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RESEARCH ARTICLE

Dynamic Monitoring of Apoptosis in Chemotherapies with Multiple Fluorescence Reporters

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Abstract

Purpose: The aim of the study is to dynamically and non-invasively monitor the temporal relationship among caspase-3, BID, and cytochrome c in chemotherapy.

Procedures: ASTC-a-1 cells expressing the corresponding fluorescence reporters were treated with Taxol or cisplatin and imaged using FRET and fluorescence overlapping technique. Western blot was performed to validate the fluorescence analysis.

Results: In fluorescence imaging analysis, Taxol-induced apoptosis showed caspase-3 activation (13 h 50 min) was prior to BID cleavage (15 h 10 min) and subsequent significant cytochrome c release (17~18 h 20 min), whereas the cisplatin-induced apoptosis showed BID cleavage (5 h 40 min) and significant cytochrome c release (7~8 h 20 min) were prior to caspase-3 activation (14 h 20 min). Western blot further validated the results above.

Conclusions: The new approach successfully reveals the difference in temporal signaling apoptosis events between Taxol and cisplatin. It may help us come to a better understanding of the detailed mechanisms in chemotherapeutic-agents-induced apoptosis.

Key words: Caspase-3, BID, Cytochrome c, FRET, Taxol, Cisplatin

Introduction

Apoptosis is an important biological event that is associated with pathogeny and therapy of many diseases [1, 2]. It is well established that there are two main signal pathways in the process of apoptosis: intrinsic and extrinsic signal pathway. In the mitochondrion-dependent intrinsic signal pathway, full-length BID is cleaved into tBID by activated caspase-8. The tBID is translocated to mitochondrion, resulting in cytochrome c release, which in turn activates caspase-3 [3, 4]. The extrinsic signal pathway involves death receptor activation at cell surface. In this pathway, instead of directly cleaving BID, caspase-8 activates the downstream caspases including caspase-3 [5–7]. In either case, the activated caspase-3 can cleave BID and, similar to that in the mitochondrion-dependent intrinsic signal pathway, result in further amplification of caspase-3 activation. This pathway acts as a potential feedback-loop for amplification of apoptosis and is named the acceleration cycle or feedback-loop [8, 9].

The temporal sequence of the caspase-3 activation, BID cleavage, and cytochrome c release is distinctively different among these pathways and may be used as a marker to identify each specific apoptotic event(s). The commonly used western blot technique, however, is insufficient to dynamically study the temporal sequence. For each data point, it requires a fresh sample to be analyzed following a series of rather complicated technical steps. In comparison, fluorescence reporters can be expressed in living cells and provide us a means to dynamically monitor the activities of signal factor (s) with a much higher temporal resolution and sensitivity.

With a spectrum of fluorescence reporters such as SCAT-3 [10], pFRET-Bid [11], BKAR [12], CKAR [13] available, the fluorescence resonance energy transfer (FRET) technique has been widely used in the research of signal

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pathways of cellular apoptosis and proliferation [14–16]. In general, these reporters consist of a donor cyan fluorescent protein (CFP) and an acceptor Venus (a mutant of yellow fluorescent protein). The donor and the acceptor are linked with a molecular sequence that can be recognized or cleaved by a specific signal factor. The interaction between activated signal factor and reporter results in a shift of FRET fluorescence spectrum distribution, i.e., a reduction of acceptor (YFP) fluorescence emission and, at the same time, an increase in that of the donor (CFP). YFP/CFP emission ratio dynamically indicates the activities of detected signal factor [17].

Taxol (paclitaxel) is a powerful chemotherapeutic agent, especially for cervical, breast, and ovarian cancer, among others [18, 19]. It binds to tubulin and disrupts the dynamic instability of microtubules; thus, preventing the completion of mitosis [20]. Cisplatin (cis-diammine dichloroplatinum; cis-Platinum (II)) is another important chemotherapeutic agent that leads to formation of DNA adducts and cell cycle arrest [21–23]. Cells exposed to either Taxol or cisplatin show typical characteristics of apoptosis, such as alteration of membrane asymmetry, caspases activation and poly (ADP-ribose) polymerase (PARP) cleavage [22, 24]. Although the apoptotic signal pathways involved in either mode of the chemotherapies are far from complete comprehension, it is generally agreed that caspase-3 activation, BID cleavage, and cytochrome c release by manipulation of mitochondrion are involved [25–29]. There is one distinctive difference between the signal pathways of the two therapies. In certain cells, caspase-8 activation is observed in Taxol-induced apoptosis [26, 30], but is not in that by cisplatin [31, 32]. In the current study, ASTC-a-1 cells (human lung adenocarcinoma cells) were treated with either Taxol or cisplatin. The fluorescence reporter technique was utilized as a novel approach to identify the apoptotic difference between Taxol and cisplatin.

Materials and Methods

Materials and Cell Culture

Taxol and cisplatin were purchased from Sigma (St. Louis, MO, USA) and dissolved in DMSO to a final concentration of 0.1%. ASTC-a-1 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% CO2). Only the well-attached and healthy cells were used in our experiments. Plasmid SCAT-3 reporter was kindly provided by Dr. Masayuki Miura (RIKEN Brain Science Institute, Wako, Japan). 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 Yokyo, Yayoi, Tokyo, Japan), and pE-GFP-Cyt-c plasmid was kindly provided by Dr. G. J. Gores (Center for Basic Research in Digestive Diseases, Molecular Medicine Program, Mayo Clinic, Rochester, Minnesota). All other chemicals were of analytical grade if not otherwise indicated.

Cells Transfection

For cells transfection, Lipofectamine™ 2000 reagent (Invitrogen) was used to introduce the corresponding plasmids into ASTC-a-1 cells in a petri dish. Lipofectamine™ 2000 is a commercially available product with high transfection efficiency. In our experiments, about 40~60% cells were successfully transfected and about 30% cells died due to the transfection. After the transfection, cells were cultured for 36 h to recover and to express plasmid. The cells were subsequently washed with 1× phosphate-buffered saline (PBS, pH 7.4) to remove the unhealthy cells due to the transfection. Only healthy cells were kept and used to study the Taxol or cisplatin-induced apoptosis.

Laser Scanning Microscopes

Cell imaging was performed on a commercial laser scanning microscope (LSM) combination system (LSM510/ConfoCor2, Zeiss, Jena, Germany). The transfected ASTC-a-1 cells in a petri dish were placed on the stage of the LSM microscope. The cells were maintained at 37°C throughout the imaging procedure with a built-in temperature regulator (Tempcontrol 37-2 digital, Zeiss) of the system.

For excitation in the FRET measurement, the 458 nm line of an argon ion laser (5%) 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 two separate channels: a 470–500-nm bandpass (CFP channel) and a 530-nm longpass (YFP channel). GFP was excited at 488 nm with an argon ion laser (7%) and the fluorescence emission was recorded through a 500–530-nm bandpass filter. DsRed was excited at 543 nm with a helium–neon laser (7%) and its emitted light was recorded through a 560-nm longpass filter. The fluorescence image and emission intensities of YFP, CFP, GFP, and DsRed in whole cells were recorded every 10 min in the process of experiments. Meanwhile, two image analysis channels were set respectively to get YFP/CFP images and fluorescence overlapping images of GFP and DsRed. The Zeiss Rel3.2 image processing software (Zeiss, Germany) was used to record fluorescence images and intensities.

Confirmation of Cells Apoptosis and Comet Assay

The effect of the chemotherapies on cellular apoptosis was evaluated by observation of nuclear morphology. ASTC-a-1 cells were exposed to 1 μM Taxol or 20 μM cisplatin for 24 h and then washed twice with 1× phosphate-buffered saline (pH 7.4). Subsequently, the cells were stained with 1 mM Hoechst 33342 for 10 min at room temperature. The cells were then washed twice with PBS and visualized under LSM microscope with a Zeiss C-Apochromat 100×, NA 1.3 objective. In normal cells, only the cell membrane is stained and preserves its asymmetric form. For apoptotic cells, membranes are usually disrupted and the nucleus is stained.

Comet assay was performed according to the descriptions by Lynn et al. [33]. Briefly, ASTC-a-1 cells after treatment with 1 μM Taxol for 24 h or 20 μM cisplatin for 12 h were harvested, embedded in 1%

agarose gel at a density of 1×10^6/ml, and spread onto a fully frosted slide. The slides were immersed in ice-cold lysis buffer (10 mM Tris–HCl, 2.5 M NaCl, 100 mM Na_2EDTA, 1% sodium N-lauryl sarcosinate, 1% Triton X-100, and 10% DMSO; pH 10) for 1 h at 4°C. Cellular DNA was denatured in electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA) for 20 min at room temperature and then electrophoresed for 20 min at 25 V and 300 mA. Afterward, the slides were washed in distilled water, renatured in 0.4 M Tris–HCl (pH 7.5),

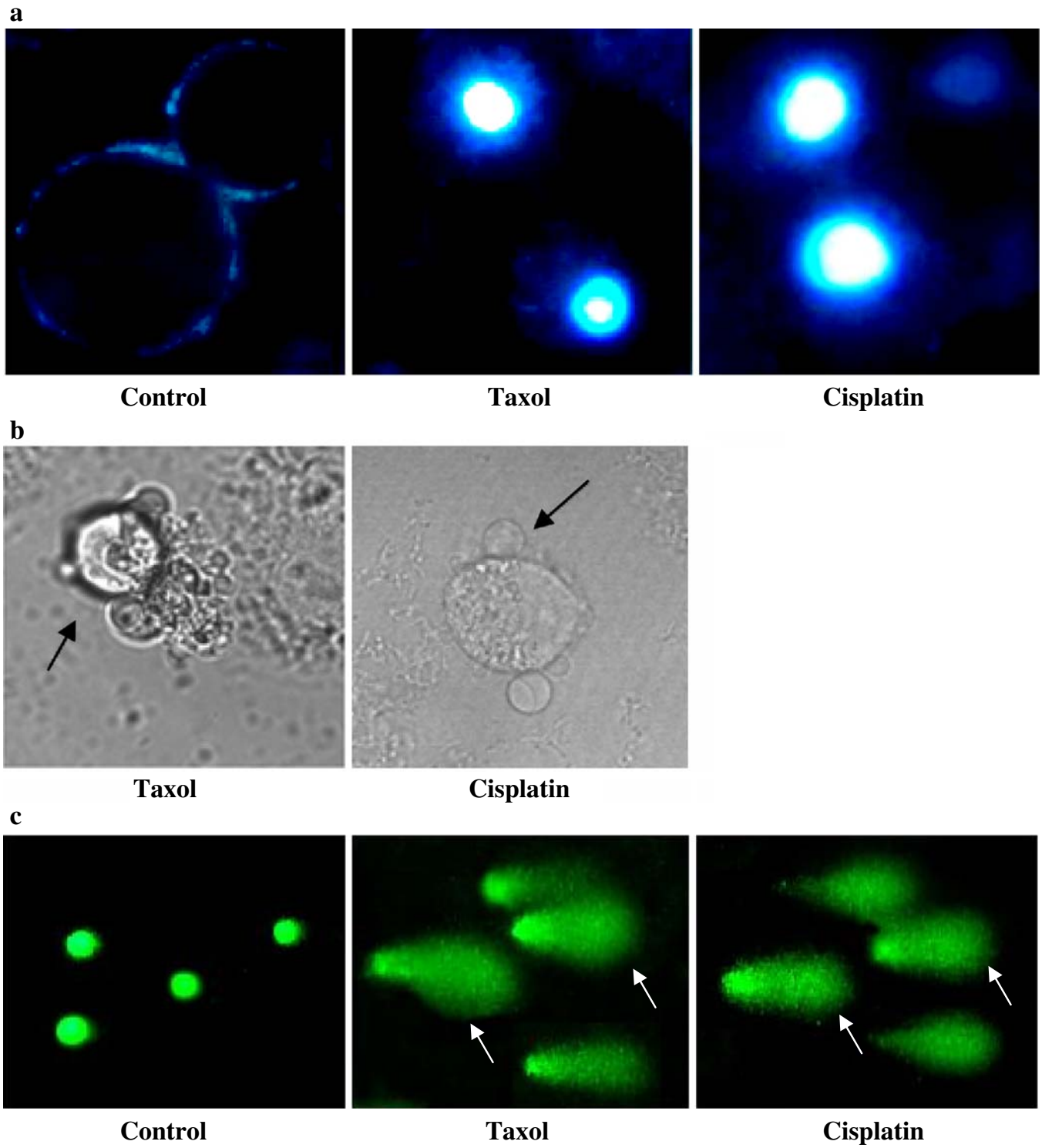


Fig. 1. Confirmation of Taxol or cisplatin-induced ASTC-a-1 cells apoptosis. **a** Hoechst 33342 nuclei staining represents cells apoptosis. **b–c** Observation of apoptotic bodies and DNA damage after treatment with Taxol or cisplatin (as pointed by *arrows*). Results represent one of three replicates.

stained with Sybr green (Molecular Probe, Eugene, OR, USA), and examined under the LSM microscope. A comet with a tail indicated that DNA was damaged while a comet with no tail indicated there was no DNA damage. Five hundred cells were examined for each treatment.

Capase-3 Activation and BID Cleavage Assay

In caspase-3 activation and BID cleavage assay, ASTC-a-1 cells were transiently transfected with SCAT-3 and pFRET-Bid respectively. In Taxol-induced apoptosis, cells were pretreated with 1 μM Taxol for 8 and 13 h, and then subjected to the fluorescence assays of caspase-3 activation and BID cleavage respectively. In cisplatin-induced apoptosis, cells were pretreated with 20 μM cisplatin for 5 and 9 h before the assays of BID cleavage and caspase-3 activation. In control groups, cells were treated with 0.1% DMSO for designated time. Fluorescence intensity increase in CFP and decrease in YFP represent caspase-3 activation or BID cleavage. Again, western blot was used to validate the FRET result. Cells were treated with 1 μM Taxol for 13 and 15 h or 20 μM cisplatin for 14 and 16 h or 0.1% DMSO for 13, 14, 15, and 16 h (control) in western blot analysis for caspase-3. Cells were treated with 1 μM Taxol for 15 and 17 h or 20 μM cisplatin for 5 and 7 h or 0.1% DMSO for 5, 7, 15, and 17 h (control) in western blot analysis for BID. To quantify the results, average fluorescence intensity of YFP and CFP were obtained in whole cells for each image in the time series. After background subtraction, YFP/CFP emission ratios were obtained by dividing the average fluorescence intensity values of single cells. For direct comparisons, the YFP/CFP emission ratio was normalized to that at the first time point. The images are representative results from three independent experiments. Each curve represents an average of 10–15 cells obtained from three independent experiments.

Cytochrome c Release Assay

In cytochrome c release assay, ASTC-a-1 cells were transiently co-transfected with E-GFP-Cyt-c and DsRed-Mit. Before imaging, cells were treated with either 1 μM Taxol for 16 h or 20 μM cisplatin for 5 h, respectively. Cells were also treated with 0.1% DMSO (control). The fluorescence images overlay between pE-GFP-Cyt-c and DsRed-Mit in the time course dynamically revealed cytochrome c release from mitochondrion. For dynamic analysis of cytochrome c release, a small region containing only cytosol was marked with a rectangular box. After background subtraction, the average fluorescence intensity of GFP-Cyt-c in the region was obtained from each image in the time series. These values were directly proportional to the concentrations of GFP-Cyt-c in cytosol. For each time point, the relative value of GFP-Cyt-c fluorescence intensity (I) was calculated as $I=I\alpha/I_1*100$ (where I_1 was the average fluorescence intensity of GFP-Cyt-c at the first time point and $I\alpha$ was the average fluorescence intensity of GFP-Cyt-c at a later time). For direct comparison of the kinetics of GFP-Cyt-c release under different treatments, the onset times of GFP-Cyt-c redistribution were set to zero. To describe the release of cytochrome c into the cytoplasm, "significant increase" was defined as a process from the beginning of cytochrome c release to the maximized cytochrome c release. Each curve represents an average of 10–15 cells obtained from three independent experiments.

Fig. 2. Signal pathways analysis in Taxol-induced ASTC-a-1 cells apoptosis. **a**–**b** Shift in CFP and YFP fluorescence represents caspase-3 activation and BID cleavage. **c** Cytochrome c (*green*) releases from the disrupted mitochondrion (*red*). **d** Normalized YFP/CFP emission ratio indicates that caspase-3 activation is prior to BID cleavage. **e** Time courses of cytochrome c release. Results represent one of three replicates. *Scale bar*=10 μm.

Sample Preparations and Western Blot

After treatment with 1 μM Taxol or 20 μM cisplatin for designated time, cells were washed with ice-cold 1× phosphate-buffered saline (PBS, pH 7.4) twice, and then lysed in lysis buffer containing 50 mM/L Tris–HCl (pH 8.0), 150 mM/L NaCl, 1%TritionX-100, 100 ug/ml PMSF for 15 min on ice, followed by sonication and heat denaturation. Fifty micrograms protein/lane was loaded onto SDS-PAGE gel, transferred to a polyvinylidene difluoride immobilon membrane. The membrane was first incubated with the primary antibodies (1:1,000 *v*/*v*) overnight at 4°C. The primary antibodies used in western blot were mouse polyclonal β-actin antibody (Santa Cruz Biotechnology), rabbit polyclonal BID, and caspase-3 antibody (Cell Signaling Technology). Then the membrane was incubated with goat anti-rabbit IRDye™800 secondary antibodies (1:500 *v*/*v*) (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) for 2 h before visualization with the LI-COR Odyssey scanning infrared fluorescence imaging system (LI-COR, Inc., Lincoln, NE, USA). β-actin control blot was used to evaluate equal protein loading in each blot.

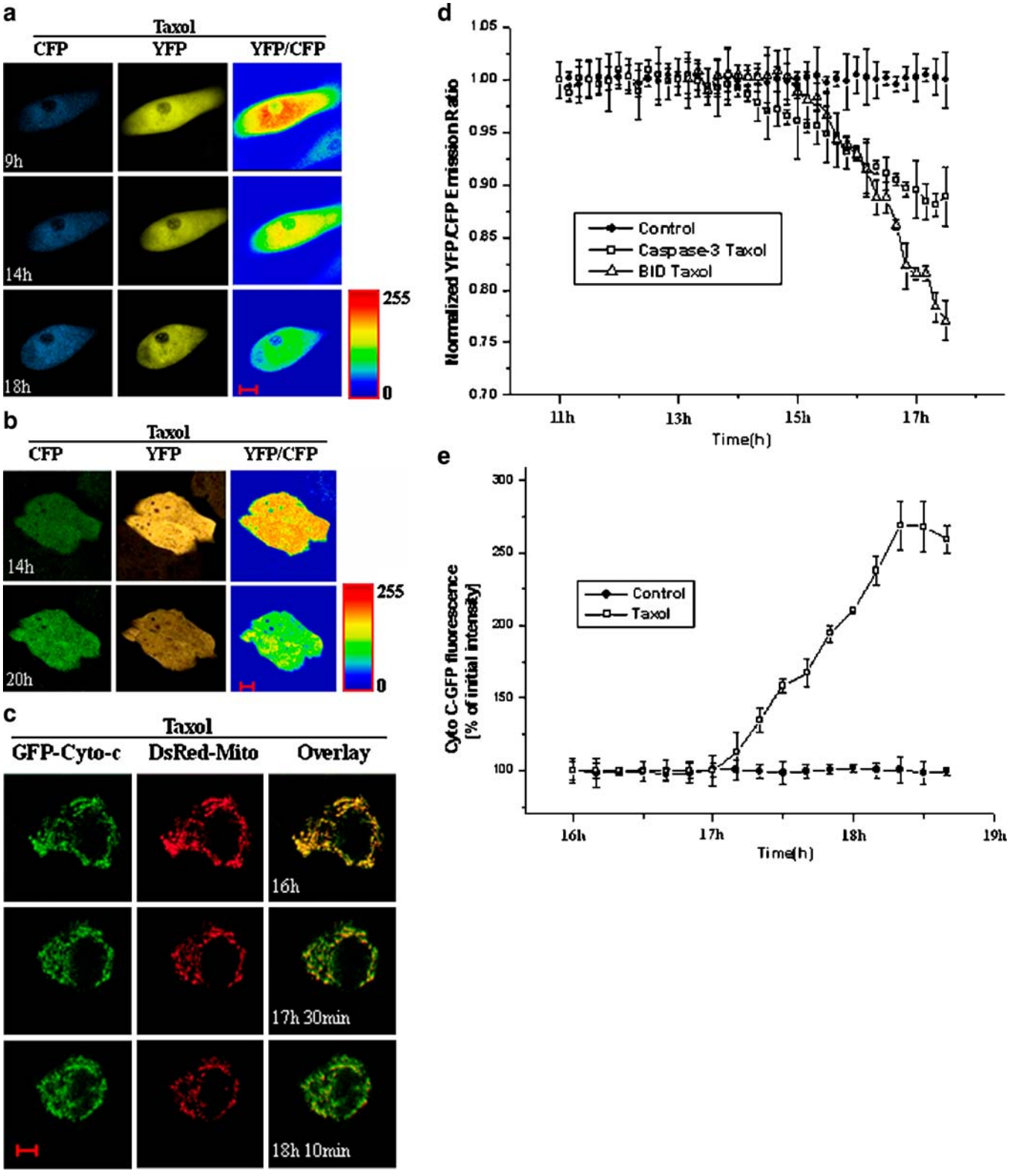
Results

Taxol or Cisplatin-Induced ASTC-a-1 Cells Apoptosis

To confirm 1 μM Taxol or 20 μM cisplatin-induced ASTC-a-1 cells death was apoptotic, cells subjected to the treatments were stained by Hoechst 33342. In control, only the cell membrane was stained. In contrast, cell nuclei were stained after treatment with Taxol or cisplatin (Fig. 1a). This indicates that either 1 μM Taxol or 20 μM cisplatin can induce apoptosis in ASTC-a-1 cells. The result was further confirmed morphologically that, apoptotic bodies were observed in Taxol or cisplatin-induced final cell death (Fig. 1b), Furthermore, DNA damage, a key factor in apoptosis, was also observed in comet assay (Fig. 1c).

Signal Pathways Analysis in Taxol-Induced Apoptosis

To determine and analyze signal pathways in 1 μM Taxol-induced ASTC-a-1 cells apoptosis, the activity of caspase-3,


a
Taxol
CFP
YFP
YFP/CFP
9h
14h
18h
255
0
b
Taxol
CFP
YFP
YFP/CFP
14h
20h
255
0
c
Taxol
GFP-Cyto-c
DsRed-Mito
Overlay
16h
17h 30min
18h 10min
d
Normalized YFP/CFP Emission Ratio
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
Control
Caspase-3 Taxol
BID Taxol
11h
13h
15h
17h
Time(h)
e
Cyto C-GFP fluorescence [% of initial intensity]
300
250
200
150
100
Control
Taxol
16h
17h
18h
19h
Time(h)

cytochrome c, and BID was dynamically analyzed with corresponding fluorescence reporters. The results showed that, with the completion of Taxol treatment, caspase-3 activation was prior to BID cleavage and subsequent cytochrome c release (Fig. 2). The normalized YFP/CFP ratio from treated cells was compared to that of untreated cells to identify the beginning of caspase-3 activation and BID cleavage (student t test, significance level $p<0.05$). As shown in Fig. 2d, BID cleavage and caspase-3 activation started at approximately 15 h 10 min and 13 h 50 min, respectively. Significant cytochrome c release was observed at 17~18 h 20 min (Fig. 2e). In addition, significant cytochrome c release was observed within 2 h after BID cleavage. This indicates that the cytochrome c release caused by BID cleavage is relatively quick (within 2 h). The fluorescence images from the control groups are shown in Fig. 4.

Signal Pathways Analysis in Cisplatin-Induced Apoptosis

Potential apoptotic signal pathways of ASTC-a-1 cells treated with 20 μm cisplatin were investigated in a similar manner, as described above. Unlike the Taxol-induced apoptosis, BID cleavage and cytochrome c release was prior to caspase-3 activation in cisplatin-induced apoptosis (Fig. 3). The normalized YFP/CFP ratio from treated cells and untreated cells was compared mutually to identify the beginning of caspase-3 activation and BID cleavage (student t test, significance level $p<0.05$). As shown in Fig. 3d, BID cleavage and caspase-3 activation started at approximately 5 h 40 min and 14 h 20 min, respectively. Significant cytochrome c release occurred at 7~8 h 20 min (Fig. 3e). Again, a significant cytochrome c release was observed within 2 h after BID cleavage. This indicates that BID cleavage induced cytochrome c release after cisplatin treatment was also a relatively quick process, similar to that in Taxol treatment. The fluorescence images in control groups are shown in Fig. 4.

Western Blot Analysis of Signal Pathways in Taxol or Cisplatin-Induced Apoptosis

To confirm the results revealed by the FRET technique, western blot was used to check the apoptotic events above. In western blot, both caspase-3 activation and BID cleavage were observed in Taxol or cisplatin-induced apoptosis. Furthermore, the time range of caspase-3 activation and BID cleavage in western blot was consistent with that of the FRET analysis, as shown in Fig. 5.

Discussion

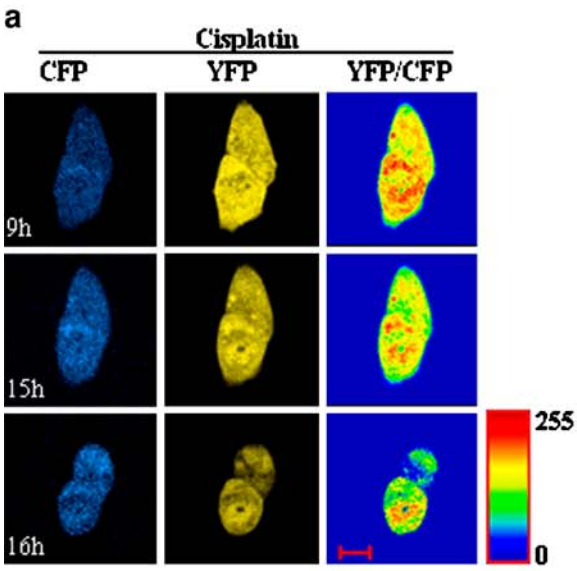
Conventional western blot is considered as golden standard in cellular signal pathways research. Determined by its intrinsic principle, the technique requires a series of lengthy steps that ultimately leads to the physical destruction of each sample. Clearly, although very reliable, western blot is not best suited for dynamic study of cellular events. In the current study, we have introduced a novel approach to solve the problem. Fluorescent reporters SCAT-3, pFRET-Bid, pDsRed-Mit, and pE-GFP-Cyt-c are used to monitor the activities of BID, cytochrome c and caspase-3 in response to chemotherapy. By determining the temporal orders among BID cleavage, cytochrome c release and caspase-3 activation, the activation of mitochondrion-dependent intrinsic and feedback-loop signal pathway are well differentiated in chemotherapy. The results are positively confirmed by the conventional western blot technique at various time points. This suggests that apoptotic factor specific fluorescence reporter could serve as a reliable means for evaluating signal pathways in apoptosis.

In Taxol-induced ASTC-a-1 cells apoptosis, caspase-3 activation is prior to BID cleavage, as shown in the real-time fluorescence analysis (Fig. 2). This indicates that feedback-loop apoptotic signal pathway (caspase-3-BID-cytochrome c-caspase-3) is involved in Taxol-induced ASTC-a-1 cells apoptosis [34]. This result is consistent with that in western blot (Fig. 5a). This suggests that the fluorescence method is as reliable as, and more feasible than, the conventional western blot for dynamic investigation of apoptotic signal pathways. The results from the current study show that cytochrome c release is behind the BID cleavage (Fig. 2b–d). This indicates that cytochrome c release is caused via BID cleavage in Taxol-induced ASTC-a-1 cells apoptosis.

In cisplatin-induced ASTC-a-1 cells apoptosis, caspase-3 activation is shown to be a downstream event of BID cleavage and cytochrome c release (Fig. 3 and Fig. 5b). Thus, our data is consistent with the report that the cisplatin-induced apoptosis is mainly via mitochondrion-dependent intrinsic signal pathway [31, 32]. The results further show that BID cleavage-induced cytochrome c release occurs rapidly after the chemotherapy in ASTC-a-1 cells (Fig. 3b–d).

In summary, a new approach for investigation of cellular apoptosis using fluorescence reporters is established in the current study. The new technique is validated by conventional western blot, and found reliable and suitable for dynamic study of the cellular events. With the new technique, we have determined that the feedback-loop signal pathway (caspase-3-BID-cytochrome c-caspase-3) is involved in Taxol-induced ASTC-a-1 cells apoptosis, as shown in Fig. 6a. For cisplatin,

Fig. 3. Signal pathways analysis in cisplatin-induced ASTC-a-1 cells apoptosis. **a–b** Shift in CFP and YFP represents caspase-3 activation and BID cleavage. **c** The cytochrome c (*green*) releases from the disrupted mitochondrion (*red*). **d** Normalized YFP/CFP emission ratio indicates that BID cleavage is prior to caspase-3 activation. **e** Time courses of cytochrome c release. Results represent one of three replicates. *Scale bar*=10 μm. ▶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Cisplatin
CFP
YFP
YFP/CFP
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16h
255
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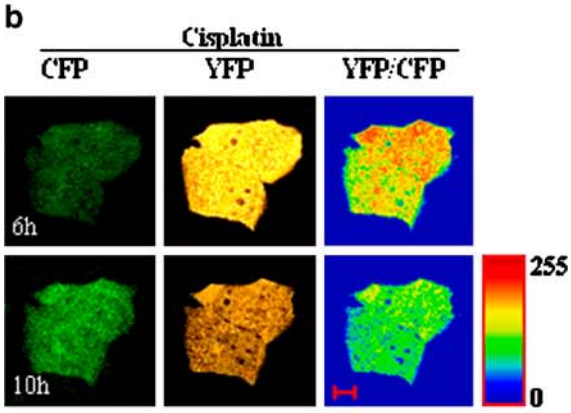
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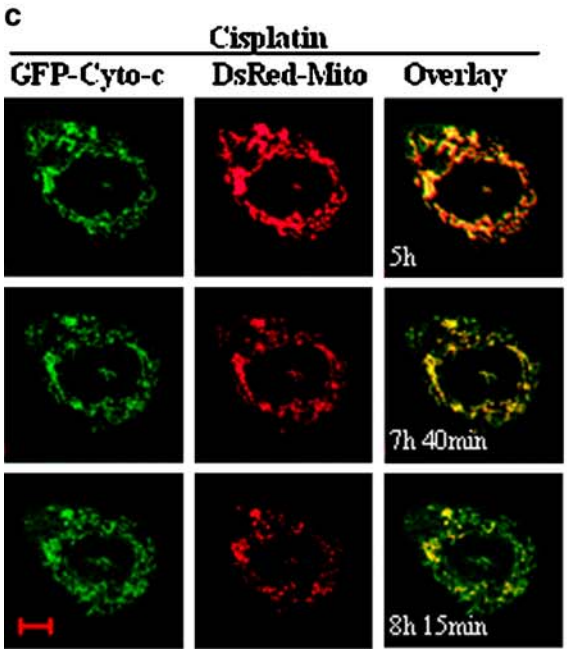
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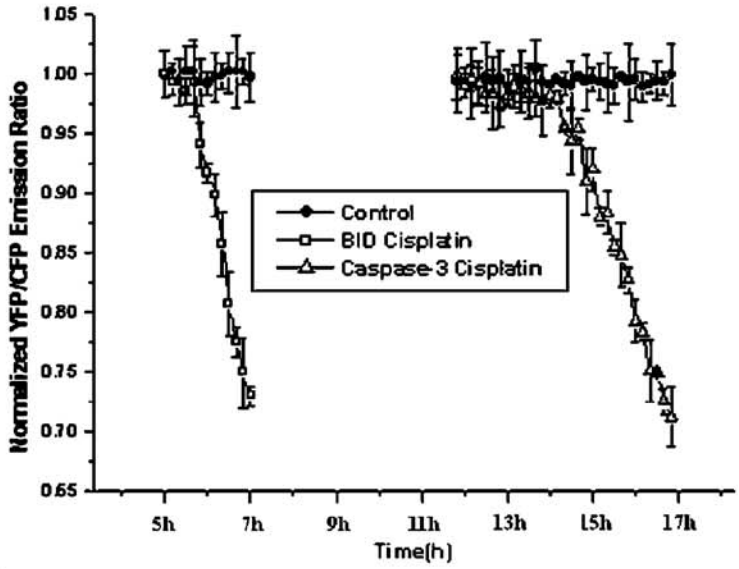
Cisplatin
GFP-Cyto-c
DsRed-Mito
Overlay
5h
7h 40min
8h 15min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1.00
0.95
0.90
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0.75
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Caspase-3 Cisplatin
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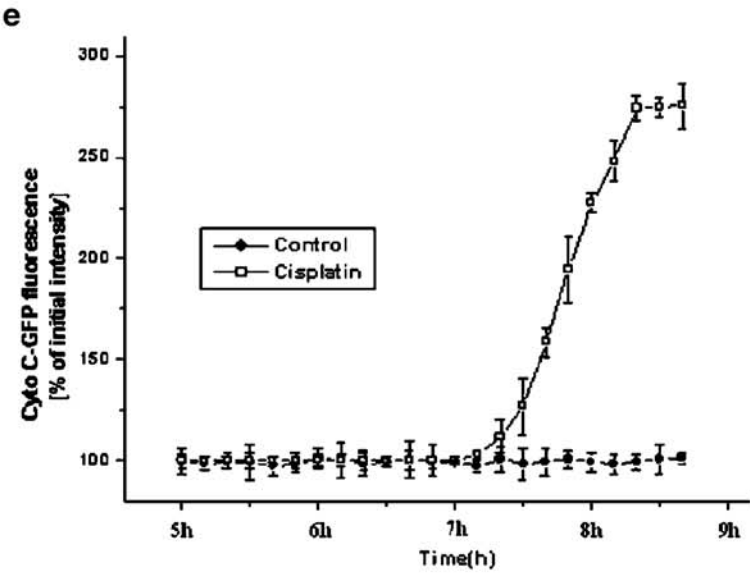
Time(h)


e
Cyto C-GFP fluorescence
[% of initial intensity]
300
250
200
150
100
Control
Cisplatin
5h
6h
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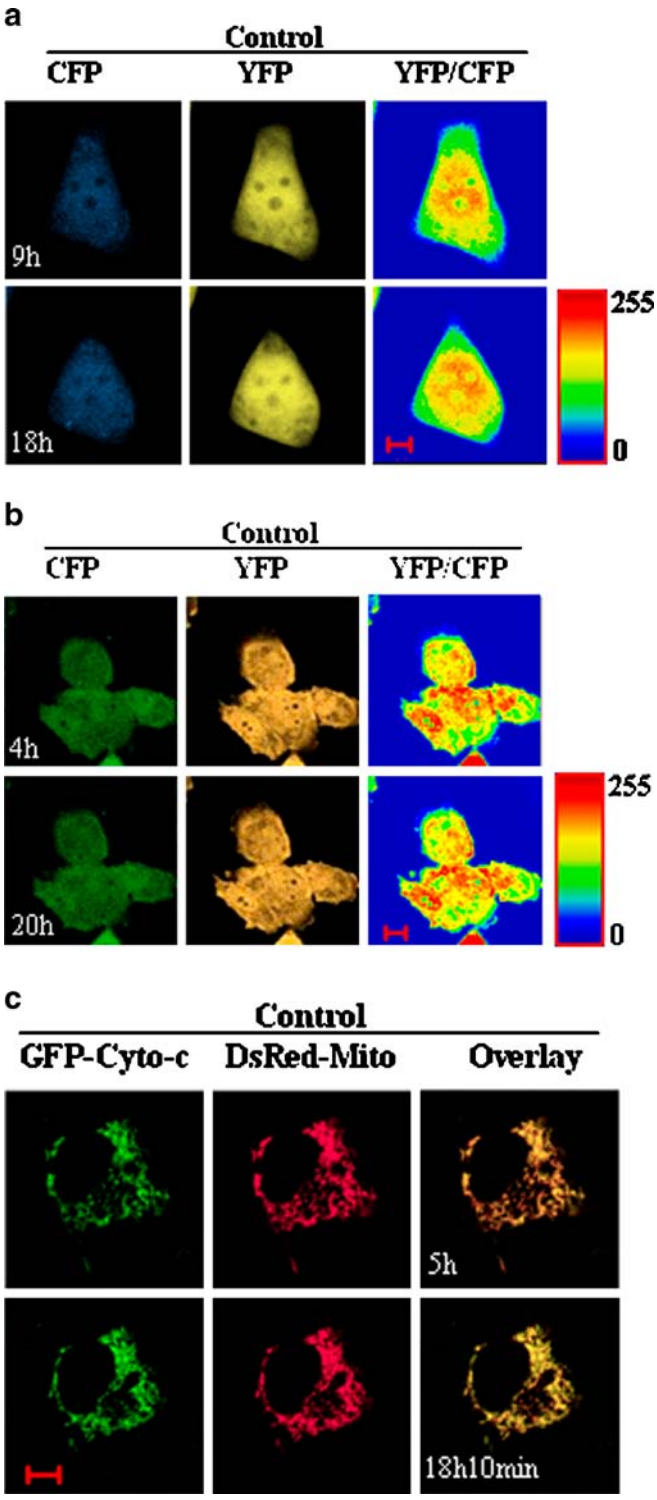

Fig. 4. Control groups in fluorescence reporter analysis. **a** No caspase-3 activation. **b** No BID cleavage. **c** No cytochrome c release. Results represent one of three replicates. *Scale bar*=10 μm.

only the mitochondrion-dependent intrinsic signal pathway (BID-cytochrome c-caspase-3) is activated, as shown in Fig. 6b. The results clearly demonstrate that, in either case, the apoptotic signal pathways are completely different for tumor cells control. It is likely that, by enhanced activation of either feedback-loop signal pathway in Taxol or mitochondrion-dependent intrinsic signal pathway in cisplatin, the efficiencies of the chemotherapies are likely to be improved clinically.

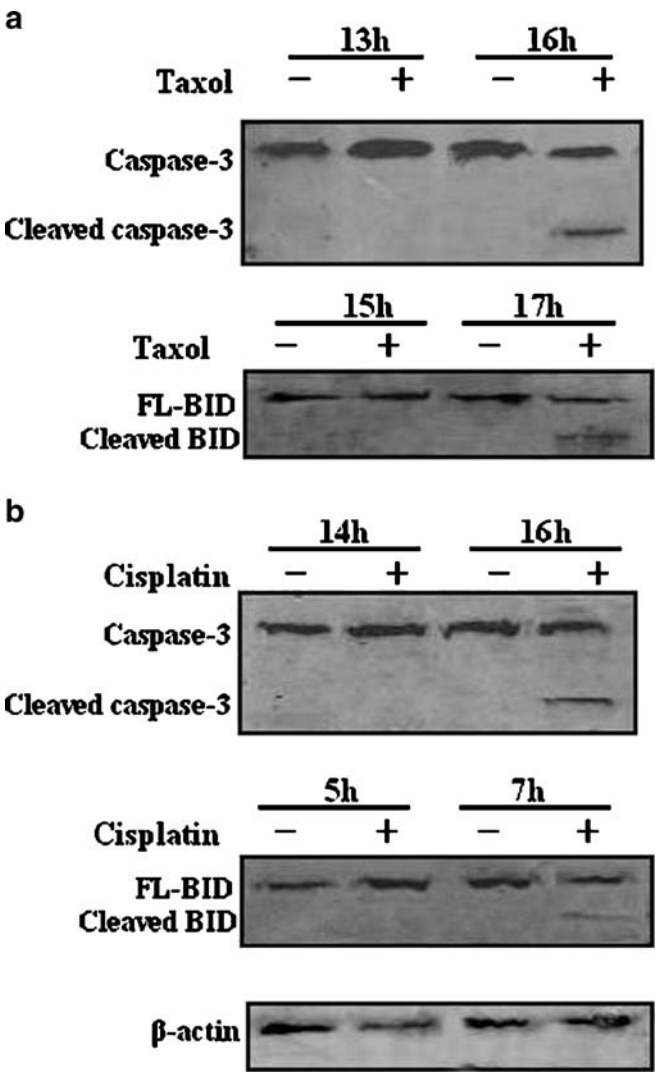

Fig. 5. Western blot analysis in Taxol or cisplatin induced ASTC-a-1 cells apoptosis. **a**–**b** The time range of caspase-3 activation and BID cleavage in western blot is consistent with that of the FRET analysis. Results represent one of three replicates.

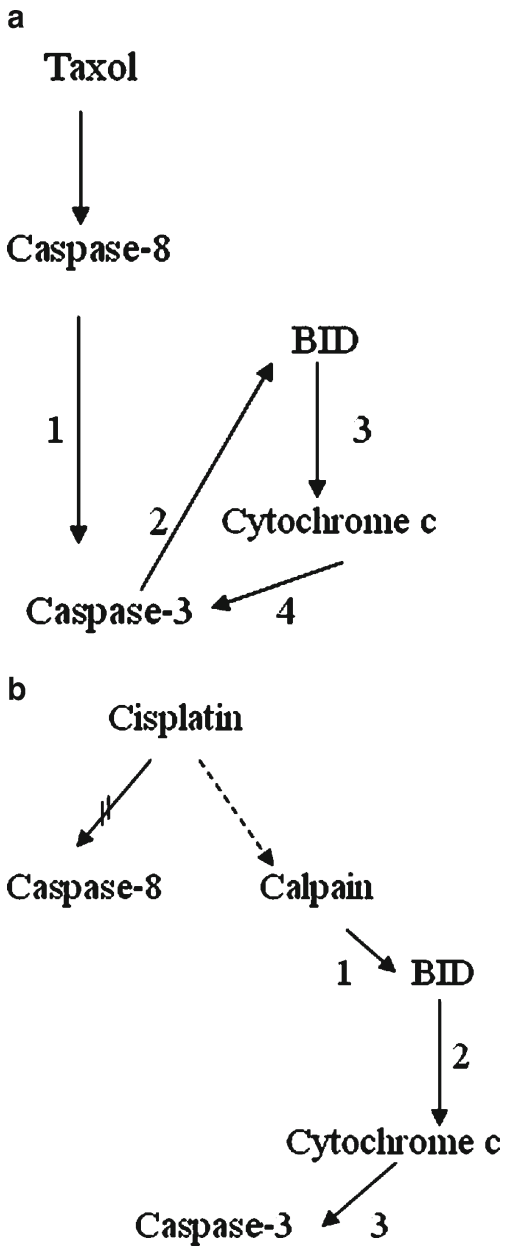

Fig. 6. Proposed mechanisms in Taxol or cisplatin-induced ASTC-a-1 cells apoptosis. **a** A feedback-loop, caspase-8-caspase-3-BID-cytochrome c-caspase-3, is involved in Taxol-induced apoptosis. **b** In cisplatin-induced apoptosis, mitochondrion-dependent intrinsic signal pathway (BID-cytochrome c-caspase-3) is activated. The apoptotic factors like calpain cause BID cleavage, but not caspase-8.

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