Preclinical Development

Therapeutic Potential and Molecular Mechanism of a Novel, Potent, Nonpeptide, Smac Mimetic SM-164 in Combination with TRAIL for Cancer Treatment

Jianfeng Lu1, Donna McEachern1, Haiying Sun1, Longchuan Bai1, Yuefeng Peng1, Su Qiu1, Rebecca Miller1, Jinhui Liao1, Han Yi1, Meilan Liu1, Anita Bellail2, Chunhai Hao2, Shi-Yong Sun3, Adrian T. Ting4, and Shaomeng Wang1

Abstract

Smac mimetics are being developed as a new class of anticancer therapies. Because the single-agent activity of Smac mimetics is very limited, rational combinations represent a viable strategy for their clinical development. The combination of Smac mimetics with TNF-related apoptosis inducing ligand (TRAIL) may be particularly attractive because of the low toxicity of TRAIL to normal cells and the synergistic antitumor activity observed for the combination. In this study, we have investigated the combination synergy between TRAIL and a potent Smac mimetic, SM-164, in vitro and in vivo and the underlying molecular mechanism of action for the synergy. Our study shows that SM-164 is highly synergistic with TRAIL in both TRAIL-sensitive and TRAIL-resistant cancer cell lines of breast, prostate, and colon cancer. Furthermore, the combination of SM-164 with TRAIL induces rapid tumor regression in vivo in a breast cancer xenograft model in which either agent is ineffective. Our data show that X-linked IAP (XIAP) and cellular IAP1 (cIAP1), but not cIAP2, work in concert to attenuate the activity of TRAIL; SM-164 strongly enhances TRAIL activity by concurrently targeting XIAP and cIAP1. Moreover, although RIP1 plays a minimal role in the activity of TRAIL as a single agent, it is required for the synergistic interaction between TRAIL and SM-164. This study provides a strong rationale to develop the combination of SM-164 and TRAIL as a new therapeutic strategy for the treatment of human cancer. Mol Cancer Ther; 10(5); 902–14. ©2011 AACR.

Introduction

Evasion of apoptosis is a hallmark of human cancers (1, 2), and targeting key apoptosis regulators with a goal to overcome apoptosis resistance of tumor cells is being pursued as a new cancer therapeutic strategy (3, 4).

Inhibitor of apoptosis proteins (IAP) are a family of key apoptosis regulators, characterized by the presence of one or more baculovirus IAP repeat domains (5, 6). Among them, X-linked IAP (XIAP), by binding to effector caspase-3 and caspase-7 and an initiator caspase-9 and inhibiting the activities of these caspses, blocks death receptor-mediated and mitochondria-mediated apoptosis, whereas cellular IAP1 (cIAP1) and cIAP2 inhibit death receptor-mediated apoptosis by directly binding to TNF receptor–associated factor 2 (TRAF2; refs. 5, 6). XIAP, cIAP1, and cIAP2 have been found to be overexpressed in human cancer cell lines (7, 8) and human tumor tissues (9–11), and their overexpression confers on cancer cells resistance to various anticancer drugs (12–15). IAP proteins are attractive cancer therapeutic targets (12–15).

Smac/DIABLO (second mitochondria-derived activator of caspases or direct IAP-binding protein with low isoelectric point) is a proapoptotic molecule (16, 17) and promotes apoptosis, at least in part, by directly antagonizing cIAP1/2 and XIAP (12–15). Smac protein interacts with XIAP and cIAP1/2 via its N-terminal AVPI terapeptide binding motif (18). In recent years, there have been intense research efforts in developing small-molecule Smac mimetics as a new class of anticancer drugs (19, 20) and several such compounds are now in early clinical development (21–24). Smac mimetics can effectively induce apoptosis as single agents in certain cancer cell lines (25–29), but their anticancer activity...
as single agents seems to be limited to approximately 10% of human cancer cell lines in vitro (27, 30). Therefore, for the successful clinical development of Smac mimetics as a new class of anticancer drugs, rational combination strategies are clearly needed.

To this end, the combination of TNF-related apoptosis inducing ligand (TRAIL) with Smac mimetics seems to be a particularly attractive strategy for several reasons. First, TRAIL is a member of the TNF-α family, but unlike TNF-α, TRAIL shows very low toxicity to normal cells and tissues and is well tolerated in clinical trials (31-33). Second, despite its good safety profile, TRAIL has minimal single-agent anticancer activity in clinical trials (31), and this has hampered its clinical development. Third, there is very strong synergy between TRAIL and Smac mimetics in cancer cell lines of diverse of tumor types (34-40).

Despite the strong synergy shown between TRAIL and Smac mimetics, the precise underlying molecular mechanism of action for their synergy is not fully understood. Because Smac mimetics have been designed on the basis of the interaction between XIAP and Smac, previous investigations have focused on XIAP as the primary cellular target for Smac mimetics when combined with TRAIL (36-39). However, our data clearly showed that although knockout of XIAP or efficient knockdown of XIAP by short interfering RNA (siRNA) can modestly sensitize cancer cells to apoptosis induction by TRAIL, the sensitization effect is far less than that achieved by Smac mimetics. In addition, although one would expect that the underlying molecular mechanism for the synergistic interaction between TRAIL and Smac mimetics may be very similar to that between TNF-α and Smac mimetics, our data showed that TNF-α fails to induce apoptosis in cancer cell lines of diverse tumor types which are very sensitive to TRAIL as a single agent and Smac mimetics can dramatically sensitize TRAIL in both TRAIL-sensitive and -resistant cancer cell lines. Finally, despite the strong synergy between TRAIL and Smac mimetics in vitro, tumor regression has not been reported in vivo for the combination when both agents are ineffective.

We previously reported the design and evaluation of SM-164 as a potent, bivalent Smac mimic (28, 41). SM-164 binds to XIAP, cIAP1, and cIAP2 with K_i values of 0.56, 0.31, and 1.1 nmol/L, respectively (28, 41). It potently antagonizes XIAP in cell-free functional assays and in cells and induces rapid degradation of cIAP1 and cIAP2 in cancer cells at concentrations as low as 1 to 10 nmol/L (28, 41). In this study, we used SM-164 (Fig. 1) and evaluated its combination with recombinant TRAIL protein in a panel of 19 human breast, prostate, and colon cancer cell lines in vitro and in a breast cancer xenograft model in vivo. Our study provides further insights into the molecular mechanism of action for the strong synergy between TRAIL and Smac mimetics and suggests that the combination of TRAIL with SM-164 or other Smac mimetics should be evaluated in the clinic as a new strategy for the treatment of human breast, prostate, and colon cancer.

Materials and Methods

Reagents and antibodies
SM-164 was synthesized as described previously (41) and the purity was more than 95% by high-performance liquid chromatography analysis. The chemical structure of SM-164 is shown in Fig. 1. Native recombinant human TRAIL (rhTRAIL; residues 114-281) construct without His tag was a kind gift of Dr. Arul Chinnaiyan at the Michigan Center for Translational Medicine and Department of Pathology, University of Michigan. TRAIL 114-281aa (amino acid) was cloned into a pHis-TEV vector in our laboratory. The resulting construct was transformed into Escherichia coli BL21 DE3. Cells harboring the construct were cultured at 37°C, 250 rpm, in Luria Bertani media with 50 μg/mL of kanamycin until the OD600 was 0.4 to 0.6. Protein expression was induced with 0.1 mol/L isopropyl-l-thio-B-D-[14C]galactopyranoside at 30°C, 250 rpm overnight. The cells were harvested by centrifugation (7,000 x g, 12 minutes, and 4°C) and cell pellet was stored in –80°C or directly used for protein purification. Cell pellet was resuspended in 40 mL lysis buffer (50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 50 μmol/L ZnAc, and 1 mmol/L dithiothreitol) for sonication to release soluble proteins. TRAIL 114-281aa protein was purified by Ni-NTA affinity chromatography. The Ni-NTA resin was washed with 50 mL lysis buffer and recombinant protein was eluted with lysis buffer with 80 mmol/L imidazole. The protein was further purified with size-exclusion chromatography by using an Amersham Biosciences P-920 FPLC (fast protein liquid chromatography) equipped with a Superdex 200 column (Amersham Biosciences). The protein was eluted in a buffer containing 50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 50 μmol/L ZnAc, and 1 mmol/L dithiothreitol for sonication to release soluble proteins. TRAIL 114-281aa protein was purified by Ni-NTA affinity chromatography. The Ni-NTA resin was washed with 50 mL lysis buffer and recombinant protein was eluted with lysis buffer with 80 mmol/L imidazole.

The following primary antibodies were used in this study: cleaved caspase-8, XIAP, PAPR, and TRAF2 (Cell Signaling Technology); caspase-3, caspase-9, FADD (Stressgen Biotechnologies), and caspase-8 clone 6E (Epitomics, Inc.); DR4 and DR5 (ProSci), cIAP1 (J. Silke, La Trobe University) and cIAP2 (R&D Systems).

Cell lines
Human breast cancer MDA-MB-436, SK-BR-3, MDA-MB-453, MDA-MB-468, SUM159, SUM52, and T47D cell lines, human colon cancer HCC116, SW620, SW480, RKO, HT29, and DLD1 cell lines, and human prostate cancer PC-3, DU-145, CL-1, 22RV1, and LNCaP cell line were purchased from American Type Culture Collection. Human breast cancer 2LMP cell line was a subclone of
the MDA-MB-231 cell line and was a kind gift of Dr. Dajun Yang, Department of Internal Medicine, University of Michigan. All 19 cell lines were passaged fewer than 6 months in our laboratory either after receipt or resuscitation. Isogenic XIAP+/− and XIAP−/− HCT116 colon cancer cell lines were a kind gift from Dr. Fred Bunz, Johns Hopkins University, Baltimore, MD.

**Cell viability, cell death, and apoptosis**

Cell viability was evaluated by a lactate dehydrogenase-based WST-8 assay (Dojindo Molecular Technologies) as described previously (23). Cell death was quantitated by microscopic examination in a trypan blue exclusion assay. Apoptosis analysis was done using an Annexin V/propidium iodide apoptosis detection kit (Roche Applied Science) by flow cytometry according to the manufacturer’s instructions. Total Annexin V (+) cells plus Annexin V (−)/propidium iodide (+) were counted as apoptotic cells.

**Western blot analysis**

Cells or xenograft tumor tissues were lysed using radioimmunoprecipitation assay lysis buffer (PBS containing 1% NP40, 0.5% Na-deoxycholate, and 0.1% SDS) supplemented with 1 μmol/L phenylmethylsulfonyl fluoride and 1 protease inhibitor cocktail tablet per 10 mL on ice for 20 minutes, and lysates were then cleared by centrifugation before determination of protein concentration by using the Bio-Rad protein assay kit according to the manufacturer’s instructions. Proteins were electrophoresed onto 4% to 20% SDS-PAGE gels (Invitrogen) as described in the Supplementary information. Membranes were incubated with a specific primary antibody, washed, and incubated with horseradish peroxidase–labeled secondary antibody (GE Healthcare). Signals were visualized with chemiluminescent horseradish peroxidase antibody detection reagent (Denville Scientific). Where indicated, the blots were stripped and reprobed with a different antibody.

**Comununoprecipitation**

TRAIL–receptor complex was immunoprecipitated with Flag-tagged TRAIL on the basis of the reported protocol (28). A total of 5 × 10^7 cells of each sample in 10 mL culture medium were treated with the mixture of 100 ng/mL of Flag-tagged TRAIL and anti-Flag M2 IgG (3 μg/mL for each sample) at 37°C and then lysed for 30 minutes on ice with the lysis buffer. The soluble fraction was pulled down with sepharose 4B beads overnight at 4°C and subjected to Western blot analysis. For the analysis of the TRAIL-dependent secondary signaling complex, after immunoprecipitation of the death-inducing signaling complex (DISC), DISC-depleted cell lysates were subjected to a second round of immunoprecipitation by using anti-RIP1, followed by Western blotting analysis of communoprecipitated procaspase-8 and cIAP1.

**RNA interference**

RNA interference was done as described previously (23). Briefly, siRNA was used to knock down XIAP, cIAP1, cIAP2, and caspase-8, -9, and -3 (Dharmacon Research, Inc.). Nontargeting control siRNA was purchased from Ambion. Transfections were done using Lipofectamine RNAiMAX (Invitrogen) in the reverse manner according to the manufacturer’s instructions. Between 5 and 10 pmol siRNA and 5 μL Lipofectamine RNAiMAX were mixed in each well of 6-well plates for 20 minutes, followed by culturing 3 × 10^5 cells in the siRNA mix for 24 to 48 hours; knockdown efficacy was assessed by Western blotting.

**In vivo studies**

Nude athymic mice bearing subcutaneous 2LMP xenograft tumors were used. For determination of PARP cleavage, caspase activation and cIAP1 degradation in vivo, tissues were harvested, and tissue lysates were analyzed by Western blotting as described in the Supplementary information. For apoptosis, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and histopathology analysis of tissues, paraffin-embedded tissues were examined as described in the Supplementary information. For the efficacy experiment, nude bearing 2LMP xenograft tumors (8–12 mice per group) were treated with 5 mg/kg of SM-164 i.v., 10 mg/kg of TRAIL i.p., or their combination daily, 5 times a week for 2 weeks. Tumor volume was measured 3 times per week. Data are represented as mean tumor volumes ± SEM. All animal experiments were done under the guidelines of the University of Michigan Committee for Use and Care of Animals.

**Combination index calculation**

Synergy was quantified by combination index (CI) analysis. CI value was calculated by equation as described previously (42, 43): CI = (IC_{A,B}/IC_{A}) + (IC_{A,B}/IC_{B}) - 1. IC_{A} and IC_{B} are the concentrations of drug A and drug B used in combination to achieve x% drug effect. IC_{A,B} and IC_{B,A} are the concentrations for single agents to achieve the same effect. We used IC_{50} values (x% = 50%) to calculate the CI in this study. A CI of less than 0.3 indicates very strong synergy, 0.3 to 0.7 strong synergy, 0.7 to 1 modest synergy, and more than 1 antagonism, respectively.

**Statistical analysis**

Statistical analyses were conducted by 2-way ANOVA and unpaired 2-tailed t test, using Prism (version 4.0; GraphPad). The value of P < 0.05 was considered statistically significant.

**Results**

SM-164 greatly enhances the anticancer activity of TRAIL in both TRAIL-sensitive and TRAIL-resistant cancer cell lines

We tested the combination of SM-164 with TRAIL in a panel of 8 breast, 6 colon, and 5 prostate cancer cell lines
by using the cell viability inhibition assay. SM-164 had no or minimal single-agent activity at concentrations up to 100 nmol/L in these cancer cell lines but showed the strong synergistic activity in combination with TRAIL in 15 cancer cell lines (Fig. 2, and Supplementary Figs. S2–S4). At concentrations of 100 nmol/L, SM-164 reduced the IC₅₀ values of TRAIL by 1 to 3 orders of magnitude in 12 of these 19 cancer cell lines. SM-164 greatly enhanced the anticancer activity of TRAIL, not only in TRAIL-sensitive but also in TRAIL-resistant cancer cell lines in the cell viability assay. The interaction between SM-164 and TRAIL in these 12 cancer cell lines was highly synergistic on the basis of the calculated CI.

SM-164 enhances TRAIL-induced apoptosis in cancer cells through amplification of the caspase-8–mediated extrinsic apoptosis pathway

To gain insights into the underlying mechanism of action for the strong synergy between TRAIL and SM-164, we selected 2LMP, a TRAIL-sensitive breast cancer cell line, and MDA-MB-453, a TRAIL-resistant breast cancer cell line, for further investigations.

SM-164 at 10 nmol/L effectively induced rapid degradation of both cIAP1 and cIAP2 in 2LMP and MDA-MB-453 cancer cell lines (Fig. 3A and Supplementary Fig. S5A). Although either TRAIL or SM-164 (10 and 100 nmol/L) had a minimal effect in induction of apoptosis in both cell lines, the combination was very effective (Fig. 3B and Supplementary Fig. S5B). Although either TRAIL or SM-164 at indicated concentrations had little or no effect on caspase-8, caspase-3, and PARP cleavage, their combination effectively induced robust processing of these proteins at 8- and 16-hour time points in both cell lines (Fig. 3C and Supplementary Fig. S5C). Interestingly, the combination had minimal effect on caspase-9 processing in both cell lines. These data show that SM-164 can greatly enhance apoptosis induction by TRAIL in both TRAIL-sensitive and -resistant cancer cell lines.

We next examined whether the anticancer activity of TRAIL and of the combination depended on caspase activity. We found that knockdown of either caspase-8 or caspase-3 effectively attenuated the cell viability inhibition by TRAIL alone in the 2LMP cell line or by the combination in both cell lines (Fig. 3D and Supplementary Fig. S5D). Knockdown of caspase-9 by siRNA had a minimal effect on the cell viability inhibition by TRAIL alone or by the combination in both cancer cell lines; although the same siRNA effectively attenuated cell viability inhibition by ABT-737, a potent Bcl-2/Bcl-xL inhibitor whose activity is known to be dependent on caspase-9 (Fig. 3D and Supplementary Fig. S5D and E; ref. 44).

SM-164 potentiates cell viability inhibition by TRAIL in both TRAIL-sensitive and TRAIL-resistant cancer cell lines of 3 tumor types. A panel of breast cancer (2LMP, MDA-MB-436, SK-BR-3, and MDA-MB-453), prostate cancer (PC-3 and DU-145), and colon cancer (SW620 and SW480) cell lines were treated with TRAIL (T) alone, SM-164 (Sm; 100 nmol/L) alone, and their combination for 4 days. Cell viability inhibition was determined using a WST-8 assay.
To investigate whether the combination synergy depends on the TRAIL receptors DR4 and/or DR5, DR4 or DR5 was knocked down individually or concurrently by siRNA. Although knockdown of either DR4 or DR5 can attenuate the activity of TRAIL or the combination in the 2LMP cell line, knocking down both receptors was more effective (Supplementary Fig. S6A). In the MDA-MB-453 cell line resistant to TRAIL as a single agent, knockdown of DR4 clearly reduced the activity of the combination, but knockdown of DR5 alone or of both receptors blocked the combination activity (Supplementary Fig. S6B). No increase of DR4 or DR5 protein was observed with TRAIL, SM-164, or their combination in both 2LMP and MDA-MB-453 cell lines (Fig. 3C and Supplementary Fig. S5C). These data indicate that the activity of SM-164 in combination with TRAIL depends on the caspase-8–mediated extrinsic pathway.

**SM-164 enhances TRAIL activity by concurrently targeting XIAP and cIAP1**

Previous studies have focused on XIAP as the primary molecular target for Smac mimetics in combination with TRAIL (36–39). However, because Smac mimetics also effectively induce degradation of cIAP1/2 (25, 26, 28, 29), all these IAP proteins may play a role in the strong synergy of the combination of Smac mimetics and TRAIL. We thus examined the roles of XIAP and cIAP1/2 using 2LMP and MDA-MB-436, two TRAIL-sensitive cell lines, and MDA-MB-453, a TRAIL-resistant cell line. 2LMP, MDA-MB-436, and MDA-MB-453 cells were treated with SMARTpool siRNA against XIAP, cIAP1, and cIAP2, individually or in combinations. Western blotting showed that each of these siRNA or their combinations knocked down their intended target genes efficiently (Fig. 4A and Supplementary Fig. S7) and individual knockdowns, or any combination of
knockdowns, had little or no effect on cell viability. Knockdown of cIAP1 in all these 3 cell lines induced robust upregulation of cIAP2 protein, presumably because of the loss of cIAP1-mediated degradation of cIAP2 (45). Knockdown of cIAP1 or XIAP alone had a modest effect in sensitizing TRAIL in these cell lines, whereas knockdown of cIAP2 alone had little or no effect compared with the control siRNA (Fig. 4A and Supplementary Fig. S7A and B).

In contrast to the modest effect of knockdown of cIAP1 or XIAP alone in TRAIL sensitization, knockdown of both XIAP and cIAP1 greatly sensitized TRAIL in all these 3 cell lines, closely mimicking the strong synergy achieved by SM-164. However, knockdown of cIAP2, in addition to knockdown of XIAP and/or cIAP1, failed to further sensitize TRAIL as compared with the respective knockdown in all of the 3 cell lines (Fig. 4A and Supplementary Figs. S7A and B).

To further test the role of cIAP1 and cIAP2, we used siRNA oligos with different knockdown efficacy. Although the combination of efficient knockdown of XIAP and cIAP1 by 2 different siRNA oligos resulted in robust sensitization of TRAIL in 2LMP cells, closely mimicking the effect achieved by SM-164, inefficient knockdown of cIAP1 by a third oligo did not further enhance TRAIL activity as compared with XIAP knockdown alone (Fig. 4B).

Three siRNA oligos that target different segments of the cIAP2 mRNA were also used to further test the role of cIAP2 in TRAIL sensitization. All 3 oligos efficiently
downregulated cIAP2 in the 2LMP (Supplementary Fig. S8) cell line, but none of them significantly enhanced cell viability inhibition by TRAIL as compared with the nontarget control siRNA. Furthermore, as compared with the XIAP siRNA alone, none of these 3 cIAP2 siRNA oligos in combination with XIAP siRNA further enhanced cell viability inhibition by TRAIL. Taken together, our data show that cIAP2 has a minimal role in blocking TRAIL activity in these cancer cell lines.

To complement these siRNA experiments, we used HCT116 XIAP+/− and XIAP−/− isogenic cell lines (46). Consistent with the original study (46), knockout of XIAP made the HCT116 cells more sensitive than its wild-type counterpart to TRAIL-induced cell viability inhibition, but SM-164 was much more effective than the XIAP gene knockout in sensitizing TRAIL in HCT116 XIAP−/− cells (Fig. 4C). Furthermore, in the XIAP knockout HCT116 cells, SM-164 still greatly enhanced the activity of TRAIL based on cell viability inhibition assay, the activation of caspase-3 and -8, and cleavage of PARP (Fig. 4D and Supplementary Fig. S9).

To examine whether degradation of cIAP1 or cIAP2 by SM-164 accounts for the further sensitization of TRAIL in the HCT116 XIAP knockout cells, cIAP1 or cIAP2 was knocked down individually or concurrently. While knockdown of cIAP1 in the HCT116 XIAP−/− cells further sensitized the cells to TRAIL and closely mimicked the effect achieved by SM-164, knockdown of cIAP2 had a minimal effect (Fig. 4D). Interestingly, although knockdown of cIAP1 again greatly increased the levels of cIAP2 protein, simultaneous knockdown of cIAP1 and cIAP2 in the HCT116 XIAP−/− cells did not further sensitize the cells to TRAIL as compared with knockdown of cIAP1 alone (Fig. 4D). In addition, when cIAP1 was knocked down in the HCT116 XIAP−/− cells, the addition of SM-164 failed to further sensitize the cells to TRAIL (Fig. 4D).

Collectively, our data provide strong evidence that XIAP and cIAP1 are 2 nonredundant blockades of the activity of TRAIL in both TRAIL-sensitive and TRAIL-resistant cancer cell lines, whereas cIAP2 plays a minimal role. Furthermore, XIAP and cIAP1 work in concert in inhibition of the activity of TRAIL and SM-164 enhances the activity of TRAIL by concurrently targeting cIAP1 and XIAP.

**Ablation of cIAP1 markedly enhances the TRAIL-DISC formation**

Previous studies have established that cIAP1 plays a critical role in TNF-α-mediated apoptosis induced by Smac mimetics (25–28). In our case, TNF-α as a single agent was ineffective in induction of apoptosis in cancer cell lines such as 2LMP and MDA-MB-436 (data not shown) that are sensitive to TRAIL as a single agent, suggesting certain key differences between TRAIL-mediated and TNF-α-mediated apoptosis pathways. We therefore investigated the role of cIAP1 in regulation of apoptosis induction by TRAIL alone or in combination with SM-164 in both TRAIL-sensitive and TRAIL-resistant cancer cell lines.

To investigate early events in TRAIL-mediated apoptosis signaling, we treated 2LMP cancer cells for 5, 10, and 30 minutes with a Flag-tagged recombinant TRAIL protein, with or without pretreatment with SM-164. TRAIL-receptor DISC (TRAIL-DISC) was then immunoprecipitated with anti-Flag antibody from the lysates of the treated cells, and the key components in the complex were analyzed by Western blotting (Fig. 5 and Supplementary Fig. S10). Our data showed that TRAIL treatment not only time dependently induced Fas-associated death domain (FADD) and procaspase-8 to form DISC, but it also rapidly recruited TRAF2, cIAP1, and RIP1 to the complex (Fig. 5A and Supplementary Fig. S10). While similar levels of DR4 and DR5 were found in the TRAIL–DISC complex with or without treatment of SM-164, the recruitment of procaspase-8 to the TRAIL-DISC were greatly enhanced at very early time points with SM-164 (Fig. 5 and Supplementary Fig. S10).

To test directly whether SM-164 promotes the TRAIL-DISC formation through induction of cIAP1 degradation, the TRAIL–receptor complex was analyzed in 2LMP cells transfected with siRNA against cIAP1, cIAP2, or XIAP. Knockdown of cIAP1 strongly enhanced procaspase-8 recruitment following TRAIL treatment (Fig. 5B) as compared with nontargeting siRNA, whereas recruitment of procaspase-8 was essentially unchanged in cells transfected with XIAP siRNA and nontargeting siRNA. In contrast to cIAP1, knockdown of cIAP2 did not enhance, and may in fact have attenuated, recruitment of procaspase-8 (Fig. 5B). These data indicate that cIAP1 degradation by SM-164 greatly promotes TRAIL-DISC formation.

**Ablation of cIAP1 facilitates the interaction between RIP1 and caspase-8**

Ablation of cIAP1 by Smac mimetics was shown to greatly enhance recruitment of RIP1 to the TNF-α–receptor complex (25, 26), which plays a major role in activation of caspase-8 and apoptosis induction by Smac mimetics as a single agent. We therefore examined the recruitment of RIP1 to the TRAIL–receptor complex and the role of RIP1 in apoptosis induction by TRAIL alone and in combination with SM-164.

In contrast to the marked enhancement of RIP1 recruitment to TNF-α–receptor complex by Smac mimetics (25, 26), ablation of cIAP1 by SM-164 or siRNA had little or no effect on the recruitment of RIP1 to TRAIL–receptor complex at all the time points examined (Fig. 5A and B and Supplementary Fig. S10).

We next investigated the interaction between RIP1 and caspase-8 in the cytoplasm. On TRAIL treatment, RIP1 formed a complex with caspase-8 within 10 minutes, which was markedly increased at the 30-minute time point (Fig. 5A bottom panel) and TRAIL stimulation also led to an interaction of RIP1 with cIAP1 within 5 minutes. The interaction between RIP1 and cIAP1 was greatly enhanced at very early time points with SM-164 (Fig. 5 and Supplementary Fig. S10).
Figure 5. Ablation of cIAP1 markedly enhances TRAIL-DISC formation and facilitates the intracellular interaction of RIP1 and caspase-8. A, 2LMP cells were treated with a mixture of Flag-tagged TRAIL and anti-Flag M2 IgG with or without pretreatment of 100 nmol/L of SM-164 for 5, 10, and 30 minutes. Cells were lysed and the associated proteins in the lysates were pulled down with sepharose 4B beads and subjected to Western blotting assay (bottom left). B, 2LMP cells transfected with siRNA against IAPs were treated with a mixture of Flag-tagged TRAIL and anti-Flag M2 IgG for 15 minutes. Cells were lysed and the associated proteins in the lysates were pulled down with sepharose 4B beads and subjected to Western blotting as indicated (top). Secondary signaling complexes were immunoprecipitated with a RIP1 antibody from DISC-depleted lysates and analyzed by Western blotting assay (bottom right). C, 2LMP cells were transfected with siRNA against IAPs were treated with a mixture of Flag-tagged TRAIL and anti-Flag M2 IgG with or without pretreatment of 100 nmol/L of SM-164 for 5, 10, and 30 minutes. Cells were lysed and the associated proteins in the lysates were pulled down with sepharose 4B beads and subjected to Western blotting as indicated (top left). D, 2LMP cells transfected with siRNA against IAPs were treated with a mixture of Flag-tagged TRAIL and anti-Flag M2 IgG for 15 minutes. Cells were lysed and the associated proteins in the lysates were pulled down with sepharose 4B beads and subjected to Western blotting as indicated (bottom left). E, 2LMP cells transfected with siRNA against IAPs were treated with a mixture of Flag-tagged TRAIL and anti-Flag M2 IgG for 15 minutes. Cells were lysed and the associated proteins in the lysates were pulled down with sepharose 4B beads and subjected to Western blotting as indicated (top right). Secondary signaling complexes were immunoprecipitated with a RIP1 antibody from DISC-depleted lysates and analyzed by Western blotting (middle). Expression of DR4, DR5, procaspase-8, RIP1, and cIAP1 in the whole cell lysates was examined by Western blotting (top right). Secondary signaling complexes were immunoprecipitated with a RIP1 antibody from DISC-depleted lysates and analyzed by Western blotting (middle). Expression of DR4, DR5, procaspase-8, RIP1, and cIAP1 in the cell lysates was examined by Western blotting (bottom). IP, immunoprecipitation; WCL, whole cell lysates; WB, Western blotting.

and caspase-8 (Fig. 5A bottom panel). To further investigate the role of XIAP, cIAP1, and cIAP2 on the interaction of RIP1 with caspase-8, RIP1 immunoprecipitation was done using the TRAIL–receptor complex-depleted lysates from cells transfected with siRNA against each IAP (Fig. 5B). While knockdown of cIAP1 markedly enhanced the interaction between RIP1 and caspase-8, knockdown of XIAP had a minimal effect and knockdown of cIAP2 seemed even to inhibit the interaction (Fig. 5B). Western blotting showed the absence of DR4 and DR5 in the RIP1 immunoprecipitation, indicating that the interaction of RIP1 with cIAP1 and caspase-8 occurs primarily in the cytoplasm (Fig. 5A bottom panel). Collectively, these data indicate that cIAP1 markedly inhibits the interaction of RIP1 and caspase-8 on TRAIL stimulation and that degradation of cIAP1 greatly enhances this interaction in the cytoplasm and promotes the activation of caspase-8.

**RIP1 plays an essential role in TRAIL sensitization by SM-164 but not in TRAIL as a single agent**

RIP1 plays a key role in TNF-α–mediated apoptosis induction by Smac mimetics (29, 47). However, critical differences exist between TNF-α- and TRAIL-mediated apoptosis induction (48). We therefore examined the role of RIP1 in the activity of TRAIL alone or its combination with SM-164 in TRAIL-sensitive 2LMP, MDA-MB-436, and MDA-MB-468 and TRAIL-resistant MDA-MB-453 cancer cell lines (Fig. 6 and Supplementary Fig. S11–13).

2LMP cells transfected with RIP1 siRNA were treated with TRAIL, SM-164 alone, or their combination, followed by determination of cell death induction and cell viability inhibition and examination of PARP cleavage and processing of caspase-8, -3, and -9 by Western blot (Fig. 6A–C). Efficient RIP1 knockdown had only a modest effect on cell death induction, cell viability inhibition, activation of caspases, and cleavage of PARP by TRAIL as a single agent in 2LMP cells. In contrast, RIP1 knockdown effectively blocked the robust sensitization to TRAIL by SM-164 in 2LMP cells (Fig. 6A–C). Similarly, efficient RIP1 knockdown in MDA-MB-436 and MDA-MB-468 cell lines had little or no effect in cell viability inhibitory activity by TRAIL, but effectively blocked the sensitization by SM-164 in both cell lines (Supplementary Fig. S11 and 12). In the TRAIL-resistant MDA-MB-453 cancer cell line, efficient RIP1 knockdown had no effect on the activity of TRAIL and SM-164 as a single agent, but it blocked the robust cleavage of PARP and processing of caspase-8 and -3 and cell viability inhibition by the combination (Supplementary Fig. S13A and B).

To complement the siRNA experiments, we next used the Jurkat cell line and its RIP1 knockout counterpart to further investigate the role of RIP1 (49). Although SM-164 clearly sensitized TRAIL in inhibition of cell
viability in the Jurkat parental cell line, it was completely ineffective in enhancing the activity of TRAIL in the Jurkat RIP1 knockout cell line (Fig. 6D).

These data show that although RIP1 plays a minimal role in the activity of TRAIL as a single agent, it is required for the synergistic interaction between SM-164 and TRAIL.

**SM-164 enhances apoptosis induction by TRAIL in xenograft tumor tissues and their combination achieves tumor regression**

To further evaluate the therapeutic potential of SM-164 in combination with TRAIL, we tested the in vivo activity for TRAIL and SM-164 as single agents and their combination using the 2LMP xenograft model.

A single dose of SM-164 at 5 mg/kg i.v. was highly effective in induction of cIAP1 degradation (Fig. 7A) but failed to induce caspase-3 activation, PARP cleavage, or apoptosis over a 6- to 24-hour time period in tumor tissues (Fig. 7A and B). Although TRAIL is very effective as a single agent in cell viability inhibition in vitro in the 2LMP cell line, a single dose of TRAIL at 10 mg/kg induced only modest caspase-3 activation, PARP cleavage, and minimal apoptosis in tumor tissues (Fig. 7A and B). In contrast, their combination induced robust caspase-3 activation, PARP cleavage, and strong apoptosis in tumor tissues (Fig. 7A and B); 50% of tumor cells were in fact TUNEL positive in tumor tissues at the 6-hour time point (Fig. 7B, right panel, Supplementary Fig. S14). Hematoxylin and eosin (H&E) staining further showed that the combination caused extensive damage to tumor tissues (Fig. 7C). In comparison, SM-164, TRAIL alone, and their combination had no effect on all normal mouse tissues examined, including highly proliferative tissues such as spleen, small intestine, and bone marrow (Supplementary Fig. S15).

We next examined the combination efficacy and potential toxicity with systemic administration of these 2 agents against established 2LMP tumors (Fig. 7D). SM-164 (5 mg/kg, i.v. daily, 5 days per week) and TRAIL (10 mg/kg i.p. daily, 5 days per week) were administered alone or in combination for 2 weeks. Although neither SM-164 nor...
TRAIL as single agent resulted in any signs of toxicity in mice, they failed to achieve any significant antitumor activity. In contrast, their combination induced tumor regression. At the end of the treatment, the combination reduced the mean tumor volume by 80% and the tumors in the combination treatment group continued to shrink after treatment ended and were undetectable on day 33 in 6 of 8 cases. This strong antitumor activity by the combination was persistent and 3 of the 8 tumors remained completely regressed 3 months after the treatment concluded. In comparison, the mean tumor volume in the control and the treatment groups with TRAIL alone or SM-164 alone were more than 1,000 mm³ on day 43 (Fig. 7D, left panel).

The combination treatment caused no gross abnormalities or other signs of toxicity. Mice treated with the combination experienced a slight but statistically insignificant body weight loss at the end of 2 weeks of treatment and all regained their weight by day 33 (Fig. 7D, right panel).

Our *in vivo* data thus show that although neither SM-164 nor TRAIL shows appreciable antitumor activity as a single agent, their combination is highly effective in induction of apoptosis and achieves tumor regression. The combination is also selectively toxic to tumor tissues over normal mouse tissues. Taken together, our *in vivo* data further suggest that the combination of SM-164 and TRAIL may have considerable therapeutic potential for the treatment of human cancers.
Discussion

Both TRAIL and Smac mimetics are being developed in the clinic as new anticancer drugs. Although TRAIL is well tolerated in patients, its single-agent efficacy is very limited (31–33). Similarly, extensive preclinical and early clinical data also suggest that Smac mimetics may have very limited single-agent activity (24, 27, 30). Therefore, rational combination strategies are clearly needed for the successful development of both TRAIL and small-molecule Smac mimetics as new anticancer drugs.

Earlier observations using Smac-based peptides and subsequent studies using potent, cell-permeable small-molecule Smac mimetics have indicated that there is a very strong synergy between Smac-based compounds and TRAIL against human cancer cell lines originating from different tissues (34–40). The data obtained from this study using SM-164, a highly potent Smac mimetic, with TRAIL also clearly show that SM-164 is capable of dramatically enhancing the anticancer activity of TRAIL in vitro in more than 70% of human breast, prostate, and colon cancer cell lines and in both TRAIL-sensitive and TRAIL-resistant cancer cell lines. For the first time, we have shown that although both TRAIL and SM-164 alone have no single-agent activity in a xenograft model of human breast cancer, their combination can achieve rapid tumor regression while showing no toxicity to animals. Our in vitro and in vivo data, together with those from previous studies (34–40), strongly suggest that combination of Smac mimetics with TRAIL warrants clinical investigation as an attractive new cancer therapeutic strategy.

The design of Smac mimetics was based on the interaction between XIAP and Smac and previous investigations on the combination of Smac mimetics with TRAIL have focused on XIAP as the primary molecular target for Smac mimetics (34–39). However, Smac mimetics also bind to cIAP1 and cIAP2 with very high affinities and induce rapid degradation of these cIAP proteins. Degradation of cIAP1/2 is an early and key event in TNF-α-mediated apoptosis induction by Smac mimetics as single agents. However, the role of cIAP1/2 proteins in the combination of Smac mimetics with TRAIL has not been defined. Using siRNA technology and XIAP knockout cells, our data show that XIAP and cIAP2 are 2 nonredundant inhibitors of TRAIL-induced apoptosis. Although ablation of either XIAP or cIAP1 can modestly sensitize TRAIL in both TRAIL-sensitive and TRAIL-resistant cancer cell lines, targeting both XIAP and cIAP1 can achieve a much stronger effect. Furthermore, although knockdown of cIAP1 by siRNA can robustly upregulate cIAP2, knockdown of cIAP2 does not further sensitize cancer cells to TRAIL. Moreover, ablation of cIAP1 by siRNA or by SM-164 greatly enhances the recruitment of caspase-8 and FADD to the TRAIL death–receptor complex, promoting the interaction of RIP1 with caspase-8 in the cytoplasm and activation of caspase-8, but ablation of cIAP2 fails to lead to any of these outcomes, indicating a differential role for cIAP1 and cIAP2 in TRAIL sensitization. The strong sensitization of TRAIL achieved by SM-164 is closely mimicked by concurrent ablation of both cIAP1 and XIAP. In cancer cells, when both XIAP and cIAP1 are removed, SM-164 is unable to further sensitize the cells to TRAIL. Collectively, these data provide strong evidence that XIAP and cIAP1 are the primary cellular targets for SM-164 in its synergistic interaction with TRAIL. Because both XIAP and cIAP1 proteins are overexpressed in tumor cells, the ability of SM-164 to concurrently and potently target XIAP and cIAP1 may prove to be a unique advantage in achieving a strong synergy with TRAIL.

Our data supported that similar to TNF-α, TRAIL induces apoptosis through both RIP1-dependent and -independent pathways (47). As a single agent, TRAIL induces apoptosis in a RIP1-independent manner. The synergy between TRAIL and Smac mimetics, however, relies on the interaction of procaspase-8 with RIP1 and is RIP1 dependent. Knockdown of RIP1 abrogates or dramatically attenuates cell viability inhibition by the combination (Figs. 5B and C and 6A–D). This RIP1-dependent event takes place once cIAP1 is removed by a Smac mimetic or siRNA (Fig. 5A and C and Fig. 6A–D). The interaction of procaspase-8 and RIP1 is detected in the cytoplasm (Fig. 5B and C), showing that RIP1-dependent caspase-8 activation primarily takes place in cytoplasm. However, removal of cIAP1 also markedly increases the procaspase-8 recruitment to TRAIL–receptor complex (Fig. 5A).

Although SM-164 potently antagonizes XIAP and induces efficient downregulation of cIAP1 in all the cancer cell lines we have evaluated in this study, SM-164 enhances the anticancer activity of TRAIL in the majority but not all of cancer cell lines examined. These data show that although XIAP and cIAP1 effectively inhibit the activity of TRAIL, they are not the only proteins that mediate the resistance of cancer cells to TRAIL. For example, cFLIP (40, 50) and Mcl-1 (51) can also effectively attenuate the activity of TRAIL and may play a role in mediating the resistance of cancer cells to the combination of Smac mimetics and TRAIL.

In summary, this study furthers our understanding on the underlying molecular mechanism of the strong synergy between Smac mimetics and TRAIL and provides strong support that the combination of Smac mimetics and TRAIL should be evaluated in the clinic as a new cancer therapeutic strategy for the treatment of a variety of human cancers.

Disclosure of Potential Conflicts of Interest

S. Wang serves as a consultant for Ascenta Therapeutics and owns stocks and stock options in Ascenta Therapeutics, which is developing a Smac mimetic for cancer treatment.
Combination of Smac Mimetic SM-164 with TRAIL

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 September 17, 2010; revised January 19, 2011; accepted February 12, 2011; published OnlineFirst March 3, 2011.

Grant Support

The work was supported by the Breast Cancer Research Foundation (S. Wang), the Susan G. Komen Foundation (H. Sun), National Cancer Institute grants R01CA109253 (S. Wang) and R01CA127551 (S. Wang), and University of Michigan Cancer Center Core grant from the National Cancer Institute P30CA046592.

References


# Molecular Cancer Therapeutics

## Therapeutic Potential and Molecular Mechanism of a Novel, Potent, Nonpeptide, Smac Mimetic SM-164 in Combination with TRAIL for Cancer Treatment

Jianfeng Lu, Donna McEachern, Haiying Sun, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-10-0864</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://mct.aacrjournals.org/content/suppl/2011/03/03/1535-7163.MCT-10-0864.DC1">http://mct.aacrjournals.org/content/suppl/2011/03/03/1535-7163.MCT-10-0864.DC1</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 47 articles, 21 of which you can access for free at: <a href="http://mct.aacrjournals.org/content/10/5/902.full.html#ref-list-1">http://mct.aacrjournals.org/content/10/5/902.full.html#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 8 HighWire-hosted articles. Access the articles at: /content/10/5/902.full.html#related-urls</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</td>
</tr>
</tbody>
</table>