Smac mimetics in combination with TRAIL selectively target cancer stem cells in nasopharyngeal carcinoma

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Running Title: Targeting CSCs in Nasopharyngeal Carcinoma

Keywords: Cancer stem cells, Smac mimetic, TRAIL, Nasopharyngeal Carcinoma

Abbreviation List:
CSCs, Cancer Stem Cells; IAPs, Inhibitors of Apoptosis Protein Family; TRAIL, TNF-Related Apoptosis Inducing Ligand; ILP2, IAP-like Protein; ML-IAP, Melanoma IAP; XIAP, X-link IAP; cIAP1, Cellular IAP1; cIAP2, Cellular IAP2; NAIP, Neuronal Apoptotic Inhibitor Protein; BRUCE, BIR-containing Ubiquitin Conjugating Enzyme; Smac/DIABLO, Second mitochondria-derived activator of caspasies /Direct IAP Binding Protein with Low Isoelectric Point; FTC, fumitremorgin C; RTV, relative tumor volume.

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No potential conflicts of interest were disclosed.
Abstract

Nasopharyngeal carcinoma is a common malignancy in Southern China. After radiotherapy and chemotherapy, a considerable proportion of nasopharyngeal carcinoma patients suffered tumor relapse and metastasis. Cancer stem cells (CSCs) have been demonstrated with the resistance against therapies and thus considered as the initiator of recurrence and metastasis in tumors, where the anti-apoptotic property of CSCs play important role. Smac/DIABLO is an inverse regulator for the inhibitors of apoptosis protein family (IAPs), which have been involved in the apoptosis. Here, the effects of Smac mimetics on the CSCs of nasopharyngeal carcinoma were studied both in vitro and in vivo, using two clones of nasopharyngeal carcinoma cell line CNE2 as models. We found that one of the clones, S18, had CSC-like properties and IAPs were overexpressed. The combination of Smac mimetics and TNF-related apoptosis inducing ligand (TRAIL) can reduce the percentage of SP cells and inhibit the colony- and sphere-forming abilities of S18 cells, indicating their ability to attenuate the CSCs. Moreover, in a nasopharyngeal carcinoma xenograft model, the administration of Smac mimetics in combination with TRAIL also led to the elimination of nasopharyngeal carcinoma stem cells. Furthermore, the Smac mimetics in combination with TRAIL induced the degradation of cIAP1 and XIAP and thus induced apoptosis in vitro and in vivo. Taken together, our data show that Smac mimetics exerted an antitumor effect on nasopharyngeal carcinoma cancer stem cells, and this combination treatment should be considered as a promising strategy for the treatment of nasopharyngeal carcinoma.
Introduction

Nasopharyngeal carcinoma is an endemic malignancy that occurs predominantly in populations from southern China, Southeast Asia, North Africa, and the Arctic Circle (Eskimos and other Arctic natives) (1). The application of chemotherapeutic drugs becomes more important in the treatment for nasopharyngeal carcinoma in recent years, and cisplatin, 5-fluorouracil and taxel are the most commonly used drugs (2, 3). However, some patients still suffered from failure of treatment including relapse and metastasis, which thought to be originated from cancer stem cells (3-9).

Cancer stem cells (CSCs) have the properties of self-renewal, differentiation and resistance to chemotherapy or radiotherapy (7, 10). Although the CSCs represent a small proportion of the tumor cells, they are key players in tumor initiation, recurrence and metastasis (8-12). Therefore, CSCs has been considered as the important therapeutic target in anticancer treatments (6, 9, 13-16). Many compounds have been shown to selectively target CSCs in cancers, such as salinomycin (6), TβRI inhibitors (9), lupeol (14), sulforaphane (15), thioridazine (16), and others.

As in other cancers, nasopharyngeal carcinoma contains a small fraction of tumor cells with properties of CSCs. Studies have demonstrated that the Side population (SP) cells, identified by having ability to pump out a fluorescent dye (Hoechst 33342), have certain characteristics of CSCs similar with those in liver cancer and gastrointestinal system cancer, suggesting that SP phenotype can be a marker of CSC for nasopharyngeal carcinoma (13, 17, 18). However, no effective compound has been discovered to target nasopharyngeal carcinoma CSCs.

CSCs are thought as the key players in the resistance to chemo- or radio-therapy (11, 12), while their ability to escape from the apoptosis pathway may render them the resistance
property to the therapies (19, 20). It has been shown that the inhibitors of apoptosis protein (IAP) family members are important anti-apoptotic proteins to regulate the apoptosis processes (19-21). Among the eight proteins in the family, namely survivin, IAP-like protein (ILP2), melanoma IAP (ML-IAP), X-link IAP (XIAP), cellular IAP1 (cIAP1), cellular IAP2 (cIAP2), neuronal apoptotic inhibitor protein (NAIP) and BIR-containing ubiquitin conjugating enzyme (BRUCE), XIAP is the most well characterized one, and cIAP1 and cIAP2 are the two closest XIAP paralogs (22, 23). Moreover, XIAP is the only IAP protein that binds directly to caspases, and inhibits their activities, which result in promoting resistance to apoptosis in cancer cells (20, 24, 25). In contrast, the cIAPs can bind to caspases without inhibiting their activities (23).

On the other hand, second mitochondria-derived activator of caspases /direct IAP binding protein with low isoelectric point (Smac/DIABLO) is a negative regulator of IAP proteins and released in response to apoptotic stimuli (26). By binding to XIAP and cIAPs, Smac can release the inhibition of caspase or lead to the degradation of cIAPs, which turn the cells into apoptosis process (26, 27). Therefore, regarding the potency of Smac to revert the anti-apoptosis of cancer cells, several Smac mimetics have been designed and synthesized as antitumor drugs in recent years (28-31). These compounds can induce cIAP1/2 degradation and prevent XIAP from binding to caspases, which induce the apoptosis of tumor cells with little effect on normal cells (27, 32). In addition, synergy effect has been reported for Smac mimetics and TNF-related apoptosis-inducing ligand (TRAIL), which is a TNF family ligand with ability to induce apoptosis in cancer cells (33-36).

Attempting to provide more effective treatment for nasopharyngeal carcinoma patients, we evaluated two Smac mimetics AT-406 and SM-164, in combination with TRAIL (37, 38), for their
abilities to selectively target CSCs in nasopharyngeal carcinoma. Our study showed that IAP proteins were overexpressed in nasopharyngeal carcinoma cancer stem cells and Smac mimetics can selectively reduce CSCs both in vitro and in vivo. The results suggest that the Smac mimetics AT-406 and SM-164 may be promising drugs for the effective treatment of nasopharyngeal carcinoma.

Materials and Methods

Cell Culture

S18 and S26 cells, clones of the human nasopharyngeal carcinoma cell line CNE2, were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 units/ml penicillin G and 100 μg/ml streptomycin at 37°C in 5% CO₂. These two clones of CNE2 were kind gifts from Dr. Chaonan Qian (Sun Yat-sen University Cancer Center, China). All cell lines were passaged less than six months.

SP Detection

S18 and S26 cells were treated with the test compounds (negative control, 5 ng/ml TRAIL, 5 μM AT-406, 0.1 μM SM-164, 5 μM AT-406 + 5 ng/ml TRAIL, or 0.1 μM SM-164 + 0.1 ng/ml TRAIL) for 48 hours, harvested and then resuspended in an ice-cold DMEM (supplemented with 2% fetal bovine serum) at a density of 1×10⁶ cells/ml. Then, the cells were incubated at 37°C in 5% CO₂ for 10 minutes. The DNA binding dye Hoechst 33342 (Sigma-Aldrich) was then added to the cells at a final concentration of 5 μg/ml (as a negative control, cells were incubated with 10 μM fumitremorgin C (FTC, an inhibitor of ABCG2 which could block the pumping out of Hoechst
33342 in CSCs, Sigma-Aldrich) for 5 minutes prior to the addition of the Hoeches dye), and the cells were incubated at 37°C in 5% CO₂ in the dark for 90 minutes and mixed every 15 minutes. Then, the cells were washed twice with PBS, resuspended in PBS and kept at 4°C in the dark before flow cytometric analysis (Experience Xtremes MoFlo XDP cell Sorter, Beckman Coulter).

**RNA extraction, reverse transcription and quantitative real-time PCR**

Total RNA of S18 and S26 cells were extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized using Thermo Scientific Maxima First cDNA Synthesis Kit (Thermo). Real-time PCR amplification was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) on a Hard-Shell PCR Plates (Bio-Rad). Relative quantification of each target gene was normalized by using an endogenous control (GAPDH).

**Cell Viability Assay**

Cell viability was measured using MTT assay. S18 and S26 cells were counted, plated in triplicate at 2500 cells per well (200 μl) in 96-well plates, and allowed to grow overnight. For individual groups, cisplatin, 5-fluorouracil, taxel, or TRAIL, AT-406, SM-164 was added to the wells in a concentration gradient. For combination groups, negative control, 5 μM AT-406, 0.1 μM SM-164 were mixed with a concentration gradient of TRAIL, and then added to the wells. Cell viability was measured 48 hours later by adding MTT solution. The observation value was detected at 490 nm.

**Colony Formation Assay**
S18, S26 or treated S18 cells (treated with negative control, 5 ng/ml TRAIL, 5 μM AT-406, 0.1 μM SM-164, 5 μM AT-406 + 5 ng/ml TRAIL, or 0.1 μM SM-164 + 0.1 ng/ml TRAIL for 48 hours prior) were counted, plated in triplicate at 100 cells per well in 6-well plates (Corning), and cultured in DMEM (supplemented with 10% fetal bovine serum) for approximately 10 days. Then, the cells were washed twice with PBS and fixed in methanol for approximately 10 minutes. After two additional washes with PBS, the cells were dyed with crystal violet for 30 minutes. Then, the crystal violet was washed out and the numbers of the colonies were counted.

**Sphere Formation Assay**

S18, S26 or treated S18 cells (treated with negative control, 5 ng/ml TRAIL, 5 μM AT-406, 0.1 μM SM-164, 5 μM AT-406 + 5 ng/ml TRAIL, or 0.1 μM SM-164 + 0.1 ng/ml TRAIL for 48 hours prior) were counted, plated in triplicate at 300 cells per well in ultra-low attachment 6-well plates (Corning), and cultured in DMEM/F12 medium (Invitrogen) with 20 ng/ml recombinant human fibroblast growth factor-basic (amino acids 1-155) (Invitrogen), 20 ng/ml recombinant human epidermal growth factor (Hu EGF) (Invitrogen) and B-27 supplement (Invitrogen) for approximately 2 weeks. The spheres were counted under a light microscope.

**Cell Apoptosis Detection**

Drug-induced apoptosis was evaluated by Annexin V and Propidium Iodide (PI) staining using an Annexin V-EGFP apoptosis detection kit (KeyGEN). Treated S18 and S26 cells (treated with negative control, 5 ng/ml TRAIL, 5 μM AT-406, 0.1 μM SM-164, 5 μM AT-406 + 5 ng/ml TRAIL, or 0.1 μM SM-164 + 0.1 ng/ml TRAIL for 48 hours) were harvested, washed twice with PBS, and
resuspended in Binding Buffer (500 μl, 1-5×10^5 cells). Annexin V-EGFR and PI (5 μl each) were then added to the cells, and the mixture was incubated for 15 minutes in the dark at room temperature. The stained cells were analyzed using a Cytomics FC500 flow cytometer (Beckman Coulter).

**Western Blot Analysis**

Compound treated S18 and S26 cells or xenograft tumor tissues were lysed in lysis buffer on ice, electrophoresed in a 10% Bis-Tris gel in MOPS running buffer and transferred to polyvinylidene difluoride membranes. The membranes were then blocked in 5% milk for 1 hour and subsequently incubated with various primary antibodies at 4°C overnight, followed by incubation with secondary antibodies conjugated to horseradish peroxidase. The chemiluminescence reagent was then added, and the signals were detected using a sheet of photographic film.

**Antibodies and drugs**

The antibodies used for the western blotting were as follows: NAIP (#5782-1, Epitomics), cIAP1 (#7065, Cell Signaling Technology), cIAP2 (#3130, Cell Signaling Technology), XIAP (#2042, Cell Signaling Technology), survivin (#2808, Cell Signaling Technology), livin(#5471, Cell Signaling Technology), PARP (#9542, Cell Signaling Technology), caspase 3 (3G2) (#9668, Cell Signaling Technology), cleaved caspase 3 (Asp175) (5A1E) (#9664, Cell Signaling Technology), tubulin (AT819, Beyotime), and actin (#60008-1-lg, Proteintech). TRAIL was provided by the Ascentage Pharma Group Corp. Limited. in Shanghai, China.
Animal experiments

For the tumorigenesis assay, 4-week-old female athymic nude mice were obtained from the Animal Experimental Center of the Guangdong Academy of Medical Sciences (Guangzhou, China) and were given subcutaneous injections of $1 \times 10^3$, $5 \times 10^3$, $1 \times 10^4$, $5 \times 10^4$, $1 \times 10^5$, or $5 \times 10^5$ S18 or S26 cells in their left or right axillary area. The mice were monitored twice per week for 5 weeks.

For the compound sensitivity assay, 4-week-old female athymic nude mice were obtained from the Sino-British Sippr/BK Lab. Animal LET., Co. (Shanghai, China) and were subcutaneous injected $1 \times 10^6$ S18 or S26 cells in the right axillary area. When the xenograft tumors developed to approximately 100 mm$^3$, the mice were randomly divided into 6 groups (for each cell line) with no differences in tumor size. Then, the mice were treated with AT-406 at 100mg/kg, po, qd, 1-5week x 3weeks, or SM-164 at 3mg/kg, iv, qd, 1-5week x 3weeks alone or in combination with TRAIL at 10mg/kg, iv, qdx3weeks. Tumor volume and body weight were measured 2 times per week. The T/C rate was also used to evaluate the tumor response to these compounds. T/C rate was calculated using the ratio of the relative tumor volume (RTV) of the treated group (T) to the RTV of the control group (C). The RTV was calculated using the ratio of the average tumor volume of the day n to the average tumor volume of the day 0 when the injection of compounds began.

All animal studies were approved by the Sun Yat-sen University Cancer Center Animal Care and Ethics Committee.

TUNEL Staining

Tumor tissues from the animal experiments were formalin-fixed and embedded in paraffin.
All sample sections were dyed with hematoxylin and eosin and microscopically examined to confirm the nasopharyngeal carcinoma cell origin. The samples were dewaxed, rehydrated using xylene and ethanol, incubated with a proteinase K working solution with microwave irradiation in 0.1 M citrate buffer, and then stained with the TUNEL reaction mixture (Roche Applied Science) for 1 hour. Then, the samples were incubated with Converter-POD (Roche Applied Science) for 30 minutes at 37°C for 1 hour. All samples were visualized using diaminobenzidine (DAB) (DAKO), and the nuclei were counterstained with hematoxylin.

Statistical methods

Sigmaplot and SPSS 13.0 were used for statistical analysis. All in vitro experiments were repeated 3 times. Data were presented as mean values and standard deviations, and the differences between groups were evaluated using Student’s t-test. P<0.05 was considered to be statistically significant.

Results

Differences between the CSC properties of S18 and S26 cells

The CSC properties of two clones of nasopharyngeal carcinoma CNE2 cells, S18 and S26, were evaluated. The S18 cells have been previously reported to have greater migration and invasion abilities than S26 cells (39). It was discovered that the SP cell population in S18 cells was approximately 27-fold higher than that in the S26 cells (Fig. 1A and C). Furthermore, the S18 cells were found to be more resistant than S26 cells to three chemotherapeutic drugs (cisplatin, 5-fluorouracil and taxel) commonly used to treat nasopharyngeal carcinoma (Table 1).
expression levels of cancer stem cells markers in nasopharyngeal carcinoma, ABCG2 and CD44, were also higher in S18 cells (Fig. 1B). These observations showed that S18 cells may have cancer stem cell properties.

Then a colony formation assay was performed, and S18 cells were better able to form colonies than S26 cells (Fig. 1D and Supplementary Fig. S1A). When the sphere formation ability of these two cells was evaluated, S18 cells were found to exhibit stronger sphere formation ability, in contrast to the S26 cells (Fig. 1E and Supplementary Fig. S1B). Tumor seeding ability of these two cell lines was also examined and tumors can be generated with $5 \times 10^3$ S18 cells, whereas $1 \times 10^5$ S26 cells were required for tumor generation (Table 2). All of these findings indicate that S18 cells acted as CSCs.

Having observed that S18 cells exhibit strong resistance to chemotherapeutic drugs, we next wanted to determine whether the apoptosis pathway was inhibited in S18 cells. Apoptosis-related proteins play a very important role in the inhibition of the apoptosis pathway (21). The IAP family protein levels were measured in S18 and S26 cells and S18 cells expressed higher levels of IAPs than S26 cells (Fig. 1F). These data indicated that IAPs play important roles in nasopharyngeal carcinoma CSCs.

We also wanted to know whether TRAIL and the Smac mimetic AT-406(40) and SM-164(38) could affect S18 and S26 cells. Using the MTT assay, it was found that TRAIL had little effect on S18 cells, in contrast to the effect on S26 cells (Fig. 1G). Even when the concentration of TRAIL was increased to 1000 ng/ml, the cytotoxic effect was still weak in the S18 cells (Fig. 1G). The Smac mimetic AT-406 and SM-164 had moderate cytotoxic effects on S18 and S26 cells at high concentration, and these effects were more evident in S26 cells (Table 1).
The Smac mimetics in combination with TRAIL selectively inhibit nasopharyngeal carcinoma CSC growth and attenuate nasopharyngeal carcinoma CSCs

Based on the results above, we investigated whether two Smac mimetics, AT-406 and SM-164, could selectively inhibit S18 cell growth in vitro in combination with TRAIL (Fig. 2A). MTT assays to determine the cell growth inhibition properties of the two compounds showed that both compounds, especially SM-164, could selectively inhibit S18 cell growth when used in combination with TRAIL (Fig. 2A).

Whether AT-406 or SM-164 in combination with TRAIL could attenuate nasopharyngeal carcinoma cancer stem cells was also tested. SP detection showed that treatment with AT-406 or SM-164 decreased the proportions of SP cells in S18 cells, especially when used in combination with TRAIL. For example, the percentages of SP cells decreased from 60% in the untreated group to approximately 25% in the AT-406 or SM-164 + TRAIL treated group, whereas the percentage of SP cells in a cisplatin-treated S18 cells population was up to 90% (Fig. 2B and C). The colony formation assay and sphere formation assay also showed that AT-406 and SM-164 could inhibit the colony formation and sphere formation abilities of S18 cells, especially when combined with TRAIL (Fig. 2D, E and Supplementary Fig. S2A and B).

AT-406 and SM-164 sensitize nasopharyngeal carcinoma xenografts to TRAIL therapy

The above results indicated that treatment with AT-406 or SM-164 in combination with TRAIL selectively targeted nasopharyngeal carcinoma CSCs in vitro. We then generated tumors in mice and tested whether AT-406 and SM-164 could affect tumor growth when combined with
TRAIL in vivo.

Mice were injected with S18 or S26 cells, and when the resulting palpable tumors reached a size of 100 mm$^3$, the mice were treated with normal saline or with TRAIL (10 mg/kg, iv), AT-406 (100 mg/kg, po), and SM-164 (3 mg/kg, iv) or in combination 5 days per week for 3 weeks. Tumor volume and body weight were measured 2 times per week. We found that the tumor volumes of mice treated with AT-406 or SM-164 in combination with TRAIL were much smaller than those of mice treated with normal saline (Fig. 3A and B). Interestingly, the two Smac mimetic compounds seemed to be more effective against S18 cell xenografts than against S26 cell xenografts (Fig. 3A and B). This result indicated that Smac mimetics also have anti-cancer stem cell properties in vivo. There has not significant toxicity in mice treated with SM-164 in combination with TRAIL (Fig. 3C and D). AT-406 in combination with TRAIL resulted in a weight loss in the first 10 days (Fig. 3C and D). However, the changes were recovered after stopping the treatment (Fig. 3C and D). And the T/C ratio were 27.7 and 26.6 when treated with Smac mimetics in combination with TRAIL in S18 xenografts, while T/C ratio were 71.2 and 47.2 in S26 xenografts (Table 3).

Smac mimetics in combination with TRAIL can induce IAPs degradation and lead to apoptosis in tumor cells

As the smac mimetics are important inhibitors of IAP family. We also wanted to know whether these compounds could induce cell apoptosis in vitro. Annexin V and PI staining showed that both AT-406 and SM-164 in combination with TRAIL could induce apoptosis in nasopharyngeal carcinoma cells (Fig. 4A). Western blotting analysis also showed treatment with AT-406 or SM-164 in combination with TRAIL led to decreased levels of procaspase 3 and
pro-PARP, while the levels of cleaved caspase 3 and PARP were increased in nasopharyngeal carcinoma cells (Fig. 4B). And the apoptosis induced by these two compounds was stronger in S18 cells than in S26 cells (Fig. 4A and B). These observations indicated that the Smac mimetic AT-406 and SM-164, in combination with TRAIL, have a selective cancer stem cell killing effects in nasopharyngeal carcinoma.

Then the tissues from the compound-treated mice were evaluated to determine whether these compounds could induce apoptosis in vivo. Increased levels of cleaved caspase 3 and PARP protein determined by western blotting analysis of the tissue from mice showed that apoptosis was induced in the compound-treated mice (Fig. 4C). TUNEL staining of the tissues from mice also confirmed these results (Fig. 4D).

It has been reported that XIAP is the only IAP protein that can bind directly to and inhibit caspases, indicating that XIAP must play a critical role in the apoptosis pathway (20, 24, 25). The cIAP1 and cIAP2 proteins are two XIAP paralogs that may also play important role in apoptosis (22, 23). cIAP1 and XIAP, which are expressed at higher levels in S18 cells, were degraded when the cells were treated with Smac mimetics in combination with TRAIL, both in vitro and in vivo (Fig. 4B and C). These observations indicated that the overexpression of the cIAP1, cIAP2 and XIAP proteins in S18 cells led these cells to become much more sensitive to the inhibition of IAPs. All of these observations indicate that the Smac mimetics AT-406 and SM-164 in combination with TRAIL, can selectively target cancer stem cells in nasopharyngeal carcinoma by inhibiting the cIAP1, cIAP2 and XIAP proteins.

Discussion
Although the 5-year overall survival (OS) of patients with nasopharyngeal carcinoma has improved, the current treatments for nasopharyngeal carcinoma still have some drawbacks. Most importantly, these treatments cannot prevent nasopharyngeal carcinoma relapse due to the development of drug resistance and metastasis, and CSCs may play a critical role in these processes (3, 12). Common chemotherapeutic drugs or radiotherapy may kill differentiated cells but fail to eliminate CSCs. A drug that selectively targets CSCs may be an effective cure for nasopharyngeal carcinoma when combined with common chemotherapeutic drugs or radiotherapy.

There are many markers that allow the identification of CSCs in different cancers, such as CD24, CD44, CD133, and the side population (SP) phenotype (6, 9, 13, 17). Here, we used the SP phenotype to identify CSCs in nasopharyngeal carcinoma and found that S18, a clone of nasopharyngeal carcinoma CNE2 cells, contains high levels of SP cells. S18 cells also exhibit strong sphere and colony formation abilities. These cells are resistant to commonly used chemotherapeutic drugs, and only a small number of cells are required to seed tumors. All of these features indicate that S18 cells act as CSCs among nasopharyngeal carcinoma CNE2 cells.

Cancer stem cells have one common feature—the loss of apoptosis, and the overexpression of IAP proteins may be involved in this phenotype (19, 20). IAPs are the last safeguards against apoptosis pathway, and can inhibit the caspase activity, which result in promoting resistance to apoptosis in cancer cells (20, 22). In this study, S18 cells were found to express higher levels of cIAP1, cIAP2 and XIAP proteins than other clones. These results indicate that cIAP1, cIAP2 and XIAP may play critical roles in nasopharyngeal carcinoma CSCs. Therefore, we treated nasopharyngeal carcinoma cells with Smac mimetics in combination with TRAIL to test whether...
these molecules can target nasopharyngeal carcinoma stem cells (35, 41).

Smac mimetics, designed as negative regulators of IAP proteins, can induce apoptosis in cancer cells by antagonizing XIAP and cIAP1/2 (26, 27, 37, 38). Some of the smac mimetics are currently evaluated in phase I clinical trials as potential therapeutic drugs in the treatment of human tumors (40, 42). Tumor necrosis factor-related apoptosis inducing ligand (TRAIL), a TNF family ligand that binds to the death receptor, is currently in development as an agent that targets the apoptosis pathway (33, 34). Moreover, TRAIL-induced apoptosis seems to play an important role in the malignant transformation (43). TRAIL kills transformed cells but not normal cells, demonstrating that this compound has low toxicity (43). However, some cancers are resistant to TRAIL, making it a prime candidate for combination with other safe agents for cancer treatment (44). In a phase I dose-escalation study, TRAIL seem to be safe and well tolerated (45). This research demonstrated that, unlike other molecules that target only differentiated tumor cells, the Smac mimetics AT-406 and SM-164 in combination with TRAIL, may target cancer stem cells. We observed that AT-406 and SM-164 have the ability to target S18 cells when combined with TRAIL in vitro. The in vivo data confirm this result and show that these treatments have no toxicity in mice.

It has been reported that XIAP is the only IAP protein that can directly bind to and inhibit caspases (20, 24, 25); cIAP1/2 are two paralogs of XIAP (22, 23). In this study, we found that Smac mimetics, especially when used in combination with TRAIL, can induce the degradation of cIAP1 and XIAP. This treatment also induces the cleavage of caspase 3 and PARP, indicating the induction of the apoptosis pathway. Because nasopharyngeal carcinoma stem cells have higher levels of cIAP1, cIAP2 and XIAP than other cells, these cells may be more sensitive to treatment
with Smac mimetics in combination with TRAIL.

Smac mimetic has been reported to sensitize cancer cells to TRAIL-induced apoptosis (35-37, 46), but this study is the first, to our knowledge, to show that smac mimetic in combination with TRAIL can targeted CSCs in nasopharyngeal carcinoma. This combination treatment can lead to the degradation of cIAP1 and XIAP, which result in the release of caspase inhibition and induces apoptosis in cancer stem cells. The usage of smac mimetic and TRAIL can be a potential application for other tumor CSCs. Moreover, using Smac mimetics in combination with TRAIL as an adjuvant therapy with common chemotherapeutic drugs or radiotherapy may provide a promising new avenue for nasopharyngeal carcinoma therapy.

Grant Support

D.Yang, G.Wang, H.Wang, M.Wu, P.Min received financial support from the National Natural Sciences Foundation (201281172107), the International cooperation project of the Ministry of Science and Technology of China (2010DFB34090), "Key New Drug Creation" project of the major science and technology program (2012ZX09401005), National "863" Grant (2012AA020305), Jiangsu Provincial Science and Technology Innovation Team Grant, China (BE2010760) and Jiangsu Provincial Key Laboratory Project Grant (BM2012114). M.Wu, Z.Zhao, Y.Liang, L.Chen, Q.Feng, J.Bei, Y.X.Zeng received financial support from the National “973” Grant (2011CB504302 and 2012CB967002).

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Table 1. IC50 values for cisplatin, 5-fluorouracil, taxel, AT-406 and SM-164 in S18 and S26 cells.

Cells were treated with a range of concentrations of each compound for 48 hours, and cell survival was measured by the MTT assay.

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<th>S18</th>
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<th>S26</th>
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<tr>
<td></td>
<td>IC50</td>
<td>SEM</td>
<td>IC50</td>
<td>SEM</td>
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<tr>
<td>Cisplatin (μM)</td>
<td>41.58</td>
<td>1.38</td>
<td>19.45</td>
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<tr>
<td>5-fluorouracil (μM)</td>
<td>231.60</td>
<td>20.52</td>
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<tr>
<td>Taxel (nM)</td>
<td>4.31</td>
<td>0.30</td>
<td>1.50</td>
<td>0.37</td>
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<tr>
<td>AT-406 (μM)</td>
<td>239.90</td>
<td>21.63</td>
<td>20.92</td>
<td>2.74</td>
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<tr>
<td>SM-164 (μM)</td>
<td>5.07</td>
<td>0.71</td>
<td>1.37</td>
<td>0.52</td>
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Table 2. Tumorgenesis abilities of S18 and S26 cells. Mice were injected with serial dilutions of S18 or S26 cells and were observed twice per week for 5 weeks.

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<th>Cells injected</th>
<th>S18</th>
<th>S26</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5 \times 10^5$</td>
<td>6 / 6</td>
<td>6 / 6</td>
</tr>
<tr>
<td>$1 \times 10^5$</td>
<td>5 / 6</td>
<td>3 / 6</td>
</tr>
<tr>
<td>$5 \times 10^4$</td>
<td>3 / 6</td>
<td>0 / 6</td>
</tr>
<tr>
<td>$1 \times 10^4$</td>
<td>2 / 6</td>
<td>0 / 6</td>
</tr>
<tr>
<td>$5 \times 10^3$</td>
<td>2 / 6</td>
<td>0 / 6</td>
</tr>
<tr>
<td>$1 \times 10^3$</td>
<td>0 / 6</td>
<td>0 / 6</td>
</tr>
</tbody>
</table>

Table 3. T/C values of the in vivo treatment. T/C rate was calculated using the ratio of RTV of the treated group to RTV of the control group. The RTV was calculated using the ratio of the average tumor volume of the day n to the average tumor volume of the day 0 when the injection of compounds began.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>T / C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S18 xenografts</td>
</tr>
<tr>
<td>TRAIL</td>
<td>68.1</td>
</tr>
<tr>
<td>AT-406</td>
<td>44.3</td>
</tr>
<tr>
<td>AT-406 + TRAIL</td>
<td>27.7</td>
</tr>
<tr>
<td>SM-164</td>
<td>47.3</td>
</tr>
<tr>
<td>SM-164 + TRAIL</td>
<td>26.6</td>
</tr>
</tbody>
</table>
Figure 1. S18 cells act as cancer stem cells among nasopharyngeal carcinoma CNE2 cell lines.

(A) S18 and S26 cells were stained with Hoechst 33342 and FTC, incubated at 37°C in 5% CO₂ in the dark for 90 minutes and mixed every 15 minutes. Then, the cells were resuspended in PBS and kept at 4°C in the dark before flow cytometric analysis.

(B) mRNA expression levels of cancer stem cells of nasopharyngeal carcinoma, ABCG2 and CD44, in S18 and S26 cells. *, P<0.05; **, P<0.01; ***, P<0.001.

(C, D, E) SP detection (C), colony formation assay (D) and sphere formation assay (E) using S18 and S26 cells. Cells were counted, plated in 6-well plates and cultured for approximately 10 days. *, P<0.05; **, P<0.01; ***, P<0.001.

(F) Western blotting for NAIP, cIAP1, cIAP2, XIAP, survivin, and livin in S18 and S26 cells.

(G) The cytotoxic effects of TRAIL on S18 and S26 cells. Cells were treated with TRAIL at a range of concentrations for 48 hours, and cell survival was measured by the MTT assay.

Figure 2. Smac mimetics in combination with TRAIL attenuate cancer stem cells in vitro.

(A) The cytotoxic effect of Smac mimetics in combination with TRAIL on S18 and S26 cells. Negative control, 5 μM AT-406, 0.1 μM SM-164 were mixed with a concentration gradient of TRAIL and then added to the cells for 48 hours. Cell survival was measured by the MTT assay.

(B) SP detection of compound treated S18 cells using Hoechst 33342 with or without FTC. Cells were treated with compounds for 48 hours and then evaluated by flow cytometry.

(C, D, E) SP detection (C), colony formation assays (D) and sphere formation assays(E) of treated S18 cells. In the colony formation and sphere formation assays, cells were treated with
compounds for 48 hours, counted, plated in 6-well plates and cultured for approximately 10 days.

*, P<0.05; **, P<0.01; ***, P<0.001.

Figure 3. AT-406 and SM-164 sensitize nasopharyngeal carcinoma xenografts to TRAIL therapy.

(A and B) Tumor volumes of S18 (A) or S26 (B) xenografts treated with different compounds. Mice were injected with 5×10⁶ S18 or S26 cells. When the xenograft tumors reached approximately 100 mm³ in size, the mice were randomly divided into six groups (for each cell line), with no difference in tumor size between groups. Then, the mice were treated with normal saline, 100 mg/kg of AT-406, 3 mg/kg of SM-164, or 10 mg/kg of TRAIL alone or in combination 5 times per week for 3 weeks. Tumor volume was measured 2 times per week. *, P<0.05; **, P<0.01; ***, P<0.001.

(C and D) Body weights of mice with S18 (C) or S26 (D) xenografts that were treated with normal saline, TRAIL (10 mg/kg), AT-406 (100 mg/kg), or SM-164 (3 mg/kg) alone or in combination. Body weight was measured 2 times per week. *, P<0.05; **, P<0.01; ***, P<0.001.

Figure 4. Smac mimetics in combination with TRAIL induce apoptosis in vitro and in vivo.

(A) Cells were treated with negative control, 5 ng/ml TRAIL, 5 μM AT-406, 0.1 μM SM-164, 5 μM AT-406 + 5 ng/ml TRAIL, or 0.1 μM SM-164 + 0.1 ng/ml TRAIL. After 48 hours, the cells were double stained with propidium iodide (PI) and Annexin V and analyzed using flow cytometry to evaluate the apoptosis.

(B and C) Western blotting for apoptosis related proteins, e.g. PARP, caspase3, cIAP1 and XIAP in treated cells (B) or xenograft tumors (C). Cells were treated with compounds for 48 hours (B).
normal saline; T, TRAIL; A, AT-406; S, SM-164; AT, AT-406 + TRAIL, and ST, SM-164 + TRAIL.

(D) TUNEL staining of compound-treated xenograft tumors. Mice were treated with normal saline, or with TRAIL (10 mg/kg), AT-406 (100 mg/kg), or SM-164 (3 mg/kg) alone or in combination.
Figure 2

A

![Graph showing cell viability vs. TRAIL concentration for different cell lines: S18-NS+TRAIL, S29-NS+TRAIL, S18-AT406+TRAIL, S28-AT406+TRAIL, S18-SM164+TRAIL, S28-SM164+TRAIL. The y-axis represents cell viability (cell number/seed number), and the x-axis represents TRAIL concentration in ng/ml.]  

B

![Heat map showing the effect of different agents (NS, TRAIL, AT-406, AT-406 + TRAIL, SM-164, SM-164 + TRAIL, cisplatin) on Hoechst 33342 Red intensity for different cell lines.]

C

![Bar graph showing the percentage of dead cells for different cell lines: S18-NS, S18-AT406, S18-AT406 + TRAIL, S28-AT406, S28-AT406 + TRAIL, SM-164, SM-164 + TRAIL.]

D

![Bar graph showing the percentage of apoptosis for different cell lines: S18-NS, S18-AT406, S18-AT406 + TRAIL, S28-AT406, S28-AT406 + TRAIL, SM-164, SM-164 + TRAIL.]

E

![Bar graph showing the anti-apoptotic effect for different cell lines: S18-NS, S18-AT406, S18-AT406 + TRAIL, S28-AT406, S28-AT406 + TRAIL, SM-164, SM-164 + TRAIL.]
Figure 3

A. S18 xenografts

B. S26 xenografts

C. S18 xenografts

D. S26 xenografts
Molecular Cancer Therapeutics

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*Mol Cancer Ther* Published OnlineFirst May 22, 2013.

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