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The p53-inhibitor Pifithrin-α inhibits Firefly Luciferase activity *in vivo* and *in vitro*

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Abstract

Background: Pifithrin-α is a small molecule inhibitor of p53 transcriptional activity. It has been proposed that the use of pifithrin-α in conjunction with chemotherapeutic and radiation therapies for cancer will reduce the side effects of these treatments in normal tissue that still contains wild type p53. In addition, pifithrin-α provides a useful tool in the laboratory to investigate the function of p53 in model systems.

Results: While investigating the effects of pifithrin-α on the transcriptional activity of NF-κB, we observed a strong inhibition of reporter plasmids containing the firefly luciferase gene. Firefly luciferase is one of the most commonly used enzymes in reporter gene assays. In contrast, no inhibition of reporter plasmids containing *Renilla* luciferase or chloramphenicol acetyltransferase was observed. The inhibition of firefly luciferase activity by pifithrin-α was observed both *in vivo* and *in vitro*. Pifithrin-α did not inhibit firefly luciferase protein expression, but rather suppressed light production/emission, since addition of exogenous pifithrin-α to active extracts inhibited this activity. Furthermore, pifithrin-α also inhibited recombinant firefly luciferase protein activity.

Conclusions: Among its other biological activities, pifithrin-α is an inhibitor of firefly luciferase activity. Caution must therefore be taken when using this compound, which has been characterised as an inhibitor of p53 transcriptional activity, to investigate effects on gene expression using transiently transfected reporter plasmids. Furthermore, these results demonstrate that when using novel compounds, the choice of vectors used in the experimental procedures might be of great importance for the correct conclusions to be made.

Background

The tumour suppressor protein, p53 is one of the most intensively studied proteins throughout biomedical research. Due to its central role in genome surveillance, cell cycle arrest and apoptosis induction, compounds affecting this protein, either re-activating it or inactivating it, are of exceptional interest and use in the field of cancer, Alzheimer's disease, Parkinson's disease, stroke and brain trauma [1–3].

In recent years, a chemical inhibitor of p53, Pifithrin-α(PFT-α), has been identified and used both *in vitro* and *in vivo* to investigate p53 function [4]. PFT-α reversibly inhibits p53-transcriptional activity, inhibiting p53-induced apoptosis, cell cycle arrest and DNA-synthesis

block [4–9]. PFT-α has been successfully used *in vitro* and *in vivo* to protect normal cells from otherwise lethal doses of chemo and radiotherapy [3,4,10]. PFT-α thus provides a valuable tool for the identification of genes under the control of p53 [10]. Despite the exciting data of these reports, little or nothing is known about the mechanism of action of PFT-α, although it is thought to disrupt the nuclear transport of p53 [10]. Recently, the group that originally discovered PFT-α, reported that this compound also inhibits the heat shock and glucocorticoid pathways, suggesting that it targets a commonly used protein required for the activity of multiple transcription factors [11].

Reporter gene assays are routinely used to study the control of transcription. This involves the coupling of reporter enzymes such as firefly or *Renilla* luciferase and Chloramphenicol acetyltransferase to the gene promoter region of interest. Generally, the activity of these enzymes is unaffected by the treatment conditions and this is not considered when interpreting the data obtained from these assays. However, it is known that enzymes such as luciferase and β-Galactosidase are affected by certain stress conditions such as heat shock and oxidative stress [12,13]. The fact that these enzymes can be affected by such conditions can give rise to misinterpreted data and compromise the conclusions from these assays.

In this report, we have investigated the effect of PFT-α on different reporter genes. We find that PFT-α is a specific inhibitor of firefly luciferase. These results indicate that when performing functional experiments with this important compound, an appropriate choice of vector should be utilised. These observations also give possible insight into the mechanism of action of PFT-α *in vivo*.

Results

Effects of PFT-α on p53-dependent and independent luciferase reporter plasmids

To determine the effects of PFT-α on p53-dependent and -independent transcriptional activity U-2 OS human osteosarcoma cells, which contain wild type p53, were transiently transfected with a variety of firefly luciferase reporters. The p53-responsive reporters used were PG13 and p21-luciferase and the unrelated reporters were 3x κB and HIV-LTR-luciferase, which are both regulated by the NF-κB family of transcription factors. Previously, we have shown that the PG13 and 3x κB reporters are specifically regulated by p53 and NF-κB, even in unstimulated U-2 OS cells where there is a basal level activity of both transcription factors [14,15]. The p21 and HIV-LTR luciferase reporters are not solely regulated by p53 and NF-κB, however, and so effects could result from other DNA-binding proteins. PFT-α was added to the cells at a final concentration of 20 μM and cells were harvested 24 hours later. As expected, PFT-α strongly downregulated p53-responsive reporters (Figure 1A) but surprisingly inhibition of the NF-κB regulated luciferase reporters was also observed (Figure 1B). Further experimentation revealed a dose-dependent inhibition of both p21-luciferase and 3x κB luciferase by PFT-α (Fig. 1C). Since cross-talk between these transcription factors has been previously observed [16–19], we decided to investigate if this was an effect seen due to PFT-α mediated inhibition of p53. Using U-2 OS cells, p53 was induced by co-transfection of an expression plasmid encoding the tumour suppressor $p14^{ARF}$, in the presence or absence of PFT-α (Figure 1D). As expected, PFT-α treatment induced p53 stabilisation and repressed p53-induced endogenous p21 expression (Figure 1D). In contrast, the effects on NF-κB dependent gene expression were conflicting. While PFT-α inhibited the IκB-α luciferase reporter, no effect on induction of endogenous IκB-α protein could be detected following RelA (p65) transfection (Figure 1E &1F). Furthermore, no changes in NF-κB DNA-binding were observed (data not shown). This prompted us to investigate the effect of PFT-α on other firefly luciferase reporters. These included the Cyclin D1, Cyclin E and Bcl-2 promoters and Gal4 fusions with RelA(p65) and the p300 coactivator protein [15,20,21]. Surprisingly, we observed that PFT-α inhibited the activity of every firefly luciferase reporter utilised, regardless of the transcription factors by which they were regulated (data not shown). PFT-α is not a general inhibitor of transcription, however, since the PFT-α treated cells did not die (data not shown) and PFT-α had no effect on an *in vitro* transcription assay (L.M. Elsby and S.G.E. Roberts, personal communication). Furthermore, it had no effect on *in vitro* translation using reticulocyte lysates (data not shown).

PFT-α does not inhibit Renilla luciferase or CAT based reporters

We next wanted to determine if PFT-α-mediated inhibition was also observed using chloramphenicol acetyltransferase (CAT) reporters or a different type of luciferase. To investigate this, different cell types were transiently transfected with pRL-CMV luciferase, where the luciferase gene is from *Renilla reniformis*. Interestingly PFT-α had no effect on *Renilla* luciferase activity (Figure 2A). Furthermore, no inhibition was observed when NF-κB-dependent CAT-reporters were used (Figure 2B). Using a 2x κB CAT reporter, no inhibition following PFT-α was observed, while 3x κB-luciferase, was strongly repressed (compare Figure 1B with Figure 2B). Similar results were found when A20 CAT was compared to A20-luciferase following PFT-α treatment (data not shown).

PFT-α inhibits firefly luciferase in vitro

The results described above led us to investigate the effects of PFT-α on firefly luciferase *in vitro*. Using protein extracts

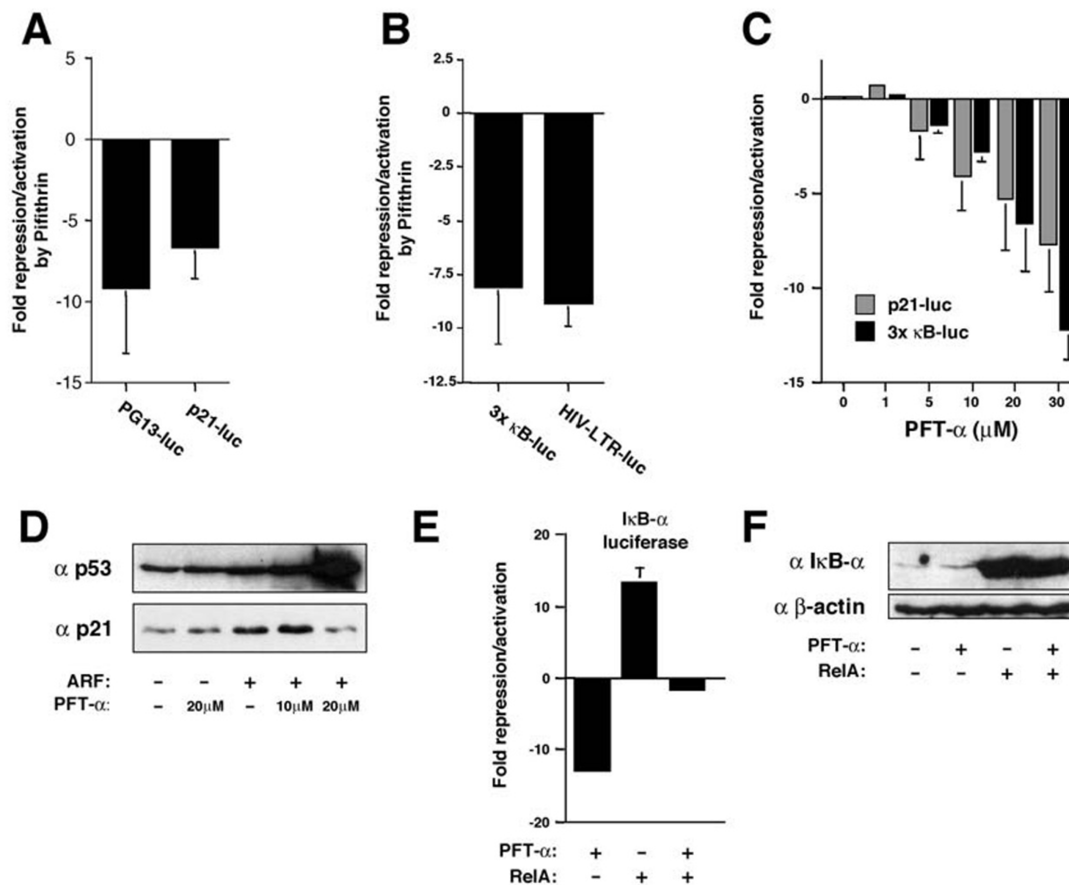

Figure 1
PFT-α inhibits both p53-dependent and -independent firefly luciferase reporters. (A) PFT-α inhibits p53-dependent luciferase reporter plasmids. U-2 OS cells were transfected with 1.5 μg of the indicated reporters and treated with 20 μM PFT-α for 24 hours. Cells were harvested and luciferase activity was measured and normalised for total protein. Results are expressed as fold repression above untreated transfected cells, and represent mean plus standard deviation of a minimum of 3 independent experiments. (B) PFT-α inhibits NF-κB-dependent luciferase reporter plasmids. U-2 OS cells were treated as (A) but transfected with 1.5 μg of 3x κB and HIV-LTR luciferase reporter plasmids. (C) PFT-α inhibition of luciferase activity is dose dependent. U-2 OS cells were transfected with 1.5 μg of the indicated reporter plasmids and treated with the indicated concentrations of PFT-α for 24 hours. Cells were harvested and luciferase activity was measured and normalised for total protein. Results are expressed as fold repression above untreated transfected cells, and represent mean plus standard deviation of a minimum of 3 independent experiments. (D) PFT-α stabilises p53 protein and inhibits p53-dependent endogenous gene expression. p53 was induced in U-2 OS cells by co-transfection of 1.5 μg of ARF expression plasmid in the presence or absence of PFT-α, for 24 hours. Nuclear protein levels were prepared and analysed by western blot using the indicated anti-p53 and anti-p21 antibodies. (E) PFT-α inhibits NF-κB-dependent IκB-α reporter gene activity. HEK293 cells were co-transfected with 1.5 μg of IκB-luciferase and 1.5 μg of RSV-p65 or empty vector. Cells were then treated with 20 μM PFT-α for 24 hours after which cells were harvested and luciferase activity was measured. (F) PFT-α does not inhibit induction of endogenous IκB-α. Western blot analysis of endogenous IκB-α using whole cell lysates from cells subjected to equivalent conditions as the luciferase assay in (E).

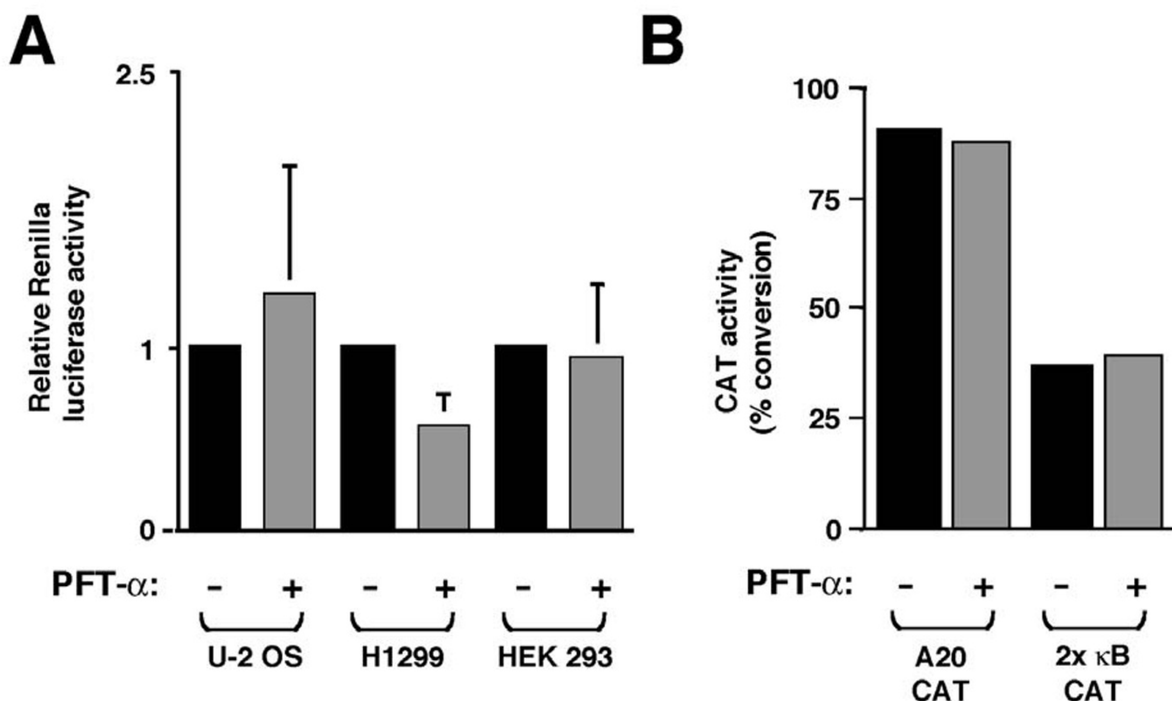

Figure 2
PFT-α does not inhibit Renilla luciferase or CAT reporters *in vivo*. (A) PFT-α does not inhibit Renilla luciferase. The indicated cell lines were transfected with 1.5 μg of pRL-CMV-luciferase reporter and treated with 20μM PFT-α for 24 hours. Cells were harvested and luciferase activity was measured and normalised for total protein. Results are expressed as fold repression above untreated transfected cells, and represent mean plus standard deviation of a minimum of 3 independent experiments. (B) PFT-α does not inhibit CAT reporter plasmids. U-2 OS cells were transfected with 5 μg of indicated reporters and treated with 20 μM PFT-α for 24 hours. Cells were harvested and CAT activity was measured and normalised for total protein. Results are expressed as percentage of conversion. Since there was a significant variation in the levels of activity of these reporters even in unstimulated conditions, although the overall conclusions were the same, the data is presented is a single experiment representative of 3 independent experiments.

from human foreskin fibroblast (HFF) cells transiently transfected with PG13 luciferase reporter plasmid, we exogenously added different dilutions of PFT-α and analysed their activity in a luminometer (Figure 3A). Increasing concentrations of PFT-α induced a dose dependent reduction in the light emission from these extracts (Figure 3A). These results were comparable to the dose dependent decrease observed when PFT-α was added to living cells transfected with firefly luciferase reporter plasmids (Figure 1C). Confirming these observations, light production/emission was also totally abolished when recombinant, purified, firefly luciferase was treated with PFT-α *in vitro* (Figure 3B). We also observed that recombinant purified luciferase activity was inhibited by PFT-α independently of substrate and enzyme concentration, suggesting a non-competitive mechanism of action (Figure 3C and data not shown). A more accurate analysis was not possible since our equipment only measured total and not the rate of light emission. It can therefore be concluded that PFT-α inhibits the production/emission of light and not the expression of active luciferase.

Discussion

The use of reporter gene assays has become a valuable tool in investigating promoter and gene activity both *in vitro* and *in vivo*. These assays rely on the expression and activity of enzymes that are not naturally expressed in mammalian systems and therefore should not be affected by

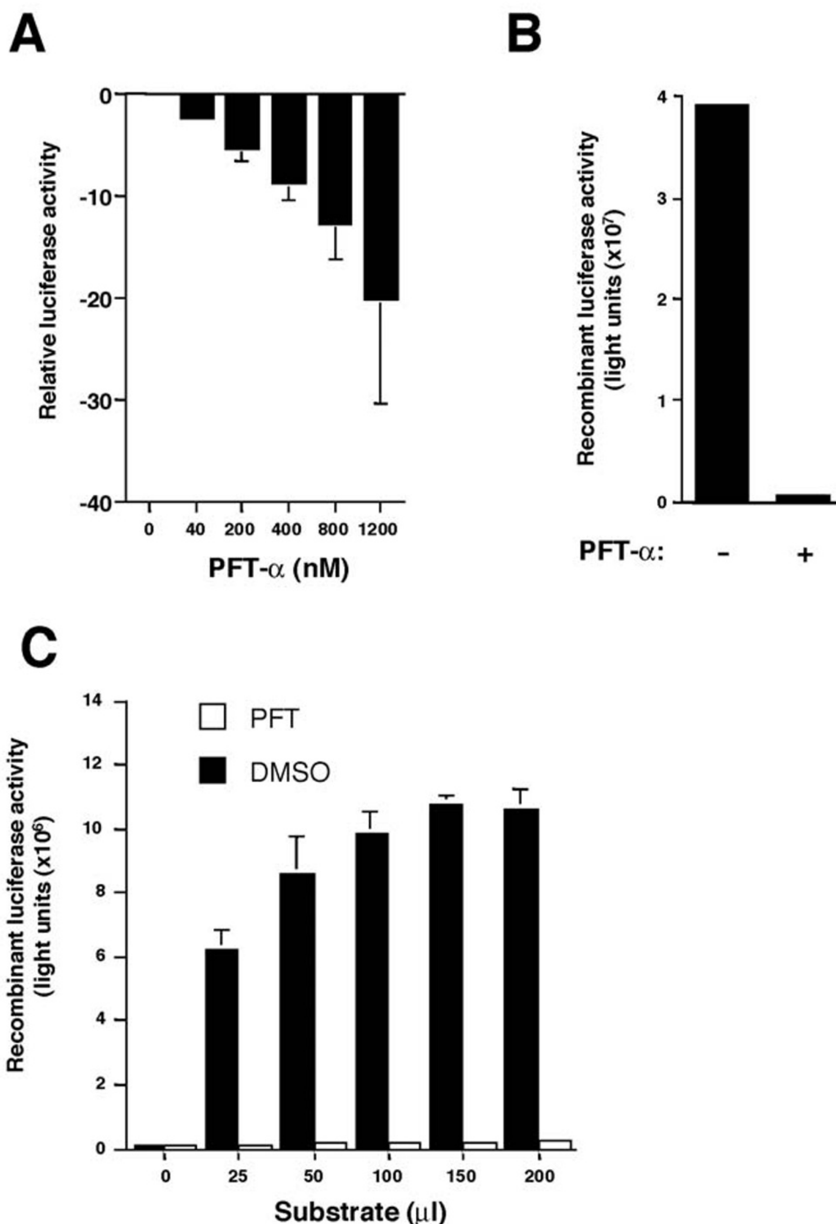

Figure 3

PFT-α inhibits active and recombinant firefly luciferase. (A) Exogenously added PFT-α inhibits cell extracts containing luciferase activity *in vitro*. HFF cells were transiently transfected with 1.5 μg of PG13 reporter plasmid. Cellular extracts were obtained, serial dilutions of PFT-α were added as indicated, and luciferase activity was measured. Results are expressed as the mean plus standard deviation of a minimum of 3 independent experiments. (B) PFT-α inhibits the activity of purified recombinant luciferase protein. Recombinant firefly luciferase was incubated with luciferase substrate in the presence or absence of 20 μM PFT-α and luciferase activity was measured. Results are expressed as light units, and represent mean plus standard deviation of a minimum of 3 independent experiments. Error bars are beneath the resolution of the graph and although calculated, cannot be seen. **(C)** PFT-α inhibits the activity of purified recombinant luciferase protein independently of substrate concentration. Recombinant firefly luciferase was incubated with increasing concentrations of luciferase substrate in the presence or absence of 20 μM PFT-α and luciferase activity was measured. Results are expressed as light units, and represent mean plus standard deviation of a minimum of 3 independent experiments. Luciferase substrate concentration is not provided by the manufacturer (Promega) and therefore is expressed as μl of substrate used.

endogenous or exogenous conditions. The enzymes used for reporter gene assays include luciferase (firefly and Renilla), β-Galactosidase and CAT. These reporter gene assays are often used to investigate the effects of novel compounds on gene or transcription factor activity. One of these compounds is the p53 inhibitor PFT-α which has proven to be a valuable tool in this field of research [4–10]. While investigating the effects of PFT-α, we found that this compound inhibited not only p53-responsive firefly luciferase reporters but also all other firefly luciferase reporters we tested (Figure 1A,1B,1C and data not shown). Further investigation revealed that although PFT-α inhibited p53 transcriptional activity, as previously described, it had no effect on endogenous levels of other genes, such as IκB-α, while repressing luciferase promoter activity of this gene when fused to firefly luciferase (Figure 1D,1E,1F). In addition, PFT-α also inhibited the activity of a chromosomally integrated luciferase reporter, indicating that it was not interfering with the stability of the reporter plasmid itself (data not shown). Additional experiments showed that PFT-α did not inhibit Renilla luciferase based reporters or CAT-based reporter plasmids (Figure 2A &2B). These results suggested specific inhibition of either firefly luciferase expression or activity. It has been recently reported that proteasome inhibitors can inactivate both firefly luciferase and β-Galactosidase activity [22]. In this report, the proteasome inhibitors were shown to prevent luciferase and β-Galactosidase protein expression. In our report, this is not the case since PFT-α inhibits active luciferase containing extracts (Figure 3A) and purified recombinant luciferase protein (Figure 3B). The exact mechanism of this inhibition is unknown, although our results suggest a non-competitive mode of action (Figure 3C). It is interesting to note that reporter enzymes such as firefly luciferase have also been shown to be inhibited by other compounds such as the general anaesthetic, bromoform [23]. It was demonstrated that anaesthetics inhibit firefly luciferase by competing with the hydrophobic substrate and not by interfering with the catalytic mechanism [23,24]. This could be a possible mechanism for PFT-α mediated inhibition of firefly luciferase although further investigations will be required to verify this hypothesis. The recent report concerning PFT-α inhibition of the heat shock and glucocorticoid signalling pathways might also provide some insights as to the mechanism of action of this compound [11]. It is interesting to observe that in this study, the authors relied on CAT based reporter gene assays and not luciferase. When CAT reporter plasmids were used, we did not observe inhibition of NF-κB activity (Figure 2B). Our preliminary data indicates that PTF-α does inhibit a p53 driven CAT reporter (data not shown). This indicates that PFT-α is at least a relatively selective inhibitor of gene expression and could be used to probe the endogenous activities of specific transcriptional regulators provided the correct experimental system is chosen. In particularly troublesome cases, Slot blots, Northern blots or quantitative PCR could be used to assay expression of the reporter gene.

Conclusions

These results, taken together with those from other groups where small molecules have been found to inhibit luciferase activity [22,23] indicate the importance of using strictly controlled experiments when using reporter gene assays and novel compounds. These effects might be of particular relevance when designing cell based assays for small molecule inhibitors of specific signalling and transcriptional regulatory pathways.

Methods

Cell Culture

Human embryonic kidney (HEK) 293, human osteosarcoma U-2 OS and human foreskin fibroblast (HFF) cells were maintained at 5% CO_2 in Dulbeccos Modified Eagle Medium (DMEM) (Sigma), supplemented with 10% FCS, 1% L-glutamine (GibcoBRL) and 1% penicillin/streptomycin (Sigma).

Reporter Plasmid and expression plasmids

Firefly (*Photinus pyralis*) Luciferase reporter plamids used were: 3x κB-luc and IκB-α-luc (provided by Prof. Ron Hay, St. Andrews), PG13-luc and p21-luc (provided by Prof. David Lane, Dundee and Dr. Tim Crook, London). *Renilla* (*Renilla reniformis*) Luciferase reporter plasmid pRL-CMV was obtained from Promega. CAT reporter plasmids: 2x κB and A20 CAT have been described previously [14,16]. The RSV-p65 expression plasmid has also been described [16]. The pCDNA3 $p14^{ARF}$ expression plasmid was provided by Dr Gordon Peters (London).

Luciferase Reporter Assays

For analysis of luciferase reporters, cells were plated in 6-well plates and transiently transfected using the Calcium Phosphate method (previously described in [16]). Luciferase activity was determined using the Luciferase assay system (Promega) according to the manufacturer's instructions and normalised for total protein. QuantiLum® Recombinant Luciferase was obtained from Promega and light units were determined according to manufacturer's instructions.

CAT Reporter Assays

Indicated cells were plated in 100 mm dishes, transfected by Calcium phosphate method and analysed as previously described [16]. Results were normalised for total protein.

PFT-α Treatments

PFT-α was obtained from Biomol and dissolved in DMSO. For *in vivo* studies, PFT-α or the equivalent amount of DMSO in controls, were added directly to cells at the final concentrations indicated. For *in vitro* studies, PFT-α was added exogenously to active cellular extracts or recombinant luciferase and compared to an equivalent DMSO treatment.

Protein Extraction, Western Blot Analysis and Antibodies

Nuclear extracts were prepared as described previously [25]. For whole protein lysates, cells were washed once with PBS, and resuspended in buffer A (20 mM Hepes pH 7.6, 400 mM NaCl, 1 mM EDTA, 25% Glycerol, 0.1% NP-40, 1 mM DTT). Cells were lysed with 10 strokes of a 26 G syringe. Cells were incubated on ice for 30 minutes before centrifugation at maximum speed for 15 minutes. All solutions used in protein extractions contained protease and phosphatase inhibitors, PMSF (1 mM), leupeptin (1 μg/μl), aprotinin (1 μg/μl), pepstatin A (1 μg/μl), NaF (5 mM), Na_3VO_4 (500 μM). Protein determination of extracts was performed using the BioRad Bradford protein assay. Following SDS-PAGE, resolved proteins were electroblotted onto PVDF membranes. The membrane was blocked in 10% blocking buffer (TBS-0.1% Tween-20, 10% milk) for 30 minutes. The membrane was then probed with the primary antibody in 5% TTBS-milk overnight at 4°C, followed by three 10 minute washes with TTBS. Incubation with the secondary antibody was performed for 1 hour at room temperature followed by 3 times 10 minutes washes with TTBS. Detection of proteins was achieved using ECL (Amersham).

Antibodies used were anti-p21 (Santa Cruz Biotechnology), anti-p53 DO-1 (gift from Dr. Sonia Lain and Prof. David Lane, Dundee) and anti-IκB-α (gift from Prof. Nancy Rice, National Cancer Institute). All of these antibodies have been previously described [20].

Authors contributions

SR performed the majority of the experimental work, initially conceived the study and wrote the earlier drafts of the manuscript. KJC and KCR performed the remainder of the experimental work, helped during the preparation of the manuscript and should be considered as having made an equal contribution to the paper. NDP participated in the design and coordination of the study and prepared the final drafts of the manuscript. All authors read and approved the final manuscript.

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