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Synthesis and evaluation of substituted dibenzo[*c,e*]azepine-5-ones as P-glycoprotein-mediated multidrug resistance reversal agents

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ABSTRACT

A series of substituted dibenzo[*c,e*]azepine-5-ones (**7a–h**) were synthesized and evaluated as P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) reversal agents. The most potent compound **7h** could significantly and selectively enhance the chemo-sensitivity of drug-resistant K562/A02 cells to the cytotoxic effect of adriamycin (ADR) in a dose-dependent manner. Further studies indicated that **7h** could markedly increase intracellular accumulation of both rhodamine 123 and ADR in K562/A02 cells and inhibit their efflux from the cells. And **7h** had little effect on the levels of P-gp mRNA and protein in K562/A02 cells. These results suggest that the anti-MDR effect of **7h** might be attributed to the inhibition of drug efflux function of P-gp, leading to the increased drug accumulation in K562/A02 cells, and thus the compound could be served as a lead for developing P-gp-mediated MDR reversal agents.

Multidrug resistance (MDR) of cancer cells impairs efficient treatment and has now become a major hindrance to successful cancer chemotherapy.[1] The mechanisms underlying MDR are very complicated, and one of the most recognized mechanisms attributes to the frequent overexpression of ATP-binding cassette (ABC) transporters on the plasma membrane of cancer cells, such as P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP).[2] P-gp, a 170-kDa transmembrane protein encoded by the MDR1 gene, is now the best characterized drug transporter which can actively efflux a wide range of structurally and functionally related or unrelated anticancer drugs out of cancer cells, thereby decreasing their intracellular levels to sublethal concentration and reducing their therapeutic efficacy.[3,4] A wide variety of investigations have suggested that inhibition of the drug efflux function and/or the overexpression of P-gp is the most important strategy to overcome P-gp-mediated MDR.[5] Thus, considerable attempts have been made to suppress MDR by developing P-gp inhibitors during past decades. And several well-known P-gp inhibitors entered clinical trials, such as Verapamil, Valspoder (PSC 833) and Tariquidar (XR9576).[6] However, up to now, no drug of this class has been marketed for many reasons, such as low selectivity, poor potency, inherent toxicity and/or unpredictable pharmacokinetic interactions with the co-administered anticancer drugs.[6] Therefore, there is a clear and urgent need to develop safe and effective P-gp inhibitors.

Nowadays, researchers have been turning to natural products and their derivatives to identify novel and effective MDR reversal agents by virtue of their advantages, for example, low toxicity, few unfavorable pharmacokinetic interactions and showing anticancer and/or MDR reversal activities, etc.[2,6,7] And a wealth of documents have reported that flavonoids, curcumins, schizandirins and their derivatives possess MDR reversal effects owing to modulating one or more ABC transporters.[8–10] Schizandrins (A, B and C) (Fig. 1) isolated from *Schisandra chinensis* Baill (Wuweizi) have attracted much attention for their potential ability to restore the sensitivity of several drug-resistant cancer cells to anticancer drugs through increasing intracellular accumulation of anticancer drugs, promoting apoptosis, down-regulating P-gp mRNA and protein and total protein kinase C (PKC) expression in MDR cells.[11–13] Dimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylenedioxybiphenyl-2,2′-dicarboxylate (DDB), an analogue of schisandrin C, is an effective liver protective drug, which has been widely used for the treatment of chronic viral hepatitis B for more than 20 years in China with few side effects.[14] It has recently been reported that DDB is also able to reverse P-gp-mediated MDR in vitro and in vivo by

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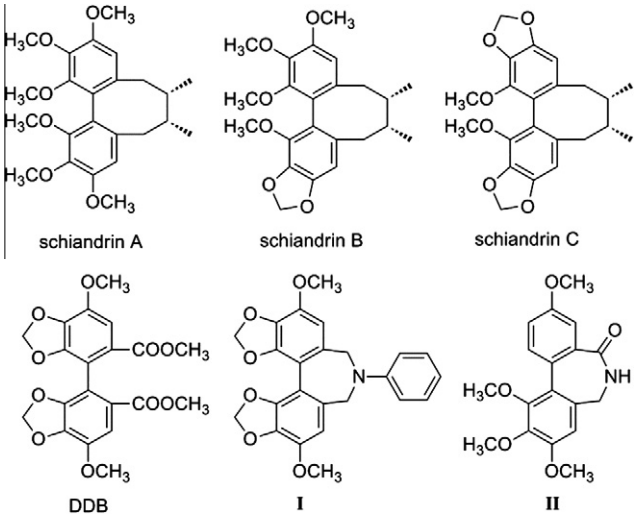
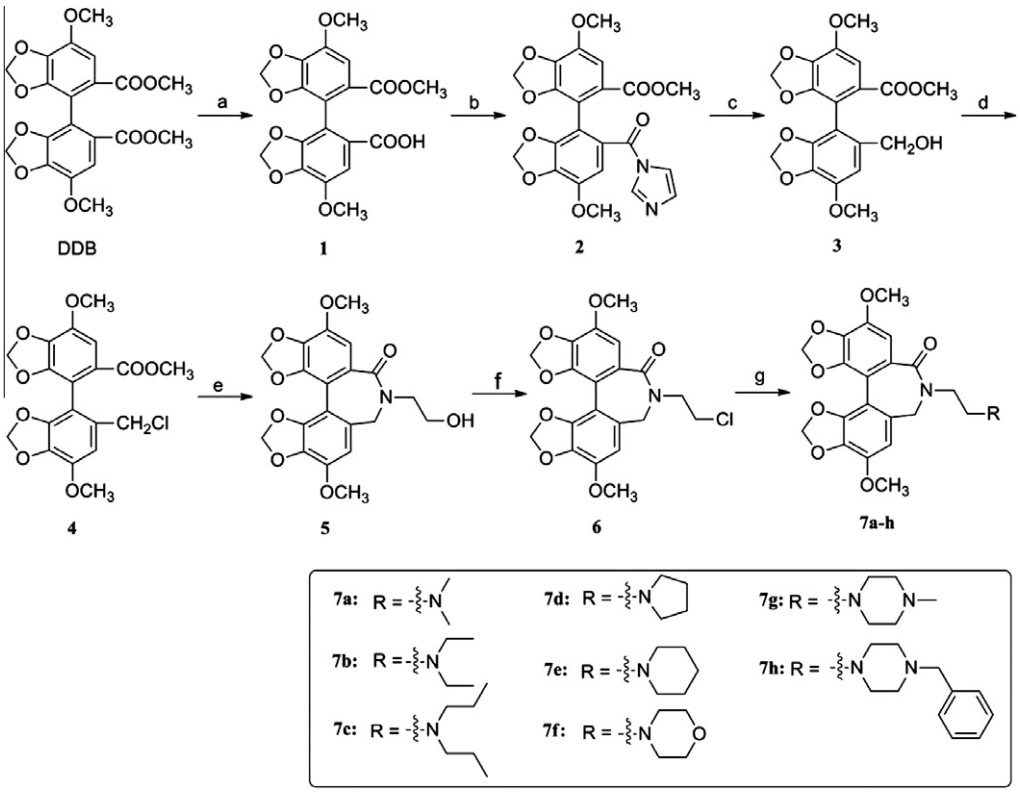


Figure 1. The structures of schiandrins (A, B and C) and their analogs (DDB, **I** and **II**).

increasing intracellular accumulation of anticancer drugs and promoting apoptosis through inhibiting P-gp, and more importantly, there is no pharmacokinetic interaction problem between DDB and co-administered anticancer drugs, at least adriamycin (ADR).[14] However, the reversal activity of DDB is weaker than verapamil (VRP), an established P-gp-mediated MDR reversal agent.[14] Therefore, improving reversal activity of DDB by proper structural modification is of great significance. Previous studies have revealed that the scaffold of alkoxyl biphenyl moiety in DDB is crucial for its pharmacological activities,[15] and when a nitrogen-containing cyclic moiety was hybridized with such scaffold to form dibenzoazepine compounds **I** and **II**, whose biological activities were improved as compared with DDB, probably attributable to the variation of ring strain and basicity.[16–18] Thus, it is of interest to examine whether the dibenzoazepine compounds like **I** and **II** possess improved MDR reversal activity relative to DDB. Additionally, a number of studies have revealed that a tertiary aminoalkyl moiety serves as a basic center which would be charged at physiological pH and has frequently been considered to be the hall mark of P-gp inhibitors.[6,19] Based on the above investigations, we synthesized and biologically evaluated substituted dibenzo[*c*,*e*]azepine-5-ones (**7a–h**) as P-gp-mediated MDR reversal agents.

The synthetic routes of target compounds **7a–h** are outlined in Scheme 1. The monoester compound **1** was prepared starting from diester compound DDB by three steps according to literature.[20] Treatment of **1** with imidazole in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) in CH_2Cl_2 afforded amide **2**, which was then selectively reduced by $NaBH_4$ to give benzylalcohol **3**. Compound **3** was treated with $SOCl_2$ to generate benzyl chloride **4**, which was nucleophilically substituted by ethanolamine and then intramolecularly aminolyzed to result in ring closure, yielding *N*-substituted aminoethanol **5**. Subsequently, the hydroxyl of **5** was chlorinated to chloride **6** which was treated with different secondary amines to provide **7a–h**. Compounds **7a–h** were purified by column chromatography, and their structures were characterized by IR, ^{1}H NMR, ^{13}C NMR, MS, and HRMS.

Firstly, the antiproliferative activity of compounds **7a–h** and DDB against human leukemic K562 cells and its drug-resistant K562/A02 cells was determined in vitro by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-



Scheme 1. Synthesis of compounds **7a–h**. Reagents and conditions: (a) (i) 10% NaOH, reflux, 5 h, 96%; (ii) $(Ac)_2O$, reflux, 8 h, 90%, (iii) CH_3OH, reflux, 3 h, 98%; (b) imidazole, EDCI, DMAP, CH_2Cl_2, rt, 2 h, 95%; (c) THF, $NaBH_4$, 0 °C, 1 h, 90%; (d) $SOCl_2$, 1H-benzotriazole, CH_2Cl_2, 0 °C, 2 h, 98%; (e) ethanolamine, Et_3N, KI, CH_2Cl_2, reflux, 3 h, 86%; (f) $SOCl_2$, CH_2Cl_2, 0 °C, 2 h, 93%; (g) secondary amines, KI, CH_2Cl_2, reflux, 2 h, 84–91%.

Table 1
The antiproliferative activities of compounds **7a–h** and DDB against K562 and K562/A02 cells in vitro by MTS assay

Compound	IC_{50}[a,b] (μM)		Compound	IC_{50}[a,b] (μM)	
	K562	K562/A02		K562	K562/A02
ADR[c]	0.43	46.69	**7d**	>100	>100
DDB	>200	>200	**7e**	89.99	91.08
7a	>100	>100	**7f**	>100	>100
7b	62.44	94.78	**7g**	>100	>100
7c	>100	>100	**7h**	49.19	56.28

[a] The IC_{50} values represent the concentration which results in a 50% decrease in cell growth after 72 h incubation.
[b] Values are the mean values of three experiments.
[c] Adriamycin (ADR) was used as a positive control.

Table 2
The effect of compounds **7a–h** on the intracellular Rh123 accumulation in K562/A02 cells at 10 μM

Compound	FAR value[a]	Compound	FAR value[a]
7a	1.44	**7f**	3.23
7b	2.57	**7 g**	1.03
7c	2.84	**7h**	14.11
7d	1.65	DDB	1.15
7e	3.46	VRP[b]	4.16

[a] The fluorescence activity ratio (FAR) values are the ratios of the mean fluorescent intensities of Rh123 in treated and untreated K562/A02 cells.
[b] VRP was used as a positive control.

tetrazolium (MTS) assay.[21] A well-known anticaner drug adriamycin (ADR) was used as a positive control. It can be seen from Table 1 that K562/A02 cells used in the present experiment displayed much serious drug resistance to ADR than parental K562 cells (IC_{50}: 46.69 vs 0.43 μM), with a resistant fold of about 108, which is reportedly caused by overexpressing P-gp in K562/A02 cells leading to drug-resistance.[22] DDB had little cytotoxicity (IC_{50} >200 μM) to both K562/A02 and K562 cells, which is consistent with previous reports.[14,23] Compounds **7a–h** had little (IC_{50} > 100 μM) or somewhat (IC_{50} = 49.19–94.78 μM) antiproliferative activity to both K562/A02 and K562 cells, suggesting these compounds are suitable candidates for the research and development of MDR reversal agents.

Subsequently, flow cytometric analysis (FACS Calibur, Becton-Dickinson, USA) was employed to evaluate the effect of compounds **7a–h** and DDB on the function of P-gp by testing their effect on rhodamine 123 (Rh123), a fluorescent substrate of P-gp, accumulation in P-gp overexpressed K562/A02 cells. The fluorescence activity ratio (FAR) values, which are the ratios of the mean fluorescent intensities of Rh123 in treated and untreated K562/A02 cells, are positively related with their MDR reversal activity. Since P-gp-mediated MDR is due to the increased efflux of its substrate or a drug from tumor cells, a FAR value larger than 1 represents that the reversal of MDR has taken place and the higher FAR value indicates stronger reversal activity.[24] All compounds **7a–h** were assayed at 10 μM, a concentration could result in less than 10% inhibition in cell growth after 72 h incubation, and the results are presented in Table 2. As we can see, **7a–h** displayed varying levels of MDR reversal activity and their FAR values ranged from 1.03 to 14.11. The activity of **7e** and **7f** is comparable to the established MDR reversal agent verapamil (VRP) (FAR = 3.46, 3.23, 4.16, respectively), and **7h** (FAR = 14.11) is more active than VRP. And all tested compounds except **7g** (FAR = 1.03) showed stronger MDR reversal activity than parental compound DDB, suggesting that the structural modification of DDB is beneficial for enhancing its MDR reversal activity.

The analysis of structure–activity reveals that different R substituents in **7a–h** (Scheme 1) could affect Rh123 accumulation in K562/A02 cells: (a) The longer *N*-alkyl chain in R might be benificial for the MDR revesal activity. For example, the FAR values of dimethylamino (**7a**), diethylamino (**7b**) and dipropylamino (**7c**) are 1.44, 2.57 and 2.84, respectively; (b) The size of one nitrogen-containing hetercycle in R is likely to affect MDR reversal activity since the FAR value of **7d** containing a five-membered pyrrolidino ring is less than **7e** with a six-membered piperidino ring (1.65 vs 3.46); and (c) The different type of six-membered hetercyclic rings in R could have different MDR reversal activity. For example, **7f** (FAR = 3.23) containing a morpholino ring in R was more active than **7g** (FAR = 1.03) with a 4-methylpiperidine moiety. However, when the 4-methylpiperazin was changed to benzylpiperazine, the corresponding compound **7h** showed the strongest activity (FAR = 14.11), suggesting that introducion of an additonal benzene ring to the molecule of **7g** could dramatically strengthen its binding affinity with P-gp, leading to more Rh123 accumulation in K562/A02 cells.

Based on the results of above experiments, the most active compound **7h** was selected to test for P-gp-mediated MDR reversal activity. Chemosensitizing effect of **7h** on drug-resistant K562/A02 cells to ADR was assayed according to previous report with minor modifications.[25] As shown in Figure 2A, 7**h** could significantly enhance the chemo-sensitivity of K562/A02 cells to the

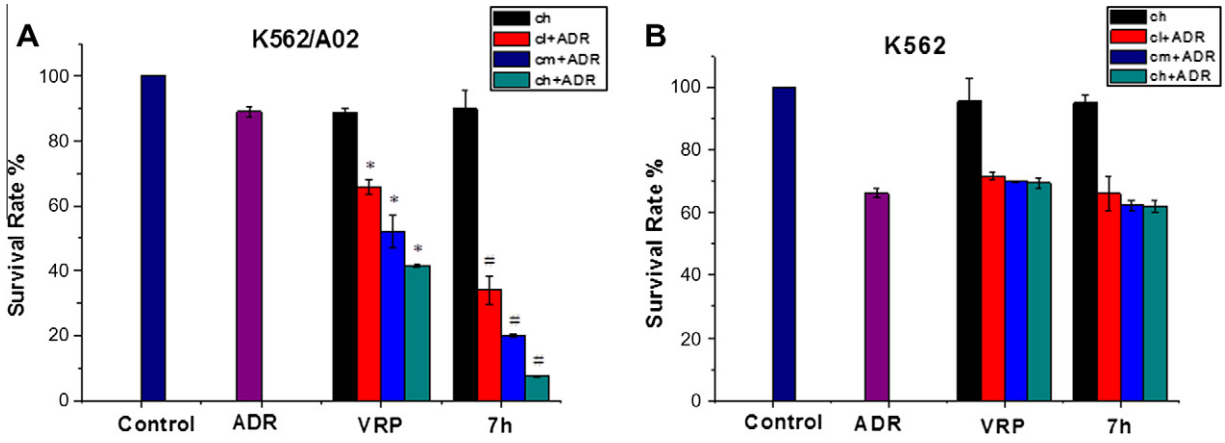


Figure 2. The effect of compound **7h** on the chemo-sensitivity of drug-resistant K562/A02 cells toward ADR. K562/A02 and K562 cells were treated with 2 and 0.04 μM ADR, respectively, combined with three concentrations (cl: 2.5 μM; cm: 5 μM; ch: 10 μM) of **7h** or VRP for 72 h. The survival rates of the cells were determined by MTS assay. The VRP was used as a positive control. $^{*}P$ <0.05, $^{\#}P$ <0.01 versus ADR treatment alone group.

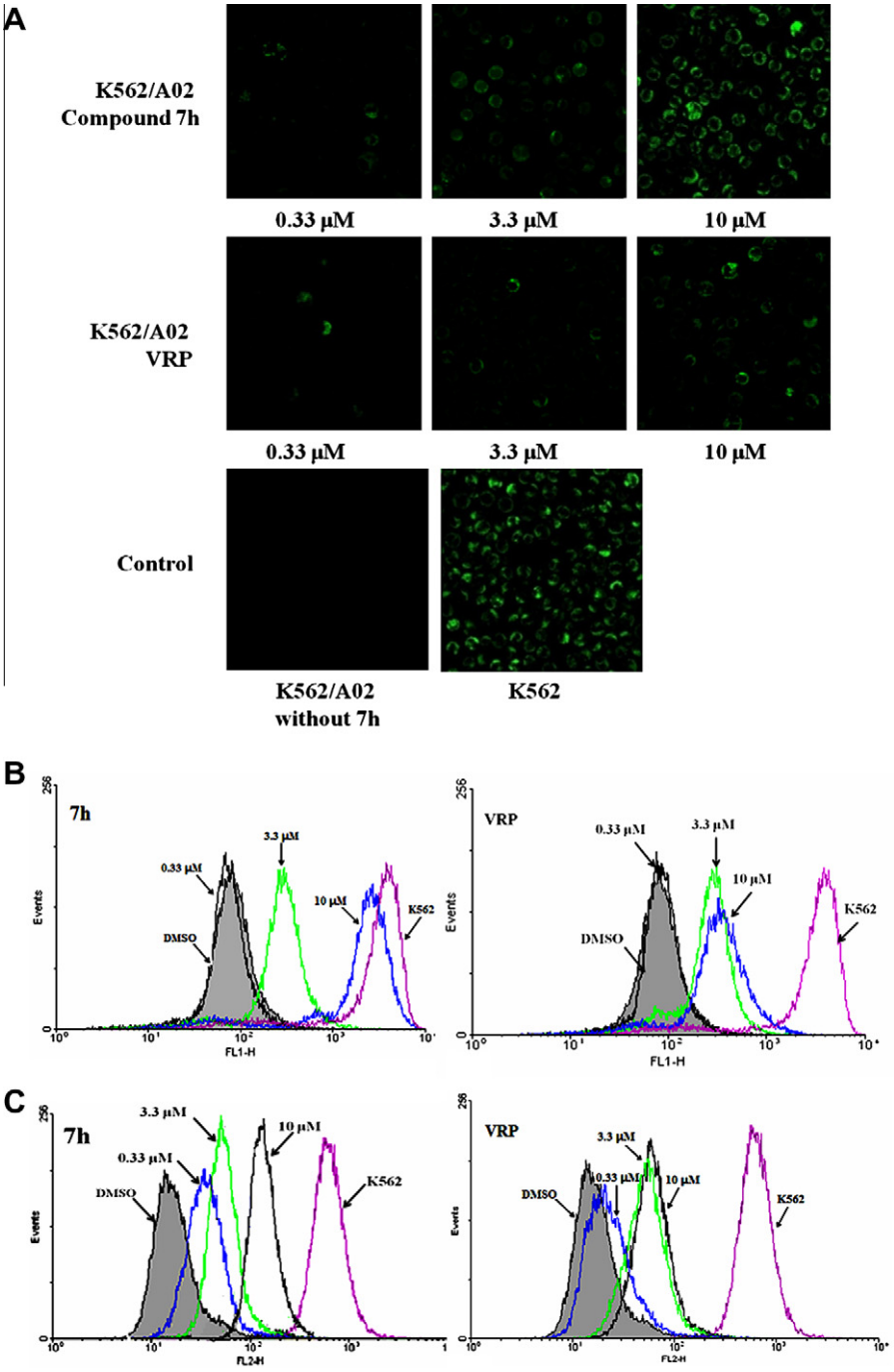


Figure 3. The dose–response effect of **7h** on the intracellular Rh123 (A and B) and ADR (C) accumulation in K562/A02 cells. K562/A02 cells were incubated with 0.5 μM Rh123 or 0.5 μM ADR in the presence or absence of 0.33, 3.3 or 10 μM of **7h** or VRP at 37 °C for 30 min. The K562 cells were treated with 0.5 μM Rh123 or ADR only. After treatment, the cells were washed twice with PBS and resuspended in medium, and then incubated at 37 °C for an additional 60 min to allow efflux of the dye. The cells were then observed and photographed under a fluorescence microscope (A), and the amount of Rh123 and ADR accumulated in the tumor cells was determined by measuring the cell-associated fluorescence. The fluorescence detection was operated at excitation and emission wavelengths of 488 and 520 nm for Rh123 (B), and of 488 and 570 nm for ADR (C), respectively, using flow cytometry. VRP was used as a positive control and 0.1% DMSO was used as vehicle control.

cytotoxic effect of ADR in a dose-dependent manner when compared with VRP, a well-known P-gp inhibitor. Interestingly, it was observed that the survival rate of K562/A02 cells was about 89.1% when treated with 2 μM ADR alone, however, when treated K562/A02 cells with a combination of 2 μM ADR and **7h** at three concentrations (cl: 2.5 μM, cm: 5 μM or ch: 10 μM), the survival rate was markedly decreased to 33.9%, 19.9% and 7.5%, respectively. Nevertheless, such chemosensitizing effect of **7h** was not observed in sensitive K562 cells (Fig. 2B), which expresses little P-gp. These results suggest that the selective chemosensitizing effect of compound **7h** could be due to the inhibition of drug efflux function of P-gp, leading to the increased intracellular accumulation of ADR in K562/A02 cells.

To validate our assumption, the dose–response effect of **7h** on inhibiting the drug efflux function of P-gp was assayed by evaluating the Rh123 accumulation in K562/A02 and K562 cells using fluorescence microscope (Fig. 3A) and flow cytometry (Fig. 3B and C). The results in Figure 3A show that K562 cells retained

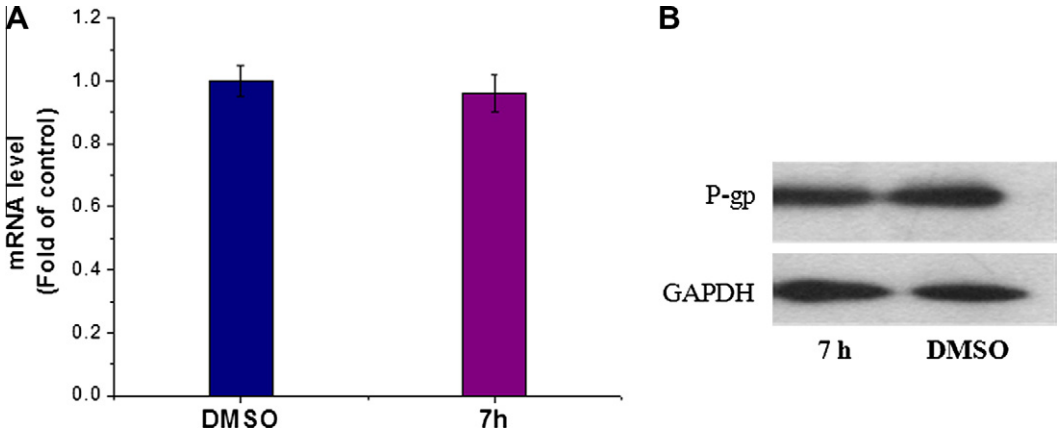


Figure 4. The effect of **7h** on P-gp mRNA level (A) and protein expression (B) in K562/A02 cells at 10 μM for 72 h treatment. K562/A02 cells with P-gp overexpression were treated with 0.1% DMSO or 10 μM compound **7h** for 72 h. The total RNA was extracted and reverse transcripted from mRNA to cDNA using the RT-PCR (Promega, WI, USA), and the data was analyzed with Bio-Rad iQ5 software.Western blot analysis: after incubation, the cells were harvested and lyzed. The cell lysates (30 μg) were separated by SDS–PAGE (12% gel) and transferred onto a PVDF membrane. After blocked in TBST containing 5% non-fat milk, the target proteins were probed with anti-P-gp, anti-GAPDH. The protein–antibody complex were visualized by the enhanced Phototope TM-HRP Detection Kit (Cell Signaling, USA) and exposed to Kodak medical X-ray processor (Kodak, USA). 0.1% DMSO was used as vehicle control. GAPDH was used as a loading control.

the most amount of Rh123, while K562/A02 cells has substantially pumped the Rh123 out of the cells and there was little fluorescence could be observed after 60-min incubation. When K562/A02 cells were treated with **7h**, the retained amount of Rh123 was significantly increased in a dose-dependent manner. These results suggest that **7h** might effectively block the drug efflux function of P-gp. And the potency of **7h** is much greater than that of VRP under the same conditions. Flow cytometry results showed similar effect of **7h** on Rh123 accumulation in K562/A02 cells. As shown in Figure 3B, the retained Rh123 level in K562 cells (purple curve) is much higher than that in K562/A02 cells treated with 0.1% DMSO, a vehicle control (black background), however, the retained Rh123 levels in K562/A02 cells elevated rapidly after treatment with **7h**, and the Rh123 retained curve shift dramatically from left to right, almost completely overlap with that from K562 cells at 10 μM. And the Rh123 accumulative effect of **7h** is much greater than that of VRP at the same dose (Fig. 3B). In addition, the effect of **7h** on ADR accumulation in K562/A02 cells was also evaluated by flow cytometry (Fig. 3C). As we can see, the accumulation amount of ADR in K562 cells (purple curve) is much larger than that in K562/A02 cells treated with 0.1% DMSO (black background). When K562/A02 cells were treated with **7h**, the ADR levels in the cells significantly increased in a dose-dependent manner. In comparison of **7h** with VRP on ADR accumulation, it was observed that the ADR retaining curve shifted from left to right markedly at the same concentration, suggesting that the effect of **7h** on ADR accumulation is much stronger than that of VRP, resulting in greater chemosensitizing effect than VRP in K562/A02 cells. Expectedly, **7h** had little effect on Rh123 or ADR accumulation in sensitive K562 cells (data not shown). All these results revealed that the MDR reversal effect of **7h** could be due to the inhibition of drug efflux function of P-gp, leading to the increased ADR accumulation in K562/A02 cells.

In order to further investigate whether the increased Rh123 and ADR accumulation in K562/A02 cells is caused by decreased P-gp expression induced by **7h**, RT-PCR and western blot experiments were carried out on the levels of P-gp mRNA and protein. The results in Figure 4 clearly show that **7h** had little affect on the levels of P-gp mRNA and protein expression in the cells.

In conclusion, eight substituted dibenzo[*c*,*e*]azepine-5-ones (**7a–h**) were synthesized based on the structural modifications of DDB and evaluated in vitro as P-gp-mediated MDR reversal agents. Compound **7h** could significantly and selectively enhance the chemo-sensitivity of drug-resistant K562/A02 cells to the cytotoxic effect of adriamycin (ADR) in a dose-dependent manner. And **7h** could markedly increase intracellular accumulation of both rhodamine 123 and ADR in K562/A02 cells, and inhibit their efflux out of these cells. Additionally, **7h** had little effect on the levels of P-gp mRNA and protein in K562/A02 cells, suggesting that the anti-MDR effect of **7h** might be implicated in the inhibition of drug efflux function of P-gp, leading to the increased drug accumulation in K562/A02 cells. Therefore, **7h** could be served as a promising lead for developing P-gp-mediated MDR reversal agents.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.03.005. These data include MOL files and InChiKeys of the most important compounds described in this article.

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