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Low toxic and high soluble camptothecin derivative 2–47 effectively induces apoptosis of tumor cells in vitro

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ABSTRACT

The cytotoxic activity of camptothecin derivatives is so high that these compounds need to be further modified before their successful application as anti-cancer agents clinically. In this study, we reported the synthesis and biological evaluation of a novel camptothecin derivative called compound **2–47**. The changes in structure did not reduce its activity to inhibit DNA topoisomerase I. Compound **2–47** induced apoptosis of many tumor cells including leukemia cells K562, Jurkat, HL-60, breast cancer cell BT-549, colon cancer cell HT-29 and liver cancer cell HepG2 with a half maximal inhibitory concentration (IC_{50}) of 2- to 3-fold lower than HCPT as a control. In particular, **2–47** inhibited the proliferation of Jurkat cells with an IC_{50} of as low as 40 nM. By making use of Jurkat cell as a model, following treatment of Jurkat cells, compound **2–47** activated caspase-3 and PARP, resulting in a decreased Bcl-2/Bax ratio. These data showed that compound **2–47** induces Jurkat cell death through the mitochondrial apoptotic pathway. In addition, compound **2–47** showed a decreased cytotoxic activity against normal cells and an improved solubility in low-polar solvent. For example, compound **2–47** solutes in $CHCl_3$ 130-fold higher than HCPT. Taken together, our data demonstrated that camptothecin derivative **2–47** notably inhibits the tumor cell proliferation through mitochondrial-mediated apoptosis in vitro.

1. Introduction

As a novel structure of alkaloids, camptothecin (CPT) was first isolated by Wall et al., in 1958 [1]. Although this compound has antitumor effects on experimental tumors severe toxicity as tested both in animal experiments and clinical trials hampered it to be used as an anticancer drug clinically [2]. It is obvious that the parent molecule has to be modified to increase its efficacy and to decrease its toxicity before it could be developed as a usable antitumor drug. Over the past decades, a series of camptothecin derivatives had been synthesized by different groups and the main camptothecin derivatives includes 10-hydroxy camptothecin [3,4], topotecan hydrochloride [5–7], irinotecan [8–11], 7-ethyl-10-hydroxycamptothecin [12,13], 9-amino camptothecin [14] and 9-nitrate based camptothecin [15]. Amongst them, 10-hydroxy camptothecin (HCPT) became an important intermediate for synthesis of camptothecin derivatives and has been attracting more and more attention. This compound has good anti-tumor activity especially for a variety of solid tumors, such as gastric cancer [16], primary liver cancer [17], chronic myeloid leukemia, colon cancer [18], colorectal cancer, lung cancer [19], bladder cancer [20] and other prevention [21] as well.

HCPT and its derivatives including topotecan hydrochloride, irinotecan, 7-ethyl-10-hydroxycamptothecin, 9-amino camptothecin and 9-nitrate camptothecin inhibit the proliferation of tumor cells through the same mechanism. These compounds target DNA topoisomerase I (Topo I) to inhibit DNA replication and transcription [22]. It has been reported that these inhibitors can bind to Topo I-DNA complex to form inhibitor-Topo I-DNA ternary complex so that the replication of DNA is thus blocked. The final outcome is

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that the tumor cells die. At present, the weakness for these compounds to be used as anti-tumor reagent is their low solubility, high toxicity and some other factors. For example, topotecan only has a short half-life [23], the cost of irinotecan production is high [24], the solubility of 9-amino camptothecin and 9-nitrate camptothecin is very low [25]. All these shortcomings severely affected the bioavailability of these compounds and then their possible usage clinically. So, the solution for camptothecin derivatives to be used as antitumor reagents is to synthesize one with 10-hydroxy camptothecin nucleus structure and meanwhile characteristic of possessing excellent antitumor activity and greatly increased bioavailability.

We previously reported the design, synthesis and in vitro cytotoxicity evaluation of a total of 39 camptothecin derivatives with 10-hydroxy camptothecin nucleus structure as anticancer agents and identified that the compound **2**–**47** exhibits excellent cytotoxic activity against three different cancer cell lines including K562, HepG2 and HT-29 [26]. Since the cytotoxic activity of compound **2**–**47** against Jurkat cells (IC_{50} = 40 nM) was found to be higher than that of K562 cells (IC_{50} = 70 nM) we thus choose Jurkat cells for further study. In the current study, we aim to clarify the molecular mechanisms how compound **2**–**47** exerts its cytotoxic activity by performing many assays including apoptotic in Jurkat cells, DNA topoisomerase I activity, toxicity, solubility and affection in vitro.

2. Materials and methods

Compound **2**–**47** *synthesis*. To a stirred solution of 10-hydroxycamptothecin (5.00 g, 13.7 mM) in dry DMF (250 ml) was added anhydrous potassium carbonate (7.59 g, 54.89 mM) and then t-chloroacetate butyl ester (7.9 ml, 54.89 mM) at 0 °C. The reaction mixture was stirred at 0 °C for 15 min, before warming to room temperature. After stirring at room temperature for another 10 h, the mixture was washed with dichloromethane (3 × 750 ml) and the organic layer was dried with anhydrous sodium sulfate. Removal of solvent under vacuum gave a brownish residue that was purified by column chromatography (200 mesh silica gel, CH_2Cl_2/CH_3OH, 200:1) to afford compound **2**–**47** (3.97 g, 60% yield) as a pale solid.

Cell lines and culture conditions. All tumor cell lines were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China) and Chinese Academy of Medical Sciences Basic Medical Institute Cell Resource Center (Beijing, China). Cells were incubated at 37 °C in 5% CO_2/95% air and the medium contains 1% penicillin/streptomycin and was replaced once every three days. Briefly, K562, HL-60, Jurkat, BT474, BT549, HCT-116 and S180 cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 2.05 mM glutamine. HepG2, DU145, PC3, SK-OV-3, MCF-7, A498, A549, NCI-322, U251 and Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 4 mM L-glutamine, 1 g/L D-glucose and 110 mg/L sodium pyruvate. HT-29 and HUV-EC cell lines were cultured in DMEM-F12 containing 10% fetal bovine serum, 2.5 mM L-glutamine and 15 mM HEPES Buffer. HEK-293 cells were cultured in MEM-basic containing 10% fetal bovine serum, Earle's salts and L-glutamine.

Cell proliferation assay. The cell proliferation activity was measured using the MTT assay [27]. In detail, 100 μl of Jurkat, K562 and HL-60 cells at a density of 5 × 10^4 cells/ml was added to each well in 96-well plates and cultured for 2 h. The same amount of BT474, BT549, HCT-116, DU145, PC3, SK-OV-3, MCF-7, A498, A549, NCI-322, U251, Hela, HepG2, HT-29, L-02, HUV-EC and HEK-293 cells was cultured in 96-well plates for 24 h. The compound with various concentrations (1–10000 nM) was added to each well and the cells continued to culture for additional 48 h. In the following, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated for additional 4 h. Finally, 100 μl dimethyl sulfoxide was added and absorbance of each well was measured by plate reader at a test wavelength of 490 nm against a standard reference solution at 630 nm by using a thermo microplate reader. The DMSO-treated controls were calculated as a cell viability value of 100%. The IC_{50} values were obtained by nonlinear regression using GraphPad Prism 5.0 (GraghPad Systems Inc. USA). Each experiment to determine IC_{50} value was conducted three times with replicate wells per concentration.

Flow cytometric analysis of apoptosis. Cell apoptosis was assayed by using an Annexin-V-FITC Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's instructions. In brief, K562 cells were treated with two different concentrations of compound for indicated periods. Cells were then harvested, washed twice with ice-cold PBS (−) and resuspended in 1 × Binding buffer at a concentration of 1 × 10^6 cells/ml. After this, cells were stained with 5 μl of Annexin-V-FITC (6 ng/μl) and 5 μl of PI (50 μg/ml) for 15 min at room temperature in the dark and analyzed by flow cytometry.

Western blot analysis. Anti-Bcl-2 (1:500) and anti-Bax (1:500) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other antibodies (1:500) were purchased from cell signaling technology (CST, Boston, MA, USA). Cells were lysed in a lysis buffer containing 10 mM Hepes-Na, 150 mM Na_2SO_4, 1 mM EDTA, 3% CHAPS, 1 mM phenylmethylsulfonyl fluoride and 10 mg/ml each of aprotinin and leupeptin. For Western blot analysis of total cell lysates, samples were prepared by mixing an aliquot of cell lysates with an equal volume of 2 × Laemmli's sample buffer and heating at 100 °C. Samples were separated by SDS-PAGE and electrotransfered to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were probed with a relevant antibody and incubated with Alexa Fluor® 680 Goat Anti-Mouse IgG (H + L) and Alexa Fluor® 680 Goat Anti-Rabbit IgG (H + L) (1:1000) followed by detection using Odyseey Western blot detection system (Amersham Pharmacia Biotech, USA).

3. Results

3.1. Synthesis route and characterization of compound 2–47

The synthesis route of compound **2**–**47** was shown in Fig. 1.

^{1}H-NMR spectra was recorded on Bruker AM-400 NMR spectrometers in deuterated chloroform and deuterated DMSO. The chemical shifts are reported in δ (ppm) relative to tetramethylsilane as internal standard.

^{1}HNMR (d_6-DMSO 400 MHz): δ/ppm 0.859–0.902 (m, 3H), 1.453 (s, 9H), 1.815–1.922 (m, 2H), 4.862 (s, 2H), 5.258 (s, 2H), 5.417 (s, 2H), 6.492 (s, 1H), 7.288 (s, 1H), 7.464 (d, 1H), 7.522–7.552 (m, 1H), 8.089 (d, 1H), 8.525 (s, 1H).

3.2. Anticancer spectrum of compound 2–47

In this study, the anticancer effect of compound 2–47 on a total of seventeen of human cancer cells was investigated. Our data showed that treatment of compound 2–47 efficiently inhibited the proliferation of 17 human cancer cell lines including the leukemia cells Jurkat, HL-60, K562, the breast cancer cells BT549, MCF-7, the colon cancer cells HT-29, HCT-116, the live cancer cells HepG2, A498, the lung cancer cells A549, NCI-H322, and the cervical cancer cells Hela, suggesting that compound **2**–**47** has a wide antitumor spectrum (Table 1). The IC_{50} values were much lower than 10 μM for the majority of the human cancer cell lines as tested. In particular, it was notable that compound **2**–**47** effectively inhibits the cell proliferation of leukemia cells Jurkat, K562, HL-60, breast

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