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Letters

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MPK-09, a Small Molecule Inspired from Bioactive Styryllactone Restores the Wild-Type Function of Mutant p53

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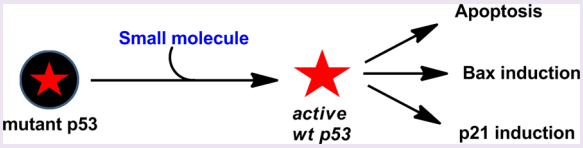
Supporting Information

ABSTRACT: In the search for more efficacious and less toxic cancer drugs, the tumor suppressor p53 protein has long been a desirable therapeutic target. In the recent past, few independent studies have demonstrated that the antitumor activity of wild-type p53 can be restored in cancer cells harboring mutant form of p53 using small molecule activators. In this study, we describe a novel small molecule **MPK-09**, which is selective and highly potent against allele specific p53 mutations mainly, R175H, R249S, R273H, R273C, and E285K . Except E285K, all other mutations tested are among the six "hot spot" p53 mutations reported in majority of human cancer. Furthermore, our study conclusively demonstrates that the apoptotic activity of the small molecule **MPK-09** against cancer cells harboring R273C and E285K mutations is due to restoration of the wild-type conformation to the corresponding mutant form of p53.



Often described as the "guardian of genome", p53 plays an important role in controlling a number of complex cellular processes including apoptosis during deoxyribonucleic acid (DNA) damage, hypoxia, and oncogenic stress. The wild-type (wt) p53 protein has a half-life of <20 min, and therefore, only small amounts of active protein reside in the cell. Upon DNA damage or oncogenic stress, the levels of p53 protein are increased, which leads to apoptosis and elimination of incipient tumor cells.[1,2] In contrary, inactivation of the tumor suppressor function of p53 occurs very frequently and ∼50% of all human cancer harbor mutations in p53 protein. The majority of the inactivating mutations reside in the central core DNA binding domain (DBD) of p53. These mutations can be divided into two main classes, DNA contact mutants, like R273H, R175H, and R273C, where the mutation alters a residue involved in making contact with DNA, and conformational mutants like R249S, which affects the stability of the DBD, resulting in loss of function, and account for ∼30% of reported cancer cases.[3] Also, the rare temperature sensitive E285K mutation of p53 leads to misfolding and loss of p53 function at the physiological temperature.[4] These mutations hamper the transcriptional regulation of various p53 target genes involved in apoptosis. Consequently, tumors harboring mutation in p53 are resistant to conventional chemo/radiotherapy. Therefore, reactivation of mutant p53 to its active form might transform the treatment of human cancer and is a big leap from the conventional treatment.

One of the approaches in restoration of wild-type p53 function in tumors that have lost p53 tumor suppressor activity is the identification of small organic molecules that can reactivate the mutant p53 to its wild-type functional protein.[5−7] Recently, this strategy has led to the identification of molecules like PRIMA-1,[8] APR-246,[9] a nine residue peptide CDB3,[10] two quinazoline based small molecules CP-31398[11] and SCH529074,[12] and ellipticine, a natural product with carbazole skeleton.[13] Majority of these molecules were identified by the screening of a synthetic chemical library and have been reported for the restoration of wild-type activity to mutant p53 protein. Very recently, while this letter was under preparation, a small molecule NSC319726, identified by *in silico* screening, was shown to restore the wild-type function of p53.[14] However, the greater challenge lies in the fact that the tumors are heterogeneous in nature and express different types of p53 mutations. Therefore, it is essential to identify molecules that can specifically be effective *in vivo* against human tumors with varied p53 mutations. In quest of finding molecules of such therapeutic significance, herein, we describe the identification of a small molecule inspired from the bioactive natural products; styryllactones, which effectively rescue the wild-type function of the most common "hot spot" p53 mutations R273C and temperature sensitive conformational mutant E285K.

Styryllactones are a class of lactone containing natural products isolated from the plant *Annonaceae* and are shown to possess good cytotoxic activity.[15,16] We have been interested in the synthesis of a number of styryllactones and their analogues in the quest of identifying a small molecule with potent cytotoxic activity against cancer cells harboring mutations in p53 protein. In this context, our group has developed a high yielding synthetic strategy for the synthesis of styryllactones such as goniofufurone, goniopypyrone, and goniotriol inspired by the proposed biogenesis.[17,18] The synthetic strategy is based on elaboration of γ-hydroxy butyramides derived from tartaric acid amide involving controlled addition of Grignard reagent

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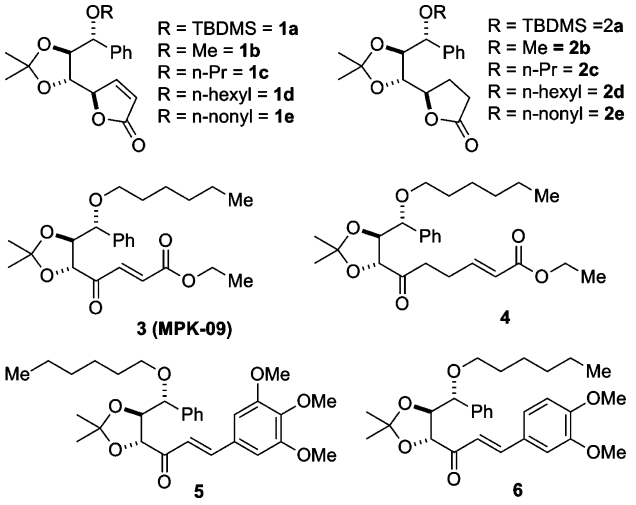
followed by stereoselective reduction. During the course of this synthetic investigation, we investigated the sensitivity of cancer cell lines mainly, BT474 (mut p53, E285K), A549 (wt p53), and HeLa (wt p53) toward the intermediate compounds and analogues (Chart 1)[19] by *in vitro* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[20] Notably, HeLa cells are infected with HPV (Human papillomavirus), which degrades the wild-type p53 proteins. Consequently, low levels of p53 protein is expressed in HeLa cell line.[21]

Chart 1. Styryllactone Analogues



As depicted in Chart 1 and Table 1, we found that the α,β-unsaturated lactone **1a** exhibited promising activity (IC_{50} = 5 μM) against the breast carcinoma cell line BT474. Further, evaluation of a similar analogue with a methyl substituent **1b** exhibited no appreciable activity. We anticipated that suitable hydrophobic substitution equivalent to that of the TBDMS (*tert*-butyldimethysilyl ether) is essential for the activity. We reasoned that an alkyl substitution with appropriate chain length will be an ideal surrogate for the TBDMS group. Indeed, this was the case with lactone **1d** comprising *n*-hexyl side chain, which displayed IC_{50} = 1.7 μM against BT474 cell line. Decreasing the chain length to C3 (compound **1c**) lowered the activity drastically, while increasing the chain length to C9 (compound **1e**) did not show much difference in the cytotoxic activity toward BT474 cells (Table 1) compared to **1d**. Examination of compound **1a** for its activity against cancer cell lines A549 and HeLa with wt p53 status exhibited IC_{50} of 4.2 and 4.0 μM, respectively. Corresponding saturated lactones **2a–e** did not show any appreciable cytotoxic activity indicating that the unsaturation is essential for the desired activity. To our surprise, analogue **3** (**MPK-09**) possessing a linear α,β-unsaturated keto ester, synthesized by elaboration of the γ-alkoxy butyramide derived from tartaric acid involving a Wittig–Horner olefination exhibited potent cytotoxic activity against BT474 cell line (IC_{50} = 0.6 μM) as compared to A549 (IC_{50} = 2.8 μM) and HeLa (IC_{50} = 4 μM) cells.

Table 1. Sensitivity of Tumor Cell Lines against Compounds 1–6 Harboring Varied p53 Status (Indicated in the Parentheses); Cell Growth Inhibition Was Analyzed by MTT Assay As Described in General Procedures

	IC_{50} in μM		
compound	BT474 (E285K)	A549 (wt p53)	HeLa (wt p53)
1a	5	4.2	4
1b	>100	>100	>100
1c	28	18	32
1d	1.7	12.8	7.5
1e	1.6	2.5	2
2a	64	>100	50
2b	>100	>100	>100
2c	>100	>100	>82
2d	22.5	80	26.5
2e	18	14	7.5
3(MPK-09)	0.6	2.8	4
4	>100	>100	>100
5	1.7	4.7	1.7
6	3	7.3	2.8

Compound **4** possessing mere conjugation of the ester did not show any activity clearly indicating that the conjugation of both ketone and ester functionalities are essential for the cytotoxic activity. Replacing the ester with aromatic groups retained promising activity. Taken together, results summarized in Table 1 suggested that **MPK-09** displayed potent cytotoxic activity against cancer cell line BT474 expressing allele specific E285K mutation of p53 as compared to wt p53 expressing cancer cell lines A549 and HeLa.

To further corroborate our findings, sensitivity of **MPK-09** was evaluated on additional mutant p53 expressing cell lines originating from the same tumor type, as shown in Figure 1. **MPK-09** indeed displayed approximately 8- to 10-fold increase in activity compared to its corresponding null or wild-type counterpart (Figure 1A–C). In addition, we ectopically expressed wild-type p53 along with mutant p53 expressing plasmids, namely, R249S, R273H, and R175H in hepatocellular carcinoma cell line, Hep3B (p53−/−). Twenty-four hours post-transfection, cells were exposed to 2 μM concentration of **MPK-09**, and cell viability was measured 48 h post-treatment using MTT. Interestingly, cells expressing hot spot p53 mutations resulted in 41% (R249S), 50% (R273H), and 39% (R175H) viability as compared to 80% viable cells expressing wt p53. These results suggested that **MPK-09** indeed was a potent cytotoxic agent toward allele specific mutations of p53 (Figure 1D).

To determine if the inhibition of cell growth was mediated through induction of apoptosis, we evaluated the effect of **MPK-09** on cell cycle. Treatment of BT474 cells with **MPK-09** displayed increase in subG1 peak as analyzed through flow cytometry. However, MCF-7 cells harboring wt p53, on treatment with the same concentration of **MPK-09**, did not show any accumulation of cells in the subG1 phase (Figure 2A). In good correlation, **MPK-09** increased AnnexinV positive cells in BT474 cells. Dot plot analysis revealed 54.3% and 89.4% of Annexin V positive cells at 4 and 8 μM concentrations of **MPK-09**, respectively (Figure 2B). This was further evidenced by the presence of Annexin positive cells as observed by immunostaining (Figure 1, Supporting Information). In concordance, treatment of cells with increasing concentration of **MPK-09** concomitantly increased DNA damage as captured by enhanced laddering of the fragmented DNA (Figure 2C) followed with increased tailing of the cleaved DNA as demonstrated by Comet assay[22] (Figure 2D). Exposure of cells to **MPK-09** leads to enhanced PARP (poly(ADP-ribose) polymerase) cleavage and activated caspase 3 expressions (Figure 2E). It is likely that the apoptotic activity induced by

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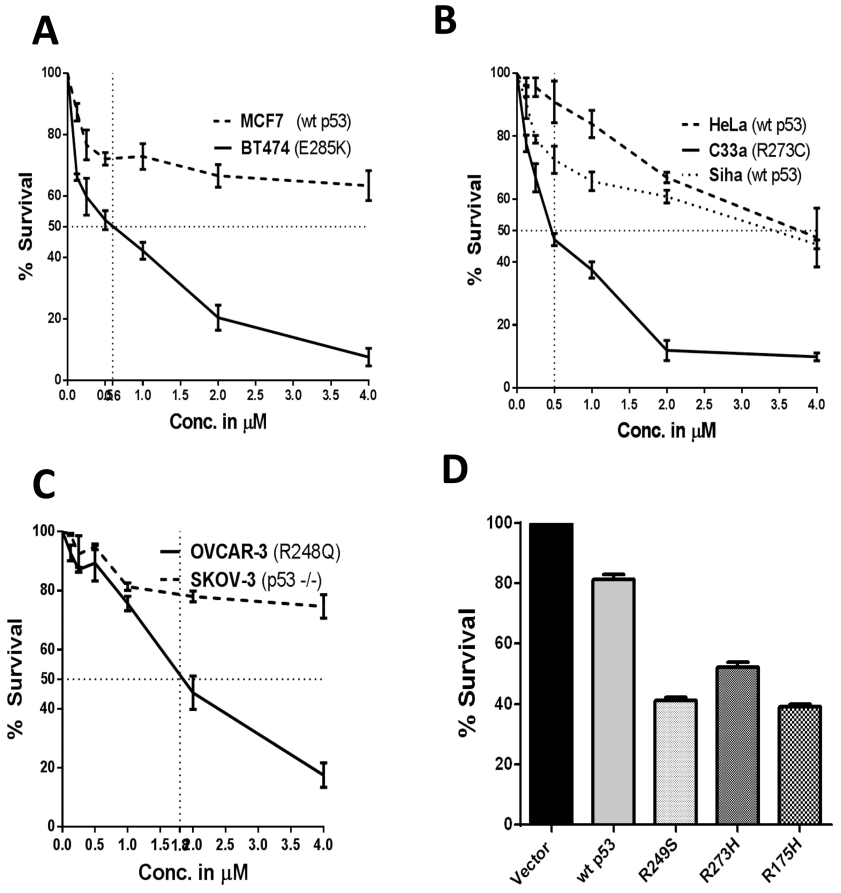

Figure 1. (A–C) Sensitivity of **MPK-09** against indicated human tumor cell lines with varied p53 mutations. Cell growth inhibition was analyzed by MTT assay. Cells were treated with six different dilutions of the compound **MPK-09** (0.025 to 4 μM) for 48 h. (D) Effect of **MPK-09** (2 μM) on Hep3B (−/− p53) cells ectopically expressing empty vector, wt p53, R249S, R273H, and R175H proteins.

MPK-09 could be a consequence of increased p53 protein stability. To test this, we determined the levels of p53 protein. As evident, no change in the levels of p53 protein was detected (Figure 2E), indicating that the actions of **MPK-09** does not have any influence on the stability of the p53 protein. Collectively, these results suggest **MPK-09** as a potent inducer of apoptosis in mutant p53 expressing BT474 cells.

It is worth noting that apoptosis is a function attributed to the wild-type p53 protein, whereas gain of function mutations like R249S and R273C are associated with aggressive tumor behavior like enhanced tumorigenesis, invasion, and metastasis.[23−25] Surprisingly, MPK-09 exhibited apoptotic effect on mutant p53 expressing BT474 cells. This made us to reason that the preferential activity of **MPK-09** against mutant p53 expressing cells might be the result of restoration of the wild-type function of the mutant p53 protein. To substantiate this hypothesis, an indirect immunoflourescence experiment with the conformation-specific monoclonal antibodies PAb1620 and PAb240, which recognizes wt and mutant p53 conformation, respectively, was undertaken. We observed that **MPK-09** induced a conformation change in C33a cells expressing R273C mutant to a structure that was recognized by the wt specific antibody (PAb1620) and was no longer recognized by the mutant-specific antibody (PAb240; Figure 3A(e,m)). Similar results were obtained with BT474 cells harboring E285K mutation (Figure 3A(g,o)). This experiment indicated that **MPK-09** can result in a profound conformational change of mutant p53 protein to its wild-type conformation.

We further confirmed the conformation change of mutant p53 by performing immunoprecipitation of wt p53 from **MPK-09** treated BT474 and C33a lysates (Figure 3B), which resulted in a significant increase in PAb1620 immunoreactivity. Presence of p53 in the lysates was analyzed using polyclonal antibody DO-7, which recognizes both mutant and wild-type p53. The data unequivocally demonstrates restoration of the wild-type conformation of mutant p53 upon exposure of mutant p53 expressing cells to **MPK-09**.

To determine if the conformation change observed with the E285K and R273C mutation of p53 resulted in restoration of wt p53 transcriptional function, we examined the expression of its downstream target p21 and apoptotic protein Bax. We found that **MPK-09** indeed induced p21 and Bax expression in both the p53 mutant expressing cell lines (Figure 3C). Induction of Bax is also seen by confocal microscopy (Supporting Information Figure 2).

In conclusion, we have demonstrated that a small molecule **MPK-09** is more cytotoxic toward cancer cell lines harboring p53 mutation E285K and R273C. We also propose that the increased activity of **MPK-09** is due to the restoration of wild-type conformation of mutant p53. However, the exact mechanism of cell death mediated by **MPK-09** needs further experimentation. Moreover, by ectopic expression of p53 expression plasmids harboring various hotspot mutations, we demonstrate that the **MPK-09** is effective on the tested p53 mutants compared to the wt p53 harboring cells. The restoration of p53 function is further corroborated by the

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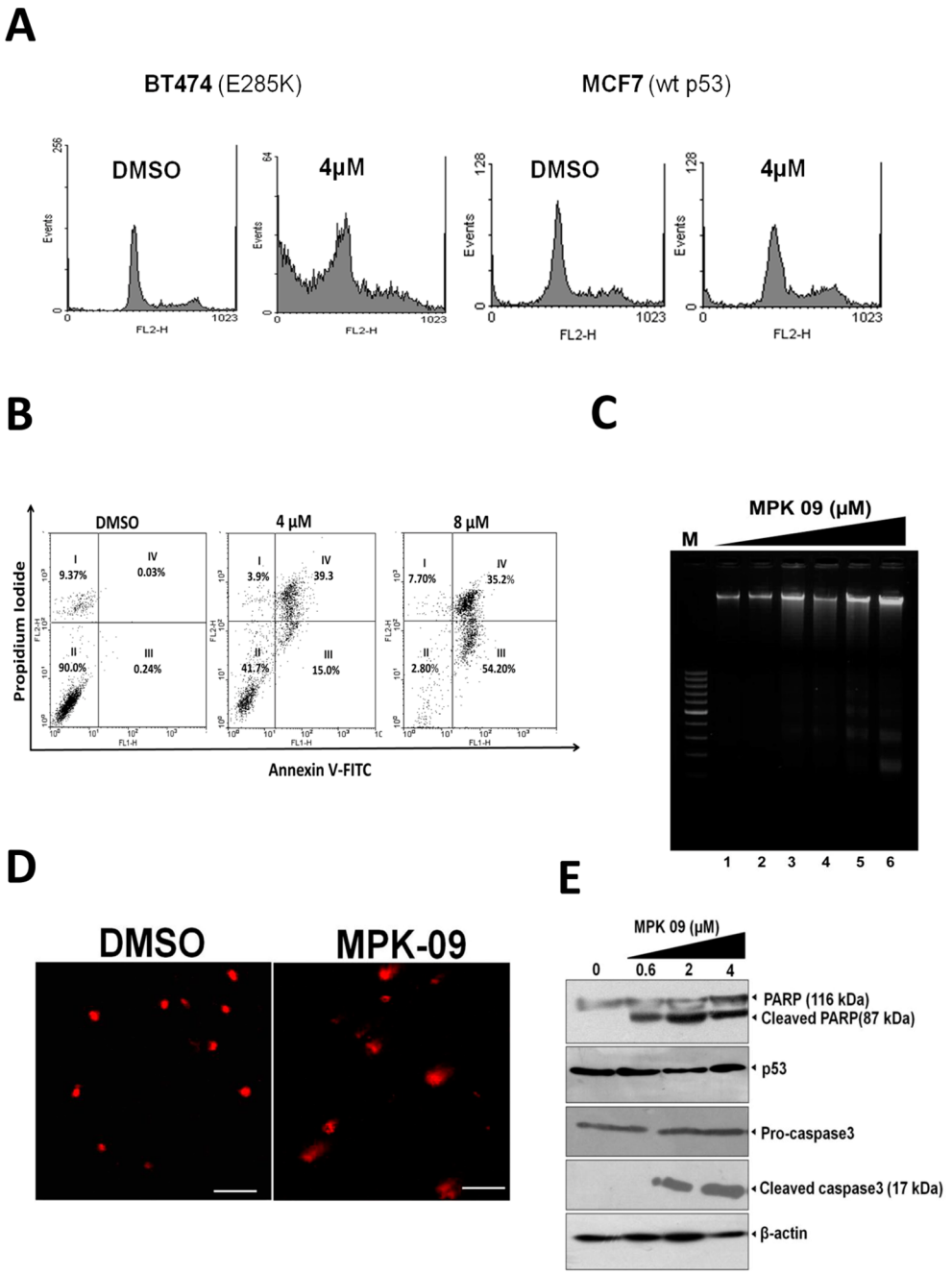

Figure 2. (A) Flow cytometric analysis showing subG1 peak in BT474 and MCF7 cell lines treated with **MPK-09** for 48 h. (B) Dot plot analysis showing percentage induction of early and late apopotosis in BT474 cells treated with **MPK-09** and analyzed by Annexin V/PI (propidium iodide) dual staining under flow cytometer. (C) Electrophoresed cleaved DNA fragments as assessed by DNA fragmentation assay upon treatment with **MPK-09**. (1) Control, (2) DMSO, (3) 0.6 μM, (4) 2 μM, (5) 4 μM, and (6) 8 μM. (D) Comet assay showing tailing of the PI stained cleaved DNA fragments under electrophoresis treated with **MPK-09** (2 μM). (E) Immunoblot analysis of BT474 cells treated with indicated concentrations of **MPK-09** showing the expression of cleaved PARP and caspase3. β-actin was used as a loading control.

transactivation of its pro-apoptotic signaling pathways as shown by the induction of p21 and Bax protein expression. Our study thus forms the basis for the generation and effective synthesis of more potent and allelic specific p53 activation for better therapeutics. Quest for further analogues for selective killing of cancer cells with p53 mutations will provide a platform for an array of investigations in cancer chemotherapy. Our findings are also a big leap in the direction of personalized cancer medicine in which the efficacy of future cancer drugs will be decided based on the knowledge of the patient's tumor genotype.

METHODS

Transfection. Cells were plated in a 96-well plate and midi prep (Qiagen), p53-Cep4 (kind gift from Prof. Kumaravel Somasundaram, Indian Institute of Science), and R249S (generous gift from Dr. Bert Vogelstein, John Hopkins University) expressing plasmids were transfected using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were treated with **MPK-09**, and cell survival was analyzed using MTT assay.

Immunoprecipitation. Immunoprecipitation of p53 were performed according to the method of Nie et al.[26] using conformational epitope monoclonal antibody PAb1620 (Calbiochem). Protein was

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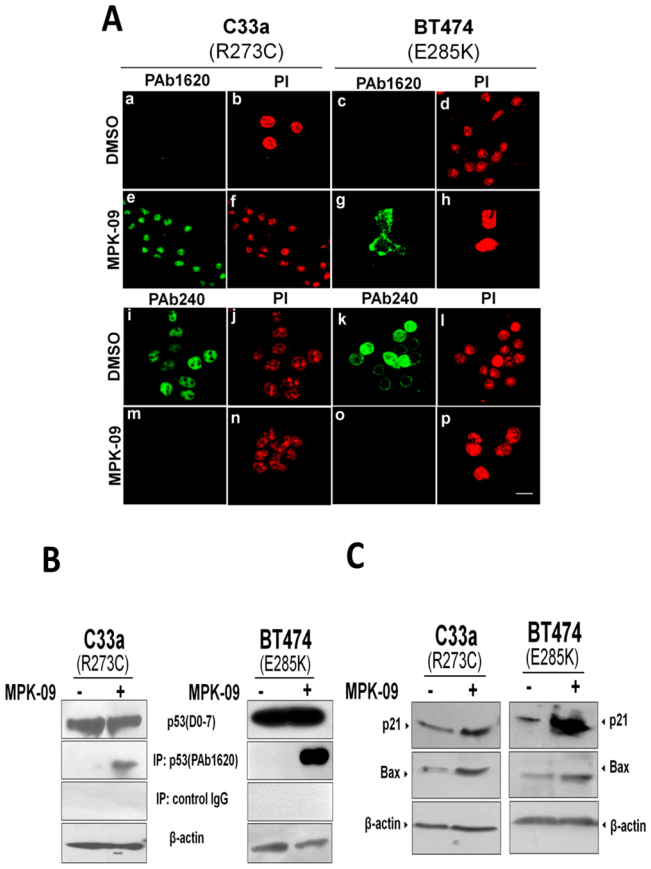

Figure 3. (A) Immunoflorescence staining showing presence of wild-type conformation of p53 in C33a (panel e) and BT474 (panel g) cells upon exposure to **MPK-09** as detected by monoclonal antibody PAb1620. Immunoflorescence staining showing loss of mutant conformation in C33a (panel m) and BT474 (panel o) cells upon exposure to **MPK-09**, as detected by PAb240. Red indicates nuclear staining with PI. (B) Immunoprecipitation of wild-type p53 indicated lysates after exposure to **MPK-09** for 48 h using monoclonal antibody PAb1620 and probed with antibody (DO-7), which can detect both mutant and wild-type p53. (C) Immunoblot analysis showing the induction of p21 and Bax expression in C33a and BT474 cell lines treated with **MPK-09**.

incubated with 30 μL of protein A-agarose beads for 1 h to preclear the lysate. The supernatant from this preclear was immunoprecipitated for 12 h at 4 °C with 2 μg of p53 wild-type conformation-specific antibody (PAb1620, Calbiochem). Precleared lysates were incubated with 30 μL of proteinA-agarose beads for 1 h, and the beads were isolated by centrifugation for 1 min at 1500*g*. Products were resolved by SDS-PAGE (sodium dodecyl-polyacrylamide gel electrophoresis) and transferred on to the PVDF (polyvinylidene difluoride) membrane for immunoblotting. Antibodies used for immunoblotting to detect p53 were monoclonal p53 antibody DO-7 (Calbiochem).

Immunofluoroscence. Cells were plated on coverslips in 12-well plates and then treated with **MPK-09** for 48 h and then fixed with 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100, washed with 1× PBS (phosphate buffer saline) for 2−3 times and then blocked with 10% FBS (foetal bovine serum) for 1 h. p53 were detected with primary antibodies PAb1620 that recognize the wild-type p53 conformational epitope and PAb 240 that recognizes the mutant-type p53 conformational epitope. Antibodies used for Bax staining were the anti-Bax monoclonal antibody (Calbiochem). FITC (fluorescein isothiocynate) conjugated antimouse or rabbit antibody were used as the secondary antibodies. DNA was stained with PI, and cells were visualized using a fluorescence microscope.

Western Blotting. BT474 and C33a cells (4×10^5) were cultured in 6-well plates to 70% confluence. The cells were exposed to **MPK-09** for 48 h, washed once with ice cold PBS, and lysed in the lysis buffer using the method of Foster et al.[27] Protein concentration was determined using Biorad assay reagent. Proteins in cell lysates were separated in 8% (for p21 and Bax) or 12.5% (for p53 and actin) SDS-PAGE gel and electrophoretically transferred onto a PVDF membrane (Immobilin-P, Millipore). The membrane was blocked with 5% milk powder for 1 h and probed with antibodies to p53 (DO-7), actin (Sigma), Bax (Santa Cruz Biotechnology), or p21 (Calbiochem) using chemiluminescence substrate (West Femto Pierce).

Flowcytometry. BT474 cells were plated in 6-well plates at a density of 4×10^5/well and cultured in 10% FBS serum medium until 70% confluence was reached. Cells were treated with different concentrations of **MPK-09** for 48 h in 0.2% FBS containing medium. Floating cells were collected, and adherent cells were washed with PBS and trypsinized. Floating and adherent cells were pooled and centrifuged at 4000 rpm for 5 min. Cells were washed again with PBS, resuspended in 1 mL of PBS followed by fixing in ice cold 70% ethanol at −20 °C overnight. The cells were washed with PBS twice and then incubated with 100 μg/mL of ribonuclease A (Sigma Aldrich) overnight at 37 °C. PI was added to the cell suspension at a final concentration of 20 μg/mL and subjected to flowcytometry (FACs Scan, BD Biosciences, USA) using Cell Quest Pro software using excitation 488 nm laser and emission at 560/670 nm. A minimum of 10 000 cells were acquired per sample, and histograms were analyzed using WinMDI 2.8 software.

DNA Fragmentation Analysis. Cellular apoptosis was determined by DNA fragmentation assay as described earlier.[28] BT474 cells were cultured in 6-well plates at a density of 4×10^5/well in 10% FBS containing medium until 70% confluence was reached. Cells were treated with different concentrations of **MPK-09** for 48 h in 0.2% FBS serum medium. Cells were then pelleted, lysed in 0.4 mL lysis buffer (10 mM Tris-HCl, pH 8.0, 20 mM ethelenediamine tetra acetic acid (EDTA), and 0.2% Triton X-100) and incubated on ice for 20 min. After centrifugation to remove nuclei, soluble chromosomal DNAs including both high molecular weight DNA and nucleosomal DNA fragments were extracted with phenol/chloroform, precipitated with 1/10 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of ethanol and stored at −20 °C overnight. DNA was pelleted by centrifugation, rinsed with 70% ethanol, and then resuspended in water containing 100 μg/mL RNase A. After 2 h of incubation at 37 °C, DNA samples were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV (ultraviolet) light.

Annexin V/Propidium Iodide (PI) Staining and Confocal Microscopy. The early and late apoptotic cells were identified and quantitated using annexin V-FITC apoptosis detection kit (Sigma Aldrich, USA). BT474 cells were cultured in 6-well plates at a density of 4×10^5/well in 10% FBS containing medium until 70% confluence was reached. Cells were treated with different concentrations of **MPK-09** for 48 h in 0.2% FBS serum medium. Cells were collected, washed, and resuspended in 1× binding buffer (HEPES−NaOH 10 mM, pH 7.4, 1.4 M NaCl, and 25 mM $CaCl_2$) at a concentration of 1×10^5 cells/mL. Annexin V-FITC (0.2 μg/μL) and PI (0.05 μg/μL, Sigma) were added and incubated for 30 min. Cells were then subjected to FACS analysis using Cell Quest pro software at an excitation of 488 nm laser and emission at 530 nm. At least 10 000 events were recorded for each sample and represented as dot plots. Confocal fluorescence microscopy was performed to visualize the apoptotic cells generated by **MPK-09**. BT474 cells after 48 h of the treatment were incubated with annexin V-FITC and PI as described above. The cells were then observed under an inverted confocal laser scanning microscope (Zeiss LSM 510 MK4, Germany).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MTT assay experiments, preparation and characterization of compounds described in Chart 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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