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Marine sponge-derived sipholane triterpenoids reverse P-glycoprotein (ABCB1)-mediated multidrug resistance in cancer cells

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ABSTRACT

Previously, we reported sipholenol A, a sipholane triterpenoid from the Red Sea sponge *Callyspongia siphonella*, as a potent reversal of multidrug resistance (MDR) in cancer cells that overexpressed P-glycoprotein (P-gp). Through extensive screening of several related sipholane triterpenoids that have been isolated from the same sponge, we identified sipholenone E, sipholenol L and siphonellinol D as potent reversals of MDR in cancer cells. These compounds enhanced the cytotoxicity of several P-gp substrate anticancer drugs, including colchicine, vinblastine and paclitaxel, and significantly reversed the MDR-phenotype in P-gp-overexpressing MDR cancer cells KB-C2 in a dose-dependent manner. Moreover, these three sipholanes had no effect on the response to cytotoxic agents in cells lacking P-gp expression or expressing MRP1 (ABCC1) or MRP7 (ABCC10) or breast cancer resistance protein (BCRP/ABCG2). All three sipholanes (IC_{50} >50 μM) were not toxic to all the cell lines that were used. [^{3}H]-Paclitaxel accumulation and efflux studies demonstrated that all three triterpenoids time-dependently increased the intracellular accumulation of [^{3}H]-paclitaxel by directly inhibiting P-gp-mediated drug efflux. Sipholanes also inhibited calcein-AM transport from P-gp-overexpressing cells. The Western blot analysis revealed that these three triterpenoids did not alter the expression of P-gp. However, they stimulated P-gp ATPase activity in a concentration-dependent manner and inhibited the photolabeling of this transporter with its transport substrate [^{125}I]-iodoarylazidoprazosin. In silico molecular docking aided the virtual identification of ligand binding sites of these compounds. In conclusion, sipholane triterpenoids efficiently inhibit the function of P-gp through direct interactions and may represent potential reversal agents for the treatment of MDR.

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1. Introduction

One of the main impediments to the successful treatment of cancer is the development of resistant cancer cell variants [1,2]. Resistance can occur to particular cytotoxic drugs, but can also occur to various drugs with different chemical structures and mechanisms of action. This second form of resistance is called multidrug resistance (MDR). Overexpression of ABCB1/P-glycoprotein (P-gp) is one of the most common causes of MDR in cancer cells. P-gp, a 170-kD transmembrane glycoprotein encoded by the human *MDR1* (ABCB1) gene, is a member of the ATP-binding cassette (ABC) transporters family. P-gp is composed of two homologous halves, each containing six transmembrane domains and an ATP binding/utilization domain, separated by a flexible linker polypeptide. It functions as a drug efflux pump that extrudes a wide spectrum of compounds that are hydrophobic, amphipathic natural product drugs. This process of transport is coupled to the energy provided by ATP hydrolysis via the ATPase domains of P-gp that are stimulated in the presence of transport substrates [3]. Examples of P-gp substrates include Vinca alkaloids (vincristine, vinblastine), anthracyclines (doxorubicin, daunorubicin, epirubicin), epipodophyllotoxins (etoposide, teniposide) and taxanes [4]. As a result, inhibition of P-gp-mediated drug efflux may re-sensitize MDR cancer cells to an effective MDR tumor treatment with chemotherapeutic agents.

Presently, three generations of P-gp inhibitors have been developed to enhance the effect of chemotherapeutic drugs on

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MDR cancer cells in *vitro* and *in vivo* [5–8]. The first-generation P-gp inhibitors, including verapamil (calcium channel blocker), quinine (antimalarial), cyclosporin A (immunosuppressant) [9], tamoxifen (anti-steroid) produced disappointing results *in vivo* because their low binding affinities necessitated the use of high doses, resulting in high toxicity to normal cells. The second-generation P-gp inhibitors constituted drugs that were designed by modification of the first-generation inhibitors and such modifications were aimed at reducing their adverse effects. Some second-generation inhibitors included PSC-833 [10] (a non-immunosuppressive analogue of cyclosporin A) and *R*-verapamil [11] (*R*-enantiomer of verapamil, a weaker calcium channel blocker). Even these drugs did not have the desired range of efficacy due to their low affinity for their target transporter proteins and thus another generation of P-gp inhibitors was developed. This third-generation of P-gp inhibitors are designed specifically for high transport affinity and low pharmacokinetic interactions. These include the anthranilamide derivative tariquidar (XR-9576) [12,13], the dibenzosuberane derivative zosuquidar (LY335979) [14,15] the benzazepine derivative laniquidar (R101933) [16] and the substituted diarylimidazole ONT-093 [17,18]. Despite having diverse chemical structures and origins, these agents have in common a high potency, affinity and selectivity for the P-gp transporter at low nanomolar range and subsequently low toxicity toward normal cells.

The development of marine compounds is one of the most important approach of global drug discovery and development due to the fact that the marine ecosystem has an abundant number of species. In the last three decades, thousands of new marine compounds and their derivatives have been discovered and many of them appear to show effective anticancer activity. A number of marine compounds are currently in various phases of human clinical trials to treat different cancers, such as ecteinascidin-743 [19–21], synthadotin [22,23], bryostatin-1 [24,25] and kahalalide F [26,27], etc. Of great interest, is another class of marine compounds known as sipholane triterpenoids that were isolated from the Red Sea sponge *Callyspongia siphonella*. Earlier, we reported the potent reversal of multidrug resistance (MDR) activity of the sipholane triterpene sipholenol A in P-gp-overexpressing cells [28,29]. The present study reports the potent ability of the three novel sipholane triterpenoids sipholenone E, sipholenol L and siphonellinol D to reverse P-gp-mediated MDR in cancer cells [30], and further investigate their reversal mechanisms.

2. Materials and methods

2.1. Materials

[^{3}H]-paclitaxel (37.9 Ci/mmol) was purchased from Moravek Biochemicals Inc (Brea, CA, USA). IAAP (2200 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA, USA). Sipholenone E (Fig. 1A), sipholenol L (Fig. 1B), siphonellinol D (Fig. 1C) and sipholenol J (Fig. 1D) were isolated from the Red Sea sponge *Callyspongia siphonella* as previously described [30]. MK571 was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Cepharanthine was generously given by Kakenshoyaku Co. (Tokyo, Japan). Fumitremorgin C (FTC) was synthesized by Thomas McCloud Developmental Therapeutics Program, Natural Products Extraction Laboratory, NCI, NIH (Bethesda, MD, USA). The monoclonal mouse antibody against P-gp (P7965), colchicine, vinblastine, paclitaxel, cisplatin, vincristine, mitoxantrone were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anti-GAPDH monoclonal antibody was obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). The secondary horseradish peroxidase-labeled anti-mouse IgG was acquired from Thermo Scientific Pierce (Rockford, IL).

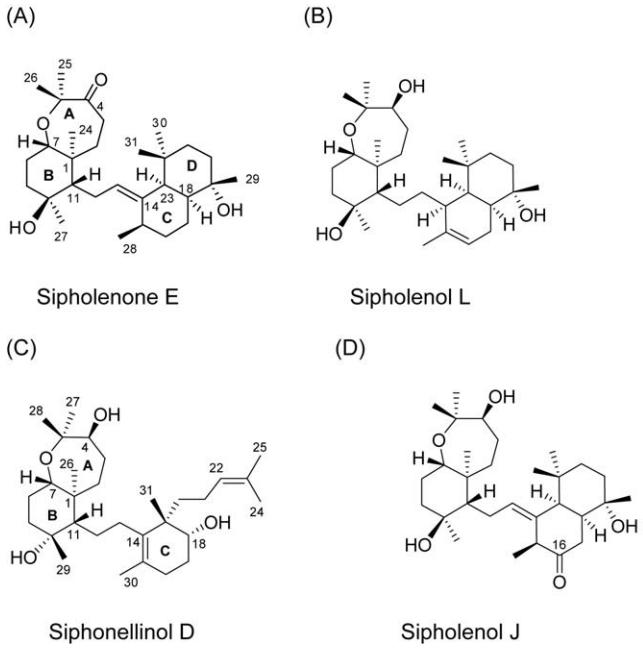

Fig. 1. The chemical structures of sipholenone E (A), sipholenol L (B), siphonellinol D (C) and sipholenol J (D).

2.2. Cell lines

The P-gp-overexpressing drug-resistant cell line KB-C2 was established from the human epidermoid carcinoma cell line KB-3-1 by exposing them to increasing concentrations of colchicine up to 2 μg/ml in a gradual manner [31]. Another P-gp-overexpressing MDR cell line, KB-V1, was also isolated from KB-3-1 cells and maintained in medium with 1 μg/ml of vinblastine. Both KB-3-1 and KB-V1 cells were kindly provided by Dr. Michael M. Gottesman (NCI, NIH, Bethesda). An MRP1-overexpressing MDR cell line KB-CV60, was also cloned from KB-3-1 cells and maintained in the medium with 1 μg/ml of cepharanthine and 60 ng/ml of vincristine. Both KB-C2 and KB-CV60 cells were generously given by Dr. Shin-ichi Akiyama (Kagoshima University, Japan). We also used transfected HEK293 with MRP7 expression vector (HEK-MRP7-C18) and parental vector-transfected control cells (HEK293-pcDNA3.1) previously described by Chen et al. [32]. HEK293/pcDNA3.1 and wild-type ABCG2-482-R2 cells were established by transfecting HEK293 with either the empty pcDNA3.1 vector or pcDNA3.1 vector containing the full length *ABCG2* coding arginine (R) at amino acid 482 and maintained in medium with 2 mg/ml of G418 [33]. All the cell lines were grown as adherent monolayers in flasks with DMEM culture medium (Hyclone Co., UT) supplemented with 10% bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator containing 5% CO_2 at 37 °C.

2.3. Cell cytotoxicity by MTT assay

Drug sensitivity was analyzed using an MTT colorimetric assay [34]. Cells were harvested with trypsin and resuspended in a final concentration of 2.5×10^4 cells/ml for KB-3-1 and 4×10^4 cells/ml for KB-C2 (and KB-V1) and 2.5×10^4 cells/ml for all the other cell lines. Cells were seeded into 96-well plates in triplicate. After incubation in DMEM supplemented with 10% bovine serum at 37 °C for 24 h, three different concentrations of each of sipholenone E, sipholenol L and siphonellinol D (10, 3 and 1 μM) were added 1 h prior to the addition of the anticancer drug. After 72 h of incubation, 20 μl of MTT solution (4 mg/ml) was added to each well, and the plate was further incubated for 4 h, allowing viable

cells to convert the yellow-colored MTT into dark-blue formazan crystals. Then, the medium was discarded, and 100 μl of dimethylsulfoxide (DMSO) was added into each well to dissolve the formazan crystals. The absorbance was determined at 570 nm by an OPSYS microplate Reader from DYNEX Technologies, Inc (Chantilly, VA). The degree of resistance was calculated by dividing the IC_{50} of the MDR cells by that of the parental sensitive cells. The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from survival curves using the Bliss method [35].

2.4. [^{3}H]-paclitaxel accumulation and efflux

Accumulation of paclitaxel in KB-3-1 and KB-C2 was measured using [^{3}H]-paclitaxel as previously described [36,37]. The confluent cells in 24-well plates were preincubated with or without the reversal agents (sipholenone E, sipholenol L, siphonellinol D and verapamil) for 1 h at 37 °C. To measure drug accumulation, the cells were then incubated with 0.1 μM [^{3}H]-paclitaxel in the presence or absence of the reversal agents for 2 h at 37 °C. To measure drug efflux, the cells were treated the same as in the drug accumulation experiment and then incubated in the fresh medium at 37 °C at various times in the presence or absence of the reversal agents. After washing three times with ice-cold PBS, the cells were trypsinized and placed in scintillation fluid to measure the radioactivity by a Packard TRI-CARB 1900CA liquid scintillation counter (Packard Instrument Inc., Downers Grove, IL). Also, a duplicate set of 24-well plates were seeded and used for cell counting.

2.5. Fluorescent drug accumulation assay

Efflux assays were carried out using a FACSort flow cytometer equipped with Cell Quest software (Becton-Dickinson, Franklyn Lakes, NJ) as described previously [38,39]. Fluorescent calcein was used to study P-gp-mediated efflux. Briefly, cells were harvested after trypsinization by centrifugation at 500 × *g* and then resuspended in IMDM (Gibco, Invitrogen) supplemented with 5% fetal bovine serum. 0.25 μM of calcein-AM was added to 3×10^5 cells in 4 ml of IMDM in the presence or absence of tested compound or the known inhibitors. In the efflux studies, the cells were incubated in a 37 °C water bath in the dark for 10 min prior to being pelleted by centrifugation at 500 × *g*. The cell pellet was then suspended in 300 μl PBS containing 0.1% FBS and analyzed immediately by flow cytometry.

2.6. Preparation of total cell lysates

Cells in T-25 flask treated with sipholenone E, sipholenol L or siphonellinol D for different time periods (0, 36, 72 h), then were harvested and rinsed twice with cold PBS. The cell extracts were prepared by incubating the cells with Radioimmunoprecipitation assay (RIPA) buffer [1 × PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 μM leupeptin, 100 μM *p*-aminophenyl-methylsulfonyl fluoride (*p*-APMSF), and 10 μM aprotinin] for 30 min on ice with occasional rocking, followed by centrifugation at 12,000 rpm at 4 °C for 15 min. The supernatant containing total cell lysates was stored at −80 °C until future experiments. The protein concentration was determined by bicinchoninic Acid (BCA™)-based protein assay (Thermo Scientific, Rockford, IL).

2.7. Immunoblotting

Equal amounts of total cell lysates (50 μg of protein) were resolved by 4–12% sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto PVDF membranes. After being incubated in blocking solution (5% skim milk in TBST) to block nonspecific binding for 1 h at room temperature, the membranes were immunoblotted overnight with primary antibodies anti-P-gp (1:400 dilution) and anti-GAPDH (1:500 dilution) at 4 °C. The next day, the membranes were washed three times with TBST buffer (0.3% Tris, 0.8% NaCl, 0.02% KCl, 0.05% Tween 20) and then incubated for 3 h with horseradish peroxidase (HRP)-conjugated secondary anti-mouse IgG for P-gp (1:1000 dilution). Protein-antibody complexes were detected by enhanced chemoluminescence detection system (Amersham, NJ). GAPDH was used to confirm that the cell lysates were equally loaded in each well.

2.8. ATPase assay of P-gp

ATPase activities of P-gp in High Five insect cell crude membranes were measured by endpoint P_i assay as described previously [40,41,42]. P-gp-specific ATPase activity was recorded as vanadate (Vi)-sensitive ATPase activity. Briefly, the membrane vesicles (10 μg of protein) were incubated in ATPase assay buffer (50 mM MES-Tris, pH 6.8, 50 mM KCl, 5 mM NaN_3, 1 mM EGTA, 1 mM ouabain, 2 mM dithiothreitol and 10 mM $MgCl_2$) over 20 min at 37 °C in the absence and presence of 0.3 mM vanadate or 2.5 mM NaF and 0.2 mM beryllium sulfate. The reaction was initiated by the addition of 5 mM ATP and terminated with SDS (2.5% final concentration). The amount of P_i released was quantified using a colorimetric method as described [40,41].

2.9. Photoaffinity labeling of P-gp with [^{125}I]-IAAP

The photoaffinity labeling of P-gp with [^{125}I]-IAAP was carried out as previously described [43]. Crude membranes (1 mg protein/ml) made from High five insect cells expressing P-gp were incubated with increasing concentrations of sipholenone E, sipholenol L or siphonellinol D for 10 min at room temperature in 50 mM Tris–HCl, pH 7.5, and then 3–6 nmol/l [^{125}I]-IAAP (2200 Ci/mmol) was added. The samples were incubated for an additional 5 min under subdued light. The samples were photo-cross-linked with UV lamp (365 nm) at room temperature for 10 min. The labeled samples were electrophoresed on SDS-PAGE in a 7% Tris-acetate NuPAGE gel; the gels were dried and exposed to Bio-Max MR film (Eastman Kodak Co.) at −80 °C. The radioactivity incorporated into P-gp band was quantified using the STORM 860 PhosphorImager system and ImageQuaNT (Molecular Dynamics).

2.10. Molecular modeling

Three-dimensional structure building and all modeling were carried out using components within the SYBYL program package; version 8.0 [44] installed on Dell desktop workstation equipped with dual 2.0 GHz Intel® Xeon® processor running the Red Hat Enterprise Linux (version 5) operating system. Energy minimizations were performed using the Tripos force field with a distance-dependent dielectric and the Powell conjugate gradient algorithm with a convergence criterion of 0.01 kcal/(mol-Å). Partial atomic charges were computed using semiempirical AM1 approach within the program MOPAC 6.0. P-gp structure was prepared using Biopolymer-Prepare protein structure-module within SYBYL 8.0; hydrogen atoms were added, protonation state of charged residues was inspected within the QZ59-RRR binding site of P-gp, and AMBER7 FF99 charges were loaded to P-gp residues. Molecular docking studies were conducted using Surflex-Dock version 2.0, interfaced with SYBYL 8.0. Surflex-Dock is a fully automatic flexible molecular docking algorithm that employs an idealized active site ligand (protomol) as a target to generate putative poses of molecules or molecular fragments. It combines the scoring function from the Hammerhead docking system with a search engine that relies on a surface-based molecular similarity method as a means to rapidly generate suitable putative poses for molecular fragments [45].

2.11. Statistical analysis

All experiments were repeated at least three times and differences were determined by using the Student's *t*-test. Results are presented as means ± standard deviations (SD). The statistical significance was determined at $P < 0.05$.

3. Results

3.1. Effect of sipholenone E, sipholenol L and siphonellinol D on the sensitivity of anticancer drugs in the P-gp-overexpressing MDR cancer cells

To establish the pharmacological characteristics of sipholenone E, sipholenol L and siphonellinol D, their cytotoxicity was first examined using MTT assay in three cancer cell lines: KB-3-1 and its drug-selected MDR cell line KB-C2 and KB-V1. Both KB-C2 and KB-V1 are highly expressing P-gp (Fig. 5A, and data not shown). The IC_{50} values of these compounds in three cell lines were more than 50 μM (data not shown). Then, whether sipholenone E, sipholenol L or siphonellinol D could potentiate the sensitivity of chemotherapeutic drugs in P-gp-overexpressing MDR cancer cells was determined. As shown in Table 1 and Supplementary Data (Fig. 1A–I), KB-C2 cells, compared to parental KB-3-1 cells, exhibited a significant resistance to various P-gp substrates such as colchicine (508-fold), vinblastine (13.6-fold) and paclitaxel (506-fold). The three sipholane triterpenoids at 1 μM, 3 μM and 10 μM, dose-dependently decreased the IC_{50} values of colchicine, vinblastine and paclitaxel of KB-C2 cells (Table 1) and KB-V1 (data not shown). In the parental KB-3-1 cells, the IC_{50} values of colchicine, vinblastine and paclitaxel with or without sipholenone E, sipholenol L and siphonellinol D showed no significant difference (Table 1). The effects of the P-gp inhibitor verapamil at 10 μM on increasing the cytotoxicity of colchicine, vinblastine and paclitaxel in KB-C2 and KB-V1 cells were moderately stronger than the three triterpenoids, while it had no significant effect on the parental KB-3-1 cells (Table 1). Though, when the non-P-gp substrate cisplatin was used, KB-3-1, KB-C2 and KB-V1 cells showed similar sensitivity to cisplatin, and none of the three sipholane triterpenoids changed the sensitivity of these three cell lines to cisplatin (Supplementary Data Fig. 1J–L and data not shown). These findings indicated that sipholenone E, sipholenol L and siphonellinol D enhanced the sensitivity of P-gp substrate anticancer drugs against KB-C2 and KB-V1 cells, whereas, had no effect on KB-3-1 cells, supporting the notion that these compounds could reverse P-glycoprotein-mediated resistance of KB-C2 and KB-V1 cells.

Table 1
Effect of sipholenone E, sipholenol L and siphonellinol D on the cytotoxicity of colchicine, vinblastine and paclitaxel in the P-gp overexpressing multidrug resistance (MDR) cancer cells.[a].

Compounds	IC_{50} ± SD[b] (nM)	
	KB-3-1	KB-C2
Colchicine	6.691 ± 0.076 (1.00)[c]	3402 ± 120.5 (508.44)
+Sipholenone E 1 μM	6.499 ± 0.069 (0.97)	551.9 ± 2.934 (82.48)
+Sipholenone E 3 μM	6.203 ± 0.075(0.93)	208.4 ± 4.365 (31.15)
+Sipholenone E 10 μM	6.032 ± 0.103 (0.90)	81.61 ± 1.647 (12.20)
+Sipholenol L 1 μM	6.412 ± 0.026 (0.96)	731.1 ± 5.644 (109.27)
+Sipholenol L 3 μM	6.034 ± 0.110 (0.90)	311.5 ± 3.931 (46.56)
+Sipholenol L 10 μM	5.567 ± 0.029 (0.83)	189.4 ± 2.375 (28.31)
+Siphonellinol D 1 μM	6.308 ± 0.052 (0.94)	783.0 ± 7.666 (117.2)
+Siphonellinol D 3 μM	6.117 ± 0.056 (0.91)	339.9 ± 1.694 (50.80)
+Siphonellinol D 10 μM	5.751 ± 0.110 (0.86)	218.4 ± 2.946 (32.64)
+Verapamil 10 μM	5.929 ± 0.048 (0.89)	37.04 ± 0.614 (5.54)
Vinblastine	52.58 ± 2.977 (1.00)[c]	712.8 ± 16.47 (13.56)
+Sipholenone E 1 μM	52.37 ± 2.455 (1.00)	188.4 ± 13.94 (3.58)
+Sipholenone E 3 μM	49.66 ± 2.414 (0.94)	118.6 ± 8.063 (2.26)
+Sipholenone E 10 μM	48.46 ± 2.898 ((0.92)	86.26 ± 6.733 (1.64)
+Sipholenol L 1 μM	53.98 ± 4.393 (1.03)	440.0 ± 7.082 (8.37)
+Sipholenol L 3 μM	51.54 ± 3.603 (0.98)	351.3 ± 8.003 (6.68)
+Sipholenol L 10 μM	49.78 ± 4.518 (0.95)	205.6 ± 5.156 (3.91)
+Siphonellinol D 1 μM	54.56 ± 5.246 (1.04)	471.8 ± 10.37 (8.97)
+Siphonellinol D 3 μM	52.33 ± 3.809 (1.00)	386.0 ± 12.42 (7.34)
+Siphonellinol D 10 μM	48.87 ± 3.517 (0.93)	236.1 ± 2.596 (4.49)
+Verapamil 10 μM	41.37 ± 2.108 (0.79)	64.29 ± 4.420 (1.22)
Paclitaxel	7.716 ± 0.221 (1.00)[c]	3908 ± 263.7 (506.48)
+Sipholenone E 1 μM	7.039 ± 0.282 (0.91)	406.5 ± 31.09 (52.68)
+Sipholenone E 3 μM	6.929 ± 0.264 (0.90)	130.4 ± 15.91 (16.90)
+Sipholenone E 10 μM	6.784 ± 0.137 (0.88)	45.33 ± 1.828 (5.87)
+Sipholenol L 1 μM	7.252 ± 0.057 (0.94)	938.1 ± 18.93 (121.58)
+Sipholenol L 3 μM	7.185 ± 0.046 (0.93)	191.8 ± 16.68 (24.86)
+Sipholenol L 10 μM	6.875 ± 0.255 (0.89)	106.8 ± 16.27 (13.84)
+Siphonellinol D 1 μM	7.309 ± 0.206 (0.95)	1059 ± 29.46 (137.25)
+Siphonellinol D 3 μM	7.016 ± 0.088 (0.91)	221.5 ± 21.66 (28.71)
+Siphonellinol D 10 μM	6.885 ± 0.168 (0.89)	130.5 ± 24.03 (16.91)
+Verapamil 10 μM	6.652 ± 0.279 (0.86)	19.04 ± 1.027 (2.47)

[a] Cell survival was determined by MTT assay as described in Section 2.
[b] Data are means ± SD of at least three independent experiments performed in triplicate.
[c] Fold-resistance was determined by dividing the IC_{50} values of colchicine, vinblastine and paclitaxel in KB-C2 cells in the absence or presence of reversal agents, or KB-3-1 cells with reversal agents, by the IC_{50} of colchicine, vinblastine and paclitaxel in KB-3-1 cells without reversal agents.

3.2. Effect of sipholenone E, sipholenol L and siphonellinol D on the sensitivity of anticancer drugs in the MRP1-, MRP7- or ABCG2-overexpressing MDR cancer cells

It was hypothesized that since P-gp shares some common substrates and functions with other members of the ABC family, modulators that overcome P-gp-linked MDR may also decrease MRP1-, MRP7-, and ABCG2-mediated drug resistance. Sipholenone E, sipholenol L and siphonellinol D significantly potentiated the sensitivity of P-gp substrate chemotherapeutic drugs in the P-gp-overexpressing MDR cancer cells. Afterward, it was determined whether these sipholanes could similarly increase the sensitivity of chemotherapeutic drugs in the MRP1-, MRP7- or ABCG2-overexpressing cells. The IC_{50} values of sipholenone E, sipholenol L and siphonellinol D in KB-CV60, HEK293-pcDNA3.1, HEK-MRP7-C18 and ABCG2-482-R2 cell lines were more than 50 μM (data not shown). As shown in Table 2, the overexpressing MRP1 KB-CV60 cells were highly resistant to vincristine, compared with the parental KB-3-1 cells. All three compounds at 10 μM did not change the sensitivity of vincristine in both KB-3-1 and KB-CV60 cells, but 2.5 μM of MK571, an inhibitor of MRP1 [46], greatly reduced the resistance of vincristine in KB-CV60 (Table 2). Meanwhile, HEK-MRP7-C18 showed high resistance to paclitaxel in comparison with the parental HEK293-pcDNA3.1. Although the three triterpenoids did not lower the IC_{50} values of paclitaxel in both cells, the MRP7 inhibitor cepharanthine at 2.5 μM [47] significantly decreased the resistance of paclitaxel in HEK-MRP7-C18 cells (Table 2). Likewise, compared with the parental HEK293-pcDNA3.1 cells, the wild-type ABCG2-482-R2 showed high resistance to mitoxantrone, and the three sipholanes had no effect on the IC_{50} values of mitoxantrone in both cells; though, the ABCG2 inhibitor FTC [48] at 2.5 μM completely reversed the resistance of mitoxantrone in ABCG2-482-R2 cells (Table 2). These data suggests that these three sipholane triterpenoids may be specific inhibitors of P-gp.

3.3. The effects of sipholenone E, sipholenol L and siphonellinol D on the intracellular accumulation of [^{3}H]-paclitaxel

In order to determine the effects of sipholenone E, sipholenol L and siphonellinol D on the function of P-gp as a drug-efflux pump, the accumulation of the P-gp substrate [^{3}H]-paclitaxel in the presence or absence of these three compounds was

Table 2
Effect of sipholenone E, sipholenol L, siphonellinol D on the cytotoxicity of vincristine, paclitaxel and mitoxantrone in the MRP1-, MRP7- and ABCG2-overexpressing multidrug resistance (MDR) cancer cells, respectively.[a].

Compounds	$IC_{50} \pm SD$[b] (nM)	
	KB-3-1	KB-CV60
Vincristine (nM)	7.436 ± 0.102 (1.00)[c]	278.8 ± 3.281 (37.49)
+Sipholenone E (10 μM)	7.196 ± 0.088 (0.97)	259.5 ± 2.932 (34.90)
+Sipholenol L (10 μM)	7.504 ± 0.106 (1.01)	307.1 ± 2.044 (41.30)
+Siphonellinol D (10 μM)	7.291 ± 0.067 (0.98)	254.3 ± 2.826 (34.20)
+Sipholenol J (10 μM)	7.619 ± 0.121 (1.02)	287.6 ± 2.457 (38.68)
+MK571 (2.5 μM)	6.112 ± 0.083 (0.82)	53.02 ± 1.12 (7.13)
Compounds	HEK293-pcDNA3.1	HEK-MRP7-C18
Paclitaxel (nM)	18.63 ± 0.067 (1.00)[d]	151.4 ± 2.632 (8.13)
+Sipholenone E (10 μM)	18.39 ± 0.161 (0.99)	142.2 ± 2.476 (7.63)
+Sipholenol L (10 μM)	18.39 ± 0.137 (0.99)	143.6 ± 2.677 (7.71)
+Siphonellinol D (10 μM)	18.53 ± 0.161 (0.99)	155.5 ± 4.134 (8.35)
+Sipholenol J (10 μM)	18.74 ± 0.059 (1.01)	157.0 ± 4.410 (8.43)
+Cepharanthine (2.5 μM)	15.52 ± 0.123 (0.83)	29.35 ± 0.262 (1.58)
Compounds	HEK293-pcDNA3.1	ABCG2-482-R2
Mitoxantrone (nM)	46.45 ± 1.044 (1.00)[e]	623.5 ± 9.597 (13.42)
+Sipholenone E (10 μM)	43.60 ± 1.126 (0.94)	619.1 ± 7.099 (13.33)
+Sipholenol L (10 μM)	44.94 ± 0.618 (0.97)	621.0 ± 5.693 (13.37)
+Siphonellinol D (10 μM)	48.93 ± 1.453 (1.05)	634.7 ± 8.284 (13.66)
+Sipholenol J (10 μM)	47.92 ± 2.048 (1.03)	640.7 ± 10.43 (13.79)
+FTC (2.5 μM)	40.10 ± 1.061 (0.86)	65.03 ± 2.963 (1.40)

[a] Cell survival was determined by MTT assay as described in Section 2.
[b] Data are means ± SD of at least three independent experiments performed in triplicate.
[c] Fold-resistance was determined by dividing the IC_{50} values of vincristine in KB-CV60 cells in the absence or presence of reversal agents, or KB-3-1 cells with reversal agents, by the IC_{50} of vincristine in KB-3-1 cells without reversal agents.
[d] Fold-resistance was determined by dividing the IC_{50} values of paclitaxel in HEK-MRP7-C18 cells in the absence or presence of reversal agents, or HEK293-pcDNA3.1 cells with reversal agents, by the IC_{50} of paclitaxel in HEK293-pcDNA3.1 cells without reversal agents.
[e] Fold-resistance was determined by dividing the IC_{50} values of mitoxantrone in ABCG2-482-R2 cells in the absence or presence of reversal agents, or HEK293-pcDNA3.1 cells with reversal agents, by the IC_{50} of mitoxantrone in HEK293-pcDNA3.1 cells without reversal agents.

measured. Our data showed that the intracellular concentration of [^{3}H]-paclitaxel in P-gp-overexpressing KB-C2 cells was significantly lower (34.24%) than that in KB-3-1 cells (Fig. 2). After the cells were incubated with either sipholenone E, sipholenol L or siphonellinol D at 10 μM for 2 h, intracellular [^{3}H]-paclitaxel accumulation was significantly increased in KB-C2 cells by 2.36-fold, 1.66-fold and 2.16-fold, respectively. Of the three compounds, sipholenone E produced the highest increase in the intracellular [^{3}H]-paclitaxel which was comparable to that of 10 μM of verapamil. However, the intracellular level of [^{3}H]-paclitaxel in KB-3-1 cells was not altered by either of the three sipholanes or verapamil (Fig. 2).

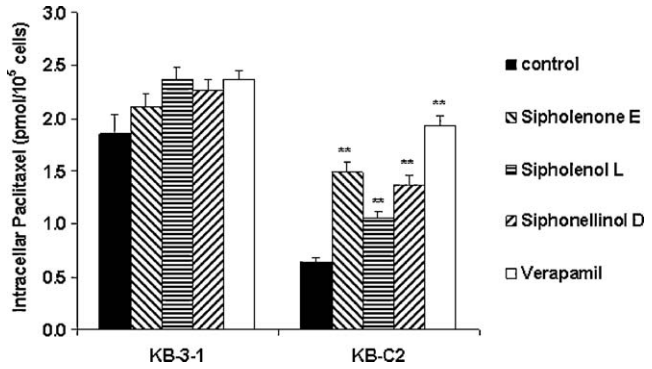

Fig. 2. The effects of sipholenone E, sipholenol L and siphonellinol D on the accumulation of [^{3}H]-paclitaxel in KB-3-1 and KB-C2 cells. The accumulation of [^{3}H]-paclitaxel was measured after preincubation with or without the reversal agents (10 μM) for 1 h at 37 °C and then incubation with 0.1 μM [^{3}H]-paclitaxel in the presence or absence of the reversal agents for 2 h at 37 °C. Afterwards, the cells were collected and the intracellular levels of [^{3}H]-paclitaxel were determined by scintillation counting. Verapamil was used as a positive control. Data points represent the means ± SD of triplicate determinations. Experiments were performed at least three independent times. $^*P < 0.05$ and $^{**}P < 0.01$, for values versus those in the control group.

3.4. The effects of sipholenone E, sipholenol L and siphonellinol D on the efflux of [^{3}H]-paclitaxel

To establish whether the increase in the intracellular [^{3}H]-paclitaxel accumulation caused by sipholenone E, sipholenol L and siphonellinol D was due to an inhibition of [^{3}H]-paclitaxel efflux, we performed a time course study to determine [^{3}H]-paclitaxel in the presence of each of these compounds. KB-C2 cells rapidly released intracellular [^{3}H]-paclitaxel, with increasing time compared with KB-3-1 cells. When the three sipholanes at 10 μM were added to both cells, they significantly blocked the intracellular [^{3}H]-paclitaxel efflux at different time periods (0, 30, 60, 120 min) from KB-C2 cells, but not in the parental KB-3-1 cells. The accumulation of [^{3}H]-paclitaxel at 0 min was set at 100%, and at 30, 60, 120 min, the percentages were 72.98%, 55.11%, 40.08%, respectively, of the intracellular [^{3}H]-paclitaxel that remained in KB-C2 cells in the absence of the three compounds. When KB-C2 cells were incubated with sipholenone E, the percentages at 30, 60 and 120 min increased to 82.31%, 68.67%, 64.32%, respectively (Fig. 3A). Sipholenol L increased the percentage of the intracellular remaining [^{3}H]-paclitaxel at 30, 60 and 120 min to 81.59%, 61.04% and 56.35%, respectively (Fig. 3B). Meanwhile, siphonellinol D, at 30, 60 and 120 min increased the percentage of [^{3}H]-paclitaxel accumulation to 82.74%, 63.57% and 58.93%, respectively (Fig. 3C). Sipholenone E was the most potent of the three compounds, which is consistent with the MTT colorimetric assay and [^{3}H]-paclitaxel accumulation experiment. In addition, sipholenone E had a closely similar effect to verapamil, the positive control, on the efflux of [^{3}H]-paclitaxel from KB-C2 cells.

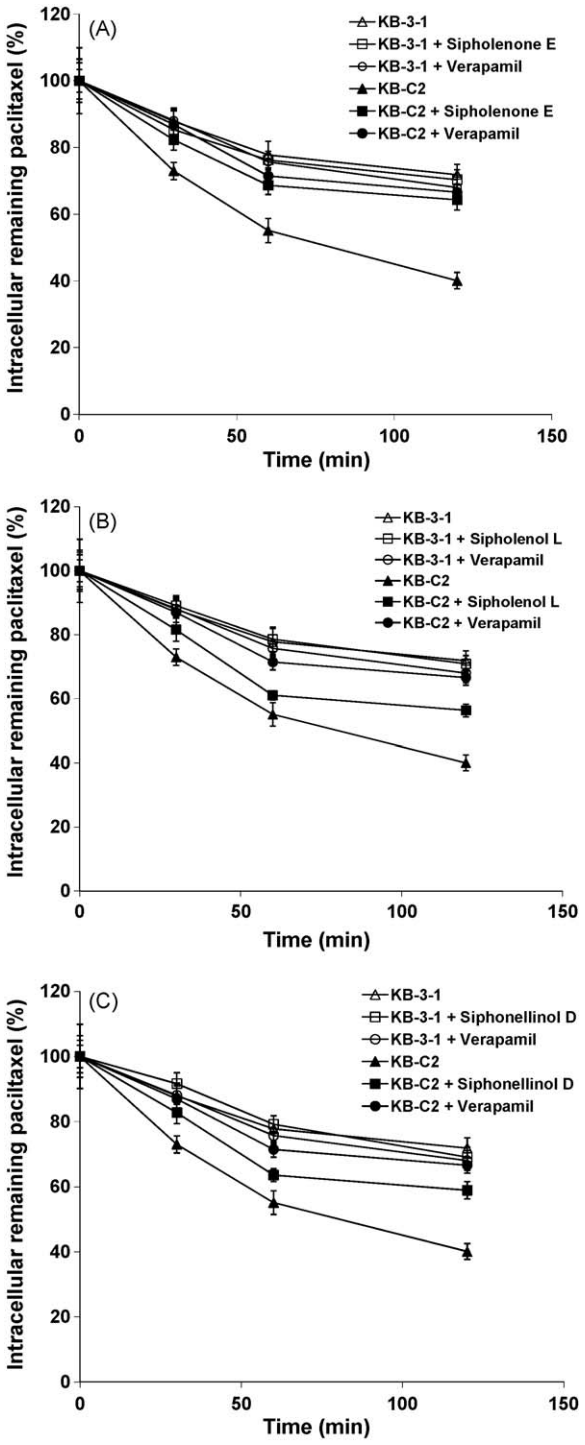

Fig. 3. The effects of sipholenone E, sipholenol L and siphonellinol D on the efflux of [^{3}H]-paclitaxel from KB-3-1 and KB-C2 cells. Cells were pre-treated with or without sipholenone E (A), sipholenol L (B) and siphonellinol D (C) at 10 μM for 1 h at 37 °C and further incubated with 0.1 μM [^{3}H]-paclitaxel at 37 °C for 2 h. Cells were then incubated in the fresh medium with or without the reversal agents for different time periods at 37 °C. Cells were then collected and the intracellular levels of [^{3}H]-paclitaxel were determined by scintillation counting. A time course versus percentage of intracellular [^{3}H]-paclitaxel was plotted (0, 30, 60, 120 min). Verapamil (10 μM) was used as a positive control. Data points represent the means ± SD of triplicate determinations. Experiments were performed at least three independent times.

3.5. Effect of sipholenone E, sipholenol L and siphonellinol D on the accumulation of fluorescent calcein

To determine whether sipholenone E, sipholenol L and siphonellinol D are able to inhibit P-gp-mediated efflux we performed FACS/calcein assay. Calcein-AM is a highly lipid-soluble dye that rapidly penetrates the plasma membrane, and which is converted to the hydrophilic fluorescent dye calcein by intracellular esterases. Whereas calcein-AM is a substrate of P-gp, the anionic calcein is not. Because the transporting capacity of P-gp is inversely proportional to the accumulation of intracellular calcein fluorescence, inhibition of P-gp will lead to increased accumulation of calcein. P-gp-overexpressing cells (3×10^5 cells) were incubated with the three sipholanes and tariquidar (XR-9576). Verapamil and sipholenol J were used as positive and negative controls, respectively. The fluorescence intensity of accumulated calcein was analysed by fluorescence-activated cell sorter (FACS). Sipholenone E, siphonellinol D and sipholenol L increased the intracellular calcein accumulation in P-gp-over-expressing cells in a concentration-dependent manner, with concentrations needed to achieve 50% inhibition of calcein accumulation between 10–20 μM, 20–50 μM, 50–100 μM, respectively (Fig. 4B). Siphonelinol J had no effect on the accumulation of intracellular calcein even at the highest concentration of 100 μM (Fig. 4B). Since, sipholenone E had the strongest inhibitory effect on P-gp-mediated efflux, we compared it to two known P-gp inhibitors, verapamil and tariquidar. As shown in Fig. 4A, sipholenone E at 50 μM increased the accumulation of intracellular calcein in P-gp-overexpressing cells more than verapamil at the same concentration of 50 μM. Meanwhile, tariquidar at 3 μM had similar effect on the calcein accumulation as sipholenone E at 50 μM (Fig. 4A). These results suggest that all three sipholane triterpenoids were able to inhibit the P-gp-mediated efflux, though sipholenone E proved to be the best compound at inhibiting the P-gp transport. This is consistent with the [^{3}H]-paclitaxel accumulation and efflux experiments.

3.6. Effect of sipholenone E, sipholenol L and siphonellinol D on the expression of P-gp

Reversal of P-gp-mediated MDR can be achieved by either decreasing P-gp expression or by inhibiting P-gp function. To evaluate the effect of sipholenone E, sipholenol L and siphonellinol D on P-gp expression, KB-C2 cells were treated with these three sipholanes at 10 μM for 0, 36 and 72 h. The result shown in Fig. 5B indicates that all three compounds do not alter the protein expression levels in KB-C2 cells at 10 μM concentration.

3.7. Effect of sipholenone E, sipholenol L and siphonellinol D on P-gp ATP hydrolysis

To examine whether sipholenone E, sipholenol L and siphonellinol D behave as P-gp substrates, we investigated the effect of these three compounds on vanadate (Vi)-sensitive P-gp ATPase activity (Fig. 6). In general, P-gp basal ATPase activity will be stimulated in the presence of P-gp substrate as P-gp utilizes energy derived from ATP hydrolysis to actively transport substrates across membranes. The assays were carried out using crude membranes isolated from P-gp expressing High Five insect cells in the absence or presence of sipholenone E, sipholenol L or siphonellinol D as described in Materials and Methods. Vi-sensitive P-gp ATPase activity was stimulated by sipholenone E, sipholenol L and siphonellinol D in a concentration-dependent manner with maximum stimulation of over 2.1-, 1.7- or 1.5-fold, respectively. The apparent K_m values for sipholenone E, sipholenol L or siphonellinol D were ~14 μM, 5 μM and 4 μM, respectively

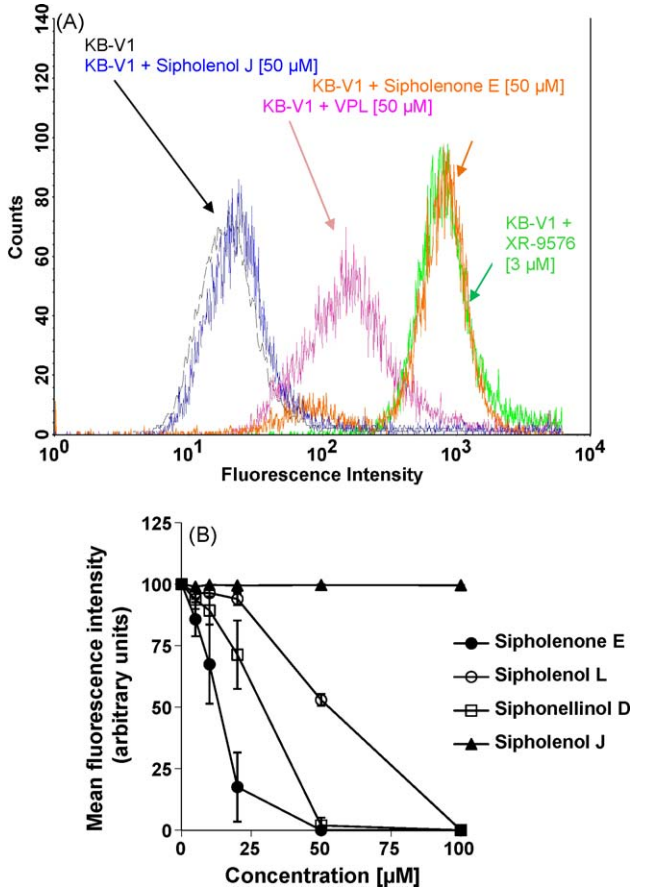

Fig. 4. Effect of sipholenone E, sipholenol L and siphonellinol D on the fluorescent calcein accumulation in P-gp-ovexpressing cells. Cells (KB-V1) were resuspended in IMDM supplemented with 5% fetal bovine serum. 0.25 μM calcein-AM was added to 3×10^5 cells in 4 ml of IMDM in the presence or absence of tested compound or the known inhibitors. The cells were incubated at 37 °C in the dark for 10 min. The cells were pelleted by centrifugation at 500 × *g* and resuspended in 300 μl PBS containing 0.1% FBS and analyzed immediately by using flow cytometry. (A) Light blue (light grey in the hard copy) line represents KB-V1, dark blue, pink, green, orange lines (dark grey in the hard copy) represent KB-V1 in the presence of 50 μM sipholenol J, 50 μM verapamil (VPL), 3 μM tariquidar (XR-9576) and 50 μM sipholenone E with arrows, respectively. Representative histograms of three independent experiments are shown. (B) Concentration-response curve where KB-V1 cells were treated with sipholenone E (●), sipholenol L (■), siphonellinol D (▲) and sipholenol J (◆) at various concentrations. Data points represent the means ± SD of triplicate determinations. Experiments were performed at least three independent times. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

(Fig. 6). Sipholenol J was used as a negative control. Our data suggest all three tested compounds behave as P-gp substrates.

3.8. Sipholenone E, sipholenol L or siphonellinol D inhibit photo cross-linking of [^{125}I]-IAAP to P-gp

To confirm the direct interaction of sipholenone E, sipholenol L and siphonellinol D to P-gp substrate binding site(s), we performed [^{125}I]-IAAP assay to examine the relative binding affinity to P-gp. [^{125}I]-IAAP is a photoaffinity analogue of prazosin that has been previously used to characterize the substrate-binding site(s) of P-gp. [^{125}I]-IAAP photolabels P-gp, but the labeling can be inhibited competitively by P-gp substrates or inhibitors [43]. The crude membranes from P-gp-expressing High five cells (Fig. 7A) were incubated with increasing concentrations of sipholenone E, sipholenol L and siphonellinol D at 21 °C for 10 min. [^{125}I]-IAAP (5 μM) was added into the reaction mixture and incubated for an additional 5 min under subdued light. Samples were then

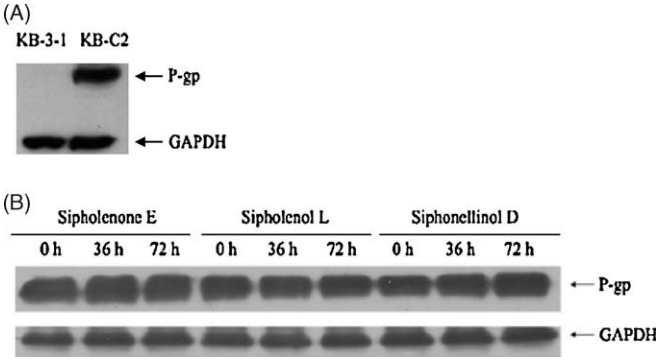

Fig. 5. Immunoblot detection of P-glycoprotein (P-gp) and the effect of sipholenone E, sipholenol L and siphonellinol D on P-glycoprotein (P-gp) expression. (A) Expression of P-gp in KB-3-1 and KB-C2 cells. (B) Effect of sipholenone E, sipholenol L and siphonellinol D at 10 μM on the expression level of P-gp in KB-C2 cells for 36 h and 72 h. Equal amounts (50 μg protein) of total cell lysate of each sample was loaded and separated using sodium dodecyl sulfate-polycrylamide gel electrophoresis. Then proteins were then transferred onto polyvinylidene fluoride membrane. The membranes were immunobloted overnight with primary antibodies against P-gp (1:400 dilution) and GAPDH (1:500 dilution) at 4 °C, then incubated for 3 h with horseradish peroxidase-conjugated secondary antibody at 1:1000 dilution. Protein-antibody complexes were detected by enhanced chemoluminescence detection system. GAPDH was used as an internal control for equal loading. Results from a representative experiment are shown. Similar results were obtained in two other trials.

illuminated with a UV lamp (365 nm) for 10 min as described in Section 2. Sipholenone E, sipholenol L and siphonellinol D inhibited the photolabeling of P-gp (Fig. 7B) in a concentration-dependent manner, with calculated IC_{50} values of 17.8 ± 3.8, 13.6 ± 3.1 and 10.7 ± 2.1 μM, respectively. The negative control, sipholenol J, did not inhibit the photolabeling of P-gp (Fig. 7A and B). Results demonstrated that all three sipholanes bind to P-gp substrate-binding site(s) with comparable affinities.

3.9. Identification of the in silico ligand binding site of sipholenone E, sipholenol L, siphonellinol D and sipholenol J by docking analysis

Aller et al. identified three binding sites in the crystallographic structure of P-gp; QZ59-RRR, QZ59-SSS and verapamil binding sites [49]. Sipholenone E, sipholenols L and J, and siphonellinol D were docked at each of these binding sites. The docking scores in the binding site of QZ59-RRR were found to be most consistent with inhibitory activity of sipholenols. QZ59 compounds are two stereoisomers of cyclic hexapeptide inhibitors of P-gp [49].

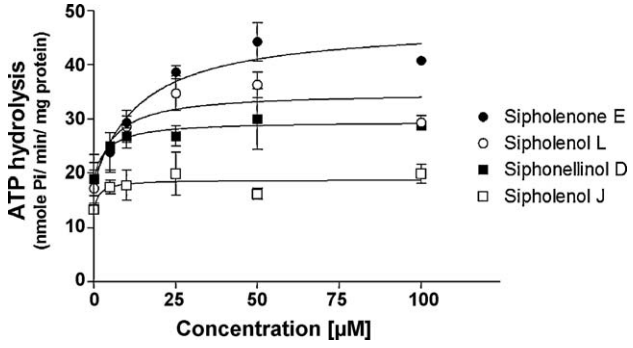

Fig. 6. Effect of sipholenone E, sipholenol L and siphonellinol D on the ATPase activity of P-gp. The vanadate-sensitive ATPase activity in crude membranes of High Five insect cells expressing P-gp was measured with different concentrations of sipholenone E, sipholenol L, siphonellinol D and sipholenol J as decribed in Section 2. Sipholenol J was used as a negative control. The lines represent the best fit for the data either by linear or non-linear least-squares regression analysis using GraphPad Prism version 2.0. Values are means and the error bars represent standard error from at least three independent experiments.

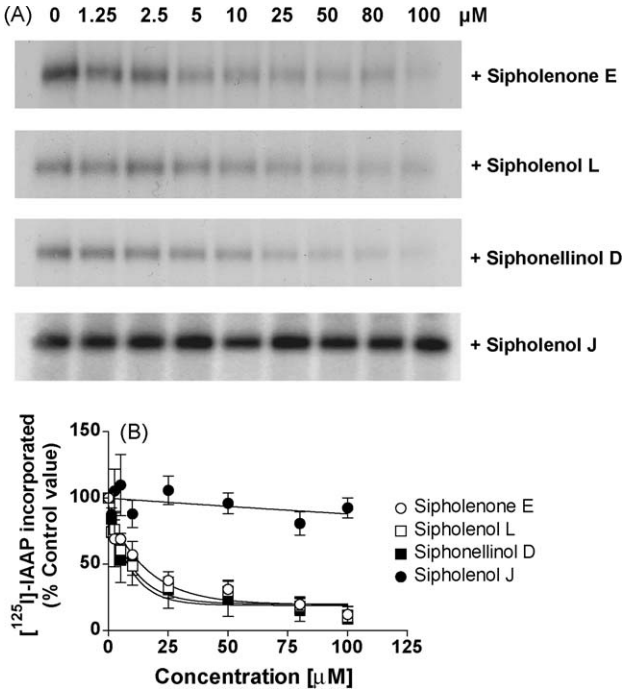

Fig. 7. Effect of sipholenone E, sipholenol L and siphonellinol D on the photoaffinity labeling of P-glycoprotein (P-gp) by [^{125}I]-iodoarylazidoprazosin (IAAP). The photoaffinity labeling of P-gp with [^{125}I]-IAAP (3–6 nmol/L) was performed in the presence of different concentrations of sipholenone E, sipholenol L and siphonellinol D. Sipholenol J was used as a negative control for photolabeling of P-gp by [^{125}I]-IAAP. The radioactivity incorporated into P-gp was determined by exposing the gels to X-ray films at −80 °C. (A) Autoradiograms of sipholenone E, sipholenol L, siphonellinol D, sipholenol J, respectively, and (B) quantification of incorporation of IAAP into the P-gp band from at least three independent experiments.

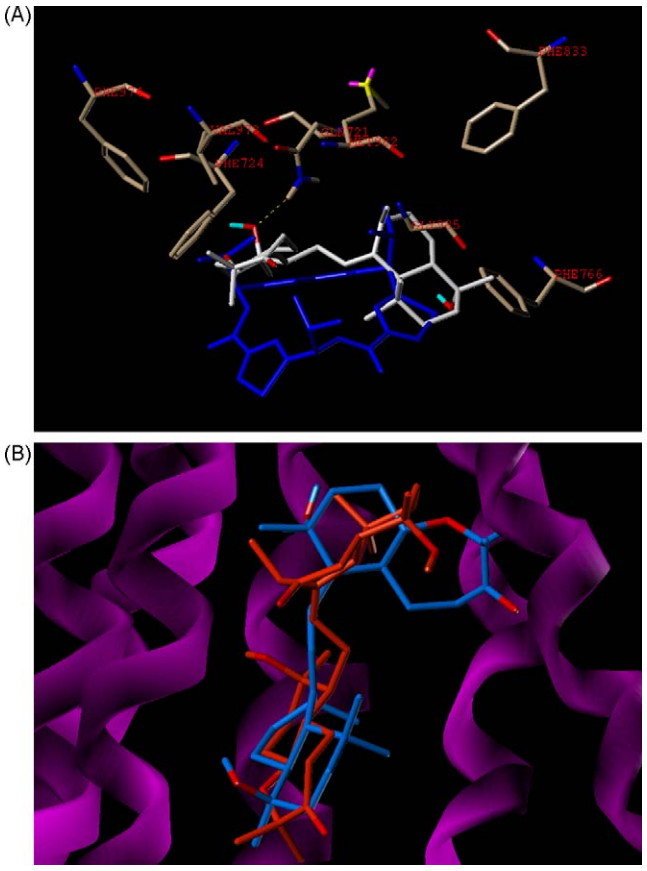

Fig. 8. Docked poses of sipholenone E, sipholenol J and QZ59-RRR. (A) The docked pose of sipholenone E (white) as predicted by Surflex-Dock compared to the crystallographic structure of QZ59-RRR (blue, but dark grey in the hard copy). (B) The alignment of docked poses of sipholenone E (red, dark grey in the hard copy) and sipholenol J (blue, light grey in the hard copy) in QZ59-RRR binding site. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

Sipholenone E showed the highest docking score (6.43, Table 3) and sipholenol J showed the lowest docking score (4.62), compared to the ligand QZ59-RRR (6.03). The binding mode of sipholenone E as predicted by Surflex-docking simulation, aligned with the crystallographic structure of QZ59-RRR, is depicted in Fig. 8A. Rings C and D of sipholenone E (Fig. 1A) [30] are aligned with the isopropyl moieties of QZ59-RRR and filling the hydrophobic pockets (Phe 724, Phe 974, and Val 978) and (Phe 766, Phe 833, Gly 985, and Met 982), respectively. Although it is not reported that QZ59-RRR form any hydrogen bonding (HB) interactions with the internal cavity of P-gp, the docked pose of sipholenone E showed a HB interaction of C-10 hydroxyl group with the Gln 721; which might justify its higher activity and binding score versus QZ59-RRR (Table 3). This interaction was not observed with the less active sipholenols L and J. Therefore, it is expected that the upper positioning of rings A and B are favorable for maximum interaction through this H-bond.

The ligand binding pocket of P-gp comprises mostly hydrophobic and aromatic residues [49]. Of the 73 solvent accessible residues in the internal cavity, 15 are polar and only two (His 60 and Glu 871) are charged or potentially charged [49]. Therefore, hydrophobic ligands are expected to bind more preferably and inhibit the P-gp activity more strongly compared to the less hydrophobic ligands. This fact can partly explain the lack of activity of sipholenol J, the C-16 ketone and the more hydrophilic analogue of sipholenone E [30]. On the other hand, the introduction of ketone group in C-16 of sipholenol J's ring C will sterically clash with the aromatic ring of Phe 833, totally changing the sipholenol J position into inactive orientation (Fig. 8B), where rings C and D are projected upward [30].

Table 3
The binding scores of sipholenols analogues as predicted by Hammerhead scoring function of Surflex-Dock software.

Compound	Total score (-logK_d)
Sipholenone E	6.43
Siphonellinol D	5.78
Sipholenol L	5.55
Sipholenol J	4.62
QZ59-RRR	6.03

4. Discussion

Marine natural products are among the most important resources for global drug discovery and development due to the fact that the marine ecosystem has an abundant number of species with unique chemical diversity. In the last three decades, thousands of new marine compounds and their derivatives have been discovered and many of them appear to show effective anticancer activity. Meanwhile, there are only a few marine compounds that were able to reverse MDR in cancer cells. Previously, we reported the ability of marine-derived sipholane triterpenoid sipholenol A isolated from *Callyspongia siphonella*, to strongly reverse P-gp-mediated MDR in MDR cancer cells [28]. Sipholenol A potentiated the cytotoxicity of established P-gp substrates such as colchicine, vinblastine and paclitaxel in P-gp-overexpressing MDR cancer cells. Also, it increased the intracellular accumulation of [^{3}H]-paclitaxel by

directly inhibiting P-gp-mediated drug efflux, stimulated the activity of ATPase of P-gp and inhibited the photoaffinity labeling of the transporter with [^{125}I]-IAAP.

In the present study, we screened a series of related sipholane triterpenoids that have been isolated from the same sponge *C. siphonella* or their semisynthetic analogues with established chemical and pharmacological characteristics [30]. Three of these sipholane triterpenoids, namely sipholenone E, sipholenol L and siphonellinol D were investigated for potential as novel inhibitors of P-gp. Our results showed that all three compounds, dose-dependently, enhanced the sensitivity of P-gp-overexpressing MDR cancer cells (KB-C2) to several P-gp substrates such as colchicine, vinblastine and paclitaxel, whereas there was no effect on the parental KB-3-1 cells (Table 1 and Supplementary Data Fig. 1A–I). In addition, none of the compounds altered the sensitivity of KB-3-1 and KB-C2 cells to cisplatin, a chemotherapeutic agent that is not a P-gp substrate (Supplementary Data Fig. 1J–L). Also, these three compounds did not have any effect on the response to cytotoxic agents in MRP1-, MRP7- and ABCG2-overexpressing multidrug resistance (MDR) cancer cells (Table 2). These findings suggest that the efficacy of these three sipholane triterpenoids to reverse MDR is specific to P-gp-mediated drug resistance.

As the MTT results alone cannot be used as a direct evidence of P-gp-mediated drug resistance, we determined the effect of sipholenone E, sipholenol L and siphonellinol D on the accumulation and efflux of [^{3}H]-paclitaxel, a known chemotherapeutic substrate of P-gp transporter. All three triterpenoids at 10 μM significantly enhanced the intracellular accumulation of [^{3}H]-paclitaxel (Fig. 2) and decreased the intracellular [^{3}H]-paclitaxel efflux (Fig. 3) in P-gp-overexpressing KB-C2 cells but not in parental KB-3-1 cells. The results of the accumulation and efflux were consistent with the results from the FACS/calcein assay, where sipholenone E proved to be the best compound at inhibiting the P-gp-mediated efflux (Fig. 4A and B). Also, the reversal effect of P-gp-mediated MDR by sipholenone E was stronger than that of the other two triterpenoids. When P-gp-overexpressing cells were treated with sipholenone E, sipholenol L and siphonellinol D at 10 μM for 36 h and 72 h, there was no change in protein expression of P-gp (Fig. 5B). Therefore, the reversal effect of these triterpenoids on P-gp-mediated MDR is not due to their effect on expression but most likely related to the inhibition of drug efflux by P-gp.

P-gp-mediated drug transport is coupled to ATP hydrolysis, and P-gp has both basal and drug-stimulated ATPase activity [1,3]. The basal activity is thought to be due to endogenous lipid or other endogenous substrates like hydrophobic peptides. Meanwhile, drug-stimulated ATPase activity is believed to reflect the nature of interaction of P-gp with drug substrates. Compounds that interact with P-gp are categorized into three different groups based on their effects on the ATPase activity of P-gp and degrees of affinity for both the active and inhibitory sites [50]. Our results showed that all three sipholane triterpenoids stimulated P-gp ATPase activity in a concentration-dependent manner, and according to the classification, these compounds interact only with the active site. Meanwhile, sipholenone E had the strongest effect on ATP hydrolysis but the lowest binding affinity.

One of the direct approaches to find the regions of P-gp that interact with drugs is the use of photoaffinity analogues of drug substrates. [^{125}I]iodoarylazidoprazosin (IAAP), an analogue of prazosin [51] is one of the most commonly used photoaffinity substrates. The photoaffinity interaction sites of [^{125}I]-IAAP are localized at least at three sites, amino acids 248–312 (TM4-TM5), 758–800 (beyond TM8), and 1160–1218, which are found within the second cytosolic NBD of hamster P-gp [52]. In human P-gp, the IAAP binding was localized to residues 1135–1164, which correspond to TM11 and the sixth extracellular loop [1]. Our results showed the ability of the sipholanes triterpenoids to inhibit the photolabeling of P-gp in a concentration-dependent manner, while the negative control sipholenol J did not (Fig. 7A and B). Siphonellinol D had the highest binding affinity for the P-gp substrate binding site(s), followed by sipholenol L and sipholenone E.

Because the P-gp structure has been elucidated [49] we were able to perform a docking analysis [44] of these sipholane triterpenoids. Sipholenone E and sipholenol J showed the highest and lowest docking score respectively, compared to the ligand QZ59-RRR (Table 3). The docked pose of sipholenone E showed a hydrogen bonding interaction of C-10 hydroxyl group with the Gln 721 which may explain its higher binding score (Table 3). This interaction was not observed with the other compounds. Another factor that has to be taken into consideration is the hydrophobicity of the ligand. Because the ligand binding pocket of P-gp has mostly hydrophobic residues, hydrophobic ligands bind more strongly than the less hydrophobic ones [49]. This might explain the lack of activity of sipholenol J. Sipholenone E, sipholenol L and J contain a perhydrobenzoxepine (rings A and B) and a *cis*-decalin (rings C and D) system connected through an ethylene bridge (Fig. 1A, B and D) [30]. Introduction of ketone group in C-16 as in sipholenol J's ring C not only will increase the hydrophilicity of the molecule compared to sipholenone E but also induce conformational changes leading to steric clash with the aromatic ring of Phe 833, loss of the possible important C-10 HB with Gln 721, and inversion of sipholenol J's to inactive orientation (Fig. 8B), where rings C and D are projected upward. Meanwhile, siphonellinol D possesses a siphonellane (rings A, B and C) skeleton (Fig. 1C) [30]. The long carbon chain at position C-19 (ring C) caused an increase in hydrophobicity which probably resulted in a higher binding affinity for the P-gp substrate binding site(s). Of all three sipholane triterpenoids, sipholenone E was the most effective reversal of multidrug resistance in P-gp-overexpressing cells, inhibiting the efflux of [^{3}H]-paclitaxel and stimulating the ATPase activity and consistent with the molecular docking and in silico data. The potency of sipholanes compared to existing first- and second-generation P-gp inhibitors bode well for future optimization and structure-activity relationship studies to develop them as possible new effective P-gp inhibitor scaffolds.

In conclusion, sipholenone E, sipholenol L and siphonellinol D potently reversed P-gp-mediated drug resistance in MDR cancer cells by directly inhibiting the drug efflux function of P-gp, resulting in an increase in the accumulation of [^{3}H]-paclitaxel and calcein. All three compounds stimulated P-gp ATPase activity and inhibited the photolabeling of P-gp with IAAP, suggesting that these triterpenoids directly interact with P-gp. In silico molecular docking study suggested the ability of sipholanes to target the binding site of QZ59-RRR at the crystallographic structure of P-gp. Overall, our findings suggest that sipholenone E, sipholenol L and siphonellinol D, which belong to a class of sipholane triterpenoids, represent potential reversal agents for the treatment of MDR in P-gp-overexpressing tumors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.08.001.

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