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Cytotoxic effects and pro-apoptotic mechanism of TBIDOM, a novel dehydroabietylamine derivative, on human hepatocellular carcinoma SMMC-7721 cells

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Abstract

We have investigated the antiproliferative effects of TBIDOM (N-(4-(2,2,2-trifluoroethyl) benzylidene) (7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthren-1-yl) meth-anamine) and have explored its possible mechanisms on human hepatocellular carcinoma SMMC-7721 cells. The proliferative status of cells treated with TBIDOM was measured by the colorimetric MTT assay. Cellular apoptosis was analysed using Hoechst 33342 staining and flow cytometry. Reduction of mitochondrial membrane potential ($\Delta\psi_m$) was also detected by flow cytometry. Western blotting assay was used to evaluate the release of cytochrome c and expression of p53, Bcl-2 and Bax proteins. It was shown that TBIDOM displayed a significant inhibitory effect on growth of SMMC-7721 cells in a dose- and time-dependent manner. Hoechst 33342 staining and flow cytometry analysis showed an increase of apoptosis rate and decrease of mitochondrial membrane potential after SMMC-7721 cells were exposed to TBIDOM for 24 h. Pretreatment of SMMC-7721 cells with TBIDOM significantly induced a decrease of Bcl-2 protein expression and an increase of caspase-3 activity and Bax protein expression. The results indicated that TBIDOM could effectively inhibit proliferation by induction of apoptosis and could be a promising candidate in the development of a novel class of antitumour agent.

Introduction

Hepatocellular carcinoma (HCC) is a frequent malignancy worldwide with a high rate of metastasis, resulting in more than 500 000 deaths per year (Bruix et al 2004). Although surgical management or non-surgical therapeutic modalities have been employed, either separately or in combination, treatment for HCC is rarely curative. Therefore, the development of more effective drugs is urgently required.

Dehydroabietylamine was one of the important reshaping products of rosin and a main component of disproportionated rosin amine, which has a phenanthrene structure with optical activity. It has been reported that dehydroabietylamines have many biological activities and applications, including chiral separation, metal ion froth flotation, surface activity, sterilization, antibiosis, as a medicine and as a colouring agent (Xiaoping et al 2006). Wilkerson & Deluccai (1993) synthesized many dehydroabietylamine derivatives, which were potential antagonists of phosphatidase-A_2, and had inhibitory hyperthyreosis activity.

N-(4-(2,2,2-Trifluoroethyl)benzylidene)(7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10 a-octahydrophenanthren-1-yl)meth-anamine, TBIDOM (Figure 1), is a novel derivative of dehydroabietylamine. Notwithstanding the widespread study of dehydroabietylamines, the pharmacological effects including the antitumour activity and mechanism of action for TBIDOM have remained unexplored. We have studied the cytotoxicity of TBIDOM on human hepatocellular carcinoma SMMC-7721 cells, and have investigated, by observation, the possible mechanisms of its effects on proliferation, apoptosis, reduction of mitochondrial membrane potential, and expressions of p53, Bcl-2, Bax and caspase-3 proteins.

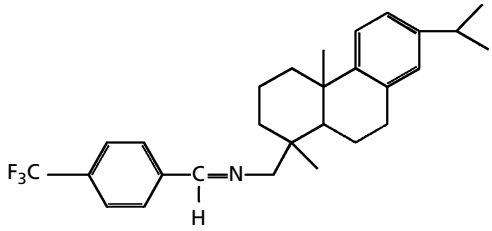

Figure 1 Molecular structure of TBIDOM.

Materials and Methods

Chemicals and reagents

TBIDOM (purity ≥ 99.68%) was kindly provided by Dr Xiao-Ping Rao (Institute of Chemical Industry of Forest Products, Chinese Academy of Forestry, Nanjing). It was dissolved in acetone as stock solutions and stored at −20°C until used. RPMI 1640 medium, trypsin/EDTA, 3-(4, 5-dimethyl thizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Hoechst 33342 were purchased from Sigma Chemical Co. (St Louis, MO). Annexin V-FITC Apoptosis Detection kit was purchased from Molecular Probes (Invitrogen, USA). Caspase-3 activity assay kit was purchased from R&D Systems Inc. (Minneapolis, MN). Anti-Bcl-2, anti-Bax, anti-p53 and anti-actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The Pierce SuperSignal West Pico chemiluminescent substrate and BCA Protein Assay Kit were from Pierce (Pierce Biotechnology, USA). All other chemicals were of analytic grade and commercially available.

Cell culture

Cell lines of human hepatocellular carcinoma (SMMC-7721), obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), were cultured in RPMI-1640 supplemented with 10% heat-inactivated calf serum, 100 U mL^{-1} penicillin, 100 U mL^{-1} streptomycin, 2 g L^{-1} $NaHCO_3$ and 2 mmol L^{-1} L-glutamine. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Cytotoxicity analysis by colorimetric MTT assay

Inhibition of cell proliferation by TBIDOM was measured by colorimetric MTT assay. Briefly, cells were incubated in 96-well plates containing 200 μL fresh medium. Cells were permitted to adhere for 8–12 h, washed with phosphate-buffered saline (PBS, pH 7.4), and then treated with various concentrations of TBIDOM (50, 100, 150, 200 or 250 μmol L^{-1}) for 12, 24, or 48 h. After exposure, the drug-containing medium was replaced by fresh medium. The cells in each well were then incubated at 37°C in 50 μL MTT (5 mg mL^{-1}) for 4 h. After the medium and MTT were removed, 150 μL DMSO was added to each well. Absorbance at 570 nm of the dissolved solutions was detected by a microplate ELISA reader (EL800, BIO-TEK Instruments Inc.). Cell inhibition ratio (I%) was calculated by the following equation:

$$I\% = (A_{control} - A_{treated})/A_{control} \times 100$$

where $A_{treated}$ and $A_{control}$ were the average absorbance of three parallel experiments from treated and control groups, respectively.

Cell morphological assessment

Apoptotic cell death was evaluated by observing morphological changes typical of apoptosis, such as cell shrinkage, chromatin condensation, membrane blebbing and formation of apoptotic bodies (Singh et al 1995), by a phase-contrast microscope (Olympus IX71, Japan). To assess the DNA chromatin, morphologic features were detected by Hoechst 33342 staining (Diaz-Ruiz et al 2001). The procedures followed the manufacturer's guidelines. Briefly, cells were cultured on glass slides and analysed 24 h after treatment. Cells growing on the slides were fixed with cold 3.7% formaldehyde, washed twice in PBS, and incubated in cold permeabilization solution (0.1% Triton X-100 + 0.1% sodium citrate) after inactivating endogenous peroxidase with 3% H_2O_2. The cells were washed with PBS again and incubated with Hoechst 33342 (10 μM) at 37°C for 1 h. After washing with PBS, the resulting fluorescence was detected at 480 nm under a fluorescence microscope (Olympus IX71, Japan). For quantification of chromatin-changed cells, at least 1000 cells from more than 10 random microscopic fields were counted by two observers.

Annexin-V/propidium iodide (PI) double-staining assay

A modified method used for detection of externalized inadherent cell lines was described by van Engeland et al (1998) and Clarke et al (2002). Externalization of phosphatidylserine and membrane integrity was quantified using Annexin V-FITC Apoptosis Detection kit. In brief, TBIDOM (100, 150 and 200 μM) was directly added into the flasks and incubated for 24 h, after which time the cells were harvested and washed with ice-cold PBS, and then incubated with annexin V and PI at room temperature for 15 min. After the incubation period, 400 μL 1× annexin-binding buffer was added, mixed gently and kept on ice. The stained cells were analysed by flow cytometry (BDFACS Canto flow Cytometer).

Mitochondrial membrane potential analysis ($\Delta\psi_m$)

Mitochondrial membrane potential (MMP) was assessed by the retention of rhodamine 123 (Rh123), a specific fluorescent cationic dye that is readily sequestered by active mitochondria, depending on their transmembrane potential (Ristow et al 2000; Hong & Liu 2004). SMMC-7721 cells were pre-incubated with different concentrations of TBIDOM (100, 150 or 200 μM) for 24 h and then washed with ice-cold PBS. After this, 500 μL 10 μM Rh123 was added and the cells incubated at 37°C in a 5% CO_2 incubator for 1 h. The fluorescence intensity was determined at excitation wavelength

485 nm, emission wavelength 535 nm with BDFACS Canto flow cytometer.

Caspase-3 activity assay

Activity of caspase-3 was detected using a fluorometric assay kit according to the manufacturer's protocol. In brief, $3–5\times10^6$ control or treated cells were lysed in 50 μL cold lysis buffer and incubated on ice for 30 min, and then centrifuged at 10 000 rev min^{-1} at 4°C for 1 min. The supernatant extracts were quantified for protein using a BCA Protein Assay Kit. Added to the cell lysates were 50 μL 2× reaction buffer and 5 μL caspase-3 substrates. After incubation at 37°C for 4 h, the fluorescence was detected by a fluorescence microplate reader (Fluoroskan Ascent, Labsystems) with excitation at 400 nm.

Western blotting

Cells (8×10^6/dish) were seeded in a 10-cm dish. After 8–12 h incubation, cells were treated with 0, 100, 150 or 200 μM TBIDOM for 24 h, washed with pre-ice cold PBS, and were harvested by scraping them in 1 mL PBS. The pelleted cells were lysed with 200 μL ice-cold lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM EGTA, 0.5% CHAPS, 10% (v/v) glycerol, 5 mM β-2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min, and then centrifuged at 13 000 g at 4°C for 10 min. The supernatant extracts were quantified for protein using a BCA Protein Assay Kit. Protein samples were separated with 4–15% SDS–polyacrylamide gel (SDS–PAGE) and transferred onto PVDF membranes (Minipore). The membranes were subsequently incubated with primary antibodies of anti-Bcl-2, anti-Bax, anti-p53 or anti-actin overnight at 4°C. Antibody recognition was detected with the respective secondary antibody, anti-mouse or anti-rabbit IgG_1 linked to horseradish peroxidase at room temperature for 2 h. The expression levels were normalized by actin expression. Antibody-bound proteins were detected by Pierce's SupeSignal West Pico chemiluminescent substrate. The experiment was repeated three times with similar results.

Mitochondrial cytochrome c release assay

Cells were trypsinized, washed once with 1×PBS and pelleted by centrifugation. Thereafter cells were lysed with lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 1 mM PMSF, 5 μg mL^{-1} leupeptin, 21 μg mL^{-1} aprotinin and 12.5 μg mL^{-1} digitonin) for 30 s. After centrifugation at 10 000 g for 1 min, the supernatant was collected and resolved in 15% denaturing SDS-PAGE minigel. Cytochrome c level was detected by probing with anti-cytochrome c antibody using standard Western blot analysis protocol as described above.

Statistical analysis

Data were expressed as means ± s.e. Statistical comparisons of the results were made using analysis of variance. Significant differences, $*P < 0.05$, $**P < 0.01$, between the means of control and TBIDOM-treated cells were analysed by Dunnett's test.

Results

Cell growth inhibition assessed by colorimetric MTT-assay

The antiproliferative effect of TBIDOM was tested in a liver cancer cell line, SMMC-7721. As shown in Figure 2, TBIDOM inhibited the proliferation in a dose- and time-dependent manner. The maximum proliferative inhibition of TBIDOM was 93.1% that occurred at 48 h using 250 μM TBIDOM.

Cell morphological assessment

Morphology of human hepatocellular carcinoma SMMC-7721 cells changes after treatment with TBIDOM for 24 h are shown in Figure 3B–D. Compared with untreated cells (Figure 3A), an increasing number of TBIDOM-treated cells displayed apoptotic features such as chromatin condensation and formation of apoptotic bodies. This indicated that cells were damaged after TBIDOM exposure. The effects

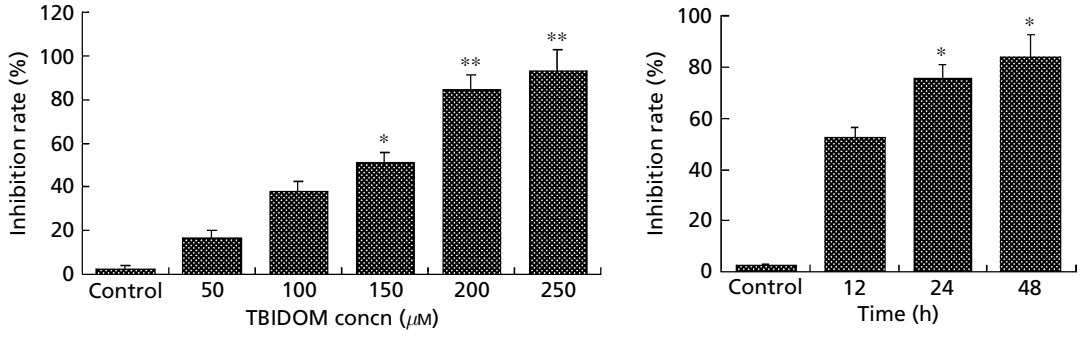

Figure 2 Inhibitory effects of TBIDOM on the proliferation of SMMC-7721 cells. SMMC-7721 cells were treated with acetone alone (control) or with 50, 100, 150, 200 or 250 μM TBIDOM for 12, 24 or 48 h. Data are shown as mean ± s.d. (n = 3). $*P < 0.05$, $**P < 0.01$, compared with control.

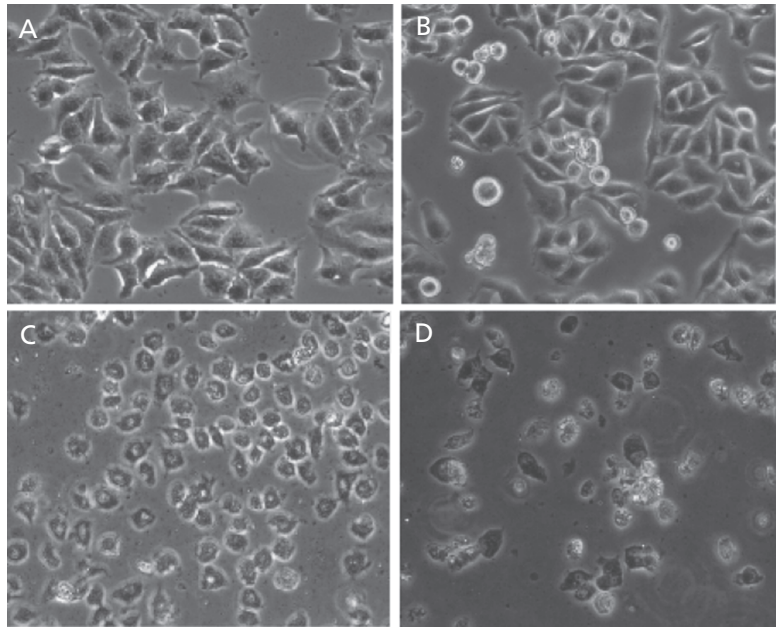

Figure 3 Morphological changes in cultured SMMC-7721 cells treated with TBIDOM. Morphological changes of SMMC-7721 cells treated with acetone alone (control, A) or with 100 (B), 150 (C) or 200 μM (D) TBIDOM for 24 h were observed under the phase-contrast microscope and photographed.

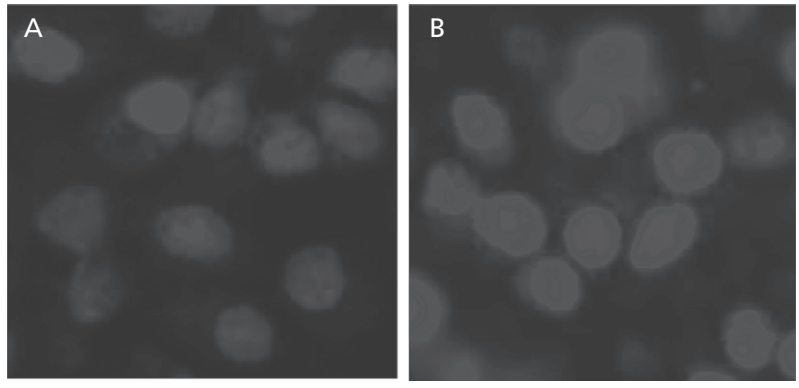

Figure 4 Apoptosis in hepatocellular carcinoma SMMC-7721 cells by fluorescence staining (Hoechst 33342 staining, original magnification $\times$200). A. Control, almost no apoptotic nuclei were observed. B. Cells treated with 150 μM TBIDOM for 24 h, some nuclear fragments were seen.

of TBIDOM-induced chromatin changes and apoptosis of SMMC-7721 cells were observed by Hoechst 33342 DNA staining. As shown in Figure 4, the cells treated with 150 μM TBIDOM for 24 h showed nuclear morphological changes (Figure 4B), while almost no apoptotic nuclei were observed in control cells (Figure 4A).

Apoptosis assessment by annexin-V and PI double-staining assay

The percentage of apoptotic cells (annexin-V^+/PI^-) in the control group was 5.2% (Figure 5A). After treatment with 100, 150 or 200 μM TBIDOM for 24 h, the percentages of apoptotic cells were increased to 7.4% (Figure 5B), 36.1% (Figure 5C), and 36.6% (Figure 5D), respectively.

Mitochondrial membrane potential ($\Delta\psi_m$) determination

The disruption of mitochondrial integrity is one of the early events leading to apoptosis. To assess whether TBIDOM affected the function of mitochondria, potential changes in mitochondrial membrane were analysed by employing a mitochondrial fluorescent dye, Rh123. As shown in Figure 6, a drop in the mitochondrial membrane potential was observed. The data showed that changes in the membrane potential were induced in treated SMMC-7721 cells after incubation

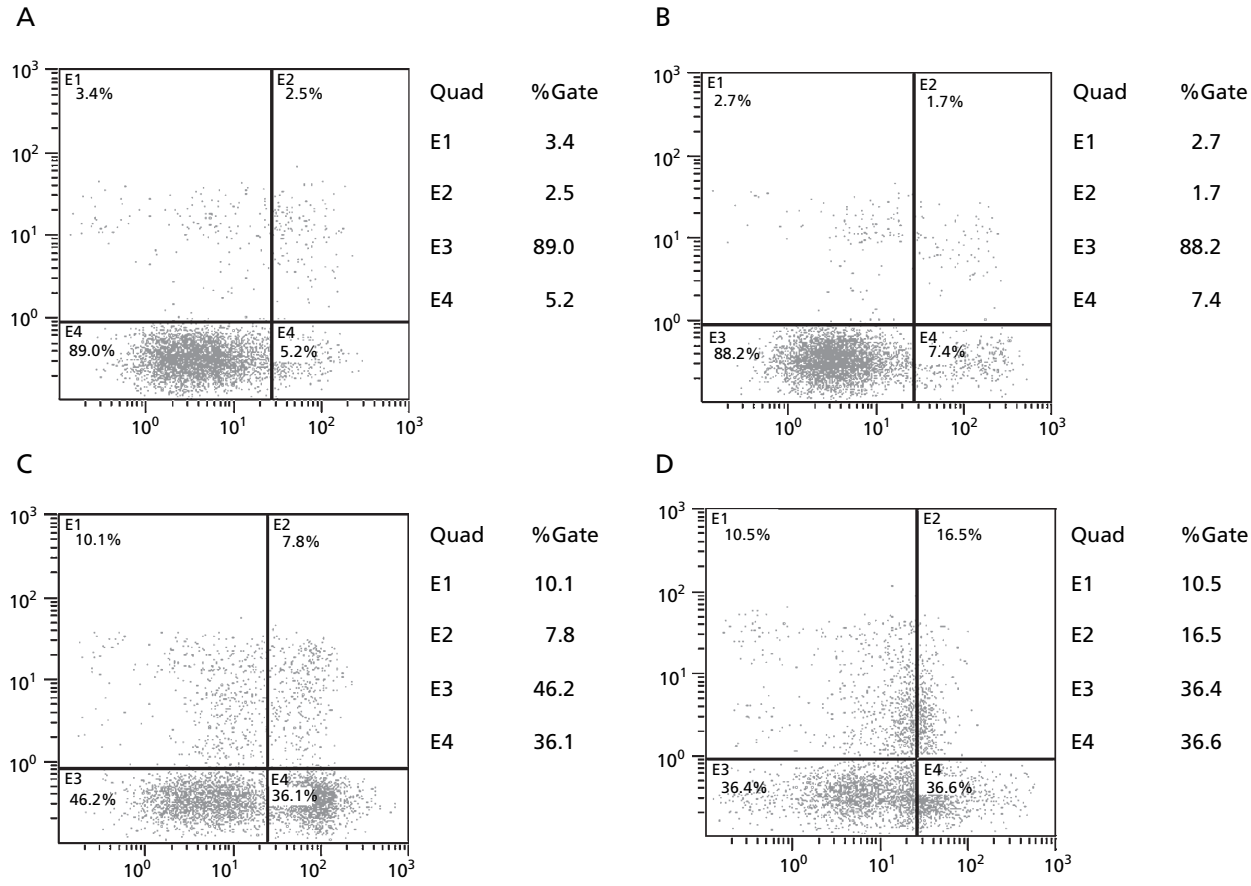

Figure 5 Fluorescence-activated cell sorter analysed by annexin-V and PI staining. A, acetone (control); B, 100 μM; C, 150 μM; and D, 200 μM TBIDOM. Upper right: necrotic cells and late apoptotic cells labelled with PI and annexin V–FITC. Lower left: fully viable cells. Lower right: early apoptotic cells labelled with annexin V–FITC but not with PI. After exposure to TBIDOM, the cells of the lower right had increased from 5.2 (control) to 7.4% (100 μM), 36.1% (150 μM), and 36.6% (200 μM).

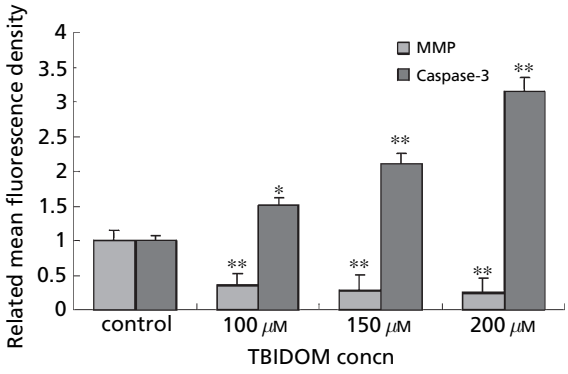

Figure 6 Effects of TBIDOM on mitochondrial membrane potential and on the activity of caspase-3 in SMMC-7721 cells. Compared with control, the mean fluorescence densities of the mitochondrial membrane potential (MMP) were reduced to approximately 37% (100 μM), 29% (150 μM) and 25.9% (200 μM), and the mean fluorescence densities of caspase-3 showed increases as much as 51.2% (100 μM), 111% (150 μM) and 214% (200 μM), respectively. Mean ± s.d., n = 3; $^*P < 0.05$, $^{**}P < 0.01$, compared with control.

with 100, 150 or 200 μM TBIDOM. A significant decrease of $\Delta\psi_m$ was detected in cells which were treated with 100, 150 or 200 μM TBIDOM for 24 h. Compared with control, the mean fluorescence densities were reduced to approximately 37%, 29% and 25.9%, respectively. The results suggested that TBIDOM could induce $\Delta\psi_m$ dissipation in a dose-dependent manner. A drop in the mitochondrial membrane potential is usually accompanied by release of cytochrome c into the cytosol. As shown in Figure 7, cytochrome c levels in the cytosol of TBIDOM-treated cells showed a rapid increase. Compared with control, the increase in the mean density was approximately 50%, 100% and 200%, respectively. The data indicated that TBIDOM increased the release of cytochrome c to cytosol in treated tumour cells.

Expressions of Bcl-2, Bax and p53 protein

The expression of proteins including p53, Bcl-2 and Bax may be involved in an intrinsic apoptosis pathway. In our case, TBIDOM (100, 150, 200 μM) could increase the expression of Bax by approximately 21.1%, 42.1%, 64.1% and decrease the expression of Bcl-2 by approximately 15.8%, 29.4%, 58.4% in a dose-dependent manner, but had little effect on p53.

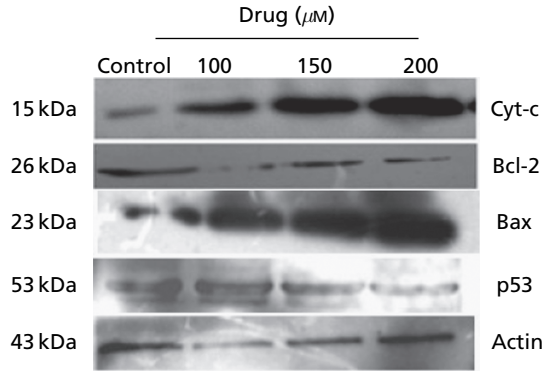

Figure 7 Effects of TBIDOM on the expressions of cytochrome c (Cyt-c) , Bcl-2, Bax, and p53 proteins in SMMC-7721 cells.

TBIDOM induces activation of caspase-3

The activity of caspase-3 was detected in SMMC-7721 cells treated with TBIDOM. The data are shown in Figure 6. TBIDOM significantly stimulated the activity of caspase-3 in a dose-dependent manner. The activity of caspase-3 showed as much as 1.5-, 2- and 3-fold increases at 24 h after treatment with 100, 150 and 200 μM TBIDOM compared with the control, respectively.

Discussion

TBIDOM (Figure 1) is a derivative of dehydroabietylamine with novel structure. Our acute toxicity data (data not shown) indicated that TBIDOM is sufficiently safe. In our work, the treatment of a tumour cell line (SMMC-7721 cells) with TBIDOM resulted in the inhibition of the cell growth in a dose- and time-dependent manner. Annexin-V/PI double-staining assay demonstrated that TBIDOM induced apoptosis in a dose-dependent manner.

Apoptosis is the result of an active, gene-directed process, and we should eventually be able to manipulate this process by developing drugs that interact with cell death proteins. The Bcl-2 family of proteins can be divided into two groups: suppressors of apoptosis (e.g. Bcl-2, Bcl-XL, and Mcl-1) and activators of apoptosis (e.g. Bax, Bok, Hrk, and Bad). Pathological increases in the amounts of one or more of the apoptosis-suppressing proteins have been observed in several types of cancer, though generally the mechanisms that bring about their increased expression are poorly understood (Tamm et al 2001; Marsden et al 2002). Previous studies indicated that an increase in pro-apoptotic Bcl-2 family proteins and a decrease in anti-apoptotic Bcl-2 family proteins were involved in the process of apoptosis (Kluck et al 1997; Yang et al 1997). Thus, the ratio of Bc1-2/Bax might be a critical factor of a cell's threshold for apoptosis. This study showed a decrease in Bcl-2 and increase in Bax protein detected in TBIDOM-treated SMMC-7721 cells, leading to a decrease in the Bc1-2/Bax ratio. Thus, we propose that the Bcl-2 protein mainly participated in TBIDOM-induced apoptosis in SMMC-7721 cells.

Caspase cascade plays a key role in the apoptosis procedure (Oubrahim et al 2001; Lakhani et al 2006). Caspase-3 typically functions at the downstream of other caspases and directly activates enzymes that are responsible for DNA fragmentation in an intrinsic apoptosis pathway. Our data suggested that activation of caspase-3 was involved in the TBIDOM-induced apoptosis of the tumour cells. At the same time, stress-mediated apoptosis is often triggered by mitochondrial function loss and subsequent cytochrome c release from mitochondria to cytosol (Tournier et al 2000; Park et al 2006). The role of mitochondria in TBIDOM-mediated apoptosis was also explored in this work. TBIDOM induced a loss of mitochondrial potential and the release of cytochrome c to cytosol. Taken together, these results indicated that the apoptosis induced by TBIDOM was through an intrinsic pathway related to mitochondrial dysfunction.

In many cases, the p53 protein has been identified as the effector of apoptosis signals (Chipuk et al 2004). It is a regulator of cell cycle progression and mediator of apoptosis in various cases. The key role of p53 in the G_1/S checkpoint was its response to DNA damage. The activation of apoptosis by TBIDOM was independent of p53 since the expression levels of p53 did not show any significant increase after TBIDOM treatment (Figure 7). We also studied the effect of TBIDOM on the cell cycle of SMMC-7721 (data not shown). Our data indicated that TBIDOM had little effect on cell cycle and may be an anticancer drug independent of p53.

Conclusion

TBIDOM exhibited significant antiproliferative effects by induction of apoptosis in hepatocellular carcinoma SMMC-7721 cells. Its mechanism may have been related to the decrease in the expression of anti-apoptotic protein, Bcl-2, accompanied by a drop in the mitochondrial membrane potential and the activation of caspase-3, that led to apoptotic body formation and finally apoptosis. TBIDOM could be a promising candidate in the development of a novel class of antitumour agent.

References

Bruix, J., Boix, L., Sala, M., Llovet, J. M. (2004) Focus on hepatocellular carcinoma. *Cancer Cell* **5**: 215–219

Chipuk, J. E., Kumana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., Green, D. R. (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* **303**: 1010–1014

Clarke, R. G., Lund, E. K., Johnson, I. T., Pinder, A. C. (2002) Apoptosis can be detected in attached colonic adenocarcinoma HT29 cells using annexin V binding, but not by TUNEL assay or sub-G0 DNA content. *Cytometry* **39**: 141–150

Diaz-Ruiz, C., Mlontaner, B., Perez-Tomas, R. (2001) Prodigiosin induces cell death and morphological changes indicative of apoptosis in gastric cancer cell line HGT-1. *Histol. Histopathol.* **16**: 415–421

Hong, H., Liu, G. Q. (2004) Protection against hydrogen peroxide-induced cytotoxicity in PC12 cells by scutellarin. *Life Sci.* **74**: 2959–2973

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., Newmeyer, D. D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**: 1132–1136

Lakhani, S. A., Masud, A., Kuida, K., Porter, G. A., Jr., Booth, C. J., Mehal, W. Z., Inayat, I., Flavell, R. A. (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* **311**: 847–851

Marsden, V. S., O'Connor, L., O'Reilly, L. A., Silke, J., Metcalf, D., Ekert, P. G., Huang, D. C., Cecconi, F., Kuida, K., Tomaselli, K. J., Roy, S., Nicholson, D. W., Vaux, D. L., Bouillet, P., Adams, J. M., Strasser, A. (2002) Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature* **419**: 634–637

Oubrahim, H., Stadtman, E. R., Chock, P. B. (2001) Mitochondria play no roles in Mn(II)-induced apoptosis in HeLa cells. *Proc Natl Acad Sci U S A* **98**: 9505–9510

Park, J., Lee, S. B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., Kim, J. M., Chung, J. (2006) Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature* **441**: 1157–1161

Ristow, M., Pfister, M. F., Yee, A. J., Schubert, M., Michael, L., Zhang, C. Y., Ueki, K., Michael, M. D., Lowell, B. B., Kahn, C. R. (2000) Frataxin activates mitochondrial energy conversion and oxidative phosphorylation. *Proc Natl Acad Sci U S A* **97**: 12239–12243

Singh, N., Sun, Y., Nakamura, K., Smith, M. R., Colburn, N. H. (1995) C-JUN/AP-1 as possible mediators of tumor necrosis factor-alpha-induced apoptotic response in mouse JB6 tumor cells. *Oncol. Res.* **7**: 353–362

Tamm, I., Schriever, F. S., Dorken, B. (2001) Apoptosis: implications of basic research for clinical oncology. *Lancet Oncol*. **2**: 33–42

Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., Davis, R. J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* **288**: 870–874

van Engeland, M., Nieland, L. J., Ramaekers, F. C., Schutte, B., Reutelingsperger, C. P. (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31**: 1–9

Wilkerson, W. W., Deluccai, G. W. (1993) Topical antiinflammatory dehydroabietylamine derivatives. *Bioorg. Med. Chem. Lett.* **3**: 2087–2092

Xiaoping, R., Zhanqian, S., Hong, G. (2006) Research and application progress on dehydroabietylamine and its derivatives. *Chemistry* **3**: 168–172

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**: 1129–1132