Predominant enhancement of apoptosis induced by methyl jasmonate in bladder cancer cells: therapeutic effect of the Antp-conjugated Smac peptide

Xing-yuan Xiao, Guo-song Jiang, Liang Wang, Lei Lv and Fu-Qing Zeng

Methyl jasmonate (MJ) has recently attracted attention as a promising antitumoral compound because of its highly specific proapoptotic properties in a wide range of malignancies. However, the high doses required to achieve a therapeutic benefit have limited its clinical development. Here, we hypothesize that the family of inhibitor of apoptosis proteins (IAPs) may inhibit MJ-mediated apoptosis in cancer cells. We combined MJ with the IAPs inhibitor, the second mitochondria-derived activator of caspases (Smac) peptide to treat bladder cancer cells. The results showed that the combination of MJ and Smac peptide enhanced the apoptosis-inducing effect in a synergistic manner by releasing and activating IAPs-bounding caspase-3. These findings suggest that the inhibition of IAPs could overcome the resistance of cancer cells to MJ. *Anti-Cancer Drugs* **22:853–863 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Department of Urology, Wuhan Union Hospital, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Correspondence to Professor Fu-Qing Zeng, Department of Urology, Wuhan Union Hospital, Huazhong University of Science and Technology, Surgery Building, 24th Floor, 1277 Jiefang Street, Wuhan 430022, Hubei Province, China
Tel: +86 27 85726869; fax: +86 27 85726821;
e-mail: zengfuqingunion@gmail.com

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Introduction

Methyl jasmonate (MJ) is a biologically active derivative of jasmonic acid and has recently shown specific anticancer activity in in-vivo and in-vitro cancer models while sparing normal cells [1,2]. Its cytotoxic effect on cancer cells relies on the induction of apoptosis [3] by the release of cytochrome *c* from the mitochondria [4], thereby activating effector caspases [5], ultimately leading to cell death [6,7].

The lethal concentration of MJ in tumor cells ranges from 1 to 10 mmol/l [5], which is higher than that of conventional cytotoxic agents. Although this concentration is nontoxic to normal cells *in vitro* [8], potential side effects can be expected in a clinical setting. Therefore, it is of interest to reduce the dose of MJ required for cancer therapy.

Generally, the efficacy of chemotherapy is reduced by an inhibitor of apoptosis (IAP) proteins, including X-linked IAP (XIAP) and survivin, which inhibit the activation of procaspases and mature caspases [9–11]. IAPs have been found to contribute to the progression and chemoresistance of bladder cancer, in which they have been proposed as a prognostic marker. In this context, numerous experimental treatments targeting IAPs, such as vaccinations, small interfering RNA, or combination therapies are currently being evaluated for bladder cancer [12–14].

The mitochondrial proapoptotic protein Smac is an endogenous IAP antagonist [15,16]. Its N-terminal seven-conserved amino acid residues (AVPIAQK) bind to a surface groove of baculovirus IAP repeat 3 (BIR3) of XIAP, thus disinhibiting caspase activation [17–19]. Previous reports have demonstrated that Smac and Smac mimetics sensitized breast and prostate cancer cells to chemotherapeutic drugs and irradiation by overcoming apoptosis resistance [20,21]. By itself, however, Smac only displays a weak antitumor effect [22,23].

Here, we hypothesize that the inhibitory effect of IAPs may result in the high dose of MJ needed to induce apoptosis in cancer cells, and combining MJ and Smac peptide may enhance the antitumor efficacy of MJ. We investigated the effect of MJ, combined with Smac peptide in two bladder cancer cell lines. Our results indicated that Smac peptide significantly enhances the apoptosis-inducing effect of MJ through a caspase-3-dependent way.

Materials and methods

Materials

Reagents were obtained from the following suppliers: MJ from Sigma (St Louis, Missouri, USA) at a concentration of 1 mol/l in anhydrous dimethylsulfoxide (DMSO; Sigma) and stored at 4°C. 2-(4,5-Dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Hoechst 33258, Ac-Leu-Glu-His-Asp-AFC, Ac-Asp-Glu-Val-Asp-AFC, and z-VAD-fmk were also obtained from Sigma. Anti-caspase-9 and caspase-3 antibodies were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Antibodies against XIAP, Smac, survivin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz,

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California, USA). The enhanced chemiluminescence substrate kit was obtained from Amersham Biosciences (Piscataway, New Jersey, USA).

Cell cultures

Human bladder cancer cell lines (EJ, T24) and human embryonic kidney cell line (HEK-293) from American Type Culture Collection (Manassas, Virginia, USA) were cultured in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml; all from Life Technologies, Gaithersburg, Maryland, USA). Cells were grown in tissue culture dishes at 37°C with 5% CO_2 in a completely humidified atmosphere. During exposure to the compounds, the medium was replaced with treatment medium containing different agents. Control cells were cultured in medium with DMSO (0.5%).

Design and synthesis of peptides

On the basis of the findings of the previous report [24], the N-terminal seven amino acids of Smac, which are critical for its binding to IAPs, were conjugated with the penetratin sequence of the *Drosophila* transcription factor, antennapedia (Antp), to form the Smac mimetic, SmacN7. Antp was chosen for its capacity to translocate through cell membranes and for its low toxicity. C-terminal lysine of Antp was labeled with biotin. The purity of the peptides was more than 95% as determined by reversed-phase high-performance liquid chromatography analysis.

Coimmunoprecipitation

EJ and T24 were incubated with SmacN7 or Antp for 30 min. Antp was used as a negative control. At the completion of incubation, cells were collected, washed twice with ice-cold phosphate buffer solution (PBS), and lysed in 1 × cell lysis buffer (Pierce, Rockford, Illinois, USA). Then, cell lysate was incubated with precleaned streptavidin agarose beads for 4 h at 4°C. The complex, which contained streptavidin–biotin-labeled SmacN7, was pulled down. Proteins in the complex were dissociated and analyzed by western blot with anti-XIAP antibody.

2-(4,5-Dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Detection of cell viability by the MTT colorimetric assay was performed as follows. In brief, cells (1×10^4 in 100 μl of culture media per well) were seeded in 96-well flat-bottom plates, and treated with varying MJ concentrations for 24 h in the absence or presence diverse doses of SmacN7. Then, 20 μl of MTT (5 mg/mol) was added to each well. After incubation for 4 h at 37°C, the supernatants were discarded. The precipitate in each well was dissolved with DMSO and the absorbance was measured at 570 nm (A570 nm) through a microplate reader (Tecan Ultra; Research Triangle Park, North Carolina, USA). Cell viability was calculated as follows: (%) = (average A570 nm value of treated cells/average A570 nm value of untreated control cells) × 100%. Each experiment was performed with six wells and all experiments were repeated at least three times.

Hoechst 33258 staining

Cells were treated with SmacN7 and only buffer as indicated, followed by incubation in the absence or presence of MJ. After fixation with 4% formaldehyde at 4°C for 10 min, cells were rinsed three times with PBS and stained with Hoechst 33258 (10 mg/l) in PBS solution at 4°C in the dark for 10 min. Morphological changes such as cell shrinkage and nuclear condensation were examined by fluorescence microscopy (Olympus, Tokyo, Japan).

Measurement of apoptosis rates

Apoptosis rates were measured by annexin V–fluorescein isothiocyanate (BD Pharmingen, San Diego, California, USA) and propidium iodide (PI)-staining flow cytometry. Cells treated with Antp, SmacN7, MJ, MJ and Antp, or MJ and SmacN7 were trypsinized and collected, washed twice with ice-cold PBS, and resuspended with 100 μl of binding buffer at a concentration of $2–5 \times 10^5$ cells/ml. Then, cells were incubated with annexin V–fluorescein isothiocyanate at room temperature for 10 min. After washing with binding buffer, the cells were resuspended in 400 μl of binding buffer containing 10 μl of PI (20 μg/ml), and incubated on ice for 15 min. Apoptosis was analyzed by a flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). This method can be used to distinguish living cells (annexin V−/PI−), early apoptotic/primary apoptotic cells (annexin V+/PI−), late apoptotic/secondary necrotic cells (annexin V+/PI+), and necrotic cells (annexin V−/PI+) [25].

Western blot

Cells treated with different agents were harvested and lysed in appropriate amounts of lysis buffer. Lysates were sonicated for 10 s, and centrifuged for 20 min at 10 000 × *g*. Crude proteins from each sample were immunoblotted with corresponding antibodies. In brief, nitrocellulose blots were blocked with 6% nonfat dry milk in Tris-buffered saline [TBS; Tris–HCl (20 mmol/l, pH 7.4); NaCl (500 mmol/l), and 0.01% Tween 20] for 1 h, then rinsed three times with TBS–Tween 20, and incubated with primary antibodies at 4°C overnight. After three washes with TBS–Tween 20, membranes were incubated with secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h. Finally, an enhanced chemiluminescence system was used for luminescent detection of protein bands with autoradiography films (Amersham Biosciences). The expression of GAPDH was used as control.

Measurement of caspase activity in a cell-free system

Cells were harvested and lysed with buffer A [sucrose (250 mmol/l), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–KOH (10 mmol/l, pH 7.4), KCl (10 mmol/l),

EDTA (1 mmol/l), ethylene glycol tetra-acetic acid (1 mmol/l), dithiothreitol (1 mmol/l), phenylmethanesulfonylfluoride (0.1 mmol/l), and cytochalasin B (20 mmol/l)]. Nuclei and mitochondria in cell lysates were removed by centrifugation at 12 000 $\times g$ for 20 min at 4°C and the supernatant was used as the cytosolic fraction. Fluorigenic substrates (50 μmol/l) (Ac-Leu-Glu-His-Asp-AFC, for caspase-9 or Ac-Asp-Glu-Val-Asp-AFC, for caspase-3) were added to each crude protein sample (50 μg) for the activation reaction. After incubation at 37°C for 1 h, the fluorescence of the fluorigenic substrates was examined with a Synergy-2 fluorometer (Bio-Tek, Winooski, Vermont, USA) at 400/505 nm.

Reverse transcription and real-time PCR for X-linked inhibitor of apoptosis protein and survivin

Reverse transcription (RT)-PCR and real-time PCR were performed to determine the XIAP and survivin expression. Total RNA was extracted with an RNeasy Mini Kit (Qiagen Inc., Valencia, California, USA). RT reactions were performed following the instructions of the Transcriptor First-Strand DNA Synthesis Kit (Roche, Indianapolis, Indiana, USA). Primers were designed by Premier Primer 5.0 software (Premier Biosoft International, Palo Alto, California, USA): for human XIAP, 5′-GAACCTTGTGATCGTGCCT-3′ and 5′-AGGGTCTT CACTGGGCTTC-3′ (318 bps); for human survivin, 5′-CAAGGACCACCGCATCTCTA-3′ and 5′-TTCTTCGC AGTTGCCTCAA-3′ (348 bps); and for human GAPDH, 5′-AGAAGGCTGGGGCTCATTTG-3′ and 5′-AGGGGC CATCCACAGTCTTC-3′ (238 bps). The ratios between the amplified DNA fragments and GAPDH for each sample RNA were quantified by Phoretix 1D software (Phoretix International Ltd., Newcastle upon Tyne, UK). Real-time RT-PCR with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems). The fluorescent signals were collected during the extension phase, Ct values of the sample were calculated, and XIAP and survivin transcript levels were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

The experimental results were presented as mean ± standard error of the mean of replicate analysis accompanied by the number of independent experiments. Statistical analyses were performed using the *t*-test or analysis of variance (SPSS Inc., Chicago, Illinois, USA and Microsoft Excel). A *P* value of less than 0.05 was considered statistically significant.

Results

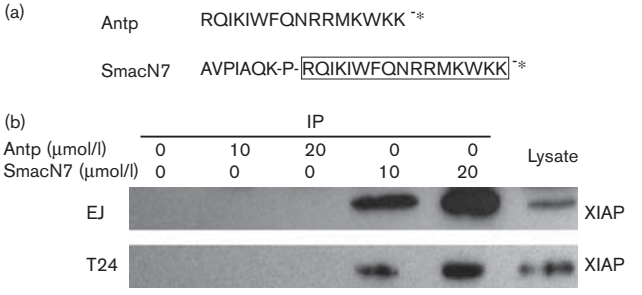
Validation of interaction between SmacN7 and X-linked inhibitor of apoptosis protein

Previous reports have shown that the N-terminal seven amino acids of Smac were sufficient to mediate the effects of Smac [15,19], and Antp can serve as a carrier for the internalization of Smac peptide [24]. SmacN7 was designed by fusing the N-terminal seven amino acids of Smac to the Antp (Fig. 1a). To determine the specific binding of SmacN7 to XIAP, we performed an immunocoprecipitation with biotin-labeled Antp and biotin-labeled SmacN7 in EJ and T-24 cell lines. As shown in Fig. 1b, SmacN7 was bound with XIAP in both cell lines tested. The binding effect of SmacN7 was associated with the dose of SmacN7. In contrast, biotin-labeled Antp showed no binding to XIAP. These results indicate that SmacN7, but not Antp, potently interacted with XIAP in a dose-dependent manner in EJ and T-24 cells.

SmacN7 enhanced the viability inhibition effect of methyl jasmonate in EJ and T-24 cells

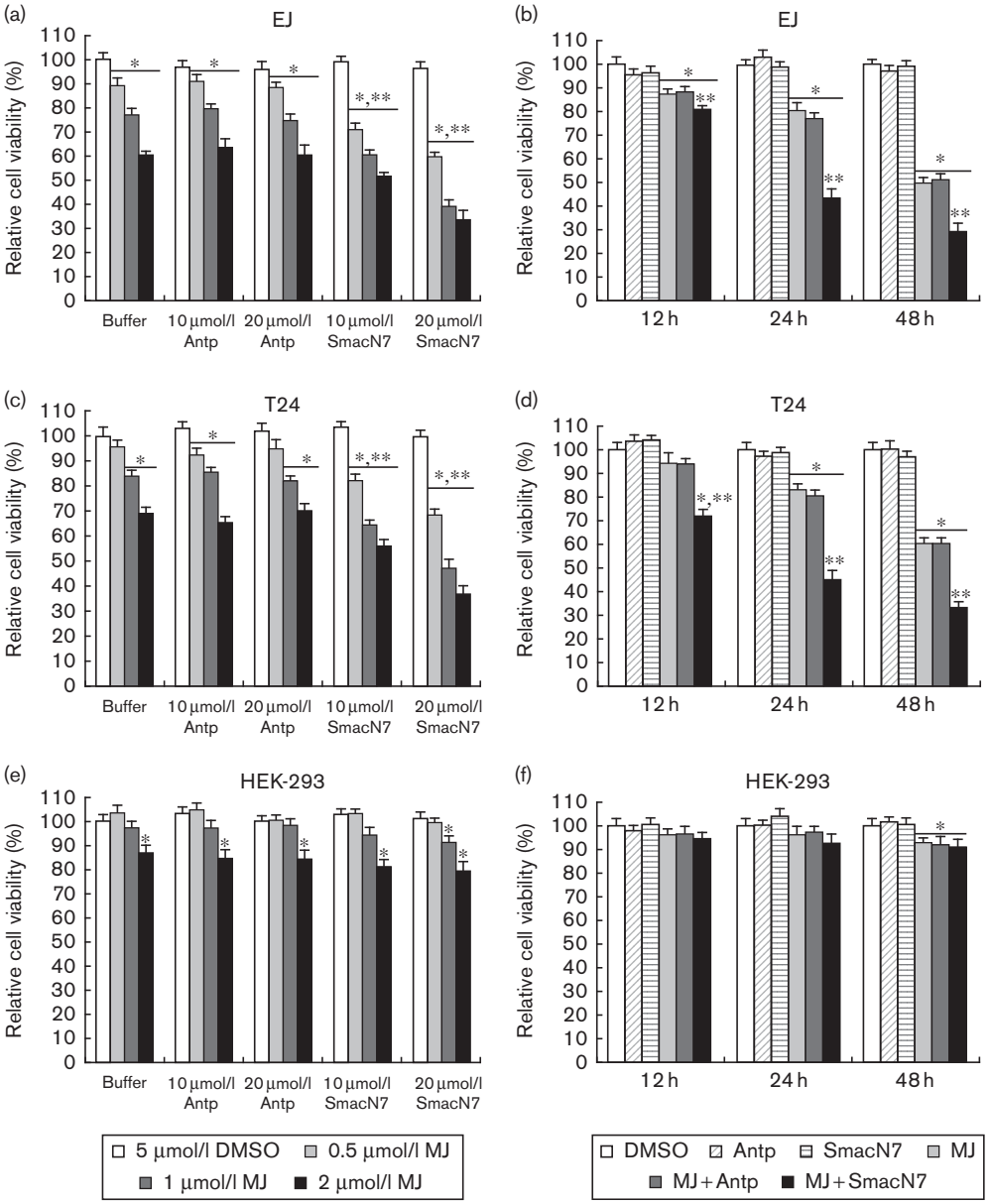
Earlier studies have shown that Smac enhanced the therapeutic potential of chemotherapeutic drugs and irradiation [20,24]. We took two approaches in EJ, T24, and HEK-293 cells. As shown in Fig. 2a and c, the results of approach one indicate that the administration of MJ, but not Antp or SmacN7, results in cell viability decrease in EJ and T24 cells in a dose-dependent manner. Moreover, the levels of cell viability were attenuated by MJ in cells pretreated with SmacN7 to a higher extent than cells that had not been pretreated, and SmacN7 showed a dose-dependent effect on MJ sensitization in EJ and T-24 cells. Similar results were observed in the two approaches, the dose-dependent manner and the time-dependent manner. Antp had no effect on cell viability, and SmacN7 showed time-dependent effects on MJ-induced cell viability decrease (Fig. 2b and d). Furthermore, administration of MJ either individually or together with SmacN7 almost had no effect on the viability of HEK-293 cells (Fig. 2e and f). These data

Fig. 1



Validation of interaction between Smac-mimetic peptides SmacN7 and X-linked inhibitor of apoptosis protein (XIAP). (a) Schematic of constructs used. Cell membrane permeability of SmacN7 was designed through linking seven N-terminal amino acids of Smac/DIABLO to 16-mer antennapedia (Antp) peptide by a proline linker. The penetratin sequence is boxed. * Indicates lysine that was biotinylated. (b) Whole-cell lysates of EJ and T24 cells were treated with 0, 10, or 20 μmol/l Antp or biotin-labeled SmacN7, followed by incubation with precleared streptavidin agarose beads. To examine the capability of SmacN7 interacting with XIAP, eluted beads were subjected to western blot analysis with anti-XIAP antibody. IP, immunoprecipitation.

Fig. 2



Interactive effects of Smac with methyl jasmonate (MJ) on cell viability. EJ (a), T24 (c), and human embryonic kidney (HEK-293; e) cells were pretreated with SmacN7 (10/20 μmol/l), antennapedia (Antp, 10/20 μmol/l), or buffer for 3 h, followed by treatment with dimethylsulfoxide (0.5 mmol/l) or different concentrations of MJ (0.5/1/2 mmol/l) for 24 h. Cell viability was measured by a 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. In a second step, EJ (b), T24 (d), and HEK-293 (f) cells were pretreated with Antp (20 μmol/l) or SmacN7 (20 μmol/l) for 3 h, followed by treatment with MJ (1 mmol/l) for different periods of time (12, 24, and 48 h). Cell viability was also detected by the MTT assay. *Significantly different from the respective control at $P<0.05$; **significantly different from MJ alone at $P<0.05$. Results are the mean ± standard deviation in triplicate.

suggested that SmacN7 enhances the effects of MJ in a dose-dependent and time-dependent manner *in vitro* in bladder cancer cells.

SmacN7 sensitizes methyl jasmonate-induced apoptosis

To detect whether apoptosis was involved in anticancer activity of MJ combined with SmacN7, we examined apoptotic nuclear changes induced by MJ with pretreatment of SmacN7 or Antp by Hoechst 33258 staining. As shown in Fig. 3a and b, SmacN7, rather than Antp, augments MJ-induced eccentric nuclei and nuclei condensation, which are characteristic changes of apoptosis, compared with MJ and SmacN7 alone. Furthermore, the annexin V/PI-staining flow cytometry analysis was performed to quantify the number of apoptotic cells induced by different stimulation functions. As shown in Fig. 3c, SmacN7 obviously enhanced MJ-induced apoptosis. The

cellular apoptotic rates were 53.6 versus 22.3% in SmacN7 pretreated versus without SmacN7 pretreated EJ cells. The same result was obtained in T-24 cells. In contrast, Antp had no effect on MJ-induced apoptosis, and SmacN7 alone showed no increased apoptotic cell population compared with control.

SmacN7 enhanced methyl jasmonate-induced downregulation of X-linked inhibitor of apoptosis protein and survivin

Our previous study demonstrated that MJ downregulated XIAP and induced apoptosis in neuroblastoma cell lines [26]. In this study, we hypothesized that SmacN7 might augment the MJ-induced downregulation of XIAP and survivin, two important members of IAPs [27,28], in EJ and T-24cells, and consequently release the inhibition of IAPs and increase the cleaved caspase peptides. To test this hypothesis, we measured the expression of procaspase, cleaved-caspase, XIAP, and survivin by western blotting. As shown in Fig. 4a and b, SmacN7 obviously enhanced MJ-induced downregulation of XIAP and survivin, and also enhanced upregulation of cleaved capase-3 and cleaved capase-9 compared with MJ alone in both EJ and T-24 cell lines. Meanwhile, endogenous Smac protein level was not affected by MJ alone or MJ combined with SmacN7 treatment.

SmacN7 enhanced methyl jasmonate-induced caspase-3 activation

Previous studies reported that caspase activation seemed to be a common pathway in stress-induced apoptosis in many systems [29–31]. As indicated above, SmacN7 increased MJ-induced apoptosis in EJ and T-24 cells. We sought to examine the mechanism of this interaction by testing caspase-3 and caspase-9 activation. As shown in Fig. 5a, MJ induced caspase-3 activity in EJ and T-24 cells. Treatment of cells with SmacN7 obviously augmented MJ-induced caspase-3 activation. In contrast, SmacN7 alone had no effect on caspase-3 activity. Moreover, caspase-3 activity induced by MJ in combination with SmacN7 can be totally blocked by caspase inhibiter z-VAD-fmk. Although processing of caspase-9 was enhanced, the activity of capase-9 was not dramatically increased by the combination treatment of Smac and MJ compared with MJ alone. Furthermore, viability inhibition effects of MJ and MJ combined with SmacN7 were notably reduced by caspase inhibitor z-VAD-fmk (Fig. 5c). These data suggest that SmacN7 enhanced the apoptosis-inducing potential of MJ through caspase-3 activation.

SmacN7 has no effect on methyl jasmonate-induced downregulation of transcription of X-linked inhibitor of apoptosis protein and survivin

Our previous study demonstrated that MJ suppressed the transcription of XIAP in human neuroblastoma cells [26]. We thought that it was important to evaluate the effect of SmacN7 on the modulation of IAPs transcription induced by MJ in bladder cancer cells. As shown in Fig. 6, MJ significantly attenuated the transcription of XIAP and survivin in a time-dependent manner in EJ and T-24 cells. However, SmacN7 alone had no effect on XIAP and survivin transcription or on MJ-induced downregulation of XIAP and survivin. These results indicate that SmacN7 did not affect the transcription of XIAP and survivin. As XIAP can be cleaved into the amino-terminal BIR1-2 and BIR3-ring finger fragments by activated caspase-3 during apoptosis [32], SmacN7 probably modulates MJ-induced apoptosis possibly not only by interacting with these IAPs in cytoplasm but also by exposing the IAPs to proteasomal degradation in a caspase-3-dependent manner.

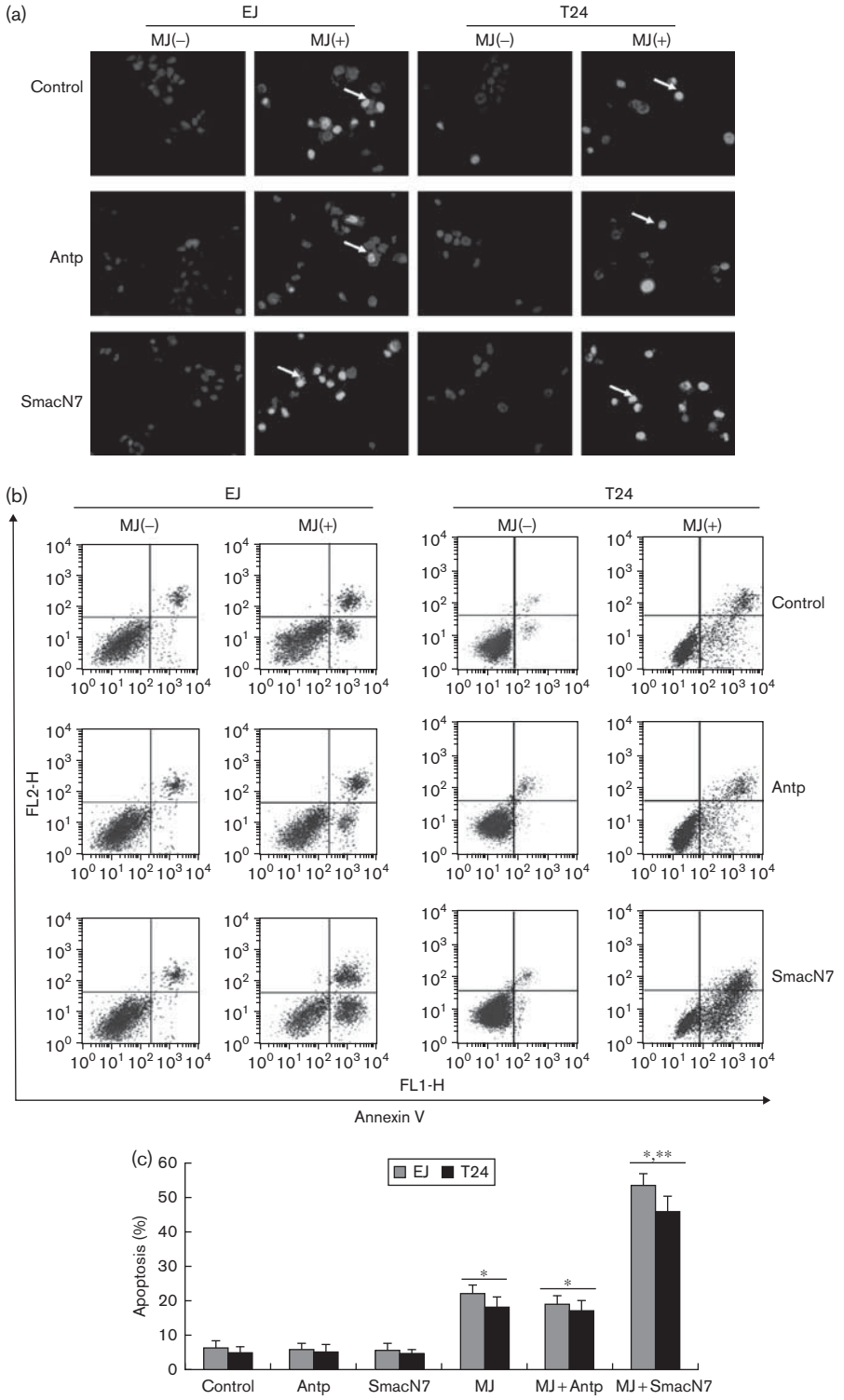
Discussion

Naturally occurring jasmonates include MJ, *cis*-jasmone, and jasmonic acid and have been tested on several cancer cell lines, namely prostate, breast, melanoma, leukemia, lymphoma, and neuroblastoma [4,6,26]. The therapeutic potency of jasmonates can be ranked from MJ, over cis-jasmone to jasmonic acid. MJ is currently being evaluated in preclinical trials for the treatment of cancer, either alone or in combination with other drugs [8,33]. The antitumor effect of MJ relies on selective induction of apoptosis by caspase activation in cancer cells [5] while having relatively little effect on benign cells [4], which makes it an attractive agent for cancer therapy. In this study, we demonstrated that MJ showed a dose-dependent and time-dependent anticancer activity on human bladder cancer cell lines EJ and T24 at concentrations starting from 0.5 mmol/l while leaving the benign cell line HEK-293 unaffected at doses of less than 2 mmol/l. Furthermore, our study confirmed that the viability inhibition effect of MJ relies on the induction of apoptosis in bladder cancer cell lines.

However, the relatively high therapeutic doses of MJ have been a drawback in the development of MJ, as they can be expected to elicit adverse reactions *in vivo*. In this context, we have hypothesized that the effect of MJ is thwarted by IAPs, which are known to inhibit MJ-induced effector caspases [34]. IAPs have been shown to play a critical role in the progression and chemoresistance of bladder cancer. Several preclinical trials that target the IAP family have been applied in bladder cancer [12–14]. The rationale of our study was therefore to combine the IAP-inhibitor SmacN7 with MJ. We hypothesized that by inhibiting IAPs, MJ will develop a stronger therapeutic effect on tumor cells without leading to increased toxicity on benign cells. We have evaluated SmacN7, the exogenous N-terminal peptides of AVPIAQK form of Smac that shows enhanced cellular uptake and is more potent than unmodified Smac [35].

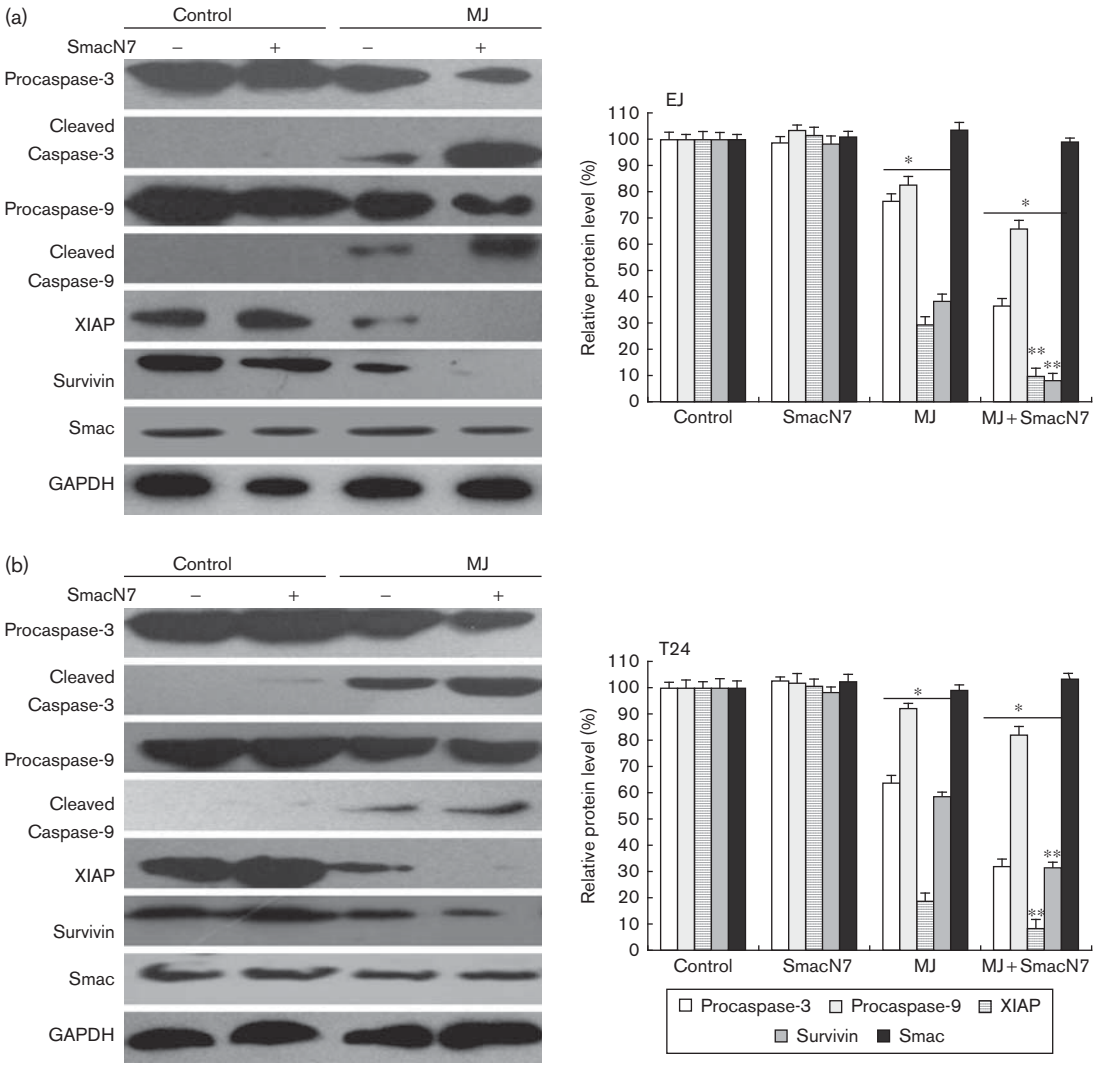
We confirmed that SmacN7 binds to the IAP family member XIAP in the cytoplasm. Similarly, Vucic *et al.* [21]

Fig. 3



Interactive effects of Smac peptides with methyl jasmonate (MJ) on cell apoptosis. EJ (a) and T24 (b) cells were pretreated for 3 h with control buffer, antennapedia (Antp, 20 μmol/l) or SmacN7 (20 μmol/l). Cells were then treated for 24 h with 1 μmol/l of MJ (+) or without MJ (–), harvested, fixed, stained with Hoechst-33528, and examined for nuclear morphological changes indicative of apoptosis. Apoptosis cells are pointed out by an arrow. (c) EJ cells (gray bars) or T24 cells (black bars) were cultured in control buffer, Antp (20 μmol/l), or SmacN7 (20 μmol/l) for 3 h, and then treated with MJ (+) (1 mmol/l) or without MJ (–). After 24 h, cells were harvested and apoptosis was examined by annexin V–fluorescein isothiocyanate and propidium iodide staining flow cytometry. *Significantly different from the respective control at $P<0.05$; **significantly different from MJ alone at $P<0.05$. Means ± standard deviations of hypodiploid (apoptotic) cells from triplicates are shown.

Fig. 4



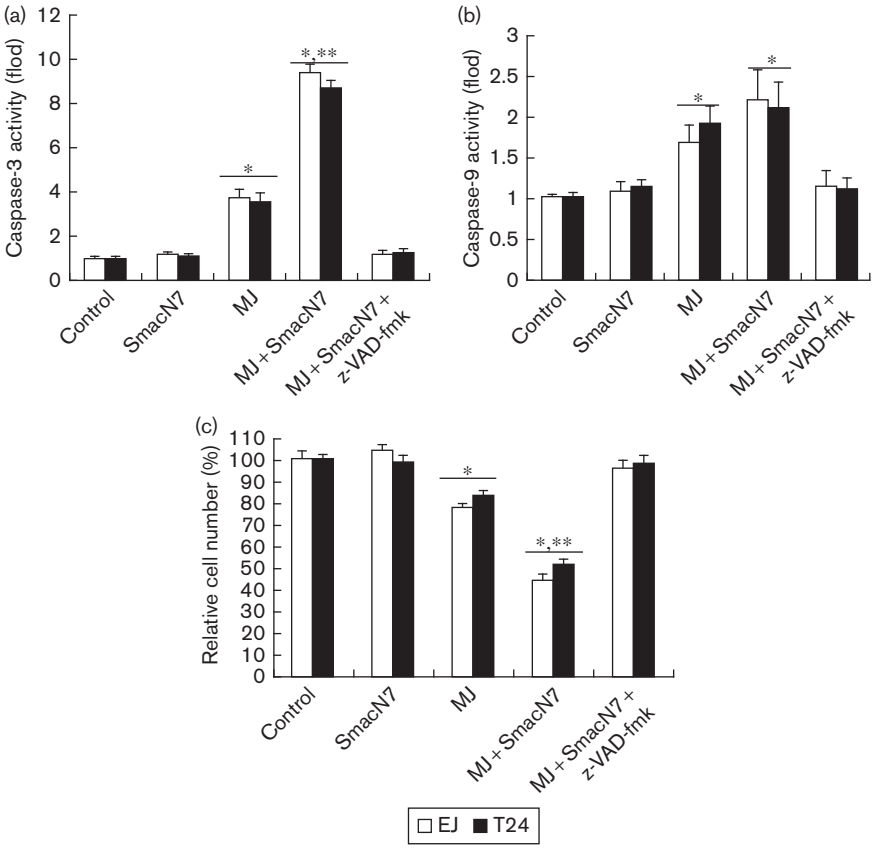
Western blot analysis for caspases and inhibitor of apoptosis proteins (IAPs) expression. EJ (a) and T24 (b) cells were pretreated in the presence or absence of SmacN7 (20 μmol/l) for 3 h, followed by culturing in medium alone (control) or treatment with methyl jasmonate (MJ, 1 mmol/l). Cells were harvested 24 h after treatment and caspase-3 (processing), caspase-9 (processing), X-linked IAP (XIAP), survivin, and endogenous Smac protein expression were determined by the western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

reported that a related Smac-derived fusion peptide could bind to exogenously expressed ML-IAP in MCF-7 cells. Moreover, SmacN7 did not show a cytotoxic effect by itself. These results are consistent with earlier studies in other cell lines, where Smac attenuated the inhibitory effect of IAPs on the processing and activity of effector caspases [23], and where Smac by itself did not induce apoptosis in either cancer cells or normal cells at moderate doses.

Then, we evaluated the combined effect of MJ and SmacN7 on bladder cancer cells *in vitro*. In a dose-dependent and time-dependent manner, SmacN7 promoted the apoptotic response of bladder cancer cell lines to MJ while sparing benign cells (HEK-293). MTT assays showed equivalent growth inhibition rates of 2 and 0.5 mmol/l of MJ combined with 20 μmol/l of SmacN7 in EJ and T24 cell lines. Benign urothelium was not affected by the combined treatment. Annexin V/PI-staining flow cytometry showed that apoptosis increased from 22 to 25% with MJ alone to 50–55% in both cell lines. Similar results have been found in combination treatments of Smac with tumor necrosis factor-related apoptosis-inducing ligand, chemotherapeutic drugs, and irradiation in the previous reports [20,22]. Thus, our results demonstrate that IAPs are valid molecular targets for modulating MJ sensitivity in bladder cancer cells.

Furthermore, our results showed that the synergy of SmacN7 and MJ ultimately relied on the activation of

Fig. 5



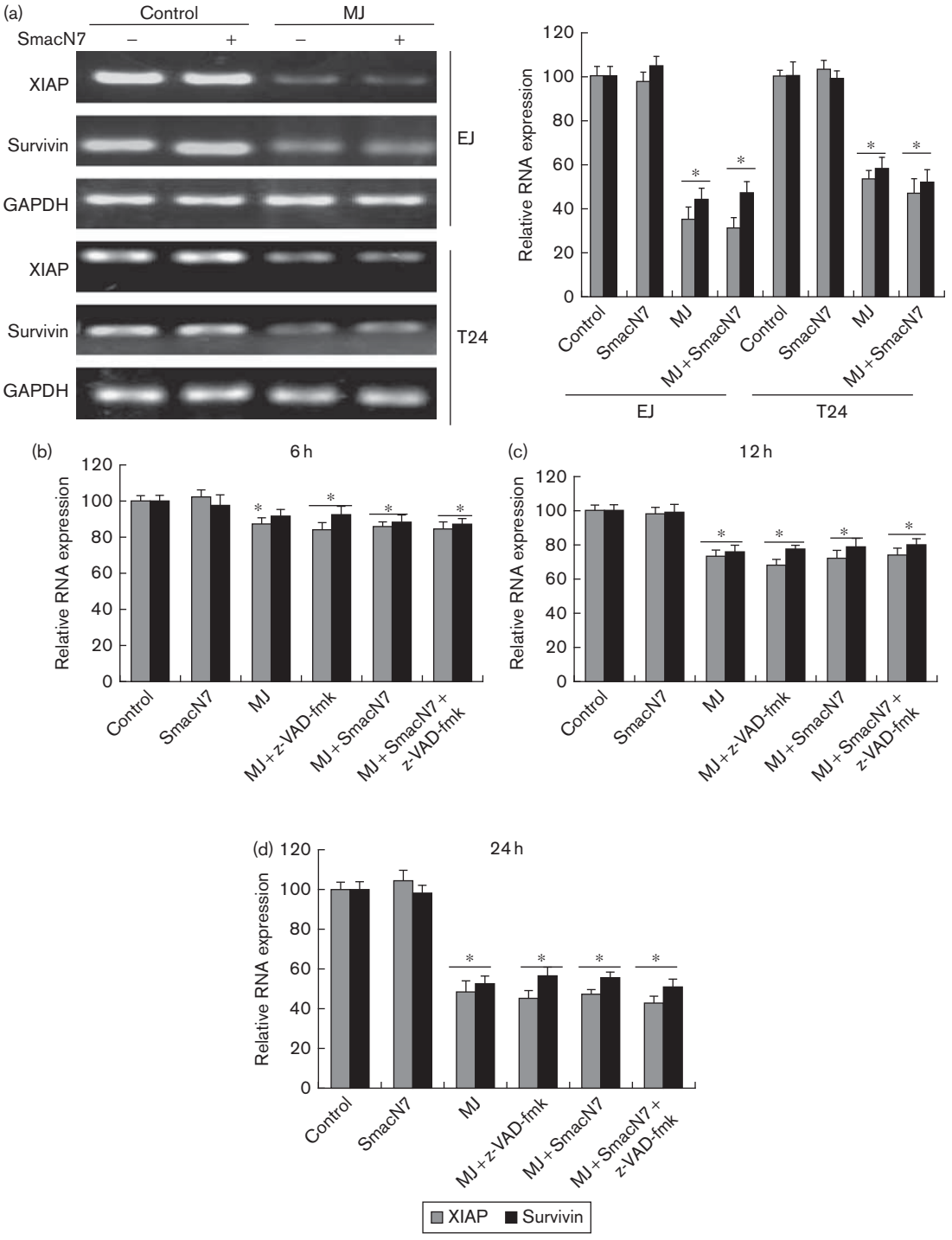
Interactive effects of SmacN7 with methyl jasmonate (MJ) on caspase-3 and caspase-9 activities. EJ (white bars) and T24 (black bars) cells were treated with SmacN7 (20 μmol/l) for 3 h, and then treated with control buffer, MJ (1 mmol/l), or MJ (1 mmol/l) with z-VAD-fmk (pan-caspase inhibitor) for 24 h. Lysates from EJ (white bars) and T24 (black bars) cells were mixed with specific fluorogenic substrates. Caspase-3 (a) and caspase-9 (b) activities were measured with a Synergy-2 fluorometer. MJ significant enhanced the activity of caspase-3 and caspase-9 from the respective control ($P<0.05$). Caspase-3 activity was increased by SmacN7 compared with MJ alone, whereas caspase-9 was not significantly affected by SmacN7. (c) Cell viability after different treatments was detected by 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide. Caspase inhibitor z-VAD-fmk dramatically reduced the downregulation of cell viability induced by MJ alone or combined with Smac in two cell lines. *Significantly different from the respective control at $P<0.05$; **significantly different from MJ alone at $P<0.05$. Data represent mean ± standard error.

caspase-3, suggesting that SmacN7 released the inhibitory effect of IAPs on the effector caspase. The activation of caspase-9 was higher after the combined treatment of MJ and SmacN7, but the differences to MJ alone treatment were not statistically significant. Caspase inhibitor z-VAD-fmk completely abolished apoptosis induced by MJ alone or MJ with SmacN7. These results indicate that the proapoptotic effect of SmacN7 depends on the activation of caspases, especially caspase-3 and cleaved caspase-9, which may be induced by activation of caspase-3. It is consistent with a previous study that caspase-3 can activate caspase-9 [36]. These results indicate that the proapoptotic effect of SmacN7 depends on the activation of caspase-3. This is consistent with a previous study in which cytochrome c release and apoptosis occur in a strictly caspase-3-dependent manner in human carcinoma cells [36].

In addition, we found that MJ also suppressed the transcription of XIAP and survivin in bladder cancer cells. Previous studies have shown that the introduction of the CP moiety increases this inhibitory effect, which induces the activation of transcription factor nuclear factor-kB [37]. However, SmacN7 had no effect on this interaction, although it enhanced MJ-induced downregulation of XIAP and survivin protein levels. Previous reports described that Smac exerted its effect in the cytoplasm by binding to a surface groove on IAPs and by blocking the binding site of the caspase [15,23], and that the XIAP protein can be cleaved by activated caspase-3 during apoptosis [32]. Thus, SmacN7 probably downregulates the levels of XIAP and survivin protein in a caspase-dependent manner, rather than suppressing its transcription.

In summary, this study established the sensitizing potential of Smac (SmacN7) on MJ-induced apoptosis in two bladder cancer cell lines in a caspase-3-dependent way. The combination treatments of SmacN7 and MJ allowed us to reduce the therapeutically active dose of MJ on bladder cancer cell lines. These are potentially

Fig. 6



Methyl jasmonate (MJ) downregulates X-linked inhibitor of apoptosis protein (XIAP) and suvivin expression of bladder cancer cells. (a) Confluent monolayers of EJ and T24 cells were pretreated with or without SmacN7 (20 μmol/l) for 3 h, followed by treatment with MJ (1 mmol/l) or control buffer for 24 h. Reverse transcription-PCR demonstrated that MJ decreased XIAP and survivin mRNA in both cell lines. Administration of SmacN7, however, resulted in no change in XIAP and survivin expression. Triplicate experiments were performed with essentially identical results. (b–d) EJ cells were pretreated with control buffer, SmacN7 (20 μmol/l) for 3 h, and then the cells were treated with control buffer, MJ (1 mmol/l), or MJ (1 mmol/l) and z-VAD-fmk for 6, 12, and 24 h respectively. The mRNA expression of XIAP and survivin were examined by real-time quantitative PCR. MJ downregulates XIAP and suvivin expression in EJ cells in a time-dependent manner. *Significantly different from the respective control at $P < 0.05$. Results are the mean ± standard error in triplicate. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

useful for future clinical application, as drug combinations help to achieve efficacy at tolerable dosages [38]. To the best of our knowledge, this is the first report showing that MJ and the combination of Smac result in growth inhibition of bladder cancer cells. As natural jasmonates exhibit a specific, but only moderate, effect against tumor cells, more potent jasmonate derivates have been developed [39,40]. Meanwhile, a series of nonpeptidic Smac mimetics have been designed, which show a higher potential to bind to IAPs than the original Smac peptide [41,42]. Thus, an improved treatment strategy relying on more potent jasmonate derivatives and Smac mimetics will possibly attain even higher therapeutic indexes.

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Conflicts of interest

There are no conflicts of interest.

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