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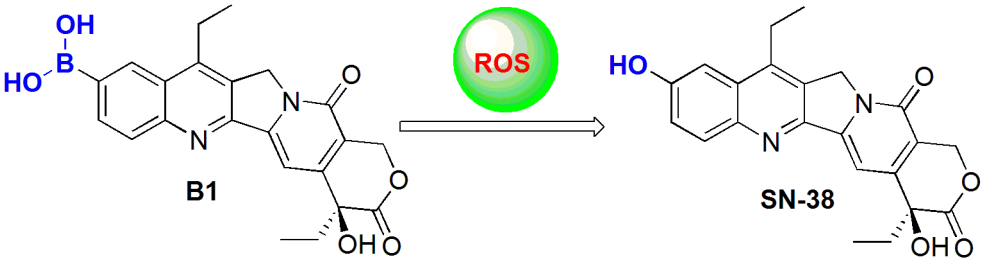


10-Boronic acid Substituted Camptothecin as Prodrug of SN-38

Lei Wang‡[a], Shao Xie‡[b], Longjun Ma[a], Yi Chen[b,*] and Wei Lu[a,*]

[a] *School of Chemistry and Molecular Engineering, East China Normal University, 3663 North Zhongshan Road, Shanghai 200062, P. R. China*

[b] *Division of Anti-Tumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, P. R. China*

* Corresponding authors:

E-mail: wlu@chem.ecnu.edu.cn; Tel.: +86-21-62602475 (W. Lu);

E-mail: ychen@simm.ac.cn; Tel.: +86-21-50801552 (Y. Chen).

‡These authors contributed equally.

Abstract

Malignant tumor cells have been found to have high levels of reactive oxygen species such as hydrogen peroxide (H_2O_2), supporting the hypothesis that a prodrug could be activated by intracellular H_2O_2 and lead to a potential antitumor therapy. In this study, the 7-ethyl-10-boronic acid camptothecin (**B1**) was synthesized for the first time as prodrug of SN-38, by linking a cleavable aryl carbon−boron bond to the SN-38. Prodrug **B1** selectively activated by H_2O_2, converted rapidly to the active form SN-38 under favorable oxidative conditions in cancer cells with elevated levels of H_2O_2. The cell survival assay showed that prodrug **B1** was equally or more effective in inhibiting the growth of six different cancer cells, as compared to SN-38. Unexpectedly, prodrug **B1** displayed even more potent Topo I inhibitory activity than SN-38, suggesting that it was not only a prodrug of SN-38 but also a typical Topo I inhibitor. Prodrug **B1** also demonstrated a significant antitumor activity at 2.0 mg/kg in a xenograft model using human brain star glioblastoma cell lines U87MG.

Keywords: boric acid; hydrogen peroxide; prodrug; camptothecin; antitumor.

1. Introduction

Natural products have been the source of most of the active ingredients of medicines. This is widely accepted to be true when applied to drug discovery in 'olden times' before the advent of high-throughput screening and the post-genomic era: more than 80% of drug substances were natural products or inspired by a natural compound [1]. In the area of cancer, over the time frame from around the 1940's to 2007, of the 155 small molecules, 73% are other than synthetic, with 47% actually being either natural products or directly derived therefrom [2]. What is noteworthy is that lots of natural products and its derivatives contain the phenol hydroxyl as the key pharmacophore, such as combretastatin A-4 (CA-4, **1**, Fig. 1), etoposide (**2**, Fig. 1) and SN-38 (the active metabolite of irinotecan, **3**, Fig. 1).

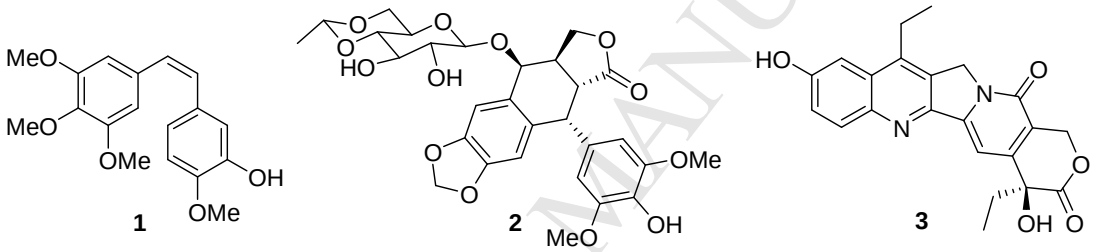


Figure 1. Representative natural products which contain the phenol hydroxyl.

Boron is an essential trace element for plants that occurs naturally in the environment. There is increasing evidence that boron may be important for animals and humans, as it is in plants [3]. Boron has a wide range of applications in chemistry, material science, energy research, and electronics, as well as in life science. The drug bortezomib (Velcade®), a drug in clinical use with boron as an active element, was approved in 2003 as a proteasome inhibitor for the treatment of multiple myeloma and non-Hodgkins lymphoma [4].

It is well established that alkyl- or aryl boronic acid and their esters can be easily oxidated by H_2O_2 [5]. Moreover, cancer cells are known to exhibit elevated intrinsic oxidative stress [6-8]. The increased amounts of reactive oxygen species (ROS) can be a therapeutic advantage, because it is an exclusive feature of cancer cells [9-10]. Therefore, it would be advantageous to develop novel anticancer prodrugs that can be activated by the high level of ROS in cancer cells. The most common ROS include

hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radical. Among these, H_2O_2 has a pivotal role because it is a stable ROS and generated from nearly all sources of oxygen radicals [11]. These factors make H_2O_2 an ideal candidate as a target to develop new ROS-inducible prodrugs with high selectivity to cancer cells. In addition, the cell surface is coated with a dense forest of polysaccharides known as the glycocalyx [12]. Boronic acids readily form boronate esters with the 1, 2- and 1, 3-diols of saccharides [13], including those in the glycocalyx [14-19]. Boronates have attributes that enhance drug selectivity toward tumor cells [20]. Meanwhile, Boronic acids and esters do not appear to have intrinsic toxicity issues, and the end product, boric acid, is considered nontoxic to humans [21].

Recently, various boron-based derivatives of 4-hydroxytamoxifen (**4**-**6**, Fig. 2) were studied for the treatment of tamoxifen-resistant breast cancer. These prodrugs share common boron-aryl carbon bonds and are converted into the active form 4-hydroxytamoxifen under favorable oxidative conditions in breast cancer. These bioisosteres were found to inhibit the growth of two breast cancer cell lines, MCF-7 and T47D, to a comparable or greater extent than that achieved by 4-hydroxytamoxifen [22]. Moreover, Peng's group has also shown that the prodrug of nitrogen mustard coupled with an arylboronate (**7** and **8**, Fig. 2) can be triggered by H_2O_2 to release active drugs that can kill cancer cells [23-24].

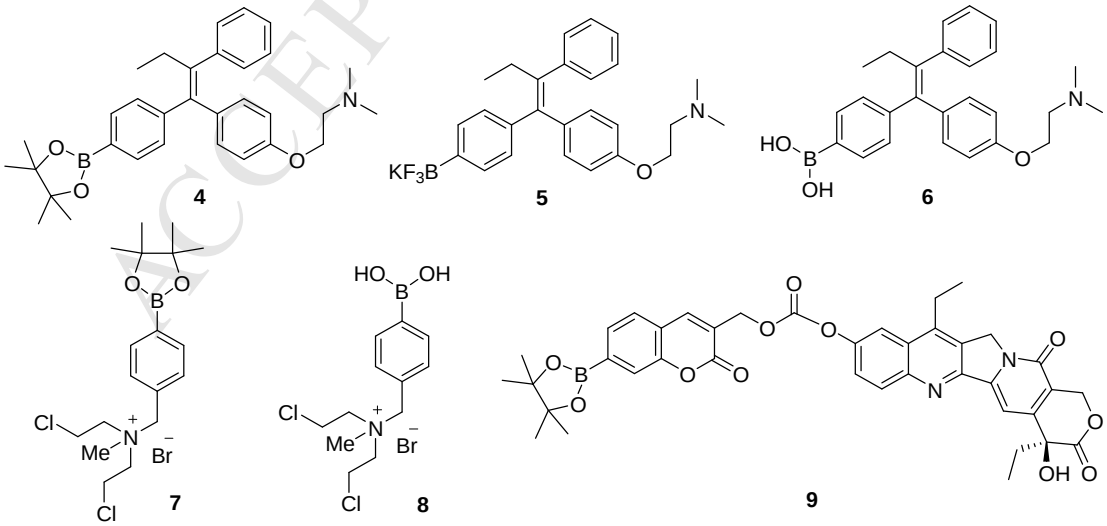
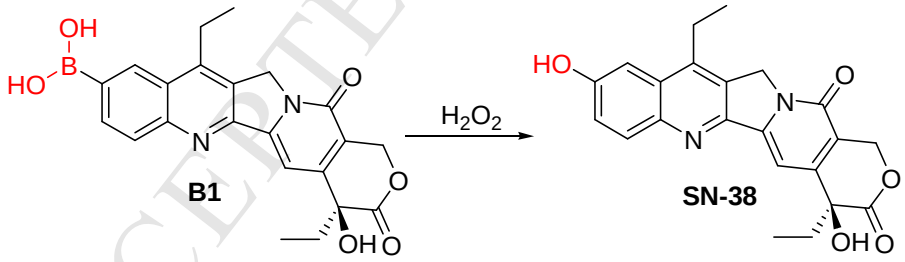


Figure 2. Structures of representative boron-based compounds.

Camptothecin (CPT) is a highly effective anti-tumor and antibiotic alkaloid that was first isolated by Wall and Wani in 1966 [25]. Its antitumor activity is induced by directly binding to topoisomerase I (Topo I), which results in interference with the catalytic cycle of DNA-Topo I and stabilization of the DNA-Topo I binary complex [26-27]. According to the previous studies of CPT derivatives, the SAR had summarized that hydroxyl substitution 10-position enhance their antitumor activity, such as topotecan, 10-hydroxy-CPT and SN-38 (the active metabolite of irinotecan, which gained FDA approval in 1994) [28]. Although a number of camptothecin derivatives were synthesized, but boronic acid substituted analogs have not been reported [29-42]. Kim et al. developed a new theranostic prodrug (**9**, Fig. 2), which contains a H_2O_2-induced cleavable boronate ester for activating a fluorescent moiety (coumarin) and releasing a potent anticancer drug, SN-38 [43]. This prodrug reacted with physiological levels of H_2O_2, resulting in dissociation of the conjugates and delivery of chemotherapeutic agent, providing a powerful new tool for cancer therapy.

In this study, we report 7-ethyl-10-boronic acid camptothecin (**B1**, Scheme 1) for the first time, it is anticipated that upon entering cancer cells, the prodrug **B1** will undergo facile oxidization reaction to release SN-38 that can kill cancer cells.

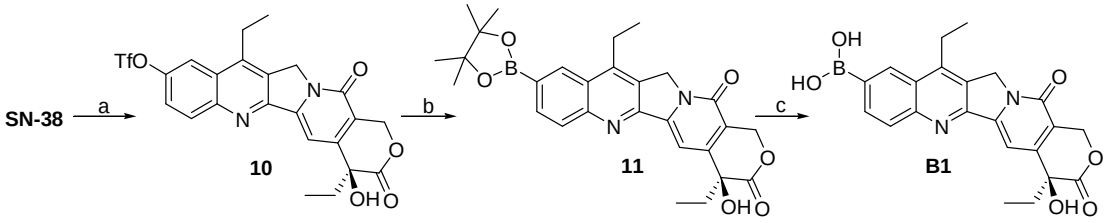


Scheme 1. Design of prodrug **B1** and H_2O_2-promoted release of SN-38.

2. Results and discussion

2.1. Chemistry

Detailed synthetic strategy to 7-ethyl-10-boronic acid camptothecin **B1** was illustrated in Scheme 2. According to published procedures, SN-38 was first converted into triflate **10** in high yield (95%) [44], then triflate **10** was borylated using palladium catalyzed Miyaura chemistry [45]. Finally, boronate ester **11** was oxidated to give boronic acid **B1** in a 50% yield [46].



Scheme 2. Reagents and conditions: (a) N,N-bis(trifluoromethylsulfonyl)aniline, TEA, DMF, rt, 98%; (b) Pd(dppf)Cl_2, KOAc, bis(pinacolato)diboron, 1,4-dioxane, 80°C, 80%; and (c) $NaIO_4$, NH_4OAc, acetone : water = 1 : 1, rt, 50%.

2.2. H_2O_2 and ROS selectivity test

With the prodrug **B1** available, firstly, **B1** was investigated its ability to release SN-38 by treating different concentration of H_2O_2 in phosphate buffer (pH=7.4). SN-38's transformation was analyzed via high performance liquid chromatography. In the presence of H_2O_2, SN-38 was observed at a concentration of H_2O_2 as low as 0.5 mM (22%), in a concentration-dependent manner. This clearly shows that the prodrug **B1** can be activated by H_2O_2 to form SN-38 (Fig. 3a). As H_2O_2 is not the only ROS in biological system, we also studied the inducible activity of **B1** toward other ROS, such as *tert*-butylhydroperoxide (TBHP), hypochlorite (OCl^-), hydroxyl radical and *tert*-butoxy radical. The results shown that **B1** was highly selective for H_2O_2 over other ROS. In the presence of H_2O_2, compounds **B1** displayed efficient SN-38 release (100%), while less than 8% release of SN-38 were observed with other ROS (Fig. 3b).

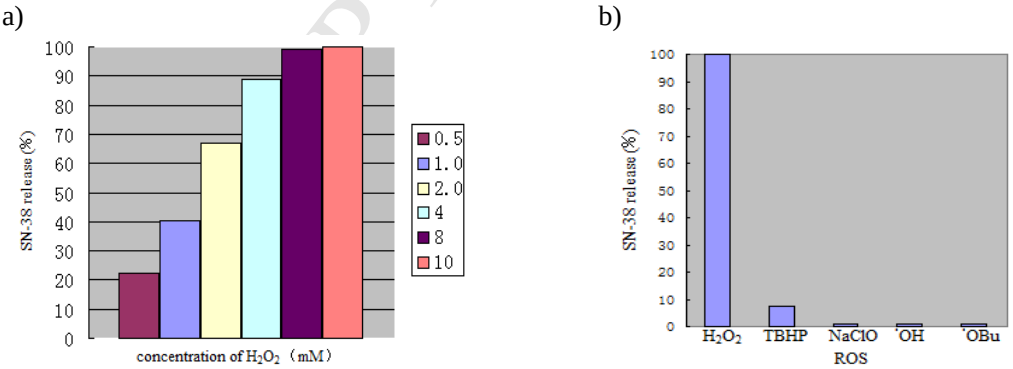


Figure 3. (a) Concentration dependence of compounds **B1** (1 mM) for SN-38 release upon H_2O_2 activation (0.5, 1.0, 2.0, 4.0, 8.0 and 10 mM, respectively); (b) Release of SN-38 induced by **B1** (1 mM) upon treatment with various ROS at 10 mM.

2.3. Relative drug release by MCF-7 cells

To determine if the prodrug **B1** undergo oxidative cleavage by intracellular hydrogen peroxide present at elevated concentration levels in the cancer cells, we

analyzed the cell culture media for concentrations of the prodrug and the active form, SN-38. Using HPLC, we were able to separate, identify, and quantify the prodrugs and its active product. As shown in Figure 4, the boronic acid prodrug **B1** was observed at 12.87 min, while the desired active drug form, SN-38, eluted at 8.85 min. The relative peak areas were measured at 59% (prodrug **B1**) and 41% (SN-38), respectively, indicating that nearly half of the prodrug has been converted to SN-38 in the media after incubation with MCF-7 cells for 48 h.

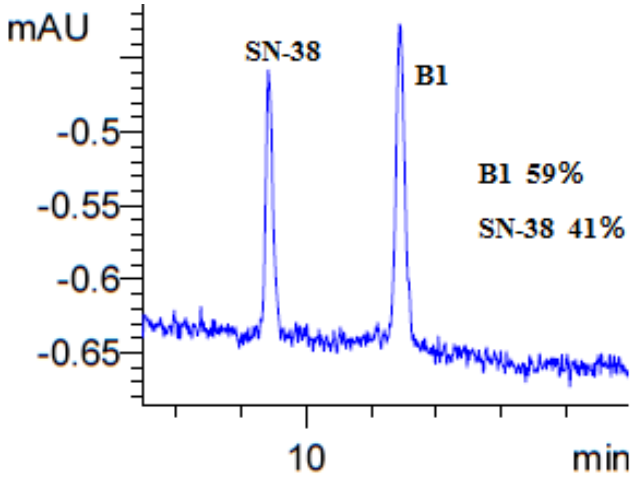


Figure 4. Relative concentrations of prodrug and its oxidative product, SN-38, as determined by HPLC after incubation with MCF-7 cells.

2.4. Cytotoxicity studies

Following, prodrug **B1** was assessed *in vitro* antitumor activities against human colon cancer cell lines HCT-15 and HT-29, human breast cancer cell lines MCF-7 and MDA-MB-231, human brain star glioblastoma cell lines U87MG and U251, with SN-38 as the reference compound. As shown in Table 1, compound **B1** exhibited significant *in vitro* cytotoxic activities against these six tested tumor cell lines, displayed a comparable (MCF-7, U251) or greater (HT-29) cytotoxic activity than that of SN-38, indicating that the 10-boric acid groups of the SN-38 might influence the cytotoxic activities of the new derivative.

Table 1. Cytotoxicity assay against HCT-15, HT-29, MCF-7, MDA-MB-231, U87MG and U251 cell lines.

compounds	IC_{50} (μM)					
	HCT-15	HT-29	MDA-MB-231	MCF-7	U87MG	U251
B1	0.018	0.047	4.284	0.628	0.047	0.036
SN-38	0.008	0.054	1.970	0.515	0.030	0.021

2.5. Topo I inhibition studies

As shown in Figure 5, prodrug **B1** was also tested for its inhibitory effects on Topo I, using CPT and SN-38 as the reference drug. Compound **B1** significantly inhibited Topo I at 100 μM, even more potent inhibitory activity than SN-38. The above result suggested that this novel boric acid substituted derivative was also a typical Topo I inhibitor.

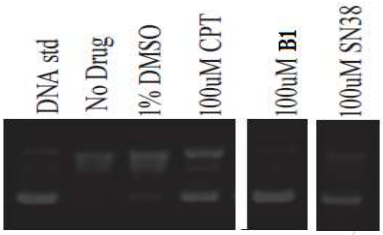


Figure 5. Topo-I-mediated supercoiled pBR322 DNA relaxation was inhibited by **B1** compared to the reference compound CPT and SN-38 at 100 μM.

In general, anticancer drugs increase ROS and increase oxidative stress in cancer cells to kill the cancer cells. Herein, as prodrug of SN-38, compound **B1** displayed significant *in vitro* cytotoxic activity and Topo I inhibitory activity. One possible explanation was that compound **B1** itself might kill cancer cells to increase ROS and oxidative stress. Meanwhile, the increasing ROS activated **B1** to release SN-38 in turn, to enhance anticancer activity.

*2.6. Antitumor activity of **B1** in vivo*

Finally, the effect of prodrug **B1** on inhibiting tumor growth was evaluated in xenograft model. The xenograft model antitumor assay was performed using human brain star glioblastoma cell lines U87MG, with irinotecan (CPT-11) being used as the reference drug (Table 2). Prodrug **B1** was administered intraperitoneally (i.p.) at 0.5 and 2.0 mg/kg doses three times a week to the end (q3w/14). Only at doses of 2.0 mg/kg, it exhibited a weakly relative tumor increment rate (T/C) against that achieved with CPT-11 at a dose of 15 mg/kg (28.10 *vs.* 8.39), at doses of 0.5 mg/kg, it lost of inhibiting tumor proliferation activity. On the basis of Student's t-test evaluation, **B1** at 2.0 mg/kg ($P < 0.01$) exhibited a significant antitumor activity i*n vivo* without overt signs of symptoms or an anaphylactic reaction.

Table 2. Assessment of antitumor efficacy of compound **B1** *in vivo*.

Group	Dose (mg/kg)	Letha toxicity[a]	BWC (%)[b]	RTV (mean±SD)[c]	T/C (%)
control	-	0/12	23.12	16.69±7.52	-
CPT-11	15	0/6	15.88	1.40±0.32**	8.39
B1	2.0	0/6	2.11	4.69±1.92*	28.10
	0.5	0/6	12.69	17.69±11.39*	105.99

[a] Number of dead mice/total number of mice.

[b] Percentage of mouse body-weight change (BWC) after drug treatment: BWC%=(mean $BW_{final\ day}/BW_{first\ day} \times 100$) −100.

[c] * P < 0.01, **P < 0.001.

3. Conclusion

In conclusion, to our best knowledge, the 7-ethyl-10-boronic acid camptothecin **B1** was synthesized for the first time, as a prodrug by linking a cleavable aryl carbon−boron bond to the SN-38. The utility of this prodrug was based on the fact that it can be converted rapidly to the active form under favorable oxidative conditions in cancer cells with elevated levels of hydrogen peroxide. The cell survival assay showed that the prodrug was equally or more effective in inhibiting the growth of six different cancer cells, as compared to SN-38. Unexpectedly, prodrug **B1** displayed even more potent Topo I inhibitory activity than SN-38, suggesting that it was not only a prodrug of SN-38 but also a typical Topo I inhibitor. Prodrug **B1** also demonstrated a significant antitumor activity at 2.0 mg/kg in a xenograft model using human brain star glioblastoma cell lines U87MG. On the basis of these positive results, our study demonstrated the feasibility of using boron chemistry as a prodrug strategy for SN-38, and this strategy would be applied to the research and development of natural products.

4. Experimental Section

4.1. Chemistry

All reagents and solvents were purchased from the suppliers and purified/dried if anhydrous one was necessary. The ^{1}H NMR spectra was recorded on Bruker-400 (400 MHz) using TMS as internal standard. ^{1}H chemical shifts were reported as δ (ppm) and spin-spin coupling constants as J (Hz) values. The mass spectra (MS) were

recorded on a Finnigan MAT-95 mass spectrometer. Melting points were taken on a Fisher–Johns melting point apparatus, uncorrected and reported in degrees Centigrade. HPLC analyses were performed on an HP 1100 series LC system.

*4.2. General procedure for the synthesis of compound **B1***

*4.2.1. 7-ethyl-10-(trifluoromethylsulfonyloxy)camptothecin (**10**)*

To a solution of SN-38 (1.0 g, 2.5 mmol) and N, N-bis(trifluoromethylsulfonyl)aniline (1.3 g, 3.6 mmol) in anhydrous dimethyl formamide (5 mL) was added triethylamine (1.0 mL, 7.5 mmol) and the reaction mixture was stirred under argon at 60 ^{o}C for 3 h. The reaction mixture was cooled and concentrated under reduced pressure to remove the solvents. The crude product was purified by flash chromatography to give compound **10** (1.27 g, 95%). White solid, m.p. 242–244^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (d, J = 9.3 Hz, 1H), 7.97 (d, J = 2.2 Hz, 1H), 7.72 (s, 1H), 7.65 (dd, J = 9.3, 2.2 Hz, 1H), 5.72 (d, J = 16.4 Hz, 1H), 5.48 – 5.09 (m, 3H), 4.27 (s, 1H), 3.19 (q, J = 7.5 Hz, 2H), 2.00 – 1.80 (m, 2H), 1.42 (t, J = 7.6 Hz, 3H), 1.01 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.77, 157.62, 153.52, 150.27, 148.27, 148.01, 146.27, 146.16, 133.57, 128.27, 127.38, 123.74, 120.51, 119.54, 117.32, 115.74, 98.79, 72.93, 66.34, 49.48, 31.81, 23.37, 14.12, 7.94.

*4.2.2. 7-ethyl-10-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)camptothecin (**11**)*

The compound **10** (550 mg, 1.0 mmol), powdered KOAc (300 mg, 3.0 mmol), and bis(pinacolato)diboron (320 mg, 1.1 mmol) were added to a sealed tube that had been purged with argon. A solution of $Pd(dppf)Cl_2$ (80 mg, 0.1 mmol) in degassed anhydrous dioxane (10 mL) was added, and the reaction mixture was stirred under argon at 80 ^{o}C for 12 h. The reaction mixture was cooled and filtered through a short celite pad. The filtrate was concentrated under reduced pressure to remove the solvents. The crude product was purified by flash chromatography to give compound **11** (0.4 g, 80%). Faint yellow solid, m.p. 263–265^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (s, 1H), 8.22 – 8.13 (m, 2H), 7.67 (s, 1H), 5.75 (d, *J* = 16.3 Hz, 1H), 5.35 – 5.23 (m, 3H), 3.81 (s, 1H), 3.27 (q, *J* = 7.7 Hz, 2H), 1.96 – 1.82 (m, 2H), 1.49 – 1.37 (m, 15H), 1.03 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.11, 157.83, 152.81, 151.11, 150.31, 147.22, 146.69, 135.12, 131.38, 129.78, 126.95, 126.41, 118.73,

98.34, 84.51, 72.91, 66.52, 49.59, 31.76, 25.09, 23.15, 14.53, 7.97.

*4.2.3. 7-ethyl-10- boronocamptothecin (**B1**)*

To a solution of compound **11** (400 mg, 0.8 mmol) in acetone:water = 1:1 (10 mL) was added NH_4OAc (125 mg, 1.6 mmol) and $NaIO_4$ (500 mg, 2.4 mmol), then the reaction mixture was stirred at room tempereture for 24 h. The reaction mixture was concentrated under reduced pressure to remove acetone, the aqueous phase was extracted with chloroform: isopropanol=3: 1 and concentrated to afford crude produce, which was further purified by column chromatograpgy to give pure **B1** (200 mg, 50%). Faint yellow solid, m.p.>300^oC; ^1H NMR (400 MHz, DMSO) δ 8.76 (s, 1H), 8.45 (s, 2H), 8.17 (d, *J* = 8.5 Hz, 1H), 8.10 (d, *J* = 8.5 Hz, 1H), 7.34 (s, 1H), 6.53 (s, 1H), 5.44 (s, 2H), 5.34 (s, 2H), 3.26 (q, *J* = 7.6 Hz, 2H), 1.94 – 1.80 (m, 2H), 1.35 (t, *J* = 7.6 Hz, 3H), 0.88 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 172.52, 156.87, 152.27, 150.04, 149.46, 146.29, 146.02, 134.69, 130.70, 128.46, 127.95, 125.89, 119.05, 96.85, 72.42, 65.32, 49.53, 30.36, 22.26, 14.14, 7.78; HRMS (ESI) *m/z* calcd for $C_{22}H_{22}BN_2O_6$ $[M+H]^+$: 421.1571; found: 421.1574.

4.3. H_2O_2 and ROS selectivity test

ROS induced drug release was assessed at pH of 7.4 using HPLC assays. Prodrug stock solutions were prepared in DMSO at concentrations of 10 mM. Aliquots of 10 μL of DMSO prodrug stocks was mixed with 50 mM H_2O_2 (1μL, 2 μL, 4μL, 8μL, 16μL and 20μL, respectively) and appropriate amount of phosphate buffer (pH=7.4) to give a final volume of 100 μL. The reaction was incubated at 37 °C for 8 h and quenched by an equal volume of Sodium thiosulfate solution (50 mM), then subjected to HPLC analysis. Reactive oxygen species were administered to reaction tube as follows. H_2O_2, *tert*-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%, 70%, and 14.5% aqueous solutions respectively. Hydroxyl radical (•OH) and *tert*-butoxy radical (•OBu) were generated by reaction of 1mM Fe^{2+} with H_2O_2 or TBHP, respectively.

4.4. Relative drug release by MCF-7 cells

For measurement of drug release by breast cancer cells, MCF-7 cells were incubated with **B1** at 10^{-5} M. After 2 days, the cell culture media were collected, centrifuged to

remove cells, and diluted to a fixed volume for relative quantitation by HPLC. Chromatographic peak areas corresponding to the prodrug **B1** and SN-38 were used as concentrations in the media.

4.5. Cytotoxicity assays

Cytotoxicity assays of prodrug **B1** was performed on human colon cancer cell lines HCT-15 and HT-29, human breast cancer cell lines MCF-7 and MDA-MB-231, human brain star glioblastoma cell lines U87MG and U251, which were obtained from the American Type Culture Collection (Manassas, VA). Cells (6,000–10,000) in 100μL culture medium per well were seeded into 96-well microtest plates (Falcon, CA). Cells were treated in triplicate with gradient concentrations of test drugs and incubated at 37 °C for 72 h. The growth inhibitory effects on the cell lines were measured with a sulforhodamine B (SRB; Sigma, St. Louis, MO) assay. The drug concentration required for 50% growth inhibition (IC_{50}) of tumor cells was determined from dose–response curves.

4.6. Assay of enzyme activity inhibiting Topo I

The Topo I we used was purchased from Amersham Biosciences. One unit is defined as the amount of enzyme that completely relaxed 0.5 mg of supercoiled pBR322 DNA in 20 μL of the reaction mixture for 30 min at 37 °C. The reaction buffer contained 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM spermidine, 0.01% bovine serum albumin, 0.25 μg supercoiled pBR322, and 2 units of Topo I in a total volume of 20 μL. Relaxation was induced at 37 °C for 30 min and terminated by the addition of 1/10 volume of 10% SDS. The mixture was then treated with proteinase K at a final concentration of 50 μg/mL and incubated at 37 °C for 30 min. An equal volume of chloroform:isoamyl alcohol (24:1) was used to extract the reaction mixture, and the supernatant was then removed onto 1% agarose gel for analysis. Electrophoresis was carried out in 1 × TAE at 4 V/cm for 2 h, and the gel stained with ethidium bromide (0.5 μg/mL), and photographed through a gel document system, ChemiGenius 2.

4.7. Antitumor activity of B1 in vivo

A xenograft model antitumor assay was performed using human brain star glioblastoma cell lines U87MG. When the tumor graft volume reached about 130

mm^3, mice were randomly separated into four groups (n = 12 for control and n = 6 for treatment). Prodrug **B1** was administered intraperitoneally (i.p.) three times a week to the end. These results were statistically significant ($P < 0.001$, Student's t-test).

Acknowledgements

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Table 1. Cytotoxicity assay against HCT-15, HT-29, MCF-7, MDA-MB-231, U87MG and U251 cell lines.

Table 2. Assessment of antitumor efficacy of compound **B1** *in vivo*.

Figure 1. Representative natural products which contain the phenol hydroxyl.

Figure 2. Structures of representative boron-based compounds.

Figure 3. (a) Concentration dependence of compounds **B1** (1 mM) for SN-38 release upon H_2O_2 activation (0.5, 1.0, 2.0, 4.0, 8.0 and 10 mM, respectively); (b) Release of SN-38 induced by **B1** (1 mM) upon treatment with various ROS at 10 mM.

Figure 4. Relative concentrations of prodrug and its oxidative product, SN-38, as determined by HPLC after incubation with MCF-7 cells.

Figure 5. Topo-I-mediated supercoiled pBR322 DNA relaxation was inhibited by **B1** compared to the reference compound CPT and SN-38 at 100 μM.

Scheme 1. Design of prodrug **B1** and H_2O_2-promoted release of SN-38.

Scheme 2. Reagents and conditions: (a) N,N-bis(trifluoromethylsulfonyl)aniline, TEA, DMF, rt, 98%; (b) Pd(dppf)Cl_2, KOAc, bis(pinacolato)diboron, 1,4-dioxane, 80°C, 80%; and (c) $NaIO_4$, NH_4OAc, acetone : water = 1 : 1, rt, 50%.

Highlights

- A boronic acid substituted camptothecin (**B1**) was synthesized for the first time.
- Compound **B1** selectively activated by cancer cells with elevated levels of H_2O_2, converted rapidly to the hydroxy form and displayed potent antitumor activity.
- This strategy would be applied to the research and development of natural products.