# Synthesis of novel SN38-aspirin prodrugs for the treatment of hepatocellular carcinoma

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**Abstract:** Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and is a leading cause of cancer-related death worldwide. However, there is no effective chemotherapeutic treatment for HCC and its prognosis remains poor. Consequently, it is urgent to find an efficient antitumor agent to treat HCC. In this study, 7-ethyl-10-hydroxycamptothecin (SN38), the bioactive metabolite of the anticancer drug irinotecan (CPT-11), which is 100–1000 times more potent than CPT-11, was coupled with aspirin to give 4 prodrugs. Their structures were characterized by ^{1}H NMR and elemental analysis. The in vitro anticancer activities of these compounds on two human hepatocellular carcinoma cell lines (BEL-7404 and HepG2) and preliminary mechanisms of action were explored. Our data indicated that these compounds decreased the viability of cancer cell lines in a concentration- and time-dependent manner. Among them, compound **4b** significantly inhibited cell viability of HepG2 cells (IC$_{50}$ = 0.1208 $\mu$M) when compared with CPT-11 (IC$_{50}$ = 18.4267 $\mu$M). Furthermore, compound **4b** blocked HepG2 cell migration and invasion in vitro. These findings suggest that compound **4b** may be used as a promising anticancer agent against HCC.

**Key words:** 7-Ethyl-10-hydroxycamptothecin, aspirin, prodrug, hepatocellular carcinoma

## 1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second leading cause of cancer-related deaths worldwide.[1] In contrast to most solid cancers, the incidence of HCC and HCC-related deaths have increased over the last several decades in many parts of the world, including the United States. In the United States, more than 39,230 new cases of HCC and more than 27,170 HCC-related deaths were predicted in 2016.[2] The prognosis of HCC is extremely poor, with a median survival of 9 months and a 5-year survival rate of 9.1%.[3] Therefore, it is urgent to discover an efficient drug to overcome HCC.

CPT-11, inhibiting topoisomerase I, is one of the key anticancer drugs in chemotherapy for several cancers such as colorectal cancer, lung cancer, gastric cancer, and gynecologic cancers.[4−7] CPT-11 was also an attractive drug to evaluate in hepatomas and biliary tract malignancies for several reasons. First, there are in vitro data indicating the utility of irinotecan in a variety of gastrointestinal cell lines, including human HCC.[8−10] Second, CPT-11 undergoes activation in the liver to its active minor metabolite, 7-ethyl-10-hydroxycamptothecin (SN38), which leads to high local concentrations of SN38 in the biliary system.[11] SN38 also undergoes enterohepatic

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recycling, which may further contribute to sustained local SN38 levels in the hepatobiliary tree. Therefore, a phase II clinical trial was conducted to determine the response rate, toxicity, and overall patient survival for irinotecan in advanced hepatocellular carcinoma.[12] Based on these characteristics of irinotecan, it was evaluated in phase II studies in patients with advanced HCC. Unfortunately, the antitumor activity of single-agent CPT-11 was not significant in advanced HCC.[13]

SN38 has high antiproliferation potency for many kinds of tumor cells such as ovarian cancer, mammary cancer, colon cancer, and lung cancer. According to in vitro results, SN38 is 100–1000 times as potent as CPT-11.[14−17] However, because of the very narrow therapeutic window of SN38, which results in clinical side effects, and its poor water solubility in any physiologically compatible and pharmaceutically acceptable solvents, SN38 could not be directly applied to clinical therapy of cancer.[18] Thus, discovery of novel derivatives based on SN38 for HCC treatment has significant clinical application value.

Aspirin (acetylsalicylic acid) is the best-known salicylate and belongs to the pharmacologic category of nonsteroidal antiin?ammatory drugs (NSAIDs). It has been widely used for the treatment of inflammation, fever, and pain for over a century.[19] Recently, aspirin has received another boost due to its potential benefits in the prevention/treatment of cancers and cancer prevention.[20,21] Furthermore, aspirin may reduce the risk of HCC,[22] inhibit HepG2 cell growth and induce HepG2 apoptosis,[23−25] increase the sensitivity of anticancer drugs, and overcome anticancer drug resistance in hepatocellular carcinoma cells.[26,27]

Prompted by the above-mentioned encouraging results, we designed and synthesized novel SN38-aspirin prodrugs to simultaneously release SN38 and aspirin. The structures of all compounds were confirmed by ^{1}H NMR and elemental analysis. Then the in vitro anticancer activity against HCC cancer cell lines and preliminary mechanisms of action were explored.

## 2. Results and discussion

### 2.1. Chemistry

Commercially available acetylsalicylic acid was first reacted with oxalyl chloride in anhydrous dichloromethane at room temperature to give compound **1**. Then compound **1** was reacted with ethylene glycol, diethylene glycol, triethylene glycol, and tetraethylene glycol, respectively, in the presence of triethylamine to produce compounds **2a**–**2d**. Compounds **3a**–**3d** were prepared by reacting compounds **2a**–**2d** with 4-nitrophenyl chloroformate using triethylamine as a base. We tried to purify compounds **3a**–**3d** with column chromatography, but no pure products were obtained. Therefore, crude compounds **3a**–**3d** were used directly for the next preparations, which were carried out in the same flask. SN38 and $N,N$-diisopropylethylamine were added to the above-mentioned flask and the reactions were stirred at room temperature overnight. Compounds **4a**–**4d** were obtained by column chromatography (dichloromethane : methanol = 100:1 to 50:1). The HPLC purities of compounds **4a**–**4d** are greater than 97%.

### 2.2. In vitro cytotoxicity screening and antiproliferative properties

The anticancer activities of compounds **4a**–**4d** were evaluated in vitro on two human hepatocellular carcinoma cell lines (BEL-7404 and HepG2) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to previous studies,[28,29] with SN38, CPT-11, and aspirin as positive controls. Data are expressed as $IC_{50}$ (50% inhibitory concentration) and are shown in the Table. As can be seen in the Table, all synthesized compounds exhibited marked anticancer activity against BEL-7404 and HepG2 cell lines, and especially HepG2

cells. Among these compounds, **4b** has an IC$_{50}$ of 0.1208 $\mu$M, while SN38 and CPT-11 have IC$_{50}$ values of 0.1450 $\mu$M and 18.4267 $\mu$M, respectively. Compound **4** showed 152-fold increase in activity when compared with CPT-11. However, aspirin indicates weak anticancer activity at the experimental concentration. The above-mentioned results suggest that compound **4b** has potent antitumor activity.

**Table.** Anticancer activities of compounds **4a**–**4d**.[a]

| Compounds | IC$_{50}$ ($\mu$M)[b] | |
|---|---|---|
| | HepG2 | BEL-7404 |
| 4a (n = 1) | 0.1676 $\pm$ 0.0257 | 4.0600 $\pm$ 0.2701 |
| 4b (n = 2) | 0.1208 $\pm$ 0.0081 | 2.5295 $\pm$ 0.7884 |
| 4c (n = 3) | 0.1707 $\pm$ 0.0245 | 5.0250 $\pm$ 1.0757 |
| 4d (n = 4) | 0.2028 $\pm$ 0.0021 | 3.0255 $\pm$ 0.8549 |
| SN38 | 0.1450 $\pm$ 0.0468 | 0.2553 $\pm$ 0.0458 |
| CPT-11 | 18.4267 $\pm$ 1.6279 | > 40 |
| Aspirin | > 40 | > 40 |

[a] Cell lines were treated with gradient concentrations of compounds for 72 h. All assays were carried out in triplicate, and the results were expressed as mean $\pm$ SD.

[b] 50% growth inhibition as determined by MTT assay.

To further determine whether compound **4b** has direct effects on cancer cells' viability, gradient concentrations (0, 0.0391, 0.0781, 0.1563, 0.3125, 0.6250, 1.2500, 2.5000 $\mu$M) of compound **4b** were used to treat HepG2 cells for 24, 48, and 72 h, respectively. As shown in Figure 1, significant decreases in cell viability were observed as the concentration of compound **4b** increased from 0.0391 to 2.5000 $\mu$M. Meanwhile, treatment with compound **4b** for 24, 48, and 72 h decreased the HepG2 cells' viability in a time-dependent manner. These data suggested that compound **4b** inhibited HepG2 cells viability in a concentration- and time-dependent manner.
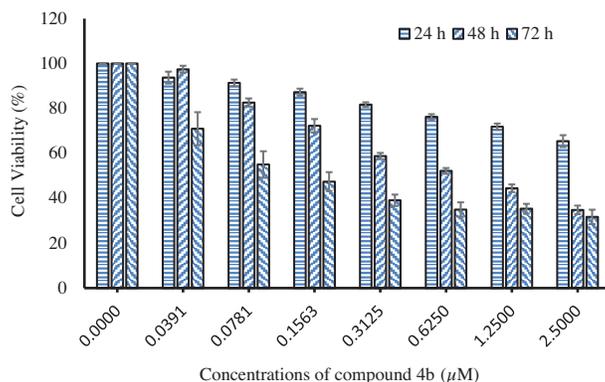


**Figure 1**. Proliferation of HepG2 cells treated with gradient concentrations (0–2.5000 $\mu$M) of compound **4b** for 24, 48, and 72 h, respectively. Cell viability was evaluated by MTT assay. Values represent mean $\pm$ SD (n = 3).

## 2.3. Compound 4b inhibits HepG2 migration and invasion

HCC is a highly aggressive form of carcinoma with poor prognosis, and HCC-associated mortality primarily occurs due to migration and invasion of HCC cells.[30] Tumor metastasis begins with the breakdown of epithelial integrity, followed by malignant cells invading into the surrounding stroma and lymphovascular space, by which cancer cells travel to distant target organs.[31] Hence, inhibition of tumor cell migration and invasion is a critical

approach in anticancer treatment.[32] Therefore, we performed assays to detect the antimetastatic ability of compound **4b** in vitro. As shown in Figure 2, the number of migrated HepG2 cells decreased with increasing concentration of the drug in a dose-dependent manner. At a dose of 0.125 $\mu$M, only about 33% of cells passed through the 8-$\mu$m bottom membrane of the chamber compared with the control group. The Matrigel matrix mimics the environment of the basilar membrane in vivo and it is a structure that must pass through during distant metastasis of cancer cells. Only about 45% cells were inhibited from being invaded when the concentration was 0.5 $\mu$M. The results showed that compound **4b** had the ability to inhibit metastasis of HepG2 cells, but the ability of inhibiting invasion of HepG2 cells was not significant.
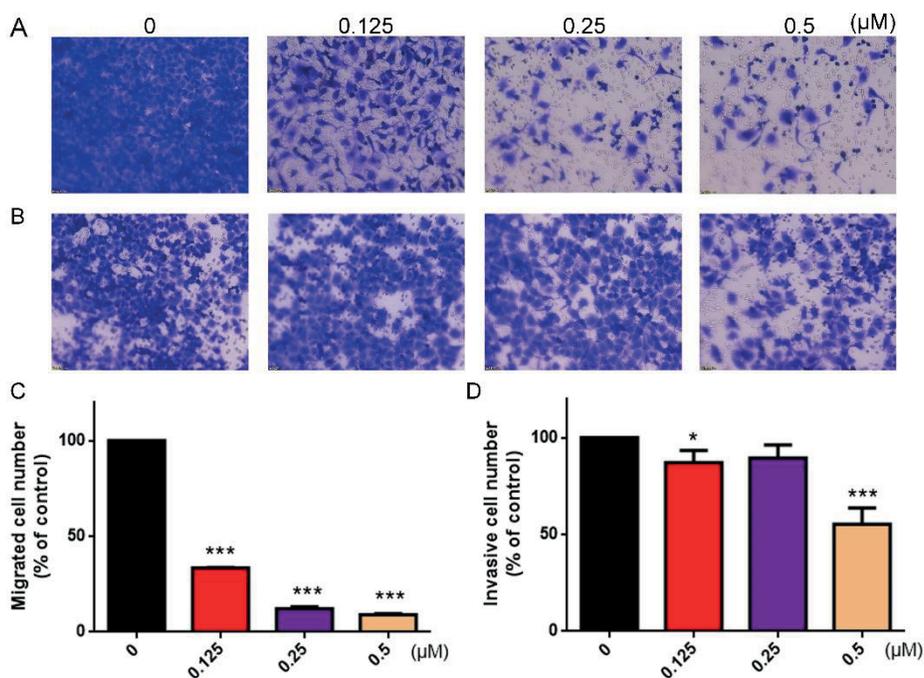


**Figure 2**. Compound **4b** inhibits HepG2 cells migration (A and C) and invasion (B and D). Migrated and invaded cells were fixed, stained, photographed, and quantified (20×). *P < 0.05, ***P < 0.001 compared with control.

## 2.4. Conclusions

In this work, four novel SN38-aspirin prodrugs were synthesized and the in vitro antitumor activities against BEL-7404 and HepG2 cancer cell lines were assessed by MTT assay. Among them, compound **4b** exhibited the most potent activity in a concentration- and time-dependent manner with an IC$_{50}$ value of 0.1208 $\mu$M against HepG2 cells, which displayed a 152-fold increase in activity when compared with CPT-11. The results of preliminary mechanisms of action showed that compound **4b** can block HepG2 cells' migration and invasion. These results imply that compound **4b** might be a potential therapeutic agent for blocking HCC growth and metastasis.

## 3. Experimental

### 3.1. Materials and methods

All chemicals were commercially available and used without further purification unless otherwise stated. All solvents were dried according to the standard methods prior to use. Column chromatography was carried out on

silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China). Thin-layer chromatography (TLC) was performed on TLC silica gel 60 F254 plates. The purity of the compound screened in biological assays was determined to be $\geq 97\%$ by HPLC analysis with a photodiode array detector (Waters, Milford, MA, USA). An Atlantis $C_{18}$ (150 mm $\times$ 4.6 mm, i.d. 5 $\mu$m) (Waters, Milford, MA, USA) was used with a gradient elution of methanol (MeOH) and HPLC-grade water as mobile phase at a flow rate of 1 mL/min. $^1$H NMR spectra were measured on a Bruker AV-400 (400 MHz) NMR spectrometer and chemical shifts ($\delta$) are reported in parts per million relative to tetramethylsilane (TMS) used as an internal standard, where ($\delta$) TMS = 0.00 ppm. Elemental analyses were carried out on a Carlo Erba-1106 analyzer.

## 3.2. Chemistry

The synthetic route for SN38-aspirin prodrugs is shown in the Scheme.
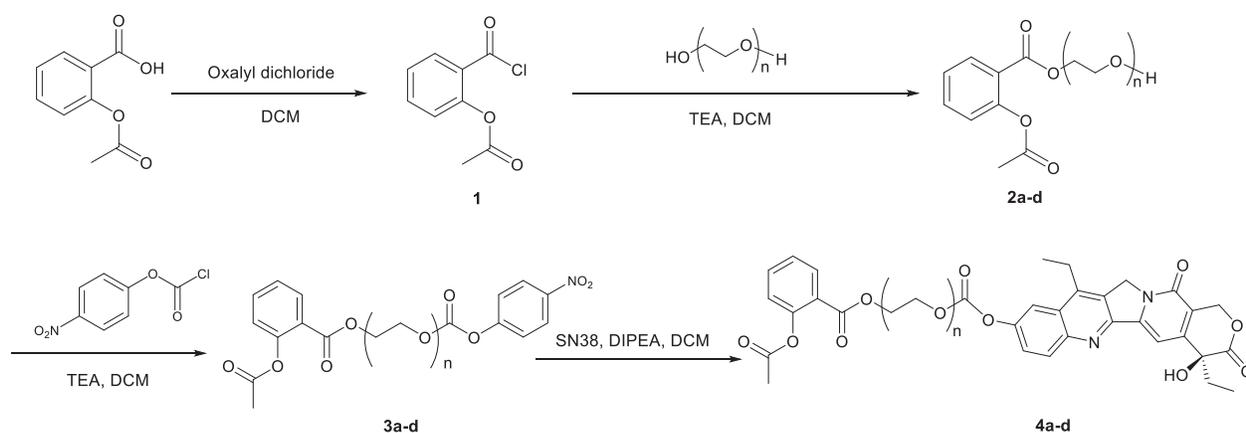


**Scheme.** Synthesis route for SN38-aspirin prodrugs. a: n = 1; b: n = 2; c: n = 3; d: n = 4.

### 3.2.1. Synthesis of compound 1

To a solution of acetylsalicylic acid (5 g, 27.8 mmol) in 50 mL of anhydrous dichloromethane (DCM), 3.3 mL of oxalyl chloride and a drop of dimethylformamide were added slowly and the mixture was stirred for 3 h at room temperature. Then the mixture was concentrated and 5.4 g of compound **1** was obtained (yield: 98.5%).

### 3.2.2. General procedure for the synthesis of compounds 2a–2d

Ethylene glycol (5.39 g, 86.9 mmol) and 10 mL of triethylamine were dissolved in 100 mL of DCM, and newly prepared compound **1** (5.4 g) in the first step was added slowly. The mixture was stirred overnight at room temperature and washed with water (2 $\times$ 50 mL), and the organic phase was dried over anhydrous sodium sulfate and concentrated. The residue was purified by column chromatography (DCM : MeOH = 100:1 to 70:1) to give 1.7 g of compound **2a** with a yield of 27.9%. Compounds **2b–2d** were prepared using the same method described above. Diethylene glycol, diethylene glycol, and tetraethylene glycol were used instead of ethylene glycol.

### 3.2.3. General procedure for the synthesis of compounds 4a–4d

Compound **2a** (224 mg, 1 mmol) and 202 mg of triethylamine (2 mmol) were dissolved in 5 mL of DCM. After the mixture was cooled to 0 °C, 400 mg of 4-nitrophenyl chloroformate (2 mmol) was added slowly to the

mixture. The reaction was complete after 24 h as monitored by TLC (petroleum ether : ethyl acetate = 1:1). SN38 (392 mg, 1 mmol) and 258 mg of $N, N$-diisopropylethylamine (2 mmol) were added to the above reaction. The mixture was stirred overnight at room temperature. Water (10 mL) was added to the reaction, and then the solution was partitioned by DCM and water. The inorganic layer was then extracted by DCM twice (2 × 25 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was purified by column chromatography (DCM : MeOH = 100:1 to 70:1) to give 129.7 mg of compound **4a** with a yield of 20.2%. Compounds **4b–4d** were synthesized according to the method described above. The ^{1}H NMR spectra of compounds **4b–4d** are shown in Figures 3–6.
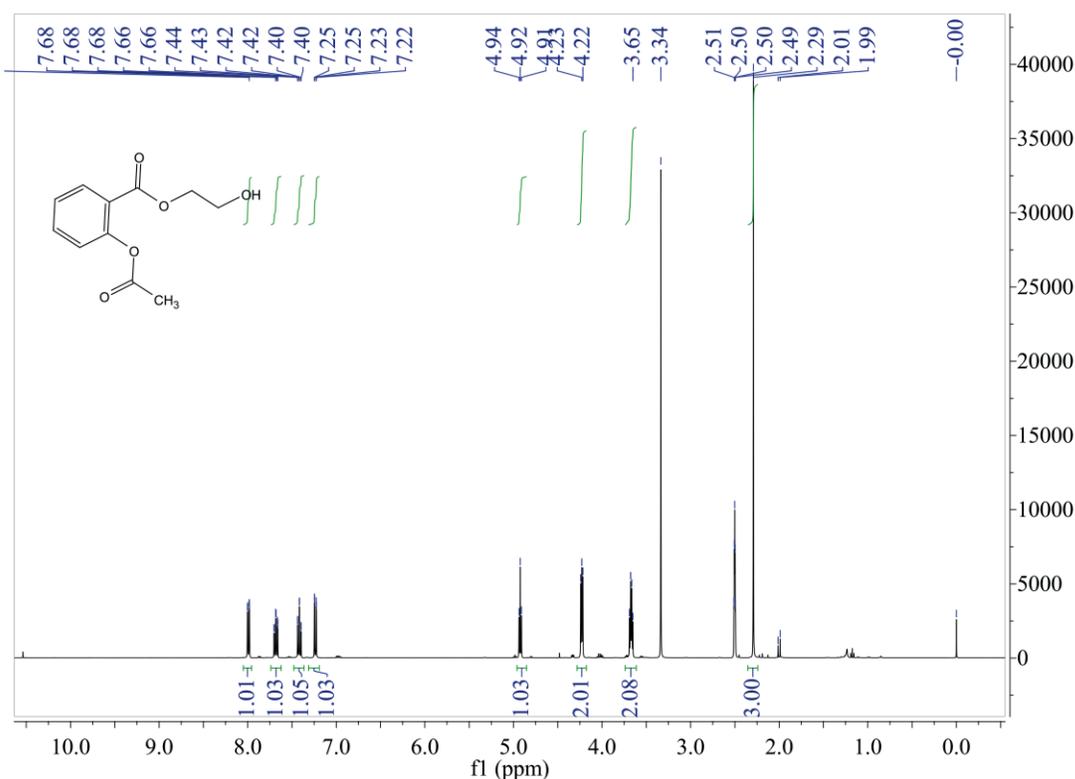


**Figure 3**. ^{1}H NMR spectrum of compound **4a**.

**Compound 2a:** n = 1, light yellow liquid, yield: 27.9%; ^{1}H NMR (400 Hz, DMSO-$d_6$) $\delta$ 7.99 (dd, $J$ = 8.0 Hz, 1.6 Hz, 1H), 7.66–7.70 (m, 1H), 7.40–7.44 (m, 1H), 7.24 (dd, $J$ = 8.2 Hz, 1.0 Hz, 1H), 4.92 (t, $J$ = 5.6 Hz, 1H), 4.22–4.24 (m, 2H), 3.65–3.69 (m, 2H), 2.29 (s, 3H).

**Compound 2b:** n = 2, light yellow liquid, yield: 27.4%; ^{1}H NMR (400 Hz, DMSO-$d_6$) $\delta$ 7.95 (dd, $J$ = 8.0 Hz, 1.6 Hz, 1H), 7.67–7.71 (m, 1H), 7.40–7.44 (m, 1H), 7.24 (dd, $J$ = 8.0 Hz, 0.8 Hz, 1H), 4.61 (t, $J$ = 5.4 Hz, 1H), 4.33–4.35 (m, 2H), 3.69–3.72 (m, 2H), 3.46–3.52 (m, 4H), 2.29 (s, 3H).

**Compound 2c:** n = 3, light yellow liquid, yield: 29.4%; ^{1}H NMR (400 Hz, DMSO-$d_6$) $\delta$ 7.95 (dd, $J$ = 7.8 Hz, 1.8 Hz, 1H), 7.67–7.71 (m, 1H), 7.40–7.44 (m, 1H), 7.24 (dd, $J$ = 8.0 Hz, 0.8 Hz, 1H), 4.58 (t, $J$ = 5.4 Hz, 1H), 4.33–4.35 (m, 2H), 3.69–3.72 (m, 2H), 3.52–3.59 (m, 4H), 3.46–3.50 (m, 2H), 3.40–3.43 (m, 2H), 2.29 (s, 3H).
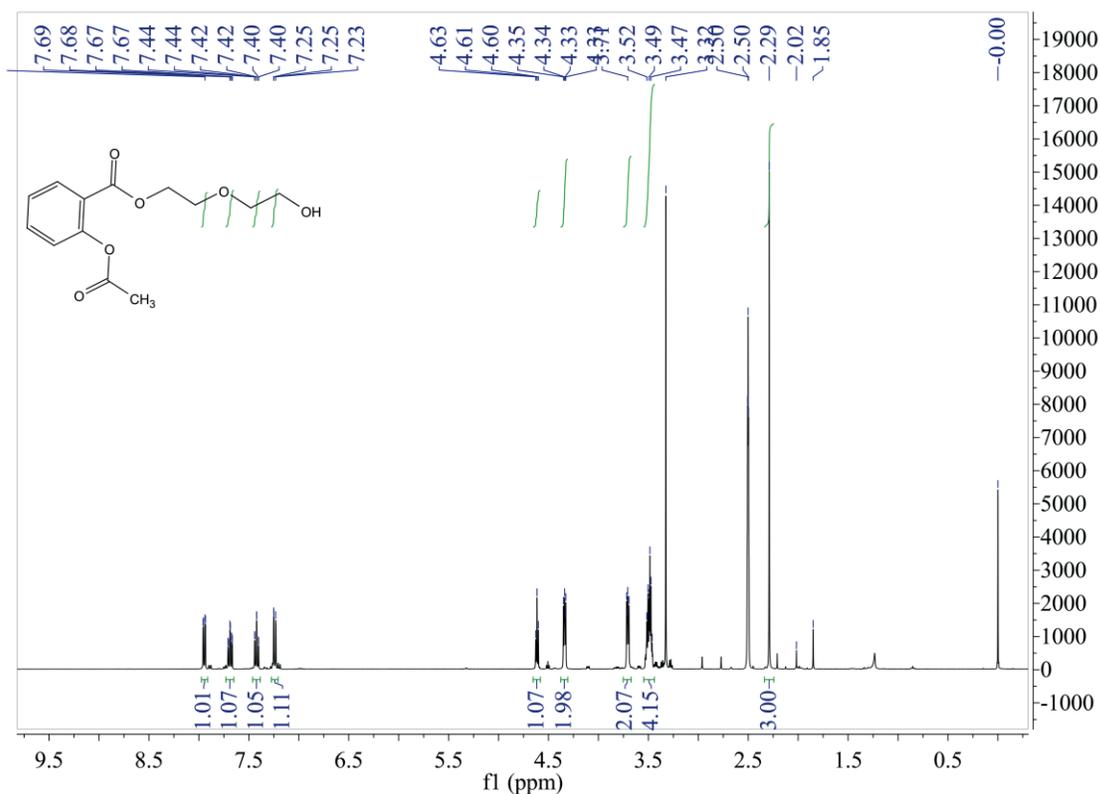
**Figure 4.** ^{1}H NMR spectrum of compound **4b**.

**Compound 2d:** n = 4, light yellow liquid, yield: 18.5%; ^{1}H NMR (400 Hz, DMSO-$d_6$) $\delta$ 7.95 (dd, $J$ = 8.0 Hz, 1.2 Hz, 1H), 7.67–7.71 (m, 1H), 7.43 (t, $J$ = 7.6 Hz, 1H), 7.24 (d, $J$ = 8.0 Hz, 1H), 4.52–4.65 (m, 1H), 4.35 (t, $J$ = 4.6 Hz, 2H), 3.71 (t, $J$ = 4.4 Hz, 2H), 3.47–3.58 (m, 10H), 3.39–3.42 (m, 2H), 2.30 (s, 3H).

**Compound 4a:** n = 1, light yellow solid, yield: 20.2%; HPLC purity: 98.2%. ^{1}H NMR (400 Hz, DMSO-$d_6$) $\delta$ 8.22 (d, $J$ = 9.2 Hz, 1H), 8.15 (d, $J$ = 2.5 Hz, 1H), 7.99 (dd, $J$ = 7.8, 1.6 Hz, 1H), 7.77 (dd, $J$ = 9.2, 2.5 Hz, 1H), 7.69–7.74 (m, 1H), 7.42–7.46 (m, 1H), 7.33 (s, 1H), 7.28 (dd, $J$ = 8.0, 0.8 Hz, 1H), 6.54 (s, 1H), 5.44 (s, 2H), 5.33 (s, 2H), 4.59 (s, 4H), 3.14–3.18 (m, 4H), 2.33 (s, 3H), 1.84-1.91 (m, 2H), 1.27 (t, $J$ = 7.6 Hz, 3H), 0.88 (t, $J$ = 7.3 Hz, 3H). Anal. Calcd. for $C_{34}H_{30}N_2O_{11}$: C, 63.55; H, 4.71; N, 4.36. Found: C, 63.58; H, 4.67; N, 4.29.

**Compound 4b:** n = 2, light yellow solid, yield 20.5%; HPLC purity: 98.7%. ^{1}H NMR (400 Hz, DMSO-$d_6$) $\delta$ 8.22 (d, $J$ = 9.2 Hz, 1H), 8.15 (d, $J$ = 2.3 Hz, 1H), 7.94 (dd, $J$ = 7.8, 1.4 Hz, 1H), 7.77 (dd, $J$ = 9.2, 2.4 Hz, 1H), 7.65–7.69 (m, 1H), 7.40 (t, $J$ = 7.4 Hz, 1H), 7.34 (s, 1H), 7.22 (d, $J$ = 8.1 Hz, 1H), 6.54 (s, 1H), 5.45 (s, 2H), 5.34 (s, 2H), 4.35–4.39 (m, 4H), 3.72–3.75 (m, 4H), 3.63 (s, 4H), 3.18–3.19 (m, 2H), 2.30 (s, 3H), 1.84–1.91 (m, 2H), 1.29 (t, $J$ = 7.5 Hz, 3H), 0.88 (t, $J$ = 7.3 Hz, 3H). Anal. Calcd. for $C_{36}H_{34}N_2O_{12}$: C, 62.97; H, 4.99; N, 4.08. Found: C, 62.85; H, 4.91; N, 4.17.

**Compound 4c:** n = 3, light yellow solid, yield: 20.5%; HPLC purity: 97.9%. ^{1}H NMR (400 Hz, DMSO-$d_6$) $\delta$ 8.22 (d, $J$ = 9.2 Hz, 1H), 8.15 (d, $J$ = 2.3 Hz, 1H), 7.94 (dd, $J$ = 7.8, 1.4 Hz, 1H), 7.77 (dd, $J$ = 9.2, 2.4 Hz, 1H), 7.65–7.69 (m, 1H), 7.40 (t, $J$ = 7.4 Hz, 1H), 7.34 (s, 1H), 7.22 (d, $J$ = 8.1 Hz, 1H), 6.54 (s, 1H), 5.45 (s, 2H), 5.34 (s, 2H), 4.35–4.39 (m, 4H), 3.72–3.75 (m, 4H), 3.63 (s, 4H), 3.18–3.19 (m, 2H), 2.30 (s,
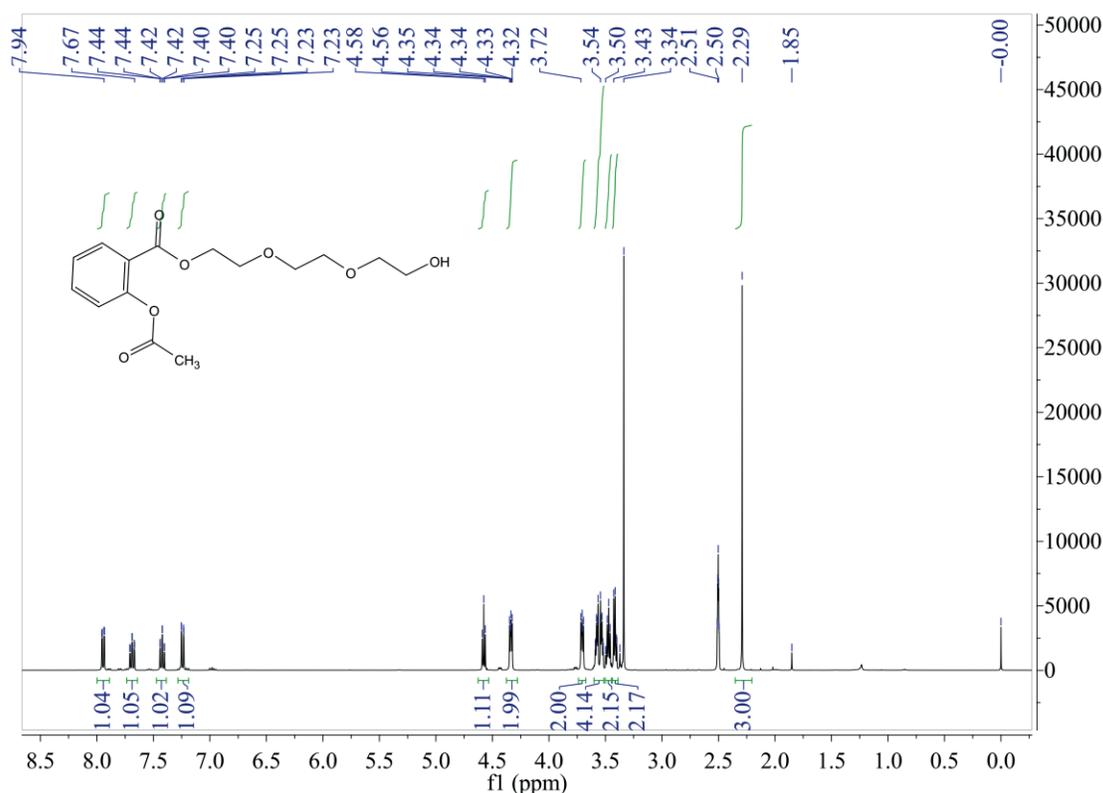
**Figure 5**. $^1$H NMR spectrum of compound **4c**.

3H), 1.84–1.91 (m, 2H), 1.29 (t, $J = 7.5$ Hz, 3H), 0.88 (t, $J = 7.3$ Hz, 3H). Anal. Calcd. for $C_{38}H_{38}N_2O_{13}$: C, 62.46; H, 5.24; N, 3.83. Found: C, 62.41; H, 5.29; N, 3.75.

**Compound 4d:** n = 4, light yellow solid, yield: 15.5%; HPLC purity: 99.1%. $^1$H NMR (400 Hz, DMSO-$d_6$) $\delta$ 8.22 (d, $J = 9.2$ Hz, 1H), 8.15 (d, $J = 2.6$ Hz, 1H), 7.93 (dd, $J = 7.8$, 1.6 Hz, 1H), 7.77 (dd, $J = 9.1$, 2.5 Hz, 1H), 7.64–7.68 (m, 1H), 7.38–7.42 (m, 1H), 7.33 (s, 1H), 7.21 (dd, $J = 8.1$, 1.0 Hz, 1H), 6.53 (s, 1H), 5.44 (s, 2H), 5.34 (s, 2H), 4.32–4.39 (m, 4H), 3.70–3.73 (m, 4H), 3.55–3.58 (m, 8H), 3.16–3.20 (m, 2H), 2.28 (s, 3H), 1.84–1.89 (m, 2H), 1.28 (t, $J = 7.6$ Hz, 3H), 0.88 (t, $J = 7.3$ Hz, 3H). Anal. Calcd. for $C_{40}H_{42}N_2O_{14}$: C, 62.01; H, 5.46; N, 3.83. Found: C, 62.08; H, 5.37; N, 3.80.

## 3.3. Cell lines and culture

The HepG2 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The BEL-7404 human hepatoma cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were propagated in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% antibiotics (penicillin and streptomycin) in 5% $CO_2$ at 37 °C.

## 3.4. Cell viability assay

The cell viability of compound-treated cells was assessed by MTT assay. Briefly, exponentially growing cells ($2 \times 10^3$ cells/well) were plated in 96-well plates (100 $\mu$L/well). After 24 h of incubation, the cells were
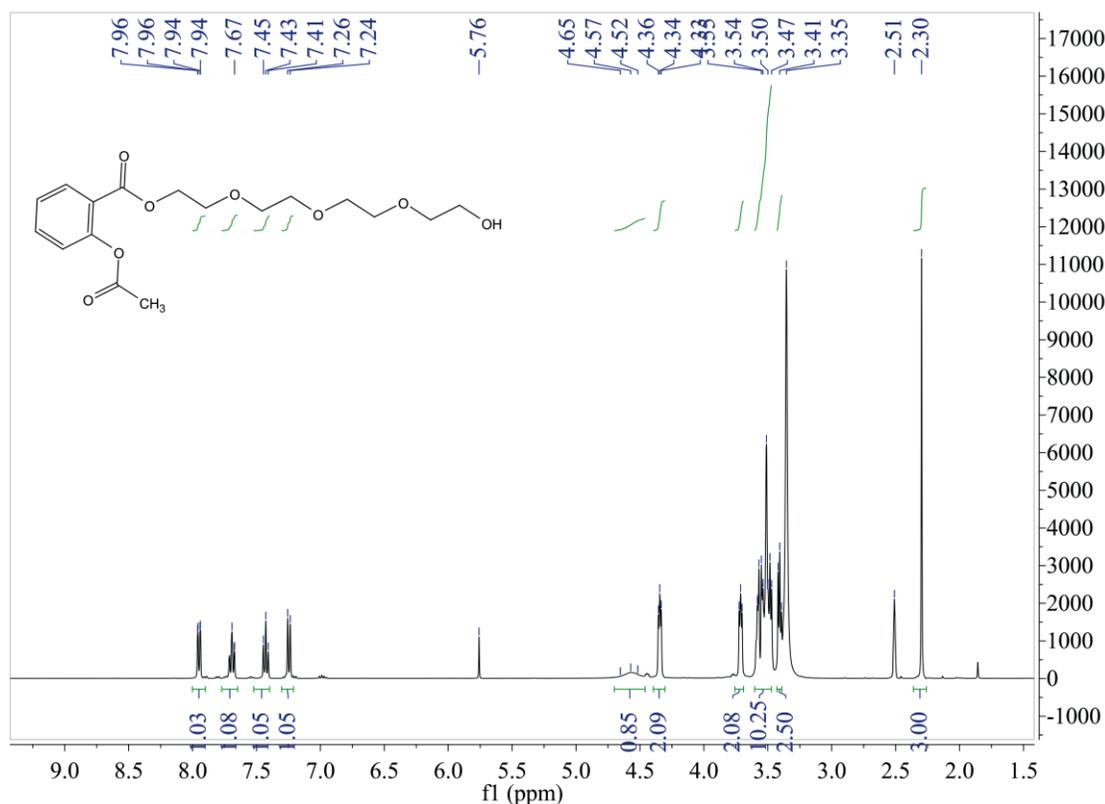
**Figure 6.** ^{1}H NMR spectrum of compound **4d**.

treated with different concentrations of compounds. After treatment for 24, 48, and 72 h, respectively, 20 $\mu$L of 5 mg/mL MTT was added to each well and the plates were incubated at 37 °C for an additional 4 h. The medium was subsequently removed and the purple-colored precipitates of formazan were dissolved in 150 $\mu$L of DMSO. The color absorbance was recorded at 570 nm using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, San Jose, CA, USA). All experiments were performed in triplicate.

### 3.5. Boyden chamber migration and invasion assay

A modified Boyden chamber (8-$\mu$m pore size) migration assay was performed according to previous reports[33] with some modification. Briefly, a total of $1 \times 10^5$ HepG2 cells in 100 $\mu$L of serum-free medium were added in the upper chamber and 600 $\mu$L of medium containing 10% FBS was added at the bottom. Different concentrations of compound **4b** were added in both chambers. Cells were allowed to migrate for approximately 24 h. Nonmigrated cells in the upper chamber were discarded using a cotton swab. The migrated cells were fixed in methanol and stained with 0.5% crystal violet. Migrated cells in 6 randomly selected fields were counted and photographed under a light microscope. Invasion assay was conducted as previously described.[34] Briefly, the upper surface of the transwell membrane was coated with serum-free diluted Matrigel (1:1, 60 $\mu$L/well, BD Biosciences). After Matrigel polymerization, the bottom chambers were filled with 600 $\mu$L of medium containing 10% FBS. HepG2 cells ($1 \times 10^5$) in 100 $\mu$L of serum-free medium were added in the upper part of each transwell and treated with 0.1% DMSO or different concentrations of compound **4b**. After incubation for 24 h, nonmigrated cells on the upper side of the filter were removed and migrated cells were fixed with

4% paraformaldehyde and stained with 0.5% crystal violet; then migrated cells were counted under a light microscope. The percentage inhibition of migrated cells by compound **4b** was expressed using 100% as the value assigned for the control group.

## 3.6. Statistical analysis

Results were represented as mean $\pm$ SD of three independent experiments. Statistical comparisons were made by 2-tailed Student t-tests. Statistically significant P-values were labeled as follows: *P $<$ 0.05, **P $<$ 0.01, ***P $<$ 0.001.

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