MDM2 antagonist Nutlin-3a protects wild-type p53 cancer cells from paclitaxel

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Received April 14, 2011; accepted July 27, 2011

The p53 pathway has an important role in cell cycle arrest and apoptosis. Downregulated levels of p53 have been shown to increase resistance to the cytotoxic effects of chemotherapy or radiotherapy. MDM2 (murine double minute 2) is able to bind p53 and modulate its transcriptional activity and stability. We studied the effect of Nutlin-3a, an MDM2 antagonist, on the response of non-small cell lung cancer cell lines, A549 (p53+/+) and H1299 (p53–/–), to paclitaxel (Taxol). A549 cells treated with Nutlin-3a plus paclitaxel showed a significant increase in MDM2 and wild-type p53 protein, a marked increase in the number of cells in the G0-G1 and G2-M phases, and a significant decrease in the percentage of cells in the S phase. The percentage of apoptotic A549 cells treated with 10 μ mol/L Nutlin-3a plus 10 nmol/L paclitaxel was significantly lower than those treated with paclitaxel alone, and was also lower than that observed in H1299 cells. MTT assays demonstrated that Nutlin-3a plus paclitaxel also significantly reduced the sensitivity of A549 cells to paclitaxel compared with that of H1299 cells. In conclusion, Nutlin-3a mediates the cytotoxic effect of paclitaxel depending on p53 status. It may also protect wild-type p53 cells from mitotic chemotherapeutics.

non-small cell lung carcinoma, p53, MDM2 antagonist, paclitaxel

Citation: Shen H C, Dong W, Gao D W, et al. MDM2 antagonist Nutlin-3a protects wild-type p53 cancer cells from paclitaxel. *Chin Sci Bull*, 2012, 57: 1007–1012, doi: 10.1007/s11434-012-4984-7

Lung cancer is the leading cause of cancer-related deaths in the world with the number of deaths more than 1180000 annually [1]. Because 65% of lung cancer patients are in the advanced stage, and about 80% of lung cancer is composed of non-small cell carcinomas (NSCLC), chemotherapy is the main treatment. However, NSCLC is more resistant to chemotherapy than other forms of cancer [2,3]. Therefore, the development of a novel and efficient therapy to sensitize NSCLC to chemotherapy is urgently required.

The p53 pathway has an important role in cell cycle arrest and apoptosis. Defects in the p53 network are thought to be involved in a massive percentage of tumors [4,5]. Restoration of the p53 tumor suppressor pathway should, in theory, trigger considerable apoptosis of the tumor. In addition, downregulated levels of p53 have been shown to in-

crease resistance to the cytotoxic effects of chemotherapy or radiotherapy [6,7]. Hence, p53 is an appealing target for novel anticancer therapeutic strategies.

Murine double minute 2 (MDM2) is the primary negative regulator of p53, and is an E3 ubiquitin ligase that transports p53 to the cytoplasm, where it promotes p53 ubiquitination and degradation by the proteasome [8]. In proliferating cells, p53 levels are tightly controlled by MDM2, which modulates its transcriptional activity and stability following binding [9–11]. MDM2 and p53 form a negative feedback loop [12]; therefore, it is possible to activate the p53 pathway by inhibiting MDM2.

Recently, small-molecule inhibitors of MDM2, Nutlins, have been developed, and found to have a high affinity for MDM2, preventing binding to p53 [13–15]. Nutlins can release p53 from the negative control of MDM2 and activate the p53 pathway to promote cell cycle arrest and apop-

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tosis in cancer cells that express wild-type p53. Nutlin-3a increases radiosensitivity of H460 and Val138 cells [16], and sensitizes cancer cells to chemotherapeutic agents such as cisplatin, and etoposide [17]. However, some researchers found that Nutlins can protect non-malignant cells and tissues [18,19]. Therefore, it is not clear whether Nutlins are effective agents for chemosensitization, or are able to protect cells from chemotherapy in wild-type p53 and p53-deficient cancer cells.

The objective of this study was to evaluate the effects of Nutlins on NSCLC cell lines, A549 (wild-type p53) and H1299 (p53-deficient), when used in conjunction with a commonly used chemotherapeutic, paclitaxel.

1 Materials and methods

1.1 Cell culture

Human NSCLC cell lines A549 and H1299 were cultured in RPMI-1640 with 10% fetal calf serum (FBS, Gibco, Grand Island, NY, USA) and Dulbecco Modified Eagle Medium supplemented with 10% FBS at 37°C in 5% CO₂, respectively. Cells were used when in the exponential growth phase.

1.2 Reagents

Nutlin-3a (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO and stored as 20 mg/mL stock solution in small aliquots at -20°C to avoid repeated freeze-thaw cycles. A stock solution of 1×10⁶ nmol/L paclitaxel (Hainan Shunyuan Chemotech, Hainan, China) diluted in alcohol was stored at -20°C. Anti-MDM2 (SMP14) and anti-p53 (DO-1) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-β-actin (AC-15) antibody was from Sigma-Aldrich. All other reagents were from Sigma-Aldrich unless otherwise stated.

1.3 Western blot analysis

A549 and H1299 cells were incubated with 10 μmol/L Nutlin-3a, 10 nmol/L paclitaxel and a combination of 10 μmol/L Nutlin-3a + 10 nmol/L paclitaxel in 25 cm² flasks in 5 mL medium for 8 h. Cells were harvested and lysed in protein lysis buffer. Equal amounts of protein lysate were loaded into each well and separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis for 2 h at 100 V, followed by transferring proteins to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were incubated with primary antibodies against p53 (1:500; Santa Cruz Biotechnology), MDM2 (1:500; Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich) overnight at 4°C, followed by secondary horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000; Sigma-

Aldrich). β-actin was used for loading control in all experiments. Proteins were traced by enhanced chemiluminescence Western blot detection reagent (Amersham Biosciences). Optical density (OD) protein levels were normalized to corresponding β-actin and results were expressed as an OD ratio.

1.4 Cell cycle analysis

A549 and H1299 cells were treated with a DMSO vehicle (untreated control), 5 μmol/L Nutlin-3a, 10 nmol/L paclitaxel or a combination of 5 μmol/L Nutlin-3a and 10 nmol/L paclitaxel for 48 h. After treatment, cells were collected with 0.25% Trypsin (Gibco) and washed twice in 0.01 mol/L PBS. After centrifugation at 2000 r/min for 5 min, cells were suspended in PBS and fixed in ice-cold ethanol (70%, v/v) at 4°C overnight and stained with 1 mL propidium iodide (PI) solution (50 μg/mL PI, 100 μg/mL RNAase A, 10 mmol/L EDTA, 0.5% Triton X-100) at 4°C for 30 min in the dark. Cell cycle distribution of samples was determined using a FACScan flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and data were analyzed using ModFit and CellQuEST software (Becton, Dickinson and Company).

1.5 Cell apoptosis analysis

A549 and H1299 cells were treated with a DMSO vehicle (untreated control), 5 μmol/L Nutlin-3a, 10 nmol/L paclitaxel or a combination of 5 μmol/L Nutlin-3a and 10 nmol/L paclitaxel for 48 h. After treatment, cells were collected with 0.25% Trypsin and washed twice in chilled 0.01 mol/L PBS. After centrifugation at 2000 r/min for 5 min, cells were suspended in Flow Cytometry Buffer and adjusted to a concentration of 1×10⁶ cells/mL. Next, 5 μL AnnexinV/FITC and 10 μL PI solution were added to 100 μL cell suspension for 15 min in the dark. Samples were measured using a FACScan flow cytometer (Becton, Dickinson and Company). Cell apoptosis was analyzed using WinMDI2.9 software (Becton, Dickinson and Company).

1.6 MTT assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT, Sigma-Aldrich) assays were used to survey and evaluate cell survival in a quantitative colorimetric assay. The cells (3×10³/well) were seeded into 96-well plates and incubated at 37°C in RPMI-1640 and DMEM mediums respectively. After 24 h culture, A549 and H1299 cells were treated with varying concentrations of paclitaxel (0, 0.1, 1, 10, 100, 1000 nmol/L) and Nutlin-3a (0, 5, 10, 15 μmol/L) for 72 h, and 10 nmol/L paclitaxel and/or 5 μmol/L Nutlin-3a for variable time points (0, 24, 48, 72 h) in quintuplet. Medium alone, and medium plus DMSO acted as control

for each dilution. A 20 μ L volume of MTT was added to each well, then plates were incubated for an additional 4 h at 37°C. The medium was then removed and replaced with 150 μ L DMSO in each well. Plates were shaken for 15 min at room temperature, and read at 490 nm in a Bio-Rad 680 Microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

1.7 Statistical analysis

Data were expressed as means $\pm$ standard deviations (SD). Statistical significance between groups was determined by the analysis of variance and Student-Newman-Keuls multiple comparison test (q test) after confirming the equality of variance with the Levene test using SPSS statistical software version 13.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was deemed statistically significant.

2 Results

2.1 Expression of p53 and MDM2 protein

Expression of wild-type p53 and MDM2 protein was determined by Western blot. As shown in Figure 1, wild-type p53 is expressed in A549 cells but not in H1299 cells, while MDM2 is expressed in both A549 and H1299 cell lines. The addition of Nutlin-3a to A549 cells resulted in dose-dependent increases in p53 and MDM2 protein expressions. In contrast, there was no change in MDM2 and p53 expressions in H1299 cells. A549 cells treated with Nutlin-3a or paclitaxel demonstrated a slight increase in wild-type p53 and MDM2

protein, but those treated with Nutlin-3a plus paclitaxel showed significant upregulation. Conversely, there was almost no increase in wild-type p53 and MDM2 protein in H1299 cells treated with Nutlin-3a and/or paclitaxel.

2.2 Cell cycle arrest by Nutlin-3a and paclitaxel

To determine if Nutlin-3a inhibition of MDM2 affects progression through the cell cycle, A549 and H1299 cells were treated with a vehicle or Nutlin-3a followed by paclitaxel. The percentage of cells in each stage of the cell cycle was then determined using flow cytometry. As shown in Figure 1(a), A549 cells treated with Nutlin-3a alone showed a decrease in the proportion in the S phase (11.4% vs. 27% control), a greater increase in the percentage of cells in G0-G1 (72.1% vs. 59% control), and a slight increase in the percentage of cells in G2-M (16.5% vs. 14% control). A549 cells treated with Nutlin-3a plus paclitaxel showed a marked increase in the number of cells in G0-G1 (71.7%) and G2-M phase (20.9%), and a significant decrease in the percentage of cells in the S phase (7.4%). In contrast, there was almost no change in cell cycle distribution following administration of Nutlin-3a and/or paclitaxel (Figure 2(a)).

2.3 Nutlin-3a protects wild-type p53 cells from paclitaxel-induced apoptosis

The percentage of apoptotic A549 cells treated with 10 μ mol/L Nutlin-3a plus 10 nmol/L paclitaxel was 13.7% $\pm$ 1.67%, which was significantly lower compared with that in H1299 cells (34.8% $\pm$ 3.52%, $P < 0.05$) (Figure 2(b)).

Figure 1 (a) Expression of MDM2 and wild-type p53 in A549 and H1299 cells or (b) in A549 and H1299 cells treated with varying concentrations of Nutlin-3a (0, 5, 10, 30 μ mol/L). (c) A549 and (d) H1299 cells were incubated in 25-cm² flasks in 5-mL medium for 8 h with vehicle (untreated control), 10 μ mol/L Nutlin-3a, 10 nmol/L paclitaxel, or a combination of 10 μ mol/L Nutlin-3a and 10 nmol/L paclitaxel. Expressions of MDM2 and wild-type p53 protein were determined using Western blot. PTX, paclitaxel; Nut, Nutlin-3a.

Figure 2 Cell cycle distribution and apoptosis. (a) Effect of Nutlin-3a and paclitaxel (PTX) on cell cycle arrest. Cell cycle distribution following 24 h treatment with DMSO (control), 5 $\mu\text{mol/L}$ Nutlin-3a, 10 nmol/L PTX or a combination of 5 $\mu\text{mol/L}$ Nutlin-3a pre-treatment for 24 h and then 10 nmol/L PTX. (b) Apoptosis induced by 5 $\mu\text{mol/L}$ Nutlin-3a, 10 nmol/L PTX or a combination of 5 $\mu\text{mol/L}$ Nutlin-3a and 10 nmol/L PTX for 48 h. Percentage of apoptotic cells was determined by flow cytometry. * $P < 0.05$.

2.4 Nutlin-3a reduces sensitivity of wild-type p53 cells to paclitaxel

A549 and H1299 cell lines were exposed to increasing doses of paclitaxel alone or in combination with 5, 10 or 20 $\mu\text{mol/L}$ Nutlin-3a for 72 h. Nutlin-3a plus paclitaxel significantly reduced the sensitivity of A549 cells compared with that in H1299 cells ($P < 0.05$) (Figure 3). In another experiment, A549 and H1299 cells were pretreated with 10 $\mu\text{mol/L}$ Nutlin-3a for 24 h, and/or treated with 10 nmol/L paclitaxel for an additional 24, 48 or 72 h. Nutlin-3a protected A549 cells from paclitaxel (Figure 3).

3 Discussion

Chemotherapy is the main treatment modality for most NSCLC patients, but there are many side-effects, such as hematologic toxicity, gastrointestinal reaction, second malignancies and drug hypersensitivity. Hence, there is an urgent need to reduce the dosage of chemotherapeutic agents and to sensitize cancer cells to chemotherapy. However, caution must be exercised when choosing the right agents to sensitize cells to chemotherapy.

The p53 tumor suppressor gene controls the activity of multiple genes involved in cell aging, cell cycle arrest, and apoptosis [20]. The p53 has an important role in preventing

Figure 3 Cell survival percentage was determined using MTT assays. (a), (c) A549 and H1299 cell lines were exposed to increasing doses of paclitaxel (PTX) alone or in combination with 5, 10 and 20 $\mu\text{mol/L}$ Nutlin-3a for 72 h. Nutlin-3a significantly protects A549 cells from PTX in comparison with H1299 cells ($P < 0.05$). (b), (d) A549 and H1299 cell lines were pre-treated with 10 $\mu\text{mol/L}$ Nutlin-3a for 24 h, and/or treated with 10 nmol/L PTX for an additional 24, 48 or 72 h.

tumor formation through transcription-dependent and transcription-independent pro-apoptotic activities. One major function of p53 is the activation of G1-S and G2-M checkpoints of the cell cycle through transcriptional stimulation of p21 [21]. The p53 also can trigger mitochondrial outer membrane permeabilization and apoptosis in the absence of transcription through direct activation of Bax or Bak or through binding to Bcl-2 or Bcl-XL. The expression of wild-type p53 in most NSCLC represents a potent molecular target for therapy [22].

As a main regulator of tumorigenesis and progression, MDM2 inhibits the expression of wild-type p53, suggesting that the inhibition of MDM2 partially contributes to the elevated activity of wild-type p53 in NSCLC [23,24]. MDM2 and p53 form a negative feedback loop [12], which is very important for tumorigenesis. If the feedback loop is interrupted by an agent such as Nutlin-3a, the tumor cell will undergo apoptosis. Nutlin-3a has been studied as an effective agent to induce cell cycle arrest and apoptosis in some tumors, and has been found to increase radiosensitivity of H460 and Val138 cells [16], and to sensitize cells to chemotherapeutic agents such as cisplatin and etoposide [17]. To date, there has been no study of Nutlin-3a as a chemosensitizer for the commonly used agent paclitaxel.

In our study, we detected the expression of MDM2 and wild-type p53 in A549 and H1299 cells treated with paclitaxel or paclitaxel plus Nutlin-3a using Western blot. We found that A549 cells treated with Nutlin-3a plus paclitaxel showed a significant upregulation of MDM2 and wild-type p53 proteins, but this effect was not observed in H1299 cells undergoing the same treatment regime. These results showed that Nutlin-3a effectively inhibited the activity of MDM2 and enhanced the wild-type p53 activity, and that there was a synergistic effect of Nutlin-3a and paclitaxel in wild-type p53 cells.

Paclitaxel is a cell cycle-dependent chemotherapeutic that targets mitotic cells. A549 cells treated with Nutlin-3a alone showed a greater increase in the percentage of cells in G0-G1 phase. A549 cells treated with Nutlin-3a plus paclitaxel showed a marked increase in the number of cells in G0-G1 and G2-M phase, and a significant decrease in the percentage of cells in the S phase. Nutlin-3a could arrest cell cycle at the G0-G1 and G2-M phases in these cells. Nutlin-3a plus paclitaxel protected wild-type p53 cells from the cytotoxic effects of paclitaxel. However, Nutlin-3a had no effect on the cell cycle of p53-deficient (H1299) cells, suggesting it has no protecting effect. The percentage of apoptotic A549 cells treated with 5 $\mu\text{mol/L}$ Nutlin-3a plus 10 nmol/L paclitaxel was significantly lower than those only treated with paclitaxel, and compared with the effect observed in H1299 cells. Proliferation assay results showed that Nutlin-3a plus paclitaxel also significantly reduced A549 cell sensitivity to paclitaxel compared with H1299 cells. Considering all this evidence, Nutlin-3a appears to interrupt the MDM2-p53 feedback loop. The elevation of p53 affected the expression of down-

stream genes such as *p21*, *Bcl-2*, *Bax*, causing cell cycle arrest in the G0-G1 and G2-M phases, which results in resistance to mitotic chemotherapeutics such as paclitaxel. Therefore, the combination of Nutlin-3a and mitotic chemotherapeutics is not a wise choice for treatment of cancers expressing wild-type p53.

In conclusion, Nutlin-3a mediates the cytotoxic effect of paclitaxel depending on the p53 status. It may protect wild-type p53 cells from mitotic chemotherapeutics, and therefore it is necessary to choose combined chemotherapeutic agents with caution and to develop treatments individually.

This work was supported by the National High-Tech Research and Development Program of China (2007AA021802) and the Provincial Natural Science Foundation of Shandong (ZR2010HM067 and ZR2011HM077). There is no conflict of interest for all authors.

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