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SL-01, an oral derivative of gemcitabine, inhibited human breast cancer growth through induction of apoptosis

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ABSTRACT

SL-01 is an oral derivative of gemcitabine that was synthesized by introducing the moiety of 3-(dodecyloxycarbonyl) pyrazine-2-carbonyl at N4-position on cytidine ring of gemcitabine. We aimed to evaluate the efficacy of SL-01 on human breast cancer growth. SL-01 significantly inhibited MCF-7 proliferation as estimated by colorimetric assay. Flow cytometry assay indicated the apoptotic induction and cell cycle arrest in G1 phase. SL-01 modulated the expressions of p-ATM, p53 and p21 and decrease of cyclin D1 in MCF-7 cells. Further experiments were performed in a MCF-7 xenografts mouse model. SL-01 by oral administration strongly inhibited MCF-7 xenografts growth. This effect of SL-01 might arise from its roles in the induction of apoptosis. Immunohistochemistry assay showed the increase of TUNEL staining cells. Western blotting indicated the modulation of apoptotic proteins in SL-01-treated xenografts. During the course of study, there was no evidence of toxicity to mice. In contrast, the decrease of neutrophil cells in peripheral and increase of AST and ALT levels in serum were observed in the gemcitabine-treated mice. Conclusion: SL-01 possessed similar activity against human breast cancer growth with gemcitabine, whereas, with lower toxicity to gemcitabine. SL-01 is a potent oral agent that may supplant the use of gemcitabine.

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1. Introduction

Gemcitabine (2′,2′-difluoro-2′-deoxy-cytidine, dFdC), is an analogue of deoxycytidine with germinal fluorine atoms at the 2′-carbon of the sugar moiety [1]. Gemcitabine is active against several types of solid tumors, including non-small cell lung cancer (NSCLC), breast cancer, ovarian cancer and pancreatic carcinoma [2,3]. Gemcitabine has to be administrated intravenously for its low bioavailability when given orally. After absorption, gemcitabine is activated to its active 5-diphosphate (dFdCDP) and triphosphate (dFdCTP) by deoxycytidine kinase [4]. dFdCDP slows the synthesis and repair of DNA by inhibition of ribonucleotide reductase [5], which also subsequently leads to an increase of dCK activity. The other active form, dFdCTP, competes with deoxycytidine-triphosphate for incorporation into DNA, thereby inhibiting DNA polymerase and preventing the activity of DNA repair enzymes [5]. Gemcitabine is rapidly inactivated in plasma and liver, where its half-life is approximately only 70 min, to form a uridine (dFdU) through the deamination by deoxycytidine deaminase. Gemcitabine injection produces various toxicities, such as hematology, gastrointestinal, hepatic and pulmonary toxicity [6–8]. Therefore, attempts have been made to design new deoxycytidine derivatives of gemcitabine.

SL-01, dodecyl-3-((1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl) carbamoyl) pyrazine-2-carboxylate, is a deoxycytidine derivative of gemcitabine that designed by introducing the moiety of 3-(dodecyloxycarbonyl) pyrazine-2-carbonyl at the N4-position on cytidine ring of gemcitabine [9,10]. This modification may expect to block the deamination to dFdU, leading to reducing the first-pass metabolism and therefore improving its bioavailability. Introducing of the moiety of 3-(dodecyloxycarbonyl)pyrazine-2-carbonyl might also improve the lipophilic activity to traverse cell membranes via passive diffusion, and therefore to accumulate high levels of drug in cancer cells [11,12]. In addition, the amide linkage might be more stable as compare to the homologous ester bond [13]. SL-01 is therefore expected to be stable in gastrointestinal tract by orally. Our previous studies showed that SL-01 was stable in the simulated intestinal fluid. The result of pharmacokinetics in mice indicated that the half-life of SL-01 was 4.6 h, and the absolute bioavailability and relative bioavailability were 59.2% and 120.1%, respectively as compared to gemcitabine, when given orally. We expected that SL-01 might possess higher efficacy than gemcitabine in the inhibition of cancer growth. In this study, we

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evaluated the effect of SL-01 on the growth of human breast cancer by *in vitro* and *in vivo* studies. Its efficacy and toxicity were then compared with that of gemcitabine.

2. Materials and methods

2.1. Chemicals

The structure of SL-01 was reported in our previous study [9,10]. The purity of SL-01 as measured by high performance liquid chromatography (HPLC) was 99.9%. SL-01 was dissolved in dimethylsulfoxide (DMSO, Sigma) for *in vitro* assay and suspended in 0.5% sodium dodecyl sulfate (Shanhe Pharmaceutical Excipients Co. China) for application in mice. Gemcitabine was obtained from Eli Lilly and Co. (Indianapolis, USA), and was dissolved in PBS before use.

2.2. Cell line and cell culture

Human breast cancer cell line MCF-7 was purchased from the American Type Cell Culture Collection (ATCC). Cells were maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine at 37 °C in a humidified atmosphere (5% CO_2-95% air). Cell proliferation was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2.3. Cell cycle analysis

Cancer cells (1.5×10^5 per well) seeded in 6-well plates were synchronized by 24 h of growth in 0.5% serum medium, and then were exposed to 10% serum medium containing different concentrations of SL-01 or gemcitabine for 24 h. Cells were harvested and fixed in cold 70% ethanol overnight. Cells were suspended in 0.6 ml propidium iodide (PI) solution for 30 min. Cell cycle was analyzed by using a FACScan Flow cytometer.

2.4. Annexin V/FITC/PI staining analysis

Cancer cells (1.5×10^5 per well) seeded in 6-well plates were exposed to increasing concentrations of SL-01 or gemcitabine for 24 h. Cells were harvested and cell surface of phosphatidylserine in apoptotic cells was quantitatively estimated by using Annexin-V/FITC and PI apoptosis detection kit (Labtek, China). The analysis of apoptotic cells was performed on a FACScan flow cytometry.

2.5. MCF-7 xenograft model

The efficacy of SL-01 was assessed in mice bearing MCF-7 xenografts. The research protocol was approved by the Committee of Animal Care and Use at Shandong University. Female athymic Balb/c athymic mice, 6 weeks of age, were purchased from the Colab Animal Center (Beijing, China). Cancer cell implantation was performed under surgical sterile conditions. MCF-7 cells (1×10^7 in 200 μl of PBS) were injected subcutaneously in the flanks of one mouse. Two weeks later, mouse was sacrificed and the cancer tissue was cut into 1.5 mm thick pieces and inoculated subcutaneously into left armpit of mice with puncture needle. When tumor volume reached approximately 100 mm^3, mice were given different dosages of SL-01 and equal volume of vehicle by oral administration. Gemcitabine was given through tail vein. Drugs were given once every 2 days for three weeks. Mice were weighed every 3 days and checked daily for any signs of illness. Tumor volumes were determined every 3 days by measuring diameters of tumors. The volumes were calculated using the formula, $V = (ab^2)/2$, where *a* is the long axis and *b* is the short axis.

Mice were sacrificed by anesthetizing with chloral hydrate. Blood samples were collected by exsanguination from inferior vein. Tumors were dissected and weighed. 200 μl blood samples were mixed with 20 μl of 0.5 M EDTA (pH 8.0) in a 1.0-μl eppendorf tube. An automated hematology analyzer was used to count the blood elements. Blood was centrifuged and serum was obtained for analysis of aspartate aminotransferases (AST) and alanine aminotransferase (ALT). Analysis was performed using commercial kits (Biosino Bio-Technology, China).

The visceral organs including liver and stomach were removed from each mouse. Organs were fixed in formalin and embedded in paraffin. Serial 4-μm sections were prepared and stained with H&E for microscopic assessment.

2.6. TUNEL staining for apoptosis detection

Apoptotic cells in the MCF-7 xenografts were determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using in situ cell death detection kit (Roche, Germany). Serial 4-μm sections were cut from formalin fixed xenografts. The staining was performed according to manufacturer's instruction. Cancer cells with brown staining in nuclei were considered as TUNEL positive cells. The proportion of positive cells in three mice per group was scored randomly under a microscope.

2.7. Western blotting analysis

Western blotting assay was performed to analyze the expression of p53, pATM, p21, cyclin D1 in MCF-7 cells and the apoptotic proteins in MCF-7 xenografts. For *in vitro* assay, MCF-7 cells (2×10^5) seeded in 6-well plates were treated with SL-01 for 24 h. Cells were harvested and cell lysates were fractionated by 10% SDS–PAGE as described below. In MCF-7 xenografts, cancer tissues were dispersed mechanically in PBS and dissolved with RIPA lysis buffer. The supernatants were collected and total protein was determined using BCA protein assay kit (Beyotime, China). Tumor lysates (30 μg of protein per lane) were separated by 10% SDS–PAGE. The proteins were electro-transferred onto PVDF membranes and then detected using the primary antibodies. The primary antibodies included those for caspase-9 (9502), caspase-3 (9662), cleaved PARP (9541), Bcl-2 (2872), Bax (2772), cyclin D1 (2922), p53 (9282), pATM (5883, Cell Signaling), p21 (2990-1), β-actin (5779-1, Epitomics). The bound antibodies were visualized using an enhanced chemiluminescence reagent and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad). Densitometric analyses of bands were adjusted with β-actin as loading control.

2.8. Statistical analysis

Data were presented as mean ± S.D. and analyzed by independent Student's *t* test. Multiple comparisons between groups were performed using S-N-K method. The limit of statistical significance was $P < 0.05$. Statistical analysis was performed with SPSS/Win13.0 software (SPSS, Inc., Chicago, Illinois).

3. Results

3.1. Inhibition of cancer growth in vitro

MCF-7 cells were exposed to SL-01 and then were subjected to the MTT assay. As shown in Fig. 1A, SL-01 at ranging from 0.125 to 2.0 μM, the percentage of inhibition were increased from 12.3% to 40.7%, for 24 h exposure; from 15.8% to 55.2%, for 48 h exposure;

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