MDM2 inhibitor Nutlin-3a suppresses proliferation and promotes apoptosis in osteosarcoma cells

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Restoring p53 activity by inhibiting the interaction between p53 and the mouse double minutes clone 2 (MDM2) offers an attractive approach to cancer therapy. Nutlin-3a is a small-molecule inhibitor that inhibits MDM2 binding to p53 and subsequent p53-dependent DNA damage signaling. In this study, we determined the efficacy of Nutlin-3a in inducing p53-mediated cell death in osteosarcoma (OS) cell lines both in vivo and in vitro. Targeted disruption of the p53-MDM2 interaction by Nutlin-3a stabilizes p53 and selectively activates the p53 pathway only in OS cells with wild-type p53, resulting in a pronounced anti-proliferative and cytotoxic effect due to G1 cell cycle arrest and apoptosis both in vitro and in vivo. p53 dependence of these alternative outcomes of Nutlin-3a treatment was shown by the abrogation of these effects when p53 was knocked-down by small interfering RNA. These data suggest that the disruption of p53-MDM2 interaction by Nutlin-3a might be beneficial for OS patients with MDM2 amplification and wt p53 status.

Keywords osteosarcoma; Nutlin; MDM2; p53; p21

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Introduction

Osteosarcoma (OS) is among the commonest of primary tumors of bone, with one of the most morphologically heterogeneous characteristics seen in histopathology, appearing in distinct clinical forms with different degrees of malignancy [1]. Most variants of OS are extremely aggressive as characterized by their rapid growth and early development of distant metastasis, mostly to lung and bones. Death from OS is usually the result of respiratory failure due to progressive pulmonary destruction from metastasis [2,3].

Standard treatment involves the use of ’up-front’ multi-agent chemotherapy, definitive surgery of the primary tumor, and postoperative chemotherapy. Introduction of adjuvant and neoadjuvant cytotoxic treatment and improvements in surgical procedures have increased 5-year survival to over 60% compared to <20% when only surgery and/or radiation therapy had been used [4,5]. Despite attempts to further increase the disease-free survival for poorly responding patients through administration of more intensified therapy, no survival benefit has been convincingly shown [6–8].

Gene therapy is a treatment for disease by the introduction of a therapeutic gene or by the manipulation of a disease-related gene, such as abrogation of an activated oncogene. The type of tumor suppressor gene therapy tries to restore the function of a tumor suppressor gene which is lost or functionally inactivated in cancer cells. In other words, tumor suppressor genes can be defined as genes in which loss-of-function mutations are oncogenic. They usually regulate diverse cellular activities, for example, cell-cycle checkpoint responses, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, cell specification, differentiation, migration, and tumor angiogenesis [9].

The p53 gene has an attractive apoptotic tumor suppressor profile as a therapeutic agent. Introduction of wild-type (wt) p53 leads to specific death of tumor cells, including OS cells [10].

MDM2 is a master regulator of p53 [11] which controls p53 function by inhibiting p53-mediated transcriptional activity [12,13] and by promoting p53 degradation [14,15]. Binding of MDM2 to p53 is essential for this effect. Importantly, the mdm2 gene is a target gene for p53. p53 binds to p53-responsive elements located within the mdm2 gene and promotes the production of mdm2 transcripts [16,17]. Consequently, an autoregulatory negative feedback loop exists, wherein p53 induces MDM2 expression and
MDM2 represses p53 activity. This serves as an important mechanism for restraining p53 function in the absence of stress. Cellular stress induces the modifications of both p53 and MDM2 proteins, which results in a reduced avidity of p53 for MDM2 and, thereby an inhibition of ubiquitination and a degradation of p53. An inappropriate excess of MDM2 may result in exaggerated silencing of p53, which abrogates its tumor suppressor effects.

Nutlin-3 inhibits p53-MDM2 interaction and activates p53 signaling in cancer cells [17–20]. Nutlin-3, an active emantiomer of Nutlin-3a, is an MDM2 antagonist and proves to be beneficial in the treatment of Ewing’s sarcoma cells [21,22]. Here we report the effect of Nutlin-3a on the proliferation and apoptosis of OS cells and its relationship with p53 status.

Materials and Methods

Cell culture and reagents

Human OS cell lines U-2 OS (wt p53) and MG63 (mut p53) cell lines were purchased from the American Type Culture Collection (Manassas, USA). OS SaOS2 cells (null p53) cell line was a kind gift from Dr Qiulin Zhang (Department of Orthopaedic, Changhai Hospital, Second Military Medical University, Shanghai, China). U-2 OS cells were maintained in McCoy’s 5A medium (Biowish, Co. Ltd., Hangzhou, China) supplemented with 10% fetal bovine serum (FBS; Biowish). MG63 cells, which have a rearrangement mutation of p53 gene resulting in disruption of p53 protein function [23], were maintained in Eagle’s minimum essential medium (EMEM) supplemented in 10% FBS. SaOS2 cells were maintained in Dulbecco’s modified eagle’s medium (DMEM) (Sigma-Aldrich, St Louis, USA) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 U/ml) at 37°C in 5% CO2.

Nutlin-3a was purchased from Cayman Chemical (Shanghai, China) was dissolved in ethanol as a 10 M stock solution, and stored at −20°C. Confluent serum-starved U-2 OS (wt p53), MG63 (mut p53) and SaOS2 (null p53) cells were exposed to 0–100 μM Nutlin-3a (active) for the time indicated. Final ethanol concentration was kept constant in each experiment.

Western blot analysis

Cells were harvested in lysis buffer containing 1% Triton X-100, 1 mM Pefablock, 10 μg/ml aprotinin, 1 μg/ml peptatin, 10 μg/ml leupeptin, 10 mM NaF, and 1 mM Na2VO4. Protein was determined using Bradford assay kit (Bio-Rad, Richmond, USA). Equal amounts of protein (40 μg) for each sample were separated on polyacrylamide gels and blotted onto nitrocellulose membranes. The membranes were detected with mouse monoclonal antibodies against human MDM2 (Ab-1, 1 : 100), p53 (DO-1, 1 : 100), p21 (Ab-1, 1 : 100), and anti-β-actin (C-2, 1 : 500) (Santa Cruz Biotechnology, Santa Cruz, USA). The targeted protein was revealed by enhanced chemiluminescence (ECL). The membrane was incubated with an ECL solution and exposed to ECL film (Eastman Kodak, Rochester, USA) to visualize specifically labeled proteins. The resulting exposed films were then analyzed by densitometry. All experiments were performed at least three times.

Viability assays

Cells were seeded in duplicate in 96-well plates (104 per well), incubated for 6 h, and treated with 10 μM Nutlin-3a for 24, 48, and 72 h, respectively, or exposed to 0–10 M Nutlin-3a for 48 h. Cell viability was determined using the Luminescent cell viability kit (Promega, Madison, China).

Measurement of apoptosis in vitro by flow cytometry

Apoptotic cells were detected using an Annexin V-FITC/PI apoptosis detection kit (Oncogene, Boston, USA). Briefly, cells were seeded in 6-cm dishes and treated with Nutlin-3a (0–10 μM) for 48 h prior to analysis. Floating and trypsinized adherent cells were collected and prepared for detection according to the manufacturer’s instructions. A total of 1 × 104 events were analyzed per assay by FACSArray™ flowcytometer (Becton Dickinson, Franklin Lakes, USA) using CellQuest software (Becton Dickinson). Cells positive for early apoptosis (Annexin V-FITC stained only) and for late apoptosis (Annexin V-FITC and PI stained) were combined. Data were obtained from triplicate experiments.

Cell cycle analysis

Cells were treated with Nutlin-3a (0–10 μM) for 48 h, labeled with 20 μM bromodeoxyuridine (Sigma-Aldrich) and fixed with 70% ethanol. Goat anti-bromodeoxyuridine FITC-conjugated monoclonal antibody (20 μl; Becton Dickinson) was added and incubated for 30 min in the dark at room temperature. Bromodeoxyuridine incorporation was analyzed by dual-color FACScan (Becton Dickinson). Cell number in each phase was expressed as the percentage of the total cell number.

siRNA-mediated transient knock-down

Regents for p53 RNA interference (RNAi) and control RNAi were purchased from Dharmacon (Beijing, China) and transfection was carried out according to the manufacturer’s guidelines. Twenty-four hours before transfection, U-2 OS cells (at 50–80% confluence) were trypsinized and resuspended in DMEM without antibiotics and plated into a 24-well plate at a density of 2 × 104 cells per well. Cells were resuspended in McCoy’s 5a medium. Then p53 RNAi or control RNAi (0.5 μg) and 90 μl Lipofectamine™ 2000 were added into U-2 OS cells and incubated for 24 h at 37°C. Then cells were exposed to 2–10 M Nutlin-3a for 48 h.
Tumor xenografts
A total of 48 female severe combined immunodeficiency mice (4–6 weeks old) were obtained from the Second Hospital of Beijing Corps of Chinese People’s Armed Police Force (Beijing, China) and maintained under specific pathogen-free conditions. Mice were injected subcutaneously with U-2 OS (wt p53), MG63 (mt p53) and SaOS2 (null p53) cells at 6 × 10⁶ cell per well, respectively, and divided randomly into two equal treatment groups. After the tumors were established, Nutlin-3a (25 mg/kg) or the vehicle was administered daily for 14 days. Weight of mice was measured twice weekly. Tumor volumes were measured daily with a caliper and calculated using the formula: 
\[ V = \frac{width \times height \times depth}{2} \]. For western blotting, histological and TUNNEL analyses, tumors were snap frozen or fixed in 10% formalin solution and processed routinely. Terminal transferase dUTP nick end labeling (TUNEL) assay was performed using ApopTag kit (Chemicon International, Temecula, USA) according to the standard protocol of manufacturer. Animal studies were conducted in accordance with the guidelines of the ethics committee at the Second Hospital of Beijing Corps of Chinese People’s Armed Police Force.

Statistical analysis
Data were present as the mean ± standard deviation (SD). Experiments were repeated three times with triplicate samples for each. Data were analyzed by analysis of variance and the Mann–Whitney U-test. Statistical significance was defined as \( P < 0.05 \).

Results

**Nutlin-3a inhibits growth of U-2 OS cells**
Nutlin-3 inhibited U-2 OS cell growth in a time- and dose-dependent manner. Nutlin-3 at 2–10 μM showed a gradual effect on cell growth [Fig. 1(A)]. When the concentration increased to 100 μM, cell proliferation was nearly completely inhibited after incubation for more than 48 h [Fig. 1(B)]. This effect was specific for U-2 OS cells containing wt p53, however, MG63 and SaOS2 cells without wt p53 were insensitive with Nutlin-3a treatment. Thus, the anti-proliferative and cytotoxic activity of the antagonist differed between cell lines depending on their p53 status.

**Nutlin-3 induces apoptosis of U-2 OS cells**
It has been reported previously that Nutlin-3a induces either cell cycle arrest or apoptosis in tumor cell lines with wt p53 [24]. In the present study, the ability of Nutlin-3a to induce apoptosis in different cell lines was detected with Nutlin-3a treatment for 48 h. About up to 37% apoptotic cells were detected in U-2 OS cells [Fig. 2(A)]. We also found that U-2 OS cells treated with 2–10 μM Nutlin-3a arrested in phase G1 in dose-dependent way [Fig. 2(B)]. No obvious change was found in MG63 and SaOS2 cells (data not shown). Cell apoptosis was dependent on the dose, as similar to that for growth inhibition [Fig. 1(A)].

![Figure 1](image1.png)

**Figure 1 Effect of Nutlin-3a on cell viability of OS cell lines**
Exponentially growing OS cells U-2 OS (wt p53), MG63 (mt p53) and SaOS2 (null p53) were exposed to 2–10 μM Nutlin-3a for 48 h (A) or to 10 μM for 24, 48 and 72 h, respectively (B). The percentage cell viability with respect to controls was determined. *\( P < 0.05 \) and **\( P < 0.01 \).

![Figure 2](image2.png)

**Figure 2 Nutlin-3a induces apoptosis in U-2 OS cells**
(A) Nutlin-3a induces apoptosis in OS cells. Cells were incubated for 48 h and the apoptotic cells were measured by flow cytometry and compared with control. *\( P < 0.05 \) and **\( P < 0.01 \). (B) U-2 OS cells were treated with Nutlin-3a followed by incubation with 2 μM bromodeoxyuridine. PI/FITC antibody to bromodeoxyuridine was used to determine cell cycle distribution.
Nutlin-3a up-regulates p53 and p21 levels

Western blot analysis was used to determine whether the antagonist affected the protein levels of p53 and its transcriptional target p21. Treatment with Nutlin-3a for 48 h significantly up-regulated p53 and p21 levels in U-2 OS cells in a dose-dependant manner [Fig. 3(A)]. However, there was no significant up-regulation of p53 or p21 in MG63 and SaOS2 cells [Fig. 3(B,C)].

Nutlin-3a activity is p53-dependent

Cell viability and apoptosis assays on U-2 OS (wt p53), SaOS2 (null p53) and MG63 (mt p53) cells showed that SaOS2 and MG63 cells were resistant to Nutlin-3a. To determine specifically whether OS cells required p53 for sensitivity, we developed a U-2 OS cell line that transiently expressed small interfering RNA (siRNA) directed at p53 and a control line infected with a control siRNA vector. According to a previously report, multiple p53 bands can be detected by western blotting, which may represent previously described p53 splice-variants [25]. The U-2 OS cell line expressing siRNA showed ~70% reduction of p53 protein, affecting two isoforms [Fig. 4(A)]. After incubation with Nutlin-3a for 48 h, cells expressing siRNA exhibited significant resistance to drug compared with the control [Fig. 4(B)].

Nutlin-3a treatment inhibits growth of U-2 OS tumors harboring wt p53 in vivo

To investigate the therapeutic potential p53 activation mediated by Nutlin-3a, a human xenograft OS animal model was used with U-2 OS (wt p53), SaOS2 (null p53) and MG63 (mt p53) cell lines in severe combined immunodeficiency mice. The antagonist was well tolerated without significant weight loss or other obvious toxicity signs. After 2-week treatment, for U-2 OS cell, the mean tumor volume in Nutlin-3a-treated group was 480 mm³, while 1751 mm³ in control group [Fig. 5(A)]. In addition, two of the Nutlin-3a-treated mice had no palpable tumors at the end of treatment. However, for SaOS2 and MG63 lines, the mean tumor volume in treated group was 1681 and 1871 mm³, respectively [Fig. 5(A)]. Western blot analysis showed the up-regulation of p53 and p53 transcriptional target p21 in Nutlin-3a-treated U-2 OS group, indicating the activation of p53 pathway [Fig. 5(B)]. The average apoptotic index assessed by TUNEL assay was 3.7 and 16.3 in treated group and the control, respectively [Fig. 5(C)]. Taken together, these data indicated that Nutlin-3a treatment induces p53-mediated cell cycle arrest and apoptosis in wt p53-positive U-2 OS cells in vivo.

Discussion

The tumor suppressor p53 is a powerful anti-tumoral molecule frequently inactivated by mutations or deletions in cancer. However, half of the human tumors express wild-type p53, and its activation by antagonizing its negative regulator MDM2 might offer a new therapeutic strategy [26]. Proof-of-concept experiments have demonstrated the feasibility of this approach in vitro, but the development of pharmacological inhibitors has been challenging. Recently,
potent and selective small-molecule MDM2 inhibitors have been identified [27,28]. Studies of these compounds have strengthened the concept that selective, non-genotoxic p53 activation is a viable alternative to current cytotoxic chemotherapy, however, the clinical validation is still pending.

Nutlin-3a isoforms are currently under extensive investigation as a selective small molecule MDM2 antagonist that could be used as an alternate treatment to current cytotoxic chemotherapy by selectively activating p53 in a non-genotoxic way [29]. Pishas et al. [22] reported that the viability of all Ewing sarcoma cell lines expressing wild-type p53 was markedly reduced in a dose-dependent manner after exposure to Nutlin-3a. But the viability of cell lines expressing mutant p53 was unaffected. Sonnemann et al. [21] reported similar results. In this study, our results showed a dose-dependent anti-proliferative and cytotoxic action that differed in three cultured cell lines depending on their p53 status (Fig. 1). It was established that the p53 pathway is only activated in cells with wild-type p53. These results suggest a role for Nutlin-3a as a non-genotoxic antagonist of MDM2 in OS cells.

However, Valentine et al. [30] have recently reported in HCT116 cells lacking p53 (HCT116p53−/−) and in MDM2 deficient cells (MEFMDM2−/−) that Nutlin-3a treatment could also lead to G2/M arrest, demonstrating the ability of Nutlin-3a to induce cell cycle checkpoint controls in a p53-independent fashion, which was contrary to the present study. These results suggest a secondary role for Nutlin-3a as a DNA-damaging agent, contrary to its proposed mechanism of action as a non-genotoxic antagonist of MDM2.

With regard to the effect of Nutlin-3a on apoptosis in OS cancer cells, Vassilev et al. [17] found that SJSA-1 OS cells (wt p53) treated with 10 μM Nutlin-3a for 48 h showed significant apoptosis by using TUNEL staining. Tovar et al. [24] found that similar treatment induced few apoptotic in U-2 OS cells. However, we found that treatment with 2–10 μM Nutlin-3a for 48 h induced significant apoptosis in U-2 OS cells. In MG63 cells with mutant p53 and SaOS2 cells with null p53, little apoptosis was detected after 48-h exposure to Nutlin-3a. However, Kitagawa et al. [31] found that HCT116 human colon cancer cells with wt p53 are insensitive to the induction of apoptosis by Nutlin-3a alone, which could also be a tumor-specific phenomenon.

Previous studies have reported that Nutlins can not only induce apoptotic cell death when exposed to primary leukemic cell cultures, but also induce strong MDM2 expression [32]. Nutlins might exert their therapeutic effects by two distinct mechanisms: a direct cytotoxic effect on leukemic cells, and an indirect non-cell autonomous effect on tumor stromal and vascular cells. This latter effect might also be therapeutically relevant for treatment of haematological malignancies with p53 mutations [32]. In lung cancer H460 (wt p53) cells treated with Nutlin-3a, there was also a significant induction of MDM2, p53 and p21 proteins. In contrast, protein levels in the p53-defective Val138 cells were relatively constant [33], which indicated

Figure 5 Nutlin-3a inhibits growth of U-2 OS cell tumors with wild-type p53 in vivo (A) Severe combined immunodeficiency mice with palpable subcutaneous U-2 OS, SaOS2 and MG63 tumors were treated daily with Nutlin-3a or vehicle for 2 weeks, and tumor volume was recorded. *P < 0.01. (B) Western blot analysis showed the protein levels of p53 and p21, a p53 transcriptional target. (C) Apoptotic index assessed by TUNEL assay. *P < 0.05.
that Nutlin-3a induced MDM2 inhibition is dependent on p53 status.

We found that Nutlin-3a decreased MDM2 expression in a dose-dependent manner in U-2 OS cells [Fig. 3(A)]. The reason for the discrepancies in our findings with those in previous studies [32,33] is unknown. It needs further investigation whether MDM2 is inhibited by active p53 under stress. Therefore, other molecular mechanisms may contribute to these differences at cellular level.

In this study, short-term cell cycle assays showed that 10 μM Nutlin-3a significantly increased G1 phase and decreased S phase fractions in U-2 OS cells [Fig. 2(B)]; however, no significant increase in G1 phase fraction and decrease S phase fractions occurred in MG63 or SaOS2 cells (data not shown). After p53 had been silenced by siRNA, U-2 OS cells were significantly resistant to the drug compared with control [Fig. 4(B)].

Vassilev et al. [17] showed that Nutlin-3 treatment on mice bearing tumors of 100–300 mm³ resulted in 90% inhibitory effect compared with control [Fig. 5(B)]. The mice did not lose weight significantly and showed no gross abnormalities at necropsy. However, for MG63 and SaOS2 tumors, no significant inhibition of tumor growth was found [Fig. 5(A)]. SaOS2 cells were less sensitive than U-2OS cells, perhaps due to an amplified mdm2 gene; and MG63 cells were less sensitive than U-2OS cells, which might due to an amplified mutant p53 gene.

We conclude that, as a single agent, Nutlin-3a only increases apoptosis and decreases survival preferentially in wt p53-expressing cells. Although our data strengthen the notion that unleashing the growth suppressive and pro-apoptotic activity of p53 by MDM2 antagonists is a potentially valuable strategy for treating OS with wt p53, further studies will be required to address the true therapeutic potential of the approach.

References