RESEARCH ARTICLE

Possible Involvement of Cyclophilin A Processing in Fumagillin-Induced Suppression of Cholangiocarcinoma Cell Proliferation

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Abstract

Methionine aminopeptidase 2 (MetAP2), a proteolytic enzyme that removes the N-terminal methionine from newly synthesized cellular proteins, plays roles in the development of various cancers and has been found to be over-expressed in cholangiocarcinoma (CCA). Fumagillin, a specific MetAP2 inhibitor, suppresses CCA cell proliferation. In order to determine the molecular mechanisms involved in the suppression of CCA cell proliferation caused by fumagillin, proteomic analysis was performed on fumagillin-treated CCA cells. Proteins affected by fumagillin were analyzed using 2D gel electrophoresis and matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry (MALDI-TOF/TOF). The results showed that the processed form of cyclophilin A (CypA) was greatly decreased in parallel with the suppression of CCA cell proliferation. These results suggest that CypA is possibly a protein substrate of MetAP2 cleavage. Removal of N-terminal methionine by MetAP2 may be essential for proper function of CypA in CCA cell proliferation. MetAP2 and CypA may thus serve as potential therapeutic targets for CCA treatment.

Keywords: Cholangiocarcinoma - bile duct cancer - methionine aminopeptidase 2 - fumagillin - cyclophilin A

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Introduction

Cholangiocarcinoma (CCA), is a bile duct cancer which is a leading cause of death in Northeast Thailand. Although CCA is relatively infrequent, attention to CCA is now growing because both incidence and mortality rates of CCA are increasing in the United States, the United Kingdom Australia and overall worldwide (Patel, 2001; Taylor-Robinson et al., 2001; Khan et al., 2002). CCA is a highly infiltrative and rapidly growing cancer which exhibits lymphatic and intrahepatic metastases (Sripa and Pairojkul, 2000), there is now an urgent need to focus on developing specific therapeutic strategies aimed at exploiting select molecular targets aberrantly expressed during carcinogenesis and metastasis of CCA.

Methionine aminopeptidase 2 (MetAP2) is a protease involved in the removal of the N-terminal methionine from newly synthesized proteins. This allows essential post-translational modifications, such as myristoylation and acetylation, to take place thus influencing localization, stability and activity. MetAP2 has been reported to play a critical role in the proliferation of endothelial cells and certain cancer cells (Ingber et al., 1990; Catalano et al., 2001; Hou et al., 2009) and serves as a promising target as anti-angiogenesis and anti cancer drugs. A class of drugs targeting this protein are fumagillin and it derivatives (e.g. ovalicin and TNP470) which bind MetAP2 covalently, and inhibit its enzymatic activity (Sin et al., 1997; Griffith et al., 1998). A defect in initiator methionine removal caused by MetAP2 inhibition therefore might lead to aberrant levels of proteins important for cell proliferation and result in tumor growth and angiogenesis inhibition which serve as an attractive target for cancer therapy. The specific MetAP2 substrates that may be important targets for CCA therapy have yet to be identified.

We previously reported the aberrant expression of MetAP2 during development of CCA and inactivation of MetAP2 activity by fumagillin resulted in reduction of CCA cell proliferation (Sawanyawisuth et al., 2007). In this study, we performed a proteomic analysis of fumagillin-treated CCA cells and revealed cyclophilin A (CypA) as a possible substrate of MetAP2. After treatment of CCA cells with fumagillin, the degree of processing alteration of CypA corresponded well with the degree of proliferation inhibition. These data suggest that CCA cell proliferation could be inhibited by interfering with MetAP2 function, in other words, MetAP2 seems to have a regulatory role in CCA for tumor cell growth.

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Materials and Methods

Preparation of fumagillin- treated cell proteins

CCA cells (KKU-M213) were seeded into a 10 cm-culture dish and incubated in the presence of fumagillin (5 μg/ml) or vehicle (0.25% ethanol) for 72 h as described previously (Sawanyawisuth et al., 2007). Cells were washed twice in 0.25 M. sucrose and then scraped in 0.25 M sucrose containing a complete protease inhibitor cocktail (Roche Molecular Biochemicals, Germany) and centrifuged at 2,000 rpm for 10 min at 4°C. The pellets were collected, resuspended in a lysis solution (8 M Urea, 4% CHAPS containing a complete protease inhibitor cocktail) and incubated on ice for 30 min. Samples were then centrifuged for 20 min at 20,000 xg at 4°C to remove cellular debris. The final protein concentration of the sample was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Cell proliferation assay

CCA cells were treated with vehicle (0.25% ethanol) or varied concentrations of fumagillin for 72 h. The number of cells was determined using the sulforhodamine (SRB) assay as described previously (Sawanyawisuth et al., 2007).

Two- dimensional (2D) gel electrophoresis

Protein was applied onto an Immobiline drystrip pH 3-10 non-linear 13 cm long for proteomic analysis or 7 cm long for 2D western blotting (Amersham Bioscience, UK) using an Ettan IPGphor II (Amersham Bioscience) following the manufacturer's protocol. After focusing, strips were incubated in equilibration buffer and then loaded onto a 12.5% SDS-polyacrylamide gel electrophoresis (PAGE). Gels were stained with SYPRO ruby (Molecular Probes) and visualized using a Typhoon 9400 imager (Amersham Biosciences). Images were analyzed with Progenesis Workstation version 2005 (PerkinElmer Japan/Nonlinear Dynamics, Newcastle uponTyne, UK).

Protein identification by MALDI-TOF/TOF MS

In-gel digestion of the protein spot on gels was performed. After the completion of staining, the gel slab was washed twice with water for 10 min. The spots of interest were excised and put into 1.5 ml microtubes. Enough 50 mM ammonium bicarbonate buffer, pH 8.0/50% acetonitrile (ACN) (1:1) (Sigma) was added and incubated at room temperature for 15 min. After dehydration by soaking the gel in 100% ACN for 30 min, the ACN was then removed. The gels were dried in Speed-Vac and incubated with trypsin solution at 37°C for 16 h. Tryptic peptides were sequentially extracted using 30%, 50% and 80% of ACN in 0.1% of Trifluoroacetic acid (TFA), and combined in one tube. After removal of ACN by centrifugation in a Speed-Vac., the peptides were concentrated by using C18Zip-Tip micro (Millipore Corp.,Bedford, MA) and eluted with 0.5 μl of 50% ACN, 0.1 % TFA and directly dropped on MALDI plate. Finally, alpha-cyano-4-hydroxycinnamic acid (0.5 μl of 10 mg/ml in 50% of ACN and 0.1% of TFA) was applied to each spot, and the spots were air-dried at room temperature prior to acquiring mass spectra. The TOF-MS and tandem MS/MS spectra of the peptides were analyzed by 4700 MALDI-TOF/TOF MS (Applied Biosystems). For MALDI peptide mapping and fragment ion analysis, Mascot Server (Matrix Science) and GPS Explorer (Applied Biosystems) search engines were employed.

2D western blotting of CypA

Protein spots on 2D gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) which were then blocked with 1% BSA for 1 h. CypA was detected by incubation overnight at 4°C with rabbit polyclonal anti-human CypA antibody at a 1:5,000 dilution (Upstate Biotechnology, Inc., Lake Placid, NY). β-actin signal was detected by mouse monoclonal anti-human β -actin antibody at a 1:5,000 dilution (Sigma-Aldrich, St. Louis, MO) and used as the internal control. The membranes were probed with horseradish peroxidase- conjugated antibody at a 1:10,000 dilution (Amersham biosciences) for 1 h, and detected by Western Lightning Chemiluminescence Reagents (PerkinElmer Ltd., Boston, MA).

Results

Proteomic analysis shows CypA processing defect in fumagillin-treated CCA cells

We sought to identify proteins that were altered when MetAP2 activity was inhibited by fumagillin. Protein extracts from CCA cells (KKU-M213) treated with either vehicle or fumagillin were analyzed using 2D gel electrophoresis followed by SYPRO ruby staining (Figure 1A). There were approximately 1,000 spots obtained in each gels. The protein spots were matched and the intensities of each spots were compared. Using Progenesis software analysis, a limited number of differences were obtained after fumagillin treatment. Two protein spots of molecular weights of 18 kDa were obviously detected. As shown in Figure 1B, the intensity of protein spot #1 increased 1.8 fold whereas that of spot #2 decreased 2.1

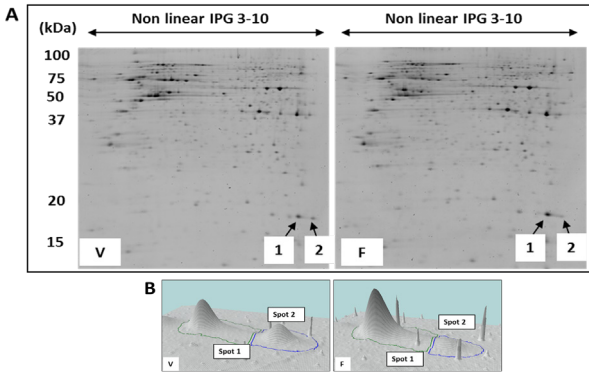

Figure 1. Comparative Proteomic Analysis of CCA Cells Following Fumagillin Treatment. Proteomic patterns of cells treated with vehicle (V) or fumagillin 5 ug/ml (F) for 72 h. The first dimension was performed on a 13 cm immobilized drystrip pH 3–10 NL, followed by a second dimension on 12.5% SDS-PAGE. A) Proteomic pattern, SYPRO Ruby staining. B) Three-dimensional intensity profiles of the 18 kDa protein spot #1 and spot #2

fold upon fumagillin treatment. These two spots were further identified using MALDI-TOF/TOF analysis of the in-gel tryptic digests.

Mass spectra of tryptic peptides from the 18 kDa spots

A

Spot 1 Match to: gb|AAH07104.1 Score: 99 Expect: 1.2e-005
peptidylprolyl isomerase A (Cyclophilin A)
Nominal mass (Mr): 18000; Calculated pI value: 7.68

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  1 MVNPTVFFDI AVDGEPLGRV SFELFADKVP KTAENFRALS TGEKGFGYKG
 51 SCFHRIIPGF MCQGGDFTRH NGTGGKSIYG EKFEDENFIL KHTGPGILSM
101 ANAGPITNGS QFFICTAKTE WLDGKHVVFG KVKEGMNIVE AMERFGSRNG
151 KTSKKITIAD CGQLE
```

Spot 2 Match to: gb|AAH07104.1 Score: 153 Expect: 5.3e-011
peptidylprolyl isomerase A (Cyclophilin A)
Nominal mass (Mr): 18000; Calculated pI value: 7.68

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  1 MVNPTVFFDI AVDGEPLGRV SFELFADKVP KTAENFRALS TGEKGFGYKG
 51 SCFHRIIPGF MCQGGDFTRH NGTGGKSIYG EKFEDENFIL KHTGPGILSM
101 ANAGPITNGS QFFICTAKTE WLDGKHVVFG KVKEGMNIVE AMERFGSRNG
151 KTSKKITIAD CGQLE
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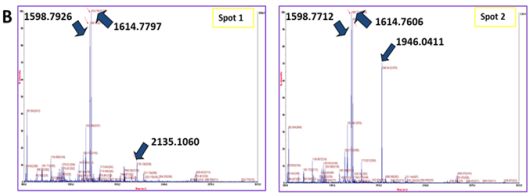
B



C

Spot	Observed	Mr(expt)	Mr(calc)	Ions score	Peptide
1	1598.7	1597.7	1597.7	44	IIPGFMCQGGDFTR + Carbamidomethyl (C)
	1614.7	1613.7	1613.7	26	IIPGFMCQGGDFTR + Carbamidomethyl (C); Oxidation (M)
	2135.1	2134.0	2134.0	17	MVNPTVFFDIAVDGEPLGR + Acetyl (N-term); Oxidation (M)
2	1598.7	1597.7	1597.7	52	IIPGFMCQGGDFTR + Carbamidomethyl (C)
	1614.7	1613.7	1613.7	47	IIPGFMCQGGDFTR + Carbamidomethyl (C); Oxidation (M)
	1946.0	1945.0	1944.9	86	VNPTVFFDIAVDGEPLGR

Figure 2. MALDI-TOF-TOF Analysis of the two 18 kDa Proteins. A) Amino acid sequence of CypA and the identified peptides are indicated by underlined bold. B) The mass spectra identified spot #1 as CypA with the retained N-terminal methionine and spot #2 as CypA without N-terminal methionine. C) Molecular masses and amino acid sequences of the identified peptides

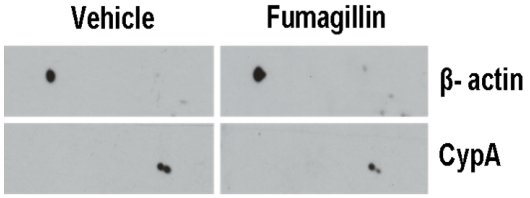


Figure 3. Verification of MALDI-TOF/TOF MS Analysis by 2D Western Blotting of CypA. Immunoblots with anti-CypA antibody of 2 spots of CypA. β-actin was used as the internal control

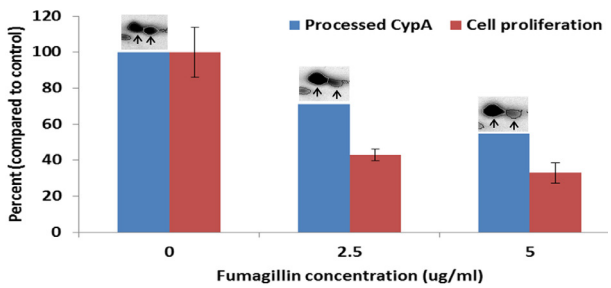


Figure 4. Inhibition of CCA Proliferation by Fumagillin Correlates with CypA Processing. CCA cells (KKU-M213) were treated with various concentrations of fumagillin for 72 h and measured the cell proliferation by SRB assay. Protein was collected and subjected to 2D gel analysis. Two spots of CypA (indicated arrows) were quantified by densitometry analysis, calculated as the ratio of the processed to unprocessed form and shown as a percentage of control group (without fumagillin)

#1 and #2 revealed three peptides, which were identified by searching against the NCBI database using Mascot and GPS. The peptides from both spots were matched with the enzyme peptidylprolyl cis-trans isomerase cyclophilin A (CypA) as seen in Figure 2A, of which spot #1 had initiator methionine residues and acetylation at the amino terminus whereas in spot #2 the N-terminus methionine was removed (Figure 2B and 2C). These results indicate that the mobility shift of CypA following drug treatment is due to retention of the initiator methionine and the proteins from untreated cells were the mixture of CypA with the methionine retention and with the methionine cleavage. To ascertain that these two spots were CypA, the 2D gels from cells with or without fumagillin treatment were subjected to western blotting with anti-CypA antibody. The results confirmed that these two 18 kDa spots were CypA (Figure 3).

Correlation between CypA processed form and CCA cell proliferation

Since we have previously reported the anti-proliferation effect of fumagillin on KKU-M213, we further questioned as to whether the defect in N-terminal methionine processing of CypA related to anti-proliferation was observed in fumagillin treated cells. CCA cells (KKU-M213) were treated with various concentrations of fumagillin and quantitated the spot intensities of both processed and unprocessed form of CypA on 2D gels. The ratio of intensity of processed CypA to unprocessed CypA was shown in Figure 4. These data demonstrated that the decrease of processed CypA correlated with proliferation inhibition induced by fumagillin in a dose-dependent fashion.

Discussion

Our previous study illustrated that MetAP2 is overexpressed in CCA clinical samples and fumagillin inhibits cell proliferation in CCA cell lines (Sawanyawisuth et al., 2007). These data led us to investigate further the cellular protein changes due to inhibition of MetAP2 activity. We studied the proteomic analysis of fumagillin treated CCA cells and identified the proteins affected by MetAP2 inhibition. 2D gel analysis combined with MALDI-TOF/ TOF MS data showed peptidylprolyl cis-trans isomerase (PPIA) or Cyclophilin A (CypA) had altered protein processing. Increased N-terminal methionine retention and acetylation which is the unprocessed form of CypA was found in fumagillin treated cells, therefore, CypA is one of the MetAP2 specific substrates. Our findings agreed well with previous reports that studied the cellular protein alteration after MetAP2 inhibitors were used to treat endothelial cells and a HT-1080 fibrosarcoma cell line (Turk et al., 1999; Warder et al., 2008).

To our knowledge, there are at least three proteins which have been proven as a substrate for MetAP2: CypA, glyceraldehyde-3-phosphate dehydrogenase (Turk et al., 1999) and 14-3-3 γ (Towbin et al., 2003). The mobilities of these proteins on 2D gels were shifted after MetAP2 activity was inhibited. However, in the present study,

only the mobility shift of CypA was obviously observed. Since CypA has significant role in CCA cell proliferation (Obchoei et al., 2011), we therefore selected CypA for further study in relation to MetAP2 action on proliferation.

Identification of MetAP2 substrates by 2D gel analysis has limitations. Cleavage of the N-terminal methionine from a protein causes a minimal change in molecular weight and is not expected to lead to large changes of isoelectric points unless accompanied by acetylation or other post-translational processing such as myristoylation of the newly exposed amino group. This possibility may explain why so few changes were observed in the current study as well as in the earlier reports (Turk et al., 1999; Towbin et al., 2003). A combination of undigested cell culture-derived proteomes by MALDI-/SELDI-MS profiling and a biochemical method using ^{35}S-methionine labeled protein lysates was applied to discover the novel MetAP2-specific substrates including thioredoxin-1 (Trx-1), SH3 binding glutamic acid rich-like protein (SH3BGRL), and eukaryotic elongation factor-2 (eEF2) (Warder et al., 2008). These proteins have diverse properties; their protein processing changes may result in the inhibition of MetAP2 dependent cell proliferation. These proteins can serve as novel targets for cancer therapy.

CypA, an 18 kDa cytosolic protein that is thought to be the major intracellular target of the immunosuppressive drug cyclosporin A, is conserved throughout the phylogenetic tree from yeasts to humans and as such, CypA is believed to be a key molecule in many biological functions including molecular chaperoning, protein folding, protein trafficking, immune modulation and cell signaling (Obchoei et al., 2009). Unusually high levels of CypA have been reported in several types of cancers and related to cancer cell proliferation, anti-apoptotic phenotype, migration/invasion, and drug resistance in various cancer cell types. In CCA, CypA was up-regulated in a majority of patients' tissues (Obchoei et al., 2011). Suppression of CypA expression decreased proliferation of CCA cell lines in vitro and reduced tumor growth in the nude mouse model. In the present study, the correlation between the level of CypA processing defect and the proliferation inhibition was observed. The underlying mechanism of this phenomenon is still unclear. Since we did not observe the apoptotic cells under fumagillin treatment, the cell cycle arrest might be the possible mechanism. Several studies demonstrated that inhibition of MetAP2 activity induces G1 cell cycle arrest via p53 activation, p21 accumulation, phosphorylated retinoblastoma tumor suppressor protein (pRb) inhibition, reduction of cyclin dependent kinases (CDK2, CDK4) and cyclins A and E (Abe et al., 1994; Yeh et al., 2000; Zhang et al., 2000; Wang et al., 2003). The suppression of CCA cell proliferation via MetAP2 inhibition could be the outcome of several biological pathways, one of which is via CypA action. It is more likely that the inhibition of MetAP2 action is possibly via cell cycle arrest.

We postulated that the unprocessed form of CypA may not fully function and affect the CCA cell proliferation. The N-terminal status of CypA may be a monitoring specific protein changes in response to MetAP2 inhibition.

In conclusion, our study illustrated that inhibition of MetAP2 activity affected CCA cell proliferation and protein processing of CypA which is a MetAP2 specific substrate. Removal of the initiator methionine from CypA seems to be crucial for its proper function, and this may be one of the molecular mechanisms by which inhibition of MetAP2 leads to cell proliferation inhibition. These results indicate that MetAP2 might be a regulator of proliferation in CCA cells and suggest to us that MetAP2 and CypA may serve as a potential target for therapeutic intervention in human CCA.

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References

Abe J, Zhou W, Takuwa N, et al (1994). A fumagillin derivative angiogenesis inhibitor, AGM-1470, inhibits activation of cyclin-dependent kinases and phosphorylation of retinoblastoma gene product but not protein tyrosyl phosphorylation or protooncogene expression in vascular endothelial cells. *Cancer Res*, **54**, 3407-12.

Catalano A, Romano M, Robuffo I, et al (2001). Methionine aminopeptidase-2 regulates human mesothelioma cell survival: role of Bcl-2 expression and telomerase activity. *Am J Pathol*, **159**, 721-31.

Griffith EC, Su Z, Niwayama S, et al (1998). Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. *Proc Natl Acad Sci USA*, **95**, 15183-8.

Hou L, Mori D, Takase Y, et al (2009). Fumagillin inhibits colorectal cancer growth and metastasis in mice: in vivo and in vitro study of anti-angiogenesis. *Pathol Int*, **59**, 448-61.

Ingber D, Fujita T, Kishimoto S, et al (1990). Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature*, **348**, 555-7.

Khan SA, Taylor-Robinson SD, Toledano MB, et al (2002). Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol*, **37**, 806-13.

Obchoei S, Weakley SM, Wongkham S, et al (2011). Cyclophilin A enhances cell proliferation and tumor growth of liver fluke-associated cholangiocarcinoma. *Mol Cancer*, **10**, 102.

Obchoei S, Wongkhan S, Wongkham C, et al (2009). Cyclophilin A: potential functions and therapeutic target for human

cancer. *Med Sci Monit*, **15**, RA221-32.
Patel T (2001). Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology*, **33**, 1353-7.
Sawanyawisuth K, Wongkham C, Pairojkul C, et al (2007). Methionine aminopeptidase 2 over-expressed in cholangiocarcinoma: potential for drug target. *Acta Oncol*, **46**, 378-85.
Sin N, Meng L, Wang MQ, et al (1997). The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. *Proc Natl Acad Sci USA*, **94**, 6099-103.
Sripa B, Pairojkul C (2000). Pathology of cholangiocarcinoma, in Liver cancer in Thailand: Epidemiology, diagnosis and control (Vatanasapt V and Sripa B eds) pp 65-100, Siriphan Press, Khon Kaen.
Taylor-Robinson SD, Toledano MB, Arora S, et al (2001). Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968-1998. *Gut*, **48**, 816-20.
Towbin H, Bair KW, DeCaprio JA, et al (2003). Proteomics-based target identification: bengamides as a new class of methionine aminopeptidase inhibitors. *J Biol Chem*, **278**, 52964-71.
Turk BE, Griffith EC, Wolf S, et al (1999). Selective inhibition of amino-terminal methionine processing by TNP-470 and ovalicin in endothelial cells. *Chem Biol*, **6**, 823-33.
Wang J, Sheppard GS, Lou P, et al (2003). Tumor suppression by a rationally designed reversible inhibitor of methionine aminopeptidase-2. *Cancer Res*, **63**, 7861-9.
Warder SE, Tucker LA, McLoughlin SM, et al (2008). Discovery, identification, and characterization of candidate pharmacodynamic markers of methionine aminopeptidase-2 inhibition. *J Proteome Res*, **7**, 4807-20.
Yeh JR, Mohan R, Crews CM (2000). The antiangiogenic agent TNP-470 requires p53 and p21CIP/WAF for endothelial cell growth arrest. *Proc Natl Acad Sci USA*, **97**, 12782-7.
Zhang Y, Griffith EC, Sage J, et al (2000). Cell cycle inhibition by the anti-angiogenic agent TNP-470 is mediated by p53 and p21WAF1/CIP1. *Proc Natl Acad Sci USA*, **97**, 6427-32.