Efficacy of MDM2 Inhibitor MI-219 Against Lung Cancer Cells Alone or in Combination with MDM2 Knockdown, a XIAP Inhibitor or Etoposide

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Abstract. *The p53 tumor suppressor is negatively regulated by murine double minute 2 (MDM2), which binds to p53 and promotes p53 ubiquitination and degradation. MDM2 inhibitor-219 (MI-219), a small molecule MDM2 inhibitor, was recently reported to disrupt p53-MDM2 binding, leading to p53 activation and suppression of tumor cell growth both in vitro and in vivo. This study tested the efficacy of MI-219 against a panel of lung cancer cell lines alone or in combination with MDM2 knockdown, an X-linked inhibitor of apoptosis protein (XIAP) inhibitor, or a chemotherapeutic drug, etoposide. When acting alone, MI-219 selectively inhibited growth of wild-type (wt) p53-containing lung cancer cells by induction of G_1 or G_2 arrest in a p53-dependent manner, but had a minor effect on wt p53-bearing immortalized cells. MDM2 knockdown had a minimal effect on MI-219 induced growth suppression. Although MI-219 increased XIAP expression, blockage of XIAP via SM-164, a Smac mimetic compound, did not selectively enhance MI-219 cytotoxicity. Significantly, MI-219 sensitized lung cancer cells to etoposide-induced cell killing. This study revealed that, when acting alone, MI-219 selectively inhibits the growth of lung cancer cells harboring a wt p53. In combination, MI-219-induced cytotoxicity was not affected by MDM2 knockdown nor by a XIAP inhibitor, but MI-219 sensitized cancer cells to etoposide, suggesting MI-219 could serve as a chemosensitizing agent.*

p53 tumor suppressor is induced and activated by a variety of stresses, particularly DNA-damaging agents (1). Upon activation, p53 prevents tumor formation *via* inducing growth arrest to repair the damage or apoptosis to eliminate irreparable cells (2-4). These biological functions of p53 are achieved through transcriptional activation or repression of p53 downstream targets and/or through direct binding of p53 to cellular proteins that regulate cell proliferation and death (5). p53 is inactivated in human cancer by two major means: point mutation which occurs in 50% of human carcinomas (6) and murine double minute 2 (MDM2) binding and degradation, which eliminates wild-type (wt) p53 (7). *MDM2* or its human homolog, *HDM2* is an oncogene with a major function as a p53 inhibitor. MDM2 inactivates p53 mainly through two mechanisms: binding to p53 transactivation domain to block its transcription activity and ubiquitinating p53 to promote its degradation (8, 9). Thus, disruption of MDM2-p53 binding to reactivate p53 is an attractive pharmacological approach against human cancer harboring a wt *p53* (10).

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In the past few years, several classes of small molecule inhibitors that disrupt p53-MDM2 binding, such as Nutlin-3 and MI-43/63/219/319 series, have been discovered (11-14). While these compounds induce a significant increase in p53 levels, they may be associated with some side-effects due to MDM2 accumulation in response to p53 activation. Accumulated MDM2 was not able to promote p53 degradation in the presence of these inhibitors, which disrupt MDM2-p53 binding, but may act on other cellular proteins such as RB, E2F and ribosomal proteins, just to name few, to execute its p53-independent functions (15). This may explain why this class of drugs, when used as a single agent, often induces p53 to cause only growth arrest, not apoptosis (except with use of a high toxic dose) in a number of human cancer cell lines, including lung cancer lines (16-18). Thus, combination of this type of MDM2/p53-disrupting drug with siRNA-mediated *MDM2* knockdown may eliminate any undesirable effects derived from MDM2 accumulation. In addition, MDM2 has been recently shown to increase X-linked inhibitor of apoptosis protein (XIAP) levels *via* enhancing IRES-dependent *XIAP* translation (19). It is well established that: (a) XIAP is a cellular survival protein that inhibits apoptosis *via* binding to and inhibiting both active caspase-9 and caspase-3 (20), and (b) disruption of XIAP-caspase binding by Smac or Smac mimetic compound activates caspases, leading to apoptotic cell killing (21). Thus, rational combination of an MDM2 inhibitor (*e.g.* MI-219) (16) with a Smac-mimetic compound (*e.g.* SM-164) (21) would, in theory, synergistically kill lung cancer cells *via* apoptosis by activation of both p53 and caspases.

It has been recently shown that MI-43 selectively kills lung cancer cells harboring wt *p53* (17). In the present study, a more potent analog, MI-219 (16), was used to further investigate the efficacy of MI-219 against lung cancer cells when acting alone or in combination with MDM2 knock-down (to abrogate MDM2 p53-independent functions), with SM-164 (to block XIAP), or with etoposide (for chemosensitization).

Materials and Methods

Compound. MI-219, MI-43 and their inactive analog MI-61 were synthesized as described previously (22, 23). SM-164 and its inactive analogue, SM-173 were synthesized as described previously (21, 24). Etopside was obtained from Sigma (St. Louis, MO, USA). The drugs were dissolved in DMSO before use.

Cell culture. Four lung cancer cell lines, A549, H1299, H460 and SKLU-1 were obtained from ATCC (Manassas, VA, USA). All of them were cultured in DMEM supplemented 10% FBS. Human immortalized lung bronchial epithelial NL20 cells were cultured in Ham's F12 medium supplemented with 1.5 g/l sodium bicarbonate, 2.7 g/l glucose, 2.0 mM L-glutamine, 0.1 mM nonessential amino acids, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 1 μg/ml transferrin, 500 ng/ml hydrocortisone and 4% FBS. Human lung fibroblast MRC5 cells were a gift from Dr. Rehemtulla and grown in RPMI-1640 containing 10% FBS.

Western blotting analysis. The cells were allowed to grow in their respective media until they reached 70% confluency. Cells were then treated with MI-219, SM-164, etoposide, alone or in combination and subjected to Western blotting using antibodies against p53, MDM2 and PUMA (EMD Chemical, Gibbstown, NJ, USA), p21 (BD Pharmingen, San Jose, CA, USA), MDMX (Bethyl, Montgomery, TX, USA), NOXA (Oncogene Science, Cambridge, MA, USA), pRB (Santa Cruz, Santa Cruz, CA., USA). β-Actin (Sigma, St. Louis, MO, USA) was used as a loading control.

ATPlite 5-day growth assay and IC_{50} determination. The lung cancer cells were seeded in 96 well white plates in the center 60 wells at 3,000 cells per well in triplicate, except for DMSO controls in 6 wells in two separate locations. Cells were treated the next day with MI-219 at doses ranging from 0.1 to 30 μM for 5 days. The viability of the cells was then measured using a one-step ATPlite kit (Perkin Elmer, Waltham, MA, USA) as described previously (25). The results were calculated by setting the value of DMSO control cells as 100% and then plotted in Prism 4.0 (Graphpad, La Jolla, CA, USA) to generate IC_{50} curves.

siRNA silencing. A lentivirus-based siRNA construct was used to silence *p53*, as described previously (17). For *MDM2* silencing, ON-TARGETplus SMARTpool oliognucleotides (Dharmacon, Lafayette, CO, USA; L-003279-00) was used (26), along with a scrambled control siRNA (27). Cells were transfected with siRNA using Lipofectamine 2000 and split 48 hours later. One portion was used for cell growth assay and the other portion for Western blotting.

Fluorescence-activated cell sorting (FACS) analysis. Cells were treated with MI-219 alone or in combination with *MDM2* knockdown, SM-164, or etoposide for 24 or 48 hours. Both detached and attached cells were harvested and subjected to FACS analysis (25).

Real-time cell growth assay. Real-time monitoring of live cell proliferation was performed using a RT-CES system (Real-Time Cell Electronic Sensing, ACEA Biosciences, San Diego, CA, USA). The system allows for label-free, dynamic, and quantitative detection of live cells (28). Cells were seeded in triplicate in 96-well ACEA E-plates (ACEA) and cell growth was continuously monitored on a RT-CES system every 15 minutes for a period up to 4 days. The dynamic cell proliferation and cell growth inhibition were presented by cell index, which is an arbitrary unit, reflecting the cell-sensor impedance from each well. The cell index is proportionally related to the cell numbers, cell size and the adhesion ability of cells (28).

SA-β-Gal staining. The expression of SA-β-Gal in cells was determined by SA-β-Gal staining as described previously (29).

Caspase-3 activation assay. The activity of caspase-3 was analyzed using a fluorogenic caspase-3 assay with Ac-DEVD-AFC as a substrate (Biomol, Plymouth Meeting, PA, USA) as described previously (25). The results were expressed as fold change compared to control.

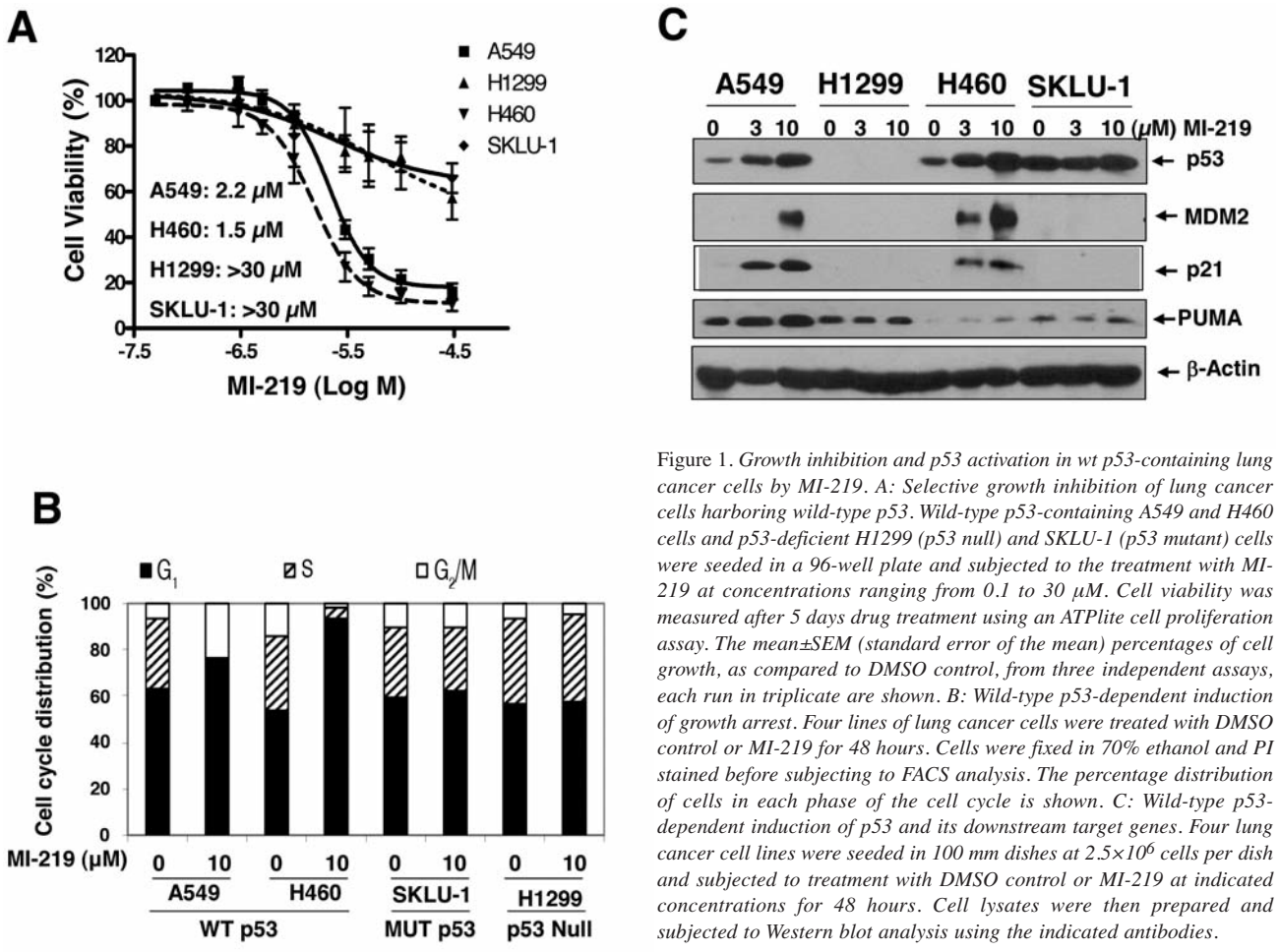

Figure 1. *Growth inhibition and p53 activation in wt p53-containing lung cancer cells by MI-219. A: Selective growth inhibition of lung cancer cells harboring wild-type p53. Wild-type p53-containing A549 and H460 cells and p53-deficient H1299 (p53 null) and SKLU-1 (p53 mutant) cells were seeded in a 96-well plate and subjected to the treatment with MI-219 at concentrations ranging from 0.1 to 30 μM. Cell viability was measured after 5 days drug treatment using an ATPlite cell proliferation assay. The mean±SEM (standard error of the mean) percentages of cell growth, as compared to DMSO control, from three independent assays, each run in triplicate are shown. B: Wild-type p53-dependent induction of growth arrest. Four lines of lung cancer cells were treated with DMSO control or MI-219 for 48 hours. Cells were fixed in 70% ethanol and PI stained before subjecting to FACS analysis. The percentage distribution of cells in each phase of the cell cycle is shown. C: Wild-type p53-dependent induction of p53 and its downstream target genes. Four lung cancer cell lines were seeded in 100 mm dishes at 2.5×10^6 cells per dish and subjected to treatment with DMSO control or MI-219 at indicated concentrations for 48 hours. Cell lysates were then prepared and subjected to Western blot analysis using the indicated antibodies.*

Results

MI-219 selectively inhibited the growth of lung cancer cells harboring wt p53 by induction of the G_1 or G_2 arrest, following p53 activation. Recently, it was reported that the small molecule MDM2 inhibitor, MI-219, disrupted MDM2-p53 binding and suppressed the growth of a number of human cancer cell lines both in *in vitro* cultured cells and *in vivo* xenograft tumors (16). However, the effect of MI-219 against lung cancer cells has not been characterized yet. The present study determined the efficacy of MI-219 against two pairs of human lung cancer lines which differ in their p53 status: adenocarcinoma lines A549 (p53 wt), and SKLU-1 (p53 mut) and non-small cell lung carcinoma (NSCLC) lines H460 (p53 wt) and H1299 (p53 null) (30). A 5-day ATPlite luminescence assay was used to measure cell proliferation or viability. As shown in Figure 1A, MI-219 induced dose-dependent growth suppression in wt p53-containing A549 and H460 cells, with an IC_{50} value of 2.2 μM and 1.5 μM, respectively. MI-219 was much less potent against p53 null H1299 cells and p53 mutant SKLU-1 cells with IC_{50} values at least one magnitude higher (>30 μM). The study further determined this wt p53-dependent selective killing by MI-61, an MI-219 structural analog inactive in disrupting MDM2-p53 binding (16) against A549, H460 and H1299 cells and found MI-61 was much less potent, with an IC_{50} value of 30 μM approximately, regardless of p53 status (data not shown). Thus, MI-219 selectively inhibited the growth of lung cancer cells harboring wt p53 in a manner dependent on its MDM2 inhibitory activity.

The nature of MI-219-induced growth suppression was determined by FACS analysis. As shown in Figure 1B, in A549 cells, MI-219 treatment of 48 hours at 10 μM caused a moderate G_1 arrest (62% to 76%), but a significant G_2 arrest (from 6.5% to 23.4%). In H460 cells, MI-219 mainly induced G_1 arrest (from 53.3% to 93.4%). No obvious induction of apoptosis at this drug concentration and treatment time period was observed in A549 and H460 cells (data not shown). In contrast, the drug had no effect on cell cycle progression of SKLU-1 and H1299 cells. Thus, MI-219 induced growth

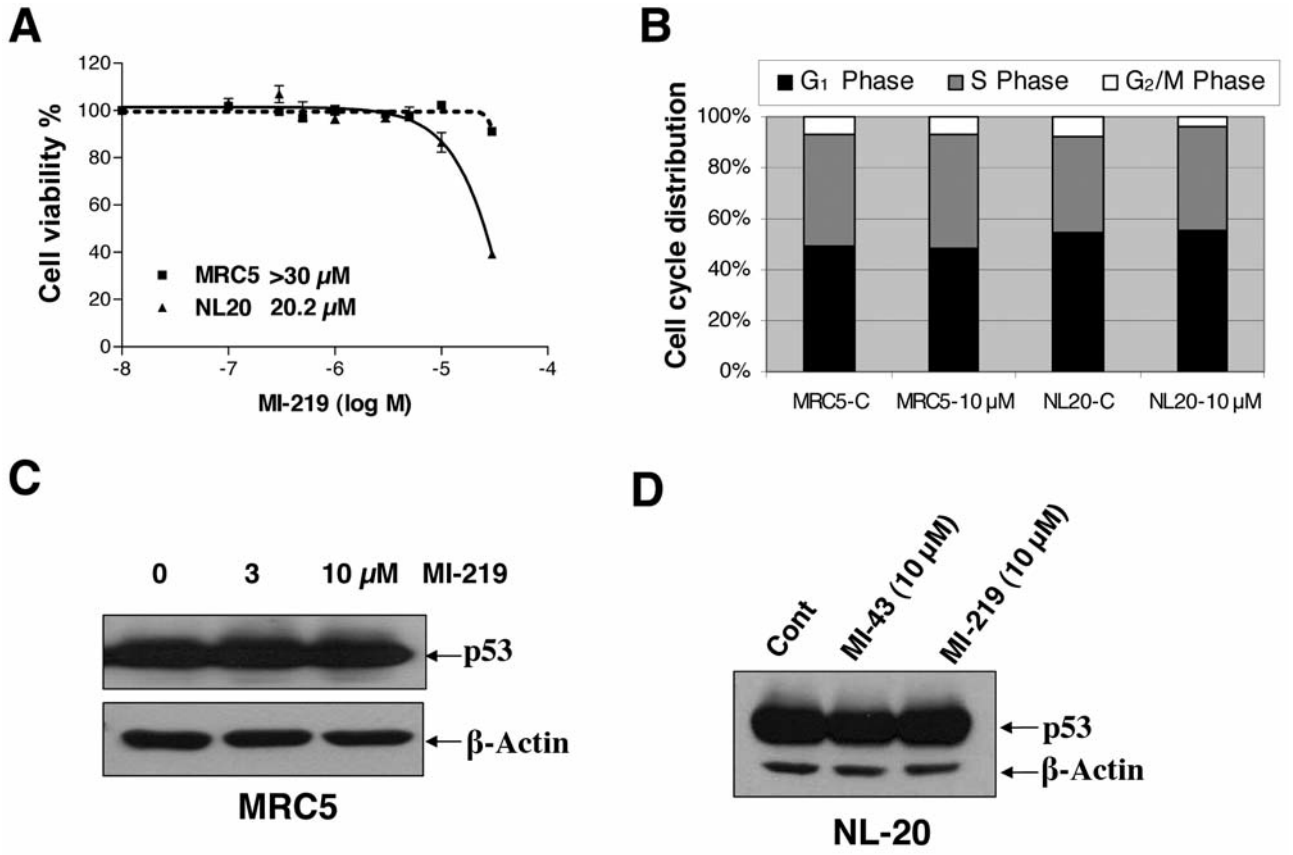

Figure 2. *Immortalized lung cells are resistant to MI-219. A: IC_{50} determination. Immortalized lung NL20 bronchial epithelial cells and MRC5 fibroblast cells were seeded in a 96-well plate and subjected to IC_{50} determination. The mean±SEM (standard error of the mean) percentages of cell growth, as compared to DMSO control, from three independent assays, each run in triplicate are shown. B: MI-219 failed to induce growth arrest in immortalized lung cells. NL20 and MRC5 cells were treated with MI-219 (10 μM) for 48 hours, followed by FACS analysis. The percentage distribution of cells in each phase of the cell cycle is shown. C, D: MI-219 and MI-43 failed to induce p53. MRC5 and NL20 cells were treated with MI-219 (C, D) or MI-43 (D) for 48 hours, followed by Western blot analysis using antibodies against p53 or β-actin for loading control.*

suppression is mainly caused by growth arrest at the G_1 and G_2 phases of the cell cycle in wt p53 containing lung cancer cells. Finally, it was confirmed that MI-219 indeed activated p53. MI-219 caused a dose-dependent induction of p53 in A549 and H460 cells, but not in H1299 and SKLU-1 cells. Activated p53 was transcriptionally active, as demonstrated by a dose-dependent induction of p53 target genes, including *MDM2*, *p21*, and *PUMA* (Figure 1C). Thus, by disrupting MDM2-p53 binding, MI-219 activates p53 to induce growth arrest at the G_1 or G_2 phase of cell cycle, leading to growth suppression.

MI-219 has minor cytotoxic effect on immoralized lung fibroblast and bronchial epithelial cells. Next it was determined whether wt p53-expressing immortalized cells would be more resistant to MI-219. MRC5 human fetal lung fibroblasts and NL20 immortalized bronchial cells were examined. As shown in Figure 2A, the drug at concentrations up to 30 μM had no effect on MRC5 cell growth. NL20 cells were also very resistant to MI-219, with an IC_{50} value of 20.2 μM, which is one magnitude higher than that for wt p53-expressing lung cancer cells. The FACS analysis confirmed that MI-219 treatment at 10 μM for 48 hours had no effect on cell cycle progression in MRC5 and NL20 cells (Figure 2B). To determine whether MI-219 would activate p53 as seen in wt p53-expressing lung cancer cells, Western blot analysis was performed. Surprisingly, in both MRC5 cells and NL20 cells, the basal levels of p53 were very high, and could not be further induced by the drug (Figure 2C and 2D). In addition, neither basal nor induced levels of MDM2, p21 and PUMA were detectable in either lines (data not shown). Thus, unlike what was observed in wt p53-expressing lung cancer cells, MI-219 was unable to activate p53 in immortalized lung fibroblasts and bronchial epithelial cells, resulting in their resistance to MI-219.

MI-219-induced growth arrest of lung cancer cells is p53 dependent. To determine whether the drug sensitivity of

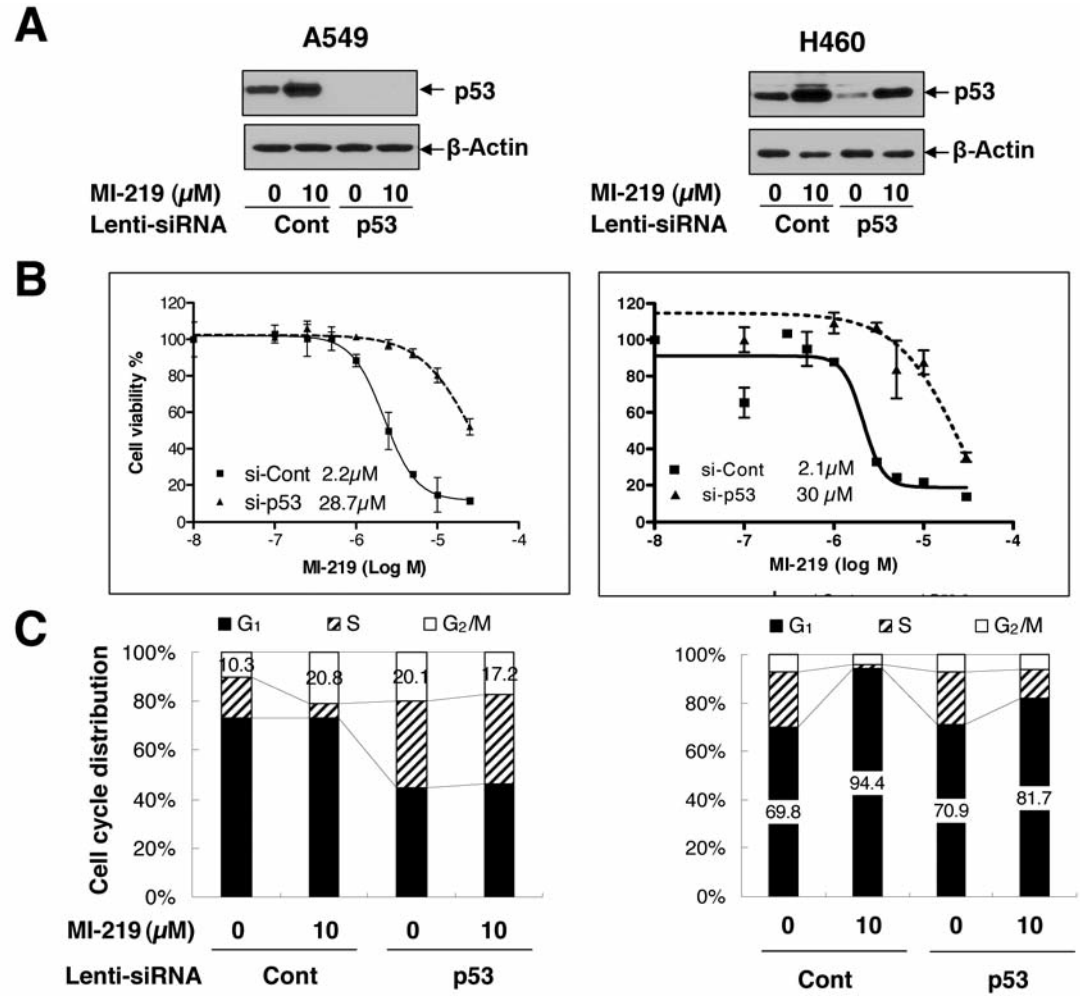

Figure 3. *p53-dependent growth inhibition by MI-219. A: siRNA silencing of endogeneous wild-type p53. A549 and H460 cells were infected with lenti-si-Cont or lenti-si-p53. Forty-eight hours later, cells were left untreated or treated with MI-219 for 24 hours. Cell lysates were prepared and subjected to Western blot analysis for p53 and β-actin as a loading control. B: p53-dependent growth inhibition. A549 and H460 cells were infected with lenti-si-Cont or lenti-si-p53 for 72 hours. Cells were then seeded in 96-well plates for IC_{50} determination as described above. The mean±SEM (standard error of the mean) percentages of cell growth, as compared to DMSO control, from three independent assays, each run in triplicate are shown. C: p53-dependent growth arrest at G_1 or G_2. H460 or A549 cells were infected with lenti-si-Cont or lenti-si-p53 for 72 hours, followed by drug treatment (10 μM) for 48 hours and subsequent FACS analysis. The percentage distribution of cells in each phase of the cell cycle is shown.*

A549 and H460 cells is strictly p53 dependent, the endogenous p53 was silenced *via* a lenti-virus based siRNA construct (Figure 3A) with a complete silencing in A549 cells and partial silencing in H460 cells. Upon *p53* silencing, the drug sensitivity was decreased up to 10-fold, with an IC_{50} of 30 μM, a level similarly seen in *p53* null H1299 and p53 mutant SKLU-1 cells (Figure 3B). FACS analysis also showed that upon *p53* silencing, MI-219-induced growth arrest seen in control silenced cells was completely abolished in A549 and partially abolished in H460 cell, correlating with the degree of p53 silencing (Figure 3C). Drug-induced p53 activation and induction of p53 target genes was also largely abrogated (data not shown).

Combination of MI-219 with MDM2 knockdown induces senescence-like phenotype without enhanced growth suppression. As shown in Figure 1C, MI-219 treatment induced a robust accumulation of MDM2 in both A549 and H460 cells. Since MDM2 interacted with many cellular proteins, such as RB, independently of p53 (15), it was hypothesized that simultaneous *MDM2* knockdown and MI-219 treatment may alter cellular response to the drug. Knockdown of *MDM2* was attempted in both A549 and H460 cells. While it was possible to silence endogenous *MDM2* in untreated A549 cells completely, only a partial knockdown was achieved upon drug treatment (Figure 4B). H460 cells were refractory to oligonucleotide siRNA

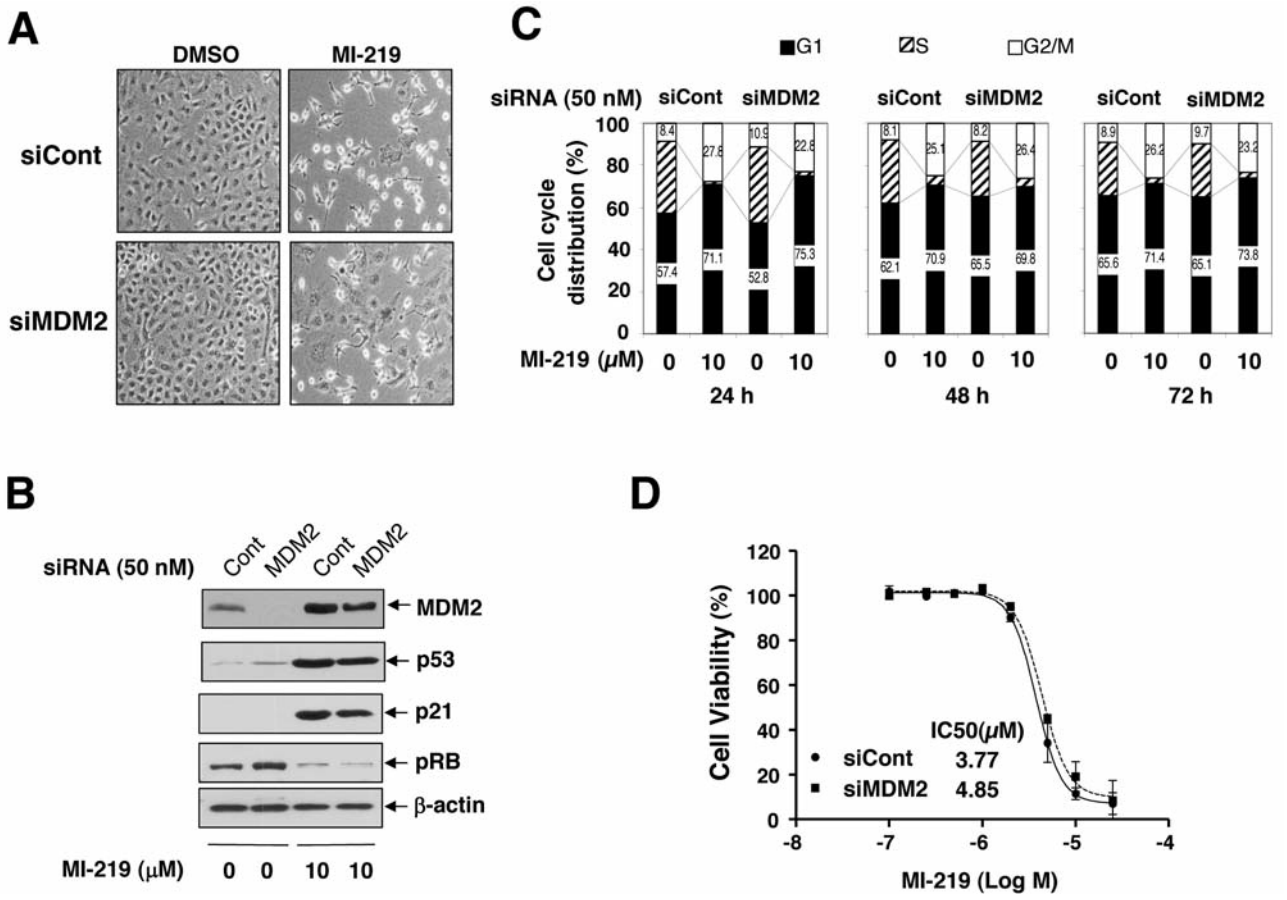

Figure 4. *MDM2 knockdown enhances MI-219 induced senescence-like phenotype, but does not increase MI-219 efficacy. A549 cells were transfected with Smart-pool siRNA oligoes targeting MDM2, along with scrambled control siCont. Forty-eight hours post transfection, cells were treated with MI-219 (10 μM), followed by: (A) SA-β-Gal staining 72 hours later, (B) Western blot analysis 48 hours later, (C) FACS analysis at the indicated time points post drug treatment, or (D) a 5-day ATPlite growth assay with different drug concentrations for IC_{50} determination (n=3).*

transfection and no knockdown was achieved (data not shown). Therefore further investigations were focused on A549 cells and found that combination of *MDM2* knockdown and MI-219 treatment induced a senescence-like phenotype as demonstrated by flattened cell morphology and positive staining for senescence-associated β-galactosidase (SA-β-gal) (Figure 4A). It was also found that *MDM2* silencing increased the basal level of pRB, whereas MDM2 accumulation (by MI-219) reduced pRB levels (Figure 4B). The fact that A549 cells with and without *MDM2* silencing had similar levels of p53, p21 and pRB after MI-219 treatment (Figure 4B) suggested that senescence-like phenotype induced by combination of *MDM2* knockdown and MI-219 is independent of p53, p21 and pRB. On the other hand, combination-induced cell senescence had a minor effect on cell cycle progression (Figure 4C) and on the sensitivity to MI-219-induced growth suppression (Figure 4D). It can be concluded from these results that *MDM2* knockdown has a minimal effect on MI-219 efficacy. However, it may still be possible that lack of significant biological consequence upon combination is due to insufficient *MDM2* knockdown, particularly after MI-219 treatment with a robust *MDM2* induction.

Inhibition of XIAP by SM-164 had no effect on MI-219 efficacy. A recent publication reported that MDM2 was able to increase expression of XIAP *via* enhancing an IRES-dependent XIAP translation (19). It was therefore determined whether XIAP levels were increased upon MI-219 treatment, which increased MDM2 levels in H460 and A549 cells. As shown in Figure 5A, drug-induced MDM2 accumulation had no effect on XIAP levels in H460 cells, but did increase XIAP about 2-fold in A549 cells. It was next found that A549 cells were very resistant, with an IC_{50} value greater than 50 μM, to SM-164 (Figure 5B), a Smac mimetic that is a very potent inhibitor of XIAP-caspases binding with an estimated K_i value of 0.2 nM

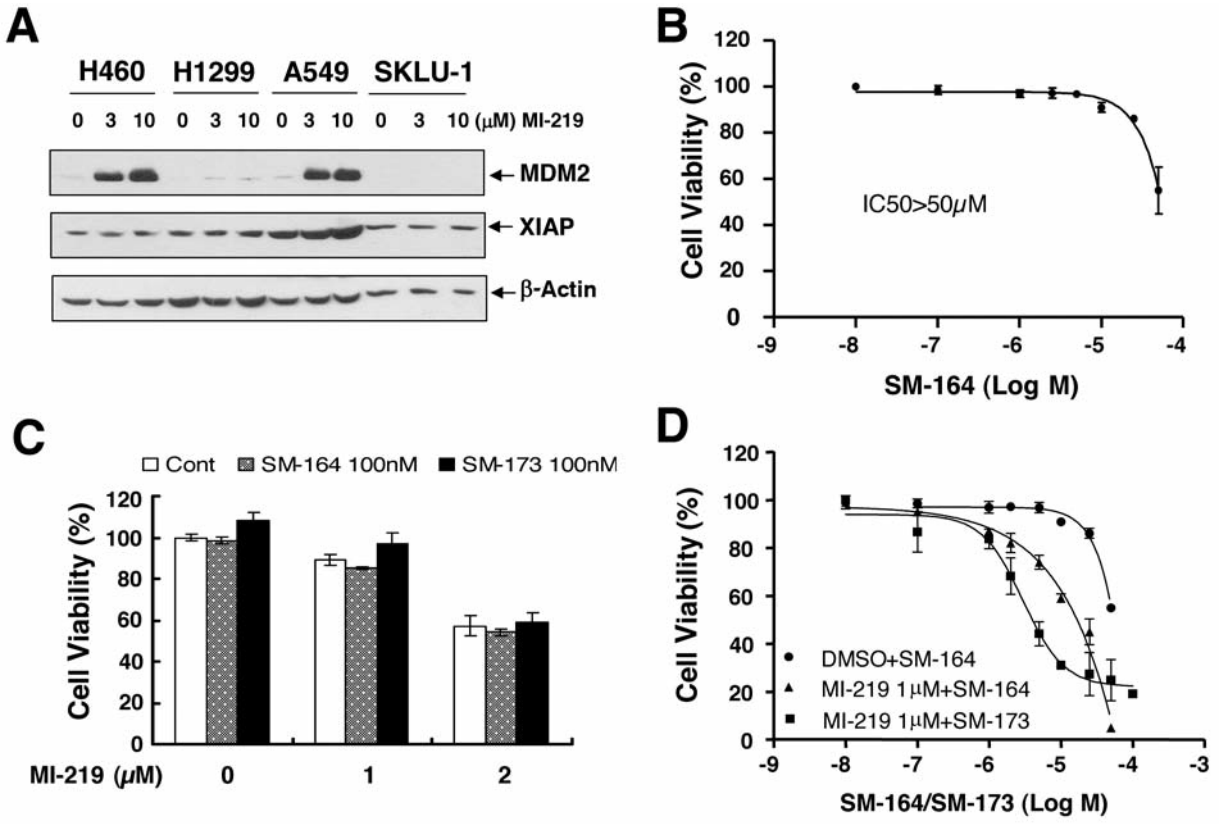

Figure 5. *XIAP does not play a major role in preventing apoptosis induction by MI-219. A: Four lines of lung cancer cells were treated with MI-219 for 48 hours, followed by Western blotting analysis. B: A549 cells were subjected to a 5-day ATPlite growth assay with different drug concentrations for IC_{50} determination (n=3). C: A549 cells were treated with MI-219 in combination with SM-164 or SM-173 at the indicated drug concentrations for 5 days, followed by ATPlite assay (n=3). D: A549 cells were subjected to a 5-day ATPlite growth assay with 1 μM MI-219 in the absence or presence of different concentrations of SM-164 or SM-173 for IC_{50} determination (n=3).*

(31). In the present study, SM-164 at a concentration sufficient to disrupt XIAP-caspase binding was found to have no effect on MI-219-induced growth suppression, nor its inactive analog, SM-173 (21,24) (Figure 5C). An additive effect on growth suppression was detected when SM-164 was used at a concentration equal to or greater than 25 μM (data not shown), likely due to non-specific cytotoxicity. Furthermore, whether MI-219 could sensitize A549 cells to SM-164 was determined and a limited sensitization was found in a manner independently of XIAP inhibition, since SM-173, an inactive XIAP inhibitor (21,24) had an even better sensitization activity (Figure 5D). Thus, inhibition of XIAP by SM-164 had no effect on MI-219-induced growth suppression.

MI-219 sensitized A549 cells to the chemotherapeutic drug, etoposide. Between the two wt p53-expressing lung cancer cell lines, H460 cells were sensitive, whereas A549 cells were resistant both *in vitro* and *in vivo* to etoposide (32-34), a chemotherapeutic drug used for the treatment of lung cancer (35). Whether MI-219 could sensitize A549 and H460 cells to etoposide was determined. Using an RT-CES system (28), cell growth was monitored for up to 3 days after treatment with MI-219 or etoposide, alone or in combination. Cells seeded in the plate were first monitored on the system in real time for 24 hours before addition of MI-219. Once the cells reached exponential growth phase (between 24 to 30 hours), MI-219 was added to the cells at different concentrations, followed by continuous monitoring for an additional 3 days. In A549 cells, growth inhibition started to appear 12 hours post treatment. Single drug treatment caused about 50% growth inhibition, whereas the combinatory treatment led to a complete growth inhibition (Figure 6A). Furthermore, A549 cells with combinatory treatment showed faster cytotoxic kinetics than single compound treatment, suggesting that MI-219 may not only enhance the overall cytotoxicity induced by etoposide but also accelerate the etoposide-mediated cell killing process (Figure 6A, top). Similarly, in H460 cells, growth inhibition started to appear 12 hours post treatment. A limited growth inhibition by MI-219, but dramatic inhibition by etoposide was observed

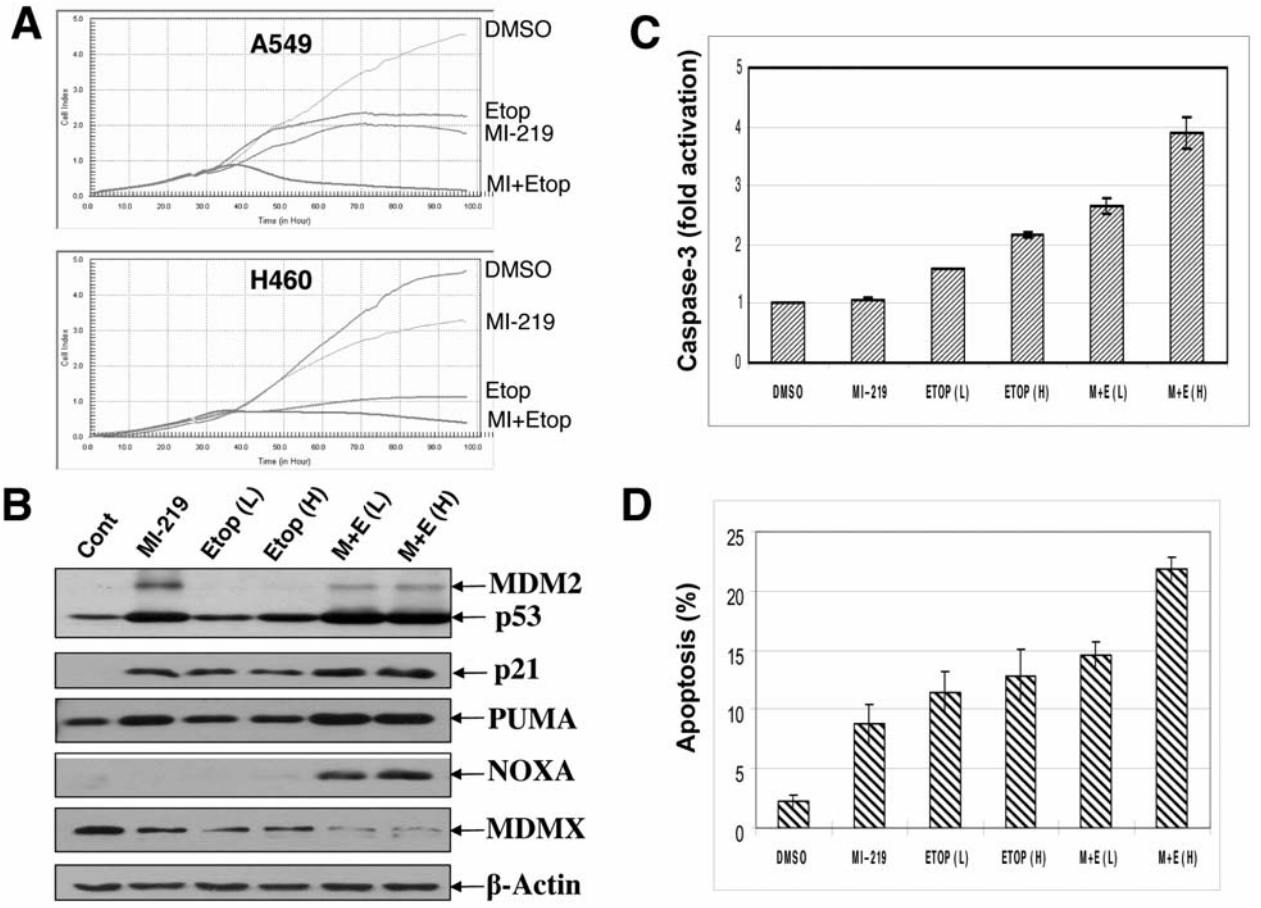

Figure 6. *Sensitization of lung cancer cells to etoposide-induced killing by MI-219. A: Growth inhibition of A549 (top) and H460 (bottom) by MI-219 or etoposide, alone or in combination. Cells seeded in triplicate in 96-well ACEA E-plates were monitored in real time on the RT-CES system. The drugs were added alone or in combination 24 hours later at the indicated concentrations. Cell growth was monitored continuously for the indicated periods of time. B: Activation of p53 and induction of p53 target genes. A549 cells were treated with MI-219 (10 μM), etoposide (L, 10 μM or H, 20 μM) or in combination for 48 hour, followed by Western blot analysis using indicated antibodies. C, D: Activation of caspase-3 and induction of apoptosis by combinational treatment. C: A549 cells were seeded in 96-well plate and subjected to treatment of MI-219 or etoposide (L, 10 μM or H, 20 μM) alone or in combination for 48 hours, followed by caspase-3 activity assay. The means±SEM (standard error of the mean) from two independent experiments, each run in triplicate are shown. D: A549 cells were seeded in 100 mm dish and subjected to treatment of MI-219, etoposide (L, 10 μM or H, 20 μM) alone or in combination, followed by FACS analysis. Apoptosis is shown by the percentage of cells in the sub-G_1 region. The means±SEM from two independent experiments are shown.*

thereafter. Again, the combination of both drugs caused near complete inhibition (Figure 6A, bottom). To understand the molecular basis of these synergistic effects, the activation of p53 and its downstream target genes were measured in A549 cells. As shown in Figure 6B, MI-219 induced *p53* and its downstream targets, including *MDM2*, *p21* and *PUMA*, but not *NOXA*, whereas etoposide induced *p53* and its targets, *p21*, but not *MDM2*, *PUMA*, and *NOXA*. Interestingly, both MI-219 and etoposide reduced the expression of MDMX, an MDM2 family member also implicated in targeting p53 for degradation (36). Combinatory treatment caused even higher levels of induction of *p53* as well as of its targets, *PUMA*, *p21* and, remarkably, *NOXA*. On the other hand, combinational treatment nearly completely eliminated *MDMX* expression. It was then determined whether increased levels of PUMA and NOXA in combinatory treatment could lead to an enhanced activation of caspase 3. Indeed, as shown in Figure 6C, while single treatment had no (by MI-219) or slight (by etoposide) activation of caspase 3, combinatory treatment caused up to 4-fold induction of caspase-3. Finally, FACS analysis was performed to determine the degree of apoptosis induction upon single and combined treatments. As shown in Figure 6D, single treatment with MI-219 or etoposide caused limited induction of apoptosis with an apoptotic population of around 10% while combinatory treatment significantly enhanced it,

reaching an apoptotic population of up to 22%. Thus, in A549 cells, MI-219 at a concentration of 10 μM mainly induces growth arrest; it also enhances apoptosis when combined with the anticancer drug etoposide as a result of elevated induction of *PUMA* and *NOXA* and activation of caspase-3. It can be concluded that MI-219 could serve as a chemosensitizer in lung cancer cells.

Discussion

Recently it was shown that MI-43, a MI-219 analog, selectively kills lung cancer cells harboring wt p53 and sensitizes lung cancer cells to etoposide-induced apoptosis (17). However, MI-43 has low anticancer potency and poor oral bioavailability. The concentration required for MI-43 to induce apoptosis is rather high (30 μM), inducing, subsequently, p53-independent killing (17). All these features of MI-43 make it impossible to be developed further in clinical settings. The present study used MI-219, a more potent and orally bioavailable MDM2 inhibitor and reported that MI-219 selectively inhibits wt p53-expressing lung cancer cells, H460 and A549 with a potency 20-fold higher than lung cancer lines, H1299 and SKLU-1 harboring defective *p53*. The mechanism of drug action was through p53 activation, followed by induction of *p21* with a minor induction of pro-apoptotic *PUMA*. The p53-dependent drug action was further confirmed by siRNA silencing of endogenous p53, which largely abolishes drug-induced growth inhibition. Mechanistically, the growth inhibition induced by MI-219 at 10 μM, as a consequence of p53-p21 activation, was mainly attributable to growth arrest either at the G_1 (H460 cells) or G_2 (A549 cells) phase of cell cycle with a minor induction of apoptosis.

Interestingly, it was found that MI-219 has a miminal growth suppressive effect on two wt p53-expressing immortalized lung cell lines, NL20 bronchial epithelia cells and MRC5 fibroblast cells. This observation appeared to be contradictory to an earlier study, reporting that Nutlin-1, a different class of MDM2 inhibitor, was able to induce G_1 arrest of the primary human fibroblasts (1043SK) as a result of *p21* induction (37). The discrepancy is likely derived from the fact that although p53 levels are extremely high in these two immortalized lines, p53 appears to be inactive regardless of MI-219 treatment, since the expression of the three common p53 targets, namely *MDM2*, *p21* and *PUMA*, were not detectable even after drug treatment.

Furthermore, the efficacy of MI-219 against lung cancer cells was investigated in combination with *MDM2* knockdown, with a XIAP inhibitor, SM-164, and with the chemotherapeutic drug etoposide. It is well-established that MDM2 has many p53-independent functions (15). For example, MDM2 binds to pRB and inhibits pRB growth regulatory function (38). MDM2 also promotes the degradation of p21 (39) and of ribosomal proteins L26 (40) and S7 (41) to modulate cell growth and proliferation (42). Therefore, it was hypothesized that MDM2 accumulation upon p53 activation by MI-219 could confer additional growth advantage and therefore simultaneous *MDM2* knockdown could block this side-effect to increase the efficacy of MI-219. On testing this hypothesis, an induction of cellular senescence-like phenotype was observed when combining MI-219 and *MDM2* knockdown within 3 days in a mechanism independent of p53, RB and p21. It has been recently reported that Nutlin-3 reversibly induced senescence-like phenotype in A549 cells after 6 days drug exposure (43). Thus, *MDM2* knockdown shortened the process by 3 days. The present observation that MI-219 reduced pRB levels is consistent with recent reports using Nutlin-3 to induce *MDM2* (43, 44). Unexpectedly, *MDM2* knockdown did not change the cell cycle profile, nor enhance the efficacy of MI-219, which may be explained by the reversible nature of senescence induction and insufficient silencing of *MDM2* under treatment conditions.

Since XIAP was found to be up-regulated by MDM2 (19) and XIAP is a known apoptosis inhibitor (45), it was further hypothesized that lack of apoptosis induction by MI-219 in lung cancer cells is due to XIAP induction by accumulated MDM2 and inhibition of XIAP-caspase binding by SM-164 would therefore reactivate caspases and sensitize cells to MI-219 for apoptosis induction. On testing this hypothesis, it was found that out of two lung cancer lines with MDM2 accumulation by MI-219, an increased XIAP level up to two-fold was detected only in A549 cells. Combination of SM-164 and MI-219, however, did not specifically enhance the efficacy of MI-219, indicating that XIAP does not play a major role in preventing apoptosis upon MI-219 treatment.

Finally, it was shown that MI-219 significantly sensitizes otherwise resistant A549 cells to etoposide. This is achieved through enhanced induction of two pro-apoptotic proteins, PUMA and NOXA, leading to caspase-3 activation to induce apoptosis. One interesting observation was that either MI-219 or etoposide caused a minor reduction of MDMX, a MDM2 family member also implicated in p53 degradation (46, 47), but combinational treatment caused a further decrease of MDMX levels. It has been previously shown that etoposide reduces MDMX level by promoting its degradation (48). Although it is not clearly at the present time how MI-219 causes MDMX reduction, reduction of MDMX, a p53 negative regulator, would certainly further activate p53 to enhance growth arrest or apoptosis, as also demonstrated in a recent study using combination of *Nutlin* and *MDMX* siRNA (49).

In conclusion this study showed that MI-219 is a potent activator of p53, leading to a selective growth inhibition of lung cancer cells harboring wt p53. Neither *MDM2* knockdown nor SM-164 combination increased the efficacy of MI-219, suggesting that accumulated MDM2 and XIAP

do not play a major role in MI-219 action. MI-219 did sensitize otherwise resistant lung cancer cells to etoposide, suggesting it could act as a chemosensitizer.

References

1 Giaccia AJ and Kastan MB: The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev *12*: 2973-2983, 1998.

2 Ko LJ and Prives C: p53: puzzle and paradigm. Genes Dev *10*: 1054-1072, 1996.

3 Levine AJ: p53, the cellular gatekeeper for growth and division. Cell *88*: 323-331, 1997.

4 Vogelstein B, Lane D and Levine AJ: Surfing the p53 network. Nature *408*: 307-310, 2000.

5 Sun Y: p53 and its downstream proteins as molecular targets of cancer. Mol Carcinog *45*: 409-415, 2006.

6 Greenblatt MS, Bennett WP, Hollstein M and Harris CC: Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res *54*: 4855-4878, 1994.

7 Freedman DA, Wu L and Levine AJ: Functions of the MDM2 oncoprotein. Cell Mol Life Sci *55*: 96-107, 1999.

8 Lozano G and Montes de Oca Luna R: MDM2 function. Biochim Biophys Acta *1377*: M55-59, 1998.

9 Momand J, Wu HH and Dasgupta G: MDM2 – master regulator of the p53 tumor suppressor protein. Gene *242*: 15-29, 2000.

10 Chene P: Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. Nat Rev Cancer *3*: 102-109, 2003.

11 Brown CJ, Lain S, Verma CS, Fersht AR and Lane DP: Awakening guardian angels: drugging the p53 pathway. Nat Rev Cancer *9*: 862-873, 2009.

12 Wang Z and Sun Y: Targeting p53 for novel anticancer therapy. Translat Oncol 3, 2010.

13 Shangary S and Wang S: Targeting the MDM2-p53 interaction for cancer therapy. Clin Cancer Res *14*: 5318-5324, 2008.

14 Mohammad RM, Wu J, Azmi AS, Aboukameel A, Sosin A, Wu S, Yang D, Wang S and Al-Katib AM: An MDM2 antagonist (MI-319) restores p53 functions and increases the life span of orally treated follicular lymphoma bearing animals. Mol Cancer *8*: 115, 2009.

15 Ganguli G and Wasylyk B: p53-independent functions of MDM2. Mol Cancer Res *1*: 1027-1035, 2003.

16 Shangary S, Qin D, McEachern D, Liu M, Miller RS, Qiu S, Nikolovska-Coleska Z, Ding K, Wang G, Chen J, Bernard D, Zhang J, Lu Y, Gu Q, Shah RB, Pienta KJ, Ling X, Kang S, Guo M, Sun Y, Yang D and Wang S: Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. Proc Natl Acad Sci USA *105*: 3933-3938, 2008.

17 Sun SH, Zheng M, Ding K, Wang S and Sun Y: A small molecule that disrupts Mdm2-p53 binding activates p53, induces apoptosis, and sensitizes lung cancer cells to chemotherapy. Cancer Biol Ther *7*: 845-852, 2008.

18 Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N and Liu EA: *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science *303*: 844-848, 2004.

19 Gu L, Zhu N, Zhang H, Durden DL, Feng Y and Zhou M: Regulation of XIAP translation and induction by MDM2 following irradiation. Cancer Cell *15*: 363-375, 2009.

20 Shi Y: Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell *9*: 459-470, 2002.

21 Lu J, Bai L, Sun H, Nikolovska-Coleska Z, McEachern D, Qiu S, Miller RS, Yi H, Shangary S, Sun Y, Meagher JL, Stuckey JA and Wang S: SM-164: a novel, bivalent Smac mimetic that induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP. Cancer Res *68*: 9384-9393, 2008.

22 Ding K, Lu Y, Nikolovska-Coleska Z, Qiu S, Ding Y, Gao W, Stuckey J, Krajewski K, Roller PP, Tomita Y, Parrish DA, Deschamps JR and Wang S: Structure-based design of potent non-peptide MDM2 inhibitors. J Am Chem Soc *127*: 10130-10131, 2005.

23 Lu Y, Nikolovska-Coleska Z, Fang X, Gao W, Shangary S, Qiu S, Qin D and Wang S: Discovery of a nanomolar inhibitor of the human murine double minute 2 (MDM2)-p53 interaction through an integrated, virtual database screening strategy. J Med Chem *49*: 3759-3762, 2006.

24 Sun H, Nikolovska-Coleska Z, Lu J, Meagher JL, Yang CY, Qiu S, Tomita Y, Ueda Y, Jiang S, Krajewski K, Roller PP, Stuckey JA and Wang S: Design, synthesis, and characterization of a potent, nonpeptide, cell-permeable, bivalent Smac mimetic that concurrently targets both the BIR2 and BIR3 domains in XIAP. J Am Chem Soc *129*: 15279-15294, 2007.

25 Bockbrader KM, Tan M and Sun Y: A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL- and etoposide-induced apoptosis in breast cancer cells. Oncogene *24*: 7381-7388, 2005.

26 Maguire M, Nield PC, Devling T, Jenkins RE, Park BK, Polanski R, Vlatkovic N and Boyd MT: MDM2 regulates dihydrofolate reductase activity through monoubiquitination. Cancer Res *68*: 3232-3242, 2008.

27 Jia L, Soengas MS and Sun Y: ROC1/RBX1 E3 ubiquitin ligase silencing suppresses tumor cell growth *via* sequential induction of G_2-M arrest, apoptosis, and senescence. Cancer Res *69*: 4974-4982, 2009.

28 Solly K, Wang X, Xu X, Strulovici B and Zheng W: Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. Assay Drug Dev Technol *2*: 363-372, 2004.

29 Itahana K, Campisi J and Dimri GP: Methods to detect biomarkers of cellular senescence: the senescence-associated beta-galactosidase assay. Methods Mol Biol *371*: 21-31, 2007.

30 O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, Scudiero DA, Monks A, Sausville EA, Weinstein JN, Friend S, Fornace AJ Jr. and Kohn KW: Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Res *57*: 4285-4300, 1997.

31 Nikolovska-Coleska Z, Wang R, Fang X, Pan H, Tomita Y, Li P, Roller PP, Krajewski K, Saito NG, Stuckey JA and Wang S: Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. Anal Biochem *332*: 261-273, 2004.

32 Kraus-Berthier L, Jan M, Guilbaud N, Naze M, Pierre A and Atassi G: Histology and sensitivity to anticancer drugs of two human non-small cell lung carcinomas implanted in the pleural cavity of nude mice. Clin Cancer Res *6*: 297-304, 2000.

33 Andriani F, Perego P, Carenini N, Sozzi G and Roz L: Increased sensitivity to cisplatin in non-small cell lung cancer cell lines after FHIT gene transfer. Neoplasia *8*: 9-17, 2006.

34 Hatta Y, Takahashi M, Enomoto Y, Takahashi N, Sawada U and Horie T: Adenosine triphosphate (ATP) enhances the antitumor effect of etoposide (VP16) in lung cancer cells. Oncol Rep *12*: 1139-1142, 2004.
35 Albain KS, Crowley JJ, Turrisi AT, 3rd, Gandara DR, Farrar WB, Clark JI, Beasley KR and Livingston RB: Concurrent cisplatin, etoposide, and chest radiotherapy in pathologic stage IIIB non-small-cell lung cancer: a Southwest Oncology Group phase II study, SWOG 9019. J Clin Oncol *20*: 3454-3460, 2002.
36 Parant J, Chavez-Reyes A, Little NA, Yan W, Reinke V, Jochemsen AG and Lozano G: Rescue of embryonic lethality in *Mdm4*-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. Nat Genet *29*: 92-95, 2001.
37 Carvajal D, Tovar C, Yang H, Vu BT, Heimbrook DC and Vassilev LT: Activation of p53 by MDM2 antagonists can protect proliferating cells from mitotic inhibitors. Cancer Res *65*: 1918-1924, 2005.
38 Xiao ZX, Chen J, Levine AJ, Modjtahedi N, Xing J, Sellers WR and Livingston DM: Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature *375*: 694-698, 1995.
39 Jin Y, Lee H, Zeng SX, Dai MS and Lu H: MDM2 promotes $p21^{waf1/cip1}$ proteasomal turnover independently of ubiquitylation. Embo J *22*: 6365-6377, 2003.
40 Ofir-Rosenfeld Y, Boggs K, Michael D, Kastan MB and Oren M: Mdm2 regulates *p53* mRNA translation through inhibitory interactions with ribosomal protein L26. Mol Cell *32*: 180-189, 2008.
41 Zhu Y, Poyurovsky MV, Li Y, Biderman L, Stahl J, Jacq X and Prives C: Ribosomal protein S7 is both a regulator and a substrate of MDM2. Mol Cell *35*: 316-326, 2009.
42 Zhang Y and Lu H: Signaling to p53: ribosomal proteins find their way. Cancer Cell *16*: 369-377, 2009.
43 Huang B, Deo D, Xia M and Vassilev LT: Pharmacologic p53 activation blocks cell cycle progression but fails to induce senescence in epithelial cancer cells. Mol Cancer Res *7*: 1497-1509, 2009.
44 Du W, Wu J, Walsh EM, Zhang Y, Chen CY and Xiao ZX: Nutlin-3 affects expression and function of retinoblastoma protein: role of retinoblastoma protein in cellular response to nutlin-3. J Biol Chem *284*: 26315-26321, 2009.
45 Salvesen GS and Duckett CS: IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol *3*: 401-410, 2002.
46 Marine JC and Jochemsen AG: Mdmx as an essential regulator of p53 activity. Biochem Biophys Res Commun *331*: 750-760, 2005.
47 Toledo F and Wahl GM: MDM2 and MDM4: p53 regulators as targets in anticancer therapy. Int J Biochem Cell Biol *39*: 1476-1482, 2007.
48 Okamoto K, Kashima K, Pereg Y, Ishida M, Yamazaki S, Nota A, Teunisse A, Migliorini D, Kitabayashi I, Marine JC, Prives C, Shiloh Y, Jochemsen AG and Taya Y: DNA damage-induced phosphorylation of MdmX at serine 367 activates p53 by targeting MdmX for Mdm2-dependent degradation. Mol Cell Biol *25*: 9608-9620, 2005.
49 Hu B, Gilkes DM, Farooqi B, Sebti SM and Chen J: MDMX overexpression prevents p53 activation by the MDM2 inhibitor Nutlin. J Biol Chem *281*: 33030-33035, 2006.

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