Triptolide circumvents drug-resistant effect and enhances 5-fluorouracil antitumor effect on KB cells

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Triptolide, a diterpenoid triepoxide derived from the Chinese herb *Tripterygium wilfordii*, exerts an antitumor effect in KB cancer cells through the induction of apoptosis. In this study, we show that triptolide possesses an anticancer effect on drug-sensitive parental KB cells and multidrug-resistant KB-7D and KB-tax cells that overexpress multidrug resistance protein and MDR, respectively. Our data revealed that triptolide decreases the expression of multidrug resistance protein and MDR in both KB-7D and KB-tax cells. It also induces apoptosis in these multidrug-resistant cancer cells by activating caspase-3, and decreasing Mcl-1 and XIAP. Triptolide not only inhibits tumor growth but also induces apoptosis of these drug-resistant cancer cells in xenograft mouse models. Moreover, we also show that triptolide combined with 5-fluorouracil could be an alternative strategy for chemotherapy enhancement. These results indicate the therapeutic value of triptolide on multidrug-resistant cells, and when combined with 5-fluorouracil for the enhancement of cancer therapy. *Anti-Cancer Drugs* 21:502–513 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2010, **21**:502–513

Keywords: 5-fluorouracil, apoptosis, multi-drug resistance, synergistic effect, triptolide

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Received 10 August 2009 **Revised form accepted** 5 January 2010

Introduction

Triptolide (TPL) is a diterpenoid triepoxide derived from the herb *Tripterygium wilfordii* [1]. Recently, TPL has been found to possess the ability to reduce proliferation and induce apoptosis in cancer cells [2–4]. Furthermore, TPL has also been reported to exhibit an inhibitory effect on tumor growth and metastasis in mouse xenografts of melanoma, breast cancer, gastric carcinoma, bladder cancer [4], and pancreatic cancer [5]. Our earlier investigation found that TPL possesses an excellent antitumor effect against oral cancer cells, SCC25 and OEC-M1, and human cervical carcinoma KB cells through caspase-mediated apoptosis [6]. However, the effect of TPL on drug-resistant cancer cells has not been explored.

p38 MAP kinase (p38-MAPK) was found to be responsible for chemotherapy resistance in human gastric cancer cells [7]. This study showed that MDR is overexpressed in vincristine-resistant gastric cancer cells, and that these cells are resistant to P-glycoprotein (P-gp)-related and P-gp-unrelated drugs. The investigators also observed an increase in phosphorylated p38-MAPK and activator protein-1 (AP-1) activities in drug-resistant cells. Moreover, the inhibition of p38-MAPK by SB202190 reduced AP-1 activity and *MDR1* expression levels and increased the sensitivity of vincristine-resistant cells to chemotherapy. TPL has been reported to induce dendritic cell apoptosis through sequential p38-MAPK phosphorylation and caspase-3 activation [8]. TPL has also been shown to have an inhibitory effect on phosphorylation of c-Jun NH_2-terminal kinase [9]. Reactive oxygen species (ROS) have also been reported to possibly act as negative regulators of MDR [10], and TPL treatment can increase the generation of ROS in RAW264.7 cells [11]. Earlier study showed that TPL induced apoptosis in CML cells, and also elevated the generation of ROS, although this is not the major factor that initiates apoptosis [12]. Taken together, these findings suggest that TPL may possess the capacity to overcome multidrug resistance through the inhibition of phosphorylation p38-MAPK and reduction of AP-1 activity or through elevation of ROS in cancer cells. On the basis of this hypothesis, we examined whether TPL treatment could exhibit a similar antitumor effect on drug-resistant cancer cells as it does on nondrug-resistant cells.

The conventional anticancer drug, 5-fluorouracil (5-FU), is a thymidylate synthase inhibitor and is widely used in the treatment of gastrointestinal, breast, head and neck, and colorectal cancer [13]. However, the use of 5-fluorouracil (5-FU) alone in cancer chemotherapy elicits many side effects and evokes poor responses [14–18], mainly because

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328337337c

of bystander cytotoxicity. Thus, candidate therapeutics that can be combined with reduced 5-FU dosage as a chemotherapeutic strategy should successfully reduce the side effects of 5-FU; moreover, they may synergistically exert an ability to induce apoptosis in cancer cells.

In this study, we used the drug-resistant cancer cell lines, KB-7D and KB-tax, which overexpress multidrug resistance protein (MRP) and MDR, respectively, to assess the potential efficacy of TPL against drug-resistant cancer cells. KB-7D and KB-tax are drug-resistant cancer cell lines derived from KB cells. KB-7D cells were generated by VP-16-driven selection, which results in the downregulation of topoisomerase II and overexpression of MRP [19]. KB-tax cells, which are characterized by the overexpression of P-gp170/MDR, were generated by taxol-driven selection [19]. Our results show that TPL exhibits a significant therapeutic effect on KB-tax and KB-7D cells and has potential therapeutic value for clinical chemoresistant cancers. Moreover, the combination of TPL and 5-FU reveals a synergistic therapeutic effect on KB cancer cells. Our results also show the potential role of TPL for establishing a therapeutic strategy enhancing the antitumor effect on 5-FU.

Materials and methods

Cells and chemicals

KB cancer cells were purchased from American Type Culture Collection (CCL-17; American Type Culture Collection, Rockville, Maryland, USA). KB-7D cells were generated by VP-16-driven selection, which displayed downregulation of topoisomerase II and overexpression of MRP. KB-tax cells were generated by taxol-driven selection, which displayed overexpression of P-gp170/MDR. These drug-resistant cancer cells were kindly provided by Dr Jang-Yang Chang (Cancer Research Division of National Health Research Institutes, Taiwan) [19]. TPL (Calbiochem, San Diego, California, USA) was dissolved in dimethyl sulfoxide as a 10-mmol/l stock and added to cells at the indicated concentrations. 5-FU (Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) at a concentration of 10 mmol/l and added to cells at the indicated concentrations.

Protein extraction and western blot analysis

Protein was extracted from cultured cells (KB, KB-7D, and KB-tax) lysed in a cell lysis containing protease inhibitors (50 mmol/l Tris (pH 7.5), 30 mmol/l $MgSO_4$, 8 mmol/l EDTA, 2 mmol/l DTT, and 2% Triton X-100). Cell lysates were clarified by centrifugation at 13 000 rpm, at 4°C for 15 min. Protein concentrations of cell lysates were measured by a BCA assay (Thermo Scientific, Rockford, Illinois, USA). For western blot analysis, 50 μg protein extracts from control cancer cells and cells treated with 20 nmol/l TPL were loaded and separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electrotransferred onto a polyvinyldifluoride (PVDF) membrane (Millipore, Billerica, Massachusetts, USA). The PVDF membrane was then blocked with a blocking solution containing 5% non-fat powdered milk in PBS plus 0.2% Tween 20 (PBST) for 1 h at room temperature. After blocking, the membrane was washed three times with PBST. The PVDF membrane was blotted with the following primary antibodies in PBS: anti-XIAP; Mcl-1 (Cell Signaling Technology, Danvers, Massachusetts, USA); anti-MDR; MRP (Millipore); and α-actinin (Santa Cruz, California, USA). After 2 h at room temperature, the membrane was again washed in PBST. The PVDF membrane was then incubated with peroxidase-linked anti-mouse or anti-rabbit IgG antibodies for 1 h, developed using an enhanced chemiluminescence detection kit (Millipore), and analyzed with a Las-3000 imaging system (Fujifilm, Tokyo, Japan).

Growth inhibition assay

Cells in logarithmic growth phase were cultured at a density of 10 000 cells/well in 24-well plates. The cells were exposed to various concentrations of the tested drugs for 72 h. The methylene blue dye assay was used to evaluate the effect of tested drugs on cell growth, as described earlier [6,20]. The IC_{50} value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with control growth [19].

Determination of caspase-3 activity

Caspase-3 activity was measured by a CaspACE Assay System Fluorometric Kit (Promega Corporation, Madison, Wisconsin, USA). Cells were initially seeded at a density of 1×10^6 cells per 100 mm^2 dish. After treatment with 40 nmol/l TPL for 24 h, caspase-3 activity was measured by the cleavage of the fluorometric substrate according to the manufacturer's instructions.

Xenograft tumor model

Eight-week-old NOD/SCID (NOD.CB17 *Prkdc* ^{scid}/J, National Laboratory Animal Center, Taiwan) mice were maintained in microisolators under specific pathogen-free conditions. These mice were fed with sterile food and chlorinated sterile water. Thirty-six mice were divided into three groups; each group of mice was subcutaneously injected with 2×10^6 KB, 2×10^6 KB-7D, and 2×10^6 KB-tax cells, respectively. Six mice in each group were further treated with TPL [0.15 mg/kg BW/day/intraperitoneal (i.p.)], and six mice in each group were daily injected with vehicle control. TPL was first injected on day 3, before any tumor was palpated, in each group of mice, and continuously administered until day 15 in KB, KB-7D, and KB-tax-bearing mice, respectively. Two million KB, KB-7D, and KB-tax cells were transplanted into the dorsum of the mice, respectively. Three days later, the mice were then randomized into four groups. TPL (0.075 mg/kg/day) and 5-FU (12 mg/kg/day) alone or in combination and a vehicle control were then separately administered to these four groups by i.p. injection. The size of the transplanted

tumors was measured by gauged calipers every 3 days and the tumor volume was calculated using the formula: volume (V) = 1/2 × (length × width^2). At the end of treatment, the mice were killed, and the tumors were removed, weighed, and photographed.

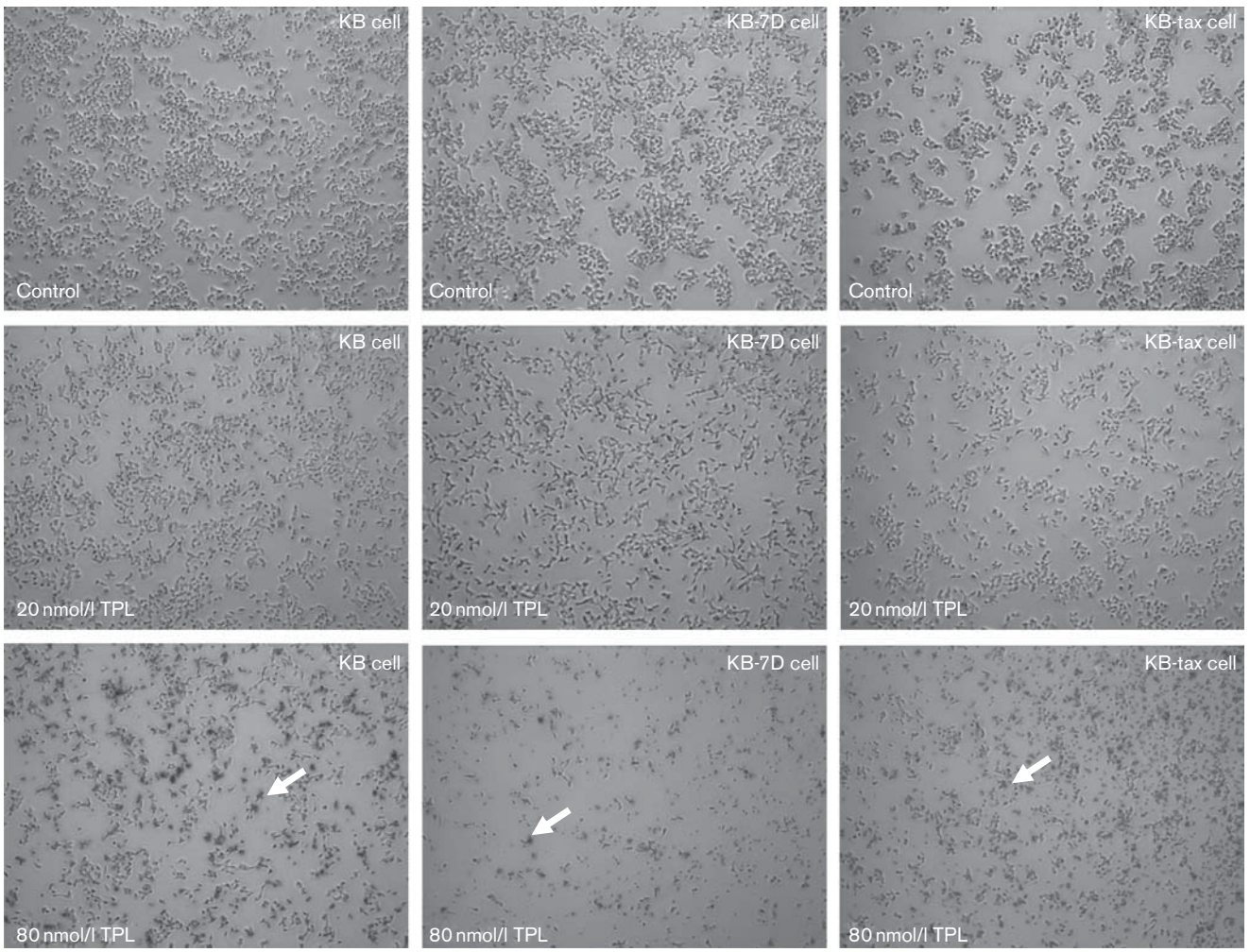
Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay

Tumors removed from the mice were formalin fixed and paraffin-embedded. Five-micrometer-thick sections were stained and examined. For in-situ staining of apoptotic cells, the TUNEL method was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). The staining procedures used followed the manufacturer's instructions. One thousand cells (1000 cells/field × 10 fields) in slide-mounted tumor sections were evaluated under a ×400 magnification. The nucleated cells were counted using hematoxylin and eosin counterstaining in a light field. The nuclei that stained fluorescent green with fluorescein as seen in the dark field were determined to be apoptotic cells. The apoptotic index (AI) was calculated as the percentage of positive staining cells: AI = number of apoptotic cells (fluorescent green)/total number of nucleated cells.

Statistical analysis

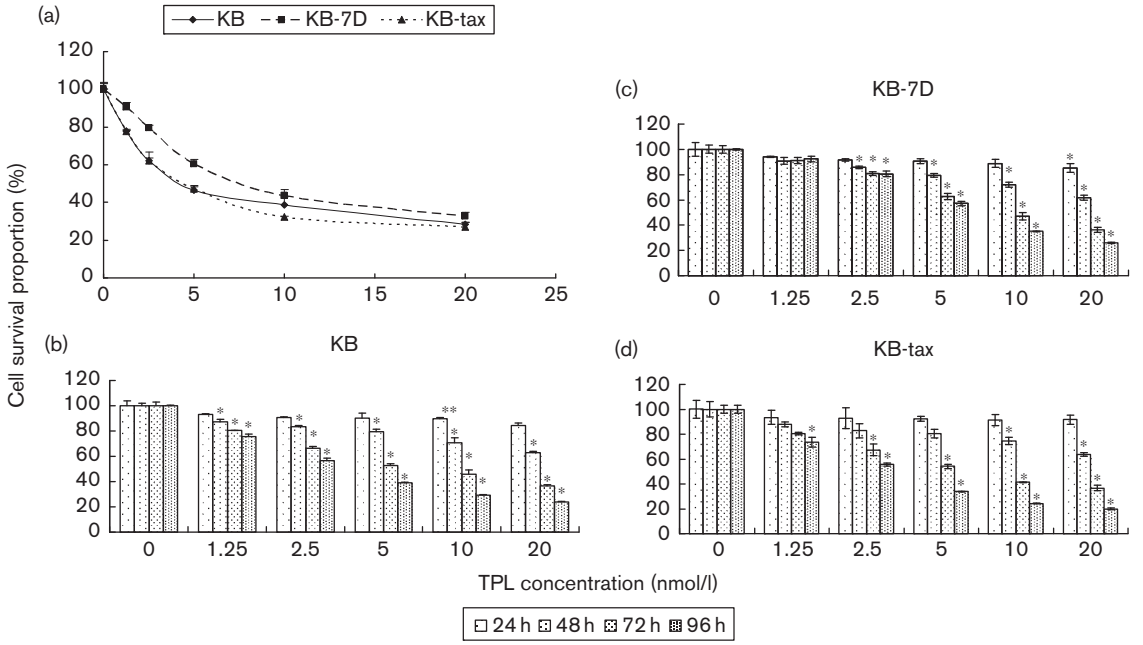
The Statistical Package for the Social Sciences (SPSS version 10.0 for Microsoft Windows, Chicago, Illinois, USA) was used to complete the analysis of the collected data. A t-test and a one-way analysis of variance with Scheffe's post hoc test were used to determine whether any significant relationships exist among quantitative results. Values of P less than 0.05 were considered significant.

Fig. 1



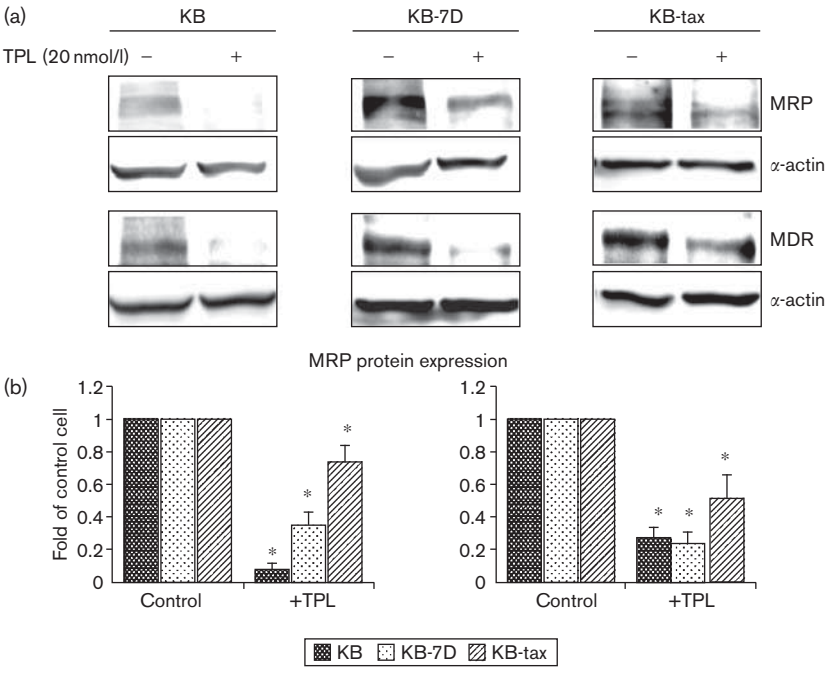
Triptolide (TPL) inhibits drug-resistant KB cells growth *in vitro*. Morphological variations of KB, KB-7D, KB-tax cells treated with 0, 20, 80 nmol/l TPL for 24 h (arrows indicate dead cells).

Fig. 2



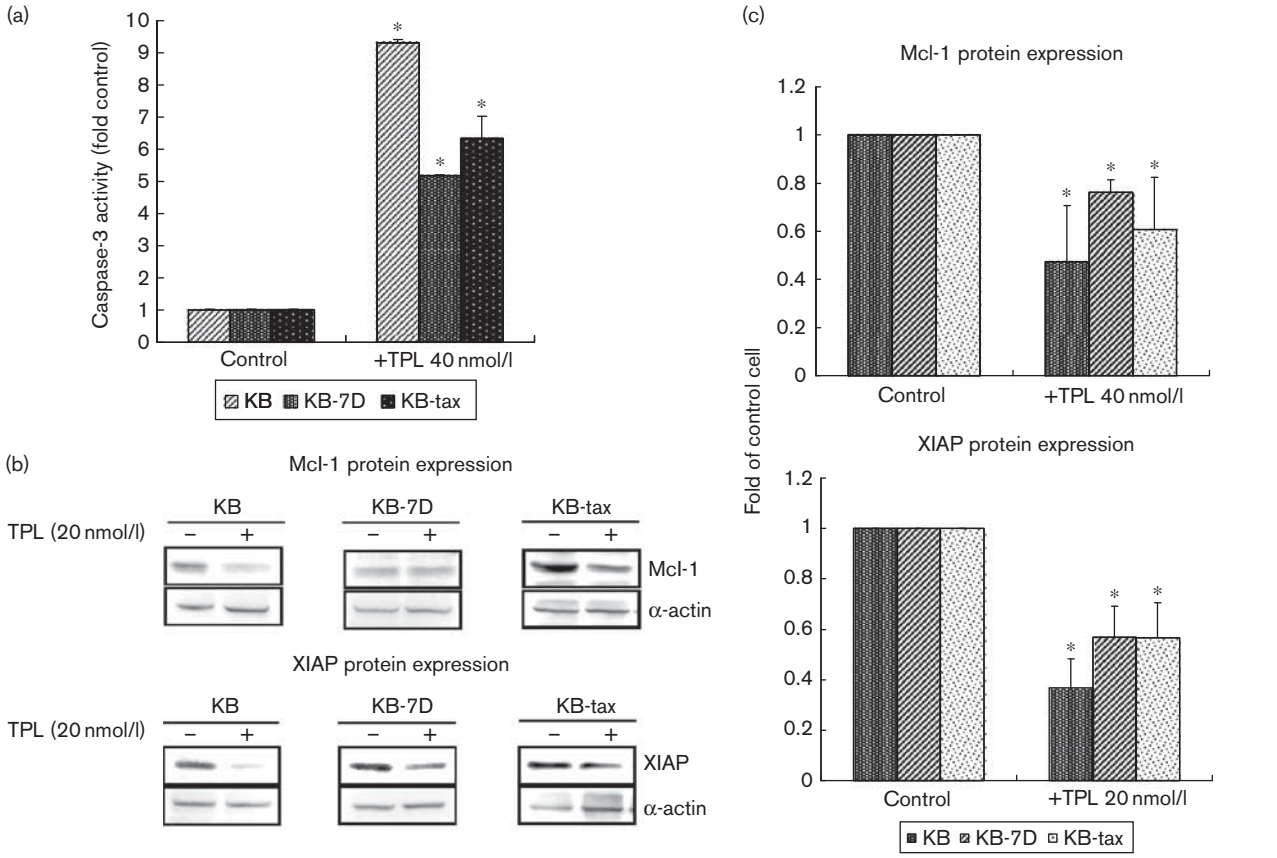
Triptolide (TPL) inhibits drug-resistant KB cells growth *in vitro*. (a) After 72 h treatment, the IC_{50} of TPL was 4.41 nmol/l in KB, 8.1 nmol/l in KB-7D, and 4.52 nmol/l in KB-tax cells. KB (b) KB-7D (c) KB-tax (d) were treated with various concentrations (0–20 nmol/l) of TPL for various time periods (24–96 h), and growth inhibition was measured using the methylene blue assay. Survival proportion (%) indicates the relative value compared with vehicle control groups in KB, KB-7D, and KB-tax ($n=3$; $^*P<0.05$).

Fig. 3



Triptolide (TPL) downregulated multidrug resistance protein (MRP) and MDR gene expression *in vitro*. (a) The results of immunoblotting for MRP and MDR proteins in KB, KB-7D, and KB-tax cells, before and after TPL treatment. Both MRP and MDR protein expression decreased in TPL-treated cells. (b) Compared with control cells, MRP proteins were expressed by 7.41 ± 4.29, 34.38 ± 8.01, and 61.0 ± 7.05% in TPL-treated KB, KB-7D, and KB-tax cells, respectively. MDR proteins were expressed by 27.36 ± 6.66, 24.45 ± 6.78, and 51.73 ± 14.55% in TPL-treated KB, KB-7D, and KB-tax cells, respectively ($n=3$; $^*P<0.05$).

Fig. 4



Triptolide (TPL) induces apoptosis *in vitro*. (a) KB, KB-7D, and KB-tax cells were treated with 40 nmol/l TPL for 24 h, after which caspase-3 level were measured and compared with control cells. Caspase-3 increased 9.31 ± 0.10-fold, 5.17 ± 0.03-fold, and 6.32 ± 0.70-fold in KB, KB-7D, and KB-tax cells, respectively ($n=3$; $^*P<0.05$). (b) Immunoblotting results for Mcl-1 and XIAP proteins in KB, KB-7D, and KB-tax cells before and after TPL treatment. Both Mcl-1 and XIAP protein expression had decreased in KB and drug-resistant KB cells. (c) Compared with control, Mcl-1 proteins were expressed by 47.3 ± 23.4, 76.27 ± 5.14, and 60.71 ± 21.75% in TPL-treated KB, KB-7D, and KB-tax cells, respectively. XIAP proteins were expressed by 36.36 ± 11.94, 56.91 ± 12.35, and 56.67 ± 13.91% in TPL-treated KB, KB-7D, and KB-tax cells, respectively ($n=3$; $^*P<0.05$).

Results

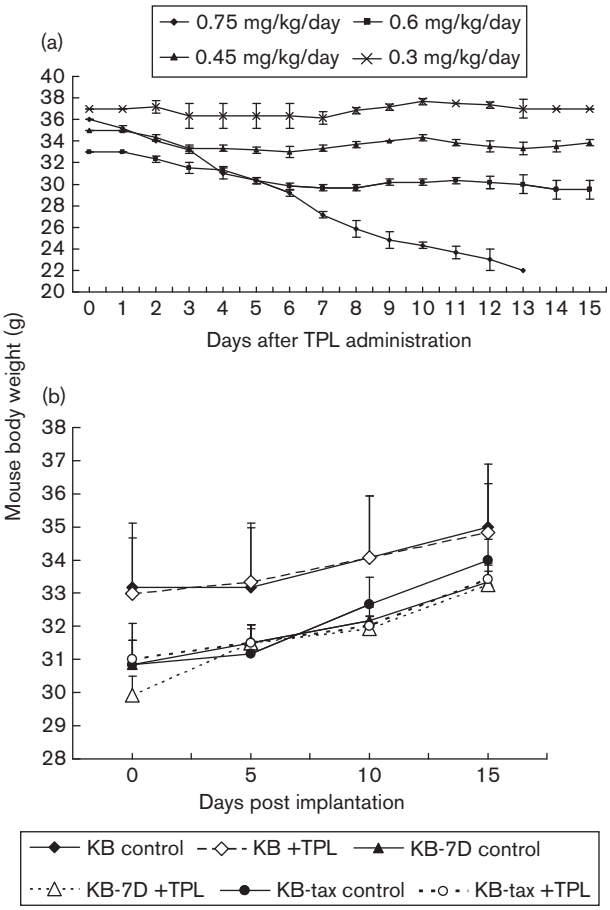
Overexpression of MRP and MDR in drug-resistant cells

KB-7D and KB-tax are drug-resistant cancer cell lines derived from KB cells. KB-7D cells were generated by VP-16-driven selection, which displays downregulation of topoisomerase II and overexpression of MRP [19]. KB-tax cells, which display an overexpression of P-gp170/MDR, were generated by taxol-driven selection [19]. To confirm the overexpression of MRP and MDR in these cell lines, we evaluated the expression of MRP and MDR in KB-7D and KB-tax, respectively, using quantitative real-time PCR. We found that mRNA of MRP and MDR were highly expressed in KB-7D and KB-tax, respectively, compared with KB parental cells (data not shown).

Growth inhibitory effect of TPL on drug-resistant cells

To examine the growth inhibitory effect of TPL on drug-resistant cells, we treated KB, KB-7D, and KB-tax cancer cells with different concentrations of TPL for 24 h and observed their morphological change under a phase-contrast microscope (Nikon, Tokyo, Japan). Under TPL treatment, the density of cells was deceased in a dose-dependent manner (Fig. 1). To evaluate the IC_{50} value of TPL on these drug-resistant cancer cells, we treated KB, KB-7D, and KB-tax cells with different doses of TPL. The IC_{50} of TPL was 4.41 nmol/l on KB; 8.1 nmol/l on KB-7D cells; 4.52 nmol/l on KB-tax cells after 72 h treatment (Fig. 2a). These results show that TPL possesses a growth inhibitory effect on drug-resistant KB-7D and KB-tax cells. To compare the growth inhibitory effect of TPL between parental and drug-resistant KB cancer cell lines, we evaluated the growth of these cells under the treatment of TPL at various doses (0–20 nmol/l) and time points (0–96 h) (Fig. 2b and d). The results revealed that effect of TPL is similar between these three cell lines, indicating a potential role for TPL against multidrug-resistant cancers.

Fig. 5



The effects of triptolide (TPL) in mouse body weight. (a) Treatment with TPL (0.75 mg/kg/day; closed diamond) (0.6 mg/kg/day; closed square) (0.45 mg/kg/day; closed triangle) (0.3 mg/kg/day; cross) affects mouse body weight in the KB xenograft tumor-bearing model. The body weight and survival rate of the mice were checked daily. Body weights significantly decreased in the group of mice treated with TPL (0.75 mg/kg/day), and all mice died on day14 ($n=4$/each group). (b) Treatment with TPL (0.15 mg/kg/day) did not affect mouse body weight in KB (control: closed diamond; $n=6$, TPL: open diamond; $n=6$), KB-7D (control: closed triangle; $n=6$, TPL: open triangle; $n=6$), and KB-tax (control: closed circle; $n=6$, TPL: open circle; $n=6$) xenograft tumor-bearing model ($n=6$/each group).

In-vitro downregulation of MRP and MDR genes by TPL treatment

To examine the effect of TPL on MRP and MDR gene expression in drug-resistant cells, we treated KB, KB-7D, and KB-tax cancer cells with 20 nmol/l TPL for 48 h, and then determined their change in gene expression by immunoblotting (Fig. 3a). Compared with control cells, MRP proteins were expressed by 7.41 ± 4.29, 34.38 ± 8.01, and $61.0 \pm 7.05\%$ in TPL-treated KB, KB-7D, and KB-tax cells, respectively. MDR proteins were expressed by 27.36 ± 6.66, 24.45 ± 6.78 and $51.73 \pm 14.55\%$ in TPL-treated KB, KB-7D, and KB-tax cells, respectively (Fig. 3b). These results indicate that TPL-induced cell death of these drug-resistant cancer cells is, at least, partially attributable to downregulation of the MRP and MDR genes.

Induction of apoptosis by the activation of caspase-3 and inhibition of Mcl-1 and XIAP

One of our aims was to determine whether TPL treatment could activate apoptosis in cancer cells. We therefore investigated whether TPL initiates the caspase-signaling cascade. KB, KB-7D, and KB-tax cancer cells were treated with 40 nmol/l TPL for 24 h, after which cell lysates were examined for caspase-3 activity. Compared with control cells, caspase-3 activity increased 9.31 ± 0.10-fold, 5.17 ± 0.03-fold, and 6.32 ± 0.70-fold in TPL-treated KB, KB-7D, and KB-tax cells, respectively (Fig. 4a). To elucidate the mechanism of TPL-induced apoptosis, we evaluated the expression of apoptosis-related proteins. Western blot analysis showed that both Mcl-1 and XIAP were down-regulated (Fig. 4b). Mcl-1 proteins were expressed by 47.3 ± 23.4, 76.27 ± 5.14, and $60.71 \pm 21.75\%$ in TPL-treated KB, KB-7D, and KB-tax cells, respectively. XIAP proteins were expressed by 36.36 ± 11.94, 56.91 ± 12.35, and $56.67 \pm 13.91\%$ in TPL-treated KB, KB-7D, and KB-tax cells, respectively (Fig. 4c). These results indicate that TPL induces cell apoptosis by activating caspase-3 and inhibiting Mcl-1 and XIAP protein expression.

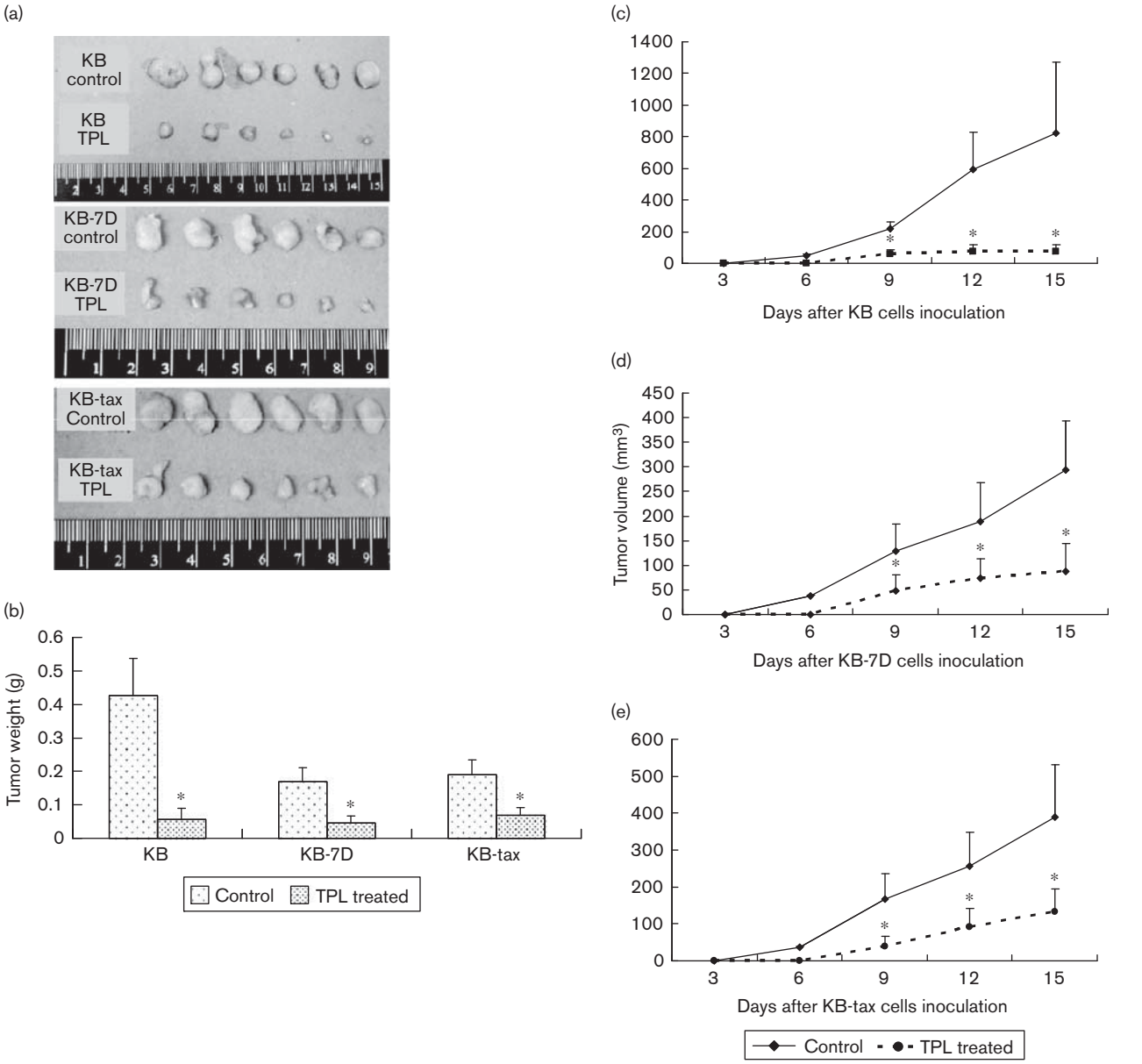
The effects of TPL on body weight in mice

To examine whether TPL treatment causes body weight loss, we monitored the alteration of body weight in experimental mice. A significant cytotoxic effect of TPL was shown on daily administration into NOD/SCID at a higher dose than 0.75 mg/kg/day (Fig. 5a). The body weight of mice treated at this higher dosage was significantly decreased, and all mice died by day 14. In these studies, we found that the maximum tolerated dose of TPL in mice was 0.6 mg/kg/day administered i.p. (Fig. 5a). On this basis, we chose a treatment dose for mice of 25% of the maximum tolerated dose or 0.15 mg/kg/day, i.p. This dosage has been proven to be safe for mice in earlier studies [4,12]. The body weight of mice was monitored at 5 day intervals during the course of the experiment (Fig. 5b). There were no obvious signs of sickness after 15 days, and treatment with TPL (0.15 mg/kg/day) did not affect mouse body weight in KB, KB-7D, and KB-tax xenograft tumor-bearing models (Fig. 5b). Our findings indicated that daily treatment of TPL at 0.15 mg/kg/day i.p. did not elicit an adverse effect in mice.

Antitumor effect on drug-resistant cells *in vivo*

To investigate the antitumor effect of TPL on drug-resistant cells *in vivo*, we established a xenograft tumor-bearing model by separately transplanting KB, KB-7D, and KB-tax cancer cells into NOD/SCID mice. TPL was first injected on day 3 in each group of mice, before any tumor was palpated, and administered daily until day

Fig. 6



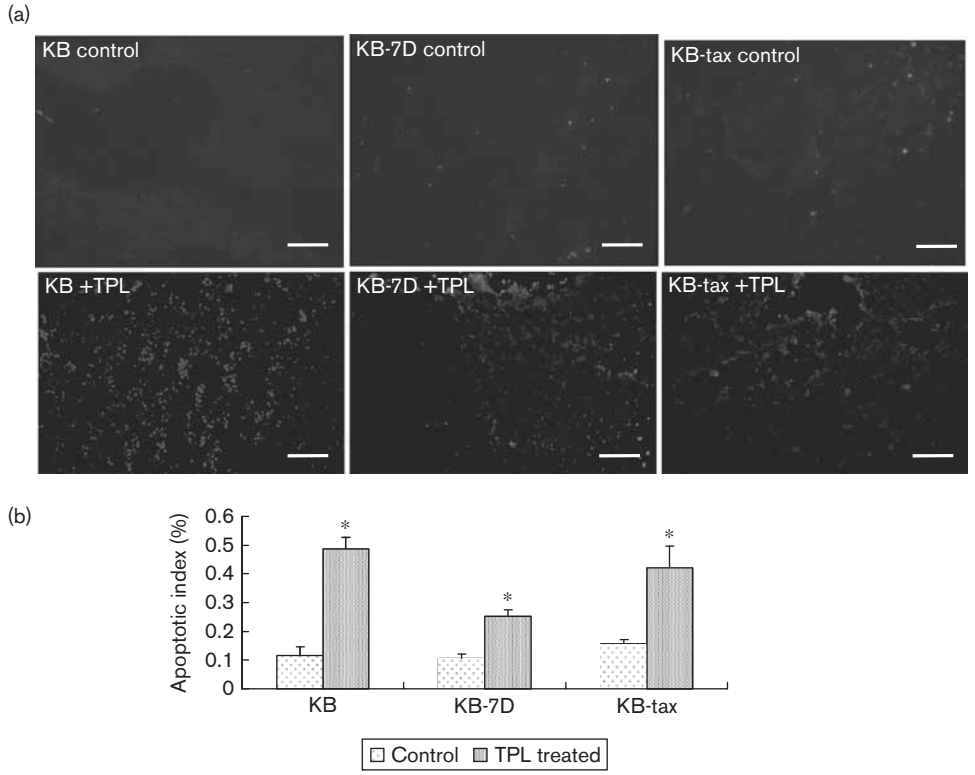
Triptolide (TPL) inhibits drug resistance KB cells growth *in vivo*. (a) Day 3 to day 15, the mice were administered with TPL (0.15 mg/kg) after tumor xenograft transplantation, and this reduced tumor size compared with vehicle. (b) The average of tumor weight was compared between the TPL treated or untreated tumor-bearing mice on day 15 ($n=6$; $^*P<0.01$). Tumor volume of KB (c), KB-7D (d), and KB-tax (e) xenografts treated with TPL *in vivo* or not was measured at indicated times ($n=6$; $^*P<0.01$).

15 in KB, KB-7D, and KB-tax-bearing mice. The tumor size was significantly reduced in the treated mice compared with tumor-bearing control mice that were given vehicle alone (Fig. 6a). KB xenografts reduced in weight by 87.35 ± 5.78, KB-7D by $72.4 \pm 6.15\%$, and KB-tax by $63.3 \pm 5.9\%$ upon TPL treatment (Fig. 6b). The in-vivo growth rates of these cells were recorded, as KB, KB-7D, and KB-tax tumors were palpable in each group of mice on day 6. However, by dynamically measuring tumor volume, TPL clearly showed an inhibitory effect on KB, KB-7D, and KB-tax cancers (Fig. 6c, d, e). These results revealed that TPL is as effective against drug-resistant KB cancer cells *in vivo* as it is to the KB cells.

Induction of tumor cell apoptosis *in vivo*

To directly observe the apoptotic effect of TPL on tumor cells *in vivo*, we performed in-situ TUNEL staining to evaluate the status of apoptosis in xenografts. After 15 days of transplantation, the xenografts were removed,

Fig. 7



Triptolide (TPL) induces apoptosis of drug resistance KB cells *in vivo*. (a) KB, KB-7D, or KB-tax xenograft-bearing mice, which were treated daily with TPL or vehicle *in vivo*, were examined on day 15 for tumor apoptosis incidence using a TUNEL assay observed at ×400 magnifications (scale bars, 100 μm). (b) The apoptotic index (AI) was calculated as the percentage of positive staining cells: AI = number of apoptotic cells/total number of nucleated cells. The apoptotic index indicates the relative value compared with control groups (*$P < 0.05$).

measured for size, and examined by TUNEL staining. The tumors from TPL-treated mice exhibited a markedly higher count of fluorescent green (TUNEL positive) apoptotic cells compared with the control tumors (Fig. 7a). The AI was significantly increased by treatment with TPL compared with the control group (Fig. 7b). Altogether, our results show that TPL reduces the proliferation of drug-resistant cancer cells, KB-7D and KB-tax, and exerts its in-vivo antitumor effect by the induction of apoptosis.

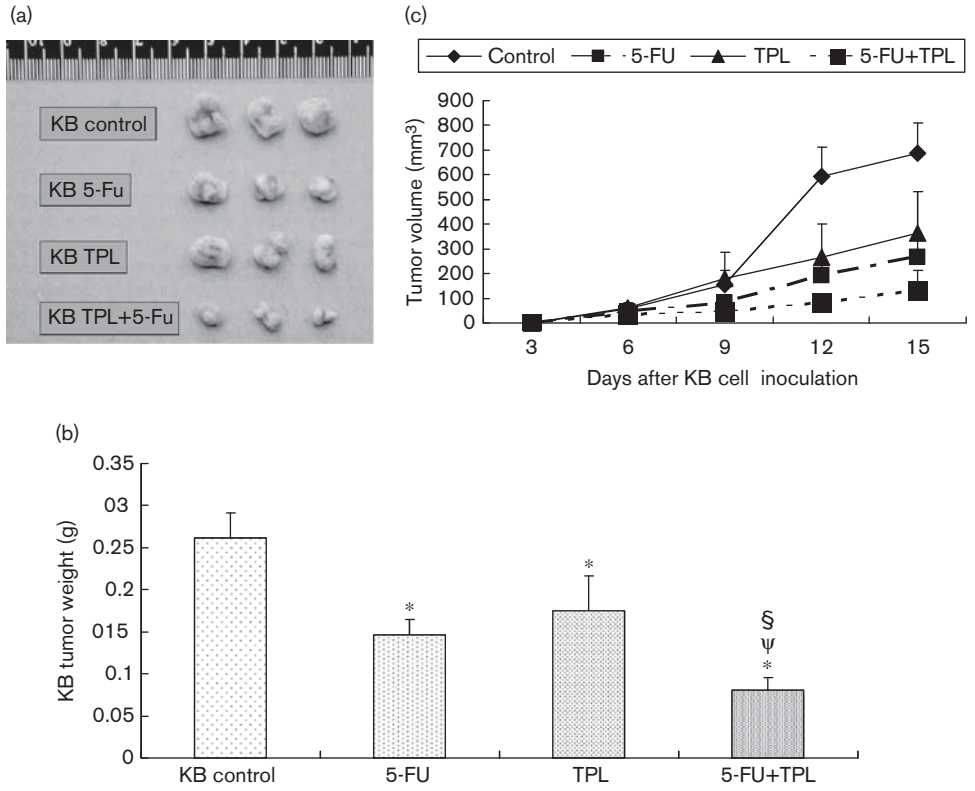
Synergistic effect of TPL combined with 5-FU on drug-resistant cells *in vivo*

We are interested in discovering whether potential therapeutic drugs, such as TPL, can enhance the effect of 5-FU in combined chemotherapy for cancers. To evaluate the therapeutic effects of these two drugs, TPL (0.075 mg/kg) alone, 5-FU (12 mg/kg) alone, and a combination of both, were administered daily into established KB, KB-7D, and KB-tax tumor-bearing mice. After 15 days administration, the xenografts were removed and measured for their size. The results showed that tumor size had been significantly reduced in combined drug-treated group (Fig. 8a, 9a, 10a) compared with treatment with TPL or 5-FU alone. Treatment with TPL or 5-FU alone significantly reduced tumor growth (by weight) in the KB xenograft; however, no profound effects on drug-resistant cancer cells (KB-7D and KB-tax) were shown in the xenograft models. In contrast, TPL combined with 5-FU had a prominent inhibitory effect on tumor growth, as measured by tumor weight (Fig. 8b, 9b, 10b) either on KB, KB-7D, or KB-tax. These results revealed that TPL combined with 5-FU exhibits a synergistic effect *in vivo* against KB cells and drug-resistant cells derived from them. Moreover, by measuring the volume of tumors, it was shown that TPL combined with 5-FU had a significant inhibitory effect on KB cells and the drug-resistant cells derived from them, KB-7D and KB-tax (Fig. 8c, 9c, 10c). These data revealed that TPL combined with 5-FU had a significantly greater anticancer effect than TPL or 5-FU used alone in both KB and drug-resistant KB-7D and KB-tax cancer cells.

Discussion

Chemotherapy is based on cytostatic and cytotoxic agents targeting the cellular mechanisms involved in the control of cell growth and division [13]. Therefore, it is frequently used in conjunction with surgery in such circumstances. One

Fig. 8



Determination of the effect of triptolide (TPL) combined with 5-fluorouracil (5-FU) on KB cells *in vivo*. (a) KB xenograft-bearing NOD/SCID mice were treated daily with TPL (0.075 mg/kg), 5-FU alone (12 mg/kg), or combination of both, and with vehicle in control mice, and the tumors were examined on day 15. (b) The average of tumor weight shown in (a) was compared between TPL, 5-FU treated alone or combination ($n=3$; $^{*}P<0.05$ compared with control, $^{\Psi}P<0.05$ compared with 5-FU; $^{\S}P<0.05$ compared with TPL). (c) Tumor volume of KB xenograft treated *in vivo* with TPL, 5-FU or not was measured at indicated times ($n=3$).

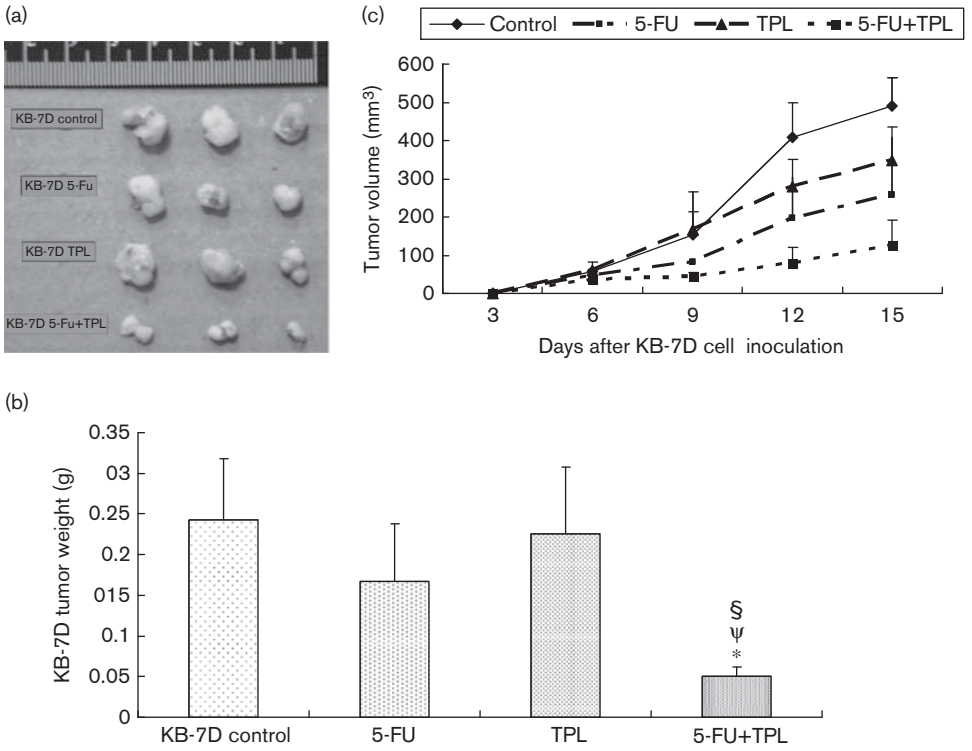
of the major problems encountered during the treatment of tumors with chemotherapeutic agents is that of drug resistance, which results from the expression of transporters that remove drugs from the cytoplasm [21]. Of particular relevance to cancer chemotherapy are the transporters P-gp encoded by the multidrug resistance 1 gene, MRP, and breast cancer resistance protein, which have been proved to play a role in drug-resistance in clinical therapy [21–24].

The component monoterpenoids of the essential oil of *Zanthoxyli fructus*, show strong inhibition of P-gp [25]. TPL is a diterpenoid triepoxide derived from the herb *Tripterygium wilfordii*, and is proposed to have similar molecular structure to the monoterpenoids. Our experimental results reveal that TPL has a growth inhibitory effect on KB-tax (P-gp) and KB-7D (MRP) cells, and their parental drug-sensitive KB cells *in vitro* (Figs 1, 2) and *in vivo* (Fig. 6). TPL treatment resulted in a decrease in expression of MRP and MDR in both KB-7D and KB-tax cells (Fig. 3). These results indicate that TPL-induced cell death of drug-resistant cancer cells is, at least in part, mediated by the downregulation of MDR and MRP genes.

We investigated whether TPL induced apoptosis by activation of caspase-3 (Fig. 4a). Earlier studies have shown that Mcl-1 and XIAP, which are predominantly localized to mitochondria, are involved in antiapoptosis in myeloma cells undergoing drug treatment [26,27]. Our results revealed that both Mcl-1 and XIAP were down-regulated by TPL treatment (Fig. 4b). It is suggested that TPL induces apoptosis by damaging mitochondria.

The toxic effect of TPL in experimental mice is shown on daily administration of a dose higher than 0.75 mg/kg/day into NOD/SCID mice (Fig. 5b). Weakness, molting, severe body weight loss, and death were obvious in our experiment. However, the tolerated dose of less than 0.6 mg/kg/day of TPL i.p. did not appear to adversely affect the mice. There are no apparent signs of sickness and no difference in the body weight loss between mice treated with TPL (0.15 mg/kg/day) and vehicle-treated

Fig. 9



Determination of the effect in triptolide (TPL) combined with 5-fluorouracil (5-FU) on KB-7D cells *in vivo*. (a) KB-7D xenograft-bearing NOD/SCID mice were treated daily with TPL (0.075 mg/kg), 5-FU alone (12 mg/kg) or combination of both as well as solvent control, and the tumors were examined on day 15. (b) The average of tumor weight shown in (a) was compared between TPL, 5-FU treated alone or combination ($n=3$; $^{*}P<0.05$ compared with control, $^{\psi}P<0.05$ compared with 5-FU; $^{\S}P<0.05$ compared with TPL). (c) Tumor volume of KB-7D xenograft treated *in vivo* with TPL and 5-FU or not was measured at indicated times ($n=3$).

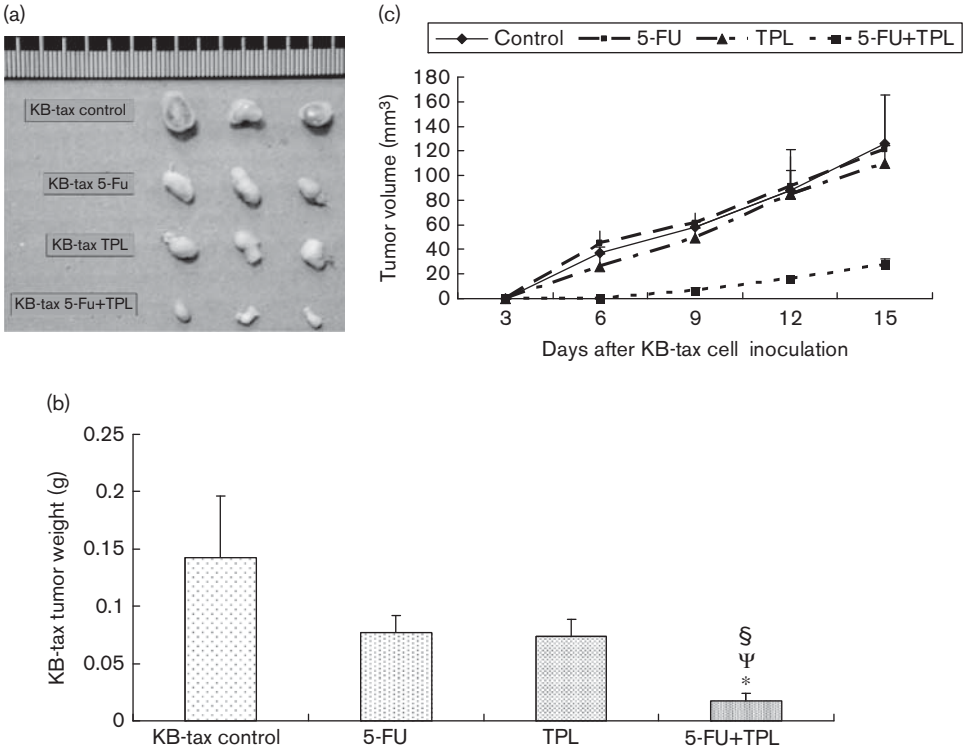
controls in 2 weeks' treatment (Fig. 5a). Daily treatment with TPL is required because of its short half-life [4,12].

In the xenograft tumor-bearing mouse model, the tumors exhibit degrees of susceptibility to TPL treatment that correlated with a growth inhibition assay (Fig. 2). Moreover, we also found that TPL exerts a significant antitumor effect on these drug-resistant cancer cells, at least in part, through apoptosis induction as determined by TUNEL staining of tumor sections (Fig. 7). Our earlier study showed that TPL exhibits an antitumor effect through the induction of apoptosis [28]. In this study, our results imply that induction of apoptosis is also a critical function of TPL in modulation of its antitumor effect on the drug-resistant cancer cells.

Exploring new anticancer drugs combined with 5-FU to counteract resistance transporters and enhancing its cytotoxicity against tumors is imperative. 5-FU is the most frequently used chemotherapeutic agent against cancers. It causes DNA damage during the S phase and in turn affects cell proliferation and survival. However, a proportion of patients have many side effects and have poor responses evoked upon 5-FU treatment. The results of our earlier study [6] indicate that TPL has an antitumor effect through the induction of apoptosis. Moreover, recent studies have shown that TPL even possesses antiangiogenesis properties [29,30]. We tested whether TPL, combined with 5-FU, could exert a synergistic anticancer effect *in vivo*.

Our results further show the advantages of TPL in combination usage with 5-FU. Earlier study has shown that TPL combined with 5-FU had synergistic antiproliferative effects in HT-29 colon cancer cells [31]. We found that TPL alone significantly inhibited the proliferation of KB cancer cells in a dose-dependent manner (Fig. 3b). Combined TPL and 5-FU chemotherapy exerts synergistic activity *in vitro* and significant antitumor activity against these cancer-resistant xenografts (Figs 8, 9, 10). In our experiment, no apparent signs of sickness such as weakness, molt, death, or severe body weight loss between groups treated with TPL (0.075 mg/kg) and 5-FU (12 mg/kg) in combination and vehicle controls were evident after 2 weeks. However, a higher dose of 5-FU

Fig. 10



Determination of the effect of triptolide (TPL) combined with 5-fluorouracil (5-FU) on KB-tax cells *in vivo*. (a) KB-tax xenograft-bearing NOD/SCID mice were treated daily with TPL (0.075 mg/kg) or 5-FU alone (12 mg/kg), or in combination, or vehicle in control mice, and the tumors were examined on day 15. (b) The average of tumor weight shown in (a) was compared between TPL, 5-FU treatment alone or in combination ($n=3$; $^{*}P<0.05$ compared with controls; $^{\Psi}P<0.05$ compared with 5-FU; $^{\S}P<0.05$ compared with TPL). (c) Tumor volume of KB-tax xenograft treated *in vivo* with TPL; 5-FU or not was measured at indicated times ($n=3$).

at 20 mg/kg led to severe body weight loss because of 5-FU-induced cytotoxicity. The two drugs show synergistic effects in tumor growth inhibition at lower concentrations, without causing side effects from chemotherapy.

In conclusion, our study indicates the potential anticancer role of TPL, a compound that is effective in the induction of apoptosis in the drug-resistant cancer cells, KB-7D, and KB-tax. Moreover, our study also showed that TPL possesses the ability to synergistically enhance the cytotoxicity of 5-FU against drug-resistant cancer without obvious side effects.

Acknowledgements

This study was supported by research grant from the National Science Council, Taiwan, ROC (NSC96-2628-B-016-002-MY3, NSC98-3112-B-016-002, and NSC99-3112-B- 016-001 to H.K. Sytwu), Tri-Service General Hospital, Republic of China (Grant No. TSGH-C99-32 and TSGH-C98-12-S01) and in part by the C.Y. Foundation for Advancement of Education, Science and Medicine.

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