Smac Mimetics in Combination with TRAIL Selectively Target Cancer Stem Cells in Nasopharyngeal Carcinoma

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Abstract

Nasopharyngeal carcinoma is a common malignancy in Southern China. After radiotherapy and chemotherapy, a considerable proportion of patients with nasopharyngeal carcinoma suffered tumor relapse and metastasis. Cancer stem cells (CSC) have been shown with resistance against therapies and thus considered as the initiator of recurrence and metastasis in tumors, where the antiapoptotic property of CSCs play an important role. Smac/DIABLO is an inverse regulator for the inhibitors of apoptosis protein family (IAP), which have been involved in 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. Mol Cancer Ther; 12(9); 1–10. ©2013 AACR.

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; ref. 1). The application of chemotherapeutic drugs has become 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 is thought to originate from cancer stem cells (CSC; refs. 3–9).

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 have been considered as an 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), TJ1RI 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 shown that the side population (SP) cells, identified by having ability to pump out a fluorescent dye (Hoechst 33342), have certain characteristics of CSCs similar to those in liver cancer and gastrointestinal 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 of as the key players in the resistance to chemo- or radiotherapy (11, 12), while their ability to escape from the apoptosis pathway may render them resistant to the properties of the therapies (19, 20). It has been shown that the inhibitors of apoptosis protein (IAP) family members are important antiapoptotic proteins to regulate the apoptosis processes (19–21). Among the 8 proteins in the family, namely survivin, IAP-like protein...
(ILP2), melanoma IAP (ML-IAP), X-linked 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 2 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 in turn activates the apoptosis process in cells (26, 27). Therefore, regarding the potency of Smac to revert the antiapoptotic process 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 patients with nasopharyngeal carcinoma, we evaluated 2 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 medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), 100 U/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, Guangzhou, China). All cell lines were passaged for less than 6 months.

SP detection

S18 and S26 cells were treated with the test compounds (negative control, 5 ng/mL TRAIL, 5 μmol/L AT-406, 0.1 μmol/L SM-164, 5 μmol/L AT-406 + 5 ng/mL TRAIL, or 0.1 μmol/L SM-164 + 0.1 ng/mL TRAIL) for 48 hours, harvested, and then resuspended in an ice-cold DMEM (supplemented with 2% FBS) at a density of 1 × 10^6 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 μmol/L fumitremorgin C (FTC, an inhibitor of ABCG2 which could block the pumping out of Hoechst 33342 in CSCs; Sigma-Aldrich) for 5 minutes before the addition of the Hoechst 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 Scientific). Real-time PCR amplification was conducted using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) on 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 2,500 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, and SM-164 were added to the wells in a concentration gradient. For combination groups, negative control, 5 μmol/L AT-406, and 0.1 μmol/L 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 μmol/L AT-406, 0.1 μmol/L SM-164, 5 μmol/L AT-406 + 5 ng/mL TRAIL, or 0.1 μmol/L 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% FBS) 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 μmol/L AT-406, 0.1 μmol/L SM-164, 5 μmol/L AT-406 + 5 ng/mL TRAIL, or 0.1 μmol/L 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% FBS) 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.
0.1 μmol/L 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 basic fibroblast growth factor (amino acids 1–155) (Invitrogen), 20 ng/mL recombinant human EGF (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-EGFR apoptosis detection kit (KeyGEN). Treated S18 and S26 cells (treated with negative control, 5 ng/mL TRAIL, 5 μmol/L AT-406, 0.1 μmol/L SM-164, 5 μmol/L AT-406 + 5 ng/mL TRAIL, or 0.1 μmol/L 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 × 10⁵ 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 Cytomix 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 SDS-PAGE electrophoresis 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 (#6008-1-lg, Proteintech). TRAIL was provided by the Ascentage Pharma Group Corp. Limited.

**Animal experiments**

For the tumorigenesis assay, 4-week-old female athymic nude mice were obtained from the Sino-British Sippr/BK Lab Animal LET., Co. and were subcutaneous injected 1 × 10⁶ S18 or S26 cells in the right axillary area. When the xenograft tumors grew approximately 100 mm³ in size, 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 100 mg/kg, per os, every day, 1–5 week × 3 weeks or SM-164 at 3 mg/kg, i.v., every day, 1–5 week × 3 weeks alone or in combination with TRAIL at 10 mg/kg, i.v., every day × 3 weeks. 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 day n to the average tumor volume of 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 xylen and ethanol, incubated with a proteinase K working solution with microwave irradiation in 0.1 mol/L citrate buffer, and then stained with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction mixture (Roche Applied Science) for 1 hour. Then, the samples were incubated with Con- verter-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 analysis**

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 SDs, and the differences between groups were evaluated using Student 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 2 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 3 chemotherapeutic drugs (cisplatin, 5-fluorouracil, and taxel) commonly used to treat nasopharyngeal carcinoma (Table 1). The mRNA expression levels of CSC markers in nasopharyngeal carcinoma, ABCG2, and CD44, were also higher in S18 cells (Fig. 1B). These observations showed that S18 cells may have CSC properties.

Then, a colony formation assay was conducted, 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 could 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 with the effect on S26 cells (Fig. 1G). Even when the concentration of TRAIL was increased to 1,000 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

On the basis of the results mentioned above, we investigated whether 2 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 percentage of SP cells in untreated group was 60%, whereas the percentage of SP cells in a cisplatin-treated group was up to 90% (Fig. 2B and C). These observations indicated that the cell formation and sphere formation abilities of S18 cells, especially when combined with TRAIL (Fig. 2D and E and Supplementary Fig. S2A and S2B).

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 mm³, the mice were treated with normal saline or with TRAIL (10 mg/kg, i.v.), AT-406 (100 mg/kg, per os), and SM-164 (3 mg/kg, i.v.) 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 anticancer stem cell properties in vivo. No significant toxicity was seen 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, whereas T/C ratio were 71.2 and 47.2 in S26 xenografts (Table 3).

Smac mimetics in combination with TRAIL can induce IAP 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 vivo. 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, whereas 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 selective CSC 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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ S18</th>
<th>SEM</th>
<th>IC₅₀ S26</th>
<th>SEM</th>
</tr>
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<tr>
<td>Cisplatin (μmol/L)</td>
<td>41.58</td>
<td>1.38</td>
<td>19.45</td>
<td>4.69</td>
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<tr>
<td>5-Fluorouracil (μmol/L)</td>
<td>231.60</td>
<td>20.52</td>
<td>13.34</td>
<td>1.52</td>
</tr>
<tr>
<td>Taxel (nmol/L)</td>
<td>4.31</td>
<td>0.30</td>
<td>1.50</td>
<td>1.52</td>
</tr>
<tr>
<td>AT-406 (μmol/L)</td>
<td>239.90</td>
<td>21.63</td>
<td>20.92</td>
<td>2.74</td>
</tr>
<tr>
<td>SM-164 (μmol/L)</td>
<td>5.07</td>
<td>0.71</td>
<td>1.37</td>
<td>0.52</td>
</tr>
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</table>

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

Table 2. Tumorigenesis abilities of S18 and S26 cells

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>S18</th>
<th>S26</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10³</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>1 x 10³</td>
<td>5/6</td>
<td>3/6</td>
</tr>
<tr>
<td>5 x 10⁴</td>
<td>3/6</td>
<td>0/6</td>
</tr>
<tr>
<td>1 x 10⁴</td>
<td>2/6</td>
<td>0/6</td>
</tr>
<tr>
<td>5 x 10⁵</td>
<td>2/6</td>
<td>0/6</td>
</tr>
<tr>
<td>1 x 10⁶</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

NOTE: Mice were injected with serial dilutions of S18 or S26 cells and were observed twice per week for 5 weeks.
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 CSCs 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 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.

CSCs 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

Table 3. T/C values of the in vivo treatment

<table>
<thead>
<tr>
<th>Compounds</th>
<th>S18 Xenografts</th>
<th>S26 Xenografts</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL</td>
<td>68.1</td>
<td>73.8</td>
</tr>
<tr>
<td>AT-406</td>
<td>44.3</td>
<td>87.9</td>
</tr>
<tr>
<td>AT-406 + TRAIL</td>
<td>27.7</td>
<td>71.2</td>
</tr>
<tr>
<td>SM-164</td>
<td>47.3</td>
<td>90.8</td>
</tr>
<tr>
<td>SM-164 + TRAIL</td>
<td>26.6</td>
<td>47.2</td>
</tr>
</tbody>
</table>

NOTE: 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 day n to the average tumor volume of day 0 when the injection of compounds began.

and the 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.

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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). 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, showing 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, TRAILs seem to be safe and well tolerated (45). This research showed 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 induce apoptosis in CSCs. 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.
Disclosure of Potential Conflicts of Interest

D. Yang has ownership interest (including patents) in Ascentage Pharma Group Corp. Limited. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M.-S. Wu, Y.-X. Zeng, D. Yang
Development of methodology: Y. Liang, H.-B. Wang, Y.-X. Zeng, D. Yang
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-S. Wu, G.-F. Wang, P. Min, D. Yang
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References


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