Minocycline and cisplatin exert synergistic growth suppression on hepatocellular carcinoma by inducing S phase arrest and apoptosis

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Abstract. Minocycline, a semisynthetic tetracycline, is a highly lipophilic molecule capable of infiltrating tissues and blood. Previous studies have revealed the functions and mechanisms of minocycline in anti-inflammation, protection of the nervous system and certain tumors. The role of minocycline has never been investigated in hepatocellular carcinoma (HCC). The functions of minocycline on HCC cells were investigated using immunohistochemical staining and western blotting. Minocycline was applied to L02, HepG2 and Huh7 cells, and the growth characteristics were studied. Cisplatin was administered in combination with minocycline in this study. Cell cycle and apoptosis analyses were employed to investigate the mechanisms underlying the growth regulation associated with minocycline and(or) cisplatin. Minocycline caused S phase cell cycle arrest and an increase in the apoptotic rate associated with upregulation of p27, cleaved-caspase8, cleaved-caspase3 and cleaved-PARP-1. Low dose of cisplatin promoted cell cycle arrest and apoptosis, whereas minocycline was mainly associated with upregulation of cleaved-PARP-1. The combination of cisplatin and minocycline increased the rate and extent of cell cycle arrest and increased the apoptosis rate caused by minocycline. A novel mechanism was revealed. Minocycline functions as an antitumor drug in HCC by regulating p27, caspase-3 and PARP-1. Cisplatin enhanced minocycline's effect on PARP-1.

Introduction

Hepatocellular carcinoma (HCC) is the fifth leading cause of all cancer-related deaths worldwide, and most of these deaths occur in developing countries (1). In recent years, studies regarding the pathogenesis of HCC, from microbial metabolites, a new type of oncogene to microRNAs (1-4), have weaved an intricate network. However, little progress in curing this cancer has been achieved. HCC is also characterized by its insensitivity to chemotherapy and radiotherapy. Although several targeted therapeutic drugs have been discovered (5), their clinical effects on HCC still require further observation. For example, sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis (6). Based on a previous study (7), PJ34, an inhibitor of PARP-1, a nuclear enzyme that not only responds to DNA damage and facilitates DNA repair, but also mediates cell death through a caspase-independent pathway (8), was found to effectively suppress proliferation of HepG2 cells. The inhibitory effect of minocycline at nanomolar concentrations on PARP-1 was revealed nearly 10 years ago (9).

Minocycline, a semisynthetic tetracycline, is a highly lipophilic molecule capable of infiltrating tissues and blood (10). As a replacement of earlier tetracycline, minocycline, which has a low propensity to produce antibiotic resistance, is commonly used to treat many types of infections. With further exploration of its functions and mechanisms, particular aspects of anti-inflammation (11) and protection of the nervous system (12-14), minocycline has found a wider and deeper utilization. Only recently has research examined how minocycline inhibits proliferation and promotes apoptosis (15). The dosage required to induce effective apoptosis was tens of folds higher than the dosage used for treatment of inflammation. Because of this, there is substantial toxicity associated with minocycline treatment (16). Therefore, the combination of chemotherapeutics with minocycline is a new method by which to combat the toxicity issues associated with high dosages of minocycline alone.

Cisplatin crosslinks DNA in several different ways, interfering with mitotic cell division. Cells undergo DNA damage...
responses that induce apoptosis when repair is not possible. Cisplatin elicits a complex response in the cell, including apoptosis, DNA repair and drug resistance involving the ATR, p53, p73 and MAPK pathways (17). The effects of cisplatin treatment on apoptosis and DNA repair are enhanced when used in combination with minocycline. Cisplatin treatment is also limited by toxicity (18,19).

Herein, we present our in vitro and in vivo results to show that minocycline treatment induces cell cycle arrest and apoptosis. Additionally, when low-dose cisplatin was used in combination with minocycline, the suppression of HCC cell proliferation and apoptosis was enhanced above the level presented when using a single agent alone.

Materials and methods

Cell lines, cell culture and transient transfection. The human embryonic liver cell line L02 and HCC cell lines Huh7 and HepG2 were purchased from the Typical Training Content Preservation Committee Cell Bank of the Chinese Academy of Sciences (China). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO₂.

p27 siRNAs (RiboBio Co. Ltd., China) were used to transiently transfect HepG2 cells. The transfection was performed using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. Cells were seeded at a density of 5x10⁴ per well in a 6-well plate.

Cell proliferation assay. Cells were seeded in 6-cm plates at 5x10⁴ cells per well. The cells were cultured with minocycline and/or cisplatin at different concentrations. Cell numbers were counted on days 3, 6, and 9 after seeding. The assay was repeated three times.

Soft agar colony formation assay. A soft agar colony formation assay was performed for the transfected and control cells. Briefly, 1x10⁴ cells were seeded into a 6-well plate in a medium containing 0.3% noble agar and cultured for 14 days. The number of colonies was determined by direct counting using an inverted microscope (Nikon, Japan).

Flow cytometry to analyze the cell cycle and apoptosis. For cell cycle analysis, 1x10⁶ HepG2 cells were washed three times in PBS and serum starved for 48 h. The cells were stimulated with DMEM containing 10% FBS for 24, 48, or 72 h. Cells (1x10⁵) were analyzed from each sample on a FACSCalibur flow cytometer (Becton Dickinson).

For the apoptosis analysis, 1x10⁵ cells per well were seeded in 6-well plates and incubated with DMEM containing 10% FBS. After 48 h, cells were stained for 15 min with FITC-Annexin V and propidium iodide in binding buffer and then analyzed by flow cytometry within 1 h.

Xenograft assay. Male Balb/c nude mice (n =18) were provided by the Experimental Animal Center of the Tongji Medical College. HepG2 cells (2x10⁶) were injected into each of the bilateral flanks of the mice. On day 14 post injection, the mice were randomly divided into three groups: the minocycline, minocycline and cisplatin, and control groups.

Minocycline at 6 mg/kg in 0.1 ml was injected intraperitoneally, and cisplatin was injected intraperitoneally at 0.3 mg/kg. The same volume of saline was injected into the control group. The tumors were measured every 3 days and tumor sizes were calculated on day 21.

TUNEL assay. Sections of tumor tissues were deparaffinized in xylene, rehydrated, washed with PBS, and treated with 20 μg/ml of Protease K (Roche) for 10 min at room temperature. Then reactions were conducted using the TUNEL Apoptosis Assay Kit® (Roche) according to the manufacturer’s instructions, and detected using DAB. The percentage of TUNEL-positive cells was assessed in five randomly selected fields for each tissue section.

Western blotting. Western blotting was performed with specific primary antibodies against CDK2, CDK4, p53, PARP-1, p21, caspase-3, caspase-8, ku80 and p27 (rabbit anti-human antibody; 1:1000; Epitomics, Burlingame, CA, USA), followed by the appropriate secondary HRP-conjugated antibodies (1:5000; Pierce, Rockford, IL, USA). The protein bands were visualized using an enhanced chemiluminescence detection system (Pierce).

Statistical analysis. ANOVA was performed to determine the statistical significance among the groups. A P-value <0.05 was considered to indicate a statistically significant result. All experimental data were analyzed using the SPSS statistical software (version 16.0).

Results

Minocycline inhibits the proliferation of HCC cell lines, but not the normal liver cell line. When HepG2 or Huh7 cells were cultured with minocycline at concentrations of 50, 75, and 100 μM, cell proliferation was significantly suppressed compared to the control cells cultured without minocycline (Fig. 1A). The suppressive effects of minocycline were dose-dependent, and the number of cells in the treated group was significantly lower than the number of cells in the control group on days 6 and 9. However, when the normal human liver cell line L02 was cultured with minocycline at 100 μM, no obvious suppressive effect was observed on days 3, 6 and 9 (Fig. 1D). At a concentration of 200 μM, minocycline strongly suppressed the proliferation of HCC cells, but it also weakly suppressed L02 cell proliferation. Furthermore, the soft agar assay revealed that minocycline treatment significantly inhibited colony formation by 50.5-64.5% compared to the control clones (Fig. 1B).

Inhibition of HCC cell proliferation by minocycline is enhanced by low-dose cisplatin. When HepG2 cells were cultured with minocycline at a concentration of 100 μM in combination with 2 μM or 5 μM cisplatin, the level of suppression of cell proliferation was comparable to that of treatment with 200 μM minocycline. The suppression was greater than with treatment of 2 μM or 5 μM cisplatin alone (Fig. 1C). There was no effect on L02 cells. Although treatment of HepG2 cells...
with a concentration of 200 µM minocycline in combination with 2 µM or 5 µM cisplatin decreased proliferation even further, this concentration also suppressed the proliferation of L02 cells.

Cisplatin enhances the effects of minocycline in regards to increased cell apoptosis and cell cycle arrest. HCC cells were divided into four treatment groups: the control; 100 µM minocycline; 2 µM cisplatin; and 100 µM minocycline and 2 µM
cisplatin groups. At 24 h, minocycline had no effect on cell cycle arrest. However, at 48 and 72 h, more cells were arrested in the S phase, with distribution rates of 49.1±0.54, 57.6±0.39 and 71.2±1.2% at the three time points, respectively. Moreover, low-dose cisplatin induced G0/G1 cell cycle arrest in almost all cells. The combination of minocycline and cisplatin significantly accelerated the S phase arrest at all three time points (Fig. 2A and B). Minocycline treatment alone only induced S phase arrest after 72 h (40±0.94%), while the combination of minocycline and cisplatin induced S phase arrest after 24 h (49.1±0.54%). After 72 h, the combination treatment induced S phase arrest at a rate of 71.2±1.2%. Additionally, the G0/G1 phase arrest induced by cisplatin treatment was eliminated with combination therapy, with rates decreasing from 97.6±1.38 to 4.1±1.22%.

Flow cytometry was used to analyze the induction of apoptosis in these cells. The apoptotic rates in the control, 100 µM minocycline, 200 µM minocycline, 2 µM cisplatin, 100 µM minocycline and 2 µM cisplatin, and 200 µM minocycline and 2 µM cisplatin groups were 5.81±0.15, 43.83±0.25, 51.44±1.52, 20.31±0.44, 60.26±1.72 and 67.34%, respectively (Fig. 3A and B). Late stage apoptotic rates in the groups treated with 100 µM minocycline, 200 µM minocycline, or 2 µM cisplatin were much higher than the rate in the control group. In the group treated with 200 µM minocycline, the apoptotic rate reached 51.44±1.52%, but in the group that was treated with the combination of 100 µM minocycline and 2 µM cisplatin, the apoptotic rate reached 60.26±1.72%. The group that was treated with combination therapy also had a significantly higher rate of apoptosis than the control group.
Minocycline induces apoptosis in HCC cells through a mitochondrial-independent pathway and by suppressing the DNA repair process. Cleaved caspase-3 was upregulated in HCC cells treated with minocycline. Since cleaved caspase-8 was also upregulated in HCC cells treated with minocycline, the extrinsic apoptosis pathway was activated. The downregulation of PARP-1 elucidated another pathway associated with apoptosis induced by minocycline. In addition to PARP-1, the
expression of another DNA repair factor, Ku80, was suppressed by minocycline treatment (Fig. 4A and B).

**Minocycline induces HCC cell cycle arrest through the p27 pathway.** In HepG2 cells, the expression levels of CDK2, CDK4, cyclin D, and cyclin E were significantly decreased in cells treated with minocycline (Fig. 4C and D), while minocycline treatment had no effect on the expression of p53 and p21. However, the expression of p27 was dose-dependently upregulated with minocycline treatment (Fig. 4C and D). To verify this result, p27 expression was knocked down by siRNA, When the p27 expression was blocked, the minocycline-induced suppression of downstream factors, such as CDK2, was reversed (Fig. 5A).

Cell proliferation assay demonstrated that the proliferation of the HepG2 cells treated with minocycline was significantly increased after transfection with p27-siRNA compared with the mock-transfected cells or the vector-transfected control cells on days 6 and 9 of culture (Fig. 5B). There was no significant difference in cell numbers between the mock-, vector- and p27-siRNA-transfected cells which were not treated by minocycline at all time points after transfection.

**Cisplatin enhances the effect of minocycline on cell cycle arrest and apoptosis.** Cisplatin can induce apoptosis through both the extrinsic and intrinsic pathways. Cisplatin can also increase the expression of p53, which activates p21 to induce cell cycle arrest in the G0/G1 phase, and it also can downregulate the expression of PARP-1 and suppress the expression of p27. In vitro assays showed that the combination of cisplatin and minocycline enhanced the levels of apoptosis in HCC cells when compared with the application of a single drug. The expression of cleaved caspase-3 and p53 was upregulated following treatment with the combination therapy when compared to that induced by treatment with minocycline alone (Fig. 6A and B). Although CDK2 and CDK4 expression levels were decreased, that was not enough to induce the suppressive effect on HCC cells observed at early time points. Therefore, we hypothesized that the S phase arrest induced by the combination of the two drugs was caused mainly by suppression...
of the PARP-1 DNA repair pathway. The downregulation of PARP-1 verified our deduction (Fig. 6A and B).

Minocycline and cisplatin treatment inhibits HepG2 tumor growth in nude mice. Nude mice received injections of 2x10⁶ HepG2 cells in each flank. Tumors formed after two weeks, and tumor-bearing mice were randomly divided into three groups. As shown in Fig. 7A, the mean volumes of the tumors from mice treated with minocycline (0.70 cm³) or cisplatin in combination with minocycline (0.45 cm³) were significantly smaller than those from the control group (1.66 cm³), and tumors from mice treated with the combination therapy were significantly smaller than tumors from mice treated with minocycline alone. In addition, when compared to tumors from the control group, tumors from mice that received any treatment grew at significantly slower rates and were smaller at
all time points examined from day 24 post-injection (Fig. 7A). The mass of tumors from mice that received minocycline alone or combination therapy was significantly lower than that of tumors from mice in the control group (Fig. 7B and C), and the mass of tumors from the mice that received combination therapy was significantly smaller than that from tumors from the mice treated with minocycline alone. Western blot analysis (Fig. 7D) verified the effect of minocycline and/or cisplatin on the expression of PARP-1, caspase-3, and Ku80 in vitro. As shown by TUNEL assay, the rate of apoptosis was calculated. As shown in Fig. 7E, treatment with minocycline alone or minocycline in combination with cisplatin resulted in a significantly higher apoptotic index (32.7 and 56.4% respectively), when compared to the control group.

**Discussion**

Minocycline is a second-generation tetracycline antibiotic with favorable pharmacological properties. It is also used as an anti-inflammatory and neuroprotective agent. In this report, we present novel and direct evidence that minocycline markedly induces apoptosis and cell cycle arrest in liver cancer cells.

Minocycline inhibits PARP-1 expression at nanomolar concentrations (9), and this research suggests that minocycline could be useful to treat HCC. Based on a previous study (7), an inhibitor of PARP-1 suppressed the proliferation of HCC cells. We hypothesized that minocycline could function similarly, and should be investigated further given its ability to infiltrate tissues and blood. We treated several HCC cell lines with minocycline at different concentrations. We found that the proliferation of liver cells was suppressed when the suitable dosage of minocycline was applied. However, minocycline had no suppressive effect on the proliferation of L02 cells at the same dosage. Moreover, minocycline treatment changed the morphology of HepG2 and Huh7 cells from smooth and plump to shrinking. The cell nucleus also appeared to undergo distinctive changes, including pyknosis and karyolitic and apoptotic body formation, a typical feature of apoptosis (20). Using flow cytometry, we determined that minocycline treatment induced S phase arrest with an increase in concentration, especially after 72 h. Moreover the late stage apoptotic cells increased with its concentration.

Western blot analysis was used to explore the underlying mechanism responsible for this phenomenon. Treatment with increasing concentrations of minocycline increased the expression levels of CDK2, CDK4, cyclin D, and cyclin E, which explains the observed cell cycle arrest noted with treatment (21). We then examined the expression of the upstream factors of CDK2 and CDK4: p53, p21 and p27. Minocycline treatment had no effect on the expression of p53 or p21, the downstream factor of p53 (22). However, p27, which also

![Figure 7](image-url)
regulates CDKs and cyclins (23,24), was induced following minocycline treatment, and the suppression of CDKs and cyclins by minocycline was reversed by blocking p27 expression. We, therefore, concluded that minocycline regulates cell cycle arrest by activating the p27 pathway, but the mechanism by which minocycline induced apoptosis of HCC cell lines remain unclear.

We then tested the effects of minocycline treatment on other apoptotic factors induced by mitochondria (25,26). Minocycline did not affect the intrinsic mitochondrial apoptotic pathway. Minocycline treatment induced both the expression of cleaved caspase-3 and cleaved caspase-8, which indicated the activation of the extrinsic apoptotic pathway (27,28).

A decrease in PARP-1 expression, as noted with minocycline treatment, has been shown to affect both cell cycle arrest and apoptosis in other pathways (8,29,30). Through suppressed PARP-1, minocycline induced HCC cells into apoptosis directly.

Although minocycline induces apoptosis and cell cycle arrest, the high doses needed also have toxicity to normal cells (16). Therefore, we wanted to use another drug in combination with minocycline so that we could reduce the dosage but still have a similar effect on cancer cell death. Cisplatin can induce apoptosis through both the extrinsic and intrinsic apoptotic pathways (31,32), which has some overlap with the functions of minocycline. Then we chose it to continue our research. The combination of cisplatin and minocycline enhanced the apoptosis of HCC cells when compared with treatment with either minocycline or cisplatin alone. The expression of p53 was also increased, suggesting that the combination of these two drugs may have synergy in inducing apoptosis.

Based on the findings of other studies, cisplatin induced the expression of p21 while decreasing p27 expression (33), thereby antagonizing the suppressive effects of minocycline on CDK2 and CDK4 complexes. Cisplatin treatment induced G0/G1 arrest, but the combination of minocycline and cisplatin induced S phase arrest. Minocycline induced S phase arrest after 72 h, thus we concluded that p27 upregulation was not the primary mechanism. The strong suppressive effect of this combination on PARP-1 expression blocked the repair of HCC cells and inhibited damaged cells from entering the G2/M phase, thus inducing cell accumulation in the S phase and apoptosis. This synergy of drugs was enhanced over time. In the animal model, subcutaneous tumors were implanted in Balb/c nude mice. The tumors in the group treated with a single drug and the control group, combination therapy were much smaller than the tumors in Balb/c nude mice. The tumors in the group treated with the combination of these two drugs may have synergy in inducing apoptosis. These data need further evaluation in clinical conditions and in other types of cancer, particularly in cancers that have a tight relation with PARP-1 expression.

References