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Imide cantharidin derivatives: Synthesis and HBV-DNA inhibitory properties

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ABSTRACT: Cantharidin and its analogs are potent serine/threonine protein phosphatase inhibitors with rare reports on inhibiting expression of Hepatitis B virus (HBV) DNA. Five imide cantharidin derivatives were synthesized, and their properties were studied. The inhibition efficiency was determined, and preliminary studies on the structure-activity relationships were conducted. The results indicated that the compounds characterized by low Log P values and the cantharidin moieties containing nitrogen exhibited low cytotoxicity and helped inhibit the expression of HBV DNA. Compound **2** and the non-cytotoxic compound **3** significantly inhibited the expression of HBV DNA. The IC_{50} values recorded for both compounds were 2.0 ± 1.2 and 19.2 ± 2.0 nM, respectively.

KEYWORDS: imide cantharidin derivatives, anti-HBV, structure-activity relationships

INTRODUCTION

The Chinese *Mylabris* was recognized as a traditional Chinese medicine approximately 2000 years ago [1]. Studies conducted with *Mylabris* revealed that cantharidin was the primary active component present in the system [2]. The biological activities of cantharidin and its derivatives have been widely studied with a focus on the anticancer activity. The effects of cantharidin on liver cancer [3], lung cancer [4], gastric cancer [5], bladder cancer [6], breast cancer [7], colon cancer [8], and cervical cancer [9] have been studied in detail. However, the anti-HBV properties of the compound have been rarely studied.

We have previously isolated the naturally occurring imide cantharidin derivatives from *Mylabris phalerata* Pallas. It was observed that some of the natural compounds exhibited anti-HBV activities. They could be used to detect the secretion of the HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) in the cell culture medium. The cantharimides fall under the class of these natural compounds [10]. The most direct and specific index reflecting HBV infection is HBV DNA [11]. The continuous monitoring of HBV DNA can significantly help in determining the incidence of HBV reactivation. The detection and quantification of HBV DNA present in whole blood collected on dried blood spots (DBS) can potentially facilitate the diagnosis and treatment of HBV infection in resource-poor settings [12, 13]. We aimed to identify the cantharimides that inhibited the expression of HBV DNA. The extraction of cantharidin derivatives from *Mylabis phalerata* Pallas is a time-consuming process, and it was observed that the amount extracted was not enough to determine the activity of the compounds. Based on the results of our previous studies [10], we selected several representative compounds, which were designed and synthesized by us, to study the inhibitory effects of the compounds on the expression of HBV DNA.

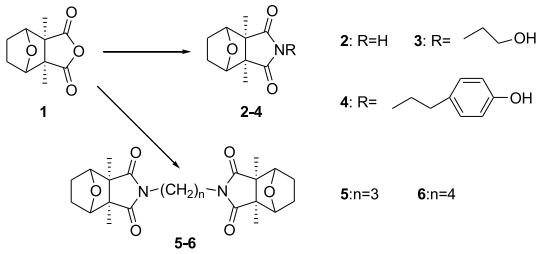

Fig. 1 Synthetic routes for the preparation of imide cantharidin derivatives.

We synthesized the imide cantharidin derivatives **2**–**6** (Fig. 1). Compounds **2**–**6** were prepared by reacting active amines with cantharidin. While compound **1** is devoid of a nitrogen unit, the other cantharidin compounds (**2**–**4**) bear nitrogen units at the anhydride site. The inhibitory effects of the nitrogen-containing compounds on the expression of HBV DNA were determined. Alkyl chains were introduced in compounds **5**–**6** to study the effects of the number of functional rings on the activity of the compounds. The anti-HBV activities of compounds **1**–**6** were tested by quantifying their effects on the expression of HBV DNA. The structure-activity relationship of these compounds was also analyzed.

MATERIALS AND METHODS

Bacteria, plasmids, and chemicals

Compound **1** was prepared following the method described by the references [14, 15]. The HepG2.2.15

cells were provided by Fudan University. Entecavir (ETV) was provided by National Drug Reference Standards of China. All reagents were purchased from companies in Shanghai, China: Merck Chemical Technology Co., Ltd., Sigma-Aldrich Trading Co., Ltd., Sinopharm Chemical Reagent Co., Ltd., and Fluka China general agent. Nuclear magnetic resonance (NMR) data were recorded on an Agilent Technologies 600 MHz DD2 (Santa Clara, CA, USA). Electrospray ionization mass spectrometry (ESI-MS) data were recorded on a Waters Acquity® SQD (Milford, MA, USA).

General procedure for compound 2 [16]

A mixture consisting of cantharidin (0.5 g, 2.5 mmol) and NH_3—H_2O (28–30%, 7 ml) was heated at 60 °C for 6 h. The solvent was evaporated in vacuo, followed by recrystallization using acetone to obtain 0.4 g (80%) of yellow needle solid, mp 203–204 °C; $[\alpha]_D^{27} + 38$ (c 0.1, MeOH); IR (KBr) ν_{max} 3189, 3063, 2985, 1773, 1704, 1471, 1388, 1350, 1306, 1265, 1231, 1122, 993, 960, 924, 896, 840, 734, 540, 464 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.2 (s, 6H), 1.7 (m, 4H), 4.60 (s, 2H), 8.6 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.5, 23.6, 55.2, 83.5, 181.8. ESI–MS: 218 $[M+Na]^+$.

General procedure for compound 3 [17]

To a solution of cantharidin (0.5 g, 2.5 mmol)) in 10 ml of CH_3OH, aminoethanol (0.18 g, 3.0 mmol) was added while stirring, and the mixture was refluxed for 3 h. The temperature of the mixture was brought down to room temperature, following which it was placed in an ice bath. Under these conditions, crystals were formed. The crystals were collected by filtering and washed using ethanol to afford 0.49 g (70%) of compound **3**, yellow needle solid, mp 187–188 °C; [α]27 D+38 (c 0.1, MeOH); IR (KBr) ν_{max}. 3453, 2981, 2932, 1770, 1697, 1435, 1407, 1339, 1268, 1234, 1208, 1153, 1061, 996, 961, 927, 899, 856, 554 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ ppm:1.2 (s, 6H), 1.6 (m, 2H), 1.9 (t, J=6 Hz, 2H), 3.6 (m, 4H), 4.5 (s, 2H), 4.8 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ ppm: 12.6, 24.5, 42.2, 55.2, 59.3, 85.1, 183.4. FT–MS m/z 240.1236 $[M+H]^+$.

General procedure for compound 4 [18]

A mixture consisting of cantharidin (0.5 g, 2.5 mmol), toluene (10 ml), and 4-(2-aminoethyl)-2-ethoxyphenol hydrochloride (2.2 mmol) was refluxed for 8 h. The solvent was evaporated in vacuo, followed by recrystallization using a solvent mixture of acetone-methanol to obtain 0.66 g (84%) of the colorless solid, mp 270–271 °C; ^1H NMR (400 MHz, CD_3OD) δ ppm: 1.0 (s, 6H), 1.6 (m, 4H), 2.8 (t, J=8 Hz, 2H), 4.4 (m, 2H), 4.9 (s, 2H), 6.7 (m, 2H), 6.9 (d, J=4 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ ppm: 12.6, 24.5, 33.2, 41.1, 55.2, 84.9, 116.2, 129.8, 131.1, 157.2, 183.2. HRMS: 316.1543 $[M+H]^+$.

General procedure for compounds 5 and 6 [19]

To a mixture consisting of cantharidin (0.5 g, 2.5 mmol) and C_2H_5OH (10 ml), binary amine (1.2 mmol) was added, and the mixture was refluxed for 5 h. The solvent was evaporated in vacuo, followed by recrystallization using a solvent mixture of ethyl acetate-acetone to obtain the desired products.

Characterization data obtained for compound **5** (0.43 g; colorless needle solid, mp 263–264 °C; yield=80%); ^1H NMR (400 MHz, CD_3OD) δ 1.2 (s, 12H), 1.6 (m, 4H), 1.9 (m, 4H), 3.4 (t, J=8, 4H), 4.5 (m, 4H), 4.8 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 12.6, 24.6, 26.4, 37.1, 55.3, 85.1, 183.3. ESI–MS: 453.3$[M+Na]^+$.

Characterization data obtained for compound **6** (colorless needle solid, mp 268–269 °C; yield=82%): ^1H NMR (400 MHz, $CDCl_3$) δ 1.1 (s, 12H), 1.55 (m, 4H), 1.61 (m, 4H), 1.8 (d, J=4 Hz, 4H), 3.5 (s, 4H), 4.5 (s, 4H); ^{13}C (100 MHz, $CDCl_3$) δ ppm: 12.6, 23.7, 24.4, 38.3, 53.8, 83.5, 181.4. ESI–MS m/z 445.2 $[M+H]^+$.

Anti-HBV activities

The anti-HBV activities of compounds **1**–**6** were studied *in vitro* using the HepG 2.2.15 cells. The studied were conducted following previously reported protocols [20]. Entecavir (ETV101248-201804) was dissolved in DMSO, and the solution was diluted to produce solutions of various concentrations (0.19, 0.75, and 3 nmol/l). The diluted solutions were used as the positive controls.

Cytotoxicity assay

The *in vitro* cytotoxicity of the different concentrations of compounds **1**–**6** was assessed by conducting a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The HepG2.2.15 cells were incubated for 48 h in 96-well plates at 37 °C in an incubator containing 5% CO_2. After 48 h, the cells were treated with different concentrations of the compounds (12.5, 25, 50, 100, and 200 μg/ml). Following this, the cells were cultured over a period of 9 days at 37 °C in an incubator containing 5% CO_2. The culture was refreshed every 3 days. Subsequently, 20 μl of the MTT solution (5 mg/ml) (Shanghai Aladdin Biochemical Technology Co., Ltd., China) was added to each well, and the cells were incubated for another 4 h. The culture medium was replaced with DMSO (200 μl/well), and the cells were incubated at 37 °C for 10 more minutes. Following this, the solution was mixed thoroughly using a pipette and transferred to 96-well plates (density: 100 μl per well). The absorbance was measured at 490 nm using the iMark™

microplate Absorbance Reader (Bio-Rad, Inc., USA). Each experiment was repeated 3 times.

Quantification of HBV DNA

The HepG2.2.15 cells were treated with the compounds and ETV over a period of 9 days following the procedure described above. On day 9, the supernatants were collected and lysed to conduct the intracellular HBV DNA analyses. HBV DNA was extracted from the supernatants of the culture using the HBV fluorescence quantitative PCR diagnostic kit (DaAn Gene Corp. of Sun Yat-sen University, China) to isolate HBV DNA from the HepG2.2.15 cells. The guidelines outlined by the manufacturer were followed. The amount of HBV DNA under each condition was determined using the real-time PCR technique using an icycler (Bio-Rad). The amplification primers used were HBV FP (5'-ATCCTGCTGCTATGCCTCATCTT-3') and HBV RP (5'-ACAGTGGGGGAAAGCCCTA-CGAA T-3'). FAM-5'-TGGCTAGTTTAC-TAGTGCCATTTG-3'-TAMRA was used as the TaqMan probe. The reaction tube was heated to 93 °C, and the temperature was maintained for 2 min for pre-denaturation and further maintained for 45 s. Subsequently, the temperature was brought down to 55 °C, and maintained there for 60 s. These temperature conditions were maintained for the first 10 cycles. In the next 30 cycles, the samples were heated to 93 °C, and the temperature was maintained for 30 s. Subsequently, the temperature was brought down to 55 °C, and was maintained for 45 s. The copies of HBV DNA were obtained from the HepG2.2.15 cells, and the Ct value and standard curves were analyzed.

RESULTS AND DISCUSSION

In the previous work [10], it was observed that some of the natural cantharimides exhibited anti-HBV activities and the compounds could be used to detect the secretion of the HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) in the cell culture medium. In this study, we synthesized the imide cantharidin derivatives. The anti-HBV activities of the imide cantharidin derivatives were tested by quantifying their effects on the expression of HBV DNA, and the structure-activity relationship of these compounds was also analyzed. In short, current study has made up for the deficiencies of previous work [10].

Inhibition assay

HBV is a partially double-stranded genomic DNA virus belonging to the Hepadnaviridae family [21]. The cytotoxicity of the synthetic compounds **2**–**6** was evaluated, and the anti-HBV activities were determined *in vitro* using the HepG2.2.15 cell line. ETV, a frequently used clinical anti-HBV agent, was used as the positive control. The results revealed that compound **1** exhibited strong cytotoxicity against the HepG 2.2.15 cells (Table 1) and exhibited low activity against HBV *in vitro*. Compound **2** significantly inhibited the growth of the HepG 2.2.15 cells and exhibited anti-HBV activity, hindering the expression of HBV DNA ($IC_{50} = 2.0 \pm 1.2$ nM). Compound **3** was less cytotoxic than the other compounds against the HepG 2.2.15 cells. It exhibited expression of HBV DNA ($IC_{50} = 19.2 \pm 2.0$ nM). The other compounds did not exhibit significant activities.

Table 1 *In vitro* anti-HBV activities of the synthesized compounds (**1**–**6**)[e].

Entry	Log P[a]	CC_{50} (nM)[b]	IC_{50} (nM)[c]
		HepG 2.2.15	HBV-DNA
1	0.98	0.8 ± 1.2	NC
2	0.30	38.9 ± 2.4	2.0 ± 1.2
3	0.02	NC	19.2 ± 2.0
4	2.16	71 ± 1.7	66 ± 1.5
5	0.93	NC	NC
6	1.39	NC	NC
ETV[d]	–	NC	1.1 ± 0.1

[a] Log P quoted from ChemicalDraw; [b] CC_{50}: concentration inducing a 50% reduction in host cell viability; [c] IC_{50}: concentration inducing a 50% inhibition in HBVâĂŞDNA release; [d] ETV: Entecavir, an antiviral agent used as a positive control; [e] data were expressed as mean ± standard deviation (S.D., $n = 3$); NC: if inhibitory activity < 40%, IC_{50} value not calculated.

Structure-activity relationships

The cantharidin analogs and their structure-activity relationships were studied [22, 23]. The compounds that inhibit the Ser/Thr protein phosphatases were used as the evaluation indices [24–26]. Herein, the inhibition of the expression of HBV DNA was used as the evaluation index.

Effects of nitrogen on the inhibition efficiency

The introduction of nitrogen had a significant effect on the activity of the cantharidin derivatives [27]. It was observed that cantharidin did not inhibit the replication of HBV DNA (Table 1). However, nitrogen-containing compounds **2**–**4** could inhibit the replication of HBV DNA. It was also observed that the introduction of nitrogen could reduce the toxicity of cantharidin. The cytotoxicity of compounds **2**–**6** against the HepG 2.2.15 cells was lower than that of cantharidin. Cantharidin exhibited strong cytotoxicity against the HepG 2.2.15 cells ($CC_{50} = 0.8 \pm 1.2$ nM).

Effects on the activity of compounds by different substituents on the nitrogen atom

In Fig. 1, the substituents on the nitrogen atoms in compounds **2** → **3** → **4** were varied as N–H → N–C_2H_4OH → N–C_2H_4–p**–Ph–OH (** indicates that the substituent is in the para position). Under these conditions, the IC_{50} values recorded for the

inhibition of the expression of HBV DNA varied as 2.0 ± 1.2 nM $\rightarrow$ 19.2 ± 2.0 nM $\rightarrow$ 66 ± 1.5 nM. Thus, the $-C_2H_4OH$ and $-C_2H_4-P-ph-OH$ substituents present on the nitrogen atom can reduce the anti-HBV activities of the compounds (activity changes are similar to those in reference [27].) The benzene ring present in compound **4** was more lipophilic than that in compound **3**. Thus, compounds **3** and **4** differed in their activities.

Effects on the activity of compounds by binary alkyl chain cantharides

Compounds **5** and **6** bear binary alkyl chains (Fig. 1). It was observed that the abilities of these compounds to inhibit the expression of HBV DNA were significantly lower than that of compound **2**. It can be stated that compounds **5** and **6** rarely exhibit the HBV-DNA inhibition property. The reason behind this is not fully understood.

Effects on the activity of compounds by the Log *P*

It was observed that the compounds (such as **2** and **3**) characterized by Log P values < 0.5 could inhibit the expression of HBV DNA, and the compounds with Log P values > 0.5 rarely exhibited the HBV-DNA inhibition ability (exception: compound **4**). For instance, compounds **2** and **3** (Log P $= 0.30$ and 0.02, respectively) exhibited anti-HBV activities, but compounds **1**, **5**, and **6** (Log P $= 0.98$, 0.93, and 1.39, respectively) did not.

CONCLUSION

We successfully synthesized the imide cantharidin derivatives **2–6**. Results from inhibition studies revealed that compound **2** exhibited significant anti-HBV activity and inhibited the expression of HBV DNA (IC_{50} $= 2.0 \pm 1.2$ nM). Compound **3** could effectively inhibit the replication of HBV DNA in HepG2.2.15 cells. It was also observed that the compound did not exhibit cell cytotoxicity. Preliminary studies were conducted to understand the structure-activity relationships of the compounds. The results revealed that the anti-HBV activities of the imide cantharidin derivatives characterized by low Log P values were significantly higher than those of the compounds characterized by high Log P values. The introduction of nitrogen could significantly inhibit the expression of HBV DNA and reduce the cytotoxicity of cantharidin. The presence of the $-C_2H_4OH$ and $-C_2H_4-p-Ph-OH$ groups on the nitrogen atom could reduce the anti-HBV activity of the compounds. The cantharides with binary alkyl chains rarely inhibited the expression of HBV DNA, and the reason is not fully understood. The results presented herein can potentially help design compounds with anti-HBV properties.

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