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ORIGINAL ARTICLE

MI-219-zinc combination: a new paradigm in MDM2 inhibitor-based therapy

AS Azmi[1], PA Philip[2], FWJ Beck[2], Z Wang[1], S Banerjee[1], S Wang[3,4], D Yang[4], FH Sarkar[1] and RM Mohammad[2]

[1]*Department of Pathology, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, USA;* [2]*Division of Hematology and Oncology, Department of Internal Medicine, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, USA;* [3]*Departments of Internal Medicine and Medicinal Chemistry, University of Michigan, Ann Arbor, MI, USA and* [4]*Ascenta Therapeutics, Inc., Malvern, PA, USA*

Zinc has a crucial role in the biology of p53 in that p53 binds to DNA through a structurally complex domain stabilized by zinc atom. The p53 negative regulator MDM2 protein also carries a C-terminal RING domain that coordinates two zinc atoms, which are responsible for p53 nuclear export and proteasomal degradation. In this clinically translatable study, we explored the critical role of zinc on p53 reactivation by MDM2 inhibitor, MI-219, in colon and breast cancer cells. $ZnCl_2$ enhanced MI-219 activity (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), apoptosis and colony formation), and chelation of zinc not only blocked the activity of MI-219, but also suppressed reactivation of the p53 and its downstream effector molecules $p21^{WAF1}$ and Bax. *N*,*N*,*N*′*N*′-tetrakis(−)[2-pyridylmethyl]-ethylenediamine (TPEN), a specific zinc chelator, but not 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Bapta-AM), a calcium chelator, blocked MI-219-induced apoptosis. Nuclear localization is a prerequisite for proper functioning of p53 and our results confirm that TPEN, and not Bapta-AM, could abrogate p53 nuclear localization and it interfered with p53 transcriptional activation. Addition of zinc suppressed the known p53 feedback MDM2 activation, which could be restored by TPEN. Co-immunoprecipitation studies verified that MI-219-mediated MDM2-p53 disruption could be suppressed by TPEN and restored by zinc. As such, single-agent therapies that target MDM2 inhibition, without supplemental zinc, may not be optimal in certain patients owing to the less recognized mild zinc deficiency among the 'at-risk population' as in the elderly who are more prone to cancers. Therefore, use of supplemental zinc with MI-219 will benefit the overall efficacy of MIs and this potent combination warrants further investigation.

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Correspondence: Professor RM Mohammad, Division of Hematology and Oncology, Department of Internal Medicine, Karmanos Cancer Institute, Wayne State University School of Medicine, 732 HWCRC, 4100 John R Street, Detroit, MI 48201, USA.
E-mail: mohammar@karmanos.org
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Introduction

The tumor suppressor *p53* is the most frequently mutated gene in human cancers. However, ~50% of all human tumors retain normal or wild-type p53 (wt-p53; Lane and Fischer, 2004). Direct activation of p53 in these tumors could, in principle, be used as a means to eradicate tumor cells (Brown *et al*., 2009). p53 is activated in response to a variety of stresses, such as DNA damage, nutrient deprivation or oncogenic activation, resulting in the transcriptional activation of target genes involved in growth arrest and apoptosis (Feng *et al*., 2008). To protect healthy cells from the deleterious effects of uncontrolled p53 activation, p53 is subject to a negative feedback loop activated by the protein product of one of its target genes, *MDM2* (Marine and Lozano, 2010). The protein MDM2 binds to p53, inhibits its transcriptional activation, causes nuclear export and acts as an E3 ligase to target p53 for proteasomal degradation (Kubbutat *et al*., 1997). Thus, there is a fine balance between MDM2, p53 and the need for p53 activation to promote cell survival or apoptosis following DNA damage or other cellular stresses. Unfortunately, in many cancers, the MDM2 protein is overexpressed and suppresses the activation of even the functional wt-p53, thereby disrupting the finely tuned balance of cell survival versus cell death. The end result is a loss of control of the normal apoptotic processes and contributes to drug resistance. One potential approach for reactivating p53 in tumor cells is to disrupt the interaction between MDM2 and p53, with the MDM2-targeting small molecule MDM2 inhibitor (MI)-219 or related inhibitors (Vassilev, 2007; Shangary *et al*., 2008; Shangary and Wang, 2009; Verma *et al*., 2010). MI-219 binds to MDM2, thereby preventing the interaction with p53 and causing p53 to be stabilized. We and others have shown that MI-219 can induce growth inhibition and apoptosis in multiple cancer cell lines and can also induce growth arrest in corresponding tumor xenografts (Shangary *et al*., 2008; Canner *et al*., 2009; Mohammad *et al*., 2009; Shangary and Wang, 2009; Yu *et al*., 2009).

Wt-p53 is one of the best recognized 'zinc-finger' transcription factors and binds DNA through a sequence-specific DNA-binding domain (p53DBD)

extending from amino acid (aa) residues 96–308 (Bargonetti *et al*., 1993). The p53DBD incurs an unusually high number of mutations that consequently result in a failure to bind DNA and prevention of p53-induced transcription (Levine *et al*., 1995; Levine, 1997). This fact strongly suggests that sequence-specific DNA binding and transactivation are the key activities that control the biological functions of p53 (Meek, 1998). The crystal structure of p53DBD reveals that the p53 core domain structure consists of a beta sandwich that serves as a scaffold for two large loops (L2 and L3) and a loop–sheet–helix motif (L1; Pavletich *et al*., 1993). Zn^{2+} is coordinated to C176 and H179 of the L2 loop, and to C238 and C242 of the L3 loop (Pavletich *et al*., 1993; Cho *et al*., 1994). Zinc coordination has been demonstrated to be necessary for the proper folding of the p53 core domain *in vitro* and disruption of this interaction greatly reduces or abrogates p53 DNA binding and transactivation of target genes (Meplan *et al*., 2000). Nuclear magnetic resonance spectral analyses reveal that the DNA-binding surface is altered by removing zinc ion and fluorescence anisotropy studies show that zinc ion removal abolishes site-specific DNA-binding activity (Butler and Loh, 2003, 2007). Using a cell-permeable metal chelator, previous investigators were able to show that depletion of intracellular zinc could induce a change in p53 protein conformation with loss of DNA-binding capacity that was reversible upon removal of the chelator from the culture medium or the addition of zinc to the media (Verhaegh *et al*., 1998). The amount of supporting information certainly highlights the crucial role of zinc in the biology of p53 protein and its importance for DNA binding as well as stability of this important tumor suppressor.

The p53 negative regulator MDM2 protein contains a C-terminal RING domain (aa 430–480) which coordinates two molecules of zinc (Wallace *et al*., 2006; Wawrzynow *et al*., 2009). This unique RING finger is responsible for shuttling p53 out of the nucleus for proteasomal degradation and is also responsible for MDM2 auto-ubiquitination (Alshatwi *et al*., 2006; Itahana *et al*., 2007; Lindstrom *et al*., 2007). The intrinsic E3 activity of MDM2 is dependent on its zinc-coordinated RING finger domain. The capacity to mediate MDM2's own ubiquitination requires no eukaryotic proteins other than E1 and E2. Therefore, zinc acts as a 'double-edged sword' promoting p53 activity, while simultaneously causing MDM2 auto-ubiquitination.

Mild zinc deficiency is a common occurrence in the elderly, a population that is well known to be more prone to cancers (Prasad *et al*., 1993; Prasad, 2001, 2004). The observed deficiency of zinc in the elderly has been attributed to a combination of factors that includes poor zinc absorption as well as consumption of low-zinc diets (Hambidge *et al*., 1998). Low intracellular zinc has been found in different tumors and has been correlated to induction of oxidative DNA damage, disruption of p53, AP1 DNA binding and also affects DNA repair (Ho and Ames, 2002; Ho, 2004; Ho and Song, 2009). Therefore, therapies that are based on using small molecule inhibitors to target MDM2 may not be fully successful in the clinical setting or in patients owing to unrecognized mild-to-moderate zinc deficiency. The studies presented in this paper demonstrate that zinc is crucial for the activity of p53 reactivated by MI-219 and provide evidence for using combination therapies with MI-219 that include supplemental zinc for the treatment of wt-p53 tumors. Before MIs make their way into the clinic for patients, we believe that the results of our findings will have high impact towards the design of novel treatment strategies (that is, combination with zinc) for achieving better survival outcome.

Results

Metal chelation in general and zinc-specific depletion suppresses MI-219-induced growth inhibition and apoptosis

To verify the role of metal ions on the activity of MI-219, we performed growth inhibition and apoptosis assays in media chelated to remove trace and non-trace metals (chelexed media). Assessment of chelation using atomic absorption spectroscopy confirmed chelation of zinc (and minimal chelation of copper; Supplementary Table 1). Our laboratory has previously standardized the procedure of zinc chelation that minimally alters the status of other trace metal ions such as iron, copper and magnesium (Prasad *et al*., 2001, 2002; Bao *et al*., 2006). HCT-116 and MCF-7 cells (wt-p53) were passaged in chelexed media and then treated with MI-219 (0–10 μM for 72 h) followed by analysis of growth inhibition by MTT assay. In normal media, the IC50 of MI-219 in HCT-116 and MCF-7 cells is 4 and 3.5 μM, respectively. However, in chelexed media depleted of trace metals, the IC50 increased to >10 μM (Figure 1a). Similar to the results obtained from MI-219-induced growth inhibition (0–10 μM for 72 h), MI-219 was also less effective in inducing apoptosis in chelexed media (Figure 1b). We did not observe any appreciable toxicity represented as change in growth patterns in cells grown in chelexed media versus those grown in normal media (Figure 1c). Based on our preliminary studies to define the optimal conditions, we found that specifically chelating Ca (using 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (Bapta-AM)) or copper (using bathocuprione disulfonate) had no effect on the events studied here and these specific chelators were used as negative controls in studies designed to determine the specific effect of zinc in MI-219-induced p53 reactivation. To delineate the crucial role of zinc on the efficacy of MI-219, we first evaluated the effect of $ZnCl_2$ (0–32 μM) on both HCT-116 and MCF-7 cells. As can be seen from results in Figure 2a, $ZnCl_2$ alone did not induce any appreciable growth inhibition in both cell lines. We then performed dose kinetics experiment to verify the effect of $ZnCl_2$ on MI-219-mediated cell growth inhibition. As can be seen from our novel results in Figure 2b, $ZnCl_2$ at increasing doses progressively enhanced cell growth inhibition by MI-219. We also tested the effect of this combination on colony formation capability in HCT-116 cells. Figure 2c shows colony

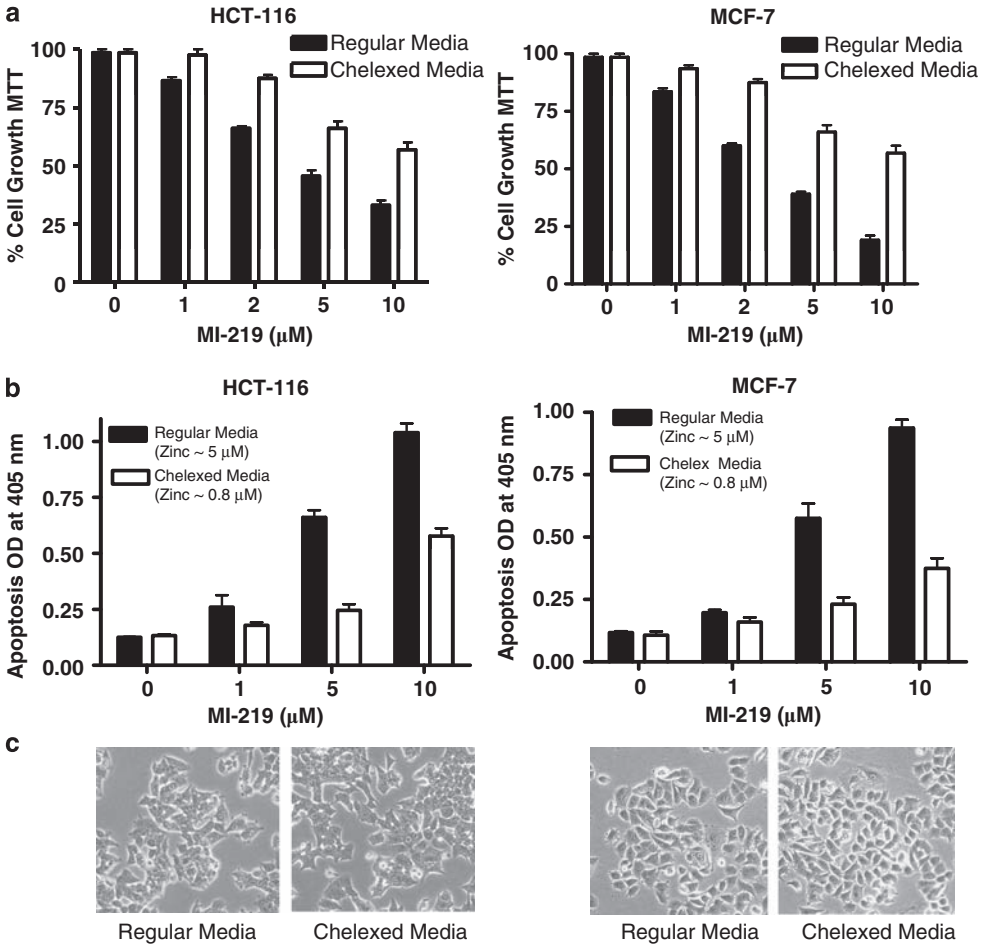

Figure 1 Chelation suppresses MI-219-mediated growth inhibition and apoptosis. (**a**) wt-p53 HCT-116 and MCF-7 cells were treated with MI-219 (0–10 μM for 72 h) in either regular or chelexed media. Note: growth inhibition (MTT assay) by MI-219 is diminished in chelexed media. (**b**) Chelation reduced media zinc levels from 5 to 0.8 μM (atomic absorption spectroscopy analysis Supplementary Table 1) and this was associated with reduced MI-219-induced apoptosis (detected by histone/DNA enzyme-linked immunosorbent assay (ELISA) assay) in both cell lines (right and left panels). (**c**) Microphotographs of HCT-116 and MCF-7 cells showing no apparent toxicity reflected as changes in growth pattern of cells grown in either regular or chelexed media. All experiments were performed in triplicate, data presented represents mean of three independent experiments.

formation in control and zinc-alone treated cells. MI-219 alone, as expected, suppressed colony formation. However, the most important and clinically relevant results are observed in the combination treatment where no colonies were visible even after 4 weeks. Taken together, these results indicate that MI-219 requires trace metal ions, specifically zinc, for its proper activity. As Chelex is non-specific metal chelator, in the next experiments, we used TPEN (a well-recognized membrane permeable zinc-specific chelator) to verify the exclusive role of zinc in mediating MI-219-induced apoptotic effects. Histone DNA enzyme-linked immunosorbent assay results shown in Figure 3a confirm that addition of TPEN (1 μM) significantly blocks MI-219-mediated apoptosis and this could be restored by the addition of 16 μM $ZnCl_2$ to both HCT-116 and MCF-7 cells. We also performed Annexin V fluorescein isothiocyanate apoptosis analysis to verify that MI-219-mediated apoptosis could only be blocked by TPEN and not other metal chelators such as the calcium-specific Bapta-AM or copper chelator, bathocuproine (Figure 3b). Taken together, these results highlight a significant role of zinc in the activity of MI-219 on wt-p53 cells. We then tested the role of zinc on the reactivation of p53 pathway in both HCT-116 and MCF-7 cells.

Zinc depletion blocks MI-219-mediated p53 reactivation

It is well known that for its transcriptional activity, p53 requires a specific DNA-binding sequence that is coordinated by a complex helix domain motif stabilized by a zinc atom. It is therefore expected that, by modifying zinc levels, one would affect the DNA-binding capacities of p53, which would result in altering the reactivation of p53 induced by MI-219. We performed western blot analysis to determine the specific role of zinc on p53 reactivation. As can be seen from results in Figures 4a and b, in regular media, MI-219 treatment (0–10 μM for 24 h) in both HCT-116 and MCF-7 cells resulted in sequential upregulation of p53 along with downstream effecter molecules such as the cell cycle regulator, p21, and the pro-apoptotic Bax. However, using chelexed media, we found negligible to

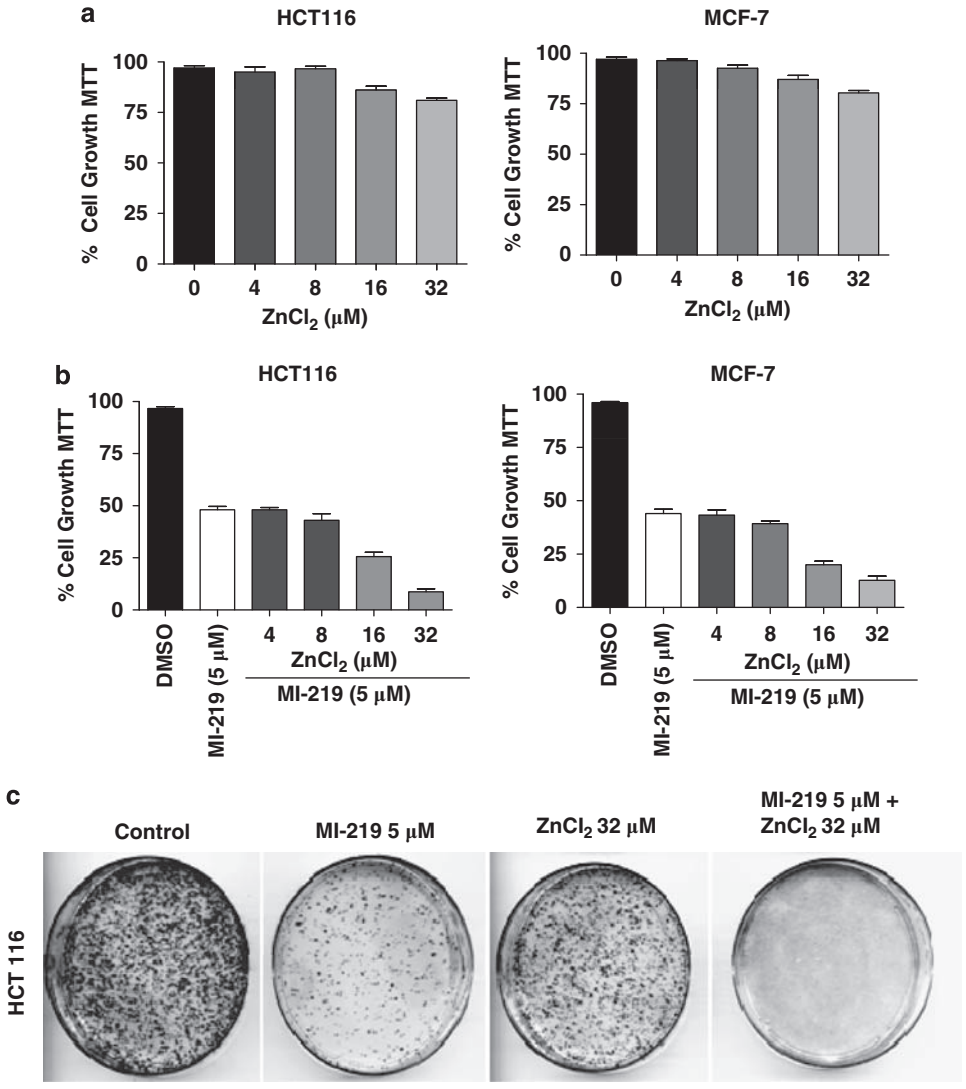

Figure 2 Zinc enhances MI-219-mediated inhibition of cell growth and colony formation. (**a**) HCT-116 and MCF-7 cells were exposed to increasing concentrations of $ZnCl_2$ (0–32 μM) for 72 h and cell growth inhibition was detected using MTT assay. (**b**) Cells were exposed to either MI-219 5 μM in combination with $ZnCl_2$ (0–32 μM) for 72 h and cell growth inhibition was detected by MTT assay. (**c**) Microphotographs of cell survival of HCT-116 cells at indicated treatments and evaluated by the clonogenic assay. Note significant reduction in the colony formation in the combination compared with cells treated with either MI-219 (5 μM) or $ZnCl_2$ (32 μM) alone. All experiments were performed in triplicate, data presented represents mean of three independent experiments.

modest expression of p53. Furthermore, the downstream effectors (p21 and Bax) were also not upregulated. We then used TPEN to evaluate the exclusive role of zinc on p53 reactivation on cell growth in normal media. In line with our cell growth inhibition and apoptosis studies using chelexed media, TPEN (1 μM) significantly blocked p53 pathway reactivation in both HCT-116 and MCF-7 cells, which could be partially restored by addition of 16 μM $ZnCl_2$ (Figure 4c). These results confirm our hypothesis that zinc is required for the proper reactivation of p53 by MI-219.

Zinc chelation blocks p53 nuclear localization

Nuclear localization of p53 is a necessary prerequisite for its proper function (O'Brate and Giannakakou, 2003). Therefore, we tested whether zinc chelation could alter the nuclear localization of p53. To achieve this, we used a fluorescent microscopy p53-Cell-Based Activation/Translocation assay kit coupled with a highly specific p53 primary monoclonal antibody and DyLight conjugated secondary antibody. As can be seen from results shown in Figure 5a, in both HCT-116 (upper panel) and MCF-7 (lower panel) cells, MI-219 (5 μM) could induce p53 nuclear localization in normal media but not in chelexed media. Chelation of zinc with TPEN could suppress p53 localization that again could be restored by addition of $ZnCl_2$ (16 μM). To further confirm that MI-219 activity is specifically directed towards wt-p53, we used small interfering RNA (siRNA) against p53 and as can be seen from results in Figure 5b, in both HCT-116 and MCF-7 cells, p53 knockdown shows neither p53 activation nor translocation induced by MI-219. To reaffirm siRNA silencing of

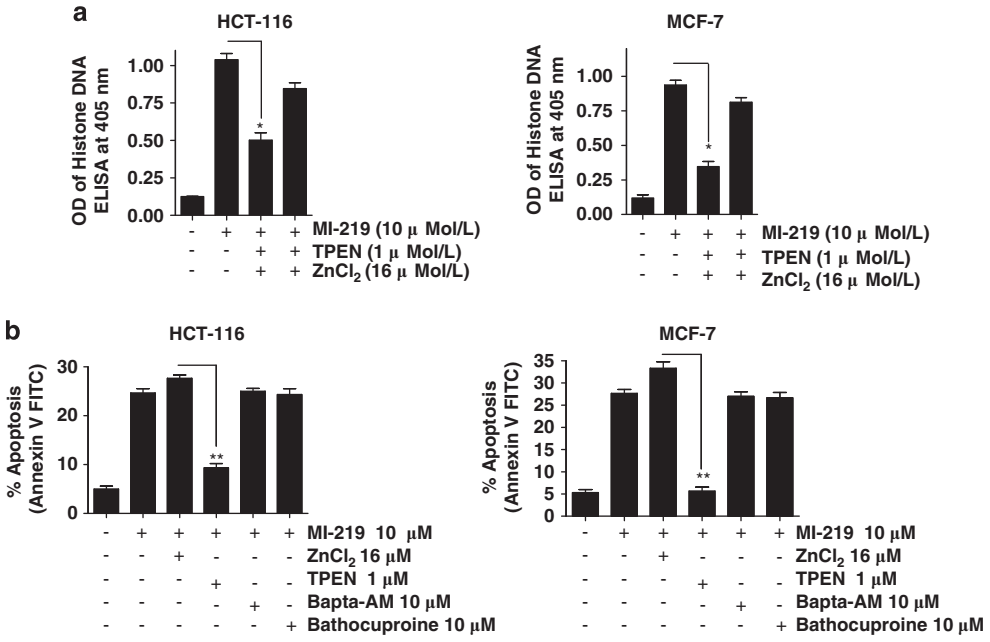

Figure 3 TPEN, a zinc-specific chelator, blocks apoptosis by MI-219. (**a**) Cells were treated with MI-219 at indicated concentration for 72 h. TPEN blocked MI-219-induced apoptosis (histone/DNA ELISA) which could be restored by addition of physiological levels (16 μM) of $ZnCl_2$. (**b**) Zinc augments MI-219-induced apoptosis (Annexin V fluorescein isothiocyanate assay) which can be abrogated by TPEN. Depletion of calcium using the calcium-specific chelator, Bapta-AM or copper, using a specific copper chelator, bathocuproine, had no effect on MI-219-induced apoptosis in either cell line. All experiments were performed in triplicate, data presented represents mean of three independent experiments. *Represents $P<0.05$ and **$P<0.01$.

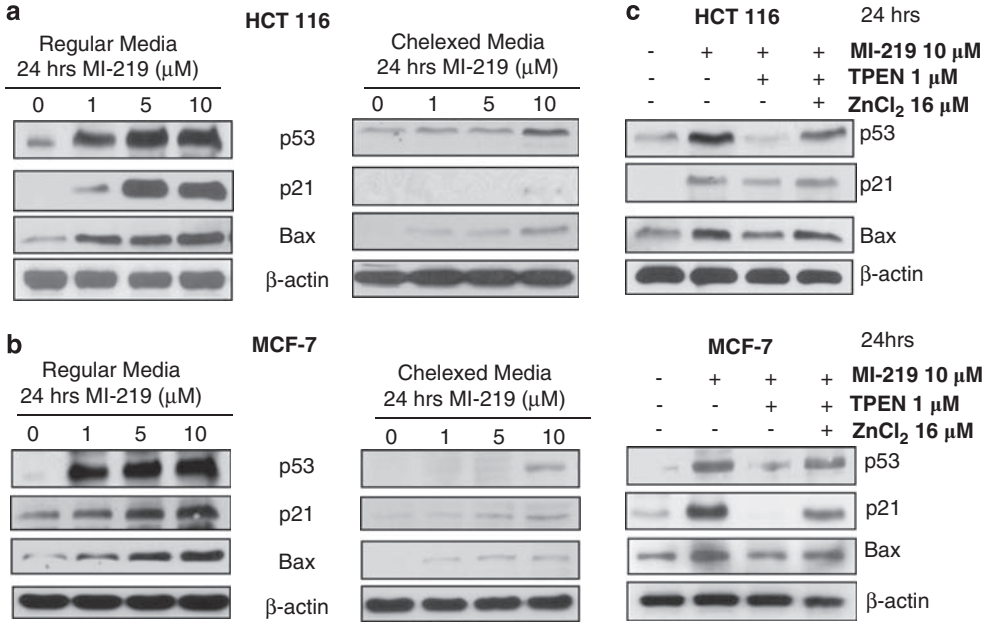

Figure 4 Zinc chelation blocks p53 reactivation by MI-219. HCT-116 and MCF-7 cells were grown in either regular or chelexed media and treated with increasing doses of MI-219 (0–10 μM for 24 h). Protein lysates were analyzed using western blot analysis for activation of the p53 pathway. (**a**, **b**) Cells grown in chelexed media exhibited insignificant amounts of p53 or its downstream p21 and Bax compared with cells grown in regular media. (**c**) MI-219 inhibition of MDM2 allowed for increased p53, and downstream Bax and p21 expression which could be abrogated by TPEN (a zinc-specific chelator) in normal (non-chelexed) media and then reversed by the addition of physiological levels of $ZnCl_2$. HCT-116 and MCF-7 cells were exposed to either dimethyl sulfoxide (DMSO); MI-219 (10 μM); MI-219 (10 μM) + TPEN (1 μM) or MI-219 (10 μM) + TPEN (1 μM) + $ZnCl_2$ (16 μM; 24 h) and western blot analysis was performed on lysates isolated from treated cells. Blots are representative of three independent experiments. β-Actin was used as loading control.

p53, we also performed western blot analysis to demonstrate negative expression of p53 in p53 siRNA-treated and not in control siRNA-treated cells (Figure 5c). These results for the first time, confirm that zinc is crucial for the proper reactivation of p53 as well as its nuclear localization induced by the MI-219.

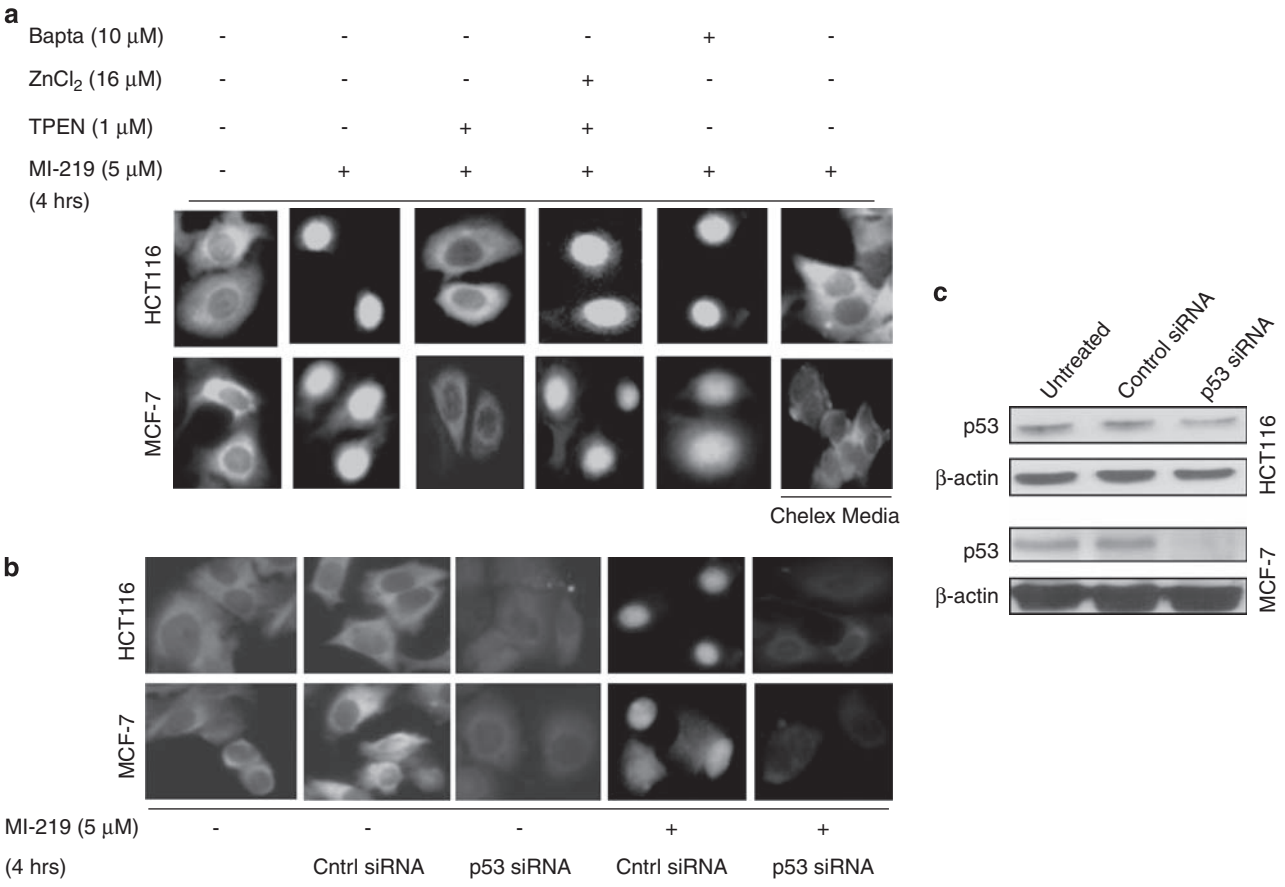

Figure 5 TPEN suppresses p53 nuclear localization. (**a**) MCF-7 and HCT-116 cells (1000) were grown in triplicate in 96-well plates and treated with either vehicle (DMSO); MI-219 (5 μM); MI-219 (5 μM) + TPEN (1 μM); MI-219 (5 μM) + TPEN (1 μM) + $ZnCl_2$ (16 μM) or MI-219 (5 μM) + Bapta-AM (10 μM) for 4 h. p53 activation/localization assay was performed using p53 localization kit according to manufacturer's protocol (Cayman Chemicals). Note: MI-219-induced p53 localization in the nucleus is blocked by TPEN. Zinc treatment restores p53 in the nucleus even in the presence of TPEN. Bapta-AM, a calcium chelator, had no effect on p53 nuclear localization. Cell grown in chelex media are also resistant to MI-219-mediated p53 nuclear localization. (**b**) p53 siRNA suppresses p53 nuclear localization/activation. HCT-116 and MCF-7 cells were grown in 96-well plates and treated with either control siRNA or p53 siRNA for 5 h in the absence or presence of MI-219 5 μM for 4 h and p53 localization was detected. SiRNA-treated MCF-7 and HCT-116 cells show reduced p53 in the nucleus even in the presence of MI-219. (**c**) Western blot analysis showing siRNA-mediated suppression of p53 in both HCT-116 and MCF-7 cells. A full colour version of this figure is available at the *Oncogene* journal online.

Zinc augments p53 transcription in HCT-116 and MCF-7 cells

We tested the p53 transcription activity using a sensitive non-radioactive method for detecting specific transcription factor DNA-binding activity in nuclear extracts. In this assay, a specific double-stranded DNA sequence containing the p53 response element is immobilized onto the wells of a 96-well plate. p53, present in a nuclear extract, binds specifically to the p53 response element and is detected by addition of a specific primary antibody directed against p53. A secondary antibody conjugated to horseradish peroxidase is added to provide a sensitive colorimetric readout at 450 nm. As can be seen from results in Figure 6a, MI-219 (10 μM) induces p53 transcription that is significantly augmented by addition of $ZnCl_2$ (16 μM). In line with our growth inhibition, apoptosis, western blot and p53 nuclear localization results, TPEN suppressed MI-219-mediated p53 transcription that could be restored by addition of zinc. Furthermore, the MI-219 inactive analog, MI-10, did not induce p53 transcriptional activity. These results are in further support of our hypothesis that zinc has an important role in the biology of p53 and its use in conjunction of such a targeted therapy.

Zinc suppresses p53-MDM2 regulatory feedback mechanism

MDM2 contains an auto-regulatory RING finger domain that coordinates zinc atoms. Therefore, we sought to assess the role of zinc addition and chelation on the activity of MDM2. As can be seen from results shown in Figure 6b, MI-219 alone at 10 μM induces MDM2 expression which was expected, as activated p53 positively regulates MDM2 (Levine, 1997). However, in the presence of zinc ($ZnCl_2$ 16 and 32 μM), the induction of MDM2 was suppressed but could be restored by addition of TPEN. These results provide evidence that zinc is required for proper positive regulation of MDM2 by p53. We further performed co-immunoprecipitation experiments to verify the effect of zinc chelation/addition on disruption of MDM2–p53 complex. As can be seen from the results shown in Figure 6c , MI-219 disrupts the

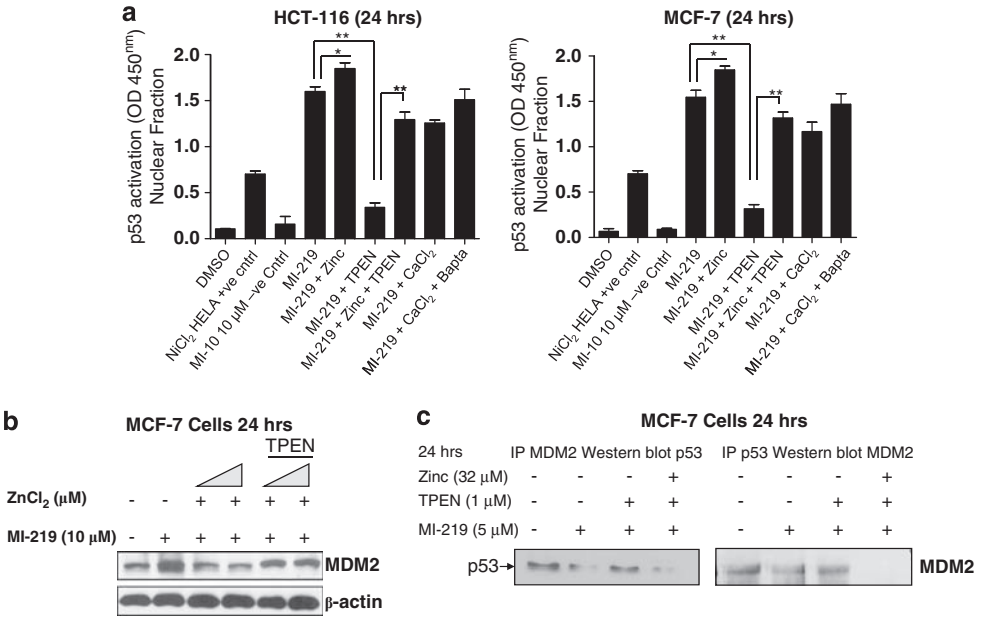

Figure 6 Zinc enhances MI-219-mediated p53 transcription that is blocked by TPEN. (**a**, **b**) Semi-confluent HCT-116 and MCF-7 cells grown in triplicate were exposed to either vehicle (DMSO); MI-219 (10 μM); MI-219 (10 μM) + $ZnCl_2$ (16 μM); MI-219 (10 μM) + $ZnCl_2$ (16 μM) + TPEN (1 μM); MI-219 (10 μM) + $CaCl_2$ (15 μM) or MI-219 (10 μM) + $CaCl_2$ (15 μM) + Bapta-AM (10 μM) for 24 h in six-well plates. Nuclear lysates were isolated and p53 transcription assay was performed in 96-well plates according to manufacturer's protocol (Cayman Chemicals). $ZnCl_2$ (16 μM) enhances MI-219 (10 μM)-mediated p53 transcription that is blocked by TPEN. Calcium does not induce p53 transcription and the calcium chelator, Bapta-AM, has no affect. $NiCl_2$-treated HeLa cell lysates were used as positive control (provided in kit) whereas MI-10, the inactive analog of MI-219, was used as negative control. *Represents $P<0.05$ and **$P<0.01$. (**c**) $ZnCl_2$ suppresses MDM2 protein expression and enhances MI-219-induced MDM2-p53 disruption. MCF-7 cells were exposed to either vehicle (DMSO); MI-219 (10 μM); MI-219 (10 μM) + $ZnCl_2$ (16 μM); MI-219 (10 μM) + $ZnCl_2$ (32 μM); MI-219 (10 μM) + $ZnCl_2$ (16 and 32 μM) in the presence of TPEN (1 μM) for 24 h, and protein lysates were subjected to western blot analysis followed by probing with MDM2 antibody. $ZnCl_2$ (16 and 32 μM) suppressed MDM2 expression (**d**) Co-immunoprecipitation studies of MCF-7 cells demonstrate reduced disruption of MDM2–p53 interaction in the presence of TPEN. Again $ZnCl_2$ abrogates the effects of TPEN. Blots are representative of three independent experiments.

MDM2–p53 interaction (with less protein precipitated in the treated samples) and this could be reversed by the addition of TPEN. The addition of supplemental $ZnCl_2$ (32 μM) could negate the effect of zinc chelator TPEN, thereby restoring the MI-219-induced disassociation between MDM2 and p53.

Discussion

This clinically translatable study, for the first time, provides insight into the mechanisms by which zinc affects the activity of the MI-219 in mediating proper reactivation of p53, leading to apoptosis in wt-p53 cancer cells. MI-219 is entering phase I clinical trials. Before MIs make their way for treatment of cancer patients, we believe that our findings will positively impact the design of MI therapy.

According to the International Zinc Nutrition Consultative Group, 20% of the world's population lack sufficient zinc in their diet, while one-third live in countries considered at high risk of zinc deficiency (Brown *et al*., 2004). Zinc is required by at least 200 different proteins and transcription factors (Christianson, 1991). Zinc binding by p53 was confirmed when the partial crystal structure of the protein was published (Collins *et al*., 1997). The zinc atom has an essential structural role in stabilizing the architecture of the DBD of p53 and it has clearly been shown that zinc is an important cofactor for p53 DNA-binding activity *in vitro*. Most of this evidence relies on the fact that metal chelators can remove zinc from p53, turning the protein to a 'mutant-like' form with loss of such sequence-specific DNA-binding activity (Meplan *et al*., 1999). Likewise, the p53 regulator MDM2 carries a RING domain that coordinates two zinc atoms (Wawrzynow *et al*., 2009). The major functions of this RING domain are to mediate nuclear export of p53 for proteasomal degradation and also induce MDM2 auto-ubiquitination. Therefore, we believe that zinc acts as a double-edged sword, on one hand promoting p53 reactivation and on the other causing MDM2 degradation. Most of intracellular zinc is immobilized in proteins, however, cells also contain a pool of labile zinc, which is in dynamic equilibrium with the extracellular medium (Zalewski *et al*., 1993, 1994). This labile zinc can undergo fluctuations (three–fivefold) depending on the availability of zinc. Such changes in zinc levels (1–10 μM range) might have regulatory effects on specific, intracellular metalloproteins including all important p53.

Strategies that utilize MDM2 inhibition to reactivate p53 pathway may not be fully successful in zinc-deficient environments/patients. Based on this assumption, we investigated in wt-p53 cancer cells (HCT-116 and

MCF-7) and demonstrated that zinc is necessary for the activity of MI in mediating enhanced reactivation of p53 leading to apoptosis. Our findings confirmed that in the absence of zinc, the efficacy of MI-219 is diminished. Following that, we verified the effect of zinc chelation on MI-219-induced p53 reactivation. As expected, p53 pathway including downstream effector p21 and Bax, reactivation was markedly suppressed by zinc chelation and most significantly, this activity was restored by the addition of zinc. These results provide irrevocable evidence for the crucial requirement of zinc on the activity of MI-219 in restoring superior p53 functioning.

Cytoplasmic p53 is rapidly degraded by MDM2-mediated ubiquitination; a reason for inhibiting MDM2 activity using MI-219. However, under stress, p53 shuttles to nucleus where it transactivates numerous genes regulating cell cycle, apoptosis and senescence (Chen *et al*., 2006). It is logical to hypothesize that by blocking MDM2-p53 binding, p53 would be allowed to localize into the nucleus and initiate transcription of target genes. To demonstrate that this is a zinc-dependent phenomenon, we found that in the presence of zinc chelator TPEN, p53 nuclear localization was significantly diminished and that the addition of zinc could restore this function (Figure 5). Using siRNA against p53 to silence the p53 expression reaffirmed the nuclear localization data observed above, which further re-instates that zinc is a crucial component of the p53 localization signaling pathways. On a cautionary note, we believe that it is too early to fully explain how zinc chelation blocks p53 nuclear localization. One possibility is that zinc deficiency may induce p53 conformational change that would hinder proper alignment of p53 to the nuclear localization signal sequence and thus block its nuclear translocation. Another explanation for the observed effects comes from an earlier study done by Giannakakou *et al*., (2002), where it was shown that in low-zinc environment, the microtubule assembly required to position p53 on nuclear localization signal sequence is disturbed and results in reduced nuclear localization. However, it is understood that more work is needed in this direction before concluding to the exact mechanism behind such reduced p53 nuclear localization. Pavletich *et al*., (1993) have reported that for proper transcription, p53 requires specific DNA-binding sequence that is regulated by zinc atoms. In this study, we also found that by lowering zinc levels, p53 transcription activity was suppressed (Figure 6). Fortified with the above strong evidence, we correctly hypothesized that zinc was required for the efficacy of MI-219 on the reactivation of p53.

It is well recognized that MDM2 expression is under a positive feedback loop of p53. However, overexpression of MDM2 as may occur in cancer cells inhibits the p53 reactivation. This would make the cancerous/tumor cells to multiply continuously or become resistant to chemotherapy. To this end, we explored whether zinc could affect the MI-219-induced inhibition of MDM2 and cause p53 release and thus, p53 reactivation. Our results show that the addition of zinc can significantly enhance MI-219-induced suppression of MDM2–p53 interaction and that this suppression could be reversed by a specific zinc chelator, TPEN, which provides clear proof for the role of zinc in this process. We believe that zinc chelation may change the p53-binding pocket in MDM2. It is also possible that zinc binding to the sites on MDM2 may alter its conformation and thus its p53-binding affinity (summarized in Figure 7). Such binding studies using X-ray crystallography are currently under study in our laboratory. In conclusion, therapies which target p53 reactivation using MI approach may still not be fully successful in zinc-deficient environment that is commonly found in a sizable patient population (Prasad *et al*., 1993; Prasad, 2001, 2004). Therefore, a logical step forward is to use supplemental zinc to enhance the efficacy of MI-219 for treatment of cancers.

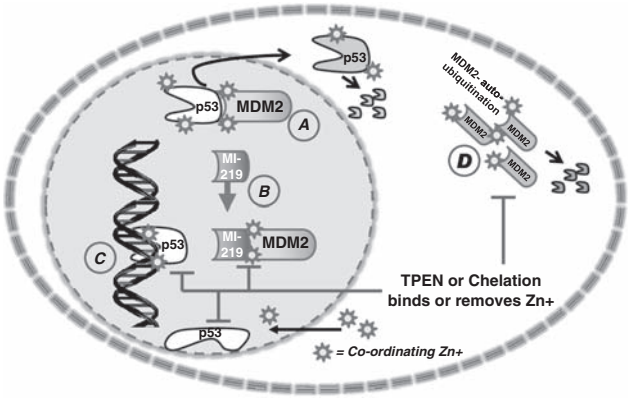

Figure 7 Zinc has a crucial role in the biology of p53 (a mechanistic summary) (A). MDM2 binds to p53 and targets p53 for nuclear export and proteasomal degradation (B). MI-219's ability to block this interaction is enhanced by the presence of zinc, releasing p53 to bind to DNA (C). p53 sequence-specific DNA binding requires crucial zinc atoms. If present, p53-initiated transcription blocks p53-MDM2 feedback loop and promotes MDM2 auto-ubiquitination. Chelation or TPEN, a membrane permeable metal chelator, abrogates zinc-mediated effects by removing zinc from p53 and causing a conformational change to prevent DNA binding. Changes in binding affinity induced by zinc (or its removal) may also apply to MI-219–MDM2 and MDM2–p53 interaction as well (D). TPEN also blocks zinc-mediated MDM2 auto-ubiquitination. A full colour version of this figure is available at the *Oncogene* journal online.

Materials and methods

Cell culture, experimental reagents and chemicals

HCT-116 (wt-p53) was obtained from Dr Bert Vogelstein's lab. MCF-7 (wt-p53) breast cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines have been tested and authenticated in our core facility, Applied Genomics Technology Center at Wayne State University, as late as 13 March 2009. The method used for testing was short tandem repeat profiling using the PowerPlex 16 System from Promega (Madison, WI, USA). Primary antibodies for p53, Bax, MDM2 and p21 were purchased from Cell Signaling (Beverly, MA, USA). All secondary antibodies were obtained from Sigma (St Louis, MO, USA). MI-219 was synthesized by using our previously published methods (Ding *et al*., 2005, 2006). Chelex 100 resin, the zinc chelator, *N*,*N*,*N*′*N*′-tetrakis(−)[2-pyridylmethyl]-ethy-

lenediamine (TPEN), the calcium chelator, BAPTA-AM, the copper chelator, bathocuprione disulfonate, and $ZnCl_2$ were purchased from Sigma.

Metal-deficient media
Chelation of media and other solution with chelex 100 resin (Sigma, St Loius, MO, USA) is a non-specific method of removing unbound or excess trace and, to some extent, non-trace metals. The advantage of its use is that the resin bound to the metals can be physically removed from the media. Metal ions were removed from the culture media, Dulbecco's modied Eagle's medium or McCoy 5A, by incubating regular media (Dulbecco's modified Eagle's medium supplemented with fetal bovine serum penicillin and streptomycin, and 10% glutamine, or McCoys 5A media supplemented with fetal bovine serum penicillin and streptomycin, 10% glutamine and 10% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with chelex 100 resin (1 g per 50 ml media) for 1 h at 37 °C. After incubation, the media was vacuum filtered to remove the metal-bound resin. The zinc and copper content was determined using atomic absorption spectroscopy using our previously established methods (Beck *et al*., 2004) and other electrolytes plus iron determined by the clinical laboratories at the Detroit medical center. Chelation of particular metal ions was accomplished by adding metal-specific chelators to normal media and includes TPEN for zinc, BAPTA-AM for calcium, bathocuprione disulfonate for copper and desferroxamine for iron.

Cell growth inhibition studies by MTT and clonogenic assay
HCT-116 and MCF-7 cells (3×10^3) were seeded in a 96-well culture plate either in regular or chelexed media, treated with MI-219 (0 to 10 μM) for 72 h and MTT assay was performed as described earlier (Azmi *et al*., 2008). The results were plotted as means ± s.d. of three separate experiments using six determinations per experiment for each experimental condition. Clonogenic assay for cell survival on HCT-116 cells was performed according to previously described methods (Azmi *et al*., 2010).

Quantification of apoptosis by annexin V fluorescein isothiocyanate flow cytometry and enzyme-linked immunosorbent assay
Apoptosis in HCT-116 and MCF-7 cells was determined using Annexin V fluorescein isothiocyanate apoptosis kit (Biovision Research Products, Mountain View, CA, USA) and enzyme-linked immunosorbent assay detection kit (Roche, Palo Alto, CA, USA) according to manufacturer's protocol.

siRNA and transfections
To study the effect of MI-219 on reactivating p53 in the presence of silenced wt-p53, we utilized siRNA techniques in both HCT-116 and MCF-7 cells. The p53 siRNA and control siRNA were obtained from Cell Signaling. Cells were transfected with respective siRNAs for 5 h using LipofectAMINE 2000 according to the manufacturer's protocol (Cell Signaling).

Western blot analysis
HCT-116 or MCF-7 were exposed to different treatments for 24 h followed by extraction of protein for western blot analysis. Procedure for cells lyses, protein concentration determination and SDS–polyacrylamide gel electrophoresis analysis has been described in our previous publication (Mohammad *et al*., 2009).

P53 activation and translocation
Activation and translocation of p53 post MI-219, chelation or siRNA treatments was detected using 'Cayman's p53 Cell-Based Activation/Translocation Assay Kit' (Ann Arbor, MI, USA) according to the manufacturer's protocol.

p53 transcript DNA-binding assay
Specific transcription factor DNA binding in nuclear extract post-treatments was detected using the sensitive non-radioactive 'Cayman's p53 Transcription Factor Assay kit'. The procedure for nuclear extract preparation and transcription activity was done according to the manufacturer's protocol.

Conflict of interest

Dr Ramzi M Mohammad, Dr Fazlul H Sarkar and Dr Shaomeng Wang are funded from the NIH. Dr Shaomeng Wang and Dr Dajun Yang own equity in Ascenta Therapeutics and are consultants for the company and receive compensation for their services. The rest of the authors in this paper have no potential conflict of interest.

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