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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.

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;

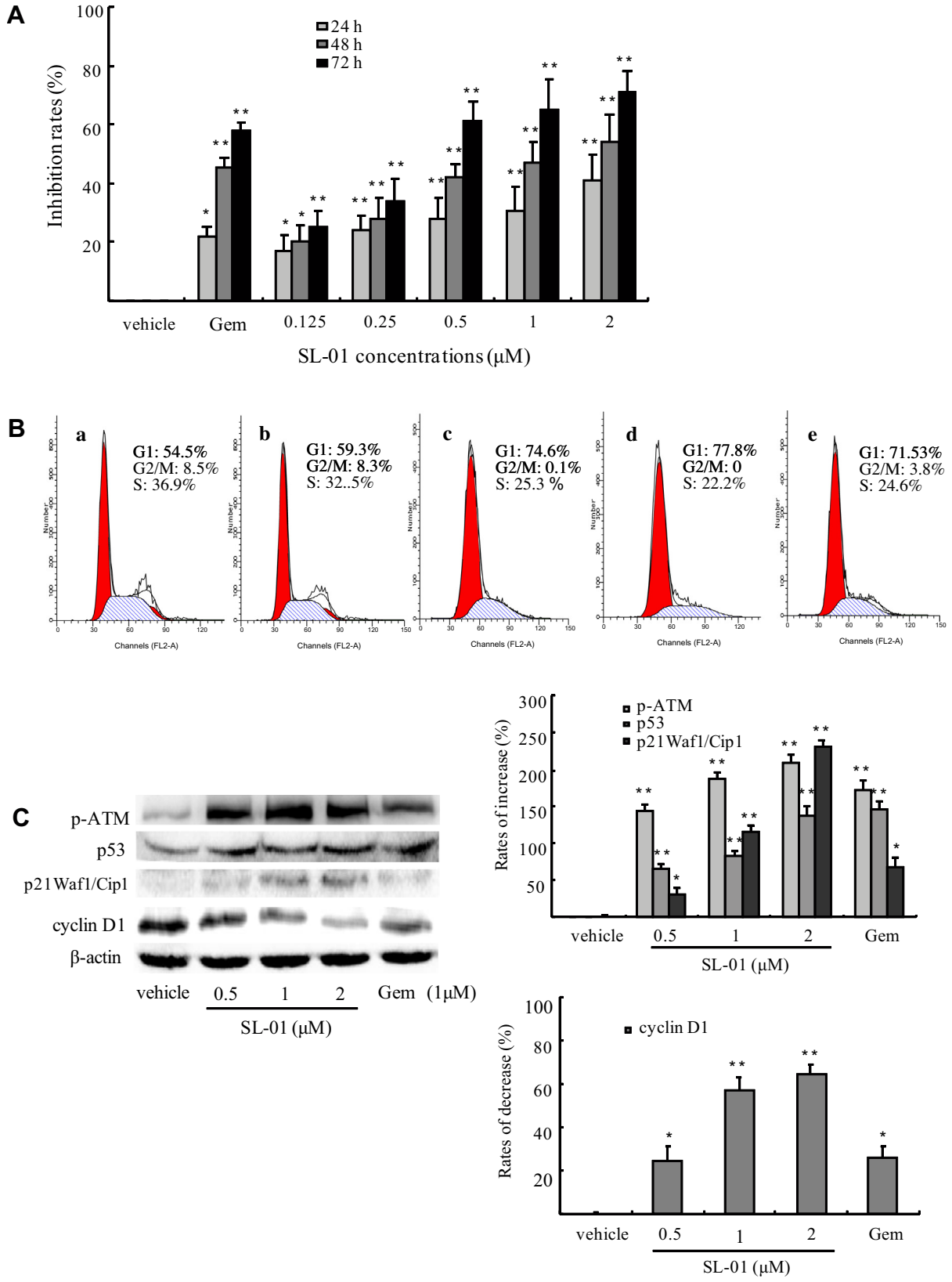

Fig. 1. The inhibition of SL-01 on the growth of MCF-7 cells by *in vitro* assays. (A) The inhibition of SL-01 on the growth of MCF-7 cells as evaluated by MTT assay. The bars indicate mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the vehicle control. (B) SL-01 arrested cell cycle in G1 phase as evaluated by flow cytometry analysis. (B)-a, the vehicle control; (B)-(a–d), SL-01 at concentrations of 0.5, 1.0, and 2.0 μM for 24 h; (B)-e, gemcitabine 1 μM. (C) Western blotting analyzed levels of p21 in p-ATM, p53, p21, and cyclin D1 in MCF-7 cells. The bars represent mean ± S.D. of triplicate experiments with triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the vehicle control.

and from 16.4% to 63.0%, for 72 h exposure, respectively (0.125 μM, $P < 0.05$; 0.25–2.0 μM, $P < 0.01$ *vs.* the vehicle control). Statistical analysis indicated that the IC50 value that based on the inhibition rates of 72 h exposure was 0.64 μM. This profile of inhibition in SL-01 was similar to that of gemcitabine. Using 1 μM for 72 h exposure, the inhibition rate was 62.9% for SL-01 and 58.3% for gemcitabine ($P > 0.05$, between SL-01 and gemcitabine).

3.2. Cell cycle arrest in G1 phase

The inhibitory effect of SL-01 was further evidenced by its activity in the regulation of cell cycle distribution. SL-01 was found to arrest cell cycle in G1 phase. As shown in Fig. 1(B)-b–d, SL-01 at 0.5, 1.0, and 2.0 μM for 24 h, the percentages of cell population in G1 phase were significantly increased from 59.3% in the vehicle-treated cells (Fig. 1(B)-a) to 77.8% (0.5 μM, $P < 0.05$; 1 μM, $P < 0.01$ *vs.* the vehicle control). Gemcitabine at 1 μM increased G1 phase from 59.3% to 71.53% (Fig. 1(B)-e, 1 μM, $P < 0.01$ *vs.* the vehicle control).

Western blotting showed that the expressions of p53, p-ATM and $p21^{Waf1/Cip1}$ were significantly increased, and cyclin D1 was decreased in the SL-01-treated cells. As shown in Fig. 1C, SL-01 at 0.5, 1, and 2 μM for 24 h, the levels of p-ATM were significantly increased by 135.0%, 183.7%, 203.3%, respectively (0.5, 1 and 2 μM, $P < 0.01$ *vs.* the vehicle control); p53 by 54.9%, 86.5%, 141.5%, respectively, (0.5, 1 and 2 μM,$P < 0.01$ *vs.* the vehicle control); $p21^{Waf1/Cip1}$ by 38.5%, 122.6%, and 232.8%, respectively (0.1 μM, $P < 0.05$; 1 and 2 μM, $P < 0.01$*vs.* the vehicle control). In the same sample, cyclin D1 was reduced by 25.8%, 58.4%, 70.4%, respectively (0.1 μM, $P < 0.05$; 1 and 2 μM, $P < 0.01$ *vs.* the vehicle control). Statistical analysis showed that SL-01 might possess more potential in regulation of these cell cycle associated proteins than gemcitabine (2 μM, $P < 0.05$ between SL-01 and gemcitabine).

3.3. Inhibition of cancer growth in mice

The activity of SL-01 was confirmed in mice bearing MCF-7 xenografts. The grafted MCF-7 tissue was strongly inhibited by SL-01 after 3 weeks oral administration (Fig. 2A, Table 1). SL-01 at 12.5, 25 and 50 μmol/kg, the growth of MCF-7 xenografts was reduced by 10.1%, 34.5% and 66.5% (25 μmol/kg, $P < 0.05$; 50 μmol/kg, $P < 0.01$ *vs.* the vehicle control), respectively. Gemcitabine at 25 μmol/kg by injection inhibited the growth of MCF-7 xenografts by 68.4% ($P < 0.01$ *vs.* the vehicle control). The effect of SL-01 was also observed by the delayed increase of tumor volume (Fig. 2B). SL-01 by oral administration was generally well tolerated by mice with no significant loss of body weight ($P > 0.05$ *vs.* the vehicle control). In contrast, a loss of body weight was observed in the gemcitabine-treated mice (Fig. 2C, $P < 0.05$ *vs.* the vehicle control).

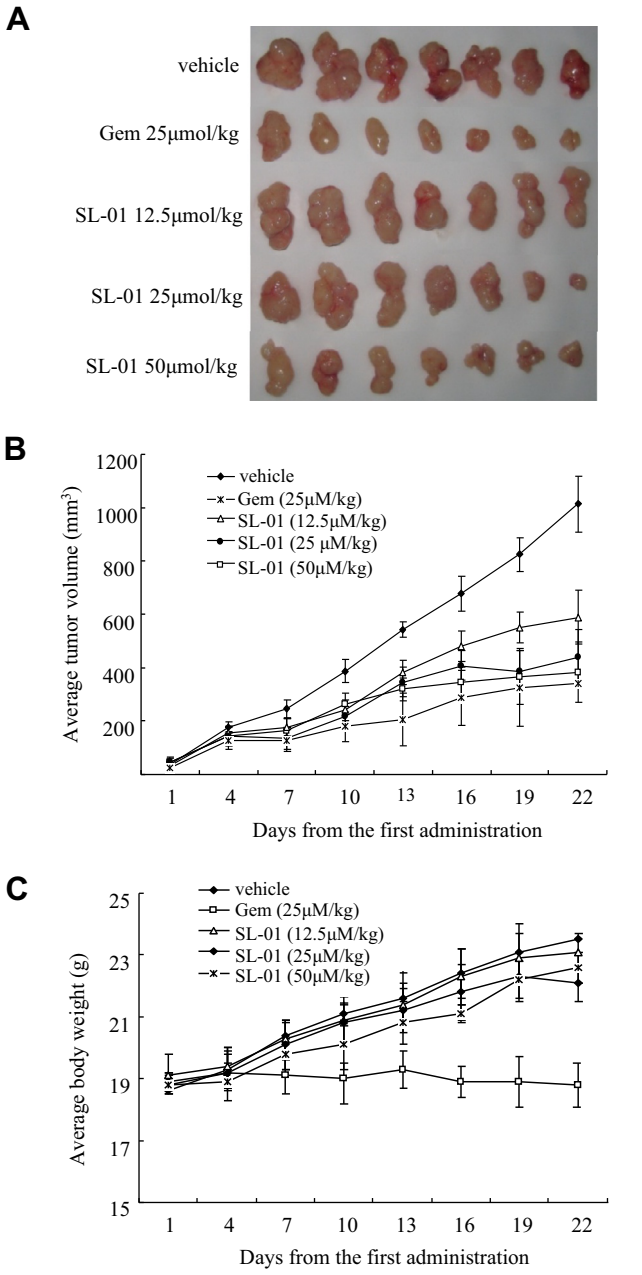

Fig. 2. SL-01 prevented the growth of MCF-7 xenografts in mice. SL-01 was administered orally once every 3 days for three weeks. The volume of the grafted cancer tissues (B) and body weight (C) were measured every three days. The inhibition of cancer growth was defined as a percentage of control tumor weight. Data are mean ± S.D. ($n = 7$). Gem: gemcitabine. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the vehicle control.

3.4. Induction of apoptosis by in vitro and in vivo studies

The inhibition of SL-01 on cancer growth was associated with its role in the induction of apoptosis. As shown in Fig. 3 of the cultured cells, SL-01 at 0.5, 1 and 2.0 μM, the percentages of apoptotic cells were significantly increased by 20.4%, 30.93% and 34.3%, respectively (Fig. 3(A)-b–d). Gemcitabine at 1 μM increased apoptotic cells by 33.2% (Fig. 3(A)-e).

The induction of apoptosis by SL-01 was confirmed in the grafted cancer tissue. We performed the TUNEL staining assay to analyze the apoptotic cells in MCF-7 xenografts. The percentages of TUNEL staining cells in the vehicle-treated MCF-7 xenografts were 7.1%. SL-01 at 12.5, 25 and 50 μmol/kg significantly increased the percentages of TUNEL staining cells by 19.4%, 23.5% and 33.8% (Fig. 3(B)-b–d,$P < 0.05$ *vs.* the vehicle control), respectively. Gemcitabine (25 μmol/kg) induced TUNEL staining positive cells by 32.3% (Fig. 3(B)-e,$P < 0.05$ *vs.* the vehicle control).

Western blotting indicated that SL-01 also activated the caspase cascade pathway as demonstrated by the increase of cleaved caspase-9, and PARP in MCF-7 xenografts (Fig. 3C). SL-01 at 12.5, 25, 50 μmol/kg increased the level of cleaved caspase-9 by 26.5%, 61.9% and 89.8%, respectively (12.5 μmol/kg, $P < 0.05$; 25 and 50 μmol/kg, $P < 0.01$ *vs.* the vehicle control). The percentages of increase in cleaved PARP were strongly increased by 61.2%, 100.8% and 132.3%, respectively ($P < 0.01$ *vs.* the vehicle control). The percentage of increase in cleaved PARP by 25 μmol/kg of gemcitabine was 123.6% ($P < 0.05$ *vs.* the vehicle control). Analysis of Bax and Bcl-2 indicated that SL-01 significantly modulated the ratio of

Table 1
The inhibitory effect of SL-01on MCF-7 xenografts growth in nude mice ($n = 7$).

Dosage (μmol/kg)	Number of mice (n)	Body weight (g)[a] (initial/22 days)	Tumor weight[b] (g, mean ± S.D)	Tumor growth inhibition (%)
SL-01				
Vehicle	7	18.1 ± 1.7/23.9 ± 1.6	1.11 ± 0.43	–
12.5	7	18.4 ± 1.1/22.7 ± 1.5	1.00 ± 0.31	10.1
25	7	18.4 ± 1.4/22.5 ± 3.4	0.73 ± 0.32	34.5
50	7	18.9 ± 1.8/22.2 ± 2.2	0.37 ± 0.16**	66.5
Gemcitabine				
25	7	19.0 ± 1.6/20.2 ± 2.4**	0.35 ± 0.23**	68.4

Established tumors were treated with SL-01 by oral administration or gemcitabine by injection through tail vein once every other day.
[a] Body was weighed before and after drug administration.
[b] Tumor weight was measured after mice were sacrificed.
** $P < 0.01$ *vs.* the vehicle control.

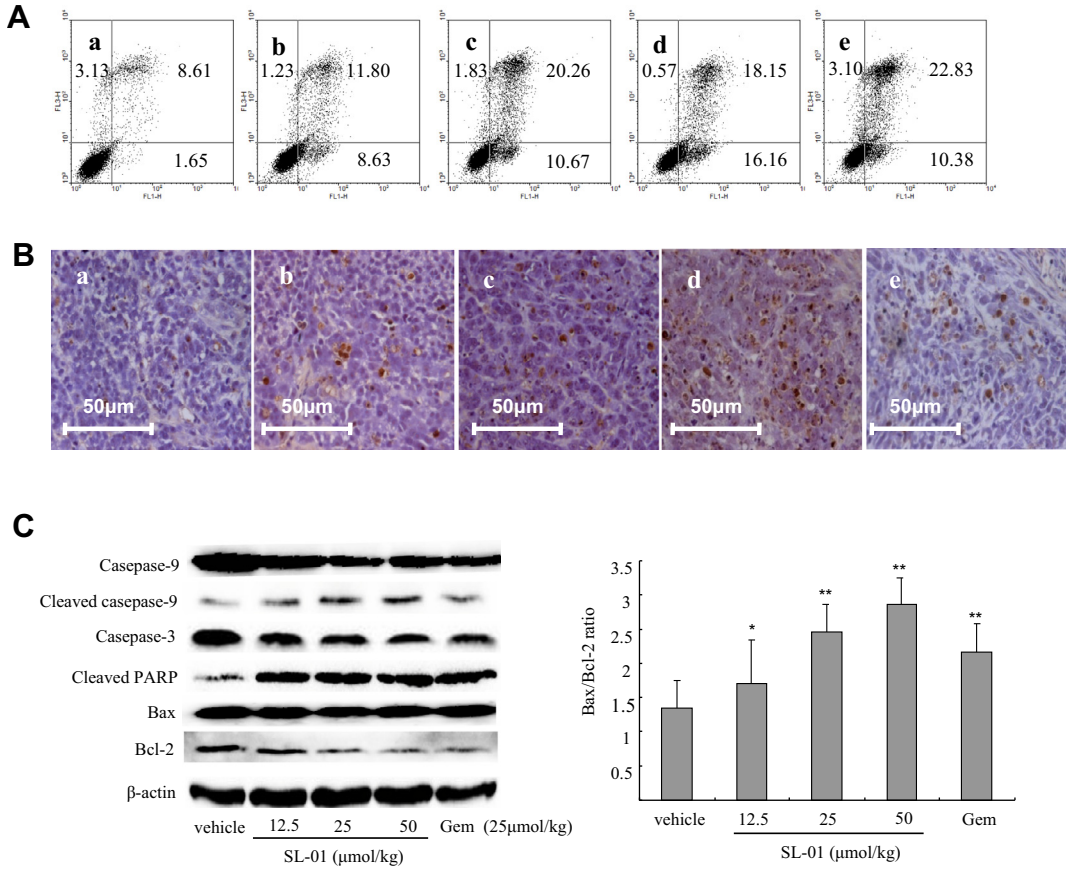

Fig. 3. SL-01 induced apoptosis in cultured MCF-7 cells and xenografts. (A) SL-01 induced MCF-7 cells to apoptosis as assayed by flow cytometry. (A)-a: vehicle control; (A)-b–d: 0.5, 1, 2 μM of SL-01; (A)-e: gemcitabine (1 μM). $P < 0.05$ *vs.* the vehicle control for SL-01 (1, 2 μM); $P < 0.05$ *vs.* the vehicle control for gemcitabine. (B) SL-01 increased TUNEL staining cells in MCF-7 xenografts. Cancer cells with brown staining in nuclei were considered as TUNEL-positive cells. The proportion of the positive cells in three mice per group was scored randomly under a microscope. a: the vehicle control; b–d: 12.5, 25, 50 μmol/kg of SL-01; e: gemcitabine (25 μmol/kg). $P < 0.01$ *vs.* the vehicle control for SL-01 (25, 50 μmol/kg); $P < 0.05$ *vs.* the vehicle control for gemcitabine. (C) SL-01 regulated the apoptotic proteins in MCF-7 xenografts. The levels of caspase-9, caspase-3, cleaved PARP, Bax and Bcl-2 were evaluated by western blotting assay. Data were mean ± S.D. ($n = 3$). **$P < 0.01$ *vs.* the vehicle control.

Bax/Bcl-2 in MCF-7 xenografts. Gemcitabine was also showed the activity in the induction of caspase cascade pathway (Fig. 3C).

3.5. The toxicity of SL-01 and gemcitabine in mice

We finally evaluated the toxicities of SL-01 in the MCF-7 xenografts mouse model and then compared them with gemcitabine. We counted the peripheral blood elements, including total white blood cells (WBC), neutrophils, lymphocytes, red cells and platelets, and also examined the serum ALT and AST. There was no significant difference in blood elements counts and serum ALT and AST between the vehicle-and SL-01-treated mice (Tables 2 and 3, $P > 0.05$). In the gemcitabine-treated mice, no significant changes were observed in total WBC, lymphocyte and platelet. However, a significant decrease of neutrophil cells was accounted. Moreover, higher levels of AST and ALT were observed in the gemcitabine-treated mice as compared with those of the vehicle-treated mice (Tables 2 and 3, $P < 0.05$).

Table 2
Effects of SL-01 on serum aminotransferases in nude mice ($n = 7$).

Groups (μmol/kg)	Number of mice (n)	ALT (U/L)	AST (U/L)
Vehicle	7	28.5 ± 2.9	38.2 ± 5.1
SL-01			
12.5	7	29.3 ± 3.2	40.4 ± 3.0
25	7	28.9 ± 4.2	39.4 ± 3.8
50	7	30.1 ± 5.2	41.1 ± 4.4
Gemcitabine			
25	7	42.4 ± 3.1*	51.8 ± 2.9

The mice were treated with SL-01 by oral administration or gemcitabine by injection through tail vein once every other day.
* $P < 0.05$ *vs.* the vehicle control.

Histopathologic examination showed no abnormalities in liver and stomach in the SL-01-treated mice. Gemcitaine also did not cause damage mucosa in stomach. In contrast, there was a relatively increase of inflammatory cells scattered in the liver as compared to the vehicle-treated mice. However, no serious spotty or piecemeal necrosis was observed (Fig. 4).

4. Discussion

SL-01 is a gemcitabine derivative designed by introducing the moiety of 3-(dodecyloxycarbonyl) pyrazine-2-carbonyl at the N4-position on cytidine ring. This design of SL-01 was considered to render it stable to both chemical and enzymatic hydrolysis in

Table 3
Effect of SL-01 on peripheral blood elements in nude mice ($n = 7$).

Dosage (μmol/kg)	Number of mice (n)	Total WBCs (×109/L)	Neutrophils ($\times 10^9$/L)	Lymphocytes ($\times 10^9$/L)	Platelets ($\times 10^9$/L)
Vehicle	7	7.06 ± 1.31	1.51 ± 0.43	5.04 ± 1.14	1027.65 ± 108.34
SL-01					
12.5	7	7.19 ± 3.17	1.67 ± 0.59	5.62 ± 1.09	1038.37 ± 117.61
25	7	6.96 ± 4.64	1.50 ± 0.76	5.10 ± 1.74	989.95 ± 120.75
50	7	6.95 ± 3.29	1.46 ± 0.68	4.96 ± 1.02	976.45 ± 137.61
Gemcitabine					
25	7	6.81 ± 2.06	1.07 ± 0.26*	4.98 ± 1.73	901.32 ± 103.45*

Balb/c athymic (nu+/nu+) mice bearing MCF-7 xenografts were treated with SL-01 by oral administration or gemcitabine by injection through tail vein once every other day. Blood was drawn from the postorbital venous plexus. An automated hematology analyzer was used to count the elements.
* $P < 0.05$ *vs.* the vehicle-treated mice.

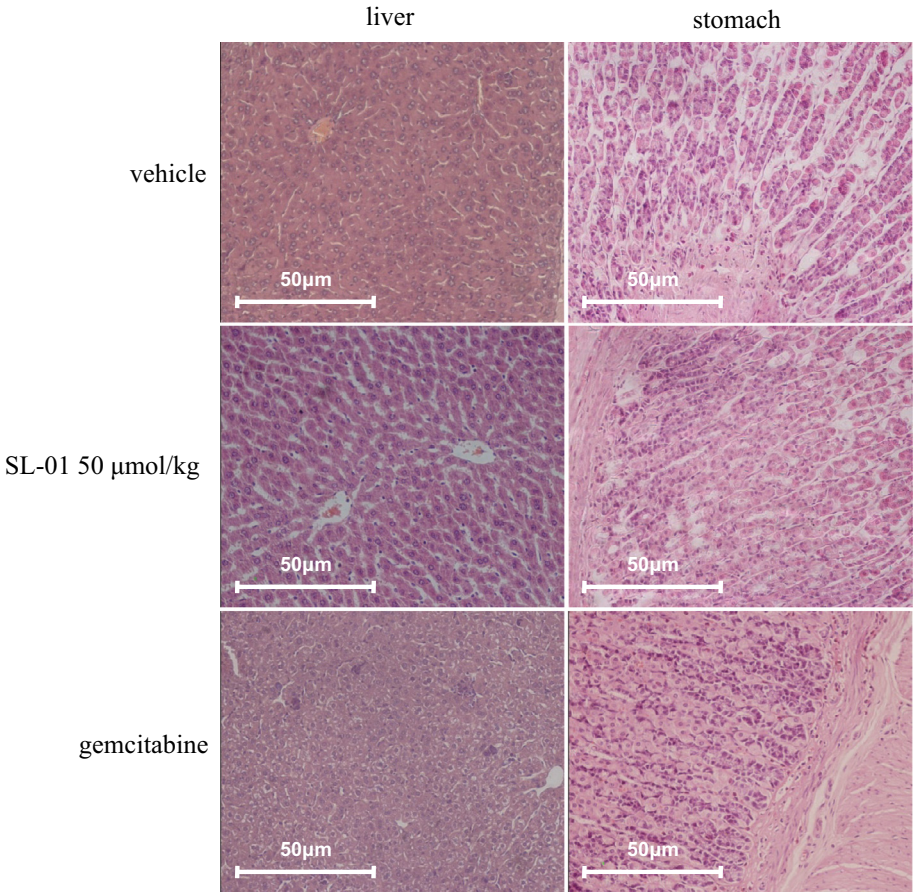

Fig. 4. H&E-stained sections of livers and stomachs from SL-01- and gemcitabine-treated mice. There were no histological abnormalities in liver and stomach in the SL-01-treated mice. In the gemcitabine-treated mice, no change was observed in stomachs. However, there was an increase of inflammatory cells scattered in liver as compared with the vehicle-treated mice (200×).

intestines. We expect that SL-01 was metabolized mostly in liver by microsomal enzymes and then released the active compound gemcitabine [9,10]. SL-01 is therefore suggested to possess higher efficacy than that of gemcitabine. In previous studies, we examined the stability of SL-01 in the simulated intestinal fluid. SL-01 was found to be more stable in chemical and enzymatic hydrolysis than gemcitabine. Further analysis of its pharmacokinetics in mice suggested that the introduction of an amide group to gemcitabine could greatly improve its bioavailability. The mean retention time (MRT) was 3.3 h, and the absolute bioavailability and relative bioavailability of SL-01 were 59.2% and 120.1%, respectively, when given orally in mice, as compared with gemcitabine [9,10]. These results indicated that SL-01 could be developed as an oral anticancer agent, potentially replacing gemcitabine for use on human cancers. This design of SL-01 based on gemcitabine was confirmed by evaluation of its activity both *in vitro* and *in vivo* studies. Our previous studies showed that SL-01 possessed the activity against human lung cancer NCI-H460 and colon cancer HCT-116 cells. SL-01 exhibited higher efficacy in mice bearing the grafted human cancer xenografts than gemcitabine. SL-01's efficacy might associate with its ability in the inhibition of cell proliferation and induction of apoptosis [9,10].

In this study, SL-01 was found to possess an inhibitory effect on human breast cancer growth. SL-01 with oral administration in mice inhibited the growth of MCF-7 xenografts without significant toxicity. The inhibitory effects of SL-01 might associate with its activity in the regulation of cell cycle and induction of apoptosis. However, these activities of SL-01 did not show more potently than gemcitabine in the inhibition rates in MCF-7 xenografts, although SL-01 might possess greater activity in the induction of apoptosis than gemcitabine ($P < 0.05$ between SL-01 and gemcitabine). Further assays were carried out in the molecular analysis levels. The expressions of p53, p-ATM and $p21^{Waf1/Cip1}$ in MCF-7 xenografts were significantly increased, and cyclin D1 was obviously decreased after treatment with SL-01. SL-01 induced cancer cells to apoptosis through activating the expression of cleaved caspase-9, and PARP in MCF-7 xenografts. SL-01 also had the role to modulate Bax/Bcl-2 ratio in MCF-7 xenografts. These results supported our previous findings that SL-01 might induce cancer cells to apoptosis through activating caspase-dependent pathway.

The toxicity of gemcitabine remains the problem that limited further use in clinic. The most commonly reported adverse drug reactions associated with i.v. treatment of gemcitabine are nausea with or without vomiting, elevated liver transaminases (AST/ALT) and alkaline phosphatase, all reported in approximately 60% of patients. Gemcitabine was also found to induce hepatistis and gastrointestinal mucosal inflammation. Gemcitabine's toxicity in myelosuppression is dose-limited, with grade III or IV hematologic toxicity developing in approximately 25% and approximately 5% of patients requiring discontinuation therapy for anemia, leukopenia, or thrombocytopenia. Other common side effects included nausea and vomiting (65%), rash (30%), fever (41%), flulike symptoms (20%) [14–16]. In this study, gemcitabine was found to induce higher levels of liver transaminases (AST/ALT), implying that the liver cells might be damaged after long time exposure to gemcitabine. This phenomenon was evidenced by histopathologic examination in liver. Gemcitabine was also found to decrease the account of neutrophils in peripheral blood. In contrast, we did not find significant changes in liver and blood elements in the SL-01-treated mice. We suggested that SL-01 might be safer than gemcitabine.

The mechanism of toxicity in gemcitabine has not been understood. It has been suggested that 2-difluorodeoxyuridine (dFdU), the main metabolite of gemcitabine, might be cause of the most toxicities, especially toxicities in liver and intestine. dFdU is formed by deoxycytidine deaminase, which is present at high levels in plasma, red blood cells and liver [17,18], and then taken up by cancer cells with high affinity by the human concentrative nucleoside transporter type 1 (hCNT1, highly expressed in liver and intestine) [19,20]. In this study, we suggested that gemcitabine might be metabolize and accumulate a high level of dFdU in a very short time in the liver and intestine and therefore cause the toxicities to these organs. In contrast, in SL-01, because of the maintenance conversion of prodrug to gemcitabine, dFdU might be produced at relatively low level. We therefore observed low toxicities in mice after treatment with SL-01.

There are two previously published clinical studies with an oral dosage form of gemcitabine, one of which with oral administration of gemcitabine itself [20]. This study was terminated because of high presystemic conversion of dFdC to dFdU and accompanying accumulation of dFdU, which most likely contributed to severe liver toxicity. The second dosage form tested orally was the prodrug LY2334737, which was designed to overcome the presystemic deamination of gemcitabine during first pass metabolism. LY2334737 has valproic acid bound to the metabolically unstable amine group and is therefore considered not to be a substrate for deaminase [21,22]. In our study, SL-01 is a derivative of gemcitabine designed by introducing the moiety of 3-(dodecyloxycarbonyl) pyrazine-2-carbonyl at the N4-position on the cytidine ring of gemcitabine. This modification may expect to block the deamination to dFdU. Like LY2334737, SL-01 appears not to be a substrate for deaminase and therefore might alleviate the adverse reaction resulted from accumulation of dFdU.

In summary, SL-01, an oral derivative of gemcitabine, exhibited similar activity against human breast cancer growth to that of gemcitabine, however, with lower toxicity than gemcitabine. SL-01 is expected to be a potent anticancer agent that may supplant the use of gemcitabine.

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