B5H7, a Morpholine Derivative of 23-Hydroxybetulinic Acid, Reverses Doxorubicin Resistance in HepG2/ADM

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Abstract: Multidrug resistance (MDR) is the major cause of the failure of cancer chemotherapy. Development of MDR reversers is an important strategy to improve the efficacy of cancer chemotherapy. Here, we have found a morpholine derivative of 23-hydroxybetulinic acid, B5H7, with a reversal effect on MDR cancer cells. Our studies showed that B5H7 enhanced cytotoxicity of doxorubicin, but no cisplatin in MDR cancer cells HepG2/ADM. And we found that B5H7 not only increased the intracellular accumulation of P-glycoprotein substrates doxorubicin and rhodamine123, but also reduced the efflux of rhodamine123 in HepG2/ADM cells. Further studies showed B5H7 did not alter the protein level of P-glycoprotein and it also had no effect on P-glycoprotein ATPase activity. Taken together, we have found that B5H7 could reverse doxorubicin resistance in HepG2/ADM cells by inhibiting the transport function of P-glycoprotein. These findings contribute to developing B5H7 as an adjuvant to anticancer chemotherapy with doxorubicin.

Keywords: Doxorubicin resistance, ABC transporter, P-glycoprotein, 23-HBA derivative B5H7, HepG2/ADM.

INTRODUCTION

Chemotherapy is considered to be an effective way to treat human malignancy, but multidrug resistance (MDR) remains an essential impediment to chemotherapy. Molecular mechanisms of MDR have been studied for several decades. ATP-binding cassette transporters (ABC transporters), the most important "efflux pumps" frequently over-expressed in MDR cancer cells, are considered to be the major factors to drug resistance [1]. P-glycoprotein (P-gp), encoded by multidrug resistance protein 1 (*MDR1*) or ATP-binding cassette sub-family B member 1 (*ABCB1*) gene, is a glycoprotein associated with multidrug resistance [2]. P-gp can pump out a wide range of anti-cancer drugs which are structurally unrelated, leading to MDR. Thus, resensitizing MDR cancer cells to chemotherapeutics by inhibiting P-gp is the most effective strategy to overcome MDR [3]. Though many known P-gp modulators are selective and potent *in vitro* and *in vivo*, many difficulties occurred in pre-clinic or clinic trials due to their toxicity and side effects [4]. Up to now, none of P-gp modulators have been approved by FDA as an adjuvant of chemotherapy. Thus, searching for novel drugs resistance reversal agents remains to be urgent and important.

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23-hydroxybetulinic acid (23-HBA), a lupane-type pentacyclic triterpene isolated from Chinese medicinal herb *Pulsatilla chinensis*, exhibited potent antitumor activity *in vitro* and *in vivo* [5,6]. However, the properties of low bioactivity and poor water solubility greatly limited its clinical implications. We have developed a series of derivatives by structural modification, which exhibited potential anti-cancer activity and remarkably improved pharmacokinetic properties [7]. In the previous studies, we found that three bipiperidinyl derivatives of 23-HBA (BBA, DABB and DHBB) which are not the transported substrates of P-gp, could markedly reverse P-gp-mediated MDR by binding to P-gp and modulating its ATPase activity [8,9]. At the present studies, we found a morpholine derivative of 23-HBA, B5H7, could also enhance the sensitivity of doxorubicin (DOX) in HepG2/ADM cells, and explored the underlying mechanisms.

MATERIALS AND METHODS

Materials

B5H7 (purity>98%, Figure **1A**) was synthesized as described previously [7]. It was dissolved in DMSO to give a stock solution of 20 mM and stored at -20 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 123 (Rhm-123), DOX and cisplatin were purchased from Sigma Aldrich (St. Louis, MO, USA). Recombinant human P-gp membranes were purchased from BD (Franklin Lakes, NJ, USA). P-gp-Glo™ assay kit was purchased from Promega

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(Fitchburg, Wisconsin, USA). Anti-P-gp antibody and verapamil (VRP) were acquired from Merck Calbiochem (Darmstadt, Germany). Anti-β-actin antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Other chemical reagents were purchased from Sigma Aldrich.

Cell Lines and Cell Culture

Human hepatocellular carcinoma cell line HepG2 and its parental multidrug resistant HepG2/ADM cell line were kindly provided by Prof. Kowk-Pui Fung (The Chinese University of Hong Kong, Hong Kong). P-gp is overexpressed in HepG2/ADM cells which are developed from HepG2 cells treated with step-by-step increasing DOX concentration. HepG2 cells were maintained in RPMI-1640 containing 10% NBS and 1% penicillin streptomycin in a humidified incubator with 5% CO_2 at 37 °C. HepG2/ADM cells were maintained in RPMI-1640 with 10% FBS and 1% penicillin streptomycin containing DOX (1.2 μM) to keep the characteristic of MDR.

MTT Assay

Cells were plated in 96-well plates at a density of 5000 cells/well and incubated for about 24 h. To detect the cytotoxicity of B5H7, cells were incubated with different concentrations of B5H7 for 72 h. The concentrations, at which B5H7 should be non-toxic to HepG2 and HepG2/ADM cells, were used in reversal experiments. To detect the reversal effect of B5H7, B5H7 in combination with DOX or cisplatin was added and incubated for 72 h. After that, cells were cultured with MTT (5 mg/mL) for another 4 h. The produced formazon was dissolved in DMSO and the optical density was measured at 595 nm using DTX 880 microplate reader (Beckman Coulter, USA).

DOX Accumulation

Intracellular accumulation of DOX was evaluated by flow cytometry as previous described [8]. Briefly, HepG2 and HepG2/ADM cells were incubated with B5H7 (2 μM and 4 μM) and VRP (5 μM) for 2 h at 37 °C, then DOX (5 μM) was added to the medium and cultured for another 2 h. After being washed with the ice-cold PBS buffer for three times, cells were resuspended in the PBS buffer and the fluorescence migration was measured by a Epics XL flow cytometry (Beckman Coulter) at excitation wavelength 488 nm and emission wavelength 590 nm.

Rhm-123 Retention

HepG2/ADM cells were treated with various concentrations of B5H7 and VRP (5 μM) for 2 h at 37 °C and then cultured with Rhm-123 (10 μM) for another 1 h. After washing with the ice-cold PBS buffer, the green fluorescence of Rhm-123 was observed by a KX41 fluorescence microscope (Olympus, Japan) with excitation wavelength 488 nm and emission wavelength 535 nm.

Rhm-123 Efflux Assay

The effect of B5H7 on the intracellular efflux of Rhm-123 in HepG2/ADM cells was determined as previously described [8]. Cells (1×10^4 cells/well) were plated in black wall 96-well plates overnight, after being preincubated with medium-only (control), B5H7 (4 μM) and VRP (5 μM) at 37 °C for 2 h, Rhm-123 (5 μM) was added then incubated for another 2 h. Cells were washed three times with medium at various time points (0, 15, 30, 60, 120 min). The fluorescence intensity of intracellular Rhm-123 was measured by a DTX 880 microplate reader at excitation wavelength 488 nm and emission wavelength 535 nm.

Western Blot Analysis

Western blot analysis was used to detect P-gp expression in HepG2 and HepG2/ADM cells after B5H7 treatment for 72 h. HepG2/ADM cells with or without B5H7 treatment were lysed and the protein concentrations were quantified using a BCA protein assay kit. 50 μg of protein was separated by 10% SDS-PAGE and transferred to PVDF membranes. After being blocked with 5% non-fat milk, the membranes were probed overnight with primary antibodies (P-gp antibody and β-actin antibody) at 4 ^{o}C and incubated with secondary rabbit antibodies for 1 h at room temperature. At last, the membranes were performed using the enhanced chemiluminescence kit (Thermo Scientific, USA) and imaged on the photographic film (Kodak, USA).

P-gp ATPase Activity Assay

Pgp-Glo™ ATPase Assay Kit was used to detect the influence of B5H7 in P-gp ATPase activity according to the manufacturer's protocol. Firstly, the diluted ABCB1 membranes (1.25 mg/mL) were incubated in Glo™ ATPase assay buffer containing 0.25 mM Na_3VO_4, 0.5 mM VRP and various concentrations of B5H7 for 5 min at 37 °C. Then 25 mM MgATP was added and incubated for 40 min at 37

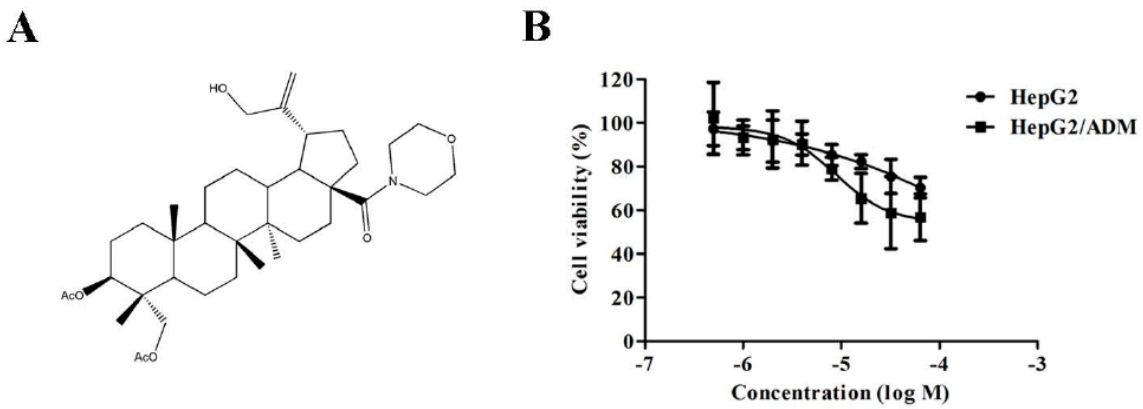

Figure 1: (**A**) Chemical structure of B5H7. B5H7 is a derivative of 23-Hydroxybetulinic acid with a morpholine at C28 site. (**B**) Cytotoxicity of B5H7 on HepG2 and HepG2/ADM cells. HepG2 and HepG2/ADM cells were treated with different concentrations (0, 1, 2, 4, 8 μM) of B5H7 for 72 h. Cell viability was measured by MTT assay. Data were shown as means ± SD of three independent experiments performed in at least triplicate.

°C. Finally, the reaction was stopped by addition of ATP detection reagent. After incubation at room temperature for 20 min, the luminescence was measured using a DTX 880 microplate luminometer.

Statistical Analysis

Each experiment was performed for at least three times and the results were shown as the mean ± standard deviation (S.D.). Graphpad Prism 4.0 was used for statistical analysis. A difference was considered significant when $P < 0.05$.

RESULTS

B5H7 Enhanced Cytotoxicity of DOX on HepG2/ADM Cells

Given that non-toxic concentrations of reversers need to be fixed before drug resistance reversal assay, we firstly determined the cytotoxicity of B5H7 in HepG2 and HepG2/ADM cells. As shown in Figure **1B**, the subcytotoxic concentration of B5H7 with 90% of cell survival was calculated to be 4 μM for HepG2 and HepG2/ADM cells. Therefore, concentrations of 2 μM and 4 μM were adopted in the following experiments to explore the resistance-reversal activity of B5H7 and underlying mechanisms. Reversal fold calculated by dividing the IC_{50} of cells in the absence of the reversers by that in the presence of the reversers is a most important index to value the reversal activity of MDR reversers *in vitro* [10]. Our results showed the obvious resistance of HepG2/ADM cells to DOX indicated by the IC_{50} value of 85.67 μM compared with HepG2 cells with the IC_{50} value of 0.20 μM. Interestingly, B5H7 at 2 μM and 4 μM significantly enhanced the cytotoxicity of DOX, a substrate of P-gp, in HepG2/ADM cells, with reversal fold of 3.70 and 95.11, respectively (Figure **2A** and Table **1**). But B5H7 did not alter the sensitivity of parental cells HepG2 to DOX, ruling out their synergetic effects. In addition, B5H7 (4 μM) had a better reversal effect on DOX resistance than a positive control, VRP (5 μM). On the contrary, B5H7 treatment had no effects on IC_{50} of cisplatin, a non-substrate of P-gp, in both HepG2 and HepG2/ADM cells (Figure **2B**). These results indicate that the reversal effect of B5H7 on DOX resistance may be associated with P-gp.

B5H7 Induced DOX Accumulation in HepG2/ADM Cells

As HepG2/ADM cells with overexpression of P-gp which can pump DOX out of cells, there is much lower concentration of DOX in HepG2/ADM cells than HepG2 cells [9]. The increase of the intracellular drug concentration is one of the common mechanisms of reversers to overcome ABC transporter-dependent drug resistance. Next, we detected whether B5H7 enhanced the cytotoxicity of DOX in HepG2/ADM cells by triggering the intracellular DOX accumulation. Flow cytometry analysis showed that B5H7 enhanced the intracellular level of DOX in HepG2/ADM cells in a dose-dependent manner, but not in HepG2 cells, and had a better effect than VRP (Figure **3**).

B5H7 Triggered the Retention of Rhm-123 and Inhibited its Efflux in HepG2/ADM Cells

In order to investigate whether B5H7-enhanced DOX accumulation is associated with inhibition of P-gp function, we used Rhm-123, a fluorescent substrate of P-gp, to evaluate the effect of B5H7 on P-gp transport activity. Enhanced the retention of Rhm-123 is now considered to be a surrogate marker for P-gp inhibitors

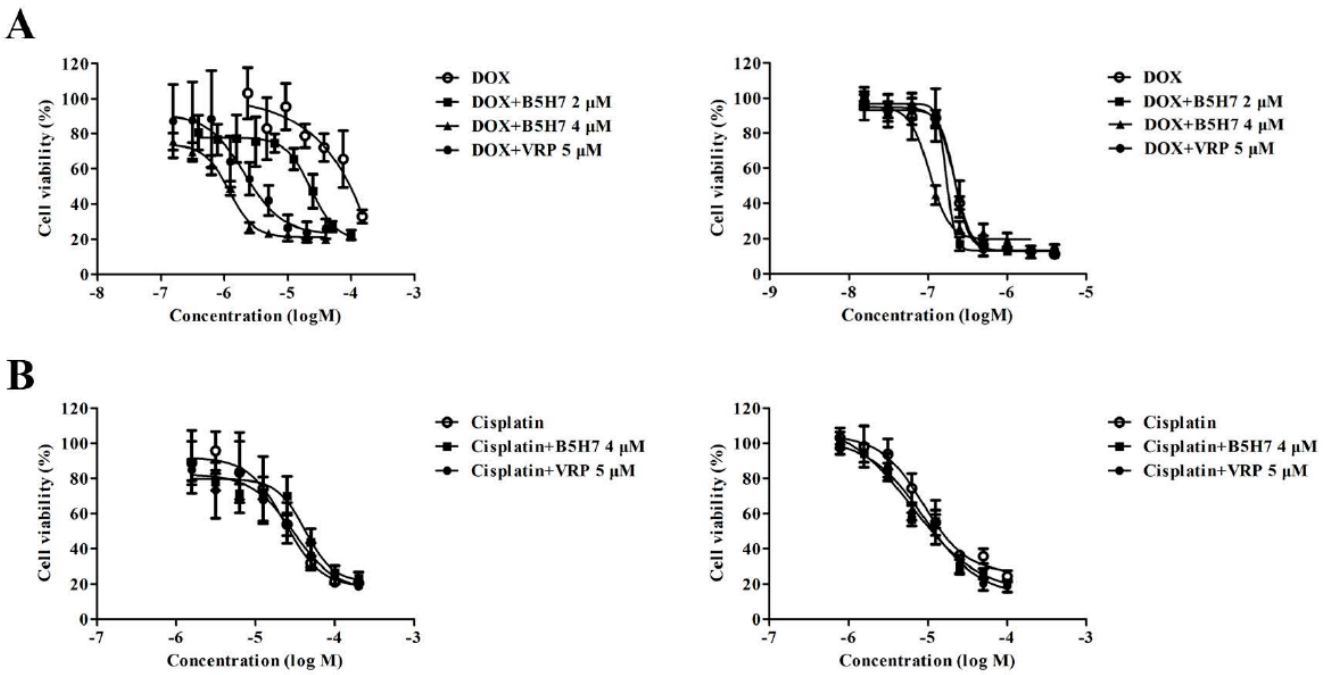

Figure 2: Drug resistance reversal effect of B5H7 on HepG2 and HepG2/ADM cells. (**A**) Effect of B5H7 on cytotoxicity of DOX to HepG2/ADM (left) and HepG2 cells (right). (**B**) Effect of B5H7 on cytotoxicity of cisplatin to HepG2/ADM (left) and HepG2 cells (right). VRP was used as a positive control. Data were shown as means ± SD of three independent experiments performed in at least triplicate.

Table 1: Reversal Effect of B5H7 in HepG2/ADM and HepG2 Cells

	IC_{50} ± SD[a] (μM) (fold-reversal)	
	HepG2/ADM	**HepG2**
DOX	85.6739 ± 17.7758 (1.00)	0.2023 ± 0.0234 (1.00)
+ B5H7 2 μM	23.1278 ± 5.4502 (3.70)	0.1847 ± 0.0039 (1.10)
+ B5H7 4 μM	0.9008 ± 0.1040 (95.11)	0.1862 ± 0.0224 (1.09)
+VRP[b] 5 μM	1.0292 ± 0.1103 (83.24)	0.2095 ± 0.0137 (0.97)
Cisplatin	29.2311 ± 4.6118 (1.00)	15.0727 ± 2.4028 (1.00)
+ B5H7 4 μM	26.9317 ± 3.3243 (1.09)	14.4337 ± 0.7576 (1.04)
+VRP 5 μM	29.8162 ± 6.5874 (0.98)	13.6663 ± 0.4438 (1.10)

[a]Data in the table are shown as means ± SD of at least three independent experiments performed in six replicates.
[b]VRP was used as positive control.

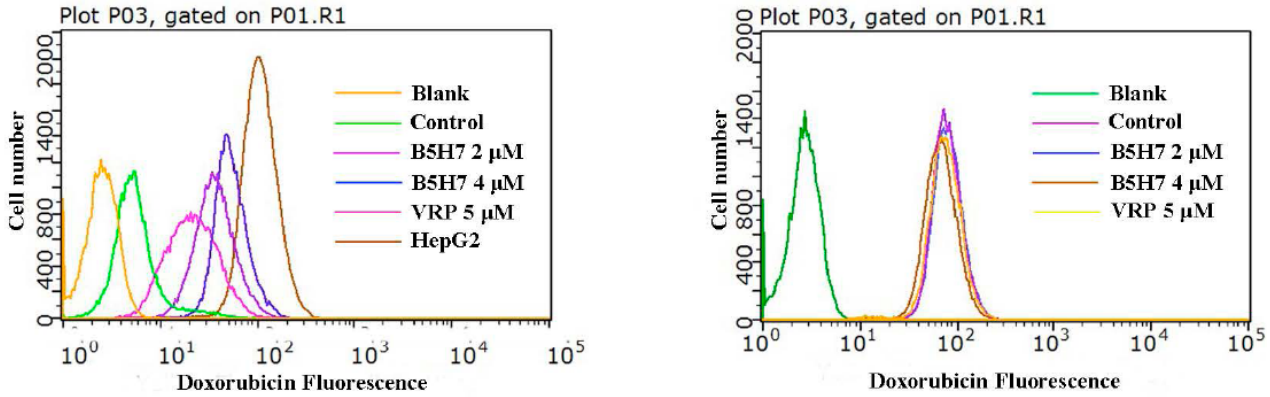

Figure 3: Effect of B5H7 on the accumulation of DOX in HepG2/ADM (left) and HepG2 cells (right) detected by flow cytometry. Cells after B5H7 treatment were incubated with DOX (10 μM) for 2 h in darkness at 37 °C, and then the intracellular accumulation of DOX was measured.

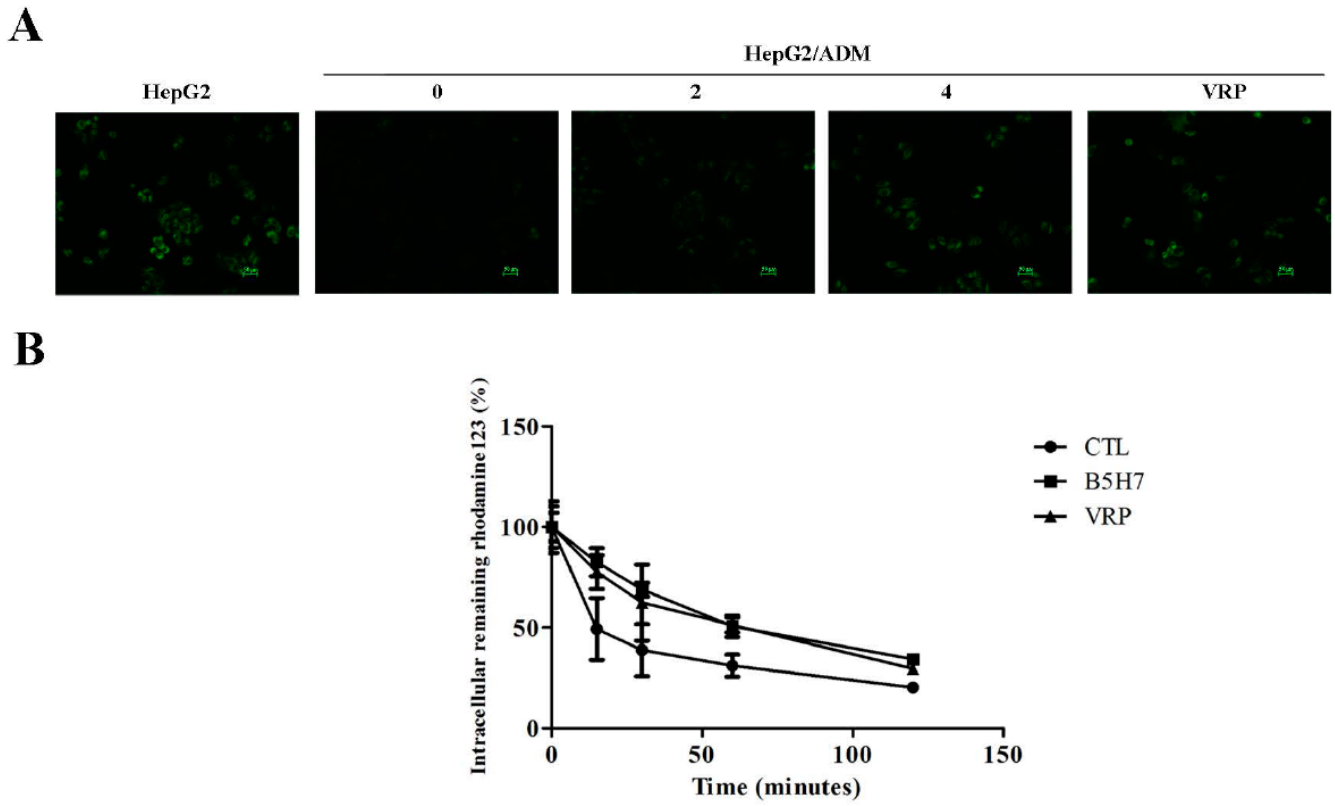

Figure 4: (**A**) Effect of B5H7 on the accumulation of rhodamine123 in HepG2 and HepG2/ADM. Cells after B5H7 treatment were incubated with rhodamine123 (10 μM) for 1 h in darkness at 37 °C, and then the intracellular accumulation of rhodamine123 was measured by a fluorescence microscope. Original amplification, 20; scale bar, 50 μm. (**B**) Effect of B5H7 on the efflux of rhodamine123 measured by a fluorescence microplate reader. Cells after B5H7 treatment were incubated with rhodamine123 (5 μM) for 2 h. After washing three times with medium at various time points (0, 15, 30, 60, 120 min), the fluorescence of intracellular rhodamine123 was measured. Each point represents the mean ± SD of three independent experiments performed in at least triplicate.

[11]. In accordance with DOX accumulation assay, B5H7 also significantly enhanced intracellular retention of Rhm-123, indicated by the stronger green fluorescence intensity of Rhm-123 in B5H7-treated HepG2/ADM cells than that of control (Figure **4A**). To better understand the direct effect of B5H7 on the efflux of drugs, we detected Rhm-123 efflux in HepG2/ADM cells treated with B5H7 or VRP for 2 h and then washed at 15, 30, 60 and 120 min, respectively. As shown in Figure **4B**, B5H7 could also attenuate the efflux of Rhm-123, just as VRP. These data suggest that B5H7 directly inhibits the drug efflux function of P-gp, resulting in the increase of the intracellular accumulation of DOX and Rhm-123.

P-gp Expression was Not Altered by B5H7

In general, inhibition of P-gp-mediated transport of anticancer drugs can be achieved either by reducing P-gp expression or by modulating P-gp activity [12]. We therefore measured P-gp expression level in HepG2 and HepG2/ADM cells in the presence or absence of B5H7 by western blot. Our results showed that the protein level of P-gp in HepG2 and HepG2/ADM cells was not altered by B5H7 treatment for 72 h (Figure **5A**), indicating that B5H7 enhanced the intracellular accumulation of DOX and its cytotoxicity in HepG2/ADM cells possibly through modulating P-gp ATPase activity.

B5H7 did Not Affect P-gp ATPase Activity

P-gp ATPase assay has been processed to directly reflect the interaction manner between P-gp and reversal agent. Most P-gp modulators, such as VRP and sildenafil, can stimulate P-gp ATPase activity of [13,14], whereas a small part of modulators like DABB and DHBB inhibit P-gp ATPase activity [9]. The results of P-gp ATPase assay showed that B5H7 did not alter the P-gp ATPase activity even at the concentrations of 0.4 μM, 4 μM and 40 μM (Figure **5B**), which was different from both ATPase stimulant VRP and inhibitor Na_3VO_4, suggesting that B5H7 is neither a competitive substrate of P-gp nor a inhibitor of P-gp ATPase.

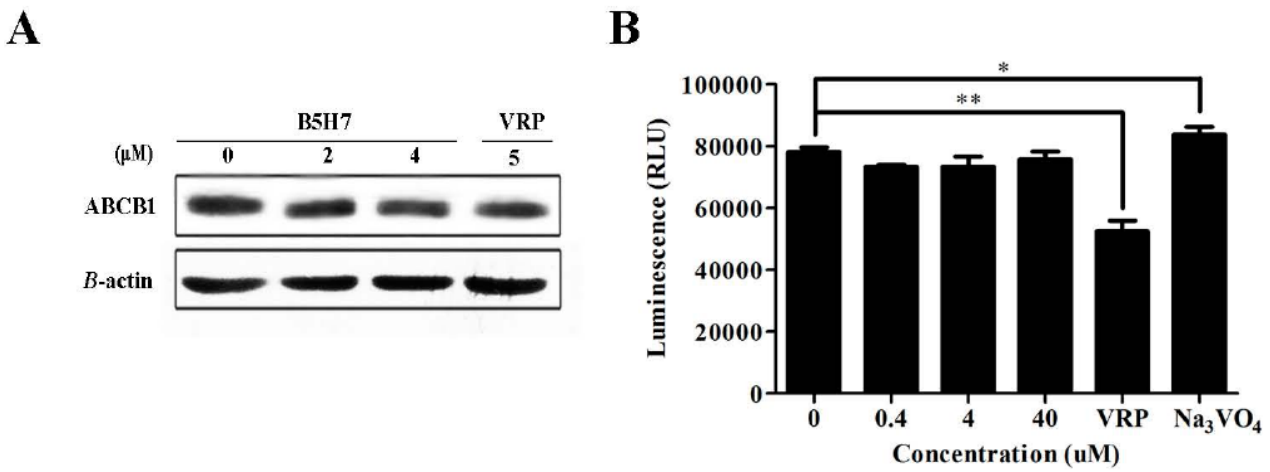

Figure 5: (**A**) Effect of B5H7 on protein level of P-gp in HepG2/ADM cells detected by western bolt. β-actin was used as a internal control. (**B**) Effect of B5H7 on the ATPase activity of P-gp was detected by Pgp-Glo™ ATPase assay kit using a fluorescence microplate reader. Na_3VO_4 was used as an inhibitor of ATPase and verapamil was used as a stimulatant. Three independent experiments were performed and the representative results were shown. Each point represents the mean ± SD of at least three replicates. * $P < 0.05$, ** $P < 0.01$ versus the control.

DISCUSSION

Most clinical studies found that cancer cells gain drug resistance by over-expressing several different types of ABC transporters such as P-gp, MRP1 and BCRP [1]. It has formed a consensus that P-gp plays a leading role in MDR cancer cells. Thus, inhibiting this efflux pump in MDR tumor cells is considered to be one of the most promising and effective approaches to combat MDR [16]. However, it still needs to look for novel MDR modulators because the majority of P-gp inhibitors failed in clinical trials. Natural products and their derivatives have been considered as the major source of chemotherapeutic drugs and MDR modulators [17].

Accumulated evidence shows that 23-HBA has anti-HIV and anti-cancer activities similar to betulinic acid [18]. We have reported a series of 23-HBA derivatives with improved anti-cancer activities previously [7]. Among them, three bipiperidinyl derivatives of 23-HBA could remarkably reverse P-gp-mediated MDR through inhibition of the activity of P-gp and its efflux function [8,9]. B5H7, a morpholine derivative of 23-HBA, exhibited cytotoxic activity in Hela, MCF-7 and HepG2 cells with IC_{50} value of 18.31 μM, 17.32 μM and 20.68 μM, respectively, and its anticancer effect was more potent than 23-HBA. In the present studies, we examined the reversal ability of B5H7 in P-gp-mediated MDR cancer cell line HepG2/ADM. Consistent with our previous studies, HepG2/ADM cells which over-expressed P-gp exhibited 428.36-fold resistance to DOX. B5H7 could significantly increase the sensitivity of DOX in HepG2/ADM cells in a concentration-dependent manner, whereas there was no significant effect on its parental cell line HepG2. Otherwise, B5H7 could not alter the cytotoxicity of cisplatin, which is not a substrate of P-gp, both in HepG2/ADM and HepG2 cells. Those results indicated that B5H7 might reverse P-gp-mediated MDR. One of the direct approaches to explore the mechanisms of reversal agents is to detect the accumulation and pump of P-gp substrates in MDR cells. It was observed that B5H7 enhanced the intracellular DOX and Rhm-123 accumulation in HepG2/ADM cells, but not in HepG2 cells. In addition, the efflux of Rhm-123 was also eliminated by B5H7 treatment. Taken as a whole, reversal activity of B5H7 is maybe obtained by targeting P-gp. Thus, B5H7 was likely to reverse the resistance to other P-gp substrates such as vincristine, paclitaxel and daunorubicin in P-gp-mediated MDR cell lines. Certainly, it still needs to further study whether B5H7 could also reverse the resistance of P-gp-transfected cancer cell lines, and P-gp RNA interference also needs to be conducted to test whether the reversal effect of B5H7 in HepG2/ADM cells is dependent on P-gp. Otherwise, conclusions about whether B5H7 inhibited other ABC transporters such as MRP1 and BCRP await more extensive analysis.

As mentioned earlier, we found three 23-HBA derivatives were potent P-gp inhibitors. BBA, 23-O-(1,4′-Bipiperidine-1-carbonyl) betulinic acid, could remarkably reverse P-gp-mediated MDR cancer cell lines HepG2/ADM, MCF-7/ADR, KB-C2 and HEK293/ABCB1 to several P-gp substrates such as DOX, vincristine and peclitaxel. Further mechanistic studies showed that BBA could affect P-gp ATPase

activity in a bidirectional regulation [8]. The other two bipiperidinyl derivatives of 23-HBA, DABB and DHBB, could also potently sensitize several P-gp substrates to HepG2/ADM and MCF-7/ADR cells by inhibiting the basal and stimulated P-gp ATPase and blocking the efflux function of P-gp [9]. Taken together, it seems that those P-gp modulators inhibited the function of P-gp related to its ATPase activity, instead of the expression of P-gp. Currently, B5H7, as a morpholine derivative of 23-HBA, also did not affect expression of P-gp, like other 23-HBA derivatives. Surprisingly, B5H7 did not alter the basal P-gp ATPase activity at the different concentrations (0.4 μM, 4 μM and 40 μM). These results indicated that the reversal mechanisms of B5H7 might completely differ from other 23-HBA derivatives reported previously, which expends our understanding of the action mechanisms of 23-HBA derivatives. The exact mechanisms of reversal effect of B5H7 remain unknown. Further investigations need to be conducted to reveal the binding sides of B5H7 on P-gp by IAAP photo-affinity label assay, molecular docking analysis and B5H7-P-gp co-crystal analysis. Recent studies indicated P-gp modulators might influence the plasma membrane fluidity, leading to the secondary and tertiary structure damage of P-gp [19]. For example, elemene and cepharanthine inhibited function of P-gp by altering the molecular arrangement of P-gp [20]. Whether B5H7 could also alter the physicochemical properties of P-gp and then affect its transport ability warrants further studies.

In conclusion, this study provide an evidence that B5H7, a morpholine derivative of 23-HBA, may inhibit the efflux function of P-gp, without affecting expression and ATPase activity of P-gp, leading to drug accumulation in HepG2/ADM cells and reverse DOX resistance. These findings contribute to developing B5H7 as an adjuvant to anticancer chemotherapy with doxorubicin.

CONFLICTS OF INTEREST STATEMENT

There are no conflicts of interest.

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