

Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation

Evguenia Strom^{1,2}, Swati Sathe², Pavel G Komarov^{1,2}, Olga B Chernova^{2,3}, Ivanda Pavlovska⁴, Inna Shyshynova⁴, Dmitry A Bosykh^{1,2}, Lyudmila G Burdelya⁴, Roger M Macklis⁵, Rami Skaliter², Elena A Komarova⁴ & Andrei V Gudkov^{1,4}

p53-dependent apoptosis contributes to the side effects of cancer treatment, and genetic or pharmacological inhibition of p53 function can increase normal tissue resistance to genotoxic stress^{1–6}. It has recently been shown that p53 can induce apoptosis through a mechanism that does not depend on transactivation but instead involves translocation of p53 to mitochondria^{7–13}. To determine the impact of this p53 activity on normal tissue radiosensitivity, we isolated a small molecule named pifithrin- μ (PFT μ , 1) that inhibits p53 binding to mitochondria by reducing its affinity to antiapoptotic proteins Bcl-xL and Bcl-2 but has no effect on p53-dependent transactivation. PFT μ has a high specificity for p53 and does not protect cells from apoptosis induced by overexpression of proapoptotic protein Bax or by treatment with dexamethasone (2). PFT μ rescues primary mouse thymocytes from p53-mediated apoptosis caused by radiation and protects mice from doses of radiation that cause lethal hematopoietic syndrome. These results indicate that selective inhibition of the mitochondrial branch of the p53 pathway is sufficient for radioprotection *in vivo*.

p53 is a tumor suppressor that is frequently inactivated in tumors¹⁴. It controls genomic stability presumably by inducing growth arrest or apoptosis in response to either genotoxic stress or activation of oncogenes, thereby ‘cleaning’ normal cell populations of potential tumor progenitors¹⁵. p53-deficient tumors are usually sensitive to ectopically expressed p53 (ref. 16) and are considered to be targets for p53 gene therapy^{17,18}. However, p53-mediated apoptosis, which occurs in several sensitive organs (for example, the gastrointestinal tract and blood) during radiation or chemotherapy, can mediate severe and well-known side effects of cancer treatment^{19–22}. To reduce these side effects, we temporarily and reversibly suppressed p53 using the small molecule pifithrin α (PFT α , 3), which was previously isolated as a suppressor of p53-mediated transactivation². The primary challenge of this strategy is achieving therapeutic benefit without affecting the important biological functions of p53 (for example,

cell-cycle checkpoints²³), many of which are exerted through its transcriptional activity.

We created a readout system for selecting apoptosis modulators that consists of p53-deficient Saos-2 cells (human osteosarcoma cells that have all components of the p53 pathway except p53 itself) transduced by a lentiviral vector expressing wild-type p53. The increase of multiplicity of lentiviral infection was accompanied by gradual elevation of p53-dependent p21 induction (Fig. 1a) followed by growth arrest. However, at a certain concentration of intracellular p53, the cells underwent rapid apoptosis without p21 induction, presumably as a result of activation of the mitochondrial branch of p53 activity (Fig. 1a). We monitored apoptotic cell death by measuring caspase activity in the culture medium. We added compounds from a chemical library 8 h after the beginning of infection, which is before newly synthesized p53 initiates the apoptotic program. We chose 214 compounds that reduced caspase activity by at least 50% as primary hits (Fig. 1b), and we selected 31 compounds for further testing after eliminating nonspecific quenchers of fluorescence, direct inhibitors of caspases 3 and/or 7 and highly toxic compounds (Fig. 1c). Of these 31, only 8 compounds inhibited p53-dependent apoptosis without affecting the transactivation function of p53. We tested these 8 compounds for their ability to inhibit apoptosis in p53 wild-type and p53-deficient primary mouse thymocytes treated with gamma radiation or dexamethasone. Only one compound, PFT μ , possessed all the desired properties (see below).

Although we selected PFT μ as an inhibitor of apoptosis induced in Saos-2 cells by lentiviral transduction of p53 (Fig. 2a,b), we could not detect any protective effect of PFT μ against cell death induced by the transduction of the proapoptotic gene *Bax* (Fig. 2c). Furthermore, PFT μ did not protect cells against staurosporine (4)-induced caspase activation (Fig. 2d) and cell death (Supplementary Figs. 1 and 2 online). In addition, we found that PFT μ had no effect on the activity of caspases 2, 8, 9 and 10 in a cell-free system (Fig. 2e). These results indicate that PFT μ is not a general inhibitor of mitochondrial apoptosis and that it is likely to act upstream of the p53-responsive mediator of cell death, Bax.

¹Cleveland BioLabs, Inc., 11000 Cedar Ave., Cleveland, Ohio 44106, USA. ²Quark Biotech, Inc., 6536 Kaiser Dr., Fremont, California 94555, USA. ³Department of Cancer Biology and ⁴Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave., Cleveland, Ohio 44195, USA. ⁵Department of Radiation Oncology, Cleveland Clinic, 9500 Euclid Ave., Cleveland, Ohio 44195, USA. Correspondence should be addressed to E.A.K. (komaroe@ccf.org) or A.V.G. (gudkov@ccf.org).

Received 30 May; accepted 29 June; published online 23 July 2006; doi:10.1038/nchembio809

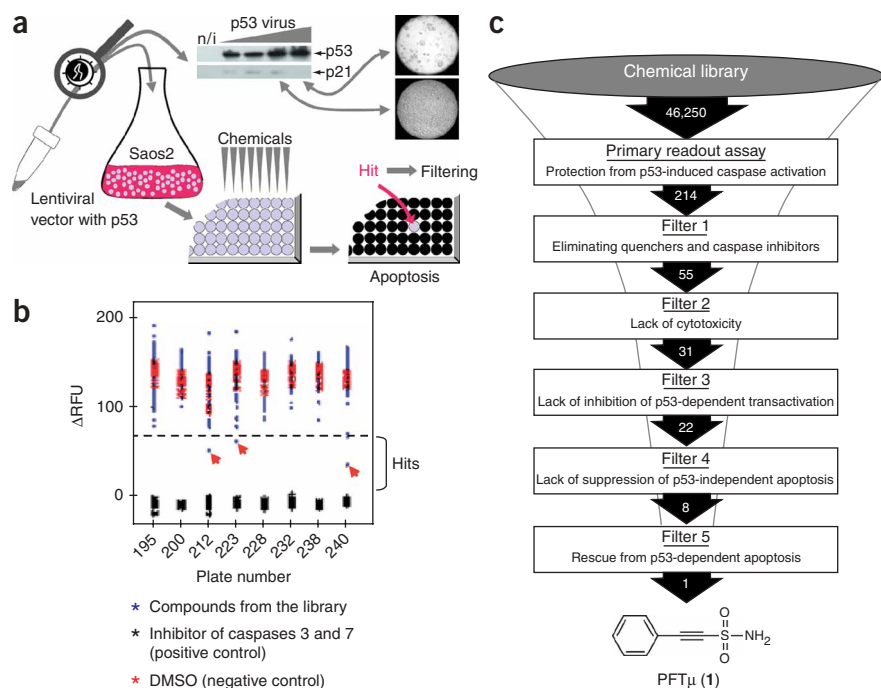


Figure 1 Principles of using a lentivirus-based readout system for screening inhibitors of p53-mediated apoptosis. **(a)** Sequential steps in the drug screening protocol are shown. We determined the levels of p53 protein expression and p21 induction in p53-deficient Saos-2 cells after transduction with increasing concentrations of p53-expressing lentiviral vector 24 h after infection. High virus concentration induced apoptosis with no effect on p21 contents. **(b)** Examples of screening results from several randomly picked plates. Δ RFU indicates the change of fluorescence signal after fluorogenic substrate cleavage. The compounds picked as primary hits are indicated by arrows. **(c)** The hits' filtering stages, showing the numbers of compounds that passed through each screening step.

doxorubicin (Dox; 5), 5-fluorouracil (5-FU; 6), methylmethane sulfonate (MMS; 7) (Fig. 3a) and UV (Fig. 3b). Also, PFT μ did not inhibit gamma irradiation-induced expression of the endogenous p53 target p21 (also known as Waf1; Fig. 3c), whereas PFT α did. Moreover, PFT μ did not affect p53-dependent checkpoint control or change the

We administered PFT μ in a range of concentrations sufficient for its suppressive effect on p53-mediated apoptosis and found that it did not affect apoptosis induced by Fas and tumor necrosis factor in human or mouse fibroblasts (Supplementary Fig. 2). Similarly, PFT μ did not affect tumor necrosis factor-induced activation of nuclear factor κ B (NF- κ B)-dependent transcription (Supplementary Fig. 3 online).

In contrast to the effects of PFT α , PFT μ did not affect the transactivation function of p53 as judged by monitoring p53-responsive lacZ reporter activity in different wild-type p53 cells treated with

cell-cycle distribution before or after gamma irradiation in a series of cell lines differing in their p53 status, including ConA, BJ, BJsip53, WI38, IMR90 (Fig. 3d). The absence of any effect of PFT μ on the protein p21 correlated with the results of real-time PCR analysis of p21 RNA expression (Fig. 3e).

We administered PFT μ to isogenic pairs of human cells having wild-type and inhibited-p53 genotypes at a concentration that effectively inhibited p53-dependent apoptosis and found that it did not suppress UV or Dox-induced transcription of proapoptotic p53-dependent genes, including *Puma* (also called *Bbc3*), *Bax* and *Noxa* (also called *Pmaip1*) (Fig. 3f). In primary mouse thymocytes, PFT μ did not suppress gamma radiation-induced p53-dependent transcription of *Puma* (Fig. 3g), the key effector of apoptosis in these cells (inactivation of *Puma* by gene knockout results in

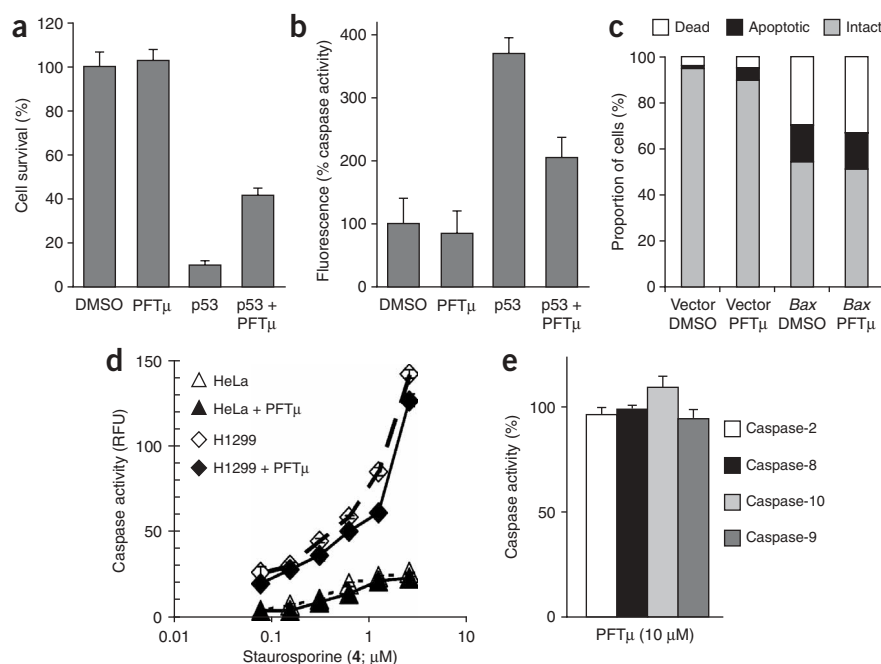


Figure 2 Effects of PFT μ on p53-dependent and p53-independent apoptosis. **(a,b)** We determined cell survival **(a)** and activity of caspases 3 and 7 (AC-DEVD-AMC fluorescent substrate) **(b)** in lysates of Saos-2 cells infected with p53-expressing lentiviral vector (48 h after infection), untreated and treated with PFT μ (10 μ M, applied 5 h after infection). Data are means \pm s.d., $n = 3$. **(c)** PFT μ (10 μ M) did not affect apoptosis induced by transduction of *Bax* (24 h after transient transfection of 293T and HT1080 cells with *pTZV3-CMV-Bax*; FACScan, annexin/PI assay). **(d)** Effect of PFT μ (10 μ M) on staurosporine-induced activity of caspases 3 and 7 in HeLa and H1299 cell lines (shown as relative fluorescence units, RFU). **(e)** Lack of effect of PFT μ (10 μ M) on the activity of caspases 2, 8, 9 and 10 (% of caspase activity) as determined by an *in vitro* assay (see Methods). Data are means \pm s.d., $n = 3$.

suppression of radiation-induced death)²⁴. PFT μ consistently had no effect on p53-mediated transactivation of the promoters of such p53-responsive genes as *Bax*, *Cyclin G* (also called *Ccng1*) and *Hdm2* (also called *Mdm2*) (**Supplementary Fig. 4** online).

Because PFT μ affected neither the transactivation function of p53 nor cell-cycle checkpoint control but effectively suppressed p53-mediated apoptosis, we analyzed its effects on p53 translocation to mitochondria, which is known to be an alternative mechanism

of induction of apoptosis in some types of cells, including primary thymocytes^{7,8,12}. PFT μ substantially reduced the concentration of p53 protein in the mitochondrial (but not in the cytosolic) protein fraction (**Fig. 4a**) of both Saos-2 cells infected with lenti-virus-transduced wild-type p53 (pLV-53) and gamma-irradiated mouse thymocytes (**Fig. 4b**). At the same time, PFT μ had no effect on the concentrations of p21, Bcl-2 or caspase 8 in these cells (**Fig. 4b**).

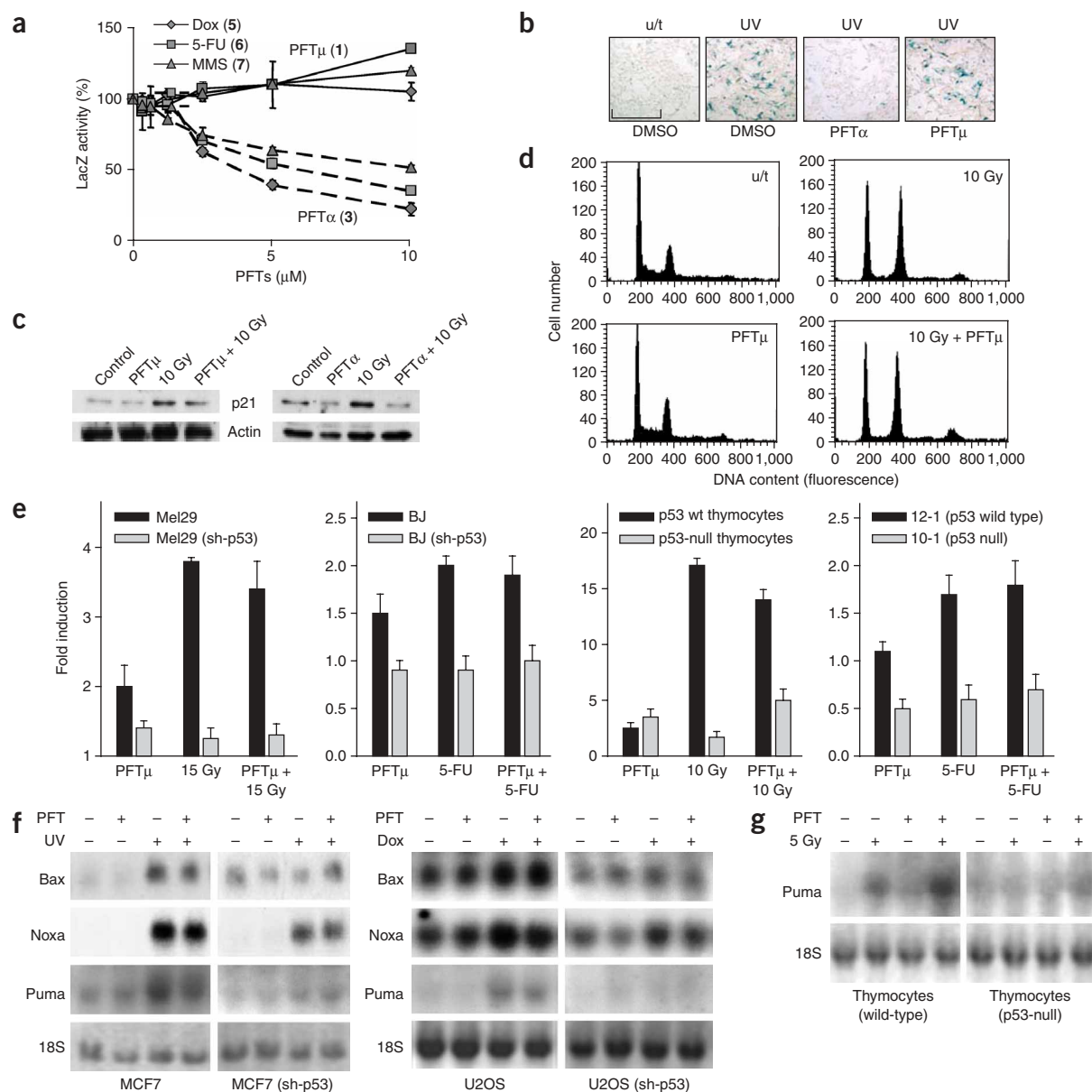
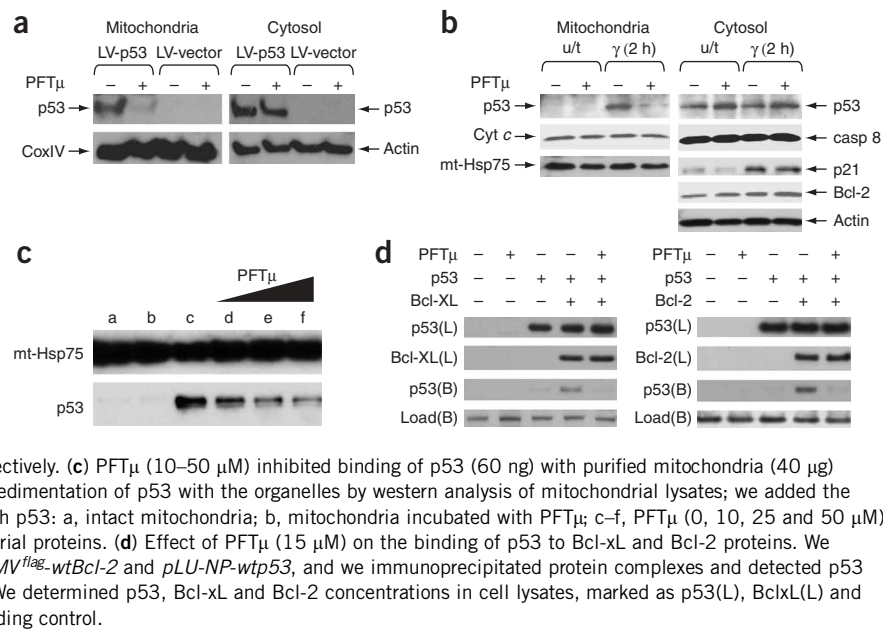


Figure 3 Lack of effect of PFT μ on p53-dependent transactivation. **(a)** Dependence of p53-responsive β -gal reporter activity on the concentration of PFT μ (solid lines) and PFT α (dashed lines) in the lysates of MCF7 'reporter' cells treated with Dox ($0.5 \mu\text{g ml}^{-1}$), 5-FU ($50 \mu\text{g ml}^{-1}$) and MMS ($35 \mu\text{g ml}^{-1}$). Data are means \pm s.d., $n = 3$. **(b)** Effect of PFT μ and PFT α on activation of p53-responsive β -gal reporter (X-gal staining) in ConA cells 24 h after treatment with UV (35 J m^{-2}). Scale bars, 100 μm ; u/t, untreated. **(c)** Comparison of PFT μ (10 μM) and PFT α (10 μM) effects on p21 activation in ConA cells. We isolated protein extracts 6 h after 10 Gy of gamma irradiation and detected p21 protein by immunoblotting. **(d)** Effect of PFT μ (10 μM) on cell-cycle distribution before and after 10 Gy of gamma irradiation (24 h) in ConA cells. **(e)** Effect of PFT μ on transactivation of p21 in wild-type and p53-deficient pairs of cells directly induced by 5-FU (50 μM) and gamma irradiation (10 or 15 Gy) detected by real-time PCR. Data are means \pm s.d., $n = 3$. **(f)** Effect of PFT μ on UV (8.5 J m^{-2})- and Dox ($125 \mu\text{g ml}^{-1}$)-induced *Bax*, *Noxa* and *Puma* mRNA in wild-type and p53-deficient cells (northern analysis). 18S rRNA is shown as loading control. **(g)** Effect of PFT μ on gamma radiation-induced *Puma* mRNA in primary mouse wild-type and p53-null thymocytes (RNA isolated 3 h after irradiation; northern analysis). Error bars at the pictures represent s.d.

Figure 4 PFT μ interferes with p53 protein binding to mitochondria. **(a,b)** PFT μ (10 μ M) reduced the concentration of p53 protein in the mitochondrial but not cytosolic protein fraction purified from *Lvp53*-infected and control Saos-2 cells **(a)** and from gamma-irradiated and control mouse thymocytes **(b)**. We applied PFT μ (10 μ M) to Saos-2 cells 5 h after infection with p53-expressing lentivirus and to primary p53 wild-type mouse thymocytes 20 min before gamma irradiation (10 Gy). We determined expression of p53 protein in the mitochondria and cytoplasm of Saos-2 cells by western blotting 48 h after virus infection and in thymocytes 2 h after irradiation. We determined expression of p53, p21, cytochrome *c*, caspase 8 and Bcl-2 proteins in total cell lysates, and we used mitochondrial heat shock protein mt-Hsp75 and β -actin as mitochondrial and cytoplasmic loading controls, respectively. **(c)** PFT μ (10–50 μ M) inhibited binding of p53 (60 ng) with purified mitochondria (40 μ g) *in vitro* in a reconstructed system. We determined cosedimentation of p53 with the organelles by western analysis of mitochondrial lysates; we added the following to the mitochondria suspension together with p53: a, intact mitochondria; b, mitochondria incubated with PFT μ ; c–f, PFT μ (0, 10, 25 and 50 μ M). We used mt-Hsp75 as a loading control of mitochondrial proteins. **(d)** Effect of PFT μ (15 μ M) on the binding of p53 to Bcl-xL and Bcl-2 proteins. We transfected 293T cells with *pCMV^{flag}-wtBcl-xL* or *pCMV^{flag}-wtBcl-2* and *pLU-NP-wtp53*, and we immunoprecipitated protein complexes and detected p53 protein by DO1-HRP antibodies, marked as p53(B). We determined p53, Bcl-xL and Bcl-2 concentrations in cell lysates, marked as p53(L), BclxL(L) and Bcl-2(L), before coimmunoprecipitation. Load(B), loading control.



To test whether the mechanism of PFT μ -mediated protection against p53-dependent apoptosis involves direct inhibition of p53 binding to mitochondria, we used an *in vitro* system consisting of isolated mitochondria and purified p53. PFT μ reduced the amount of mitochondria-bound p53 in this cell-free system (**Fig. 4c**).

Researchers have previously reported that p53-mediated permeabilization of the outer mitochondrial membrane, which results in

cytochrome *c* release, involves formation of inhibitory complexes of p53 with Bcl-xL and Bcl-2 proteins⁸. Therefore, we analyzed lysates from 293 T cells transfected with a combination of plasmids expressing p53 and either Bcl-xL or Bcl-2 for the presence of p53 in complex with Bcl-2 and Bcl-xL proteins following treatment with PFT μ . Compared with untreated control cells, treatment with PFT μ strongly reduced binding of p53 to both Bcl-xL and Bcl-2 proteins (**Fig. 4d**).

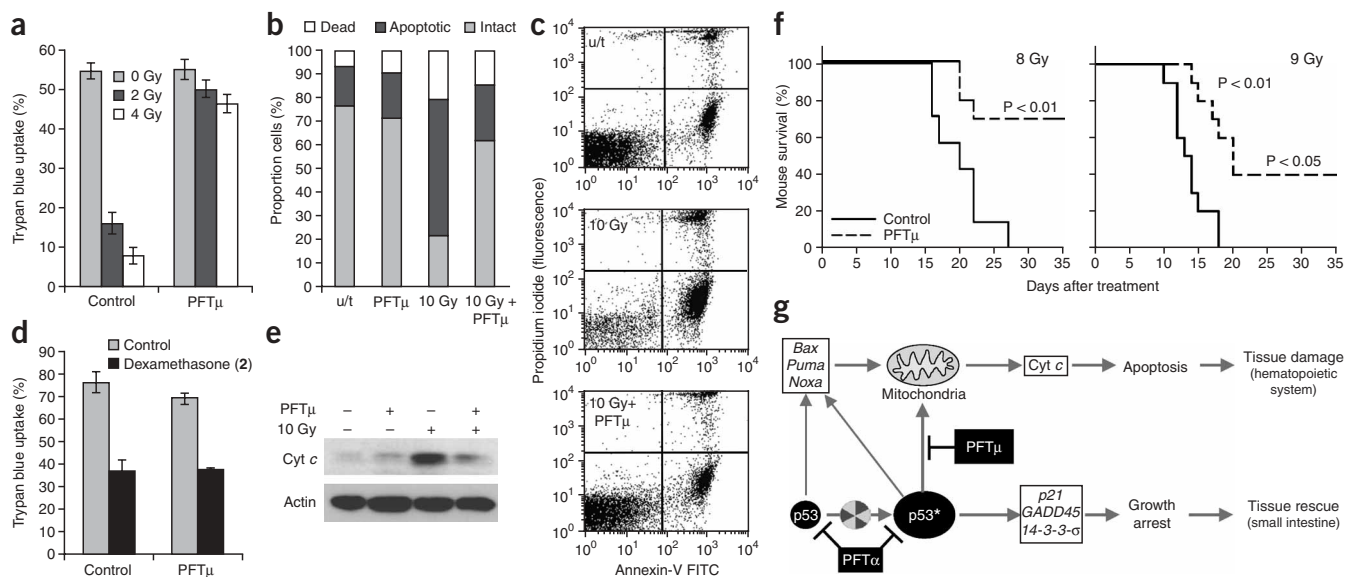


Figure 5 PFT μ protects mouse thymocytes (*in vitro*) and mice (*in vivo*) from gamma irradiation-induced death. **(a–c)** Protective effect of PFT μ (10 μ M) on survival of mouse thymocytes (24 h) treated with 2 and 4 Gy doses radiation (trypan blue uptake assay) **(a)** and 10 Gy dose of radiation (6h; FACSscan, PI and annexin): fluorescent image **(c)** and quantification **(b)** of apoptotic and live cells. **(d)** PFT μ (10 μ M) had no effect on dexamethasone-treated (0.5 μ M, 24 h) p53-null mouse thymocytes. Data are means \pm s.d., $n = 3$. **(e)** Effect of PFT μ on cytochrome *c* release in mouse thymocytes. We gamma irradiated (10 Gy) thymocytes from 5-week-old C57Bl/6J mice (20×10^6) in the absence or presence of PFT μ (20 μ M), and we isolated cytosolic fractions 5 h after irradiation and analyzed them for cytochrome *c* by western blot. **(f)** A single intraperitoneal injection of 40 mg kg⁻¹ body weight of PFT μ reduced lethality in C57Bl/6J mice of 8 or 9 Gy of total body gamma radiation. Results of a representative experiment from three independent repeats are shown, each involving ten mice per experimental subgroup. **(g)** Scheme of pharmacological dissection of p53 activities. In contrast to PFT α , which shuts down both branches of the p53 pathway, PFT μ blocked only the mitochondrial branch of the pathway, thereby protecting from lethal hematopoietic radiation syndrome without affecting transactivation-mediated growth arrest, which has a rescuing effect in the epithelium of the small intestine²³. p53*, activated p53.

To analyze the effect of PFT μ on p53-mediated apoptosis in a natural cell system, we used primary cell cultures of mouse thymocytes, which undergo rapid p53-dependent apoptosis in response to gamma radiation²⁵. PFT μ strongly inhibited cell death induced by gamma radiation (Fig. 5a–c). However, PFT μ did not affect the survival of gamma radiation-treated p53-deficient thymocytes (Supplementary Fig. 5 online) and had no protective effect against dexamethasone-induced apoptosis (Fig. 5d), which is known to be p53 independent²⁵. In addition, PFT μ inhibited cytochrome *c* release from mitochondria detected in the cytosolic fractions of gamma-irradiated thymocytes (Fig. 5e).

A single intraperitoneal injection of mice with PFT μ (40 mg kg⁻¹) 30 min before lethal gamma-irradiation rescued 70% and 50% of mice, respectively, from 8 Gy and 9 Gy radiation doses (Fig. 5f). As expected, PFT μ had no protective effect against doses of radiation that cause gastrointestinal syndrome (Supplementary Fig. 6 online), a condition in which p53 is not a sensitizing factor^{23,26}. Radioprotection *in vivo* by PFT μ is an encouraging result that validates the main hypothesis of this study: p53-mediated apoptosis exerted through the mitochondrial branch of p53 activity is responsible for radiosensitivity of mammals to hematopoietic radiation syndrome (Fig. 5g).

METHODS

Chemicals. We purchased Dox, 5-FU, camptothecin (8), MMS and staurosporine from Sigma and PFT α from Calbiochem. The chemical library DIVERSet consisting of 46,250 compounds was manufactured by Chembridge Corporation.

Cell cultures. We grew tumor- and normal tissue-derived cell lines, including Saos-2, HeLa, BJ, Bjsip53, Mel29, Mel sh-p53, W138, W138hTERT, Balb/c-3T3, LNCap, HT1080, C4-2, H1299, MCF7, ACHN, RCC45 and 293T cells, in DMEM supplemented with 10% FBS. We generated reporter cell lines expressing either lacZ under the control of a p53-responsive promoter or luciferase under the control of an NF- κ B-responsive promoter by introducing reporter constructs into HCT116, ConA, MCF7 and H1299 cell lines as described previously^{2,27}. We prepared short-term cultures of primary thymocytes from the thymus of 4-week-old C57Bl/6 wild-type and p53-null mice, which we purchased from Jackson Laboratory.

Recombinant constructs. Recombinant constructs expressing the complementary DNAs for human wild-type *TP53* (encoding p53) and *EGFP* (encoding enhanced green fluorescent protein, under the control of the EF1 promoter) were provided by Tranzyme, Inc., and *Bax* (under the control of the CMV promoter) was generated based on lentiviral vectors provided by I. Verma (Salk Institute). *pCMV^{flag}-wt Bcl-xL* and *pCMV^{flag}-wt Bcl-2* plasmids were provided by A. Almasan (Cleveland Clinic Foundation). We received the reporter plasmids *pGL3 Basic Super Cyclin G Luc*, *pGL3 Basic Bax Luc* and *pPD Mdm2 LacZ*, expressing lacZ or luciferase under the control of promoters of p53-responsive genes, from M. Oren (Weizmann Institute of Science). For large-scale screening, we concentrated p53-expressing virus to 10⁹ IU ml⁻¹ using ultracentrifugation. We obtained the viruses from culture media collected 48–72 h after cotransfecting both packaging and vector plasmids into 293T cells, and we stored frozen p53-expressing viruses in aliquots at -70 °C. We estimated the conditions of infection of target cells using EGFP-expressing viruses.

Apoptotic assays. We performed the effector-caspase activation assay using 30 μ M of Ac-DEVD-AMC (a fluorogenic substrate specific for caspases 3 and 7) and a Fluroskan Ascent FL microplate reader (Labsystems). We performed the initiator caspase assay using active human recombinant initiator caspases 2, 8, 9 and 10 from a MultiCasPak-3 kit. We performed the biochemical assay as recommended by the manufacturer, using 30 μ M Ac-LEHD-AMC as a substrate for the enzymes (BIOMOL International, L.P.). We applied Z-VAD-FMK (9; 2 μ M) as a control inhibitor.

Primary screening. We infected confluent cultures of Saos-2 cells with lentiviral vector expressing p53 in the presence of 7.5 μ g ml⁻¹ polybrene. Six hours after virus infection, we collected cells by trypsinization and seeded them into 384-well plates, 15,000 cells per well. We delivered library compounds 2 h after plating as DMSO solution to a final concentration of 5 μ g ml⁻¹ using Biomek 2000 (Beckman). We determined apoptotic activity 36 h after delivery of the chemicals.

Hit confirmation and filtering assays. We performed these assays manually in 96-well plates. We plated 25,000 Saos-2 cells infected with p53-expressing lentivirus in each well and treated them in triplicate with a range of concentrations of the selected compounds. To test chemicals for fluorescence-quenching ability, we added them 44 h after the infection (before adding caspase substrate), thereby ruling out the chemicals' effect on p53. We tested the effect of the compounds on cell viability using immortalized human fibroblast WI38h-Tert and Saos-2 cells.

Detection of lacZ and luciferase activity. We detected lacZ (β -galactosidase) expression in cells using X-gal staining, or alternatively we estimated expression using a colorimetric assay in the cell extracts after 24 h of incubation with the drugs, as described previously². We performed the luciferase assay using a Promega kit according to the manufacturer's protocol²⁷.

Cell viability assays. We estimated the number of attached cells by staining with 0.5% methylene blue² and measuring optical density using a Multiscan Ascent microplate reader (Labsystems). We determined cell viability in suspension of short-term culture of primary thymocytes by staining them with 0.1% trypan blue² or by analysis of annexin- or propidium iodide (PI)-positive cells (BD Biosciences) by FACScan.

Transient transfections and lentiviral infections. We performed transient transfections of cells with plasmid constructs using the Lipofectamine Plus system (Invitrogen). We prepared high-titer lentiviral stocks and infected target cell cultures as described previously²⁸.

Gamma and UV irradiation. The animals were treated in accordance with animal care protocol approved by the Institution for Animal care and Use Committee. We injected male C57Bl/6J mice (Jackson Laboratory) age 8–10 weeks with different concentrations of PFT μ (up to 75 mg kg⁻¹) and gamma irradiated them using the Shepherd 4,000-Ci cesium 137 source at a dose rate of 4 Gy min⁻¹. We irradiated cells with 2–10 Gy gamma irradiation doses and 25 J m⁻² UV irradiation doses.

Western blot analysis. We performed western blot analysis on mitochondrial, cytosolic and crude cell lysates that were equalized by protein concentration as previously described²⁹. We used the following antibodies: DO-1 and horseradish-conjugated DO1 for human p53 (Santa Cruz Biotechnology) and 421 for mouse p53 (Oncogene); monoclonal antibodies against mitochondrial Hsp70 (Affinity Bioreagents), cytochrome *c* (BD Biosciences Pharmingen), cytochrome oxidase IV (Molecular Probes), PCNA, Bcl-2, Bax, caspase 3, caspase 8 and p21; and polyclonal antibodies against β -actin, Bcl-xL (Santa Cruz Biotechnology) and Flag tag M2 (Sigma-Aldrich).

RNA analysis. We performed northern blot hybridizations according to ULTRA hyb protocol (Ambion Inc). We hybridized 10 μ g of RNA (isolated 18 h after treatments and purified from MCF7 and U2OS cells and similar cells with p53 expression knocked down by lentivirus-driven short hairpin RNA against p53) with ³²P-labeled cDNA full-length probes of human or mouse *p21* (also known as *Cdkn1a*), *Bax*, *Puma*, *Noxa* and *Bid* as previously described²¹. We measured expression of p21 mRNA by real-time PCR as previously described²⁷ using primers and protocols recommended by Applied Biosystems, Inc.

Coimmunoprecipitation. We performed coimmunoprecipitation of p53–Bcl-2 and p53–Bcl-xL proteins using anti-Flag M2 affinity gel and antibodies to Flag M2 according to the manufacturer's recommendations (Sigma-Aldrich). We added anti-Flag M2 affinity gel to cell lysates (1 mg of protein) of 293T cells treated with PFT μ (15 μ M) 24 h after transfection with *pCMV^{flag}-wt Bcl-xL* or

pCMV^{flag}-wt Bcl-2 and *pLV-wt-p53*. We eluted Flag fusion protein using 3xFlag peptide and analyzed it by western blot.

Preparation of mitochondrial fractions. We prepared mitochondria by sucrose density gradients as previously described³⁰. To test the effect of PFT μ on mitochondrial p53 association *in vitro*, we incubated freshly isolated mitochondria (40 mg) from HeLa and Saos-2 cells at 37 °C for 1 h with purified baculoviral human wild-type p53 (60 ng; BD Pharmingen) and different concentrations of PFT μ (15–120 μ M) in MS buffer containing 150 μ M CaCl₂. We recovered p53 associated with mitochondria using centrifugation followed by three washes in MS buffer.

Note: Supplementary information is available on the Nature Chemical Biology website.

ACKNOWLEDGMENTS

We thank P. Chumakov, A. Almasan and M. Oren for providing recombinant constructs. This work was supported by grant CA75179 to A.V.G., CA103283 grant to Quark Biotech, Inc. and contract HHSN261200422015C to Cleveland BioLabs, Inc. from the US National Institutes of Health.

AUTHOR CONTRIBUTIONS

E.S. generated data, performed data analyses and prepared the manuscript; S.S. generated data and performed data analyses; P.G.K. generated data and performed data analyses; O.B.C. managed part of the work; I.P., I.S., D.A.B. and L.G.B. generated data; R.M.M. performed data analysis; R.S. organized the research and performed data analysis; E.A.K. generated data, performed data analyses, prepared the manuscript and organized the research; and A.V.G. performed data analyses, prepared the manuscript and managed the project overall.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at <http://www.nature.com/naturechemicalbiology>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

- Komarova, E.A. & Gudkov, A.V. Could p53 be a target for therapeutic suppression? *Semin. Cancer Biol.* **8**, 389–400 (1998).
- Komarov, P.G. *et al.* A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* **285**, 1733–1737 (1999).
- Botchkarev, V.A. *et al.* p53 is essential for chemotherapy-induced hair loss. *Cancer Res.* **60**, 5002–5006 (2000).
- Komarova, E.A. & Gudkov, A.V. Chemoprotection from p53-dependent apoptosis: potential clinical applications of the p53 inhibitors. *Biochem. Pharmacol.* **62**, 657–667 (2001).
- Gudkov, A.V. & Komarova, E.A. The role of p53 in determining sensitivity to radiotherapy. *Nat. Rev. Cancer* **3**, 117–129 (2003).
- Gudkov, A.V. & Komarova, E.A. Prospective therapeutic applications of p53 inhibitors. *Biochem. Biophys. Res. Commun.* **331**, 726–736 (2005).
- Marchenko, N.D., Zaika, A. & Moll, U.M. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J. Biol. Chem.* **275**, 16202–16212 (2000).
- Mihara, M. *et al.* p53 has a direct apoptogenic role at the mitochondria. *Mol. Cell* **11**, 577–590 (2003).
- Sansome, C., Zaika, A., Marchenko, N.D. & Moll, U.M. Hypoxia death stimulus induces translocation of p53 protein to mitochondria. Detection by immunofluorescence on whole cells. *FEBS Lett.* **488**, 110–115 (2001).
- Dumont, P., Leu, J.I., Della Pietra, A.C.III, George, D.L. & Murphy, M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nat. Genet.* **33**, 357–365 (2003).
- Chipuk, J.E. *et al.* Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* **303**, 1010–1014 (2004).
- Erster, S., Mihara, M., Kim, R.H., Petrenko, O. & Moll, U.M. *In vivo* mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol. Cell. Biol.* **24**, 6728–6741 (2004).
- Erster, S. & Moll, U.M. Stress-induced p53 runs a transcription-independent death program. *Biochem. Biophys. Res. Commun.* **331**, 843–850 (2005).
- Soussi, T. & Beroud, C. Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat. Rev. Cancer* **1**, 233–240 (2001).
- Vousden, K.H. p53: death star. *Cell* **103**, 691–694 (2000).
- May, P. & May, E. Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene* **18**, 7621–7636 (1999).
- Willis, A.C. & Chen, X. The promise and obstacle of p53 as a cancer therapeutic agent. *Curr. Mol. Med.* **2**, 329–345 (2002).
- Fang, B. & Roth, J.A. Tumor-suppressing gene therapy. *Cancer Biol. Ther.* **2**, S115–S121 (2003).
- Cui, Y.F. *et al.* Apoptosis in bone marrow cells of mice with different p53 genotypes after gamma-rays irradiation *in vitro*. *J. Environ. Pathol. Toxicol. Oncol.* **14**, 159–163 (1995).
- Song, S. & Lambert, P.F. Different responses of epidermal and hair follicular cells to radiation correlate with distinct patterns of p53 and p21 induction. *Am. J. Pathol.* **155**, 1121–1127 (1999).
- Komarova, E.A. *et al.* Transgenic mice with p53-responsive lacZ: p53 activity varies dramatically during normal development and determines radiation and drug sensitivity *in vivo*. *EMBO J.* **16**, 1391–1400 (1997).
- Chow, B.M., Li, Y.Q. & Wong, C.S. Radiation-induced apoptosis in the adult central nervous system is p53 dependent. *Cell Death Differ.* **7**, 712–720 (2000).
- Komarova, E.A. *et al.* Dual effect of p53 on radiation sensitivity *in vivo*: p53 promotes hematopoietic injury, but protects from gastro-intestinal syndrome in mice. *Oncogene* **23**, 3265–3271 (2004).
- Jeffers, J.R. *et al.* Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* **4**, 321–328 (2003).
- Lowe, S., Schmitt, E., Smith, S., Osborne, B. & Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**, 847–849 (1993).
- Merritt, A.J., Allen, T.D., Potten, C.S. & Hickman, J.A. Apoptosis in small intestinal epithelial from p53-null mice: evidence for a delayed, p53-independent G2/M-associated cell death after gamma irradiation. *Oncogene* **14**, 2759–2766 (1997).
- Komarova, E.A. *et al.* p53 is a suppressor of inflammatory response in mice. *FASEB J.* **19**, 1030–1032 (2005).
- Pfeifer, A., Ikawa, M., Dayn, Y. & Verma, I.M. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc. Natl. Acad. Sci. USA* **99**, 2140–2145 (2002).
- Williams, G.T. & Morimoto, R.I. Maximal stress-induced transcription from the human HSP70 promoter requires interactions with the basal promoter elements independent of rotational alignment. *Mol. Cell. Biol.* **10**, 3125–3136 (1990).
- Bogenhagen, D. & Clayton, D.A. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. *J. Biol. Chem.* **249**, 7991–7995 (1974).