

## Pifithrin- $\alpha$ Inhibits p53 Signaling after Interaction of the Tumor Suppressor Protein with hsp90 and Its Nuclear Translocation\*

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Pifithrin- $\alpha$  (PFT $\alpha$ ) was originally thought to be a specific inhibitor of signaling by the tumor suppressor protein p53. However, the laboratory that discovered pifithrin recently reported that the compound also inhibits heat shock and glucocorticoid receptor (GR) signaling, and they suggested that PFT $\alpha$  targets a factor common to all three signal transduction pathways, such as the hsp90/hsp70-based chaperone machinery (Komarova, E. A., Neznanov, N., Komarov, P. G., Chernov, M. V., Wang, K., and Gudkov, A. V. (2003) *J. Biol. Chem.* 278, 15465–15468). Because it is important for the mechanistic study of this machinery to identify unique inhibitors of chaperone action, we have examined the effect of PFT $\alpha$  on transcriptional activation, the hsp90 heterocomplex assembly, and hsp90-dependent nuclear translocation for both p53 and the GR. At concentrations where PFT $\alpha$  blocks p53-mediated induction of p21/Waf-1 in human embryonic kidney cells, we observed no inhibition of GR-mediated induction of a chloramphenicol acetyl transferase reporter in LMCAT cells. PFT $\alpha$  did, however, cause a left shift in the dexamethasone dose response curve by increasing intracellular dexamethasone concentration, apparently by competing for dexamethasone efflux from the cell. The assembly of p53 or GR heterocomplexes with hsp90 and immunophilins was not affected by PFT $\alpha$  either *in vivo* or *in vitro* and did not affect the nuclear translocation of either transcription factor. Thus, we conclude that PFT $\alpha$  does not inhibit GR-mediated induction or the function of the chaperone machinery, and, as originally thought, it may specifically inhibit p53 signaling by acting at a stage after p53 translocation to the nucleus.

The tumor suppressor protein p53 is a transcription factor that induces growth arrest or apoptosis in response to a variety of stress signals (1–3). The *p53* gene is lost or mutated in more than half of all human tumors (4), and inactivation of *p53* is the

most common alteration found in human cancer (5, 6). Activation of p53-mediated apoptosis in response to a stress, such as DNA damage, results in the elimination of potential tumor cell precursors. Thus, p53 is referred to as a tumor suppressor, and p53 knock-out mice have a high incidence of spontaneous tumors (7). Although p53-mediated apoptosis is important for tumor suppression, it may contribute to side effects of therapy such as myelosuppression (reviewed in Ref. 8).

It was with the intention of suppressing the side effects of cancer treatment that Komarov *et al.* (9) screened a library of 10,000 synthetic chemicals for inhibitors of p53-dependent apoptosis. A stable, water-soluble inhibitor of p53-dependent apoptosis was identified that was also shown to reduce the activation of p53-regulated genes, including cyclin G, *p21/Waf-1*, and *mdm2* (9). The compound was named pifithrin- $\alpha$  (PFT $\alpha$ ),<sup>1</sup> an abbreviation for p-fifty three inhibitor, and it was shown to protect mice from the lethal genotoxic stress associated with cancer treatment without promoting the formation of tumors (9). Subsequently, PFT $\alpha$  has been shown to protect against doxorubicin-induced apoptosis in mouse heart (10) and camptothecin-, ischemia-, and dopamine-induced apoptosis in neurons (11, 12), cisplatin-induced apoptosis in cochlear and vestibular hair cells (13), and endotoxin-induced apoptosis in liver tissue (14).

The p53 protein itself or perhaps factors required for p53-mediated transcriptional activation have been thought to be the molecular target of PFT $\alpha$ . However, Komarova *et al.* (15) recently published a report revealing that PFT $\alpha$  suppresses signaling through the heat shock transcription factor HSF1 and the glucocorticoid receptor (GR) but not signaling by NF- $\kappa$ B. This finding indicated that PFT $\alpha$  may not specifically target p53 but targets some unknown cellular component that is common for three major signal transduction pathways. Inasmuch as p53, HSF1, and the GR are all bound to and regulated by hsp90 (see Ref. 16 for review), it was suggested that heat shock proteins of the hsp90/hsp70-based machinery are obvious candidate targets for PFT $\alpha$  (15).

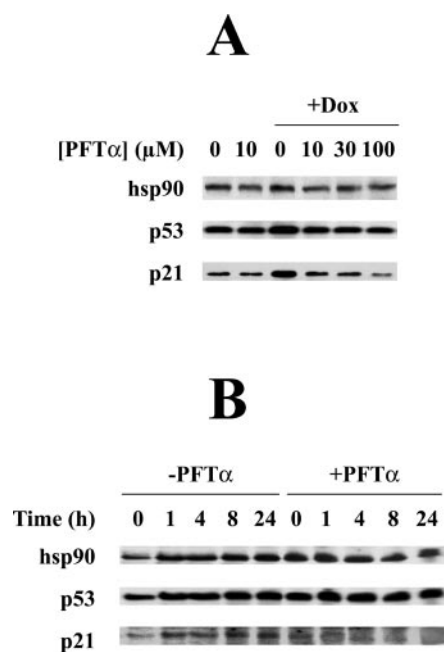
We have recently demonstrated that p53-hsp90 and GR-hsp90 complexes exist in identical large heterocomplexes containing dynein and one of three immunophilins, namely FKBP52, cyclophilin 40 (CyP-40), or PP5 (17). The association of either transcription factor with hsp90 is critical both for maintenance of its inactive state and for rapid translocation to

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<sup>1</sup> The abbreviations used are: PFT $\alpha$ , pifithrin- $\alpha$ ; Ab, antibody; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; FKBP, FK506-binding protein; GR, glucocorticoid receptor; HEK, human embryonic kidney; hsp, heat shock protein; LMCAT, L929 cells stably transfected with MMTV-CAT; MMTV, mouse mammary tumor virus; PP5, protein phosphatase 5; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

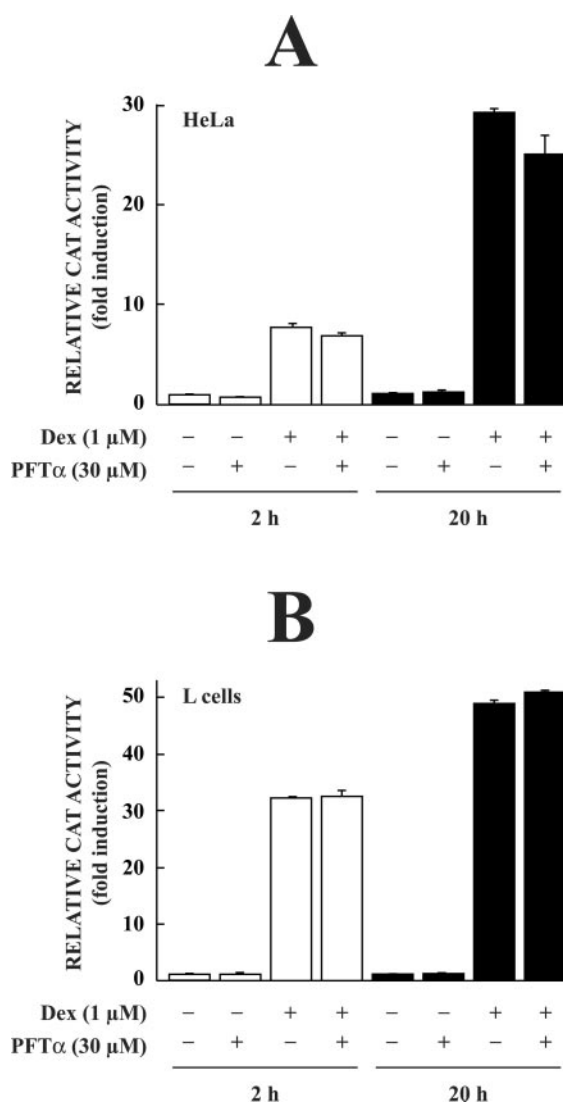


**FIG. 1. PFT $\alpha$  inhibits p53-mediated transcription in HEK cells.** *A*, exponentially growing HEK cells were incubated with 0–100  $\mu$ M PFT $\alpha$  for 4 h in the presence or absence of 500 nM doxorubicin (*Dox*). Cells were harvested, and whole cell cytosols were prepared. Hsp90, p53, and p21/Waf-1 were resolved by electrophoresis and immunoblotting. *B*, exponentially growing HEK cells were incubated for 1–24 h with 500 nM doxorubicin in the presence (+) or absence (–) of 30  $\mu$ M PFT $\alpha$ . Cells were harvested, and whole cell cytosols were prepared. Hsp90, p53, and p21 were resolved by electrophoresis and immunoblotting as in *panel A*.

the nucleus after its activation by stress or steroid (16–19). Because it would be very useful to have additional inhibitors of the hsp90/hsp70-based chaperone machinery, we have tested the proposal of Komarova *et al.* (15) by examining PFT $\alpha$  effects on p53-mediated and GR-mediated transcriptional activation, the formation of heterocomplexes with hsp90 and immunophilins, and the nuclear movement of the two transcription factors. We find that, at concentrations where PFT $\alpha$  abrogates p53-dependent induction of p21/Waf-1, there is no inhibition of dexamethasone-dependent activation from a GR-dependent reporter construct. However, PFT $\alpha$  did cause a left shift in the dose response curve of dexamethasone, apparently by competing for dexamethasone efflux from the cell. PFT $\alpha$  did not affect assembly of either p53-hsp90-immunophilin or GR-hsp90-immunophilin complexes *in vivo* or *in vitro*, and it did not affect nuclear translocation of either transcription factor. Our observations indicate that PFT $\alpha$  may very well be a specific inhibitor of p53 signaling that does not affect the hsp90/hsp70-based chaperone machinery and that inhibits p53 function at a stage after its translocation to the nucleus.

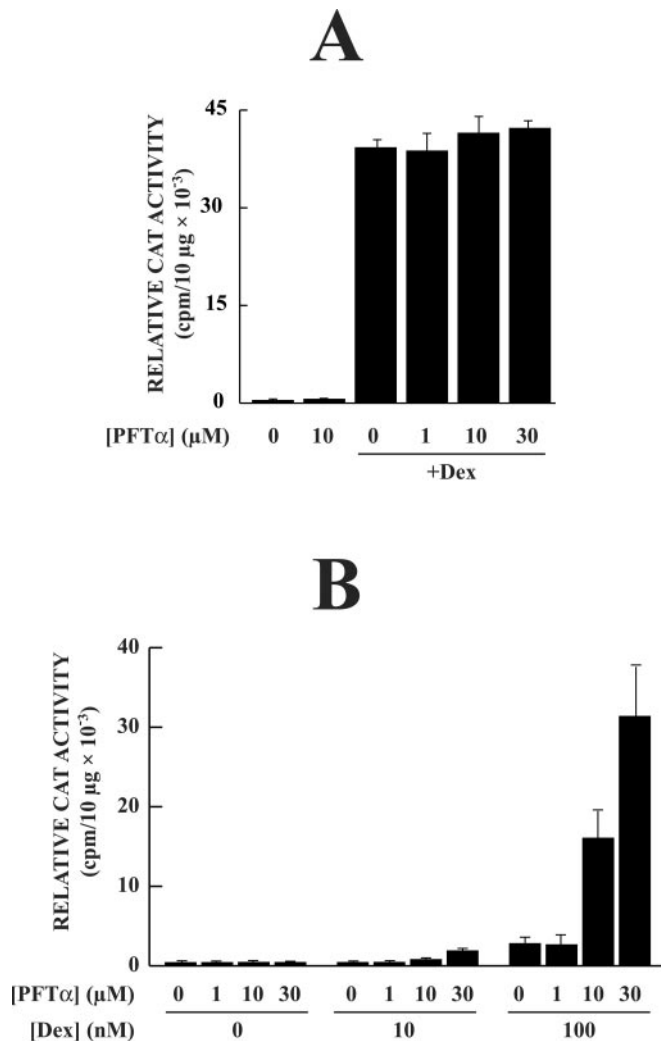
#### EXPERIMENTAL PROCEDURES

**Materials**—PFT $\alpha$  (1-(4-methylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone hydrobromide) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). PFT $\alpha$  and the cyclic PFT $\alpha$  analog were also purchased from Alexis Biochemicals (San Diego, CA), and similar results to those seen with PFT $\alpha$  from Biomol were observed. Untreated rabbit reticulocyte lysate was purchased from Green Hectares (Oregon, WI). [6,7- $^3$ H]Dexamethasone (40 Ci/mmol), [ring-3,5- $^3$ H]chloramphenicol (38 Ci/mmol), and  $^{125}$ I-conjugated goat anti-mouse and goat anti-rabbit IgGs were obtained from PerkinElmer Life Sciences (Boston, MA). Protein A-Sepharose, doxorubicin, nonradiolabeled dexamethasone, charcoal-stripped calf serum, pristane, mixed xylenes, and goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated antibodies were from Sigma. Dulbecco's modified Eagle's medium (DMEM) was from Bio-Whittaker (Walkersville, MD). Rhodamine-conjugated donkey anti-mouse IgG was



**FIG. 2. PFT $\alpha$  does not affect GR-mediated transcription from a transiently transfected reporter in HeLa or L929 cells.** *A*, transcription in HeLa cells. HeLa cells transiently transfected with MMTV-CAT reporter plasmid as described under "Experimental Procedures" were treated for 2 (*open bars*) or 20 h (*solid bars*) with vehicle, 30  $\mu$ M PFT $\alpha$ , 1  $\mu$ M dexamethasone (*Dex*), or dexamethasone and PFT $\alpha$ . The data represent the mean  $\pm$  S.E. of three samples expressed as fold induction over the vehicle-treated sample set at 1. *B*, transcription in L cells. L cells transiently transfected with MMTV-CAT were treated as described above.

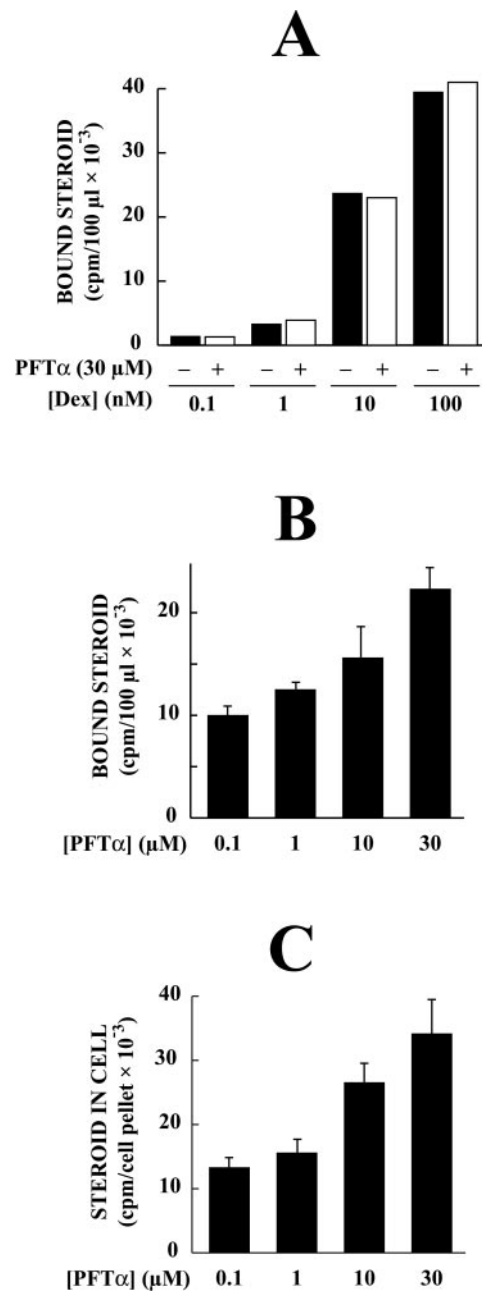
from Jackson ImmunoResearch (West Grove, PA). The BuGR2 monoclonal IgG used to immunoblot the GR and the rabbit polyclonal antibody against cyclophilin 40 were from Affinity Bioreagents (Golden, CO). The AC88 monoclonal IgG against hsp90 was from StressGen Biotechnologies (Victoria, British Columbia, Canada). The anti-p21/Waf-1 monoclonal antibody (Ab-1) was from Oncogene Research Products (San Diego, CA). The Ab421 pan-specific mouse monoclonal antibody used to immunoadsorb p53 was kindly provided by Dr. Michael Clarke (University of Michigan Medical School). The Ab-4 mouse monoclonal IgG used for localization of p53 by indirect immunofluorescence and the Ab-7 sheep antiserum used to immunoblot p53 were purchased from Calbiochem. The FiGR monoclonal IgG used to immunoadsorb the GR was generously provided by Dr. Jack Bodwell (Dartmouth Medical School, Lebanon, NH). The UPJ56 rabbit antiserum against FKBP52 was provided by Dr. Karen Leach (Pfizer) and the rabbit antiserum against PP5 was provided by Dr. Michael Chinkers (University of South Alabama, Mobile, AL). Rhodamine-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch. The mouse mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter plasmid and the mouse fibroblast L929 cell line stably transfected with MMTV-CAT (LMCAT cells) were provided by Dr. Edwin Sanchez (Medical College of



**FIG. 3. Effect of PFT $\alpha$  on GR-mediated transcription from a stably transfected reporter by receptor saturating and subsaturating concentrations of steroid.** **A**, PFT $\alpha$  does not affect transcription at a GR saturating concentration of dexamethasone (*Dex*). Mouse fibroblast cells stably transfected with the MMTV-CAT reporter plasmid (LMCAT cells) were incubated with 0–30  $\mu$ M PFT $\alpha$  for 20 h in the presence or absence of 1  $\mu$ M dexamethasone. Cells were washed in PBS and harvested by cell scraping. Whole cell cytosols were prepared and assayed for expression of the translated reporter gene by assaying CAT activity. **B**, PFT $\alpha$  potentiates GR-mediated transcription at subsaturating concentrations of dexamethasone. LMCAT cells were incubated with 0–30  $\mu$ M PFT $\alpha$  for 2 h in the presence of 0–100 nM dexamethasone. Whole cell cytosols were assayed for expression of the translated reporter gene by assaying CAT activity.

Ohio, Toledo, OH). DLD-1 human colon adenocarcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). HT29-tsp53 (formerly referred to as ts29-G cells) human colorectal cancer cells overexpressing a temperature-sensitive mutant of mouse p53 were described previously (20, 21).

**Cell Culture and Cytosol Preparation**—Cultures of mouse fibroblast L929 cells, L929 cells stably transfected with an MMTV-CAT reporter plasmid (LMCAT cells), HeLa cells, human embryonic kidney (HEK) cells, and NIH 3T3 mouse fibroblast cells, all expressing wild-type p53, were cultured in DMEM supplemented with 10% bovine calf serum. DLD-1 cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1.5 g/liter NaHCO<sub>3</sub>, 4.5 g/liter glucose, and 1 mM pyruvate. Cells were harvested by scraping into Hanks' buffered saline solution and centrifugation. Cell pellets were washed in Hanks' buffered saline solution, resuspended in 1.5 volumes of HEM buffer (10 mM NaOH-Hepes, 1 mM EDTA, and 20 mM sodium molybdate, pH 7.4) with 1 mM phenylmethylsulfonyl fluoride and 1 tablet of Complete-Mini protease inhibitor mixture (Roche Applied Science) per 3 ml of buffer and ruptured by Dounce homogenization. The lysate was then centrifuged at 100,000  $\times g$  for 30 min, and the supernatant, referred to as



**FIG. 4. Effect of PFT $\alpha$  on steroid binding.** **A**, PFT $\alpha$  does not affect steroid binding *in vitro*. Cytosol from LMCAT cells was incubated overnight on ice with radiolabeled dexamethasone (*Dex*) in the presence (+) or absence (-) of 30  $\mu$ M PFT $\alpha$ . Steroid binding was assayed by charcoal assay, and specific binding activity was calculated by subtracting the activity of a duplicate sample incubated with 1,000-fold excess non-radioactive dexamethasone. **B**, PFT $\alpha$  treatment increases steroid binding to GRs *in vivo*. LMCAT cells were incubated for 2 h with 100 nM [<sup>3</sup>H]dexamethasone in the presence of 0–30  $\mu$ M PFT $\alpha$  and the presence or absence of a 1,000-fold excess of non-radioactive dexamethasone. Cytosols were prepared, and steroid binding was determined by charcoal assay. **C**, PFT $\alpha$  treatment increases [<sup>3</sup>H]dexamethasone retention in cells. LMCAT cells were incubated for 2 h with 100 nM [<sup>3</sup>H]dexamethasone in the presence of 0–30  $\mu$ M PFT $\alpha$ , and the amount of radioactivity in washed cell pellets was assayed.

“cytosol,” was collected, aliquoted, flash-frozen, and stored at  $-70^{\circ}\text{C}$ . Mouse GR was expressed in Sf9 cells, and cytosol was prepared as described previously (22).

**Indirect Immunofluorescence of GR and p53**—NIH 3T3 cells were grown on 11  $\times$  22 mm coverslips placed in 35-mm culture wells in 2 ml of DMEM supplemented with bovine calf serum. When the cells were ~50% confluent, the culture medium was replaced with phenol red-free DMEM supplemented with 10% charcoal-stripped calf serum, and the

cells were grown for an additional 24 h. Cells were incubated with 0.05% Me<sub>2</sub>SO or 100 μM PFTα for 2 h prior to the addition of 0.1% ethanol or 1 μM dexamethasone for 10 min to permit nuclear translocation of the GR. HT29-tsp53 cells were grown at 39 °C (non-permissive temperature) on coverslips placed in 35-mm culture wells in 2 ml of DMEM containing 10% bovine calf serum and 2 mM L-glutamine. Nuclear translocation of p53 was triggered by reducing the incubation temperature of HT29-tsp53 cells from 39 to 32 °C. Cells were fixed and permeabilized by immersion in cold (−20 °C) methanol and immunostained by inverting the coverslip on a 50-μl solution of phosphate-buffered saline with 1% bovine serum albumin containing 1 μl of the Ab-4 mouse monoclonal IgG against p53 or 1 μl of FiGR mouse monoclonal IgG overnight at 4 °C in a humid chamber. The coverslips were incubated with a washing buffer (20 mM Tris-HCl, pH 8, 630 mM NaCl, 0.05% Tween 20, and 1% bovine serum albumin) for 30 min at room temperature, and reincubated with 1:100 dilution of rhodamine-conjugated donkey anti-mouse IgG counter-antibody for 2 h. Coverslips were

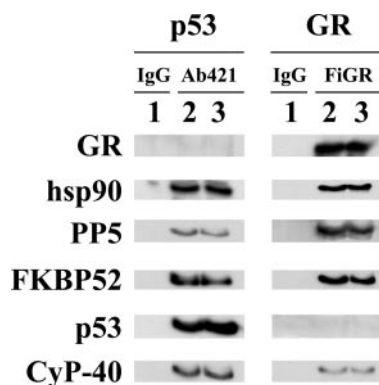


FIG. 5. PFTα does not affect the composition of p53 or GR heterocomplexes formed in cells. DLD-1 cells were incubated for 12 h with (lane 3) or without (lanes 1 and 2) 30 μM PFTα; cytosol was prepared and immunoadsorbed with non-immune IgG (lane 1, IgG) or with antibody against p53 (Ab421) or the GR (FiGR) (lanes 2 and 3). The immunopellets were washed, and proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by immunoblotting for the indicated proteins.

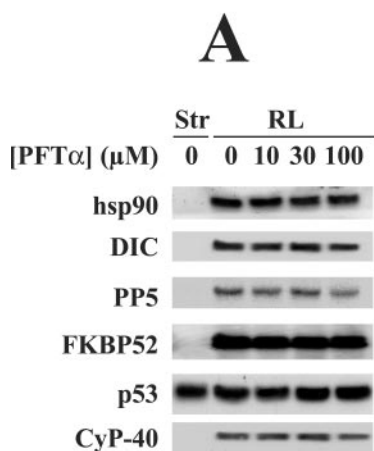


FIG. 6. PFTα does not affect cell-free assembly of p53 or GR heterocomplexes with hsp90 and immunophilins. Replicate immunopellets of p53 (A) or GR (B) were stripped of associated chaperones, and the stripped immune pellets (Str) were incubated for 20 min at 30 °C with reticulocyte lysate (RL), an ATP-regenerating system, 20 mM molybdate, and various concentrations of PFTα. Proteins in the washed immune pellets were resolved by electrophoresis and immunoblotting. Replicate GR immune pellets were incubated with [<sup>3</sup>H]dexamethasone to determine steroid binding activity. Lane 1 (counting from the left), stripped immune pellet; lanes 2–5, stripped pellet incubated with reticulocyte lysate and Me<sub>2</sub>SO vehicle (lane 2) or 10 (lane 3), 30 (lane 4), or 100 μM PFTα (lane 5).

rinsed in washing buffer for 30 min and mounted on microscope slides using a drop of mounting medium (1 mg/ml phenylenediamine in 10% phosphate-buffered saline and 90% glycerol, pH 9).

Cells were observed with a Leitz Aristoplan epi-illumination microscope and scored for GR or p53 translocation as described previously (23) using a score of 4 for nuclear fluorescence much greater than cytoplasmic fluorescence, 3 for nuclear fluorescence greater than cytoplasmic fluorescence, 2 for nuclear fluorescence equal to cytoplasmic fluorescence, 1 for nuclear fluorescence less than cytoplasmic fluorescence, and 0 for nuclear fluorescence much less than cytoplasmic fluorescence. The translocation scores represent the mean ± S.E. of three experiments in which ≥ 50 cells per condition per experiment were counted.

**Transient Transfection of MMTV-CAT Reporter**—L929 or HeLa cells were grown as monolayer cultures in 35-mm culture wells to ~50% confluency, washed, and incubated for 1 h with 1 ml of serum-free medium containing 5 μg of plasmid DNA and 15 μl of TransFast transfection reagent (Promega). The transfection medium was replaced with regular medium, and the cells were incubated for 48 h. During the incubation, cells were treated for 2 or 20 h with dexamethasone or PFTα or both as indicated in the legend of Fig. 2.

**GR-mediated Transcriptional Activation**—Dexamethasone-induced CAT gene expression was assayed by measuring CAT enzymatic activity in HeLa, L cell, and LMCAT cell cytosol, using a modified version of the CAT assay described in Kwok *et al.* (24). LMCAT cells were grown in 35-mm culture wells to ~50% confluence and incubated with varying concentrations of dexamethasone and PFTα for up to 24 h. Cells were washed, harvested, resuspended in potassium phosphate buffer (100 mM potassium phosphate and 1 mM dithiothreitol, pH 7.8), and ruptured by exposing the cell suspensions to three freeze-thaw cycles. Cell suspensions were centrifuged at 18,000 × g for 10 min, and the protein concentration of the supernatants was measured by a Bradford assay. Aliquots of the supernatants containing 10 μg of total protein were incubated for 15 min at 70 °C in 150 mM Tris-HCl buffer, pH 7.4. The aliquots were added to a CAT reaction mixture (50 nM purified [<sup>3</sup>H]chloramphenicol, 150 mM Tris-HCl, pH 7.4, and 0.25 mM butyryl CoA) and incubated for 2 h at 37 °C. An organic phase mixture consisting of 2 parts pristane and 1 part mixed xylenes was added and samples were thoroughly subjected to a vortex. The reaction mixture was centrifuged at 20,000 × g for 10 min, and 150 μl of the organic phase was counted by liquid scintillation spectrometry.

**Immunoadsorption of GR and p53**—Receptors were immunoad-

## B

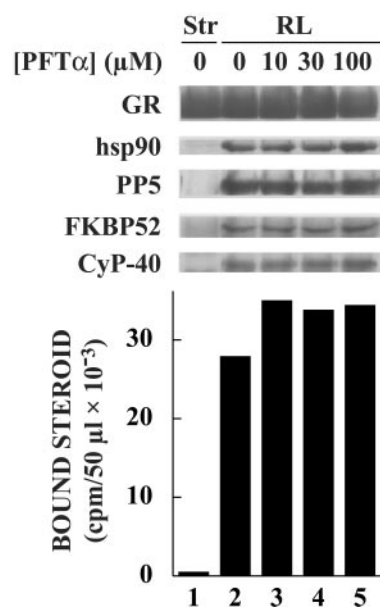
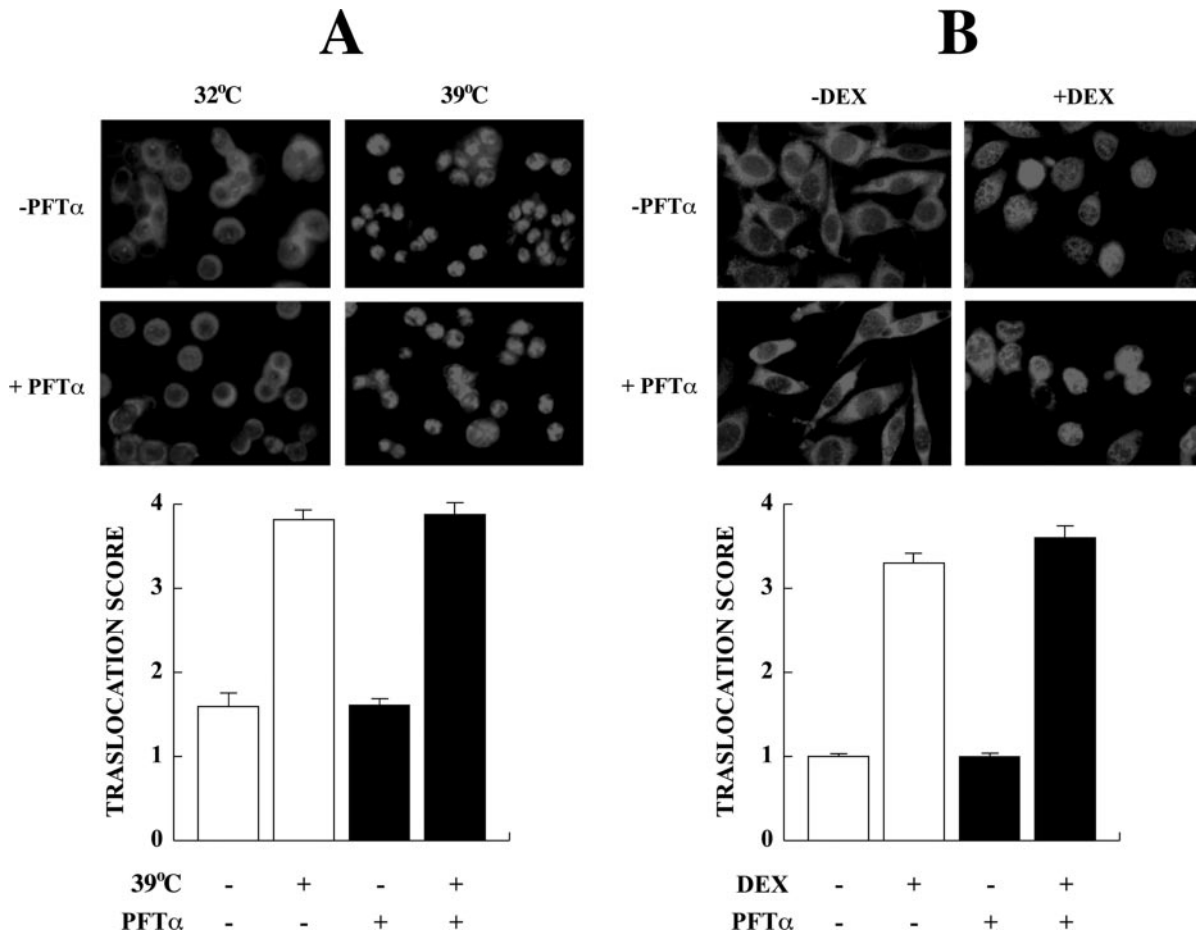


FIG. 6. PFTα does not affect cell-free assembly of p53 or GR heterocomplexes with hsp90 and immunophilins. Replicate immunopellets of p53 (A) or GR (B) were stripped of associated chaperones, and the stripped immune pellets (Str) were incubated for 20 min at 30 °C with reticulocyte lysate (RL), an ATP-regenerating system, 20 mM molybdate, and various concentrations of PFTα. Proteins in the washed immune pellets were resolved by electrophoresis and immunoblotting. Replicate GR immune pellets were incubated with [<sup>3</sup>H]dexamethasone to determine steroid binding activity. Lane 1 (counting from the left), stripped immune pellet; lanes 2–5, stripped pellet incubated with reticulocyte lysate and Me<sub>2</sub>SO vehicle (lane 2) or 10 (lane 3), 30 (lane 4), or 100 μM PFTα (lane 5).



**FIG. 7. PFT $\alpha$  does not affect nuclear translocation of p53 or the GR.** *A*, p53 translocation. HT29-tsp53 colon cancer cells were preincubated for 1 h in the presence of 100  $\mu$ M PFT $\alpha$  (+PFT $\alpha$ ) or Me<sub>2</sub>SO vehicle (-PFT $\alpha$ ), and p53 translocation to the nucleus was triggered by shifting the temperature from 39 to 32 °C. After 1 h at 32 °C, the cells were fixed and permeabilized and immunostained for p53. *B*, GR translocation. NIH 3T3 mouse fibroblasts were pretreated with 100  $\mu$ M PFT $\alpha$  or with Me<sub>2</sub>SO vehicle for 1 h and then incubated with (+DEX) or without (-DEX) 1  $\mu$ M dexamethasone for 10 min to allow nuclear translocation. Cells were fixed, and GR was visualized by immunostaining. The graphs show data compiled from  $\geq$  50 cells for each condition scored for nuclear translocation on a scale of 0 for completely cytoplasmic fluorescence to 4 for completely nuclear fluorescence. Bars represent the average score  $\pm$  S.E. for each condition.

sorbed from aliquots of 50 (for measuring steroid binding) or 100  $\mu$ l (for Western blotting) of Sf9 cell cytosol by rotation for 2 h at 4 °C with 18  $\mu$ l of protein A-Sepharose precoupled to 9  $\mu$ l of FiGR ascites suspended in 200  $\mu$ l of TEG buffer (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, and 10% glycerol). p53 was immunoadsorbed from 250- $\mu$ l aliquots of DLD-1 cytosol with 10  $\mu$ l of Ab421 antibody. Prior to incubation with reticulocyte lysate, immunoadsorbed p53 and GR were stripped of associated hsp90 by incubating the immunopellet for an additional 2 h at 4 °C with 350  $\mu$ l of 0.5 M NaCl in TEG buffer. The pellets were then washed once with 1 ml of TEG buffer followed by a second wash with 1 ml of Hepes buffer (10 mM Hepes, pH 7.4).

**GR-hsp90 and p53-hsp90 Heterocomplex Reconstitution**—Immunopellets containing GR or p53 stripped of chaperones were incubated with reticulocyte lysate and 5  $\mu$ l of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase). The assay mixtures were incubated for 20 min at 30 °C with suspension of the pellets by shaking the tubes every 2 min. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEG with 20 mM sodium molybdate) and assayed for steroid binding capacity and for GR- or p53-associated proteins.

**Assay of Steroid Binding Capacity**—For measuring hormone retention in intact cells, exponentially growing LMCAT cells ( $\sim 2 \times 10^6$  cells/25 cm<sup>2</sup> flask) were incubated with 100 nM [<sup>3</sup>H]dexamethasone at 37 °C for 2 h in the absence or presence of 1,000-fold excess unlabeled dexamethasone and the presence of Me<sub>2</sub>SO or PFT $\alpha$ . Cells were washed, harvested, and suspended in cold phosphate-buffered saline and counted by liquid scintillation spectrometry. Specific binding activity was calculated by subtracting the radioactivity in the cells incubated in the presence of excess unlabeled dexamethasone.

Immune pellets to be assayed for steroid binding were incubated

overnight at 4 °C in 50  $\mu$ l of HEM buffer plus 100 nM [<sup>3</sup>H]dexamethasone. Samples were then washed three times with 1 ml of TEGM buffer and counted by liquid scintillation spectrometry. The steroid binding is expressed as counts/min of [<sup>3</sup>H]dexamethasone bound/FiGR immunopellet prepared from 50  $\mu$ l of cytosol.

For cytosols to be assayed for steroid binding, a 50  $\mu$ l aliquot of cytosol was incubated overnight at 4 °C in 50  $\mu$ l of HEM buffer with 100 nM [<sup>3</sup>H]dexamethasone plus or minus a 1,000-fold excess of non-radioactive dexamethasone. Samples were mixed with dextran-coated charcoal, centrifuged, and counted by liquid scintillation spectrometry. The steroid binding is expressed as counts/min of bound [<sup>3</sup>H]dexamethasone/100  $\mu$ l of cell cytosol.

**Gel Electrophoresis and Western Blotting**—Immune pellets were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were probed with 0.1% Ab-7 for p53, 0.25  $\mu$ g/ml BuGR2 for GR, 1  $\mu$ g/ml AC88 for hsp90, 1  $\mu$ g/ml anti-p21/Waf-1, 0.1% UPJ56 for FKBP52, 0.1% anti-cyclophilin 40, or 0.1% anti-PP5. The immunoblots were then incubated a second time with the appropriate <sup>125</sup>I-conjugated or horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands. p53 was revealed by enhanced chemiluminescence. Because PP5, FKBP52, and p53 migrate in the same region upon gel electrophoresis, we electrophoresed replicate samples of both non-immune and immune pellets and probed replicate immunoblots with an antibody specific for each protein. Thus, the Western blots of Figs. 5 and 6 are necessarily composites prepared from two or more replicate immunoblots.

## RESULTS AND DISCUSSION

**PFT $\alpha$  Effect on p53- and GR-mediated Transcription**—To confirm the activity of the PFT $\alpha$  preparation as an inhibitor of

p53 signaling, HEK cells were treated with doxorubicin to cause DNA damage and activate p53. As shown in Fig. 1A, doxorubicin treatment causes an increase in the amount of p21/Waf-1 that is prevented by 10  $\mu\text{M}$  PFT $\alpha$ . The PFT $\alpha$  inhibition of p21/Waf-1 induction is sustained over 24 h of doxorubicin treatment (Fig. 1B).

To determine the effect of PFT $\alpha$  on GR-mediated transcription, HeLa cells (Fig. 2A) or L929 mouse fibroblasts (Fig. 2B) were transiently transfected with an MMTV-CAT reporter plasmid and treated for 2 or 20 h with 1  $\mu\text{M}$  dexamethasone or 30  $\mu\text{M}$  PFT $\alpha$  or both prior to the assay of CAT activity. In both cell lines, there was a robust induction of CAT by dexamethasone that was not affected by PFT $\alpha$ . To further test any possible effect of PFT $\alpha$  on dexamethasone-dependent induction, we used a subline of L929 mouse fibroblasts stably transfected with an MMTV-CAT reporter plasmid (LMCAT cells) that was developed by Ning and Sanchez (25). When the LMCAT cells are treated with dexamethasone, there was an induction of CAT driven by the endogenous L cell GR. As shown in Fig. 3A, PFT $\alpha$  did not inhibit GR-mediated CAT induction when cells were treated with a high concentration (1  $\mu\text{M}$ ) of dexamethasone. At lower concentrations of dexamethasone and a short induction time, PFT $\alpha$  stimulated dexamethasone-dependent CAT induction (Fig. 3B).

*PFT $\alpha$  Increases the Concentration of Dexamethasone in Cells*—Potentiation of a dexamethasone response like that shown in Fig. 3B has been reported with immunosuppressant drugs (reviewed in Ref. 26). It is known that both dexamethasone and the immunosuppressant drugs, such as FK506, are transported out of the cell by the multidrug transporter Mdr1, and Kralli and Yamamoto (27) showed that FK506 potentiates dexamethasone responsiveness in L cells by increasing dexamethasone accumulation without altering the hormone binding properties of the GR. Here, we show that PFT $\alpha$  did not affect the binding activity of the GR when it was added to cytosol (Fig. 4A), but treatment of LMCAT cells with PFT $\alpha$  increased the amount of [<sup>3</sup>H]dexamethasone bound to GRs *in vivo* (Fig. 4B). As shown in Fig. 4C, treatment with PFT $\alpha$  increased the amount of [<sup>3</sup>H]dexamethasone retained in the cell. This increase in intracellular dexamethasone is likely due to PFT $\alpha$  competition for efflux in the same manner as that seen with the immunosuppressant drugs, and it forms the basis for a potential drug interaction.

*PFT $\alpha$  Does Not Inhibit Assembly of Heterocomplexes with hsp90 and Immunophilins*—To examine the effects of PFT $\alpha$  on the assembly of p53-hsp90-immunophilin complexes *in vivo*, we selected the DLD-1 human colorectal cancer cell line, which possesses a point mutation that converts serine 241 of p53 to phenylalanine (28). This mutant p53 is localized in the cytoplasm where it exists in cytosolic heterocomplexes with hsp90 (29). We have shown previously that the p53-hsp90 heterocomplexes contain the immunophilins that are present in GR-hsp90 heterocomplexes (17). Fig. 5 is an immunoblot of hsp90 and the tetratricopeptide repeat domain immunophilins co-immunoadsorbed with p53 or the GR from cytosol prepared from untreated DLD-1 cells (Fig. 5, lanes 2) or DLD-1 cells treated with PFT $\alpha$  (lanes 3). In neither case did PFT $\alpha$  affect heterocomplex formation *in vivo*.

Heterocomplexes with hsp90 and the tetratricopeptide repeat domain immunophilins that bind hsp90 can be assembled *in vitro* by incubating immunoadsorbed p53 or GR that has been stripped of endogenous bound chaperones with rabbit reticulocyte lysate. Fig. 6 presents immunoblots of hsp90 and immunophilins that associate with p53 (Fig. 6A) and the GR (Fig. 6B) during such cell-free heterocomplex assembly. It is clear that PFT $\alpha$  did not affect assembly in either case. When

the GR is assembled into GR-hsp90 heterocomplexes, it is converted from a non-steroid binding state to a steroid binding state, and, as shown in the bar graph in Fig. 6B, the generation of steroid binding activity was not affected by pifithrin.

*PFT $\alpha$  Does Not Inhibit Nuclear Translocation of p53 or GR*—Both p53 and the GR utilize an hsp90-dependent movement system for translocation to the nucleus along microtubular tracts driven by the cytoplasmic dynein motor protein (17–19, 23, 30, and reviewed in Ref. 31). In the movement system, the immunophilins bind via their tetratricopeptide repeat domain to hsp90 and via their peptidylprolyl isomerase domain to the dynamitin component of the dynein-associated dynactin complex. Thus, rapid nuclear translocation of either the GR or p53 requires dynamic assembly of heterocomplexes with hsp90, and movement is impeded by geldanamycin and radicicol, which inhibit hsp90 heterocomplex assembly (17, 18, 23). Nuclear translocation of p53 or the GR is also inhibited when immunophilin binding to dynein is competed by expression of a peptidylprolyl isomerase domain fragment or when dynein linkage to cargo is dissociated by the expression of dynamitin (17, 18, 30).

To examine the movement of p53 from the cytoplasm to the nucleus, we chose HT29-tsp53 cells, a stable human colon carcinoma cell line expressing a temperature-sensitive allele of murine p53 (20). This temperature-sensitive mutant of p53 is fully active and nuclear at the permissive temperature of 32 °C, but it is inactive and located in the cytoplasm at the non-permissive temperature of 39 °C (32–34). When the incubation temperature of HT29-tsp53 cells is shifted from 39 to 32 °C, p53 translocates to the nucleus, with 1 h being required for complete translocation (17). As shown in Fig. 7A, nuclear translocation of p53 was not affected by 100  $\mu\text{M}$  PFT $\alpha$ . We routinely examine GR translocation in 3T3 mouse fibroblasts, where the receptor translocates to the nucleus within 10 min after the addition of steroid (18). As shown in Fig. 7B, dexamethasone-dependent GR translocation was not affected by 100  $\mu\text{M}$  PFT $\alpha$ .

*Status of Pifithrin Inhibition of Transcription*—The data of this paper differ from the observations of Komarova *et al.* (15) in that we do not see PFT $\alpha$  inhibition of GR-dependent induction. Inasmuch as we have exposed cells to much higher concentrations of PFT $\alpha$ , we do not know the basis for the discrepancy. The notion of Komarova *et al.* (15) that PFT $\alpha$  might inhibit the chaperone machinery that is common to p53, GR, and HSF1 signaling (15) is intriguing and well worth testing. Here, we do not find PFT $\alpha$  inhibition of hsp90 heterocomplex assembly with p53 or the GR, inhibition of functional opening of the steroid binding cleft in the GR, or inhibition of the nuclear transfer of either transcription factor. The absence of PFT $\alpha$  inhibition shows that PFT $\alpha$  must be inhibiting some step in the induction mechanism after p53 transfer to the nucleus. Because, in our hands, induction by the GR is not affected, we would suggest that PFT $\alpha$  is not inhibiting a coactivator common to both p53-dependent and GR-dependent responses.

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