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ORIGINAL ARTICLE

Syl611, a novel semisynthetic taxane derivative, reverses multidrug resistance by p-glycoprotein inhibition and facilitating inward transmembrane action

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Abstract
Purpose To investigate the reversal mechanisms of a novel semisynthetic taxane derivative, Syl611. Syl611 is a structurally modified compound from Sinenxan A, and the chemical structure is entirely new. It was found to significantly increase paclitaxel-induced cytotoxicity in drug-resistant cells, while presenting a low level of cytotoxicity.

Methods The in vitro cytotoxic and MDR-reversing activities of the Syl611 were determined by MTT assays. The cytotoxicity enhancement of paclitaxel was performed using the acridine orange/ethidium bromide double staining. Rhodamine 123 accumulation and retention assay in KB/V cells, Caco-2 monolayer model were used to find mechanism of action.

Results The cytotoxicity of Syl611 was wondrously lower in all tested cell lines than that of paclitaxel. Cytotoxicity enhancement from Syl611 was dramatically higher than that of verapamil of the same concentration (10 μM): the reversal fold index for A549/Paclitaxel, KB/V, and Bel7402/5-FU were 45.95, 73.56, and 107.13 (Syl611) and 11.36, 23.92, and 70.42 (verapamil). AO/EB double staining assay equally showed that Syl611 could enhance the cytotoxicity induced by paclitaxel. Furthermore, Syl611 could also increase the intracellular accumulation of Rhodamine 123 in KB/V cells without affecting P-gp's expression, and this accumulation was reversible. In bidirectional permeability assay, Syl611 increased the permeability of paclitaxel but decreased the net secretory of paclitaxel.

Conclusions Syl611 is an effective and potential agent in reversing multidrug resistance (MDR) by multiple actions, which attributed to p-glycoprotein inhibition and drug permeability enhancement.

Keywords Multidrug resistance · P-glycoprotein · Sinenxan A · Syl611 · Reversing agents

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Introduction

The most common obfuscation in face of successful cancer chemotherapy is multidrug resistance (MDR). MDR

tumors show cross-resistance to multiple anticancer drugs of different chemical structures and mechanisms of action, which eventually results in the shortage of effective chemotherapy for patients [1]. The main but not unique mechanical cause of MDR is the overexpression of P-glycoprotein (P-gp), which occurs when cancer cells are exposed to a single hydrophobic cytotoxic agent [2]. P-gp, a 170-kDa transmembrane glycoprotein encoded by the ABCB1 (MDR1) gene, is attributed to the excretion of several anticancer agents including anthracyclines, vinca alkaloids, and taxanes [3, 4].

The first generation of P-gp inhibitor, verapamil, is identified as a calcium channel blocker which can inhibit P-gp-mediated drug efflux, accordingly, increasing the accumulation of anticancer drugs in tumor cells [5]. However, due to verapamil's calcium channel blocking effect on heart toxicity and its adverse effects presented during animal studies and clinical trials, verapamil was not authorized for clinical use eventually [6]. After verapamil's failure, several other MDR-reversing agents such as valspodar, LY335979, and ONT-093 have been studied for their inhibitory effects on P-gp, but all of them were found to be of dose-limiting toxicity [7, 8]. Therefore, a novel compound of promising MDR-reversing activity and low toxicity was urgently explored upon.

Paclitaxel, an important anti-cancer agent against breast, ovarian, and non-small cell lung cancers, has been widely used by many medical practitioners in the past century. However, this drug has fallen into disuse and is in need of urgent chemical structural modification or biological recomposition in order to improve its pharmacological profile upon its progressively increasing drug resistance and administration route embarrassment [9–11]. Sinenxan A (2α, 5α, 10β, 14β-tetraacetoxy-4(20), 11-taxadiene) is a taxoid, which is isolated from the callus cultures of *Taxus yunnanensis* (Fig. 1a) [12]. The rich resources of Sinenxan A and its taxane skeleton provide the possibility for the semisynthesis of paclitaxel and other bioactive taxane derivatives. Through our continuing search for new drugs from natural products, we used Sinenxan A as the starting material for reorganizing the structure and obtained a series of new compounds to study their valuable potential for drugs. Some research results for these studies have been reported [13, 14]. Syl611 (9α-cinnamoyloxy-10β-hydroxy-2α, 5α, 14β-triacetoxy-taxa-4β-methyl-11(12)-ene) was screened out from 67 compounds for its outstanding MDR-reversing activity and impressive low cytotoxicity (Fig. 1b). Here, we report the reversing ability of Sy1611 against P-gp-mediated MDR. Mechanism study showed that Sy1611 increased the intracellular accumulation of drugs by inhibiting the function of P-gp and facilitating inward transmembrane action, thus potentially making Syl611 a new agent in cancer chemotherapy.

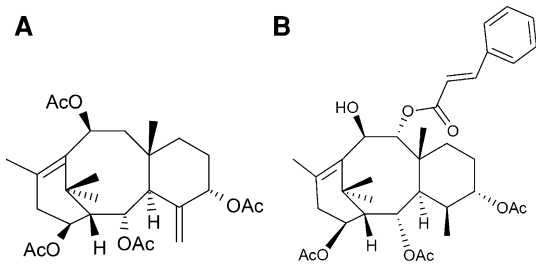

Fig. 1 Chemical structures of Sinenxan A (**a**) and Syl611 (**b**)

Materials and methods

Chemicals and materials

Syl611, a biosynthetic taxane, is obtained as pale yellow powder and has the molecular formula $C_{35}H_{46}O_9$ and a molecular weight of 610.31. In vitro, Syl611, paclitaxel, and vincristine were dissolved in DMSO and stored at −20°C for less than 1 month before use. Paclitaxel, vincristine, and 5-fluorouracil (5-FU), which were used for keeping the drug resistance, were obtained from Beijing Union Pharmaceutical Factory (Beijing, China). Verapamil was obtained from Sigma. Cell culture media, RPMI-1640, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA).

Cell culture

The human lung adenocarcinoma cell line A549 and its paclitaxel-resistant subline A549/Paclitaxel, the human oral epithelium cancer cell line KB and vincristine-resistant subline KB/V, the human hepatocellular carcinoma cell line Bel7402 and its 5-FU-resistant subline Bel7402/5-FU were cultured at 37°C in a 5% CO_2 and 95% air atmosphere using RPMI-1640 with 10% FBS and penicillin (100 U/ml). The human colon carcinoma cell line Caco-2 were used between passage number of 25–35 and were cultured using Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. The A549, KB, Bel7402, and Caco-2 cells were purchased from the American Type Culture Collection (ATCC) and the Cell Culture Center of Institute of Basic Medical Science, Chinese Academy of Medical Sciences. The drug-resistant cell lines were established by in vitro continuous stepwise exposure of drugs and identified by the Department of Pharmacology at our institute. The 160-fold 5-FU-resistant cell line Bel7402/5FU and 175-fold vincristine-resistant cell line KB/V have been reported before [15, 16]. The A549/Paclitaxel showed about 70-fold higher resistance to paclitaxel than A549 cells.

Cytotoxicity assay and MDR-reversing assay

The in vitro cytotoxic and MDR-reversing activities of the Syl611 were determined by MTT (Sigma) assays. Briefly, cells were seeded into 96-well plates at a density of $1–2\times10^3$/well (depended on the cell growth rate). Twenty-four hours later, the cells were exposed to the tested agents. The cytotoxicity of Syl611 in A549, KB, Bel7402, A549/Paclitaxel, KB/V, and Bel7402/5-FU was analyzed after incubation of cells with Syl611 for 4 days. In MDR-reversing assay, Syl611 combined with paclitaxel was treated in A549/Paclitaxel, KB/V, and Bel7402/5-FU cells to detect the reversing activities of Syl611 in those drug-resistant cells, while verapamil was used as positive control. The IC_{50} values defined as the drug concentration that resulted in a 50% reduction in the number of cells after incubation for 4 days. We used reversal fold index (RF) as the index for MDR-reversing activities and which was defined as the ratio between the IC_{50} value in the presence of paclitaxel alone and the paclitaxel combined with Syl611. The reversing activities of Syl611 on vincristine and 5-FU were tested in the same manner.

Cell viability and morphological characterization assay

Fluorescence microscopy was performed 48 h after treatment of the cells with each combination of drugs. The assessment of viability by fluorescence microscopy was performed using the acridine orange/ethidium bromide (AO/EB) assay. Briefly, 5×10^4 cells were seeded into 24-well plates for 24 h. After treatment, the cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 10 min at room temperature. The fixed cells were washed with PBS and stained with 4 μl aliquot of dye mixture consisting of 100 μg/ml AO and EB for 5 min. The stained cells were washed with PBS for 5 min thrice and viewed with a fluorescent microscope. To determine the percentage of viable (green fluorescence) versus apoptotic or necrotic cells (orange fluorescence), four microscopic fields, each containing 200 cells were examined. The quantitative visual differentiation between apoptotic and necrotic cells was based on cell size, nuclear condensation, and chromatin structure, while determination was performed according to Hotz et al. [17].

Western blotting for P-gp

MDR-reversing agent-treated and untreated drug-resistant cells were collected and prepared for cell lysates. Briefly, total cellular proteins (100 μg/lane) were electrophoresed on 10% SDS–polyacrylamide gels. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore, USA). The membranes were blocked for 2 h in Tris-buffered saline (TBS) containing 5% (w/v) dry skimmed milk powder (Sigma, USA) and incubated overnight with primary antibodies to P-gp (mouse monoclonal IgG_1, Santa Cruz, USA) and actin (goat polyclonal IgG, Santa Cruz, USA). The membranes were then washed with TBS-0.05% Tween 20 for 15 min thrice and incubated with secondary antibodies and were visualized with an enhanced chemiluminescence detection kit (Amersham Life Sciences, IL, USA).

RT–PCR for MDR1

The RT–PCR was performed by RT–PCR kit (Takara, Japan) and operated according to the manufacturer's instruction. Briefly, total RNA was extracted by TRIZOL (Invitrogen, USA). The integrity and purity of the RNA were checked by UV Spectrophotometer for OD260 and OD280, then reverse transcripted from mRNA to cDNA using the RT–PCR kit. The cDNA then was used as the template for PCR. The primer sets for MDR1 were 5′-AG AAGGTTCTGGGAAGATCGC-3′ (sense) and 5′-GG CACCAAAGACAACAGCTG-3′ (anti-sense); and the primer sets for actin were 5′TGTTTGAGACCTTCAACA CCC-3′ (sense) and 5′-AGCACTGTGTTGGCGTACAG-3′ (anti-sense). The PCR product of MDR1 was 286 bp, and the actin was 529 bp. The PCR profile was as follows: 10 min at 95°C, followed by 30 cycles of 30 s at 95°C and 1 min at 60°C. The PCR product was separated by 2% agarose gel electrophoresis, and the gels were stained using ethidium bromide and viewed by UV transillumination.

Rhodamine 123 (Rh123) accumulation and retention

Fluorescence intensity of intracellular Rh123 (Sigma, USA) was determined by flow cytometry. Briefly, after treatment of exponentially growing drug-resistant cells with 10 μM Rh123 (with and without MDR-reversing agents) in serum-free RPMI1640 for 3 h, cells were harvested and then washed with PBS. The retention group cells were incubated in PBS for another 1 h followed by harvesting. The mean fluorescence intensity of intracellular Rh123 was detected using flow cytometry. The mean fluorescence intensity was then automatically calculated from the fluorescence intensity of 1×10^4 cells by the flow cytometry machine.

Caco-2 monolayer model and bidirectional permeability assay

The Caco-2 cells (passage No. 25–35) were seeded on top of Millicell Cell Culture Insert polycarbonate filters (pore size, 0.4 μm; diameter, 13 mm; growth area, 0.6 cm^2) (Millipore, USA) at a cell density of

40,000 cells/cm^2, which was inserted in 24-well plate (Corning, USA). The culture medium was replaced every 2 day and essentially everyday if the cells almost fully covered the top of filters. The transepithelial electrical resistance (TEER) was tested after changed the medium. About 21 days later, the monolayer would be used for permeability studies when the TEER values were greater than 1,000 ohm cm^2.

The transport medium was Hank's balanced salt solution (HBSS) buffer containing 10 mM HEPES. After washed the warm medium (37°C) twice, the test compounds (paclitaxel alone or combined with Syl611 otherwise verapamil) were added into either the apical (for apical to basolateral transport; AP to BL) or basolateral (for basolateral to apical transport; BL to AP) side of the monolayer. The monolayers were then placed in a swing incubator at 37°C. Samples were taken from both the apical and basolateral compartment during the test process (no more than 3 h), and the concentration of paclitaxel were analyzed by a high-performance liquid chromatography–UV (HPLC–UV) as previously described [18, 19]. The detection of paclitaxel was at 227 nm for UV absorption.

Statistical analysis

The results are expressed as mean ± standard error of the mean. Statistical comparisons were made using one-way ANOVA by SPSS (version 10.0, SPSS Inc., Chicago, IL, USA). P value < 0.05 was considered statistically significant.

Results

Syl611 showed low cytotoxicity on three human tumor cell lines and their related drug-resistant cell lines

Considering the action modality of MDR-reversing agents, low cytotoxicity is the emphasis of these agents as they relate to anticancer drugs. We used three human tumor cell lines: A549, KB, and Bel7402, and their related drug-resistant cell lines: A549/Paclitaxel, KB/V, and Bel7402/5-FU to detect the cytotoxicity of Syl611 by MTT assay. The inhibition rate of each drug concentration was recorded, and paclitaxel was used to validate the feasibility of this assay. The experiments were carried out in triplicate. As the data shown, the cytotoxicity of Syl611 was wondrously lower in all tested cell lines than that of paclitaxel. As reported, all the drug-resistant cells were more resistant than their parental cells to paclitaxel, but when these drug-resistant cells were treated combined with Syl611, the chemosensitizing effects to paclitaxel increased (Fig. 2).

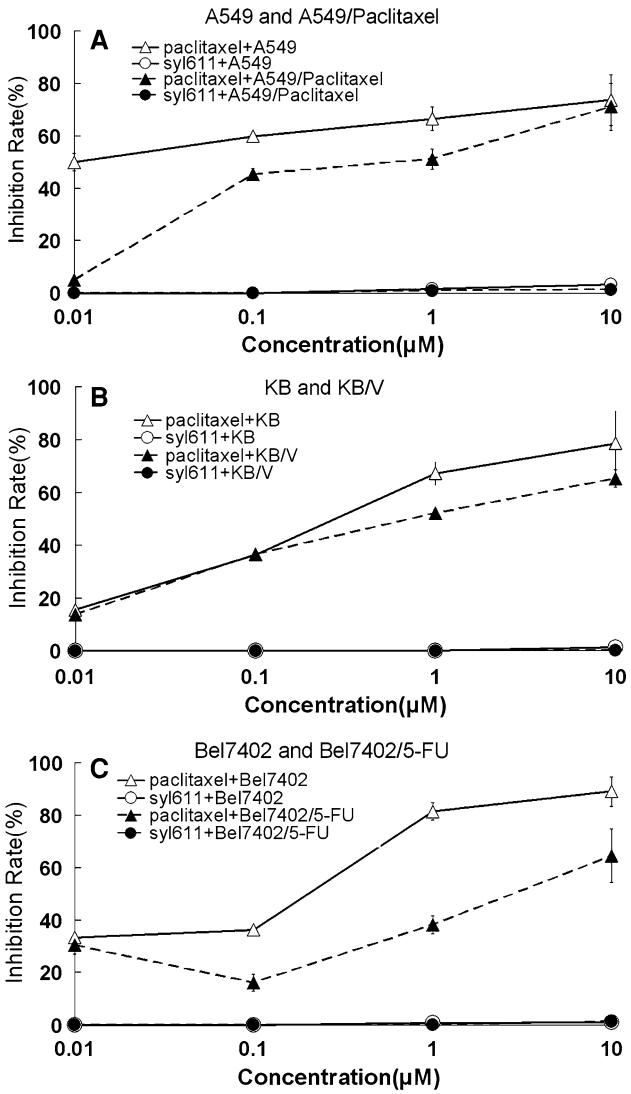

Fig. 2 The cytotoxicity of Syl611 on A549, KB, and Bel7402 and their related drug-resistant cell lines: A549/Paclitaxel (**a**), KB/V (**b**), and Bel7402/5-FU (**c**). Cell viability was determined using the MTT assay as described in "Materials and methods", and "Results" were showed as inhibition ratio (%)

Syl611 treatment increased paclitaxel-induced cytotoxicity in drug-resistant A549/Paclitaxel, KB/V, and Bel7402/5-FU cells

In order to explore the molecular mechanism for the MDR-modulating activity of Syl611, we used P-gp-overexpressed A549/Paclitaxel, KB/V, and Bel7402/5-FU cells to detect the MDR-reversing activities of Syl611. First of all, we treated these three drug-resistant cells with paclitaxel alone (100 nM) or drug combinations [paclitaxel associated with Syl611 (2.5, 5, 10 μM) and verapamil (10 μM)]. In these results, the combination treatment of Syl611 with paclitaxel markedly enhanced the paclitaxel-induced cytotoxicity in drug-resistant cells. Furthermore,

cytotoxicity enhancement from Syl611 was dramatically higher than that of verapamil of the same concentration (10 μM): the RF for A549/Paclitaxel, KB/V, and Bel7402/5-FU were 45.95, 73.56, and 107.13 (Syl611) and 11.36, 23.92, and 70.42 (verapamil), respectively. Compared to drug-resistant cells, the RFs of 10 μM Syl611 on parental cells were very low than drug-resistant cells: the RFs of A549, KB, and Bel7402 were 4.30, 8.10, and 1.22, respectively (Fig. 3a). The cytotoxicity enhancements of Syl611 on vincristine and 5-FU in their resistant cells KB/V and Bel7402/5-FU were checked in parallel. Syl611 obviously increased the cytotoxicity of vincristine (6.35, 49.83, and 55.39) and 5-FU (22.93, 54.73, and 65.34). The cytotoxicity enhancements of Syl611 were stronger than 10 μM verapamil (7.39 vincristine, 6.42 5-FU). Homogeneously, Syl611 showed very low modulating actions of vincristine on KB (6.38) and 5-FU on Bel7402 (1.07) (Fig. 3b).

The expression levels of P-gp in the three tumor cells were then determined via Western blotting. Results showed that Bel7402/5-FU had the highest level of expression for P-gp in these cells, and the level of expression for A549/Paclitaxel was the lowest. This notion was similar to the RF index results and indicated that the reversing effect of Syl611 was related to the P-gp (Fig. 3c).

Syl611 treatment increased paclitaxel-induced apoptosis in drug-resistant A549/Paclitaxel cells

It has been well known that paclitaxel can facilitate cell apoptosis by disturbing microtubule function during cell cycle progression [20]. We examined the effect of Syl611 on the morphological characterization of A549/Paclitaxel cells. A549/Paclitaxel cells were treated with 0.1 μM paclitaxel alone or in combination with 10 μM verapamil or Syl611 for 48 h (Fig. 4). Untreated A549/Paclitaxel cells displayed normal cell conformation with organized chromatin structure, an intact cytoplasm and rounded cell nuclei (Fig. 4a). Likewise, the treatment of 10 μM Syl611 alone did not affect the nuclear morphology of A549/Paclitaxel cells, and nuclei displayed no obvious change (Fig. 4b). On the other hand, a concentration of 0.1 μM paclitaxel led to a decrease in cell number and observable morphological changes. These resulting morphological changes presented as typical apoptotic features: cell shrinkage, chromatin condensation, and nuclear fragmentation (Fig. 4c). When treated with the combination of 10 μM verapamil (Fig. 4d) or Syl611 (Fig. 4e) with 0.1 μM paclitaxel, there were greater deceases in cell number and more serious apoptotic features. And even cell death was observed compared with paclitaxel alone. According to the Hotz et al., apoptosis ratio was detected; Syl611 (72.2%) and verapamil (47.8%) all induced a

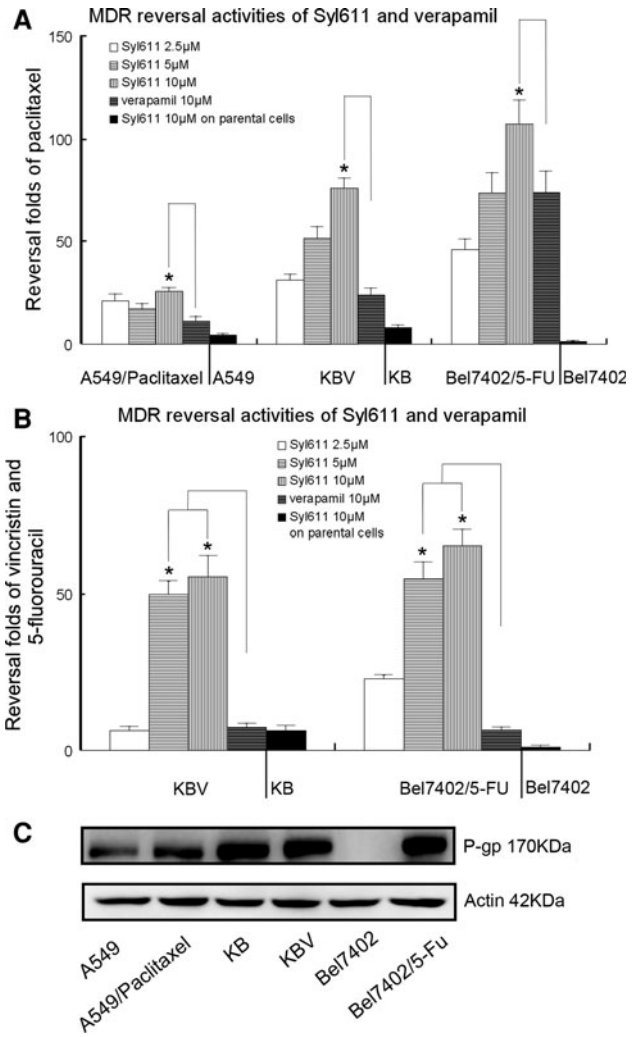

Fig. 3 Syl611 treatment increased paclitaxel-induced cytotoxicity in drug-resistant A549/Paclitaxel, KB/V, and Bel7402/5-FU cells. The action was not obvious in parental cells (**a**), Syl611 also increased the sensitivities of KB/V and Bel7402/5-FU to vincristine and 5-FU (**b**). Fold index (RF) was used as the index for MDR-reversing activities. RF index represents means ± SD of three independent experiments. The statistical difference between the Syl611-treated group and positive control was determined by one-way ANOVA (*$P < 0.05$). The expression levels of P-gp were measured by Western blotting (**c**)

greater level of apoptosis when they were used in combination with paclitaxel (Fig. 4f). The same effect of Syl611 with verapamil demonstrated that Syl611 might increase the potential of paclitaxel by allowing its intracellular accumulation, due to the inhibition of P-gp function.

Syl611 increased the accumulation of Rh123 in KB/V cells without affecting P-gp expression

The MDR phenotype is characterized by decreased accumulation and enhanced efflux of anticancer drugs, which is reversible upon treatment with P-gp inhibitors such as verapamil and cyclosporin. To find the MDR-reversing

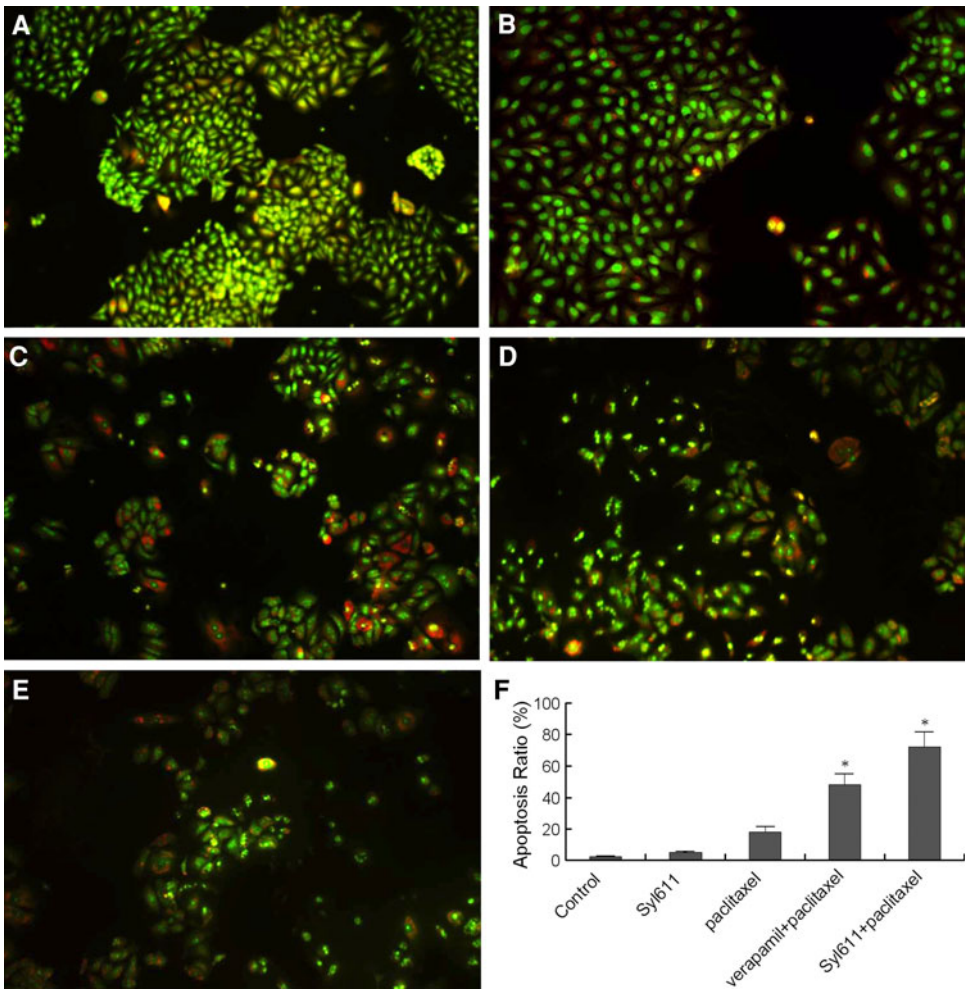

Fig. 4 Syl611 treatment increased paclitaxel-induced apoptosis in drug-resistant A549/Paclitaxel cells. Untreated A549/Paclitaxel cells displayed normal cell conformation with organized chromatin structure, intact cytoplasm, and rounded cell nuclei (**a**). Cells were treated with 10 μM Syl611 alone (**b**) or 0.1 μM paclitaxel alone (**c**) or in combination with 0.1 μM paclitaxel and 10 μM verapamil or Syl611 for 48 h (**d**, **e**). After fixation, cells were stained with AO/EB and cell morphological characterization was analyzed using a fluorescence microscope. The apoptosis ratio was calculated according to the "Materials and methods" (×100 magnifications)

mechanism of Syl611, we chose the KB/V cells (which have higher levels of P-gp expression) as the drug-resistant model. The results of RT–PCR and Western blotting showed that Syl611 treatment (10 μM) of KB/V cells could not alter the expression levels of P-gp mRNA and protein (Fig. 5a). The accumulation of P-gp's substrate Rh123, which was detected by flow cytometry, demonstrated the drug efflux function of P-gp. Relative fluorescence intensity was used to denote the effects. As shown in Fig. 5b, Syl611 increased the accumulation of Rh123 in KB/V cells compared with control and verapamil-treated cells. The enhancement of accumulation due to 10 μM Syl611 (943) was significantly higher than from treatment with 10 μM verapamil (149.8). Furthermore, we evaluated gradient concentrations of Syl611 on the accumulation of Rh123 in KB/V cells (Fig. 5c). The relative fluorescence intensity curve of Syl611 showed as a sigmoidal curve. In addition, the accumulation was reversible: the intracellular amount of Rh123 fell back (Fig. 5c) after cells incubated with PBS for 1 h. To determine whether Syl611 have the same fluorescence characteristics as Rh123, so to prevent false-positive results, we identified the fluorescence character of Syl611. The results showed that Syl611 cannot form similar fluorescence absorption compared with Rh123. Rh123 had a maximum fluorescence absorption wavelength at about 530 nm, and Syl611 cannot form fluorescence absorption in the same wavelength range between 510 and 600 nm.

Syl611 increased the bidirectional permeability of paclitaxel in Caco-2 monolayer model but decreased the efflux ratio

The bidirectional permeability assay is regarded as the gold standard in identifying P-gp substrates and also used for measuring the characteristics of P-gp inhibitors. As a supplement methods for detecting the functional mechanism of Syl611's reversal ability, the results in the presence showed that Syl611 unexpectedly increased the bidirectional permeability of paclitaxel that was completely different with the action of verapamil. As shown in Fig. 6a, paclitaxel (50 μM) is typically a P-gp substrate because of the obvious transportation from BL to AP and almost not from AP to BL. Verapamil (50 and 100 μM) could inhibit the permeability

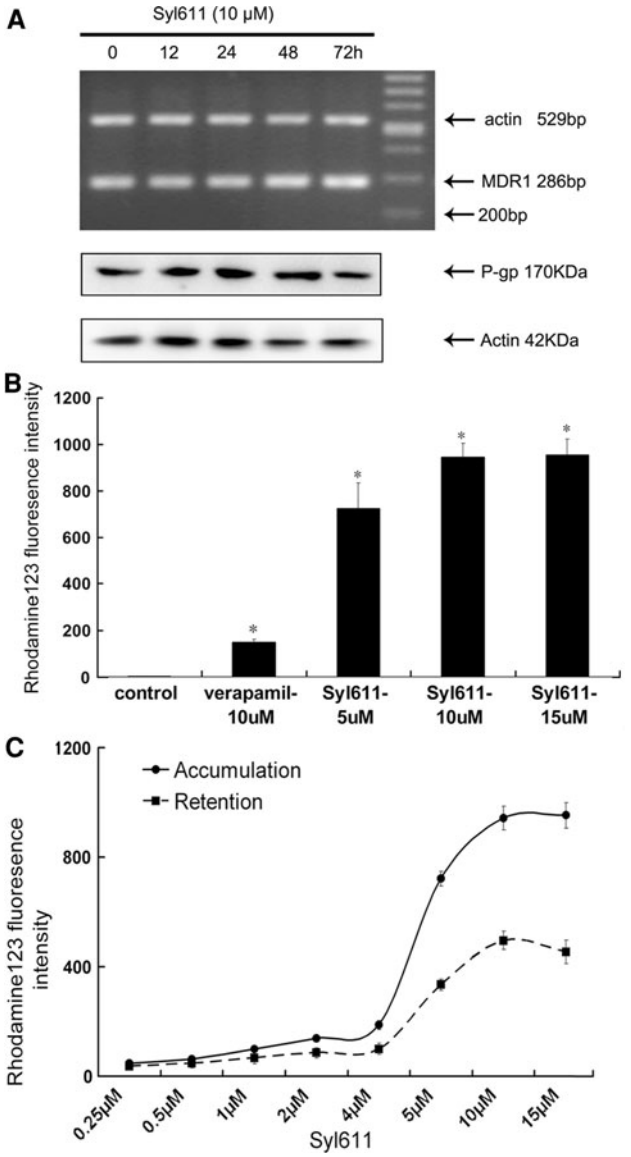

Fig. 5 Syl611 treatment increased the accumulation of Rh123 in KB/V cells without affecting P-gp expression. After treatment of KB/V cells with 10 μM Syl611 for indicated time points, the expression level of MDR1 and P-gp was analyzed by RT–PCR and Western blotting (**a**). The relative fluorescence intensity of intracellular Rh123 was determined as described in "Material and methods" and represented means ± SD of three independent experiments. The statistical difference was determined by one-way ANOVA (*$P < 0.05$) (**b**, **c**)

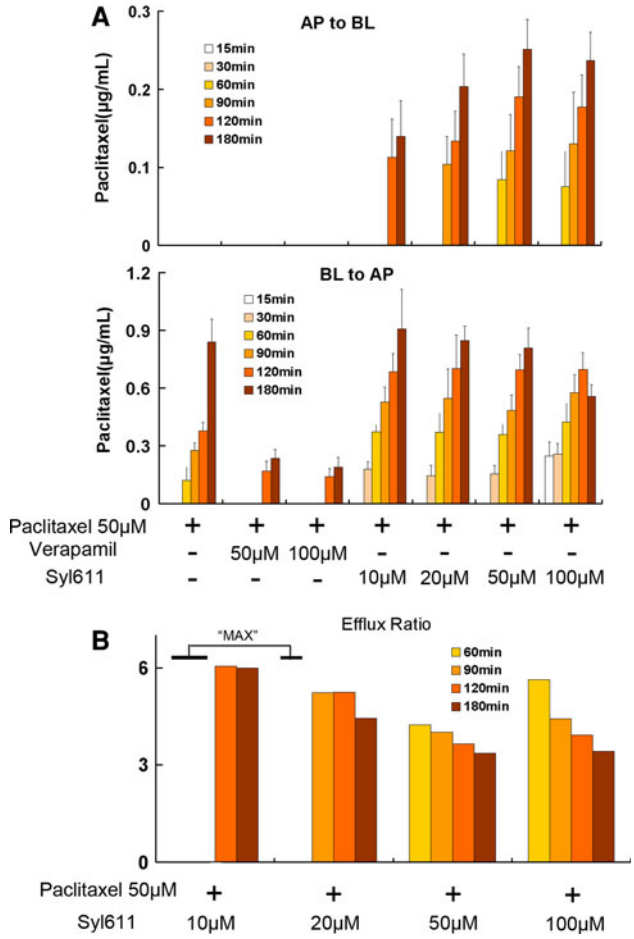

Fig. 6 Syl611 treatment increased the bidirectional permeability but decreased the efflux ratio of paclitaxel in Caco-2 monolayer model. Paclitaxel (50 μM) alone and combined with verapamil (50, 100 μM) or Syl611 (10, 20, 50, 100 μM) were treated on the Caco-2 monolayer in the basolateral or apical side. The concentration of paclitaxel in the opposite was tested at the indicated time point (15, 30, 60, 90, 120, 180 min) and represented means ± SD of three independent experiments (**a**). Efflux ratio (**b**) was the ratio of BL to AP and AP to BL, the amount of AP to BL in Syl611 10 μM at 60, 90 min and Syl611 20 μM at 60 min almost could not be detected, so the efflux ratio was indicated by "MAX". The ratios before 60 min were not showed here on the condition that they all are "MAX"

of paclitaxel from BL to AP but Syl611 gradually increased the bidirectional transportation along with the increased dosage (10, 20, 50, and 100 μM) and time lapse (15, 30, 60, 90, 120, and 180 min). Especially, in the absorptive direction phase (AP to BL), bigger dosage of Syl611 could distinctly bring forward the appearance of paclitaxel in basolateral side. In the results of Fig. 6b, Syl611 dramatically decreased the efflux ratio (ratio of BL to AP/AP to BL) in concentration-dependent and time-dependent manner. The efflux ratio indicated the bidirectional transport speed that Syl611 could decrease the net secretory of paclitaxel. Similarly, with Rh123 accumulation assay, the action of large dosage maintained saturation effect. These results would be the circumstantial evidence of Rh123 accumulation assay for accumulated intracellular paclitaxel. In order to eliminate the false-positive detection of paclitaxel, we found that the UV absorption peak of Syl611 was at 278.16 nm and the retention time was different from paclitaxel in the same test condition of HPLC–UV.

Discussion

In the past five decades, the usage of anticancer agents has become one of the most important ways of controlling malignant cancers. However, the development of multidrug resistance to anticancer agents among patients continues to pose a serious obstacle to cancer chemotherapy and those medical professionals and researchers who investigate it. The overexpression of P-gp, which is a membrane protein transporting drug, is the main mechanical cause of MDR. The reversing of MDR would be the key for overcoming drug resistance in modern cancer therapy. Currently, many MDR-reversing agents such as synthetic chemicals and natural products have been developed for anticancer therapy, but most of these products have been abolished during clinical usage for their toxicity in crux organs [8]. Thus, finding novel interference form on drug efflux effect from P-gp would be the indication of the next generation of P-gp inhibitors. The present study shows that Syl611, which is semisynthesized from the biosynthetic taxane, Sinenxan A, can lead to the reversal of MDR phenotype by inhibiting P-gp's function and facilitating inward transmembrane action.

Our data showed that Syl611 is devoid of cytotoxicity in three varieties of tumor cell lines and their parental drug-resistant cells in vitro. Syl611 has the same diterpenoid structure with paclitaxel, but the replacement and modification of the taxane structure resulted in opposite cytotoxicity. The low cytotoxicity provides the foundation property for Syl611 because the MDR agents would better not bring in side effects such as cytotoxicity effects. Several taxane-based chemicals possessing excellent MDR reversal abilities have been reported by Ojima et al. [21, 22], and modification of the special position brings about high potency against drug efflux mediated by P-glycoprotein, these research results boost our confidence to find new chemicals. Based on the abundant developing experience on taxane derivatives in our institute, Syl611 was screened out and obtained from new structural modification.

In the MDR-reversing assay, Syl611 increased the cytotoxicity of paclitaxel in a dose-dependent manner. Ten micromolar Syl611 maintained a greater than fourfold difference in RF values than the positive control verapamil in A549/Paclitaxel cell line, more than threefold in KB/V cell line and 1.5-fold in Bel7402/5-FU cell line. P-gp level in Bel7402/5-FU cells was highest in those three cell lines and that in A549/paclitaxel was the lowest. Homogeneously, the RF value in Bel7402/5-FU was the highest and the A549/Paclitaxel was the lowest in value. Furthermore, Syl611 showed the similar modulating action of vincristine and 5-FU on their resistant cells, the levels of reversing activities were associated with the amount of P-gp. Vincristine and 5-FU were also the substrate of P-gp. Consequently, this notion indicated that the reversing abilities of Syl611 are relative to the membrane drug efflux protein P-gp. The AO/EB double staining assay showed that Syl611 could enhance the cytotoxicity induced by paclitaxel. In drug-resistant A549/paclitaxel cells, the impressive low cytotoxicity of Syl611 did not show any changes between Syl611-treated drug-resistant cells and the untreated control (rounded nuclear with green fluorescence and orange in cytoplasm, complete cell size). However, when Syl611 and paclitaxel were combined to contact cells, the toxicity enhanced and was observed as an increase in apoptosis. Such apoptosis was revealed in the form of the nuclear concentration (anomalous nuclear with green fluorescence), the shrinkage of cell size, and even the necrosis of cells (red fluorescence with shrink size). These phenomena confirmed the results of the MTT assay and indicated that Syl611 could increase the intracellular accumulation of paclitaxel. All of the former results led us to believe that the P-gp would be the target of Syl611's reversing mechanism, but how did Syl611 act on P-gp?

It is unlikely that the MDR-reversing activity attributes to the diminution of P-gp, as Syl611 did not change the expression of P-gp and its mRNA. As shown in Fig. 5b, Syl611 is very potent at reversing the accumulation deficit and blocking the efflux of Rh123 from P-gp-overexpressed cell line KB/V. Furthermore, the accumulation abilities of gradient concentration Syl611 presented as a sigmoidal curve demonstrating the dose saturate property of the effect. According to the dose–response relationship of Syl611 and Thomas's review [8], we hypothesize that Syl611 may bond to P-gp and take up the functional position, sequentially disturbing the efflux of drugs. This hypothesis would need further researched in relation to its chemical structure and protein affinity. The finding that the result was not affected by the Sy611's own fluorescence characterization further indicated that the reverse of drug resistance by Syl611 is probably attributable to the inhibition of P-gp-mediated drug efflux. Meanwhile, the accumulation of Rh123 was reversible, which response the mechanism of Syl611 and low side effects.

In the bidirectional permeability assay, Syl611 unexpectedly increased the transmembrane of paclitaxel in Caco-2 monolayer model, but the net secretory amount of paclitaxel was decreased, these results gave corroborative evidence of the increasing accumulation of intracellular paclitaxel in Rh123 accumulation assay. In the drug-resistant cells, drug permeability crossing the cell membrane is in the same manner with monolayer model cells, but the extracellular concentration is much higher than intracellular. The net secretory of paclitaxel that goes down would obviously increase the intracellular accumulation. Drug transportation across the cell membrane is a complex multi-pathway process. In the Caco-2 model, besides the

function of P-gp or other efflux pumps, passive transcellular, active transcellular, facilitated diffusion, paracellular, and so on would mediate the drug transportation together. Besides the interference of P-gp, Syl611 also manifested a facilitated inward transmembrane action of paclitaxel to increase the intracellular accumulation of paclitaxel. Additionally, the action of large dosage maintained saturation effect. The phenomena were similar with Rh123 accumulation assay thus the following research on P-gp affinity of Syl611 is the key to reveal the mechanism of action. Consequently, Syl611 would be used as a novel and effective agent for improving oral administration obfuscation of paclitaxel, which exhibits a well developing perspective.

In conclusion, our current study demonstrates that Syl611, a novel semisynthetic taxane derivative, is efficacious in reversing MDR by combined action, which attributes to the inhibition of the drug efflux function of P-gp and facilitation inward transmembrane action. The low cytotoxicity, non-interference on the expression of P-gp, and reversible blocking action of P-gp suggest its favorable pharmacology profiles. Our studies suggest that Syl611 is an attractive chemotherapeutic agent for treating malignant tumors with P-gp-mediated MDR, Therefore, in order to follow up the results obtained about Syl611 and Sinenxan A derivatives, it is advised that further research be taken to investigate Syl611 and other potential MDR reversal agents.

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