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IJC
International Journal of Cancer

Enhancement of chemotherapeutic agent-induced apoptosis by inhibition of NF-κB using ursolic acid

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NF-κB activation is known to reduce the efficiency of chemotherapy in cancer treatment. Ursolic acid, a minimally toxic compound, has shown the capability to inhibit NF-κB activation in living cells. Here, for the first time, we investigated the effects and mechanisms of NF-κB inhibition by ursolic acid on chemotherapy treatment (Taxol or cisplatin) of cancer. ASTC-a-1 (human lung adenocarcinoma), Hela (human cervical cancer) cells, primary normal mouse cells of lung and liver and mouse *in vivo* model were used. Activity of signal factors (NF-κB, Akt, Fas/FasL, BID, Bcl-2, cytochrome *c* and caspase-8, 3) was used to analyze the mechanisms of ursolic acid-chemo treatment. Ursolic acid-mediated suppression of NF-κB drastically reduced the required dosage of the chemotherapeutic agents to achieve identical biological endpoints and enhanced the chemotherapeutic agent-induced cancer cells apoptosis. Chemosensitization by ursolic acid in cancer cells was dependent on the amplified activation of intrinsic pathway (caspase-8-BID-mitochondria-cytochrome *c*-caspase-3) by augmentation of BID cleavage and activation of Fas/FasL-caspase-8 pathway. Prolonged treatment with relatively low doses of ursolic acid also sensitized cancer cells to the chemotherapeutic agents through suppression of NF-κB. Chemosensitization by ursolic acid was observed only in cancer cells, but not in primary normal cells. The inhibitive effect of ursolic acid on NF-κB was reversible, and the reversal was not accompanied by a loss in cells viability. By supplementing chemotherapy with minimally toxic ursolic acid, it is possible to improve the efficacy of cancer treatment by significantly reducing the necessary drug dose without sacrificing the treatment results.

Activation of NF-κB is believed to promote cell survival and upregulate the expression of genes for tumor proliferation and metastasis, including antiapoptotic factors (cIAP, TRAF, bcl-2 and bcl-xl), COX-2, MMP-9, adhesion molecules, chemokines, inflammatory cytokines, iNOS and cell cycle-regulatory factor (cyclin D1).[1,2] The chemotherapeutic agent-induced NF-κB activation in cancer cells upregulates survival signals, thus reduced the effectiveness of a treatment.[3,4] Taxol (Paclitaxel) and cisplatin (*cis*-diammine dichloroplatinum, *cis*-Platinum (II)) are important clinical chemotherapeutic agents. Both are shown to cause NF-κB activation in cancer cells.[5,6]

Ursolic acid is a compound derived from berries, leaves, flowers and fruits of medicinal plants and is shown to have minimal toxicity to the human body. It has been reported that ursolic acid can effectively prohibit NF-κB activation, and this inhibitive effect is not cell type specific.[2] We thus hypothesize that, with a pretreatment with ursolic acid, it is possible to improve the efficacy of the chemotherapeutic agents by prohibiting NF-κB activation. This will likely reduce the required chemotherapy dosage for a certain therapeutic endpoint and the associated side effects commonly associated with the therapy.

In most cases, chemosensitization caused by suitable adjuvants is often associated with Fas/FasL signal pathway reactivation, because cancer cells are often resistant to Fas/FasL-mediated apoptosis.[7–9] Formation of Fas/FasL signaling complex stimulates caspase-8 activation that induces cells apoptosis by activating caspase-3 either directly (extrinsic pathway) or indirectly (intrinsic pathway) *via* the mitochondrial apoptotic signal pathway. In intrinsic pathway, BID is cleaved by caspase-8, and its activated form tBID translocates to the mitochondria and disrupts organelles. This results in the release of apoptogenic molecules such as cytochrome *c* and apoptosis-inducing factor. Released cytochrome *c* can bind to

Key words: Taxol, cisplatin, ursolic acid, Western blot, FRET

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Basic Research Program of China; **Grant number:** 2010CB732602; **Grant sponsor:** Program for Changjiang Scholars and Innovative Research Team in University; **Grant number:** IRT0829; **Grant sponsor:** National Natural Science Foundation of China; **Grant numbers:** 30870676, 30870658

DOI: 10.1002/ijc.25044

History: Received 13 Aug 2009; Accepted 3 Nov 2009; Online 11 Nov 2009

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Apaf-1 and caspase-9, forming a complex that activates caspase-3. Activated caspase-3 results in final cell apoptosis.[10–12]

FRET (fluorescence resonance energy transfer) technique has been used to study protein–protein interaction in living cells for highly temporal sensitivity.[13–15] Several FRET fluorescence reporters, such as SCAT-3,[16] pFRET-Bid[17] and BKAR,[18] have been constructed to dynamically analyze signal pathways of cell apoptosis and proliferation.[19,20]

In this study, we investigated the effect and potential mechanism of chemosensitization by ursolic acid in chemotherapy treatment of cancer. Our data show that pretreatment with ursolic acid chemosensitizes cancer cells, but not normal cells, and significantly reduces required chemotherapeutic drug dose without sacrificing treatment outcome. By further exploring the mechanism of this combination therapy, it is revealed that chemosensitization by ursolic acid is dependent on the inhibition of NF-κB, leading to amplified activation of intrinsic pathway *via* augmentation of BID cleavage and activation of Fas/FasL-caspase-8 pathway.

Material and Methods

Materials and cell culture

Taxol, cisplatin and ursolic acid were purchased from Sigma (St. Louis, MO) and were dissolved in DMSO. The final DMSO concentration in culture was 0.1% in all experiments. ASTC-a-1 cells were obtained from the Department of Medicine, Jinan University. Normal primary cells were isolated from the liver and lung of Kunming white mice. Cells were grown in DMEM supplemented with 15% fetal calf serum (FCS) and maintained at 37°C in a humidified atmosphere (95% air and 5% CO_2). Antibodies against caspase-8 (1C12), caspase-3 (8G10), phospho-Akt(Thr308), cytochrome *c*, BID and Bcl-2 were purchased from Cell Signaling (Beverly, MA). Antibodies against NF-κB p65 (C-20), sc-372, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human FasL neutralizing antibody (Nok-1), anti-human Fas (DX-2) and mouse IgG1, κ isotype antibody, were purchased from Biolegend (San Diego, CA). Z-IETD-fmk was purchased from BioVision (Mountain View, CA). All other chemicals were analytical grade if not otherwise indicated. For cells transfection, Lipofectamine™ 2000 reagent (Invitrogen) was used to introduce the corresponding plasmids cells. pECFP-IκBαM was constructed by X. Li in our lab.[21] pNF-κB-Luc was kindly provided by Dr. X. Shen (Institute of Biophysics, Chinese Academy of Sciences); pFRET-Bid was kindly provided by Dr. K. Taira (University of Tokyo, Hongo, Tokyo, Japan); pDsRed-Mit plasmid was kindly provided by Dr. Y. Gotoh (University of Tokyo, Yayoi, Tokyo, Japan) and pE-GFP-Cyt-c plasmid was provided by Dr. G.J. Gores (Center for Basic Research in Digestive Diseases, Molecular Medicine Program, Mayo Clinic, Rochester, MN).

Colonogenic survival and CCK-8 cytotoxicity assay

For colonogenic survival studies, cells (300 cells per 60 mm Petri dishes) in different treatment groups were cultured in regular medium for 12 days. The colonies were fixed for 10 min in 100% methanol and stained with a 10% Giemsa solution for 10 min. Percent colony formation was calculated by assigning untreated cultures as 100%. Percent colony formation of treated cells was determined by (colony formation of treated cells/colony formation of untreated cells)×100. Cells transfected with pECFP-IκBαM or pNF-κB-Luc were screened in the presence of 0.8 mg/ml G418 for 2 weeks, and stable clones were selected and cultured before CCK-8 cytotoxicity assay. The growth rate of IκBαM-cells was slower than that of nontransfected cells.

In CCK-8 cytotoxicity assay, cells were seeded in 96-well plates at 3×10^3 cells/well and exposed to Taxol, cisplatin, ursolic acid or their combinations of designated concentrations. At designated times, cell vitality was assayed with CCK-8 (Cell Counting Kit, Dojindo Laboratories, Kumamoto, Japan) using a 96-well plate reader (DG5032, Huadong, Nanjing, China), and absorbance at 450 nm was obtained.

Western blot assay for p65 translocation, caspase-8, 3, cytochrome c, BID and Fas/FasL

After various treatments, floating and attached cells were harvested and combined, washed twice with ice-cold PBS and lysed in lysis buffer (50 mmol/l Tris·HCL pH 8.0, 150 mmol/l NaCl, 1% Trition X-100 and 100 μg/ml PMSF) for 15 min on ice. Briefly, equal protein/lane was separated by SDS-PAGE and then blotted onto a polyvinylidene difluoride immobilon membrane. The membrane was incubated with the solution of diluted primary antibodies (1:000 v/v) overnight at 4°C. Subsequently, the membrane was incubated with secondary antibodies, goat anti-rabbit conjugated to IRDye™ 800 (Rockland Immunochemicals, Gilbertsville, PA) for 2 hr, and then detected using the LI-COR Odyssey Scanning Infrared Fluorescence Imaging System (LI-COR, Lincoln, NE).

For p65 translocation assay, the treated cells were homogenized with NEBA buffer (10 mM HEPES (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF) supplemented with 2.5% NP40 and then homogenized in NEBB (20 mM HEPES (pH 7.8), 0.4 mM NaCl, 1 mM EDTA and 1 mM EGTA) to obtain nuclear extracts for Western blot analysis. For cytochrome *c* release assay, treated cells were harvested and resuspended in lysis buffer for 10 min [80 mM KCl, 250 mM sucrose and 50 μl/ml digitonin (Sigma-Aldrich, St. Louis, MO), 1 mM DTT and Protease Inhibitor Cocktail Set I (Calbiochem, San Diego, CA)]. Then, samples were centrifuged at 10,000*g* for 5 min, and then cytosol was isolated. To extract mitochondrial proteins, the pellet was resuspended in lysis buffer, lysed by freeze/thaw and centrifuged at 20,000*g* for 10 min. Last, total proteins from cytosol and mitochondria were analyzed by Western blot. For assay of caspaes-8, 3, BID and Fas/FasL, the treated cells were analyzed by Western blot using appropriate antibodies.

Luciferase assay of pNF-κB-Luc

The luciferase assay of pNF-κB-Luc is based on the description by Nelson *et al.*[22] For microtiter plate-based living cell

luciferase assays, 1 mM luciferin was added to the medium, and luminescence was recorded a further 12 hr after the addition of luciferin. The 96-well microplate reader (DG5032, Huadong, Nanjing, China) was used to monitor the luminescence. Measurements of luminescence from 96-well plates were performed using an integration time of 10 sec per well per time point. The luminescence of pNF-κB-Luc in different time points represents the transcriptional activity of NF-κB. Treatment cells (ASTC-a-1) with chemotherapeutic agents for 1 hr caused significant p65 nuclear translocations; therefore, the luminescence of pNF-κB-Luc was monitored within 1-hr treatment.

Flow cytometry assay

For FACS (fluorescence-activated cell sorting) analysis, Annexin-V-FITC conjugate was used, and binding buffer was included as standard reagents. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) with excitation at 488 nm. Fluorescent emission of FITC was measured at 515–545 nm and that of DNA-PI complexes at 505–606 nm. Cell debris was excluded from analysis by appropriate forward light scatter threshold setting. Compensation was used wherever necessary. Apoptosis and necrosis were analyzed by quadrant statistics on the propidium iodide-negative, fluorescein isothiocyanate-positive cells and propidium iodide-positive cells, respectively.

Laser scanning microscope

FRET was performed on a commercial laser scanning microscope system (LSM510/ConfoCor2, Zeiss, Jena, Germany). For excitation, the 458 nm line of an Ar-Ion Laser was attenuated with an acousto-optical tunable filter, reflected by a dichroic mirror (main beam splitter HFT458). The emission fluorescence was split by a second dichroic mirror (secondary beam splitter NFT515) into 2 separate channels: the 470–500 nm bandpass (CFP channel) and the 530 nm long pass (YFP channel). The fluorescence intensities of YFP and CFP from the cells and the corresponding YFP/CFP emission ratio were obtained and analyzed to observe the dynamics of BID cleavage. GFP was excited at 488 nm with an argon ion laser, and its fluorescence was recorded through a 500–530 nm IR bandpass filter. DsRed was excited at 543 nm with a helium-neon laser, and its emitted light was recorded through a 560 nm long-pass filter. The overlapping of fluorescence images of pE-GFP-Cyt-c and DsRed-Mit plasmid dynamically indicates the release of cytochrome *c* from mitochondria. For intracellular measurements, the desired measurement position was chosen in the LSM image. Zeiss image processing software (Rel3.2, Zeiss, Germany) was used to record fluorescence images and fluorescence intensities.

shRNA interference on BID

BID suppression was accomplished using shRNA constructs with specific sequences of BID. To generate BID knockdown plasmids, the corresponding oligonucleotides were cloned into pGPU6/GFP/Neo vector (GenePharma, Shanghai, China). The oligonucleotides for shRNA-BID were 5′-CAC CGCGCCGTCCTTGCTCCGTGATTCAAGAGATCACGGA GCAAGGACGGCGTTTTTTG-3′. The negative control shRNA-NC was also generated, and the oligonucleotides sequences were 5′-CACCGTTCTCCGAACGTGTCACGTC AAGAGATTACGTGACACGTTCGGAGAATTTTTTG-3′. In Western blot and CCK-8 cytotoxicity assay, cells were cultured for 48 hr after shRNA-BID or shRNA-NC transfection and then subjected to the analysis.

Mode of ursolic acid-chemo treatment

In combination treatment of ursolic acid and chemotherapeutic agents (Taxol or cisplatin), cells were first treated with ursolic acid for designated times. The ursolic acid was removed by washing the cells with PBS 3 times. The treated cells were subsequently treated with the chemotherapeutic agents. When 100 μM ursolic acid was used, pretreatment time of ursolic acid was 8 hr. When 1 and 10 μM ursolic acid was used, pretreatment time of ursolic acid was 48 hr. For the ursolic acid treatment alone (negative control), the cells pretreated with ursolic acid were cultured in regular cell culture medium for designated times when the pretreatment time of ursolic acid reached. In colonogenic survival assay, cells were first treated with 100 μM ursolic acid and then exposed to various doses of Taxol for 24 hr or cisplatin for 16 hr. In flow cytometry assay, cells were pretreated with 100 μM ursolic acid and then exposed to 1 μM Taxol or 20 μM cisplatin for 16 hr. In Western blot assay for caspase-3, cells were pretreated with 100 μM ursolic acid and then exposed to 1 μM Taxol for 13 and 16 hr or 20 μM cisplatin for 14 and 16 hr. In fluorescence assay of BID, cells were first treated with 100 μM ursolic acid and then exposed to 1 μM Taxol for 6 hr or to 20 μM cisplatin for 3 hr before imaging. In fluorescence assay of cytochrome *c* assay, cells were pretreated with 100 μM ursolic acid for 8 hr and then exposed to 1 μM Taxol for 6 hr or 20 μM cisplatin for 4 hr before imaging. In Western blot assay for caspase-8, cells were pretreated with 100 μM ursolic acid and then exposed to 1 μM Taxol or 20 μM cisplatin for 12 and 24 hr. In cells viability assay with pECFP-IκBαM transfection, shRNA interference on BID, Nok-1 or Z-IETD-fmk, cells were first pretreated with 100 μM ursolic acid and then exposed to 1 μM Taxol for 24 hr or 20 μM cisplatin for 16 hr. In Western blot analysis on Fas or FasL expression, cells were first treated with 100 μM ursolic acid and then exposed to 1 μM Taxol or 20 μM cisplatin for 16 hr. In Western blot analysis on earlier cleavage of BID, ASTC-a-1 cells with 100 μM ursolic acid pretreatment were exposed to 1 μM Taxol for 10 hr or 20 μM cisplatin for 5 hr. In Fas/FasL-caspase-8 pathway assay, ASCT-a-1 cells were first treated with 1 μg/ml anti-FasL (Nok-1) or 10 μM Z-IETD-fmk for 1 hr before adding Taxol, cisplatin or ursolic acid. Treatment with 1 μg/ml IgG isotype served as a control to FasL antibody. In the assay of p65 translocation of low-doses ursolic acid-chemo treatment,

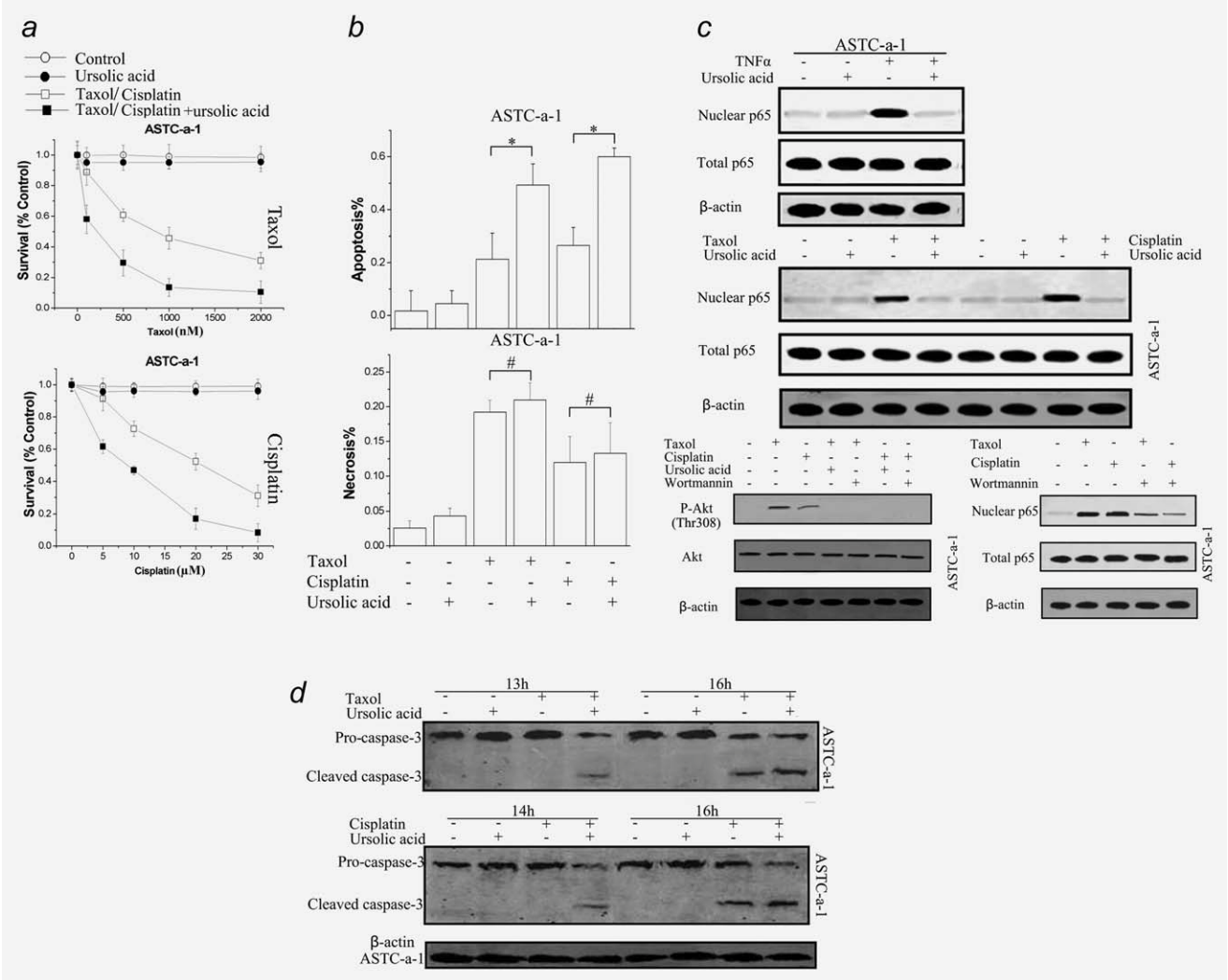

Figure 1. Evaluation of ursolic acid-chemo treatment on cancer cells growth. (*a*) Cells survival rates under different treatments. ASTC-a-1cells were first treated with 100 μM ursolic acid for 8 hr. After removing ursolic acid, the treated cells were exposed to 0 nM, 100 nM, 500 nM, 1 μM and 2 μM Taxol for 24 hr or 0, 5, 10 and 20 μM cisplatin for 16 hr, respectively. After 12 days, colonies were stained with a 10% Giemsa solution and counted in each group. (*b*) ASTC-a-1 cells were pretreated with 100 μM ursolic acid for 8 hr and then treated alone with 1 μM Taxol or 20 μM cisplatin for 16 hr, respectively, before flow cytometry assay. (*c*) Effects of ursolic acid-chemo treatment on NF-κB p65 and Akt in Western blot analysis. Before Western blot assay on NF-κB p65 or Akt, ASTC-a-1 cells were pretreated with 100 μM ursolic acid or 1 μM wortmannin (30 min) and then exposed alone to TNFα (10 ng/ml) for 30 min or chemotherapeutic agents (1 μM Taxol and 20 μM cisplatin) for 1 hr. (*d*) Ursolic acid accentuates 1 μM Taxol- or 20 μM cisplatin-induced caspase-3 activation. The β-actin blot was used to evaluate equal protein loading in each blot for capsase-3 activation in ursolic acid-Taxol treatment, and the similar results were observed in ursolic acid-cisplatin treatment, but not shown. Treatment with Taxol, cisplatin or ursolic acid served as a comparison. Results represent 1 of 3 replicates. *$p < 0.05$, significantly different, #$p > 0.05$, not significantly different.

cells were pretreated with 1, 10 and 100 μM ursolic acid and then treated with 10 nM Taxol or 2 μM cisplatin for 1 hr. In cells viability assay of low-doses ursolic acid-chemo treatment, cells were pretreated with 1, 10 and 100 μM ursolic acid and then treated with 10 nM Taxol or 2 μM cisplatin for 48 hr. Cells were also treated alone with Taxol, cisplatin or 0.1% DMSO for designated times to make a comparison with combination treatment.

Results

Effects of ursolic acid-chemo treatment on cancer cells growth

In all experiments of our study, 2 cancer cell lines were used: ASTC-a-1 and Hela cells. The results of ASTC-a-1 cells are similar to that of Hela cells. Only data on ASTC-a-1 are given in this study. To analyze combined effects of ursolic acid and chemotherapeutic agents (Taxol and cisplatin) on

Table 1. Dose response analysis for the effect of Taxol or cisplatin on survival fraction in the presence or absence of ursolic acid during colonogenic survival assay (Fig. 1*a*)

	ASTC-a-1-LD_{50}
Taxol	800 nM
Taxol (ursolic acid)	142 nM
Cisplatin	20.3 μM
Cisplatin (ursolic acid)	7.4 μM

Ursolic acid significantly decreases the LD_{50} of Taxol- or cisplatin-mediated chemotherapy in ASTC-a-1 cells.

cancer cells growth, colonogenic survival assay was performed on ASTC-a-1 cells at 24-hr incubation with Taxol and 16-hr incubation with cisplatin. The use of ursolic acid is referred to the description by Shishodia *et al.* to suppress Taxol- or cisplatin-induced NF-κB activation[2]: 100 μM ursolic acid was used to pretreat cells for 8 hr and then removed to avoid the cytotoxicity induced by ursolic acid itself. After that, the treated cells were exposed to the chemotherapeutic agents alone. Our data were nearly consistent with the conclusions described by Shishodia *et al. in vitro*: (*i*) Continuous and long-time treatment with ursolic acid caused cytotoxicity to cells (Supporting Information Fig. S1). (*ii*) Treatment with ursolic acid for certain times (*e.g.*, 100 μM for 8 hr or 10 and 1 μM for 48 hr) and then removing ursolic acid did not lead to a decrease of cells viability at various durations (Supporting Information Fig. S2). Colonogenic survival assay (Fig. 1*a*) shows that cancer cells treated with ursolic acid exhibit a modest sensitizing effect to Taxol- or cisplatin-induced cytotoxicity (20–30%). LD_{50} was determined by analyzing the data of colonogenic survival assay (Fig. 1*a*). It is noted that ursolic acid drastically reduces LD_{50} in chemotherapy (Table 1). LD_{50} of Taxol is reduced from 800 to 142 nM. Similarly, LD_{50} of cisplatin is reduced from 20.3 to 7.4 μM. Our data from *in vivo* mouse model (Supporting Information Fig. S3) show that ursolic acid drastically reduces the necessary drug dose without sacrificing the treatment results, and that ursolic acid/chemotherapeutic agents combination treatment resulted in tumor regression (6 day), whereas the corresponding single drug treatments only resulted in tumor growth suppression. The combination treatment was well tolerated with no weight loss (Supporting Information Fig. S3) or observable toxicities in the combination treatment group. Based on the colonogenic survival assay above, 1 μM Taxol and 20 μM cisplatin were used in the below cellular experiments to induce detectable amounts of cell death.

In Annexin-V-FITC binding assay, ursolic acid-chemo treatment resulted in an early apoptotic cells increase (>25%) *in vitro* when compared with the chemotherapies alone, but not necrosis (Fig. 1*b*). We subsequently analyzed the effects of ursolic acid on NF-κB inhibition in chemotherapy. Two standards were used to evaluate the activation of NF-κB: p65 nuclear translocation (Fig. 1*c*) and transcriptional activation of NF-κB by pNF-κB-Luc luciferase reporter assay (Supporting Information Fig. S4). TNFα treatment group serves as a positive control. Our data *in vitro* show that ursolic acid significantly inhibits the activation of NF-κB induced by chemotherapeutic agents. Meanwhile, the results are consistent with immunofluorescence assay of NF-κB p65 (Supporting Information Fig. S5). A strong activation of NF-κB was observed in cells treated with TNFα or chemotherapeutic agents. However, the fraction of nuclear p65 and transcriptional activation of NF-κB were minimal in nontreated cells. The results indicate that NF-κB is not constitutive active in cancer lines used for this study. In addition, ursolic acid could completely inhibit chemotherapeutic agent-induced Akt activation (Fig. 1*c*). The specific inhibition of Akt alone by wortmannin (a specific Akt inhibitor) partially inhibited chemotherapeutic agent-induced NF-κB activation (Fig. 1*c* and Supporting Information Fig. S4). Caspase-3 activation induced by Taxol or cisplatin was accentuated by ursolic acid *in vitro*, which by itself alone did not induce any caspase-3 activation, as shown in Figure 1*d*. Thus, ursolic acid-mediated chemosensitization was NF-κB inhibition and caspase-3 dependent.

Effects of ursolic acid-chemo treatment on cytochrome c release

To examine the contribution of mitochondria-mediated apoptosis for the activation of caspase-3 under different treatment conditions, the activities of cytochrome *c* in ASTC-a-1 cells were assayed. Western blot analysis shows that ursolic acid increases the chemotherapeutic agent-induced cytochrome *c* release, as shown in Figure 2*a*. Real-time fluorescence analysis was used to determine the initial time of cytochrome *c* release. By dynamically observing fluorescence overlapping of cytochrome *c* (green) and mitochondria (red), we found that the initial time of chemotherapeutic agent-induced cytochrome *c* release was advanced by ursolic acid (Figs. 2*b* and 2*c*). The earlier release of cytochrome *c* might be a reason to explain the increasing release of cytochrome *c* in ursolic acid-chemo treatment.

Effects of ursolic acid-chemo treatment on BID cleavage

To determine the relationship between the release of cytochrome *c* and the cleavage of BID, the activities of BID in ASTC-a-1 cells were assayed. Western blot analysis shows that ursolic acid augments chemotherapeutic agent-induced BID cleavage (Fig. 3*a*). Real-time fluorescence analysis was used to determine the initial time of BID cleavage. The decrease of YFP emission and increase of CFP emission in FRET-Bid reporter indicate BID cleavage. Thus, dynamic changes of YFP/CFP allow us to determine initial time of BID cleavage, because YFP/CFP declines when BID is cleaved. By dynamically observing the changes of YFP/CFP emission ratio, we found that the initial time of chemotherapeutic agent-induced BID cleavage was advanced by ursolic acid (Figs. 3*b* and 3*c*). The earlier cleavage of BID might be a

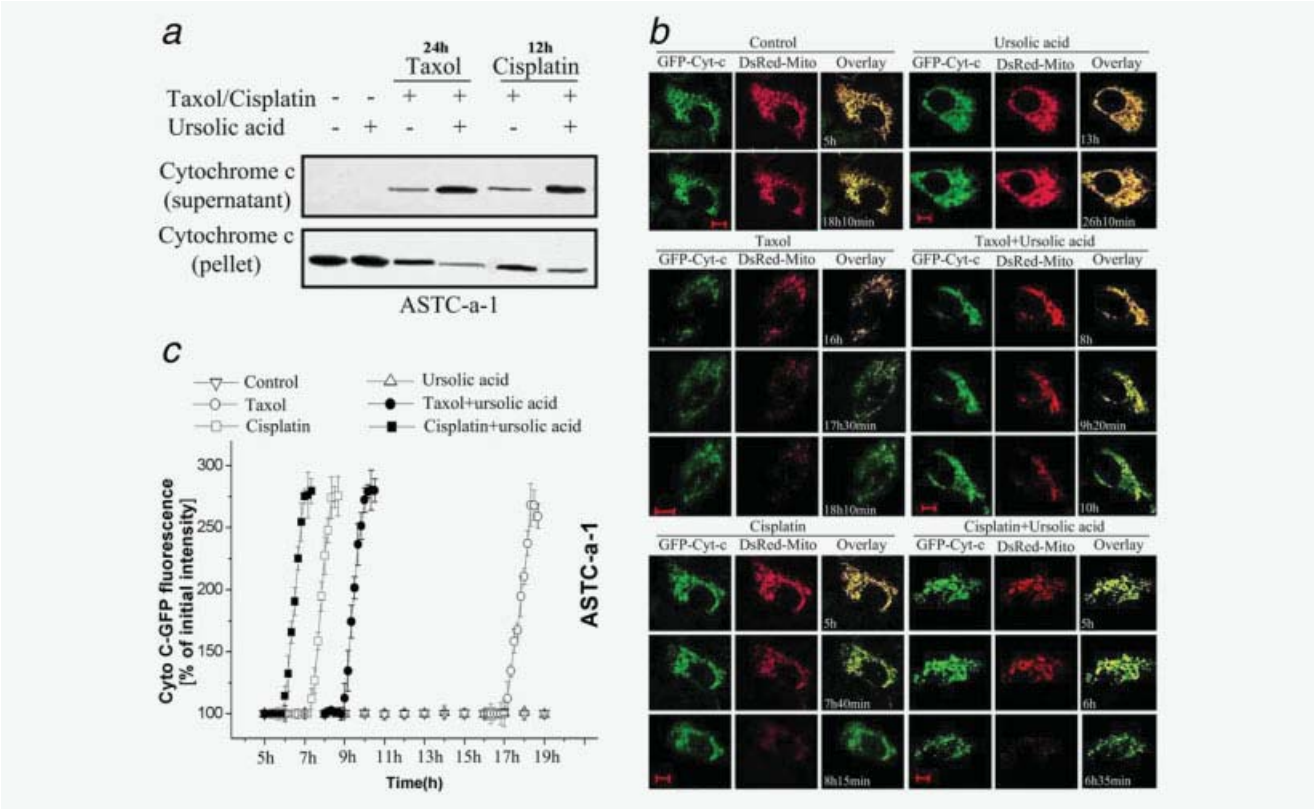

Figure 2. Effects of ursolic acid-chemo treatment on cytochrome *c* release. (*a*) Ursolic acid increases chemotherapeutic agent-induced cytochrome *c* release. ASTC-a-1 cells were first treated with 100 μM ursolic acid for 8 hr, and then the treated cells were treated alone with 1 μM Taxol for 24 hr or 20 μM cisplatin for 12 hr before Western blot analysis on cytochrome *c*. (*b*) Fluorescence images of ASTC-a-1 cells under different treatments. Cells were transiently cotransfected with DsRed-Mit and E-GFP-Cyt-*c*, which were used to mark mitochondria and cytochrome *c*, respectively. (*c*) Intensities of fluorescent emission from ASTC-a-1 cells under different treatments. The initial time of cytochrome *c* release from mitochondria in cells treated with ursolic acid-chemo treatment was much earlier than with Taxol or cisplatin alone. For each cell expressing GFP-Cyt-*c*, a small region containing only cytosol was marked by a rectangular box. After background subtraction, the average fluorescence intensity of GFP-Cyt-*c* in the region was obtained for each image in the time series. These emission intensity values are directly proportional to the concentrations of GFP-Cyt-*c* in cytosol. The cytochrome *c* release is reflected by the sharp increase of the emission intensity. Each curve represents an average of 10–15 cells obtained from 3 independent experiments. Scale bar = 10 μm. β-actin blot was used to evaluate equal protein loading in each blot. Treatment with ursolic acid, Taxol or cisplatin served as comparisons. Results represent 1 of 3 replicates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

reason to explain the augmentation of BID cleavage in ursolic acid-chemo treatment.

Effects of ursolic acid-chemo treatment on caspase-8 and Fas/FasL signaling

To determine whether the enhanced apoptosis in ursolic acid-chemo treatment was caspase-8 dependent, Western blot analysis was performed using treated ASTC-a-1 cells. Using Taxol alone, caspase-8 was activated with or without ursolic acid pretreatment, and caspase-8 activation was enhanced in ursolic acid-Taxol treatment (Fig. 4*a*). In cisplatin-treated cells, caspase-8 activation was observed only when cells were pretreated with ursolic acid (Fig. 4*a*). The results indicate that ursolic acid-chemo treatment upregulates the activity of caspase-8. Meanwhile, chemotherapeutic agent-induced caspase-3 activation, cytochrome *c* release and BID cleavage were potentiated by ursolic acid. Thus, 2 possibilities existed in chemosensitization by ursolic acid: (*i*) activation of both intrinsic (caspase-8-BID-mitochondria-caspase-3) and extrinsic (caspase-8-caspase-3) pathway was amplified. (*ii*) Only activation of intrinsic pathway was amplified.

To determine contribution of extrinsic or intrinsic pathway in chemosensitization by ursolic acid, the intrinsic pathway was blocked by knocking down BID with shRNA-BID. In CCK-8 cytotoxicity assay, the chemosensitization by ursolic acid was almost inhibited by transfecting cells with shRNA-BID, whereas the cells transfected with shRNA-NC (negative control) were still sensitive to the ursolic acid-chemo treatment (Fig. 4*b*). The results indicate that chemosensitization by ursolic acid depends on the amplified

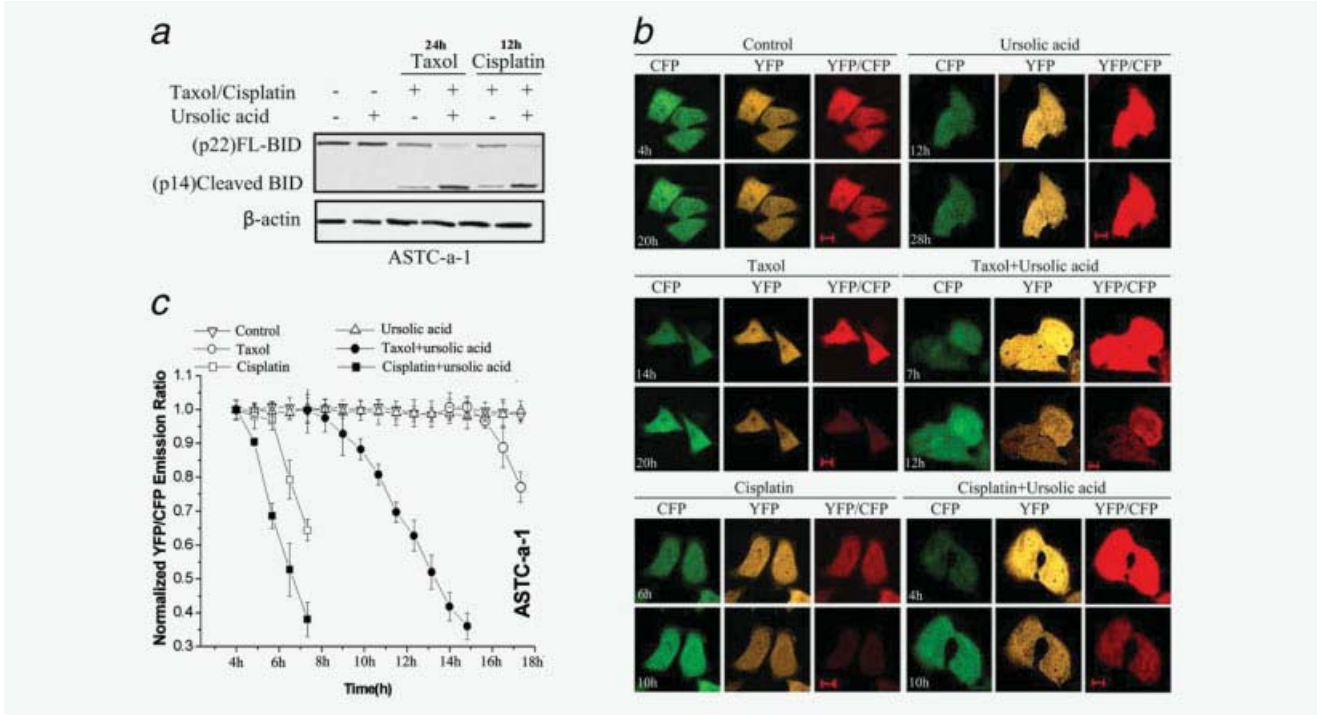

Figure 3. Effects of ursolic acid-chemo treatment on BID cleavage. (*a*) Ursolic acid augments chemotherapeutic agent-induced BID cleavage. ASTC-a-1 cells were first treated with 100 μM ursolic acid for 8 hr and then treated alone with 1 μM Taxol for 24 hr or 20 μM cisplatin for 12 hr before Western blot analysis. (*b*) ASTC-a-1 cells were transiently transfected with pFRET-Bid. Decrease of YFP fluorescent emission and increase of CFP fluorescent emission represent BID cleavage in living cells. No cleavage of BID was detected in fluorescence images of ASTC-a-1 cells treated with 0.1% DMSO (control) or ursolic acid. BID cleavage was observed in images of ASTC-a-1 cells treated with Taxol alone or Taxol plus ursolic acid and with cisplatin alone or cisplatin plus ursolic acid. (*c*) Temporal profiles of YFP/CFP emission ratio of FRET-Bid from ASTC-a-1 cells show that Taxol plus ursolic acid or cisplatin plus ursolic acid caused an earlier BID cleavage than by Taxol or cisplatin alone. Each curve represents an average of 10–15 cells obtained from 3 independent experiments. Scale bar = 10 μm. β-actin blot was used to evaluate equal protein loading in each blot. Treatment with ursolic acid, Taxol or cisplatin served as comparisons. Results represent 1 of 3 replicates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

activation of intrinsic pathway by augmentation of BID cleavage, and that the extrinsic pathway was minimally affected by ursolic acid. Western blot assay was performed to determine the effects of chemo-ursolic acid treatment on Bcl-2 (a BID interacting protein). The results (Fig. 4*b*) show that expression of Bcl-2 was upregulated by Taxol or cisplatin, and that ursolic acid significantly reduced the chemotherapeutic agent-induced upregulation of Bcl-2. Bcl-2 is a negative regulator of BID. Thus, enhancement of BID cleavage by ursolic acid is associated with downregulation of Taxol- or cisplatin-induced Bcl-2 expression.

The aforementioned results show that NF-κB inhibition, caspase-8 activation and BID cleavage are involved in chemosensitization by ursolic acid. Meanwhile, it is classic that Fas/FasL-mediated caspase-8 activation can result in BID cleavage, and activation of Fas/FasL signal pathway plays a key role in NF-κB inhibition-induced cells apoptosis or chemosensitization. To determine whether augmentation of BID cleavage by ursolic acid involves Fas/FasL-caspase-8 pathway, FasL neutralizing antibody (Nok-1) and Z-IETD-fmk (caspase-8 inhibitor) were used. Nok-1 is an anti-human FasL neutralizing antibody, which can effectively inhibit Fas/FasL signal pathway by binding and sequestering FasL. No protection of chemotherapeutic agent-induced cells death by Nok-1 (Fig. 4*c*) shows that Fas/FasL signaling does not mediate ASTC-a-1 cells apoptosis induced by chemotherapy alone. The addition of Nok-1 antibody or Z-IETD-fmk significantly protected cell death and inhibited the earlier cleavage of BID in ursolic acid-chemo treatment (Figs. 4*c* and 4*d*). The protein levels of either Fas or FasL were upregulated in ursolic acid-chemo treatment, and no marked changes were observed in Fas and FasL levels in chemotherapy alone (Fig. 4*d*). The results indicate that ursolic acid augments BID cleavage by activation of Fas/FasL-caspase-8 pathway, and that the activation of Fas/FasL signaling is likely to be caused by the upregulation of Fas/FasL by ursolic acid.

Effects of relatively low-doses ursolic acid-chemo treatment on cancer and normal cells

Until now, it was still unclear whether this inhibitive effect on NF-κB could be induced by other ursolic acid doses (more specifically, <100 μM) with prolonged pretreatment

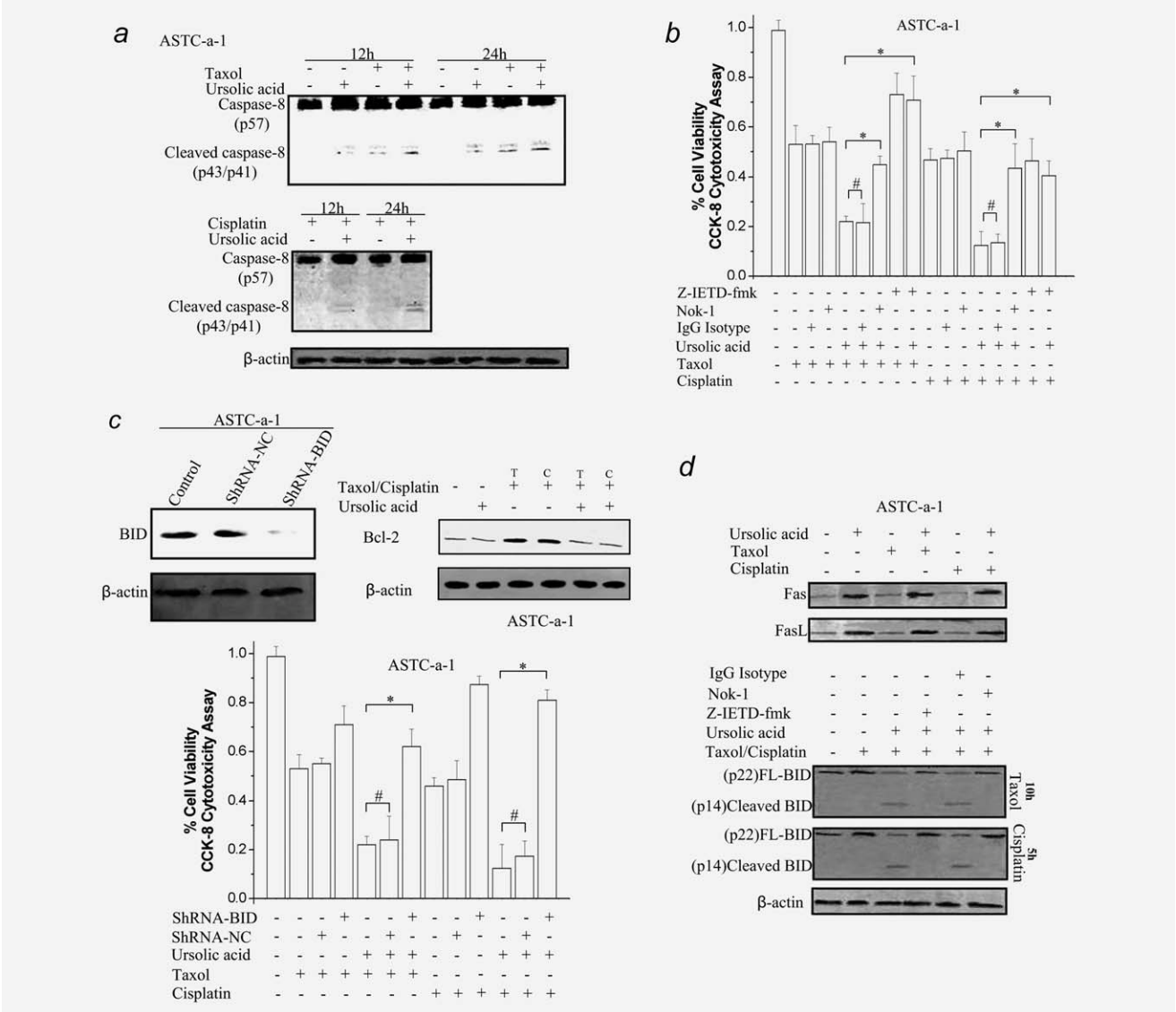

Figure 4. Effects of ursolic acid-chemo treatment on caspase-8 and Fas/FasL Signaling (*a*) The activity of caspase-8 was upregulated by ursolic acid. Ursolic acid-Taxol treatment enhanced caspase-8 activation when compared with Taxol alone. Activation of caspase-8 was only observed in ASTC-a-1 cells treated with cisplatin plus ursolic acid, but not with cisplatin alone. (*b*) Chemosensitization by ursolic acid was dependent on the augmentation of BID cleavage and Bcl-2. In CCK-8 cytotoxicity, ASTC-a-1 cells transfected with shRNA-BID exhibited no response to ursolic acid-mediated chemosensitization. (*c*, *d*) The chemosensitization and earlier cleavage of BID by ursolic acid were suppressed by FasL neutralizing antibody (Nok-1) or Z-IETD-fmk. Fas and FasL were upregulated by ursolic acid. In CCK-8 cytotoxicity assay, cells treated with 1 μg/ml Nok-1 or 10 μM Z-IETD-fmk exhibited no sensitization to ursolic acid-chemo treatment. In Western blot analysis, pretreatment with 100 μM ursolic acid upregulated Fas and FasL; treatment with Taxol or cisplatin alone did not influence the levels of either Fas or FasL. The earlier cleavage of BID was inhibited by Nok-1 or Z-IETD-fmk in ursolic acid-chemo treatment. IgG isotype served as a control to FasL neutralizing antibody. The β-actin blot was used to evaluate equal protein loading in each blot for caspase-8 (Taxol) and protein expression assay of Fas and FasL, and the similar results were observed in other treatment groups, but not shown. Results represent 1 of 3 replicates. *$p < 0.05$, significantly different, #$p > 0.05$, not significantly different.

time. To address the above concerns and examine the possibility of ursolic acid-chemo treatment *in vivo*, relatively low doses of ursolic acid (1 and 10 μM) and chemotherapeutic agents (10 nM Taxol and 2 μM cisplatin) were used. Cells were pretreated with 1 and 10 μM ursolic acid for 48 hr, because the time exposures had no influence on cells viability (Supporting Information Fig. S2). Pretreatment of cancer cells with 10 μM ursolic acid for 48 hr completely suppressed the chemotherapeutic agent-induced p65 nuclear translocations and transcriptional activation of NF-κB to a level like the untreated cells, and pretreatment of cells with 1 μM ursolic acid for 48 hr partially suppressed the activation of NF-κB,

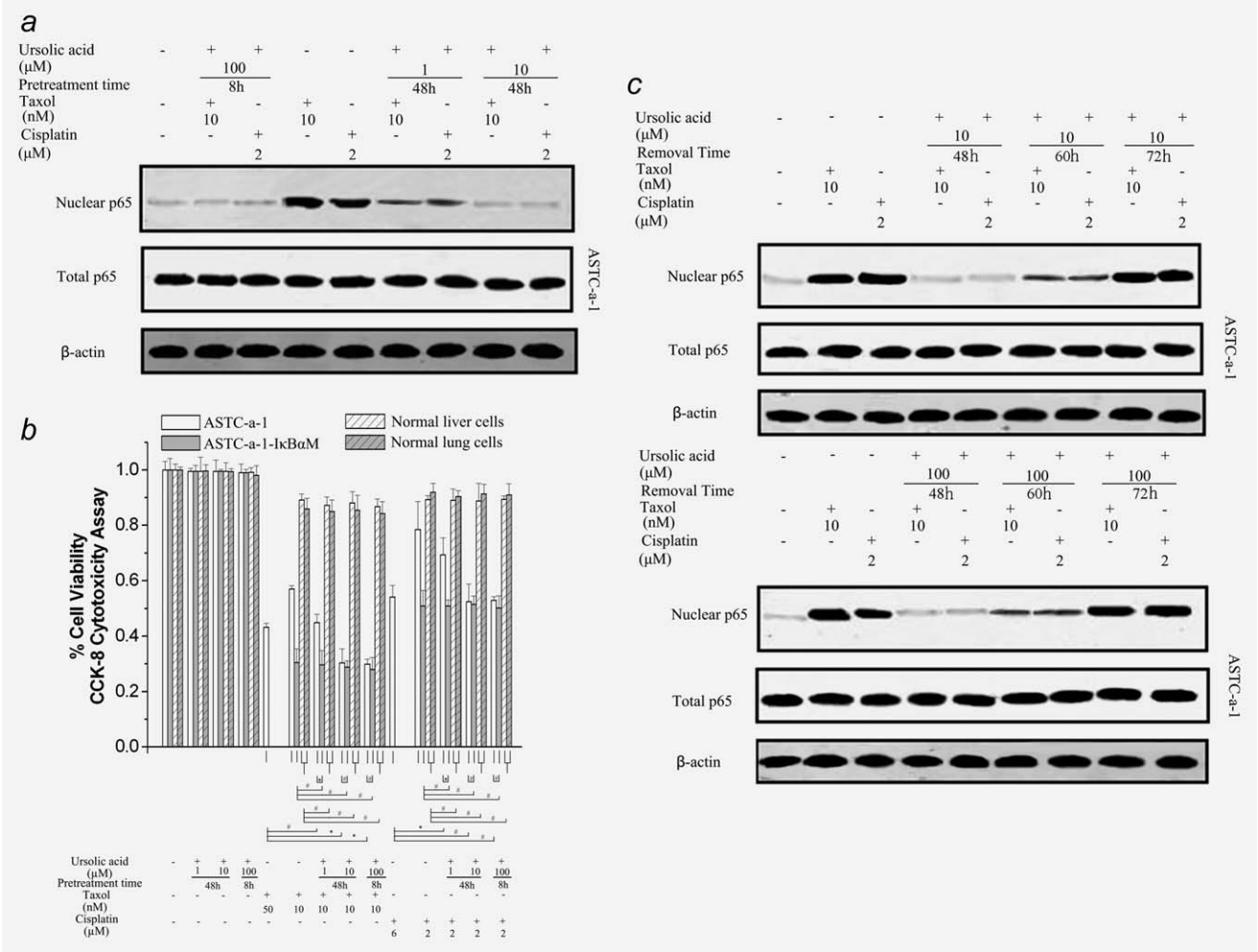

Figure 5. Effects of relatively low-doses ursolic acid-chemo treatment on ASTC-a-1 and normal cells. (*a*) ASTC-a-1 cells were first pretreated with 1 and 10 μM ursolic acid for 48 hr or 100 μM ursolic acid for 8 hr and then treated alone with chemotherapeutic agents (10 nM Taxol or 2 μM cisplatin) for 1 hr before Western blot assay. (*b*) ASTC-a-1 cells and primary normal cells of mouse (lung and liver) were first treated with various doses of ursolic acid. After removal of ursolic acid, cells were treated with Taxol or cisplatin for 48 hr before CCK-8 cytotoxicity assay. (*c*) Treatment with ursolic acid first and then removing ursolic acid made ASTC-a-1 cells insensitive to chemotherapeutic agent-induced p65 nuclear translocation for at least 48 hr. Cells were first treated with 100 μM ursolic acid for 8 hr or 10 μM ursolic acid for 48 hr (The doses make a complete inhibition of NF-κB). After removal of ursolic acid, cells were cultured in fresh medium for various durations (48, 60 and 72 hr) and then treated with chemotherapeutic agents (10 nM Taxol or 2 μM cisplatin) for 1 hr before Western blot analysis. β-actin blot was used to evaluate equal protein loading in each blot. Treatment with Taxol, cisplatin or ursolic acid served as comparisons. Results represent 1 of 3 replicates. *$p < 0.05$, significantly different, #$p > 0.05$, not significantly different.

as shown in Figure 5*a* and Supporting Information Figure S6. The inhibitive effects of ursolic acid were also visually observed in immunofluorescence assay of NF-κB p65 (Supporting Information Fig. S5). A total of 10 μM ursolic acid-mediated suppression of NF-κB needs relatively long pretreatment time (48 hr), which is unlike 100 μM ursolic acid (8 hr). Expression product of pECFP-IκBαM is a mutant of IκBα, which can effectively inhibit NF-κB activation.[23,24] The CCK-8 cytotoxicity assay shows that the pretreatment with different doses of ursolic acid chemosensitize cancer cells and significantly reduce the effective doses of chemotherapy, as shown in Figure 5*b*. It is noted that chemosensitization induced by 10 or 100 μM ursolic acid is very similar due to the complete suppression level of NF-κB, although the pretreatment time is different. A total of 1 μM ursolic acid-mediated chemosensitization was lower than that of 10 μM ursolic acid, because pretreatment with 1 μM ursolic acid partially suppressed the activation of NF-κB. Generally, the dose of 100 μM ursolic acid was not a required dose to sensitize cancer cells to chemotherapeutic agents, because 10 μM ursolic acid (a relatively low dose) can also sensitize cancer cells to the chemotherapeutic agents *via* suppression of NF-κB with prolonged pretreatment time. Furthermore, pretreatment with ursolic acid did not chemosensitize the primary

normal cells of mouse (Fig. 5*b*). The chemosensitization by ursolic acid was not observed in normal cells, possibly due to the inactivation of NF-κB after treatment of normal cells with Taxol or cisplatin.[25,26]

In addition, the inhibitive effect of ursolic acid on NF-κB was reversible. By evaluating the p65 nuclear translocation (Fig. 5*c*) and transcriptional activation of NF-κB (Supporting Information Fig. S7), we found that, after removal of ursolic acid, the inhibitive effect of ursolic acid on NF-κB still remained at 48 hr and completely reversed at 72 hr. The results are also visually confirmed by immunofluorescence assay of NF-κB p65 (Supporting Information Fig. S5). After removal of ursolic acid, no decrease of cells viability was observed at various durations (Supporting Information Fig. S2), and we also observed increase in cell numbers of the treated cells under microscope. Thus, the inhibitive effect of ursolic acid on NF-κB was reversible, and the reversal was not accompanied by a loss in cells viability.

Discussion

It has been reported that by combining various agents with chemotherapy, it is possible to improve the overall effectiveness of the treatment.[25,27,28] One potential adjuvant, ursolic acid, is investigated in our study for its demonstrated inhibiting effect on NF-κB activation.[2]

Clinical studies demonstrated that hypersensitivity reactions are related to the rate of chemotherapeutic agents infusion. As a result, the time span of intravenous (IV) infusion of Taxol or cisplatin is routinely prolonged to 3 hr or beyond. In addition, undesirable side effects, such as the occurrence of myelosuppression, neurotoxicity or renal failure, are associated with the concentration and the total dosage of the chemotherapeutic agents.[29,30] Our results clearly indicate for the first time that, by supplementing ursolic acid, the required dosage of the chemotherapy agents (Taxol or cisplatin) to achieve identical biological endpoints can be drastically reduced (80 or 50% of the original dosage, respectively) (Fig. 1*a*, Table 1 and Supporting Information Fig. S3). Given the fact that ursolic acid is a relatively nontoxic component and is already approved for routing clinical applications, it may have great potential for use in combination with Taxol or cisplatin to reduce the required drug dose and the severe side effects commonly associated with the chemotherapy without sacrificing the therapeutic results. With the effectiveness of the combination therapy demonstrated, the mechanism of the combination therapy is also investigated in our study.

The p65 nuclear translocation and transcriptional activation of NF-κB were inhibited by ursolic acid (Fig. 1*d* and Supporting Information Fig. S4). The cancer cells apoptosis were enhanced by ursolic acid (Fig. 1*b*). In CCK-8 cytotoxicity assay, cells treated with ursolic acid and/or transfected with pECFP-IκBαM show similar sensitizing effects to Taxol or cisplatin (Fig. 5*b*). Our studies provided *in vitro* evidence of the synergistic effects between ursolic acid and chemotherapeutic agents through apoptosis increase by NF-κB inhibition. However, the *in vivo* effects of ursolic acid are still unclear. This point is very important, and thereby, we plan to conduct future animal studies to determine the functions of ursolic acid *in vivo*.

The upregulated activation of caspase-8, 3 (Figs. 1*d* and 4*a*), increasing release of cytochrome *c* from mitochondria (Fig. 2*a*) and increasing cleavage of BID (Fig. 3) were observed in cells pretreated with ursolic acid in Taxol- or cisplatin-induced apoptosis. Ursolic acid-mediated chemosensitization is not observed in BID knockdown cells (Fig. 4*b*). These results strongly suggest that activation of intrinsic pathway was amplified by augmentation of BID cleavage during ursolic acid-chemo treatment. Cells pretreated with anti-FasL neutralizing antibody or Z-IETD-fmk did not show sensitizing effects or earlier BID cleavage to the ursolic acid-chemo treatment (Figs. 4*c* and 4*d*). Thus, the augmentation of BID cleavage by ursolic acid was due to the activation of Fas/FasL-caspase-8 pathway. The additions of anti-FasL neutralizing antibody did not protect cell death induced by the chemotherapy alone (Fig. 4*c*). These results indicate that chemosensitization by ursolic acid is dependent on activation of an additional pathway (Fas/FasL-caspase-8), which subsequently amplified activation of the intrinsic pathway by augmentation of BID cleavage. To the best of our knowledge, this is the first time that Fas/FasL-caspase-8 pathway activation and the amplified activation of such an intrinsic pathway are linked to the enhancement of chemotherapy by ursolic acid.

Caspase-3 can be activated by the active caspase-8 either directly (extrinsic pathway) or indirectly (intrinsic pathway). The indirect activation of caspase-3 by caspase-8 is dependent on the activation of BID-mediated intrinsic pathway.[11] The chemosensitization by ursolic acid was almost inhibited by shRNA-BID (Fig. 4*b*) or Z-IETD-fmk (caspase-8 inhibitor) (Fig. 4*c*), and the augmentation of BID cleavage by ursolic acid was suppressed by Z-IETD-fmk (Fig. 4*d*). Furthermore, the activity of BID, cytochrome *c* and caspase-3 is potentiated by ursolic acid (Figs. 1*d*, 2 and 3). The results indicate that the activation of BID-mediated intrinsic pathway was amplified in ursolic acid-chemo treatment, and that the extrinsic pathway (caspase-8-caspase-3) was minimally affected by ursolic acid. In mitochondrial events, the increased accumulation of proapoptotic proteins (Bax, Bak) and downregulation of antiapoptotic proteins (Bcl-2, Bcl-XL and Mcl-1) have been found in cells treated with ursolic acid.[31,32] The active BID (tBID) can induce the activation of Bax and Bak, resulting in the release of cytochrome *c*. Bax and Bak can be antagonized by Bcl-2, Bcl-XL and Mcl-1.[10,32] Considering the augmentation of BID cleavage (Fig. 4*b*) and amplified activation of intrinsic pathway (Figs. 1*d*, 2 and 3) by ursolic acid, it is possible that, after the augmentation of BID cleavage, the balance of bcl-2 family proteins (Bax, Bak, Bcl-2, Bcl-XL and Mcl-1) was more effectively altered and then caused a more efficient activation of mitochondrial-dependent intrinsic pathway in ursolic acid-chemo treatment.

Ca^{2+} release has been found in cells treated with ursolic acid.[33] Ca^{2+} release is linked with activation of caspase-12, which activates procaspase-9 without cytochrome *c* release and in turn activate caspase-3.[34] However, our results show that chemosensitization by ursolic acid involved the increasing release of cytochrome *c* (Fig. 2) and augmentation of BID cleavage (Fig. 4*b*). Thus, the apoptotic pathway associated with Ca^{2+} release and caspase-12 not seems to be affected in ursolic acid-chemo treatment. It has also been found that ursolic acid treatment downregulates the expression of survivin[32] and cIAP,[35] which can antagonize the activation of capsases. In our study, ursolic acid upregulates the activity of caspase-8 and caspase-3 (Figs. 1*d* and 4*a*). Thus, it is possible that the activation of apoptotic pathways associated with survivin and cIAP is enhanced in chemosensitization by ursolic acid. The aforementioned factors associated with ursolic acid have been found to be linked with NF-κB,[36,37] which is consistent with our findings that chemosensitization by ursolic acid is NF-κB inhibition dependent. In addition, inhibition of STAT3 activation by ursolic acid also sensitizes myeloma cells to thalidomide- or bortezomib-mediated chemotherapy.[32] Some studies show that the NF-κB-mediated gene expression requires the complex formation of STAT3 and NF-κB.[38] Thus, it is possible that chemosensitization by ursolic acid involves regulation of the interactions between NF-κB and STAT3. Our studies proved that chemosensitization by ursolic acid is through NF-κB (Figs. 1 and 5*b*). Akt activation by chemotherapeutic agents could be completely inhibited by ursolic acid, and the inhibition of Akt alone by wortmannin partially inhibited the NF-κB activation in chemotherapy (Fig. 1*c* and Supporting Information Fig. S4). Thus, the inactivation of Akt survival signal by ursolic acid contributes to the enhancement of chemotherapeutic efficacy, which is associated with partial downregulation of chemotherapeutic agent-induced NF-κB activation.

Our data first show that the required dose of ursolic acid to suppress NF-κB activation can be reduced by prolonging the pretreatment time, and that the chemosensitization by 10 or 100 μM is very similar and only observed in cancer cells, but not in normal cells (Fig. 5*b*). Until now, there is no exact data on bioavailability of ursolic acid (metabolism and absorption) *in vivo*. Thus, although ursolic acid-chemo treatment shows synergistic effects in our studies of *in vivo* (Supporting Information Fig. S3), we could not determine whether the effective concentrations of 10–100 μM ursolic acid in our studies of *in vitro* can be achieved in animal models. Considering the importance of bioavailability of ursolic acid *in vivo*, an intense study on this point is made in our lab now. Meanwhile, ursolic acid is well tolerated in animal studies, with little toxicity.[39,40] There are numerous studies on ursolic acid toxicity in animal models. LD_{50} dose of ursolic acid in rodent-mouse (oral) is 8,330 mg/kg (acute toxicity). Hypodermic injection of ursolic acid (3,500 mg/kg) resulted in neither death nor observable side effects in mouse. No toxicity was observed in the chronic toxicity assay of ursolic acid (500 mg/kg daily for 30 days).[41] In addition to the reported minimal toxicity, ursolic acid has been demonstrated to inhibit toxicant activation and enhances the body defense systems.[42] Thus, ursolic acid-chemo treatment of cancer has a great potential to be used clinically. Recent studies show that proinflammatory responses are associated with the use of ursolic acid.[43] Although the precise impact of such inflammatory response is not clear, it should be considered when ursolic acid is used; further studies are needed.

In summary, our results show that ursolic acid-mediated NF-κB inhibition can sensitize ASTC-a-1cells to chemotherapeutic agents (Taxol or cisplatin), and that the sensitizing effects are not observed in primary normal cells of mouse lung and liver. The chemosensitization by ursolic acid in cancer cells depends on the amplified activation of intrinsic pathway by augmentation of BID cleavage and activation of Fas/FasL-caspase-8 pathway. The ursolic acid-mediated chemosensitization and its mechanism could provide guidance for optimal application of chemotherapy.

Acknowledgements

The authors thank Dr. X. Shen (Institute of Biophysics, Chinese Academy of Sciences) for providing the pNF-κB-Luc, Dr. K. Taira (University of Tokyo, Hongo, Tokyo, Japan) for providing the pFRET-Bid and Dr. Y. Gotoh (University of Tokyo, Yayoi, Tokyo, Japan) for providing the pDsRed-Mit plasmid. They also thank Dr. G.J. Gores (Center for Basic Research in Digestive Diseases, Molecular Medicine Program, Mayo Clinic, Rochester, MN) for kindly providing the pE-GFP-Cyt-c plasmid used in our research.

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