P53 Inhibitor Pifithrin α Can Suppress Heat Shock and Glucocorticoid Signaling Pathways*

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Pifithrin α (PFTα) is a chemical compound isolated for its ability to suppress p53-mediated transactivation. It can protect cells from p53-mediated apoptosis induced by various stimuli and reduce sensitivity of mice to gamma radiation. Identification of molecular targets of PFTα is likely to provide new insights into mechanisms of regulation of p53 pathway and is important for predicting potential risks associated with administration of PFTα-like p53 inhibitors in vivo. We found that PFTα, in addition to p53, can suppress heat shock and glucocorticoid receptor signaling but has no effect on nuclear factor-κB signaling. PFTα reduces activation of heat shock transcription factor (HSF1) and increases cell sensitivity to heat. Moreover, it reduces activation of glucocorticoid receptor and rescues mouse thymocytes in vitro and in vivo from apoptotic death after dexamethasone treatment. PFTα affected both signaling pathways in a p53-independent manner. These observations suggest that PFTα targets some unknown factor that is common for three major signal transduction pathways.

Based on the analysis of p53-dependent effects caused by ionizing radiation and chemotherapeutic drugs in mice, p53-mediated apoptosis was defined as a determinant of organism sensitivity to systemic genotoxic stress associated with cancer treatment (1). Temporary reversible pharmacological suppression of p53 was suggested as an approach to reduce cancer treatment side effects. This hypothesis was supported by isolation of a small molecule inhibitor of p53, pifithrin α (PFTα)1 that was capable of rescuing mice from lethal genotoxic stress caused by gamma radiation (2). Furthermore, inhibition of p53 was suggested as a therapeutic approach to treatment of other pathological conditions associated with p53 activation (3), some of which have already been experimentally confirmed. Thus, PFTα was shown to protect neurons from death induced by DNA-damaging agents, hypoxia and dopamine (4, 5); it had therapeutic effects in animal models of Parkinson disease (6) and acute renal failure (7). In all these works, biological effects of PFTα were attributed to its anti-p53 function, although not in all of them has this conclusion been confirmed by genetic approaches. Accurate interpretation of biological effects of PFTα requires identification of its molecular target(s) and determination of molecular mechanisms of its activity. PFTα was isolated by screening of the National Cancer Institute/SIAT Cell Line Library in a cell-based readout system for its ability to reduce p53-dependent transactivation (2). This biological effect could be reached by affecting p53 pathway at numerous points and therefore PFTα could act by targeting one of numerous factors cooperating with p53 function. Biological effects of PFTα on p53 pathway suggested that it acted by interfering with nuclear accumulation of p53 (2). Many transcription factors involved in other signal transduction pathways have the same principles of regulation as p53: after activation in cytoplasm they are translocated to the nucleus, followed by modulation of transcription of the target genes. We, therefore, were interested to test whether PFTα would have an effect on other signal transduction pathways besides p53. We found that, in fact, PFTα can also interfere with heat shock (HS) and glucocorticoid receptor (GR) signaling but shows no effect on the activity of NF-κB. This finding indicates that PFTα is not solely specific to p53 and presumably targets some unknown cellular component that is common for three major signal transduction pathways.

EXPERIMENTAL PROCEDURES

Reagents—1-(4-Methylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-9H2B)-benzothiazolylmethanone hydrobromide, molecular weight 367 (known as PFTα), was provided by Chenbridge Corporation (San Diego, CA) and stored as 10 mM Me2SO frozen solution at −70 °C. TNF, cycloheximide (CHI) and dexamethasone (Dex) were purchased from Sigma and used at a concentration of 1.5 ng/ml, 1 μg/ml, and 0.5–2 μM, respectively.

Cell Lines and Animals—Mouse fibroblast cell line ConA carries the wild type p53 gene and the bacterial lacZ reporter gene under the control of a p53-responsive promoter (2). Two isogenic human colon cancer cell lines HCT116 p53 (wt-p53) and its p53-deficient derivative, developed from the parental cell line by targeted homologous recombination (8), were provided by I. Roninson (University of Illinois at Chicago). HeLa cells and human prostate cancer cell line PC3 (p53-deficient) were purchased from ATCC. Short term cultures of primary thymocytes were prepared from the thymus of 4-week-old C57BL/6 mice (wild type and p53-deficient), which were purchased from Jackson Laboratory (Bar Harbor, ME).

Cell Viability Assay—At the end of cell treatments, the number of attached cells was estimated by staining with 0.25% crystal violet in 50% methanol, followed by elution of the dye with 1% SDS. Optical density (530 nm) reflecting the number of stained cells was determined with a Bio-Tek EL311 microplate reader. Cell viability in suspension of short term culture of primary thymocytes was determined by their staining with 0.1% of methyl blue and microscopic counting of blue (dead) cells.

Gel Shift Assay—Gel shift assay was performed as described earlier (9). Nuclear and total cellular extracts were prepared from untreated or

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‡ The abbreviations used are: PFTα, pifithrin-α; TNF, tumor necrosis factor; CHI, cycloheximide; HS, heat shock; HSF, heat shock transcription factor; HSP, heat shock protein; Dex, dexamethasone; ConA, concanavalin A; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; NF, nuclear factor; LTR, long terminal repeat; MMTV, murine mammary tumor virus; CAT, chloramphenicol acetyltransferase.
HS-treated (30 min, 42 °C) ConA cells with and without PFTα (10 μM), or HeLa cells, untreated and treated with TNF (1.5 ng/ml) in the presence or in the absence of 15 μM of PFTα or Dex (0.05–0.1 μM) for 4 h. Labeled double-stranded oligonucleotides, corresponding to the sequences of the HSF1-binding site in HSP70 promoter (5′-TGCAAGGCCTGGATTCCACCCTGCAGCAAAGCT-3′), NF-κB-binding region of the mouse B cell light chain enhancer (9′-AGTCTGAGGACACCTAGGAAGGAGAAGGAGAGG-3′) and glucocorticoid-responsive element (GRE) in LTR MMTV (5′-GATCCACCTATTTAATTACCAAAGG-3′) (10) were used as the probes.

CAT Assay—CAT assay was done as previously described (11). ConA and HeLa cells were transfected with plasmids, containing CAT gene under the control of a minimal thymidine kinase promoter alone (Promega) or combined with HSF1-binding or GRE-binding sequences from HSP70 (12) and LTR MMTV (13) promoters, respectively. Cells were treated with HS (42 °C, 30 min) or Dex (0.1 μM, 1 h) with and without PFTα (15 μM).

Western Blot Analysis—Western blot analysis was done as described previously (12). Wild type and p53-deficient HCT116 cells were incubated 15 min at 43 °C in the presence or in the absence of PFTα (15 μM) and total cell lysates were prepared 3 and 6 h later. HSP70 was detected using goat polyclonal antibodies K-20 (Santa Cruz Biotechnology).

RESULTS AND DISCUSSION

PFTα and Heat Shock Response—HS induces expression of a large family of heat shock proteins (HSP70, HSP90, HSP43, HSP27, etc.), many of which function as either molecular chaperones or proteases that assist the cell in recovery either by repairing damaged proteins (protein refolding) or by degrading them (14). Hsp gene promoters contain heat shock elements responsible for binding with HSFs mediating HS-inducible transcription, among which HSF1 seems to play a major role in Hsp gene regulation (15). Under normal conditions, HSF1 exists as an inactive monomer bound to multichaperone complexes (HSF90, HSP70, and others) (16) but is readily activated after HS by forming active trimers that are translocated into the nucleus where they bind heat HS-responsive elements in cellular DNA and stimulate HS genes transcription (14). Thus, there are obvious similarities in regulation of HSF1 and p53 response pathways that justified testing effects of PFTα on HS signaling.

Activation of HSF1-containing transcription complex was determined by gel-shift assay using 32P-labeled heat shock element-derived oligonucleotides and total or nuclear extracts of ConA cells growing under normal conditions or subjected to HS (42°C, 20 min) in the presence and in the absence of 15 μM of PFTα. The intensity of HSF1-specific band in the lysates from HS-treated cells was substantially decreased if the cells were incubated with PFTα during treatment (Fig. 1a).

The results of gel-shift experiments were confirmed in a functional transcription assay using HSF1-responsive construct with CAT reporter. PFTα (15 μM) caused a 2-fold reduction in CAT activity in ConA cells under conditions of HS (Fig. 1b), suggesting that it might have similar effect on the expression of endogenous Hsp genes. In fact, application of PFTα was accompanied by an increased susceptibility of ConA cells to heat shock determined by colony assay (data not shown) and reduction in accumulation of HSP70 (Fig. 1d).

To determine whether the effect of PFTα on cell sensitivity to HS is p53-dependent or p53-independent, we compared the effect of the compound on HS sensitivity of two isoegenic variants of human colon cancer cell line HCT116 differing in their p53 status (8). Presence of PFTα during HS treatment (45°C, 30 min) significantly increased HS-induced cytotoxicity in both p53-wt and p53-deficient cell lines in a dose-dependent manner; similar doses of PFTα had no toxic effect on either cell line under normal growth conditions (Fig. 1c). Consistently, PFTα caused a reduction in HS-induced accumulation of HSP70 protein in both p53 wild type and p53-deficient HCT116 cells (Fig. 1d). In addition to ConA and HCT116, similar results were obtained with p53-deficient human prostate cancer cell line PC3 (data not shown). These observations indicate that PFTα has a p53-independent mechanism of activity directed against HSF1-mediated HS response.

PFTα and GR Signaling—Glucocorticoid hormones are involved in regulation of many important functions in the organism, including development and function of the immune system. Signaling is mediated by interaction of glucocorticoids with their receptor (GR), ligand-dependent transcription factor, that is, as p53 and HSF, regulated at the level of nuclear transport (17, 18). In the absence of ligands, GR resides in the cytoplasm in a monomeric form bound to cytoplasmic chaperones, such as HSP70 and HSP90. Binding of the ligand typically results in a conformational change in GR, dimerization and translocation to the nucleus, where GR homodimer binds to a DNA motif termed a GRE and transactivates glucocorticoid-responsive genes. In thymocytes, this results in activation
of proapoptotic genes and subsequent death that is consistent with anti-inflammatory role of glucocorticoids (19).

To analyze whether PFTα has an effect on GR signaling we used the same strategy as described above for HS signaling. GR activation was tested in HeLa cells treated for 4 h with a range of concentrations of Dex. Results of gel-shift assay with oligonucleotide specific for GRE showed no affect of Dex-induced DNA binding activity of GR. GR responsive genes that determine physiological cell response (23). Consistently, presence of PFTα reduced CAT activity in the lysates of ConA and HeLa cells transfected with the glucocorticoid-responsive construct with CAT reporter and treated with Dex (Fig. 2b).

To test whether biochemical indications of inhibition of GR activity by PFTα reflect alterations of physiological function of GR, we analyzed cell response to glucocorticoid in the presence and in the absence of PFTα (15 μM) that was intraperitoneally injected three times, 0, 2, and 6 h (3.6 mg/kg each) after Dex (Fig. 2c). The Dex-induced degeneration of the thymus. Subcutaneous injection of Dex (4.5 mg/kg) in the size of mouse thymus as early as 24 h after hormone administration. This effect was almost completely reverted by PFTα that was intraperitoneally injected three times, 0, 2, and 6 h (each dose was 3.6 μg/kg) after Dex (Fig. 2d).

PFTα also had a prominent protective effect in vivo, inhibiting Dex-induced degeneration of the thymus. Consistently, presence of PFTα reduced CAT activity in the lysates of ConA and HeLa cells transfected with the glucocorticoid-responsive construct with CAT reporter and treated with Dex (Fig. 2b).

In our study of PFTα effect on NF-κB activation, the lysates of both cell lines and labeled oligonucleotide, corresponding to NF-κB-binding region of the mouse B cell light chain enhancer, showed no affect PFTα on the induction of NF-κB (Fig. 3a, shown for ConA cells). Similarly, PFTα had no effect of NF-κB transactivation as judged by reporter transfection assays (data not shown).
Inhibition of transactivation ability of p53, HSF1, and GR by PFTα was accompanied by suppression of their biological functions resulting in suppression of apoptosis caused by genotoxic stress (p53), sensitization to HS (HSF1), and resistance to Dex-mediate cell killing (GR). We tested whether PFTα would affect the ability of activated NF-κB to protect cells from TNF-induced apoptosis (23). CHI, an inhibitor of translation, suppresses induction of NFκB and makes cells highly sensitive to TNF-mediated apoptosis. If PFTα would suppress NFκB activation (as CHI does), its application should sensitize cells to TNF. Analysis of three cell systems (ConA, NIH 3T3 cells, and short term primary culture of thymocytes), all known to be TNF-resistant due to activation of NFκB, did not show any effect of PFTα on their sensitivity to TNF, while treatment with CHI had strong sensitizing effect presumably by blocking NFκB activation (23) (Fig. 3b). These observations are well in line with lack of PFTα effect on activity of the activation of NFκB transcription factor found in biochemical assays.

The dramatic differences between the effects of PFTα on p53, HSF1, and GR on one hand and NFκB, on the other, suggest the existence of a common regulatory component(s) in those pathways that are affected by the compound, which is not part of NFκB signaling. Moreover, this putative PFTα target is likely to act by affecting nuclear accumulation of sensitive transcription factors as it was previously shown for p53 (2). Although at this stage it is impossible to precisely define the molecular target of PFTα, we can base our speculations on the known properties of the studied pathways focusing on what differs PFTα-sensitive pathways from NFκB signaling. Cellular factors belonging to this category are HSP complexes that participate in holding inactive HSF1, GR, and p53 proteins in the cytoplasm but are not likely to be involved in regulation of NFκB that couples instead with its “own” specific inhibitor IκB. Many properties of HSPs and HSP inhibitors (such as quercetin, an HSP inhibitor, is known to enhance apoptosis in a variety of systems (24, 27). Thus, HSPs are obvious candidate targets of PFTα, and this hypothesis remains to be experimentally tested.

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