

Pifithrin- α provides neuroprotective effects at the level of mitochondria independently of p53 inhibition

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Abstract Impaired mitochondrial integrity and function are key features of intrinsic death pathways in neuronal cells. Therefore, key regulators of intrinsic death pathways acting upstream of mitochondria are potential targets for therapeutic approaches of neuroprotection. The tumor suppressor p53 is a well-established regulator of cellular responses towards different kinds of lethal stress, including oxidative stress. Recent reports suggested that p53 may affect mitochondrial integrity and function through both, transcriptional activation of mitochondria-targeted pro-death proteins and direct effects at the mitochondrial membrane. In the present study, we compared the effects of pharmacological inhibition of p53 by pifithrin- α with those of selective p53 gene silencing by RNA interference. Using MTT assay and real-time cell impedance measurements we confirmed the protective effect of both strategies against glutamate-induced oxidative stress in immortalized mouse hippocampal HT-22 neurons. Further, we observed full restoration of mitochondrial membrane potential and inhibition of glutamate-induced mitochondrial fragmentation by pifithrin- α which was, in contrast, not achieved by p53 gene silencing. Downregulation of p53 by siRNA decreased p53 transcriptional activity and reduced expression levels of p21 mRNA, while pifithrin- α did not affect these endpoints. These results suggest a neuroprotective effect of pifithrin- α which occurred at the level of mitochondria and independently of p53 inhibition.

Keywords p53 · Mitochondria · Pifithrin- α · Oxytosis · Neuroprotection

Abbreviations

AIF	Apoptosis inducing factor
AD	Alzheimer's disease
ANOVA	Analysis of variance
Bid	BH3 interacting-domain death agonist
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
DRP1	Dynamin-related protein 1
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
LPS	Lipopolysaccharide
MDM2	Mouse double minute 2 homolog
MMP	Mitochondrial membrane potential
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PCD	Programmed cell death
PD	Parkinson's disease
PFT α	Pifithrin- α
pMCAO	Permanent middle cerebral artery occlusion
PUMA	p53 upregulated modulator of apoptosis
ROS	Reactive oxygen species
TIF-IA	Pol I-specific transcription initiation factor IA
TMRE	Tetramethylrhodamin ethyl ester

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Introduction

Degeneration and death of neurons are the major underlying causes for impaired brain functions in many neurological disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and stroke [1]. Although these

diseases show significant differences in their pathogenesis and symptoms, they all share common mechanistic features such as the involvement of oxidative stress which can trigger neurodegeneration [2]. Further, there is increasing evidence for a pivotal role of increasing mitochondrial impairments during processes of neurodegeneration and aging [3]. In fact, the loss of mitochondrial integrity and function is the hallmark of intrinsic pathways of programmed cell death (PCD). The translocation of the proapoptotic protein Bid to mitochondria [4] as well as the detrimental generation of reactive oxygen species (ROS) results in the permeabilization of the mitochondrial membrane [5]. Once this point of mitochondrial demise has been reached, the death of neuronal cells is inevitable [6]. Thus, the protection of mitochondria is considered to be a potential therapeutic target in neurological disorders. For example, restoration of mitochondrial function improved performance in a transgenic mouse model of AD [7], and even in humans beneficial effects of “mitoprotection” against progression of neurodegenerative processes were observed [8].

The tumor suppressor protein p53 is regarded as the guardian of the genome. Hence, it plays a crucial role in sensing DNA damage, cell cycle regulation and cellular response to oxidative stress affecting DNA integrity, also in neurons. Dysfunction of p53 contributes to neuronal impairment in AD and PD [9]. Depending on the nature and the extent of cellular stress and associated DNA damage, p53 can either mediate DNA repair via transcriptional induction of p21 [10] or cell death by induction of e.g. the Bcl-2-family protein BAX and the BH3-only proteins Bid, PUMA and NOXA [1, 11, 12]. On the one hand, a loss of function in the p53 gene is often associated with an enhanced responsiveness to cancer [13], but on the other hand, p53 knockout or pharmacological inhibition of p53 was shown to be beneficial in cases of neuronal damage [14–16]. These findings underline the importance of a well controlled p53 expression in terms of disease. Besides transcriptional activity, p53 can also directly act at the level of mitochondria to induce PCD [17–19].

As the induction of apoptosis is one of the most important functions of p53, its pharmacological inhibition has been studied for a long time as a potential target in neurodegenerative disease e.g. Alzheimer and stroke [11, 20, 21]. One of the most frequently used p53-inhibitors is pifithrin- α (PFT α), a small molecule which was first described by Komarov and coworkers [22]. PFT α was shown to be beneficial in rodent models of PD [23, 24], epileptic seizures [25] and stroke [15, 20]. Although PFT α is claimed to be a specific inhibitor of p53, reports on PFT α interaction with the aryl hydrocarbon receptor [26], inhibition of nuclear translocation of p53 [27] and suppression

Fig. 1 PFT α prevents glutamate-induced cell death. **a** PFT α (10 μ M) preserves HT-22 cells from glutamate-induced (5 mM, 15 h) morphological changes compared to non-treated controls ($\times 10$). **b** MTT assay shows dose-dependent protection of PFT α against glutamate (7 mM, 16 h) toxicity. (#### $p < 0.001$ compared to untreated control; * $p < 0.05$ and *** $p < 0.001$ compared to glutamate-treated control). **c** Neuroprotective effect of PFT α (10 μ M) was determined by AnnexinV/PI staining (5 mM glutamate, 16 h). Data shown as mean + SD. (#### $p < 0.001$ compared to untreated control; *** $p < 0.001$ compared to glutamate-treated control). **d** PFT- μ does not protect against glutamate toxicity (3 mM, 15 h). (#### $p < 0.001$ compared to untreated control). **e** Impedance was measured in post-treatment conditions when PFT α (10 μ M) was added 2, 4 and 6 h after the onset of glutamate (4 mM). PFT α attenuated cell death compared to glutamate treated controls when applied up to 4 h after the onset of glutamate ($n = 7$). **f** Bar graph evaluation at the 14 h time point from the xCELLigence recordings of Fig. 1e (right black arrow). (#### $p < 0.001$ compared to untreated control; ** $p < 0.01$ and *** $p < 0.001$ compared to glutamate-treated control)

of heat shock and glucocorticoid receptor signaling [28] question the specificity of PFT α .

Therefore, the aim of this study was to elucidate the effects of PFT α on cell viability and mitochondrial function in a model of glutamate-induced oxidative cell death in immortalized hippocampal HT-22 cells compared to a selective downregulation of p53 by using p53 siRNA. In HT-22 cells lacking NMDA-receptors, glutamate induces a depletion of glutathione and the subsequent accumulation of ROS which therefore results in lethal oxidative stress and mitochondrial fragmentation [4]. We report that PFT α provides neuroprotection in hippocampal HT-22 neurons by protecting mitochondria against glutamate-induced oxytosis independent of p53 inhibition. In contrast, p53 depletion only partly abolishes cell death and does not affect mitochondrial damage in intrinsic cell death triggered by oxidative stress.

Results

PFT α mediates neuroprotective effects against oxidative stress

Millimolar concentrations of glutamate induce detrimental amounts of ROS in hippocampal HT-22 cells causing cell death 12–15 h after the glutamate challenge [5]. The damaged cells shrink, round up and detach from the bottom of the dish. Treatment with PFT α prevented these morphological changes completely (Fig. 1a). The morphological changes after glutamate exposure were also reflected in a loss of cell viability detected by the MTT assay. Co-treatment with PFT α rescued HT-22 cells from glutamate induced cell death in a concentration dependent manner (Fig. 1b). This neuroprotective effect of PFT α was also confirmed by Annexin V/PI staining and subsequent FACS

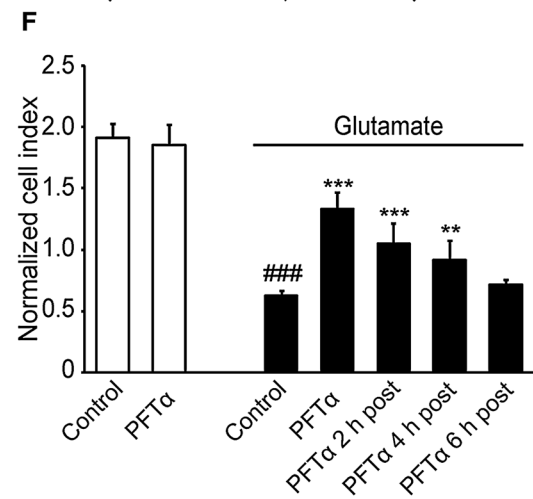
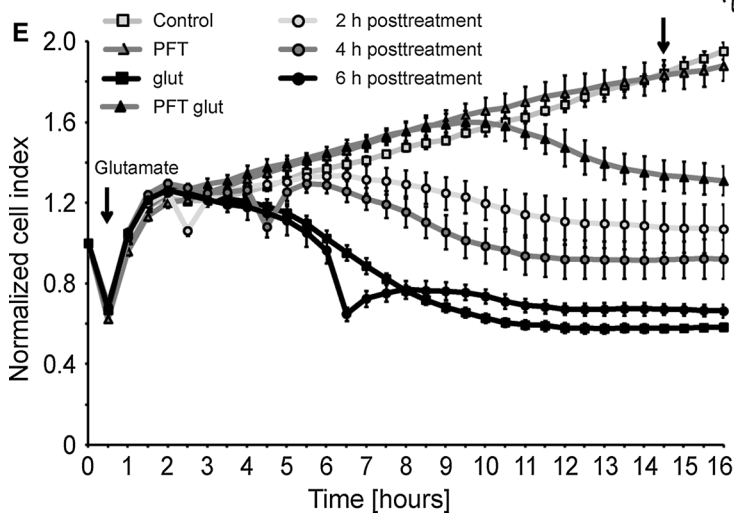
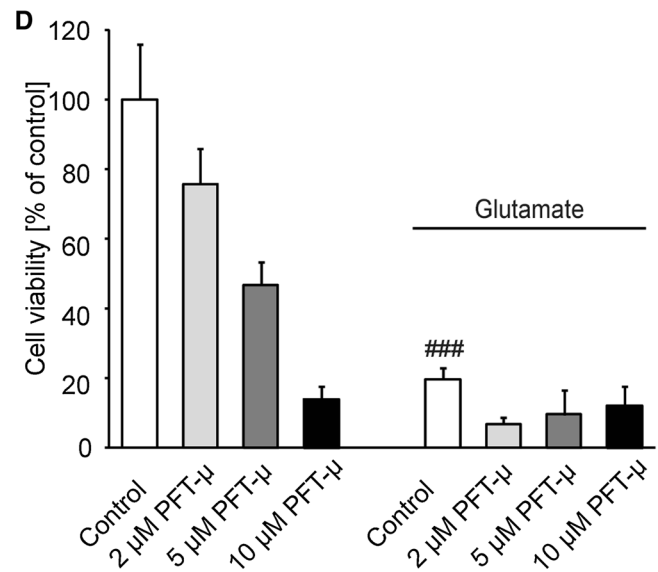
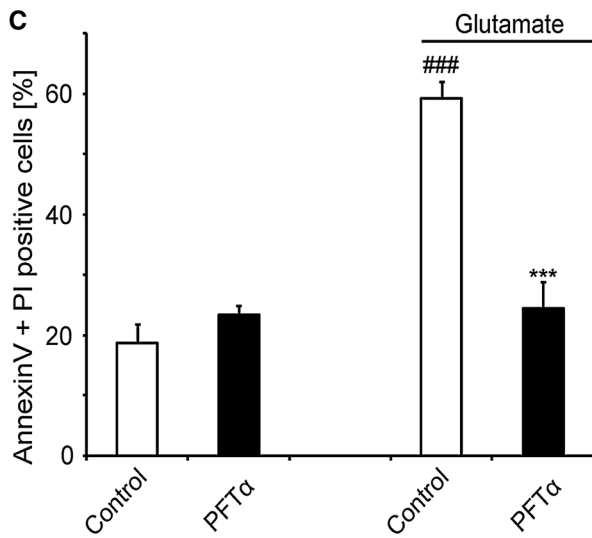
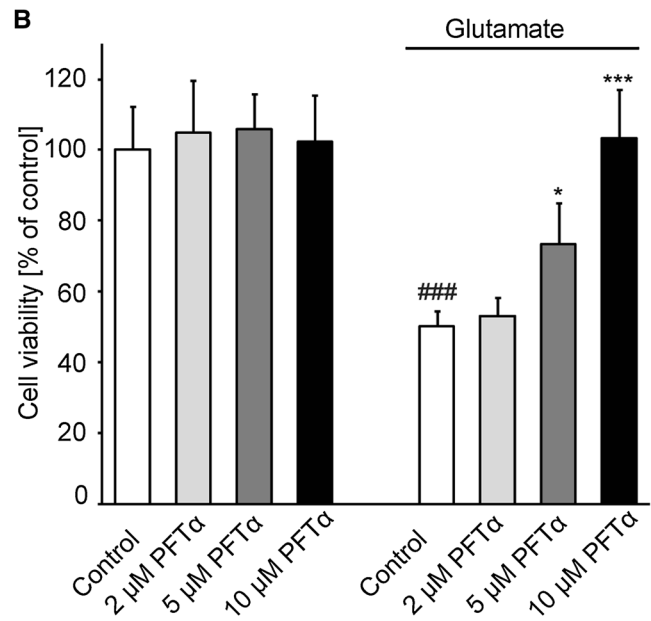
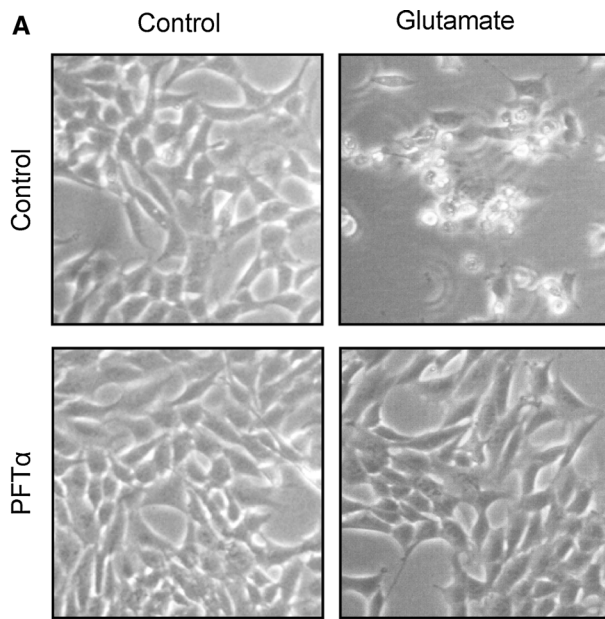
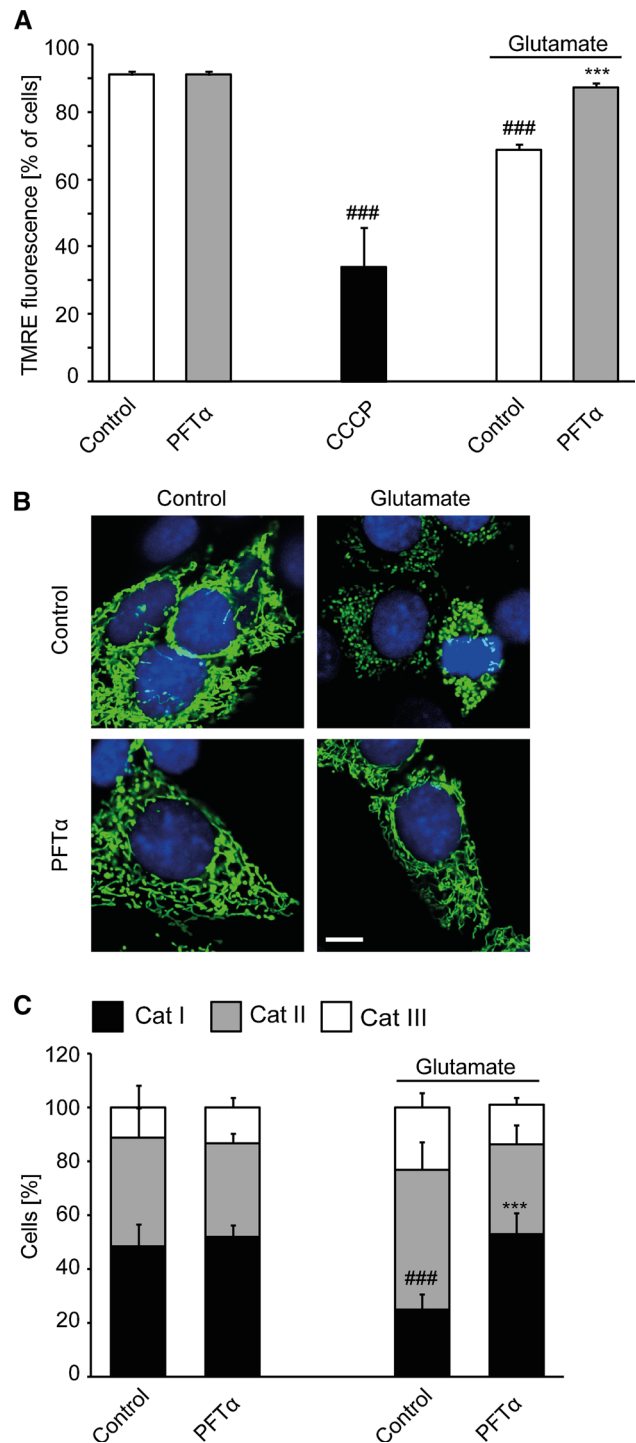


Fig. 2 PFT α preserves MMP and morphology. **a** MMP measured by TMRE fluorescence is fully restored by PFT α (10 μ M) after 3 mM glutamate, 14 h. Data shown as mean \pm SD. (### p < 0.001 compared to untreated control; *** p < 0.001 compared to glutamate-treated control). **b** Fluorescence pictures show conservation of mitochondrial morphology after PFT α (10 μ M) in combination with glutamate (5 mM, 14 h). Cells were stained with Mitotracker green and DAPI. Scale bar 20 μ m. **c** Quantification of mitochondrial morphology; Category I (Cat I): elongated, Category II (Cat II): intermediate, Category III (Cat III): fragmented. Values are given from five independent experiments with at least 500 cells per condition counted blind. (### p < 0.001 compared to Cat I of untreated control; *** p < 0.001 compared to Cat I of glutamate-treated control). Statistics were obtained using ANOVA, Scheffé Test

analysis. In this assay, early apoptotic cells appear Annexin V-positive while late apoptotic and necrotic cells are Annexin V- and PI-positive [29]. Healthy cells remain unstained. Total cell death was determined by adding the values for both populations of dying cells showing positive staining for Annexin V and Annexin V/PI. Glutamate significantly increased the number of dead cells compared to control levels. The p53 inhibitor PFT α was able to abolish the increase in Annexin- and PI-positive cells after glutamate challenge almost to control level (Fig. 1c). In contrast, Pifithrin- μ (PFT- μ), another p53 inhibitor, which inhibits p53 binding to mitochondria [42], failed to protect HT-22 cells from glutamate induced toxicity. It even showed a dose-dependent toxicity (Fig. 1d). A post-treatment with PFT α up to 4 h after the glutamate challenge also significantly prevented the decrease in cell viability, but was not as protective as the simultaneous administration of glutamate and PFT α (Fig. 1e, f).

PFT α preserves mitochondrial integrity

Mitochondrial damage is a key feature of cell death induced by oxidative stress in the present model system [4, 5, 30]. Hence, we examined mitochondrial morphology and functional hallmarks such as mitochondrial membrane potential (MMP) and ATP production. To this end, HT-22 cells were treated with glutamate and stained with TMRE, a lipophilic cationic dye indicating intact MMP by red fluorescence. Loss of MMP due to glutamate toxicity was determined by the loss of red fluorescence detected by FACS analysis. Carbonyl cyanide 3-chlorophenylhydrazine (CCCP), which acts as an uncoupler of the oxidative phosphorylation, was applied as a positive control for the loss of MMP. Glutamate did not affect the MMP to the same extent as CCCP, but induced a significant reduction of MMP compared to untreated control cells. PFT α preserved such MMP loss induced by glutamate almost at control levels. PFT α treatment itself did not influence the membrane potential at all (Fig. 2a). Next, we examined the mitochondrial morphology. As established in our earlier



work, glutamate toxicity in HT-22 cells was accompanied by mitochondrial fragmentation [4, 30]. These fragmented mitochondria appear in a dotted pattern and are located in close vicinity to the nucleus of the damaged cells. Treatment with PFT α significantly prevented this glutamate-induced mitochondrial fragmentation. Mitochondria of PFT α -treated cells remained elongated and appeared as a

tubular network all over the cell (Fig. 2b). Overall, these findings pointed to a protective mechanism of action of PFT α at the level of mitochondria.

PFT α blocks lipid peroxidation

Lipid peroxidation is a well-established trigger mechanism of mitochondrial demise and neuronal cell death in the present model of oxytosis induced by glutamate in HT-22 cells [5, 31, 32]. Accordingly, we detected lipid peroxidation using fluorescent Bodipy staining and subsequent FACS analysis. Bodipy fluorescence is shifted from red to green upon oxidation. At both time points, at 7 and 15 h after the glutamate challenge, PFT α attenuated the formation of lipid peroxides in a concentration-dependent manner (Fig. 3a, b) which correlated well with the previous results from the MTT assays where the protective effect also showed a concentration dependency (Fig. 1b).

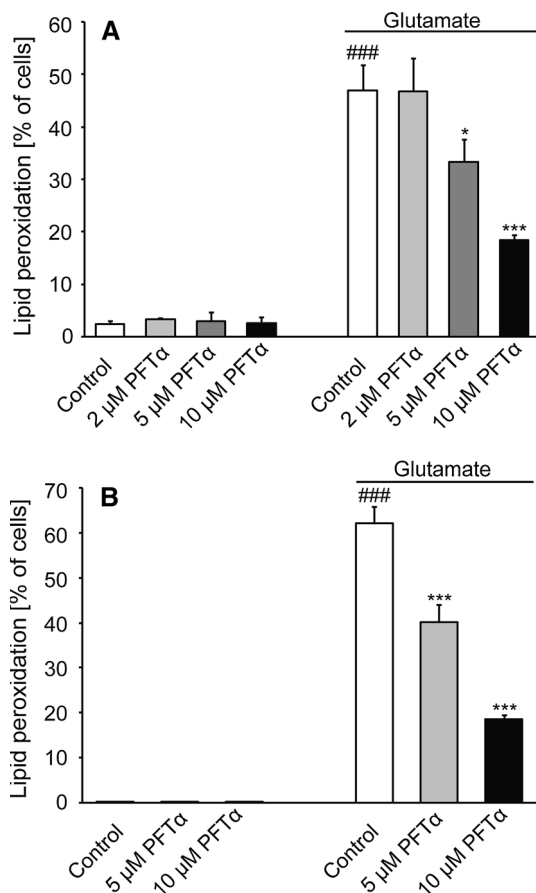


Fig. 3 PFT α reduces lipid peroxidation. **a** PFT α lowers lipid peroxidation after glutamate challenge (7 mM, 15 h) in a concentration dependent manner detected by Bodipy staining and FACS analysis. PFT α was added 1 h before glutamate treatment. **b** Bodipy staining after 7 h glutamate (7 mM) and PFT α pretreatment. Data shown as mean + SD. (### p < 0.001 compared to untreated control; *** p < 0.001 and * p < 0.05 compared to glutamate-treated control)

Selective p53 silencing attenuates glutamate-induced toxicity

PFT α is a widely used inhibitor of p53 [22]. To link our previous results to this proposed inhibition we investigated the effects of a siRNA-mediated downregulation of p53 protein levels in HT-22 cells on cell viability and mitochondrial integrity. The knockdown of p53 was achieved by siRNA transfection and confirmed at mRNA and protein levels using RT-PCR and Western Blot analysis, respectively (Fig. 4a). Gene silencing of p53 attenuated glutamate-induced cytotoxicity as detected by the MTT assay while transfection of controls with the scrambled siRNA did not alter cell viability. PFT α co-treatment was used as a positive control for cell survival and appeared more potent than p53 downregulation (Fig. 4b). To rule out off target effects we confirmed these observations by using another p53 siRNA as shown in the supplements (online resource 1). As the MTT assay is just an endpoint measurement, we also detected cell viability over time using the real time impedance measurement system (xCELLigence). Here we could show a delay of cell death by selective p53 silencing for about 2 h compared to cells transfected with a non-specific scrambled siRNA (Fig. 4c) which could explain a lesser effect than in comparison to PFT α in the MTT. PFT α administration to p53 silenced cells was still protective against glutamate induced cytotoxicity and even more than p53 silencing alone (Fig. 4d, e).

p53 gene silencing fails to preserve mitochondrial integrity after the glutamate challenge

As p53 silencing had similar but only transient protective effects on cell viability compared to PFT α treatment, we further investigated the impact of p53 knockdown on mitochondrial integrity. The FACS analysis of cells stained with TMRE revealed that p53 siRNA-mediated knockout failed to prevent mitochondrial depolarization after the glutamate challenge for 14 h (Fig. 5a). Transfection of p53 siRNA in control cells did not change MMP at all. After 7 h of glutamate treatment there were no changes in the MMP detectable in cells transfected with scrambled siRNA as well as in p53 silenced cells (Fig. 5a). Further, after the glutamate challenge we observed similar increases in mitochondrial fragmentation (category II and III) and decreasing percentages of tubular mitochondria (category I) in cells treated either with scrambled siRNA or p53 targeting siRNA, suggesting that the depletion of p53 did not prevent mitochondrial fragmentation. Notably, siRNA transfection itself did not alter the shape of the mitochondria in control cells which predominantly contained elongated and tubular mitochondria distributed in a tubular network all over the cell (Fig. 5b).

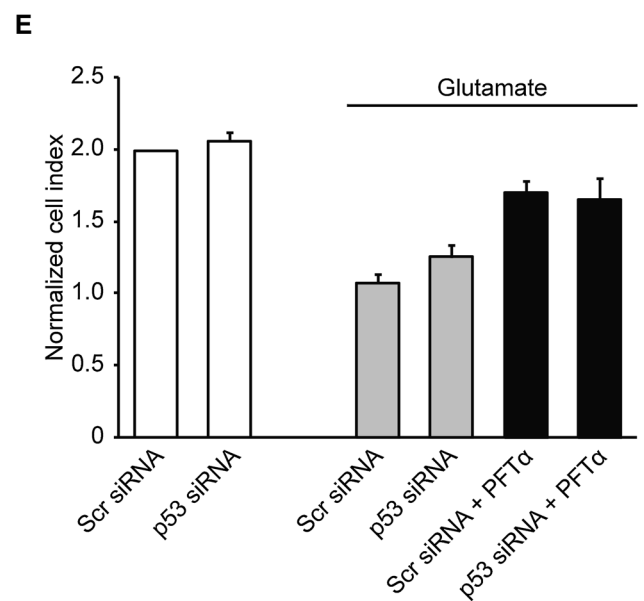
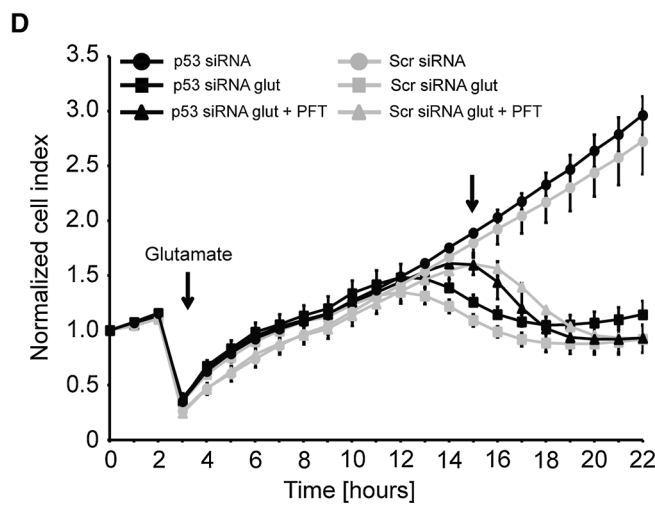
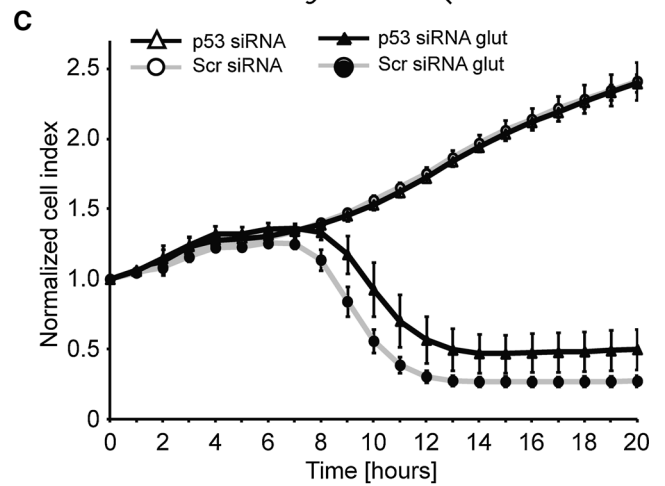
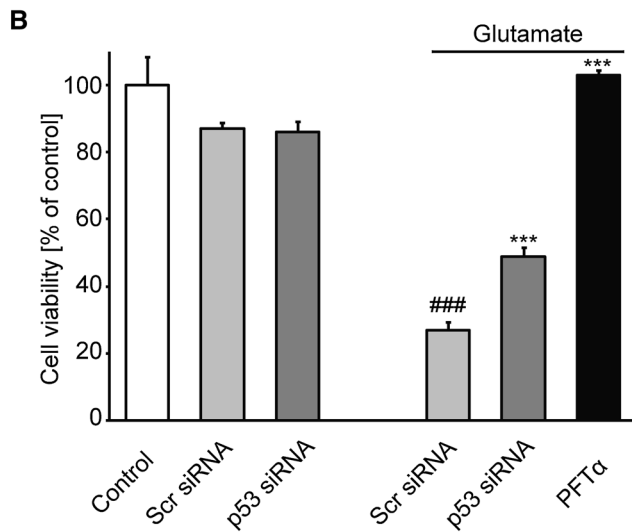
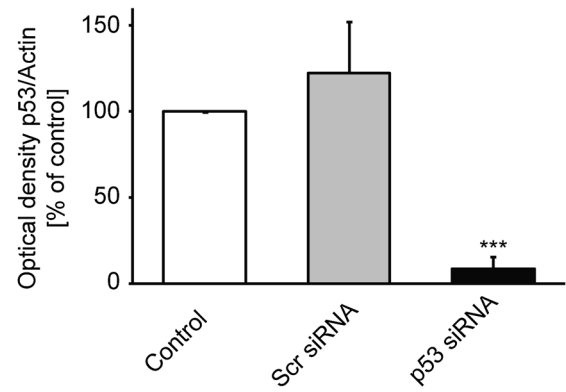
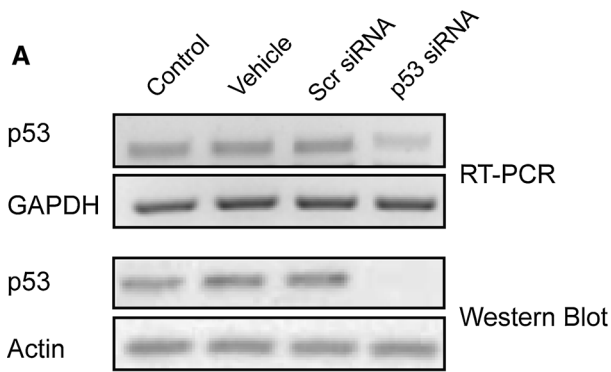


Fig. 4 Selective p53 knockdown attenuates glutamate toxicity. **a** Knockdown of p53 by siRNA (20 nM) was verified by RT-PCR and Western Blot analysis. Quantification of 7 independent Western Blots shows a significant reduction of p53 protein level. (** $p < 0.001$ compared to scrambled siRNA). **b** MTT assay shows increased cell viability of cells transfected with p53 siRNA (20 nM) compared to cells transfected with scrambled control siRNA (20 nM) after glutamate challenge (3 mM, 14 h). (### $p < 0.001$ compared to untreated scrambled siRNA; *** $p < 0.001$ compared to glutamate-treated scrambled siRNA). **c** xCELLigence real-time measurement: Cells transfected with p53 siRNA (20 nM) show a short transient protection against treatment with 3 mM glutamate (glut) compared to cells transfected with scrambled siRNA. **d** xCELLigence real-time measurement reveals protection of knocking down p53 and PFT α treatment at the same time against glutamate (5 mM) toxicity. **e** Bar graph evaluation at the 15 h time point from the xCELLigence recordings of Fig. 4d (right black arrow)

p53 knockdown blocks p53 transcriptional activity

It is well established that p53 can induce cell death through induction of different proapoptotic target genes [33]. It has recently been shown that the inhibition of PUMA and

NOXA upregulation by PFT- μ was associated with reduced cerebral damage in a model of hypoxic-ischemic brain damage. In contrast to that, the beneficial effect of PFT α in this model was much smaller although it fully blocked p53 transcriptional activity [19]. To address the question, whether downregulation of p53 was also able to diminish p53 transcriptional activity in HT-22 cells despite the small effect on cell viability, we created a p53-Luc reporter plasmid. This plasmid was transfected into p53 silenced HT-22 cells and p53 transcriptional activity was assessed in the presence or absence of glutamate to measure luciferase activity. As expected, siRNA-mediated p53 gene silencing reduced p53 transcriptional activity compared to the controls. Unfortunately, this assay is not suitable to investigate the described inhibitory effect of PFT α on p53 transcriptional activity because a direct inhibition of firefly luciferase has been reported for PFT α concentrations higher than 1 μ M [34], and this unspecific inhibition of luciferase activities was also confirmed in our current experimental setup (data not shown). To further compare

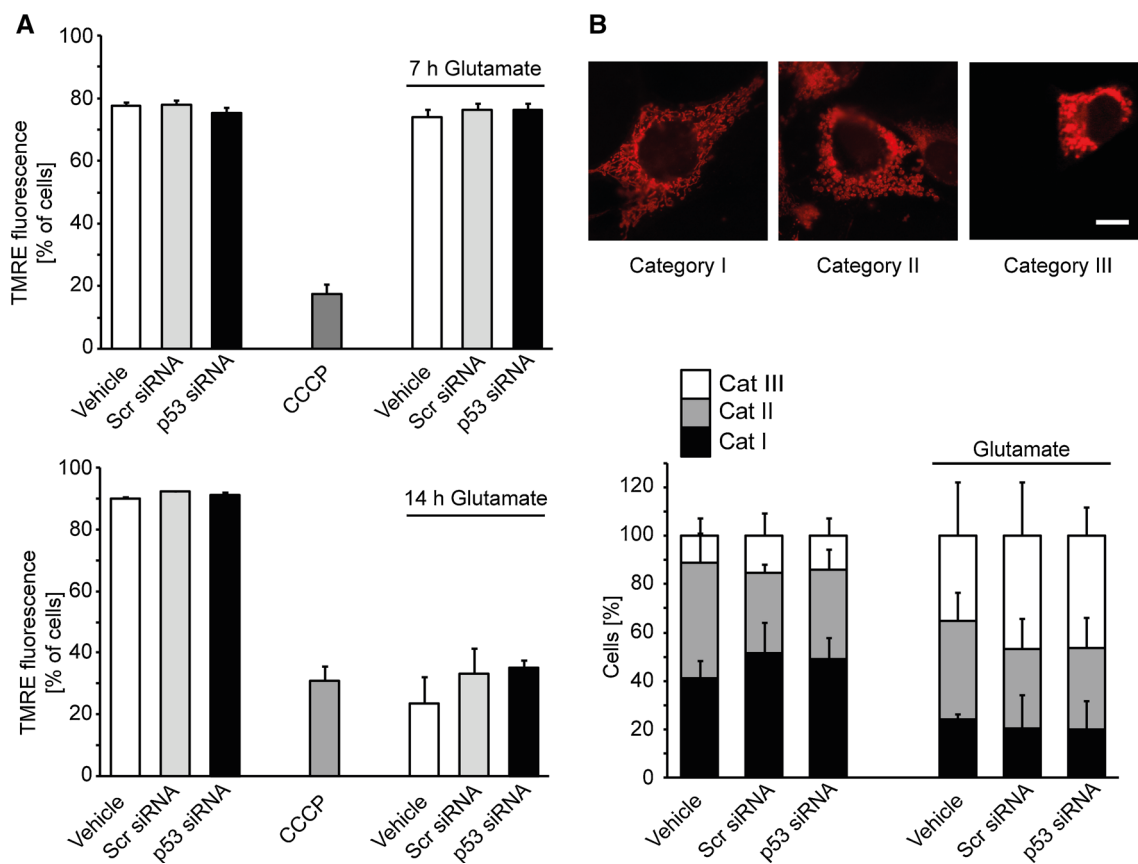


Fig. 5 p53 siRNA does not keep mitochondrial integrity. **a** MMP was determined after 7 and 14 h glutamate (3 mM). CCCP was used as positive control for breakdown of MMP. **b** Fluorescence photomicrographs show categories of mitochondrial morphology: Category I (Cat I): elongated, Category II (Cat II): intermediate,

Category III (Cat III): fragmented; scale bar 20 μ m. Cells were stained with Mitotracker Red before glutamate challenge (3 mM, 14 h) and quantified by counting at least 500 cells per condition blind to treatment

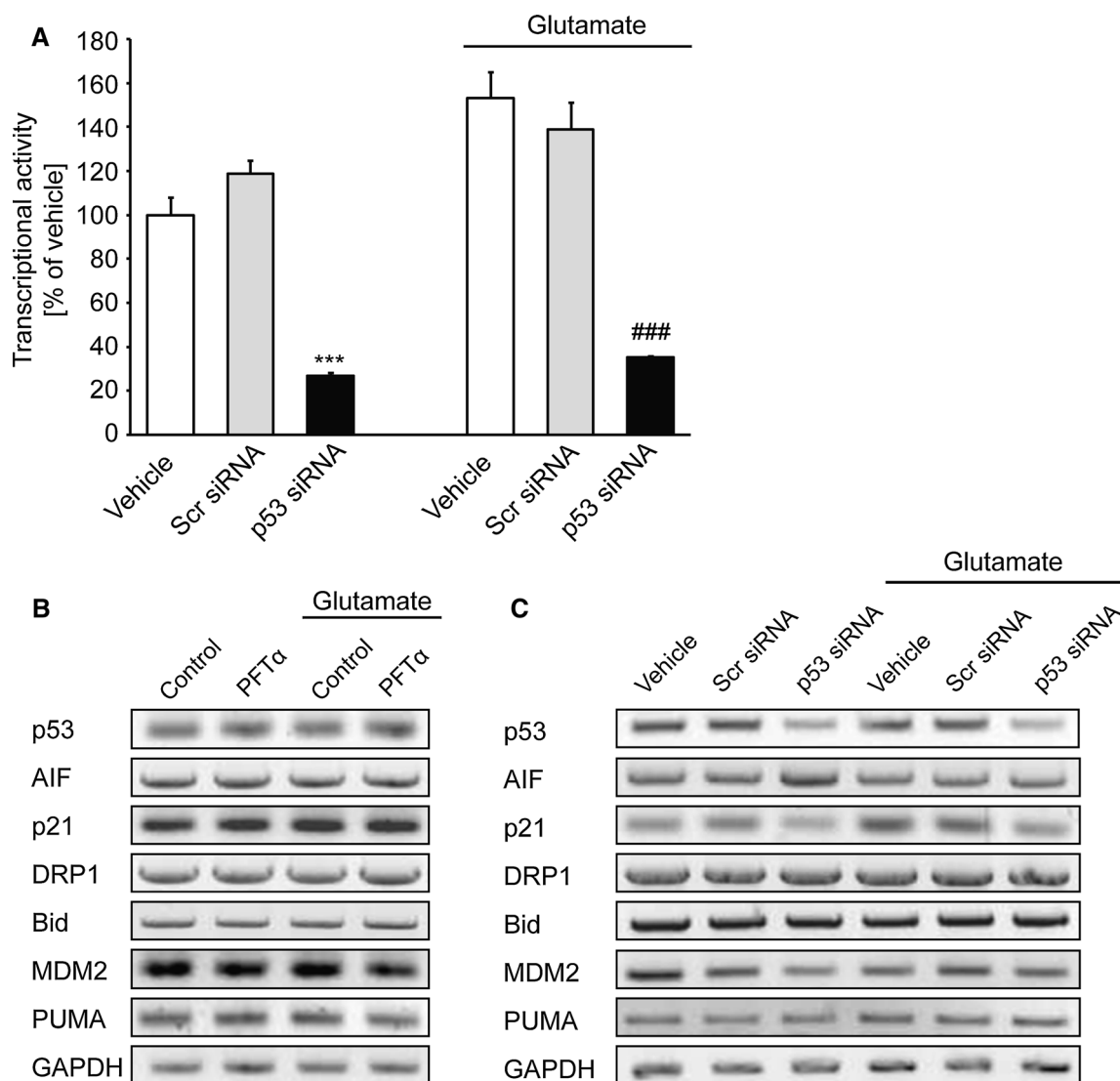


Fig. 6 p53 siRNA blocks p53 transcriptional activity. **a** p53 silencing diminishes p53 transcriptional activity. Reporter assay was performed after glutamate challenge (3 mM, 14.5 h). Data shown as mean + SD. (***) $p < 0.001$ compared to untreated vehicle;

$p < 0.001$ compared to glutamate-treated vehicle.). **b** RT-PCR analysis of p53 target genes after PFT α -treatment and glutamate challenge (7 mM, 16 h). **c** RT-PCR analysis of p53 target genes after p53 knockdown and glutamate challenge (7 mM, 15 h)

the effects of pharmacological p53 inhibition and selective p53 gene silencing on transcriptional p53 activity, we determined the mRNA expression levels of p53 target genes known to be associated with mitochondrial function and regulation of apoptosis. Here, we confirmed the functionality of the p53 siRNA on p53 mRNA expression, while PFT α did not affect p53 mRNA levels. The amount of the proapoptotic proteins AIF, DRP1, PUMA and Bid remained unchanged after treatment with both, PFT α or p53 siRNA, in the presence or absence of glutamate. Notably, the induction of oxytosis by glutamate itself was not accompanied by changes in the mRNA level of any of these proteins. The mRNA levels of the negative p53 regulator MDM2 were decreased in cells with p53

silencing but not in the presence of PFT α . Further, we found a downregulation of the cell cycle regulator p21 after p53 knockdown both with and without induction of oxidative stress, whereas this was not observed with PFT α treatment (Fig. 6b, c).

Discussion

The present study shows that the inhibition of p53 protects neuronal HT-22 cells against glutamate-induced oxytosis. Regarding cell viability, PFT α was more potent than p53 silencing via RNA interference. The observed protection of HT-22 cells by both, PFT α -mediated pharmacological p53

inhibition and p53 gene silencing, is in line with findings reported in other model systems of neuronal cell death in the literature. Yonekura and coworkers observed reduced neuronal death in p53^{-/-} mice after global cerebral ischemia compared to p53^{+/+} mice [35]. Further, in a model of permanent middle cerebral artery occlusion (pMCAO), p53 knockout reduced infarct size [36]. PFT α also reduces contusion volume in mice after traumatic brain injury [16]. Considering these findings one would not expect such a notable difference between p53 gene silencing and pharmacological p53 inhibition with PFT α regarding cell viability as described in the present study. It should be considered that we do not achieve a sustained full knock out of p53 levels and transcriptional activity using RNA interference while a concentration of 10 μ M PFT α is rather high and probably able to fully block p53 action leading to a more pronounced effect, particularly at the level of mitochondria which would explain our observations.

Differential effects of pharmacological p53 inhibition and genetic knockout were also described in previous studies. For example, in a model of Pol I-specific transcription initiation factor IA (TIF-IA) depleted dopaminergic neurons the inhibition of p53 by PFT α decreased the amount of apoptotic cells [37] while in TIF-IA depleted medium spiny neurons p53 knockout led to an increase of apoptotic cells [38]. Mendjargal and coworkers also demonstrated in a murine macrophage-like cell line that a siRNA-mediated down regulation of p53 did not affect lipopolysaccharide (LPS)-induced iNOS expression whereas PFT α strongly reduced iNOS levels after LPS treatment also in p53-silenced cells which points towards a p53-independent action of PFT α in this model system as well [39].

In contrast to inhibition of p53 by PFT α the gene silencing of p53 was not able to preserve mitochondrial integrity after the glutamate challenge. Thus, the present data suggest an action of PFT α upstream of mitochondria preventing glutamate-induced oxytosis indicated by a pronounced protection of MMP and mitochondrial morphology (Fig. 2). This is in line with earlier findings in cultured primary neurons [20, 40] and in synaptosomes [40] where PFT α also preserved mitochondrial function after insults induced by DNA damage, glutamate or oxidative stress.

Our diverse findings regarding effects of PFT α and p53-siRNA on mitochondria are important to explain the differences in the respective effects on cell viability. Our findings indicate pleiotropic effects of PFT α beyond the inhibition of p53 that mediate protection of neurons against oxidative stress. This conclusion is supported by previous findings on interactions of PFT α with the aryl hydrocarbon receptor [26] or HSP70 [28]. Further, PFT α may also

induce mitochondrial protection and neuroprotection in ischemic brain tissue via activation of Akt and enhanced NF- κ B signaling [15, 41]. The beneficial effects of PFT α on mitochondria and the sustained neuroprotective effect compared to mere p53 silencing detected in the present study underline once more the pivotal role of mitochondrial integrity for neuroprotection.

The full mitochondrial protection is also accompanied by the reduction of lipid peroxide formation in the presence of PFT α (Fig. 3). This stands in contrast to the observations of Nijboer et al. who did not see a reduction in lipid peroxidation in a model of hypoxic-ischemic brain injury by PFT α , but by PFT- μ [19] which inhibits mitochondrial association of p53 [42], thus mediating its protective effect. However, PFT- μ also failed to protect HT-22 cells against glutamate-induced oxytosis (Fig. 1d) supporting our hypothesis of a p53-independent mitochondrial mechanism of action of PFT α in the present model system of oxytosis.

In order to gain deeper insights into the mechanisms of protection at the level of mitochondria we screened for several target genes of p53 that were previously associated with mitochondrial pathways of cell death. Although there are numerous target genes of p53 involved in cell death it is still controversially discussed which of these are the most important for p53-mediated cell death [33]. So far, it seems to depend on the kind of stress and cell type which target gene is the most relevant for p53-dependent induction of apoptosis [43].

MDM2 is one of the major regulators of p53 activity and also one of its transcriptional targets acting in a negative feedback loop [33] thereby controlling p53 levels during normal growth [44]. This described negative regulation is consistent with our observed decrease in MDM2 expression levels after siRNA-mediated p53 gene silencing (Fig. 6c).

While we could not observe any differences in expression levels of AIF, DRP1, Bid or PUMA after both, PFT α treatment or p53 downregulation, the most striking effect was the decrease of p21 levels after p53 silencing in the presence as well as in the absence of glutamate. p21 is the major p53-dependent mediator of cell cycle arrest [10, 33]. Yu et al. showed that p21^{-/-} cancer cells are more susceptible to p53-induced apoptosis than wild type cells [45, 46] leading to the conclusion that in the present model system less p21 attributed to p53 gene silencing may render the HT-22 cells more sensitive to the imposed oxidative stress. In contrast, Leker and colleagues claimed that a reduction of p21 by PFT α decreased the amount of apoptotic cells in ischemic brain tissue due to the inhibition of p53 transcriptional activity [47]. In the present study the reduced transcriptional activity of p53 in p53 silenced cells (Fig. 6a) led to a decline of p21 expression levels which was not found after PFT α treatment (Fig. 6b, c). Together

with the differential outcome on cell viability after p53 gene silencing versus the effects of PFT α , in the present setting of experiments lower levels of p21 correlated with enhanced susceptibility of the cells for oxidative stress.

PUMA is one of the most prominent pro-apoptotic p53 target genes which act at the level of mitochondria in p53-dependent apoptosis [48]. Recent studies showed an upregulation of PUMA in damaged neurons after transient global cerebral ischemia [49] and after status epilepticus triggered by intra-amygdala kainic acid in mice [25], these effects were abolished by PFT α . As there is no change in PUMA expression in our model we can conclude that glutamate-induced oxytosis in HT-22 cells is not reliant on p53-dependent transcriptional activation of PUMA, which is also supported by the limited effect of p53 downregulation on cell survival compared to the pronounced and sustained protection achieved by PFT α . According to previous results in cancer cells, conserved PUMA levels and depletion of p21 [50] are required for mitochondrial damage and subsequent cell death while disruption of both PUMA and p21 prevented p53-induced apoptosis [45]. Since p53 gene silencing resulted in a similar expression pattern of reduced p21 levels and stable PUMA levels, this may also explain why p53 silencing failed to protect the HT-22 cells more efficiently.

In the present study, we showed a strong neuroprotective effect of the p53 inhibitor PFT α in HT-22 cells contrary to p53 gene silencing which only had partial effects on neuronal viability in a model of oxidative stress and did not affect mitochondrial integrity at all. The current data suggest a protective mechanism of PFT α upstream or at the level of mitochondria which must be either independent of p53 inhibition or accompanied by additional effects which could be the subject of future studies. All in all our results again confirm the neuroprotective potential of PFT α in neurodegenerative disease associated with oxidative stress and the importance of a well controlled p53 expression.

Materials and methods

Cells

HT-22 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Karlsruhe, Germany) with the addition of 10 % heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine (all Sigma-Aldrich, Munich, Germany).

For inducing cell death, 3–7 mM glutamate (Sigma-Aldrich, Munich, Germany) was added to the medium for the indicated amount of time.

SiRNA transfections were performed by using Lipofectamine RNAiMax (Invitrogen, Karlsruhe, Germany) following the manufacturers protocol. After complex

formation, an adequate number of cells were added in antibiotic-free medium to the transfection mix according to the following experimental procedure. Cells were treated after growing for 48 h. The following siRNA sequences were used: CCACUUGAUGGAGAGUAUU (p53 siRNA), UAAUGUAUUGGAACGCAUA (scrambled siRNA).

Pifithrin- α (Sigma-Aldrich, Munich, Germany) was dissolved in DMSO and used in a concentration of 10 μ M as pretreatment (1 h) and cotreatment with glutamate.

Pifithrin- μ (Sigma-Aldrich, Munich, Germany) was dissolved in DMSO.

Cell viability

Cell viability was quantified by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 1 h at 37 °C. The dye was dissolved in an appropriate amount of DMSO and absorbance measured at 570 nm versus 630 nm with FluoStar (BMG Labtech, Offenburg, Germany).

For real time measurement of cell viability, the xCELLigence system (Roche, Penzberg, Germany) was used as described earlier [51].

Additionally, cell viability was detected by an Annexin V/PI staining using an Annexin-V-FITC Detection Kit (Promokine, Heidelberg, Germany) followed by fluorescence-activated cell sorting (FACS) analysis. Data were collected from 10,000 cells from at least four wells per condition.

Mitochondrial membrane potential

MMP was measured by FACS analysis using a MitoPTTM TMRE Kit (Immunochemistry Technologies, Hamburg, Germany). Cells were treated with glutamate for 7–15 h and harvested by trypsin. After staining for 20 min with tetramethylrhodamin ethyl ester (TMRE) at 37 °C, cells were washed with PBS and resuspended in assay buffer. Data were collected from 10,000 cells from at least four wells per condition.

Mitochondrial morphology

For analyzing mitochondrial morphology, cells were stained with Mitotracker red/green and DAPI. After glutamate treatment, cells were fixed with 4 % paraformaldehyde. At least 500 cells per condition were counted blind to treatment. Images were acquired using a fluorescence microscope (Leica, Wetzlar, Germany).

Lipid peroxidation

The formation of lipid peroxides was determined by staining the cells with 2 μ M BODIPY 581/591 C11

(Invitrogen) for 1 h at 37 °C in culture medium after glutamate treatment at indicated time points. After collecting the cells, flow cytometric analysis was done in PBS with an excitation at 488 nm and an emission at 530 and 585 nm. Data were collected from 10,000 cells from at least four wells per condition.

Protein analysis and Western Blotting

For protein analysis, cells were treated as indicated. After washing with PBS, cells were lysed with buffer containing 0.25 M Mannitol, 0.05 M Tris, 1 M EDTA, 1 M EGTA, 1 mM DTT, 1 % Triton-X (all Sigma-Aldrich), supplemented with Complete Mini Protease Inhibitor Cocktail and PhosSTOP (both Roche Diagnostics, Penzberg, Germany). To eliminate membrane fragments and other insoluble components, extracts were centrifuged at 13000xg for 15 min at 4 °C. The amount of protein was measured by Pierce[®] BCA Protein Assay Kit (Perbio Science, Bonn, Germany).

For Western Blot analysis, samples were run on a 12.5 % SDS-Gel and blotted onto a PVDF-membrane at 15 V for 90 min. Blots were incubated with primary antibody at 4 °C overnight. The following primary antibodies were used: Anti-p53 (1C12) (Cell Signaling, Danvers, MA, USA) and Anti-Actin C4 (MB Biomedicals, Illkirch Cedex, France). After incubation with proper secondary HRP-labeled antibody (Vector Laboratories, Burlingame, CA, USA) Western Blot signals were detected by chemiluminescence with Chemidoc software (Bio-Rad, Munich, Germany).

RT-PCR

Total RNA amount was extracted after indicated treatment by InviTrap[®] Spin Universal RNA Kit (Strattec molecular, Berlin, Germany). RT-PCR was performed with SuperScript III One-Step RT-PCR Kit with Platinum *Taq* (Invitrogen). Following primers were used p53: forward 5'-GACCGCCGTACAGAAGAAGA-3' and reverse 5'-GCC CCACTTTCTTGACC-3'; GAPDH: forward 5'-AGGCCG GTGCTGAGTAT-3' and reverse 5'-TGCCTGCTTACC ACC TTCT-3'; AIF: forward 5'-GCGTAATACGACT CACTATAGGGAGATCCAGGCAACTTGT TCCAGC-3' and reverse 5'-CGTAATACGACTCACTATAGGGAGAC CTCTGCTCCAGCC CTATCG-3'; p21 forward 5'-GCAG ATCCACAGCGATATCC-3' and reverse 5'-CAACTGC TCACTGTCCACGG-3'; DRP1 forward 5'-ACAGGAGA AGAAAATGGAGTTTGAAG CAG-3' and reverse 5'-AA CAAATCCTAGCACCACGCAT-3'; Bid forward 5'-GGG A ACTGCCTGTGCAAGCTTAC-3' and reverse 5'-CAGT GAGGFCCTTGTCTCTGAA-3'; MDM2 forward 5'-CCA GCTTCGGAACAAGAGAC-3' and reverse 5'-ACACAAT

GTGCTGCTGCTTC-3'; PUMA forward 5'-CAGACTGT GAATCCTGTGCT-3' and reverse 5'-ACAGTATCTTAC AGGCTGGGG-3'. For visualization of the DNA products, UV illumination on a 1.5 % agarose gel was used.

Luciferase-reporter-assay

To investigate the effect of p53 siRNA on p53 transcriptional activity we have generated a luciferase reporter gene construct harboring six consensus p53 DNA binding elements [52]. The oligonucleotides containing p53 DNA binding elements used in this study were sense 5'-CTAGC AGACATGCCTAGACATGCCTAGACATGCCTAGAC ATGCCTAGACATGCCT AGACATGCCTCGA-3' and anti-sense 5'-GATCTCGAGGCATGTCTAGGCATGTCT AGGCATG TCTAGGCATGTCTAGGCATGTCTAGG CATGTCTG-3' and were flanked by sites for *Nhe*1 and *Bgl*2 restriction enzymes. One microgram of each oligonucleotide was mixed together and heated at 95 °C for 5 min in a water bath and allowed to cool slowly to room temperature. These annealed oligonucleotides were ligated into *Nhe*1 and *Bgl*2 restriction sites of the pTAL-Luciferase (Luc) reporter plasmid yielding p53-Luc reporter vector. pTAL-Luc vector contains a TATA-like promoter region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter to drive the luciferase gene expression upon p53 binding to its enhancer element.

For detection of p53-transcriptional activity, cells were transfected with p53 siRNA as previously described. After 1 day, Renilla-plasmid and p53-Luc-plasmid were cotransfected using Attracten[®] (Qiagen, Hilden, Germany). Twenty-four hours later cells were treated as indicated and luciferase assay was performed with Dual-Luciferase[®] Assay Kit (Promega, Madison, USA) following manufacturers protocol. Luminescence was measured with FluoStar (BMG Labtech, Offenburg, Germany).

Statistical analysis

All data are given as mean + standard deviation (SD). Statistical comparison between treatment groups was performed by analysis of variance (ANOVA) followed by Scheffé's post hoc test. Calculations were executed with Winstat standard statistical software (R. Fitch Software, Bad Krozingen, Germany).

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Conflict of interest The authors declare that they have no conflict of interests.

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