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Vitamin E reverses multidrug resistance *in vitro* and *in vivo*

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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.

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, consequently minimizing their intracellular concentrations [2]. Thus, P-gp inhibitors may re-sensitize 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 [5–7]. 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* [8–13]. 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 [17–19].

TPGS is one of the most potent and commercially available surfactants that serves as a P-gp inhibitor [20]. TPGS (as shown in Fig. 1) is a water-soluble derivative of natural Vitamin E (VE), which is formed by esterification of VE succinate with

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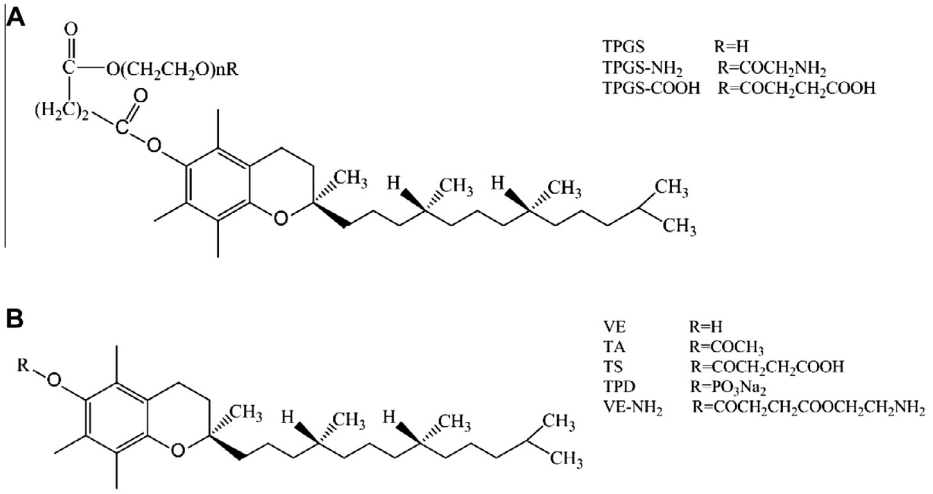

Fig. 1. (A) The structure of TPGS and modified TPGS and (B) VE and its derivatives.

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 [21–23]. 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 (Fig. 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, 20–22 g) 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