

Effects of an irinotecan derivative, ZBH-1208, on the immune system in a mouse model of brain tumor and its antitumor mechanism

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Abstract. The present study aimed to evaluate the inhibitory effects of an irinotecan derivative, ZBH-1208, on brain tumors, and to explore the underlying molecular mechanisms. To determine the effects of ZBH-1208, a brain tumor mouse model was established by transplanting B22 cells. Subsequently, the visceral indices of immune organs and white blood cell counts were determined, and the effects of ZBH-1208 on the expression levels of cell cycle-related proteins were assessed by western blotting. The tumor inhibition rates of 20 and 40 mg/kg ZBH-1208 were 11.7 and 54.1%, respectively. Compared with the negative control group, ZBH-1208 barely affected visceral indices or white blood cell count. Furthermore, the expression levels of p53, p21, cyclin-dependent kinase 7 (CDK7), Wee1, phosphorylated (p)-cell division cycle 2 (CDC2) (Tyr15), p-CDC2 (Thr161) and cyclin B1 proteins were upregulated, whereas the expression levels of cyclin E were downregulated, and those of CDC2, CDK2 and CDC25C were barely altered. In conclusion, the present study demonstrated that ZBH-1208 suppressed the growth of B22 mouse brain tumor xenografts, but did not affect their visceral indices or white blood cell counts. It was suggested that ZBH-1208 exerted its effects by regulating the expression of p53, p21, Wee1, p-CDC2 (Tyr15) and cyclin E proteins.

Introduction

Tumors are associated with high incidence rates and cannot be easily treated. Currently available treatment strategies that are used in clinical practice include surgery, chemotherapy, radiotherapy, targeted drug therapy and gene therapy. Although

numerous novel antitumor drugs have been developed, these drugs inevitably injure healthy tissues and cells, thus inducing severe adverse reactions that greatly affect clinical use (1). Therefore, it is of great significance to identify novel antitumor agents characterized by high pharmacological efficiency, few adverse events and low toxicity, and high specificity.

As a semi-synthetic camptothecin derivative, irinotecan overcomes low aqueous solubility, promotes gastrointestinal absorption and exerts evident therapeutic effects on various tumors, including colorectal cancer, gastric cancer, cervical cancer, ovarian cancer, lung cancer and esophageal cancer (2-5). In addition, irinotecan induces cytotoxicity predominantly by affecting DNA topoisomerase I (DNA Topo I), which participates in intracellular DNA replication, transcription, recombination and repair. This enzyme resolves topological restrictions inside DNA molecules and relaxes excessively coiled DNA double strands, in order to maintain normal DNA replication and transcription (6). Irinotecan only works after being catalyzed by carboxylesterase, when it is converted into the active SN-38 form; however, the conversion process is characterized by low efficiency. Furthermore, it strongly inhibits acetylcholinesterase (AChE) activity. Long-term or high-dose use of irinotecan may trigger gastrointestinal reactions, liver and kidney toxicity, and bone marrow suppression (7,8). To reduce such adverse reactions, it is of great significance to structurally modify irinotecan. We previously synthesized a series of irinotecan derivatives by analyzing the structure-property relationships of camptothecin derivatives and selecting proper carriers (unpublished data). The screened compound, ZBH-1208 (Fig. 1), exhibits higher *in vitro* antitumor activity and reduced AChE-inhibiting activity compared with irinotecan (9). Therefore, it is necessary to study the antitumor activity and the underlying mechanisms of ZBH-1208, in order to develop more eligible drugs. The present study aimed to evaluate the inhibitory effects of ZBH-1208 on B22 mouse brain tumor xenografts, and to clarify the underlying molecular mechanism.

Materials and methods

Drugs and reagents. ZBH-1208 was provided by the Institute of Pharmacology and Toxicology of Academy of Military Medical Sciences (Beijing, China). Irinotecan was purchased

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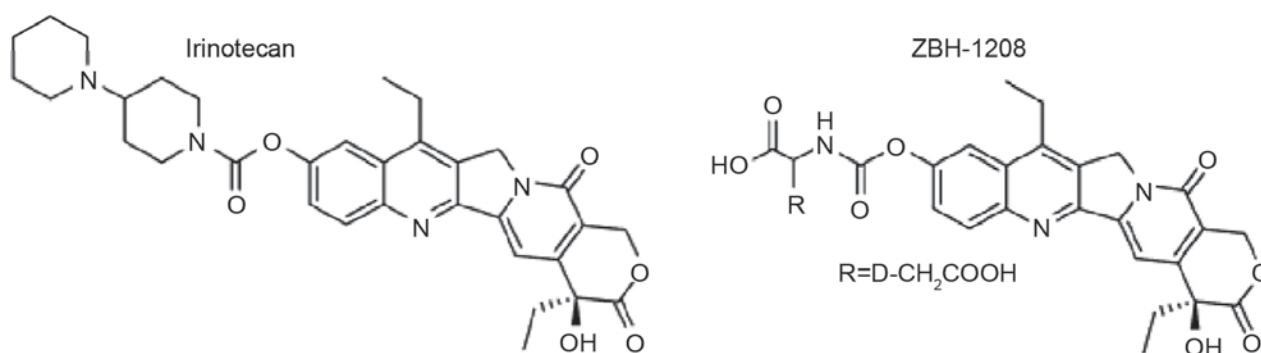


Figure 1. Structures of irinotecan and ZBH-1208.

from Shandong Qilu Pharmaceutical Co., Ltd. (Jinan, China; National Medicine Permit no. H20068128). Sodium chloride was obtained from Sichuan Kelun Pharmaceutical Co., Ltd. (National Medicine Permit no. H20056626; Sichuan, China). TRIzol reagent, bicinchoninic acid (BCA) protein quantification kit and enhanced chemiluminescent (ECL) reagent were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Primers were designed by and purchased from Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Rabbit anti-mouse p53 (cat. no. sc-126), rabbit anti-mouse p21 (cat. no. sc-6246), rabbit anti-mouse cyclin B1 (cat. no. sc-245), mouse anti-human cyclin E (cat. no. sc-481), rabbit anti-mouse cell division cycle 2 (CDC2) (cat. no. sc-54), rabbit anti-mouse phosphorylated (p)-CDC2 (Tyr15) (cat. no. sc-12340), rabbit anti-mouse p-CDC2 (Thr161) (cat. no. sc-12341), rabbit anti-mouse cyclin-dependent kinase 2 (CDK2) (cat. no. sc-6248), rabbit anti-mouse CDK7 (cat. no. sc-56284), rabbit anti-mouse Wee1 (cat. no. sc-5285) and rabbit anti-mouse CDC25C (cat. no. sc-327) antibodies, and horseradish peroxidase-labeled goat anti-rabbit (cat. no. sc-2030) and goat anti-mouse immunoglobulin G (cat. no. sc-2005) secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Apparatus. The 7700 fluorescence quantitative polymerase chain reaction (qPCR) system was purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). Medonic CA620 hematology analyzer was obtained from Boule Medical AB (Spånga, Sweden). High-speed refrigerated centrifuge was purchased from Heraeus (Hanau, Germany), and centrifuge was obtained from Eppendorf AG (Hamburg, Germany).

Animals. Specific pathogen-free-grade male ICR mice (age, 56-70 days; weight, 18-22 g) were purchased from the Experimental Animal Center of Peking University Health Science Center (Beijing, China; certificate of conformity: SCXK (Beijing) 2006-0008). Mice were maintained at a temperature of 20-22°C, humidity of 40-60% under a 12-h light/dark cycle with free access to food and water. The present study has been approved by the Ethics Committee of Liaocheng People's Hospital (Liaocheng, China), and efforts were made to minimize the suffering of animals.

Cell line. The B22 mouse brain tumor cell line was provided by the China Center for Type Culture Collection (Wuhan, China). Cells were cultured in Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified 5% CO₂ atmosphere at 37°C. Cells were inoculated into 30 ICR mice with a 2 ml intraperitoneal injection of 1x10⁶ cells/ml.

Animal grouping and treatment. Under sterile conditions, B22 tumor tissues, which had been subcutaneously inoculated into the right axillary region for 2 weeks, were collected, cut into small pieces, ground, screened and centrifuged at 1,200 x g for 5 min at 4°C. Subsequently, tumor cells were resuspended by adding 5X volume of normal saline and the cells (1x10⁶ cells/ml) were subcutaneously inoculated into the right axillary region of male ICR mice (0.2 ml/mouse). On the subsequent day, mice were randomly divided into the negative control (normal saline) group (i.p., once daily), the low-dose and high-dose ZBH-1208 administration groups (20 and 40 mg/kg, respectively; i.p., once daily) and the positive control (irinotecan) group (25 mg/kg; i.p., once daily) (n=8 mice/group). The irinotecan and low-dose ZBH-1208 groups were administered freshly prepared solutions at the same molar concentration. The mice were weighed daily and sacrificed by cervical dislocation following 12 days of treatment, after anesthesia with 1.5% pentobarbital sodium (35 mg/kg) intravenously. The solid tumors were weighed; tumor inhibition rate was calculated as follows: Tumor inhibition rate (%) = (Average tumor weight of negative control group - average tumor weight of administration group)/average tumor weight of negative control group x 100%. Weight difference was calculated, as follows: Weight difference = Weight on the last day - weight on the first day.

Measurement of visceral indices and counting of white blood cells. Prior to sacrifice of tumor-bearing mice by cervical dislocation, 2-5 ml peripheral blood was collected from the orbital sinus to evaluate the effects of ZBH-1208 on mouse white blood cell counts using the Medonic CA620 hemocytometer. After the mice were sacrificed, spleen and thymus gland tissues were collected, in order to calculate the visceral index, as follows: Visceral index (mg/g) = Visceral weight/body weight.

Reverse transcription-qPCR (RT-qPCR). Total RNA was extracted from peripheral blood cells using TRIzol, and its integrity was identified by electrophoresis. Subsequently, 1 µg total RNA was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Inc., Valencia,

CA, USA), as previously described (10). The total reaction volume was 10 μ l. qPCR was performed using the 7700 fluorescent qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primer sequences were as follows: p53 sense, 5'-TACTCCCCTGCCCTCAACAAGA-3' and antisense, 5'-ACAACCTCCGTCATGTGCTGTG-3'; p21 sense, 5'-TAC TCCCCTGCCCTCAACAAGA-3' and antisense, 5'-CGC TATCTGAGCAGCGCTCAT-3'; cyclin B1 sense, 5'-GCA ACCTCCAAGCCCGGACTG-3' and antisense, 5'-AAATAG GCTCAGGCGAAAGTT-3'; Wee1 sense, 5'-ATTTCTCTG CGTGGGCAGAAAG-3' and antisense, 5'-CAAAAGGAG ATCCTTCAACTCTGC-3'; CDC2 sense, 5'-TCTATCCCT CCTGGTCAAGTTC-3' and antisense, 5'-TGTCCACTGGAG TTGAGTAGC-3'; CDC25c sense, 5'-ACCTCTTTCATACCG TTGCTGG-3' and antisense, 5'-AACTC CTTGTATCCGCC CTTCA-3'; CDK7 sense, 5'-GTGGGCTGTTTGCTGTAT-3' and antisense, 5'-TTCTTGGGCAATCCTCCT-3'; cyclin E sense, 5'-CAGGGTATCAGTGGTGCAGACA-3' and antisense, 5'-TCTTTGCTCGGGCTTTGTCC-3'; CDK2 sense, 5'-CCA CCGAGACCTTAAACC-3' and antisense, 5'-GTGTAAGTA CGAACAGGG-3'; and GAPDH sense, 5'-AAGGTGGTG AAGCAGGCGGC-3' and antisense, 5'-GAGCAATGCCAG CCCAGCA-3'. The PCR reaction system (25 μ l) contained 12.5 μ l 2X Premix Ex Taq (Takara Bio, Inc., Otsu, Japan), 1 μ l each sense and antisense primer, 1 μ l cDNA and ddH₂O. The thermocycling conditions for PCR were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for 20 sec. The expression ratio of target gene to internal reference gene (GAPDH) was calculated based on the fluorescent curve and C_q values were used to determine the difference in gene expression between the samples (11).

Western blot analysis. Total protein was extracted from tumor tissues by chemical lysis using lysis buffer (Abcam, Cambridge, MA, USA) and protein concentration was determined using the BCA method. Total proteins (25-50 μ g) were separated by 8% SDS-PAGE and were transferred to a nitrocellulose membrane. The membrane was then stained with Ponceau S (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), blocked in 1% skimmed milk at room temperature for 1 h and incubated overnight with primary antibodies diluted at 1:800 in 1.5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) at 4°C. Subsequently, the membrane was washed three times with TBS containing 10% Tween (TBST; 10 min/wash) and incubated with 1.5% BSA-diluted secondary antibodies (1:1,500) at room temperature for 1 h. The membrane then underwent four further washes with TBST (15 min/wash), after which the blot was visualized with an ECL reagent for ~1 min, exposed to X-ray film for 5-30 min and scanned. The gray-scale values were analyzed by ImageJ software version 1.41 (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as the housekeeping protein.

Statistical analysis. All data were analyzed using Microsoft Excel version 2012 (Microsoft Corporation, Redmond, WA, USA) and expressed as mean \pm standard deviation of 3 independent experiments. Data were analyzed using a two-way analysis of variance, followed by Dunnett's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Inhibitory effects of ZBH-1208 on B22 solid tumors (n=8; mean \pm standard deviation).

Group	Dose (mg/kg)	Average tumor weight (g)	Inhibition rate (%)
Negative control	-	3.094 \pm 1.022	-
Irinotecan	25	0.391 \pm 0.112 ^a	87.7
ZBH-1208	20	2.739 \pm 1.131	11.7
	40	1.422 \pm 0.224 ^b	54.1

^a $P < 0.01$, ^b $P < 0.05$ compared with the negative control group.

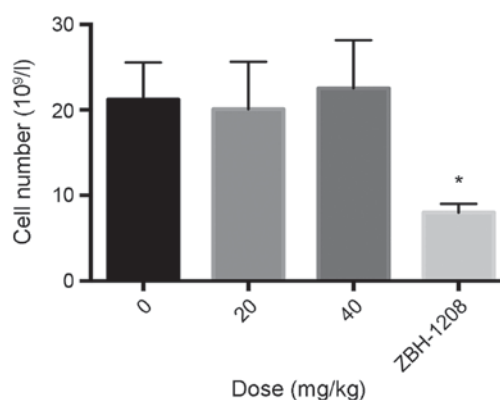


Figure 2. Effects of ZBH-1208 on white blood cell counts (n=8). * $P < 0.01$, compared with the negative control (0 mg/kg) group.

Results

Inhibitory effects of ZBH-1208 on B22 solid tumor. As presented in Table I, compared with the negative control group, the average tumor weight of the irinotecan group was significantly decreased ($P < 0.01$), with a high tumor inhibition rate. In addition, the average tumor weight of the 40 mg/kg ZBH-1208 group was significantly reduced compared with the negative control group ($P < 0.05$), with an inhibition rate of >50%.

Effects of ZBH-1208 on immune organs and white blood cell count. The average white blood cell count of the irinotecan group ($4.5 \times 10^9 \text{ l}^{-1}$) was significantly lower than that of the negative control group ($21.3 \times 10^9 \text{ l}^{-1}$); however, the average white blood cell count of the 20 and 40 mg/kg ZBH-1208 groups was similar to the negative control group (20.7×10^9 and $21.5 \times 10^9 \text{ l}^{-1}$ respectively) (Fig. 2).

As presented in Table II, the irinotecan group exhibited a significantly reduced weight difference, and reduced thymic and splenic indices, compared with the negative control group ($P < 0.05$). However, the values of the ZBH-1208 groups were similar to those of the negative control group.

mRNA expression levels of cell cycle-related genes, as detected by RT-qPCR. Compared with the negative control group, the mRNA expression levels of p53, p21, cyclin B1, Wee1 and CDK7 in the ZBH-1208 groups were upregulated to different extents in a dose-dependent manner. Conversely, the mRNA

Table II. Effects of ZBH-1208 on visceral indices (n=8, mean \pm standard deviation).

Group	Dose (mg/kg)	Weight difference (g)	Splenic index (mg/g)	Thymic index (mg/g)
Negative control	-	18.7 \pm 2.1	11.405 \pm 4.234	2.425 \pm 0.421
Irinotecan	25	15.1 \pm 2.1 ^a	6.237 \pm 1.002 ^a	1.235 \pm 0.112 ^b
ZBH-1208	20	17.3 \pm 1.1 ^b	12.325 \pm 5.210	4.157 \pm 0.112
	40	18.4 \pm 2.2	10.535 \pm 2.117	2.279 \pm 0.471

^aP<0.01, ^bP<0.05 compared with the negative control group.

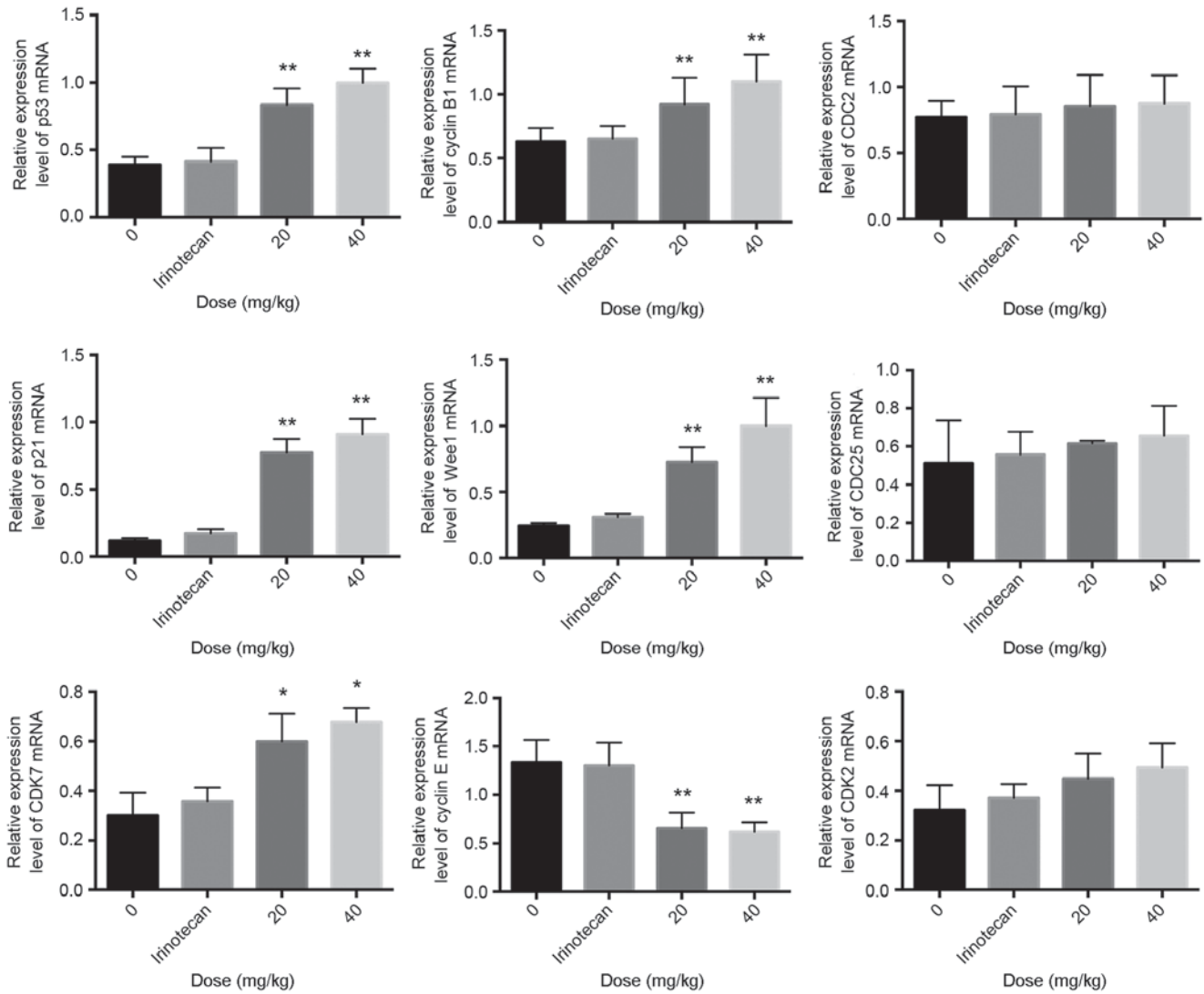


Figure 3. Effects of ZBH-1208 on the mRNA expression levels of cell cycle-related genes, as determined by quantitative polymerase chain reaction. *P<0.05 and **P<0.01, compared with the negative control (0 mg/kg) group. CDC, cell division cycle; CDK, cyclin-dependent kinase.

expression levels of cyclin E were significantly downregulated in response to ZBH-1208, in a dose-independent manner. In addition, ZBH-1208 treatment did not affect the mRNA expression levels of CDC2, CDK2 or CDC25C (Fig. 3).

Protein expression levels of cell cycle-related proteins, as detected by western blotting. Compared with the negative control group, the expression levels of p53, p21, CDK7, Wee1, p-CDC2 (Tyr15), p-CDC2 (Thr161) and cyclin B1 were

upregulated, whereas the expression of cyclin E was downregulated in the ZBH-1208 groups. However, the expression levels of CDC2, CDK2 and CDC25C were barely altered in response to ZBH-1208 treatment (Fig. 4).

Discussion

Irinotecan and its derivatives commonly exist as non-bioactive hydroxylated forms or bioactive lactone forms *in vivo*. At low

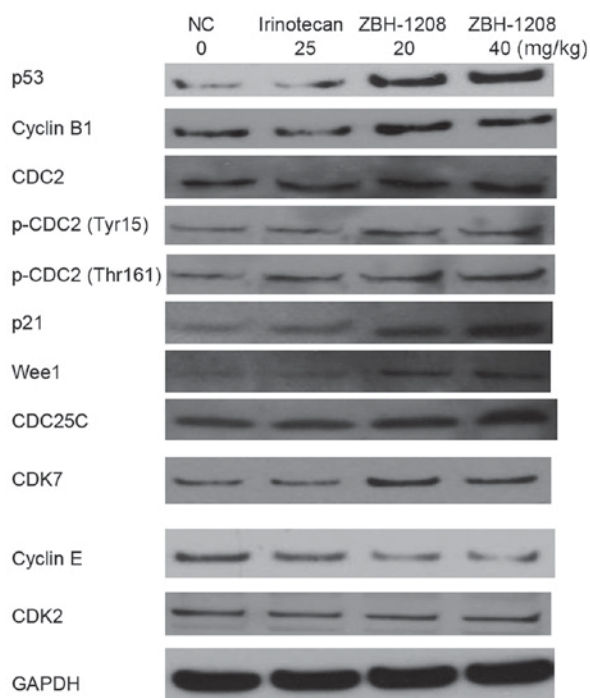


Figure 4. Effects of ZBH-1208 on the expression levels of cell cycle-related proteins, as determined by western blotting. NC, negative control; CDC, cell division cycle; p-, phosphorylated; CDK, cyclin-dependent kinase.

pH, the non-bioactive hydroxylated forms can easily transform into bioactive lactones, following an interconvertible, dynamically balanced reaction. SN-38 is the active metabolite of irinotecan *in vivo*; previous unpublished data from our group suggested that SN-38 may predominantly affect the S phase of tumor cells and target DNA Topo I. In the liver, irinotecan is metabolized by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) into the nonactive glucuronide product SN-38G (7), which is secreted into the intestinal tract through bile and converted into active SN-38 by β -glucuronidase. As a result, the intestinal mucosa is injured, thus causing toxic side effects, such as delayed diarrhea. Furthermore, UGT1A1 catalyzes SN-38 into SN-38G for detoxification (12,13). Notably, irinotecan and its derivatives only serve biological roles following *in vivo* metabolism; therefore, the present study performed *in vivo* experiments. Since drugs for the treatment of brain tumors remain limited in clinical practice, the present study focused on the anticancer effects of ZBH-1208 on B22 mouse xenografts. As a novel derivative of irinotecan, ZBH-1208 inhibited subcutaneously implanted B22 tumors in mice; however, its role in treating orthotopically implanted tumors requires further verification. Furthermore, the present study observed the immunotoxic effects of ZBH-1208. Briefly, irinotecan (25 mg/kg) decreased weight, visceral index and white blood cell count, whereas ZBH-1208 at the same concentration (20 mg/kg) or higher (40 mg/kg) did not induce discernible toxicity.

Suppression of cell cycle progression is of great significance to the inhibition of tumor growth. In eukaryotes, there are two crucial regulatory points in the cell cycle: G_1/S and G_2/M phases. Cell cycle progression is regulated by cyclins and CDKs, which are negatively regulated by CDK inhibitors (CKIs) (14,15). As a CKI, p21 is markedly expressed in the early

G_2/M phase, which induces temporary arrest in the G_2 phase, blocks cell division and proliferation, and finally suppresses tumor cell growth. In addition, it inhibits the activities of various cyclin-CDK complexes and negatively regulates their functions to affect cell cycle progression. For example, p21 is able to independently bind cyclin E and CDK2 and deactivate them, thereby arresting cells in the G_1/S phase (15-18). The product of the antioncogene p53, i.e., the p53 protein, serves a critical role in the G_1/S phase transition. As an upstream gene of p21, it initiates the transcription and expression of p21 following transcription (16-19). It has previously been reported that irinotecan may affect the expression levels of cell cycle-related proteins, including cyclin D and cyclin B, to nonspecifically block the cell cycle (20). To clarify the mechanism by which ZBH-1208 inhibits the growth of B22 brain tumor xenografts, the present study assessed its effects on the expression levels of cell cycle-related proteins. In a dose-dependent manner, ZBH-1208 upregulated the expression levels of p53 and p21, which may lead to p53/p21 pathway-mediated cell cycle arrest. However, whether ZBH-1208 affects other CKIs, such as p16 and p27, requires further study.

Cyclin E-CDK2 has an essential role in G_1/S transition. Synthesized in the middle of the G_1 phase, cyclin E has a high content in S phase and gradually degrades thereafter. The expression levels of CDK2 remain unchanged throughout the cell cycle. After binding CDK2, cyclin E can activate it and form a complex to promote phosphorylation of retinoblastoma protein, thus releasing transcription factor E2F and accelerating the progression of G_1/S phase (21,22). In the present study, ZBH-1208 downregulated cyclin E expression; however, it did not affect CDK2 expression. Regulation of the G_2/M phase depends on formation of the cyclin B1/CDC2 complex, i.e., mitosis promoting factor (MPF). Cyclin B1 is specifically synthesized from the S phase, which gradually increases and peaks in the G_2/M phase. Activation of CDC2 requires dephosphorylation of Thr14 and Tyr15 residues, as well as phosphorylation of Thr161. In addition, a decrease in the activity of Wee1 kinase and CDC25 can dephosphorylate Thr14 and Tyr15 of CDC2, and Thr161 is phosphorylated in the presence of CDK7 (23,24). In a dose-dependent manner, ZBH-1208 upregulated cyclin B1 expression in B22 xenografts; however, CDC2 expression remained unchanged. Although the resulting upregulated expression of CDK7 elevated that of p-CDC2 (Thr161), which may benefit MPF activation, the expression levels of Wee1 were upregulated and those of CDC25C were unaltered. Accordingly, phosphorylation of CDC2 Tyr15 was markedly enhanced to inhibit the activation of CDC2 and the activity of MPF.

In conclusion, ZBH-1208 moderately suppressed B22 mouse brain tumor growth, without significantly reducing visceral indices of immune organs or white blood cell counts. In addition, it exerted its effects by upregulating the protein expression levels of p53, p21, Wee1 and p-CDC2 (Tyr15), and by downregulating cyclin E expression. Nevertheless, the detailed mechanism remains unclear. For example, whether ZBH-1208 induces DNA damage via *in vivo* metabolism, similar to irinotecan; whether it activates the p53 protein via the ataxia telangiectasia mutated serine/threonine kinase/checkpoint kinase 2 pathway; and whether it may induce immune system toxicity requires further investigation.

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