PG490-88, a derivative of triptolide, causes tumor regression and sensitizes tumors to chemotherapy

John M. Fidler,[1] Ke Li,[3] Cathie Chung,[2] Ke Wei,[2] Jessica A. Ross,[2] Mingxing Gao,[2] and Glenn D. Rosen[2]

[1]Pharmagenesis, Inc., Palo Alto, CA; [2]Department of Medicine, Stanford University Medical School, Stanford, CA; and [3]Applied Biosystems, Foster City, CA

Abstract

Treatment of solid tumors with combinations of chemotherapeutic agents has not led to significant increases in long-term survival. Recent studies support a role for inhibitors of checkpoint arrest as a means to enhance the cytotoxicity of chemotherapy. We have shown previously that triptolide (PG490), an oxygenated diterpene derived from a Chinese medicinal plant, induces apoptosis in cultured tumor cells and sensitizes tumor cells to topoisomerase inhibitors by blocking p53-mediated induction of p21. Here we extend our studies to a tumor xenograft model and evaluate the efficacy and safety of PG490-88 (14-succinyl triptolide sodium salt), a water-soluble prodrug of PG490. We also look at the combination of PG490 or PG490-88 with CPT-11, a topoisomerase I inhibitor, in cultured cells and in the tumor xenograft model. We show that PG490-88 is a safe and potent antitumor agent when used alone, causing tumor regression of lung and colon tumor xenografts. We also show that PG490-88 acts in synergy with CPT-11 to cause tumor regression. A phase I trial of PG490-88 for solid tumors began recently and safety and optimal dosing data should accrue within the next 12 months. Our findings that PG490-88 causes tumor regression and that it acts in synergy with DNA-damaging chemotherapeutic agents suggest a role as an antineoplastic agent and chemosensitizer for the treatment of patients with solid tumors. (Mol Cancer Ther. 2003;2:855–862)

Received 2/6/03; revised 5/9/03; accepted 6/17/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Grant support: Pharmagenesis and an ALA Career Investigator Award to G.D.R.

Note: J. M. Fidler, K. Li, and C. Chung contributed equally to the work presented in this manuscript. J. M. Fidler and K. Li contributed to the *in vivo* tumor biology studies and C. Chung contributed to the *in vitro* tumor biology studies.

Requests for reprints: Glenn D. Rosen, Pulmonary and Critical Care Medicine, Stanford University Medical School, 300 Pasteur Drive, Stanford, CA 94305-5236. Phone: (650) 725-9536; Fax: (650) 725-4071. E-mail: grosen@stanford.edu

Introduction

PG490-88 (14-succinyl triptolide sodium salt) is a semisynthetic compound derived from the diterpene triepoxide, triptolide (PG490). PG490 was first isolated and structurally characterized in 1972 (1) when it was extracted from the Chinese medicinal herb, *Tripterygium wilfordii* Hook F (TWHF), a member of the Celastraceae family. Historically, extracts of TWHF have been used for centuries in traditional Chinese medicine but in the 1970s, they were identified as being effective in the treatment of inflammatory/autoimmune disorders such as rheumatoid arthritis (2). Since then, more rigorous attempts were made to better identify biologically active constituents of TWHF responsible for its various clinical properties. We now know, for example, that diterpenoid components of TWHF, especially PG490, exert their anti-inflammatory and immunosuppressant effects by inhibition of cytokine production (*e.g.*, IL-2, IL-4, IFN-γ) by T lymphocytes (3, 4). These effects of PG490 have also been explored in mouse models where it was shown that PG490 prevents graft *versus* host disease (GVHD) (5, 6) and prolongs skin, heart, and kidney allograft survival (7, 8).

The isolation of PG490 has also led to studies supporting its potential development as an antineoplastic agent. Shamon *et al.* (9), for example, showed that PG490 inhibited growth of several human cancer-derived cell lines (including breast, prostate, and lung) grown in culture. PG490 was also shown to induce apoptosis of human promyelocytic leukemia, T-cell lymphoma, and hepatocellular carcinoma cell lines grown in culture (4). Interestingly, the inhibitory effects of PG490 on the growth of tumor cells in culture were enhanced in the presence of other inducers of apoptosis such as tumor necrosis factor-α (TNF-α) (10) and chemotherapeutic agents (11). When combined with chemotherapeutic drugs, PG490 enhanced apoptosis through signaling pathways involving both p53 and p21 (11).

Data on the effects of PG490 on tumor cell growth *in vivo*, however, are limited. Previous reports have shown that PG490 inhibits tumor development in a hamster model of cholangiocarcinoma (12) and in a murine breast cancer model (9). These beneficial effects of PG490, however, were counterbalanced by toxicity that was observed at high doses. In the present studies, we further examined the role of PG490 in inhibition of tumor cell growth both *in vitro* and in a tumor xenograft model. We show that PG490-88, a water-soluble prodrug of PG490, suppresses tumor cell growth *in vivo* without toxicity. We also show that PG490 acts in synergy with chemotherapy. Our results suggest a potential role of PG490-88 alone and in combination with chemotherapy as a novel antineoplastic regimen for the treatment of patients with solid tumors.

Materials and Methods

Reagents

PG490 was used for *in vitro* studies, and a water-soluble prodrug of PG490, PG490-88, was used for the *in vivo* animal studies. Both PG490 and PG490-88 conform to standard preparations by proton nuclear magnetic resonance, were determined by reverse-phase HPLC to be 97–98% pure, and produced single spots on TLC. For *in vivo* studies, stock solutions of PG490-88 (1 mg/ml) were prepared in 0.9% saline and sterilized by microfiltration through 0.2-μm-pore size filters. CPT-11 (Irinotecan, Pharmacia, Piscataway, NJ) was purchased commercially as 100 mg/5 ml vials and diluted in 0.9% NaCl for administration to mice.

Cell Culture and Plasmids

H23 (non-small cell lung carcinoma), HT1080 (fibrosarcoma), and COLO 205 (colon carcinoma) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM with 10% FCS supplemented with L-glutamine, penicillin, and streptomycin. Inducible expression of p21 in HT1080 cells was performed by transfection with a clone of the full-length coding region of human p21 cDNA into the pIND, ecdysone-inducible vector (Invitrogen, Carlsbad, CA), as previously described (9, 11). The p53 wild-type (+/+) and null (−/−) mouse embryonic fibroblasts (MEFs) were a gift from Dr. Amato Giaccia's laboratory (Stanford University) and cultured as described previously (11). The cDNA of β-galactosidase (LacZ) was cloned into the same vector as a control. For the inducible expression of exogenous p21 or LacZ, stable transfectants were cultured in the presence of 5 μM Ponasterone A for 16 h. Cells were then left untreated (control), or were treated with PG490 alone (5 ng/ml for 40 h), CPT-11 alone (10 μM for 36 h), or a combination of PG490 and CPT-11 (pretreated with 5 ng/ml PG490 for 4 h then concurrently with 10 μM CPT-11 for an additional 36 h).

Cell Viability Assays

HT1080 cells were seeded onto six-well plates at 2×10^5 cells/well the day before treatment. For the inducible expression of p21 and LacZ, stable transfectants were cultured in the presence of 5 μM Ponasterone A for 16 h before treatment with PG490 and CPT-11. Three fields from each well were chosen, marked, and counted to ensure similar numbers of cells (greater than 200) per field before initiation of treatment. Cells were then treated as described in the text and cell viability was determined by trypan blue exclusion with a 2% trypan blue solution. Cell death was confirmed as apoptotic by Annexin V staining followed by fluorescence-activated cell sorting (FACS) analysis and DNA laddering.

Immunoblotting

Cells were treated and harvested as indicated and lysed in HNET buffer [50 mM HEPES (pH 7.5), containing 100 mM NaCl, 1 mM EGTA, and 1% Triton X-100] supplemented with 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN). Total cellular protein was then loaded onto 10% SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes. Immunoblotting was performed as previously described (11) using antibodies against p53 (Oncogene Research Products, Cambridge, MA), p21, MDM2, and protein phosphatase-1 (PP1; Calbiochem, La Jolla, CA).

Cell Cycle Analysis

HT1080 cells (2×10^6) and p53 MEFs were treated with PG490 and CPT-11, as described in Tables 1 and 3. Cells were then harvested, washed in PBS, resuspended gently in 5 ml of 100% ethanol, and fixed at 25°C for 1 h. After washing with PBS, cells were incubated with DNase-free RNase A (200 μg/ml) at 37°C for 1 h and washed with PBS. Propidium iodide (PI; 10 μg/ml) was added and the cells were incubated at 37°C for 5 min. Cells were then separated by sonication and analyzed by FACS. Cell cycle analysis was done with FlowJo (version 3.0.3; Tree Start Inc., San Carlos, CA).

Nude Mouse Tumor Xenograft Model

Female NCr nude mice were purchased from Taconic Farms (Germantown, NY) and weighed 20–24 g at the time of experimentation. Mice were maintained in autoclaved filter-top microisolator cages with autoclaved water and sterile food *ad libitum*. Cages were kept in an isolator unit provided with filtered air (Lab Products Inc., Maywood, NJ). Tumor cells used for inoculation were grown in culture and harvested as described above. Mice received i.d. injections of 5×10^6 cells from a human tumor cell line. Tumor sizes determined using micrometer calipers were monitored and mice with similar tumor sizes were grouped together and treated as indicated in the text. Mice that received tumor cell implants were treated with PG490-88 and/or CPT-11 when tumors reached a volume of approximately 100 mm^3. Mice were weighed 3–5 times/week and were inspected daily for possible signs of toxicity.

Results

PG490 Cooperates with CPT-11 to Reduce Cell Viability

We have previously shown that PG490 acts in synergy with the topoisomerase II inhibitor, doxorubicin, to induce apoptosis of cultured HT1080 cells (11). We showed that PG490 sensitized HT1080 cells to doxorubicin, at least in part, by inhibiting doxorubicin-mediated up-regulation of $p21^{cip1/waf1}$, one of several downstream proteins normally under p53 control that causes cell cycle arrest. Our data, like those of Bunz *et al.* and others, suggest that blocking chemotherapy-induced growth arrest enhances apoptosis (13–15). In the present studies, we examined if PG490 also cooperates with the topoisomerase I inhibitor, CPT-11, to reduce cell viability in culture and to examine its efficacy and safety in a tumor xenograft model. At 24 h, PG490 (5 ng/ml) and CPT-11 alone reduced viability of HT1080 cells by 10–15% and the combination of PG490 and CPT-11 reduced cell viability by 70% (Fig. 1). The reduction in viability with PG490 alone and in combination with CPT-11 was shown to be apoptotic by Annexin V staining followed by FACS analysis and DNA laddering (data not shown). At 24 h, therefore, the cytotoxicity of PG490 plus CPT-11 is

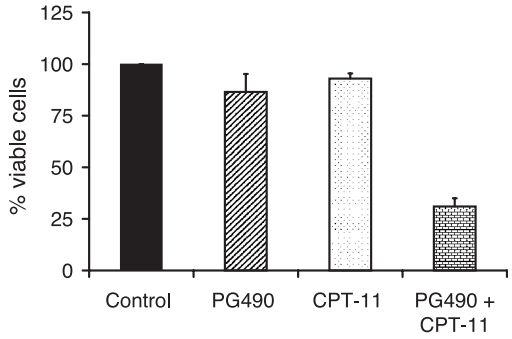

Figure 1. Synergy between PG490 and CPT-11. HT1080 cells were treated for 24 h with PG490 (5 ng/ml) or CPT-11 (10 μM) alone or in combination. The percentage of viable cells was determined by trypan blue exclusion. *Columns,* average of three experiments; *bars,* SD.

synergistic. We saw similar synergy between PG490 and other DNA-damaging agents such as *cis*-platinum, but PG490 did not sensitize tumor cells to radiation (data not shown). Also, the combination of PG490 and taxol produced additive but not synergistic cytotoxicity.[1]

PG490 Induces p53 but Inhibits Expression of p21

We showed previously that PG490 induces p53 but blocks doxorubicin-mediated induction of p21 (11). Here we examined the effect of PG490 on CPT-11-mediated p53 signal transduction in HT1080 cells. We found that both PG490 and CPT-11 induced p53 by 2-fold and the combination of PG490 and CPT-11 induced p53 3-fold (Fig. 2). CPT-11 induced a 2-fold increase in MDM2 and p21, both of which are also downstream of p53 transcriptional activation. However, PG490 reduced p21 basal levels to 60% of unstimulated levels, and did not induce increased MDM2 levels (Fig. 2). Also, the combination of PG490 and CPT-11 reduced both MDM2 and p21 levels to 40% of unstimulated levels (Fig. 2). We showed previously that PG490 down-regulates doxorubicin-induced p21 mRNA and we observed that PG490 also down-regulates CPT-11-mediated induction of p21 mRNA, suggesting that PG490 inhibits transcription of p21 in response to topoisomerase inhibitors (9, 11 and data not shown). PG490 did not, however, affect endogenous or chemotherapy-induced Bax levels.[2] Moreover, higher doses of PG490 (20 ng/ml) further induced p53 but suppressed basal and CPT-11-induced levels of p21 (data not shown). These data show that PG490 blocks induction of some p53-downstream genes despite inducing p53.

PG490 Inhibits Cell Cycle Arrest Induced by CPT-11

To further characterize the cooperativity between PG490 and CPT-11, we evaluated the effects of these agents on cell cycle progression in HT1080 cells. Treatment of cells with PG490 (5 ng/ml) alone increased the number of cells in S phase from 15% to 22% and treatment of cells with CPT-11 (10 μM) increased the

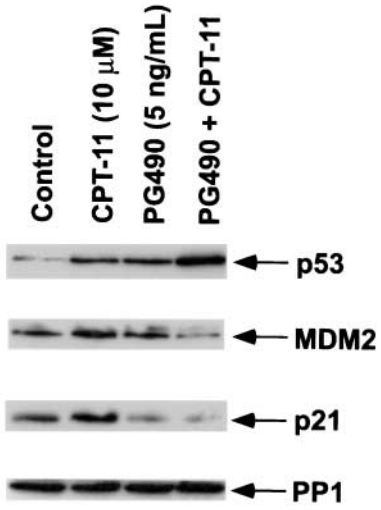

Figure 2. PG490 induces p53 but inhibits p21 and MDM2 expression. HT1080 cells were treated for 24 h as shown and cellular extract was harvested for Western blot analysis with antibodies recognizing p53, p21, MDM2, and PP1. PP1 is used as a loading control.

number of S-phase cells from 15% to 43% (Table 1). CPT-11 increased the number of cells in G_2-M from 9% to 21% (Table 1). PG490, however, inhibited both S- and G_2-M-phase accumulation induced by CPT-11, reducing the number of S-phase cells from 43% to 23% and G_2-M cells from 21% to 17% (Table 1). Because CPT-11-mediated arrest of cells in S and G_2-M is mediated by p21, our results suggest that PG490 blocks CPT-11-mediated growth arrest by blocking induction of p21.

To determine if these effects were mediated by p53, we examined whether PG490 inhibits CPT-11 arrest in S or G_2-M in wild-type and null p53 MEFs. We found that PG490 alone induces accumulation of p53 +/+ MEFs in S and G_2-M and that CPT-11 induces accumulation of these MEFs in G_2-M (Table 2). However, CPT-11-treated MEFs in G_2-M decreased from 71.6% to 41.3% in the presence of PG490 (Table 3). In p53 −/− MEFs, more untreated cells were in S phase consistent with the loss of the p53 cell cycle checkpoint and CPT-11 or PG490 alone still caused accumulation of cells in G_2-M (Table 2). However, PG490

Table 1. PG490 inhibits CPT-11-induced S and G_2-M arrest

Treatment	Cell Cycle Distribution (%)		
	G_1	S	G_2-M
Control	74.9	15.4	8.7
CPT-11 (10 μM)	35.1	43.0	21.1
PG490 (5 ng/ml)	65.1	21.7	12.4
CPT-11 + PG490	61.2	22.9	16.8

Note: HT1080 cells were treated as shown and harvested 16 h later for cell cycle analysis by PI staining and data analysis with the FlowJo program (Stanford FACS facility). Data shown are from a single representative experiment. The analysis was repeated three times with similar results.

[1] K. Wei and G. D. Rosen, unpublished results.
[2] C. Chung and G. D. Rosen, unpublished results.

Table 2. PG490 requires p53 to inhibit CPT-11-induced G_2-M arrest

	Cell Cycle Distribution (%)					
	MEFs (p53 +/+)			MEFs (p53 −/−)		
Treatment	G_1	S	G_2-M	G_1	S	G_2-M
Control	50.1	18.8	34.1	24.8	45.2	31.1
CPT-11 (10 μM)	6.76	22.5	71.6	2.35	33.6	61.6
PG490 (5 ng/ml)	15.6	34.2	54.3	10.7	40.6	49.0
CPT-11 + PG490	33.4	24.5	41.3	3.01	36.8	58.9

Note: MEF p53 knockout cells were treated as shown and harvested 8 h later for cell cycle analysis by PI staining and cell cycle analysis with the FlowJo program (Stanford FACS Facility). Data shown are from a single representative experiment. The analysis was repeated twice with similar results.

did not inhibit CPT-11-mediated accumulation of cells in G_2-M. A recent article showed that CPT-11 triggering of G_2-M arrest is p53 independent but maintenance of G_2-M arrest requires p53 (14, 16). Therefore, our data suggest that PG490 inhibits the maintenance of CPT-11-mediated G_2-M arrest.

Inducible Expression of p21 Blocks the Effects of PG490

To examine whether the synergistic effects of PG490 and CPT-11 on HT1080 cells also involve p53- and p21-mediated signaling pathways, we introduced an inducible p21 vector (pIND-p21) into HT1080 cells. We showed previously that p21 is induced by ecdysone in these cells (11). PG490 (5 ng/ml) alone reduced cell viability by 14–15% after 48 h (Table 2). In cells transfected with a control vector [pIND-LacZ, pIND-LacZ(I)] or in uninduced cells transfected with the p21 vector (pIND-p21), CPT-11 (10 μM) reduced viability by 44–50% and cell viability, in the presence of CPT-11, increased to 61% following induction of p21 [pIND-p21(I)]. HT1080 cell viability was reduced by 85% in vector-transfected cells with both PG490 and CPT-11. In the presence of PG490 plus CPT-11, the induction of p21 increased HT1080 cell viability from 14% to 45% (Table 3). These data support our previous finding with doxorubicin that PG490 sensitizes tumor cells to DNA-damaging chemotherapeutic agents by blocking induction of p21 (11).

PG490-88 Induces Regression of Tumor Xenografts

To explore the potential of PG490 as a novel therapeutic agent to treat solid tumors, we examined the effects of PG490-88 in the nude mouse human tumor xenograft model with tumors derived from H23, HT1080, or COLO 205 human tumor cell lines.

H23 tumor cells were implanted i.d. in nude mice. When the tumors reached approximately 100 mm^3, daily i.p. treatment with PG490-88 was initiated. PG490-88 inhibited tumor growth in a dose-dependent manner and induced tumor regression at both the 0.5 and 0.75 mg kg^{-1} day^{-1} dosages (Fig. 3). By day 15, mean tumor volume increased over 8-fold with saline but decreased by 87% with the 0.75 mg/kg dose of PG490-88. PG490-88 at 0.25 mg/kg markedly decreased tumor growth and the 0.5 and 0.75 mg/kg doses caused profound tumor regression, while the higher dose eradicated the tumor in two of five animals. No change in weight, skin color, appetite, or respiratory rate was observed at these dosages (data not shown).

PG490-88 and CPT-11 Act in Synergy Causing Tumor Regression in HT1080 Xenografts

As shown in Fig. 4, both PG490-88 (0.75 mg/kg i.p., days 0–5 and 7–11) and CPT-11 (11 mg/kg i.v., days 1, 5, and 9) as single agents inhibited tumor growth even after treatment was stopped but tumor regression was incomplete. In mice treated concurrently with PG490-88 and CPT-11, however, tumors started to regress by day 7 of treatment and showed complete regression by day 20 in all four mice (Fig. 4), even though day 11 was the last day of dosing. Mice treated with PG490-88 and CPT-11 were followed for a year after cessation of treatment and no tumors recurred. These results are consistent with a synergistic antitumor effect of the combination of PG490-88 and CPT-11.

Regression of COLO 205 Tumors by PG490-88 and Cooperation between PG490-88 and CPT-11

Although our cell culture and tumor xenograft studies showed cooperativity between PG490 and CPT-11 in HT1080 cells, the clinical use of CPT-11 as first-line therapy for the treatment of metastatic colorectal cancer led us to investigate the COLO 205 cell line in our *in vivo* human tumor xenograft model. Mice bearing COLO 205 tumors were treated with two different dosages of PG490-88 (0.25 mg/kg i.p. 5 days/week and 0.75 mg/kg

Table 3. p21 inhibits synergy between PG490 and CPT-11

	Percentage of Viable Cells			
Treatment	pIND-LacZ	pIND-LacZ(I)	pIND-p21	pIND-p21(I)
Control	100 ± 1.9	100 ± 1.4	100 ± 1.7	100 ± 0.3
PG490 (5 ng/ml)	86 ± 2.1	85.4 ± 7.1	82.8 ± 3.5	91.7 ± 4.2
CPT-11 (10 μM)	53.3 ± 0.6	56.3 ± 4.0	42.7 ± 1.9	60.9 ± 3.4
PG490 + CPT-11	14.3 ± 0.1	16.5 ± 0.2	13.8 ± 0.2	45.5 ± 2.0

Note: HT1080 cells were treated for 48 h as shown followed by analysis of cell viability by trypan blue exclusion. pIND-LacZ (vector control, uninduced); pIND-LacZ(I) (vector control, induced); pIND-p21 (p21 expression vector, uninduced); pIND-p21(I) (p21 expression vector, induced). Data are average of two experiments ± SD.

i.p. 5 days/week, for 3 weeks) alone and in combination with CPT-11 (22 mg/kg i.v. twice a week for 3 weeks). PG490-88 alone caused dose-dependent inhibition of COLO 205 tumor growth at 0.25 mg/kg and tumor regression at 0.75 mg/kg (Fig. 5). CPT-11 (22 mg/kg i.v. twice a week) inhibited COLO 205 tumor growth but tumors did not regress (Fig. 5). The results of combined CPT-11 and PG490-88 at 0.75 mg/kg were similar to treatment with PG490-88 0.75 mg/kg alone, and the combination of CPT-11 and low-dose PG490-88 (0.25 mg/kg) caused 49% tumor regression (Fig. 5). Although the tumors in several dose groups were greatly reduced in size, all tumors were palpable and none completely regressed.

PG490-88-Treated Tumors Continue to Regress after Suspension of Dosing

To determine whether continuous dosing of PG490-88 was necessary to maintain tumor cell regression in COLO 205 xenografts, dosing of the mice described above with both PG490-88 and CPT-11 was suspended after 21 days and mice were then followed for an additional 35 days. After CPT-11 dosing was stopped at day 21, tumors rapidly regrew from day 28 to 56 (Fig. 6). Mice treated with the lower dose of PG490-88 alone also demonstrated regrowth of tumors after cessation of treatment, although at a slower rate compared to animals treated with CPT-11 alone (Fig. 6). Mice receiving the higher, 0.75 mg/kg dose of PG490-88 alone, however, continued to show tumor regression for 3 weeks after the cessation of treatment after which time, tumors started to regrow (Fig. 6). Similarly, in mice treated with both PG490-88 (0.25 mg/kg i.p. 5 times/week) and CPT-11, tumors continued to show regression for 3 weeks after suspension of treatment before regrowth (Fig. 6). These data suggest that unlike CPT-11, continuous dosing of PG490-88 is not required to achieve therapeutic results.

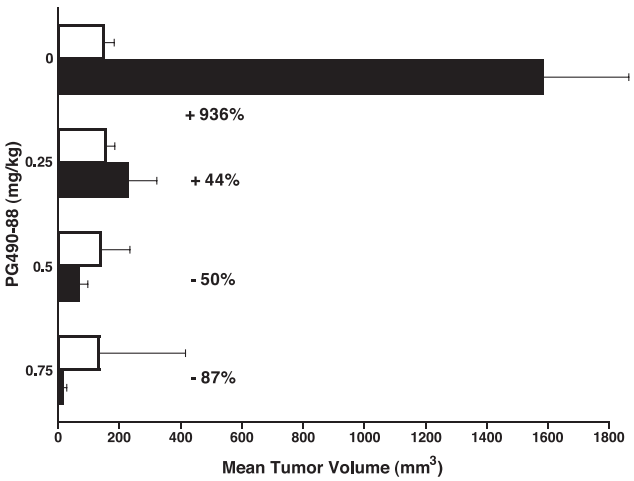

Figure 3. PG490-88 induces regression of established H23 colon carcinoma tumor xenografts. Nude mice bearing xenografts of H23 human colon tumor cells were treated daily with saline or PG490-88 at 0.25, 0.5, or 0.75 mg/kg saline. Data are mean tumor volumes on days 0 (□) and 15 (■) for each treatment group; *bars,* SE. There were five mice per group.

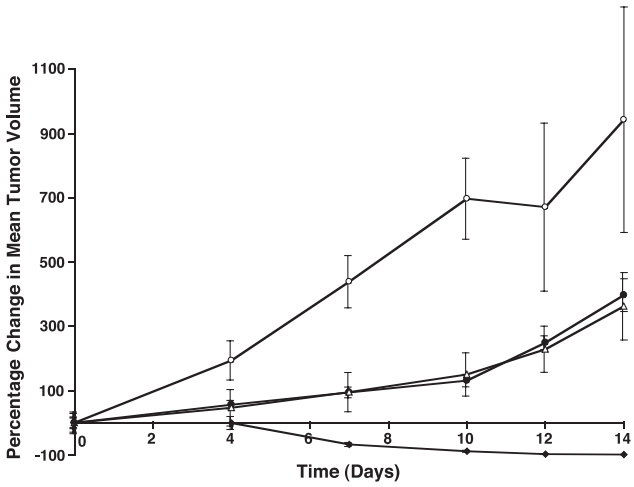

Figure 4. Combination dosing with PG490-88 and CPT-11 induces complete regression of HT1080 tumor xenografts. Nude mice bearing established HT1080 tumors were grouped together to constitute a similar mean tumor size in each group. Saline (○) or PG490-88 (●) at 0.75 mg/kg was given i.p. on days 0 – 5 and 7 – 11. CPT-11 was administered i.v. on days 1, 5, and 9 at 11 mg/kg (△), and mice in the combination treatment group (◆) received PG490-88 i.p. and CPT-11 i.v. at the appropriate times. The mean tumor volumes on day 0 were: Saline, 103 ± 15 mm^3; PG490-88 (0.75 mg/kg), 107 ± 20 mm^3; CPT-11 (11 mg/kg), 107 ± 36 mm^3; and PG490-88 (0.75 mg/kg) + CPT-11 (11 mg/kg), 107 ± 31 mm^3. *Points,* percentage change in mean tumor volume from day 0 for each treatment group; *bars,* SE. There were four mice per group.

Reinstatement of PG490-88 Combination Therapy with CPT-11 Demonstrates Continued Effectiveness

On day 56 (the start of week 0 for Fig. 7), dosing was reinitiated in the group receiving the combination of PG490-88 0.25 mg/kg and CPT-11. Mean tumor volume initially rose but then decreased from day 4 of week 1 through the end of dosing at week 3 (Fig. 7). Despite a 5-week cessation of exposure to the agents, the tumors remained sensitive to combination dosing. When dosing was discontinued a second time (from week 3 to 8) and again reinstated, the tumors retained sensitivity to combination dosing. Therefore, tumors did not become resistant to PG490-88 plus CPT-11 after treatment was stopped. Also, we found that tumors regressed when PG490-88 therapy was initiated 7 days after starting CPT-11, suggesting that CPT-11 does not confer resistance to PG490-88 (data not shown).

PG490-88 Is Effective with a Less Frequent Dosing Schedule

To determine whether a less frequent dosing schedule of PG490-88 could be achieved without compromising efficacy, we tested the effects of PG490-88 in mice bearing COLO 205 tumors at twice a week rather than 5 times/week dosing. PG490-88 caused dose-dependent tumor regression when given twice a week at 1.0 or 1.25 mg/kg i.p. alone or concurrently with CPT-11 (11 mg/kg i.v. twice a week) (Fig. 8). Also, we observed tumor regression with the combination of PG490-88 and a lower dose CPT-11 (11 mg/kg) instead of 22 mg/kg. The untreated tumors

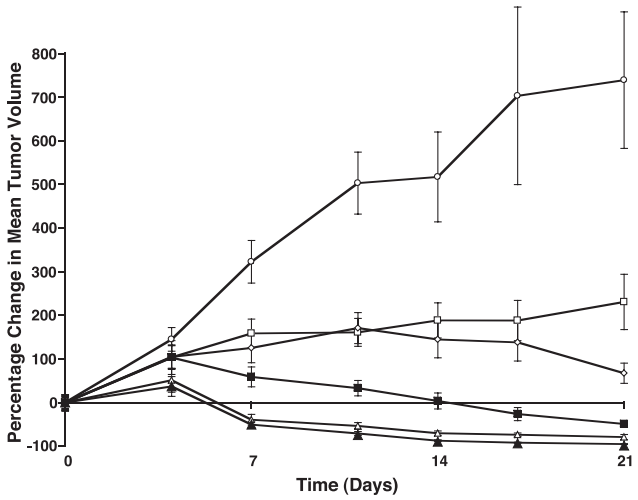

Figure 5. Cooperation between PG490-88 and CPT-11 in regression of COLO 205 tumor xenografts. Nude mice with established COLO 205 tumors were grouped together to constitute a similar mean tumor size in each group. Saline (○) or PG490-88 was given at 0.25 (□) or 0.75 mg/kg (△) i.p. daily 5 days/week, CPT-11 was administered i.v. twice a week at 22 mg/kg starting on day 1 (◇), and mice in the combination treatment groups received CPT-11 i.v. and PG490-88 i.p. at 0.25 (■) or 0.75 (▲) mg/kg at the appropriate times. Treatment continued for 3 weeks. The mean tumor volumes on day 0 were: Saline, 101 ± 12 mm^3; PG490-88 (0.25 mg/kg), 102 ± 14 mm^3; PG490-88 (0.75 mg/kg), 103 ± 18 mm^3; CPT-11 (22 mg/kg), 102 ± 18 mm^3; and PG490-88 (0.25 mg/kg) + CPT-11 (22 mg/kg), 102 ± 15 mm^3. *Points,* percentage change in mean tumor volume from day 0 for each treatment group; *bars,* SE. There were five mice per group.

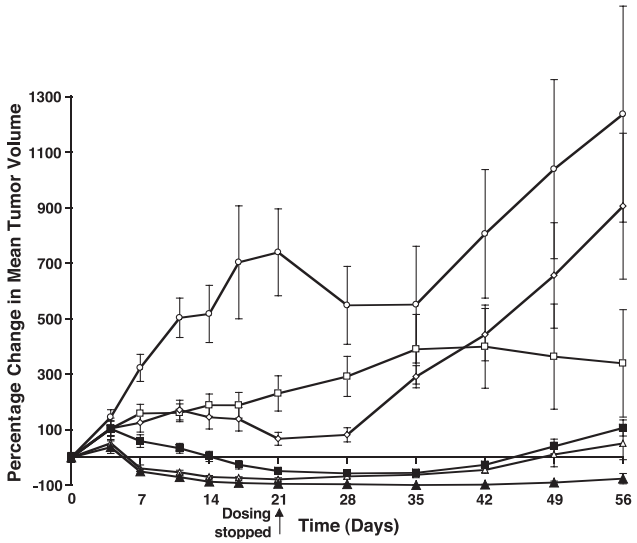

Figure 6. Cooperation between PG490-88 and CPT-11 in regression of COLO 205 tumor xenografts. The experiment shown in Fig. 5 was continued with no treatment between days 21 and 55. *Points,* percentage changes in mean tumor volumes through day 56; *bars,* SE.

regressed after day 26 due to necrosis as tumor growth outstripped the blood supply (Fig. 8). TUNEL staining of tumors revealed increased numbers of TUNEL-positive cells in tumors treated with the combination of PG490-88 and CPT-11 compared to PG490-88 or CPT-11 alone, suggesting increased apoptosis with combination therapy.[3] Taken together, these data show that clinically practical dosing schemes for PG490-88 will not sacrifice efficacy, after adjusting for dose level and treatment frequency.

Discussion

We have shown previously that PG490 sensitizes cultured tumor cells to the topoisomerase II inhibitor doxorubicin, at least in part, by blocking doxorubicin-mediated induction of p21 and thereby abrogating the G_1-S arrest (11). Here we extend our findings to show that p53 is required for PG490 to block G_2-M arrest induced by CPT-11, a topoisomerase I inhibitor, and that reconstitution of p21 blocks the PG490 inhibition of G_2-M arrest. Also, we show in tumor xenograft models using CPT-11, that PG490 acts in synergy with CPT-11. As with doxorubicin, PG490 inhibition of p21 by CPT-11 drives cells into apoptosis instead of growth arrest. Our findings agree with those of Bunz *et al.* and others who have shown that blocking checkpoint function sensitizes tumor cells to chemotherapy by blocking p21-mediated growth arrest (13–15). Additionally, we show that PG490-88 alone is a potent antitumor agent *in vivo* and that PG490-88 acts in synergy with CPT-11 in tumor xenograft models.

A recent study showed that PG490 requires functional p53 to kill tumor cells (17). Our experience with cultured cells and tumor xenografts suggests a more complex model of how PG490 kills tumor cells. We have found

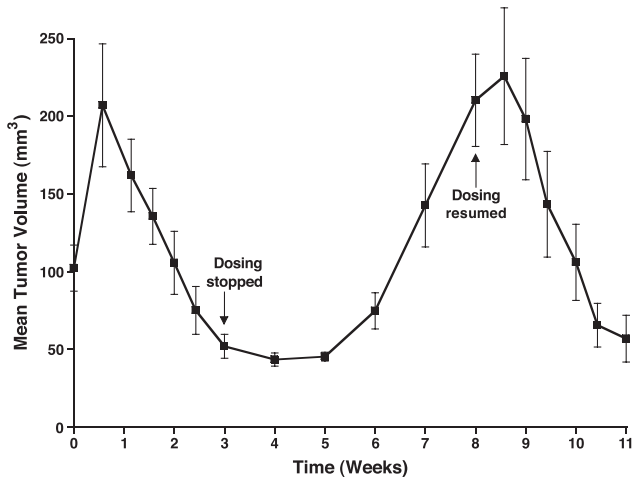

Figure 7. Reinitiation of PG490-88 combination therapy with CPT-11 demonstrates continued effectiveness. Treatment in the group having received 0.25 mg/kg of PG490-88 and CPT-11 (Figs. 5 and 6) was reinstated on day 56 (the beginning of week 0 in this figure) and continued for 3 weeks. PG490-88 treatment was given on day 0 of week 0 and CPT-11 was administered on day 1 of week 0. Treatment was suspended after week 3, reinitiated at the beginning of week 8 (PG490-88 treatment beginning on day 0 of week 8 and CPT-11 administrations beginning on day 1 of week 8), and continued until the end of the experiment. *Points,* mean tumor volumes for week 0 through week 11; *bars,* SE.

[3] K. Li, C. Chung, J. Fidler, and G. D. Rosen, unpublished results.

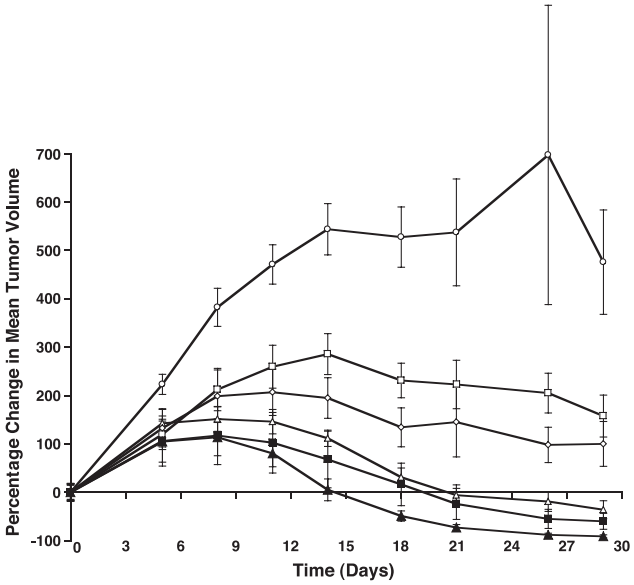

Figure 8. PG490-88 treatment with reduced frequency. Nude mice with established COLO 205 tumors were grouped together to constitute a similar mean tumor size in each group. Saline (○) or PG490-88 [1.0 (□) or 1.25 (△) mg/kg] was given i.p. twice a week starting on day 0. CPT-11 was administered i.v. twice a week at 11 mg/kg (◇) starting on day 1, and mice in the combination treatment groups received CPT-11 i.v. and PG490-88 i.p. at 1.0 (■) or 1.25 (▲) mg/kg at the appropriate times. Dosing was conducted for 3 weeks. The mean tumor volumes on day 0 were: Vehicle, 106 ± 18 mm^3; PG490-88 (1.0 mg/kg), 108 ± 17 mm^3; PG490-88 (1.25 mg/kg), 107 ± 20 mm^3; CPT-11 (11 mg/kg), 108 ± 16 mm^3; PG490-88 (1.0 mg/kg) + CPT-11 (11 mg/kg), 108 ± 18 mm^3; and PG490-88 (1.25 mg/kg) + CPT-11 (11 mg/kg), 107 ± 17 mm^3. *Points,* percentage change in mean tumor volume from day 0 for each treatment group; *bars,* SE. There were five mice per group.

that the most PG490-sensitive tumor cell line is the H23 non-small cell lung carcinoma cell line that harbors mutant p53. In support of this, a recent study showed that PG490 inhibits the growth and metastasis of solid tumor xenografts regardless of their p53 status (18). In the study, Yang *et al.* (18) show that PG490 is also cytotoxic in multidrug-resistant (MDR) tumor cells. We have also found that MDR tumor cells are as sensitive to PG490 as their non-MDR parental cell counterpart.[4] It is possible then that PG490 activates the p53 apoptotic pathway in cells with functional p53 but also can kill tumor cells by a mechanism independent of p53.

How does PG490 kill tumor cells? The molecular target(s) for PG490 is currently unknown. Clues to the cellular target, however, are emerging from its effect on transcriptional activity. For example, we have shown along with Qiu *et al.*, that PG490 blocks transcriptional activation of NF-κB by blocking transcriptional activation of p65 but without affecting DNA binding by p65 (3, 10). Additionally, we have found that PG490 blocks transcriptional activation by AP-1 and p53 without affecting DNA binding by Jun/Fos or p53.[5] Recent studies show that the transcriptional activity of AP-1, NF-κB, and p53 is regulated by a chromatin structure that is controlled, in part, by histone acetylation (19–21). In support of this, a recent study showed that p65 interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate NF-κB transcriptional activity (20). Also, silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) was shown to inhibit transactivation of AP-1, NF-κB, and serum response factor (SRF) by binding to their cognate transcription factors (17). Recent studies also show that p53-mediated transcriptional activity is regulated by histone acetylation (19, 22). However, we have not observed an effect of PG490 on histone acetyltransferase (HAT) activity or histone acetylation.[6]

PG490 at doses of 5–10 ng/ml does not repress basal transcriptional activity mediated by AP-1, NF-κB, and p53[7] but it does block induction of NF-κB by TNF-α and p53 transcriptional activity induced by chemotherapy. Also, PG490 does not affect topoisomerase I or II activity or increase topoisomerase cleavage complexes.[8] Therefore, its synergy with chemotherapy may in large part be due to its inhibition of p21-mediated growth arrest, which activates an apoptotic pathway.

The treatment of solid tumors is evolving to more targeted treatments that may be helped by genetic profiling of tumors and targeting tumor-specific angiogenic and growth factor pathways. Also, several recent studies have shown that disrupting checkpoints in tumors drives tumor cells into apoptosis by abrogating checkpoint arrest (reviewed in 23). Here we show that PG490-88, a water-soluble derivative of PG490, reduces tumor growth, induces marked regression, or completely eradicates human tumor xenografts. Moreover, PG490-88 is a potent and well-tolerated antitumor agent that acts in synergy with DNA-damaging agents and is effective in a clinically relevant dosing schedule. PG490-88 is now in phase I clinical trials for patients with solid tumors. A recent study showing that PG490 inhibits metastasis of solid tumors (17) coupled with our findings that PG490-88 markedly enhances the cytotoxicity of DNA-damaging agents suggests that PG490 or PG490-88 alone or in combination with chemotherapy may become an effective therapy for patients with solid tumors. Also, our finding that PG490 sensitizes tumor cells to TNF-α by blocking NF-κB suggests a role for the combination in treating patients with TNF-sensitive tumors such as melanoma. Identification of the target of PG490 and its mechanism of action will complement the ongoing clinical trials, and will provide insight into potential mechanisms of toxicity and the design of compounds that may be more selective and more potent.

[4] K. Wei and G. D. Rosen, unpublished results.

[5] M. Gao and G. D. Rosen, unpublished results.

[6] M. Gao and G. D. Rosen, unpublished results and personal communication from Dr. Marty Mayo, University of Virginia.

[7] W. Chang and G. D. Rosen, unpublished results.

[8] M. Gao and G. D. Rosen, unpublished results.

Acknowledgments

We thank Ed Lennox from Pharmagenesis for helpful discussions and reading of the manuscript. We also thank Kendall Stewart for help with preparation of the figures.

References

1. Kupchan, S. M., Court, W. A., Dailey, R. G., Jr., Gilmore, C. J., and Bryan, R. F. Triptolide and tripdiolide, novel antileukemic diterpenoid triepoxides from *Tripterygium wilfordii*. J. Am. Chem. Soc., *94:* 7194–7195, 1972.

2. Tao, X. and Lipsky, P. E. The Chinese anti-inflammatory and immunosuppressive herbal remedy *Tripterygium wilfordii* Hook F. Rheum. Dis. Clin. N. Am., *26:* 29–50, viii, 2000.

3. Qiu, D., Zhao, G., Aoki, Y., Shi, L., Uyei, A., Nazarian, S., Ng, J. C., and Kao, P. N. Immunosuppressant PG490 (Triptolide) inhibits T-cell interleukin-2 expression at the level of purine-box/Nuclear factor of activated T-cells and NF-κB transcriptional activation [In Process Citation]. J. Biol. Chem., *274:* 13443–13450, 1999.

4. Chen, B. J. Triptolide, a novel immunosuppressive and anti-inflammatory agent purified from a Chinese herb *Tripterygium wilfordii* Hook F. Leuk. Lymphoma, *42:* 253–265, 2001.

5. Chen, B. J., Liu, C., Cui, X., Fidler, J. M., and Chao, N. J. Prevention of graft-*versus*-host disease by a novel immunosuppressant, PG490-88, through inhibition of alloreactive T cell expansion. Transplantation, *70:* 1442–1447, 2000.

6. Fidler, J. M., Ku, G. Y., Piazza, D., Xu, R., Jin, R., and Chen, Z. Immunosuppressive activity of the Chinese medicinal plant *Tripterygium wilfordii*. III. Suppression of graft-*versus*-host disease in murine allogeneic bone marrow transplantation by the PG27 extract. Transplantation, *74:* 445–457, 2002.

7. Yang, S. X., Gao, H. L., Xie, S. S., Zhang, W. R., and Long, Z. Z. Immunosuppression of triptolide and its effect on skin allograft survival. Int. J. Immunopharmacol., *14:* 963–969, 1992.

8. Wang, J., Xu, R., Jin, R., Chen, Z., and Fidler, J. M. Immunosuppressive activity of the Chinese medicinal plant *Tripterygium wilfordii*. I. Prolongation of rat cardiac and renal allograft survival by the PG27 extract and immunosuppressive synergy in combination therapy with cyclosporine. Transplantation, *70:* 447–455, 2000.

9. Shamon, L. A., Pezzuto, J. M., Graves, J. M., Mehta, R. R., Wangcharoentrakul, S., Sangsuwan, R., Chaichana, S., Tuchinda, P., Cleason, P., and Reutrakul, V. Evaluation of the mutagenic, cytotoxic, and antitumor potential of triptolide, a highly oxygenated diterpene isolated from *Tripterygium wilfordii*. Cancer Lett., *112:* 113–117, 1997.

10. Lee, K. Y., Chang, W., Qiu, D., Kao, P. N., and Rosen, G. D. PG490 (Triptolide) cooperates with tumor necrosis factor-α to induce apoptosis in tumor cells [In Process Citation]. J. Biol. Chem., *274:* 13451–13455, 1999.

11. Chang, W. T., Kang, J. J., Lee, K. Y., Wei, K., Anderson, E., Gotmare, S., Ross, J. A., and Rosen, G. D. Triptolide and chemotherapy cooperate in tumor cell apoptosis. A role for the p53 pathway. J. Biol. Chem., *276:* 2221–2227, 2001.

12. Tengchaisri, T., Chawengkirttikul, R., Rachaphaew, N., Reutrakul, V., Sangsuwan, R., and Sirisinha, S. Antitumor activity of triptolide against cholangiocarcinoma growth *in vitro* and in hamsters. Cancer Lett., *133:* 169–175, 1998.

13. Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W., and Vogelstein, B. Disruption of p53 in human cancer cells alters the responses to therapeutic agents [see comments]. J. Clin. Invest., *104:* 263–269, 1999.

14. Wang, Y. A., Elson, A., and Leder, P. Loss of p21 increases sensitivity to ionizing radiation and delays the onset of lymphoma in atm-deficient mice. Proc. Natl. Acad. Sci. USA, *94:* 14590–14595, 1997.

15. Li, W., Fan, J., Banerjee, D., and Bertino, J. R. Overexpression of p21(waf1) decreases G2-M arrest and apoptosis induced by paclitaxel in human sarcoma cells lacking both p53 and functional Rb protein. Mol. Pharmacol., *55:* 1088–1093, 1999.

16. Magrini, R., Bhonde, M. R., Hanski, M. L., Notter, M., Scherubl, H., Boland, C. R., Zeitz, M., and Hanski, C. Cellular effects of CPT-11 on colon carcinoma cells: dependence on p53 and hMLH1 status. Int. J. Cancer, *101:* 23–31, 2002.

17. Jiang, X. H., Wong, B. C., Lin, M. C., Zhu, G. H., Kung, H. F., Jiang, S. H., Yang, D., and Lam, S. K. Functional p53 is required for triptolide-induced apoptosis and AP-1 and nuclear factor-κB activation in gastric cancer cells. Oncogene, *20:* 8009–8018, 2001.

18. Yang, S., Chen, J., Guo, Z., Xu, X-M., Wang, L., Pei, X-F., Yang, J., Underhill, C. B., and Zhang, L. Triptolide inhibits the growth and metastasis of solid tumors. Mol. Cancer Ther., *2:* 65–72, 2003.

19. Lee, S. K., Kim, J. H., Lee, Y. C., Cheong, J., and Lee, J. W. Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-κB, and serum response factor. J. Biol. Chem., *275:* 12470–12474, 2000.

20. Wang, T., Kobayashi, T., Takimoto, R., Denes, A. E., Snyder, E. L., el-Deiry, W. S., and Brachmann, R. K. hADA3 is required for p53 activity. EMBO J., *20:* 6404–6413, 2001.

21. Ashburner, B. P., Westerheide, S. D., and Baldwin, A. S., Jr. The p65 (RelA) subunit of NF-κB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol. Cell. Biol., *21:* 7065–7077, 2001.

22. Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol. Cell, *8:* 1243–1254, 2001.

23. Owa, T., Yoshino, H., Yoshimatsu, K., and Nagasu, T. Cell cycle regulation in the G1 phase: a promising target for the development of new chemotherapeutic anticancer agents. Curr. Med. Chem., *8:* 1487–1503, 2001.