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Pifithrin-α enhances chemosensitivity by a p38 MAPK dependent modulation of the eukaryotic initiation factor 4E in malignant cholangiocytes

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Abstract

Pifithrin-α is the lead compound for a novel group of small molecules that are being developed for use as anticancer agents.   The eukaryotic initiation factor 4E (eIF-4E) is over-expressed in many cancers, can mediate sensitivity to therapy and may be regulated by p53. We examined the utility of pifithrin-α as an adjunct to therapy for the treatment of human cholangiocarcinoma, a tumor that is highly refractory to therapy, and assessed the involvement of p53 dependent eIF-4E regulation in cellular responses to pifithrin-α. The expression of eIF-4E was increased in human cholangiocarcinomas compared to normal liver.   Modulation of eIF-4E expression by RNA interference enhanced the efficacy of gemcitabine in KMCH cholangiocarcinoma cells.   Pre-incubation of KMCH cells with pifithrin-α enhanced gemcitabine-induced cytotoxicity in an eIF-4E dependent manner.   Furthermore, pifithrin-α increased eIF-4E phosphorylation at Serine 209 via activation of p38 MAPK.   Pifithrin-α was shown to activate aryl hydrocarbon receptor (AhR) signaling and p38 mitogen activated protein kinase (MAPK) activation.   Sequencing analysis indicated the presence of a functionally inactivating p53 mutation in KMCH cells, and siRNA to p53 did not modulate chemosensitization by pifithrin-α. Thus, pifithrin-α enhances chemosensitivity by a mechanism independent of p53 and involving AhR and p38 MAPK deregulation of eIF-4E phosphorylation. Thus, pifithrin-α may prove useful for the enhancing chemosensitivity in tumors with mutated p53.   Moreover, modulation of eIF-4E is an attractive therapeutic target for intervention in cancer treatment.

Introduction

Pifithrin-α is a small molecule that was originally identified as a p53 inhibitor on chemical screens and represents a promising new class of therapeutic agents that are being developed and evaluated for use in cancer and neurodegenerative diseases (Gudkov and Komarova, 2005;Komarov, et al., 1999).   The utility of pifithrin-α in anti-cancer therapy remains undefined, partly due to a lack of knowledge regarding the mechanisms and targets by which it may exert cellular effects.   Although originally identified as an inhibitor of p53, recent studies have shown that pifithrin-α can modulate intracellular signaling independent of p53 by acting as a potent agonist of the Aryl hydrocarbon receptor (AhR) (Hoagland, et al., 2005). Moreover, there is limited information about the potential interactions with other chemotherapeutic agents.

We examined the utility of pifithrin-α as an adjunct to therapy for the treatment of human cholangiocarcinomas, malignancies arising from the biliary tract.   These tumors are highly refractory to conventional treatments and consequently are associated with a poor prognosis. Recent reports indicate that global incidence and mortality of this aggressive cancer are increasing (Patel, 2006).   Due to a limited ability to detect the tumor early, cholangiocarcinoma is often diagnosed at an advanced disease stage when curative resection is not possible.   Thus, there is an urgent need for newer, more effective anti-cancer approaches for this cancer.   An understanding of signaling mechanisms regulating chemotherapy resistance in cholangiocarcinoma may eventually result in the development of more effective therapies and enhance patient long-term survival.

Dysregulation of protein synthesis has recently been recognized as a contributing event in tumorigenesis. The eukaryotic translation initiation factor-4E (eIF-4E) is an mRNA cap binding

protein that can regulate the initiation of translation and is becoming increasingly recognized as a critical mediator of tumor cell growth and responses to environmental stresses such as chemotherapy. eIF-4E binds to the m^7-G cap on mRNA and is a rate-limiting determinant of cap-dependent translation.   Overexpression of eIF-4E has been shown to contribute to tumorigenesis by enhancing the growth and survival of transformed cells in many cancers (reviewed in (De and Graff, 2004)). Increased eIF-4E expression has been correlated with the increased rate of translation of several genes, including many known malignancy-associated proteins such as ornithine decarboxylase, cyclin D1, c-myc, VEGF and FGF (reviewed in (Mamane, et al., 2004)).   eIF-4E may be an important contributor of resistance to chemotherapeutic agents by activating anti-apoptotic or cell survival pathways.   Indeed, modulation of eIF-4E by antisense approaches to decrease expression of eIF-4E or by altered eIF-4E binding to the mRNA cap have been shown to modulate cell survival as well as to decrease tumor cell growth. Translational regulation of survival proteins can result in resistance to therapy, and increased eIF-4E can modulate apoptotic responses to hypoxia in human malignant biliary tract epithelia (cholangiocytes) (Marienfeld, et al., 2004).

The activity of eIF-4E can be modulated by eIF-4E inhibitory proteins (4E-BPs). The activity of these proteins can be modulated by phosphorylation with a decrease in phosphorylation enhancing binding to eIF-4E and an increase in phosphorylation resulting in their detachment from eIF-4E, thereby enabling eIF-4E to bind to mRNAs. Phosphorylation of eIF-4E at serine 209 occurs once eIF-4E is bound to the scaffolding protein eIF-4G by the MAP-kinase signal-integrating kinases Mnk-1 and Mnk-2, which can be activated by Erk or p38 MAPK kinase. We have recently shown that p38 MAPK signaling is aberrantly activated in malignant cholangiocytes and moreover can directly regulate protein translation and eIF-4E

(Tadlock and Patel, 2001;Yamagiwa, et al., 2003).   Thus, aberrant p38 MAPK signaling can stimulate eIF-4E activity and promote either tumor proliferation or survival. Thus, targeting eIF-4E may be an effective strategy in developing successful treatments for human cholangiocarcinoma.   Developing such strategies necessitates understanding molecular mechanisms of regulation of eIF-4E in cancer cells.

Recent studies have demonstrated that eIF-4E can be regulated by the p53 transcription factor (Constantinou and Clemens, 2005;Horton, et al., 2002;Tilleray, et al., 2006). To elucidate potential mechanisms involved in eIF-4E-mediated chemoresistance, we evaluated the effect of aberrant eIF-4E expression to pifithrin-α exposure. We asked the following questions: Is eIF-4E over-expressed in human cholangiocarcinoma? What is the cellular effect of pifithrin-α on responses to chemotherapy? Is eIF-4E involved in the pifithrin-α response?   If so does this involve p38 MAPK signaling?   Our studies show that eIF-4E is a target of pifithrin-α and support the potential utility of pifithrin-α to improve the cellular response to chemotherapeutic agents.

Methods

  Cell Lines and Culture. H69 nonmalignant and KMCH malignant human cholangiocytes were obtained as previously described (Park, et al., 1999b;Park, et al., 1999a). KMCH cells were stably transfected with dominant negative MKK3 as previously described and the stable transfectants designated as KM-MKK3dn (Yamagiwa, et al., 2003). H69, KMCH and KM-MKK3dn cells were cultured in Dulbecco's modified Eagle medium with necessary supplements as previously described, and unless otherwise noted, culture media was supplemented with 10% fetal bovine serum.

  Tissue Microarray and analysis. Tissue microarray slides containing samples from normal livers and human cholangiocarcinoma were obtained from Abxis (Seoul, Korea). Each unique tumor sample was represented in duplicate. Immunohistochemistry was performed following the manufacturer's protocol and using primary monoclonal antibodies for eIF-4E at a dilution of 1:500, and a biotinylated secondary antibody with detection using Streptavidin-HRP (Zymed, San Francisco, CA). Each section was scored using a numerical value of 1-4 for none, mild, moderate or intense staining intensity. The number of positive cells in each section were determined and assigned numerical values of 1-5 for 0, <25%, 25-50%, 50-75%, or >75% positive cells respectively. The values for the staining intensity and positive cells were multiplied and values of 1-5, 6-10, 11-15 and 16-20 were assigned an expression score of 0, 1, 2 and 3 respectively.

  RNA interference. The design and validation of siRNA to eIF-4E was previously described (Yamagiwa, et al., 2003). Validated siRNA to p53 and scrambled nucleotide control siRNA were obtained from Ambion (Austin, TX). Transfection of siRNA was performed using the Nucleofector system (Amaxa Biosystems, Koln, Germany). One million cells were

resuspended in 100 µl Nucleofector solution T at room temperature followed by addition of 1.5

µg of siRNA eIF-4E, p53, or scrambled nucleotide control, and transfected by electroporation

with Nucleofector. Cells were then resuspended in DMEM containing 10% serum prior to use

for subsequent studies.

Cytotoxicity Assay.   Cells were transfected with siRNA to either eIF-4E or p53 or

scrambled nucleotide control and seeded into 96-well plates at a cell density of 10,000 cells/well

in 200 µl DMEM with 10% fetal bovine serum. After 24 hours, the cells were washed with 1X

phosphate buffered saline and pre-treated for 24 hours with varying concentrations of pifithrin-α

in serum-free DMEM. The media was then changed and replaced with one containing 0 (diluent

control) or 100 µM Gemcitabine.   Cell viability was assessed after 24 hours using a colorimetric

assay (Cell Titer 96 Aqueous; Promega Corp. Madison, WI). Cytotoxicity was expressed as a

percentage of control. For studies to evaluate the involvement of aryl hydrocarbon receptor

signaling, cells were pre-treated with 0.1 and 1 µM α-naphthoflavone, prior to incubation with

pifithrin-α and gemcitabine as noted above.

Clonogenic growth assay.   Cells were seeded in 96-well plates (10,000 cells/well) in an

agar suspension containing media with 20% fetal bovine serum, and incubated at 37°C with

varying concentrations of pifithrin-α and gemcitabine. Growth in soft agar was assessed after

seven days, following addition of Alamar Blue (Biosource, Camarillo, CA) and measurement of

fluorescence using a CytoFluor Multiwell Plate Reader with excitation at 530 nm and emission at

580 nm.

Immunoblot Analysis. KMCH and KM-MKK3dn cells were grown to ~70% confluency

in 100 mm dishes. Cells were washed twice with phosphate buffered saline (PBS) prior to

incubation with varying concentrations of pifithrin-α in serum-free DMEM for 48 hours.

Subsequently, cells were washed with ice-cold PBS and then lysed with 0.5 ml lysis buffer containing protease inhibitors for total protein (Tadlock and Patel, 2001). Equivalent amounts of protein samples were mixed with 4x sample buffer, separated on 4% to 12% gradient polyacrylamide gels (Novex; San Diego, CA) and then transferred to nitrocellulose membrane (Millipore; Bedford, CA). The membranes were blocked with 5% nonfat dry milk in TBST (Tris-buffered saline, pH 7.4, containing 0.05% Tween 20) for 1 hour and then incubated overnight at 4°C with the respective anti-human primary antibody (1:1000). The membrane was washed 3 times for 5 minutes with TBST and then incubated with IRDye700 (Molecular Probes; Eugene, OR) and IRDye800-labeled (Rockland, Inc.; Gilbertsville, PA) secondary antibodies (1:2000) for 30 minutes. Blots were stripped and re-probed with β-actin (1:2000 primary antibody; 1:4000 secondary antibody). Protein expression was visualized and measured using a LI-COR Odyssey Infrared Imaging System (LI-COR Bioscience; Lincoln, NE).

MAPK Assay. KMCH cells (1.5×10^6) in 100 mm dishes were incubated with 25 μM SB203580 or diluent control for 24 hours prior to the addition of 25 μM pifithrin-α or diluent control for 48 hours. After 48 hours, total protein was obtained and total and active site phosphorylation specific p38 MAPK activity was measured using a commercially available assay kit from BioSource International, Inc. (Camarillo, CA), and following the manufacturer's instructions.

Cell Cycle Analysis. KMCH cells (1.5×10^6) in 100 mm dishes were serum starved for 24 hrs. Subsequently, cells were incubated with 0 or 25 μM pifithrin-α in 10% serum containing medium. After 24 hours, cells were harvested and re-suspended (0.5 to 1×10^6 cells/ml) in propidium iodide solution containing 0.1 mM propidium iodide, 0.1% Triton X-100, and 0.25 mg/ml RNase A in PBS at 4°C for 1 hour in the dark. Cell cycle analysis was performed using a

BD FACSAria flow cytometer (BD Biosciences, San Jose, CA).   Ten thousand events were recorded and the percentage of cells in the cell cycle phases was analyzed using the BD FACSDiva software version 4.1 (BD Biosciences, San Jose, CA).

Reverse Transcription-PCR.   Total RNA was isolated using the ToTALLY RNA kit (Ambion, Austin, TX).   cDNA was prepared from 2 μg RNA using the M-MLV Reverse Transcriptase kit (Invtrogen; Carlsbad, CA) and random primers.   The PCR primer sequences were CYP1A1: 5'-TCT TTC TCT TCC TGG CTA TC-3' (sense) and 5'-CTG TCT CTT CCC TTC ACT CT-3' (antisense); β-actin: 5'-CAG AGC AAG AGA GGC ATC CT-3' (sense) and 5'-TTG AAG GTC TCA AAC ATG AT-3' (antisense).   The PCR mixture contained 1 μl of cDNA, 1X PCR SuperMix High Fidelity Buffer (Invitrogen; Carlsbad, CA) containing a mixture of *Taq* and *Pyrococus* GB-D DNA polymerase, Mg^{++} and dNTPs, 200 nM each primers in a final volume of 50 μl.   Amplification was performed on a Techne Genius Thermal Cycler (Techne Inc.; Princeton, NJ).   Reactions were hot-started at 95°C for 5 min, then amplified for 35 cycles (30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C), followed by a final 10 min extension at 72°C.   Controls with total RNA were performed for each set of primers. 1 μl of each PCR reaction was then resolved on a 500 DNA chip and analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany).

DNA Sequencing. KMCH cells were grown in 100 mm dishes in 10% serum containing DMEM till confluency. Cells were washed twice with cold PBS and genomic DNA was isolated from the cells using a commercially available DNA extraction kit (Chemicon; Temecula, CA).   PCR amplification was performed as described above using published sequencing primers for p53 exons 4 to 9 (Rassidakis, et al., 2005).   Reactions were hot-started at 95°C for 5 min, then amplified for 35 cycles (30 sec at 95°C, 30 sec at 56°C and 1 min at 72°C), followed by a final 5

min extension at 72°C. PCR reaction products were confirmed by on a 500 DNA chip with Agilent 2100 Bioanalyzer. p53 sequencing was performed at the TGen DNA Sequencing Facility (Phoenix, AZ) and sequence data analyzed using Mutation Surveyor™ (SoftGenetics, LLC; State College, PA).

Materials. Cell culture media, supplements and PCR primers were obtained from Invitrogen (Carlsbad, CA). Pifithrin-α and SB203580 were obtained from Calbiochem (San Diego, CA) α-naphthoflavone from Sigma (St Louis, MO). Antibodies to eIF-4E, CYP1A1 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to p53, Ser209-eIF-4E, and Phospho-Mnk-1 were obtained from Cell Signaling, Inc. (Beverly, MA). All other reagents were obtained from Sigma (St Louis, MO).

Statistical Analysis. Data are expressed as the mean ± 95% confidence limits from at least 3 separate experiments. The difference between groups was analyzed using a double-sided Student's *t* test. Statistical significance was considered as $p < 0.05$.

Results

Is eIF-4E expression increased in human cholangiocarcinoma?   We have recently shown that the expression of eIF-4E is increased in malignant human cholangiocarcinoma cell lines compared to that in non-malignant cholangiocytes (Marienfeld, et al., 2004). However, it is unknown if eIF-4E is over-expressed in human cholangiocarcinoma. Therefore we assessed the expression of eIF-4E in 46 human cholangiocarcinoma using a tissue microarray. Compared to expression in normal liver, there was an increase in eIF-4E expression in cholangiocarcinoma samples (Fig. 1A). A semi-quantitative analysis indicated low expression in all benign tissue samples.   However increased expression (score of $\geq$ 2) was observed in 85.9%, and was markedly increased (score $\geq$ 3) in 51.1% of cholangiocarcinomas (Fig. 1B).   Thus, eIF-4E expression is increased in human cholangiocarcinoma.

Can pifithrin-α alter the sensitivity of malignant cholangiocytes to gemcitabine? Pifithrin-α is currently under investigation as an adjunct to chemotherapy to minimize the side effects of anti-cancer therapies or as the basis for the development of new drugs (Zhu, et al., 2002). Although pifithrin-α may be potentially useful for the treatment of chemoresistant tumors such as cholangiocarcinoma, there is a lack of information about the effect of pifithrin-α in cholangiocytes, and its utility in enhancing chemotherapeutic response is unknown.   In order to assess the cellular responses of pifithrin-α, we first assessed the cytotoxicity of pifithrin-α. Indeed, pifithrin-α did not alter KMCH cell viability over a concentration range from 0-100 µM (Fig. 2A).   Gemcitabine is the most effective single-agent currently available for cholangiocarcinoma. However, treatment responses are poor. Thus, we next evaluated the potential of pifithrin-α as an adjunct to gemcitabine. Incubation of KMCH cells with 50 µM pifithrin-α for 24 hours enhanced sensitivity to gemcitabine compared to controls (Fig. 2B).

Similarly, an increase in anchorage-independent growth in soft agar was also observed in cells incubated with pifithrin-α (Fig. 2C). These observations suggest that pifithrin-α is a potentially useful adjunct to chemotherapeutic agents such as gemcitabine for the treatment of cholangiocarcinoma. Chemosensitization by pifithrin-α was not observed in H69 non-malignant human cholangiocytes (Fig 2D) suggesting that the observed effects were specific to malignant cells and supporting the potential use of pifithrin-α as an adjunct to chemotherapeutic agents.

Is eIF-4E involved in the chemosensitization effects of pifithrin-α? Having established that pifithrin-α can enhance the effects of gemcitabine, we next evaluated the involvement of eIF-4E in mediating this effect. Pifithrin-α enhanced the response to gemcitabine in KMCH cells transfected with scrambled nucleotide control siRNA (Fig. 3A). However, in cells transfected with siRNA to eIF-4E, the effect of pifithrin-α on enhancing gemcitabine sensitivity was decreased. Thus, eIF-4E is involved in pifithrin-α chemosensitization.   These observations indicated a previously unrecognized mechanism by which pifithrin-α can sensitize cancer cells to chemotherapeutic agents. To further investigate the mechanisms involved, we next assessed the effect of pifithrin-α on eIF-4E phosphorylation (Fig. 3B). An increase in eIF-4E phosphorylation at serine 209 was observed during incubation with pifithrin-α suggesting that pifithrin-α chemosensitization may involve modulation of eIF-4E phosphorylation by pifithrin-α.

Does pifithrin-α modulate eIF-4E phosphorylation via a p38 MAPK dependent mechanism? Phosphorylation of eIF-4E can occur via a signaling cascade involving modulation of phosphorylation of 4E-binding proteins by the Mnk-1 kinase, a downstream substrate of p38 MAPK activation (Pyronnet, 2000). We assessed the contribution of this p38 MAPK dependent kinase-signaling pathway in the modulation of eIF-4E phosphorylation by pifithrin-α.   KMCH cells were stably transfected with dominant negative MKK3, an upstream activator of p38

MAPK which decreases p38 MAPK activation. Compared to control cells with functionally active p38 MAPK activation, pifithrin-α did not increase eIF-4E phosphorylation in KM-MKK3dn cells which are stably transfected with dominant negative MKK3 (Fig. 4A). Incubation with pifithrin-α increased phospho-p38 MAPK activity by ~5-fold and moreover, pifithrin-α induced phospho-p38 MAPK activity was reduced in the presence of SB203580 (Fig. 4B). Furthermore, we found an increase in phospho-Mnk-1 (data not shown) consistent with the activation of p38 MAPK and eIF-4E phosphorylation. Additionally, the pifithrin-α dependent increase in eIF-4E phosphorylation was inhibited by pre-incubation of cells with SB203580, a pharmacological inhibitor of p38 MAPK activation (Fig. 4C). These findings indicate that activation of p38 MAPK is required for pifithrin-α dependent phosphorylation of eIF-4E.

Does pifithrin-α mediate chemosensitization by a mechanism involving aryl hydrocarbon receptor activation? Pifithrin-α has recently been recognized as an agonist of the aryl hydrocarbon receptor (AhR) that can modulate cell cycle progression. Therefore we next evaluated the effect of AhR blockade using α-naphthoflavone (αNF) on pifithrin-α chemosensitization. Cells pre-treated with 0.1 or 1 μM αNF for 24 hours prior to pifithrin-α treatment had decreased sensitivity to gemcitabine consistent with the postulate that the effects of pifithrin-α are mediated through the AhR (Fig. 5A). To further confirm this we evaluated the effect of pifithrin-α on the activation of CYP1A1, a well-characterized downstream target gene of AhR activation. By reverse-transcription PCR, we observed a significant increase in CYP1A1 mRNA and protein levels in cells incubated with pifithrin-α (Fig. 5B,C). However, pre-treatment of KMCH cells with αNF increased CYP1A1 mRNA and protein levels compared to untreated controls (Figure 5B,C). We speculate that αNF may enhance the binding of pifithrin-α to AhR and enhance receptor activation. Thus, further studies on the biochemical interactions

between pifithrin-α and exogenous ligands for AhR will be required to clarify the precise

mechanism by which pifithrin-α can modulate the expression of downstream targets such as

CYP1A1.   Aryl hydrocarbon receptor mediated signaling has been shown to involve p38 MAP

kinase (Chen, et al., 2003).   Indeed, CYP1A1 protein levels were decreased in KM-MKK3dn

cells (Fig. 5D) suggesting that p38 MAPK signaling mediates AhR signaling during pifithrin-α

chemosensitization.   We then evaluated whether pifithrin-α exposure altered cell cycle

progression.   The percentage of KMCH cells in the G0/G1 phase increased from 64.4% ± 1.6%

of untreated cells to 70.3% ± 1.0% of pifithrin-α treated cells (n = 4, P = 0.0004), indicating that

pifithrin-α abrogates cell cycle progression into S phase.

Does pifithrin-α chemosensitization involve p53 dependent mechanisms?   Although

our results show that pifithrin-α chemosensitivity could result from the activation of aryl

hydrocarbon receptor signaling, pifithrin-α is an inhibitor of p53 raising the potential that

inhibition of p53 dependent responses to chemotherapy may contribute to the observed effects.

Wild-type p53 is a transcription factor that can regulate the expression of proteins involved in

cell cycle regulation.   To examine the potential contribution of p53 dependent mechanisms in the

observed effects of pifithrin-α, we examined the expression of p53, analyzed for mutations by

direct sequencing, and evaluated the effects of modulation of p53.   First, we noted that p53

expression was decreased in KMCH cells compared to nonmalignant H69 cells (Fig. 6A) by

immunoblot analysis.   Next, we sequenced p53 exons 4 – 9 and analyzed for the presence of any

mutations.   In KMCH malignant cholangiocytes, a single missense mutation TAT > TGT : Y > C

was identified in exon 6 at codon 220.   This mutation results in a total loss of p53 transactivation

activity (Mitsumoto, et al., 2004).   This functionally inactive mutation has been noted in several

other cancers such as colorectal, lung, liver, ovarian and pancreatic (Andriani, et al., 2004;el-

Mahdani, et al., 1997;Kalthoff, et al., 1993;Kohler, et al., 1993;Kubicka, et al., 1995). No p53

mutations were identified in the non-malignant H69 cells.   In addition, the effect of p53

dependent responses to chemotherapy response was assessed in KMCH cells.   Transfection of

KMCH cells with siRNA to p53 did not alter cellular resistance to gemcitabine (Fig. 6B). These

findings that p53 is mutated, functionally inactive, and does not contribute to chemosensitivity to

gemcitabine in KMCH cells exclude the possibility that the observed effects of pifithrin-α are

mediated by p53 dependent mechanisms.

Discussion

In the present study, we have identified a novel function for pifithrin-α in enhancing the response of human cholangiocarcinoma cells to gemcitabine. The mechanism involves activation of aryl hydrocarbon receptor (AhR) signaling and modulation of eIF-4E phosphorylation involving p38 MAPK signaling pathway. Enhanced expression of eIF-4E is common in human cholangiocarcinoma and these studies identify eIF-4E as a potential target that can be modulated by pifithrin-α. This study has several important clinical considerations that warrant further study given their relevance to treatments of solid tumors. Pifithrin-α has been reported to sensitize refractory tumors to chemotherapy (Xu, et al., 2005;Yin, et al., 2006). This effect has been attributed to inhibition of p53, and it has been postulated that chemosensitization arises from failed cell cycle arrest with insufficient time for DNA repair. These studies have not specifically evaluated the possibility that pifithrin-α may act in a p53 independent mechanism. Thus, these observations need to be re-evaluated and the potential involvement of p53 independent mechanisms such as AhR mediated signaling considered.

eIF-4E may be characterized as an oncoprotein and is over-expressed in many different cancers. Over-expression of eIF-4E has been associated with enhanced translation of several factors promoting tumor survival, angiogenesis and metastasis. Thus, manipulation of eIF-4E expression or function is a valid therapeutic strategy for tumors in which this protein is over-expressed. However, the regulation of eIF-4E expression and function are not completely understood, and eIF-4E has received scant attention as a potential therapeutic target. As a rate limiting factor for protein translation, modulation of eIF-4E may globally influence protein translation, as well as the translation of downstream targets characterized by extensively structured 5' UTR that are selectively translated by over-expressed eIF-4E and may contribute to

tumor behavior. Small molecule drugs, inhibitory peptides and proteins, anti-sense and gene therapy are all potential approaches by which eIF-4E expression could be manipulated or eIF-4E function inhibited to abrogate eIF-4E-mediated effects on tumor growth.

Although we have shown that pifithrin-α enhances eIF-4E phosphorylation at serine-209, via a p38 MAPK – Mnk-1 kinase pathway, and that pifithrin-α can modulate chemosensitivity, the mechanisms by which eIF-4E phosphorylation contributes to chemosensitivity and the mediators involved are unknown. The mechanistic and functional significance of eIF-4E phosphorylation remains enigmatic (Scheper and Proud, 2002;Raught and Gingras, 1999). Whereas early reports showed that eIF-4E phosphorylation resulted in an increased affinity of eIF-4E for capped mRNA, more recent studies have reported decreased affinity of eIF-4E to the mRNA cap structure following phosphorylation of eIF-4E at serine 209 (Minich, et al., 1994;Scheper, et al., 2002). Furthermore, the role of eIF-4E phosphorylation in the regulation of protein translation is not completely understood and varies between different conditions. We speculate that eIF-4E phosphorylation results in the separation of the eIF-4E-mRNA cap complex, thereby decreasing the efficiency of translation of mRNA that enhance cell cycle progression and are selectively translated by over-expression of eIF-4E.   Future studies to clarify the protein translation machinery in response to pifithrin-α may be useful to clarify the functional role of eIF-4E phosphorylation.

Our observations showing activation of AhR and p38 MAPK signaling suggest the possibility that p38 MAPK signaling can be directly activated by AhR signaling, consistent with a recent study by Weiss et al. showing AhR dependent activation of p38 MAPK in response to the polyhalogenated aromatic hydrocarbon dioxin (Weiss, et al., 2005). In this context, it is noteworthy that exposure to dioxins predisposes to cholangiocarcinoma formation in rats, and

that the p38 MAPK signaling pathway is aberrantly activated and contributes to tumor growth in human cholangiocarcinoma (Walker, et al., 2005;Tadlock and Patel, 2001).   Targeting downstream mediators of p38 MAPK such as eIF-4E is therefore an appropriate strategy for intervention in cholangiocarcinoma.

The clinical utility of pifithrin-α has been signified by its potential for use as an adjunct to chemotherapy to reduce adverse effects by reversibly inhibiting p53 dependent responses to chemotherapy-induced genotoxic stress in normal cells and tissues (Komarov, et al., 1999). Our study indicates that pifithrin-α may have additional benefits by enhancing chemosensitivity in tumor cells with functionally inactive mutated p53 responses.   The role of p53 in tumor cell responses to chemotherapy is complex and variable and the loss of p53 dependent responses can enhance as well as reduce resistance to chemotherapy in a tumor specific context (Gudkov and Komarova, 2005).   Although we have focused on studies in malignant cholangiocytes, we believe that studies in other tumor cell types are also justified based on our observations.   More importantly, pifithrin-α analogs that have the ability to modulate p53 independent responses without inhibition of p53 should be developed.   Such analogs would be extremely useful therapeutics as they may decrease the potential risk of allowing survival of genetically altered cells which would otherwise be eliminated via p53 dependent apoptosis. Moreover, tumor cells that have functional p53 responses may respond differently to pifithrin-α. In this context it is worth noting the observations of Hoagland et al. that pifithrin-α is structurally similar to many ligands of the aryl hydrocarbon receptor (Hoagland, et al., 2005).   Thus, the development of pifithrin-α analogs which can selectively target aryl hydrocarbon receptor signaling independent of effects on p53 are likely to be valuable as biological tools and for clinical use in cancer treatment.

Acknowledgments

References

Andriani F, Conte D, Mastrangelo T, Leon M, Ratcliffe C, Roz L, Pelosi G, Goldstraw P, Sozzi G and Pastorino U (2004) Detecting lung cancer in plasma with the use of multiple genetic markers. *Int J Cancer* **108**:91-96.

Chen S, Nguyen N, Tamura K, Karin M and Tukey RH (2003) The role of the Ah receptor and p38 in benzo[a]pyrene-7,8-dihydrodiol and benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide-induced apoptosis. *J Biol Chem* **278**:19526-19533.

Constantinou C and Clemens MJ (2005) Regulation of the phosphorylation and integrity of protein synthesis initiation factor eIF4GI and the translational repressor 4E-BP1 by p53. *Oncogene* **24**:4839-4850.

De BA and Graff JR (2004) eIF-4E expression and its role in malignancies and metastases. *Oncogene* **23**:3189-3199.

el-Mahdani N, Vaillant JC, Guiguet M, Prevot S, Bertrand V, Bernard C, Parc R, Bereziat G and Hermelin B (1997) Overexpression of p53 mRNA in colorectal cancer and its relationship to p53 gene mutation. *Br J Cancer* **75**:528-536.

Gudkov AV and Komarova EA (2005) Prospective therapeutic applications of p53 inhibitors. *Biochem Biophys Res Commun* **331**:726-736.

Hoagland MS, Hoagland EM and Swanson HI (2005) The p53 inhibitor pifithrin-alpha is a potent agonist of the aryl hydrocarbon receptor. *J Pharmacol Exp Ther* **314**:603-610.

Horton LE, Bushell M, Barth-Baus D, Tilleray VJ, Clemens MJ and Hensold JO (2002) p53 activation results in rapid dephosphorylation of the eIF4E-binding protein 4E-BP1, inhibition of ribosomal protein S6 kinase and inhibition of translation initiation. *Oncogene* **21**:5325-5334.

Kalthoff H, Schmiegel W, Roeder C, Kasche D, Schmidt A, Lauer G, Thiele HG, Honold G, Pantel K, Riethmuller G and . (1993) p53 and K-RAS alterations in pancreatic epithelial cell lesions. *Oncogene* **8**:289-298.

Kohler MF, Marks JR, Wiseman RW, Jacobs IJ, Davidoff AM, Clarke-Pearson DL, Soper JT, Bast RC, Jr. and Berchuck A (1993) Spectrum of mutation and frequency of allelic deletion of the p53 gene in ovarian cancer. *J Natl Cancer Inst* **85**:1513-1519.

Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV and Gudkov AV (1999) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* **285**:1733-1737.

Kubicka S, Trautwein C, Schrem H, Tillmann H and Manns M (1995) Low incidence of p53 mutations in European hepatocellular carcinomas with heterogeneous mutation as a rare event. *J Hepatol* **23**:412-419.

Mamane Y, Petroulakis E, Rong L, Yoshida K, Ler LW and Sonenberg N (2004) eIF4E--from translation to transformation. *Oncogene* **23**:3172-3179.

Marienfeld C, Yamagiwa Y, Ueno Y, Chiasson V, Brooks L, Meng F and Patel T (2004) Translational regulation of XIAP expression and cell survival during hypoxia in human cholangiocarcinoma. *Gastroenterology* **127**:1787-1797.

Minich WB, Balasta ML, Goss DJ and Rhoads RE (1994) Chromatographic resolution of in vivo phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: increased cap affinity of the phosphorylated form. *Proc Natl Acad Sci U S A* **91**:7668-7672.

Mitsumoto Y, Nakajima T, Marutani M, Kashiwazaki H, Moriguchi M, Kimura H, Okanoue T, Kagawa K and Tada M (2004) Loss of p53 transcriptional activity in hepatocellular carcinoma evaluated by yeast-based functional assay: comparison with p53

immunohistochemistry. *Hum Pathol* **35**:350-356.

Park J, Gores GJ and Patel T (1999a) Lipopolysaccharide induces cholangiocyte proliferation via an interleukin-6-mediated activation of p44/p42 mitogen-activated protein kinase. *Hepatology* **29**:1037-1043.

Park J, Tadlock L, Gores GJ and Patel T (1999b) Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. *Hepatology* **30**:1128-1133.

Patel T (2006) Cholangiocarcinoma. *Nat Clin Pract Gastroenterol Hepatol* **3**:33-42.

Pyronnet S (2000) Phosphorylation of the cap-binding protein eIF4E by the MAPK-activated protein kinase Mnk1. *Biochem Pharmacol* **60**:1237-1243.

Rassidakis GZ, Thomaides A, Wang S, Jiang Y, Fourtouna A, Lai R and Medeiros LJ (2005) p53 gene mutations are uncommon but p53 is commonly expressed in anaplastic large-cell lymphoma. *Leukemia* **19**:1663-1669.

Raught B and Gingras AC (1999) eIF4E activity is regulated at multiple levels. *Int J Biochem Cell Biol* **31**:43-57.

Scheper GC and Proud CG (2002) Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? *Eur J Biochem* **269**:5350-5359.

Scheper GC, Van KB, Hu J, Luo Y, Goss DJ and Proud CG (2002) Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. *J Biol Chem* **277**:3303-3309.

Tadlock L and Patel T (2001) Involvement of p38 mitogen-activated protein kinase signaling in transformed growth of a cholangiocarcinoma cell line. *Hepatology* **33**:43-51.

Tilleray V, Constantinou C and Clemens MJ (2006) Regulation of protein synthesis by inducible

wild-type p53 in human lung carcinoma cells. *FEBS Lett* **580**:1766-1770.

Walker NJ, Crockett PW, Nyska A, Brix AE, Jokinen MP, Sells DM, Hailey JR, Easterling M, Haseman JK, Yin M, Wyde ME, Bucher JR and Portier CJ (2005) Dose-additive carcinogenicity of a defined mixture of "dioxin-like compounds". *Environ Health Perspect* **113**:43-48.

Weiss C, Faust D, Durk H, Kolluri SK, Pelzer A, Schneider S, Dietrich C, Oesch F and Gottlicher M (2005) TCDD induces c-jun expression via a novel Ah (dioxin) receptor-mediated p38-MAPK-dependent pathway. *Oncogene* **24**:4975-4983.

Xu GW, Mymryk JS and Cairncross JG (2005) Pharmaceutical-mediated inactivation of p53 sensitizes U87MG glioma cells to BCNU and temozolomide. *Int J Cancer* **116**:187-192.

Yamagiwa Y, Marienfeld C, Tadlock L and Patel T (2003) Translational regulation by p38 mitogen-activated protein kinase signaling during human cholangiocarcinoma growth. *Hepatology* **38**:158-166.

Yin B, Kogan SC, Dickins RA, Lowe SW and Largaespada DA (2006) Trp53 loss during in vitro selection contributes to acquired Ara-C resistance in acute myeloid leukemia. *Exp Hematol* **34**:631-641.

Zhu X, Yu QS, Cutler RG, Culmsee CW, Holloway HW, Lahiri DK, Mattson MP and Greig NH (2002) Novel p53 inactivators with neuroprotective action: syntheses and pharmacological evaluation of 2-imino-2,3,4,5,6,7-hexahydrobenzothiazole and 2-imino-2,3,4,5,6,7-hexahydrobenzoxazole derivatives. *J Med Chem* **45**:5090-5097.

Footnotes

Legends for Figures

Figure 1. Overexpression of eIF-4E contributes to chemoresistance in human cholangiocarcinoma. (A) Immunohistochemistry for eIF-4E was performed on a human cholangiocarcinoma tissue microarray containing samples from 46 cholangiocarcinomas and 4 normal livers. Representative histological sections from normal liver and cholangiocarcinoma are shown; magnification X 100 (upper panels), X 400 (lower panels). Compared to normal biliary tract epithelia, there was a marked increase in eIF-4E staining in malignant biliary tract epithelia. (B) eIF-4E expression was quantitated using a semi-quantitative 4-point score ranging from 1 (least) to 4 (greatest) as described in the Methods section.

Figure 2. Pifithrin-α is non-cytotoxic but increases sensitivity to gemcitabine. KMCH cells (1 x 10^4) in 96-well plates were incubated with (A) pifithrin-α at the indicated concentrations for 24 hours or (B) diluent control or 50 µM pifithrin-α for 24 hours followed by 100 µM gemcitabine 24 hours. The effect of pifithrin-α on KMCH cells was assessed using a viable cell assay and expressed as a percentage of the control. (C) The effect of pifithrin-α on transformed cell growth was determined using a short-term anchorage independent growth assay. KMCH cells (1x10^3) were treated with diluent controls or 50 µM pifithrin-α and/or 100 µM gemcitabine and plated in soft agar in 96-well plates. Growth was quantitated by determining Alamar Blue fluorescence after 7 days. (D) H69 non malignant human cholangiocytes (1 x 10^4 cells/well) in 96-well plates were incubated with diluent control or 50 µM pifithrin-α for 24 hours followed by 100 µM gemcitabine 24 hours. Cell viability was assessed and expressed as a percentage of the control. The results represent the mean ± 95% confidence value of at least four experiments. *$P < 0.05$ relative to control untreated cells.

Figure 3. eIF-4E is involved in the chemosensitization effects of pifithrin-α. (A) KMCH cells (1×10^6) were transfected with 1.5 µg siRNA to eIF-4E or scrambled nucleotide control siRNA. A representative immunoblot demonstrating reduced eIF-4E levels under these conditions is shown. 24 hours post-transfection cells were treated with diluent control or 50 µM pifithrin-α for 24 hours followed by 100 µM gemcitabine for 24 hours. The bar graph represents the viability of cells treated with 50 µM pifithrin-α and expressed as a percentage of the control cells which were not pretreated with pifithrin-α. Results represent the mean ± 95% confidence of at least four separate experiments. (B) KMCH cells were incubated with pifithrin-α at the indicated concentrations for 24 hours prior to isolation of total protein. Immunoblot analysis was performed for total and phospho-serine 209 eIF-4E (1:1000). The blots were stripped and re-probed with antibodies for β-actin (1:2000) for equal loading. Representative immunoblots are shown in addition to quantitative data which is expressed as a percentage of control of phospho-serine209-eIF-4E against total eIF-4E. Results represent the mean ± 95% confidence of four separate blots. * $P < 0.05$ relative to control.

Figure 4. Pifithrin-α modulates p38 MAPK dependent regulation of eIF-4E. (A) KMCH cells and dominant negative MKK-3 cells were treated with diluent control or 25 µM pifithrin-α for 48 hours followed by isolation of total protein and immunoblot analysis for phosphorylation levels of serine209-eIF-4E (1:1000); The blots were stripped and re-probed with antibodies for β-actin (1:2000) for equal loading. (B) KMCH cells were incubated with diluent control or 25 µM SB203580 for 24 hours followed by the addition of diluent control or 25 µM pifithrin-α for 48 hours. Protein lysates were obtained and total p38 MAPK and phosphoT-phosphoY 180/182 p38 MAPK was assayed. The data represents phospho-p38 MAPK expressed as a percentage of total p38 MAPK expression, and represents data from 4 separate

determinations. (C) Immunoblot analysis was performed for phospho-serine209-eIF-4E (1:1000)

levels. The blots were stripped and re-probed with antibodies for β-actin (1:2000) for equal

loading. Representative immunoblots are shown in addition to quantitative data which is

expressed as a percentage of control of probed proteins against β-actin.. Results represent the

mean ± 95% confidence of four separate experiments. *$P < 0.05$ relative to control.

**Figure 5.   Chemosensitization effects of pifithrin-α involves aryl hydrocarbon
receptor mediated signaling.** (A) KMCH cells (1 x 10^4) in 96-well plates were pre- treated

with diluent control, 0.1 or 1 μM α-naphthoflavone for 24 hours, After 24 hours, 25 μM pifithrin-

α was added. The response to incubation with 100 μM gemcitabine was assessed after 24 hours.

The effect of α-naphthoflavone on pifithrin-α chemosensitization of KMCH cells was assessed

using a viable cell assay and expressed as a percentage of the control. (B) KMCH cells (1.5 x

10^6) were incubated with diluent control or 0.1 μM α-naphthoflavone for 24 hours followed by

diluent control or 25 μM pifithrin-α in 100 mm dishes for 48 hours prior to isolation of total

RNA. cDNA was generated and PCR was performed to amplify β-actin and CYP1A1, a

downstream target of aryl hydrocarbon signaling. 1 μl of each PCR reaction products were

resolved on a 500 DNA chip and analyzed on an Agilent 2100 Bioanalyzer. A representative gel

image is shown along with the quantitative data of CYP1A1 normalized against β-actin and

expressed as a percentage of the control. (C) Total protein was isolated from KMCH cells

similarly treated with α-naphthoflavone and pifithrin-α and immunoblot analysis of CYP1A1

was performed (1:1000). (D) KMCH cells stably transfected with dominant negative MKK-3

cells or control cells were treated with diluent control or 25 μM pifithrin-α and immunoblot

analysis of CYP1A1 was performed (1:1000). The blots were stripped and re-probed with

antibodies for β-actin (1:2000) for equal loading. Results represent the mean ± 95% confidence

of four separate experiments. *$P < 0.05$ relative to untreated controls. **$P < 0.05$ relative to KMCH treated control.

Figure 6. Modulation of p53 does not alter the responses of KMCH malignant cholangiocytes to chemotherapy. (A) Total protein was isolated from H69 nonmalignant and KMCH malignant cholangiocytes and immunoblot analysis was performed for total p53 levels (1:1000). The blots were stripped and re-probed with antibodies for β-actin (1:2000) for equal loading. Representative immunoblots are shown in addition to quantitative data which is expressed as p53 relative to β-actin. (B) KMCH cells (1 x 10^6) were transfected with 1.5 µg siRNA to p53 or scrambled nucleotide control siRNA. A representative immunoblot showing p53 expression under these conditions is shown. After 48 hours cells were treated with diluent control or 100 µM gemcitabine and cell viability assessed after 24 hours. Gemcitabine sensitivity was assessed using a viable cell assay and expressed as a percentage of the control. The results represent the mean ± 95% confidence value of at least four experiments. *$P < 0.05$ relative to control.
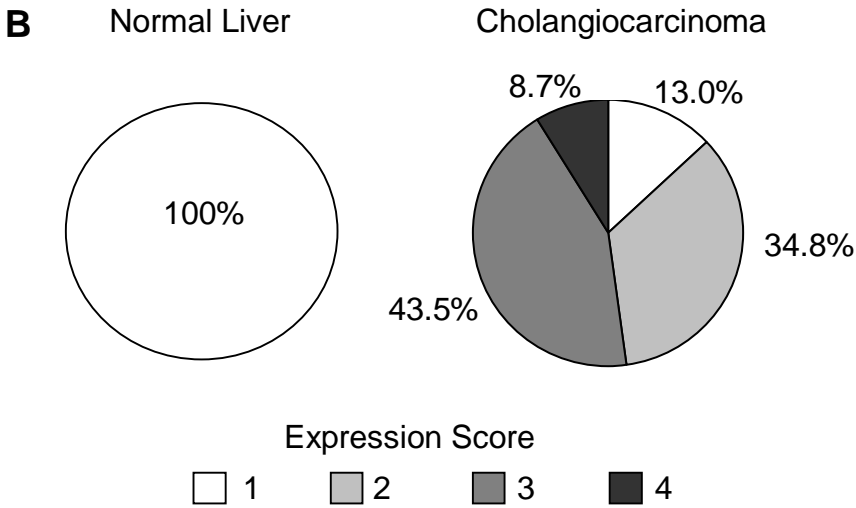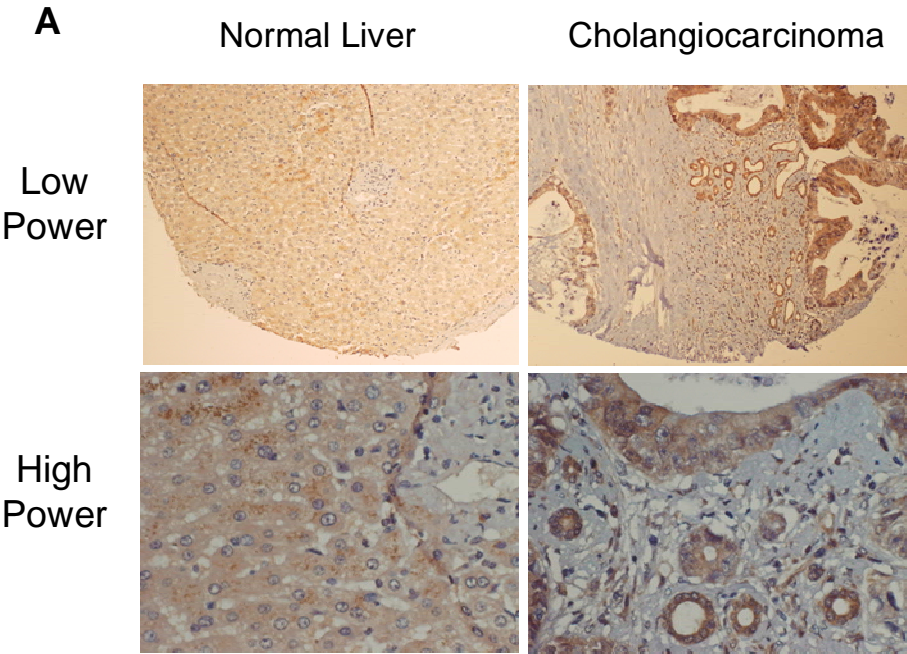
Figure 1

A

	Normal Liver	Cholangiocarcinoma
Low Power		
High Power		

B

Normal Liver

Cholangiocarcinoma

100%

8.7%   13.0%

34.8%

43.5%

Expression Score

□ 1   ▨ 2   ▨ 3   ■ 4

Figure 2

A

B

C

D

Non-Malignant Human Cholangiocytes

Figure 3

A

Percent of Control

	DN MKK3	PIF-α
	—	—
	—	+
	+	—
	+	+

P-Ser209 eIF4E

ß-actin

B

Phospho-p38, Normalized Percent of Control

	SB203580	PIF-α
	—	—
	—	+
	+	—
	+	+

C

Percent of Control

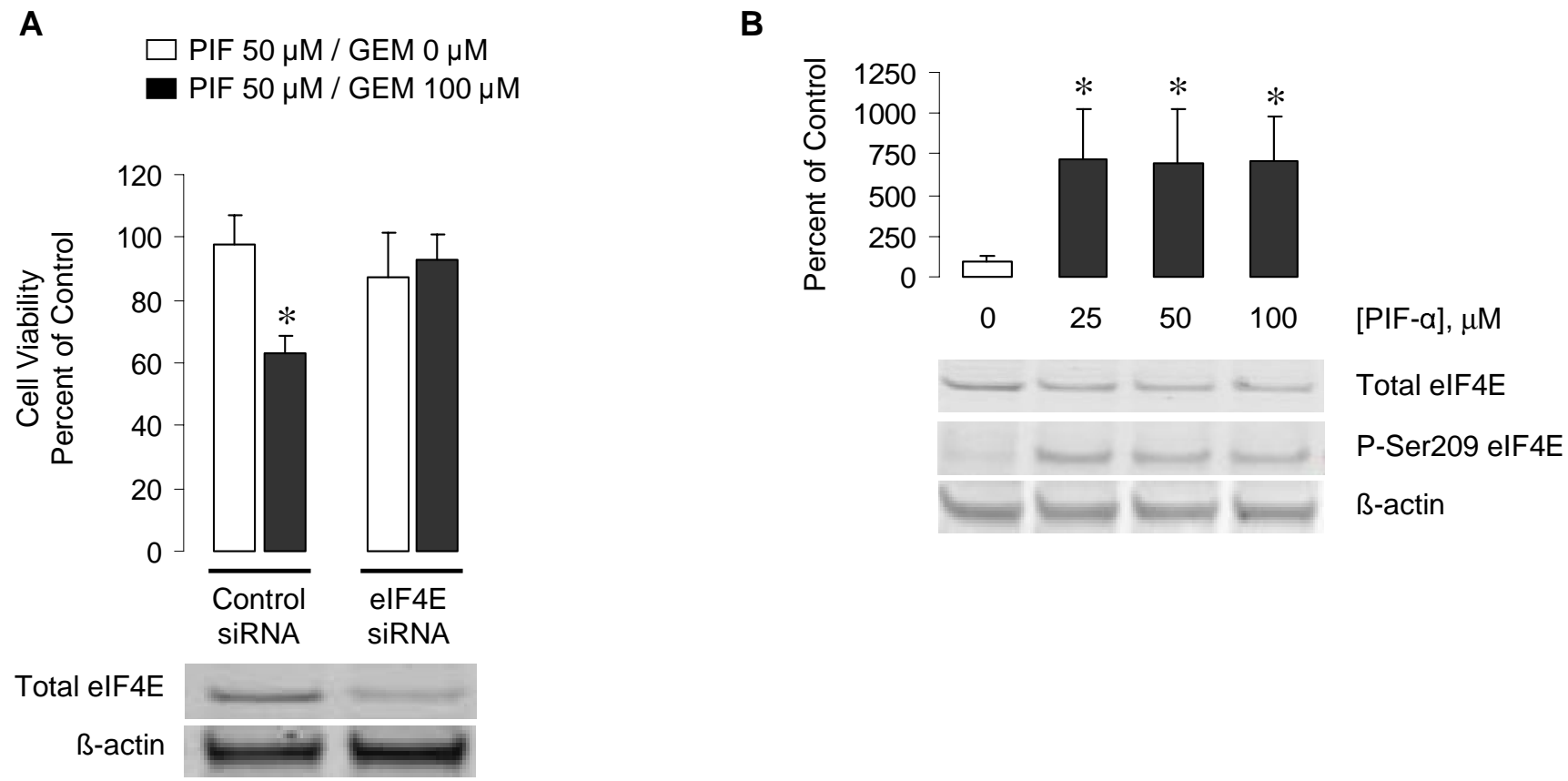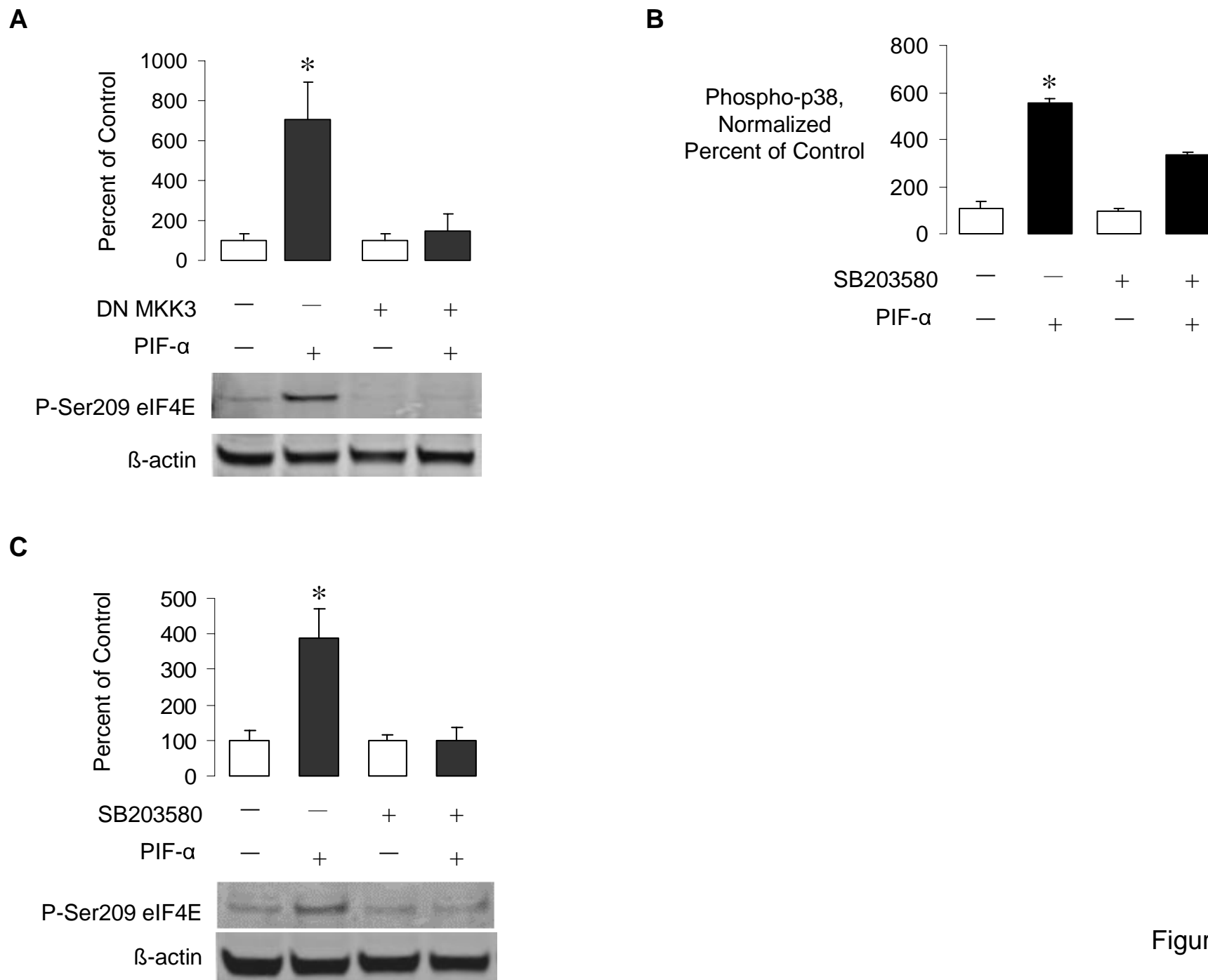|	SB203580	PIF-α
	—	—
	—	+
	+	—
	+	+

P-Ser209 eIF4E

ß-actin
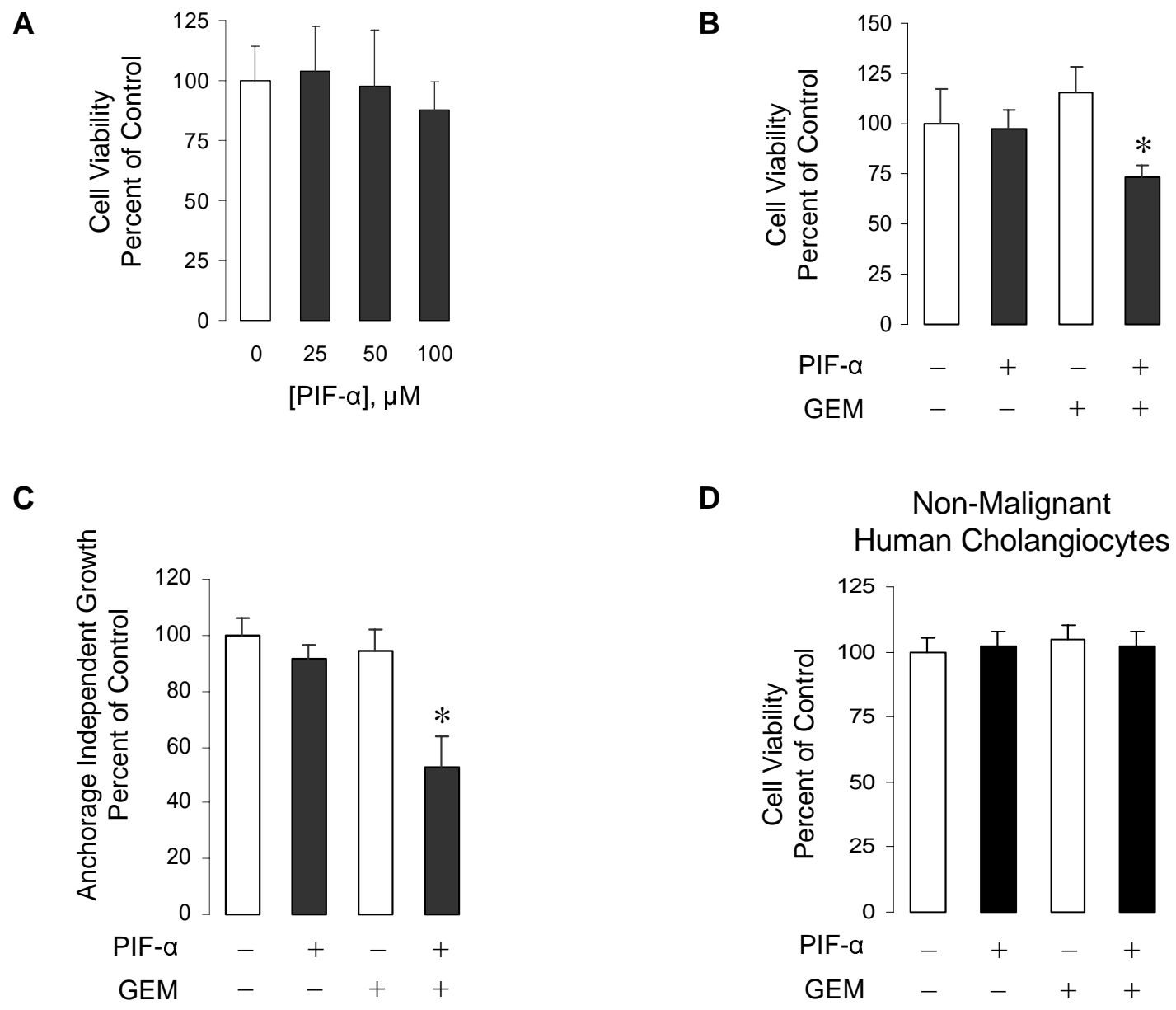
Figure 4

Figure 5

Figure 6

A

Relative p53 expression (y-axis: 0, 25, 50, 75, 100, 125, 150)

H69   KMCH

*

p53

β-actin

B

Cell Viability Percent of Control (y-axis: 0, 20, 40, 60, 80, 100, 120)

Control siRNA   p53 siRNA

p53

β-actin