SLXM-2, a derivative of cyclophosphamide: mechanism of growth inhibition on hepatocarcinoma 22 cells

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Restructuring of cyclophosphamide (CPA) is a promising method for the development of antineoplastic therapy. This study investigated the inhibitory effects of a derivative of CPA, SLXM-2, on hepatocarcinoma 22 (H_{22}) transplanted into ICR mice as well as its effects on the survival time of mice transplanted with the ascitic fluid-type H_{22}. We found that SLXM-2 inhibited tumor growth and prolonged survival time. Moreover, the compound had little effect *in vivo* on leukocytes and body weight and a higher lethal dose 50 than CPA. The cell cycle analysis by flow cytometry revealed that SLXM-2 arrested tumor cells in both the S and G_2 phases, and the arrest in the G_2 phase increased in a dose-dependent manner. Western blotting and reverse transcription-PCR experiments indicated that the observed G_2 arrest was associated with an increase of cyclin B1, whereas cell division cycle protein 2 (Cdc2) remained constant. The results, however, showed an accumulation of tyrosine 15 phosphorylated Cdc2 and a reduction of threonine 161 phosphorylated Cdc2. In addition, SLXM-2 led to a decrease in cyclin-dependent kinase 7 and Cdc25c kinase, which participated in inhibiting the G_2/M transition. Our data identified two upstream targets leading to the inactivity of the cyclin B1/Cdc2 complex, which explained the arrest in the G_2/M phase following SLXM-2 treatment. These results demonstrated the antitumor activity of SLXM-2 and its potential use as an antineoplastic drug. *Anti-Cancer Drugs* 19:167–174 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: cell cycle arrest, cyclophosphamide, derivative, hepatocarcinoma H_{22}, lethal dose 50, leukocytotoxic effect

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Introduction

Hepatocarcinoma is a tumor with a particularly high incidence and mortality rate [1]. Current therapies include radiofrequency ablation, chemoembolization and systemic chemotherapy. Although there are various therapies for hepatocarcinoma, the chemotherapeutic agents are relatively ineffective. Accordingly, screening compounds for potential use as effective therapeutic agents for hepatocarcinoma is an important undertaking.

Cyclophosphamide (CPA) is a widely used antineoplastic drug for treating malignant lymphoma, multiple myeloma, leukemia and other malignant diseases [2]. CPA is also used for treating systemic lupus erythematosus [3]. CPA is a cytotoxic drug that results in the death of rapidly growing cells. During the process of metabolism, CPA liberates two metabolites, phosphoramide mustard and acrolein, the former generally believed to be the ultimate DNA alkylator [2,4,5]. On the contrary, acrolein is responsible for hemorrhagic cystitis. Various side effects, such as heart inflammation, anorexia, nausea and vomiting, result from the effects of the CPA metabolites on normal cells. CPA also suppresses the production of blood cells from the bone marrow. To reduce the side effect and find more potent anticancer drug, scientists are constantly seeking new methods and drugs to treat tumors and increase the survival rate of patients, such as targeted therapies, neoadjuvant chemotherapy [6,7], gene radiotherapy [3,8] and immunotherapy [9]. The molecule of CPA has also been modified numerous times. All modifications, however, focused on the cyclic part of CPA, whereas the mustard part has remained unaltered. Recently, we designed and synthesized a series of cyclophosphamide piperazine quaternary ammonium (CPQA) compounds [10] in which the mustard part is altered (Fig. 1).

Preliminary experiments show that many CPQAs exhibit in-vivo anticancer activity; however, these effects are yet to be confirmed and quantified. In this study, we selected one of the most potent compounds, SLXM-2 (CPQA, R,R′ = $MeN(CH_2CH_2\text{-})_2$, chloride3-methyl-9-(2-oxa-2λ^5-2H-1,3,2-oxazaphosphorine-2- cyclohexyl)-3,6,9-triazaspiro [5.5]undecane) [10], to investigate its potential anticancer effects, toxicity and possible mechanism of growth inhibition on hepatocarcinoma 22 (H_{22}) cells (Fig. 1).

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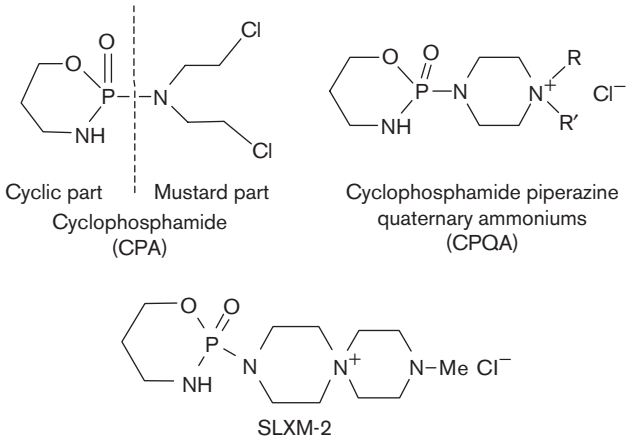
Fig. 1



The structures of CPA, CPQA and SLXM-2.

Materials and methods

Materials

CPA was purchased from Shanghai Hualian Pharmaceutical Co. Ltd (Shanghai, China). SLXM-2, provided by Professor Run-tao Li (Peking University), is a white solid with a melting point of 177–179°C. ^{1}H-NMR (D_2O, 300 MHz): 4.15–4.27 (m, 2H, OCH_2), 3.39–3.43 (m, 12H, N-CH_2, N^+-CH_2), 3.04–3.18 (m, 2H, NH-CH_2), 2.70–2.72 (m, 4H, CH_2-N-CH_2), 2.23 (s, 3H, CH_3), 1.72–1.73 (m, 2H, O-C-CH_2-C-NH). Anal. $C_{12}H_{26}ClN_4O_2P{\cdot}1.5H_2O$, C, 41.75%; H, 8.05%; N, 15.71%. SLXM-2 was dissolved in normal saline (NS) to the concentration needed. Saline was purchased from China Double-Crane Pharmaceutical Business Co. Ltd (Beijing, China). Antibodies against cyclin B1, cyclin-dependent kinase 7 (Cdk7), cell division cycle protein 25c (Cdc25c) and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA); antibodies against Cdc2, phospho-Cdc2 (Tyr^{15}) and phospho-Cdc2 (Thr^{161}) were from Cell Signaling Technology (Beverly, Massachusetts, USA); antibody against p53 was from Lab Vision Corporation (Fremont, California, USA); and antibody against β-actin was from Sigma (St Louis, Missouri, USA).

Hepatocarcinoma 22 cell line and experimental animals

H_{22} cell line was provided by the cell bank of the pharmacology group of National Key Laboratory of Natural and Biomimetic Drugs (Peking University). Animal procedures were approved by the Department of Laboratory Animal Science of Peking University Health Science Center (Beijing, China). The Center for Experimental Animals of Peking University Health Science Center (Beijing, China) provided ICR mice. Only male mice (specific pathogen-free, 4–6 weeks, weight 18–22 g) were used in this study. All the mice were maintained under clear conditions and were fed with clear food and water at room temperature and a humidity of 45–55%.

Solid-type hepatocarcinoma 22 mouse model

Effectiveness of SLXM-2 was first investigated in a solid-type H_{22} mouse model. Ascitic fluid was drawn from the abdominal cavities of mice in which H_{22} had been inoculated for 3–7 days; fluid was then diluted with NS to 1×10^9/l cell solution. We injected 0.2 ml of diluted solution into the left armpits of 50 mice by using an aseptic manipulation. The mice implanted with H_{22} were randomized into three SLXM-2 groups (15, 30 and 60 mg/kg/day), a CPA group (30 mg/kg/2 days) and an NS control group (0.1 ml/10 g/day), with 10 mice in each group. Drug administration began on the day after tumor implantation and was delivered by intraperitoneal (i.p.) injection once per day for 10 days. Body weight was recorded once per 2 days. On the 11th day, mice were euthanized, and tumors were harvested and weighed. The average tumor inhibition rate, standard deviation and *P* values were calculated in comparison with the NS control group. The tumor growth inhibition rate of drugs on H_{22} was calculated using the following formula: Tumor growth inhibition rate (%) = [mean tumor weight of control group (g) – mean tumor weight of treatment group (g)]/mean tumor weight of control group (g) × 100%.

To further determine side effects of SXLM-2, we investigated leukocytotoxic effects of SXLM-2 on a relative high dosage level. A total of 40 mice transplanted with the solid-type H_{22} were randomized into two SLXM-2 groups (60 and 120 mg/kg/day), a CPA group (30 mg/kg/2 days) and an NS control group (0.1 ml/10 g/day), with 10 mice in each group. Drug administration began on the day after tumor implantation and was delivered by i.p. injection once per day for 10 days. We drew blood on the 11th day of treatment from the eyepits of mice. Leukocytes were analyzed with MEDONIC CA620-20 VET whole-blood analyzer (Boule Medical AB, Stockholm, Sweden).

Ascitic fluid-type hepatocarcinoma 22 mouse model

Effectiveness of SLXM-2 was also evaluated in an ascetic fluid tumor model. H_{22} ascitic fluid was diluted to 5×10^9/l cell solution with NS. A total of 40 mice inoculated with the H_{22} cell solution (0.2 ml) by i.p. injection were randomly divided into two SLXM-2 groups (30 and 60 mg/kg/day), a CPA group (30 mg/kg/2 days) and an NS control group (0.1 ml/10 g/day), with 10 mice in each group. All drugs were dissolved in saline and administered i.p., once a day, for 9–10 days or until the mouse died. General reactions and the death time were observed every day after drug administration, body weight was recorded and prolongation of survival time was calculated as follows:

(mean survival days of experimental group – mean survival days of control group)/mean survival days of control group × 100%.

Flow cytometric assay

To clarify the mechanism of any antitumor activity with SLXM-2, we also investigated the cell cycle arrest of hepatocarcinoma in the ascitic fluid model. Cell cycle change was analyzed using flow cytometry [11] with the FACSCalibur (BD, Franklin Lakes, New Jersey, USA). After treatment with SLXM-2 and CPA for 10 days, mice bearing the ascitic fluid-type H_{22} were euthanized. Cells from the ascitic fluid of mice were washed with phosphate-buffered saline, fixed with 70% ethanol and resuspended for staining with propidium iodide (Sigma) to determine the distribution of cells in different phases of the cell cycle. The results were analyzed with ModFIT software (BD). Cell cycle assay by flow cytometry was representative of at least three independent experiments.

Western blotting

Western blotting was used to quantify protein products of tumor cells. The cells from the ascitic fluid of mice bearing H_{22} were washed twice with ice-cold phosphate-buffered saline, and then were lysed on ice with a solution containing 9.99 mmol/l Tris–HCl (pH 7.4), 0.15 mol/l NaCl, 2.55 mmol/l ethylenediaminetetra-acetic acid, 1% NP-40, 2 μg/μl both aprotinin and leupeptin and 10 μg/μl pepstatin. The cell lysates were clarified by centrifugation at 14 000 rpm after incubation at 4°C for 30 min. The supernatants were recovered, and their protein concentrations were measured using the Pierce protein assay reagent (Pierce Biotechnology, Rockford, Illinois, USA). Equivalent amounts of cell lysate protein (40 μg) were separated on a 12.5% sodium dodecyl sulfate–polyacrylamide gel and electrotransferred onto a nitrocellulose membrane (Pall Corporation, New York, New York, USA). After having been blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween-20, the membrane was incubated with the desired primary antibody in 1.5% bovine serum albumin in Tris-buffered saline containing 0.05% Tween-20 overnight at 4°C with the following dilutions: cyclin B1 (1 : 200 dilution), Cdc2 (1 : 500 dilution), phospho-Cdc2 (Tyr^{15}) (1 : 500 dilution), phospho-Cdc2 (Thr^{161}) (1 : 500 dilution), Cdk7 (1 : 200 dilution), Cdc25c (1 : 100 dilution) and β-actin (1 : 2000 dilution). Subsequently, the membrane was incubated with appropriate secondary antibodies, and the immunoreactive protein bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. All proteins were detected by enhanced chemiluminescence-based autoradiography. Western blots were representative of at least three independent experiments.

Reverse transcription-PCR

Reverse transcription (RT)-PCR was then used to quantify mRNA expressions of tumor cells. Total RNA was prepared from frozen ascitic fluid samples using Trizol reagent (Invitrogen Life Technologies, Carlsbad, California, USA) according to the general protocol. To prepare cDNA samples, 1 μg of total RNA was reverse-transcribed with a ReverTra Ace reverse transcriptase using a high-fidelity RT-PCR kit (TOYOBO Co. Ltd., Osaka, Japan). RT-PCR amplification was carried out for one cycle of 94°C for 2 min, followed by 30 cycles of 98°C for 10 s, T_m – 5°C for 30 s, and 68°C for 30 s, then held by 4°C. PCR primers used were as follows [12]: cyclin B1, 5′-GTAATCCTTGCAGTGAGTGACG and 3′-GGTCT AGTCTGTCTACCTCTAC; Cdc2, 5′-AGAAGGTACT TACGGTGTGG and 3′-CGCAAACCTTATGGCTAT; glyceraldehyde 3-phosphate dehydrogenase, 5′-ACCAC AGTCCATGCCATCAC and 3′-ATGTCGTTGTCCCAC CACCT. The PCR products were analyzed by gel electrophoresis on 1.2% agarose gels, stained with ethidium bromide and visualized on the UV transilluminator.

Determination of lethal dose 50

To help characterize toxicity of SLXM-2, 50 mice, randomly divided into five groups, were used to determine the lethal dose 50 (LD_{50}). Five concentrations of SLXM-2 were given by i.p. injection. Behaviors and death time were recorded for 7–10 days after injection. Necropsies were performed to determine the changes in the hearts, livers, spleens, lungs and kidneys.

Statistical analysis

Each experiment was repeated at least three times. All experimental data were analyzed using the statistical software Macrocal Origin 6.0. (Microcal Software Inc., Northampton, Massachusetts, USA) Differences between groups were analyzed with paired Student's *t*-tests. Differences were considered statistically significant at $P < 0.05$.

Results

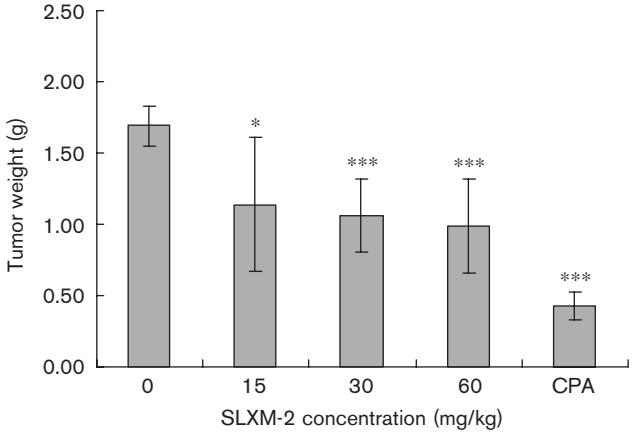
Effect of SLXM-2 on solid-type H_{22} tumors

SLXM-2 significantly inhibited the growth of H_{22} cells in ICR mice (Fig. 2). The tumor weights of the experimental groups were substantially lower than those of the NS control group. Significant differences were observed between control mice and the treatment group. CPA reduced body weight significantly, whereas mice in all SLXM-2 groups have no obvious change (Fig. 3). Leukocyte levels in both SLXM-2 groups were significantly higher than those in the CPA group (Table 1).

Effect of SLXM-2 on ICR mice with ascitic fluid-type H_{22}

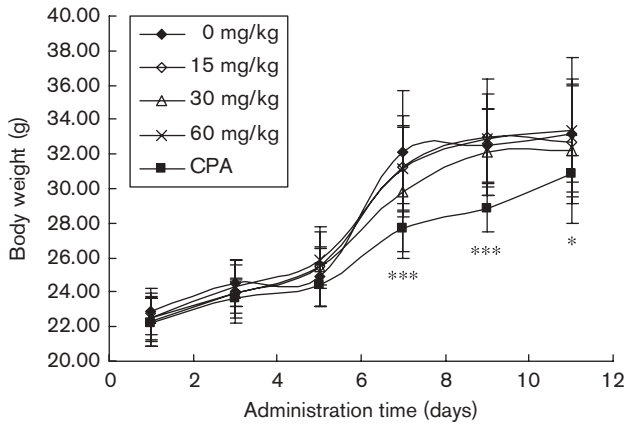
Figure 4 shows that SLXM-2 at different doses significantly prolonged the survival time of mice transplanted with ascitic fluid-type H_{22} compared with the NS control

Fig. 2



The effects of SLXM-2 on the growth of H_{22} transplanted into ICR mice. Ten mice in each group were given intraperitoneal injection. Dosages of 15, 30 and 60 mg/kg/day produced inhibition rates of 32.6, 37.5 and 41.5%, respectively. The inhibition rate of the CPA group (30 mg/kg) was 74.8%. *$P<0.05$; ***$P<0.001$, compared with the NS group by paired Student's *t*-test ($n=10$). CPA, cyclophosphamide; H_{22}, hepatocarcinoma 22; NS, normal saline.

Fig. 3



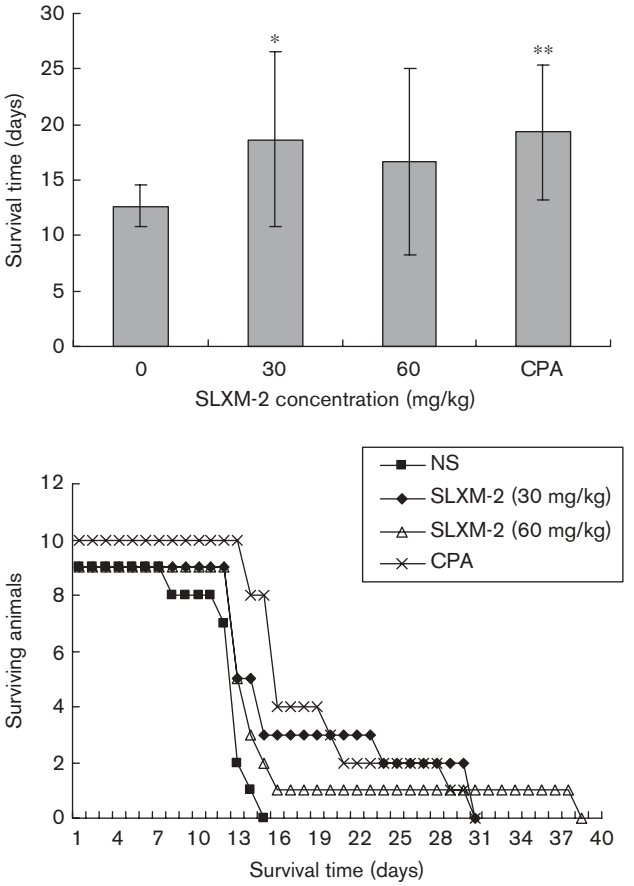
The change of body weight in solid-type H_{22} mouse models. Dosages of 0, 15, 30 and 60 mg/kg/day produced body weight change. The body weight change of the CPA group was examined as a control. Significant differences were observed between NS control mice and the CPA group. *$P<0.05$; ***$P<0.001$, compared with the NS group by paired Student's *t*-test ($n=10$). CPA, cyclophosphamide; H_{22}, hepatocarcinoma 22; NS, normal saline.

Table 1 The effect of SLXM-2 on leukocytes in H_{22}-implanted mice

Groups	Dose (mg/kg)	Leukocyte count (mean ± SD)
Control	/	11.10 ± 4.03
CPA	30	4.46 ± 2.70[a]
SLXM-2	60	10.10 ± 2.39
SLXM-2	120	10.00 ± 3.47[b]

CPA, cyclophosphamide; H_{22}, hepatocarcinoma 22; NS, normal saline.
[a]$P<0.001$, compared with the NS group by paired Student's *t*-test;
[b]$P<0.001$, compared with the CPA group by paired Student's *t*-test ($n=10$).

Fig. 4



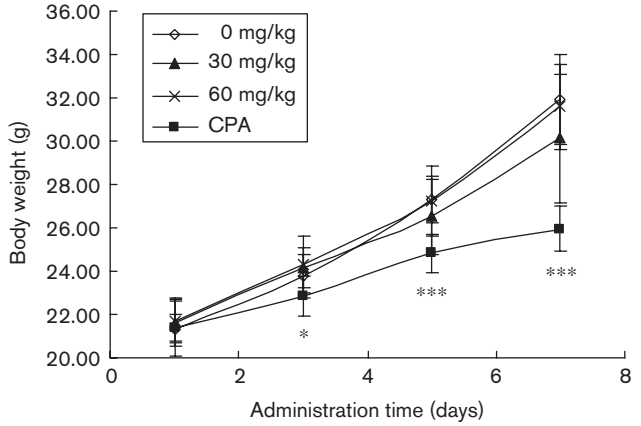
The effect of SLXM-2 on the survival of ICR mice with ascitic fluid-type H_{22}. SLXM-2 prolonged the survival times of mice with ascitic fluid tumors by 47.4 and 37.3%, respectively, at doses of 30 and 60 mg/kg; CPA prolonged survival times by 52.3%. Mice in the NS control group died within 16 days, whereas mice in both SLXM-2 groups lived up to 31 days. **$P<0.01$; *$P<0.05$, compared with the NS group by paired Student's *t*-test ($n=10$). CPA, cyclophosphamide; H_{22}, hepatocarcinoma 22; NS, normal saline.

group; this prolonged survival time was consistent with the tumor inhibitory effect. Moreover, the time of death in the control group was relatively concentrated, whereas the times of death in the treatment groups were dispersed and delayed. All mice in the control group died within 16 days after injection, whereas mice in both SLXM-2 groups lived up to 31 days. SLXM-2 did not influence the change of body weight, although CPA induced body weight loss significantly (Fig. 5).

Cell cycle analysis

To determine whether the antitumor effect of SLXM-2 was mediated through an alteration of the cell cycle, we analyzed the cell cycle distribution of cells from the

Fig. 5



The change of body weight in ascitic fluid-type H_{22} mouse models. Dosages of 0, 30 and 60 mg/kg/day produced body weight change. The body weight change of the CPA group was examined as a control. Significant differences were observed between NS control mice and the CPA group. *$P<0.05$; ***$P<0.001$, compared with the control group by paired Student's *t*-test ($n=10$). CPA, cyclophosphamide; H_{22}, hepatocarcinoma 22; NS, normal saline.

ascitic fluid of mice bearing H_{22}. Cell cycle analysis by FACS showed that after i.p. injection with 30 mg/kg SLXM-2, the percentages of H_{22} cells in the G_1 phase decreased significantly, while the percentages of cells in the S and G_2/M phases increased substantially (Table 2). At the 60 mg/kg dose, SLXM-2 further increased the percentage of cells in the G_2/M phase. This finding shows that cell cycle was arrested in both the S phase and G_2/M phase with SLXM-2, and G_2/M arrest increased in a dose-dependent manner. Cell cycle arrest of SLXM-2 was consistent with that of CPA.

Effects of SLXM-2 on cyclin B1 protein, cell division cycle protein 2 and its activity

To understand the mechanisms of the SLXM-2-induced G_2/M arrest in ascitic fluid-type H_{22}, we performed Western blotting to examine the expression of cell cycle regulatory proteins at the G_2/M boundary, including cyclin B1, Cdc2 and the phosphorylated form of Cdc2. As shown in Fig. 6, the level of cyclin B1 increased in a dose-dependent manner. On the contrary, although SLXM-2 did not affect the protein level of Cdc2, it decreased the activity of Cdc2. The level of phospho-Cdc2 (Tyr^{15}) increased in ascitic fluid-type H_{22}, whereas the level of phospho-Cdc2 (Thr^{161}) decreased.

Effects of SLXM-2 on cyclin-dependent kinase 7 and cell division cycle protein 25c kinases

To further investigate the underlying reasons for the prevention of Cdc2 activity in ICR mice with ascitic fluid-type H_{22} treated with SLXM-2, we examined the possible involvement of two known Cdc2 regulators, Cdk7 and Cdc25c kinases. In these experiments, the levels of Cdk7 and Cdc25c were reduced in a dose-dependent manner (Fig. 7).

Effects of SLXM-2 on the mRNA expression of cyclin B1 and cell division cycle protein 2

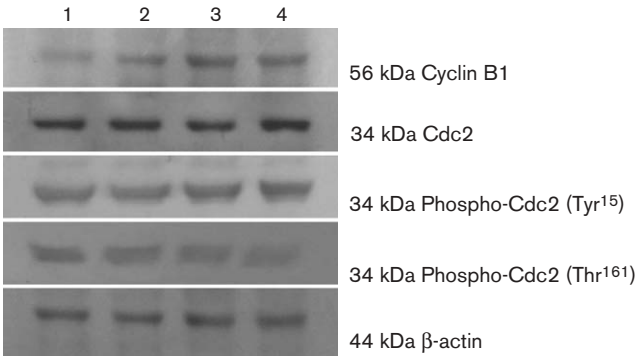
The results described above suggest that SLXM-2 increased cyclin B1 protein synthesis and did not influence the expression of Cdc2 at the translational level. To further understand the mechanisms of the SLXM-2-induced protein changes, we analyzed the expression of cyclin B1 and Cdc2 genes in ICR mice with ascitic fluid-type H_{22} hepatocarcinoma by using RT-PCR. The expression of cyclin B1 mRNA increased in a dose-dependent manner; however, the difference in Cdc2

Table 2 Cell cycle analysis of H_{22} ascitic fluid treated with different concentrations of SLXM-2

Group	Dose (mg/kg)	Cell cycle phase (%) (X ± SD)		
		G_1	S	G_2/M
Control	/	79.41 ± 8.50	14.13 ± 8.12	6.46 ± 2.58
CPA	30	11.89 ± 4.51[b]	42.67 ± 5.35[c]	45.44 ± 1.08[b]
SLXM-2	30	16.73 ± 2.52[b]	44.67 ± 6.75[b]	38.60 ± 9.27[c]
SLXM-2	60	5.54 ± 1.32[b]	23.12 ± 1.77	71.35 ± 2.64[a]

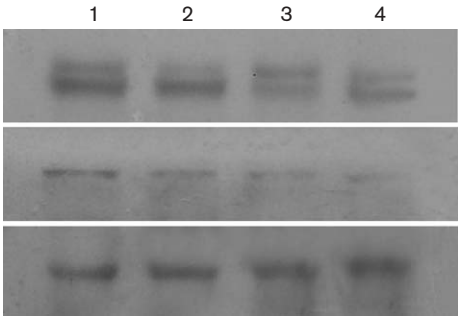
Ten mice in each group were given intraperitoneal injection. We analyzed cell cycle distribution of three mice in each group.
CPA, cyclophosphamide; H_{22}, hepatocarcinoma 22; NS, normal saline.
[a]$P<0.001$; [b]$P<0.01$; [c]$P<0.05$, compared with the NS group by paired Student's *t*-test ($n=3$).

Fig. 6



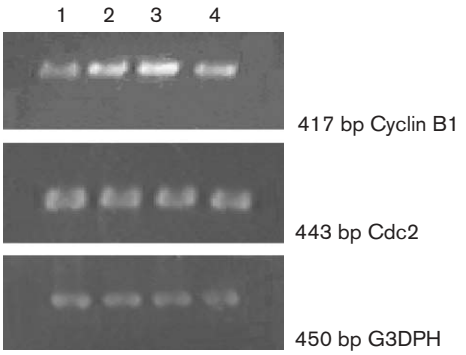
Effects of SLXM-2 on expressions of cyclin B1, Cdc2 and phosphorylated Cdc2 on ICR mice with ascitic fluid-type H_{22}. Lanes 1–4: 0, 30, 60 mg/kg SLXM-2 and 30 mg/kg CPA. ICR mice bearing asciticfluid-type H_{22} were randomized into two SLXM-2 groups (30 and 60 mg/kg/day), a CPA group (30 mg/kg/2day) and an NS control group (0.1 ml/10 g/day), with 10 mice in each group. After treatment for 10 days, H_{22} ascitic cells were harvested and the protein lysates subjected to 12.5% SDS–PAGE for analysis of the main cell cycle regulators. The expression level of Cdc2 was not affected, but the expression of phospho-Cdc2 (Tyr^{15}) increased and the expression of phospho-Cdc2 (Thr^{161}) decreased in a dose-dependent manner. Cyclin B1 also accumulated in a dose-dependent manner. β-actin was examined as a loading control. Cdc, cell division cycle protein; CPA, cyclophosphamide; H_{22}, hepatocarcinoma 22; NS, normal saline.

Fig. 7



Effects of SLXM-2 on the expression of Cdk7 and Cdc25c kinases on ICR mice with ascitic fluid-type H_{22}. Lanes 1–4: 0, 30, 60 mg/kg SLXM-2 and 30 mg/kg CPA. ICR mice bearing ascitic fluid-type H_{22} were randomized into two SLXM-2 groups (30 and 60 mg/kg/day), a CPA group (30 mg/kg/2days) and an NS control group (0.1 ml/10g/day), with 10 mice in each group. After treatment for 10 days, H_{22} ascitic cells were collected and the protein lysates subjected to 12.5% SDS–PAGE for analysis of Cdk7 and Cdc25c kinases. The levels of Cdk7 and Cdc25C were reduced in a dose-dependent manner. β-actin was examined as a loading control. Cdc, cell division cycle protein; Cdk, cyclin-dependent kinase; CPA, cyclophosphamide; H_{22}, hepatocarcinoma 22; NS, normal saline.

Fig. 8



The mRNA expression of cyclin B1 and Cdc2 on ICR mice with ascitic fluid-type H_{22} when treated with SLXM-2. Lanes 1–4: 0, 30, 60 mg/kg SLXM-2 and 30 mg/kg CPA. ICR mice bearing ascitic fluid-type H_{22} were randomized into two SLXM-2 groups (30 and 60 mg/kg/day), a CPA group (30 mg/kg/2days) and an NS control group (0.1 ml/10 g/day), with 10 mice in each group. After treatment for 10 days, H_{22} ascitic cells were collected and the PCR products were analyzed by gel electrophoresis on 1.2% agarose gels for analysis of mRNA expression of cyclin B1 and Cdc2. The expression of cyclin B1 mRNA increased in a dose-dependent manner; however, the difference in Cdc2 mRNA expression was not detected between the CPA group and the control group. G3DPH was examined as a loading control. These results were consistent with those of Western blotting. Cdc, cell division cycle protein; CPA, cyclophosphamide; G3DPH, glyceraldehyde 3-phosphate dehydrogenase; H_{22}, hepatocarcinoma 22; NS, normal saline.

mRNA expression was not detected between the CPA group and the control group (Fig. 8).

Lethal dose 50 of SLXM-2

The LD_{50} of SLXM-2 was 1202.0 mg/kg, and the 95% confidence limit was 1202.0 ± 27.0 mg/kg (Table 3), whereas the LD_{50} of CPA was about 300 mg/kg [13]. A few mice that received high doses of SLXM-2 died within a few minutes of injection. Even in the high-dose groups, however, most mice survived longer than 30 min. No abnormalities were visible in the hearts, livers, spleens, lungs or kidneys.

Table 3 The LD_{50} of SLXM-2 in ICR mice

Groups	Dosage (mg/kg)	No. dead animals	Mortality rate (%)
1	1050	0	0
2	1110	1	10
3	1170	3	30
4	1230	6	60
5	1300	8	80

Ten mice in each group were given intraperitoneal injection of SLXM-2. The LD_{50} of SLXM-2 was 1202.0 mg/kg, and the 95% confidence limit was 1202.0 ± 27.0 mg/kg.
LD_{50}, Lethal dose 50.

Discussion

CPA is a classic anticancer drug used in chemotherapy that has robust inhibitory effects on a variety of tumors. It, however, has many side effects, such as bone marrow suppression, leukocytopenia and inducement of secondary tumors. We found that the CPA derivative SLXM-2 has potent antineoplastic effects with low toxicity and less potential to cause leukocytopenia in mice with H_{22} hepatocarcinoma than that of CPA. In the solid tumor model, SLXM-2 significantly inhibited tumor growth but did not decrease leukocyte counts and body weight. SLXM-2 also prolonged survival time in mice with the ascitic fluid-type H_{22} and did not significantly cause body weight loss compared with the NS group even at the dose of 60 mg/kg. Mice in the CPA group, however, lost weight greatly compared with those in the NS group. In the solid-type model, the effect at the 60 mg/kg dose is similar to that at the 30 mg/kg dose. Additionally, effects in the ascitic fluid-type model were more robust at the 30 mg/kg dose than at the 60 mg/kg dose, which indicates that 30 mg/kg may be the optimal dosage for inhibitory effects.

We also explored the mechanism of cell cycle arrest in mice with the ascitic fluid-type H_{22}. SLXM-2 arrested cells in the S phase and G_2/M phase at 30 mg/kg and arrested more cells in the G_2/M phase at 60 mg/kg. These results suggested that SLXM-2 arrested the cell cycle in a dose-dependent manner; mechanisms for this arrest include upregulation of cyclin B1, inactivity of Cdc2 and downregulation of Cdk7 and Cdc25c kinases.

The cell cycle is mediated by the activation of a highly conserved family of protein kinases, the Cdks [14–16].

Activation of a Cdk requires binding to a specific regulatory submit, the cyclins. Together, these cyclin/Cdk complexes are the cell cycle regulators. The entry into mitosis is under the control of cyclin B1, which also associates with Cdc2. The cyclin B1/Cdc2 complex was originally defined as the maturation-promoting factor or M phase-promoting factor [17,18]. The cyclin B1/Cdc2 complex is activated by phospho-Cdc2 (Thr^{161}) and the dephosphorylation of phospho-Cdc2 (Thr^{14}-Tyr^{15}) [19,20]. Thr^{161} is phosphorylated by cyclin activation kinase (CAK), after activation of CAK by a CAK-activating kinase (CAKAK). At G_2/M transition, these inhibitory phosphates are removed by Cdc25c phosphatase [14,21]. M phase-promoting factor catalyzes the phosphorylation of lamins and histone1, and is involved in the regulation of events proceeding cell division, such as spindle formation, chromatin condensation and fragmentation of the nuclear envelope and of organelles such as the Golgi and endoplasmic reticulum [22–26]. Thus, G_2 arrest is considered a period of repair and recovery following DNA damage. It has been shown that inhibitory phosphorylations are maintained during this period, resulting in the accumulation of the cyclin B1/Cdc2 complex. Our results showed the accumulation of the cyclin B1/Cdc2 complex and consequent G_2/M arrest, after treatment with various concentrations of SLXM-2 on mice bearing ascitic fluid-type H_{22}. We observed that cyclin B1 increased but Cdc2 remained constant. Moreover, the upregulation of phospho-Cdc2 (Tyr^{15}) and downregulation of phospho-Cdc2 (Thr^{161}) led to inactivation of the cyclin B1/Cdc2 complex, resulting in G_2/M arrest.

To better understand the mechanism by which regulators decreased the activity of Cdc2, we explored the effects of several related proteins on the regulation of Cdc2 activity. The cyclin B1/Cdc2 complex involved in the regulation of mitosis in eukaryotic cells is subject to multiple levels of control [27–30]. Among these, the regulation of the catalytic subunit by phospho-Cdc2 (Tyr^{15}) is the best understood. Tyrosine phosphorylation inhibits the Cdc2/cyclin B complex, whereas tyrosine dephosphorylation, which occurs at the onset of mitosis, directly activates the cyclin B1/Cdc2 complex. The Cdc25 gene serves as a rate-limiting mitotic activator, apparently owing to its action as the Cdc2 tyrosine phosphatase. In the absence of Cdc25, Cdc2 accumulates in a tyrosine phosphorylated state. We observed a substantial reduction in the Cdc25c level after treatment with SLXM-2, which correlated with the observed accumulation of Tyr^{15} phosphorylated cyclin B1/Cdc2 complex in the animal model. Moreover, the activity of Cdc2 is also dependent upon Cdk7 kinase [31,32]. Further experiments indicated that Cdk7 activity was inhibited by SLXM-2 and failed to phosphorylate Thr^{161} of Cdc2 but the Thr^{14} and Tyr^{15} residues of Cdc2 remained phosphorylated. Concomitantly, the down-regulation of Cdk7 contributed to the G_2/M arrest.

The toxic side effects of CPA include hematopoietic depression, fulminant cardiac toxicity, hemorrhagic cystitis, gonadal dysfunction, alopecia, nausea, gastrointestinal toxicity, renal toxicity, antidiuresis and vomiting [33]. To explore the toxicity of SLXM-2, we investigated its LD_{50}. In the experiments of acute toxicology, the LD_{50} of SLXM-2 was found to be 1202.0 ± 27.0 mg/kg, whereas that of CPA was about 300 mg/kg [13], which indicates the lower toxicity of SLXM-2. Additionally, we observed no obvious change in organs after SLXM-2 injection. Taken together with the finding that SLXM-2 did not suppress leukocytes and body weight, these results show that SLXM-2 has fewer toxic effects than CPA.

A limitation to this study was that we do not know the pharmacokinetics of this compound and are not aware of the actual metabolites of SLXM-2 presenting in the serum during administration. CPA is not the active compound for its biological activity, and its metabolite, phosphoramide mustard, makes DNA alkylated and consequently inhibits tumor growth. It also generally produces its effect *in vivo* because of metabolism. Therefore, we evaluated the effect of SLXM-2 in the animal level. The observation that administration of SLXM-2 had an antineoplastic effect on animal models, however, confirmed that the regimen we used was successful.

In summary, SLXM-2 is a CPA derivative formed by converting the mustard group into a suitable spiropiperazinium structure. SLXM-2 inhibits the growth of H_{22} tumors and prolongs the survival of mice bearing the ascitic fluid-type H_{22}. Its antineoplastic effects are slightly weaker than those of CPA, but its decreased potential for leukocytopenia and body weight loss, and higher LD_{50} indicate that it is less toxic than CPA. SLXM-2 may have a similar mechanism as CPA, as the cell cycle was arrested in the S phase and the G_2/M phase. As pharmacological interference with G_2/M arrest is known to increase the fraction of cells undergoing apoptosis after irradiation, understanding the mechanism of SLXM-2 on G_2/M checkpoint may be helpful in designing more effective cancer preventive and therapeutic strategies. These observations suggest that SLXM-2 may emerge as an effective antineoplastic agent with further development and exploration.

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