Schisandrin B: A dual inhibitor of P-glycoprotein and multidrug resistance-associated protein 1

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Abstract

We recently reported that schisandrin B (Sch B) was a novel P-glycoprotein (P-gp) inhibitor. In this study, we revealed that Sch B was also an effective inhibitor of multidrug resistance-associated protein 1 (MRP1). The activities of Sch B to reverse MRP1-mediated drug resistance was tested using HL60/ADR and HL60/MRP, the human promyelocytic leukemia cell lines with the overexpression of MRP1 but not P-gp. Sch B resumed daunorubicin and carboxyfluorescein diacetate (CFDA, a specific substrate for MRP1) accumulation and retention in HL60/ADR cells in a time and concentration dependent manner. At the equimolar concentration, Sch B demonstrated significantly stronger potency than probenecid, a MRP1 inhibitor. This study, together with the previous findings, demonstrated that Sch B was a dual inhibitor of P-gp and MRP1, a type suggested to be preferable to the use of combination of two specific modulators to prevent drug–drug interaction and cumulative toxicities.

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1. Introduction

Cancer multidrug resistance (MDR) is one of the major causes for the failure of clinical chemotherapy. While there are many mechanisms underlying the MDR, the ATP binding cassette members, particularly P-glycoprotein (P-gp, ABCB1) and multidrug resistance-associated protein 1 (MRP1, ABCC1), are responsible for most of the clinical cancer MDR [1,2].

Several lines of evidence have proved that MRP1 conferred cancer with multiple drug resistance. MRP1 is a transmembrane protein, functioning mainly as a (co-)transporter of amphipathic organic anions [2]. While it transports hydrophobic drugs conjugated to the anionic tripeptide glutathione, the efficient extrusion of non-conjugated drugs by this protein depends on a normal cellular supply of glutathione. MRP1 has a broad substrate specificities, including anthracyclines, vinca alkaloids, epi/dipodophyllotoxins, mitoxantrones, methotrexates, and to a less extent, taxanes [2]. The expression of MRP1 was found in almost every tumor type, including the solid tumors (lung, gastrointestinal and urothelial carcinomas, neuroblastoma, glioma, retinoblastoma, melanoma, cancers of the breast endometrium, ovary, prostate, and thyroid) and hematological malignancies [3–21]. It has been implicated that the expression of MRP1 is negatively correlated with the prognosis in certain types of cancers [3,22,23].

One of the ways to overcome MRP1 mediated MDR is to use an inhibitor to block the function of MRP1. Although the roles of MRP1 in MDR were known a decade ago, the discovery and development of the
inhibitors to MRP1 with high efficacies and appreciable safety apparently have been much more difficult than to P-gp, most probably because MRP1 is an anionic transporter. Therefore, in principle, the MPR1 inhibitor should be anionic in nature. The anionic molecule, however, enters intact cells poorly, so that the effective intracellular concentration was difficult to be attained. Another potential problem related to the anionic transporter inhibitors is that these molecules may cause a widespread inhibition of the anionic transporter system in human body and hence a series consequences [2]. Probenecid is anionic in nature and a MRP1 inhibitor. The development of this compound as a clinical MDR modulator appears to be not optimistic due to its dose-limiting toxicities [2]. On the other hand, several P-gp inhibitors were found to cross-react with MRP1, among which, VX-710 (a pipecolate derivative) and MS-209 (a quinoline derivative) are of particular interest [24–26]. Since the clinical MDR appears to be multifactorial, the MDR inhibitors with broad specificity are apparently preferable to the use of combination of several specific modulators to reduce the drug–drug interaction and cumulative toxicities [24].

Sch B (Fig. 1), the most abundant dibenzocyclooctadiene lignan present in Schisandra chinensis (Turcz.) Baill, is of multiple biological functions to protect against carbon tetrachloride-induced hepatotoxicity [27], myocardial ischemia/reperfusion injury [28], and brain oxidative damage [29]. We recently discovered that this compound functioned as a P-gp inhibitor [30,31]. In addition, we proved that this compound could enhance doxorubicin-induced apoptosis in cancer cells but not in normal cells [32]. In this study, we further demonstrated that Sch B could effectively reverse MRP1-mediated cancer MDR.

Fig. 1. The chemical structures of Sch B and dibenzocyclooctadiene.

2. Materials and methods

2.1. Cell lines

Human promyelocytic leukemia MDR cell line HL60/ADR was obtained from the Institute of Hematology, Chinese Academy of Medical Sciences, Tianjin, China. HL60/MRP was a generous gift from Dr Jean-Pierre Marie (Hopital Hotel-Dieu AP-HP, France) [33]. HL60/ADR and HL60/MRP were grown in RPMI-1640 containing 10% FBS and 100 ng/ml doxorubicin. The drug sensitive parental cell line HL60 was maintained in RPMI-1640 containing 10% FBS. The expression of MRP1 but not P-gp was confirmed by labeling HL60/ADR cells with either a FITC-conjugated mouse anti-human MRP1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or a R-phycocerythin (R-PE)-conjugated mouse anti-human P-gp monoclonal antibody (Becton-Dickinson, Holbrook, NY, USA), and then analyzed using a Becton Dickinson FACScan (BD Immunocytometry Systems, San Jose, CA). At least 50,000 cells were counted in each assay. The non-specific labeling of cells was corrected by the monoclonal immunoglobulin isotype control (FITC or R-PE-conjugated mouse IgG2b, BD PharMingen).

2.2. RT-PCR of mdr1 and mrp1 mRNA

Total RNA was isolated from HL60/ADR, HL60/MRP, or MCF-7/ADR cells by TRIZol reagent (Invitrogen, Rockville, MD, USA) according to the manufacturer’s instruction. The first strand cDNA was synthesized from extracted RNA using an Oligo dT as primer. The PCR primers were 5-agt ggt tca ggt ggc tct-3(forward) and 5-ttc tgt ctt ggg ctt gtg-3(reverse) for mdr1, 5-ggt gct tcc cac gga gg-3(forward) and 5-tca acc aca aaa ctg cag cc-3(reverse) for mrp1, and 5-ttc cag cct tcc ttg gg-3(forward) and 5-ttg cgc tca gga gga gca at-3(reverse) for β-actin. Amplification was performed in a DNA thermal cycler (Perkin–Elmer, CA, USA) according to the following protocol: (a) for mdr1, initial denaturation for 5 min at 95 °C; 35 cycles of denaturation for 15 s at 95 °C, primer annealing for 15 s at 58 °C, polymerization for 15 s at 72 °C, and final extension for 5 min at 72 °C; (b) for mrp1 and β-actin, initial denaturation for 5 min at 95 °C; 35 cycles of denaturation for 15 s at 95 °C, primer annealing for 15 s at 58 °C, polymerization for 15 s at 72 °C, and final extension for 5 min at 72 °C. PCR products were separated on ethidium bromide-stained 1.5% agarose gels. Expected RT-PCR product sizes were 341 bp for mdr1, 182 bp for mrp1, and 250 bp for β-actin.

2.3. Drug accumulation and retention assays

For drug accumulation assays, cells (1×10⁶) were incubated in RPMI-1640 medium containing 2 μg/ml daunorubicin (DNR) or 1 μM CFDA in the presence or absence of Sch B or probenecid, and incubated in a CO₂ incubator at
37 °C. At appropriate intervals, cells were collected and washed with ice-cold PBS. The intracellular DNR and CFDA were determined at the excitation wavelength (488 and 492 nm) and emission wavelength (530 and 517 nm) using a Becton Dickinson FACScan. A minimum of 10,000 cells was counted for each sample, which was corrected by subtracting cells’ auto-fluorescence.

For drug retention assays, cells (1 × 10^6) were incubated in RPMI-1640 medium containing 2 μg/ml DNR or 1 μM CFDA and incubated in a CO2 incubator at 37 °C for 60 or 40 min. Cells were collected, washed with ice-cold PBS twice, and resuspended in RPMI-1640 medium containing 10% FBS with or without Sch B or probenecid, and incubated in 5% CO2 at 37 °C for appropriate intervals. Cells were then washed with ice cold PBS, and immediately subjected for intracellular DNR fluorescence (λ_exλ_em 488/530 nm) and CFDA fluorescence (λ_exλ_em 492/517 nm) determination using a Becton Dickinson FACScan. A minimum of 10,000 cells was counted for each sample, and corrected by subtracting cells’ auto-fluorescence.

2.4. Growth inhibition assays

Assays were performed as described previously [31]. Briefly, cells were seeded into 24-well plates at the density of 40,000 cells/well. Anticancer drugs at series concentrations

Fig. 2. (A) A representative FACScan profiles of MRP1 and P-gp expression in HL60, HL60/ADR, and HL60/MRP. Solid line, the MRP1 or P-gp associated fluorescence; dotted line, the isotype control. (B) Detection of mdr1 and mrp1 mRNA using rtPCR. MCF7/ADR [30,31], a cell line with P-gp overexpression, was used as a positive control for P-gp, and a negative control for MRP1.
were added into wells in quadruplicates in the presence or absence of Sch B or probenecid, and the cultures were placed in a humidified CO2 incubator at 37 °C. After 72 h incubation, cells in each well were counted using a Becton Dickinson FACSCan. The cell numbers versus drug concentration in the presence and absence of MRP1 modulators were plotted to calculate the IC50.

3. Results and discussion

3.1. HL60/ADR and HL60/MRP express MRP1 but not P-gp

Since Sch B is a P-gp inhibitor, to test its activities against MRP1, it is essential that the expression of MRP1 but not P-gp be confirmed. As shown in Fig. 2, the HL60/ADR and HL60/MRP displayed a strong peak corresponding to MRP1. The profiles of P-gp related fluorescence were virtually the same as those of isotype controls, indicating no appreciable amount of P-gp in these cell lines. In addition, the two cell lines expressed mrp1 mRNA but not mdr1 mRNA (Fig. 2). These results validated the cell lines for testing Sch B as a MRP1 modulator.

3.2. Sch B increases intracellular drug accumulation in HL60/ADR

For the drug accumulation and retention assays, we chose DNR and CFDA, because DNR is one of the major agents against acute myelogenous leukemia, and CFDA was a specific substrate for MRP1, one of the best fluorescent molecule to probe the activities of MRP1 [33].

Fig. 3. The effect of Sch B on the intracellular DNR or CFDA accumulation and retention in HL60/ADR. (A) Cells were incubated with 2 μg/ml DNR or 1 μM CFDA in the presence or absence of Sch B at indicated concentrations for 60 (DNR) or 40 (CFDA) minutes and collected for the determination of intracellular DNR as described in Section 2. (B) Cells were incubated with 2 μg/ml DNR in the presence or absence of Sch B at 50 μM for appropriate intervals and collected for determination of the intracellular DNR. (C) Cells were loaded with 2 μg/ml DNR or 1 μM CFDA, washed with ice-cold PBS, resuspended in fresh RPMI-1640 medium containing Sch B at indicated concentrations. After 30 min incubation, the retained intracellular DNR or CFDA was determined. (D) Cells were loaded with 2 μg/ml DNR, washed with ice-cold PBS, resuspended in fresh RPMI-1640 medium containing Sch B (50 μM). At appropriate intervals, cells were collected and the retained intracellular DNR was determined. Data are mean ± SD of at least four determinations from two separate experiments.
As shown in Fig. 3A, intracellular DNR accumulation in HL60/ADR increased with the increment of the concentrations of Sch B. The optimal concentration of Sch B for restoring the intracellular DNR accumulation appears to be 50 μM, at which the intracellular DNR increased for more than threefold. We then further tested if Sch B could restore the intracellular accumulation of CFDA, a specific substrate of MRP1. The results showed that Sch B enhanced the CFDA uptake by HL60/ADR in a dose-dependent manner (Fig. 3A). In addition to the drug accumulation, Sch B significantly increased the intracellular retention of DNR and CFDA in HL60/ADR cells, and this activity was concentration dependent (Fig. 3C). Likewise, in time-dependent DNR accumulation and retention assays, Sch B at 50 μM demonstrated strong activities inhibiting MRP1-mediated drug efflux, resulting in a pronounced increased intracellular drug concentration (Fig. 3B and D). These results provided strong evidence that Sch B could effectively inhibit MRP1 mediated drug efflux.

3.3. Sch B demonstrated better activities than probenecid

Probenecid has been proved to be a MRP1 modulator and has been often used as a reference for novel MRP1 inhibitors [24,29,30]. We compared the efficacies of Sch B with probenecid on the accumulation of DNR and CFDA in HL60/ADR cells. The results showed that at the same concentration (50 μM), Sch B is significantly stronger than probenecid in restoring the drug accumulation in HL60/ADR (Fig. 4A and B). The effect of Probenecid at 500 μM on DNR accumulation is comparable to that of Sch B at 50 μM, but significantly stronger on CFDA accumulation than the latter. Similar results were obtained when HL60/MRP was assayed for the intracellular CFDA accumulation in the presence of Sch B or probenecid (Fig. 4C).
3.4. Sch B reverses the drug resistance of HL60/ADR and HL60/MRP

Firstly, we did the dose-dependent effect of Sch B on the reversal of DNR resistance of HL60/ADR. As shown in Fig. 5, in the concentration of Sch B ranging from 2.5 to 25 μM, the IC50 of HL60/ADR against DNR reduced linearly. Therefore, Sch B at 25 μM appears to be the optimal concentration for the reversal of drug resistance of HL60/ADR. Table 1 summarized the effects of Sch B at 25 μM on the reversal of drug resistance of HL60 against a panel of anticancer drugs. The drug sensitivities of HL60/ADR toward DNR, vincristine, mitoxantrone, and taxol in the presence of Sch B were comparable to those of the drug sensitive HL60, indicating the fully reversal of MRP1-mediated drug resistance. The sensitivities of HL60/ADR toward VP-16 increased for about 20-fold in the presence of Sch B.

Probenecid at 500 μM or over were previously used to reverse MRP1 mediated drug resistance in vitro [24,34,35]. However, we found that the growth rate of HL60/ADR was inhibited for 50% after incubation with this compound at 500 μM for a 3-day period (data not shown). Therefore, this concentration is not suitable for the assay condition. Since probenecid at 100 μM did not exhibit significant toxicities toward HL60/ADR, this concentration was used for the subsequent assays. As shown in Tables 1 and 2, probenecid showed a very limited potency to reverse the drug resistance of both HL60/ADR and HL60/MRP, much weaker than Sch B.

3.5. Sch B is a dual inhibitor P-gp and MRP1

Sch B is a dibenzocyclooctadiene derivative, whose chemical structure is distinctive from all other reported P-gp and MRP1 modulators [30,31]. The optimal concentration of Sch B to inhibit P-gp and MRP1 appears to be comparable with each other, ranging between 20 and 50 μM [30 and this study]. A compound as a dual inhibitor of P-gp and MRP1 was not surprising, since P-gp and MRP1 shares structural and functional similarity [36]. VX-710 and MS209 are good examples as dual inhibitors [24–26], both of which have entered clinical studies. Since the clinical MDR appears to be multifactorial, the MDR inhibitors

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**Table 1**
The reversal of the drug resistance of HL60/ADR by Sch B or probenecid

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (ng/ml)</th>
<th>Vehicle&lt;sup&gt;a&lt;/sup&gt;</th>
<th>+ Sch B&lt;sup&gt;b&lt;/sup&gt;</th>
<th>+ Probenecid&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>VP-16</td>
<td>473 ± 42</td>
<td>24,869 ± 3150</td>
<td>1250 ± 81 (20)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14,061 ± 1131 (2)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>39 ± 9</td>
<td>351 ± 31</td>
<td>43 ± 7 (8)</td>
<td>209 ± 29 (2)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.3 ± 0.1</td>
<td>4.4 ± 1.3</td>
<td>0.5 ± 0.1 (9)</td>
<td>3.2 ± 1.4 (1)</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.2 ± 0.1</td>
<td>3.6 ± 1.3</td>
<td>0.3 ± 0.1 (12)</td>
<td>2.5 ± 0.9 (1)</td>
</tr>
<tr>
<td>Taxol</td>
<td>0.2 ± 0.1</td>
<td>3.1 ± 0.6</td>
<td>0.5 ± 0.1 (8)</td>
<td>3.0 ± 0.1 (1)</td>
</tr>
</tbody>
</table>

Data are mean ± SD from three separate experiments.

<sup>a</sup> Cells treated with anticancer drug only.

<sup>b</sup> Cells treated with anticancer drugs in the presence of 25 μM Sch B.

<sup>c</sup> Cells treated with anticancer drugs in the presence of 100 μM probenecid.

<sup>d</sup> The reversal folds of drug resistance, the IC50s in the absence of Sch B divided by the IC50 in the presence of Sch B or probenecid.

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**Table 2**
The reversal of the drug resistance of HL60/MRP by Sch B or probenecid

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (ng/ml)</th>
<th>Vehicle&lt;sup&gt;a&lt;/sup&gt;</th>
<th>+ Sch B&lt;sup&gt;b&lt;/sup&gt;</th>
<th>+ Probenecid&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>VP-16</td>
<td>12,645 ± 1140</td>
<td>901 ± 67 (14)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3868 ± 211 (3)</td>
<td></td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>232 ± 28</td>
<td>82 ± 9 (3)</td>
<td>93 ± 8 (3)</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>9.3 ± 0.5</td>
<td>2.9 ± 0.4 (3)</td>
<td>7.1 ± 0.3 (1)</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SD from three separate experiments.

<sup>a</sup> Cells treated with anticancer drug only.

<sup>b</sup> Cells treated with anticancer drugs in the presence of 25 μM Sch B.

<sup>c</sup> Cells treated with anticancer drugs in the presence of 100 μM probenecid.

<sup>d</sup> The reversal folds of drug resistance, the IC50s in the absence of Sch B divided by the IC50 in the presence of Sch B or probenecid.
of dual functions are probably preferable to the use of combination of two specific modulators in terms of reducing cumulative toxicities and drug–drug interaction.

The efficacies and safety are the two most important criteria to evaluate if a lead compound should be placed onto a development track. Although a lead could be of high efficacies, its development to a clinical MDR modulator could be failed due to its dose-limiting adverse effects [1,2]. At equimolar concentration, Sch B exhibited significant stronger potency than probenecid in inhibiting P-gp and comparable activities to verapamil in inhibiting P-gp [30]. While probenecid and verapamil are not suitable for clinical MDR modulators due to the dose-limiting adverse effects at their effective concentrations, Sch B appears to be not affected by this problem. The previous reports from other laboratories indicated that in their protocols using Sch B to protect carbon tetrachloride-induced hepatotoxicity in laboratories indicated that in their protocols using Sch B affected by this problem. The previous reports from other laboratories indicated that in their protocols using Sch B to protect carbon tetrachloride-induced hepatotoxicity in mice, the dose of Sch B is 3 mmol/kg (1.2 g/kg) [37,38], implicating the well-tolerance of this compound. The toxicological data as reviewed in [39] revealed the high-safety of this compound. In addition, there is also evidence to show that this compound is safe to human beings. Sch B is the most abundant dibenzocycloocta-diene derivative in *S. chinensis* (Turcz.) Baill, a Chinese traditional medicinal herb, which has been used as a medication for the last several thousand years and are being still actively practiced in China. There is no report about the side effects of this herb, an indicative of the safety of Sch B. Taken together, Sch B is a dual inhibitor of P-gp and MRP1 with relatively strong potency but without dose-limiting side effects at its effective concentrations. The future direction would focus on the pharmacokinetic interaction between Sch B and daunorubicin or other major anti-cancer agents.

Acknowledgements

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References


