Triptolide and Chemotherapy Cooperate in Tumor Cell Apoptosis

A ROLE FOR THE p53 PATHWAY

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Triptolide (PG490), a diterpene triepoxide, is a potent immunosuppressive agent extracted from the Chinese herb Tripterygium wilfordii. We have previously shown that triptolide blocks NF-κB activation and sensitizes tumor necrosis factor (TNF-α)-resistant tumor cell lines to TNF-α-induced apoptosis. We show here that triptolide enhances chemotherapy-induced apoptosis. In triptolide-treated cells, the expression of p53 increased but the transcriptional function of p53 was inhibited, and we observed a down-regulation of p21<sub>waft/cip1</sub>, a p53-responsive gene. The increase in levels of the p53 protein was mediated by enhanced translation of the p53 protein. Additionally, triptolide induced accumulation of cells in G<sub>2</sub>/M and blocked doxorubicin-mediated accumulation of cells in G<sub>2</sub>M and doxorubicin-mediated induction of p21. Our data suggest that triptolide, by blocking p21-mediated growth arrest, enhances apoptosis in tumor cells.

The p53 tumor suppressor protein plays a critical role in regulating cell cycle checkpoints and apoptosis (reviewed in Ref. 1). Various post-translational modifications and protein-protein interactions stabilize the level of the p53 protein (1). For example, DNA-dependent protein kinase and mutated in ataxia telangiectasia phosphorylate p53 at serine 15 activating p53 following DNA damage, but Mdm2, through association with p53, negatively regulates p53 by translocating p53 from the nucleus to the cytosol and mediating the ubiquitin/proteosome degradation of p53 (2, 3). A less well characterized mechanism is translational regulation of p53 (4). Translational regulation of p53 expression is controlled by a negative autoregulatory feedback (4, 5). The p53 protein interacts with its 3' untranslated region (UTR) by binding to a 330-nucleotide region at the distal end of the 3'-UTR. For example, following γ-irradiation, there is enhanced translation of p53 mRNA without an increase in p53 stability. This is mediated, at least in part, by the increased binding of p53 mRNAs to polysomes (6, 7).

The increase in p53 protein in response to various stresses is an important regulator of cell cycle and apoptosis (1). In transcription-dependent p53 activation, p53 functions as a site-specific transcription factor that induces p53-inducible genes such as p21<sub>waft/cip1</sub>, bax, gadd45, and mdm2. This in turn initiates the program of growth arrest or apoptosis in a stress-specific and/or cell type-specific manner (reviewed in Ref. 8). p53 also acts as a transcriptional repressor by inhibiting the expression of genes such as c-fos, DNA polymerase α, microtubule-associated protein 4, insulin-like growth factor-1 receptor, presenilin-1, RelA, and bel-2 (reviewed in Refs. 8–10). However, the exact mechanism(s) by which p53 mediates apoptosis is still elusive. Also, recent studies suggest that the transactivation function of p53 is not required for p53-dependent apoptosis and that transrepression may play a role in inducing apoptosis (8, 10–12). For example, Bel-2, adenovirus E1B 19K, and the tumor suppressor WT1 inhibit apoptosis and suppress p53-dependent transcriptional repression but not transactivation (10). In addition, inhibition of p53-mediated apoptosis has been demonstrated through overexpression of MAP4 that supports a potential role for p53-dependent transrepression in mediating apoptosis (9).

The p53 gene is inactivated in ~50% of tumors. The effect of p53 inactivation on the response of tumors to chemotherapy or radiation is conflicting with some studies showing enhanced sensitivity and others showing increased resistance to the same compounds. Two recent studies have shown that p53-mediated induction of p21 inhibits the apoptotic response by inducing growth arrest (13, 14). Bunz et al. (13), for example, show that p53- or p21-deficient cells are more sensitive to adriamycin (doxorubicin)-induced apoptosis than wild-type cells because they do not induce p21 in response to doxorubicin. Therefore, a compound that blocks chemotherapy-mediated growth arrest may accelerate and enhance apoptosis.

A crude extract from the Chinese herb, Tripterygium wilfordii, also called T2, has been used as an immunosuppressant for the treatment of inflammatory diseases such as rheumatoid arthritis. One purified component of T2, the diterpene triepoxide triptolide is immunosuppressive and cytotoxic to tumor cells, and a recent study showed that triptolide inhibits cytokine-mediated activation of NF-κB in immune cells (15–17). Recently, our laboratory has shown that triptolide sensitizes tumor cells to TNF-α-induced apoptosis through inhibition of NF-κB (18). We also found that triptolide, alone, induces apo-
potosis in solid tumor cells. To elucidate further the mechanism of triptolide-induced apoptosis, we investigated a potential role for p53 in this pathway.

In this study, we show that triptolide and doxorubicin act in synergy to kill tumor cells. Interestingly, we find that triptolide induces translation and phosphorylation of p53, but triptolide-modified p53 is transcriptionally inactive. Triptolide also blocks doxorubicin-mediated induction of p21 and doxorubicin-mediated growth arrest. Our results suggest that triptolide enhances doxorubicin-mediated apoptosis, at least in part, by blocking p21-mediated growth arrest.

**MATERIALS AND METHODS**

**Reagents**—PG490 (triptolide, MW 360) was obtained from Pharmagenesis (Palo Alto, CA). A549 (non-small cell lung cancer) and HT1080 (fibrosarcoma) cell lines were from ATCC. Mouse embryonic fibroblasts (p53+/+ and p53−/−) cell lines were provided by Dr. Amato J. Giaccia (Stanford University). Doxorubicin, cycloheximide, and the anti-FLAG (M2) antibody were obtained from Sigma. Antibodies for p53, p21, p21vR2/cip2 and protein phosphatase-1 were from Calbiochem.

**Cell Culture and Plasmids**—A549 and HT1080 cells were cultured in the appropriate media with 10% fetal calf serum supplemented with l-glutamine, penicillin, and streptomycin. p53 wild-type (+/+) and null (−/−) mouse embryonic fibroblasts (MEFs) transfected with the EIA/Ras were grown in Dulbecco's modified Eagle's medium containing 15% fetal calf serum supplemented with l-glutamine, penicillin, and streptomycin.

The full-length coding region of human p21 cDNA was amplified by RT-PCR from HT1080 cells with oligo primers 5′-GGATCCGCCACCA-TGTCCGAGAAGGCTTCCTCTTGGAGAAGATCAG-3′ and 5′-GTCGACTCCTTTGCCCTATCGCCTCTTGTTAGTCCTCGAGGGGCTTCCTCTTGGAGAAGATCAG-3′. The 3′ primer was manipulated to add an in-frame FLAG-tag sequence before the stop codon. Subsequently, the cDNA fragments were cloned into the pcDNA, edcysone-inducible vector (Invitrogen, Carlsbad, CA). The full-length sequence of human p21 coding region was confirmed by DNA sequencing. The cDNA of β-galactosidase (LacZ) was cloned into the same vector as a control. Transfection into HT1080 cells was performed with the LipofectAMINE Plus kit (Life Technologies, Inc.) according to the manufacturer's protocol. Briefly, HT-1080 cells at 70% confluence were cotransfected with 1 μg of pcDNA2F1-flag or pcDNA-LacZ-FLAG plus 1 μg of pVgRκ (Invitrogen, Carlsbad, CA) in 6-well plates. After incubation for 3 h at 37 °C, the medium was replaced with fresh media. Then 48 h after transfection, cells were trypsinized and transferred into three 10-cm culture dishes. After overnight culture, stable transfectants were selected by adding 600 μg/ml of zeocin (Invitrogen) and 800 μg/ml G-418 (Life Technologies). The selection was carried on for 2 weeks. Individual clones were isolated and tested for protein expression induced by the addition of 5 μM pantostone A (Invitrogen).

**Cell Viability Assay**—HT-1080 cells were seeded into 6-well plates at 2 × 10⁵ per well the day before the treatment. For the inducible expression of exogenous p21 and LacZ, the stable transfectants were cultured in the presence of 5 μM pantostone A for 16 h. Three fields from each well were carefully selected, marked, and counted to ensure a similar number greater than 300 cells per field before the treatment. The cells were untreated or treated with doxorubicin or triptolide, or the combination of both at indicated dosages for 8 h at 37 °C. Subsequently, the medium was replaced with new media plus 5 μM pantostone A. After 16 h incubation at 37 °C, the number of viable cells within the same fields were determined by trypsin blue exclusion with a 2% trypan blue solution. Cell death was confirmed as apoptotic by annexin V/propidium iodide (PI) staining followed by FACS analysis as described previously (19).

**Northern Blot Analysis**—RNA was prepared from HT1080 cells using RNeasy Mini Kit from Qiagen Inc. (Valencia, CA). cDNAs for Northern blot analysis for p21 and p53 were prepared using RT-PCR with 2 μg of total RNA. The following primer pairs were used: p53, 5′-AGTCGATCGTCGAGATCGGAGGTAACACGAGGAG-3′ and 5′-AGTCGATCGTCGAGATCGGAGGTAACACGAGGAG-3′; 5′-ATGACCTTGCCCACAGCC-3′ and 5′-ATGACCTTGCCCACAGCC-3′. Northern blot analysis was performed as described previously (18).

**Electromobility Shift Assay (EMSA)**—HT1080 cells were treated as described previously. The EMSA was performed as described previously using an end-labeled [32P]p53 consensus binding site (Santa Cruz Biotechnology, Santa Cruz, CA) (18).

**Immunoblotting**—Cells were harvested at the conditions and times indicated and lysed using HENET buffer (50 mM HEPES, pH 7.5, 10 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors mixture (Roche Molecular Biochemicals). 100 μg of protein was loaded on 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed as described previously using a p53 mouse monoclonal antibody from Oncogene Research Products (19). To measure p53 half-life, cycloheximide (30 μg/ml) was added to HT1080 cells 30 min after the addition of triptolide and harvested at the times shown for immunoblot analysis of p53. Immuno blot analysis using other antibodies was performed as described above. The band intensity was measured by NIH Image 1.62.

**Cell Cycle Analysis**—HT1080 cells (2 × 10⁶) were treated with triptolide (20 ng/ml) and/or doxorubicin (100 nM) for 16 h. Cells were then harvested and washed with cold PBS. The cells were resuspended gently in 5 ml of 100% ethanol and fixed at 25 °C for 1 h. After washing with PBS, the cells were incubated with DNase-free RNase A (20 μg/ml) at 37 °C for 1 h and washed with PBS. Propidium iodide (10 μg/ml) was added, and the cells were incubated at 37 °C for 5 min. Cells were separated by sonicating at 20% output level for 15 s using a Virsonic 50 sonicator (Vitis Inc., NY). The samples were then sorted by FACScan, and cell cycle analysis was done with FlowJo (version 3.0.3) (Tree Start, Inc, San Carlos, CA).

**Fluorescent and Labeling of HT1080 Cells**—Cells were grown to 80% confluence before the trypetreatment with triptolide (20 ng/ml) for 6 h in the appropriate media. Cells were washed twice with short term labeling medium (RPMI with 5% dialyzed fetal calf serum supplemented with l-glutamine, penicillin, and streptomycin). To deplete intracellular pools of methionine, short term labeling medium was added for 15 min at 37 °C and then replaced by short term labeling medium containing 0.1 μCi/ml [35S]methionine (Amersham Pharmacia Biotech). Cells were then labeled for 30 min at 37 °C and washed with ice-cold PBS before harvesting for immunoprecipitation. The cells were lysed using RIPA buffer supplemented with protease inhibitors and immunoprecipitated using an agarose-conjugated p53 mAb (Ab-6, Oncogene Research Products) followed by 10% SDS-PAGE. The intensity of labeled p53 protein was measured by NIH Image 1.62.

**In Vivo Orthophosphosphate Labeling**—Subconfluent HT1080 cells were pretreated with 20 ng/ml triptolide for 2 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. These cells were washed twice with 37 °C labeling medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum dialyzed against phosphate-free) lacking sodium phosphate. Cells were then labeled for an additional 1 h with the labeling medium containing 1 mM [32P]orthophosphate and 20 ng/ml triptolide. The labeling medium was removed, and the cells were washed three times with cold Tris-buffered saline (TBS). The cells were scraped in 1 ml of lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15% NaCl, 0.01% sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, and 100 μM aprotinin) and kept on ice for 15 min. Cell lysates were lysates were passed through 27-gauge needles and spun at 11,000 × g for 10 min, and 100 μg of the supernatants were subjected to immunoprecipitation with p53 mAb (Ab-6) conjugated with agarose for 4 h. Samples were then spun at 2500 rpm at 4 °C for 5 min and washed three times with the lysis buffer. The pellets were boiled in SDS sample buffer and analyzed on 10% SDS-PAGE. The bands were visualized and quantified by Optiquant PhosphorImager.

**RESULTS**

**Triptolide Enhances Chemotherapy-induced Apoptosis**—Triptolide is a diterpene triepoxide extracted from a Chinese herb with potent immunosuppressive effects (15–17). We have recently shown that triptolide sensitizes several solid tumor cell lines to TNF-α-induced apoptosis through inhibition of NF-κB. To examine if triptolide also sensitizes tumor cells to chemotherapy, we examined if triptolide enhances cell death induced by doxorubicin, a well-known DNA damaging II inhibitor. After 24 h of doxorubicin (100 nM) treatment, doxorubicin (50 nM) alone did not reduce HT1080 cell viability (Fig. 1). The combination of triptolide (5 ng/ml) plus doxorubicin reduced cell viability by 65% (Fig. 1). Triptolide alone at 20 ng/ml reduced HT1080 cell viability by 84% (Fig. 1). Cytotoxic synergy between triptolide and doxorubicin was also observed in A549 lung cancer cells, and triptolide also enhanced cell death by carboplatinum, an
other topoisomerase II inhibitor, in A549 and HT1080 cells (data not shown). Doxorubicin did not induce NF-kB transcriptional activity in HT1080 cells so that triptolide is not enhancing doxorubicin-mediated apoptosis through inhibition of NF-κB (data not shown).

Tripotolide Induces p53 but Inhibits p21 Expression—p53 mediates cell death responses to cytotoxic stimuli such as hypoxia, irradiation, and DNA-damaging chemotherapeutic agents. Since triptolide alone is cytotoxic and it cooperates with DNA-damaging chemotherapeutic agents, we hypothesized that triptolide-induced apoptosis may be mediated by p53. In HT1080 cells that contain wild-type p53, triptolide (20 ng/ml) increased p53 steady-state protein levels 4-fold for 9 h, and triptolide (5 ng/ml) induced a 2.4-fold increase in p53 (Fig. 2A). Doxorubicin induced a 4.9-fold increase in p53, and the combination of doxorubicin plus triptolide induced a 4-fold increase in p53 protein (Fig. 2A). In A549 cells, the combination of triptolide (20 ng/ml) plus doxorubicin (100 nM) at 24 h showed greater than 5-fold increase in p53 (data not shown). We next examined if the increase in the p53 protein level was due to an increase in the p53 mRNA. The level of p53 mRNA was not affected by triptolide (data not shown). These data suggest, therefore, that triptolide induces post-transcriptional accumulation of p53.

A current model of p53-mediated apoptosis is that upon cellular stresses (such as DNA damage), p53 is stabilized, and this increases expression of genes such as mdm2, bax, p21<sup>kip1</sup>/wdm2, and gadd45. Recent studies show that doxorubicin and γ-irradiation-mediated activation of p53 in p53 wild-type cells induces p21 and causes growth arrest which inhibits apoptosis (13, 14). To determine whether triptolide enhances doxorubicin-mediated apoptosis by blocking p21-mediated growth arrest, we examined the effect of triptolide on p21 expression.

Triptolide (20 ng/ml) reduced basal p21 levels by 50% despite inducing p53 (Fig. 2A). Doxorubicin induced a 14.5-fold increase in p21 that was completely blocked by triptolide (20 ng/ml), and triptolide (5 ng/ml) reduced doxorubicin-mediated induction of p53 by 58%.

We planned to examine the effect of triptolide on p21 expression in p53 wild-type and null MEFs, but p21 basal expression is almost absent in p53<sup>−/−</sup> MEFs, and it is not inducible by doxorubicin (Fig. 2B and data not shown).

Tripotolide Inhibits p21 mRNA Expression—Tripotolide (20 ng/ml) also completely blocked doxorubicin-mediated induction of p21 mRNA (Fig. 3A). Triptolide or doxorubicin did not affect p21 expression in the p53 mutant HT29 colon cancer cell line (data not shown). Triptolide also blocked doxorubicin-mediated induction of mdm2 mRNA, but it did not affect gadd45 or map4 mRNA expression (Fig. 3B). These data show that triptolide alone inhibits transcription of p21, and it blocks doxorubicin-mediated transcriptional induction of p21 despite increasing p53 levels.

Tripotolide does not inhibit DNA binding of p53—Tripotolide induces p53 but inhibits p21 expression. Also, triptolide blocks doxorubicin-mediated induction of p21. We then performed EMSA to determine whether triptolide, alone or in combination with doxorubicin, inhibits DNA binding of p53 to a p53 consensus binding site in the p21 promoter. Triptolide alone slightly enhanced DNA binding of p53, and it did not block doxorubicin-mediated induction of DNA binding (Fig. 4). These data suggest triptolide represses expression of p21 by blocking transactivation but not DNA binding of p53.

Tripotolide Inhibits p21-mediated Growth Arrest—We then evaluated the effect of triptolide alone and in combination with chemotherapy on cell cycle progression. Triptolide (20 ng/ml) alone increased the number of cells in S phase from 23.7% in unstimulated cells to 46% in triptolide-treated cells (Fig. 5). Doxorubicin induced accumulation of cells in G2/M from 11 to

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**Fig. 1.** Tripotolide acts in synergy with doxorubicin. HT1080 cells were treated with doxorubicin (100 nM) and/or triptolide (5 ng/ml or 20 ng/ml) for 8 h and then removed. Cell viability was analyzed at 24 h by trypan blue exclusion. Data represent mean of triplicates from two experiments ± S.D.

**Fig. 2.** Tripotolide induces p53 but inhibits p21 expression. A, subconfluent HT1080 tumor cell lines were treated as shown. After 9 h, total cellular lysate was harvested followed by immunoblot analysis with a p53 mAb. The blot was then stripped and reprobed with a p21 mAb. B, MEFs (p53<sup>+/+</sup> and p53<sup>−/−</sup>) were immunoblotted with a p21 mAb. The gel was scanned, and the p53 bands were measured using a densitometry program. Protein phosphatase-1 (PP1) is used as a loading control.
49.8%, but triptolide inhibited doxorubicin-mediated G2/M accumulation from 49.8 to 22.6% (Fig. 5). A recent study showed that potent inhibition of tumor survival is achieved by combining drugs with different cell cycle checkpoints (20). Li et al. (20) show that β-lapachone inhibits taxol-mediated G2/M arrest which leads to enhanced apoptosis through generation of conflicting signals regarding cell cycle progression versus arrest. Since p21 mediates G2/M arrest in p53 wild-type cells in response to chemotherapy, our data suggest that triptolide inhibits doxorubicin-mediated G2/M arrest by blocking induction of p21.

**Overexpression of p21 Inhibits Cytotoxic Synergy between Triptolide and Doxorubicin**—To determine whether triptolide-mediated inhibition of p21 is involved in the cytotoxic synergy between triptolide and doxorubicin, we overexpressed p21 in HT1080 cells using a ponasterone-inducible p21 vector (pIND-p21). The addition of ponasterone A (5 µM) strongly induced exogenous p21 expression that was slightly reduced by triptolide plus doxorubicin (Fig. 6A). The combination of triptolide (5 ng/ml) and doxorubicin (100 nM) reduced cell viability to 30% in the induced vector control, and viability increased to 58% following the induction of exogenous p21 (Fig. 6B).

**Triptolide Induces Translation of p53**—To determine the mechanism by which triptolide induces p53, we examined the effect of triptolide on p53 protein stability and translation. To examine the effect on stability, we examined levels of p53 in the presence of cycloheximide (30 µg/ml) in HT1080 cells, a dose that blocks translation. In cells that were pretreated with

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**Fig. 3. Triptolide inhibits p21 mRNA expression.** A, total RNA was harvested from HT1080 cells after the indicated treatments followed by Northern blot analysis with a 32P-radiolabeled p21 cDNA probe. B, RT-PCR analysis of p21, mdm2, p53, map4, and gadd45 mRNA expression is shown following treatment with triptolide (20 ng/ml) for the indicated times. mRNA expression is expressed relative to glyceraldehyde phosphate-3-dehydrogenase (GAPDH).

**Fig. 4. Triptolide does not affect DNA binding of p53.** EMSA in HT1080 cells were treated as shown. 100X corresponds to a 100-fold excess of unlabeled p53 oligonucleotide probe, which is a consensus p53-binding site. The * denotes a nonspecific band.
triptolide (20 ng/ml) for 0.5 h prior to the addition of cycloheximide, there was a slight increase in p53 stability at 30 min, but there was no difference from untreated cells at 60 min (Fig. 7A). These data suggested that the increased steady-state level of the p53 protein in response to triptolide did not result from an increase in the half-life of the p53 protein. We then examined if triptolide induces translation of p53 by \textit{in vivo} [35S]methionine metabolic labeling of HT1080 cells. We found, interestingly, that triptolide induced a 4.9-fold increase in p53 translation (Fig. 7B). Thus, triptolide-induced p53 accumulation is mediated by an increase in p53 translation.

**Triptolide Induces Phosphorylation of p53**—Many studies have shown that phosphorylation of p53 in response to DNA damage regulates p53-mediated apoptosis and transcriptional activity (21–23). Therefore, we examined if p53 undergoes hyperphosphorylation upon triptolide treatment in HT1080 cells. There was a 2–3-fold increase in phosphorylated p53 in triptolide-treated cells compared with p53 from the untreated cells (Fig. 8). Western blot analyses of the samples show equivalent levels of immunoprecipitated p53 in both samples (Fig. 8). These data show that triptolide induces phosphorylation of p53 at 3 h that is prior to the triptolide-mediated induction of p53 protein expression.

**DISCUSSION**

We have recently shown that triptolide cooperates with TNF-\(\alpha\) to enhance apoptosis in solid tumor cells (18). We found that triptolide sensitizes tumor cells to TNF-\(\alpha\) by blocking TNF-\(\alpha\)-mediated activation of NF-\(\kappa\)B. Triptolide, however, blocked transactivation but not DNA binding of NF-\(\kappa\)B. Triptolide is an oxygenated diterpene purified from the Chinese herb \textit{T. wilfordii} with anti-inflammatory and tumoricidal properties \textit{in vitro} and \textit{in vivo} (15–17). Triptolide with its three epoxides may act as an electrophile possibly inactivating target molecules through alkylation and reaction with exposed cysteines. We are presently looking for targets of triptolide in tumor cells. Here we show that triptolide enhances chemotherapy-induced cell death in p53 wild-type cells. Triptolide induced p53 protein expression but inhibits basal p21 expression and doxorubicin-mediated induction of p21. We observed that p21 levels are almost undetectable in p53 \(-/-\) cells suggesting that p53 is also required for basal p21 (Fig. 2B). These data likely reflect the inhibitory effect of triptolide on transcriptional activity but not DNA binding of p53 which is analogous to triptolide blocking transactivation and not DNA binding of NF-\(\kappa\)B. Triptolide, however, is not a general transcriptional inhibitor because it does not inhibit growth arrest and DNA damage-inducible (\textit{gadd45}) elongation factor-\(\alpha\) (EF-\(\alpha\)) or glyceraldehyde-3-phosphate dehydrogenase expression (GAPDH) (Fig. 3B). Additionally, we observed that triptolide induces phosphorylation of p53, but this is the first example of a modification of p53 that inhibits p21 expression. We are presently mapping the site(s) of p53 that is phosphorylated by triptolide and that may provide insight into the mechanism of triptolide-mediated transcriptional repression of p21.

A recent study showed that doxorubicin-mediated activation of p53 induces p21 in tumor cells which inhibits apoptosis by inducing growth arrest. Bunz \textit{et al.} (13) show that inhibition of p21-mediated growth arrest sensitizes these tumor cells to doxorubicin-mediated apoptosis (13, 14). We show here that triptolide inhibits p21-mediated accumulation of cells in G2/M and induces accumulation of cells in S phase. Also, in combi-
Role of p53 in Triptolide-induced Apoptosis

Fig. 7. Triptolide induces translation of p53. A, prior to cycloheximide (30 μg/ml) addition, HT1080 cells were pretreated with 20 ng/ml triptolide for 0.5 h. At the times indicated, cells were collected, and total lysate was prepared. 35 S of total protein was used for immunoblot analysis with a p53 antibody. The gel was scanned, and the p53 bands were measured using a densitometry program. The values are an average of three experiments ± S.D. B, in vivo metabolic labeling was performed using [35S]methionine. HT1089 cells were pretreated with 20 ng/ml triptolide for 6 h followed by the addition of 0.1 mCi/ml [35S]methionine for 30 min. Cells were collected and lysed for immunoprecipitation with a p53 mAb and 10% SDS-PAGE.

Fig. 8. Triptolide induces phosphorylation of p53. HT1080 cells were labeled for 3 h with [32P]orthophosphate followed by immunoprecipitation (IP) with an agarose-conjugated p53 mAb. The samples were boiled in SDS sample buffer and analyzed by 10% SDS-PAGE followed by transfer to polyvinylidene difluoride membrane. The bands were visualized and quantified by Optiquant PhosphorImager. Western blot (WB) analysis on the same membrane was then done with a p53 polyclonal antibody.

nation with doxorubicin triptolide enhances apoptosis in p53 wild-type tumor cells, and overexpression of p21 inhibits the cytotoxic synergy between triptolide and doxorubicin. Triptolide, however, does not repress p21 expression in p53 mutant tumor cells, but it does induce accumulation of p53 mutant cells in S phase. A possible explanation for the triptolide-mediated accumulation of cells in late G1/S would be inhibition of cyclin-dependent kinase-2 (cdk-2) activity, but triptolide does not affect cdk-2 activity in HT1080 cells. Therefore, triptolide may directly inhibit cyclins required for G1/S transition such as cyclin A, cyclin D, and cyclin E or block cdk-4 or cdk-6 activity. Li et al. (20), for example, recently showed that cells treated with drugs that activate different cell cycle checkpoints, β-lapachone and taxol, produce conflicting signals that enhance tumor cell apoptosis (20). We show here that not only do triptolide and doxorubicin affect different cell cycle checkpoints but triptolide blocks p21-mediated G1/M arrest which likely enhances apoptosis by blocking growth arrest. Topoisomerase I inhibitors such as β-lapachone and CPT-11 also induce accumulation of cells in S phase in a p53-independent manner. Triptolide, however, does not affect topoisomerase I activity.4 Our data presented here suggest that triptolide enhances doxorubicin-mediated apoptosis, at least in part, by blocking p21-mediated G1/M arrest.

We observed that triptolide induces translation of p53. A recent study demonstrated that p53 translation is inhibited in the presence of increased levels of p53 protein by binding of p53 protein to its corresponding mRNA in the 5'-untranslated region which inhibits further translation of p53 (24). Also, there are unidentified 3'-UTR binding factor(s) that are essential for regulating translation of p53 (24). Thus, we are examining if triptolide directly binds to p53 or modifies factors that regulate translation of p53. We are also in the process of determining if triptolide enhances the redistribution of p53 messages to polysomes as seen in DNA damage induced by γ-irradiation. In addition, a recent study showed that N-acetylcysteine induces apoptosis in tumor cells via a p53-translational-dependent mechanism involving the cellular redox potential (25). Since triptolide shows enhanced cytotoxicity in combination with DNA-damaging agents, it may also interfere with DNA repair. It has been reported (26) that casein kinase II phosphorylates p53 at serine 386 which causes p53-mediated repression. The proline-rich region of the p53 protein has been shown to be important in chromatin remodeling and is required to overcome p53-mediated transcriptional repression (27). The cytotoxic activity of triptolide alone and its ability to cooperate with other cytotoxic agents may represent a novel method to enhance cytolysis of solid tumor cells in vivo. In support of this observation, we have found that PG490-88, a water-soluble derivative of triptolide, cooperates with chemotherapy to cause tumor regression in a tumor xenograft model.5

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