An evaluation of the ability of pifithrin-α and -β to inhibit p53 function in two wild-type p53 human tumor cell lines

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Abstract

The small-molecule compound pifithrin-α (PFT-α) has been reported to inhibit p53 function and protect against a variety of genotoxic agents. We show here that PFT-α is unstable in tissue culture medium and is rapidly converted to its condensation product PFT-β. Both compounds showed limited solubility with PFT-α precipitating out of tissue culture medium at concentrations >30 μmol/L. PFT-α and -β exhibited cytotoxic effects *in vitro* towards two human wild-type p53–expressing tumor cell lines, A2780 ovarian and HCT116 colon (IC_{50} values for both cell lines were 21.3 ± 8.1 μmol/L for PFT-α and 90.3 ± 15.5 μmol/L for PFT-β, mean ± SD, *n* = 4). There was no evidence of protection by clonogenic assay with either compound in combination with ionizing radiation. Indeed, there was some evidence that PFT-α enhanced cytotoxicity, particularly at higher concentrations of PFT-α. Neither compound had any effect on p53, p21, or MDM-2 protein expression following ionizing radiation exposure and there was no evidence of any abrogation of p53-dependent, ionizing radiation–induced cell cycle arrest. Similarly, there was no evidence of cellular protection, or of effects on p53-dependent gene transcription, or on translation of MDM-2 or p21 following UV treatment of these human tumor cell lines. In addition, there was no effect on p53 or p21 gene transactivation or p38 phosphorylation after UV irradiation of NIH-3T3 mouse fibroblasts. In conclusion, neither PFT-α nor -β can be regarded as a ubiquitous inhibitor of p53 function, and caution should be exercised in the use of these agents as specific p53 inhibitors. [Mol Cancer Ther 2005;4(9):1369–77]

Introduction

The loss of tumor suppressor proteins is a hallmark of human cancer (1). The most commonly mutated tumor suppressor protein is p53, which is functionally compromised in >70% of all human cancers (2, 3). Wild-type p53 can be transcriptionally transactivated following a wide variety of stimuli including ionizing radiation, UV, DNA damage, hypoxia, and nucleotide depletion (4, 5). This can lead to a number of possible outcomes depending on cellular context, including cell cycle arrest, DNA repair, senescence, or apoptosis, functions which are central to the ability of p53 to prevent the propagation of genetically damaged cells, and hence prevent tumor formation (5–8). Loss of p53 could therefore promote tumorigenesis and, in addition, could also cause chemoresistance in a number of situations, probably through the loss of critical p53-regulated proapoptotic processes (9–11). Consequently, it has been shown that a variety of common cytotoxic treatments require higher doses in p53-deficient cell lines compared with wild-type lines to achieve comparable levels of cytotoxicity (12, 13). Similarly, p53-deficient mice are more resistant to ionizing radiation than their wild-type counterparts (14, 15). Other studies have shown that lymphomas expressing mutant p53 require higher doses of drugs to achieve comparable responses to p53 wild-type tumors (11, 13). However, dose-limiting normal tissue toxicity frequently restricts treatments to suboptimal doses, which can lead to treatment failure. This is probably due to the ubiquitous expression of wild-type p53 in normal tissues and its activation following drug treatment, leading to extensive apoptotic cell death (8, 11, 15). Consequently, temporary inhibition of wild-type p53 function may ameliorate normal tissue toxicity, allowing improved therapeutic selectivity and higher doses of cytotoxic treatments to be administered (16).

An *in vitro* screen for agents that blocked doxorubicin-induced, p53-dependent, lacZ-encoded β-Gal expression led to the identification of a small-molecule inhibitor of p53, pifithrin-α [PFT-α, 2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-(4-methylphenyl)ethanone; Fig. 1; ref. 17]. This compound has been reported to inhibit a number of p53-dependent processes *in vitro*, including UV-induced, p53-dependent β-gal expression, UV-induced cyclin G, p21, and MDM-2 protein expression (17). Moreover, 10 μmol/L PFT-α also protected murine C8 cells from the cytotoxic effects of several anticancer drugs. A similar concentration was

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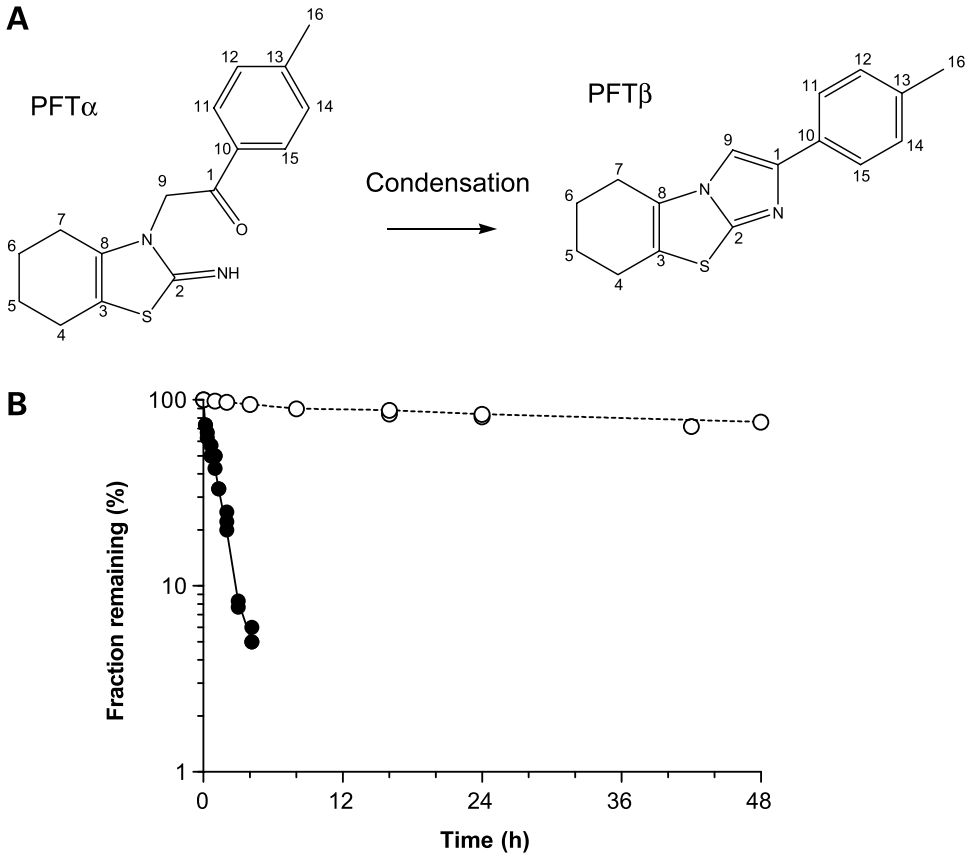

Figure 1. **A**, structure of PFT-α and its condensation product PFT-β. **B**, stability of PFT-α in DMSO at room temperature (○) and in tissue culture medium (DMEM containing 10% fetal bovine serum, 5 mmol/L L-glutamine, and nonessential amino acids) at 37°C (●). Individual data points from two independent experiments.

shown to abrogate ionizing radiation–induced G_1-S checkpoint control in murine concanavalin A cells. *In vivo* studies showed that a single i.p. dose of PFT-α protected mice from a lethal dose of whole-body irradiation (17). Following the initial report of the discovery of PFT-α, this agent has been investigated in various murine model systems (18–20). In view of the fact that the initial studies were carried out in murine models, and that there is no clearly defined molecular mechanism of action for the agent, we have carried out a detailed study of the effects of PFT-α in two human p53+/+ cancer cell lines. We show that PFT-α is unstable *in vitro* and is converted to PFT-β. We also find that there is minimal evidence of any inhibitory effect of either of these two compounds on p53 activity in the two human tumor cell lines or in the murine fibroblast NIH-3T3 cell line. Thus, caution should be exercised in the use of PFT-α as a modulator of p53 function.

Materials and Methods

Tissue Culture

The human ovarian carcinoma cell line A2780 was obtained from the European Collection of Cell Cultures (Porton Down, Wilts, United Kingdom). The human colon carcinoma cell line HCT116 and the murine immortalized fibroblast cell line NIH-3T3 were obtained from the American Type Culture Collection (Rockville, MD). These cell lines had previously been shown to express functional wild-type p53 and to harbor wild-type p53 gene sequences (12, 21). Cells were grown as attached monolayers in DMEM containing 10% fetal bovine serum, 5 mmol/L glutamine, and essential amino acids under an atmosphere of 5% CO_2/95% air at 37°C. Cell lines were routinely screened for *Mycoplasma* expression using a nested PCR–based assay from the American Type Culture Collection.

Drug Synthesis and Characterization

The iminotetrahydrobenzothiazole PFT-α was synthesized in a two-step reaction starting with cyclohexanone and thiourea plus iodine. The subsequent intermediate was reacted with bromomethylacetophenone to form the HBr salt. This was triturated with ether, filtered, and the solid dissolved in chloroform/methanol and precipitated with ether, filtered, and dried to give a product of 100% purity (18, 22). ^{1}H-NMR (250 MHz, DMSO-d_6); δ 9.50 (s, 1H, NH), 7.95 (d, 2H, J = 8.2 Hz, C11,15), 7.44 (d, 2H, J = 8.1 Hz, C12,14), 5.70 (s, 2H, C9), 2.54 (t, 4H, C4,7), 2.42 (s, 3H, C16), 1.72 (m, 4H, C5,6). Relevant protons quoted with reference to the attached carbon number (see Fig. 1, PFT-α). Accurate mass $[M+H]^+$ for $C_{16}H_{19}N_2OS$: calculated 287.1218, measured 287.1223. The cyclized imidazol[2,1-b]thiazole PFT-β was unexpectedly identified from the small-molecule X-ray crystal structure determination of a sample of PFT-α following recrystallization in aqueous

methanol (see Supplementary information).[1] Details of the crystal structure of PFT-β have been published (23). To confirm this initial observation, heating a solution of PFT-α in methanol and water (2:1) to reflux resulted in complete conversion into PFT-β. ^{1}H-NMR (250 MHz, DMSO-d_6); δ 8.47 (s, 1H, C9), 7.70 (d, 2H, *J* = 8.1 Hz, C11,15), 7.30 (d, 2H, *J* = 8.0 Hz, C12,14), 2.75 (t, 4H, C4,7), 2.33 (s, 3H, C16), 1.88 (m, 4H, C5,6). Relevant protons quoted with reference to the attached carbon number (see Fig. 1, PFT-β). Accurate mass $[M+H]^+$ for $C_{16}H_{17}N_2S$: calculated 269.1112, measured 269.1059. Comparison of the spectral data obtained using synthetic PFT-α with commercially sourced material (Sigma-Aldrich, Poole, United Kingdom) unambiguously confirmed its identity (see Supplementary data).[1] ^{1}H-NMR (250 MHz, DMSO-d_6); δ 9.50 (s, 1H, NH), 7.95 (d, 2H, *J* = 8.2 Hz, C11,15), 7.44 (d, 2H, *J* = 8.1 Hz, C12,14), 5.71 (s, 2H, C9), 2.54 (t, 4H, C4,7), 2.42 (s, 3H, C16), 1.72 (m, 4H, C5,6). Accurate mass $[M+H]^+$ for $C_{16}H_{19}N_2OS$: calculated 287.1218, measured 287.1159. Nuclear magnetic resonance spectra were run in DMSO-d_6 (10 mg/mL) on a Bruker Avance DPX250 and referenced to the multiplet for DMSO (δ 2.49 ppm). Accurate mass measurement was carried on a Waters LCT using electrospray ionization with leucine-enkephelin as a lock spray reference. The stability of PFT-α was monitored by determining PFT-α concentrations remaining in the chosen incubation medium at selected time points using quantitative liquid chromatography-mass spectrometry.

Cytotoxicity Studies

Both PFT-α and -β were white crystalline powders which were made up as stock solutions (10 mmol/L) in DMSO immediately prior to dilution into medium to the required final concentration. Other drugs were obtained from Sigma-Aldrich. Cells were plated in 96-well plates at defined densities (5,000 cells/well) 36 hours prior to drug treatment under exponential growth conditions. Cytotoxicity was determined by 96-hour sulforhodamine B assays (24) equivalent to four cell doublings. Previous studies had established that these conditions gave linear growth curves. Briefly, drugs were added in 20 or 40 μL of medium to give a final volume of 200 μL. After 96 hours, plates were fixed in 10% trichloroacetic acid for 30 minutes at 4°C, then washed four times in water and dried. Subsequently, 100 μL of 0.04% sulforhodamine B in 1% acetic acid was added to each well and left for 15 minutes. Excess stain was removed by washing with 1% acetic acid four times and then dried. Cellular stain was solubilized in 100 μL of 10 mmol/L Tris base and read at 540 nm. UV and ionizing radiation sensitivity was determined by clonogenic assay at three plating densities of 100, 1,000, and 10,000 cells/plate using 3, 10, and 30 μmol/L concentrations, respectively (25). Following colony formation (7–10 days), cells were fixed in methanol for 30 minutes and then washed twice in PBS and stained in 0.05% methylene blue. Colonies (>100 cells) were counted and survival expressed relative to 100% survival in controls. Plating efficiency was typically 20%. For studies involving UV irradiation, cells were plated onto 9 cm Petri dishes and drug-containing or drug-free medium removed immediately prior to irradiation and replaced immediately following UV exposure. UV irradiation was carried out using a UV Stratolinker 1800 (Stratagene, La Jolla, CA). Combination studies were carried out using 10 μmol/L PFT-α or -β as a standard concentration unless otherwise stated. PFT-α or -β was administered 1 hour prior to cytotoxic therapy as this has been reported to be the maximally effective treatment protocol (Supplemental data in ref. 17) unless otherwise stated. For clonogenic assays, PFT-α or -β remained in contact with the cells for 24 hours following irradiation to allow any protective effects to occur prior to plating out.

Western Blotting

Samples were lysed in ice-cold lysis buffer (containing 50 mmol/L HEPES, pH 7.4, 250 mmol/L NaCl, 0.1% NP40, 1 mmol/L DTT, 1 mmol/L NaF, 10 mmol/L β-glycerophosphate, 0.1 mmol/L sodium orthophosphate and one "Complete" protease inhibitor cocktail tablet containing EDTA, Roche, England, United Kingdom) and subsequently stored at −70°C prior to analysis. Lysates (50 μg) were boiled in Laemmli buffer prior to separation on gradient (4–20%) or 10% linear gels (Invitrogen, Paisley, United Kingdom) and transferred to polyvinylidene difluoride membranes. Primary antibodies were incubated overnight in 3% milk with membranes at 4°C. Proteins were detected using the following antibodies: p53 DO-1 at 1:1,000, SC126 and total p38, SC7972 at 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA); $p21^{WAF1}$, OP-64 at 1:250 and MDM-2, OP46 at 1:500 (Calbiochem, Darmstadt, Germany); phospho-p38, Thr^{180}/Tyr^{182}, CS9211S at 1:1,000 (Cell Signaling Technologies, Beverly, MA), and glyceraldehyde-3-phosphate dehydrogenase, MAB374 at 1:100,000 (Chemicon International, Temecula, CA). Protein expression was subsequently visualized using appropriate HRPO conjugated secondary antibodies (goat antimouse or antirabbit, Bio-Rad, Hemel Hempstead, United Kingdom) with enhanced chemiluminescence reagents (Pierce, Cheshire, United Kingdom) and Hyperfilm (Amersham, Bucks, United Kingdom) film.

Results

Stability of PFT-α and Conversion to PFT-β

The stability of PFT-α was examined in DMSO at room temperature and full tissue culture medium (10% fetal bovine serum in DMEM) at 37°C, conditions under which the drug was likely to be handled during the course of the biological studies. Figure 1 clearly shows that PFT-α was relatively stable in DMSO with a half-life of 18.5 hours (10.8–65.2 hours, 95% confidence limits). By contrast, PFT-α was rapidly converted by condensation to the ring-closed product PFT-β in tissue culture medium at 37°C with a half-life of 59.0 minutes (46.5–80.4 minutes, 95% confidence limits). Consequently, it is unlikely that the reported biological effects of PFT-α on cells *in vitro* (17) are due

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

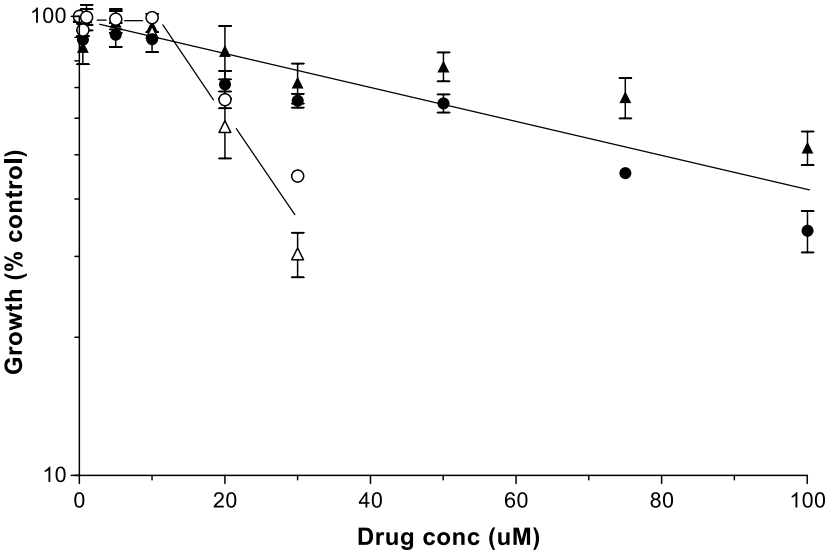

Figure 2. Graph showing 96-h sulforhodamine B assay for PFT-α (○, △) and PFT-β (●, ▲) in wild-type p53 A2780 human ovarian cancer cells (○, ●) and wild-type p53 HCT116 human colon cancer cells (△, ▲). Similar results were obtained in repeat experiments (data not shown). *Bars,* ± SD.

exclusively to this compound and may be due to a mixture of both PFT-α and -β. We therefore included PFT-β as well as PFT-α in all of our subsequent experiments. For characterization of chemical structure of both agents, see Materials and Methods.

In vitro Cytotoxicity of PFT-α and -β

The cytotoxicity of PFT-α and -β were determined in human, functional wild-type p53 expressing HCT116 colon and A2780 ovarian tumor cell lines. PFT-α was more cytotoxic than PFT-β in both cell lines with 96-hour sulforhodamine B assay IC_{50} values of 17 and 29 μmol/L for PFT-α and 74 and 80 μmol/L for PFT-β in A2780 (two independent experiments; Fig. 2). Corresponding values for HCT116 were 12 and 27 μmol/L for PFT-α and 102 and 105 μmol/L for PFT-β, also as determined in two independent experiments. The solubility of PFT-β was greater than that for PFT-α as evidenced by the appearance of drug crystals in the medium at concentrations >30 μmol/L for the latter, and we have therefore omitted data >30 μmol/L for PFT-α. These studies established a maximum nontoxic concentration of 10 μmol/L and a maximum usable concentration of 30 μmol/L PFT-α.

We subsequently investigated the ability of PFT-α and -β to modulate the cytotoxicity of four commonly used anticancer drugs in A2780 and HCT116 cells. The results are summarized in Table 1 and indicate the complete absence of any robust protective effect, i.e., no consistent ratios ≥2-fold when comparing results with and without PFT-α or PFT-β. In fact, these data suggest that both PFT-α and PFT-β potentiate the cytotoxicity of doxorubicin, etoposide, and paclitaxel in some cases. Similar results were obtained at the higher concentration of 30 μmol/L PFT-α and PFT-β (data not shown). There was also no evidence of protection when PFT-α and -β at 20 μmol/L were combined with the four cytotoxic drugs in NIH-3T3 murine fibroblast cells (data not shown). In addition,

Table 1. Effects of PFT-α and -β on the cytotoxicity of a number of anticancer drugs in human, wild-type p53 – expressing HCT116 and A2780 cancer cell lines as assessed by 96-hour sulforhodamine B assays

Treatment	A2780 IC_{50} values (μmol/L)					HCT116 IC_{50} values (μmol/L)				
	Alone	+PFT-α	Ratio	+PFT-β	Ratio	Alone	+PFT-α	Ratio	+PFT-β	Ratio
Cisplatin	0.32, 0.38	0.33	0.94	0.51	1.45	0.54, 0.4	1.0	2.12	0.54	1.14
	0.50, 1.00	0.78	0.62	2.05	1.64	1.4, 1.9	1.95	1.18	2.1	1.27
Doxorubicin	0.0039, 0.0033	0.001	0.27	0.0069	1.91	0.012, 0.009	0.005	0.48	0.007	0.67
	0.008, 0.0061	0.0048	0.68	0.0032	0.45	0.023, 0.030	0.022	0.83	0.018	0.68
Etoposide	0.074, 0.070	0.032	0.44	0.027	0.38	0.19, 0.31	0.19	0.76	0.21	0.84
	0.019, 0.128	0.0091	0.12	0.0091	0.12	1.9, 2.1	2.2	1.1	2.8	1.4
Paclitaxel	1.0, 1.3	0.28	0.24	0.21	0.18	0.4, 0.9	0.23	0.35	0.65	1.0
	0.51, 1.3	0.27	0.31	0.37	0.42	0.3, 0.4	0.33	0.94	0.34	0.97

NOTE: PFT-α and -β were used at 10 μmol/L, the maximum nontoxic dose. Similar results were obtained at 30 μmol/L (data not shown). Cells were pretreated with PFT-α and -β for 1 hour prior to drug treatment, and then for a subsequent 96 hours. Similar results were obtained with a 16- and 22-hour pretreatment (data not shown). Ratios >1 indicate protection, and ratios <1 indicate enhanced cytotoxicity. For other details, see Materials and Methods.

preincubation of HCT116 cells with 10 μmol/L PFT-α or -β for 22, 16, or 1 hour(s) had minimal protective effects with cisplatin, doxorubicin, etoposide, or paclitaxel (data not shown).

Effect of PFT-α and -β on Ionizing Radiation Cytotoxicity in Human Cells

The original publication describing PFT-α identification and characterization reported that this compound could protect cells from ionizing radiation–induced cytotoxicity *in vitro* (1). We have therefore carried out clonogenic assays using an approximate IC_{50} dose of ionizing radiation (1 Gy) in combination with different concentrations of PFT-α or -β. Figure 3A shows that there was no protection afforded by PFT-α and that this compound seemed to enhance radiation cytotoxicity. Similar results were obtained with PFT-β (Fig. 3B). Figure 3C shows that 4 hours following ionizing radiation, p53 was stabilized and p21 and MDM-2 induced in A2780 cells, confirming their p53+/+ functional status. There was no evidence of an effect of PFT-α or -β on ionizing radiation–induced p53, p21, or MDM-2 protein expression in A2780 cells (Fig. 3C). Similar results were obtained in HCT116 cells (data not shown).

Effect of PFT-α and -β on Ionizing Radiation–Induced Cell Cycle Arrest in Human Cells

Figure 4 shows the cell cycle effects in A2780 cells treated with 5 Gy ionizing radiation in combination with different concentrations of PFT-α and a concentration of 30 μmol/L PFT-β. An HPV16 E6 knock-out cell line was also included as a control for loss of p53 function. It can be seen that there is a marked G_1 and G_2 arrest in A2780 cells 16 hours following irradiation, consistent with a wild-type p53 phenotype. By contrast, there is a much smaller G_1 arrest and correspondingly larger G_2 block in E6-transfected A2780 cells following irradiation, confirming that E6 transformation has compromised the G_1-S checkpoint and p53 function. The addition of PFT-α or -β had minimal effects on the ionizing radiation–induced G_1-S checkpoint in wild-type p53 expressing A2780 cells.

Effect of PFT-α and -β on UV-Induced Cytotoxicity in Human Cells

The initial report of PFT-α also described its ability to protect from UV-induced cytotoxicity and suppress p53-dependent UV-induced gene transcription (17). Figure 5A shows that there were no protective effects of PFT-α on UV radiation–induced cytotoxicity in human A2780 cells. In fact, there was a suggestion that there was a PFT-α concentration–dependent increase in cytotoxicity. Similar results were obtained with PFT-β (Fig. 5B). Figure 5C shows that UV-induced p53 and MDM-2 protein expression in A2780 and HCT116 cells were unaffected by combination with either PFT-α or -β.

Effects of UV and PFT-α or -β on p38 Expression in NIH-3T3 Cells

Although PFT-α and -β do not seem to directly modulate p53-dependent gene expression or response in the human cell lines studied, it was possible that PFT-α might alter a

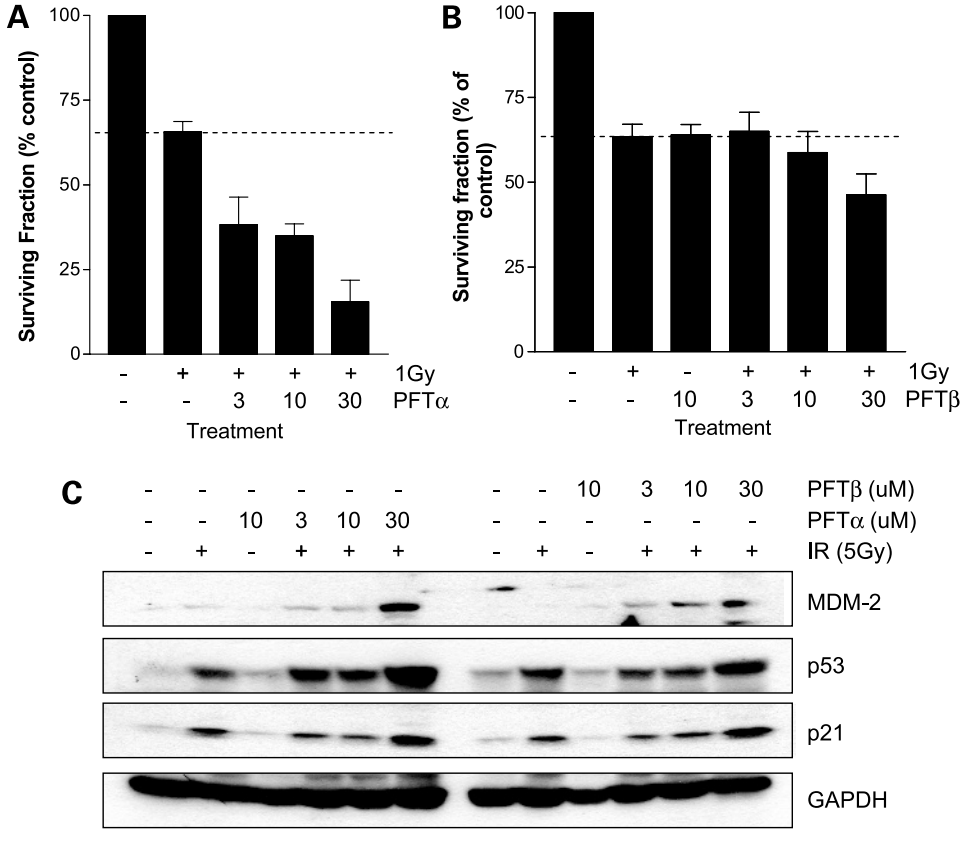

Figure 3. Clonogenic survival of A2780 cells exposed to 1 Gy of ionizing radiation alone or in combination with increasing doses of PFT-α (**A**) or PFT-β (**B**). Values above the dotted line indicate protection, and values below the dotted line indicate enhanced cytotoxicity by PFT compounds. *Bars*, ± SD. **C,** Western blot for MDM-2, p53, and p21 from A2780 cells treated 4 h previously with 5 Gy ionizing radiation alone or in combination with increasing concentrations of PFT-α or PFT-β. Similar results were obtained in repeat experiments. For further details, see Materials and Methods.

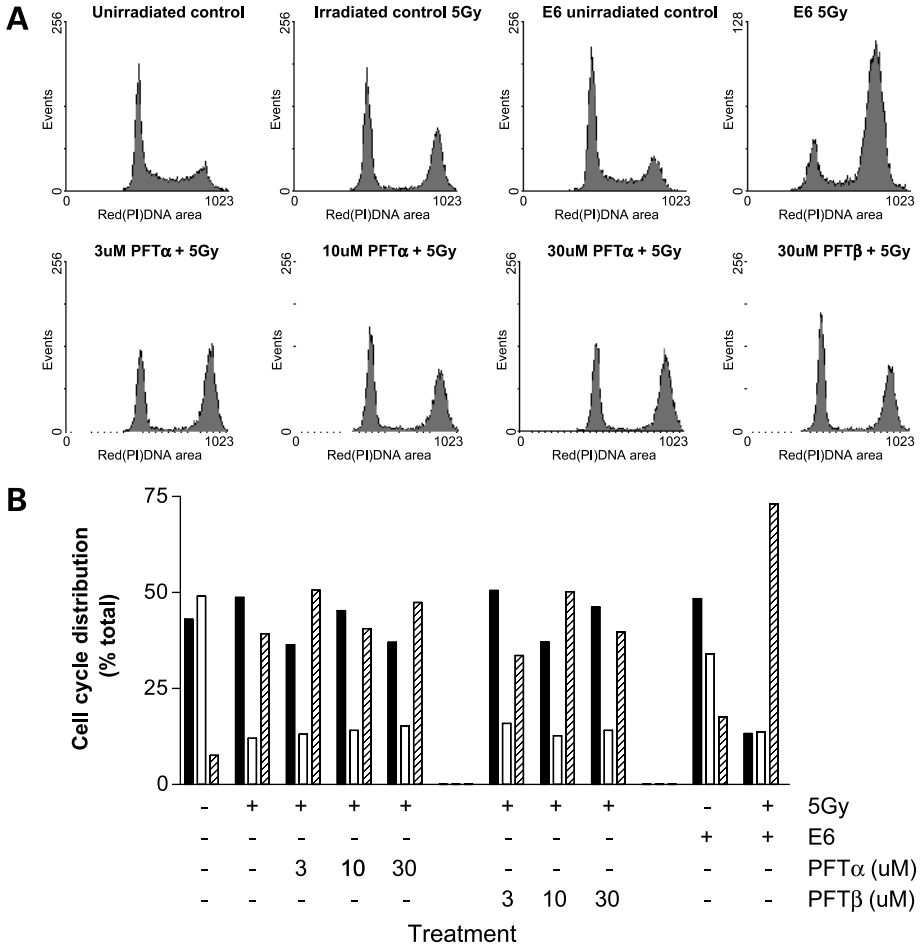

Figure 4. **A**, effects of ionizing radiation alone or in combination with PFT-α or PFT-β on cell cycle distribution 16 h after radiation treatment in human wild-type p53 expressing human A2780 ovarian cancer cell lines. A2780 cell lines expressing HPV16E6 were included as controls for loss of p53 function. **B**, quantification of the cell cycle data shown in **A**. ■, G_1/G_0 phase; □, S phase; ▨, G_2-M phase. Similar results were obtained in repeat experiments. For further details, see Materials and Methods.

pathway that interacts with p53 function. In addition, the original studies described protection by PFT-α in rodent cells (17) and it was possible that such an interaction is predominantly involved in modulating rodent cell survival. One possible mechanism of action of PFT-α or -β is as a p38 kinase inhibitor. Inhibition of the p38 pathway has been shown to enhance survival of wild-type p53–expressing cells following a variety of genotoxic stresses and this pathway is known to be active in rodent cell lines (26, 27). We therefore determined the effects of PFT-α and -β on UV-induced p38 signaling pathway in NIH-3T3 cells. Figure 6A shows that phospho-p38 was maximally induced within 30 minutes of a 25 J m^{-2} dose of UV radiation in NIH-3T3 cells (although this signal was relatively weak), whereas p53 induction occurred at 2 hours and lasted until 16 hours post-irradiation. Subsequent experiments were therefore carried out using 50 J m^{-2} UV and samples harvested 30 minutes following irradiation. Figure 6B shows that there was no effect of either PFT-α or -β on phosho-p38 induction in NIH-3T3 cells, indicating that these compounds are not inhibitors of p38 kinase activity. Figure 6C confirms that there were also minimal effects of PFT-α or -β on p53 or p21 induction in this cell line at either an early or late time point following UV irradiation.

Discussion

The development of a small-molecule inhibitor of p53 function could provide a means of enhancing the therapeutic index of a number of conventional cytotoxic therapies by decreasing the dose-limiting normal tissue toxicity. It would be envisaged that this could allow higher doses of cytotoxic therapies to be employed and may yield an increased number of tumor responses or cures. Furthermore, a selective p53 inhibitor might be valuable for validating a number of therapeutic strategies designed to exploit loss of p53 function, such as the use of G_2 checkpoint abrogators (28, 29). Consequently, the report of such an agent, PFT-α, was of considerable interest (17) and this compound has been the subject of a number of follow-up studies (18–20, 30–32). Because of the potential therapeutic significance of such a p53 modulator, we have undertaken a detailed study of its pharmacology in an attempt to understand its mechanism of action. Our studies have focused on the use of human wild-type p53–expressing lines as these are clinically the most relevant with respect to PFT-α mechanism of action. Following the synthesis of PFT-α, described here, we have shown that PFT-α is unstable in tissue culture medium and is rapidly condensed to PFT-β. This observation suggests that, in

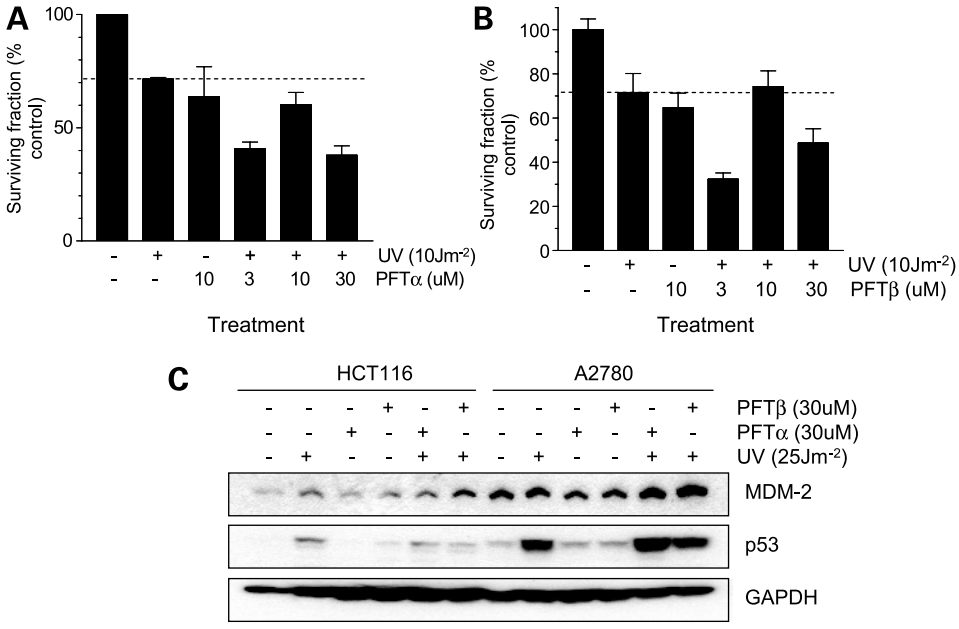

Figure 5. **A,** effects of PFT-α on clonogenic survival in response to UV irradiation in human wild-type p53 A2780 ovarian tumor cell lines. *Columns,* mean; *bars,* ± SD; *n* = 3. Survival above the line shows protection, whereas survival below the line shows enhanced cytotoxicity. **B,** similar data for the human wild-type p53 expressing HCT116 colon adenocarcinoma. **C,** Western blot for MDM-2 and p53 in A2780 and HCT116 cell lines 8 h after UV irradiation (25 J m^{-2}) with or without different doses of PFT-α or -β. For further details, see Materials and Methods.

most tissue culture experiments, any pharmacologic effects will be mediated by a mixture of these two compounds. Moreover, in contrast to the original report, we provide a complete physical chemical characterization of both the PFT-α and -β that were synthesized for this study.

PFT-α was shown to be relatively cytotoxic in its own right with a mean IC_{50} of 21.3 μmol/L in human A2780 ovarian and HCT116 colon cancer cells, both of which have functionally active wild-type p53. This value is only slightly higher than the active concentration of 10 μmol/L described previously (17), suggesting that this agent may have a very narrow therapeutic window. Importantly, we have shown that both PFT-α and -β do not alter cell survival following exposure to etoposide, cisplatin, paclitaxel or doxorubicin in p53 functionally proficient human A2780 or HCT116 cells. This observation was consistent despite the use of preincubation times of 1 to 22 hours and a number of different modulator concentrations. Furthermore, survival was not enhanced by either PFT-α or -β following UV or ionizing radiation treatment in the same human cell lines, but rather there was evidence of a concentration-dependent cytotoxicity induced by PFT-α and -β. There was also no apparent protection afforded by PFT-α or -β with the same drug combinations in a non–tumor-derived, wild-type p53–expressing, murine fibroblast cell line (NIH-3T3). PFT-α has been shown to result in cleavage of caspase 3 and to induce apoptosis and cytotoxicity in a p53-dependent manner in murine JB6 cells (19). This contrasts with studies

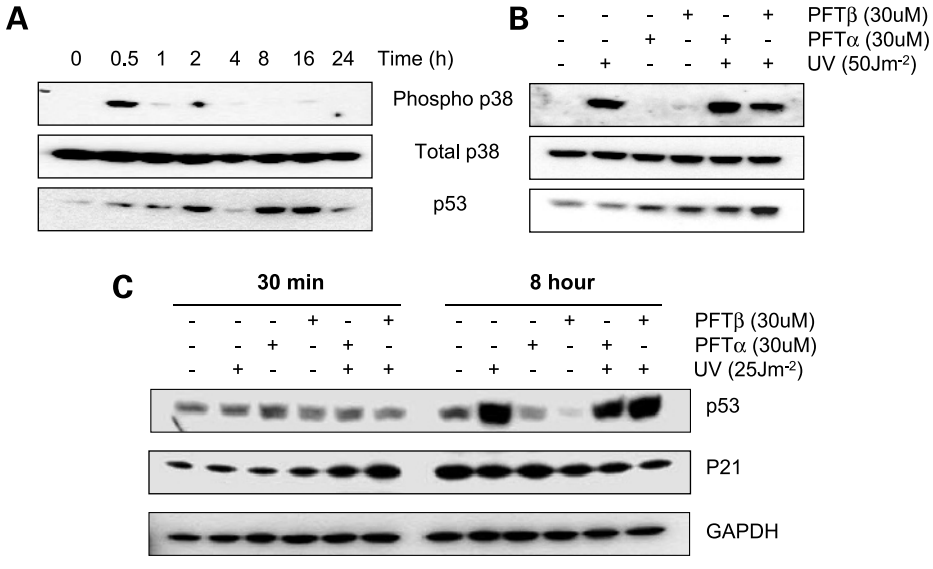

Figure 6. **A,** Western blots showing a time course for phospho-p38 induction in murine, wild-type p53–expressing NIH-3T3 fibroblast cells following UV irradiation (25 J m^{-2}). **B,** effect of UV irradiation (50 J m^{-2}) alone or in combination with PFT-α or -β on phospho-p38 induction measured 30 min after radiation exposure in murine wild-type p53, NIH-3T3 fibroblast cells. **C,** effect of UV exposure in combination with PFT-α or -β on p53 and p21 expression in murine (p53+/+) NIH-3T3 fibroblasts for either 30 min or 8 h after radiation treatment. For further details, see Materials and Methods.

in neuronal cells and models where PFT-α has been shown to protect against the lethal effects of camptothecin and etoposide, although mechanistic evidence of direct inhibition of p53 is lacking in these studies (18, 20).

In contrast with the original observations by Komarov et al. (17), we did not find any marked effects of either PFT-α or -β on G_1-S checkpoint control, a well-characterized p53-dependent event (33–35), in either of our wild-type p53–expressing lines. Together with our demonstration of p21 induction following ionizing radiation in the presence of the agents, this suggests that neither PFT-α nor -β is inhibiting p53-dependent p21 function, which is important in the regulation of cell cycle control (2, 8). Other studies have also reported minimal inhibitory effects of PFT-α on p53 function; although these investigations have not usually been carried out in human wild-type p53–expressing cell lines (19, 32). In addition, we failed to show any effect of PFT-α on wild-type p53 transactivation of p21 in wild-type p53–expressing CH1 human ovarian xenografts following irradiation (data not shown), nor were there any significant effects of exposure of A2780 cells to 10 μmol/L PFT-α on gene expression as measured by cDNA microarray analysis.[2] It has been suggested that PFT-α may exert some of its effects by acting on the molecular chaperone HSP90 (31). However, we found that there was no direct inhibitory effect of PFT-α or -β on purified yeast HSP90 ATPase activity (data not shown), as measured by a malachite green assay for inorganic phosphate release (36), and Murphy et al. (32) found no effect of PFT-α on the chaperone machinery in intact cells (32).

A number of studies have shown that down-regulation of the p38 kinase pathway can protect a variety of wild-type p53–expressing cell lines from genotoxic stress (26, 37). UV irradiation has been shown to activate p38, which phosphorylates p53 at S392 or S389, and disrupts p53-dependent apoptosis (26, 38). Consequently, we studied the effects of PFT-α or PFT-β on UV-induced p38 phosphorylation in a murine wild-type p53–expressing fibroblast cell line, NIH-3T3. We show that this pathway is unaffected by PFT-α or PFT-β and cannot therefore directly account for any protective effects of these agents. Furthermore, in murine JB cells where PFT-α was shown to have no inhibitory effects, it was reported to activate p38 kinase and extracellular signal–regulated kinase pathways, particularly at higher concentrations (19).

The precise mechanism of the reported inhibition of p53 function by PFT-α is still unclear. It seems highly likely that any protective effects may operate through inhibition of apoptosis, most probably through disruption of any one of the large number of p53-dependent proapoptotic pathways, and this could potentially be tissue context–dependent. Some hint of this possibility has already been suggested in certain p53+/+ neuronal cell lines in which PFT-α was reported to protect against a variety of toxic insults with concomitant decreases in the proapoptotic proteins, Bax and caspase-3 (20). Despite this possibility, we show that neither PFT-α nor -β protect against UV, ionizing radiation, or anticancer drug–induced, p53-dependent cytotoxicity nor does it show any p53-dependent gene expression effects.

In conclusion, we have shown that PFT-α is unstable *in vitro* and is rapidly converted to PFT-β. Both of these compounds, at nontoxic doses, failed to protect two human wild-type p53 cancer cell lines from a variety of genotoxic insults. Moreover, the expression of several well-characterized p53-dependent genes was shown to be unaffected by PFT-α or -β following p53 stimulation. In addition, minimal effects were seen in NIH-3T3 mouse fibroblasts. Consequently, PFT-α and -β cannot be regarded as ubiquitous inhibitors of p53 function and caution should be exercised in using these agents as p53-specific tools.

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References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.

2. Kirsch DG, Kastan MB. Tumour-suppressor p53: implications for tumour development and prognosis. J Clin Oncol 1998;16:3158–68.

3. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science 1991;253:49–53.

4. Oren M. Regulation of the p53 tumour suppressor protein. J Biol Chem 1999;274:36031–4.

5. Giaccia AJ, Kastan MB. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev 1998;12:2973–83.

6. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307–10.

7. Rubbi CP, Milner J. p53: Guardian of a genome's guardian. Cell Cycle 2003;2:18–9.

8. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594–604.

9. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957–67.

10. El-Deiry WS. The role of p53 in chemosensitivity and radiosensitivity. Oncogene 2003;22:7486–95.

11. Fridman JS, Lowe SW. Control of apoptosis by p53. Oncogene 2003;22:9030–40.

12. O'Connor PM, Jackman J, Bae I, et al. Characterisation of the p53 tumour suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Res 1997;57:4285–300.

13. Fan S, El-Diery WS, Bae I, et al. P53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. Cancer Res 1994;54:5824–30.

14. Westphal CH, Hoyes KP, Canman CE, et al. Loss of atm radiosensitizes multiple p53 null tissues. Cancer Res 1998;58:5637–9.

15. Gudkov A, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. Nat Rev Cancer 2003;3:117–29.

16. D Ferber. A new way to combat therapy side effects. Science 1999;285:1651, 1653.

17. Komarov PG, Komarova EA, Kondratov RV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999;285:1733–7.

18. Zhu X, Yu Q-S, Cutler RG, et al. Novel p53 inactivators with neuroprotective action: syntheses and pharmacological evaluation of 2-imino-2,3,4,5,6,7-hexahydrobenzothiazole and 2-imino-2,3,4,5,6,7-hexahydrobenzoxazole derivatives. J Med Chem 2002;45:5090–7.

19. Kaji A, Zhang Y, Nomura M, et al. Pifithrin-α promotes p53-mediated apoptosis in JB6 cells. Mol Carcinog 2003;37:138–48.

[2] R. te Poele, M. Walton, and P. Workman, unpublished data.

20. Culmsee C, Zhu X, Yu Q-S, et al. A synthetic inhibitor of p53 protects neurons against death induced by ischaemic and excitotoxic insults, and amyloid β-peptide. J Neurochem 2001;77:220 – 8.

21. Brown R, Clugston C, Burns P, et al. Increased accumulation of p53 protein in cisplatin-resistant ovarian carcinoma cells. Int J Cancer 1993; 55:678 – 84.

22. Bhattacharyra AK. Interaction of bis(aryl formamidine) disulfides (salts) with ketones: formation of thiazoles, thiazines, and pyrimidine-thiones. Indian J Chem 1967;5:62 – 7.

23. Clegg W, Jamieson C. Pifithrin β. Acta Crystallogr 2005;E61:1486 – 8.

24. Skehan P, Storeng R, Scudiero D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107 – 12.

25. Wilson AP. Cytotoxicity and viability assays. In: Freshney IR, editor. Animal cell culture, a practical approach, 2nd ed. Oxford: Oxford University Press; 1992. p. 263 – 303.

26. Huang C, Ma W-Y, Maxiner A, Sun Y, Dong Z. p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. J Biol Chem 1999;274:12229 – 35.

27. Sanchez-Prieto R, Rojas JM, Taya Y, Gutkind JS. A role for the p38 mitogen-activated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. Cancer Res 2000;60:2464 – 72.

28. Kawabe T. G_2 checkpoint abrogators as anticancer drugs. Mol Cancer Ther 2004;3:513 – 9.

29. Dixon H, Norbury CJ. Therapeutic exploitation of checkpoint defects in cancer cells lacking p53 function. Cell Cycle 2002;1:362 – 8.

30. Rocha S, Campbell KJ, Roche KC, Perkins ND. The p53-inhibitor pifithrin-α inhibits firefly luciferase activity *in vivo* and *in vitro*. BMC Mol Biol 2003;4:9 – 17.

31. Komarova EA, Neznanov N, Komarov PG, Chernov MV, Wang K, Gudkov AV. p53 inhibitor pifithrin α can suppress heat shock and glucocorticoid signalling pathways. J Biol Chem 2003;278:15465 – 8.

32. Murphy PJM, Galigniana MD, Morishima Y, et al. Pifithrin-α inhibits p53 signaling after interaction of the tumor suppressor protein with hsp90 and its nuclear translocation. J Biol Chem 2004;279:30195 – 201.

33. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991;51:6304 – 11.

34. El-Diery WS, Harper JW, O'Connor PM, et al. WAF1/CIP1 is induced in p53-mediated G_1 arrest and apoptosis. Cancer Res 1994;54:1169 – 74.

35. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. Cell 1993;75:805 – 16.

36. Aherne W, Maloney A, Prodromou C, et al. Assays for HSP90 and inhibitors. In: Buolamwini JK, Adjei AA, editors. Methods in molecular medicine. Vol. 85, Novel anticancer drug protocols. Totowa, NJ: Humana Press Inc; 2003. p. 149 – 61.

37. Bulavin DV, Saito S, Hollander MC, et al. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J 1999;23:6845 – 54.

38. Keller D, Zeng X, Li X, et al. The p38MAPK inhibitor SB203580 alleviates ultraviolet-induced phosphorylation at serine 389 but not serine 15 and activation of p53. Biochem Biophys Res Commun 1999;261: 464 – 71.