Vitamin E Reverses Multidrug Resistance *In Vitro* and *In Vivo*

Jingling Tang[1],[†], **Qiang Fu**[†], **Yongjun Wang**[2], **Kelly Racette**, **Dun Wang**[2],[*], and **Feng Liu**[*]
Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7360, USA

[1]School of Pharmacy, Harbin Medical University, Harbin, 150086, P. R. China

[2]Shenyang Pharmaceutical University, Shenyang, 110016, P.R. China

Abstract

Multidrug resistance (MDR) is a major obstacle to successful and effective chemotherapeutic treatments of cancers. This study explored the reversal effects of vitamin E on MDR tumor cells *in vitro* and *in vivo*, elucidating the potential mechanism of this reversal. VE at a concentration of 50 μM exhibited a significant reversal of the MDR effect (compared to only PTX in DMSO, $p <$ 0.05) in two human MDR cell lines (H460/taxR and KB-8-5). The MDR cell xenograft model was established to investigate the effect of VE on reversing MDR *in vivo*. Mice intravenously injected with Taxol (10 mg/kg) with VE (500 mg/kg, IP) showed an ability to overcome the MDR. VE and its derivatives can significantly increase intracellular accumulation of rhodamine 123 and doxorubicin (P-gp substrate), but not alter the levels of P-gp expression. These treatments also did not decrease the levels of intracellular ATP, but were still able to inhibit the verapamil-induced ATPase activity of P-gp. The new application of VE as an MDR sensitizer will be attractive due to the safety of this treatment.

Keywords

multidrug resistance (MDR); P-glycoprotein; TPGS; Vitamin E

1. Introduction

Multidrug resistance (MDR) is a major obstacle to successful and effective chemotherapeutic treatments of cancers [1]. MDR involves multiple mechanisms, the most important being those associated with the overexpression of various members of the ATP-binding cassette, (ABC)-family of transport proteins [2]. Among them, P-glycoprotein (P-gp) is the most extensively studied. P-gp belongs to ABC subfamily B and is encoded by the ABCB1 gene. In tumor cells, P-gp recognizes a large variety of antineoplastic agents (e.g., anthracyclines, vinca alkaloids, taxanes) as substrates for an ATP-dependent efflux,

[*]**Corresponding author:** 1. Prof. Feng Liu Division of Molecular Pharmaceutics, University of North Carolina, Eshelman School of Pharmacy, Chapel Hill, NC 27599-7360, USA. Tel: (919)843-2277. Fax: (919)966-0197. fliu@email.unc.edu. 2. Associated Prof. Dun Wang Shenyang Pharmaceutical University, Shenyang, 110016, P.R. China. wangduncn@hotmail.com..
[†]both authors contributed equally

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consequently minimizing their intracellular concentrations [2]. Thus, P-gp inhibitors may resensitize MDR cells.

Since 1981, when it was discovered that verapamil had the capability to reverse MDR[3], P-gp inhibitors have been intensively studied as potential MDR reversers [4]. Initially, drugs to reverse MDR were not specifically developed for inhibiting P-gp; in fact, they had other pharmacological properties, as well as a relatively low affinity for MDR transporters. An example of the first-generation of P-gp inhibitors is verapamil. The second-generation was made up of more specific inhibitors that created fewer side-effects, including dexverapamil or dexniguldipine. A third-generation of P-gp inhibitors was comprised of compounds such as tariquidar, which have a high affinity to P-gp at nanomolar concentrations. Inhibitors of P-gp have been examined in preclinical and clinical studies, but these trials have largely failed to demonstrate an improvement in therapeutic efficacy [5; 6; 7]. These limitations have spurred efforts to search for new, more effective compounds with low toxicity and fewer side effects. Several non-ionic surfactants, such as Pluronic and D-α-tocopheryl polyethylene glycol succinate (TPGS) have been shown to modulate the sensitivity of certain antitumor agents *in vitro* and *in vivo* [8; 9; 10; 11; 12; 13]. Non-ionic surfactants have been shown to reverse MDR by inhibiting membrane transporters, most reports of the phenomenon have focused on P-gp.

Paclitaxel (PTX) is a potent antineoplastic agent against a wide variety of malignancies [14], which has been applied for patients with breast cancer, ovarian cancer and non-small-cell lung cancer (NSCLC) [15]. However, MDR developed by cancer cells still represents a major challenge in the clinical cure of cancer by PTX or PTX in combination with other antineoplastic agents, especially advanced and metastatic forms. PTX has been shown to be a high affinity substrate of P-gp which hinders its successful therapy in cancers [15; 16]. Our laboratory has developed PTX nanocrystal (NC) formulations using TPGS as the sole excipient for overcoming MDR [17; 18; 19].

TPGS is one of the most potent and commercially available surfactants that serves as a P-gp inhibitor [20]. TPGS (as shown in Figure 1) is a water-soluble derivative of natural Vitamin E (VE), which is formed by esterification of VE succinate with polyethylene glycol (PEG) [21]. In recent years TPGS has been applied extensively in developing the various drug delivery systems. TPGS has been used as an absorption enhancer, emulsifier, solubilizer, additive, permeation enhancer and stabilizer [21; 22; 23]. TPGS is also an excipient responsible for overcoming MDR and an inhibitor of P-gp that increases the bioavailability of orally administered anticancer drugs [24].

While TPGS has been well established as a P-gp inhibitor, its structure-activity relationship has not been fully elucidated. The aim of this study was to pinpoint the key structure of TPGS which enables it to overcome MDR. In our study, the PEG component and linker, succinate acid, cannot, by themselves, reverse MDR. Therefore, we hypothesized that VE plays an important role in the reversal of MDR by TPGS. We examined the hypothesis both *in vitro* and in tumor-bearing mice, simultaneously investigating the possible mechanism of VE's MDR reversal activities.

2. Experimental Section

2.1 Materials

PTX was purchased from Lc Laboratories (Woburn, MA). TPGS was purchased from Eastman (Anglesey, U.K.). Dichloromethane (DCM), Dimethylaminopyridine (DMAP), (+)-α-Tocopherol (VE), D-α-Tocopherol succinate (TS), (+)-α-Tocopherol acetate (TA), (±)-α-Tocopherol phosphate disodium salt (TPD) and Succinic acid (SA) were purchased from

Sigma-Aldrich (St. Louis, MO). Dimethyl succinate (DS), Mono-methyl succinate (MS) were purchased from Acros (New Jersey). TPGS-COOH, TPGS-NH$_2$ and VE-NH$_2$ (Figure. 1) were prepared by our laboratory. CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) were from Promega Corporation (Madison, WI). The ATPlite™ Luminescence ATP Detection Assay System was purchased from PerkinElmer (Waltham, MA). Monoclonal antibodies including MDR1 (sc-55510), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-20357) and the secondary antibody, anti-mouse or anti-rabbit IgG with HRP, were products of Santa Cruz Biotechnology, Inc.

2.2 Synthesis

2.2.1 TPGS-COOH—TPGS-COOH (succinoylated TPGS) was synthesized according to the method described by Si-Shen Feng (2010) [25]. TPGS (0.77 g), SA (0.10 g) and DMAP (0.12 g) were mixed and allowed to react at 100 °C under a nitrogen atmosphere for 24 h. The mixture was then cooled to room temperature and taken up in 5.0 ml cold DCM. The mixture was filtered to remove excess SA and precipitated overnight in 100 ml diethyl ether at −10°C. The resulting white precipitate was filtered and dried in a vacuum to obtain TPGS-COOH.

2.2.2 TPGS-NH$_2$—At room temperature under a nitrogen atmosphere, TPGS (0.30 g) was reacted with N-(tert-butoxycarbonyl)glycine (0.10 g) in the presence of N,N'-dicyclohexylcarbodiimide (0.12 g) and a catalytic amount of dimethylaminopyridine in anhydrous dichloromethane (5 ml). The reaction was completed over a period of 7 h. The resulting mixture was filtered to remove N,N-dicyclohexylurea. Trifluoroacetic acid (2 ml) was added dropwise, with stirring, into the filtrate at 4°C. The mixture was allowed to react for 3 h. Trifluoroacetic acid was then removed by concentration under a vacuum. The residue was dissolved in dichloromethane (10 ml) and then washed successively with 5% sodium bicarbonate solution and water. The organic phase was separated and dried over anhydrous sodium sulfate overnight and then concentrated to dryness to yield white waxy solid (TPGS-NH$_2$).

2.2.3 VE-NH$_2$—Tert-butyl N-(2-hydroxyethyl)carbamate was synthesized according to the method described previously by Qinhua Wu (2011) [26]. D-α-tocopherol succinate (6.32 g) and N,N'-dicyclohexylcarbodiimide (3.68 g) were dissolved in dichloromethane (70 ml) with dimethylaminopyridine as a catalyst; then 2.30 g of Tert-butyl N-(2-hydroxyethyl)carbamate was added under stirring. The reaction mixture was stirred over night at room temperature under nitrogen atmosphere in the darkness. Dicyclohexylurea was filtered out and the filtrate was dried under vacuum. The residue was purified using silica-gel column chromatography, eluting with a chloroform- methanol solution with increasing methanol content gradually. The eluting solvent was removed in vacuo to produce conjugate, which was then unprotected by treatment with trifluoroacetic acid in dichoromethane to obtain VE-NH$_2$.

2.3 Tumor cell lines and experimental animals

A resistant, human-lung-cancer cell line, H460/taxR, was obtained from the National Cancer Institute. H460/taxR cells were maintained in RPMI-1640 medium supplemented with heated 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). KB-8-5 cells were originally selected from KB-3-1 cells, obtained from American Type Culture Collection. KB-8-5 cells were cultured in an RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) containing 10 ng/ml colchicine.

Female BALB/c nude mice (5 weeks old, 20–22g) were purchased from the National Cancer Institute, U.S. National Institute of Health (NCI, Frederick, MD). All work performed on animals was in accordance with and permitted by the University of North Carolina Institutional Animal Care and Use committee.

2.4 Cytotoxicity on Resistant Cancer Cells

An MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed on H460/taxR and KB-8-5 cells for cytotoxicity. Cells were seeded into 96-well plates at a concentration of 1×10^4 cells/well in a volume of 200 µl per well. After 24 h, various test compounds (15 µM TPGS or modified TPGS and 50 µM VE or VE derivatives) and 5 µM PTX was added for H460/taxR cells and 50 nM PTX for KB-8-5 cells. PTX, VE, TS and TA dissolved in DMSO, TPGS, TPGS-NH$_2$, TPGS-COOH and TPD dissolved in water were diluted using water before being added to the medium. The final vol% of DMSO or water added into each well was less than 0.5%. Following a 48 h period of incubation, the medium was removed and 100 µl of fresh medium and 20 µL of the combined MTS/PMS solution was added into each well of a 96-well assay plate. The plates were incubated for an additional 2 h at 37°C in a humidified, 5% CO_2 atmosphere. The absorbance values were read on a microplate reader using a Bio-Rad microplate imaging system (Hercules, CA) at a wavelength of 490 nm. Cell viability was calculated using the following formula: cell viability (%) (A490 nm for the treated cells/A490 nm for the control cells) × 100, where A490 nm is the absorbance.

2.5 In vivo anticancer effects

Nude mice were inoculated with 5×10^6 H460/taxR cells or KB-8-5 cells injected subcutaneously into their right flanks. Once the tumor mass in the xenograft was established, mice were randomly assigned to treatment groups (5 mice per group) and control group. To complete an *in vivo* study, VE was IP administered daily (500 mg/kg in 150 µL PBS containing 0.5 mg Pluronic F127) in emulsion, beginning 3 days before the IV injection of Taxol (10 mg/kg). Pluronic F127 in the present concentration cannot reverse MDR according to our previous study [19]. The IP injection of a dose of VE 500 mg/kg (or higher) has been reported in several studies [27; 28; 29]. Tumor volumes were calculated as (length × width^2)/2 from measurements taken every other day. Mice were sacrificed when the length of the tumor reached 2 cm.

2.6 Cellular uptake of rhodamine 123 (Rh 123) in H460/tax-R cells using flow cytometry

The effects of VE and its derivatives on the cellular accumulations of Rh 123 were measured using flow cytometry. Overnight, 2.5×10^5 H460/taxR cells were incubated in 12-well plates and allowed to attach. The cells were then treated with TPGS, VE and its derivatives at the same concentration as was used in the cytotoxicity experiment at 37°C for 5 h. Five µM Rh 123 was then added and the cells were incubated for another hour. Following this incubation period, the cells were collected and washed twice with ice-cold PBS buffer. Finally, the cells were resuspended in PBS buffer for analysis with a flow cytometer (BD FACS Canto flow cytometer, BD Bio sciences, San Jose, CA).

2.7 Intracellular accumulation of doxorubicin (DOX) in H460/tax-R cells using fluorescence microscopy

In order to perform cellular uptake studies, H460/taxR cells were seeded at densities of 2.5×10^5 cells per well in 12-well plates. TPGS, VE and its derivatives at the same concentration as in the cytotoxicity experiment were applied to H460/taxR cells and incubated for 5 h. Following the incubation, DOX was added to each well and cells were incubated for an additional hour. After incubation, the cells were washed twice with ice-cold

PBS buffer, and then visualized using a Nikon Eclipse Ti fluorescence microscope (Nikon Corp.) equipped with a SPOT RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

2.8 Western Blot Analysis

Total protein in the lysate was quantified using a Pierce BCA protein assay kit (Thermo Scientific Inc., Rockford, IL). Approximately 40 µg of protein from each sample was separated using NuPAGE 12% gel and then transferred onto an Immobilon-P Transfer Membrane (Millipore, Billerica, MA). The mouse monoclonal antibody to P-gp was used at a 1:2000 dilution and the rabbit monoclonal anti-GAPDH clone was used at a 1:200 dilution. An anti-mouse antibody conjugated with HRP at a dilution of 1:10000 or an anti-rabbit IgG at a dilution of 1:2000 served as the secondary antibodies in the experiment. The specific protein bands were visualized using a chemiluminescence kit (Pierce, Rockford, IL). Chemiluminiscent signals were detected with the use of high-performance chemiluminescence film (GE Healthcare).

2.9 Determination of intracellular ATP

The intracellular ATP content was measured using an ATPlite™ Luminescence ATP Detection Assay System. We modified the manufacturer's protocols to obtain the correct measurements. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight. VE and its derivatives were added to cells and incubated at 37°C for 2 h. After treatment, cells were washed twice with ice-cold PBS and then an equal volume of the one-step reagent provided by the kit was added to each well. The plates were then rocked for 15 minutes at room temperature. Cellular ATP content was measured using a luminescent plate reader.

2.10 P-gp ATPase assay

ATPase activity is potentially affected by VE and its derivatives; therefore, it was measured using a P-gp-Gloμ assay system (Promega). The System provides the necessary reagents for performing luminescent P-gp ATPase assays. These assays are valuable screening tools for determining if a drug interacts with P-gp. The activity of P-gp ATPase is measured in the presence or absence of Na_3VO_4 and Verapamil (positive reference). The assay method is based on the dependence of ATP on the light-generating reaction of firefly luciferase. This reaction is quantified through the detection of decreases in luminescence as an indication of ATP consumption. The effect of VE and its derivatives on the ATPase activity of P-gp was measured using the manufacturer's protocols. In a 96 well plate, recombinant human P-gp (25 µg) was incubated with P-gp-Gloμ assay buffer (20 µl), verapamil (200 µM), sodium orthovanidate (100 µM), and test compounds (TPGS, VE, TS and TPD at 50 µM). Each compound was loaded in five individual wells. If the inhibitory effects of test compounds are being examined against a Verapamil-stimulated P-gp ATPase activity, then Verapamil is included in the test wells. In this case, 10µl of Verapamil and 10µl of 5× concentrated test compound, both dissolved in P-gp-Glo™ Assay Buffer are added to test wells. The reaction was initiated by the addition of MgATP (10 mM), and then halted 40 min later by the addition of 50 µl of the firefly luciferase reaction mixture (ATP detection reagent) that initiated an ATP-dependent luminescence reaction. Signals were measured 60 min later using a Bio-Rad microplate imaging system (Hercules, CA).

2.11 Statistics

All data were represented as mean value ± standard deviation (SD). Statistical comparisons were made using a one-way analysis variance (ANOVA) and Tukey's-test. Results were considered significant when a confidence interval of 95% ($p < 0.05$) was achieved.

3. Results

3.1 Identify the key structure of TPGS to overcome MDR

Of the known surfactants that act as P-gp inhibitors, TPGS was one of the most potent. It is well known that surface charge can influence the cellular uptake of drugs [30]. Therefore, we coupled TPGS with an amino and carboxyl group forming TPGS-NH$_2$ and TPGS-COOH (structure shown in Figure 1, ^{1}H-NMR spectrum shown in Figure S1) to investigate the influence of the charge on the sensitization of MDR cells by TPGS. TPGS and modified TPGS (TPGS-COOH and TPGS-NH$_2$) were measured in H460/taxR cells and KB-8-5 cells, which overexpress P-gp and are resistant to PTX, using MTS after the cells had been treated for 48 h. PTX in 5μM or 50 nM DMSO in H460/taxR or KB-8-5 cells, respectively, was virtually ineffective (Figure 2A). However, in sensitive cell lines, i.e. H460 or KB-3-1 cells, PTX in 5μM or 50 nM inhibited cell growth by 60% for H460 cells and 55% for KB-3-1 cells, respectively (Figure S3).

Modified TPGS (TPGS-NH$_2$ and TPGS-COOH) at a concentration of 15 μM, on the other hand, exhibited a sensitization effect similar to that of TPGS at the concentration 15 μM ($p > 0.05$), which inhibited cell growth by 55% for H460/taxR cells and 60% for KB-8-5 cells (Figure 2). TPGS modified using an amino or carboxyl group did not increase the reversal effect on MDR. Fundamentally, TPGS contains a hydrophile (PEG) attached to a hydrophobe (VE) through a linker (SA) (Figure 1). Each fragment of TPGS was investigated to identify the key component of this structure that enables the inhibition of P-gp. The hydrophilic PEG 1000, PEG 2000, linker (SA), SA derivatives, DS and MS, were tested (from 10 to 50 μM) for their ability to sensitize MDR cells. All tested substances showed no significant effect on sensitizing MDR cells (data not shown). Therefore, we hypothesized that the structure of hydrophobic VE was important in the sensitization of MDR cells. Indeed, VE at a concentration of 50 μM exhibited a significant reversal of the MDR effect (compared to only PTX in DMSO, $p < 0.05$). In contrast, VE showed no enhanced cytotoxicity in parent cell lines (H460 and KB-3-1) (Figure S3). As shown in Figure 2B, VE did not inhibit P-gp as efficiently as TPGS. This phenomenon may be due to the fact that a low availability of VE is taken up by the MDR cells *in vitro* because of the compound's hydrophobicity. TPGS contains a hydrophilic (water-soluble) PEG tail that greatly increases its aqueous solubility and membrane permeability [31; 32], which may enhance the reversal of MDR. In addition, TPGS, an amphipathic compound, would have increased chance to stay in the membrane and interact with P-gp.

To verify that an increase in the polarity of VE also plays a critical role in P-gp inhibition, VE derivatives, with a polar (TS or TPD), weak-polar (VE-NH$_2$, ^{1}H-NMR spectrum shown in Figure S2) or non-polar (TA) head group (Figure 1B), were examined for their ability to overcome MDR in H460/taxR and KB-8-5 cells. TA and VE-NH$_2$ had a sensitization effect similar to that of VE ($p > 0.05$). TS and TPD could more efficiently increase the sensitization compared to VE ($p < 0.05$), which demonstrated that the hydrophobicity of VE limits its activity to inhibit P-gp *in vitro*. However, hydrophobicity should not cause problems *in vivo* because VE is taken up by cells after it is bound to lipoproteins [33]. For this reason, we elected to complete an *in vivo* experiment to further evaluate the reversal of MDR by VE.

3.2 MDR reversal *in vivo* by VE

Treatment of mice bearing MDR-tumors with Taxol was performed to evaluate the inhibition of P-gp by VE. The treatment consisted of VE being IP administered (10 mg each mouse) daily, beginning 3 days before IV injection of Taxol (10 mg/kg, every other days for 10 times). The VE+Taxol, Taxol, VE and control groups were evaluated in two xenograft

models (H460/taxR, and KB-8-5 cells). The female nude mice were injected with Taxol (10mg/kg) every other day for a total of 10 injections beginning 7 days after their inoculation with 5×10^6 H460/taxR cells. As shown in Figure 3A, only VE+Taxol showed an ability to overcome the MDR. For example, on day 23 when the experiment was terminated (this tumor cell line grows very slowly), the tumor volume in the mice treated with VE +Taxol was 426.9 mm^3, but 798, 795.6 and 723.7 mm^3 in untreated (Control), treated with VE alone and PTX/Cremophor-EL (Taxol), respectively. There was a significant difference ($p < 0.05$) between VE+Taxol group and Taxol group.

For the KB-8-5 model (Figure 3B), the female nude mice, 4 days after the tumor cell inoculation, were injected (every other day for a total of 4 injections) with Taxol (10 mg/kg). The untreated tumors grew rapidly, reaching $1,796 \pm 332$ mm^3 in volume 15 days after the first injection. In contrast, the mice intravenously injected with Taxol (10 mg/kg) with VE (500 mg/kg, IP) had reached a size of only 325 ± 86 mm^3, but a size of 1253 ± 354 mm^3 for Taxol (a significant difference between these two groups, $p < 0.05$).

The body weight of mice in all groups did not change significantly compared to that on day 0 (Figure S4). There was no significant toxicity observed in the mice for all treatments.

3.3 Examine intracellular accumulation of Rh 123 and DOX in H460/taxR cells

The decrease in intracellular drug concentrations, a result of the efflux of anticancer drugs from tumor cells into the surrounding tissue, is believed to be a common cause of MDR. To investigate whether VE and its derivatives inhibit the function of P-gp, the intracellular accumulation of two substrates of P-gp, Rh 123 and DOX, in the presence or absence of VE and its derivatives was examined in H460/taxR cells. P-gp function was correlated with Rh 123 and DOX efflux, and the inhibition of P-gp resulted in the increase of the intracellular accumulation of Rh 123 and DOX. As shown in Figure 4A, TPGS, VE, TS and TPD increased the intracellular accumulation of Rh 123 by 10.6-, 0.5-, 1.0- and 1.2-fold in H460/taxR cells, respectively. After an incubation period of one hour with free DOX in drug resistant, H460/taxR cells, only low levels of fluorescence were observed in the cell, indicating a only a small amount of the drug had entered the cells (Figure 4B). When drug resistant cell lines were treated with VE or its derivatives prior to the administration of DOX, an increase in fluorescence was observed (Figure 4B).

3.4 Determine P-gp expression, intracellular ATP and P-gp ATPase activity

The reversal of P-gp mediated MDR can usually be achieved through the inhibition of its function or down-regulation of P-gp expression. To assess the effect of P-gp protein expression levels, western blot analyses were performed. After a pre-treatment with TPGS, VE and its derivatives (in the same concentration as cytotoxicity) did not significantly alter the expression level of P-gp protein (Figure 5A) compared to levels in the control H460/taxR cells. The results indicate that inhibiting the expression of P-gp is not involved in the reversal of P-gp MDR by TPGS, VE and its derivatives.

Because drug efflux is an energy-dependent process, the intracellular ATP levels of cells after treatment with VE and its derivatives without loaded drugs were investigated. It was found that the exposure of cells to VE and its derivatives could not induce a decrease in the level of drug resistant cells (Figure 5B).

P-gp ATPase activity stimulated by compounds was estimated using a P-gp-Glo assay kit (Promega, Madison, WI), which provides the necessary reagents for performing luminescent P-gp ATPase assays. This assay relies on the ATP-dependence of the light-generating reaction of firefly luciferase. ATP consumption by this reaction is detected as the levels of luminescence signal; the lower levels of the signal, the higher the P-gp activity. Verapamil

enclosed in the assay kit was used as a P-gp substrate that stimulates P-gp activity (consuming ATP). As shown in Figure 6A, the P-gp-dependent decreases in luminescence were detected only in the presence of Verapamil, which reflects the consumption of ATP by Verapamil-stimulated Pgp. TPGS, VE, TS and TPD (all tested at 50 μM) did not significantly influence ATPase activity on their own ($p > 0.05$, compared to the control). None of the compounds induced additional ATPase activity nor inhibited basal ATPase function. In order to investigate the inhibitory effects of test compounds against a Verapamil-stimulated P-gp ATPase activity, Verapamil was included in the test wells. In the presence of Verapamil (200 μM), VE, TS and TPD were able to significantly inhibit the Verapamil stimulated P-gp ATPase activity (Figure 6B). The luminescence of VE+Ver, TS +Ver and TPD+Ver increased significantly compared to that of Verapamil ($p < 0.05$). However, VE, TS and TPD still inhibit Verapamil-induced ATPase activity less than TPGS (compared to Verapamil, $p < 0.01$, Figure 6B).

4. Discussion

TPGS was initially selected for this study for several reasons. First, it has been recognized as one of the most potent P-gp inhibitors among many different surfactants and can function at a concentration well below the critical micelle concentration (CMC) [24; 34]. Second, it has a long-standing safety record in biomedical applications as a water-soluble derivative of natural VE. Additionally, TPGS lacks unrelated pharmacological effects and shows no pharmacokinetic interactions with other drugs.

Collnot et al. (2006) revealed that the rate of permeation of Rh 123 through monolayers of Caco-2 cells was influenced by the length of PEG chains, with an optimum molecular weight of these linear PEG chains occurring between 1000 and 1500 Da [35]. We modified the head group of PEG in TPGS with amino and carboxyl group forming TPGS-NH$_2$ and TPGS-COOH (structure shown in Figure 1) to investigate the influence of the charge on the sensitization effects of TPGS on MDR cells. Our data (Figure. 2A) indicated that TPGS-NH$_2$ and TPGS-COOH exhibited similar inhibition of P-gp to that of TPGS. Our further studies (Figure 2B) clearly showed that the VE is the key structure in TPGS to sensitize MDR in cancer.

VE has moderate reversal effects on MDR *in vitro*, this may be due to its poor solubility in the medium. However, *in vivo*, the hydrophobicity of VE should not be an issue because the cell uptake of VE is through a lipoprotein-mediated mechanism [33]. The reversal of MDR by VE *in vitro* indicated that only Taxol does not show therapeutic effects in H460/taxR cells xenograft models or moderate or inhibit tumor growth in xenograft models of KB 8-5 cells (no significant difference between Taxol group and Control group, $p > 0.05$). VE +Taxol exhibited significantly improved antitumor effects compared to Taxol in the PTX-resistant H460/taxR and KB-8-5.

Unfortunately, the exact mechanism through which the non-ionic surfactants inhibits P-gp remains unclear. Steric hindrance of substrate binding [11], alteration of membrane fluidity [20; 36], and inhibition of efflux pump ATPase with possible intracellular ATP depletion have been proposed as potential mechanisms [9; 12; 37; 38].

Many studies with multidrug resistant cells correlated resistance to reduced accumulation of drugs within the cells due to their increased efflux or decreased influx by P-gp [2; 39; 40]. As P-gp is an ATP-dependent transport protein, agents that inhibit ATP dependent drug transport should inhibit the efflux of drugs from resistant cells and increase intracellular accumulation. In this study, we observed that VE and its derivatives increased the intracellular accumulation of the P-gp substrates, Rh-123 and DOX. The increased

accumulation of intracellular drugs may be a result of decreased expression of P-gp or the decreased function of P-gp. VE and its derivatives did not influence P-gp protein expression. P-gp has an ATP-binding region that is essential for substrate transport, and the hydrolysis of ATP by P-gp ATPase is critical for restoring the transporter to its active conformational state. Thus, monitoring ATPase activity in cell membrane preparations or in purified membrane proteins allows for identification of those compounds that interact with the drug efflux transporters. P-gp exhibits a highly drug-dependent ATP hydrolysis activity, and a variety of P-gp inhibitors, as well as P-gp substrates, can stimulate ATPase activity [41; 42; 43].

When TPGS was incubated with artificial P-gp containing membranes, TPGS neither stimulated ATPase activity nor inhibited basal ATPase function over a concentration range, indicating no direct interaction between the transport sites and TPGS [12]. TPGS therefore appears not to be a substrate of P-gp, making the competitive inhibition of substrate binding unlikely. In the present study, we show that VE and its derivatives, like TPGS, did not stimulate ATPase activity. VE and its derivatives did not appear to be a P-gp substrate or a competitive inhibitor. Furthermore, intracellular ATP depletion by TPGS, VE and its derivatives may be excluded. However, a significant inhibition of P-gp after substrate-induced ATPase activation was indeed observed. VE, TS and TPD inhibit Verapamil-induced ATPase activity less than TPGS (Figure 6B). It was consistent with results of cytotoxicity and Rh 123 uptake. Altogether, the ATPase assay data suggests that ATPase activity inhibition is a major part in the mechanism of P-gp inhibition by TPGS, VE and its derivatives. TPGS and VE show similarity to polyoxyethylene (40) stearate, a material known to affect P-gp ATPase while not influencing intracellular ATP or mitochondrial function [44].

The P-gp plays a role in the excretion of potentially toxic xenobiotics. However, the existence of P-gp has not been able to explain all types of acquired MDR. Adaptation of cells to drug exposure involves activation of drug efflux pumps, increased cellular detoxification, and up-regulation of repair mechanisms, all of which lead to the development of multidrug resistant character in cancer cells [45].

The activity of VE in preventing cancer has been suggested by many epidemiologic studies. However, several recent, large-scale human trials with VE, failed to show a cancer preventive effect. The recently finished follow-up of the Selenium and VE Cancer Prevention Trial (SELECT) even showed higher prostate cancer incidence in subjects who took a-tocopherol supplementation. Dietary supplementation with VE significantly increased the risk of prostate cancer among healthy men. In the study, however, the men had been taking their pills with VE for a minimum of 7 and maximum of 12 years [46; 47]. In our study, VE was administrated only for short time, which is less likely to increase the risk of prostate cancer.

Since the first observation that Verapamil could inhibit P-gp many efforts have been made to develop P-gp inhibitors. Limited success has been achieved in this pursuit, mainly due to undesired toxicities. To date, it has not been possible to avoid the toxicities associated with the inhibition of P-gp activity. Although TPGS has shown more potent inhibition of P-gp activity than VE, as a surfactant, it still can cause hemolysis at the concentration of > 100 μM (Figure S5). Furthermore, TPGS-dependent toxicity of lipid nanocapsules has been reported [48]. Hence, the use of VE in the improvement of MDR chemotherapy will be safer than TPGS. It has been reported that the administration of a high dose of VE (800 mg TA) in short term (30 days) had no adverse effect on healthy older adults [49]. The tolerable upper intake of VE is 1000 mg according the safety guidance by the Food and Nutrition Board,

Institute of Medicine [50]. Therefore, the use of VE as a P-gp inhibitor, does not present a major toxicity concern.

In conclusion, in the present study, we have identified the key structure of TPGS to overcome MDR in cancer is the VE moiety. VE can enhance the therapeutic efficacy of PTX, both *in vitro* and in nude mice bearing tumors. The new application of VE to serve as a MDR sensitizer will be attractive due to its safety.

Supplementary Material

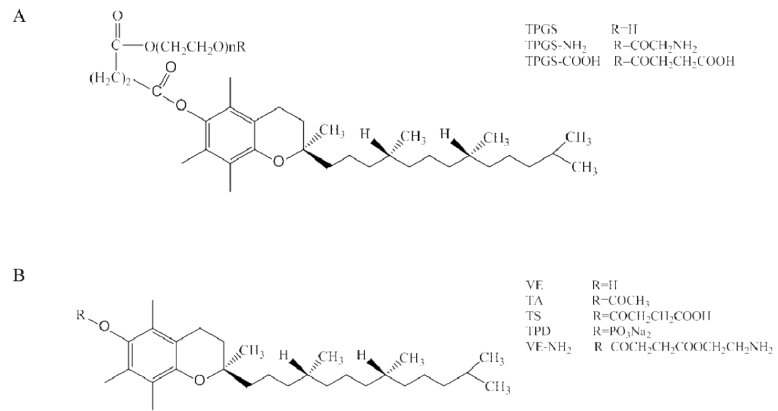Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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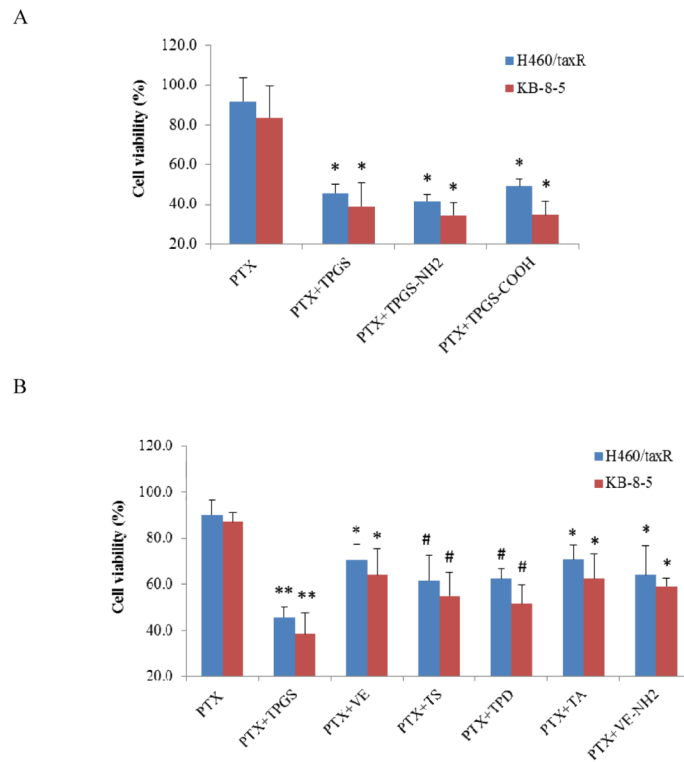
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A

TPGS	R=H
TPGS-NH$_2$	R=COCH$_2$NH$_2$
TPGS-COOH	R=COCH$_2$CH$_2$COOH

B

VE	R=H
TA	R=COCH$_3$
TS	R=COCH$_2$CH$_2$COOH
TPD	R=PO$_3$Na$_2$
VE-NH$_2$	R=COCH$_2$CH$_2$COOCH$_2$CH$_2$NH$_2$

Figure 1.
(A) The structure of TPGS and modified TPGS and **(B)** VE and its derivatives

A

B

Figure 2.
(A) The cell viability after being treated with PTX+TPGS, PTX+TPGS-NH$_2$, PTX +TPGSCOOH and PTX (DMSO) for 48 h on H460/taxR and KB-8-5 cells, PTX was at a concentration of 5 µM and 50 nM, respectively (n=6). * indicates a significant difference compared to PTX (in DMSO), $p < 0.05$.
(B) The cell viability after being treated with PTX+TPGS, PTX+VE, PTX+TS (VE succinate), PTX+TPD (phosphate disodium salt), PTX+TA (VE acetate) and PTX+VE-NH$_2$ for 48 h on H460/taxR cells and KB-8-5 cells (n=6). * indicates a significant difference compared to PTX (in DMSO), $p < 0.05$, ** indicates a highly significant difference compared to PTX (in DMSO), $p < 0.01$, # indicates a significant difference compared to PTX +VE, $p < 0.05$.
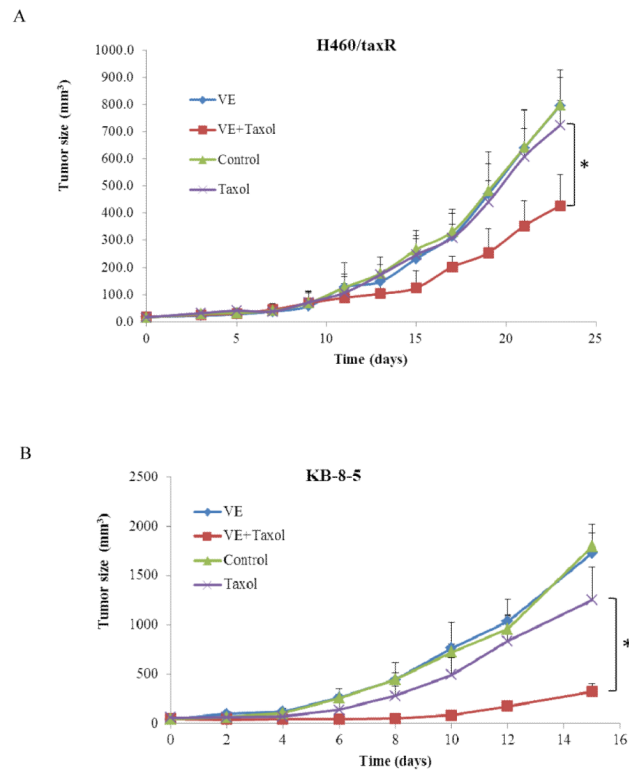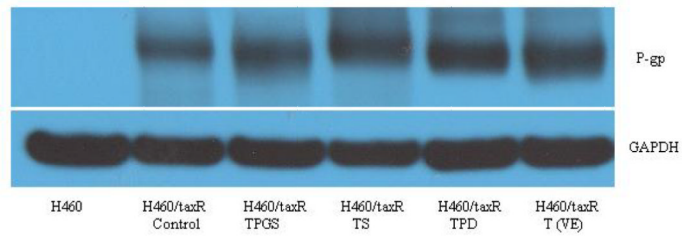
Figure 3.
Effect of Taxol, VE+Taxol and VE alone on the inhibition of tumor growth in the **(A)** H460/taxR and **(B)** KB-8-5 xenograft models (n=5). * indicates a significant difference compared to the control, $p < 0.05$.
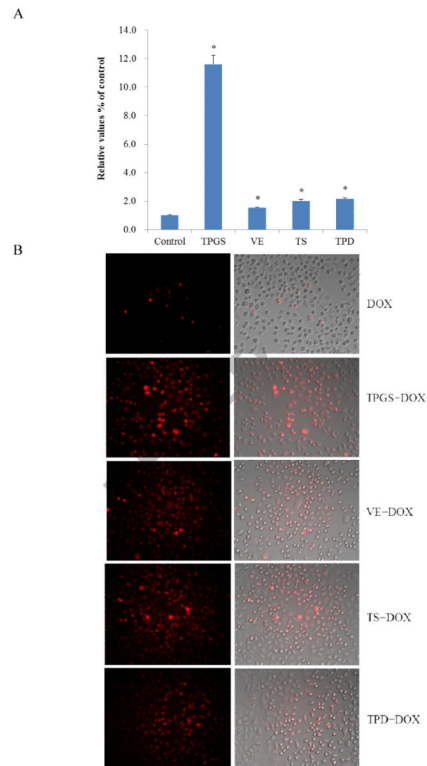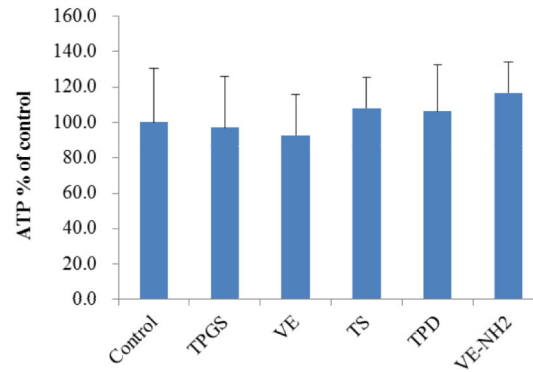
Figure 4.
(A) Intracellular accumulation of Rh 123 in H460/taxR cells. The cells were incubated with VE, TS, TPD or TPGS at 37°C for 5 h, then 5 μM Rh 123 was added and cells were incubated for another hour. Intracellular fluorescence was analyzed using flow cytometry. Cells that were not exposed to VE or TPGS were used as a positive control. The results are presented as the amount of change in fluorescence intensity relative to control cells. * indicates significant difference compared to the control, $p < 0.05$. Data are represented as the mean ± SD (n=3). **(B)** Fluorescence images of H460/taxR cells, fluorescence view (left) and normal view (right). TPGS, VE, TS and TPD were applied to H460/taxR cells and incubated for 5 h. Following the incubation, DOX was added to each well and was incubated for an additional hour.
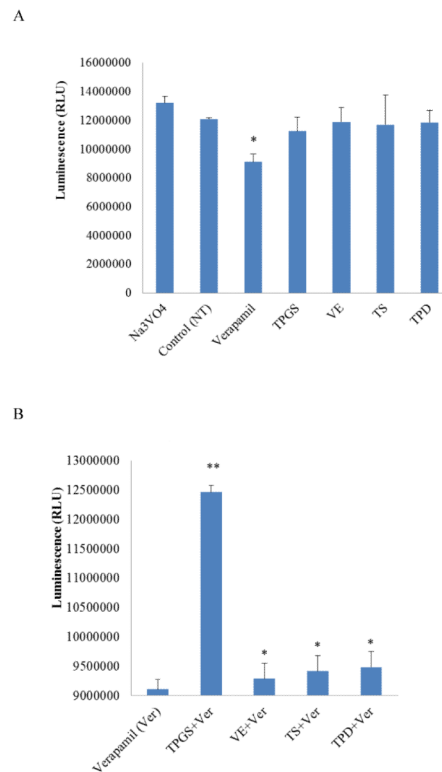
A

B

Figure 5.
(A) The effects of TPGS, VE and its derivatives on P-gp expression, as indicated by western blotting. **(B)** The effects of TPGS, VE, TS and TPD on intracellular ATP levels in H460/taxR cells. Data are represented as the mean ± SD (n=5). * indicates a significant difference compared to the control, $p < 0.05$.

A

B

Figure 6.
(A) The effects of VE, TS, TPD and TPGS on P-gp ATPase. Na_3VO_4 (sodium orthovanadate), which is contained in the assay kit, is a selective inhibitor of P-gp. * indicates a significant difference from the control, $p < 0.05$. **(B)** The effects of TPGS, VE and its derivatives on Verapamil induced ATPase activity. * indicates a significant difference from verapamil, $p < 0.05$, ** indicates a highly significant difference compared to PTX (in DMSO), $p < 0.01$. VE, TS, TPD and TPGS were administered in an amount of 50 μM. Data are represented as the mean ± SD (n=5).