

Pifithrin- α protects against DNA damage-induced apoptosis downstream of mitochondria independent of p53

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Pifithrin- α (PFT- α) was shown to specifically block transcriptional activity of the tumor suppressor p53 and was therefore proposed to be useful in preventing the severe side effects often associated with chemo- and radiotherapy. We report here that although PFT- α efficiently protected different cell types from DNA damage-induced apoptosis, it mediated this effect regardless of the presence or absence of p53. Interestingly, PFT- α blocked the apoptosome-mediated processing and activation of caspase-9 and -3 without interfering with the activation of mitochondria. Neither the DNA damage-induced activation of Bax or Bak nor the loss of the mitochondrial membrane potential or the final release of cytochrome *c* were inhibited by this compound. Instead, the ability of PFT- α to protect p53-deficient cells from DNA damage-induced caspase activation and apoptosis was greatly diminished after siRNA-mediated downregulation of cyclin-D1 expression. In contrast, downregulation of other proteins involved in cell-cycle progression, such as the retinoblastoma protein, cyclin D3, as well as the cyclin-dependent kinases, 2, 4 and 6, could not abolish this protection. Thus, our data show that PFT- α protects cells from DNA damage-induced apoptosis also by a p53-independent mechanism that takes place downstream of mitochondria and that might involve cyclin D1.

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DNA-damaging agents, such as chemotherapeutic drugs or ionizing radiation (IR), are important treatment modalities for many cancers that lead to either cell-cycle arrest or apoptosis. One of the main players mediating these responses is the tumor-suppressor protein, p53, which accumulates after DNA damage and which is known to accomplish these tasks through transcription-dependent and -independent events.¹ Although it is well appreciated that p53-mediated responses are crucial for tumor suppression, under certain circumstances p53-deficient tumors can also be killed using high-dose chemo- or radiation therapy.² However, such a treatment is especially implicated in the occurrence of severe side effects as it also results in a vigorous damage of normal p53-expressing tissues surrounding the tumor.³

To reduce the side effects associated with the treatment of p53-deficient tumors, Gudkov *et al.* developed a cell-based screen that allowed them to identify compounds capable of inhibiting p53-mediated apoptosis from a library of 10 000 synthetic chemicals.⁴ A stable water-soluble p53-inhibitor named pifithrin- α (PFT- α) was identified, which not only prevented transactivation of p53-responsive genes but also suppressed p53-dependent apoptosis. In addition, Gudkov *et al.* showed that PFT- α protected mice from the lethal genotoxic stress associated with cancer treatment without promoting the formation of tumors. As this compound was

shown in subsequent *in vitro* and *in vivo* studies to protect different cell types against p53-dependent apoptosis induced by a multitude of stimuli,^{5–11} PFT- α was considered a specific p53 inhibitor and was therefore commonly used to distinguish p53-dependent and -independent apoptosis systems. However, only a few studies applied PFT- α onto p53-deficient cells to unambiguously show its specificity in their systems.⁴ Even then, it was not too surprising to observe an unresponsiveness of drug-treated p53-deficient neuroblastoma cells toward PFT- α when these cells were only barely killed by such a treatment in the absence of this compound.¹² As the precise mechanism of PFT- α -mediated p53 inhibition is still largely unknown, such controls are particularly indispensable.

Moreover, in recent years it became apparent that PFT- α targets additional proteins besides p53, such as the aryl hydrocarbon receptor (AhR),¹³ and was found to suppress heat shock and glucocorticoid receptor signaling in a p53-independent manner.¹⁴ Although it was later shown that the PFT- α -mediated suppression of p53-induced gene activation and apoptosis does not require interaction with AhR,¹³ and is achieved with lower concentrations than those required for inhibition of heat shock and glucocorticoid receptor signaling,¹⁵ these findings shed a new light especially on those studies that failed to show p53 specificity in p53-deficient cells.

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Therefore, we compared the effect of PFT- α on DNA damage-induced apoptosis signaling in p53-proficient and -deficient cells. We found that PFT- α efficiently suppressed DNA damage-induced apoptosis in most cases regardless of their p53 status. Thereby, PFT- α acts downstream of mitochondria, but upstream of caspase-9 activation, by a mechanism that might involve cyclin D1.

Results

Pifithrin- α inhibits expression of p53-responsive genes. During our earlier study,¹⁶ we made the intriguing observation that PFT- α protected not only p53-expressing p21-deficient HCT116 cells from γ -irradiation (IR)-induced apoptosis but also HCT116 cells lacking p53. To investigate this phenomenon, we first determined the influence of PFT- α on the expression of p53-responsive genes by western blot analyses. HCT116 wild-type cells and their p53-deficient counterparts¹⁷ were exposed to 20 Gy γ -IR in the absence or presence of various concentrations of PFT- α and analyzed for their status of p53, the cyclin-dependent kinase (CDK) inhibitor p21, and for expression of the BH3-only protein Puma, which was shown to mediate the DNA damage-induced apoptotic response of p53 in several cell types including colorectal cancer cells.^{18,19} In wild-type cells, PFT- α inhibited dose-dependently the expression of both p21 and Puma, which were induced by IR in a p53-dependent manner (Figure 1a), verifying the p53-inhibitory potential of PFT- α . In contrast, p53-deficient cells lacked any detectable p21 protein and also the IR-induced expression of Puma was too weak to undoubtedly judge an influence of PFT- α (Figure 1a). On the basis of a slight toxicity of 100 μ M PFT- α , the following experiments were routinely carried out with 30 μ M PFT- α , a concentration that did not elicit a toxic response even when it was re-added in the middle of a 4–5-day incubation period.

PFT- α inhibits DNA damage-induced apoptosis and caspase activation in a p53-independent manner. Next, we irradiated wild-type and p53-deficient HCT116 cells in the absence or presence of PFT- α (30 μ M) and assayed cell-death induction by several means. Consistent with our earlier finding that irradiated wild-type cells are predominantly driven into senescence rather than apoptosis,¹⁶ only a minor release of the enzyme lactate dehydrogenase (LDH) (Figure 1b) and the high mobility group 1 (HMG1) protein (Figure 1c) into the supernatant was observed. Nevertheless, both events were completely blocked when the cells were irradiated in the presence of PFT- α . On the other hand, supernatants of similarly treated p53-deficient cells showed a marked increase in the abundance of these proteins correlating with their apoptosis sensitivity toward IR. Remarkably, even 5 days after IR, PFT- α greatly impaired the death-associated release of LDH (Figure 1b) and HMG1 protein (Figure 1c), suggesting that PFT- α also protects p53-deficient cells from IR-induced apoptosis. Together with the observation that this protection was even achieved with a suboptimal PFT- α concentration (30 μ M) that is only partially able to prevent p53-induced p21 expression (Figure 1a),

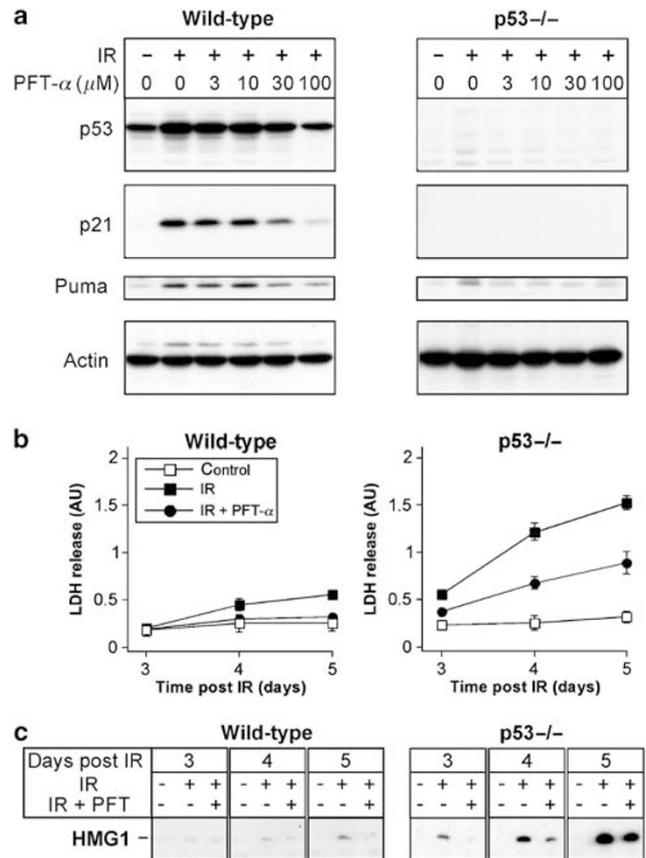


Figure 1 PFT- α inhibits expression of p53-responsive genes and prevents IR-induced cell death independently of p53. (a) Western blot analyses for p53, p21 and Puma levels in wild-type and p53-deficient HCT116 cells one day after IR in the absence or presence of the indicated PFT- α concentrations. Reprobing the membranes with an actin antibody served as loading control. (b, c) Cell-death assessment of wild-type and p53-deficient HCT116 cells that were either left untreated or were exposed to IR in the absence or presence of PFT- α (30 μ M). After the indicated days, the release of LDH (b) and HMG1 (c) into their supernatants was determined. Values given represent the mean of three independent experiments \pm S.D.

these results suggest the existence of additional p53-independent apoptosis-related targets the functions of which are modulated by this compound.

In contrast to wild-type cells, irradiated checkpoint-deficient (p21^{-/-}, p53^{-/-}) HCT116 cells die through apoptosis, as evidenced by the activation of caspases, PARP cleavage and by the fact that IR-induced death of these cells is blocked by QVD-OPH, an irreversible caspase inhibitory peptide.¹⁶ Thus, we analyzed whether the protective function of PFT- α involves inhibition of caspases. Indeed, the IR-induced processing and activation of caspase-9 and caspase-3 could be almost completely blocked by PFT- α in a dose- and time-dependent manner, not only in p53-proficient cells lacking p21 (not shown) but also in p53-deficient cells (Figure 2a and b). Incubation of either cell line with PFT- α in the absence of DNA damage had only a marginal effect on caspase-3 activation. Moreover, IR-induced caspase-mediated cleavage of PARP (Figure 2c) and intra-nucleosomal DNA fragmentation (Figure 7c) were also blocked by this compound, regardless of their p53 status.

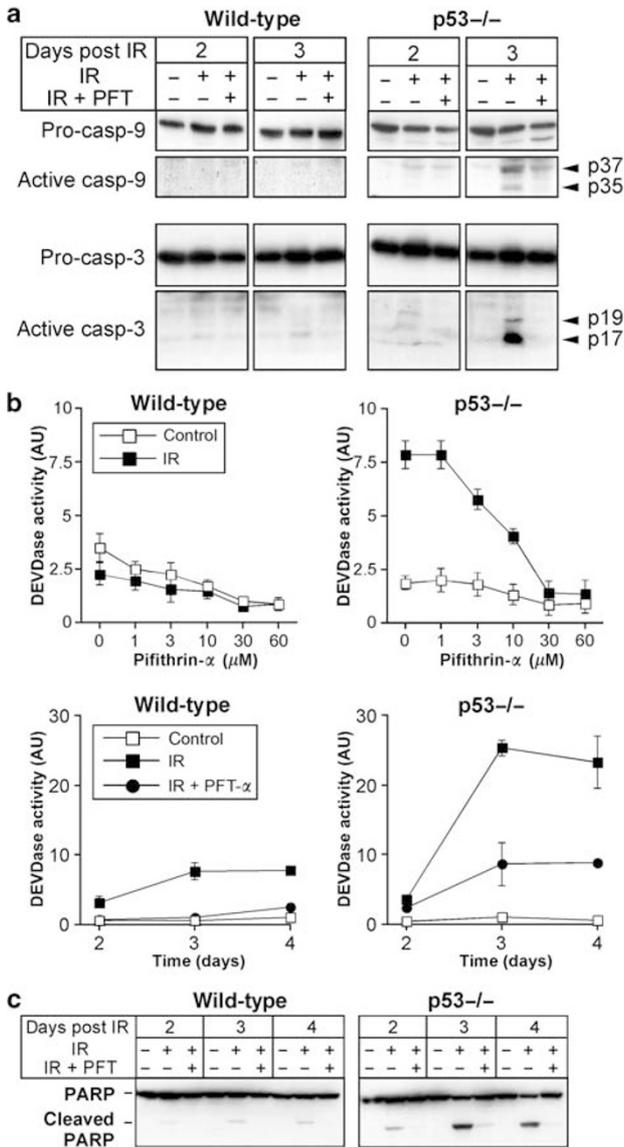


Figure 2 PFT- α inhibits IR-induced apoptosis in a p53-independent manner. (a) PFT- α blocks the processing of caspase-9 and -3. Wild-type and p53-deficient HCT116 cells were exposed to IR in the absence or presence of PFT- α (30 μ M) and analyzed after the indicated times by western blotting for the processing of caspase-9 and caspase-3. The active caspase fragments are indicated. (b) PFT- α inhibits caspase-3 (DEVDase) activity in a dose- (upper panels) and time- (lower panels) dependent manner. Wild-type and p53-deficient HCT116 cells were exposed to IR in the absence or presence of the indicated PFT- α concentrations (upper panels) or with 30 μ M PFT- α (lower panels), and analyzed after 2 days (upper panels) and after the indicated times (lower panels) for DEVDase activity. Values given represent the mean of three independent experiments \pm S.D. (c) Wild-type and p53-deficient HCT116 cells were exposed to IR in the absence or presence of PFT- α (30 μ M) and after the indicated days PARP cleavage was analyzed by western blotting

We then asked whether PFT- α can also protect cells from apoptosis induced by other DNA damaging agents such as etoposide or camptothecin. In contrast to IR, which induced apoptosis and caspase activation only in p53-deficient cells, both drugs efficiently induced these events in a dose-dependent manner also in wild-type cells (Figure 3b). More importantly, caspase-3 activation was almost completely

abrogated regardless of the p53 status when the cells were treated with these agents in the presence of PFT- α (Figure 3a and b). Thus, PFT- α not only protects cells from IR-induced apoptosis but also appears to generally block death induced by DNA damaging agents by a mechanism(s) that does not necessarily involve inhibition of p53. In addition, as PFT- α almost completely abolished IR-mediated caspase-3 activation that could also be induced in HCT116 wild-type cells after siRNA-mediated knockdown of p53 (Figure 3c and d), these results clearly argue against the possibility that the observed protection of the original p53-deficient HCT116 cells is caused by a clonal artefact.

PFT- α does not interfere with the IR-induced activation of mitochondria. To examine at which step PFT- α interferes with the apoptotic cascade, we analyzed its effect on the initial events leading to the activation of mitochondria, that is, the oligomerization-induced activation of the pro-apoptotic Bax and Bak proteins. Both proteins exert their pro-apoptotic activity upon a stress-induced conformational change leading to the exposure of the otherwise occluded N-terminus and subsequent oligomerization. Carrying out immunoprecipitation studies with antibodies that specifically detect the N-termini of such conformationally changed active Bax and Bak proteins, we visualized the activation of both proteins after camptothecin treatment in wild-type and p53-deficient cells, although to a different extent (Figure 4a). Regardless of the presence of PFT- α , both antibodies precipitated approximately similar amounts of activated Bax and Bak, indicating that PFT- α does not interfere with the activation of these proteins.

Next we exposed the two HCT116 lines to IR, and analyzed their mitochondrial membrane potential ($\Delta\Psi_m$). Consistent with our earlier report,¹⁶ IR-induced loss of $\Delta\Psi_m$, an event most likely located downstream of Bax and Bak activation and closely associated with the onset of apoptosis,²⁰ was also observed in both cell lines regardless of their p53 status (Figure 4b and c). PFT- α alone showed no obvious influence on $\Delta\Psi_m$ and, more importantly, it could not prevent IR-induced loss of $\Delta\Psi_m$ in either cell line. Neither the prolonged presence of this compound for up to 4 days after IR nor varying PFT- α concentrations ranging from 1 to 60 μ M could prevent the mitochondrial membrane depolarization in wild-type or p53-deficient cells (Figure 4b and c). In addition, mitochondrial cytochrome *c* release, which occurred in a time-dependent manner also in IR-resistant wild-type cells,¹⁶ was not affected by the presence of PFT- α in either cell line (Figure 4d). Thus, PFT- α protects cells from DNA damage-induced apoptosis by a p53-independent mechanism that takes place downstream of mitochondria, but upstream of caspase activation.

PFT- α interferes with IR-induced apoptosome signaling. We speculated that PFT- α must somehow interfere with the apoptosome-mediated signaling process that controls the activation of the caspase cascade. To this end we determined the influence of PFT- α on this process using the standard *in vitro* caspase activation assay that reflects the capability of cellular extracts to form a functional apoptosome upon addition of cytochrome *c* and dATP. We harvested p53-deficient cells 24 h post IR, as this represents

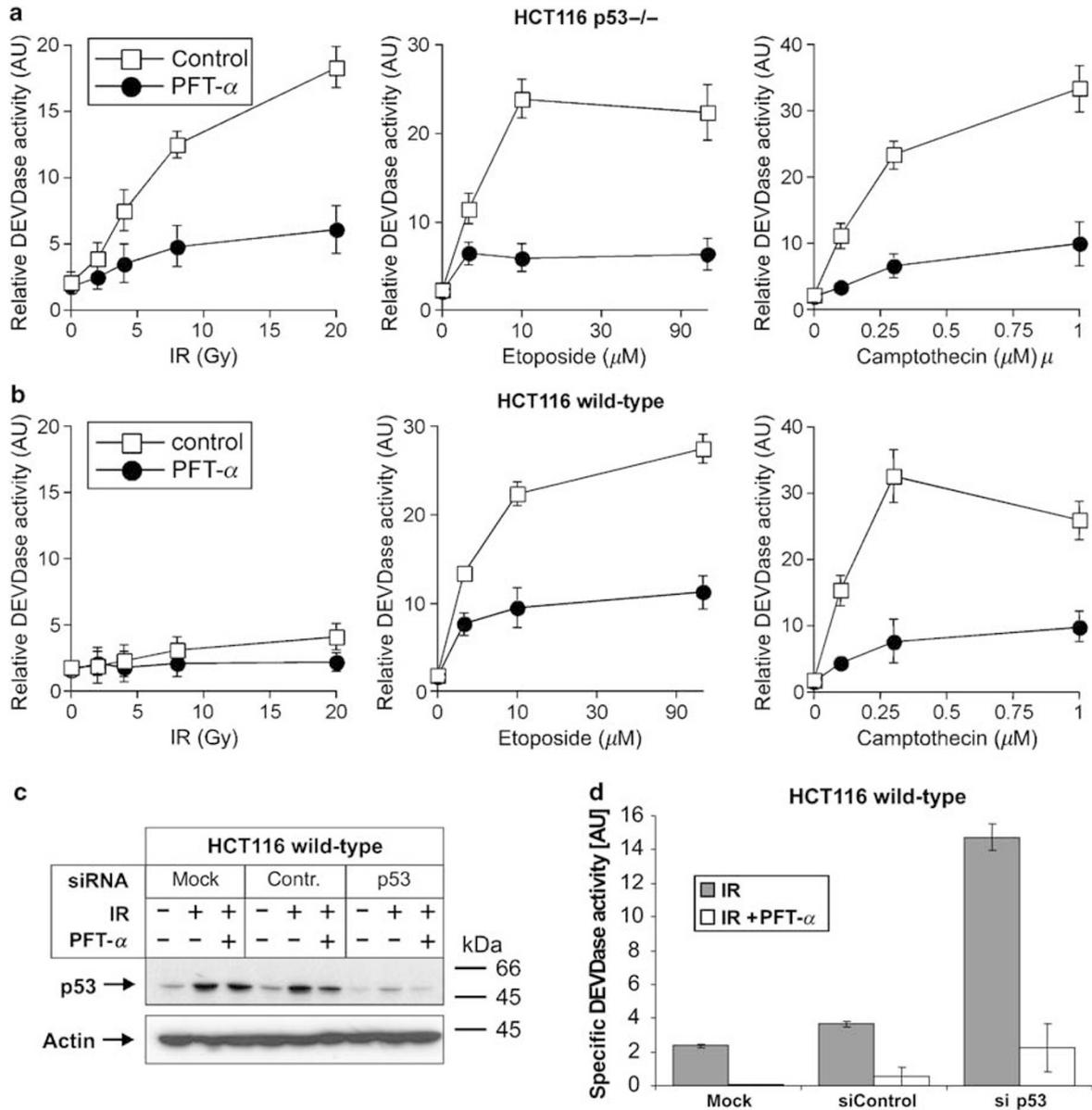


Figure 3 PFT- α inhibits DNA damage-induced apoptosis in a p53-independent manner. Caspase-3 (DEVDase) activity was determined 72 h (IR) and 24 h (etoposide, camptothecin) after the exposure of p53-deficient (a) and wild-type (b) HCT116 cells to the indicated doses of these stimuli in the absence or presence of PFT- α (30 μ M). Values given represent the mean of three independent experiments \pm S.D. (c, d) HCT116 wild-type cells were mock transfected or transfected with a control or a p53 siRNA and were either left untreated or exposed to IR with or without PFT- α (30 μ M). Cells were harvested after 72 h and the extracts were analyzed for their p53 status (c) and caspase-3 (DEVDase) activity (d). Control DEVDase activities obtained in the absence of IR were subtracted. Columns represent the mean of two independent experiments \pm S.D.

a time point at which all extracts from untreated and irradiated cells showed neglectable caspase-3 activities in the absence of cytochrome *c* (no *in vitro* activation) (Figure 5a). As expected, following addition of cytochrome *c* (*in vitro* activation), we found a substantial increase of caspase-3 activity in extracts of untreated cells, which was even further enhanced when extracts of irradiated cells were treated in a similar manner. Interestingly, this increase was completely abolished when we used extracts of cells that were irradiated in the presence of PFT- α (Figure 5a). These results indicate that IR contributes an additional signal(s)

required for an efficient apoptosome signaling that is inhibitable by PFT- α . Together with the observed lack of caspase-9 and -3 processing in the presence of PFT- α (Figure 2a), our finding implies that this compound inhibits the formation of the apoptosome. However, as caspase-9 can be activated according to an induced proximity dimerization model in the absence of proteolytic processing,^{21,22} we cannot undoubtedly deduce whether PFT- α interferes with the binding of caspase-9 to apoptotic protease-activating factor 1 (APAF-1) or with downstream signaling to caspase-3.

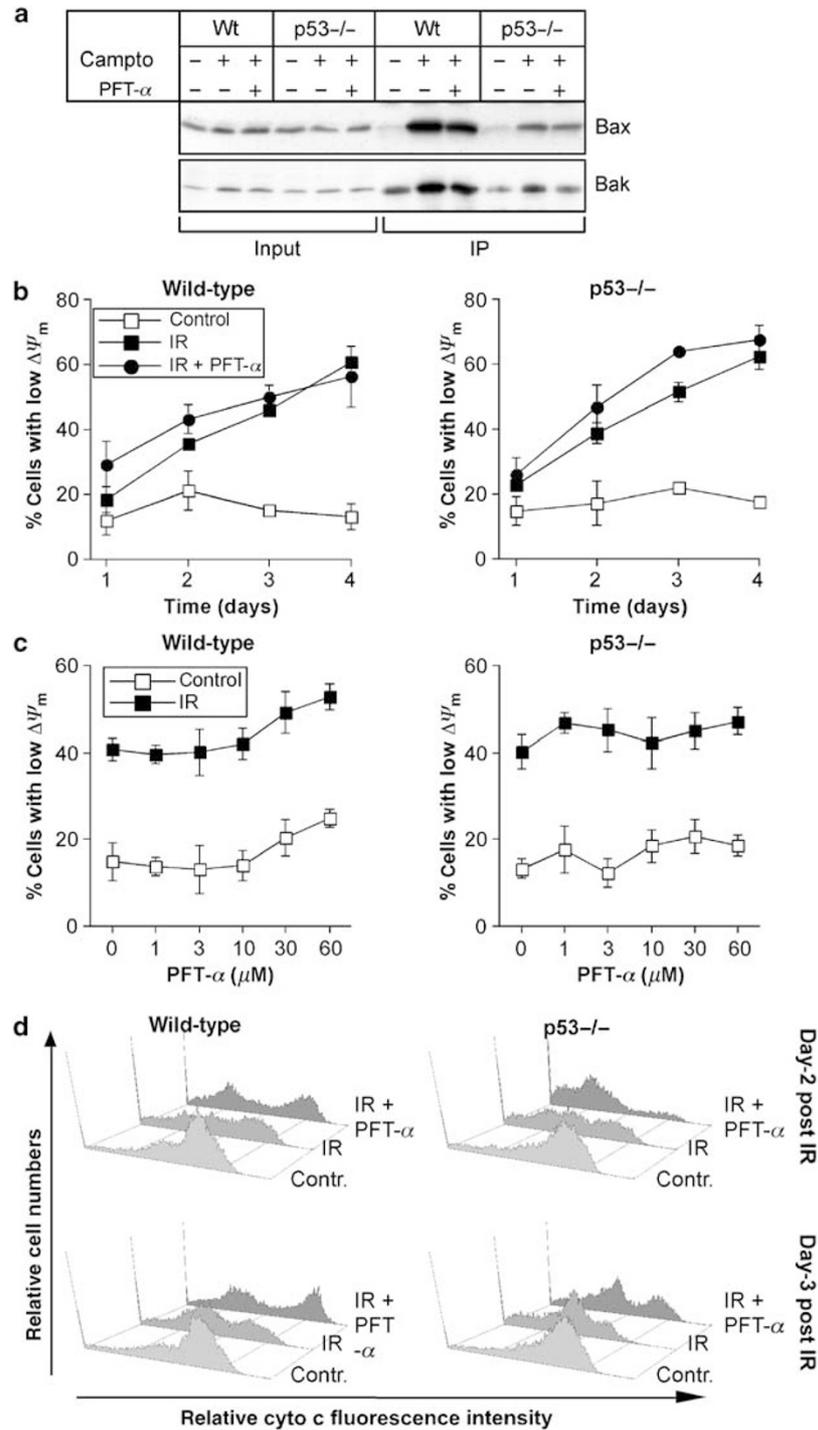


Figure 4 PFT- α does not inhibit DNA damage-induced activation of mitochondria. **(a)** PFT- α does not inhibit camptothecin-induced activation of Bax and Bak. Wild-type and p53-deficient HCT116 cells were treated for 24 h with camptothecin (1 μM) in the absence or presence of PFT- α (30 μM), and activated Bax and Bak proteins were immunoprecipitated (IP) with the conformation-specific antibodies and visualized by western blotting. Whole cell extracts (input) were included as a control. One representative experiment out of two is shown. **(b, c)** PFT- α does not inhibit IR-induced decrease of the mitochondrial membrane potential. Measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) of wild-type and p53-deficient HCT116 cells that were either left untreated or exposed to IR in the absence or presence of 30 μM PFT- α for the indicated times **(b)** or for 3 days with the indicated PFT- α concentrations **(c)**. Values given represent the mean of three independent experiments \pm S.D. **(d)** PFT- α does not inhibit IR-induced cytochrome c release. Measurement of cytochrome c release from the mitochondria of cells that were either left untreated (Contr.) or from cells 2–3 days after exposure to IR in the absence or presence of PFT- α (30 μM). One representative experiment out of three is shown

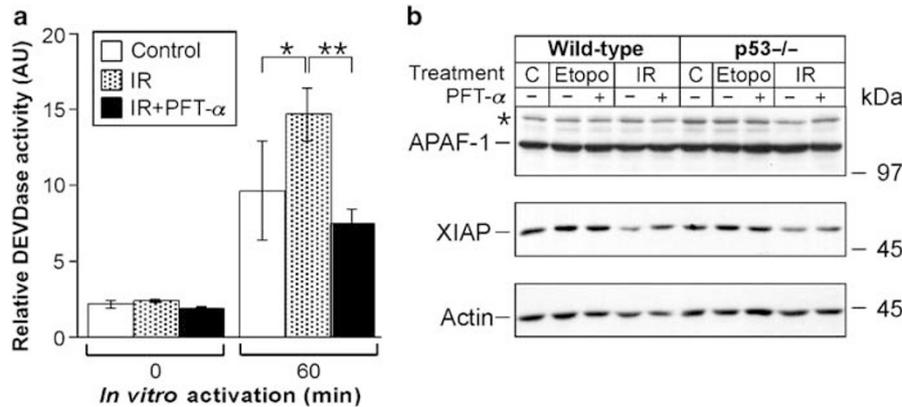


Figure 5 Influence of PFT- α on apoptosome signaling. (a) *In vitro* caspase activation assay. Extracts of untreated (control) p53-deficient cells and of cells that were irradiated in the absence or presence of PFT- α (30 μ M) were prepared 1 day post IR. After incubation for 1 h with or without cytochrome *c* and ATP, the extracts were analyzed for caspase-3 (DEVDase) enzymatic activities. Columns represent the mean of three independent experiments \pm S.D. * P < 0.05; ** P < 0.01, paired Student's *t*-test. (b) Western blot analyses for the expression of APAF-1 and XIAP in untreated (C) wild-type and p53-deficient HCT116 cells and in cells 24 h after exposure to etoposide (100 μ M) or IR in the absence or presence of PFT- α (30 μ M). One representative experiment out of two is shown

We also investigated the possibility that PFT- α inhibits caspase activation by modulating the expression of apoptosome components such as APAF-1 and the anti-apoptotic XIAP. However, PFT- α had no influence on the expression of these two proteins in either etoposide-treated or irradiated wild-type and p53-deficient cells (Figure 5b). Together with our observation that PFT- α did also not affect the expression of pro-caspases 3 and 9 (Figure 2a), these data suggest an alternative mechanism by which PFT- α controls activation of caspases.

PFT- α inhibits IR-induced phosphorylation of the retinoblastoma protein (pRb). To examine whether PFT- α can also protect other p53-deficient cells from apoptosis, we exposed Saos-2 osteosarcoma and H1299 lung carcinoma cells to different DNA-damaging agents in the absence or presence of this compound. Interestingly, whereas PFT- α was able to impair drug- and IR-induced caspase-3 activation also in H1299 cells, it had little or no effect on this process when Saos-2 cells were exposed to various doses of etoposide, camptothecin or IR (Supplementary Figure 1). As Saos-2 cells are deficient not only for p53 but also for pRb, we hypothesized that PFT- α might exert its protective effect through modulation of pRb phosphorylation. Hyperphosphorylation of pRb after IR treatment was observed in p53-deficient HCT116 cells, but not in wild-type cells (Figure 6a). This is consistent with the absence or presence of p21, which mediates cell-cycle arrest through CDK inhibition and subsequent inhibition of pRb phosphorylation. More importantly, IR-induced hyperphosphorylation of pRb in p53-deficient cells was almost completely abrogated by PFT- α . This observation was made not only when p53-deficient cells were exposed to IR but also when etoposide or camptothecin was used in the presence of this compound (Figure 6b). As pRb undoubtedly plays a critical role in cell death pathways,²³ these findings strongly supported our hypothesis of an involvement of pRb in the protective effect of PFT- α . However, siRNA-mediated knockdown of pRb expression (Figure 6a) in p53-deficient

cells neither altered the IR-induced levels of active caspase-3 nor interfered with the protective function of PFT- α (Figure 6c). Thus, although PFT- α significantly affected the phosphorylation status of pRb in stressed cells, it most likely does not exert its protective function through modulation of this tumor suppressor.

Knockdown of cyclin D1 impairs IR-induced caspase-3 activation and the protective effect of PFT- α . As the PFT- α -mediated protection closely resembled the anti-apoptotic mechanism of p21, which also inhibits caspase activation downstream of mitochondria most likely through inhibition of CDKs,^{16,24} and because pRb is a prominent CDK target, we investigated whether CDKs and related cyclins play a role in this process. Western blotting experiments showing that the IR-induced upregulation of cyclin D3 expression in p53-deficient cells was abolished by PFT- α supported this hypothesis (Supplementary Figure 2a). On the other hand, PFT- α interfered neither with the IR-induced downregulation of cyclin D1 nor with the expression of the G1-associated CDK2, CDK4 and CDK6 proteins, which mostly remained unaltered after the exposure of the cells to IR (Supplementary Figures 2a–c). Cyclin D2 is not expressed in HCT116 cells (not shown). However, similar to the results obtained with pRb (Figure 6), knockdown of cyclin D3, CDK2, CDK4 or CDK6 (Supplementary Figures 2a–c) had no effect on IR-induced caspase-3 activation and did not alter the protective effect of PFT- α compared with cells that were mock-transfected or transfected with a control siRNA (Figure 7a and b). In contrast, the siRNA-mediated downregulation of cyclin D1 (Supplementary Figure 2a) reproducibly diminished IR-induced caspase-3 activation (Figure 7a). This was observed not only in HCT116-p53^{-/-} cells but also in p53-deficient H1299 cells (Figure 7e), implying an involvement of this cyclin in IR-induced apoptosis. Consistently, DNA fragmentation and LDH release were greatly impaired after knockdown of cyclin D1, but not when cyclin D3 expression was inhibited or when HCT116-p53^{-/-} cells were transfected with a control siRNA

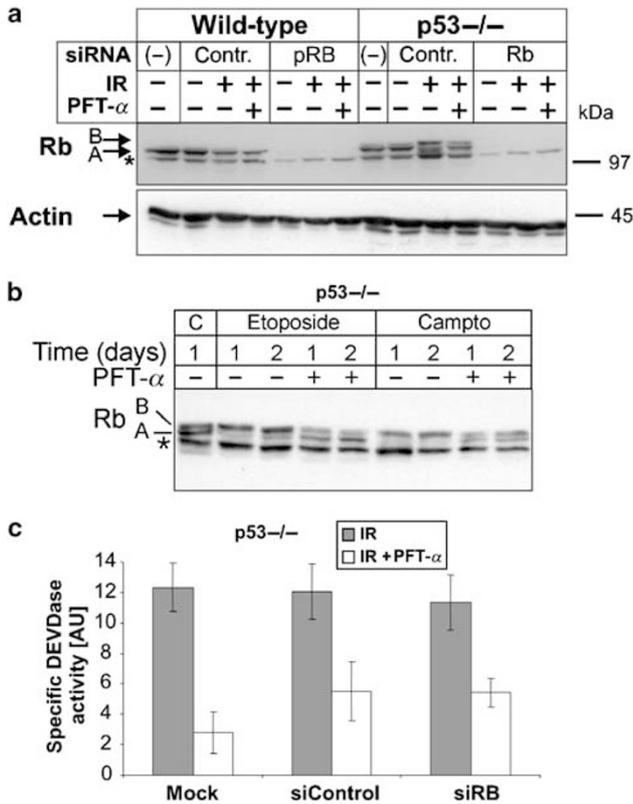


Figure 6 PFT- α prevents hyperphosphorylation of pRb, but does not mediate its protective effect through this tumor suppressor. **(a)** Western blot analysis for the status of pRb in wild-type and p53-deficient HCT116 cells that were either left untreated or transfected for 24 h with a control or the pRb siRNA. The cells were analyzed 3 days after exposure to IR in the absence or presence of PFT- α (30 μ M). Hypo- and hyperphosphorylated pRb forms are indicated as protein bands A and B, respectively, and the asterisk denotes a nonspecific band. Re-probing the membrane with an actin antibody served as loading control. **(b)** Western blot analyses for the status of pRb in p53-deficient HCT116 cells that were either left untreated (C) or that were exposed for the indicated days to etoposide (100 μ M) or camptothecin (1 μ M) in the absence or presence of PFT- α (30 μ M). Hypo- and hyperphosphorylated pRb forms are indicated as protein bands A and B, respectively, and the asterisk denotes a nonspecific band. For the western blots in A and B, one representative experiment each out of three is shown. **(c)** Determination of specific caspase-3 (DEVDase) enzymatic activities from untransfected (mock) and siRNA-transfected p53-deficient HCT116 cells. Cells were analyzed 3 days after exposure to IR in the absence or presence of PFT- α (30 μ M). Control DEVDase activities obtained in the absence of IR were subtracted. Columns represent the mean of three independent experiments \pm S.D.

(Figure 7c and d). More importantly, whereas PFT- α effectively inhibited apoptotic characteristics in those cells in which expression of pRb (Figure 6), the three CDKs or cyclin D3 was suppressed, it was much less efficient to do so after downregulation of cyclin D1 (Figure 7). Exactly such results would be expected after the knockdown of a putative PFT- α target or a component of a PFT- α -targeted signaling pathway that is involved in IR-induced apoptosis. In this case, the cells should not only be rendered more resistant toward apoptosis induction but also PFT- α should be unable to further suppress apoptosis-related events owing to the absence of its target. Therefore, our data indicate that cyclin D1 is involved in IR-induced apoptosis and might play a role in the p53-independent protective function of PFT- α .

Discussion

Radiation and chemotherapy are the most common treatment modalities for many cancers, but the frequent occurrence of severe side effects that are partially determined by p53 often jeopardize a long-term treatment required for a successful eradication of tumors. To overcome such deleterious complications, the small chemical p53 inhibitor, PFT- α , was identified, which protected mice from otherwise lethal doses of radiation.⁴ As p53 proficiency is not always associated with a better prognosis and might in some instances even protect tumor cells from DNA damage-induced apoptosis,^{24–26} the concept of p53 inactivation by PFT- α might be worth considering even in such cases.²⁷

In any case, this concept is only valid provided PFT- α prevents apoptosis solely through inhibition of p53, as an interference with additional apoptosis signaling components unrelated to p53, but common to normal and tumor cells, might adversely affect any therapeutic intervention. Our present data describe such a scenario showing an additional p53-independent mode of action for PFT- α , a conclusion reached by three independent observations. First, PFT- α protected not only p53-proficient HCT116 wild-type cells from DNA damage-induced apoptosis but, surprisingly, also their p53-deficient counterparts. In addition, PFT- α exhibited its protective effect also in p53-deficient H1299 lung carcinoma cells and in HCT116 wild-type cells after siRNA-mediated knockdown of p53. Second, although the PFT- α concentration used (30 μ M) was not sufficient to completely abrogate the transcriptional activity of p53, explaining why this compound did not block mitochondrial activation in p53-expressing cells, it efficiently prevented induction of apoptosis. Finally, we have located the anti-apoptotic activity of PFT- α for the first time downstream of cytochrome *c* release, but upstream of caspase-3 and -9 activation, indicating that PFT- α modulates an event downstream of mitochondria required for proper apoptosome signaling. As p53, however, is known to induce apoptosis through transcription-dependent and -independent events that take place either upstream or at the level of mitochondria, it is highly unlikely that its inhibition contributes to the observed protection mediated by PFT- α . Likewise, although PFT- α was recently shown to inhibit p73,²⁸ our findings most likely also exclude a participation of this p53 family member, as p73 is known to instigate apoptosis through induction of Puma upstream of the mitochondria.²⁹ Consistently, siRNA-mediated knockdown of p73 in p53-deficient HCT116 cells affected neither IR-induced caspase-3 activation nor PFT- α -mediated protection (Supplementary Figure 2e and f).

Thus, the question arises why this p53-independent protective mechanism of PFT- α was not reported earlier. Unfortunately, many studies aimed at verifying specific p53-dependent mechanisms applied PFT- α mostly on p53-expressing cells, but have not confirmed their results in cells lacking p53.^{6,7,11,30–34} In addition, studies that included these essential control experiments often used cells expressing either a non-functional p53 mutant or a p53 protein that is inactivated by the papilloma virus E6 protein.^{12,34,35} The inability, shown herein, of PFT- α to protect these cells from apoptosis might, however, be because of a competitive

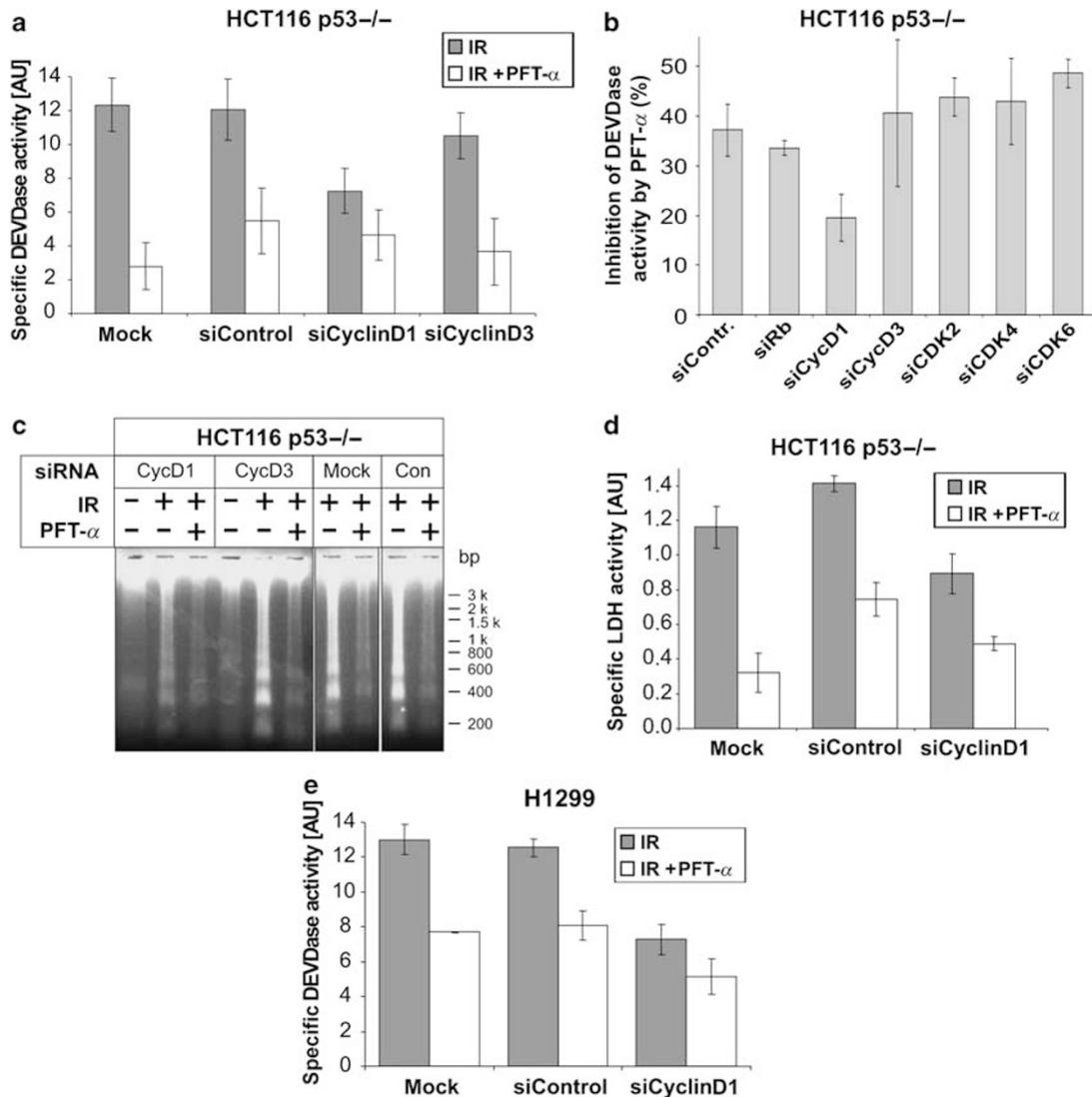


Figure 7 Downregulation of cyclin D1 impairs IR-induced apoptosis and the protective effect of PFT- α . **(a)** Determination of the specific caspase-3 (DEVDase) enzymatic activities from untransfected (mock) and siRNA-transfected p53-deficient HCT116 cells. Cells were analyzed 3 days after exposure to IR in the absence or presence of PFT- α (30 μ M). Control DEVDase activities obtained in the absence of IR were subtracted. Columns represent the mean of five independent experiments \pm S.D. **(b)** Alternative presentation of the entire DEVDase activity data from Figures 6(c) and (a) that, in addition, also includes data obtained after knockdowns of CDK2, CDK4 and CDK6. Displayed is the percentage of the PFT- α -mediated inhibition of the IR-induced DEVDase activity in the indicated siRNA-transfected cells. Columns represent the mean of two to five independent experiments \pm S.D. **(c)** Determination of DNA fragmentation in untreated and irradiated p53-deficient mock-transfected HCT116 cells and in cells transfected with the indicated siRNAs. DNA was prepared 3 days post IR in the absence or presence of PFT- α . DNA molecular size marker is given in base pairs (bp). One representative experiment out of three is shown. **(d)** Cell-death assessment of mock-transfected p53-deficient HCT116 cells and of cells transfected with a control or cyclin D1 siRNA that were irradiated in the absence or presence of PFT- α (30 μ M). Four days after IR, the release of LDH into their supernatants was determined. Control activities obtained in the absence of IR were subtracted. Values given represent the mean of two independent experiments \pm S.D. **(e)** Determination of the specific caspase-3 (DEVDase) enzymatic activities from untransfected (mock) and siRNA-transfected (control and cyclin D1) p53-deficient H1299 cells. Cells were analyzed 3 days after exposure to IR in the absence or presence of PFT- α (30 μ M). Control DEVDase activities obtained in the absence of IR were subtracted. Columns represent the mean of two independent experiments \pm S.D.

binding of the inactivated or mutated p53 protein to PFT- α , leaving none or too little of this compound behind to interfere with other p53-unrelated targets. Moreover, results showing that PFT- α does not protect p53 mutant cells from drug-induced apoptosis are especially questionable in view of the fact that these cells are only barely killed by the drug in the absence of PFT- α ¹². Even the inclusion of fibroblasts or mice completely deficient for p53⁴ might not always constitute an adequate control system to analyze

the influence of PFT- α on apoptosis induction, as, for example, fibroblasts primarily undergo cell-cycle arrest upon DNA damage, and because p53-deficient mice survive much higher doses of γ -radiation than wild-type mice,³⁶ making it almost impossible to compare the effects of PFT- α in these irradiated animals. Thus, it appears conceivable that the p53-independent protection of cells from DNA damage-induced apoptosis by PFT- α was overlooked in earlier studies.

Although several reports published the identification of p53-unrelated PFT- α targets,^{13,14} these are probably not involved in the protective effect mediated by PFT- α .^{13,15} Therefore, this investigation represents the first study clearly showing a p53-independent mechanism by which PFT- α protects cells from DNA damage-induced apoptosis. Intriguingly, this mechanism may involve cyclin D1, a cell-cycle regulatory protein earlier linked to apoptosis signaling.^{37–39} This is because knockdown of cyclin D1 inhibited IR-induced caspase-3 activation and apoptosis and, more importantly, PFT- α was clearly less efficient in preventing these events in cyclin D1-depleted cells. In contrast, loss of cyclin D3, CDKs and pRb had no effect on DNA damage-induced apoptosis and did not alter the protective effect of PFT- α . Although our data thereby imply that cyclin D1 might constitute a PFT- α target or a component of a PFT- α -targeted signaling pathway participating in DNA damage-induced apoptosis, it is still unknown how directly or indirectly cyclin D1 is involved in this process. On the other hand, the observed IR-induced decrease of cyclin D1 appears to argue against such a role. However, IR-induced alterations of expression levels or post-translational modifications of certain proteins are not always indicative of their involvement in this pathway. For instance, although expression of cyclin D3 was strongly upregulated after IR, an event efficiently counteracted by PFT- α , knockdown of cyclin D3 had no effect on apoptosis signaling. Similarly, siRNA-mediated loss of pRb could also not protect the cells even though its IR-induced phosphorylation was abrogated by PFT- α .

However, participation of other cell-cycle regulatory proteins in addition to cyclin D1 cannot be ruled out, as there exists a high-functional redundancy between various cyclins and their CDKs.⁴⁰ In addition, modulation of cyclin/CDK protein levels might reciprocally influence expression of other cyclin/CDK complexes. For instance, downregulation of CDK2 resulted in an increased expression of CDK4 (Supplementary Figure 2b), whereas a combined application of both siRNAs suppressed either protein level less efficiently than the individual siRNAs (data not shown). As, however, such practically unfeasible double or even triple knockdowns would be necessary to further decipher their role in the protective effect mediated by PFT- α , we are presently unable to rule out a participation of these proteins. On the basis of this redundancy, it is also unknown why PFT- α appears to require the presence of cyclin D1 to protect cells from DNA damage-induced apoptosis, especially because PFT- α neither modulates its expression nor interferes in the association of cyclin D1 with CDKs (not shown). In this line, it will be now particularly interesting to determine whether the complexation of cyclin D1 with CDKs or CDK-independent functions that were shown to involve physical association with more than 30 transcription factors or transcriptional co-regulators is essential for this process.⁴¹

In summary, we have shown that PFT- α protects cells from DNA damage-induced apoptosis also by a p53-independent mechanism that takes place downstream of mitochondria and that might involve cyclin D1. As a p53-independent mode of action would clearly jeopardize the originally intended goal of reducing the severe side effects of chemo- and radiation-based therapies, further studies are required to decipher the underlying mechanism(s).

Materials and Methods

Reagents and antibodies. The two HCT116 colon carcinoma cell lines were maintained in McCoy's 5A medium (PromoCell, Heidelberg, Germany), whereas H1299 lung carcinoma and Saos-2 osteosarcoma cells were cultured in DMEM High Glucose (PAA Laboratories, Cölbe, Germany). The media were supplemented with 10% heat-inactivated fetal calf serum, 10 mmol/l glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories, Linz, Austria). PFT- α as well as the fluorogenic caspase-3 substrate DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) were from Biomol (Hamburg, Germany). The polyclonal goat and rabbit caspase-3 and APAF-1 antibodies were from R&D Systems (Wiesbaden, Germany) and Chemicon International (Temecula, CA, USA), respectively. The polyclonal rabbit antibodies recognizing caspase-9 were from Cell Signaling Technology (Danvers, MA, USA). The p53 mAb (Ab-6) was from Calbiochem (Bad Soden, Germany), whereas the mAbs recognizing cytochrome *c*, CDK2, poly(ADP-ribose) polymerase (PARP), p21, XIAP and the polyclonal rabbit antibody against the HMG1 were from BD Bioscience (Heidelberg, Germany). The N-terminal conformation-specific polyclonal rabbit antibodies recognizing active Bax and Bak were from Upstate (Virginia, USA), and the mAbs for their western blot detection were from Trevigen (Gaithersburg, MD, USA) and Oncogene (San Diego, CA, USA), respectively. Poly- and monoclonal Puma, pRb (IF8) and p73 antibodies and the rabbit antibodies against CDK2, CDK4 and CDK6 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cyclin D1 and D3 mAbs were from LabVision Corporation (Fremont, CA, USA), whereas the actin mAb, the protease inhibitors PMSF, aprotinin, leupeptin and pepstatin, as well as the anticancer drugs etoposide and camptothecin were from Sigma (Deisenhofen, Germany). Peroxidase-labeled secondary antibodies were from Promega GmbH (Mannheim, Germany).

Treatment of cells, measurements of cell death and DNA fragmentation analysis.

Cells were exposed to IR (20 Gy) using a Gammacell 1000 Elite (Nordion International Inc., Fleurus, Belgium), or were treated with the indicated concentrations of the anti-cancer drugs in the absence or presence of PFT- α . For assays up to 4–5 days, PFT- α was re-added once on day 3. Cell death was assessed by microscopic examination and by determination of the LDH activity in supernatants of 10^5 cells, according to the protocol of the manufacturer (Roche). The supernatants were also analyzed on SDS-polyacrylamide gels for the death-associated release of HMG1. For DNA fragmentation analysis, 10^6 cells were suspended in a 35 μ l lysis buffer (100 mM Tris/HCl pH 8.0, 2 mM EDTA, 0.8% SDS) supplemented with 5 μ l RNase A (10 mg/ml) and incubated for 1 h at 37°C. After addition of 15 μ l proteinase K (10 mg/ml), the samples were further incubated for 2 h at 50°C and analyzed in a 1.6% agarose gel.

Preparation of cell extracts, western blotting and immunoprecipitation.

Total cell extracts were prepared as described.⁴² Protein concentrations were determined with the BioRad protein assay, separated on SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Amersham, Braunschweig, Germany). After antibody incubation, the proteins were visualized by enhanced chemiluminescent staining using ECL reagents (Amersham Biosciences). Immunoprecipitations were carried out as described.¹⁶

Determination of the mitochondrial transmembrane potential and cytochrome *c* release.

$\Delta \Psi_m$ was analyzed using the $\Delta \Psi_m$ -specific stain, TMRE (Molecular Probes), as described.⁴³ The measurement of mitochondrial cytochrome *c* release was also described earlier.¹⁶ Both assays were performed by flow cytometric analyses on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) using the FL2-histogram profile and CellQuest software.

In vitro caspase activation assay and fluorometric determination of caspase activity.

For *in vitro* activation of caspases, cell extracts were prepared¹⁶ and the reactions were started by adding 10 mmol/l DTT, 2 mmol/l dATP and 1 mmol/l MgCl₂ in the presence of 3.5 μ mol/l cytochrome *c* (from horse heart, Sigma) to 200 μ g extract aliquots and incubation at 37°C. Caspase-3 enzymatic activities were determined as described.⁴⁴ A paired Student's *t*-test was employed for statistical analysis.

Transfection of siRNAs. ON-TARGETplus SMARTpool and non-targeting control siRNAs were purchased from Dharmacon RNA technologies (Lafayette, CO, USA) and the knockdown was carried out according to the manufacturer's

instructions. At 24 h after transfection with or without (mock) siRNAs, cells were harvested and divided equally to receive either no treatment, or exposure to IR with or without PFT- α . Three days after IR, cells were harvested and directly analyzed by western blotting and by the fluorometric caspase substrate assay for a successful knockdown of the target protein and DEVDase activity, respectively.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)