an additive manner. When used alone, AT-101 resulted in slight induction in apoptosis in A549 cells (8.8% versus 5.7%, $p = 0.04$), whereas it did not significantly increase the amount of apoptosis in HCC2429 cells. However, the treatment with AT-101 alone increased significantly the amount of dead cells, especially in the HCC2429 group (up to 23%). Indeed, AT-101 results in increased overall toxicity, even though the rate of apoptosis is relatively stable. This suggests that cell death mechanisms other than apoptosis may be responsible for the observed effects.

AT-101 Reduces Cell Proliferation and Sensitizes Lung Cancer Cells to Radiotherapy

To investigate whether AT-101 would reduce cell proliferation, we performed MTT assays in both cell lines for 2 days (Figure 6A). After 14 hours of treatment, we observed significant inhibition of cell proliferation in HCC2429 cells treated with AT-101 (10 μ M) compared with DMSO control. When both AT-101 and radiation (6 Gy) were combined, a further decrease in HCC2429 cell proliferation was observed compared with radiation ($p < 0.01$) or AT-101 alone ($p < 0.01$). In A549 cells, AT-101 resulted in a modest decrease in cell proliferation when compared with the DMSO group. AT-101 was also able to modestly increase radiation cytotoxicity in A549 cells compared with radiation alone ($p = 0.02$). To determine whether the combination of AT-101 and radiation in both cell lines results in synergistic cytotoxic effects, isobologram analysis was performed (Figure 6D). In this analysis, there is synergy when the combinatorial index (CI) is < 1.0 , additivity when the CI is equal to 1.0, and antagonism when the CI is > 1.0 . As shown in Figure 6D, there is an additive interaction between radiation and AT-101 in both A549 and HCC2429 cells.

To determine the effects of AT-101 on radiosensitivity in lung cancer cells at a longer time point, we used clonogenic assays. Because a dose of 10 μ M AT-101 did not allow any colony formation in HCC2429 cells after radiation, we reduced the dose of AT-101 to 5 μ M to obtain a representative survival curve. Therefore, both A549 and HCC2429 cells were treated with 5 μ M AT-101 or DMSO control and were then irradiated with 0 to 6 Gy. Surviving colonies were counted 8 days later and graphed as survival curves, as shown in Figure 6. After AT-101 treatment, enhanced radiosensitivity was demonstrated in both A549 (Figure 6B) and HCC2429 (Figure 6C) lung cancer cells, with a DER of 1.19

($p = 0.005$) and 1.22 ($p < 0.05$), respectively, when compared with control. Interestingly, the increase in sensitivity of HCC2429 cells occurs at lower dose than in A549 cells. These results suggest that 5 μ M AT-101 can significantly sensitize HCC2429 to relatively lower dose of ionizing radiation after treatment and that A549 lung cancer cells would need higher dose to achieve a similar sensitization.

DISCUSSION

In this study, we used NSCLC cell lines to determine whether NSCLC is dependent on the apoptotic block provided by expression of BCL-2 and related proteins. We also showed the effects of AT-101, a pan-BCL-2 inhibitor, which resulted in the effective radiosensitization of lung cancer cells. This report suggests that this novel strategy has some potential for enhancing the treatment of lung tumors with radiotherapy.

Expression levels of antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-X_L, and Mcl-1) and proapoptotic (Bax, Bak, and Bid) proteins were determined in both A549 and HCC2429 cell lines. Although the levels of Bcl-2 were low and similar for both groups, Bcl-X_L expression was dramatically higher in the A549 group, whereas almost not detected in the HCC2429 group. Overall, Mcl-1 protein levels were moderate but higher in the HCC2429 group when compared with the A549 group. Notably, the levels of proapoptotic Bax and Bak were also slightly higher in HCC2429 cells. On the basis of these results, Bcl-X_L expression was the major difference between the two groups, and subsequently, one can speculate that this antiapoptotic protein may contribute the most to the observed resistance to radiation-induced apoptosis when compared with Bcl-2 and Mcl-1. Consistently, it has been shown that Bcl-X_L is overexpressed in numerous cancer types and is associated with cancer progression and with resistance to a large panel of chemotherapeutic agents.²⁴ In addition, the National Cancer Institute Anticancer Drug Screen study reported the protective effect of high basal levels of Bcl-X_L on cellular response to a wide variety of agents, suggesting that Bcl-X_L plays an important role in general resistance to cytotoxic agents.²⁵

Recently, another small molecule inhibitor, ABT-737, that targets mainly Bcl-2 and Bcl-X_L (and not Mcl-1) was shown to increase the response to radiation and chemotherapeutic agents in vitro and also effective as single agent in small cell lung cancer xenografts.²⁶ However, several studies have suggested that high basal levels of Mcl-1 expression are associated with resistance to ABT-737.^{27,28} In this study, we found that AT-101, which inhibits Mcl-1, was more effective to promote apoptosis in HCC2429 cells compared with A549 cells (Figure 5), even though the levels of Mcl-1 were slightly higher in HCC2429 than in A549 cells (Figure 4). These results may suggest that the efficacy of AT-101 is not limited by the relative levels of Mcl-1 in NSCLC. Because the difference in Mcl-1 expression levels were relatively modest and not different for both cell lines, it is likely that other mechanisms participate in AT-101-induced apoptosis. Consistently, it has been recently showed that one of the proposed proapoptotic mechanisms of AT-101 is through

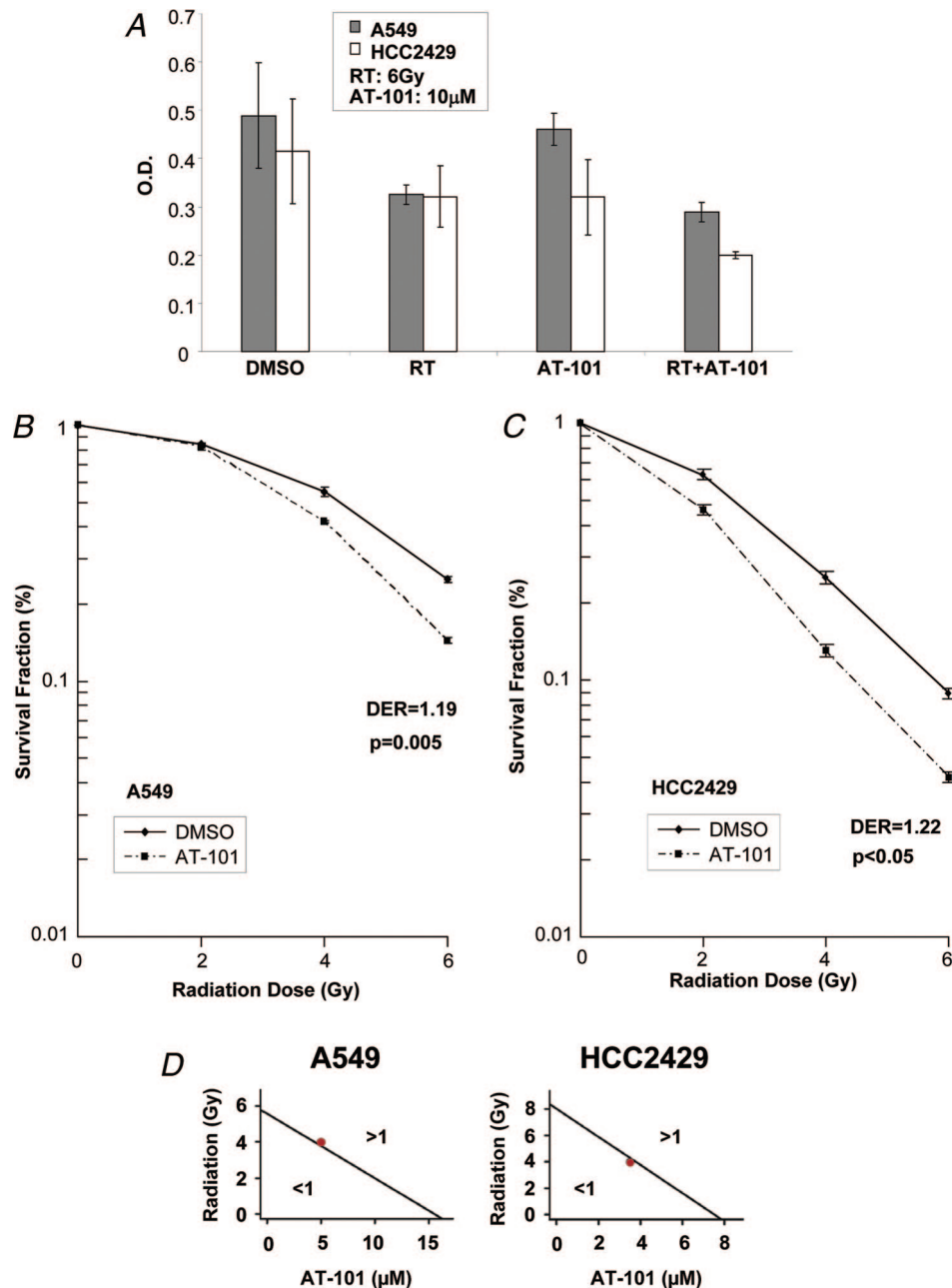


FIGURE 6. AT-101 reduces cell proliferation and sensitizes lung cancer cells to radiotherapy. **A**, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 8 hours treatment with either dimethyl sulfoxide (DMSO) or AT-101 (10 μ M) alone, radiation (6 Gy), or combination of both AT-101 (10 μ M) and radiation (6 Gy). Data are shown as mean (columns) with standard deviation (bars). **B**, MTT assay after 14 hours treatment with either DMSO or AT-101 (10 μ M) alone, radiation (6 Gy), or combination of both AT-101 (10 μ M) and radiation (6 Gy). Data are shown as mean (columns) with standard deviation (bars). Clonogenic assays showing sensitivity of A549 (**B**) and HCC2429 (**C**) lung cancer cells. Cells were treated with DMSO or AT-101 (5 μ M for 14 hours) and then irradiated with the indicated doses of radiation. After 8 days, colonies were stained and scored for colony formation, and the surviving fractions were plotted in a semi-log format. Mean \pm SD of three separate repeated experiments are shown. **D**, Isobologram analysis of the combination of AT-101 and radiation shows an additive interaction in both A549 and HCC2429 cells. The red dot represents the concentrations of both drugs that result in a MTT activity, that is, 50% of control (IC_{50}).

upregulation of Noxa and Puma.²⁹ Indeed, authors demonstrated that AT-101 induced apoptosis in both Bax/Bak-dependent and -independent manners in several human breast

and prostate cancer cell lines associated with increased expression of Noxa and Puma, whereas knockdown of Noxa or Puma reduced AT-101-induced apoptosis.²⁹ These results

suggest that the mechanisms by which AT-101 promotes apoptosis are complex and not yet fully understood.

In this study, the administration of 5 μM AT-101 markedly enhanced sensitivity of both A549 (DER = 1.19 and $p = 0.005$) and HCC2429 (DER = 1.22 and $p < 0.05$) lung cancer cells to radiation in clonogenic assays. This enhanced radiosensitization was associated with an increase in apoptosis in both lung cancer groups. Interestingly, A549 cells required increased AT-101 dose and/or longer treatment duration to achieve a similar radiocytotoxicity to HCC2429 cells. Indeed, the use of a dose of 10 μM AT-101 in a clonogenic assay against HCC2429 was too high and resulted in the killing of all colonies (data not shown because no curve could be obtained). In contrast, 1 μM was too low to achieve a significant sensitization of A549 cells (data not shown). These data confirm the results obtained with MTT assays (Figure 6A), which showed an early effect of AT-101 with radiotherapy against HCC2429 as opposed to A549 cells. Our findings are consistent with previous reports showing that AT-101 modulates Bcl-2 family balance by down-regulating the antiapoptotic members of the Bcl-2 family and upregulating the proapoptotic members in a concentration and cell line-specific fashion.³⁰ Therefore, it is necessary to determine the optimal concentrations, doses, and schedules of AT-101 in lung cancer clinical trials to maximize its potential in combination with radiotherapy. Nevertheless, it has been suggested recently that Bcl-2 inhibitors could act on a pathway that is independent of Bcl-2 in mouse embryonic fibroblasts and Jurkat T-cells.³¹ The study showed that most inhibitors, including gossypol and apogossypol, damage the mitochondria (induction of swelling and loss of cristae) and may have additional cellular targets that could participate in the observed cytotoxicity. These data are consistent with our study that showed that the lung cancer cells are sensitized to radiation therapy even though apoptosis levels remain low in A549 cells (Figure 6). In addition, our Annexin V experiment showed that AT-101 alone increased significantly the amount of dead cells independently of apoptosis (Figure 5C, D), which suggest the participation of alternate cell death mechanisms for the observed radiosensitization.

In conclusion, this preclinical study supports the therapeutic potential of AT-101 as a radiosensitizer in lung cancer. Further studies are warranted to study the clinical potential of AT-101 as an agent that may effectively raise the therapeutic ratio of radiotherapy in lung cancer.

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