Radiosensitization of Head and Neck Squamous Cell Carcinoma by a SMAC-Mimetic Compound, SM-164, Requires Activation of Caspases

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Abstract

Chemoradiation is the treatment of choice for locally advanced head and neck squamous cell carcinoma (HNSCC). However, radioresistance, which contributes to local recurrence, remains a significant therapeutic problem. In this study, we characterized SM-164, a small second mitochondria-derived activator of caspase (SMAC)-mimetic compound that promotes degradation of cellular inhibitor of apoptosis-1 (cIAP-1), also known as baculoviral IAP repeat–containing protein 2 (BIRC2) and releases active caspases from the X-linked inhibitor of apoptosis inhibitory binding as a radiosensitizing agent in HNSCC cells. We found that SM-164 at nanomolar concentrations induced radiosensitization in some HNSCC cell lines in a manner dependent on intrinsic sensitivity to caspase activation and apoptosis induction. Blockage of caspase activation via short interfering RNA knockdown or a pan-caspase inhibitor, z-VAD-fmk, largely abrogated SM-164 radiosensitization. On the other hand, the resistant lines with a high level of Bcl-2 that blocks caspase activation and apoptosis induction became sensitive to radiation on Bcl-2 knockdown. Mechanistic studies revealed that SM-164 radiosensitization in sensitive cells was associated with NF-kB activation and TNFα secretion, followed by activation of caspase-8 and -9, leading to enhanced apoptosis. Finally, SM-164 also radiosensitized human tumor xenograft while causing minimal toxicity. Thus, SM-164 is a potent radiosensitizer via a mechanism involving caspase activation and holds promise for future clinical development as a novel class of radiosensitizer for the treatment of a subset of head and neck cancer patients. Mol Cancer Ther; 10(4); 658–69. ©2011 AACR.

Introduction

Radiotherapy is the treatment of choice for locally advanced head and neck squamous cell carcinomas (HNSCC); but, intrinsic tumor radioresistance contributes to the poor, 5-year relapse-free survival (1). Both laboratory studies and clinical investigations have suggested that multiple factors contribute to radioresistance of HNSCC. A major factor is abnormal activation of epidermal growth factor receptor (EGFR) signaling pathways (2). Other contributing factors include B-cell leukemia/lymphoma expression (Bcl-2) and TP53 mutation (3), expression of survivin and Bcl-xL (4), disruption of the FAS-mediated apoptotic pathway (5), and NF-kB activation (6). A potential role of cellular inhibitor of apoptosis-1 (cIAP-1) in HNSCC carcinogenesis and radioresistance was implicated by the amplification at the chromosome 11q22 region in some HNSCC tumors, where the cIAP-1/BIRC2 gene resides (7–9), and by the association of lymph node metastasis and poor survival of patients with cIAP-1 nuclear expression (10).

cIAP-1, cIAP-2 (also known as baculoviral IAP repeat–containing protein 3, BIRC3), and X-linked inhibitor of apoptosis (XIAP) are 3 well-known family members of inhibitor of apoptosis (IAP; ref. 11). Although the main function of XIAP is to suppress apoptosis via binding to and thus inhibiting active caspase-3/7 and -9 (12), both cIAP-1 and cIAP-2 are implicated in NF-kB activation (13) and suppression of caspase-8 activation during TNFα signaling (14). On binding to TNFR1 and TNFR2, TNFα can signal both cell survival and cell death (15) through 2 separate protein complexes (16).
prosurvival complex (complex I) contains TNFα/TNFR1, TNFR1-associated death domain protein (TRADD), TNF receptor–associated factor (TRAF) 2, receptor-interacting protein, and cIAP-1/2. This complex recruits and activates the inhibitor of κB kinase, leading to the activation of NF-κB (13, 17). The death-inducing signaling complex (DISC, complex II) is also assembled following internalization of the TNFR1 and consists of TRADD and the receptor-interacting protein, which then recruits the Fas-associated death domain protein and caspase-8 to form DISC (16). Although DISC can be formed, it may be unable to induce cell death as long as there is a prosurvival signal being generated. Thus, disruption of a prosurvival complex would facilitate the activation of DISC to induce cell killing.

Survival function of IAPs is negatively regulated by the second mitochondria-derived activator of caspase (SMAC), a mitochondrial protein that is released to the cytoplasm on induction of apoptosis (18, 19). SMAC binds to XIAP, as well as to cIAP-1 and cIAP-2, via its N-terminal AVPI tetrapeptide binding motif to abrogate their inhibitory binding to both caspase-9 and -3 (7, 12). Small molecule SMAC mimetics have been designed and developed to mimic this AVPI binding motif of SMAC (20). Recently, SMAC mimetics were found to induce rapid autoubiquitination and degradation of cIAP-1, resulting in NF-κB activation and TNFα-dependent apoptosis (21–24). Thus, by eliminating cIAP-1 via SMAC-mimetic–mediated autoubiquitination, prosurvival complex I is inactivated, which facilitates activation of complex II to induce apoptosis (25).

NF-κB is activated by ionizing radiation via induced degradation of IkB (26, 27). Activated NF-κB, on one hand, induces an adaptive resistance to ionizing radiation (28) as a cellular defensive mechanism and, on the other hand, increases the production of TNFα for apoptosis induction under certain circumstances (16, 29). Radiation is also well known to induce G2 arrest and apoptosis (30).

In this study, we investigated SM-164, a potent and well-characterized SMAC mimetic (31, 32), as a radiosensitizer in HNSCC cells. Our data showed that by eliminating cIAP-1, SM-164 acts as an effective radiosensitizer both in vitro and in vivo, in a subset of HNSCC lines, through NF-κB activation that increases TNFα secretion, followed by activation of caspase-8 and -9 and induction of apoptosis. The present study lays the groundwork for clinical development of SM-164 as a novel class of radiosensitizing agent for the treatment of a subset of head and neck cancers that are sensitive to caspase activation.

Materials and Methods

Cell culture

Human HNSCC lines, including UMSCC-1, -12, -17B, and -74B, were grown in Dulbecco’s Modified Eagle’s Medium with 10% FBS. Human lung fibroblast MRC5 cells were a gift from Dr. A. Rehemtulla and cultured in RPMI 1640 with 10% fetal bovine serum. The cell line authentication is as follows: all 4 HNSCC cell lines were from Dr. T. Carey at the University of Michigan (33) and have been tested and authenticated by genetic profiling with various microsatellite loci, using Profiler Plus PCR Amplification Kit (Applied Biosystems; ref. 34). The identities of the HNSCC cell lines were last tested and confirmed in September (for UMSCC-1, -12, -74B) and November (for UMSCC-17B) of 2010, respectively. SM-164 was synthesized by us as described (31, 32).

Radiation exposure and clonogenic assay

Cells were seeded in 6-well plates and exposed to different doses of radiation (Philips RT250, Kimtron Medical) after 2 hours of pretreatment with SM-164, followed by incubation at 37°C for 7 to 9 days. Survival curves were fitted using the linear quadratic equation and the mean inactivation dose was calculated (35).

Small interfering RNA silencing of caspase-8, -9, and Bcl-2

The small interfering RNA (siRNA) oligonucleotides for silencing caspase-8 (5′-GCCCAAGUUCCAGCAUU-3′), caspase-9 (5′-CGGUGAAAGGGAUUAUAAA-3′), scrambled control siRNA (siCONT: 5′-ATTGTATTCGATCGGACCTT-3′), and SMARTpool siRNA targeting Bcl-2 were from Dharmacon. Cells were transfected with siRNA using Lipofectamine 2000 and split 48 hours later. One portion was used for clonogenic assay and the other portion for immunoblotting (36).

Immunoblotting

The assay was done as described in the work of Bockbrader and colleagues (37) using antibodies against cIAP-1 (a gift from Dr. J. Silke, La Trobe University, Melbourne, Victoria, Australia; ref. 24), caspase-8, Bcl-2, Bcl-xL, Mcl-1 (Santa Cruz), β-actin (Sigma), XIAP and caspase-9 (BD Pharmingen), cIAP-2 (Cell Signaling Technology).

ATPlite growth assay and IC50 determination

Cells were seeded in 96-well plates in triplicates and treated with SM-164 with various doses for 24 hours. Cell viability was measured with ATPlite kit (Perkin Elmer; ref. 37).

Fluorescence-activated cell sorting analysis

Cells were treated with SM-164 or exposed to radiation alone or in combination. Cells were harvested 24 or 48 hours postradiation and analyzed by flow cytometry (37).

Luciferase reporter assay

Cells were seeded into a 96-well plates and transfected with a luciferase reporter driven by NF-κB consensus sequence (pNifty plasmid; Invivogen), along with Renilla construct. Cells were treated 24 hours later with SM-164, 4 Gy, or the combination for 24 hours, followed by...
luciferase activity assay (Promega) with TNFα (10 ng/mL; BD biospheres) treatment as a positive control. The results are presented as the fold activation after normalization with Renilla.

Caspase activation assay
The activity of caspase-8, -9, -2, or -3 was analyzed using a fluorogenic caspase assay with Ac-IETD-AMC (BD Pharmingen), Ac-LEHD-AMC or Z-VDVAD-AMC or Ac-DEVD-AMC (Calbiochem) as a substrate, respectively (37). The results are expressed as fold change compared with the control.

Reverse transcriptase-PCR
Total RNA was isolated using TRIzol (Invitrogen), reverse transcribed using Superscript II (GibcoBRL) with oligod(T) from Applied Biosystems. PCR was performed with the primer sets spanning at least 2 intron–exon junctions for TNFα and TNF-related apoptosis-inducing ligand (TRAIL; ref. 38).

TNFα ELISA
Cells were plated onto 6-well plates and irradiated with 4 Gy, treated with SM-164 (100 nmol/L), or the combination. The conditioned medium was removed at 24, 48, and 72 hours posttreatment for ELISA assay using a TNFα ELISA kit (Cayman Chemicals Co.).

In vivo antitumor study
All animal studies were conducted in accordance with the guidelines established by the University Committee on Use and Care of Animals. Five million UMSCC-1 cells were inoculated s.c. in both flanks of nude mice. The mice were randomized and the treatment started when the tumor size reached 70 mm³ at 18 days after inoculation.

Results

Sensitivity of HNSCC lines to radiation or SM-164 as a single agent
We first determined radiosensitivity among 4 HNSCC lines by classic clonogenic assay after exposure to various doses of radiation. As shown in Fig. 1A (left), 2 lines (UMSCC-1 and UMSCC-17B) were relatively sensitive, whereas the other 2 lines (UMSCC-12 and UMSCC-74B) were relatively resistant to radiation. We then determined the sensitivity of these HNSCC lines to SM-164, a SMAC-mimetic small molecule compound (chemical structure shown in Supplementary Fig. S1A). Unlike MDA-MB-231 human breast cancer and SK-OV3 ovarian cancer cell lines, in which SM-164 was highly potent at low nanomolar concentrations (31, 32, 37), all 4 HNSCC lines were highly resistant to SM-164 as a single agent with the IC₅₀ values ranging from 22 to 57 μmol/L, although the 2 radiosensitive lines were relatively more sensitive to SM-164 (Fig. 1A, right).

A subset of HNSCC cell lines is sensitive to SM-164–induced radiosensitization
We next determined the potential radiosensitizing effect of SM-164 using drug concentrations much lower than the IC₅₀ value in HNSCC cells. Cells were treated with SM-164 at 10 or 200 nmol/L in combination with different doses of radiation. An SM-164 dose-dependent radiosensitization was observed in UMSCC-1 cells with an SER (Sensitivity enhancement ratio) of 1.4 or 1.5, respectively. In UMSCC-17B cells, only higher dose caused radiosensitization with an SER of 1.6 (Fig. 1B, 2 left panels). In UMSCC-12 and UMSCC-74B cells, SM-164 at 200 nmol/L showed no or minimal radiosensitization (Fig. 1B, 2 right panels). An inactive analogue of SM-164, SM-173 (31) had no effect on either group of lines at 200 nmol/L (Fig. 1C), indicating a specific effect of SM-164.

We also determined potential toxicity of SM-164 on human lung fibroblast MRC5 cells alone or in combination with radiation and found that MRC5 cells were resistant to SM-164 with an IC₅₀ value of 41 μmol/L and are resistant to SM-164 radiosensitization with an SER of 1.05 (Fig. 1D).

Radiosensitization by SM-164 is attributable to enhanced induction of apoptosis
To determine the nature of SM-164 radiosensitization, we did fluorescence-activated cell sorting (FACS) analysis of all 4 lines treated with SM-164 or radiation, alone or in combination. In sensitive UMSCC-1 cells, exposure to 200 nmol/L SM-164 or irradiation with 6 Gy induced a moderate level of apoptosis (sub-G₁ population). The combination of radiation and SM-164, but not its inactive analogue, SM-173, significantly enhanced radiation-induced apoptosis (Fig. 2A, left; P < 0.05). Importantly, apoptosis induced by combination of SM-164 and radiation was completed blocked by z-VAD, a pan-caspase inhibitor, indicating the involvement of caspase activation. Similar results were seen at 48 hours posttreatment with a higher degree of apoptosis induction (Fig. 2A, left). On the other hand, SM-164 had no effect on radiation-induced G2-M arrest (Fig. 2A, right). Consistently, in another sensitive UMSCC-17B line, SM-164 alone caused a time-dependent induction of apoptosis, which was enhanced by radiation (from 30% to 44% of
the population at 48 hours) although radiation alone had a minimal effect on apoptosis induction (Fig. 2, left). Again, SM-164 had a minimal effect on radiation-induced G2-M arrest (Fig. 2B, right). As expected, SM-164 had no or little radiation-enhancing activity for apoptosis induction and G2-M arrest in 2 resistant lines, UMSCC-12 and UMSCC-74B (Fig. 2C), consistent with the lack of SM-164 radiosensitization. Take together, these results suggest that SM-164–mediated radiosensitization in sensitive lines is associated with enhanced apoptosis.

**SM-164 radiosensitization is independent of cIAP-1 degradation but dependent on caspase activation**

We next addressed why some HNSCC lines are sensitive, whereas others are resistant to SM-164 radiosensitization. Several groups have recently reported

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**Figure 1.** SM-164 sensitizes a subset of HNSCC cell lines to radiation. A, sensitivity of HNSCC lines to radiation and SM-164. Four lines of HNSCC cells were seeded in 6-well plates in triplicate and irradiated. The colonies with more than 50 cells were counted after 9 days. Surviving fraction was calculated as the proportion of seeded cells following irradiation to form colonies relative to that of untreated cells (mean ± SEM, n = 3; left). Cells were seeded in 96-well plate in triplicate and treated the following day with various concentrations of SM-164 for 24 hours. Cells were then lysed for ATPlite assay (mean ± SEM, n = 3; right). B and C, radiosensitization by SM-164 (B) but not its inactive SM-173 (C) in HNSCC cells. Cells were seeded in 6-well plates and irradiated. SM-164 or SM-173 was added to culture media 2 hours prior to radiation. SER was calculated as the ratio of the mean inactivation dose under untreated control conditions divided by the mean inactivation dose after SM-164 treatment (mean ± SEM, n = 3). D, normal fibroblasts are resistant to SM-164. MRC5 cells were treated with SM-164 for 24 hours, followed by ATPlite cell viability assay (left). MRC5 cells were pretreated with SM-164 or its inactive analogue SM-173 for 2 hours prior to radiation exposure, followed by standard clonogenic assay (right). Mean ± SEM (n = 2). Cont, control.
posttreatment, and again, no effect on XIAP was found (Supplementary Fig. S1B). Thus, SM-164 radiosensitization is independent of the levels or degradation of cIAP-1 or XIAP.

Since the mechanism of SM-164 action is to promote cIAP-1 degradation and to disrupt XIAP binding to active caspases (31), we reasoned that removal of negative blockers of caspase activation by SM-164 would be effective only in cells that undergo caspase activation on external stimuli but not in cells which are resistant to caspase activation. We, therefore, determined potential involvement of caspase activation in
SM-164 radiosensitization. Cells were treated with SM-164, radiation alone, or the combination and the activity of caspases 8, 9, and 2 was measured. As shown in Fig. 3B, in sensitive UMSCC-1 cells, SM-164 alone treatment caused a 3-, 2.5-, and 2-fold activation of caspase-9, -8, or -2, respectively, whereas radiation alone had a minimal effect on caspase activation. Combinational treatment caused a significant increase

Figure 3. SM-164 promotes cIAP-1 degradation and caspase activation. A, Dose-dependent degradation of cIAP-1. Cells were treated with SM-164 or SM-173 for 6 hours, followed by immunoblotting. B–D, caspase activation. UMSCC-1 (B), UMSCC-17B (C), and UMSCC-12 and -74B (D) cells were treated with SM-164, radiation or both, and subjected to caspase activity assay for caspase-8, -9, -2, and -3 (for UMSCC-1). Mean ± SEM (n = 3; *, P < 0.05; **, P < 0.01). Cont, control.
in caspase activation with an up to 4-fold increases for caspase-9, -8, and -2, as well as a significant increase in caspase-3 activity. Similar results were observed in another sensitive line of UMSCC-17B cells with combination treatment causing maximal activation of caspases (Fig. 3C). Neither of the 2 resistant lines showed caspase activation after treatment with SM-164 alone or combined with radiation (Fig. 3D).

**Bcl-2 knockdown by siRNA sensitizes resistant HNSCC cells to radiation, whereas blockage of caspase activation largely abrogated SM-164 radiosensitization in sensitive cells**

We further investigated the potential mechanism by which cells were intrinsically either sensitive or resistant to caspase activation by focusing on 3 antiapoptotic Bcl-2 family members. While the Bcl-xL levels were very high in all 4 lines and Mcl-1 levels varied among lines, the Bcl-2 levels were inversely correlated to intrinsic sensitivity to caspase activation with undetectable or very low levels in the 2 sensitive lines but a high level in 2 resistant lines (Fig. 4A). Since Bcl-2 overexpression correlated with radioresistance in primary HNSCC tumor tissues (3), we tested whether Bcl-2 knockdown would sensitize these 2 Bcl-2 highly expressed lines to radiation. Indeed, Bcl-2 knockdown to about 50% of the original level sensitized otherwise resistant UMSCC-12 and UMSCC-74 cells to radiation with an SER of 1.29 and 1.22, respectively (Fig. 4B). Thus, the Bcl-2 levels correlated with radioresistance, whereas SM-164 radiosensitization is determined by intrinsic cellular sensitivity to caspase activation when Bcl-2 level is very low or undetectable.

We then determined whether caspase activation is causally related to SM-164 radiosensitization by blocking
caspases via siRNA knockdown and inhibitor treatment. In UMSCC-1 cells, transfection of siRNAs against caspase-8 and -9 caused a greater than 70% reduction of their levels and consequently decreased SM-164 radiosensitization with an SER reduction from 1.31 to 1.1 (Fig. 4C; \( P < 0.05 \)). SM-164 radiosensitization was also partially blocked by z-VAD-fmk, a pan-caspase inhibitor, with an SER reduction from 1.51 to 1.24 in UMSCC-1 cells (Fig. 3D; \( P < 0.05 \)). Thus, caspase activation plays a major role in SM-164 radiosensitization in sensitive HNSCC cells.

**SM-164 increased radiation-induced NF-κB activation and TNFα secretion in UMSCC-1 cells**

A number of studies have shown that sensitivity of cancer cells to apoptosis induced by a SMAC mimetic as a single agent directly correlates with TNFα levels (21, 22) and blockage of TNFα signals by TNFα antibody or TNFR siRNA, abolishes SMAC-mimetic–induced apoptosis (22–24). Given the fact that ionizing radiation causes activation of NF-κB (26, 27), whereas TNFα is an NF-κB target (29), which induces apoptosis if cIAP-1 is removed (14), we determined if NF-κB activation, followed by TNFα secretion, is the major mechanism for apoptosis induction and SM-164 radiosensitization in sensitive cells. As shown in Fig. 5A, irradiation of UMSCC-1 cells with 4 Gy induced a 2-fold activation of NF-κB, whereas the treatment with SM-164 induced a 4.5-fold activation. An additive 6.5-fold induction was observed with the combination. The inactive analogue SM-173 had no effect alone or in combination with radiation. TNFα, a known inducer of NF-κB activity, was measured by ELISA. The values were normalized with protein concentrations of the cell lysates. Mean ± SEM (\( n = 3 \)). C, induction of TNFα mRNA. UMSCC-1 cells were treated with SM-164 or radiation (6 Gy) or the combination. Cells were harvested 24 hours later for reverse transcriptase-PCR analysis to determine mRNA expressions of TNFα with TRAIL as a control. D, partial blockage of SM-164 radiosensitization by TNFα Ab. UMSCC-1 cells were left untreated or treated with SM-164 (200 nmol/L) for 2 hours with or without TNFα antibody (50 ng/mL) before irradiation. Cells were grown in media containing SM-164 + TNFα Ab for 9 days before the colonies were counted (mean ± SEM, \( n = 2 \)). Cont, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

**Figure 5.** NF-κB activation and TNFα secretion during SM-164 radiosensitization. A, NF-κB activity. Cells were transfected with a NF-κB luciferase reporter (pNifty plasmid), along with Renilla, in 6-well plates, and split into a 96-well plate 24 hours later. Cells were then left untreated (control) or treated with TNFα (10 ng/mL) for 3 hours as a positive control or irradiated with 4 Gy. SM-164 or SM-173 (100 nmol/L) were added immediately after radiation. Cells were lysed 24 hours later for luciferase activity assay (Promega). Results (\( n = 3 \)) are presented as the fold activation after normalization with Renilla (*, \( P < 0.05 \)). B, increased TNFα secretion. TNFα levels in conditioned media (CM) were measured by ELISA. The values were normalized with protein concentrations of the cell lysates. Mean ± SEM (\( n = 3 \)). C, induction of TNFα mRNA. UMSCC-1 cells were treated with SM-164 or radiation (6 Gy) or the combination. Cells were harvested 24 hours later for reverse transcriptase-PCR analysis to determine mRNA expressions of TNFα with TRAIL as a control. D, partial blockage of SM-164 radiosensitization by TNFα Ab. UMSCC-1 cells were left untreated or treated with SM-164 (200 nmol/L) for 2 hours with or without TNFα antibody (50 ng/mL) before irradiation. Cells were grown in media containing SM-164 + TNFα Ab for 9 days before the colonies were counted (mean ± SEM, \( n = 2 \)). Cont, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
of NF-κB used as a positive control in the assay, caused a 3-fold activation. Thus, it appears that by eliminating cIAP-1, SM-164 increased radiation-induced NF-κB activation in sensitive cells.

We next determined whether the sensitivity to SM-164 radiosensitization correlated with increased production of TNFα by measuring TNFα levels in the conditioned medium in UMSCC-1 cells. As shown in Fig. 5B, a single treatment with SM-164 or radiation induced a minor increase in TNFα secretion into the conditioned medium. In contrast, the combined treatment induced a significant time-dependent increase in TNFα secretion, reaching the peak induction of 4-fold at 72 hours. We further did reverse transcriptase-PCR analysis of mRNA expressions of TNFα and TRAIL, a ligand for death receptor 5 as a control. As shown in Fig. 5C, TNFα mRNA, but not TRAIL mRNA, was induced only by combined treatment, indicating the TNFα increase occurred at the transcriptional level. Finally, we tested if blockage of TNFα by TNFα neutralizing antibody would abolish SM-164 radiosensitization. As shown in Fig. 5D, inclusion of TNFα Ab reduced SM-164-induced SER from 1.42 to 1.18, indicating a partial rescue, which is statistically different (P < 0.05). Thus, increased TNFα secretion, likely as a result of NF-κB activation following the cIAP-1 elimination, contributes at least in part to the radiosensitizing effect of SM-164 in UMSCC-1 cells.

**SM-164 degraded cIAP-1 and radiosensitized UMSCC-1 in vivo xenograft tumors**

Finally, we determined SM-164 radiosensitization in vivo using the UMSCC-1 xenograft model. To ensure that SM-164 hits its target cIAP-1 in in vivo tumors and to determine when radiation should be delivered post-drug administration, we measured cIAP-1 levels among 4 groups of tumor samples at the indicated times after a single administration of SM-164 or a single dose of radiation or the drug–radiation combination. As shown in Fig. 6A, cIAP-1 was detectable in control tumors. SM-164 administration eliminated cIAP-1 starting at 3 hours posttreatment and for up to 24 hours. As expected, radiation alone had no effect on cIAP-1. The combination of SM-164 and radiation also eliminated cIAP-1 by 6 hours and cIAP-1 had not returned to the basal level in any tumor by 24 hours. Our results clearly showed that SM-164 indeed hits the target and suggested that the radiation should be delivered 2 to 3 hours postadministration of SM-164, when cIAP-1 is undetectable.

We next determined the in vivo radiosensitizing activity of SM-164. As shown in Fig. 6B and C, administration of SM-164 alone at a dose of 5 mg/kg i.v. d for 5 d/wk for 2 weeks had no effect on tumor growth in nude mice. Radiation treatment at the clinically relevant dose of 2 Gy/d for 5 d/wk for 2 weeks had a moderate but not statistically significant antitumor activity. In contrast, the combination of SM-164 and radiation caused a remarkable suppression of tumor growth, which is statistically significantly greater than either treatment alone beginning at day 34 (Fig. 6C). The combination treatment was well tolerated by the animals with a minimal loss of body weight (Fig. 6D). Taken together, our results indicate that SM-164 sensitizes UMSSC-1 head and neck cancer cells to radiation, as assayed in both in vitro cell culture and in vivo tumor xenograft models, and acts as a novel class of radiosensitizer.

**Discussion**

In this study, we determined the radiosensitizing activity of SM-164, a small molecule SMAC-mimetic compound that promotes a rapid degradation of cIAP-1 and disrupts inhibitory binding of XIAP to caspase-9 and -3 (31) in HNSCC cells. We found that SM-164, at nontoxic subnanomolar concentrations, significantly sensitized a subset of HNSCC cells to radiation both in vitro cell culture and in vivo xenograft tumor models. Surprisingly, although cIAP-1 degradation contributes to SM-164 radiosensitization, sensitivity of HNSCC cells to SM-164 radiosensitization is determined neither by the degradation of cIAP-1, nor by cellular levels of cIAP-1 and/or XIAP. Rather, it is determined by intrinsic cellular sensitivity to caspase activation with an inverse relationship to endogenous Bcl-2 levels. It is very likely that lack of Bcl-2 expression in sensitive cells facilitates caspase activation, whereas removal of negative blockers of cIAP-1 and XIAP, SM-164 confers full activation of caspases, leading to enhanced killing and increased sensitivity to radiation. Consistently, the high levels of Bcl-2 rendered HNSCC cells resistant to radiation and knockdown of Bcl-2 via siRNA silencing sensitized cells to radiation. Our finding that SM-164 can induce apoptosis when combined with radiation is consistent with a study showing that overexpression of SMAC protein itself enhanced radiation-induced apoptosis in several lines of cancer cells including neuroblastoma, glioblastoma, and pancreatic carcinoma (39). Our mechanistic study revealed that both sensitive lines are intrinsically sensitive to activation of a number of caspases, including caspase-8, -9, and -2 by SM-164, or radiation but to a lesser extent. The combination of SM-164 and radiation further activates these caspases, leading to an enhanced apoptosis. The causal effect of caspase activation in SM-164–induced radiosensitization was shown by complete abrogation of apoptosis induction by z-VAD treatment (Fig. 2A) and significant blockage of SM-164 radiosensitization by caspase knockdown or z-VAD treatment (Fig. 4C and D). On the other hand, in UMSCC-12 and UMSCC-74B with very high levels of Bcl-2, neither caspase-8 and -9 nor caspase-2 was significantly activated by either SM-164 or radiation, and consistently, neither cell line was radiosensitized by SM-164.

It is well established that ionizing radiation activates NF-κB (26) through degradation of IκB (27). Activated
NF-κB, on one hand, induces survival proteins, TRAF1/2 and cIAP-1/2, to inhibit apoptosis via suppressing caspase-8 activation (14). On the other hand, activated NF-κB induces its downstream target, TNFα, to initiate the death receptor pathway for apoptosis induction if survival proteins, such as TRAF-2 or cIAP-1, are removed (21–24). This notion is further supported by our recent study that UMSCC-1 cells were sensitized to radiation by siRNA silencing of TRAF-2 (36), as well as by siRNA silencing of cIAP-1 or cIAP-2 (Supplementary Fig. S1C). Here, we showed that SM-164 further activated radiation-induced NF-κB, leading to increased TNFα transcription and secretion. The role of TNFα in SM-164 radiosensitization was clearly shown by a substantial abrogation of radiosensitization in the presence of a TNFα neutralizing antibody. Thus, in UMSCC-1 cells, NF-κB activation, followed by TNFα secretion and caspase-8 activation on cIAP-1 degradation, is the major mechanism of SM-164 radiosensitization.

In summary, we report here that SM-164 is a potent and novel class of radiosensitizer in a subset of HNSCC cells. SM-164 radiosensitization is determined by intrinsic sensitivity to caspase activation in cells with undetectable or low Bcl-2 expression. Under these conditions, removal of negative blockers, such as cIAP-1 and XIAP, by SM-164 leads to a full activation of caspase-8 and -9 via NF-κB/TNFα death receptor pathway. Although multiple factors contribute to radioresistance of HNSCC, including activation of EGFR, NF-κB, and COX-2 signals (2, 6, 40, 41), our study does suggest that HNSCC patients, whose tumors lack the Bcl-2 expression (seen in 76% of primary HNSCC tumors of 85 cases; ref. 3), are intrinsically sensitive to caspase activation, might benefit from combinational therapy with SM-164 and radiation. Thus,
SM-164 shows some promise for future development as a novel class of radiosensitizing drugs for a subset of HNSCC patients, although caution should be taken for potential side effects, given the role of IAPs in modulation of inflammatory signaling and immunity in addition to regulating caspases and apoptosis (42).

**Disclosure of Potential Conflict of Interest**

No potential conflicts of interests were disclosed.

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**References**


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