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Inhibition of p73 Function by Pifithrin-α as Revealed by Studies in Zebrafish Embryos

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Abreviations: MO, antisense morpholino oligonucleotide; PFTα, pifithrin-α; Hpf, hours post fertilization; Kd, knock down; IR, ionizing radiation

Key Words: zebrafish, development, radiation effects, tumor suppressor protein p53, tumor suppressor protein p73, pifithrin-α

The p53 family of proteins contains two members that have been implicated in sensitization of cells and organisms to genotoxic stress, i.e., p53 itself and p73. In vitro, lack of either p53 or p73 can protect certain cell types in the adult organism against death upon exposure to DNA damaging agents. The present study was designed to assess the relative contribution of p53 to radiation resistance of an emerging vertebrate model organism, i.e., zebrafish embryos. Consistent with previous reports, suppressing p53 protein expression using antisense morpholino oligonucleotides (MOs) increased survival and reduced gross morphological alterations in zebrafish embryos exposed to ionizing radiation. By contrast, a pharmacological inhibitor of p53 function [Pifithrin-α (PFTα)] caused developmental abnormalities affecting the head, brain, eyes and kidney function and did not protect against lethal effects of ionizing radiation when administered at 3 hours post fertilization (hpf). The phenotypic abnormalities associated with PFTα treatment were similar to those caused by antisense MO knock down (kd) used to reduce p73 expression. PFTα also inhibited p73-dependent transcription of a reporter gene construct containing canonical p53-responsive promoter sequences. Notably, when administered at later stages of development (23 hpf), PFTα did not cause overt developmental defects but exerted radioprotective effects in zebrafish embryos. In summary, this study highlights off-target effects of the pharmacological p53 inhibitor PFTα related to inhibition of p73 function and essential roles of p73 at early but not later stages of zebrafish development.

Introduction

The genotoxic stress response is one of the most widely studied phenomena in biology and the efforts of many groups have provided a detailed understanding of the molecular determinants of this homeostatic mechanism (reviewed in refs. 1–3). Yet, the current understanding of the effects of genotoxic stress on whole organisms is curtailed by the fact that many of the mechanistic insights are based on experiments with cultured cells. These shortcomings are compounded by pitfalls associated with the preferential use of immortalized or transformed cells.[4] In recognition of these problems, many groups have resorted to studying the DNA damage response in experimental animals, particularly genetically engineered mice. These efforts have contributed considerably to the understanding of molecular determinants of the in vivo genotoxic stress response including Ku,[5,6] DNA-PK,[7] DNA ligase IV,[8] ATM,[9] ATR,[10] Chk1[11] and Chk2.[12] In addition, these studies confirmed a central role of the tumor suppressor p53 in the genotoxic stress response (reviewed in refs. 13 and 14).

The present study was undertaken to explore molecular determinants of the genotoxic stress response in an emerging animal model system, i.e., zebrafish embryos. Zebrafish represents a vertebrate species with many similarities to mammals. Yet, they breed prolifically and are amenable to large-scale phenotypic screening facilitated by the fact that they are transparent during organogenesis. Importantly, 'knockdown' strategies using antisense MOs have been developed in this species to investigate protein function in the in vivo context. Zebrafish are attractive not only to model human diseases but also as tools in drug discovery.[15] We have previously reported that zebrafish embryos provide a rapid, facile system to identify pharmacological modifiers of the radiation response.[16] Here, we extend these studies to assess the contribution of endogenous modifiers of the radiation response to radiation-induced morbidity and mortality by focusing on pharmacological and genetic inhibition of p53 function.

Results and Discussion

Time- and dose-dependent effects of ionizing radiation on zebrafish embryo survival. Previously, we observed that radiation sensitivity of zebrafish embryos was different at distinct developmental stages and progressively decreased between 2 to 8 hours post fertilization (hpf).[16] Here, we extend this earlier study by assessing embryo survival after exposure to increasing radiation doses up to 72 h after radiation. These experiments confirmed progressive radioresistance at successive stages of development (not shown). To determine the consequences of inhibiting translation of specific gene products by antisense MO kd for radiation resistance of the developing embryo we performed all subsequent experiments in embryos which were irradiated at 24 hpf. This was based on the consideration that, at this time point, target protein expression is sufficiently suppressed by antisense MO kd and remains low for extended time periods up to 4 days post fertilization (dpf).[17] Dose-dependent survival upon radiation exposure at 24 hpf revealed 100% lethality scored at 6–7 dpf (40 Gy) with an LD_{50} of 20 Gy.[18] To monitor the effects of p53 expression on radiation sensitivity as it relates to both, mortality and tissue-specific effects, we thus performed experiments at 20 or 40 Gy.

Reduced p53 expression is associated with radioprotection of zebrafish embryos. Zebrafish embryos harboring homozygous missense p53 mutations exhibit increased resistance to the deleterious effects of ionizing radiation.[19] We determined whether suppressing

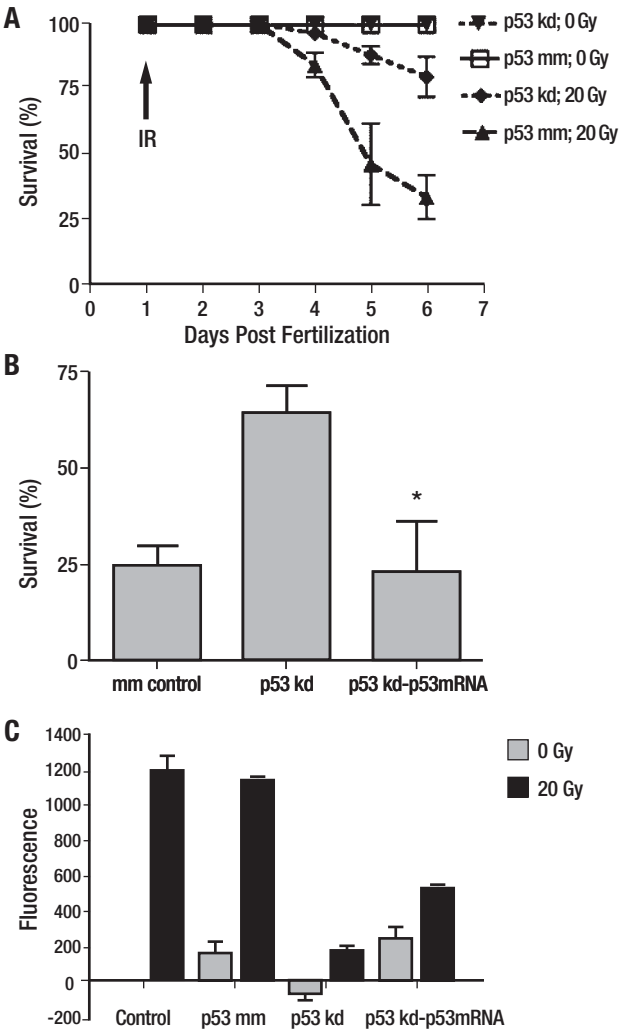

Figure 1. Antisense morpholino oligonucleotides (MOs) targeted to p53 increase embryo survival and reduce ionizing radiation-induced apoptosis evident in the head and trunk regions. **(A)** Embryo survival. Triplicate dishes of 60 embryos each were scored for live embryos daily after 20 Gy IR administered at 24 hpf. Necrotic dissolution or absent heartbeat were considered criteria for embryo death. **(B)** Restoration of IR sensitivity by restoring p53 expression using G-capped zebrafish p53 mRNA. Triplicate dishes of 60 embryos each were scored as described above at 6 dpf (5 days post IR). Asterix (*) p53kd vs p53kd-p53 mRNA; $p < 0.05$; t-test, one-tailed). **(C)** Attenuation of IR-induced apoptosis by p53 kd as assessed by quantification of acridine orange (AO) staining at 30 hpf. Sixty embryos per condition (Control, phenol red control; p53 mm, mismatch antisense MO; p53 kd, p53 antisense MO; p53 kd-p53mRNA, rescue coinjection with p53 antisense MO and p*53* G-capped mRNA) were pooled and stained with AO as described in Materials and Methods. Pooled embryos were transferred to 95% ethanol for 15 minutes to extract the AO for fluorescence determination. Triplicate measurements for each condition were performed on a FL600 microplate fluorescence reader (Bio-Tek) and normalized to control background fluorescence and reported as relative fluorescence units (RFU).

p53 expression by antisense MOs[20] similarly induced a radioresistant phenotype. We observed that *p53*-targeted MO kd markedly improved survival of zebrafish embryos irradiated with 20 Gy at 24 hpf (Fig. 1A) whereas coinjection of capped p53 mRNA restored radiation sensitivity (Fig. 1B). Similarly, *p53*-targeted MO kd markedly reduced the incidence and severity of radiation-induced morphological defects, notably defects in midline development that manifest as dorsal curvature of the body axis (Fig. S1). These results were similar to results by Duffy and Wickstrom published during preparation of this manuscript.[21] In addition, *p53*-targeted MO kd markedly reduced the extent of radiation-induced apoptosis as determined by acridine orange staining (Fig. 1C).

Effects of the pharmacological p53 inhibitor PFTα on development and radiation sensitivity of zebrafish embryos. PFTα was originally identified as an inhibitor of p53-dependent transcription[22] and it reduced the sensitivity of mice to the deleterious effects of ionizing radiation.[23] Based on these previous studies we tested whether PFTα also protected zebrafish embryos against radiation-associated toxicity (Fig. 2A). Unexpectedly, when added to zebrafish embryos at 3 hpf (sphere stage), PFTα (2 μM) caused malformations affecting the head region and led to the development of massive edema affecting the whole body of treated fish at later stages of development (Fig. 2B and Table 1). Furthermore, it has been described earlier that PFTα treatment also reduces overall survival of zebrafish embryos.[21] These results together raised the question whether PFTα exerted effects on molecular targets other than p53, which confound potential radioprotective properties of PFTα in the zebrafish embryo.

PFTα treatment mimics morphological effects associated with knockdown of p73 expression in zebrafish embryos. P73 is a likely candidate for off-target effects of PFTα because p73 binds to and transactivates p53 responsive promoters.[24] Thus, we determined whether suppression of p73 expression by antisense MO kd caused developmental defects similar to those observed in PFTα treated embryos. A previous report showed that targeting p73 adversely affected development of the head region, i.e., the olfactory system, the telencephalon and the pharyngeal arches of zebrafish embryos.[25] In addition, p73 is expressed at high levels in the developing kidneys.[26] We observed that *p73*-targeted antisense MO-mediated kd induced head region abnormalities (Fig. 3A) and led to liquid accumulation affecting the whole body of treated fish (Fig. 4 and Table 2). These changes were very similar to the morphological alterations observed in PFTα-treated fish (Fig. 2B). Furthermore, alcian blue staining revealed severe disturbances of branchial arch development associated with either PFTα treatment or *p73*-targeted antisense MO kd (Fig. 3B). These defects were, at least partially, reversed by coinjection of G-capped *p73* mRNA and not observed in embryos injected with *p73* mismatched antisense MO.

Impaired kidney function in zebrafish treated with PFTα and p73 morpholinos. This is the first report of edema formation upon treatment of developing embryos with *p73*-targeted antisense MO kd. We hypothesized that this phenotype was due to impaired renal clearance consistent with high-level expression of p73 in the developing kidneys.[26] To address this issue we used a renal function assay, which measures retention of a fluorescent dextran within 24 h after injection into the cardiac venous-sinus.[27] As compared to control fish receiving mismatch antisense MO, the *p73*-targeted antisense MO kd caused markedly reduced clearance of this contrast agent (Fig. 5). In contrast, control *p53*-specific antisense MO kd did not affect dextran retention. Importantly, PFTα treatment not only led to liquid accumulation in fish embryos in a manner similar to *p73*-targeted antisense MO kd but it also increased dextran retention in

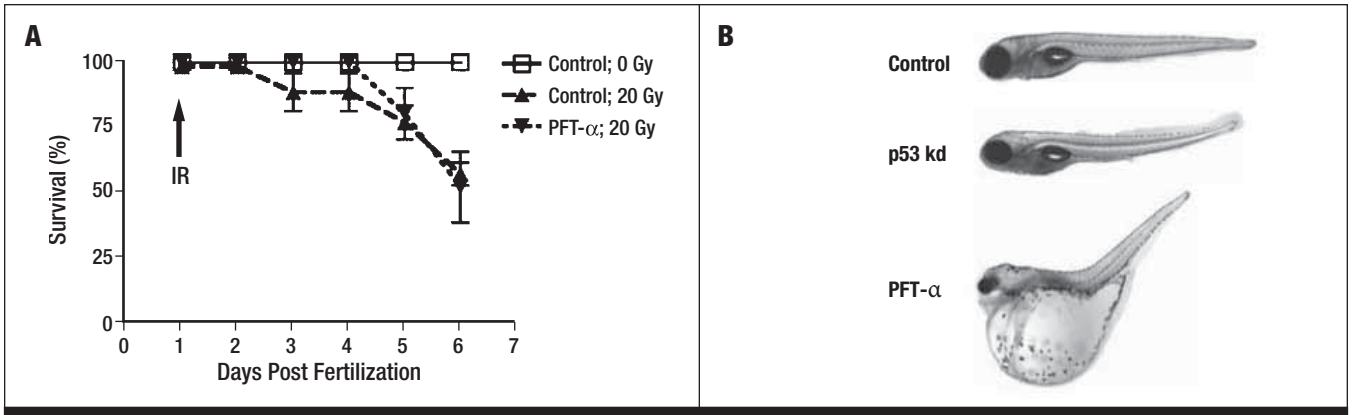

Figure 2. PFT-α (2 μM) administered to zebrafish embryos at 3 hpf does not protect against the lethal effects of IR and is associated with developmental abnormalities. **(A)** Embryo survival scored as described in Figure 1A. **(B)** Embryo morphology in the different experimental conditions as indicated. Representative embryos were digitally photographed at 4x magnification and processed using NIH ImageJ software.

Table 1.
Incidence of whole body edema caused by PFTα treatment

	Normal	Edema	Total	Edema (%)
Control	77	0	77	0
PFTα (2μM)	18	29	47	62

Triplicate dishes (30 embryos each per condition) of live embryos at 7 dpf were scored for edema as shown in Figure 2B and results expressed as percent edema.

a similar fashion. In these experiments, kidney function was tested at 3 dpf and prior to the development of edema to avoid confounding effects of the liquid accumulation on embryonal kidney function.

An alternative explanation for the profound edema in zebrafish embryos following IR exposure is that this effect was caused by reduced cardiac function.[28] To investigate this possibility, we performed time-lapse microscopy of cardiac contractility in control and irradiated fish embryos. Quantitative analysis of the images revealed only marginal effects of either PFTα treatment or *p73*-specific antisense MO kd on heart rate and blood flow (not shown). Collectively, these results suggest that the edema observed in PFTα and *p73 antisense* MO kd zebrafish embryos is due primarily to compromised renal function.

PFTα inhibits p73-dependent transactivation of a p53-responsive promoter construct. The striking similarities in developmental abnormalities caused by either PFTα treatment of suppression of p73 expression raised the question whether PFTα targeted not only p53-dependent but also p73-dependent transcription. Using a p53 responsive reporter gene construct and zebrafish p53 and p73 expression plasmids cotransfected into Saos-2 cells we observed that PFTα not only inhibited p53-dependent transcription but also p73-dependent transcription in a dose-dependent manner (Fig. 6).

It should be noted that administration of PFTα shortly before radiation (i.e., at 23 hpf) did not cause developmental abnormalities either of the craniofacial region or systemic edema and provided a measure of protection against radiation similar to that observed in *p53 antisense* MO kd fish (Fig. S1). This result indicates that p73 serves essential functions during the first 24 h of zebrafish development but is less relevant at later developmental stages and, presumably, in the adult organism. This circumstance also explains why inhibition of p73 function by PFTα has not been obvious in previous in vitro or in vivo studies in adult mice.

In summary, this report demonstrates the utility of the zebrafish model system in characterizing drug effects and highlights previously unrecognized effects of the p53 inhibitor PFTα related to inhibition of p73 function. Lack of either p53 or p73 function is associated with chemoresistance of transformed cells.[29] Furthermore, p73 is induced after DNA damage by the checkpoint kinases Chk1 and Chk2.[30] Based on these results, p73 has been considered as the "assistant" guardian of the genome that acts in concert with p53 to limit propagation of cells with damaged DNA.[31] Since we observed that PFTα inhibits not only p53-dependent but also p73-dependent transcription the overall radioprotective effect of PFTα as observed in mice may, thus, be due to inhibition of p53 and p73 function. Indeed, short-term pharmacological inhibition of both, p53 and p73 may be superior to inhibition of p53 alone to protect normal adult cells and tissues against deleterious effects of radiation.

Materials and Methods

Embryo harvesting and maintenance. Zebrafish husbandry, embryo collection, dechorionation and embryo maintenance were performed according to accepted standard operating procedures[32] and with approval by the Institutional Animal Care and Use Committee at Thomas Jefferson University. Zebrafish were maintained in the Zebrafish Core Facility of the Kimmel Cancer Center at Thomas Jefferson University at 28.5°C on a 14-h light/10-h dark cycle.

Zebrafish morphology by visual analysis. For visual analysis, zebrafish embryos were anesthetized with 0.003% tricaine, placed on 3% methylcellulose on a glass depression slide and analyzed using an Olympus BX51 microscope (Olympus, Melville, NY) at 4x magnification. Images were recorded using a SPOT camera and SPOT Advanced software (SPOT Diagnostic Instruments, Sterling Heights, MI).

Targeted knock down of gene expression. Antisense MO sequences targeting *p53, p73* and controls (5 base mismatches; p53 mm, p73 mm) were as described.[20,25] For microinjection, a 0.5 mM oligonucleotide solution was prepared in 10x phosphate-buffered saline solution, diluted 9:1

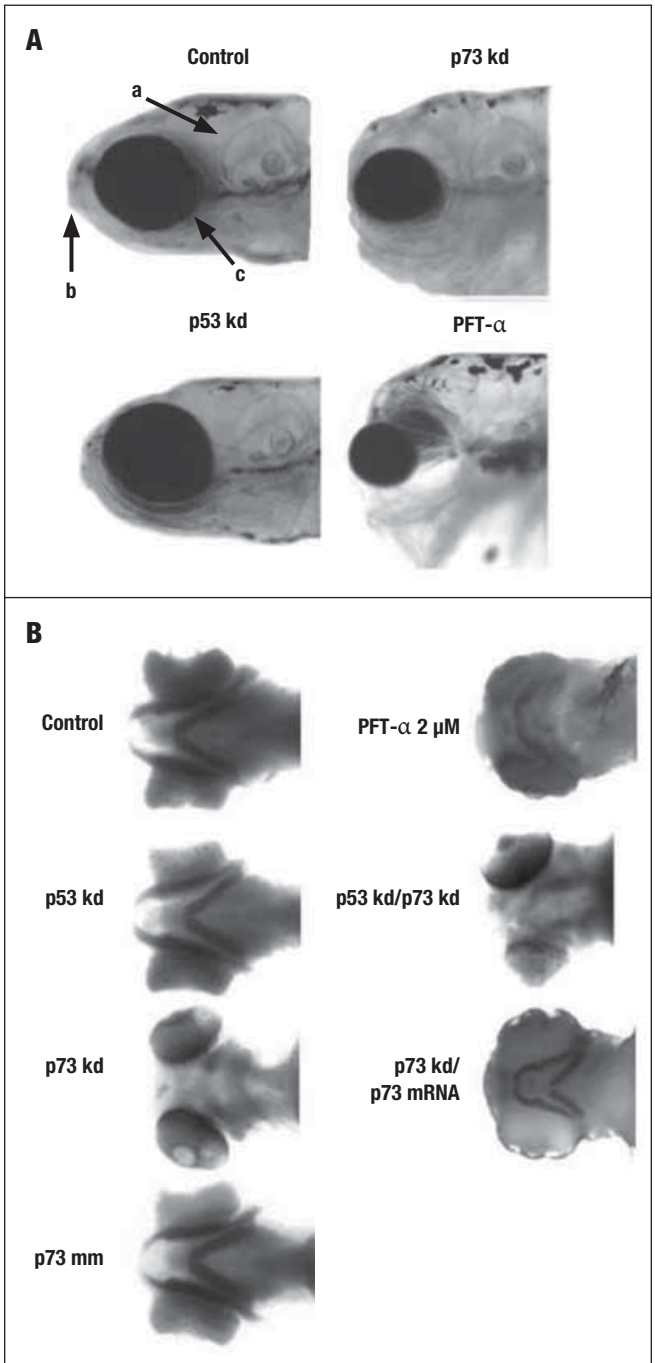

Figure 3. PFT-α treatment (2 μM at 3 hpf) affects cranio-facial development reducing brain, eye and auditory organ size. (A) Embryo head morphology at 6 dpf. Representative embryos were digitally photographed at 10x magnification and processed using NIH ImageJ software (a) snout, (b) eyes, (c) auditory cup. (B) Alcian blue staining (described in Materials and Methods) of cartilage shows markedly abnormal cranio-facial development associated with PFT-α treatment and with p73 antisense MO kd.

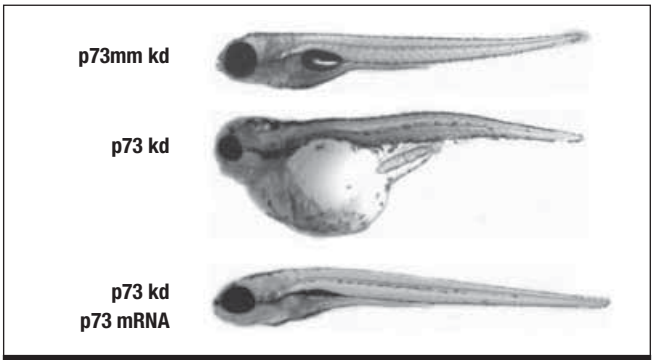

Figure 4. Phenotypic abnormalities associated with PFT-α treatment are similar to those caused by MO-mediated p73 kd. PFT-α was administered at 3 hpf. Embryo morphology and rescue by G-capped mRNA. Representative embryos were digitally photographed at 4x magnification and processed using NIH ImageJ software.

Table 2. Incidence of whole body edema caused by p73kd

	Normal	Edema	Total	Edema (%)
Control	219	1	220	0
p73 MO	121	126	247	51
p73 mm	64	4	68	6
p73 MO/mRNA	151	37	188	20

Live embryos at 7 dpf were scored for edema as described in Figure 4 and results expressed as percent edema.

(v:v) with Phenol Red dye, and ~1 nL injected into 1–4 cell embryos using a nitrogen gas pressure injector (Harvard Apparatus, Cambridge, MA). To account for non-specific effects of MO oligonucleotides, rescue experiments were carried out by coinjection of MOs with G-capped mRNA of the respective target gene. To this end, triplicate dishes of 60 embryos were injected with 4.5–7.5 pg of mRNA generated by cloning the zebrafish *p53* cDNA or p73 cDNA into the pCS2+ vector and producing mRNA with the mMessage-mMachine SP6 kit (Ambion, Austin, TX).

Radiation exposure and PFTα protection. Triplicate dishes (60 embryos each) were irradiated at 24 hpf (20 Gy) using 250 kVp X-rays (PanTak, East Haven, CT) at 50 cm source-to-skin with a 2-mm aluminum filter. Dosimetric calibration was performed before each experiment using a thimble ionization chamber (Victoreen; Elimpex-Medizintechnik, Moedling, Austria) with daily temperature and pressure correction. Pifithrin-α (EMD Biosciences, San Diego, CA) was solubilized in DMSO and diluted with embryo media. PFTα was applied 30 minutes prior to IR.

Kidney function assay. A 1% solution of rhodamine-labeled dextran (10 kDa; Molecular Probes) in PBS was injected (3 dpf) using glass micropipets into the cardiac venous sinus of embryos immobilized in 3% methyl cellulose. Prior to injection, embryos were anesthetized using a 0.003% tricaine solution in egg water.[33] After injection, the embryos were washed in egg water for 10 minutes and placed back into 3% methylcellulose on a glass depression slide. Fluorescence was quantitated using ImageJ software (NIH, USA). The analysis was repeated at 24 h after dextran injection. Percent dextran retention at 24 h was calculated using the formula: (intensity 24 h/intensity 0 h) X 100.

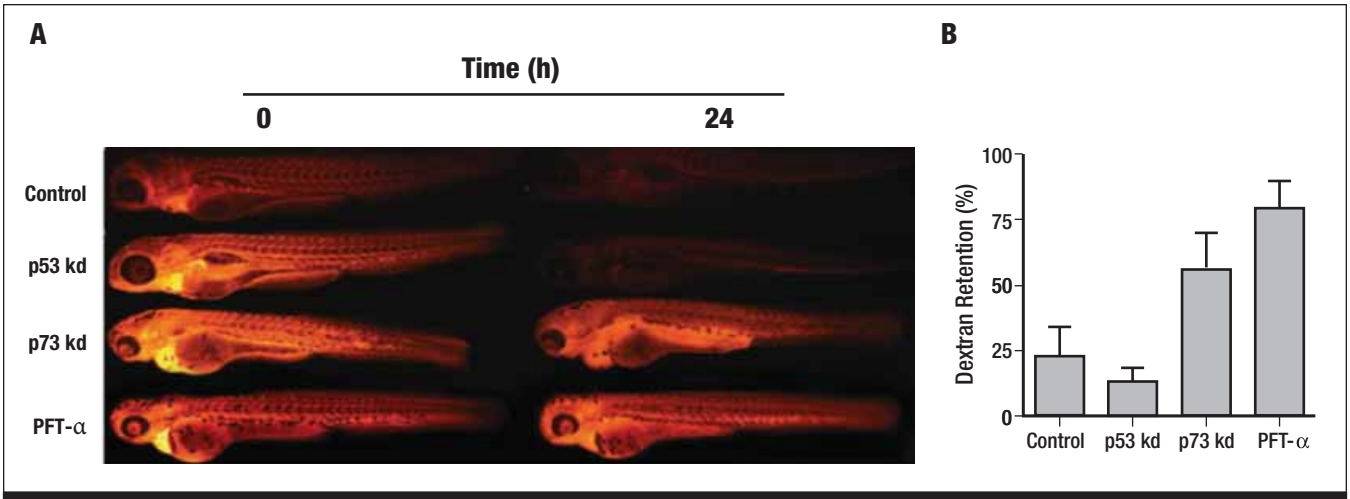

Figure 5. Reduced kidney function by either PFT-α treatment or p73 kd as determined by increased fluorescent dextran retention. **(A)** Rhodamine labeled dextran staining is described in Materials and Methods. Representative images are shown at 0 and 24 h. **(B)** Quantification of dextran label retention at 24 h after dye injection. Three embryos per condition were quantified for dye retention using NIH imageJ software.

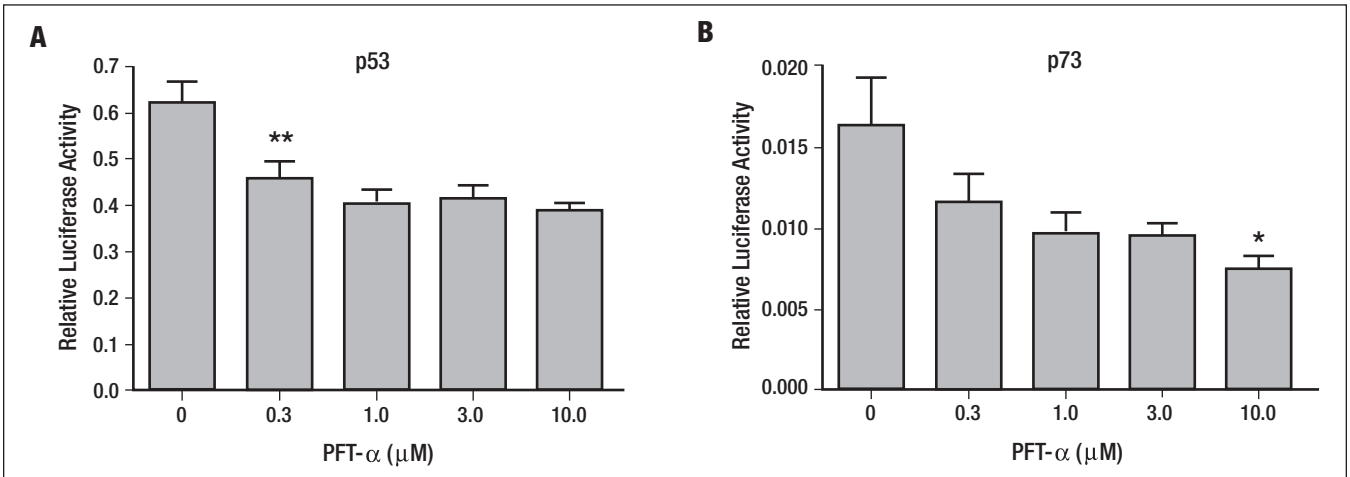

Figure 6. PFT-α inhibits p73-dependent transcription of a reporter gene construct. Triplicate wells (48-well plate) were transfected with pCMV-βgal and p53 pr-RLuc plasmids together with either a pCMV-zp53 **(A)** or a pCMV-zp73 plasmid. **(B)** Renilla luciferase activity was assayed 48 h post transfection and normalized to β-gal activity as described in the Materials and Methods. Cells were treated with PFT-α 15 minutes before transfection. Asterix (*) $p < 0.5$ (tukey test); **$p < 0.01$(tukey test).

Alcian blue staining. Alcian blue staining was performed according to Neuhauss *et al.*,[34] with the following modifications. Embryos (4 dpf) were fixed overnight in Davidson's Solution (Electron Microscopy Sciences, Hatfield, PA) and rinsed 3x for 10 min in PBS and transferred to neutral buffered formalin for 2 days at 4°C. The embryos were then transferred into distilled water and stored at 4°C. For Alcian blue staining, the samples were washed in PBT (0.1% Tween-20 in PBS) and transferred into 30% H_2O_2 (Sigma, St. Louis, MO) and bleached for 4–5 hours or until eyes became translucent. After bleaching, the embryos were rinsed in PBS for 15 min and transferred to filtered Alcian Blue solution (1% conc HCl, 70% Ethanol, 0.1% Tween-20) and stained overnight. The stain was cleared with acidic ethanol (5% conc HCl, 70% ethanol).

Acridine orange staining. Zebrafish embryos were dechorionated and placed in 50 μg/ml of acridine orange (Sigma) in fish water. After 30 min of staining, embryos were washed 3x for 10 min in PBS. Pooled embryos were transferred to 95% ethanol for 15 minutes to extract the AO for fluorescence determination. Triplicate measurements for each condition were performed on a FL600 microplate fluorescence reader (Bio-Tek) and normalized to control background fluorescence and reported as relative fluorescence units (RFU).

In vitro reporter gene assays. Saos-2 cells (ATCC Rockville, MD) were cultured in DMEM supplemented with 10% fetal calf serum. Cells were cultured to 60–70% confluence and transferred to 48-well plates at a density of 2.6×10^4 cells/well. Cells were transfected with three plasmids

using Fugene (Roche). The p53 reporter plasmid was constructed by inserting the synthetic p53-responsive promoter containing 14 tandem p53 enhancer elements and a TATA-box (Pathdetect p53-cis reporter, Stratagene) into the pRLnull plasmid (Promega) to drive the *Renilla* luciferase gene (p53 pr-RLuc). For normalization, a B-galactosidase reporter plasmid was used (pCMV-Bgal;[35]). *Renilla* luciferase activity was measured 48 hrs after transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and B-gal activity was measured using the Beta-Glo Reporter Assay System (Promega, Madison, WI) according to the manufacturer's specifications. Cells were treated with PFTα 15 minutes before transfection. Chemiluminescence was measured using a Veritas Microplate luminometer (Turner Biosystems, Sunnyvale, CA).

Acknowledgements

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/DavidsonCC7-9-Sup.pdf

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