Published in final edited form as:

Phytomedicine. 2019 February ; 53: 252–262. doi:10.1016/j.phymed.2018.09.008.

Tenulin and isotenulin inhibit P-glycoprotein function and overcome multidrug resistance in cancer cells

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Abstract

Background—Multidrug resistance (MDR) in cancer is one of the main obstacles in treatment with chemotherapy. Drug efflux through P-glycoprotein is the major mechanism involved in MDR. A potential strategy to provide the best possible clinical outcomes is to develop P-glycoprotein (P-gp) inhibitors from natural products.

Purpose—The present study investigated the effects of the natural sesquiterpene lactone tenulin and its derivative isotenulin on human P-gp; the mechanisms of kinetic interactions were also explored.

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Conflict of interest

The authors declare no competing financial interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.09.008.

Methods—The human P-gp (ABCB1/Flp-InTM-293) stable expression cells were established by using the Flp-InTM system. The effects of tenulin and isotenulin on cell viability were evaluated by SRB assays in established cell lines, sensitive cancer cell line (HeLaS3), and resistant cancer cell line (KB-vin). The transporter inhibition ability was evaluated by calcein-AM uptake assays. The P-gp inhibition kinetics of tenulin and isotenulin were evaluated by rhodamine123 and doxorubicin efflux assays. The ATPase activity was evaluated with the Pgp-GloTM Assay System.

Results—Tenulin and isotenulin significantly inhibited the P-gp efflux function by stimulating P-gp ATPase activity. Tenulin and isotenulin interacted with the effluxes of rhodamine 123 and doxorubicin through a competitive and noncompetitive mechanism, respectively. The combinations of tenulin and isotenulin with chemotherapeutic drugs significantly resensitized MDR cancer cells.

Conclusion—These results suggested that tenulin and isotenulin are potential candidates to be developed for synergistic treatment of MDR cancers.

Keywords

Tenulin; Isotenulin; Sesquiterpene lactone; P-glycoprotein; Multidrug resistance; Kinetic mechanism

Introduction

Cancer is a global health problem and the leading cause of death worldwide. Despite various advances in cancer treatments, multidrug resistance (MDR) remains a major obstacle in cancer chemotherapy. Numerous cellular mechanisms related to MDR have been proposed, including the decreased accumulation of drugs due to increased efflux by ATP-binding cassette (ABC) efflux transporters (Gillet and Gottesman, 2010). Within the human ABC transporter superfamily, *ABCB1* (P-gp), *ABCC1* (MRP1), and *ABCG2* (BCRP) are most frequently associated with MDR in cancer chemotherapy (Karthikeyan and Hoti, 2015).

P-glycoprotein (P-gp), encoded by the *ABCB1* gene, was the first identified as an ABC transporter in 1976 (Juliano and Ling, 1976). P-gp is a 170 kDas apical membrane transporter, which is expressed abundantly in the kidney, liver, intestines, placenta, and luminal blood–brain barrier, as well as in several cancer cells. Its normal function is to protect cells against xenobiotics and cellular toxicants (Bugde et al., 2017). However, several types of MDR cancers overexpress P-gp (Alfarouk et al., 2015), which plays an important role in cancer progression and therapeutic outcomes. Thus, the development of P-gp inhibitors is regarded as a promising strategy to overcome MDR cancers. Substrates of P-gp include an extensive range of neutral and cationic hydrophobic chemotherapeutic agents, such as vinca alkaloids (e.g., vinblastine and vincristine), anthracyclines (e.g., doxorubicin and daunorubicin), and taxanes (e.g., paclitaxel and docetaxel).

Several generations of P-gp inhibitors have been developed. Unfortunately, the three prior generations of P-gp inhibitors have several safety problems, such as unexpected systemic toxicities, non-targeted inhibition, and unpredictable pharmacokinetic interactions between chemotherapeutic agents and candidate P-gp inhibitors. These problems raise serious concerns regarding clinical benefits (Thomas and Coley, 2003). Because of safety

advantages, the development of fourth generation P-gp inhibitors from natural products has attracted considerable scholarly attention since 2016. Several natural compounds, including quercetin, curcuminoids, flavonoids, cordycepin, and carotenoids, possess P-gp inhibitory effects (Joshi et al., 2017; Mohana et al., 2016).

Sesquiterpene lactones are secondary plant metabolites used in traditional medicine against inflammation, hyperlipidemia, and cancer (Hall et al., 1980; Lee et al., 1977). Prior evidence has shown that sesquiterpene lactones exert cytotoxic activity in various cancer cell lines (Ren et al., 2016). Tenulin, a major sesquiterpene lactone component isolated from *Helenium amarum*, has been reported to exert cytotoxic activity through inhibition of DNA synthesis and cellular enzymatic activity. Tenulin also stimulates ATPase activity and inhibits mitochondrial oxidative phosphorylation (Narasimham et al., 1989). However, the effects of tenulin on P-gp transporter activity and MDR cancer reversal remain largely unexplored. In the present study, we evaluated the effects of tenulin and its derivative, isotenulin, on human P-gp expression and function, and further examined relevant molecular mechanisms and kinetic interactions to elucidate the underlying mechanisms of tenulin- and isotenulin-mediated transporter inhibition. Furthermore, the MDR cancer reversal potency levels of tenulin and isotenulin were evaluated in an MDR cancer cell line to demonstrate whether combinations of tenulin or isotenulin with current chemotherapy drugs could provide effective treatment against MDR cancer cell.

Materials and methods

Chemicals and reagents

Calcein-AM, doxorubicin, vincristine, paclitaxel, rhodamine123, DMSO, R-(+)-verapamil, sulforhodamine B (SRB), trichloroacetic acid (TCA), and Tris Base were purchased from Sigma Chemical Co (St. Louis, MO, USA). All cell culture media were obtained from Thermo Fisher Scientific Inc., USA. Both tenulin and isotenulin were kindly provided by Dr. Kuo-Hsiung Lee (University of North Carolina, Chapel Hill, USA). Tenulin was isolated through the extraction of *Helenium amarum* as previously reported (Hall et al., 1977). Isotenulin was derived from tenulin by the published protocol (Waddell et al., 1979). The purity assessment of tenulin and isotenulin has been performed on a Shimadzu (Kyoto, Japan) HPLC system equipped with an LA-20AT pump, a SIL-20AHT autosampler, and an SPD-M20A PDA detector. The purities of tenulin and isotenulin were determined to be 97.5% and 96.6%, respectively.

Expression plasmid construction and cell line establishment

Human P-gp stable expression cells (ABCB1/Flp-InTM-293) were established by the protocol reported in our previous studies (Teng et al., 2016a). Briefly, the constructed ABCB1/pcDNA5 and pOG44 plasmids were co-transfected into Flp-In-293 cells. The stable transfected cell line (ABCB1/Flp-InTM-293) was selected on the basis of hygromycin B resistance and parental Flp-In TM-293 cells were selected by zeocin. All cells were cultured were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C, 95% humidity, and 5% CO₂. The protein expressions of P-gp were confirmed by a surface protein detection assay as previously described (Teng et al., 2016b).

Parental and multi-drug resistant cancer cell lines

Human cervical carcinoma cell line HeLaS3 was purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). The multidrug resistant human cervical cancer cell line KB-vin was a generous gift from Dr. Kuo-Hsiung Lee (University of North Carolina, Chapel Hill, USA) and maintained with vincristine in a fixed period. All cells were cultured in RPMI-1640 containing 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂.

Cell viability assay

The MDR reversal effects of tenulin and isotenulin on human cancer cell lines (HeLaS3 and KB-vin) were evaluated by SRB assay as previously described (Teng et al., 2016a). Briefly, cells were seeded in 96-well plates, and treated with chemotherapeutic drugs in the presence or absence of tenulin and isotenulin. After 72 h incubation, the living cells were fixed with 50% TCA and stained with 0.04% SRB for 30 min, respectively. The 10 mM Tris base added to solubilize the bound stain and the absorbance was measured using a BioTek Synergy HT Multi-Mode Microplate Reader at 515 nm. The IC₅₀ values of compound–drug combinations were used to generate the combination index (CI) and the normalized isobologram using the Chou–Talalay method by CompuSyn software. The CI < 1 indicates a synergistic effect; CI = 1 indicates an additive effect; and CI > 1 indicates an antagonist effect between the two test compounds (Chou, 2010).

Calcein-AM uptake assay

The modulatory effects of tenulin and isotenulin on human P-gp efflux function were screened by a calcein-AM uptake assay. One × 10⁵ cells/well were seeded in 96-well black plates and cultured overnight. Cell were pretreated with tenulin and isotenulin for 30 min. After pretreatments, calcein-AM, the P-gp substrate, was added and incubated at 37 °C in the incubator for 30 min. The intracellular calcein fluorescence was detected by BioTek Synergy HT Multi-Mode Microplate Reader using excitation wavelength 485 nm and emission wavelength 528 nm at 37 °C every 3 min for 30 min. Each experiment was performed at least three times, each in triplicate on different days.

Intracellular rhodamine 123 accumulation assay

Flp-InTM-293 and ABCB1/ Flp-InTM-293 cells were seeded at 1 × 10⁶ cells/well in 6-well transparent plate and incubated for 24 h. Next day, cells were treated with test compounds or verapamil (standard P-gp inhibitors) at 37 °C for 30 min, and then incubated with rhodamine 123 at 37 °C for 30 min. Afterwards, cells were harvested and resuspended with cold PBS. The fluorescence was measured by FACS analysis (BD FACSCanto System with excitation laser 488 nm, measuring at emission 530 nm for rhodamine 123.)

P-gp ATPase activity determination

The effects of tenulin or isotenulin on P-gp ATPase activity were evaluated using the Pgp-GIO assay system (Promega, Madison, WI, USA) as previously described (Teng et al., 2015). Serial concentrations (from 0.1 to 20 μM) of test compounds were added to a 96-well white plate and incubated with recombinant human P-gp membranes. The Pgp-GIO assay buffer was used as the untreated control, 200 μM verapamil was used as the positive control

of drug-induced P-gp ATPase activity, and 100 μ M sodium orthovanadate was used as the selective inhibitor of P-gp ATPase activity. Five mM MgATP was added to initiate the ATPase activity. After 40 min incubation at 37 °C, the reaction was stopped with 50 μ L ATPase Detection Reagent for 20 min at room temperature. Luminescence was measured using a BioTek Synergy HT Multi-Mode Microplate Reader and data were presented as change in luminescence (RLU).

Rhodamine123 and doxorubicin efflux assay

Totally, 1×10^5 cells/well were seeded on 96-well plates. After overnight incubation, cells were pretreated with tenulin or isotenulin for 30 min, and subsequently incubated with rhodamine123 for 30 min or doxorubicin for 3 h at 37 °C. Then, the cells were washed and incubated with warm PBS to efflux rhodamine123 and doxorubicin for 10 min and 2 h, respectively. Supernatant samples were collected and transferred to 96-well black plates. The fluorescence was measured on a BioTek Synergy HT Multi-Mode Microplate Reader with excitation wavelength 485 nm and emission wavelength 528 nm for rhodamine123 and excitation wavelength 485 nm and emission wavelength 590 nm for doxorubicin. Each experiment was performed at least three times, each in triplicate on different days.

MDR1 shift assay

The conformational changes of P-gp during the transport of substrates were detected by MDR1 shift assay as previously described (Teng et al., 2015). UIC2 antibody (Abcam, Cambridge, MA, USA) is a conformation-sensitive mouse monoclonal against human MDR1, which binds preferentially to the P-gp in the presence of the transport substrate. Cells were treated with DMSO or vinblastine (positive control) or test compounds for 30 min and then treated with IgG2a (negative control antibody) or UIC2 working solution (P-gp conformational sensitive antibody) for 15 min. The fluorescence of secondary antibody Alexa Fluor® 488-conjugated AffiniPure Goat Anti-Mouse IgG was added and incubated for 15 min. The fluorescence was evaluated by FACS analysis (BD FACSCanto II System).

Real-time quantitative RT-PCR

Real-time RT-PCR was performed to quantify ABCB1 mRNA expression levels. In 6-well plates, 10^6 cells/well were seeded and treated with test compounds at 37 °C. After 72 h treatment, the cells were harvested, and total cellular RNA was isolated using a Qiagen RNeasy kit (Valencia, CA, USA). Taqman Assay on Demand reagents of primers and probes for human ABCB1 (Hs00184500_m1) and GAPDH (Hs02758991_g1) genes were purchased from Applied Biosystem (Foster City, CA, USA). The relative ABCB1 mRNA expression levels were normalized by GAPDH mRNA level and analyzed by Applied Biosystems StepOnePlus Real-Time PCR System with standard curve method.

Apoptosis assay

Apoptosis evaluation was performed with FITC Annexin V Apoptosis Detection Kit (BD Pharmingen™, Catalog No. 556547). HeLaS3 and KBvin cells were plated in 6-well plates and treated with vehicle or compounds at 37 °C for 72 h. After pre-treatment, the harvested cells were washed with cold PBS and then cells were resuspended in 1X Binding Buffer.

Afterwards, cells were incubated with 5 µl of FITC Annexin V and 5 µl PI for 15 min at room temperature in the dark. The apoptotic cells were analyzed by FACS analysis (BD FACSCanto II System with excitation laser 488 nm, measuring at emission 530 nm for FITC and 575 nm for PI, respectively).

Molecular docking simulations

The docking simulations were performed with BIOVIA Discovery Studio 4.5 (D.S. 4.5), which has been extensively tested and proved to be successful in a variety of docking experiments (Rao et al., 2007). The ligands were prepared using the “prepare ligand” module. The ligands were primarily positioned in the binding site by using CDOCKER generate random conformations by using a CHARMM-based docking engine to perform flexible ligand-based docking and docking refinement. Hence, CDOCKER is a suitable algorithm to find various conformations of the ligands within the receptor (Usha et al., 2013). Receptor-ligand interactions were further optimized by molecular dynamics using CHARMM (Brooks et al., 2009) and Clean Geometry of Discovery Studio. Force fields applied in CHARMM are energies and forces on each particle of the system and also defines the positional relationships between atoms that determine their energy. For the ascertainment of potential correlations between experimental activities and corresponding values of - CDOCKER energy values, the best docked conformations of isotenulin and tenulin were selected as preliminary binding conformations..

Data and statistical analysis

The inhibitor potency was evaluated by IC₅₀ value using the following equation:

$$E = E_0 \left(\frac{IC_{50}^s}{IC_{50}^s + I^s} \right)$$

, where E and E_0 are the efflux in the presence and absence of inhibitor. I denotes the concentration of inhibitor. IC₅₀ is the half maximal inhibitory concentration of drug and s is the slope factor.

Kinetic analysis were estimated by nonlinear regression by Scientist v2.01 (MicroMath Scientific Software, Salt Lake City, UT, USA) according to the following equation:

$$V = \frac{V_{\max} \times C}{K_m + C}$$

, where V is the efflux rate; $V_{\max}$, the maximal efflux rate; K_m , the Michaelis–Menten constant; and C , the substrate concentration.

Statistical differences were evaluated by ANOVA followed post hoc analysis (Tukey’s test) or the Student’s t -test. The statistical significance was set at p value < 0.05.

Results

Expression and function of constructed models

Our previous study demonstrated the function of human P-gp in established cell lines by using an eFluxx-ID Green Dye assay (Teng et al., 2016b). In the present study, the transporter efflux function was evaluated with an intracellular rhodamine 123 accumulation assay utilizing P-gp fluorescence substrate rhodamine123. The efflux function of stable transfected cells, ABCB1/Flp-InTM-293, was demonstrated by the intracellular fluorescence of P-gp substrate (Fig. 1(D)).

The modulating effects of tenulin and isotenulin on human P-gp

The antiproliferative effects of tenulin and isotenulin against FlpInTM-293 and ABCB1/Flp-InTM-293 cells were evaluated by SRB assay. More than 70% cell viability remained at a concentration of less than 20 μ M of tenulin or 40 μ M of isotenulin after 72 h treatment. Hence, serial concentrations below 20 μ M of tenulin and isotenulin were used to evaluate the effects on human P-gp. First, the effects of tenulin and isotenulin on P-gp efflux function were screened with a calcein-AM uptake assay. Calcein-AM is a hydrophobic P-gp substrate, which can be hydrolyzed by intracellular esterase then converted to hydrophilic fluorescent calcein. Therefore, the P-gp efflux function can be evaluated by measuring intracellular calcein fluorescence. Verapamil was used as a standard P-gp inhibitor. With tenulin and isotenulin treatments, the intracellular calcein fluorescence was enhanced in a concentration-dependent manner, which indicated that tenulin and isotenulin significantly inhibited the efflux function of human P-gp (Fig. 1(B) and (C)). This effect was also confirmed by an intracellular rhodamine 123 accumulation assay. As compared with the untreated control, tenulin and isotenulin significantly increased intracellular fluorescence of rhodamine 123 (Fig. 1(D)).

P-gp inhibitory mechanisms of tenulin and isotenulin

Initially, we confirmed whether tenulin or isotenulin would change the conformation of P-gp. UIC2, a P-gp conformation-specific antibody, was used to indicate possible structure change by the transporting substrates. The positive control vinblastine increased binding of UIC2. However, tenulin or isotenulin treatment did not influence the fluorescence intensity of UIC as compared with the untreated control (DMSO only) (Fig. 2(A)).

A Pgp-GloTM assay was performed to evaluate the effect of tenulin or isotenulin on the ATPase activity of human P-gp. At high concentration, both tenulin and isotenulin significantly stimulated basal P-gp ATPase activity (Fig. 2(B)). The verapamil-stimulated P-gp ATPase activity was inhibited by 0.1 μ M and 1 μ M of tenulin and 1 μ M of isotenulin (Fig. 2(C)). These results suggested that tenulin and isotenulin would stimulate the ATPase activity of human P-gp.

The inhibitory mechanisms of tenulin or isotenulin on human P-gp were evaluated with standard fluorescent substrate (rhodamine123 and doxorubicin) efflux assays. The efflux of rhodamine123 and doxorubicin by human P-gp followed Michaelis–Menten kinetics, and the efflux inhibition kinetics were analyzed using Lineweaver–Burk plots. The maximum

rate ($V_{\max}$) of rhodamine 123 efflux was not affected by treatment with tenulin or isotenulin, but the affinity (K_m) decreased with increasing drug concentration (Table 1; Fig. 2(D)–(G)). These results suggested that tenulin and isotenulin inhibited human P-gp via competitive inhibition. In the doxorubicin efflux assay, tenulin and isotenulin reduced the $V_{\max}$ of doxorubicin, while the K_m of doxorubicin remained the same (Table 1; Fig. 2(H)–(K)). These results indicate that tenulin and isotenulin non-competitively inhibited the efflux of doxorubicin by human P-gp.

The effects of tenulin and isotenulin on MDR cancer cell line

The effects of tenulin and isotenulin on P-gp over-expressing MDR cancer cell line were evaluated by real-time RT-PCR. The ABCB1 mRNA expression was not significantly different between the control (no treatment) cells and cells treated with isotenulin (10 μ M) for 72 h. On the other hand, ABCB1 expression level was significantly downregulated in KB-vin cells after treatment with tenulin (10 μ M) for 72 h (Fig. 3(A)).

Since previous studies have indicated that tenulin may potentially induce apoptosis (Hall et al., 1977; Nakagawa et al., 2005), we also examined the apoptosis levels in HeLaS3 and KB-vin cancer cells. Tenulin and isotenulin significantly increased the numbers of vincristine-induced apoptotic cells in MDR cancer cell lines, but not in HeLaS3 cells (Fig. 3(B)).

To study the reversal ability of tenulin and isotenulin, we compared the viability of cells treated with current chemotherapeutic agents alone with that of cells co-treated the tenulin or isotenulin and chemotherapeutic agents. Verapamil was used as a positive control. The IC_{50} values of vincristine, paclitaxel and doxorubicin against KB-vin cells were 2919.11 $\pm$ 470.26, 843.98 $\pm$ 3.9 and 6063.85 $\pm$ 20.17 nM, respectively (72 h treatment) (Table 2). When used in separate combinations with 20 μ M tenulin and 40 μ M isotenulin, the IC_{50} of doxorubicin was reduced dramatically by 6.81- and 17.1-fold, respectively. The combination effects of tenulin or isotenulin with chemotherapeutic agents were further determined based on CI values calculated using CompuSyn software. The CI values of tenulin with the three chemotherapeutic agents ranged from 0.48 to 1.06, suggesting either synergism or an additive effect of the combination treatments (Table 3). Similar CI values (0.22 to 1) were found with isotenulin and the chemotherapeutic agents, again indicating either synergism or an additive effect of the combination treatments (Table 4).

The docking model of tenulin and isotenulin on P-gp

In the present study, structure of P-gp was retrieved from RCSB PDB (PDB id: 5KPI) (Esser et al., 2017). 5KPI Mouse P-gp is a 1276-residue polypeptide and bears 87% sequence identity to human P-gp. It consists of two homologous halves connected by a flexible linker of ~75 residues. The nucleotide binding domains (NBDs) are separated by 45.50–59.64 Å. The inward facing conformation, formed from two bundles of six helices, results in a large internal cavity open to both the cytoplasm and the inner leaflet (Esser et al., 2017). The docking results showed that isotenulin and tenulin with the best binding energies active site of P-gp with -CDOCKER energy score of 23.1383 (isotenulin) and 27.5846 (tenulin) and binding energy are 67.35 (isotenulin) Kcal/mol, 166.67 (tenulin) Kcal/mol. We examined the docked pose of the most active antagonist P-gp, in terms of hydrogen-bond interactions with

the receptor and this structure to the X-ray pose of agonist isotenulin and tenulin. Fig. 4 shows the hydrogen bonding networks between isotenulin and tenulin with the P-gp. Isotenulin and tenulin forms bidentate hydrogen bonds with the transmembrane domain, which are known as key residues of ligand binding to P-gp (Loo and Clarke, 2015). The binding model of the ligands within the active site of P-gp was also analyzed using DS 4.5. It provides a 2D visualization of the drug and receptor interaction. The model clearly indicates the isotenulin of the ligand with the residues considered were GLU180, LYS177, SER176, ASP173, THR172, LYS883 and ALA879 (Fig. 4(A)). Tenulin of the ligand with the residues like GLU180, ASN353, LYS177, SER176, ASP173, THR172 and LYS883 (Fig. 4(B)).

Discussion

The development of multidrug resistance remains a major challenge in cancer chemotherapy. Chemosensitivity can be increased by resolving the high expression of ABC transporters or inhibiting their transport function. However, despite the development of many P-gp inhibitors, their clinical use has still been limited due to issues such as undesirable toxicity or nonspecific effects. Identification of P-gp inhibitors from natural products provides promising opportunities to overcome such problems. In the present study, tenulin and isotenulin exhibited P-gp inhibitory effects at nontoxic concentrations, resulting in the reduction of both expression and function of P-gp. Tenulin and isotenulin inhibited the P-gp efflux effect via stimulation of ATPase activity and interacted with P-gp transport of rhodamine 123 and doxorubicin via competitive and noncompetitive mechanisms, respectively. Furthermore, tenulin and isotenulin showed significant MDR reversal ability on KB-vin cells by restoring sensitivity to the chemotherapeutic drugs paclitaxel, doxorubicin, and vincristine. These results suggest that tenulin and isotenulin are potential candidates to be developed for synergistic treatment of cancer.

Previous studies have reported that several natural products reverse the MDR phenomenon in many types of cancer cells. Numerous compounds, such as terpenoids, alkaloids, flavonoids and polyphenols, exert inhibitory effects on ABC transporters (Wu et al., 2011). Some sesquiterpene lactones downregulate the expression of ABC transporters, accompanied by increasing the intracellular concentrations and cytotoxic effects of doxorubicin in MDR cancer cells (Yang et al., 2013). However, the mechanisms and kinetics of the P-gp interaction have not been clarified.

P-gp functional activity is affected by the expression of P-gp, the composition and fluidity of the lipid bilayer, and cytoplasmic components (Park et al., 2003). In the present study, we detected cellular accumulation of calcein-AM and intracellular fluorescence of rhodamine 123, widely used P-gp substrates, to evaluate the human P-gp inhibition ability of tenulin and isotenulin. The MDR1 shift assay was performed for the P-gp substrates identification based on the specific reactivity of UIC2 with P-gp in the process of transporting substrates.

The possible binding site of tenulin and isotenulin on P-gp was investigated through kinetic mechanism analyses with different P-gp substrates. P-gp has two substrate-binding sites, the H-site and the Rsite, identified as preferentially binding Hoechst 33342 and rhodamine123,

respectively. In addition, a modulator site (M-site) binds compounds that can block substrate efflux. P-gp standard substrates rhodamine 123 and doxorubicin recognize and bind to the R-site, whereas rhodamine 123 has an additional binding pocket on the M-site (Ferreira et al., 2013). Both tenulin and isotenulin exhibited competitive and noncompetitive inhibition kinetics on rhodamine 123 and doxorubicin efflux, respectively. These results suggested that tenulin and isotenulin might bind competitively to the M-site with rhodamine 123.

Previous study mentioned that most substrates and modulators stimulated the basal ATPase activity of P-gp. Only a few drugs with high binding affinity for P-gp, such as zosuquidar, elacridar and tariquidar, were reported to inhibit the basal ATP hydrolysis (Chufan et al., 2016). Tenulin and isotenulin enhanced ATPase activity in a dose-dependent manner. The molecular docking results showed that tenulin and isotenulin bind similarly on the NBD (ATPase binding site), indicating that the inhibition of drug efflux probably is related to ATP binding to P-gp. Consequently, when tenulin or isotenulin (0.1 – 1 μ M) was combined with 200 μ M verapamil in an ATPase assay, the consumption of ATP was decreased as compared to verapamil treatment alone. This result demonstrated that tenulin and isotenulin likely bind to the same site as verapamil on P-gp ATPase.

Tenulin, a major constituent of sesquiterpene lactone from *Helenuim amarum*, was first identified by Clark (1939). This compound and its analogs exhibit a broad spectrum of biological activities, including anti-inflammatory, antifeedant, and hyperglycemia prevention (Hall et al., 1980,1979). More recently, many research studies have focused on its anticancer effects (Li and Zhang, 2008). Evidence was presented that the anticancer effect of tenulin might relate to the suppression of chromatin protein phosphorylation and reaction with thiol groups of enzymes necessary for cell replication and differentiation (Hall et al., 1977; Lee et al., 1977). However, the effects in reversing human MDR cancer cells are still unclear. In the present study, we evaluated the reversal potency of tenulin and isotenulin, which showed either synergism or an additive effect in combination treatment with chemotherapeutic drugs. Furthermore, tenulin and isotenulin exhibited an additive apoptosis effect with vincristine on a human MDR cervical cancer cell line.

The strength of this study was the use of stable cloned human P-gp expression cells (ABCB1/Flp-InTM-293) to explore the inhibitory effects and underlying mechanisms of tenulin and isotenulin. This transporter-specific expression system avoids the interference from other transporters. Moreover, we evaluated the reversal ability in a chemotherapeutic drug-induced MDR cancer cell line to further confirm the tumor environment in clinical MDR circumstances. Even so, some limitations still cannot be overlooked in this study. Further investigation will be needed to determine whether tenulin and isotenulin reverse other types of MDR cancers. In addition, although the anticancer effects of tenulin and isotenulin have been evaluated in animal models in previous studies (Hall et al., 1977; Lee et al., 1977), the MDR reversal effect may need to be examined in further animal studies.

Conclusion

The results of this study demonstrated that tenulin and isotenulin inhibit human P-gp function via competitive and noncompetitive inhibition mechanisms through ATPase

stimulation. The two compounds also restored the sensitivity of MDR cancer cells to chemotherapeutic drugs. Additional in vivo studies regarding the MDR reversal effect and drug safety of tenulin and isotenulin may well provide evidence to support the use of these compounds as an adjuvant clinical treatment with chemotherapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Ministry of Health and Welfare, Taiwan (MOHW107-TDU-B-212-123004), Ministry of Science and Technology (MOST 106-2320-B-039-006), China Medical University and Asia University (CMU105-ASIA-24), and China Medical University (CMU105-S-16). Partial support from NIH grant CA177584 awarded to K.H. Lee is also acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This manuscript was edited by Wallace Academic Editing.

Abbreviations:

MDR	multidrug resistance
ABC	ATP-binding cassette
P-gp	P- glycoprotein
MRP1	multidrug resistance protein 1
BCRP	breast cancer resistance protein

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Fig. 1.

Primary screen for inhibitory effects of tenulin and isotenulin on human P-gp. (A) Structures of tenulin and isotenulin. (B) and (C) In the calcein-AM uptake assay, intracellular calcein accumulation was significantly increased by tenulin and isotenulin treatment in a dose dependent manner in ABCB1/Flp-In™-293 cells. Verapamil was used as a positive control. (D) Tenulin and isotenulin significantly increased intracellular fluorescence of rhodamine 123 as compared to the untreated control. Data presented as mean $\pm$ SE of at least three

experiments, each in triplicate. * in (B) and (C) denotes $p < 0.05$ as compared to intracellular calcein fluorescence in control group.

Fig. 2.

Inhibitory potency and mechanisms analyses of tenulin and isotenulin on human P-gp. (A) MDR1 shift assay showed fluorescence intensity did not shift with tenulin or isotenulin treatments a compared to the solvent, indicating P-gp conformation did not change to the open form. Vinblastine was used as a positive control. (B) and (C) P-gp ATPase activity was measured by Pgp-Glo™ Assay System and data were analyzed as RLUs. Incubation with tenulin or isotenulin (10–20 μM) could significantly increase the P-gp ATPase activity. Effect on verapamil-stimulated P-gp ATPase activity showed that tenulin and isotenulin decreased the consumption of ATP as compared to verapamil treatment alone. (D)–(G) P-gp inhibition kinetics analysis of tenulin and isotenulin on rhodamine 123 efflux. (D) and (F) The effect of tenulin and isotenulin on rhodamine123 efflux was dose-dependent following the Michaelis–Menten kinetics. (E) and (G) Lineweaver–Burk plot analysis of tenulin and isotenulin inhibitory mechanism on rhodamine123 efflux. (H)–(K) P-gp inhibition kinetics analysis of tenulin and isotenulin on doxorubicin efflux. (H) and (J) The dose-dependent

effect of tenulin and isotenulin on doxorubicin efflux by P-gp followed the Michaelis–Menten kinetics. (I) and (K) Lineweaver–Burk plot analysis of the inhibition effect of tenulin and isotenulin on doxorubicin efflux. Data presented as mean $\pm$ SE of at least three experiments, each in triplicate. * denotes $p < 0.05$ as compared to control group.

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Fig. 3.

Reversal ability of tenulin and isotenulin on MDR cancer cell line. (A) ABCB1 mRNA expression levels were quantified by real-time RT-PCR in HeLaS3 and MDR KB-vin cancer cell line with or without tenulin or isotenulin 72h treatment. (B) Apoptotic cells detection after tenulin or isotenulin 72 h treatment in HeLaS3 and KB-vin cancer cells. Apoptotic and necrotic cells were determined by Annexin V (FITC) plus propidium iodide (PI) double staining and flow cytometry. Quadrant diagrams represent cell distribution in early apoptosis

(Q1), apoptosis (Q2), live (Q3), and dead (Q4). Data presented as mean $\pm$ SE of at least three experiments, * denotes $p < 0.05$ compared with control group.

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Fig 4.
A binding mode of the interactions of isotenulin and tenulin with the residues of the P-glycoprotein. (A) Right: 2D visualization of ligand isotenulin interaction. left: 3D model representation of the adduct isotenulin with P-glycoprotein. (B) Right: 2D visualization of ligand tenulin interaction. 3D model representation of the adduct tenulin with P-glycoprotein.

Table 1

The effects of tenulin and isotenulin on human P-gp-mediated efflux of rhodamine123 and doxorubicin in *ABCB1*/Flp-In™-293 cells.

	Nonlinear kinetic parameters	
	V_m (pmol/mg protein/10 min)	K_m (μM)
Tenulin		
Nonlinear regression Rhodamine123 only	13.15 ± 0.44	30.78 ± 2.59
+Tenulin 2.5 μM	13.98 ± 1.09	49.26 ± 6.33
+Tenulin 5 μM	13.04 ± 0.66	64.25 ± 4.71 *
Isotenulin		
Nonlinear regression Rhodamine123 only	11.17 ± 0.31	22.30 ± 0.47
+Isotenulin 2.5 μM	11.49 ± 0.50	45.88 ± 2.09 *
+Isotenulin 5 Mm	11.57 ± 0.94	57.93 ± 4.49 *
	V_m (pmol/mg protein/10 min)	K_m (μM)
Tenulin		
Nonlinear regression Doxorubicin only	172.49 ± 21.44	79.17 ± 13.62
+Tenulin 2.5 Mm	85.20 ± 9.80 *	79.89 ± 15.76
+Tenulin 5 Mm	54.46 ± 20.47 *	81.24 ± 36.01
Isotenulin		
Nonlinear regression Doxorubicin only	172.49 ± 21.44	79.17 ± 13.62
+Isotenulin 2.5 μM	97.75 ± 19.62	80.62 ± 15.01
+Isotenulin 5 Mm	56.13 ± 10.14 *	81.38 ± 16.97

V_m , the maximal efflux rate; K_m , the Michaelis–Menten constant.

* $p < 0.05$ as compared with rhodamine123 or doxorubicin only.

Table 2

Effects of tenulin and isotenulin on cytotoxicity of chemotherapeutic drugs in HeLaS3 and KB-vin cells.

	HeLaS3		KBvin	
	IC 50 (nM)	RF ^a	IC 50 (nM)	RF
Paclitaxel	9.2 ± 0.07	1.00	843.98 ± 3.90	1.00
+Verapamil 2.5 µM	0.85 ± 0.01	10.83 *	76.91 ± 4.75	10.97 *
+Tenulin 10 µM	7.70 ± 0.50	1.20	631.75 ± 1.86	1.34 *
+Tenulin 20 µM	1.89 ± 0.49	4.86 *	492.25 ± 7.79	1.71 *
+Isotenulin 10 µM	9.74 ± 0.36	0.94	694.54 ± 0.77	1.22 *
+Isotenulin 20 µM	4.17 ± 0.42	2.21 *	612.54 ± 9.73	1.38 *
+Isotenulin	0.76 ± 0.05	12.17 *	405.47 ± 7.66	2.08 *
Vincristine	7.4 ± 0.37	1.00	2919.11 ± 470.26	1.00
+Verapamil 2.5 µM	0.48 ± 0.02	15.50 *	370.81 ± 9.56	7.87 *
+Tenulin 10 µM	6.12 ± 0.53	1.21	1107.23 ± 82.31	2.64 *
+Tenulin 20 µM	2.73 ± 0.56	2.71 *	1021.67 ± 116.32	2.86 *
+Isotenulin 10 µM	6.60 ± 0.43	1.12	1191.38 ± 94.17	2.45 *
+Isotenulin 20 µM	5.38 ± 0.43	1.38 *	828.48 ± 5.85	3.52 *
+Isotenulin 40 µM	1.75 ± 0.46	4.23 *	465.37 ± 13.42	6.27 *
Doxorubicin	82.61 ± 2.79	1.00	6063.85 ± 20.17	1.00
+Verapamil 2.5 µM	87.18 ± 6.32	0.95	708.17 ± 2.61	8.56 *
+Tenulin 10 µM	86.96 ± 2.01	0.95	5123.26 ± 197.40	1.18 *
+Tenulin 20 µM	26.59 ± 1.78	3.11 *	890.06 ± 99.06	6.81 *
+Isotenulin 10 µM	85.19 ± 7.07	0.97	4456.83 ± 90.35	1.36 *
+Isotenulin 20 µM	78.19 ± 4.43	1.06	1974.46 ± 34.48	3.07 *
+Isotenulin 40 µM	74.10 ± 6.27	1.11	354.36 ± 14.75	17.11 *

^aRF: Reversal fold of tenulin or isotenulin. RF = IC₅₀ of chemotherapeutic agent/IC₅₀ of combination of tenulin or isotenulin with chemotherapeutic agent.

* Denotes $p < 0.05$ as compared to chemotherapeutic drug treatment alone.

Table 3

Combination index analysis of vincristine, doxorubicin and paclitaxel combined with tenulin at a non-constant ratio in MDR KB-vin cells.

Chemotherapeutic agent (nM)	Tenulin (μM)	Fa ^a	CI ^b	Pharmacological Effect
Paclitaxel				
1000	10	0.22	0.86	Slight synergism
	20	0.14	0.73	Moderate synergism
Vincristine				
1000	10	0.53	1.06	Additive
	20	0.47	1.02	Additive
Doxorubicin				
1000	10	0.58	0.61	Synergism
	20	0.45	0.48	Synergism

^aFa: Fraction affected.

^bCI: Combination index.

Table 4

Combination index analysis of vincristine, doxorubicin and paclitaxel combined with isotenulin at a non-constant ratio in MDR KB-vin cells.

Chemotherapeutic agent (nM)	Isotenulin (μ M)	Fa ^a	CI ^b	Pharmacological effect
Paclitaxel				
1000	10	0.28	0.90	Moderate synergism
	20	0.25	0.85	Moderate synergism
	40	0.13	0.60	Synergism
Vincristine				
1000	10	0.52	1.00	Additive
	20	0.39	0.84	Moderate synergism
	40	0.16	0.53	Synergism
Doxorubicin				
1000	10	0.61	0.59	Synergism
	20	0.53	0.50	Synergism
	40	0.27	0.22	Strong synergism

^aFa: Fraction affected.

^bCI: Combination index.