Inhibition of MDM2 Re-Sensitizes Rapamycin Resistant Renal Cancer Cells via the Activation of p53

Xin Tian\textsuperscript{a} Shundong Dai\textsuperscript{b,c} Jing Sun\textsuperscript{d} Shenyi Jiang\textsuperscript{e} Chengguang Sui\textsuperscript{a} Fandong Meng\textsuperscript{a} Yan Li\textsuperscript{a} Liye Fu\textsuperscript{a} Tao Jiang\textsuperscript{a} Yang Wang\textsuperscript{a} Jia Su\textsuperscript{a} Youhong Jiang\textsuperscript{a}

\textsuperscript{a}Molecular Oncology Laboratory of Cancer Research Institute, the First Affiliated Hospital of China Medical University, Shenyang, \textsuperscript{b}Department of Pathology, the First Affiliated Hospital and College of Basic Medical Sciences of China Medical University, Shenyang, \textsuperscript{c}Institute of Pathology and Pathophysiology, Shenyang, \textsuperscript{d}Department of Immunology and Biotherapy, Liaoning Cancer Hospital and Institute, Shenyang, \textsuperscript{e}Department of Rheumatology, the First Affiliated Hospital of China Medical University, Shenyang, PR China

Key Words
Rapamycin • Renal cancer • MDM2 • p53

Abstract

\textbf{Background/Aims:} Rapamycin is a potential anti-cancer agent, which modulates the activity of mTOR, a key regulator of cell growth and proliferation. However, several types of cancer cells are resistant to the anti-proliferative effects of rapamycin. In this study, we report a MDM2/p53-mediated rapamycin resistance in human renal cancer cells. \textbf{Methods:} Trypan blue exclusion tests were used to determine the cell viability. Changes in mRNA and protein expression were measured using real-time PCR and western blot, respectively. Xenograft models were established to evaluate the in vivo effects of rapamycin combined with a MDM2 inhibitor. \textbf{Results:} Rapamycin treatment suppresses the expression of MDM2 and exogenous overexpression of MDM2 in A498 cells contributes to rapamycin resistance. By establishing a rapamycin resistant cell line, we observed that MDM2 was significantly upregulated in rapamycin resistant cells than that in rapamycin sensitive cells. Importantly, the rapamycin resistant cells demonstrated attenuated accumulation of p53 in the nucleus in response to rapamycin treatment. Moreover, the inhibition of MDM2 by siMDM2 sensitizes A498 cells to rapamycin through the activation of p53. In both \textit{in vitro} and \textit{in vivo} models, the combination of rapamycin with the MDM2 inhibitor, MI-319, demonstrated a synergistic inhibitory effect on rapamycin resistant cells. \textbf{Conclusion:} Our study reports a novel mechanism for rapamycin resistance in human renal cancer and provides a new perspective for the development of anti-cancer drugs.
Introduction

The mammalian target of rapamycin (mTOR) exists in the form of two complexes: the mTOR complex 1 (mTORC1), which plays a central role in controlling cell growth by regulating protein translation via the modulation of S6K1 and 4EBP1, and the mTOR complex 2 (mTORC2), which regulates cell survival via the phosphorylation of Akt on Ser-473, which fully activates Akt [1, 2]. Rapamycin is an FDA-approved antibiotic and immunosuppressant used to treat tumors [3]. It is currently undergoing clinical trials [4]. Rapamycin inhibits the kinase activity of mTOR by binding to the FK506 binding protein 12 (FKBP12) to dephosphorylate 4EBPs and S6K. This results in the reduction of active eIF4E and S6K levels, which inhibits protein translation [5]. Therefore, mTOR activation is common in cancers and inhibitors of mTOR have been widely studied as effective anti-cancer agents. Despite the solid rationale for developing mTOR inhibitors, rapamycin resistance is a major cause of failed clinical treatments, even with combination therapy [6]. The mechanisms of rapamycin resistance are complicated and are still under investigation. Previous studies have suggested that cells with or without mutation on S6K and 4EBP1 may acquire resistance [7, 8]. Aberrant activation of Akt [9], disruption of p53 signaling [10] and mutations at certain residues of ATM may also result in cells developing rapamycin resistance [11].

The oncoprotein MDM2 is an important negative regulator of the tumor suppressor, p53. MDM2 binds and causes destabilization of p53 [12-14]. MDM2 interacts with a variety of factors affecting RNA biosynthesis, DNA synthesis, cell cycle control, transcription, and cell surface receptor regulation [12]. Following DNA damage, the phosphorylation of MDM2 enables it to function as an E3 ligase which ubiquitinates p53 leading to changes in the protein function and stabilization of p53 [15, 16]. It has been reported that treatment with rapamycin selectively decreases MDM2 protein levels as it inhibits the translation of MDM2 [17, 18]. Moreover, this inhibition leads to an increased p53 to MDM2 protein ratio which results in the activation of p53, indicating that the MDM2-p53 interaction is regulated by rapamycin [17].

Recent reports suggest that small molecule MDM2 antagonists that activate p53 signaling by targeting the MDM2-p53 interaction might be a novel therapeutic target for the treatment of various types of cancers [18]. MI-319 is a synthetic small molecule designed to target the MDM2-P53 interaction by stabilizing p53 and promoting its activity [19]. It binds to the p53 pocket on the surface of the MDM2 molecule and specifically blocks the protein-protein interaction between MDM2 and p53 [19]. In this study, we identified MDM2 as an important regulator involved in rapamycin resistance. Rapamycin resistant renal cancer cells show upregulated MDM2 protein levels and limited activation of p53 in response to rapamycin treatment. In addition, the inhibition of MDM2 by siRNA or MI-319 significantly re-sensitizes the rapamycin resistant renal cancer cells in vitro and in vivo. Our study reveals mechanisms for the MDM2-p53 mediated rapamycin resistance and will provide a new perspective in the development of anti-cancer drugs.

Material and Methods

Cell culture

Human renal carcinoma cells RCC4 and A549 were obtained from ATCC and maintained in RPMI-1640 (Lonza, USA) and Eagle minimal essential medium (ATCC), respectively containing 10% fetal bovine serum (USA Scientific), 2 mM glutamine, and 50 μg/ml gentamycin at 37°C in 5% CO₂.

Antibodies and reagents

Antibodies were purchased from the following manufacturers: MDM2 (Pierce, #4H26L4); p53 (Santa Cruz, #sc-126); α-Tubulin (Santa Cruz, #sc-53646); β-actin (Santa Cruz, #sc-8432) and Lamin B (Santa Cruz, #sc-374015). Rapamycin was purchased from Sigma Aldrich (Shanghai, China) and MI-319 was provided by Sanofi-Aventis (Paris, France).
Isolation of nuclear and cytoplasmic protein

Nuclear and cytoplasmic protein was extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit from Thermo Scientific according to the manufacturer's instruction. Concentrations of purified proteins were measured using Bradford assays.

Cell viability assay

In 6-well plates, $5 \times 10^5$ cells/well was seeded. Eighteen hours later, the medium was replaced with fresh medium. Cells were treated with either rapamycin, MI-319 or both for 48 h at the concentrations indicated in the text. After incubation, cell viability was determined by trypan blue exclusion tests with trypan blue (0.4%) purchased from Sigma Chemical Co. (St. Louis, MO).

Plasmid DNA and siRNA transfection

Expression vectors containing wild type MDM2 were purchased from Addgene. siRNA oligonucleotides for MDM2 were purchased from Sigma, with a scrambled siRNA used as the control. Cells were transfected using the Lipofectamine 2000 Transfection reagent (Invitrogen) according to the manufacturer's protocol. Transfection was performed with 100 nmol/L of siRNA and plasmid DNA was transfected with 2μg of DNA. Forty-eight hours after transfection, whole-cell lysates were prepared for further analysis by Western blot.

cDNA Preparation and Real time RT-PCR

Total RNA was extracted after homogenization of the cells and tissues using RNeasy mini kit (Qiagen Sciences, Maryland MD). Total RNA (1 µg) was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA). The cDNA reaction was diluted to 1:10 and then used as a template for real-time RT-PCR. TaqMan Gene Expression Assay primers and probes specific to MDM2 were used to analyze mRNA expression and 18S ribosomal primers and probes (Applied Biosystems, Foster City, CA) were used as internal controls. PCR amplifications were performed in a final reaction volume of 10 μl containing, 5.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.5 μl of the primers and probes mix, and 4.5 μg of the cDNA-diluted solution. The cycling conditions were as follows: one cycle of 2 minutes at 50°C, one cycle of 10 minutes at 95°C, 40 cycles of denaturation (15 seconds at 95°C) and annealing/extension (1 minute at 60°C). All reactions were carried out in the Step 1 Plus Real-Time PCR Systems Thermocycler (Applied Biosystems, Foster city, CA). All quantitative PCR reactions were carried out in triplicate and repeated at least twice. The ΔCt for mRNA expression was calculated relative to the Ct (threshold cycle) of 18S ribosomal RNA. The relative mRNA expression was calculated using the formula $2^{-\Delta\Delta C_t}$. The primers used for MDM2 Real Time PCR were, forward: 5′AGC GAG TCC ACA GAG A 3′ and reverse: 5′ ATC CTG ATC CAG GCA ATCAC 3′.

Immunofluorescence

For immunofluorescence, the cells were washed in PBS, fixed for 10 min in 4% paraformaldehyde, and mounted on cover slides. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. Samples were blocked by 5% BSA at room temperature for 1 hour and incubated overnight in a freezer at 4°C with the primary antibody followed by the incubation of the secondary antibody (FITC) for 1 hour. The samples were mounted onto coverslips with ProLong Gold Antifade and DAPI reagent (Invitrogen) and were examined using a Zeiss LSM5 EXCITER confocal microscope.

Clonogenic assay

For the cell focus formation assay, 1 × 10³ cells were seeded onto a 10 cm dish with regular cell culture medium. A498 rapamycin resistant and parental cells treated with 600 nM of rapamycin was grown for 3 weeks. The surviving colonies were stained with gentian violet after methanol fixation and the visible colonies (>50 cells) were counted. Colonies from randomly selected image areas of three replicate wells were enumerated.

Western blots

Cells were treated as described in the text and then lysed with RIPA buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% SDS and 1% sodium deoxycholate) supplemented with 1 mM Na3VO4, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Sigma). Equal quantities of protein were loaded
and separated on SDS-polyacrylamide gels and were then transferred to nitrocellulose membranes. Using specific antibodies, the blots were probed overnight for the proteins of interest. This was then followed by a second antibody-horse radish peroxidase conjugate at room temperature for 2 hours. After three 15 min PBS-T washes, signals were detected using ECL Plus Western Blotting Detection Reagents (Amersham) or Western Lighting (PerkinElmer Life sciences).

Xenograft model

The animal experiments were conducted according to the Institutional Animal Care and Use Committee (IACUC)-approved protocol at the Molecular Oncology Laboratory of Cancer Research Institute, the First Affiliated Hospital of China Medical University. Six to eight week old athymic nude/beige mice (Charles River Labs) were implanted subcutaneously with \(1.0 \times 10^7\) A549 rapamycin resistant cells with or without siMDM2 transfection. When the tumors reached 10 mm in diameter, mice were divided into 4 groups of 8 mice and treated daily for 30 days by gavage with rapamycin (30 mg/kg), MI-319 (200 mg/kg), rapamycin+MI-319, or saline (control). Tumors were measured bidimensionally three times a week. Mice were euthanized in their home cage by filling it with CO\(_2\) for 3-5 minutes until mice stopped moving or breathing and eyes were fixed and dilated. Tumor tissue from the sacrificed mice was frozen in liquid nitrogen for western blot analysis and real-time PCR as described in the results. The tumor volumes were calculated with the following formula: volume (mm\(^3\)) = width × length/2, where W and L are the minor and major diameters (in millimeters), respectively.

Calculation of the combination index

The multiple drug effect analysis of Chou and Talaly, based on the median-effect principle, was used to calculate the combined drug effect [20]. The combination index (CI) was calculated using the CompuSyn Software for data analysis of the two-drug combination. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively.

Statistical Analysis

Statistical analyses were carried out using GraphPad StatMate software (GraphPad Software, Inc.). The unpaired Student’s t-test was used to analyze the data. All data were shown as mean ± standard error (SEM). A statistical difference of \(P < 0.05\) was considered significant.

Results

Rapamycin suppresses MDM2 to activate p53

To assess whether rapamycin treatment could regulate the expression of MDM2 we examined MDM2 protein levels in rapamycin-treated A498 and RCC4 cells (both express wild-type p53 protein). As shown in Fig. 1A, rapamycin inhibits MDM2 expression in a dose-dependent manner. As we discussed above, MDM2 negatively regulates the activity of p53 by binding to and destabilizing it [21]. Next, we asked whether rapamycin-mediated MDM2 inhibition could regulate the activity of p53. By transient transfection of MDM2 into A498 cells, we observed that endogenous p53 protein levels were decreased by overexpression of MDM2 (Fig. 1B left). In the absence of rapamycin treatment, p53 accumulated in the cytoplasm, but when subjected to rapamycin treatment, p53 translocated into the nucleus to be activated as a transcription regulator (Fig. 1B right). In addition, exogenous MDM2, in the presence of rapamycin treatment, prevented the translocation of p53 into the nucleus (Fig. 1B right), indicating that p53 is involved in the rapamycin-induced inhibition of MDM2. To investigate the role of MDM2 in rapamycin-induced apoptosis, we transiently transfected MDM2 overexpressing vectors into A498 cells. The cells were then treated with rapamycin at multiple concentrations for 48 hours. Our data demonstrated that renal cancer cells with exogenous MDM2 were more resistant to rapamycin at 100, 400 and 800 nM (Fig. 1C). The IC\(_{50}\) of control cells was about 250 nM, but the IC\(_{50}\) was elevated to 600 nM with a higher amount of MDM2, suggesting that MDM2 contributes to rapamycin resistance in renal cancer cells.
Rapamycin resistance is associated with the up-regulation of MDM2 in human renal cancer cell lines

Our above data revealed that overexpression of MDM2 rendered cancer cells resistant to rapamycin, possibly through the inhibition of p53. The potential role of MDM2 in the response of cancer cells to rapamycin was investigated by comparing MDM2 protein levels in rapamycin-sensitive (parental) and -resistant cells derived from A498 human renal cancer cell lines. Rapamycin resistant cells were established by gradual treatment with increasing concentrations of rapamycin for up to four months. Resistant cell clones were developed and pooled for the subsequent experiments in this study. Rapamycin inhibited the viability of A498 parental cells but not the viability of A498 rapamycin resistant cells (Fig. 2A). The IC50 increased from 200 nM (sensitive cells) to 1200 nM (resistant cells). To verify rapamycin resistance, clonogenic assay was performed. Figure 2B reveals that the
A498 rapamycin resistant cells survived and formed colonies, while parental cells hardly survived under the same rapamycin treatment. As we expected, the protein and mRNA levels of MDM2 were upregulated in rapamycin resistant cells compared with sensitive cells (Fig. 2C), indicating that the elevated MDM2 levels in rapamycin resistant cells might account for their resistance. Since MDM2 is a negative regulator of p53, we next asked whether the activity of p53 was involved in the resistance to rapamycin. By measuring the localization of p53, our results showed that rapamycin treatment induced the translocation of p53 into nucleus in rapamycin sensitive cells. In contrast, p53 was mainly found in the cytoplasm of resistant cells during rapamycin treatment at 200 nM (Fig. 2D). These data suggest that MDM2 is important in rapamycin resistance as it prevents the activation of p53. MDM2 may present a new therapeutic target in the development of drugs used to overcome rapamycin resistance.

**MDM2 knockdown sensitizes renal cancer cells to rapamycin**

As shown above, MDM2 was upregulated in rapamycin resistant renal cancer cells. We next explored whether suppression of MDM2 could sensitize A498 cells to rapamycin. MDM2
knockdown by siRNA specifically decreased the protein level of MDM2 in both A498 and RCC4 cells (Fig. 3A & 3B left). Cells transfected with control siRNA and siMDM2 were treated with rapamycin at the indicated concentrations for 48 h, followed by cell viability assays. Columns show the mean of three independent experiments; bars show the SEM. *, ** and *** represents P < 0.05, P < 0.01 and P < 0.001 respectively.

Fig. 4. Inhibition of MDM2 re-sensitizes rapamycin resistant cells through the activation of p53. (A) A498 rapamycin resistant cells were transfected with siMDM2 or control siRNA for 48 h, and then cells were treated with rapamycin at the indicated concentrations for 48 h, followed by a cell viability assay. (B) The cellular distribution of p53 in A498 rapamycin resistant cells with or without transfection of siMDM2 was observed using immunofluorescence staining of p-53 (FITC) and the nucleus (DAPI) under rapamycin treatment at 200 nM for 24 h. (C) In A498 rapamycin resistant cells, MI-319 inhibited the MDM2/p53 ratio in a dose-dependent manner. Cells were treated with MI-319 at 5μM or 10μM for 24 h and were then analyzed for MDM2 and p53 expression by western blot. (D) Treatment with a combination of rapamycin and MI-319 showed synergistic effects on A498 rapamycin resistant cells. Columns show the mean of three independent experiments; bars show the SEM. *, ** and *** represents P < 0.05, P < 0.01 and P < 0.001 respectively. Scale bars, 10 mm.
with rapamycin at the indicated concentrations for 72 hours, after which cell viability was measured. A498 and RCC4 cells with MDM2 knockdown responded to rapamycin with similar potency and all cell viabilities were effectively inhibited by rapamycin in a dose-dependent manner (Fig. 3A & 3B right).

**Inhibition of MDM2 re-sensitizes rapamycin resistant cells to rapamycin through the activation of p53 in vitro and in vivo**

Since MDM2 knockdown by siRNA enhanced the sensitivity of renal cells to rapamycin, we hypothesized that the inhibition of MDM2 might overcome rapamycin resistance through the activation of p53. To verify this, we transfected control siRNA or siMDM2 into A498 rapamycin resistant cells to compare cell viability under multiple treatments of rapamycin. As we expected, knocking down MDM2 significantly reversed the resistance to rapamycin (Fig. 4A). The IC50 of control cells was 1200 nM, which is about six-fold higher than the IC50 of MDM2-knockdown cells. Rapamycin induced the translocation of p53 into nucleus in control cells, leading to the inhibition of cell viability. In accordance with this, the suppression of MDM2 promoted the translocation of p53 into the nucleus (Fig. 4B), suggesting that the ablation of MDM2 led to the re-sensitization of rapamycin resistant cells to rapamycin through the activation of p53. To investigate whether our discovery is clinically significant, we specifically inhibited MDM2 using a MDM2 inhibitor, MI-319, which is known to restore the functions of p53 by disrupting the MDM2–p53 interaction. Treatment with MI-319 at 5 μM and 10 μM significantly inhibited the expression of MDM2 and inversely increased the expression of p53 (Fig. 4C). As a result of treatment with rapamycin and MI-319, we observed a synergistic inhibition of cell viabilities in A498 rapamycin resistant cell by calculating the combination index (CI) value (Fig. 4D & Table 1, 2). Our data revealed that the MDM2 inhibitor could be clinically applied with rapamycin to enhance its therapeutic efficacy. Subsequently, we sought to verify the in vitro results in vivo using a xenograft mouse model. We investigated whether the combination of rapamycin and MI-319 could re-sensitize the rapamycin resistant cells. Rapamycin resistant A498 cells transfected with control siRNA or siMDM2 were injected subcutaneously into mice. Rapamycin alone (30 mg/kg) or with MI-319 (200 mg/kg) were administered orally to the animals twice a day. At this dose, rapamycin alone displayed no major inhibitory effect on tumor formation whereas the administration of MI-319 and rapamycin resulted in a decrease in tumor size in all animals. In accordance with this, mice injected with A498-siMDM2 cells demonstrated a similar potency to rapamycin treatment (Fig. 5A & 5B). To further confirm our findings, the tumors and total protein were isolated for western blot analysis. In vivo data showed MDM2 was inhibited by MI-319 at both protein and mRNA level (Fig. 5C & 5D). Taken together, our in vivo results

### Table 1

| Concentration ratio (molar) of Rapamycin to MI-319: 1:200:1:100:1:50:1:25:1:17 | Dose: Rapamycin (nM) | MI-319 (nM) | Total (nM) | Effect: |
|---|---|---|---|---|
| 50 | 10000 | 10050 | 0.346 |
| 100 | 10000 | 10100 | 0.421 |
| 200 | 10000 | 10200 | 0.563 |
| 400 | 10000 | 10400 | 0.685 |
| 600 | 10000 | 10600 | 0.724 |

Combination Index (CI): 0.438

### Table 2

| Concentration ratio (molar) of Rapamycin to MI-319: 1:400:1:200:1:100:1:50:1:34 | Dose: Rapamycin (nM) | MI-319 (nM) | Total (nM) | Effect: |
|---|---|---|---|---|
| 50 | 20000 | 20050 | 0.495 |
| 100 | 20000 | 20100 | 0.572 |
| 200 | 20000 | 20200 | 0.691 |
| 400 | 20000 | 20400 | 0.804 |
| 600 | 20000 | 20600 | 0.913 |

Combination Index (CI): 0.804
confirmed that the combination of rapamycin and the MDM2 antagonist MI-319 exhibit synergistic effects on rapamycin resistant renal cancer.

**Discussion**

Intrinsic sensitivity to rapamycin between cancer cells may vary and the mechanisms underlying rapamycin resistance are still under investigation. In this study, we report a novel mechanism for the MDM2-p53-mediated rapamycin resistance in human renal cancer cells. We observed that MDM2 was downregulated by rapamycin treatment, consistent with a previous study that showed rapamycin increased the p53/MDM2 ratio in colon cancer cell lines through hypophosphorylation of 4EBP-1 [21]. Moreover, the decrease in MDM2 expression correlated with the increased sensitivity of cells to rapamycin: exogenous expression of MDM2 rendered cancer cells resistant to rapamycin. Interestingly, from establishing the rapamycin-resistant cell line, we found that MDM2 was significantly upregulated in rapamycin resistant cells.

p53 is a tumor suppressor controlled primarily by its protein stability through MDM2-mediated ubiquitination and degradation. Therefore, cancer cells are often resistant to apoptosis induced by chemotherapeutic reagents that downregulate p53. Although evidence suggests that rapamycin increases the sensitivity of cancer cells to DNA damage, we demonstrated that increased MDM2 protein levels represent another mechanism responsible for increased resistance in rapamycin resistant cells. It has been reported that a
constitutively active AKT signal downstream of mTOR contributes to rapamycin resistance [9]. Our data illustrated that the cells with upregulated MDM2, which is the downstream effector of rapamycin, were more resistant to rapamycin through the inactivation of p53. Although our data showed that rapamycin treatment inhibits MDM2 expression, there might also be an indirect mechanism underlying MDM2-mediated rapamycin resistance. Rapamycin resistant cells might express decreased amount of cellular p53 to suppress the expression of apoptosis-inducing genes in response to rapamycin. We report that the inhibition of MDM2 by siRNA or the inhibitor MI-319 re-sensitized rapamycin resistant cells both in vitro and in vivo, indicating a novel strategy for reversing rapamycin resistance in human renal cancer.

Both MDM2 and p53 shuttle back and forth between the nucleus and the cytoplasm. It has been proposed that MDM2 promotes p53 short ubiquitination in the nucleus, which facilitates p53 nuclear export [22]. Our results revealed that, in rapamycin resistant cells, the majority of p53 accumulated in the cytoplasm due to its interaction with MDM2, indicating that MDM2-mediated rapamycin resistance occurs through the inhibition of p53. The two cell lines in this project, A549 and CCR4, are both p53 wild-type renal cancer cell lines. However, the phenotype was not detected in 768-O cells, which is a renal cancer cell line with a mutant p53. To our knowledge, this is the first report showing that the activity of an MDM2 inhibitor in combination with rapamycin against human renal cancer cells is p53-dependent. Moreover, our in vivo experiment demonstrates that, when administered orally to the animals, the combination of MDM2 inhibitor with rapamycin showed synergistic anti-tumor activity. However, the detailed mechanisms for the regulatory correlation between rapamycin and MDM2 are not clear. In our future project, we will focus on the signal pathways downstream of mTOR/AKT to explore the mechanism behind the up-regulation of MDM2 in rapamycin resistant cells. Proteomic and bioinformatics experiments will be performed to investigate the protein-protein interactions involved in this pathway. In summary, our study provides a basis for the development of therapeutic strategies that utilize MDM2 inhibitors to reverse rapamycin resistance.

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Disclosure Statement

None.

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Tian et al.: Inhibition of MDM2 by the Activation of p53

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