Original Article

Benzaldehyde levofloxacin schiff baseinduced apoptosis of human hepatocarcinoma cells

Hong-Xia Liang1, Yuan-Yuan Fan1, Ying Zhang2, Chao-Shen Huangfu1, Guo-Qiang Hu3, Bin Liu1

1College of Nursing, Institute of Neurobiology, Henan University, Kaifeng 475004, China; 2Huaihe Clinical College, Henan University, Kaifeng 475000, China; 3Institute of Chemical Biology, Henan University, Kaifeng 475004, China

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Abstract: To study the effect of (S)-1, 8-(2-methyl phosphate ethoxy)-6-fluorine-7-(4-methyl-pi-perazine-1-base)-3-[S-benzyls-based-4-(for nitrobenzene methylene group amino)-1, 2, 4-all triazole-3 base]-quinoline (1-H)-4-ketone (M18) on apoptosis of hepatocarcinoma SMMC-7721 cells in vitro. Different concentrations of M18 at different time were used to treat SMMC-7721 cells, human breast cancer MB-231 cells, human colon cancer HCT-116 cells, human hepatocarcinoma HEPG-2 cells, mouse bone marrow mesenchymal stem cells (BMSCs) in vitro. The inhibition effects of M18 on cell proliferation were examined by MTT assay. Cell apoptosis was determined using Hoechst 33258 fluorescence staining and TUNEL method. Mitochondrial membrane potential (Δψm) was measured using a high content screening image system. Protein expression of caspase-3, p53 and cytochrome C was detected with Western blot analysis. Treatment with M18 (4~32 μmol·L⁻¹) potently inhibited the proliferation of the cancer cells in time-and dose-dependent manners (the IC50 value at 24 h in SMMC-7721 cells, MB-231 cells, HCT-116 cells and HEPG-2 cells was 8.65 μmol·L⁻¹, 9.37 μmol·L⁻¹, 12.74 μmol·L⁻¹ and 9.40 μmol·L⁻¹, respectively). In contrast, M18 had weak cytotoxicity against BMSCs with IC50 value of 38.96 μmol·L⁻¹. Levofloxacin had weak cytotoxicity against SMMC-7721 cells with IC50 value of 735.10 μmol·L⁻¹. Treatment of SMMC-7721 cells with different concentrations of M18 for 24 h increased the percentage of the apoptosis cells (P < 0.05) and decreased the mitochondrial membrane potential. In addition, M18 increased protein expression of p53, caspase-3 and the cleaved activated forms of caspase-3 in SMMC-7721 cells. Treatment of SMMC-7721 cells with M18 significantly increased cytochrome C in the cytosol, and decreased cytochrome C in the mitochondrial compartment. The mitochondrial-dependent pathways are involved in M18 induction of apoptosis of SMMC-7721 cells.

Keywords: Fluoroquinolones schiff base, hepatocarcinoma cells, cell proliferation, apoptosis, mitochondrial membrane potential, p53

Introduction

Fluoroquinolone carboxylic acid is a widely used class of chemical synthesized antibiotics in clinical. Its target is bacterial DNA gyrase (gyrase), which inhibited bacterial growth [1]. Primary structural sequence of eukaryotic topoisomerase II α and II β are very similar to bacterial gyrase, which topoisomerase II N-terminal domain (amino acids 1-670) and DNA gyrase subunit B (GyrB) have the same homology. Topoisomerase II central domain (amino acids 671-1200) and DNA gyrase A subunit (GyrA) have the same homology, the tyrosine active site contained in the structure is very necessary for catalytic DNA breakage and reconnection [2]. Currently, the target of a variety of anticancer drugs is enzyme topoisomerase II [3]. Some antimicrobial fluoroquinolone carboxylic acids had weaker inhibition on eukaryotic cell proliferation, such as ciprofloxacin, norfloxacin, ofloxacin, etc. [4]. Previous study of our research group showed that the novel fluoroquinolone derivatives synthesize by binding the C-3 carboxyl of fluoroquinolone acid with acylhydrazones had a strong inhibition on topoisomerase II [5, 6], promoting topoisomerase II-mediated PBR332 plasmid DNA helicase and fracture and inhibiting DNA re-ligation; the role was consistent with the clinical topoisomerase II inhibitor-etoposide [7, 8]. In this study, levofloxacin was used as the lead compound, and its C-3 carboxyl was replaced with 12 kinds of structural units to synthesize a series of levo-
floxacin C-3 acylhydrazone derivatives. Wherein benzaldehyde levofloxacin Schiff base (S)-1, 8-(2-methyl phosphate ethoxy)-6-fluorine-7-(4-methyl-piperazine-1-base)-3-[S-benzyls-based-4-(for nitrobenzene methylene group amino)-1, 2, 4-all triazole-3-base]-quinoline (1-H)-4-ketone.

**Figure 1.** Structure of (S)-1, 8-(2-methyl phosphate ethoxy)-6-fluorine-7-(4-methyl-piperazine-1-base)-3-[S-benzyls-based-4-(for nitrobenzene methylene group amino)-1, 2, 4-all triazole-3-base]-quinoline (1-H)-4-ketone.

**Material and methods**

**Reagents and instruments**

*Inducer* p-nitrobenzaldehyde levofloxacin Schiff base was designed, modified and synthesized by the Institute of Chemical Biology, Henan University; purity > 99% (HPLC); it was dissolved in dimethyl sulfoxide (DMSO, Solarbio company) with a concentration of 1 × 10⁻² mol·L⁻¹.

*Cell lines and reagents:* Human hepatoma cell lines SMMC-7721 and HEPG-2, human breast cancer cell MB-231, and human colon carcinoma cell HCT-116 were purchased from Institute of Basic Medicine, Chinese Academy of Medical Sciences; cells were incubated with DMEM medium (Gibco Co.) containing 10% fetal bovine serum (Hangzhou Evergreen biological Engineering materials Co., Ltd.), placed in an incubator (5% CO₂, 37°C).

Methyl thiazoly tetrazolium salt (MTT), Dead-End™ Fluorometric TUNEL System (promega company); Mitochondria isolation kit (ShangHai Beyotime Biotechnology Co., Ltd.); Caspase-3, p53, cytochrome c antibody (Cell Signaling Technology); β-actin antibody, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG (Zhongshan Gold Bridge Company); Hoechst 33258 (Sigma Co.). Other reagents were analytical grade.

*The main instruments:* Carbon dioxide incubator (Thermo Forma, 3121, USA); microplate reader (Thermo Multiskan Ascent, USA); BX51 fluorescence microscope (Olympus Company); DYY-7C transfer electrophoresis apparatus, vertical electrophoresis apparatus, semi-dry electric transfer apparatus, gel imaging system (Beijing Liuyi Instrument Factory); high-content live cell imaging system (Thermo Fisher Scientific, USA).

**Methods**

**MTT assay to detect the effect of M18 on cell proliferation:** Cells were seeded in 96-well plates at a density of 16 × 10⁴·ml⁻¹ concentrations; after treated by different concentrations of M18 for 24, 48, 72 h, culture medium was aspirated, and DMSO 0.15 ml was added into each well, shocking for 10 min to blue crystals were completely dissolved; absorbance (A) value at 570 nm was measured by a microplate reader and the inhibition rate was calculated. The absorbance of the non-cell well containing equal volume of medium and DMSO was taken as the blank control. According to the equation \[ \frac{fa}{fu} = \frac{(D/Dm)m}{C50} \] IC₅₀ was calculated [9]. Cell growth inhibition rate/% = \[ \frac{(absorbance of treated group-absorbance of the blank control group)}{(absorbance of control group-absorbance of the blank control group)} \] × 100%.

**Isolation and culture of BMSCs:** Two C57BL/6J mice were collected, aged 6-8 weeks. Under sterile conditions, double lower limbs were quickly removed; the marrow cavity was washed with low-sugar DMEM containing 10% fetal bovine serum, and then the 4th needle was used to pipet cells into single cell suspension; cells were inoculated into flasks at the density of 600 × 10⁴·ml⁻¹ to 800 × 10⁴·ml⁻¹; after 48 h culture, medium was changed every three days; cells were sub-cultured when the density reached 80%.

**Hoechst 33258 staining to observe apoptotic morphological changes:** Cells were seeded in six-well plates with coverslip at a density of 1 × 10⁵·ml⁻¹; When the cells adherent, they were treated with different concentrations of M18 for 24 h, washed twice with PBS, fixed with
paraformaldehyde for 15 min, and stained with 30 µl Hoechst 33258 solution for 10 min at RT; After rinsed twice with PBS and mounted with neutral glycerol, they were observed and recorded under a fluorescence microscope.

**TUNEL assay to detect cell apoptosis:** Cells were seeded in six-well plates with coverslip at a density of 1 × 10⁵·ml⁻¹ and treated with different concentrations of M18 for 24 h; according to Promega’s kit instructions, cell apoptosis was recorded.

**Mitochondrial membrane potential (Δψm) Determination:** Cells were seeded into six-well plates with 8 × 10⁴·ml⁻¹ and incubated with different concentrations of M18 for 24 h; 1 ml of Rh-123 dilution was added into each well for 1 h; after PBS-washing for 3 times, 5 mg·L⁻¹ Hoechst 33342 was added and cells were
darkly stained for 10 min, washed with PBS, and observed and analyzed by HCS live cell imaging system.

Isolation of mitochondrial protein: After treated by different concentrations of M18 cells for 24 h, cells were routinely collected, centrifuged at 12000 rpm for 10 min, and washed with PBS; extraction of mitochondrial proteins and cytoplasmic protein was performed according to the instructions of mitochondria isolated kit.

Western blot to detect protein expression: Cells were treated with different concentrations of M18 for 24 h, lysed by 200 μL RIPA lysis buffer sufficiently, and centrifuged at 4°C (12 000 r·min⁻¹) for 5 min to extract protein; Coomassie brilliant blue G250 method and spectrophotometric instrument were used to measure protein concentration; 12% SDS-PAGE electrophoresis was used to separate samples. Then the protein was electrically transferred to a PVDF membrane, sealed with 5% skim milk for 1 h, incubated with the primary antibody (1:2000) at 4°C overnight and incubated with the secondary antibody (HRP-labeled goat anti-rabbit or goat anti-mouse antibody 1:4000) for 1 h; chemiluminescence method was used to display the results; gel image analysis system was used for photography.

Statistical analysis

All data were statistically analyzed using SPSS 14.0 software package. Measurement data were expressed as x±s; differences between the groups were compared using t-test.

Results

Inhibition of M18 on the proliferation of various tumor cells and mouse bone marrow mesenchymal stem cell

SMMC-7721, MB-231, HCT-116 and HEPG-2 cells were treated with different concentrations of M18 for 24 h, 48 h and 72 h respectively; M18 had a stronger inhibitory effect on the proliferation of tumor cells, in a time and concentration-dependent manner; IC50 value in 24 h

Figure 3. SMMC-7721 cells apoptosis under fluorescent microscope stained by Hoechst 33258 (×200). A: Control; B: M18 (2.467 μmol·L⁻¹); C: M18 (8.650 μmol·L⁻¹); D: M18 (26.401 μmol·L⁻¹).
was 8.65 μmol·L⁻¹ (r² = 0.8841), 9.37 μmol·L⁻¹ (r² = 0.8523), 12.74 μmol·L⁻¹ (r² = 0.7994) and 9.40 μmol·L⁻¹ (r² = 0.8218); bone marrow mesenchymal stem cells were also treated with M18 for 24 h, 48 h and 72 h; the cell growth inhibition was not significant; IC50 value in 24 h was 38.96 μmol·L⁻¹ (r² = 0.7822); the effect of synthetic material-levofloxacin on human hepatoma SMMC-7721 cell proliferation was not obvious; 24 h IC50 value was 735.10 μmol·L⁻¹ (r² = 0.6854) (Figure 2).

M18-induced apoptosis of SMMC-7721 cells

Hoechst33258 staining showed that, after treated with M18 for 24 h, SMMC-7721 cells showed cell membrane shrinkage, chromatin condensation, nuclear fragments and other morphological changes of apoptosis (Figure 3). TUNEL results showed that with the increasing concentrations of M18, apoptotic cells increased, in a dose-dependent manner (Figure 4; Table 1).

Effect of M18 on mitochondrial membrane potential

Rh-123 fluorescence intensity can indirectly reflect the degree of damage to the mitochondrial membrane. SMMC-7721 cells were treated with M18 for 24 h; with the increasing concentrations of the compound, the fluorescence intensity increased, indicating that mitochondrial membrane potential decreased, which respectively decreased (8.97±4.15)%; (31.82±5.29)% and (47.25±6.03)% compared with the control group; the difference was statistically significant (P < 0.01) (Figure 5).

Effect of M18 influence on p53 and caspase-3 protein expression and the inside and outside distribution of mitochondrial cytochrome c

SMMC-7721 cells were treated with 2.467, 8.650, 26.401 μmol·L⁻¹ M18 for 24 h, and Western blot assay was used to detect the expression of p53 and caspase-3 and mitochondrial cytochrome c distribution. Compared with the control group, after M18 treatment,
p53 protein expression was significantly increased, in a concentration-dependent manner; Caspase-3 protein expression and active fragments also increased. Cytochrome c reduced within the mitochondrial but increased in the cytoplasm, suggesting that M18-induced SMMC-7721 apoptosis may be related to mitochondrial apoptosis pathway (Figure 6).

Discussion

Some fluoroquinolone antibiotics commonly used in the clinical had weaker inhibition on eukaryotic topoisomerase II [10]. The study found that fluoroquinolone carboxylic acid C-3 carboxy was essential for the antibacterial activity, but was not necessary for the anti-tumor activity, which provided ideas for searching for new anti-tumor fluoroquinolone candidates [11, 12]. Our research group took levofloxacin as substrate, used structural units to replace C-3 carboxy, and synthesized 12 kinds of derivatives of levofloxacin; the inhibition of 12 kinds of quinolone derivatives on SMMC-7721 cell proliferation was detected by MTT assay; most compounds exhibited different degrees of inhibition on SMMC-7721 cell growth; IC50 values were lower than 50 μmol·L^{-1}; wherein M18 showed the best inhibition effect on cell proliferation; IC50 value in 24 h was 8.65 μmol·L^{-1}; the effect of its synthetic material-levofloxacin on SMMC-7721 cell proliferation was not obvious; IC50 value was 735.10 μmol·L^{-1}, indicating that the compound transformation was in line with theoretical speculation, with good research and development value. In order to understand the differences in the effect of M18 on various tumor tissues, this study selected four different types of tumor cell lines (HTC-116 colon cancer cells, MB-231 breast cancer cells, and human hepatoma HEPG-2 cells, human hepatoma SMMC-7721...
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cells) to detect the inhibition effect of M18 on cell growth. The results showed that, M18 significantly inhibited the proliferation of various tumor cells, in a time and concentration-dependent manner. The study also detected the inhibitory effect of M18 on the growth of primary-cultured bone marrow mesenchymal stem cells; the IC50 value was 38.96 μmol·L⁻¹, which was significantly higher than that in SMMC-7721 cells, indicating that M18 had a good selectivity in tumor cells, which may have less side effects in future clinical applications.

Our previous experiments have proved that the target of C-3 carboxyl modified fluoroquinolone derivatives was DNA topoisomerase II, resulting in tumor cell DNA damage; This effect may activate p53 protein and induce apoptosis [13, 14]. In this study, Western-blot method was used to detect the changes in p53 expression, suggesting that M18 may kill SMMC-7721 cells through DNA damage and increasing p53, and thus induced cells into apoptosis. Hoechst 33258 fluorescence staining was used to observe SMMC-7721 nuclear morphological changes; it showed that at 24 h after M18 treatment, the apoptosis of SMMC-7721 cells increased, showing nuclear chromatin condensation, nuclear side-shift, nuclear fragmentation, apoptotic bodies and other apoptotic nuclear morphological changes. TUNEL assay showed that, with the increasing concentrations of M18, apoptosis was significantly increased. Western-blot results also proved that with the increasing concentrations of M18, Caspase-3 expression and cleavage fragments increased, indicating that the activation of Caspase-3 pathway induced apoptosis [15]. We speculated theoretically that, p53-mediated apoptosis pathway was mainly based on mitochondrial apoptosis pathway. This study used M18 to induce cytochrome c to flow out from the mitochondria of SMMC-7721 cells, resulting in mitochondrial membrane potential decrease; this indicated that M18 induced apoptosis mainly through mitochondrial apoptosis pathways.

In summary, modified fluoroquinolone derivative M18 could significantly inhibit the proliferation of tumor cells, with a better selectivity; M18 could induce the apoptosis of human hepatoma SMMC-7721 cells, which was mediated by mitochondrial apoptosis pathway.

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Disclosure of conflict of interest.

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Address correspondence to: Bin Liu, School of Nursing, Henan University Jinming Campus, Jinming Road North, Kaifeng, Henan Province, Kaifeng 475004, China. Tel: +86-0371-23880399; Fax: +86-0371-23880399; E-mail: binliu_227@163.com

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