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Ionizing Radiation Sensitizes Breast Cancer Cells to Bcl-2 Inhibitor, ABT-737, through Regulating Mcl-1

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Abstract

Breast-conserving surgery followed by radiation therapy has become the standard of care for early stage breast cancer. However, there are some patients that develop a local failure. We have previously shown that Bcl-2 overexpression was associated with an increased risk of local recurrence in patients with early stage breast cancer. The purpose of this study was to explore an approach to overcome radiation resistance by targeting pro-survival Bcl-2 family proteins in breast cancer cells. The breast cancer cell lines MCF-7, ZR-75-1 and MDA-MB231 were used in this study. siRNAs were employed to silence myeloid cell leukemia 1 (Mcl-1). A small molecule inhibitor of Bcl-2, ABT-737, was used to target anti-apoptotic Bcl-2 family proteins. Apoptosis was identified by FITC Annexin V, PI staining and Western blot analysis. The sensitivity to ionizing radiation and ABT-737 were measured by clonogenic assays. The effect of radiation and ABT-737 was also tested in a MCF-7 xenograft mouse model. Our data demonstrate that the combination of ABT-737 and radiation-induced apoptosis had an inhibitory effect on breast cancer cell proliferation. However, treatment with ABT-737 resulted in elevated Mcl-1 in breast cancer cell lines. Targeting Mcl-1 by siRNA sensitized MCF-7 cells to ABT-737. We revealed that radiation blunted Mcl-1 elevation induced by ABT-737, and that radiation downregulated Mcl-1 by promoting its degradation. Our results indicate that radiation and ABT-737 exert a synergistic effect on breast cancer cell lines through downregulating Mcl-1 and activating the bak-apoptotic pathway. These results support the combination of radiation and pro-survival Bcl-2 family inhibitor as a potential novel therapeutic strategy in the local-regional management of breast cancer.

INTRODUCTION

In 2014, an estimated 232,670 new cases of invasive breast cancer will be diagnosed and 40,000 women will die from breast cancer in the United States (American Cancer Society, 2014). Radiation therapy after breast-conserving surgery (BCS+RT) is a standard treatment strategy for early stage breast cancers (1). However, 10–20% of patients will develop local

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recurrence despite receiving BCS+RT (2, 3). Radiation therapy has been shown to induce cancer cell death by triggering apoptosis and nonapoptotic cell death (4, 5). Bcl-2 family members are the key players of the cellular apoptotic machinery (6, 7). Elevated expression of Bcl-2, Bcl-xl and Mcl-1 has been found in various subtypes of breast cancer (7). Bcl-2 overexpressing lymphomas and prostate cancer cell lines are resistant to radiation-induced apoptosis (8, 9). The Bcl-2 family encompasses two groups: the pro-survival group (e.g., Bcl-2, Bcl-xl, Bcl-w, Mcl-1, A1) and the pro-apoptotic group, which includes the effector Bcl-2 proteins, such as Bak, Bax and the BH3 only proteins (e.g., Bad, Bim, Bid, Puma, Noxa) (10). Bcl-2 family proteins are mainly located on the mitochondria membrane. In normal conditions, pro-survival proteins and pro-apoptotic proteins work together to maintain the integrity of mitochondrial membrane, which favors cellular homeostasis. Bcl-2 or Bcl-xl usually binds Bak/Bax to form a complex for cell survival. Once the binding of Bcl-2/Bcl-xl is broken down by intrinsic or extrinsic injury factors, Bak or Bax is released and activated, causing mitochondria outer membrane permeabilization (MOMP) and cytochrome *c* is released into the cytosol, thus inducing apoptotic cell death (11).

ABT-737 is a synthetic small molecular compound with a high affinity for Bcl-2, Bcl-xl and Bcl-w, but not for Mcl-1 or A1. ABT-737 is the mimic of BH3-only proteins. It cannot bind Bak or Bax directly, but can disrupt the complex protein unit of Bax/Bak and Bcl-2. It unleashes Bax/Bak, activates Bak/Bax and induces mitochondrial outer membrane permeabilization (12). ABT-737 has a specificity profile and efficacy similar to ABT-263, which can be given orally and is now in Phase I/II clinical trials to treat solid tumors (13).

Miyashita and Reed reported that overexpression of Bcl-2 in various lymphoid cell lines conveyed resistance to a broad range of DNA-damaging drugs (14, 15). Overexpression of Bcl-2, Bcl-xL and Mcl-1 reduced apoptosis induced by ionizing radiation in a variety of cancer cells (8, 16, 17). In previous studies we have shown that Bcl-2 expression was associated with an increased risk of local recurrence in patients with early stage breast cancer (18). Given these clinical findings and other studies, which suggest that overexpression of Bcl-2 is associated with resistance to radiation therapy, we hypothesized that inhibition of Bcl-2 may enhance the tumoricidal effects of radiation. The purpose of this study was to explore an approach to overcome radiation resistance by targeting pro-survival Bcl-2 family proteins using a combination of ABT-737 with radiation in breast cancer cell lines.

MATERIALS AND METHODS

Cell Lines in Culture

The human breast cancer cell lines MCF-7, ZR-75-1 and MDA-MB-231 were purchased from American Type Culture Collection (ATCC®, Manassas, VA). MCF-7, ZR-75-1 cells were cultured in RPMI-1640 medium, MDA-MB-231 cells were cultured in Dulbecco's modified Eagle medium (DMEM) and all were supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂/95% air.

Reagents and Antibodies

ABT-737 was supplied by Abbott Laboratories (Abbott Park, IL). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and anti- α -tubulin antibodies were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). FITC Annexin V Apoptotic Detection Kit II was from BD Bioscience (San Jose, CA). All cell culture media and other products were purchased from Invitrogen Corporation (Carlsbad, CA). MG132 was from Cayman Chemical (Ann Arbor, MI). Chemiluminescence Western blot reagents were obtained from Pierce Biotechnology, Inc. (Rockford, IL). The following antibodies were used in this study: anti-cleaved poly(ADP-ribose) polymerase (PARP) antibody, anti-Mcl-1 (Cell Signaling Technology® Inc., Beverly, MA), anti-BAK antibody (Lab Vision™, Fremont, CA).

Radiation and ABT-737 Treatment

To study the effect of combination treatment of ABT-737 and radiation, all cells were pretreated with ABT-737 for 18 h before radiation exposure and ABT-737 remained in the media after radiation exposure. The cells were irradiated with Cesium-137 in Gammacell® 40 Exactor irradiator (MDS Nordion, Canada). Based on dose-response studies, 5 μ M of ABT-737 and 8 Gy of radiation were used in the *in vitro* experiments, except for the clonogenic and MTT assays in which cells were seeded at a lower density.

siRNA Preparation and Transfection

Mcl-1 siRNA duplexes, 5'GAAUUGAUUACCCGCCGAA [dT][dT] (sense); 5'UUCGCGGGUAAUCAAUUC[dT][dT] (antisense) were prepared by Sigma-Aldrich. For transfection, cells in exponential phase of growth were plated in 60 mm tissue culture dishes at 5×10^5 cells per dish, grown for 24 h, then transfected with siRNA (100 nM) using Oligofectamine™ and Opti-MEM® I reduced serum medium, according to the manufacturer's protocol. The cells were harvested for further experiments 24 h after transfection.

Western Blot Analysis

Cells were harvested at varying times after treatment. Cells were pelleted at 500g for 5 min and were lysed in cold lysis buffer [20 mM/L Tris-HCl (pH 7.5), 150 mM/L NaCl, 1 mM/L EDTA, 1 mM/L EGTA, 1% Triton X-100, 2.5 mM/L sodium pyrophosphate, 1 mM/L β -glycerolphosphate, 1 mM/L Na_3VO_4 , 1 μ g/mL leupeptin, and 1 mM/L PMSF] and sonicated for 5 s. The lysates were clarified by centrifugation at 12,000g for 30 min at 4°C. Identical amounts (25 μ g of protein) of cell lysates were resolved by 4–20% SDS-PAGE, and proteins were transferred onto nitrocellulose or PVDF. Membranes were incubated in blocking solution consisting of 5% powered milk in TBST [10 mM/L Tris-HCl (pH 8.0), 150 mM/L NaCl and 0.1% Tween 20] for 1 h, then immunoblotted with the respective antibodies. Detection by enzyme-linked chemiluminescence was carried out according to the manufacturer's protocol. Bands were quantified using Scion Image software (Scion Co., Frederick, MD).

Apoptosis Evaluated by Flow Cytometry

MCF-7 cells were plated in 60 mm dishes at a density of 1×10^6 cells/dish. Cells were treated with ABT-737 for 18 h before irradiation and remained in the medium until they were harvested. The cells were detached by trypsinization at 48 h after irradiation, and were incubated with FITC Annexin V and PI staining solution for 15 min. The stained cells were then analyzed with a FACSCalibur™ flow cytometer using CellQuest™ software (Becton Dickinson, Bedford, MA).

Clonogenic Assay

Cells were plated in 100 mm cell culture dishes and incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% air for 14 days in the presence of ABT-737 after irradiation. At the end of the incubation period, cells were stained with 1% methylene blue in 50% methanol for 30 min, washed with water, and colonies > 50 cells were counted.

Trypan Blue Exclusion Assay

Cells were plated at 2.5×10^4 per well in 24-well tissue culture plates. Viable cells were counted using a Vi-CELL™ cell viability analyzer (Beckman Coulter Inc., Miami, FL).

Mouse Xenograft Studies

Ovariectomized female NCR NUNU mice (Taconic, NJ) were subcutaneously injected in a thigh with 1×10^7 MCF-7 cells with a 90-day release 17-estradiol pellet. Mice were randomly divided into 4 groups of 8 each. ABT-737 and/or radiation were administered once tumors reached an average volume of about 300 mm³. ABT-737 was given by intraperitoneal injection at 50 mg/kg two days before irradiation and for 4 days with concurrent irradiation. Only the thigh carrying the tumor was irradiated with 3 Gy for 4 days using a collimator specifically designed for the irradiator. Tumor volume was measured by caliper measurements ($V = L \times W^2/2$).

Statistical Analysis

Pairwise comparisons or comparisons to the combination were performed based on the analysis of heterogeneous variance model because of unequal variances among the treatment groups. Tukey or Dunnett's adjustment methods were used to account for the multiple comparisons. All tests of statistical significance were two-sided. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of ABT-737 and Radiation on MCF-7 and ZR-75-1 Cell Survival

To evaluate the effect of ABT-737 combined with radiation on cell viability, MCF-7 cells were treated with 8 Gy of radiation and 5 μ M ABT-737. The density of cells with combination treatment was much less than cells treated with radiation or ABT-737 alone (Fig. 1A). Viable cells were counted by trypan blue exclusion (Fig. 1B). Furthermore, clonogenic assays were performed to measure the sensitivity to radiation and ABT-737 in MCF-7 and ZR-75-1 cells. The number of surviving clones in the combination treatment

group was significantly less than the group treated with radiation or ABT-737 alone. In the MCF-7 cells, the number of surviving clones in the combination treatment group reduced to 11.2%, compared to the group treated with either ABT-737 alone [(39.7%) $P < 0.01$] or radiation alone [(65.2%) $P < 0.01$] (Fig. 1C). In the ZR-75-1 cells, for the combination treatment group receiving 2.5 μ M ABT-737 and 4 Gy irradiation, the surviving clones decreased to 11.76%, compared to the group treated with either ABT-737 alone [(78.15%) $P < 0.05$] or radiation alone [(39.5%) $P < 0.05$] (Fig. 1D). The results indicate combination treatment enhanced sensitivity of breast cancer cells.

Effect of ABT-737 and Radiation on Apoptosis

The combination of ABT-737 and radiation resulted in more breast cancer cell death according to the results shown above. Apoptotic death was determined by detecting cleaved PARP and staining of FITC Annexin V. Nuclear poly (ADP-ribose) polymerase (PARP), is involved in DNA repair in response to environmental stress, and is one of the main cleavage targets of caspase-3 *in vivo*. Cleaved PARP, which serves as a marker of cells undergoing apoptosis (19, 20), showed an increase at 4, 24 and 48 h after combination treatment in MCF-7 (Fig. 2A). Specifically, combined ABT-737 and radiation showed an increased cleaved PARP of 2.06-fold greater than ABT-737 alone and 48.54-fold greater than radiation alone at 48 h after treatment in MCF-7 cells. While ABT-737 alone induced Bak, the combined treatments of ABT-737 and radiation increased Bak by 3.3-fold compared to ABT-737 alone in MCF-7 cells (Fig. 2B).

Further, early apoptotic cells were quantified with staining of FITC Annexin V by flow cytometric analysis. The results shown in Fig. 2C illustrate that the combination of ABT-737 and radiation significantly increased the numbers of both the dead cells and early apoptotic cells compared to ABT-737 or radiation alone. The number of early apoptotic cells in MCF-7 breast cancer cells treated with a combination of ABT-737 and radiation increased by 6.13-fold compared to cells treated with ABT-737 alone, and by 3.44-fold compared to cells treated with radiation alone ($P < 0.05$).

Effect of Mcl-1 siRNA, ABT-737 and Radiation on Mcl-1

It has been known that ABT-737 targets the Bcl-2 pro-survival family except for Mcl-1. Since Mcl-1 is involved in the regulation of cancer cell growth, we used Mcl-1 siRNA to knock down the expression of Mcl-1 (Fig. 3A). The results shown in Fig. 3B illustrate that the survival of Mcl-1 siRNA-treated cells was significantly lower than nontargeting control siRNA-treated cells with ABT-737 ($P < 0.05$). Mcl-1 siRNA did increase sensitivity to ABT-737 in MCF-7 cells (Fig. 3B). However, it did not sensitize MCF-7 cells to radiation (Fig. 3C). We detected the Mcl-1 protein levels after ABT-737 treatment in breast cancer cell lines by Western blot assays. Results shown in Fig. 4A illustrate that ABT-737 enhanced the Mcl-1 protein levels in MCF-7 cells at different concentrations (2.5, 5 and 10 μ M). The Mcl-1 protein levels were further detected after the combined treatment of combined ABT-737 and radiation. The results shown Fig. 4B shows that radiation reduced the increase of Mcl-1 protein levels induced by ABT-737 in MCF-7 (40.16%), ZR-75-1 (77.2%) and MDA-MB231 (20.9%) cells. Radiation had a similar effect on Mcl-1 protein levels as Mcl-1

siRNA did. Our results therefore suggest that enhancement of Mcl-1 expression is a side effect of ABT-737 as a pro-survival Bcl-2 family inhibitor, which was blunted by radiation.

Radiation Regulates Degradation of Mcl-1 in MCF-7 Cells

We further investigated how radiation downregulates Mcl-1. MCF-7 cells were treated with the proteasome inhibitor, MG132, before irradiation. Compared with the treatment without the MG132, Mcl-1 protein was at a similar level between ABT-737 alone and the combined treatment of ABT-737 and radiation (Fig. 5). The results showed that MG132 blunts radiation-induced degradation of the Mcl-1 protein and indicates that radiation promoted the degradation of the Mcl-1 protein.

Effect of ABT-737 and Radiation on Tumor Growth In Vivo

To confirm the effect of the combined treatment of ABT-737 and radiation on tumor growth, *in vivo* studies were conducted in xenograft mouse models of MCF-7 breast cancer cells. We found that the growth of tumor xenografts was rapid and uninhibited by ABT-737 alone. Both radiation alone and combined ABT-737 and radiation inhibited tumor growth. Notably, on day 12 the combined treatment of ABT-737 and radiation significantly inhibited the growth of tumors compared to either radiation alone or ABT-737 alone [$P < 0.05$ (Fig. 6)].

DISCUSSION

Although radiation therapy is a promising method for improving the survival rate of breast cancer patients, 10–20% of early stage breast cancer patients with breast-conserving therapy develop recurrence (2, 3) and local-regional relapse even after mastectomy and postmastectomy radiation therapy continues to be a significant clinical issue. Overexpression of Bcl-2 has been observed in a variety of solid tumors (21). Studies have shown that targeting Bcl-2 may be of therapeutic benefit (11, 22, 23). We have previously shown that Bcl-2 overexpression correlated with ipsilateral breast tumor recurrence in early stage breast cancer patients treated with breast-conserving surgery and radiation therapy (18). In addition to Bcl-2, other Bcl-2 pro-survival family members might play important roles in cell survival. Therefore, we hypothesized that the combination of radiation therapy and a Bcl-2 inhibitor might sensitize breast cancer to radiation therapy.

ABT-737 has shown promise in inducing drug sensitivity to multiple chemotherapy drugs *in vitro* and in animal studies (7, 12, 24). ABT-263, which has a similar specificity profile and efficacy as ABT-737, induces complete tumor regressions in xenograft models of small cell lung cancer and acute lymphoblastic leukemia (13). Oral administration of ABT-263 is currently in clinical phase I/II trials in solid tumors (25). Therefore, ABT-737 was selected to combine with radiation in breast cancer cells in this study. We found that the combined treatment of ABT-737 and radiation caused more breast cancer cell death and increased cleaved PARP compared with radiation or ABT-737 alone (Fig. 2A). Bak enhancement also supported the combination treatment activated apoptotic pathway (Fig. 2B). We further demonstrated that the combination of ABT-737 and radiation dramatically increased early apoptotic cells quantified with staining of FITC Annexin V (Fig. 2C). Our data from mouse

xenograft studies also indicated that combination treatment is favored to controlling tumor growth.

ABT-737 does not target Mcl-1, although it is a member of Bcl-2 pro-survival family. Mcl-1 was initially identified in a human myeloblastic leukemia cell line (26). Derenne *et al.* showed that Mcl-1 was an essential survival protein of human myeloma cells (27). Mcl-1 sequesters Bak to inhibit cytochrome *c* release and favor cell survival (28). Elimination of Mcl-1 is required to initiate the apoptotic pathway by releasing Bak for the Mcl-1-Bak complex (29, 30). ABT-737 has been shown to impacted upon the growth of various cancer cell lines and improved animal survival, which caused regression of established tumors in animal models (12). However, Mcl-1 is not a target of ABT-737. Although both Mcl-1 and Bcl-2 function as anti-apoptotic proteins, the distribution of Mcl-1 in human tissues is different with Bcl-2, suggesting that Mcl-1 and Bcl-2 might play different roles and be regulated by different elements in the apoptotic pathway (31). ABT-737 has a low affinity to Mcl-1. B-cell lymphoma cells often acquire resistance to ABT-737 after long-term exposure by upregulation of Mcl-1 (32). Targeting Mcl-1 sensitizes ABT-737 in multiple cancer types (33, 34). It is essential to downregulate Mcl-1 to increase sensitivity when ABT-737 is administrated. The enhancement of Mcl-1 was observed in this study (Fig. 4). We also showed that Mcl-1 siRNA increased sensitivity to ABT-737 in MCF-7 cells (Fig. 3A and B). Furthermore, we found that radiation decreased the Mcl-1 levels induced by ABT-737. It has been reported that ultraviolet (UV) radiation and DNA damage agents cause Mcl-1 elimination, which initiates apoptosis (30). Nijhawan *et al.* reported that UV radiation decreased Mcl-1 levels by blocking protein synthesis in HeLa cells (35). Our results showed that radiation blunted the enhancement Mcl-1 induced by ABT-737. Furthermore, MG132 blocked the decrease of Mcl-1 mediated by radiation (Fig. 5), which suggests that radiation regulates Mcl-1 by promoting Mcl-1 protein degradation. However, further investigation is needed to better understand how radiation regulates Mcl-1 degradation.

ABT-737 impacts the binding of Bak and Bcl-2 or Bcl-xl to unleash Bak. However, as shown here, the Mcl-1 upregulated by ABT-737 would bind free Bak to inactivated Bak and decrease apoptosis. Our results show that radiation decreased the enhancement of Mcl-1, and radiation protects activated Bak from binding to Mcl-1 when combination treatment is applied, which promotes ABT-737 to induce cell death. Thus, the combination of ABT-737 and radiation therapy may prove to be a potential novel therapeutic strategy in the treatment of breast cancer.

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FIG. 1.

The combination of radiation and ABT-737 enhanced the sensitivity to ABT-737 or radiation in breast cancer cells. Panel A: MCF-7 cells were plated in 100 mm cell culture dishes and irradiated with 8 Gy in the presence or absence of 5 μ M of ABT-737 in MCF-7 cells. The cells were examined 48 h after irradiation under an inverted microscope. Panel B: MCF-7 cells were plated at 2.5×10^4 per well in 24-well tissue culture plates. Viable cells were counted 48 h after irradiation by trypan blue exclusion using a Vi-CELL cell viability analyzer. Panel C: MCF-7 cells and panel D: ZR-75-1 cells were plated in 100 cell culture dishes and irradiated with 4 Gy in the presence or absence of 2.5 μ M of ABT-737 for 14 days. At the end of incubation, cells were stained with 1% methylene blue in 50% methanol and colonies were counted. The results shown are the representative of three independent experiments.

FIG. 2.

The effect of radiation and ABT-737 on apoptosis in MCF-7 cells. MCF-7 cells were treated with ABT-737 (5 μ M) for 24 h and then irradiated with 8 Gy. The cells were harvested at 4, 24 and 48 h after irradiation. Apoptosis was determined by measuring the apoptotic marker, cleaved PARP (panel A), by Western blot assays. The combination of radiation and ABT-737 increased Bak (panel B) protein levels in MCF-7 cells. Bands were quantified using Scion Image software. Error bars (SD) are calculated from three independent experiments. Panel C: Apoptotic cells stained with Annexin-V detected by flow cytometry after treatment with ABT-737 (5 μ M) and 8 Gy of radiation (48 h). The results shown are the representative of three independent experiments.

FIG. 3.

The effect of Mcl-1 siRNA on cell viability in MCF-7 cells. Panel A: MCF-7 cells were transfected with Mcl-1 siRNA for 48 h. Mcl-1 and α -tubulin were detected by Western blot assays. At 24 h after Mcl-1 siRNA transfection, MCF-7 cells were plated in 100 mm dishes for clonogenic assays. Panel B: Cells were treated with 0.63, 1.25, 2.5 and 5 μM of ABT-737. Panel C: Cells were treated with 2, 4, 6 and 8 Gy of radiation. Fourteen days later, cells were stained with 1% methylene blue in 50% methanol, and colonies were counted. The results shown are the representative of three independent experiments. NT: nontargeting control siRNA. * $P < 0.05$; ** $P < 0.01$.

FIG. 4.

The effect of radiation and ABT-737 on Mcl-1 in MCF-7, MDA-MB 231 and ZR-75-1 cells. Panel A: ABT-737 upregulated Mcl-1 protein levels in MCF-7 cells. MCF-7 cells were collected after treatment with various concentrations of ABT-737 for 24 h. Panel B: MCF-7, MDA-MB 231 and ZR-75-1 cells were treated with ABT-737 (5 μ M) for 24 h. Cells were then irradiated with 8 Gy and harvested at 30 min after exposure for Western blot assays. Bands were quantified using Scion Image software. Error bars (SD) are calculated from three independent experiments.

FIG. 5.

Radiation regulates degradation of Mcl-1 in MCF-7 cells. MCF-7 cells were treated with ABT-737 (5 μ M) for 24 h in the presence or absence of MG132 (4 h). Cells were then irradiated with 8 Gy and harvested at 30 min after exposure for Western blot assays. Bands were quantified using Scion Image software. Error bars (S.D.) are calculated from three independent experiments.

FIG. 6.

Effect of ABT-737 and radiation on a xenograft mouse model. MCF-7 cells were subcutaneously injected in the thigh of ovariectomized female nude mice with a 90 day release 17-estradiol pellet. ABT-737 and/or radiation (3 Gy, 4 days) was administrated once tumors reached an average volume of about 300 mm³. * $P < 0.05$, compared with radiation alone and ABT-737 alone, respectively.