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Smac13-Tat Fusion Peptide Induces Cell Death and Sensitizes HeLa Cells to Chemotherapeutic Drugs

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Abstract

Background: The inhibitor of apoptosis proteins (IAPs) are overexpressed in a variety of cancer cells and play an important role in the inhibition of caspases, thereby suppressing programed cell death and leading to chemoresistance of cancer cells. However, the anti-apoptotic function of IAPs could be suppressed by Smac, the second mitochondria-derived activator of caspase through its direct interaction with IAPs. Smac can interact with IAPs through the N-terminal Ala-Val-Pro-Ile tetrapeptide domain.

Methods: In the present study, the first 7 amino acid sequence (residues 54-60), Smac7,and the first 13 amino acid sequence (residues 54-66), Smac13, which are conserved among mammalian homologues were transduced into cells by bonding to the HIV-1 Tat-derived cell-penetrating peptide.

Results: The Smac-Tat peptides induced cell death and increased the sensitivity to chemotherapeutic agents in HeLa cells, whereas they did not affect cell survival in normal lymphocytes. The Smac7-Tat peptide could bind to Livin but not Survivin.

Conclusion: We demonstrate here that Smac13-Tat is more effective for chemosensitization than Smac7-Tat and that its use might serve as a potent molecular targeted therapy for chemoresistant cervical cancer.

Keywords

Smac, Survivin, Livin, Drug resistance, Cervical cancer

Abbreviations

IAP: Inhibitor of Apoptosis Protein; Smac: The Second Mitochondria-Derived Activator of Caspase; HIV: Human Immunodeficiency Virus; BIR: Baculovirus IAP Repeat; PBL: Peripheral Blood Lymphocyte; MTT: 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide

Introduction

The inhibitor of apoptosis proteins (IAPs) are highly conserved anti-apoptotic proteins in various organisms ranging from yeast to mammals [1,2]. They have one or more structurally conserved domains, termed baculovirus IAP repeat (BIR) domains, which mediate the interaction with caspases. Thus far, eight human IAP members have been reported: NAIP, c-IAP1, c-IAP2, XIAP, ILP-2, Apollon, Survivin, and Livin [3]. Most of them also contain a RING finger domain that functions as E3 ubiquitin ligase [1,2] and promote proteasome-mediated degradation of caspases. Thus, they are endogenous signaling molecules that have important roles for the regulation of apoptotic pathways in normal cells. More importantly, XIAP, Survivin and Livinare are frequently over expressed in various tumor cells and cause resistance against chemotherapeutic agents and radiation [4-8]. Many reports have shown that their expression is correlated with tumor aggressiveness [9-12]. Though XIAP is expressed in normal cells and tissues, expression of Livin and Survivin is limited in most normal adult tissues [8,13]. These findings have led to great interest in Livin and Survivin as ideal therapeutic targets for cancer.

It has been reported that the expression and functions of IAP members are regulated by mitochondrial proteins such as Smac (the second mitochondria-derived activator of caspase) and HtrA2 [14]. Smac contains a mitochondria-targeting sequence at its N-terminus, which is cleaved after mitochondrial entry [15]. Upon apoptotic signals, it is released into the cytosol and binds to a BIR domain of IAP through the N-terminal Ala-Val-Pro-Ile (AVPI) tetrapeptide domain, thereby leading to inhibition of their interaction with caspases. In this context, Smac is a kind of endogenous IAP antagonist. Furthermore, it has been reported that a seven amino-acid N-terminal peptide of cleaved Smac (Smac7, AVPIAQK) is necessary and sufficient to bind to IAP [16]. Studies have shown that synthetic Smac7 peptide is transduced into cells by generating a fusion peptide through bonding of the C-terminus of the Smac7 peptide to the N-terminus of a cell-penetrating peptide such as the antennapedia peptide, polyarginine peptide or HIV-Tat peptide, and successfully induces apoptosis in tumor cells [17-20]. Since the 13 N-terminal amino acids of cleaved Smac are highly conserved among mammalian Smac proteins [21], it was speculated that the Smac13-Tat peptide might have higher capacity to induce cell death of cancer cells than Smac7-Tat.Therefore, in the present study, we analyzed the efficiency of the Smac13-Tat peptide for inducing cell death and sensitization to chemotherapeutic drugs. HeLa cells were examined because they express both Survivin and Livin and have resistance to

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Table 1: Amino acid sequences of the peptides used in the study

Peptide	Sequence
Smac7-Tat	AVPIAQKGYGRKKRRQRRR
Smac7M-Tat	MVPIAQKGYGRKKRRQRRR
Smac7-Tat-His6	AVPIAQKGYGRKKRRQRRRHHHHHH
Smac7M-Tat-His6	MVPIAQKGYGRKKRRQRRRHHHHHH
Smac13	AVPIAQKSEPHSL
Smac13-Tat	AVPIAQKSEPHSLGYGRKKRRQRRR
Smac13M-Tat	MVPIAQKSEPHSLGYGRKKRRQRRR

The cell-penetrating peptide sequence derived from HIV-Tat is underlined.

drug-induced cell death. We examined the chemosensitivity to VP-16 and paclitaxel, which are known as a topoisomerase inhibitor and an antimicrotubule agent, respectively. Since mutation of the very first amino acid Ala of Smac7 to Met leads to loss of the interaction with IAPs [15,22,23], we examined Smac7M-Tat and Smac13M-Tat as control peptides. In addition, the target IAP molecule of Smac7-Tat was defined in transfected cells.

Materials and Methods

Cell culture

The experiments were conducted with the approval of the Sapporo Medical University Study Review Board. Human cervical cancer HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma- Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100units/mL penicillin G, and 100μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Human peripheral blood lymphocytes were collected from a healthy volunteer and cultured in RPMI1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS in a 5% CO2 incubator at 37°C.Human embryonal kidney HEK293 cells and HEK293T cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in DMEM containing 10% FBS.

Synthetic peptides

The amino acid sequences of the synthetic peptides are listed in Table 1. Smac7 (AVPIAQK) and Smac13 (AVPIAQKSEPHSL) are 7 amino acid and 13 amino acid N-terminal sequences of the cleaved human Smac protein, respectively. Tat (YGRKKRRQRRR) is a cell-penetrating peptide derived from HIV-Tat protein [24-26]. All of these synthetic peptides were purchased from SigmaGenosys (Ishikari, Japan).Mean fluorescence intensity of cells with FITC-labeled peptide was analyzed using ImageJ software (Dr. Wayne Rasband, the Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA).

Cell survival assay

The cell survival rate was assessed using the MTT colorimetric assay. It is based on cleavage of the tetrazolium salt MTT to formazan by cellular mitochondrial dehydrogenases. With this assay, a decrease in the number of viable cells results in a decrease in the overall activity of the mitochondrial dehydrogenases in the sample. The decrease in the enzyme activity leads to a decrease in the formazan dye formed, which can be quantified by using a plate reader. Briefly, cells were suspended at a cell density of $2x10^4$ cells in 100μL culture medium per well on 96-well flat-bottom plates (Corning, NY, USA) and incubated with the peptides and/or chemotherapeutic reagents for the indicated time at 37°C. Then, the cells were incubated with MTT reagent (Sigma) for 4 hours, and the formazan dye was solubilized by addition of acidic isopropanol. As a positive control, cells were incubated with normal culture medium. As a background control, all cells were lysed with Triton X100 before incubation with MTT reagent. The optical density (O.D.) of each well was quantified by using a microplate reader (Model 680 microplate reader, Bio-Rad). The test wavelength was 570nm and the reference was 630nm. All the experiments were performed in triplicate wells and repeated three times. The cell survival rate was expressed as % survival, which was calculated according to the following formula:

% Survival=(Experimental O.D.-Background O.D.) / (Positive control O.D - Background O.D.) x 100

Transfection of cultured cells

cDNAs encoding a full length human Livin cDNA and a full length human Survivin cDNA with an N-terminal myc-epitope-tag were amplified by PCR respectively. The PCR products were purified, sequenced, and cloned into pcDNA3/Amp mammalian expression vector (Invitrogen, Carlsbad, CA, USA). The resulting expression plasmids, pcDNA3-Myc-Livin and pcDNA3-Myc-Survivin, were used for the transfection. HEK293T cells ($1x10^6$) were transfected with the plasmids using Lipofectamine (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Forty-eight hours after the transfection, the cells were harvested.

Pull-down assay and Western blotting

The transfected HEK293 cells were washed in ice-cold PBS, homogenized in ice-cold CHAPS lysis buffer [20mM HEPES (pH8.0), 100mM NaCl, 0.5% CHAPS, protease inhibitor cocktail (Complete, Roche Diagnostics, Basel, Switzerland)] for 30 minutes, and clarified by centrifugation at 12,000 x g for 20 minutes at 4°C. A part of the whole cell lysate was boiled for 5 minutes with SDS sample buffer and then separated by SDS-PAGE. The Smac7-Tat-His6 peptide and Smac7M-Tat-His6 peptide were each incubated with Probond Nickel-Chelating Resin (Invitrogen, Carlsbad, CA, USA) for 1 hour at 4°C, followed by washing and incubation with the whole cell lysate for 24 hours at 4°C. The beads were washed three times with ice-cold CHAPS lysis buffer, and the His6-tagged peptides were eluted by incubation with 200mM imidazole. The eluted His6-tagged peptides and proteins were boiled for 5 minutes with SDS sample buffer, separated by 12% SDS-PAGE, and then transferred electrophoretically to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membranes were incubated with blocking buffer (5% nonfat dry milk in PBS) at room temperature and then incubated for 60 minutes with the following antibodies: a mouse anti-Myc epitope tag monoclonal antibody (clone 9E10, American Type Culture Collection) and a mouse anti-His (5) antibody (Penta-His antibody, Qiagen, Valencia, CA, USA).After three washes with wash buffer (0.1% Tween 20, PBS), the membrane was reacted with a peroxidase-labeled secondary antibody (peroxidase-labeled goat anti-mouse IgG antibody; KPL, Gaithersburg, MD, USA) for 2 hours. Finally, the signal was visualized using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL) according to the manufacturer's protocol.

Statistical analysis

Data are presented as values of means ± standard deviation (SD) of at least three experiments. Statistical analysis was carried out with Student's t-test. P values of less than 0.05 were considered statistically significant.

Results

Transduction of Smac7-Tat peptide into HeLa cells

The Smac7-Tat peptide labeled with FITC (1μM) was added to the culture medium. Two hours later, the peptide was visualized using a fluorescence microscope. As shown in Figure 1, the Smac7-Tat was successfully transduced into the cytosol of HeLa cells and partially transduced into the nucleus. Mean fluorescence intensity of cells with FITC-Smac7-Tat was 98.4 ± 7.7, whereas that without peptide was 59.7 ± 2.0.

Cytotoxicity of Smac7-Tat peptide against HeLa cells

HeLa cells were incubated with various concentrations of Smac7-Tat peptide or Smac7M-Tat peptide for 24 hours (Figure 2A) or 72 hours (Figure 2B), and the cell viability was analyzed by MTT assay. Smac7-Tat reduced the cell viability significantly after incubation with 10μM and 100μM concentration of the peptide. However, Smac7M-Tat did not affect the survival rate even after incubation with the

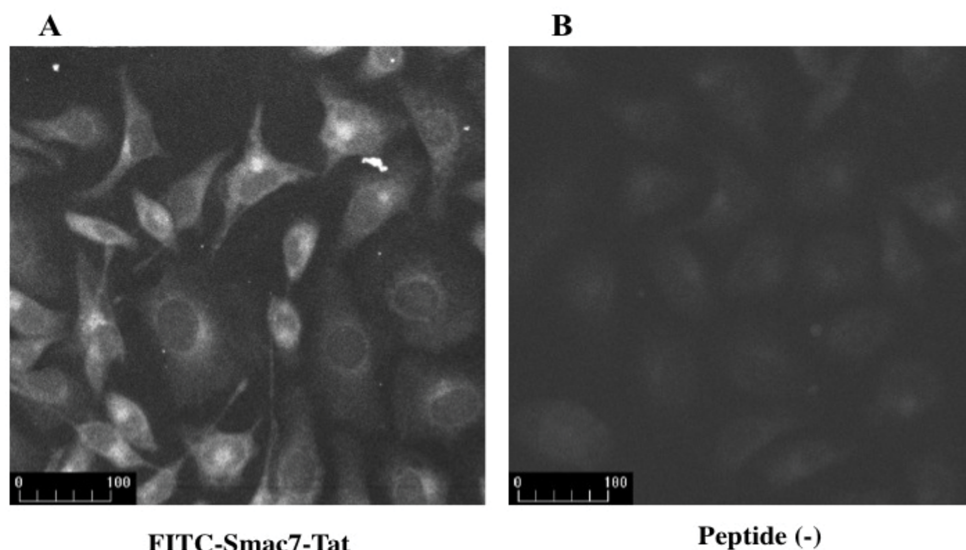

Figure 1: Transduction of Smac7-Tat peptide into HeLa cells

FITC-labeled peptide Smac7-Tat was added to the culture medium. Two hours later, the peptide was visualized by a fluorescence microscope. (A) Mean fluorescence intensity of cells with FITC-Smac7-Tat was 98.4 ± 7.7. (B) Meanfluorescence intensity of cells without peptide was 59.7 ± 2.0.

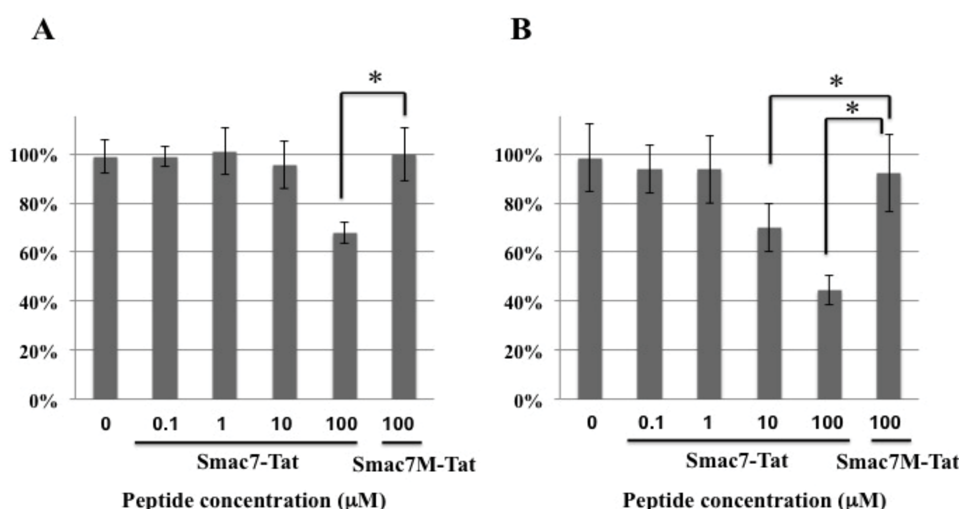

Figure 2: Cytotoxicity of Smac7-Tat peptide against HeLa cells

HeLa cells were incubated with the indicated concentrations of the Smac7-Tat peptide or Smac7M-Tat peptide for 24 hours (A) and 72 hours (B). The survival rates were accessed by MTT assay and are expressed as % survival, which was calculated according to the formula described in the methods section. Data are presented as values of eans ± standard deviations of at least three experiments. (*P<0.05)

100µM concentration for 72 hours. These results were consistent with the previous reports that the N-terminal amino acid Ala of Smac7 was essential for the binding to IAP [15,16].

Cytotoxicity of Smac13-Tat peptide against HeLa cells, normal lymphocytes, and human embryonal kidney cells

HeLa cells, normal human peripheral blood lymphocytes (PBLs), and HEK293 cells were incubated with 100µM Smac13, Smac7M-Tat, Smac7-Tat, Smac13M-Tat, or Smac13-Tat for 24 hours. Neither Smac13 nor Smac13M-Tat affected the survival rate of HeLa cells; however, Smac7-Tat and Smac13-Tat reduced the survival rate significantly (Figure 3A). Smac13-Tat had greater cytotoxicity against HeLa cells than Smac7-Tat. In contrast, neither Smac7-Tat nor Smac13-Tat affected the survival rate of normal PBLs (Figure 3B) or HEK293 cells (Figure 3C). These results indicated that Smac-Tat peptides might exert cytotoxicity against tumor cells, but not non-cancerous cells.

Then, we analyzed a half maximal inhibitory concentration (IC50) of Smac-Tat peptides in HeLa cells. HeLa cells were incubated with various concentrations of the Smac7-Tat peptide or Smac13-Tat peptide for 72 hours, and the survival rates were analyzed by MTT assay. IC50 of Smac13-Tat was 9µM, whereas that of Smac7-Tat was 62µM (Figure 3D).

Smac-Tat peptides increase chemosensitization of HeLa cells to VP-16-induced cell death

HeLa cells were incubated with or without VP-16 in the presence of 100µM Smac-Tat. The survival rates were analyzed by MTT assay after incubation for 24 hours. The survival rate was decreased to ca. 70% in the presence of the Smac7-Tat peptide. After incubation with 250µM VP-16 in the presence of 100µM Smac7M-Tat or Smac13M-Tat, the survival rates were not changed; however, they were decreased to ca. 55% and 42% in the presence of Smac7-Tat and Smac13-Tat, respectively (Figure 4). The results indicated that Smac-Tat peptides increased the sensitivity of HeLa cells to the chemotherapeutic agent VP-16, and that Smac13-Tat had a more potent sensitizing effect than Smac7-Tat.

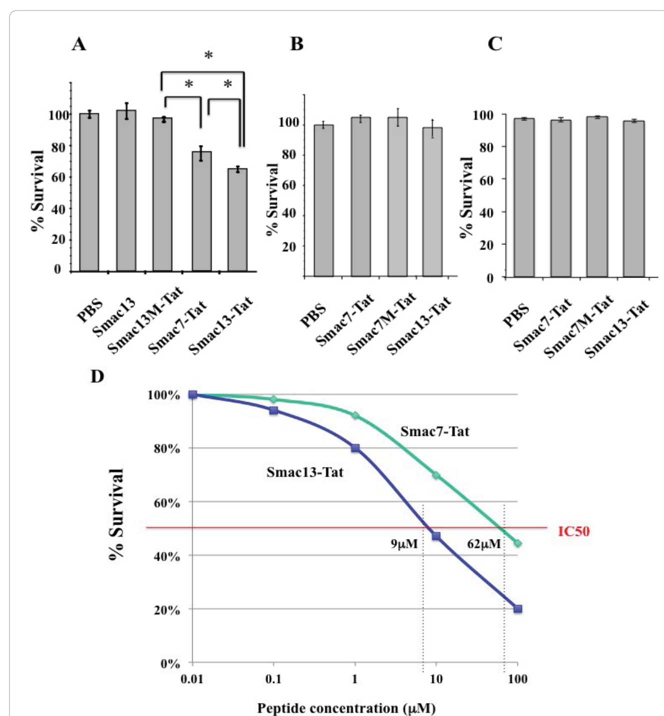

Figure 3: Cytotoxicity of Smac13-Tat peptide against HeLa cells and normal lymphocytes

(A) HeLa cells were incubated with 100μM Smac13, Smac13M-Tat, Smac7-Tat, or Smac13-Tat for 24 hours. (B) Normal human peripheral blood lymphocytesand (C) HEK293 cells were incubated with 100μM Smac7-Tat, Smac7M-Tat, or Smac13-Tat for 24 hours. The survival rates were accessed by MTT assay and are expressed as % survival, which was calculated according to the formula described in the methods section. Data are presented as values of means ± standard deviations of at least three experiments. (*P<0.05) (D) HeLa cells were incubated with the indicated concentrations of the Smac7-Tat peptide or Smac13-Tat peptide for 72 hours, and the survival rates were analyzed by MTT assay. Data are presented as mean values of triplicate experiments. IC50 of Smac13-Tat was 9μM, whereas that of Smac7-Tat was 62μM.

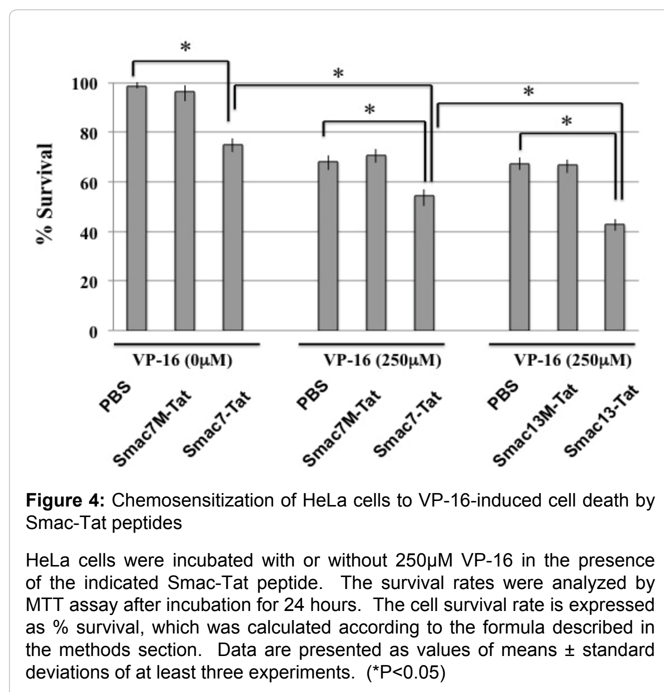

Figure 4: Chemosensitization of HeLa cells to VP-16-induced cell death by Smac-Tat peptides

HeLa cells were incubated with or without 250μM VP-16 in the presence of the indicated Smac-Tat peptide. The survival rates were analyzed by MTT assay after incubation for 24 hours. The cell survival rate is expressed as % survival, which was calculated according to the formula described in the methods section. Data are presented as values of means ± standard deviations of at least three experiments. (*P<0.05)

Chemosensitization of HeLa cells to paclitaxel-induced cell death by Smac-Tat peptides

HeLa cells were incubated with 1μM paclitaxel or 100μM concentrations of Smac-Tat peptides for 24 hours and 40 hours, and the survival rates were analyzed by MTT assay. The survival rates were decreased to ca. 90% in the presence of 1μM paclitaxel and ca. 70%

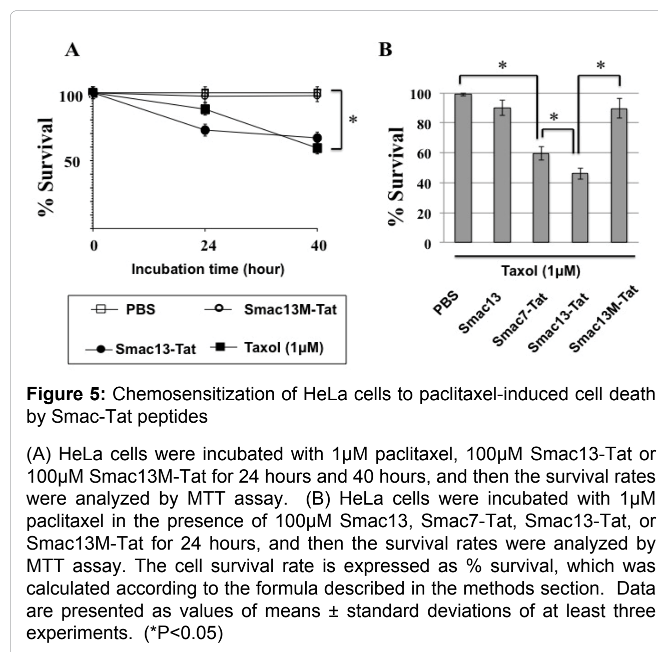

Figure 5: Chemosensitization of HeLa cells to paclitaxel-induced cell death by Smac-Tat peptides

(A) HeLa cells were incubated with 1μM paclitaxel, 100μM Smac13-Tat or 100μM Smac13M-Tat for 24 hours and 40 hours, and then the survival rates were analyzed by MTT assay. (B) HeLa cells were incubated with 1μM paclitaxel in the presence of 100μM Smac13, Smac7-Tat, Smac13-Tat, or Smac13M-Tat for 24 hours, and then the survival rates were analyzed by MTT assay. The cell survival rate is expressed as % survival, which was calculated according to the formula described in the methods section. Data are presented as values of means ± standard deviations of at least three experiments. (*P<0.05)

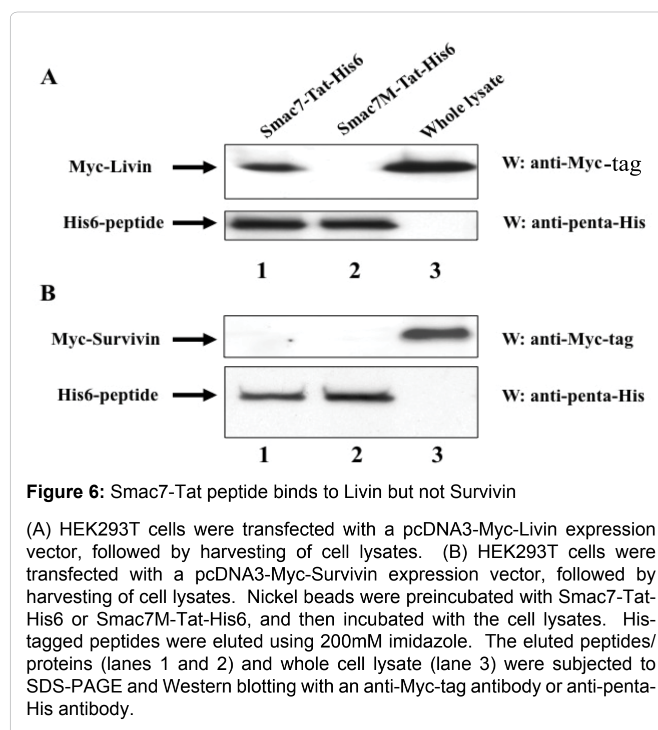

Figure 6: Smac7-Tat peptide binds to Livin but not Survivin

(A) HEK293T cells were transfected with a pcDNA3-Myc-Livin expression vector, followed by harvesting of cell lysates. (B) HEK293T cells were transfected with a pcDNA3-Myc-Survivin expression vector, followed by harvesting of cell lysates. Nickel beads were preincubated with Smac7-Tat-His6 or Smac7M-Tat-His6, and then incubated with the cell lysates. His-tagged peptides were eluted using 200mM imidazole. The eluted peptides/proteins (lanes 1 and 2) and whole cell lysate (lane 3) were subjected to SDS-PAGE and Western blotting with an anti-Myc-tag antibody or anti-penta-His antibody.

in the presence of Smac13-Tat after incubation for 24 hours (Figure 5A). It was indicated that the HeLa cells used in the present study was resistant to paclitaxel-induced cell death, since it was previously reported that IC50 of paclitaxel in HeLa cells was 2.6nM [27]. In the presence of both 1μM paclitaxel and 100μM Smac13-Tat, the survival rate was decreased to ca. 45% after incubation for 24 hours, indicating that Smac13-Tat increased the cytotoxicity of paclitaxel against HeLa cells synergistically. Neither Smac13 nor Smac13M-Tat had such a chemosensitizing effect. Smac13-Tat had a more potent chemosensitizing effect than Smac7-Tat (Figure 5B).

Smac7-Tat peptide bound to Livin but not Survivin

To determine the target IAP molecules of the Smac-Tat peptides, we analyzed whether Livin and Survivin could bind to Smac7-Tat, since both of them were expressed in HeLa cells but not in normal PBLs. HEK293T cells were transfected with a Myc-tagged Livin or Myc-tagged Survivin fusion protein-expression vector, followed by harvesting of cell lysates. Nickel beads were preincubated with the Smac7-Tat-His6 or Smac7M-Tat-His6 peptide, and then incubated

with the cell lysates. The His-tagged Smac-Tat peptides were eluted by imidazole and subjected to SDS-PAGE and Western blotting with an anti-myc tag antibody or anti-penta-His antibody. It was clearly demonstrated that Smac7-Tat could bind to Livin but not to Survivin (Figure 6). The Smac7M-Tat peptide could bind to neither Livin nor Survivin. These results indicated that Smac-Tat peptides might exert cytotoxic and chemosensitizing effects via interaction with Livin in HeLa cells.

Discussion

Smac is aproapoptotic mitochondrial factor and functions as an endogenous IAP inhibitor [28]. It binds to the BIR domain in IAP family proteins through the N-terminal AVPI tetrapeptide domain (amino acid residues 54-57) after the proteolytic cleavage in the N-terminal mitochondrial import signal sequence (amino acid residues 1-53) [15,22,28]. The structure of the tetrapeptide domain is conserved not only among mammalian Smac homologues, but also among the insect IAP binding proteins Grim, Reaper, and Hid [21]. Mutation of the very first amino acid Ala to Met leads to loss of the interaction with IAPs [15,22,23]. Therefore, we used Smac7M-Tat and Smac13M-Tat as negative control peptides. It has been reported that the first 7 amino acid sequence (amino acid residues 54-60), Smac7 is sufficient for the binding and suppression of the anti-apoptotic function of IAPs [16,22]. Several studies have shown that transduction of Smac7 or its mimetics into cells renders tumor cells sensitive to proapoptotic stimuli such as chemotherapeutic agents and irradiation [29-31]. Cell-penetrating peptides such as antennapedia peptide and HIV-Tat peptide have been used to efficiently transduce the Smac7 peptide into the cytosol [17,19]. In this study, the HIV-Tat peptide was used for the transduction. Since the 13 amino acid N-terminal sequence (amino acid residues 54-66), Smac13 is conserved among mammalian Smac homologues [15,21], we compared the cytotoxicities of Smac7-Tat and Smac13-Tat against human tumor cells and normal lymphocytes. It was clearly demonstrated that Smac13-Tat had higher cytotoxicity and a greater ability to sensitize HeLa cells to chemotherapeutic agents, VP-16 and paclitaxel. In contrast, Smac13-Tat exerted no cytotoxic effect against normal PBLs. These results suggested that Smac-Tat peptides might preferentially target IAP molecules that are expressed in tumor cells but not in normal lymphocytes. Among the IAP family proteins, expression of Survivin, Livin and ILP-2 is very limited in normal adult tissues. Since Survivin and Livin are expressed in HeLa cells, we examined whether Smac7-Tat could bind to these IAP family proteins. Our data showed that Smac7-Tat could bind to Livin but not to Survivin. The results were inconsistent with previous reports that Smac could bind and suppress various IAPs, including Survivin [29]. It is speculated that each BIR domain of IAP has distinct affinity to the Smac7 peptide, and that a larger peptide might be necessary for binding to Survivin. Actually, amino acid sequence alignment studies revealed that the homology of the BIR domain of Livin to those of XIAP-BIR3 and cIAP2-BIR3 was 50.0% and 51.5%, respectively, whereas that to the BIR domain of Survivin was 39.7% [32].

Our study suggests that Smac13-Tat might be suitable for use in molecular targeted therapy for Livin-positive cancer. A number of studies have shown that Livin is over expressed in various human solid cancers such as cervical cancer, lung cancer, colon cancer, kidney cancer and malignant melanoma, and its expression is correlated with a worse prognosis of cancer patients [5,8,10,11]. Most importantly, Livin-expressing cancer cells might be dependent on the function of Livin for cell survival, considering that inhibition of its expression or function leads to cell death in various cancer cells [11,12]. Chemoresistant cancer cells express increased levels of Livin, which suppresses the activation of caspases, thereby leading to decreased sensitivity to chemotherapeutic agents [12].

In summary, we have shown that (1) Smac7/13-Tat could sensitize HeLa cells to chemotherapeutic drugs, (2) Smac13-Tat is much better than Smac7-Tat in the chemosensitization of HeLa cells, and (3) Livin instead of Survivin may mediate the effects of Smac7. Our study suggests that Smac13-Tat and its peptidomimetics might serve as potent molecular targeted therapy agents for chemoresistant cervical cancer.

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