Radiosensitization of solid tumors by Z-VAD, a pan-caspase inhibitor

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Abstract

Despite recent advances in the management of breast and lung cancer, novel treatment strategies are still needed to further improve patient outcome. The targeting of cell death pathways has therefore been proposed to enhance therapeutic ratio in cancer. In this study, we examined the in vitro and in vivo effects of Z-VAD, a broad-spectrum caspase inhibitor, on breast and lung cancer in association with radiation. Using clonogenic assays, we observed that Z-VAD markedly radiosensitized breast and lung cancer cells, with a radiation dose enhancement ratio of 1.31 ($P < 0.003$). For both models, the enhanced tumor cytotoxicity was associated with induction of autophagy. Furthermore, we found that administration of Z-VAD with radiation in both breast and lung cancer xenograft produced a significant tumor growth delay compared with radiation alone and was well tolerated. Interestingly, Z-VAD also had dramatic antiangiogenic effect when combined with radiation both in vitro and in vivo and thus represents an attractive anticancer therapeutic strategy. In conclusion, this preclinical study supports the therapeutic potential of Z-VAD as a radiosensitizer in breast and lung cancer. This study also suggests caspase inhibition as a promising strategy to enhance the therapeutic ratio of radiation therapy in solid tumors. Therefore, clinical trials are needed to determine the potential of this combination therapy in cancer patients. [Mol Cancer Ther 2009;8(5):1270–9]

Introduction

Solid tumors present formidable challenge in the multidisciplinary management of cancer patients. Among solid tumors, breast and lung cancers account for an estimated 399,470 new cases in the United States in 2008 and, despite multimodality therapy, remain the leaders in cancer-related mortality (1). Because tumor resistance limits effectiveness of current treatments, such as radiotherapy (RT), the identification of novel therapeutic strategies is critical for improving outcome. Manipulation of radiation-induced cell death is one such strategy (2) and has been proposed to enhance therapeutic ratio in cancer. Of the different cell death processes involved in RT, apoptosis has been the most well studied (3). Apoptosis molecularly represents a cascade of caspase proteases released from the mitochondria and is characterized by condensation, chromatin margination, early nuclear condensation, and nucleosomal ladder formation. In the apoptosis pathway, caspases are the mediators of cellular destruction and can schematically be divided in two groups: “initiator” caspases (including caspase-2, caspase-8, caspase-9, and caspase-10) and effectors or executors (caspase-3, caspase-6, and caspase-7; ref. 4). However, radiation-induced apoptosis accounts for a minor portion of cell death in irradiated solid tumors (5). Consistent with these observations, we have previously shown that inhibition of apoptosis via knockdown of Bak and Bax results in an increase in breast and lung cancer radiosensitivity in vitro through up-regulation of autophagy, an alternate type of programmed cell death (6). Autophagy is an evolutionarily conserved process and a survival mechanism whose primary function is to degrade long-lived proteins and recycle cellular components (7). Under excessive stress conditions, autophagy can also lead to cell death by complete self-digestion, which is characterized by the formation of cytoplasmic double-membrane vacuoles (called autophagosomes) that fuse with lysosomes (8). The induction of cancer cell death by several agents underlines the potential of autophagy as a novel cancer treatment target (6, 9). In this study, we examine the radiosensitizing effects of Z-VAD, a broad-spectrum irreversible caspase inhibitor (10), on MDA-MB-231 breast and H460 lung cancer cells in vitro and in both breast and lung cancer xenograft models.

Materials and Methods

Cell Culture and Chemical

MDA-MB-231 cells were cultured in DMEM (Invitrogen) and H460 cells were cultured in RPMI 1640 (Invitrogen), both supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and humidified 5% CO2. Z-VAD-fmk was purchased from Axxora. Human umbilical vein endothelial cells (HUVEC) were obtained.
from Clonetics and maintained in EBM-2 medium supplemented with EGM-2 MV single aliquots (BioWhittaker).

**Clonogenic Assay**

MDA-MB-231 breast and H460 lung cancer cells were treated with DMSO, Z-VAD (50 μmol/L, for 24 h), small interfering RNAs (siRNA) against caspase-3 and caspase-7, or siRNA control. 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). After irradiation, cells were incubated at 37°C for 8 to 10 d. Cells were fixed for 15 min with 3:1 methanol/acetic acid and stained for 15 min 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) x (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 Z-VAD (normalized for Z-VAD toxicity) necessary for a surviving fraction of 0.25. Experiments were conducted in triplicate and mean, SD, and P values (using a Student's t test) were calculated. Error bars were calculated as ±SE by pooling of the results of three independent experiments.

**Immunoblotting**

Following treatment with 50 μmol/L Z-VAD or not, MDA-MB-231 breast and H460 lung cancer cells (0.5 x 10^6) were treated with various doses of radiation and collected at various time points. The cells were harvested and then washed with ice-cold PBS twice before the addition of lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 20 mmol/L EDTA, 1% NP40, 50 mmol/L NaF, 1 mmol/L Na2VO4, 1 mmol/L NaMO4, and cocktail inhibitor (5 μL/mL; Sigma)]. Protein concentration was quantified by the Bio-Rad method. Equal amounts of protein were loaded into each well and separated by 10% SDS-PAGE gel followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in PBS-Tween 20 for 1 h at room temperature. The blots were then incubated overnight at 4°C with the ATG5-ATG12 (a gift from Dr. Norboru Mizushima, Tokyo Medical and Dental University, Tokyo, Japan), Beclin-1 (Santa Cruz Biotechnology), or actin (Sigma) antibodies. After washing with PBS, membranes were incubated with goat anti-rabbit IgG secondary (1:1,000; Santa Cruz Biotechnology) antibody for 45 min at room temperature. Immunoblots were developed by using the enhanced chemiluminescence detection system (Amersham) and autoradiography.

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**Autophagy Assay**

MDA-MB-231 and H460 cancer cells were transfected with green fluorescent protein–tagged light chain 3 (GFP-LC3) plasmid (gift from Dr. Norboru Mizushima; ref. 11) using a mixture of Lipofectamine reagent (Invitrogen Life Technologies). After 24 h, cells were treated with or without 50 μmol/L Z-VAD and sham or 5 Gy irradiation. After 24 and 48 h, GFP-LC3 fluorescence was observed using confocal fluorescence microscope. Characteristic punctate GFP-LC3 signal was considered a cell undergoing autophagy. The percentage of punctate GFP-LC3 cells per total GFP-transfected cells was calculated and experiments were conducted thrice.

**siRNA Transfection**

siRNAs against mouse caspase-3 and caspase-7, Beclin, and control siRNA were purchased from Santa Cruz Biotechnology. siRNA ATG5 (mouse) was synthesized by Dharmacon Research. The sense and antisense strands of ATG5 were begun at nucleotide 5'-AACUUGCUUUACUCUCUCUAUU-3' (sense) and 3'-UUUUGACGAAAU-GAGAGAUAGU-5' (antisense). Cells were transfected with 25 nmol/L siRNAs using Lipofectamine 2000. The transfected cells were used for experiments 24 h later.

**Endothelial Cell Morphogenesis Assay: Tubule Formation**

HUVECs were used to examine tubule formation for angiogenic function in vitro. HUVECs grown to ~70% confluency were treated with 50 μmol/L Z-VAD, 5 Gy, or combination therapy. Cells were then trypsinized and counted. They were seeded at 48,000 per well on 24-well plates coated with 300 μL Matrigel (BD Biosciences). These cells undergo differentiation into capillary-like tube structures and were periodically observed by microscope. One day later, cells were stained with H&E and photographs were taken via microscope. The average number of tubules was calculated from examination of three separate microscopic fields (×100) and representative photographs were taken.

**Tumor Volume Assessment**

Human MDA-MB-231 and H460 cells were used to generate a breast and lung xenograft model, respectively, in female athymic nude mice [nu/nu, 5–6 wk old (Harlan Sprague Dawley, Inc.)]. A suspension of 2 x 10^6 cells in 50 μL volume was injected s.c. into the left posterior flank of mice using a 1-cm³ syringe with 27.5-gauge needle. Tumors were grown for 6 to 8 d until average tumor volume reached 0.28 cm³. Treatment groups consisted of vehicle control (in DMSO), Z-VAD, vehicle plus radiation, and Z-VAD plus radiation. Each treatment group contained five mice. Z-VAD was given daily by i.p. injection at doses of 2 mg/kg for 7 consecutive days. DMSO was given by daily i.p. injection as a vehicle control. In the case of combination treatment, drug or vehicle was given for 2 d before the first dose of irradiation. Mice in radiation groups were irradiated 1 h after drug or vehicle treatment with daily 2 Gy fractions given over 5 consecutive days. Tumors on the flanks of the mice were irradiated using an X-ray irradiator (Therapax, Agfa NDT, Inc.). The non–tumor-bearing parts of the mice were shielded by lead blocks. Tumors were measured two to three times weekly in three perpendicular dimensions using a Vernier caliper and volume was calculated using the modified ellipse volume formula [volume = (height x width x depth) / 2]. Growth delay was calculated for treatment groups relative to control tumors.
Histologic Sections, von Willebrand Factor, Ki67, Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling, P62, and High Mobility Group Box 1 Staining

Mice were implanted with MDA-MB-231 or H460 cells and treated as described above in the tumor volume studies. After 7 d of daily treatments, tumors from each mice group were resected and paraffin fixed. Slides from each treatment group were then stained for von Willebrand factor (vWF) using anti-vWF polyclonal antibody (Chemicon). Blood vessels were quantified by randomly selecting three separate ×400 fields and counting the number of blood vessels per field. This was done in triplicate and the average of the three counts was calculated. Ki67 (cell proliferation), terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL), P62 (autophagy assay), and necrosis assay high mobility group box 1 (HMGB1) staining were done in the Vanderbilt University Pathology Core laboratory using standard protocols. The TUNEL fluorescence method detects and quantifies apoptosis at the cellular level by labeling free 3′-OH terminals that result from cleavage of genomic DNA during apoptosis (12). P62, a scaffold protein binding to LC3, is a preferred target for autophagy, which controls its intracellular level, and a marker for autophagy (13). HMGB1 is a small acidic chromatin binding protein that is passively released from necrotic cells and a specific marker for necrosis (14). Number of positive cells per field was scored and graphed by averaging three repeated assessments. For each type of staining, representative photographs were taken.

Statistical Analysis

Analysis of study results focused on testing the differences of the mean tumor volume among treatment groups and different time points. The data analysis was completed using the restricted/residual maximum likelihood-based mixed-effect model to adjust the intracorrelation effect for the mice that had multiple measurements. The model reported in the article was selected based on the Schwarz’s Bayesian criterion. All tests of significance were two sided, and differences were considered statistically significant when $P < 0.05$. A statistical package, Statistical Analysis System v8.2, was used for all analyses.

Results

Z-VAD Radiosensitizes Both MDA-MB-231 and H460 Cancer Cells

To investigate whether inhibition of apoptosis would result in radiosensitization of MDA-MB-231 breast or H460 lung cancer cells, we used a clonogenic assay to examine the effects of Z-VAD, a pan-caspase inhibitor. In addition,
we also used siRNAs against caspase-3 and caspase-7 to study more specifically the effect of Z-VAD on radiation sensitivity. Both MDA-MB-231 and H460 cells were treated with 50 μmol/L Z-VAD, DMSO control, siRNAs against caspase-3/caspase-7, or siRNA control and were then irradiated with 0 to 6 Gy. We used a Z-VAD concentration of 50 μmol/L because this concentration has been used previously in many studies investigating apoptosis. Surviving colonies were counted 8 days later and graphed as survival curves (Fig. 1). After Z-VAD treatment, enhanced radiosensitivity was shown in both breast (Fig. 1A) and lung (Fig. 1B) cancer cells, with a DER of 1.31 (P < 0.003). As is commonly seen with DMSO treatment, which is known to possess some toxicity, there was a small reduction only in surviving fraction of DMSO-treated cells at high doses of radiation. Figure 1 also shows that caspase-3/caspase-7 siRNAs sensitize MDA-MB-231 (DER = 1.23; P < 0.004) and H460 (DER = 1.31; P < 0.003) cancer cells to ionizing radiation. These cells are unable to undergo apoptosis because caspase-3 and caspase-7, which execute the apoptotic process, are blocked. These results suggest that inhibition of caspases by their siRNAs or Z-VAD can significantly sensitize both MDA-MB-231 breast and H460 lung cancer cells to ionizing radiation.

**Z-VAD Induces Autophagy in Irradiated MDA-MB-231 and H460 Cancer Cells**

Our previous published data suggested that by inhibiting apoptosis, cells can be radiosensitized through alternative cell death pathways, such as autophagy (6, 15). To further investigate this phenomenon in breast and lung cancer, we examined the effects of blocking caspase-dependent apoptosis with Z-VAD on autophagosome formation. Microtubule-associated protein-1 LC3 is an important constituent of mammalian autophagosomes, and GFP-LC3 is an effective marker of their presence (11, 16). To determine whether autophagic cells were increased in cancer cells treated with Z-VAD and RT, GFP-LC3 plasmid was transfected into cancer cells before treatment with Z-VAD and RT (5 Gy). After MDA-MB-231 or H460 cells were exposed to the combined treatment, cells with characteristic punctate GFP-LC3 pattern were observed, suggesting the presence of autophagic cells (11). Quantitative analysis of this effect revealed only a slight increase in the punctate fluorescence pattern following radiation alone (∼10%) or Z-VAD alone (∼13–17%) at 24 hours (Fig. 2A). However, when radiation and Z-VAD treatments were combined, there was a greater than additive effect with a major increase in the number of cells with the punctate GFP-LC3 pattern (P < 0.0001) for both models. These results suggest a significant increase in autophagosome formation after combined Z-VAD/radiation treatment in MDA-MB-231 and H460 cancer cells. These results were also confirmed by assessing the level of Beclin-1, a component of a class III phosphatidylinositol 3-kinase complex, and ATG-5/ATG-12 complex, which are essential autophagy proteins (17, 18). As shown in Fig. 2B, treatment with irradiation or Z-VAD alone only resulted in a moderate increase in expression of autophagic proteins. The greatest induction, however, was seen following combination treatment, particularly at the 24-hour time point. These results suggest that cancer cell treatment with radiation/Z-VAD is associated with up-regulation of essential autophagic proteins.

**Combination Treatment of Z-VAD and Radiation Results in Extended Tumor Growth Delay and Is Well Tolerated in Solid Tumor Xenograft Models**

Having established the *in vitro* effect of Z-VAD on solid tumor radiosensitivity, mouse heterotopic xenograft models were produced to explore the radiation response by Z-VAD *in vivo*. The treatment groups consisted of a vehicle control, Z-VAD (2 mg/kg, i.p.), vehicle plus radiation, and combination Z-VAD plus radiation. MDA-MB-231 and H460 tumor xenografts in mice were treated as described in Materials and Methods.

![Graph](Image.png)

**Figure 2.** Induction of autophagosome formation and essential autophagic proteins by treatment with Z-VAD/radiation. GFP-LC3-transfected cells were treated with 5 Gy, 50 μmol/L Z-VAD, or both and then examined by fluorescence microscopy after 24 h. **A,** quantitative measurement of positive MDA-MB-231 and H460 cells, showing increased autophagosome formation in response to Z-VAD/radiation. The percentage of cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-positive cells. **Columns,** mean; **bars,** SD. **B,** MDA-MB-231 and H460 cells were treated with 5 Gy, 50 μmol/L Z-VAD, or a combination of both. Cells were collected at noted time points and protein extracts were made for Western immunoblotting. Shown are immunoblots of ATG5/ATG12 complex and Beclin-1 using lysates from the MDA-MB-231 and H460 cancer cells. Actin immunoblot was used for normalization.
Growth delay was calculated as the number of days required to reach a tumor volume of 2 cm\(^3\) for treatment groups relative to control tumors (Fig. 3A and B). In the breast xenograft model, a significant tumor growth delay was seen with combination therapy of Z-VAD and radiation compared with irradiation alone (22 versus 16 days; \(P < 0.005\)), and Z-VAD alone did also significantly affect the tumor growth compared with control (~3-day delay; \(P = 0.003\)). Similarly, combination therapy of Z-VAD/radiation resulted in a significant tumor growth delay in the lung xenograft model (9 versus 6 days; \(P = 0.008\)) compared with irradiation alone. These results suggest that Z-VAD can increase solid tumor response in combination with RT. In addition, mouse body weights were also monitored to assess whether treatment with Z-VAD, radiation, or combination treatment yielded systemic toxicity (Supplementary Fig. S1A and B). As expected, the combination treatment group, which had the most prolonged tumor growth delay, had the smallest increase in body weight. Only minimal weight loss was seen at 10 days following the combined Z-VAD and irradiation treatment, suggesting that the combined treatment was very well tolerated.
Z-VAD Reduces Tumor Proliferation Index Despite Marked Decrease in Apoptosis in Irradiated MDA-MB-231 and H460 Mouse Xenografts

To further characterize the effect of Z-VAD shown in the tumor growth delay models, we examined fixed MDA-MB-231 and H460 tumor sections in all treatment groups for proliferation (Ki67 staining) and apoptosis (TUNEL staining). The four treatment groups were identical to those used for the tumor growth delay study. As shown in Fig. 4A, Ki67 staining revealed lower breast (35 versus 59; \( P < 0.001 \)) and lung (16 versus 44; \( P < 0.0001 \)) cellular proliferation in the combination treatment group compared with the irradiation alone group. Apoptosis levels in fixed MDA-MB-231 and H460 tumor sections were assessed using TUNEL staining. As shown in Fig. 4B, combined Z-VAD and radiation treatment resulted in \( \sim 80\% \) reduction in apoptosis compared with radiation alone in both breast (\( P = 0.002 \)) and lung (\( P < 0.001 \)) tumor xenografts. These results suggest that Z-VAD can reduce radiation-induced apoptosis and further reduce tumor cell proliferation after irradiation.

Z-VAD Induces Both Autophagy and Necrosis in Irradiated MDA-MB-231 and H460 Mouse Xenografts

To explore the mechanisms of cell death resulting from Z-VAD in vivo, we examined fixed MDA-MB-231 and H460 tumor sections in all treatment groups for autophagy (P62 staining) and necrosis (HMGB1 staining). The four treatment groups were identical to those used for the tumor growth delay study. P62 interacts and binds to LC3 and is removed in lysosomes by autophagy, which controls its turnover. Representative histologic photographs of P62 staining on breast and lung tumor sections are shown in Fig. 5C and Supplementary Fig. S2A, respectively. As shown in Fig. 5A, P62 staining revealed lower breast (17.3 versus 55; \( P < 0.001 \)) and lung (19.7 versus 55.3; \( P < 0.001 \)) positive cells in the combination treatment group compared with the irradiation alone group. Necrosis levels in fixed MDA-MB-231 and H460 tumor sections were assessed using HMGB1 staining. HMGB1 is a nuclear protein that binds tightly to chromatin in apoptotic cells, whereas cytoplasmic and extranuclear staining of HMGB1 is found in the tumor tissue treated with Z-VAD, radiation, or both. As shown in Fig. 5B, combined Z-VAD and radiation treatment resulted in \( \sim 70\% \) and \( \sim 73\% \) increase in cytoplasmic HMGB1-positive cells compared with radiation alone in breast (\( P = 0.002 \)) and lung (\( P = 0.0005 \)) tumor xenografts, respectively. Taken together, these results suggest that Z-VAD induces autophagy and necrosis after irradiation in vivo.

Z-VAD Reduces Vascular Density in Irradiated Solid Tumor Models and Sensitizes HUVECs to Radiation

To determine the effect of Z-VAD on tumor vasculature, vascular density study was done using vWF staining in each breast and lung cancer xenograft treatment groups. The number of vessels per microscopic field was then quantified for each treatment group. As shown in Fig. 6A, combination therapy of Z-VAD and radiation in both xenograft models resulted in a dramatic 5-fold reduction (\( P < 0.0001 \)) in the average number of vessels per microscopic field in comparison with control and a \( \sim 2\)-fold reduction (\( P = 0.006 \)) relative to RT alone. To further investigate the effects of Z-VAD and radiation on blood vessel formation, an endothelial cell morphogenesis assay was done to examine the ability of treated HUVECs to produce capillary-like tubular structures. The mean number of counted tubules in three separate (×100) fields and a representative image are shown in Fig. 6B and C, respectively. Treatment with

Figure 4. Z-VAD reduces cell proliferation and apoptosis in irradiated MDA-MB-231 and H460 cancer models. Histologic sections were obtained from the tumors of the mice in each treatment group from the tumor volume study. Number of positive cells was scored and graphed by averaging three repeated assessments. A, average Ki67 proliferative index of each treatment group was determined by counting positive cells per microscopic field in breast and lung tumor sections. This was repeated thrice. Columns, mean; bars, SD. B, TUNEL staining was also done on breast and lung tumor sections, and apoptotic index was similarly calculated by counting positive TUNEL-stained cells per microscopic field. Columns, mean; bars, SD.
Z-VAD combined to radiation significantly decreased tubule formation compared with radiation alone (6.0 versus 23.33; \( P < 0.0001 \)). No treatment control had 47.66 tubules (SD, 0.57) per microscopic field and Z-VAD alone had 18 tubules (SD, 1.0), suggesting an antiangiogenic effect in addition to the radiosensitization effect of Z-VAD.

**Discussion**

In the present report, we study the effects of Z-VAD, a pan-caspase inhibitor, which resulted in the effective radiosensitization of breast and lung cancer cells *in vitro*. The potential therapeutic effects of caspase inhibition by Z-VAD were then shown in both breast and lung cancer xenograft models. This study also suggests that Z-VAD increases radiation effects on vasculature and thus may contribute to the extended tumor growth delay. Consistent with previous study, *in vitro* experiments also showed that Z-VAD induces autophagy, which may play a dual role, either protection or mediation of cell death. Importantly, this report suggests that this novel strategy has great potential for enhancing the treatment of solid tumors with RT.

**Figure 5.** Z-VAD induces autophagy and necrosis in irradiated MDA-MB-231 and H460 cancer *in vivo* models. Histologic sections were obtained from the tumors of the mice in each treatment group from the tumor volume study. Number of positive cells was scored and graphed by averaging three repeated assessments. A, average P62 index of each treatment group was determined by counting positive cells per microscopic field in breast and lung tumor sections. This was repeated thrice. *Columns*, mean; *bars*, SD. B, HMGB1 staining was also done on breast and lung tumor sections, and quantification was similarly done by counting positive cytoplasmic HMGB1-stained cells per microscopic field. *Columns*, mean; *bars*, SD. C, representative histologic photographs of MDA-MB-231 tumor sections following P62 staining. D, representative histologic photographs of MDA-MB-231 tumor sections following HMGB1 staining.

Z-VAD sensitizes solid cancer to radiation.
It has been shown that Z-VAD is a competitive, irreversible, and broad specificity inhibitor of all 10 caspases, except for caspase-2, which is only weakly inhibited (19). We showed that the administration of Z-VAD in combination with ionizing radiation decreased dramatically the survival of MDA-MB-231 breast and H460 lung cancer cells (Fig. 1). Moreover, siRNAs against caspase-3/caspase-7 sensitized lung cancer cells to ionizing radiation, suggesting that Z-VAD acts by inhibition of caspase-3/caspase-7. Interestingly, siRNAs against caspase-3/caspase-7 also sensitized breast cancer cells to ionizing radiation, although to a lower extent compared with Z-VAD, possibly because the latter can inhibit caspase-3 and caspase-7 and also other main caspases that are involved in apoptosis. These results suggest that Z-VAD has the potential to achieve a high degree of radiosensitization in breast and lung cancer cells in vivo.

In our breast and lung xenograft models, Z-VAD resulted in extended tumor growth delay when administered with radiation. Not surprisingly, Z-VAD decreased radiation-induced apoptosis as determined by the TUNEL staining in vivo (Fig. 4B). Indeed, Z-VAD blocks caspase protease activity required for apoptosis. More interestingly, Z-VAD was also able to reduce tumor cell proliferation (Fig. 4A). These results suggest that an alternative death pathway, other than apoptosis, might be involved in response to the combined treatment Z-VAD/radiation. Autophagy is such a death mechanism, as previously shown in irradiated cancer cells (9). We have previously shown that inhibition of apoptosis with the pan-caspase inhibitor Z-VAD or siRNA knockout of Bax/Bak up-regulates autophagy in radiosensitized breast and prostate cancer cell lines (15). Consistent with our previous studies targeting upstream apoptotic regulators (15), we observed that the combination of Z-VAD and irradiation was associated with an increase in radiation-induced autophagy in both breast and lung cancer cells (Fig. 2). Indeed, ionizing radiation has previously been shown to induce autophagy in cancers of the breast (15), lung (6), prostate (15), and malignant glioma (20, 21). In addition, autophagy was shown to enhance radiation cytotoxicity in cancer cells (22) and thus represents an attractive target for cancer therapy. Consistent with these data, it has been reported that EB 1089, a vitamin D3 analogue, was able to enhance radiation sensitivity of breast cancer cells via induction of autophagy (23). Recent studies showed the induction of autophagic cell death after exposure to Z-VAD or specific caspase-8 knockdown in L929 fibrosarcoma cells (24), suggesting that the absence of caspase activity can induce autophagic cell death. In light of these data, autophagy may explain the enhancement of radiation response in our xenograft models.

However, the role of autophagy is more complex (25). It has been implicated in cell survival by self-degradation of proteins in stress-induced conditions such as amino acid deprivation. In our study, the application of Z-VAD and irradiation resulted in a transient burst of autophagic protein expression at 24 hours (Fig. 2B). The noted overexpression of autophagic proteins may possibly represent an acute survival mechanism adopted by breast tumors subjected to stress conditions (26). In the lung model, however, the overexpression of autophagic proteins is more sustained at both 24 and 48 hours (Fig. 2B). Nevertheless, an increasing body of evidence suggests that the role of autophagy may depend on cells and circumstances, such as the type and quantity of stress exposure. Thus, in an ionizing radiation model, excessive autophagy may function as an alternate death pathway for apoptosis-inhibited cancer cells (6, 21). Based on the interconnection between autophagy and apoptosis (27), these mechanisms may serve as “backup” for each other, becoming activated when one pathway or the other is suppressed. It should also be noted that irreversibly damaged cells on
Z-VAD have alternative route to death besides autophagy, such as necrosis (28). Recent studies in different models showed that autophagy may have a role in determining cell fate in response to stress in apoptosis-deficient cells (29) toward necrosis and contributing to cellular destruction during necrosis in a yeast model (30). Here, we focused our study on the in vivo biological efficacy and effects of Z-VAD mainly to take into account the complex microenvironment of tumors. Interestingly, we found that the combined treatment radiation plus Z-VAD induces not only autophagy but also necrosis in vivo as shown by the HMGB1 staining on breast and lung tumor sections (Fig. 5B). Our findings are consistent with the previously published data showing that autophagy can promote cell death in apoptosis-deficient murine embryonic fibroblast cells (29). Of note, this is the first in vivo report showing that Z-VAD promotes both autophagy and necrosis in irradiated breast and lung cancer models.

Our results also support the potential effects of Z-VAD on the tumor microenvironment. As we know, RT induces the production of proangiogenic factors such as the vascular endothelial growth factor, fibroblast growth factor, and platelet-derived growth factor (31–34), which results in an increase in radioreistance and thus attenuates vasculature damage by radiation. This survival mechanism potentially limits the effectiveness of ionizing radiation for cancer therapy. It is also well known that excessive angiogenesis is a decisive step for tumorigenesis and cancer progression (35). In addition, microvessel density was shown to be a prognostic factor predictive of neoplastic transformation (36–39) and poor survival (40, 41). As illustrated by the vWF staining in Fig. 4, the combination Z-VAD/radiation further reduced the vessel density compared with radiation alone (P = 0.006). To verify the vascular effects of Z-VAD observed in vivo, we studied the ability of treated endothelial cells to produce capillary-like tubular structures in vitro (Fig. 4C). Our results are consistent with a report suggesting that apoptosis has an important role in angiogenesis by allowing the formation and remodeling of a new functional blood vessel network (42). More recently, it has also been shown that glioblastoma cells exhibit a basal constitutive caspase activity promoting migratory and invasive activities (43) and that caspase-8 can regulate cell adhesion and motility (44), which are characteristics needed for angiogenesis. Therefore, we speculated that Z-VAD may possibly exert its radiosensitizing activity in solid tumors at least partially via antiangiogenic effects by either inhibiting the caspase function in invasiveness and motility or disrupting the ability to form functional vascular network. Of note, the results observed in the in vivo experiment may be limited by the relative short half-life of Z-VAD. It is thus possible that higher or more frequent drug dosing may result in bigger differences between treatment groups. Nevertheless, this study strongly shows the enhancement of RT with the concurrent administration of Z-VAD and suggests an even more interesting trend for caspase inhibition.

In conclusion, this preclinical study supports the therapeutic potential of Z-VAD as a radiosensitizer in breast and lung cancer. We also showed the avascular effects of Z-VAD (in vivo and in vitro), which thus represents an attractive anticancer therapeutic strategy in combination with RT. This report suggests pan-caspase inhibition as a novel concept for the enhancement of RT in breast and lung cancer. Further clinical investigation using this proof of principle is warranted in combination with RT for breast and lung cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Molecular Cancer Therapeutics

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Luigi Moretti, Kwang Woon Kim, Dae Kwang Jung, et al.

Mol Cancer Ther Published OnlineFirst May 5, 2009.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-08-0893

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