Cancer Therapy: Preclinical

Smac Modulates Chemosensitivity in Head and Neck Cancer Cells through the Mitochondrial Apoptotic Pathway

Quanhong Sun, Xingnan Zheng, Lin Zhang, and Jian Yu

Abstract

**Purpose:** Overexpression of inhibitors of apoptosis proteins (IAP) contributes to therapeutic resistance. Second mitochondria-derived activator of caspase (Smac) promotes caspase activation by binding to IAPs upon release from the mitochondria. IAP antagonists, also called SMAC mimetics, are promising anticancer agents modeled after this mechanism. We investigated the role and mechanisms of Smac- and Smac mimetic–mediated chemosensitization in head and neck squamous cell carcinoma (HNSCC) cells.

**Experimental Design:** The effects of SMAC knockdown, SMAC overexpression, and a small molecule Smac mimetic on the chemosensitivities of HNSCC cells were determined. The mechanisms of Smac- and Smac mimetic–mediated chemosensitization were investigated by analyzing growth suppression, the mitochondrial apoptotic pathway, caspase activation, and IAP proteins. The therapeutic responses of HNSCC cells with different levels of Smac were compared in xenograft models.

**Results:** We found that Smac mediates apoptosis induced by several classes of therapeutic agents through the mitochondrial pathway. SMAC knockdown led to impaired caspase activation, mitochondrial membrane depolarization, and release of cytochrome c. A small molecule Smac mimetic, at nanomolar concentrations, significantly sensitized HNSCC cells to gemcitabine-induced apoptosis and restored gemcitabine sensitivity in SMAC knockdown cells, through caspase activation, X-linked IAP dissociation, and mitochondria-associated events, but not the TNF-α pathway. Furthermore, Smac levels modulated the therapeutic response of HNSCC cells to gemcitabine in xenograft models.

**Conclusions:** Our results establish a critical role of Smac in mediating therapeutic responses of HNSCC cells and provide a strong rationale for combining Smac mimetics with other anticancer agents to treat HNSCC.

Clin Cancer Res; 17(8); 2361–72. ©2011 AACR.

Introduction

Patients with head and neck squamous cell carcinoma (HNSCC) are often diagnosed with advanced diseases that respond poorly to chemotherapy and radiation therapy. As a result, the overall survival of HNSCC patients has not been significantly improved over the past 2 decades (1). Gemcitabine is a chemotherapeutic agent commonly used to treat HNSCC, and often in combination with other modalities such as surgery, radiation, or additional chemotherapeutic agents including cisplatin. The chemotherapeutic properties of gemcitabine are associated with severe mucositis in the majority of patients (2–4).

Therefore, novel strategies are needed to improve efficacy and reduce side effects in HNSCC treatment. Deregulation of programmed cell death (apoptosis) is a major cause of therapeutic resistance (5–7). Apoptosis is blocked through a variety of mechanisms in HNSCC cells. The tumor suppressor p53 is frequently mutated or targeted for degradation by human papillomavirus (HPV) oncoproteins (8), which prevents the induction of proapoptotic BH3-only proteins, such as PUMA and Noxa, in response to DNA damage (9). Overexpression of antiapoptotic Bcl-2 family members (10), such as Bcl-xL and Bcl-2, is also common in HNSCC (11). Widespread overactivation of growth factor pathways, such as EGFR (epidermal growth factor receptor) and STATs, can suppress apoptosis by affecting the expression of several Bcl-2 family members (12). Overexpression of inhibitor of apoptosis proteins (IAP), such as c-IAP2 and X-linked IAP (XIAP), has been reported in HNSCC (13, 14) and other malignancies (15, 16).

Second mitochondria-derived activator of caspase (Smac) is an endogenous inhibitor of IAPs (17). Upon release into the cytosol, Smac binds to IAPs through its N-terminal AVPI domain and relieves the inhibition of caspases by IAPs. However, induction of apoptosis in response to various anticancer agents is not affected by Smac deficiency in murine models (18). Studies using human colon cancer...
Translational Relevance

Patients with head and neck squamous cell carcinoma (HNSCC) are often diagnosed with advanced diseases that respond poorly to chemotherapy and radiation therapy. The overall survival of HNSCC patients has not been significantly improved over the past 2 decades. Overexpression of inhibitors of apoptosis proteins (IAP) was reported to be associated with worse prognosis in HNSCC. In this study, we establish a critical role of second mitochondria-derived activator of caspase (Smac), an endogenous inhibitor of IAPs, in mediating therapeutic and apoptotic responses of HNSCC cells in cell culture and xenograft models. Our mechanistic studies revealed that Smac or a small molecule Smac mimetic activates caspases and amplifies apoptotic signaling through mitochondrial damage, but not the TNF-α signaling, in HNSCC cells. Our studies suggest IAPs as useful therapeutic targets and provide a strong rationale for combining Smac mimetics with other anticancer agents to treat HNSCC through enhanced induction of apoptosis.

Materials and Methods

Cell culture and drug treatment

Head and neck cancer cell lines were obtained from the University of Pittsburgh Cancer Institute (UPCI) Head and Neck Cancer program. All cell lines were maintained at 37°C in 5% CO2. Cell culture media included DMEM (Dulbecco’s modified Eagle’s media; Mediatech) for 1483 cells and RPMI 1640 (Cellgro) for JHU cells and were supplemented with 10% FBS (HyClone), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Gemcitabine was purchased from Eli Lilly and Company. Other anticancer agents used in the study include cisplatin, 5-fluorouracil (5-FU), TRAIL (PeproTech), and human TNF-α and its neutralizing antibody (R&D system). Tetra-Logic Pharmaceuticals supplied the Smac mimetic, GT-A, and control compound GT-C (19). Stock solutions of all small compounds were prepared in DMSO (dimethyl sulfoxide). For treatment with adenovirus, Cells were infected with Ad-PUMA or the BH3-deleted form (Ad-ΔBH3) (27) (multiplicity of infection = 40) for 48 hours. The Noxa expression vector was constructed in pcDNA3.1, using a PCR-mediated method, and confirmed by sequencing and Western blotting.

Western blotting

Antibodies used for Western blotting included those against caspase-8, caspase-3, Myc (Cell Signaling Technology), cytochrome c, α-tubulin (BD Biosciences), caspase-9 (Stressgen Bioreagents), cytochrome oxidase subunit IV (Cox IV; Invitrogen), Bcl-2 (Dako), PUMA (27), p53 (DO1), cIAP-1, cIAP-2 (R&D system), XIAP (Invitrogen), Survivin (Cell Signaling), Bim, Noxa, and Smac (EMD Biosciences). Western blotting analysis was carried out as previously described (28).

Immunoprecipitation

Cells were harvested after 24 hours with or without Smac mimetic treatment (150 nmol/L) in T-75 flasks and resuspended in 1 mL of EBC buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 0.5% Nonidet P-40) supplemented with protease inhibitors cocktail (Roche Applied Sciences). The cells were disrupted by sonication and then spun at 10,000 x g for 10 minutes to collect the cell lysate. For immunoprecipitation (IP), 2 μg of antibodies or control IgG were added to protein G Dynabeads (Invitrogen) for 1 hour followed by incubation with 400 μL cell lysates according to manufacturer’s instructions. After the final wash, the beads were mixed with 50 μL of 1 x Laemml sample buffer, heated at 95 °C for 10 minutes, and analyzed by Western blotting.

Apoptosis assays

Adherent and floating cells were harvested, stained with Hoechst 33258 (Invitrogen), and analyzed for apoptosis by nuclear staining assay and flow cytometry (28). For detection of mitochondrial membrane potential change, harvested cells were stained by Mitotracker Red CMXRos (Molecular Probes) for 15 minutes at 37°C and analyzed by flow cytometry using the FL3 channel, according to the manufacturer’s instructions. For colony formation assays, equal numbers of cells were subjected to various treatments and plated into 12-well plates at different dilutions. Colonies were visualized by crystal violet staining 11 to 14 days after plating as previously described (28). Each experiment was carried out in triplicate and repeated at least twice.
Analysis of cytochrome c release
Mitochondrial and cytosolic fractions were isolated from treated cells by differential centrifugation as previously described (27, 29). Concentrations of cytosolic fractions obtained from different samples were normalized using a protein assay dye reagent from Bio-Rad. All fractions were mixed with equal volumes of 2× Laemmli sample buffer and subjected to Western blotting analysis.

Stable SMAC knockdown and SMAC-overexpressing cells
SMAC short hairpin RNA (shRNA) was constructed using the pSUPER vector (Oligoengine) as described (30). Pur- ormycin-resistant clones were isolated as previously described (31). Western blotting was used to identify stable clones with significant downregulation of Smac in HNSCC lines JHU-012, JHU-019, JHU-022, and 1483. For Smac-overexpressing (SO) cells, JHU-012 and 1483 cells were transfected with an expression construct encoding either Myc-tagged wild-type Smac (AVPI) or mutant Smac with deletion of alanine in the AVPI domain (AA; ref. 32), and were selected by G418 (1 mg/mL for JHU-012; 1.2 mg/mL for 1483). Stable clones expressing Smac were identified by Western blotting. Drug-resistant transfecctants without KD or SO behaved similarly to the parental cells in response to chemodrugs tested. The parental (P) cells were therefore chosen as the controls.

Xenograft tumors
All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. JHU-012 and 1483 xenografts were established and measured as described (28). In brief, 5- to 6-week-old female athymic nude mice (Harlan) were inoculated with JHU-012 or 1483 (5 × 10⁶ cells/site) on both flanks. Tumors were allowed to establish for 10 days. The tumor volumes were measured in 2 dimensions using a vernier caliper. Mice were randomized into groups (7 mice/group), such that the average tumor volume across the groups was the same. Gemcitabine or vehicle (ddH₂O) treatments were administered intraperitoneally at 80 mg/kg thrice on days 10, 13, and 16 (33). For all in vivo experiments, tumor volumes were measured every other day in 2 dimensions and volumes were determined in mm³ using the formula: \( V = \frac{1}{2} l b^2 \) (where \( l \) is the larger diameter and \( b \) is the smaller diameter of the tumor). Mice were injected i.p. 2 hours before sacrifice with a single dose of bromodeoxyuridine (BrDU) at 150 mg/kg to label cells in S phase. BrDU was dissolved in PBS to a final concentration of 30 mg/mL. Histologic and immunofluorescence analysis for apoptosis and proliferation were carried out on 5-µm frozen sections as described (28).

Statistical analysis
Statistical analysis was carried out using GraphPad Prism IV software. All \( P \) values were calculated by the Student’s \( t \) test, and \( P < 0.05 \) was considered significant. Means ± 1 SD were displayed in figures where applicable.

Results
Smac mediates apoptosis induced by therapeutic agents in HNSCC cells
To determine a potential role of Smac in chemotherapeutic agent-induced apoptosis in HNSCC cells, we first analyzed several biochemical markers of apoptosis following gemcitabine treatment. Gemcitabine was found to induce cytosolic release of cytochrome c and Smac and caspase 3 activation in 4 HNSCC lines including JHU-012, 1483, JHU-019, and JHU-022 cells (Fig. 1A and data not shown). We then generated stable SMAC knockdown (KD) cells in these 4 lines by shRNA-mediated gene silencing. Two independent SMAC-KD clones of each line were produced. SMAC-KD cells were found to be resistant to gemcitabine-induced apoptosis, compared with parental cells (Fig. 1B and Supplementary Fig. S1). SMAC-KD partially rescued long-term cell growth suppression induced by gemcitabine in JHU-012 and 1483 cells (Fig. 1C). In addition, SMAC-KD significantly blocked apoptosis induced by other therapeutic agents, including cisplatin, 5-FU, and TRAIL in HNSCC cells (Fig. 1D). These data show that Smac mediates apoptosis induced by several classes of anticancer agents in HNSCC cells.

Smac mediates gemcitabine-induced apoptosis through the mitochondrial pathway
We further examined the potential mechanism of Smac-mediated and gemcitabine-induced apoptosis. Overexpression of Bcl-2 blocked apoptosis induced by gemcitabine in both JHU-012 and 1483 cells (Fig. 2A), suggesting an important role of the mitochondrial pathway. We then compared biochemical markers of the mitochondrial pathway in parental and SMAC-KD cells following gemcitabine treatment. Consistent with reduced apoptosis, release of cytochrome c and activation of caspases-3, -8, and -9 were significantly attenuated in SMAC-KD cells, compared with parental cells (Fig. 2B and C). In addition, mitochondrial membrane depolarization was significantly blocked in SMAC-KD cells (Fig. 2D).

DNA damage is known to activate the expression of several BH3-only proteins and the mitochondrial apoptosis pathway through p53 stabilization. We found that BH3-only proteins PUMA, Bim, and Noxa were strongly induced by gemcitabine, whereas p53 was not consistently induced (Supplementary Fig. S2A). SMAC-KD blocked apoptosis induced by exogenous expression of PUMA or Noxa (Supplementary Fig. S2B). In addition, elevated expression of PUMA or Noxa sensitized HNSCC cells to apoptosis induced by gemcitabine (Supplementary Fig. S2C). These data suggest that regulation of the Bcl-2 family of proteins is likely to be upstream of Smac, and Smac mediates gemcitabine-induced apoptosis and caspase activation by promoting mitochondrial damage, such as membrane depolarization and cytochrome c release, in a positive feedback loop (19, 34, 35).
SMAC overexpression potentiates gemcitabine-induced apoptosis

The N-terminal AVPI residues of cytosolic Smac mediate caspase activation (36). To determine whether this function of Smac is important for apoptosis induced by chemotherapeutics in HNSCC cells, we stably expressed either a wild-type (AVPI) or mutant Smac, containing a deletion of alanine in the AVPI domain (ΔA).
interactions between Smac and IAPs (19, 32), in JHU-012 and 1483 cells. Expression of exogenous, mature Smac was lower than that of the endogenous Smac in the stable lines (Fig. 3A, Smac). Nonetheless, gemcitabine-induced apoptosis and caspase activation were potentiated by the expression of the wild-type, but not the mutant Smac (Fig. 3A and B). Furthermore, wild-type, but not mutant Smac, enhanced growth inhibition (Fig. 3C and data not shown) and mitochondrial membrane depolarization induced by gemcitabine (Fig. 3D). These results suggest that elevated levels of Smac sensitize HNSCC cells to anticancer drug-induced apoptosis and growth inhibition by promoting caspase activation and mitochondrial damage.

A Smac mimetic potentiates gemcitabine-induced apoptosis through the mitochondrial pathway

The requirement of the AVPI domain for Smac function prompted us to test whether pharmacologic agents that mimic this domain can enhance gemcitabine-induced apoptosis. An active Smac mimetic compound GT-A at nanomolar concentrations, but not the control compound GT-C, sensitized HNSCC cells to gemcitabine-induced apoptosis and growth inhibition by promoting caspase activation and mitochondrial damage.

Figure 2. Smac mediates gemcitabine-induced apoptosis through the mitochondrial pathway. A, Bcl-2 suppressed gemcitabine-induced apoptosis. JHU-012 or 1483 cells were transfected with a Bcl-2 expression construct or empty vector followed by 50 μmol/L gemcitabine treatment for 48 hours, respectively. Top, the expression of Bcl-2 was analyzed by Western blotting. Bottom, apoptosis was analyzed by nuclear fragmentation assay. **, P < 0.01, Bcl-2 versus vector or mock (un) transfected group. B, cytochrome c release in parental and SMAC-KD cells treated as in (A) was analyzed by Western blotting in the cytosolic fractions. Tubulin was used as control for loading. C, activation of caspases-3, -8, and -9 was analyzed by Western blotting in indicated cells treated with 50 μmol/L gemcitabine for 48 hours. D, left, mitochondrial membrane depolarization in JHU-012 parental and SMAC-KD cells was analyzed by flow cytometry 48 hours after 50 μmol/L gemcitabine treatment. Right, quantitation of depolarized cells.
Figure 3. SMAC overexpression potentiates gemcitabine-induced apoptosis. A, generation of stable wild-type (WT) SMAC-SO cells and mutant (ΔA) cells. P, parental cells; ΔA, mutant SMAC overexpression cells; SO1 and SO2, independent SMAC overexpression clones. Left, Smac expression was analyzed by Western blotting in indicated cell lines. Right, apoptosis in JHU-012 or 1483 cells following indicated treatment for 48 hours was analyzed by nuclear fragmentation assay. **, P < 0.01, SO versus P. Gem, 50 \( \mu \)mol/L and TRAIL, 100 ng/mL. B, the indicated cell lines were treated with gemcitabine (50 \( \mu \)mol/L) for 48 hours. Activation of caspase-3 was analyzed by Western blotting. C, long-term cell growth was assessed by colony formation assay as in Figure 1C. Cells were treated with 10 \( \mu \)mol/L gemcitabine for 6 hours before plating. Top, representative pictures of colonies. Bottom, quantitation of colony numbers with untreated cells set at 100%. **, P < 0.01, SO versus P. D, mitochondrial membrane depolarization was analyzed by flow cytometry 48 hours after 50 \( \mu \)mol/L gemcitabine treatment in indicated cells.
apoptosis (Fig. 4A). GT-A at 100 nmol/L markedly enhanced gemcitabine-induced apoptosis in JHU-012 cells, increasing from 32% to 69% at 48 hours with 50 μmol/L gemcitabine (Fig. 4A), which is associated with markedly enhanced caspase-3 activation and cytochrome c release (Fig. 4A and Supplementary Fig. S3A). Combinations of gemcitabine with GT-A, but not GT-C, inhibited long-term survival and growth of HNSCC cells more effectively compared with gemcitabine alone (Supplementary Fig. S3B and C). The GT-A compound also sensitized HNSCC cells to cisplatin-induced apoptosis and long-term growth suppression (Supplementary Fig. S4). GT-A or the control compound alone up to 1 μmol/L did not have detectable growth inhibitory or apoptotic effects on HNSCC cells, or on caspase activation or cytochrome c release (Fig. 4A and B and data not shown).

Smac mimetics were recently reported to induce rapid degradation of cIAP-1/2, leading to NF-κB activation, TNF-α secretion, and apoptosis in some cancer cells (23–26). To probe this potential mechanism in Smac mimetic-induced

Figure 4. The Smac mimetic potentiates gemcitabine-induced apoptosis. Cells were treated with 50 μmol/L gemcitabine with or without 100 nmol/L control (GT-C) or active (GT-A) Smac mimetic compound for 48 hours or as indicated. A, left, apoptosis induction was determined by nuclear staining in 4 HNSCC lines at 48 hours. **, *P < 0.01; *, *P < 0.05, GT-A + Gem versus GT-C + Gem. Right, caspase-3 activation was analyzed at 48 hours by Western blotting. B, JHU-012 and 1483 cells were incubated with TNF-α Ab (5 μg/mL) for 1 hour and then treated with gemcitabine alone or combined with GT-A for 48 hours. Apoptosis was determined by nuclear fragmentation assay. **, *P < 0.01, GT-A + Gem versus GT-C + Gem. C, the indicated cells were treated with 100 nmol/L GT-A. The expression of cIAP-1, cIAP-2, XIAP, and survivin was analyzed by Western blotting at indicated time points. D, the indicated cells were treated with 150 nmol/L GT-A for 24 hours. Left, the whole cell extracts (5% input) were analyzed for the expression of XIAP, survivin, and Smac. Right, the cell extracts were immunoprecipitated (IP) with IgG control or Smac antibody and blotted for XIAP or Survivin.
chemosensitization of HNSCC cells, we treated the cells with TNF-α neutralizing antibody before exposing them to gemcitabine and GT-A. However, the TNF-α antibody did not block apoptosis induced by the gemcitabine and GT-A combination in JHU-012, JHU-019, JHU-022, or 1483 cells (Fig. 4B and data not shown). In contrast, the TNF-α neutralizing antibody effectively blocked apoptosis induced by TNF-α alone or by the TNF-α and GT-A combination in both JHU-012 and JHU-019 cells (Supplementary Fig. S6A), as well as apoptosis induced by GT-A in HT-29 cells as reported before (Supplementary Fig. S5).

Other IAP proteins, such as XIAP (36) or Survivin (37, 38), can bind to Smac either in the cytosol or mitochondria to suppress apoptosis. Therefore, the Smac mimetic might promote dissociation of endogenous Smac from XIAP or survivin via competitive binding. The treatment of Smac mimetic did not affect the levels of XIAP, survivin, or Smac but induced a complete dissociation of Smac and XIAP within 24 hours in HNSCC cells (Fig. 4D). The interactions between Smac and survivin were unaffected by GT-A (Fig. 4D). These studies established that Smac mimetic–induced chemosensitization in HNSCC cells is mediated through enhanced caspase activation and mitochondrial damage but not the TNF-α signaling. Nonetheless, the TNF-α signaling is intact and synergizes with the Smac mimetic to induced apoptosis in HNSCC cells.

A Smac mimetic restores gemcitabine sensitivity in the SMAC-KD cells

SMAC-KD cells are resistant to gemcitabine-induced caspase activation and apoptosis compared with parental cells (Figs. 4A and 5A). We expected the GT-A compound to restore these events, bypassing the need for Smac protein. Indeed, the active compound GT-A, but not the control compound GT-C, restored apoptosis induced by gemcitabine in SMAC-KD cells (Fig. 5A). The GT-A compound markedly enhanced caspase activation and cytochrome c release (Fig. 5B and C) and fully restored mitochondrial membrane depolarization in SMAC-KD cells (Fig. 5D). These data suggest that activation of the mitochondrial apoptotic pathway by Smac or Smac mimetics can potentially overcome gemcitabine resistance.
Smac modulates gemcitabine sensitivity of HNSCC cells in vivo

To assess whether Smac modulates therapeutic responses in vivo, parental cells, SMAC overexpression (SMAC-SO) or SMAC-KD HNSCC cells were injected subcutaneously into the flanks of BALB/c (nu/nu) nude mice to establish xenografts. Gemcitabine was administered i.p. into tumor-bearing mice on 3 occasions. Comparing with water control, gemcitabine treatment resulted in 70.5% ($P < 0.01$) and 41.4% ($P < 0.01$) growth inhibition in 1483 parental and SMAC-KD tumors, respectively (Fig. 6A). Similarly, gemcitabine administration inhibited JHU-012 parental and SMAC-KD cells.
SMAC-KD tumors by 71.8% ($P < 0.01$) and 33.8% ($P < 0.01$), respectively (Supplementary Fig. S4A). Meanwhile, gemcitabine treatment resulted in 21.2% ($P < 0.01$) and 37.2% ($P < 0.01$) growth inhibition in parental and SMAC-SO tumors, respectively (Fig. 6B). The differences between the responses of the tumors with different SMAC genotypes were statistically significant, whereas little or no difference was found in the efficiency or growth rate in tumor establishment in the absence of treatment (Figs. 6A and B and Supplementary Fig. S7A).

Analysis of tumor sections after the second gemcitabine injection (day 15) revealed significantly lower apoptosis and higher cell proliferation in SMAC-KD tumors compared with parental tumors. In contrast, SMAC-SO tumors showed more extensive apoptosis (13% vs. 5%) and lower proliferation than parental tumors (Fig. 6D and Supplementary Fig. S7B). These results show that the levels of Smac modulate the therapeutic responses of HNSCC cells to gemcitabine in vivo through apoptosis.

**Discussion**

**Smac in anticancer agent–induced apoptosis in HNSCC cells**

IAP family members are frequently overexpressed in many solid tumors including HNSCC. Overexpression of IAPs was reported to be associated with worse prognosis in HNSCC (13, 14). Biochemical studies indicate that IAP proteins are antagonized by Smac in mammals, which promotes caspase activation and apoptosis through its N-terminal AVPI motif (21). The structural basis of such interactions led to the development of several small molecule Smac mimetics, also called IAP antagonists, which are believed to compete with caspases for IAP binding and consequently release caspases to promote cell death (20–22). Despite extensive biochemical data, SMAC-KO mice or mouse fibroblasts show limited if any alteration in apoptosis (18). Our study showed, for the first time in HNSCC cells, that Smac plays an important role in apoptosis induced by several classes of anticancer agents, and elevated Smac levels or a Smac mimetic compound potentiates therapeutic responses of HNSCC cells by promoting apoptosis.

Our observations are consistent with the notion that a requirement of Smac in apoptosis appears to be cell-type and agent-dependent (30, 35). Overexpression of Smac or Smac mimetics can potentiate anticancer effects of chemotherapeutic agents and irradiation in glioma, hepatoma, neuroblastoma, glioblastoma, or pancreatic carcinoma cells (25, 39–41). It is of interest to note that the killing or sensitizing effects of Smac or Smac mimetics appear somewhat selective toward cancer cells compared with normal or untransformed cells (16). The precise mechanisms of this differential sensitivity remain unclear, which might be explained partly by the addiction of cancer cells to overexpression of IAPs or perhaps alterations in other upstream regulators such as the Bcl-2 family of proteins and the death receptors (16).

**Signaling events in Smac-mediated apoptosis and the mitochondria**

Emerging evidence suggests that induction of BH3-only proteins by therapeutic agents might be a universal mechanism underlying favorable and apoptotic responses of cancer cells (28, 31, 42–45). In HNSCC cells, the BH3-only subfamily plays a critical role in regulating their survival, whose expression is suppressed by an oncogenic form of p53 overexpressed in majority of squamous cancers (31, 46, 47). Our data show that BH3-only proteins PUMA, Bim, and Noxa are induced by gemcitabine mostly likely through a p53-independent mechanism, and SMAC-KD blocked apoptosis induced by PUMA or Noxa. Therefore, multiple BH3-only proteins might be involved in promoting mitochondrial damage and Smac release during gemcitabine-induced apoptosis. In addition, SMAC-KD abrogated apoptotic responses to several anticancer agents, which is rescued by the Smac mimetic. Despite a clear role of Smac in activating caspases following its release from the mitochondria, our data suggest that the release of apoptotic proteins might not be independent, and a complex and positive feedback mechanism might exist to regulate mitochondrial outer membrane permeability (MOMP) and caspase activation (30, 34, 35, 48).

**Mechanisms of Smac mimetic–induced chemosensitization in HNSCC cells**

Smac mimetics can induce rapid degradation of cIAP-1/2, leading to TNF-α–dependent apoptosis through the NF-κB signaling in some cells (23–26, 49). In this study, the GT-A compound was also found to induce rapid down-regulation of cIAP-1/2, with cIAP-2 levels restored long before apoptosis. These observations coupled with failure of the TNF-α neutralizing antibody to block apoptosis induced by the gemcitabine and Smac mimetic combination would suggest that the TNF-α signaling is unlikely to be responsible for the chemosensitization effects of Smac mimetics. Rather, a more direct mechanism engaging caspase activation and mitochondrial damage is mediated, at least in part, via the dissociation of endogenous Smac and XIAP. Because Smac is found predominantly in the mitochondria of cells such as HNSCC cells, it is reasonable to predict that this dissociation occurs at the mitochondria. It is also possible that Smac mimetics activate additional proapoptotic proteins (50) by displacing them from IAPs either in the cytosol or mitochondria. The selective involvement of TNF-α signaling (23–26, 49) or the mechanism described here might reflect the cell-type–specific role of endogenous Smac and/or the structural and functional differences of the small molecule Smac mimetics.

**Implications on novel combination therapies in HNSCC**

In phase II studies, gemcitabine in combination with other chemotherapeutics or radiation has shown improved response rates in HNSCC patients with advanced diseases, compared with single-agent regimes (2–4). However, severe mucositis is a common complication in combination...
settings. Our data suggest that this side effect might be reduced without compromising therapeutic efficacies, potentially by using lower doses of gemcitabine with Smac mimetics. Smac mimetics might be useful as sensitizers for other anticancer agents to boost apoptosis in otherwise resistant tumor cells. Several apoptotic blocks exist in cancer cells and the Bcl-2 family of proteins have become promising targets with the development of a class of so called BH3 mimetics, or Bcl-2 antagonists (51, 52). In HNSCC cells, induction of BH3-only proteins in response to 5-FU or cisplatin is often blocked by defective p53 signaling (28), whereas their induction by gemcitabine appears largely p53 independent (this study). Even modest overexpression of BH3-only proteins, such as PLMA or Noxa, can sensitize HNSCC cells to these agents, independent of p53 status. Smac and BH3-only proteins act at different steps of apoptosis and participate in a positive feedback loop to activate caspase and mitochondrial damage. Therefore, targeting either or both steps with Smac and BH3 mimetics depending on the genetic background of the tumors might bring us one step closer to individualized HNSCC treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank other members of Yu and Zhang laboratories for helpful discussions.

Grant Support

This work is supported by NIH grant CA129829, American Cancer Society grant RSG-10-124-01-CCF, and FAMRI (J. Yu) and by NIH grants CA106348, CA121105 and American Cancer Society grant RSG-07-106-01-CNE (L. Zhang). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 25, 2010; revised December 23, 2010; accepted January 3, 2011; published OnlineFirst January 17, 2011.

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