Antiproliferative and Proapoptotic Activities of Triptolide (PG490), a Natural Product Entering Clinical Trials, on Primary Cultures of Human Prostatic Epithelial Cells

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INTRODUCTION

Interest in exploiting traditional medicines for prevention or treatment of cancer is increasing. Extracts from the herb *Tripterygium wilfordii* hook F have been used in China for centuries to treat immune-related disorders. Recently it was reported that triptolide (PG490), a purified compound from *Tripterygium*, possessed antitumor properties and induced apoptosis by p53-independent mechanisms in a variety of malignant cell lines. This property of triptolide attracted our attention because we have found that primary cultures of human prostatic epithelial cells derived from normal tissues and adenocarcinomas are in general extremely resistant to apoptosis. Furthermore, the properties of triptolide and the recent approval of its water-soluble form (PG490-88) for entry into Phase I clinical trials suggested that this drug was a promising candidate to test for antitumor activity against prostate cells. Experiments presented here demonstrated that triptolide had dose-dependent effects on both normal and cancer-derived primary cultures of human prostatic epithelial cells. Low concentrations of triptolide inhibited cell proliferation and induced a senescence-like phenotype. Higher concentrations of triptolide induced apoptosis that was unexpectedly associated with nuclear accumulation of p53. Paradoxically, levels of the p53 target genes, p21<sup>WAF1/CIP1</sup> and hdm-2, were reduced, as was bcl-2. Our preclinical studies suggest that triptolide might be an effective preventative as well as therapeutic agent against prostate cancer and that triptolide may activate a functional p53 pathway in prostate cells.

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sis, we have suggested that dysfunction of p53 may partially explain the resistance of prostate cancer to drug treatment. The prevalence of mutated p53 in advanced prostate cancer (14) may also impact response to chemotherapy. We therefore hypothesize that drugs using p53-independent pathways would be most efficacious against prostate cancer.

In our study, we found that triptolide exerted two qualitatively different types of effects on primary cultures of prostatic epithelial cells. Low concentrations of triptolide inhibited growth and induced senescence, an irreversible growth-arrested state that is associated with tumor suppression. Higher concentrations of triptolide triggered apoptosis. Interestingly, unlike our results with any previously tested compound, triptolide-induced apoptosis of primary cultures was accompanied by nuclear accumulation of p53. This finding was unexpected, given the original reports of p53-independent activity of triptolide, but in fact more recent studies indicate that triptolide-induced apoptosis may be at least partly mediated through activation of p53 in cells with wild-type p53 (6, 15). Down-regulation of p53 target genes hdm-2 and p21\(^{\text{WAF1/CIP1}}\), as well as bcl-2, was also observed in prostatic epithelial cells. Our preclinical findings support further investigation of chemopreventive and chemotherapeutic activity of triptolide against prostate cancer.

MATERIALS AND METHODS

**Cell Culture and Reagents.** Tissue samples were dissected from radical prostatectomy specimens. None of the patients had received prior chemical, hormonal, or radiation therapy. Histological assessment was performed as described previously (16). Epithelial cells were cultured and characterized as described previously (17). Culture medium was MCDB 105 (Sigma-Aldrich, St. Louis, MO) supplemented with 10 ng/ml bovine pituitary extract, 4 \(\mu\)g/ml insulin, 1 \(\mu\)g/ml hydrocortisone, 0.1 mM phosphoethanolamine, 30 nM selenium, 0.03 nM all-trans-retinoic acid, 2.3 \(\mu\)M \(\alpha\)-tocopherol, and 100 \(\mu\)g/ml gentamicin (Complete MCDB 105). The sources and concentration of these supplements were described previously (17). PG490 (97% pure triptolide) was provided by Pharmagenesis (Palo Alto, CA) and stored in a stock solution of 1 mg/ml in DMSO at –20°C.

Four cell strains used in this study were derived from prostatic adenocarcinomas of Gleason grade 3/3 (E-CA-11), 30% intraductal carcinoma/70% Gleason grade 4 (E-CA-12), and Gleason grade 3/4 (E-CA-13 and E-CA-14). An additional cell strain (E-PZ-10) was derived from histologically normal tissue of the peripheral zone. The characteristic traits of primary cultures of human prostatic epithelial cells have been described previously (18). Cells cultured from adenocarcinomas are distinguished from those derived from normal tissues on the basis of cytogenetic abnormalities (19) and differential expression of metabolic enzymes (20, 21).

**Clonal Growth Assay.** Cell cultures at \(\sim\)30% confluence were trypsinized, suspended in medium, and centrifuged. The cell pellet was suspended in buffered saline at a concentration of \(2 \times 10^5\) cells/ml. Five hundred cells in 100 \(\mu\)l of buffered saline were inoculated into each collagen-coated, 60-mm dish containing 5 ml of Complete MCDB 105 with experimental factors. Cells were incubated for 10 days without feeding and then fixed with 10% formalin and stained with crystal violet (17). Growth was quantitated with an Artel image analyzer (Dynatech, Chantilly, VA), which measures the total area of the dish covered by cells. This relative value has been shown to be directly proportional to cell number (22).

**High Density Growth Assay.** Cells were inoculated at \(10^5\) cells/dish into collagen-coated, 60-mm dishes containing Complete MCDB 105. One day later (day 0), various concentrations (1–100 ng/ml) of triptolide were added. Cells treated with diluent (0.001% DMSO) were included as controls. After 3 days, fresh media containing diluent or triptolide were replaced. Cells in replicate dishes were counted by hemocytometer after trypsinization on days 0, 3, and 6.

**Cell Viability.** Loss of cell viability was assessed by the trypan blue exclusion method. Cells treated with or without triptolide were harvested by trypsinization. After incubation in 0.04% trypan blue (Sigma-Aldrich) for 4 min, cells were counted under a hemocytometer. The number of cells that retained the dye (nonviable) and the total cell number were noted.

**Apoptosis.** Cells were inoculated at \(10^5\) cells/dish into collagen-coated, 60-mm dishes containing Complete MCDB 105. One day later, the medium was replaced, and cells were treated with various concentrations (1–100 ng/ml) of triptolide. Cells treated with diluent (0.001% DMSO) were included as controls. After 24, 48, and 72 h, Hoechst 33342 and propidium iodide (Sigma-Aldrich) were added to medium at 10 and 20 \(\mu\)g/ml, respectively. After incubation for 15 min at 37°C, 400 cells from each dish were counted (from 10 randomly selected fields) under fluorescence to determine the proportion of viable and apoptotic cells (23).

**Cell Cycle Analyses.** Semi confluent cell cultures were fed fresh medium containing triptolide (1 or 50 ng/ml). Cells treated with diluent (0.001% DMSO) were included as controls. At 24 and 48 h, cells were harvested by trypsinization, washed with cold PBS, and fixed by dropwise addition of ice-cold 70% ethanol. After 1 h of fixation at 4°C, cells were pelleted and then incubated with RNase A (50 \(\mu\)g/ml; Sigma-Aldrich) and stained with propidium iodide (20 \(\mu\)g/ml). Analyses of DNA content were carried out on a FACScan flow cytometer, and cell cycle phase distribution was quantified using Cellfit software.

**Staining for SA-\(\beta\)-gal.** Forty thousand cells were inoculated into each collagen-coated, 60-mm dish containing 5 ml of Complete MCDB 105. The next day, cells were treated with or without 1 ng/ml triptolide. SA-\(\beta\)-gal-positive cells were detected by the method of Dimri et al. (24) after 3, 5, and 10 days of treatment with triptolide. Cells were fed with fresh media and diluent or triptolide on day 5. The presence of positive staining was observed microscopically and photographed.

**Immunoblot Analyses.** Cells were trypsinized and centrifuged. The cell pellet was washed in ice-cold PBS, resuspended in urea-Tris buffer [9 M urea, 75 mM Tris-HCl, (pH 7.5),
and 0.15 mM 2-mercaptoethanol, and sonicated briefly. The protein concentration was determined by a Bio-Rad assay (Bio-Rad, Hercules, CA). Typically, 50 or 80 μg of protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Osmonics, Westborough, MA), and blocked in PBS with 5% nonfat milk. Proteins were detected with the following antibodies: mouse anti-p53 mAb (DO-1), mouse anti-p21WAF1/CIP1 mAb, rabbit anti-p16INK4a polyclonal antibody, rabbit anti-p27Kip1 polyclonal antibody, mouse anti-bax mAb, and mouse anti-hdm-2 mAb (SMP14; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-bcl-2 mAb (Chemicon, Temecula, CA); mouse anti-GAPDH mAb (Research Diagnostics, Inc., Flanders, NJ); and mouse anti-hdm-2 mAb (2A10; a gift from Dr. Arnold Levine; Princeton University, NJ; Ref. 25). Anti-species hors eradish peroxidase-conjugated secondary antibodies were obtained from Dako (Carpinteria, CA), and visual detection was performed using the enhanced chemiluminescence method (Amersham, Piscataway, NJ).

RESULTS

Effects of Triptolide on Growth of Prostatic Epithelial Cells. Clonal assays were used to test the effect of triptolide on the growth of primary cultures of prostatic epithelial cells derived from normal and malignant tissues. Cells were inoculated at 500 cells/dish into medium with or without triptolide (0.01–1 ng/ml), and clonal growth was evaluated after 10 days of incubation. Five cell strains (one derived from normal peripheral zone tissue and four derived from adenocarcinomas) were assayed. Fig. 1 shows that triptolide was growth inhibitory. Complete growth inhibition of all cell strains occurred with 1 ng/ml triptolide, with half-maximal growth inhibition at ~0.1 ng/ml triptolide.

The clonal growth assay served as a convenient preliminary tool to test the responses of prostatic epithelial cell strains to triptolide. However, because our experimental plans called for the use of higher cell density cultures, and density can affect cellular response, we further tested the effect of triptolide on growth of cells at higher density. Cells were inoculated into culture medium at 10⁵ cells/dish and treated 1 day later with or without triptolide (1–100 ng/ml). Cell numbers were determined on days 3 and 6. Two cell strains, one derived from normal tissue (E-PZ-10) and one derived from a primary adenocarcinoma of the prostate (E-CA-12), were assayed. As shown in Fig. 2A, treatment with triptolide inhibited cell proliferation in a concentration-dependent manner. Triptolide at 1 ng/ml, which completely inhibited clonal growth, was less inhibitory in high density cultures. After 6 days, the number of E-PZ-10 cells treated with 1 ng/ml triptolide was 38% of that of untreated cells. Slightly higher doses of triptolide (10–15 ng/ml) inhibited growth by 65% at day 3 and by ~90% at day 6. The highest...
concentrations of triptolide (50 and 100 ng/ml) caused loss of cells over time. The response of E-CA-12 cells in high density assays to triptolide was comparable with that of E-PZ-10 cells. On day 6, growth was inhibited by 1 ng/ml triptolide to 58% of control. With 50 ng/ml triptolide, the cell number on day 6 declined by 66% from day 0.

E-PZ-10 and E-CA-12 cells in this assay were also incubated with trypsin blue to evaluate the proportion of nonviable cells in treated versus untreated populations. Concentrations of triptolide ≥ 50 ng/ml created >90% nonviable normal cells by day 3 (Fig. 2B). By day 6, >90% of the normal cells treated with triptolide concentrations of ≥5 ng/ml were nonviable. Cell viability declined by 47% after 6 days of treatment with 1 ng/ml triptolide. With E-CA-12 cells, >90% were already nonviable at day 3 with concentrations of ≥10 ng/ml triptolide. Cell viability declined by 78% with 1 ng/ml triptolide at day 6 (data not shown).

**Induction of Apoptosis by Triptolide.** After observing the decline in cell viability caused by triptolide, particularly at the higher concentrations, we evaluated the induction of apoptosis by triptolide (Fig. 3). For that purpose, E-CA-12 cells (incubated at 10^5 cells/dish) were cultured for 24, 48, and 72 h with or without triptolide, and cells with apoptotic morphology were visualized with Hoechst 33342 and propidium iodide. Triptolide at 1 ng/ml did not cause apoptosis during the 3-day period of exposure. After 24 h of exposure to higher concentrations of triptolide (>50 ng/ml), a modest increase in the apoptotic rate was detected (at maximum, 19% apoptosis with 100 ng/ml triptolide compared with 7.5% in control populations). However, at 48–72 h, the number of apoptotic cells in the treated populations increased substantially, especially with 50 and 100 ng/ml triptolide. Despite the preferential effect of triptolide on the decline of E-CA-10 cell viability, we did not see higher apoptotic rates with E-CA-12 compared with E-PZ-10 cells (data not shown). Possibly cells became nonviable by other mechanisms in addition to apoptosis.

**Cell Cycle Distribution with Low and High Concentrations of Triptolide.** Progression of cells through the cell cycle was evaluated by flow cytometric determination of cellular DNA content. Cell cycle analyses were performed on E-PZ-10 and E-CA-12 cells that had been treated with 1 or 50 ng/ml triptolide for 24–72 h (Table 1). The lower concentration of triptolide (1 ng/ml) slightly increased the accumulation of the cells in S phase after 24 h. However, this effect was transient and was not present at 72 h. Triptolide at 50 ng/ml for 24 h increased the proportion of E-PZ-10 cells in S phase from 20.3% in untreated cells to 32.3% in treated cells and increased the proportion of E-CA-12 cells in S phase from 22.8% in untreated cells to 30.5% in treated cells. Whereas the percentage of cells in S phase in untreated populations declined with time, the percentage of cells in S phase in treated populations remained elevated.

**Senescence of Cells Treated with Low Concentrations of Triptolide.** We found that 1 ng/ml triptolide completely inhibited clonal growth of normal and cancer-derived prostatic epithelial cells (Fig. 1). However, inhibition was cell density dependent, and higher concentrations of triptolide were required to completely inhibit the growth of higher density cultures. We also observed that 1 ng/ml triptolide did not induce apoptosis in high density cultures (see previous sections and Fig. 3). We considered the possibility that senescence might be involved in growth inhibition of prostatic epithelial cells by low doses of triptolide. SA-β-gal assays were conducted to address this possibility. Semiconfluent cultures of normal and cancer-derived cells (E-PZ-10 and E-CA-13) were treated with or without 1 ng/ml triptolide for up to 10 days. SA-β-gal activity was found to be minimal in untreated cells throughout the course of the experiment. In contrast, significant SA-β-gal activity, as evidenced by blue cytoplasmic staining, was noted in normal cells after 5 days of triptolide treatment (Fig. 4). SA-β-gal activity became somewhat more pronounced after 10 days (data not shown). Cancer-derived cells exhibited relatively faint levels of

**Table 1 Effects of triptolide on cell cycle distribution**

Subconfluent cultures of E-PZ-10 and E-CA-12 cells were treated with indicated concentrations of triptolide and harvested for cell cycle analysis after 24 and 72 h of treatment with or without triptolide. Each entry indicates the proportion of cells (%) in the respective cell cycle compartments, as determined by flow cytometric analysis after propidium iodide staining.

<table>
<thead>
<tr>
<th>Cell phase</th>
<th>Triptolide (ng/ml) at 24 h</th>
<th>Triptolide (ng/ml) at 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-PZ-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>67.8</td>
<td>73.4</td>
</tr>
<tr>
<td>S phase</td>
<td>20.3</td>
<td>15.4</td>
</tr>
<tr>
<td>G2-M</td>
<td>11.7</td>
<td>10.3</td>
</tr>
<tr>
<td>E-CA-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>59.5</td>
<td>70.5</td>
</tr>
<tr>
<td>S phase</td>
<td>22.8</td>
<td>14.1</td>
</tr>
<tr>
<td>G2-M</td>
<td>17.7</td>
<td>14.5</td>
</tr>
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</table>

Fig. 3 Induction of apoptosis by triptolide. E-CA-12 cells were treated with various concentrations of triptolide (1–100 ng/ml) for 24–72 h. The amount of apoptosis was quantified by Hoechst 33342/propidium iodide staining of nuclear DNA in conjunction with nuclear morphology. Four hundred cells were randomly selected, and the number of cells with normal and abnormal nuclei was noted for each treatment. Data represent the mean (±SE) of two separate experiments.
SA-β-gal after 5 days of treatment with triptolide compared with normal cells. Blue staining was more evident after 10 days of treatment of cancer-derived cells (data not shown).

Modulation of Signaling Pathways by Low Concentration of Triptolide. Triptolide at 1 ng/ml inhibited clonal growth as well as high-density cell proliferation after 6 days of incubation. This concentration of triptolide also induced SA-β-gal, a marker of senescence. We wanted to further investigate the possible molecular pathways associated with the observed inhibition of cell proliferation and induction of senescence by low concentrations of triptolide. Expression of p53 protein and cell cycle regulators p16INK4a, p21\(^{WAF1/CIP1}\), and p27\(^{kip1}\) was investigated in E-CA-12 and E-PZ-10 cells after 3, 5, and 10 days of exposure to 1 ng/ml triptolide. These time points corresponded to those at which expression of SA-β-gal became apparent. None of these proteins were altered in E-CA-12 (Fig. 5) or E-PZ-10 (data not shown) cells by 1 ng/ml triptolide. Protein levels of p53 were also analyzed at earlier time points (6 and 24 h after exposure to triptolide) in E-PZ-10 and E-CA-11 cells. No changes in p53 protein levels were observed at these time points (data not shown).

Modulation of Signaling Pathways by High Concentration of Triptolide. Higher concentrations of triptolide were shown to induce apoptotic cell death. Because p53 may trigger apoptosis, we investigated the expression of p53 in E-CA-12 and E-PZ-10 cells after 6, 24, 48, and 72 h of treatment with 50 ng/ml triptolide. Triptolide increased p53 levels in E-CA-12 cells at 6 h, reaching a maximum after 24 and 48 h (Fig. 6A). Levels of p53 declined toward basal level after 72 h of exposure to triptolide. Similar patterns of p53 expression were observed in E-PZ-10 cells (Fig. 6B, 72 h time point not shown). Immunocytochemical analyses verified the nuclear localization of p53 (data not shown).

The levels of p53 target gene products (hdm-2, p21\(^{WAF1/CIP1}\), bax, and bcl-2) and other p53-independent cell cycle regulators (p16\(^{INK4a}\) and p27\(^{kip1}\)) were also analyzed in immunoblots. Triptolide decreased levels of the intact, \(M_r\) 90,000 hdm-2 protein in E-CA-12 and E-PZ-10 (data not shown) cells significantly after 24 h as detected by the hmd-2-specific 2A10 (Fig. 6A) and SMP14 mAbs (data not shown). Cleaved forms of hdm-2 (\(M_r\) 60,000) detected by the same hdm-2-specific antibodies increased steadily after 24 h of treatment with triptolide in E-CA-12 (Fig. 6A; results with mAb 2A10 against \(M_r\) 60,000 hdm-2 not shown) and E-PZ-10 cells (data not shown).

Triptolide (50 ng/ml) reduced p21\(^{WAF1/CIP1}\) levels in E-PZ-10 cells below the basal expression level of this protein at 24 h (Fig. 6B). The non-p53-regulated cell cycle inhibitor p27\(^{kip1}\) declined in triptolide-treated cells in a manner similar to that of p21\(^{WAF1/CIP1}\) (Fig. 6B). No changes in the non-p53-regulated cell cycle inhibitor p16\(^{INK4a}\) were found after triptolide treatment (Fig. 6B). Triptolide reduced levels of the anti-apoptotic protein bcl-2 after 48–72 h (data for the 72 time point not shown). The intensities of the bcl-2 bands normalized to GAPDH were quantitated by scanning densitometry, and bcl-2 was found to be reduced 5-fold in treated versus untreated cells at 48 h. The decline in bcl-2 levels corresponded with 43% and 79% of cells undergoing apoptotic cell death at 48 and 72 h, respectively, in response to 50 ng/ml triptolide (Figs. 3 and 6B). No change in expression of the p53 target and proapoptotic protein bax was found after triptolide treatment (Fig. 6B). Effects on signaling pathways (p21\(^{WAF1/CIP1}\), p27\(^{kip1}\), p16\(^{INK4a}\), and bax) by triptolide in E-CA-12 cells were similar in E-PZ-10 cells (data not shown). The decline in bcl-2 levels was not so obvious in E-CA-12 cells as compared with E-PZ-10 cells because the basal level of expression of bcl-2 in E-CA-12 cells was relatively low compared with that in E-PZ-10 cells.

DISCUSSION

Prostate cancer is the most common noncutaneous malignancy in American men and was responsible for an estimated 200,000 new cases and over 31,000 deaths in the year 2001 (26). Traditional approaches to localized disease are either radical prostatectomy or radiation therapy. Chemotherapy for advanced prostate cancer is still of limited efficacy, although the development of several promising new modalities is under way (27). Our previous studies on the role of p53 in mediating growth inhibition or apoptosis of prostate cancer cells by DNA damaging-inducing drugs or γ-irradiation led us to suggest that drugs that work through p53-independent mechanisms might be most useful for treating prostate cancer, even those cancers that retain wild-type p53. Previously, investigators reported that triptolide inhibited growth and induced apoptosis of HL-60 (promyelocytic leukemia) cancer cell lines in a p53-independent manner (4). We therefore decided to test the effects of triptolide on primary cultures of prostate epithelial cells to see if this compound would show antitumor activity. A previous study of triptolide and the prostate cancer cell line LNCaP was limited in extent but indicated that triptolide was growth inhibitory, with an ED\(_{50}\) of about 10 ng/ml (3).
Our results revealed that triptolide indeed exhibited many antitumor activities against prostate cells, in an interesting dose-dependent manner. Low concentrations of triptolide (1 ng/ml) inhibited growth completely in clonal assays and partially in high cell density assays. Growth inhibition with 1 ng/ml triptolide was accompanied by the induction of SAβ/H252-gal activity, a widely used surrogate marker of senescence. In addition to expression of SAβ/H252-gal, cells adopted several morphological changes that have been associated with a senescence-like phenotype (28, 29). These changes included an enlarged and flattened shape and the development of vacuoles.

We did not observe any regulation of p53, p21WAF1/CIP1, p27Kip1, or p16INK4a in prostatic epithelial cells in response to 1 ng/ml triptolide, despite the induction of a senescence-like phenotype. In contrast to fibroblasts, prostatic epithelial cells do not have elevated levels of p53 or p21WAF1/CIP1 after undergoing replicative senescence (30, 31). However, increased expression of p16INK4a has been associated with replicative senescence of prostatic epithelial cells (30), which was not the case in the senescent-like cells induced by triptolide. A number of factors have been reported to induce a senescence-like state in cells, but it is not known whether this phenotype is exactly equivalent to replicative senescence (28). The molecular signaling pathway mediating triptolide-induced premature senescence of prostatic epithelial cells remains to be determined but does not appear to involve p53, p21WAF1/CIP1, p27Kip1, or p16INK4a.

In normal cells, irreversible proliferative arrest may result from terminal differentiation or replicative senescence. Phenotypic alterations that resemble replicative senescence can be induced in normal as well as in tumor cells by treatment with different anticancer agents (29), as we saw with triptolide. Induction of accelerated senescence that results in terminal growth arrest is proposed to be a programmed protective response of normal cells to potentially carcinogenic insults (32). The phenomenon of senescence has attracted increasing attention recently and is believed to be relevant to cancer prevention and tumor suppression. The induction of senescence in primary cultures of prostatic epithelial cells suggests that the chemopre-
ventive activity of low doses of triptolide be further evaluated in additional experimental models.

Higher concentrations of triptolide (15–100 ng/ml) effectively inhibited high density cell growth, and 50–100 ng/ml triptolide induced apoptosis in the majority of cells after 3 days of treatment. High doses of triptolide caused a number of molecular changes in prostatic epithelial cells in conjunction with the induction of apoptosis. Interestingly, protein levels of p53 were significantly increased and predominantly accumulated in the nuclei of prostatic epithelial cells. Although the activity of triptolide was originally reported to be p53-independent, two more recent studies showed that triptolide-induced apoptosis may be at least partly mediated through p53 activation in cells with wild-type p53. Chang et al. (6) showed that triptolide-mediated enhancement of chemotherapy-induced apoptosis was accompanied by enhanced translation and accumulation of wild-type p53 protein in HT1080 (fibrosarcoma) cell lines. Jiang et al. (15) demonstrated that p53 expression was up-regulated in AGS (gastric cancer) cell lines after triptolide treatment, and suppression of p53 expression by p53 antisense oligonucleotides significantly abolished triptolide-induced apoptosis. These investigators also found that MKN-28 and SGC-7901 (gastric cancer) cell lines with mutant p53 did not show any significant changes in growth or apoptotic rates after treatment with triptolide. Taken together, these results suggest that triptolide may use multiple signaling pathways, some of which involve p53 in cells with wild-type p53, to arrest growth or induce apoptosis.

It is of interest that up-regulation of p53 downstream target genes (such as hdm-2, p21\(^{WAF1/CIP1}\), or bax) was not seen in conjunction with up-regulation of p53 by triptolide in our primary cultures of prostatic cells. This is in contrast to the up-regulation of these genes that generally accompanies p53-mediated growth arrest and/or apoptosis in response to DNA damage (33). Rather, protein levels of several p53 target genes were reduced after triptolide treatment. For example, our results showed reduction of hdm-2 (the intact, \(M_r 90,000\) hdm-2 protein) after triptolide treatment. Given that hdm-2 targets p53 for degradation (33), the reduction in hdm-2 protein could partially provide an explanation for the observed sustained elevation of p53. Reduction of \(M_r 90,000\) hdm-2 was detected by two antibodies, 2A10 and SMP14. The former antibody does not bind hdm-2 protein that is phosphorylated in the middle portion of the molecule (35). However, the fact that lower levels of hdm-2 were also detected by SMP14, whose reactivity with hdm-2 is not altered by phosphorylation, suggests that total protein was decreased.

As the intact, \(M_r 90,000\) form of hdm-2 protein decreased in response to triptolide, a \(M_r 60,000\) form of hdm-2 accumulated. It has been reported that mdm-2, the mouse homologue of hdm-2, is a substrate for proteases involved in apoptosis that share specificity with CPP32 (caspa-3). These proteases cleave mdm-2 at residue 361, generating a \(M_r 60,000\) fragment (36, 37). More recently, work from the same investigators showed that a distinct caspase activity for hdm-2 was induced by p53 before the onset of apoptosis in H1299 cells expressing a temperature-sensitive human p53 (38). The p53 binding and inhibitory functions of hdm-2 have not been reported to be affected by the cleavage. However, cleaved hdm-2 has been reported to be unable to promote p53 degradation and may function in a dominant negative fashion to stabilize p53 (36, 38). The relationship of cleaved hdm-2 to accumulation of p53 and apoptosis in prostatic epithelial cells in response to triptolide is under investigation.

Another interesting feature associated with the induction of p53 by triptolide in prostatic epithelial cells and in other cells (6) was decreased expression of p21\(^{WAF1/CIP1}\). Reduction of p21\(^{WAF1/CIP1}\) protein levels by triptolide in the HT1080 (fibrosarcoma) cell line was reported to be due to transcriptional inhibition of p21\(^{WAF1/CIP1}\) (6). These same investigators attempted to study the effect of triptolide on p21\(^{WAF1/CIP1}\) expression in p53 wild-type and p53-null mouse embryonic fibroblasts to determine whether decreased expression of p21\(^{WAF1/CIP1}\) was directly mediated by p53. However, basal expression of p21\(^{WAF1/CIP1}\) was almost absent in the p53-null cells, making this experiment impossible. However, they found that triptolide did not affect p21\(^{WAF1/CIP1}\) expression in the p53-mutant HT29 colon cancer cell line, leading them to propose that down-regulation of p21\(^{WAF1/CIP1}\) was a p53-mediated event (6). It has been reported that the p21\(^{WAF1/CIP1}\) protein can antagonize p53-mediated apoptosis because the induction of endogenous p21\(^{WAF1/CIP1}\) or overexpression of a p21\(^{WAF1/CIP1}\) transgene prevented apoptosis (39, 40). Therefore, down-regulation of p21\(^{WAF1/CIP1}\) may contribute to the ability of triptolide to induce apoptosis. However, it must be noted that apoptosis and accumulation of p53 in AGS (gastric cancer) cells in response to triptolide were accompanied by up-regulation of p21\(^{WAF1/CIP1}\) (15). This discrepancy suggests that the mechanism by which p53 induces apoptosis and the role of p21\(^{WAF1/CIP1}\) in this process are dependent on multiple different factors and are cell type specific (23, 41).

Apoptosis promoted by p53 has also been associated with regulation of bcl-2 protein family members, including induction of proapoptotic bax and repression of antiapoptotic bcl-2 (42). A p53 response element has been mapped to the bcl-2 P2 minimal promoter. The exact mechanism of p53-mediated repression is not clear, but it has been proposed that the interaction between p53 and the TATA-binding protein is most likely responsible for the repression of the bcl-2 promoter (43). Our results showed that apoptosis was associated with reduction of antiapoptotic bcl-2 protein in prostatic epithelial cells, whereas protein levels of the proapoptotic protein bax remained unchanged. The increased ratio of bax:bcl-2 may have a role in the induction of apoptosis by triptolide.

Triptolide also reduced expression of p27\(^{kip1}\), which is not a direct target of p53. Reduction in p27\(^{kip1}\) protein can be due to S-phase block and activity of ubiquitin protein ligase p45\(^{SKP2}\) that is known to promote p27\(^{kip1}\) degradation and induction of S phase (44, 45). Therefore, the reduced levels of p27\(^{kip1}\) that we observed may be a consequence of the S-phase arrest induced in prostatic cells by triptolide. Alternatively, reduction of p27\(^{kip1}\) protein levels could reflect caspase activity in conjunction with apoptosis (46).

It is tempting to speculate that p53 plays a role in induction of apoptosis in prostatic epithelial cells after triptolide treatment, but further investigation is required to determine whether up-regulation of p53 is coincidental or instrumental in this process. Certainly p53 is not essential for the activity of triptolide in
prostate cells because regression of the prostate cancer cell line PC-3 with mutated p53 (47) occurred in mice treated with PG490-88 (48). However, it is remarkable that triptolide is one of the few agents that we have found to induce p53 in primary cultures of prostatic epithelial cells. DNA-damaging drugs and γ-irradiation do not induce p53 in these cells, and the signaling pathway used by these agents to stabilize and activate p53 appears nonfunctional in prostatic cells. However, different types of stresses initiate divergent signaling pathways to activate p53 (41), so we may have identified an alternative functional pathway in prostatic epithelial cells that leads to induction of p53. Regulation of expression of the p53 target genes hdm-2, p21WAF1/CIP1, and bcl-2 in conjunction with up-regulation of p53 in prostatic cells suggests but does not prove p53 activity. We must keep in mind that accumulation of nuclear p53 protein, as we noted in prostatic cells treated with triptolide, may not be sufficient to initiate downstream signaling by p53. Generally, p53 must undergo specific posttranslational modifications to enable it to bind DNA and transactivate downstream gene targets (41). On the other hand, certain downstream effects of p53, particularly those leading to apoptosis, are known to occur in the absence of transcriptional activity of p53 (23).

It is of note that accumulation of p53 protein and S-phase arrest, as we observed in prostatic cells treated with triptolide, have been described in other types of cells in which DNA synthesis has been blocked. Agents such as hydroxyurea, which inhibits the activity of ribonucleotide reductase, or aphidicolin, which blocks DNA polymerase α, cause an early S-phase arrest independent of p53. However, p53 protein levels increase in cells treated with these compounds. Upon closer scrutiny, it has been discovered that p53 is functionally impaired, and only some p53 targets are efficiently induced when cells are arrested in S phase (49). The similarities between the effects of triptolide on prostate cells and hydroxyurea or aphidicolin on other types of cells suggest that it would be worthwhile to further investigate the possibility that triptolide blocks DNA replication.

The dose-dependent effects of triptolide that we noted are comparable with those of certain other compounds with antitumor activity. At low concentrations, the microtubule inhibitor paclitaxel was reported to induce mitotic arrest in several types of cancer cells, but at high concentrations, paclitaxel triggered rapid apoptosis (50). Another example is the induction of senescence in normal human fibroblasts by low concentrations of hydrogen peroxide and the induction of apoptosis by high concentrations of hydrogen peroxide (51). The effects of different doses of triptolide on prostatic epithelial cells might be exploited for different applications. The moderate growth-inhibitory activity and induction of senescence by low doses of triptolide might be appropriate for chemopreventive strategies against prostate cancer. The fact that normal as well as cancer-derived cells responded to triptolide is supportive of this possibility. Induction of senescence is especially relevant to slowly proliferating prostate cells that do not respond well to agents that directly target replication. The proapoptotic activity of higher doses of triptolide might be more suitable for chemotherapeutic applications. It is notable that primary cultures of prostatic cancer cells, in contrast to established cell lines, are very resistant to apoptosis. We have identified few agents capable of causing apoptosis in these cells; therefore, if this resistance to cell death is reflective of cancer in vivo, then any compound capable of inducing apoptosis in primary cultures may be particularly worthy of further investigation. If Phase I trials of triptolide that are currently being initiated show safety, as might be anticipated because of the long-time use of the parental herb in traditional medicine, then Phase II/III trials of this drug against prostate cancer should be considered.

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REFERENCES

Preclinical Activity of PG490 on Prostate Cells


Antiproliferative and Proapoptotic Activities of Triptolide (PG490), a Natural Product Entering Clinical Trials, on Primary Cultures of Human Prostatic Epithelial Cells

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