

# Triptolide Inhibits the Growth and Metastasis of Solid Tumors<sup>1</sup>

Shanmin Yang, Jinguo Chen, Zhen Guo, Xue-Ming Xu, Luping Wang, Xu-Fang Pei, Jing Yang, Charles B. Underhill, and Lurong Zhang<sup>2</sup>

Department of Oncology, Georgetown University Medical Center, Washington, DC 20007 [S. Y., J. C., X-M. X., L. W., X-F. P., J. Y., C. B. U., L. Z.], and Key Laboratory of China Education Ministry on Cell Biology and Tumor Cell Engineering, Xiamen University, Fujian, People's Republic of China 361003 [S. Y., Z. G., L. Z.]

## Abstract

**Triptolide (TPL), a diterpenoid triepoxide purified from the Chinese herb *Tripterygium wilfordii* Hook F, was tested for its antitumor properties in several model systems. *In vitro*, TPL inhibited the proliferation and colony formation of tumor cells at extremely low concentrations (2–10 ng/ml) and was more potent than Taxol. Likewise, *in vivo*, treatment of mice with TPL for 2–3 weeks inhibited the growth of xenografts formed by four different tumor cell lines (B16 melanoma, MDA-435 breast cancer, TSU bladder cancer, and MGC80-3 gastric carcinoma), indicating that TPL has a broad spectrum of activity against tumors that contain both wild-type and mutant forms of p53. In addition, TPL inhibited experimental metastasis of B16F10 cells to the lungs and spleens of mice. The antitumor effect of TPL was comparable or superior with that of conventional antitumor drugs, such as Adriamycin, mitomycin, and cisplatin. Importantly, tumor cells that were resistant to Taxol attributable to the overexpression of the multidrug resistant gene 1 were still sensitive to the effects of TPL. Studies on cultured tumor cells revealed that TPL induced apoptosis and reduced the expression of several molecules that regulate the cell cycle. Taken together, these results suggest that TPL has several attractive features as a new antitumor agent.**

Received 3/13/02; revised 8/21/02; accepted 11/26/02.

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<sup>1</sup> Supported by the Susan G. Komen Breast Cancer Foundation, National Cancer Institute/NIH (R29 CA71545), and United States Army Medical Research and Materials Command (DAMD17-98-1-8099, DAMD17-00-1-0081, and DAMD17-01-1-0708; to L. Z.). L. Zhang was a recipient of a Visiting Scholar Award from the Key Laboratory of China Education Ministry on Cell Biology and Tumor Cell Engineering, Xiamen University, Fujian, People's Republic of China. The animal protocols were reviewed and approved by the Animal Care and Use Committee of Georgetown University.

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Oncology, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington, DC 20007. Phone: (202) 687-6397; Fax: (202) 687-7505; E-mail: zhangl@georgetown.edu.

## Introduction

TPL<sup>3</sup> is a diterpenoid triepoxide ( $M_r$  360) derived from the herb *Tripterygium wilfordii* that has been used as a natural medicine in China for hundreds of years (1). TPL exerts both anti-inflammatory and antifertility activities through its ability to inhibit the proliferation of both activated monocytes and spermatocytes (2–8).

Several reports have indicated that TPL also inhibits the proliferation of cancer cells *in vitro* and reduces the growth of some tumors *in vivo* (9–12), *e.g.*, Shamon *et al.* (10) have found that TPL can block the growth of human mammary tumor cells in nude mice, and similarly, Tengchaisri *et al.* (11) have reported that it will inhibit the growth of cholangiocarcinoma cells in hamsters. In addition, clinical trials in China have demonstrated that TPL could achieve a total remission rate of 71% in mononucleocytic leukemia and 87% in granulocytic leukemia, which was more effective than any other chemotherapeutic agent currently available (13). Studies on cells grown in tissue culture suggest that TPL may be inducing apoptosis by altering pathways involving the proteins p21 and p53 (9, 12). However, at this point, the exact mechanism by which TPL is able to inhibit tumor cell growth remains unknown. In addition, TPL has not been characterized with regard to its effects on different types of solid tumors.

In this study, we have examined a highly purified preparation of TPL with regard to its activity against a variety of solid tumors and made the following observations: (a) we found that the antitumor effects of TPL were very broad, because it was able to block the growth of four tumor cells with distinct origins and of different p53 status (B16 mouse melanoma, MDA-435 human breast cancer, TSU bladder cancers, and MGC80-3 gastric cancer); (b) we found that the antitumor effects of TPL were comparable with other conventional chemotherapeutic drugs, such as Adriamycin, mitomycin, and cisplatin; (c) we found that TPL was effective against tumor cells that overexpress the MDR1 and are resistant to the effects of other chemotherapeutic compounds, such as Taxol; and (d) we have found that TPL influences the expression of key molecules that regulate apoptosis and cell cycle progression.

## Materials and Methods

**Cell Lines.** B16F10 mouse melanoma cells, MDA-435 human breast carcinoma, and TSU human bladder cancer cells were obtained from the Tumor Bank of the Lombardi Cancer Center at Georgetown University (Washington, DC). MGC80-3 human gastric carcinoma cells were obtained from

<sup>3</sup> The abbreviations used are: TPL, triptolide; MDR1, multidrug resistance 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; HPLC, high-pressure liquid chromatography.

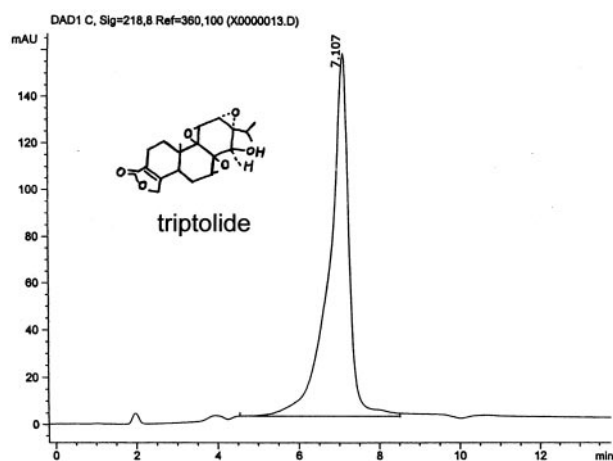


Fig. 1. The purity of TPL as assessed by HPLC. The preparation of TPL was applied to a Hypersil C18 column (reverse phase) and eluted with solution of acetonitrile and water. The single peak indicated that the preparation was >99% pure. The structure of TPL is also shown.

the Cancer Research Center of the Life Science School at Xiamen University (People's Republic of China). The tumor cells were cultured in 10% calf serum and 90% DMEM.

**Source of TPL.** The TPL used in these experiments was obtained from Fujian Institute of Medical Science. The purification was carried out using a modification of the procedure described by Kupchan *et al.* (1). Briefly, the ground roots of *Tripterygium wilfordii* Hook F were extracted with ethanol, evaporated, and partitioned with diethyl ether/ethyl acetate/water. The ester layer was subjected to column chromatography on silica gel and eluted with methanol-chloroform. The eluted fractions were evaporated, and the residue was re-chromatographed on silica gel and eluted with diethyl ether. TPL was obtained from the later-eluting fractions. The purity of the TPL was assessed by HPLC on a Hypersil C18 column using acetonitrile-water (40:60, volume for volume) as the mobile phase (14). As shown in Fig. 1, the TPL eluted as a single peak and on this basis was determined to be >99% pure. The purified TPL was dissolved at a concentration of 1 mg/ml in a mixture of 60% ethanol, 30% DMSO, and 10% phosphate buffer (pH 6.0) as recommended by Mao *et al.* (15).

**Cell Proliferation Assay.** Aliquots of complete medium containing  $1.5 \times 10^4$  cells (MDA-435, B16F10, or TSU cells) were distributed into 96-well tissue culture plates. On the following day, the media were replaced with 190  $\mu$ l of fresh media and 10  $\mu$ l of a solution containing different concentrations of the TPL or Taxol at 100 ng/ml. Three days later, 30  $\mu$ l of 0.3  $\mu$ Ci [ $^3$ H]thymidine in serum-free media were added to each well. After 12 h, the cells were harvested on a glass filter with a 96-well auto-harvester, and the amount of incorporated [ $^3$ H]thymidine was determined with a  $\beta$ -counter.

**Colony Formation Assay.** The viability of the tumor cells was determined by staining with trypan blue followed by visual examination on a hemocytometer. B16F10 cells were suspended at a concentration of  $2 \times 10^4$  cells/ml in 0.36% agarose in 10% calf serum and DMEM containing TPL (0, 2,

or 10 ng/ml) and then immediately placed on top of a layer of 0.6% solid agarose in 10% calf serum and DMEM medium in six-well plates. Two weeks later, the number of colonies >60  $\mu$ m in diameter was determined using an Omnicon Image Analysis System (Imaging Products, Chantilly, VA).

**Effect of TPL on the Growth of Primary Tumors in Mice.** Four tumor cell lines were used to establish primary tumor xenografts. The B16F10 cells ( $5 \times 10^5$  cells/site, 10 mice/group) were injected s.c. into 6-week-old C57BL/6 mice and allowed to grow for 3 days. For studies involving human tumor cells, such as TSU, MDA-435, or MGC80-3, the cells were injected s.c. into the flanks of 5–6-week-old BABL/c nude/nude mice ( $5 \times 10^6$  cells/site, 8 mice/group). After growing for 3 days, the tumor xenografts reached a size of  $\sim 100$  mm $^3$ . Thereafter, TPL (0.15 mg/kg/day) was injected i.p. into the mice on a daily basis. At the end of 2 or 3 weeks, the mice were sacrificed, and the tumor xenografts were removed, photographed, and weighed.

**Effect of TPL on Experimental Metastases in Mice.** To examine the effects of TPL on experimental metastasis, B16F10 cells ( $5 \times 10^4$  cells in serum-free DMEM) were injected into the tail veins of mice (5-week-old C57BL/6, 10 mice/group). After 3 days, TPL (0.15 mg/kg/day) was administered daily to the mice by i.p. injections. Two weeks later, the mice were sacrificed, and the lung and spleen metastases were photographed and counted under a dissecting microscope in a double blind setting.

**Comparison of TPL with Conventional Chemotherapeutic Drugs.** For the *in vitro* comparison of drug potency, MGC80-3 cells were cultured in a 96-well plate and then treated with the following agents: (a) vehicle alone (control); (b) TPL at a concentration of 10 ng/ml (28 nM); (c) Adriamycin at 360 ng/ml (663 nM); (d) mitomycin at 2700 ng/ml (8.1  $\mu$ M); and (e) cisplatin at 2490 ng/ml (8.3  $\mu$ M). Two days later, the viability of the cells was determined by the MTT method according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). For the *in vivo* comparison of drugs, mice with tumor xenografts (100 mm $^3$  in size) were divided into five groups (5 mice/group) and treated as follows: (a) vehicle alone (PBS); (b) TPL at 0.25 mg/kg daily; (c) Adriamycin at 1.2 mg/kg weekly; (d) mitomycin at 1.7 mg/kg weekly; and (e) cisplatin at 7 mg/kg weekly. The doses and injection regimens for these chemotherapeutic reagents were based on reports published previously (16). Three weeks later, the mice were sacrificed, and the tumors were dissected and weighed. The data were expressed as:

$$\text{Inhibition \%} = [1 - (\text{mean weight of tumor tests} / \text{mean weights of tumor controls})] \times 100\%$$

**Effect of TPL on Tumor Cells Overexpressing MDR1.** A pair of MDA-435 cell lines that had been transduced with either a control retrovirus vector or one containing the *MDR1* was kindly provided by Dr. Clarke of the Lombardi Cancer Center (17). These cells were tested for their sensitivities to TPL in both *in vitro* proliferation assays and colony formation assays and *in vivo* tumor growth using the procedures described above.

**Detection of DNA Fragmentation.** The induction of apoptosis in the cultured cells was determined by analysis of DNA fragmentation. For this,  $2 \times 10^5$  TSU cells were grown

to 80% confluence on tissue culture plates and then incubated in the presence and absence of 10 ng/ml TPL for 3 days. The DNA was extracted and subjected to gel electrophoresis according to the methods of Sellins and Cohen (18). The resulting gel was stained with ethidium bromide and photographed under a UV lamp.

**Western Blotting.** Cultures of MDA-435 cells at 80% confluence in 100-mm dishes were treated with TPL (2 or 10 ng/ml) for different lengths of times and then harvested with 1 ml of lysis buffer (1% Triton X-100, 0.5% Na deoxycholate, 0.5  $\mu$ g/ml leupetin, 1 mM EDTA, 1  $\mu$ g/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride). The protein concentration of the lysate was determined by the bicinchoninic acid method (Pierce, Rockford, IL), and 30  $\mu$ g of protein were loaded onto a 10% SDS-PAGE, electrophoresed, and transferred to a nitrocellulose membrane. The loading and transferring of equal amounts of protein were confirmed by staining the membrane with a solution of Ponceau S (Sigma). The membranes were blocked with 5% fat-free milk in PBS (pH 7.4) for 30 min and then incubated overnight with 0.2  $\mu$ g/ml of the different antibodies (Oncogene, Boston, MA). After washing, the membranes were incubated with alkaline phosphatase-labeled secondary antibodies for 1 h, followed by a chemo-luminescent substrate, and exposed to enhanced chemiluminescence Hyperfilm MP (Amersham, Piscataway, NJ).

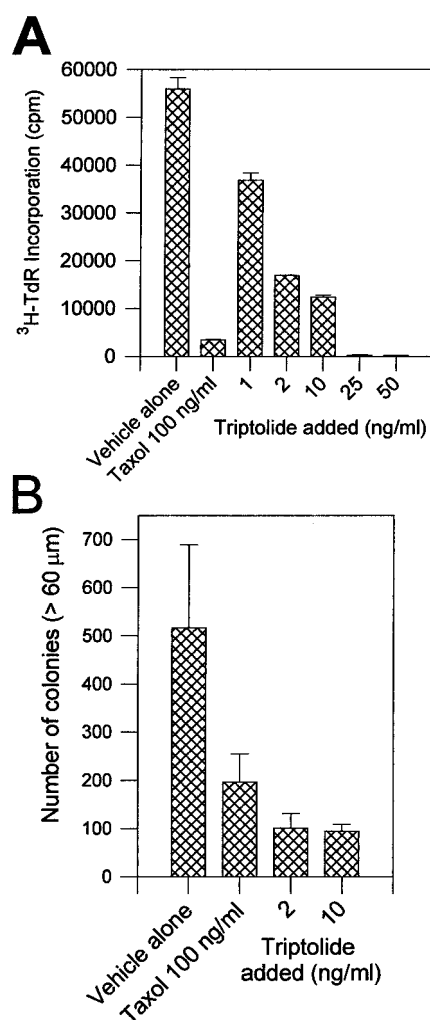
**Statistical Analysis.** The mean and SE were calculated from the raw data and then subjected to Student's *t* test. A *P* < 0.05 was regarded as statistically significant.

## Results

### Effect of TPL on the Proliferation of Tumor Cells *in Vitro*.

The TPL was extracted from the roots of *Tipterygium wilfordii* and purified by chromatography on silica gel. Analysis of the preparation by reverse-phase HPLC revealed a single peak (Fig. 1), indicating that it was  $\geq$ 99% pure. In initial experiments, we examined the effects of different doses of TPL on the proliferation of tumor cells (MDA-435, TSU, or B16) in tissue culture. As shown in Fig. 2A, after 2 days of treatment, the proliferation of the tumor cells was significantly inhibited by TPL in a dose-dependent manner as indicated by [ $^3$ H]thymidine incorporation. This was also reflected in the fact that the treated tumor cells had an unhealthy appearance in that they were round, condensed, and detached as compared with the controls (data not shown). Significantly, the inhibitory effect of TPL at 25 ng/ml (70 nM) was stronger than that of Taxol at 100 ng/ml (117 nM), suggesting that TPL is very potent. The inhibition rate increased in a time-dependent manner, and the maximum effect was observed at days 3–4 after treatment with 2 ng/ml TPL (data not shown).

TPL also influenced colony formation of several types of tumor cells in soft agar, which is one of the best *in vitro* indicators of malignant behavior. As shown in Fig. 2B, treatment of B16F10 tumor cells with TPL (2 or 10 ng/ml) resulted in fewer and smaller colonies than those treated with vehicle control. In addition, Taxol at a concentration of 100 ng/ml resulted in a 60% inhibition rate, whereas TPL at 2 ng/ml had an 80% inhibition rate, again indicating that TPL is very potent.



**Fig. 2.** Effect of TPL on proliferation and colony formation of tumor cells. In A, MDA-435 tumor cells were treated with vehicle alone, 100 ng/ml Taxol, or TPL at the indicated concentrations for 2 days, and then [ $^3$ H]thymidine was added to the cultures for 12 h. The cells were harvested, and the amount of incorporated [ $^3$ H]thymidine was determined with a  $\beta$ -counter. The proliferation of the tumor cells was inhibited by TPL in a dose-dependent manner (*P* < 0.01). Similar results were obtained with B16F10 and TSU cells. In B, B16F10 tumor cells were suspended in 0.36% agarose containing Taxol or TPL at the indicated concentrations. Two weeks later, the colonies >60  $\mu$ m were counted with Omnicon Image Analysis system. The colony formation was inhibited by TPL and Taxol (*P* < 0.01). All of the experiments were repeated three times. Similar results were obtained in other tumor cell lines, such as 4T1 and TSU cells.

It should be noted that several components with similar structure to TPL can also be purified from *Tripterygium wilfordii* Hook, such as epitriptolide, triptonide, and triptoprenolide (1, 19–21). When we compared the antitumor potency of these compounds, we found that only TPL showed significant activity and that the other three components, even at a 1000-fold higher concentration (10  $\mu$ g/ml), could not achieve an inhibition as effective as TPL at 10 ng/ml (data not shown).

**Effect of TPL on Primary Tumor Xenografts.** Next, we examined the effects of TPL on the growth of primary tumor xenografts in mice. In preliminary studies, we found that the

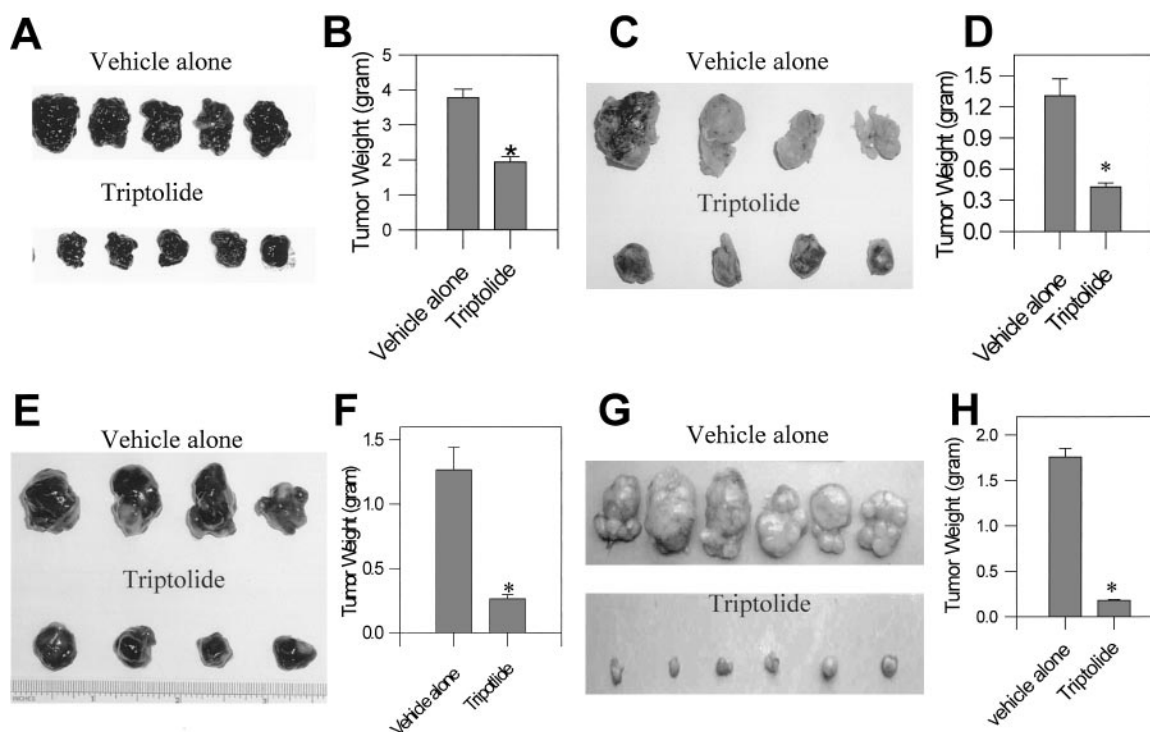


Fig. 3. Effect of TPL on the growth of primary tumors in mice. Mice were injected s.c. with the following tumor cells: (a) B16F10 mouse melanoma (A and B); (b) TSU human bladder (C and D); (c) MDA-435 human breast (E and F); and (d) MGC80-3 human gastric carcinoma (G and H). Beginning 3 days later, the mice were divided into two groups, one of which was given daily i.p. injections of TPL (0.15 mg/kg/day), whereas the other was given vehicle controls. At the end of 2 or 3 weeks, the mice were sacrificed, and the tumors were removed, photographed, and weighed. The growth of all four types of tumors was inhibited by TPL ( $P < 0.01$ ).

maximum tolerated dose for TPL in mice was 0.25 mg/kg administered daily. On this basis, we chose a dose that was 60% of this maximum tolerated dose or 0.15 mg/kg/day, i.p. The injections were given daily because TPL has a short half-life. This regimen did not appear to adversely affect the mice, because there were no obvious signs of sickness after 2 weeks, and there was no difference in the body weights between groups treated with and TPL and the controls (data not shown).

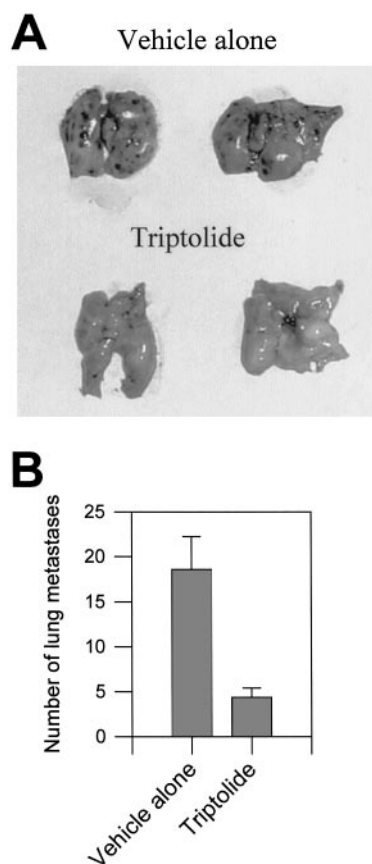
When the TPL (0.15 mg/kg/day, i.p.) was administered to mice with established tumor xenografts, the results were dramatic. Fig. 3 shows that TPL had a significant inhibitory effect on the growth of all four tumor cell lines (B16 by 50%, MDA-435 cells by 80%, TSU by 65%, and MGC80-3 by 90%). These results suggest that TPL is active against a spectrum of different tumor types, although the sensitivity varies depending on the particular tumor.

**Effect of TPL on Experimental Metastases.** In the next series of experiments, we tested the ability of TPL to inhibit experimental lung metastases. For this, B16F10 cells were injected into the tail veins of C57BL/6 mice, which were subsequently treated with TPL (0.15 mg/kg/day for 14 days). The mice were then sacrificed, and the organs were examined to determine the number of tumor nodules. As shown in Fig. 4, the number of lung metastases was dramatically reduced in the group treated with TPL as compared with the vehicle control. The B16 cells also metastasized to the

spleen but at a somewhat lower rate than to the lungs. Here again, the treatment with TPL decreased the level of obvious spleen metastases from 40% in the control group as opposed to 18% in the TPL-treated group (data not shown).

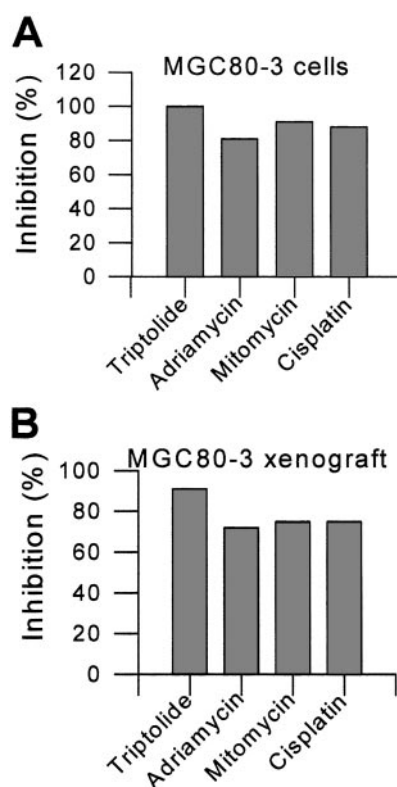
**Comparison of TPL with Other Chemotherapeutic Drugs.** Next, we compared the efficacy of TPL with that of other chemotherapeutic drugs that are in wide use. Initially, we compared the effects of these drugs on the growth of MGC80-3 tumor cells in tissue culture as determined by the MTT method. After 2 days of treatment, TPL at a concentration of 10 ng/ml (28 nM) could achieve an inhibition rate of 98% (Fig. 5A). In contrast, much higher concentrations of the other chemotherapeutic agents were required to achieve an 80–90% inhibition: (a) 360 ng/ml Adriamycin (663 nM); (b) 2700 ng/ml mitomycin (8.1  $\mu$ M); and (c) 2490 ng/ml cisplatin (8.3  $\mu$ M).

Similar effects were obtained with xenografts of MGC80-3 cells growing in nude mice. When such mice were treated with TPL at 0.15 mg/kg daily (i.p.), the tumor size was reduced by 90%, whereas those that were treated with Adriamycin (1.2 mg/kg), mitomycin (1.7 mg/kg), and cisplatin (7 mg/kg) weekly showed a reduction of 75–80%, which was less than the TPL group (Fig. 5B). These results demonstrated that the antitumor effect of TPL was comparable with or even superior to the three conventional chemotherapeutic drugs tested.



**Fig. 4.** Effect of TPL on experimental metastasis. B16F10 cells were injected into the tail veins of mice, and beginning 3 days later, TPL (0.15 mg/kg/day) and control vehicle were administered daily to the mice by i.p. injection. Two weeks later, the mice were sacrificed, and the lungs were dissected free. In **A**, representative lungs show that B16F10 metastases were reduced in mice receiving TPL as compared with vehicle controls. In **B**, the number of lung metastases was counted under a dissecting microscope in a double blind setting and indicated a significant difference between the TPL and control mice ( $P < 0.01$ ). The experiment was repeated three times with similar results.

**Effect of TPL on Cells that Overexpress MDR1.** One of the major problems encountered during the treatment of tumors with chemotherapeutic agents is the emergence of resistance caused by the expression of pumps on the plasma membrane of the tumor cells that remove drugs from the cytoplasm (17). In this experiment, we wanted to determine whether the effects of TPL were influenced by the expression of the MDR1 protein. To test this possibility, we examined a pair of MDA-435 cell lines that had been transduced with either a control retrovirus vector or one containing the MDR1 gene. As shown in Fig. 6A, although the control MDA-435 cells responded to Taxol at 10 ng/ml, this concentration of Taxol did not significantly affect the proliferation of the MDR1-overexpressing cells. However, these drug-resistant cells did show a significant response to TPL at 2 ng/ml. Similarly, TPL also inhibited the ability of MDR1-overexpressing cells to form colonies in soft agar (data not shown) and grow as tumor xenografts in nude mice (Fig. 6B). Thus, both *in vitro* and *in vivo* data strongly suggest that the TPL can circumvent the drug-resistant effects of MDR1.



**Fig. 5.** Comparison of TPL with other chemotherapeutic drugs. In **A**, to compare the drugs *in vitro*, MGC80-3 cells were treated with: (a) vehicle alone (control); (b) TPL (10 ng/ml, 28 nM); (c) Adriamycin (360 ng/ml, 663 nM); (d) mitomycin (2700 ng/ml, 8.1  $\mu$ M); and (e) cisplatin (2490 ng/ml, 8.3  $\mu$ M). Two days later, the viability of the cells was determined by the MTT method. In **B**, to compare the drugs *in vivo*, mice with tumors 100 mm<sup>3</sup> in size were divided into five groups and treated as follows: (a) vehicle alone (PBS); (b) TPL (0.25 mg/kg daily); (c) Adriamycin (1.2 mg/kg weekly); (d) mitomycin (1.7 mg/kg weekly); and (e) cisplatin (7 mg/kg weekly). Three weeks later, the mice were sacrificed, and the tumors were dissected and weighed. The data are expressed as inhibition % =  $(1 - \text{mean of tumor weights of tests} / \text{mean of tumor weights of controls}) \times 100\%$ .

#### Effect of TPL on Apoptosis and Cell Cycle Proteins.

Because TPL inhibits the proliferation of tumor cells, we examined its effects on the induction of apoptosis. As shown in Fig. 7A, treatment of MDA-435 cells with TPL resulted in DNA fragmentation, the most definitive evidence that apoptosis is taking place. Next, we tested for molecules that control apoptosis and the cell cycle. The results of Western blotting revealed that TPL activated two key molecules in the apoptosis pathway, namely caspase 3 and PARP (Fig. 7, B and C). When MDA-435 cells were treated with TPL, there was an increase in the cleaved caspase 3 within 2 days and a shift in PARP from its intact molecule to a subunit (DNA catalytic domain) of  $M_r$  89,000, which peaked on day 4 of treatment. The level of the  $M_r$  89,000 PARP was greater in the TPL-treated group than in the Taxol-treated group, indicating that the antitumor action of TPL was different from the antimicrotubule Taxol. Western blotting analysis also revealed that the treatment with TPL for 3 days caused a significant reduction in *c-myc* and two pairs of cell cycle-promoting protein complexes, cyclin A/cdk2 and cyclin B/cdc2, and

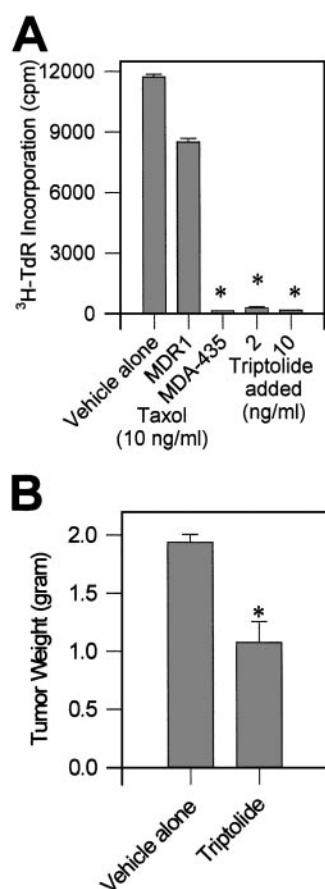


Fig. 6. Effect of TPL on cells that express the MDR1. A pair of MDA-435 cells that had been transfected with either a control retrovirus vector or one containing the MDR1 were tested for their sensitivities to TPL. In A, TPL inhibited the proliferation of MDA-435 cells that express the MDR1 gene as measured by [<sup>3</sup>H]thymidine incorporation. However, these same cells were relatively resistant to Taxol. Similar results were obtained in a colony formation assay. In B, TPL (0.15 mg/kg/day) also inhibited the growth of MDR1 expressing MDA-435 cells in nude mice (see Fig. 3 for methods). Similar results were obtained in two replications.

cyclin D1 as well as the phosphorylated nonfunctional pRb (Fig. 7D). The inhibition of the cell cycle and induction of apoptosis may be responsible for antitumor effects of TPL.

## Discussion

In this study, we have found that TPL has a number of attractive features as an antitumor agent. First, TPL appears to be active against a broad spectrum of tumors. In the mouse tumor model system, we found that the administration of TPL resulted in a 50–90% inhibition of primary tumors derived from the breast, bladder, stomach, and melanomas. In addition, other studies have shown that purified preparations of TPL were effective against cholangiocarcinomas in hamsters (11) and human breast cancer cells growing as xenografts in nude mice (10). Furthermore, TPL also blocked the formation of metastases in experimental mouse models involving the injection of B16 melanoma cells. Thus, TPL can inhibit the growth of both primary and secondary tumors.

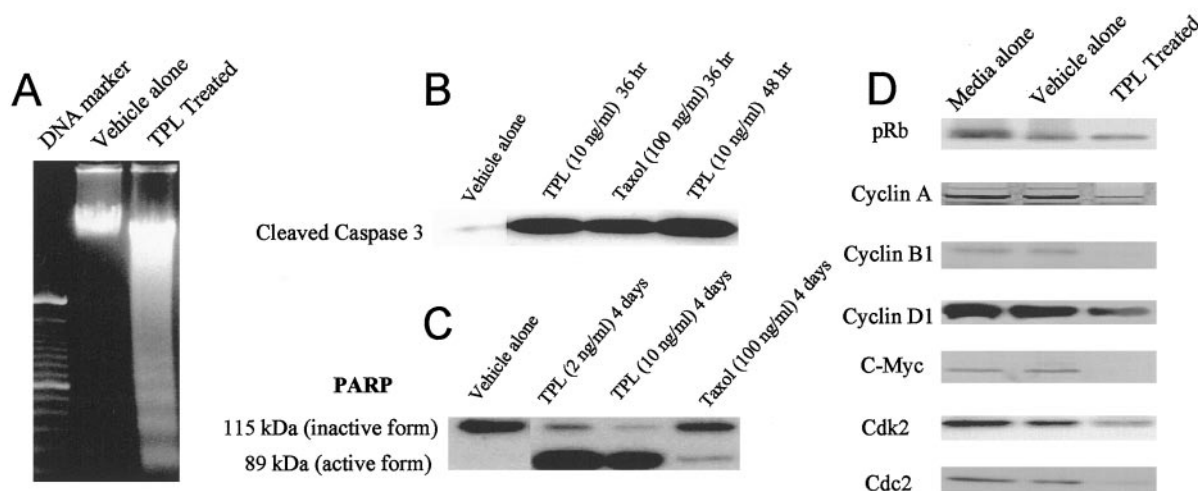
A second feature of TPL is that *in vivo*, it appears to inhibit the growth of tumor cells regardless of their p53 status. We found that in mice, TPL inhibits the growth of tumor cells that possess both wild-type (B16) and mutated forms of p53 (MDA-435 and TSU; Refs. 22–24). This observation was somewhat surprising in view of several previous studies indicating that a functional p53 was required for the inhibitory effects of TPL on the growth of cultured tumor cells (9, 12), *e.g.*, the suppression of p53 levels with antisense oligonucleotides has been reported to abrogate the induction of apoptosis by TPL *in vitro* (12). At present, the explanation for these conflicting results is unclear. Perhaps the *in vitro* results do not apply to the *in vivo* situation, which is far more complex and involves multiple factors, such as vascularization and the immune response. Regardless of the cause, the fact that TPL acts independently of p53 *in vivo* is advantageous, because a high proportion of tumors has p53 mutations or deletions and will still be targeted by TPL.

A third feature of TPL is that its antitumor activity is not adversely effected by the expression of the MDR1 protein, which inhibits the effects of other chemotherapeutic drugs. MDR1 is a transmembrane protein that is able to pump hydrophobic drugs out of the cytosol using an ATP-dependent mechanism. When we tested cells that had been induced to express high levels of MDR1 by an expression vector, these cells were still sensitive to the killing effects of TPL, although they were relatively resistant to the effects of Taxol, another commonly used chemotherapeutic agent. This feature is important because TPL will remain effective against tumors that have developed tolerance against other agents. In this regard, TPL should be considered for use against tumors that have developed tolerance or in combination with other chemotherapeutic drugs.

A fourth attractive feature of TPL is its high potency. When tested against tumor cells growing in tissue culture, TPL was much more effective on a molar basis than other chemotherapeutic agents, such as Taxol, Adriamycin, mitomycin, and cisplatin. Similarly, in the mouse model system, TPL was more potent than Adriamycin, mitomycin, or cisplatin in inhibiting the growth of tumor xenografts. The high potency of TPL greatly simplifies the administration of this drug.

However, TPL does have one major drawback as an antitumor agent, namely its toxicity. Shamon *et al.* (10) have reported that TPL had a modest antitumor effect on breast cancer cells when administered *i.p.* at a dose of 25  $\mu$ g/mouse three times per week. However, when the dose was increased to 50  $\mu$ g/mouse, it was lethal. In our hand, the therapeutic window (the ratio of lethal dose to effective dose) for TPL was about 4 (data not shown) or about twice as high as that reported by Shamon *et al.* This difference in therapeutic window between our results and that of Shamon *et al.* could be attributable to the different regimens that were used for the administration of the TPL. We injected a lower dose of TPL on a daily basis, whereas Shamon *et al.* used a higher dose three times per week.

Our studies have also suggested that TPL affects a number of pathways within the cell that could be responsible for its antitumor activity. First, TPL can induce apoptosis in cultured cells as indicated by DNA fragmentation, and in-



**Fig. 7.** Effect of TPL on apoptosis and cell regulatory molecules. Cultures of tumor cells at 80% confluence were incubated in the presence or absence of TPL (2 or 10 ng/ml) for different lengths of times, harvested, lysed, and analyzed for molecules related to apoptosis and cell cycle. In **A**, TSU cells treated with 10 ng/ml TPL for 3 days show laddering of the DNA indicative of apoptosis. In **B** and **C**, Western blotting of MDA-435 cells with antibodies specific for cleaved caspase 3 and to PARP show that these proteins were up-regulated by treatment with TPL. In **D**, Western blotting of MDA-435 cells shows that TPL treatments caused a reduction in the levels of phosphorylated (nonfunctional) pRb, cyclin A, cyclin B1, cyclin D1, *c-myc*, *cdk2*, and *cdc2*.

creased levels of caspase 3 and the cleaved form of PARP, all of which are markers of apoptosis. These results are consistent with other studies on cultured cells (9, 11, 12). At present, this appears to be the most likely mechanism by which TPL is able to block tumor growth. Although the TPL is probably acting directly on the tumor cells, it is possible that TPL also acts on the endothelial cells that are responsible for tumor vascularization as has been shown to be the case with other chemotherapeutic agents. Secondly, TPL reduces the levels of cell cycle-promoting factors, such as cyclin A/*cdk2*, cyclin B/*cdc2*, cyclin D1, and *c-myc*, as well as the phosphorylated (nonfunctional) form of pRb. These results are in keeping with those of Jiang *et al.* (12) who have shown that TPL arrests cells in the  $G_0$ - $G_1$  phase of the cycle. However, it is difficult to ascertain from this the upstream target of TPL because the reduction in these cell cycle regulators could result in cell apoptosis, and conversely, the apoptosis could reduce the synthesis of cell cycle-promoting factors.

Although TPL is known to activate a number of pathways within the cell, its specific upstream target remains unclear. Indeed, it is possible that TPL may target multiple molecules critical to cell survival. These molecules may, in turn, activate the various pathways that lead to suppression of the cell cycle and induction of apoptosis that inhibits the growth of primary and metastatic tumors. Clearly, future research should be directed toward the identification of the upstream molecules that are directly influenced by TPL.

In conclusion, TPL has very attractive features as an anti-tumor agent with regard to its broad spectrum of activity and potency. Potentially, TPL could be developed into a new antitumor agent.

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*Mol Cancer Ther* 2003;2:65-72.

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