Antitumor potential of a novel camptothecin derivative, ZBH-ZM-06

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Abstract. Camptothecin (CPT) is a cytotoxic quinoline alkaloid that is used clinically as an anticancer drug. However, the clinical application of CPT is limited due to its low solubility as well as serious and unfathomable side-effects. In the present study, we created a novel 10-hydroxy CPT prodrug, ZBH-ZM-06. Its cellular cytotoxic activity was analyzed in terms of cellular viability, acetylcholinesterase (AchE) inhibition, DNA relaxation, cellular cycling and apoptosis properties. Our results showed that the AchE inhibition rate of 10 μmol/l ZBH-ZM-06 was 12.5%, compared to 96.5% for carbonyl-oxycamptothecin (CPT-11). In a chemical stability assay, only 4.9% of ZBH-ZM-06 remained after 4 h at pH 7.4. In addition, 10 μmol/l ZBH-ZM-06 significantly inhibited the tumor cell viability of nine tumor cell lines, compared to CPT-11 and the CPT active ingredient, 7-ethyl-10-hydroxy-camptothecin (SN38) ($P<0.01$-0.05). In the apoptosis assay, ZBH-ZM-06 increased the ratio of Annexin V^+/propidium iodide $(PI)^{-/+}$ cells by flow cytometric analysis ($P<0.05$). Moreover, ZBH-ZM-06 activated caspase-3 and poly(ADP-ribose)polymerase (PARP) expression by immunoblotting. Furthermore, ZBH-ZM-06 induced a greater G_2/M phase arrest ratio, compared to CPT-11 and SN38. These results indicated that ZBH-ZM-06 had higher antitumor activity than CPT-11 and SN38, which was shown by its: i) release of the effective ingredient; ii) growth inhibition of a broad spectrum of tumor cells; iii) inhibition of DNA topoisomerase (Topo-1); and iv) promotion of apoptosis through an intrinsic signaling pathway. Thus, ZBH-ZM-06 may be applied in the preclinic study for cancer treatment.

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Introduction

Camptothecin (CPT) is a cytotoxic quinoline alkaloid isolated from *Camptotheca acuminta*, a type of tree natively growing in China, which was discovered in the 1960s (1). The antitumor activity of CPT depends on a highly specific inhibition of Topo-1. This activity is achieved by docking at the enzyme-DNA interface to stabilize the formation of Topo-1-DNA cleavable complexes, thus prohibiting DNA strand religation. Once the stable form of the cleavable complex is broken by some mechanism, such as replication or transcription caused by some cytotoxic drugs, the breakage of the Topo-1-linked DNA single-strand can cause DNA double-strand breaks (DSBs) (2). Subsequently, these DSBs will trigger a DNA damage response to activate serine-threonine kinases to drive the ATM-CHK2- and ATR-CHK1-mediated checkpoint pathways as well as H2AX phosphorylation to arrest the cell cycle at the G_1/S and G_2/M phases (3). The clinical application of CPT is limited due to its low solubility as well as serious and unfathomable side-effects (4,5). To overcome these drawbacks, several CPT derivatives have been developed to date, including topotecan (9-dimethyl amino-10-hydroxy camptothecin; TPT) and irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin; CPT-11) (6,7). The US Food and Drug Administration has approved these CPT derivatives for ovarian and colon cancer treatment (8,9). Furthermore, their anticancer potential on other tumors also has been reported, such as lung (7,10), breast (11), pancreatic cancer (12), lymphoma (13), glioma (14) and leukemia (15).

SN38, the prodrug of CPT-11, is approximately 100- to 1,000-fold more cytotoxic than CPT-11 (16). CPT-11 plays an antitumor role *in vivo* through the release of its active form SN38 by liver carboxylesterase. However, there are significant individual differences among the antitumor activity of

CPT-11 due to its low enzymatic conversion rate *in vivo* and its uncertain pharmacokinetic properties among individuals. In addition, the CPT-11 prodrug group (4-piperidinyl piperidine) causes AchE inhibition, which easily leads to acetylcholine syndrome, resulting in early severe diarrhea and other side-effects (10).

In order to overcome the above drawbacks of CPT-11, scientists have made several attempts to improve it (10,17-19). In the present study, we designed and synthesized a novel 10-hydroxy CPT prodrug with a high efficiency and low toxicity (20,21). Its cytotoxic activity, side-effects, antitumor activity and possible mechanism were analyzed in multiple assays. The results demonstrated that ZBH-ZM-06 has optimal antitumor properties and fewer side-effects than those of CPT-11 and SN38.

Materials and methods

Derivative of CPT. The new 10-hydroxy CPT prodrug, ZBH-ZM-06, was designed and synthesized by the Institute of Pharmacology and Toxicology Academy of Military Medical Sciences (20). By using a linear amino acid as a linker, the N-terminal amino acid was ligated with 10-OH of SN38 through a urethane bond. Next, through removal of the benzyl protecting group by catalytic hydrogenation, a carboxyl group was liberated. The selected *N*-methylpiperazine, which has good biocompatibility, was conjugated with the free carboxyl group of the amino acid through an amide bond. The basic nitrogen atom of *N*-methylpiperazine can form a salt with the carboxyl group to improve the water solubility of the compound.

CPT-11 and SN38 were also provided by the Institute of Pharmacology and Toxicology Academy of Military Medical Sciences. All of the compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 10 mmol/l as a stock reagent. Further dilutions were made with Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Grand Island, NY, USA) at the appropriate concentrations and stored at -20˚C.

AchE inhibition assay and stability test. The AchE inhibition test by ZBH-ZM-06 was performed as previously described (20). The stability of ZBH-ZM-06 was analyzed at 1, 2, 4, 8 and 12 h in phosphate-buffered saline (PBS; pH 7.4 and pH 5.0) by high-performance liquid chromatography (HPLC) with a C18 analytical column, as previously described (20).

Cell culture and cytotoxicity assay. Twelve human tumor cell lines, including SW1116, SAOS-2, A549, SGC-7901, 7860, HeLa, SK-OV-3, K562, NCI-H446, A375, MCF-7, SMMC-7721 and NCM460, a normal human colon mucosal epithelial cell line, were incubated with IMDM or RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 2 mmol/l glutamine, and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere incubator containing 5% CO_2. Cells were harvested during the logarithmic growth phase and plated in 96-well plates at $2.5x10^3$ cells/well in 100 μl of medium. After incubation for 24 h, 0.0032 μmol/l to 50 μmol/l ZBH-ZM-06, CPT-11 or SN38 was added to the indicated plate. The cellular viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 72 h later (22). The absorbance at 490 nm was detected by a microplate reader. The half-maximal inhibition concentration (IC_{50}) values of the analyzed drugs were calculated. Each experiment was repeated at least three times.

DNA relaxation assay. To evaluate the effects of ZBH-ZM-06 and CPT-11 on DNA relaxation, the TopoGEN Topoisomerase 1 Drug Screening kit (TopoGEN, Inc., Port Orange, FL, USA) was employed, according to the manufacturer's instructions. Closed loop superhelix plasmid DNA (pHOT1) was co-incubated with recombinant wild-type human Topo-1 (2 U, TG2005H-RC1; TopoGEN) at 37˚C for 30 min in the presence or absence of the drugs in Topo-1 reaction buffer. Reactions were quenched by incubation with sodium dodecyl sulfate (1%) and proteinase K (50 μg/ml) for 15 min at 37˚C. The DNA samples were then analyzed by electrophorese on 1% agarose gel containing 1 μg/ml ethidium bromide. Gels were visualized and photographed under ultraviolet light. Each experiment was performed in triplicate.

Cell cycle analysis. K562, SK-OV-3 and SW1116 cells were seeded in 6-well plates at $5x10^5$/well in FCS-free IMDM. After incubation for 24 h, the cells were exposed to ZBH-ZM-06, CPT-11, or SN38 at 10 μmol/l in IMDM containing 10% FCS and harvested after 24, 48 and 72 h, respectively. The cells were stained with the Coulter DNA PREP™ reagents kit (Beckman Coulter, Brea, CA, USA), according to the manufacturer's protocol. The cell cycle was analyzed by flow cytometry using a Coulter EPICS XL-MCL instrument. Data were analyzed using MultiCycle 32-bit Version Software (Phoenix Flow Systems, San Diego, CA, USA).

Measurement of tumor cell apoptosis. The FITC Annexin V Apoptosis Detection kit I (BD Biosciences, San Diego, CA, USA) and the FITC Active Caspase-3 Apoptosis kit (550480; BD Biosciences) were employed for the measurement of cell apoptosis. K562, SK-OV-3 and SW1116 cells ($5x10^5$) were seeded on 6-well plates and cultured overnight. The next day, 10 μmol/l ZBH-ZM-06, CPT-11, or SN38 was added for the indicated time (24, 48 and 72 h). After treatment, the cells were harvested, washed and resuspended in 100 μl of binding buffer containing 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI), or stained with FITC-conjugated cleaved caspase-3 antibody for 15-20 min in the dark at 20˚C. Next, viable, apoptotic, necrotic cells, and activated caspase-3^+ cells were analyzed by an Epics XL/MCL flow cytometer.

Western blot analysis. SW1116 cells were incubated with 10 μmol/l ZBH-ZM-06, CPT-11, or SN38 for 72 h. The cell lysate preparation and immunoblot analysis were performed as previously described (21).

Statistical analysis. Experimental data were analyzed by the t-test, one-way analysis of variance, and chi-squared test with SPSS 23.0 software (version 23.0; SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean ± standard deviation (SD) using a minimum of triplicate determinations. $P<0.05$ was considered to be statistically significant.

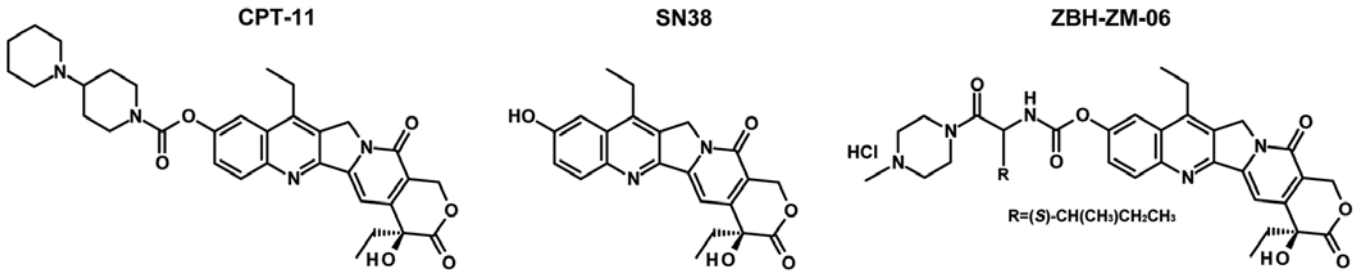

Figure 1. Molecular structures of CPT-11, SN38 and ZBH-ZM-06. ZBH-ZM-06 was constructed by: i) coupling of the 10-OH substituent of SN38 and an N-terminal amino acid via a urethane bond; ii) removal of the benzyl protecting group by catalytic hydrogenation to release a carboxyl group; and iii) conjugation of *N*-methylpiperazine with the free carboxyl group of an amino acid by an amide bond. The basic nitrogen atom of *N*-methylpiperazine can form a salt with the carboxyl group to improve the water solubility of the compound.

Table I. The AchE inhibitory activity of ZBH-ZM-06 and CPT-11.

	Inhibition (%)			
	0.1 μM	1 μM	10 μM	100 μM
ZBH-ZM-06	4.9	8.4	12.5	44.2
CPT-11	45.7	78.9	96.5	-

Table II. The chemical stability of ZBH-ZM-06 as indicated by the percentage of remaining compound after incubation for various times and pH values.

	0 h	1 h	2 h	4 h	8 h	12 h
pH 7.4	98.1	38.8	17.7	4.9	-	-
pH 5.0	99.8	-	-	97.8	95.0	92.8

Results

Decreased AchE inhibitory activity of ZBH-ZM-06. The molecular structures of CPT-11, SN38 and ZBH-ZM-06 are shown in Fig. 1. CPT-11 is a potent inhibitor of AchE and can cause acute cholinergic diarrhea. In order to test whether ZBH-ZM-06 causes this side-effect, the inhibitory activity of ZBH-ZM-06 on AchE was detected. Obviously, ZBH-ZM-06 revealed a lower potential to inhibit AchE compared with CPT-11 at different concentrations (Table I). This result indicates that the toxic side-effect of AchE inhibition of ZBH-ZM-06 is less than that of CPT-11.

ZBH-ZM-06 can fully release the active ingredient SN38 under physiological pH conditions. The stability of ZBH-ZM-06 was investigated in phosphate-buffered saline (PBS) by HPLC using a C18 analytical column. The mobile phase consisted of water and acetonitrile at a ratio of 70:30, respectively, containing 1% trifluoroacetic acid. ZBH-ZM-06 and its metabolite SN38 were separated at a flow rate of 0.8 ml/min and detected at a wavelength of 370 nm. ZBH-ZM-06 was dissolved in PBS (0.2 mg/ml, pH 7.4 and pH 5.0), incubated at 37˚C and analyzed at 0, 1, 2, 4, 8 and 12 h (20). The results are summarized in Table II. ZBH-ZM-06 showed a pH-dependent stability. ZBH-ZM-06 was relatively stable, as shown by the finding that 92.8% still remained after 12 h at pH 5.0, 37˚C. However, at pH 7.4, only 4.9% remained after incubation for 4 h. The antitumor activity of CPT-11 is achieved through the release of its active ingredient, SN38 (23), by liver carboxylesterase. Nevertheless, there are significant individual differences in the antitumor activity of CPT-11 due to its low enzymatic conversion rate *in vivo* and its uncertain pharmacokinetic properties among individuals. ZBH-ZM-06 can fully release the active ingredient, SN38, under physiological pH (pH 7.4); therefore, this non-liver release-dependent property may improve the drug efficacy.

Inhibition of tumor cell viability by ZBH-ZM-06. The inhibitory activity of ZBH-ZM-06 on cellular viability was detected by MTT assay in 12 tumor cell lines. The NCM460 cell line was used as a normal cell line control. The IC_{50} values are summarized in Table III. The most sensitive cell lines were SW1116, SAOS-2, HeLa, SK-OV-3, K562 and A375. ZBH-ZM-06 showed weak inhibitory activity against NCM460 cells.

ZBH-ZM-06 inhibited the relaxation of supercoiled DNA. To investigate whether ZBH-ZM-06 inhibits cell growth through the inhibition of Topo-1 activity, a DNA relaxation assay was performed in the presence of ZBH-ZM-06 or CPT-11 on supercoiled plasmid pHOT1 *in vitro* (Fig. 2). The results demonstrated that supercoiled DNA was relaxed by Topo-1 in the absence of drug (lane 2 of Fig. 2). In contrast, incubation with ZBH-ZM-06 or CPT-11 inhibited the relaxation of supercoiled DNA, as demonstrated by the increased intensity of the band corresponding to the supercoiled plasmid DNA. Furthermore, the inhibition of ZBH-ZM-06 occurred at a concentration as low as 1 μmol/l, compared to CPT-11 (at 5 μmol/l), indicating that ZBH-ZM-06 has a stronger inhibitory activity against Topo-1 than CPT-11.

Different patterns of tumor cell cycle arrest induced by ZBH-ZM-06, CPT-11 or SN38. The cell cycle arrest by ZBH-ZM-06, CPT-11 or SN38 at 24, 48 and 72 h was analyzed (Fig. 3). The cell cycle arresting patterns at the S and G_2/M phases induced by ZBH-ZM-06 were significantly different from those by CPT-11 or SN38 ($P<0.0251$-0.0001), except for K562 cells treated for 24 h, which did not show a significant difference among the three groups.

Table III. IC_{50} values of ZBH-ZM-06, CPT-11 and SN38 in tumor cell lines and NCM460 cells.

No.	Cell line	IC_{50} (μmol/l)			P-value	
		ZBH-ZM-06	CPT-11	SN38	vs. CPT-11	vs. SN38
1	SW1116	0.0679±0.0588	2.7742±0.7676	862.2826±151.3390	0.0563	0.0045
2	SAOS-2	0.0000±0.0000	0.3781±0.1157	2.2989±0.5484	0.0217	0.0002
3	A549	0.9331±0.9234	3.7853±0.2473	683.9418±255.6370	0.2747	0.0072
4	SGC-7901	1.3539±0.4708	19.5366±7.5020	13.2479±6.2612	0.0642	0.1331
5	7860	0.0369±0.1414	0.4515±0.1000	4.9959±2.5830	0.3400	0.0087
6	HeLa	0.3025±0.1996	68.4969±21.7217	7630.3091±6997.9112	0.0037	0.0054
7	SK-OV-3	0.0475±0.0404	3.3985±1.2949	88.4301±61.3723	0.0256	0.0004
8	K562	0.0000±0.0000	0.7551±0.3578	1.2429±0.1687	0.0298	0.0059
9	NCI-H446	0.3710±0.3924	4.5701±4.6985	26.0500±8.1501	0.1056	0.0053
10	A375	0.0000±0.0000	1.1970±0.7749	7.3358±1.3535	0.0169	0.0008
11	MCF-7	5.3157±1.3016	1.4336±0.3022	3.3145±0.0007	0.5764	0.1083
12	SMMC-7721	1.7157±2.2853	12.2966±6.0580	0.0028±0.0016	0.7424	0.4478
13	NCM460	189.1652±49.3190	31.8162±10.9849	22.7732±6.1773	0.8134	0.9053

SW1116, colon adenocarcinoma; SAOS-2, human osteosarcoma; A549, non-small cell lung cancer; SGC-7901, gastric adenocarcinoma cancer; 7860, human renal carcinoma; HeLa, cervical carcinoma; SK-OV-3, ovarian cancer; K562, chronic myelogenous leukemia; NCI-H446, non-small cell lung cancer; A375, human melanoma; MCF-7, breast cancer; SMMC-7721, hepatoma; NCM460, human colon mucosal epithelia.

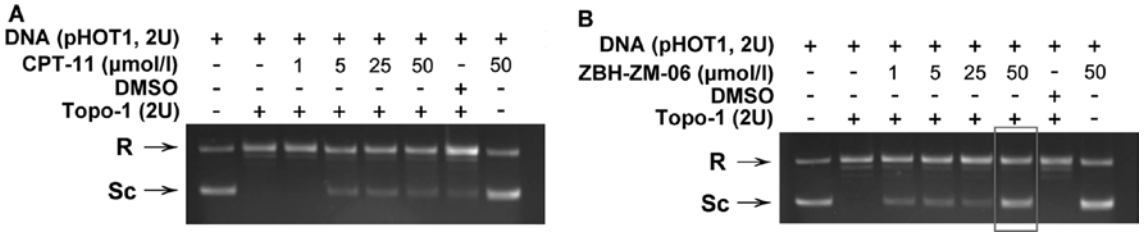

Figure 2. ZBH-ZM-06 and CPT-11 inhibited Topo-1-mediated supercoiled pHOT1-DNA relaxation. Closed loop pHOT1 plasmid DNA was incubated with the indicated concentration of CPT-11 (A) or ZBH-ZM-06 (B) (lanes 3-6) in the presence of Topo-1 at 37˚C for 30 min. Topo-1 alone (lane 2) was used as a positive control, and the absence of Topo-1 (lanes 1 and 8) was used as a negative control. Sc, supercoiled plasmid DNA; R, relaxed DNA. The assay was performed in triplicate.

Tumor cell apoptosis induced by ZBH-ZM-06. Tumor cell apoptosis induction by ZBH-ZM-06, CPT-11 and SN38 was analyzed by an Epics XL-MCL flow cytometer with FITC-Annexin V and PI staining. Fig. 4 shows the percentages of apoptotic SW1116 and SK-OV-3 cells induced by 10 μmol/l ZBH-ZM-06, CPT-11 and SN38 after 72 h. The results indicated that ZBH-ZM-06 more efficiently induced apoptosis in SK-OV-3 and SW1116 cells either early (Annexin V^+/PI^-) or late (Annexin V^+/PI^+) than CPT-11 and SN38 ($P<0.05$). To test whether the operation process influences the results, we applied the non-adherent K562 cells to detect the number of apoptotic cells after 10 μmol/l ZBH-ZM-06, CPT-11 and SN38 treatment for 24, 48 and 72 h (Fig. 5). As shown in Fig. 5, after 24 h of treatment, the number of apoptotic K562 cells in the ZBH-ZM-06 group was significantly higher than that in the CPT-11 and SN38 groups ($P<0.05$-0.0001). At this time, although cell cycle arrest had occurred (Fig. 3), there was no significant difference among the ZBH-ZM-06, CPT-11 and SN38 groups, indicating that cell apoptosis occurred earlier after drug treatment. This result is consistent with our previous conclusion (21). The number of apoptotic K562 cells treated with ZBH-ZM-06 for 48 and 72 h was significantly higher than that of cells treated with CPT-11 or SN38 ($P<0.0001$).

ZBH-ZM-06 promotes tumor cell apoptosis by activating caspase-3 and poly(ADP-ribose)polymerase (PARP). Caspase-3 is a key player in the execution of the apoptotic cascade by cleaving PARP. To determine whether ZBH-ZM-06 induced apoptosis through this pathway, the expression levels of cleaved capase-3 and PARP were examined by western blot analysis. As shown in Fig. 6, ZBH-ZM-06 efficiently activated caspase-3 to cleave PARP proteins.

The levels of the active form of caspase-3 were determined by flow cytometry in the similarly treated groups as well. Consistent with the western blot results, the percentage of activated caspase-3^+ K562, SK-OV-3 and SW1116 cells in the ZBH-ZM-06 group was higher than that of the CPT-11 and SN38 groups (Fig. 7).

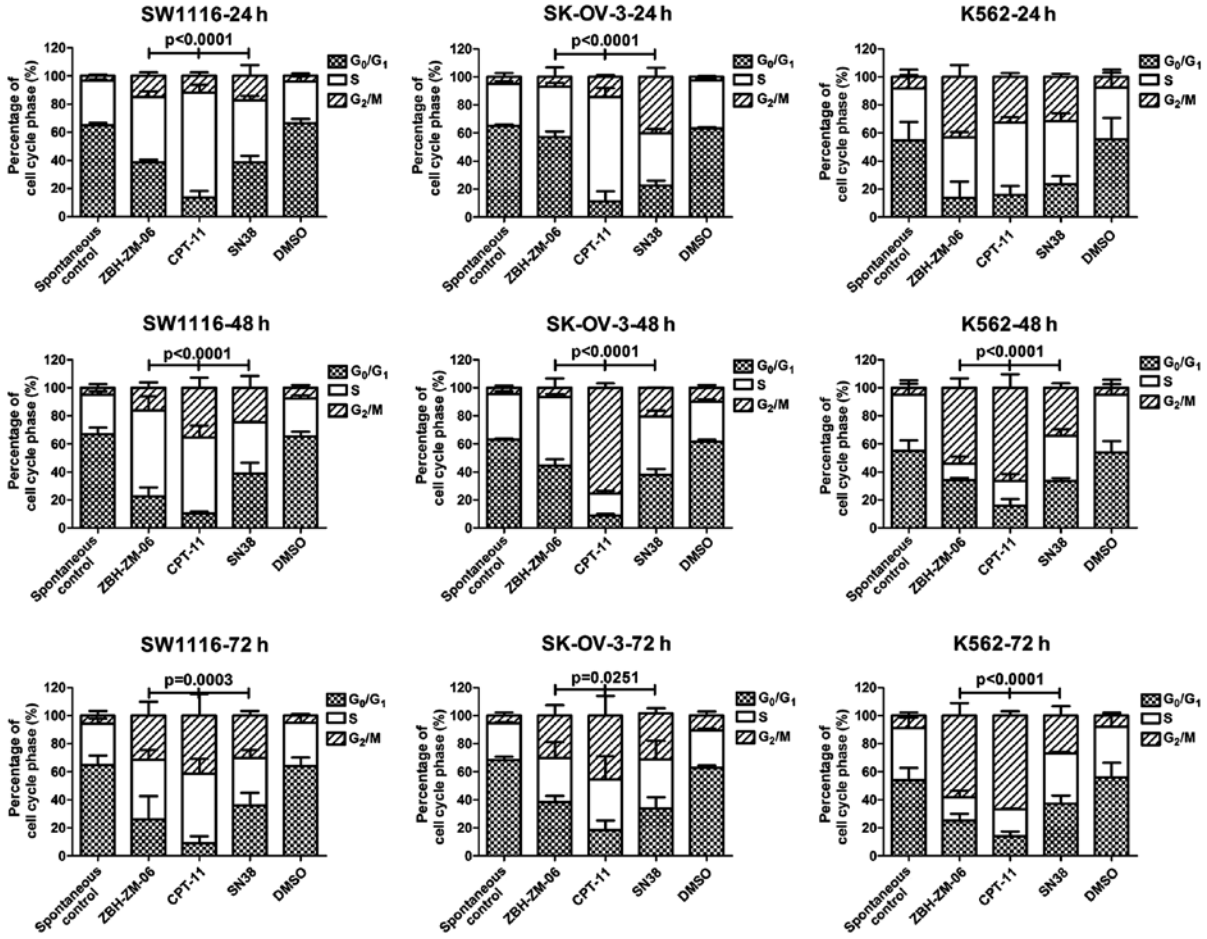

Figure 3. Tumor cell cycle arrest induced by ZBH-ZM-06, CPT-11, or SN38. The cell cycle arrest induced by ZBH-ZM-06, CPT-11 or SN38 was analyzed in SW1116 (left panels), SK-OV-3 (middle panels) and K562 (right panels) tumor cell lines. The distributions of G_0/G_1 (diagonal lines), S (open), and G_2/M (dotted) phases were normalized to 100%. Data are presented as the mean ± standard deviation from three independent experiments.

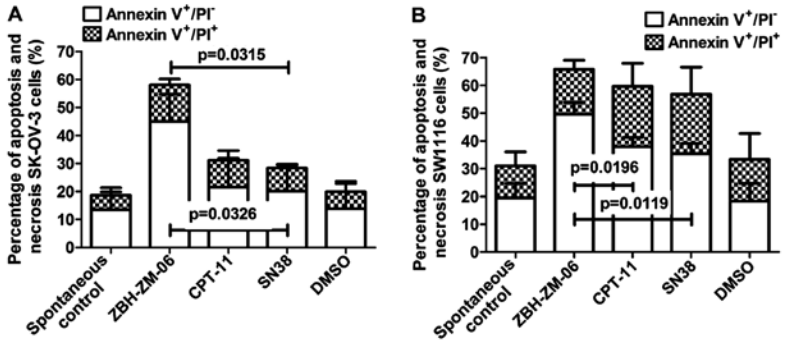

Figure 4. ZBH-ZM-06, CPT-11 and SN38 induced SK-OV-3 cell (A) and SW1116 cell (B) apoptosis. (A) SK-OV-3 cells and (B) SW1116 cells were treated with 10 μmol/l ZBH-ZM-06, CPT-11 and SN38, respectively, for 72 h. The percentages of apoptotic cells were detected by an Epics XL/MCL flow cytometer with FITC-Annexin V and PI staining. The Annexin V^+/PI^- and Annexin V^+/PI^+ cells indicated the early (dotted) and late (open) apoptosis, respectively. DMSO-treated cells were used as negative controls. Data are presented as the mean ± standard deviation from three independent experiments. The statistical analysis of early apoptosis among the ZBH-ZM-06, CPT-11 and SN38 groups is presented above the bars, and that of late apoptosis is shown inside the bars.

Discussion

The present study reports the capacity of the novel CPT derivative ZBH-ZM-06 as a potential antitumor agent *in vitro*. ZBH-ZM-06 was created by reconstruction of SN38 to reduce several of the disadvantages of CPT-11, for example, poor solubility and causing severe diarrhea. SN38 is the active metabolite of CPT-11, which is 100-1000 times more potent than CPT-11 (24). However, only a small portion (2-8%) of it is finally converted to SN38. The variability of the conversion from CPT-11 to SN38 causes substantial dangerous toxicity risks and difficulties for the clinical management of the corresponding complications. Therefore, the direct administration of SN38 might be of benefit for cancer patients. However, the poor solubility in aqueous and other pharmaceutical solvents (including ethanol, cremophor and polysorbate 80) limits its

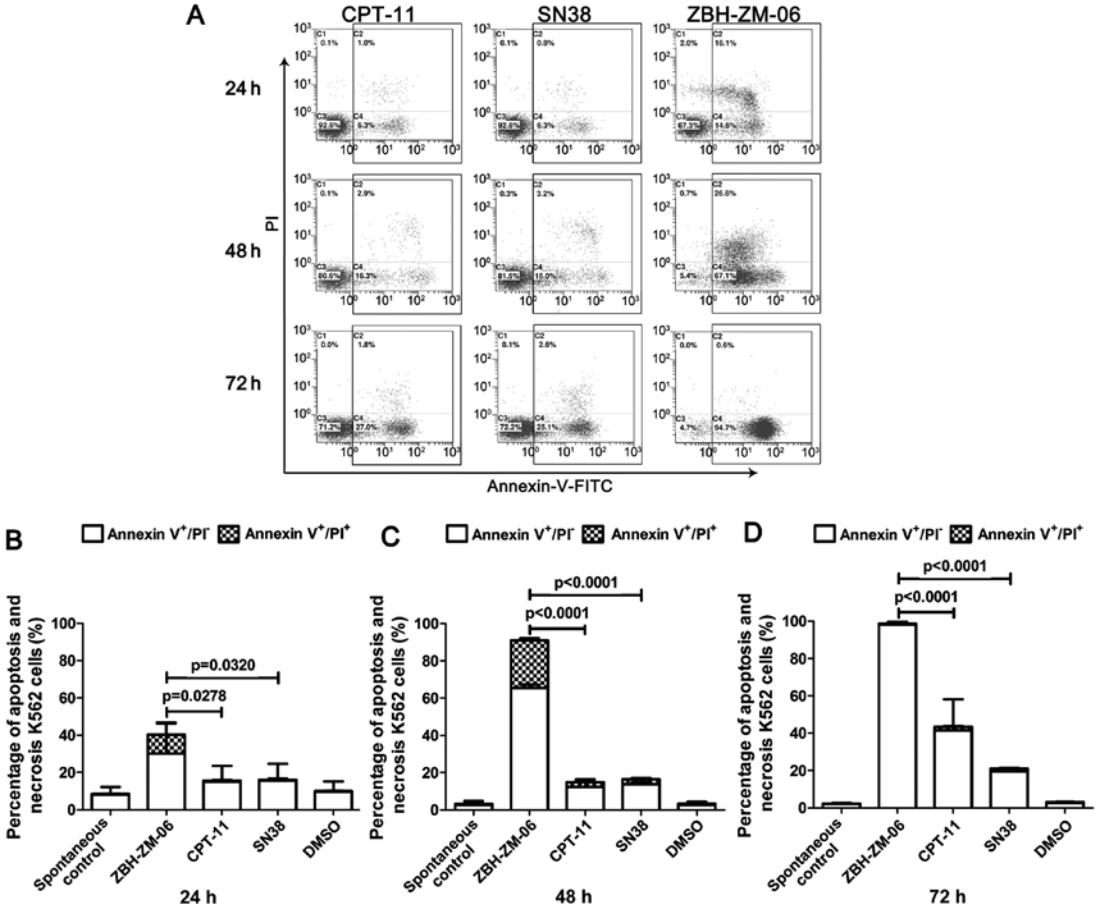

Figure 5. ZBH-ZM-06, CPT-11 and SN38 induce K562 cell apoptosis. K562 cells were treated with 10 μmol/l ZBH-ZM-06, CPT-11 and SN38, respectively, for 24, 48 and 72 h. The percentages of apoptotic cells were detected by an Epics XL/MCL flow cytometer with FITC-Annexin V and PI staining. The Annexin V^+/PI^- and Annexin V^+/PI^+ cells indicated early (dotted) and late (open) apoptosis, respectively. DMSO-treated cells were used as negative controls. The typical biparametric images provided by the flow cytometer are presented in A. The summarized data (mean ± standard deviation from three independent experiments) are presented in B, 24 h, C, 48 h, D, 72 h.

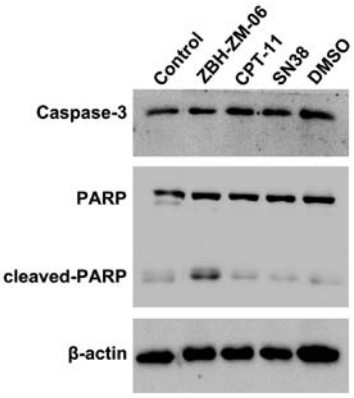

Figure 6. ZBH-ZM-06 promotes the activation of caspase-3 and PARP. SW1116 cells were treated with ZBH-ZM-06, CPT-11 and SN38 at 10 μmol/l for 72 h. Non-treated (Control) and DMSO-treated cells were used as negative controls. Each lysate sample (50 μg) was analyzed by western blot analysis with cleaved-caspase-3 and cleaved-PARP antibodies. β-actin expression was detected on the same membrane as a protein loading reference. Experiments were repeated three times.

application (25), which has been proven previously by our group and others (26,27).

Generally, CPT derivatives are expected to have improved solubility and stability. Yu *et al* (18) synthesized a series of 6-substituted indolizinoquinolinedione derivatives and evaluated them for their biochemical and biological activities. Zhou *et al* (28) evaluated the cytotoxicity of MXN-004 (a small-molecule compound of PEGylated SN38) *in vitro* and demonstrated that it has good water solubility. They further investigated the pharmacokinetics and tissue distribution of MXN-004 and its active metabolite SN38 in rats. We designed and synthesized the novel compound ZBH-ZM-06 by using a linear amino acid as a prodrug carrier. The amine of the amino acid was linked to the 10-OH of SN38 via a carbamate linkage. *N*-methylpiperazine, which has good physiological compatibility, was linked to the C-terminus of the amino acid by an amide bond. The basic nitrogen atom of *N*-methylpiperazine can form a salt with the carboxyl group to improve the water solubility of the compound. One of the disadvantages of CPT-11 is that it is too stable under physiological conditions to be converted into the active form SN38, which results in a low efficiency. ZBH-ZM-06 overcame this disadvantage. It released SN38 rapidly and completely under physiological conditions (pH 7.4), while remaining stable under acidic conditions (pH 5.0). Therefore, it could be used as a lead compound for further drug development.

The anticancer activity of ZBH-ZM-06 was evaluated in 12 cancer cell lines representing diverse histologies. The results

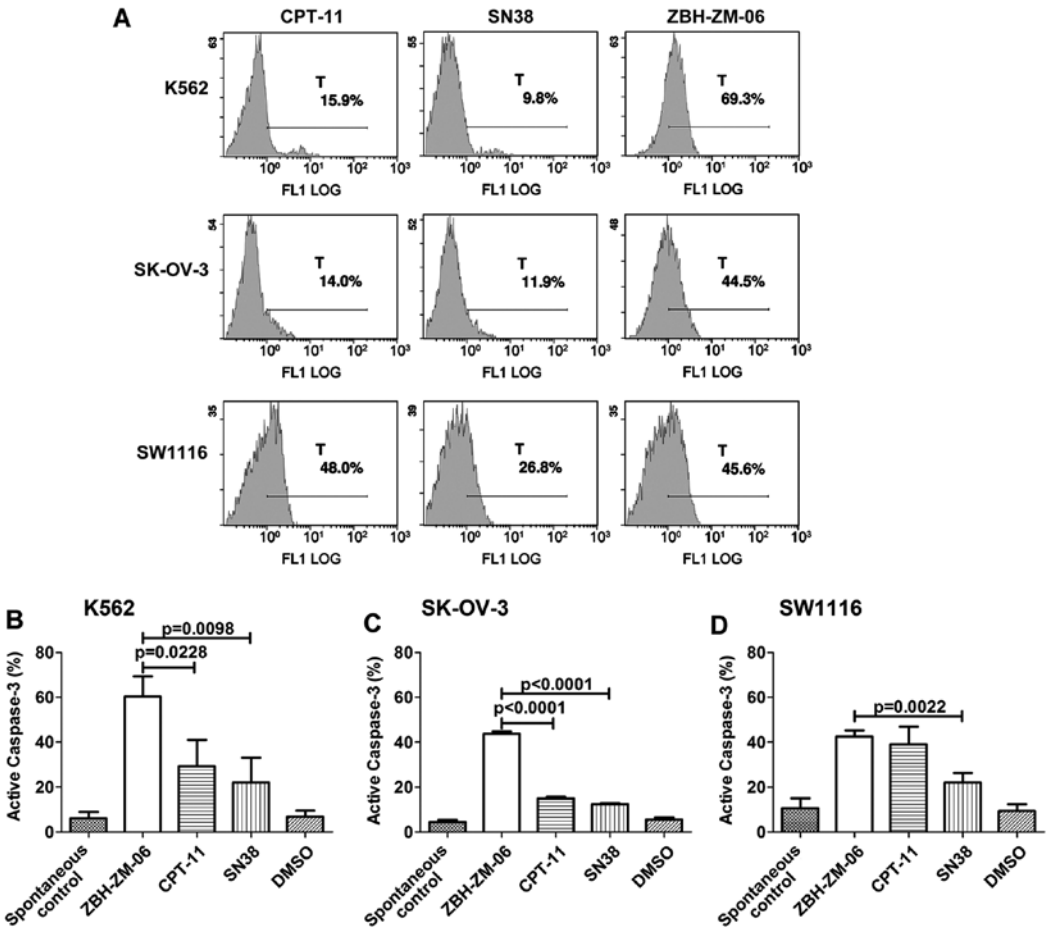

Figure 7. Increase of activated caspase-3 expression induced by ZBH-ZM-06, CPT-11 and SN38 in K562, SK-OV-3 and SW1116 cells. (A) Typical histograms of activated caspase-3$^+$ cells by flow cytometry in the groups treated with 10 μmol/l ZBH-ZM-06 (right panels), CPT-11 (left panels), or SN38 (middle panels) in K562 (upper panels), SK-OV-3 (middle panels), and SW1116 (lower panels) cells. The quantitative analysis of activated caspase-3 in K562 (B), SK-OV-3 (C) and SW1116 (D) cells was performed from three independent experiments, respectively. Data are presented as the mean ± standard deviation.

revealed that ZBH-ZM-06 had a stronger cytotoxic activity on nine cell lines (Table III). Such effects are comparable with those of previous studies. Demarquay *et al* (29) reported the characterization of BN80927, a novel CPT analog, and demonstrated that it was a very potent antiproliferative agent, as shown by the fact that the IC_{50} values were consistently lower than those of SN38 in tumor cell lines as well as in their related drug-resistant lines. Lansiaux *et al* (26) determined whether an E-ring ketone derivative such as S36272 functions as a typical Topo-1 inhibitor, in a manner similar to TPT and SN38. The cytotoxicity of S38809 was studied on a panel of 34 cell lines, including 31 human tumor cell lines, P388 and P388CPT5 sublines, and normal pulmonary artery endothelial cells. S38809 proved to be a potent cytotoxic agent against the 31 human tumor cell lines, with a mean IC_{50} value of 5.4 vs. 11.6 nM for TPT and 3.3 nM for SN38; the colon, leukemia and ovary cell lines were relatively more sensitive. Consistent with our previous report, the correlation of the drug effect with the drug concentration was not considerable in ZBH-ZM-06-treated NCM460 cells, compared with CPT-11- or SN38-treated cells (21).

The 4-piperidinyl piperidine moiety of CPT-11 is responsible for inhibiting AchE activity, causing acute cholinergic diarrhea. This fact hinted to design a novel CPT derivative without AchE inhibition (30). ZBH-ZM-06 exhibited only weak inhibitory activity against AchE, compared to CPT-11, and showed that it can reduce acute cholinergic diarrhea associated with CPT-11. This result was comparable to those of previous studies (31,32).

During DNA replication, Topo-1 primarily produces single-stranded breaks in DNA, which causes DNA relaxation to allow DNA replication. Once DNA replication is complete, Topo-1 will religate the single-stranded breaks to restore the double-stranded DNA structure. In order to explore the cytotoxic mechanism on DNA, we performed a conversional DNA relaxation assay to evaluate the inhibitory effect of ZBH-ZM-06 on Topo-1 activity. Topo-1-regulating drugs, such as CPT-11, can stabilize the covalent enzyme-DNA complex to prevent DNA religation, thereby triggering a series of unstoppable DNA replications to induce cell death eventually (33). Compared to our previously reported structure (21) of ZBH-1205, the effective inhibitory concentration of ZHB-ZM-06 for DNA relaxation is 1 μmol/l. The effective inhibitory concentration for DNA relaxation for ZBH-1205 is 50 μmol/l. This DNA relaxation activity of ZHB-ZM-06 is even higher than that of CPT-11 (at 5 μmol/l).

According to the cell cycle arrest analysis, our previously constructed compounds did not show an obvious effect on tumor cell cycle arrest, even though they induced apoptosis. In the present study, we modified the structure of ZBH-ZM-06 to improve the solubility and the stability, thus providing good

effects. We treated SW1116, SK-OV-3 and K562 cells with ZBH-ZM-06 at a lower concentration (10 μmol/l) and analyzed the cell cycle at 24, 48 and 72 h after treatment. The results suggested that ZBH-ZM-06 arrested the cell cycle of SW1116, SK-OV-3 and K562 cells at the S and G_2/M phases (Fig. 3) at 24 h after treatment. With the extension of treatment, the S-phase arrest ratio was obviously increased and accompanied with a decrease of the G_2/M-phase arrest ratio. During the cell cycle in eukaryotes, the G_2/M phase is a critical highly complex multi-stage process before cell division. Cell cycle arrest at the G_2/M phase is a common cellular response to a variety of DNA-damaging agents. Topo-1 poisons, including CPT-11, induce replication-mediated double-stranded DNA breaks by a replication-fork collision mechanism and induce cell cycle arrest of cancer cells at the G_2/M phase. Treatment of human colon cancer cells with SN38 also resulted in G_2/M cell cycle arrest (34). Our results are consistent with previous reports, suggesting that the mechanism of ZBH-ZM-06 is similar to that of CPT-11 and SN38.

Cell cycle arrest allows time for the repair of DNA lesions. Cells re-enter the cell cycle if the damage can be adequately repaired, or they die by apoptosis if the damage is too severe. The apoptotic signal is transferred through a series of signaling cascades. Activation of cell surface receptors triggers the extrinsic signaling pathway to activate caspase-8, while cytochrome *c* release from the mitochondria can initiate the intrinsic signaling pathway to subsequently induce caspase-9 and caspase-3 activation (35,36). Caspase-3 is a cysteine protease and a well-characterized effector of programmed cell death signaling. It is synthesized in normal cells as an inactive proenzyme that can be rapidly activated and transformed into cleaved caspase-3 (active form) by autoproteolytic cleavage or cleavage by other caspases. Cleaved caspase-3 allows caspase-activated DNase, also known as DNA fragmentation factor, to translocate to the nucleus where it is responsible for internucleosomal DNA cleavage, generating oligo-nucleosomal DNA fragments. Therefore, cleaved caspase-3 is the executor in apoptosis signaling. During the process of apoptosis, caspase-3 cleaves the death substrate PARP to a specific 85-kDa form.

A previous study has revealed that the pro-apoptotic activity of CPT-11 is via an intrinsic apoptotic signaling pathway (37). To validate whether ZBH-ZM-06 can trigger the intrinsic apoptotic signaling pathway, we detected activated caspase-3 and cleaved PARP. Our investigation indicated that ZBH-ZM-06 increased the protein expression level of activated caspase-3 and cleaved PARP, suggesting that similar to CPT-11, the pro-apoptotic activity of ZBH-ZM-06 is via an intrinsic signaling pathway.

According to proapoptotic activity analysis, the apoptosis of SW1116, SK-OV-3 and K562 cells induced by 10 μmol/l ZBH-ZM-06 for 72 h showed similar results as we reported previously. But the early apoptosis rate induced by ZBH-ZM-06 is higher than that induced by CPT-11 and SN38. Although there were some differences among these three tumor cell lines (Fig. 5A). Since the experimental procedures used for adherent cells may injure the cells, we analyzed K562 tumor cells in suspension at 24 and 48 h. The results revealed that the rates of apoptosis and necrosis, especially the early apoptotic rate, induced by ZBH-ZM-06 for 24 and 48 h were significantly higher than those by CPT-11 and SN38 (Fig. 5B). These data suggested that the novel constructed CPT-11 derivative has a stronger proapoptotic capacity (38). Our results are in agreement with those reported by other research groups. Cao *et al* (39) explored apoptosis induced by SN38 and anti-Fas antibody (CH11) in WR/Fas-SMS1 cells and its possible mechanisms. The results indicated that the combination of SN38 and CH11 made the cells undergo greater apoptosis by finally activating caspase-3, whereas SN38 alone was ineffective at inducing these responses at any time-point. Di Francesco *et al* (40) also reported that Gimatecan (ST1481, LBQ707; 7-t-butoxyiminomethylcamptothecin), a novel lipophilic CPT derivative, was more cytotoxic than SN38 and TPT in a panel of neuroblastoma cell lines. Gimatecan's superior cytotoxicity is likely characterized by a marked arrest at the G_2/M phase and induction of caspase 3-dependent apoptosis.

In conclusion, in this study, the CPT derivative ZBH-ZM-06 was designed and synthesized. It revealed higher water solubility and fewer side-effects than those of CPT-11 and SN38. Our results suggested that ZBH-ZM-06 had a greater ability to inhibit the viability of a broad spectrum of human tumor cells than CPT-11 and SN38, but it had less of an effect on normal cells. This antitumor activity may be obtained through Topo-1 inhibition and intrinsic apoptosis activation. This study provides experimental evidence that the novel CPT derivative is more effective and has fewer side-effects than other CPT derivatives. Therefore, it may be applied in the clinic for cancer treatment.

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