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Mechanism of Inhibition of P-Glycoprotein Mediated Efflux by Vitamin E TPGS: Influence on ATPase Activity and Membrane Fluidity

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Abstract: Efflux pump (e.g., P-gp, MRP1, and BCRP) inhibition has been recognized as a strategy to overcome multi-drug resistance and improve drug bioavailability. Besides small-molecule inhibitors, surfactants such as Tween 80, Cremophor EL, several Pluronics, and Vitamin E TPGS (TPGS 1000) are known to modulate efflux pump activity. Competitive inhibition of substrate binding, alteration of membrane fluidity, and inhibition of efflux pump ATPase have been proposed as possible mechanisms. Focusing on TPGS 1000, the aim of our study was to unravel the inhibitory mechanism by comparing the results of inhibition experiments in a Caco-2 transport assay with data from electron spin resonance (ESR) and from ATPase activity studies. ESR results, on Caco-2 cells using 5-doxyl stearic acid (5-SA) as a spin probe, ruled out cell membrane fluidization as a major contributor; change of membrane fluidity was only observed at surfactant concentrations 100 times higher than those needed to achieve full efflux inhibition. Concurrently, TPGS 1000 inhibited substrate induced ATPase activity without inducing significant ATPase activity on its own. By investigating TPGS analogues that varied by their PEG chain length, and/or possessed a modified hydrophobic core, transport studies revealed that modulation of ATPase activity correlated with inhibitory potential for P-gp mediated efflux. Hence, these results indicate that ATPase inhibition is an essential factor in the inhibitory mechanism of TPGS 1000 on cellular efflux pumps.

Keywords: P-gp; inhibition; ATPase; ESR; membrane fluidity

Introduction

Formulations of water insoluble active pharmaceutical ingredients typically require surface active agents; the latter are used as emulsifiers, solubilization enhancers, and/or wetting agents. Besides classical anionic tensides (e.g., sodium lauryl sulfate), nonionic surfactants are also widely used in oral dosage forms. Nonionic surfactants offer several advantages: (i) nonionic tensides are more hydrophobic than ionic surfactants; (ii) nonionic surfactants possess a greater capacity to dissolve poorly soluble drugs; and (iii) in general, they are less toxic to biological membranes. In addition to this purely technological benefit, several nonionic surfactants (e.g., Tweens, Spans, Cremophors (EL and RH40), Pluronic block copolymers, and Vitamin E TPGS) have been shown to influence drug pharmacokinetics (PK) by modulating cellular drug transport activity, particularly by inhibiting

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efflux pumps such as P-glycoprotein (P-gp) and/or multidrug resistance associated proteins (MRP1 and MRP2).[1−3]

Almost all epithelial tissues are known to have high P-gp expression. P-gp is the product of the MDR1 gene, an ATP-dependent multidrug efflux pump belonging to the ATP-binding cassette (ABC) superfamily of proteins.[4] P-gp is predominantly located in the apical membranes of various epithelia (e.g., on the luminal surface of small intestine, colon, capillary endothelial cells of the brain, and on kidney proximal tubules).[5−7] It protects cells from cytotoxic compounds by actively transporting them out of the cell against a concentration gradient, thereby reducing intracellular levels below their effective and/or toxic concentrations.[8] Overexpression of P-gp plays a major role in the development of multiple drug resistance (MDR) in cancer cells.[9] As a result of its enterocyte localization, and its broad substrate specificity, P-gp also significantly limits the oral absorption of a large number of drugs.[10] Therefore, inhibition of P-gp activity may be an effective way to enhance oral bioavailability and increase anticancer agent efficacy in multi-drug resistant tumors.

A vast amount of research effort has been focusing on P-gp, yet the exact inhibitory mechanism of P-gp by nonionic surfactants remains unclear. Sterically hindering substrate binding,[11] alteration of membrane fluidity,[2,12] and inhibition of efflux pump ATPase with possibly intracellular ATP depletion[13−15] have been proposed as potential mechanisms.

We focused our studies on Vitamin E TPGS (TPGS 1000), one of the most potent known P-gp inhibitors among surfactants.[2] TPGS 1000 (D-α-tocopheryl polyethylene glycol 1000 succinate; Figure 1a) is a water-soluble vitamin E derivative containing a lipophilic nonpolar (water-insoluble) head and a hydrophilic (water-soluble) PEG tail. Due to its surface active properties, TPGS 1000 may be used as a solubilizer, as an emulsifier, and as a vehicle for lipid based drug delivery formulations. In a previous structure activity relationship (SAR) study[16] on a series of homologous TPGS 1000 analogues, we observed a PEG chain length dependent inhibition effect on P-gp mediated rhodamine 123 (RHO) efflux in the Caco-2 transport assay. An optimal PEG chain length residing between 1581 ± 209 and 1182 ± 476 Da was predicted. No direct correlation was observed, however, between physicochemical properties, such as log *P* or critical micelle concentration (CMC) and inherent inhibitory potential, making a general interpretation of the SAR data difficult. Besides these vitamin E analogues, a second group of derivatives with modified hydrophobic moieties was synthesized; the α-tocopherol part of TPGS was replaced by γ-tocopherol, cholesterol, phytol, and thioctic acid. The latter formed a direct ester with the PEG chain without a succinate linker (Figure 1b). In the case of cholesteryl PEG 1000 succinate, the substitution proved to be successful as it surpassed the P-gp inhibitory potential of commercially available TPGS 1000 in Caco-2 efflux assays: cholesteryl PEG 1000 succinate > commercially available TPGS 1000 = γ-tocopheryl PEG 1000 succinate > phytyl PEG 1000 succinate > thioctic acid PEG 1000 ester.[17]

Mechanistic understanding of the inhibition of P-gp by TPGS may give new insights into the SAR and improve rational design of more potent P-gp inhibitors. The purpose

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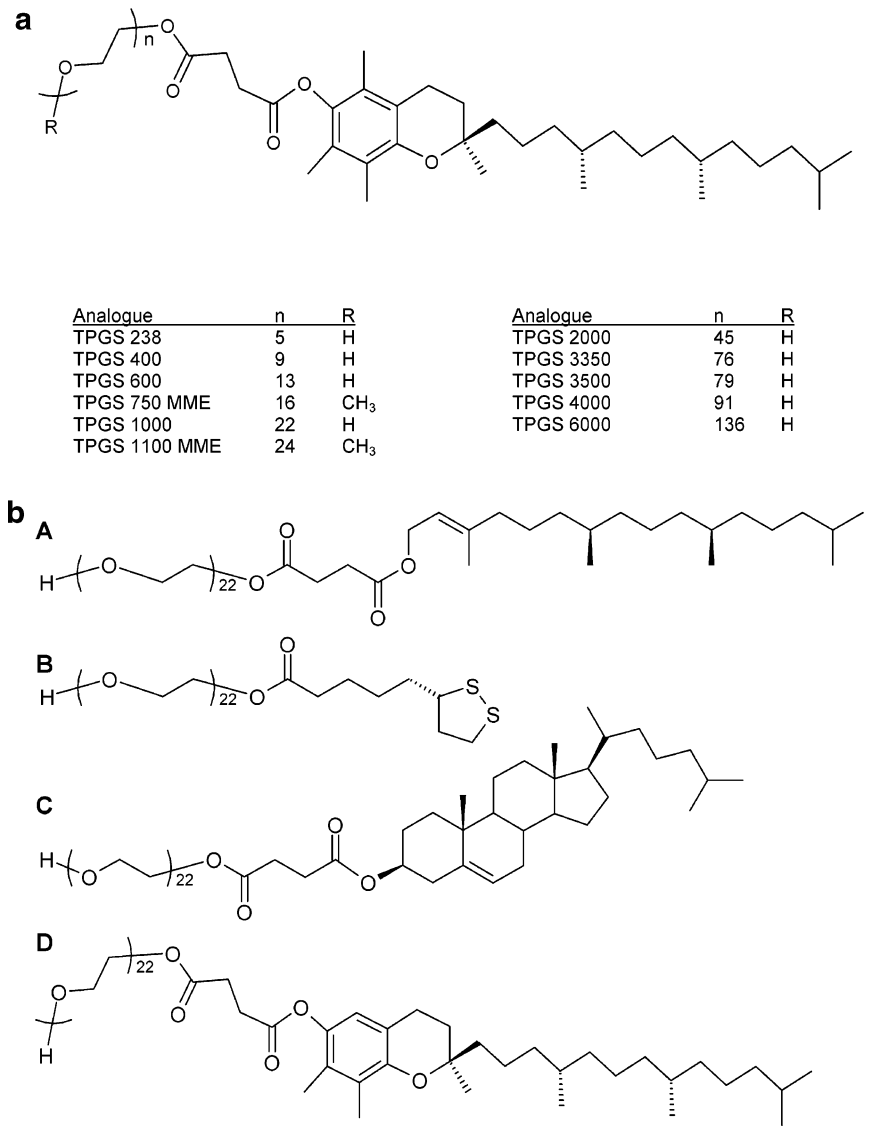

Analogue	n	R
TPGS 238	5	H
TPGS 400	9	H
TPGS 600	13	H
TPGS 750 MME	16	CH_3
TPGS 1000	22	H
TPGS 1100 MME	24	CH_3

Analogue	n	R
TPGS 2000	45	H
TPGS 3350	76	H
TPGS 3500	79	H
TPGS 4000	91	H
TPGS 6000	136	H

Figure 1. (a) General structure of D-α-tocopheryl polyethylene glycol succinate (TPGS), $n = 3-61$ depending on the PEG chain length; R = H, except for TPGS 750 monomethylether where R = CH_3. (b) Structures of TPGS analogues with modified hydrophobic moieties: A, phytyl PEG1000 succinate; B, thioctic acid PEG1000 ester; C, cholesteryl PEG1000 succinate; D, D-γ-tocopheryl PEG1000 succinate.

of this study was to investigate TPGS interactions with P-gp in its membrane environment. Alterations in membrane fluidity by TPGS were studied via electron spin resonance spectroscopy (ESR). In the absence and presence of P-gp substrates, ATPase activity was measured using an ATPase assay. The results were compared and correlated to findings from our previous inhibition experiments using a Caco-2 monolayer transport model.

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Materials and Methods

Materials. Commercial Vitamin E TPGS 1000, vitamin E, vitamin E succinate, cholesterol succinate, and all TPGS derivatives were obtained from Eastman Chemical Company (TN). Vitamin E TPGS and all analogues were prepared and purified according to the method previously described.[16] Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids (NEAA), and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen Corp., Carlsbad, CA). Reduced β-nicotinamide dinucleotide (NADH), sodium azide, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and phosphoenol-pyruvate were from Fluka (Neu-Ulm, Germany). 5-Doxyl stearic acid, ouabain, nicardipine, R+-verapamil, quinidine, progesterone, lactic dehydrogenase (LDH)/pyruvate kinase, bovine

serum albumin (BSA), PEG 1000, SDS, Triton-X 100, procaine hydrochloride, and all other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Cell Culture. Caco-2 cells, clone C2BBe1, were purchased at passage 60 from American Type Culture Collection (ATCC; Manassas, VA) and used at passages 70−92. Cells were grown to ∼90% confluence in 75 cm^2 T-flasks with DMEM supplemented with 10% FBS and 1% NEAA. Culture media were changed every second day, and cells were grown at a temperature of 37 ± 1 °C in an atmosphere of ∼85% relative humidity and ∼5% CO_2.

ESR Spectroscopy. Trypsinized Caco-2 cells were spin labeled by incubating a suspension (2.0×10^7 cells/mL) in Krebs Ringer Buffer (KRB; pH 7.4) for 30 min at 37 ± 1 °C with 0.50 mM of a spin label stock solution (10 mM 5-doxyl stearic acid (5-SA) in ethanol). Different test compound concentrations were added via stock solutions, and cell suspensions were incubated for 60 min. After suspension centrifugation (4 min at 1200 *g*), supernatant was removed and the cell pellet was washed and resuspended in KRB to give a final concentration of $\sim 1.0 \times 10^8$ cells/mL. To quench residual free spin label, chrome oxalate (final concentration 2.0 mM) was added and the ensuing cell suspension (100 μL) was filled into the test tube (WG806A Tissue cell; Rototec-Spintec, Biebesheim, Germany) and placed in a 4103 TM/ 8609 cavity (Bruker, Karlsruhe, Germany).

ESR measurements were performed at room temperature using a Bruker ESP300 E spectrometer with an ER081 (90/30 C5) magnet (Bruker, Karlsruhe, Germany). The following conditions were used: microwave power 1 mW, modulation amplitude 2 G, sweep width 100 G, modulation frequency 100 kHz, scanning time 40.96 s, and time constant 10.24 ms.

Using the equation proposed by Gaffney and Lin (eq 1),[18] cell membrane fluidity may be depicted via the cell membrane order parameter *S*. *S* can be derived from the ESR spectral line splitting. Briefly, the spin active nitroxide group of 5-SA is situated within four carbon atoms of the polar carboxyl group of the lipid acid. Reporting on molecular dynamics occurring near the membrane surface, 5-SA is located in the membrane close to the membrane head groups and the nitroxide functionality unveils molecular dynamic changes that may occur near the membrane surface. Normally, a free nitroxide radical in solution will produce a characteristic three line (isotropic) ESR spectrum because of rapid tumbling of the molecule; whereas in biological membranes, the restricted mobility of the spin label is causing an anisotropic (or powderlike) spectrum. The spin probe 5-SA becomes intercalated into the membrane bilayer and aligns itself along an axis parallel to the membrane normal. In that case the maximal hyperfine component a_{zz} of the NO moiety is oriented parallel to the long molecular axis of 5-SA and parallel to the membrane normal, and the minimal hyperfine component a_{xx} is oriented perpendicular to it. The anisotropic motion of the spin label with respect to membrane normal then leads to the experimentally observed largest hyperfine interaction $a_{||}$ ($\leq a_{zz}$) and minimal splitting $a_{\perp}$ ($\geq a_{xx}$) which thus are monitoring the dynamic behavior (Figure 2). Comparing the observed $a_{||}$ and $a_{\perp}$ values to the theoretical principal values, a_{zz} and a_{xx}, allows one to calculate *S*, defined as the ratio of the observed hyperfine anisotropy ($a_{||} - a_{\perp}$) to the 25 G theoretical maximum $a_{zz} - a_{xx} = 25$ G when the spin label is rigidly immobilized.

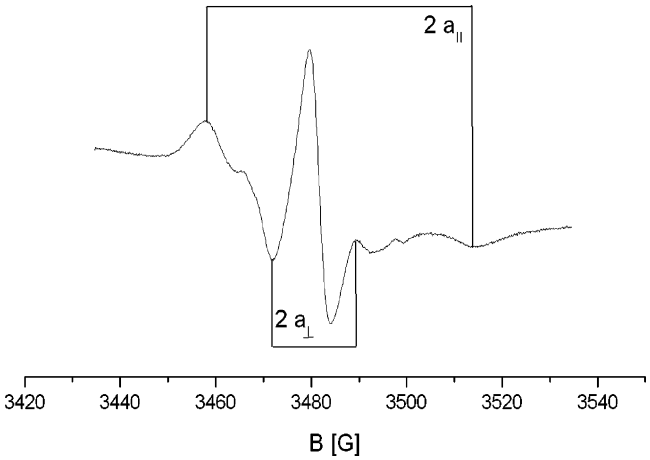

Figure 2. Typical ESR spectrum of 5-doxyl stearic acid in the cell membrane of untreated Caco-2 cells. The parameters $2a_{||}$ and $2a_{\perp}$ are directly obtained from the spectrum and are used for the order parameter equation to give a measure of membrane fluidity.

$$S = (a_{||} - a_{\perp})/(a_{zz} - a_{xx}); \quad 0 \leq S \leq 1 \qquad \text{(eq 1)}$$

When $S = 0$, molecular mobility is unhindered and fluidity is maximal. When $S = 1$, molecular motion is negligible and rigid glass spectra are obtained. In biological membranes, a membrane order gradient may be observed.[19] The gradient typically stretches from a highly ordered zone (0.60−0.80) in the polar−nonpolar interface region to the nonpolar membrane core where it may approach 0.20. As the solvent terms such as polarity are not included, the expression for *S* is only an approximation. However, in this study only relative changes in the Caco-2 system are measured and the absolute values for *S* are not essential.

ATPase Assay. Potentially affected by TPGS analogues, ATPase activity was measured in the presence and absence of different P-gp substrates (verapamil, 50 μM; quinidine, 50 μM; progesterone, 100 μM; and nicardipine, 1.0 μM). We used a modified version of a high-throughput screening assay developed by Garrigues et al.;[20] commercially available

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Table 1. Order Parameter *S* of the Spin Label 5-SA Incorporated into Untreated Caco-2 Cells and Cells Preincubated with Potential Membrane Rigidizers and Fluidizers[a]

membrane fluidizer/rigidifier	order parameter *S*
control	0.789 ± 0.007
SDS 0.10 mM	0.784 ± 0.070[b]
SDS 1.0 mM	0.728 ± 0.014[b]
Triton-X 100 0.1%	0.732 ± 0.006[b]
procaine hydrochloride 37.0 mM	0.813 ± 0.005[b]
cholesterol succinate 1.0 mM	0.805 ± 0.005[b]

[a] Mean ± SD, $n = 3-5$. [b] Significantly different from untreated control ($p < 0.05$).

human P-gp membranes from Sf9 insect cells (BD Gentest, Heidelberg, Germany) were employed. Briefly, membrane vesicles were preincubated for 30 min at 37 ± 1 °C in assay buffer that contained (i) MgATP (1.0 mM); (ii) an ATP regenerating system consisting of pyruvate kinase (0.7 units/mL) and phosphoenol-pyruvate (1.0 mM); and (iii) a coupled system using LDH (1.0 unit/mL) and NADH (0.5 mM). Ouabain (0.5 mM), sodium azide (10 mM), and EGTA (1.0 mM) were added as unspecific ion pump ATPase inhibitors. The surfactant and—depending on the experimental setup—the respective P-gp substrate were added from stock solutions, and vesicles were incubated for 60 min at 37 ± 1 °C. NADH consumption, corresponding stoichiometrically to ADP produced, was measured with a UV−vis plate reader (Spectra SLT III, TECAN, Crailsheim, Germany) at 340 nm.

Statistical Analysis. Results are expressed as mean ± SD. Significance of difference in *S* values and ATPase activity was determined by one-way analysis of variances (ANOVA) followed by Neumann−Keuls−Student or Holm−Sidak post hoc tests.

Results

ESR Studies. The influence of TPGS 1000, and other analogues, on bilipid cell membrane fluidity was investigated using the lipid soluble spin label 5-doxyl stearic acid (5-SA). Figure 2 shows a typical ESR spectrum obtained when 5-SA was incorporated into Caco-2 cell membranes. Incubating with the spin label, with brief exposure to hypoxic conditions during the ESR measurements, had no negative effect on cell viability; trypan blue viability staining results for ESR treated and untreated trypsinized Caco-2 cells revealed 72 ± 5% and 75 ± 4% viable cells, respectively (mean ± SD, $n = 3$).

The spin probe 5-SA in untreated cells gave an average *S* value of 0.789 ± 0.007 (Table 1), indicating a highly organized Caco-2 cell membrane in the head group region. The results are in line with literature values describing an *S* value between 0.60 and 0.80 in the head group region of the lipid membrane bilayer.

To validate the Caco-2 ESR technique, we studied the influence of the surfactants SDS and Triton-X 100, which are both commonly used in molecular biology to fluidize and enhance the permeability of cell membranes, and have already been shown to fluidize membranes of prokaryotic and eukaryotic cells.[21−23] SDS showed a concentration dependent effect on membrane fluidity. Compared to control, 1.0 mM SDS significantly reduced *S* to 0.728 ± 0.014 (Table 1). A similar effect was observed for Triton-X 100 0.1%, which afforded an *S* value of 0.732 ± 0.006 (Table 1).

Cholesterol succinate (1.0 mM) was used as a model membrane rigidifying compound.[24−26] The moderate but significant ($p < 0.05$) rigidification of the cell membrane in the presence of cholesterol succinate ($S = 0.813 \pm 0.005$) was comparable to the effect observed in the presence of 0.37 mM of the local anaesthetic procaine hydrochloride ($S = 0.805 \pm 0.005$; Table 1). Local anaesthetics were for a long time believed to fluidize cell membranes,[27,28] before it could be shown that they exert an amphiphilic effect on cell membrane fluidity, fluidizing the hydrocarbon core region but rigidifying the head group region.[29,30] Overall, the extent of change in the order parameter *S* observed in the validation studies is well in the "normal range" compared to findings from previous ESR studies on other membrane active substances.[21,31]

TPGS 1000 at 3.3 μM to 1.65 mM had no statistically significant ($p < 0.05$) effect on Caco-2 cell membrane

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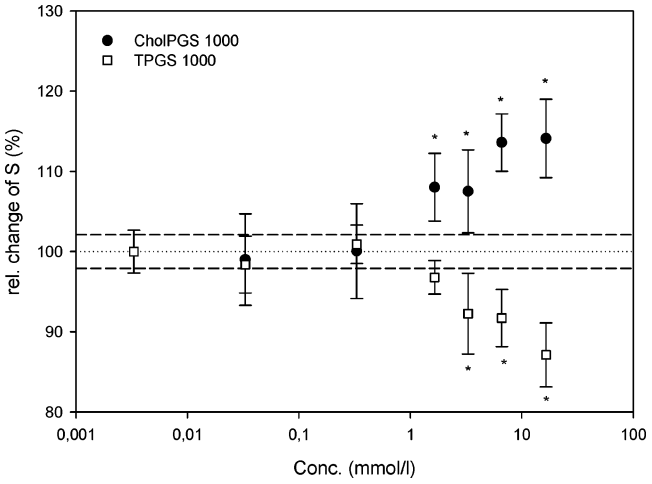

Figure 3. Relative change of the order parameter *S* of the spin label 5-SA in Caco-2 cells under the influence of different concentrations of TPGS 1000 and cholesteryl PEG 1000 succinate; mean ± SD, $n = 3-4$; * = significantly different from untreated control ($p < 0.05$).

Table 2. Order Parameter *S* of the Spin Label 5-SA Incorporated into Caco-2 Cell Membranes in the Presence of Different TPGS Analogues (at 33 μM and 3.3 mM)[a]

	order parameter $S \pm$ SD	
control	0.780 ± 0.016	
test compounds	3.3 mM	33 μM
TPGS 200	0.791 ± 0.010	0.788 ± 0.008
TPGS 600	0.779 ± 0.016	0.784 ± 0.031
TPGS 1000	0.746 ± 0.004[b]	0.783 ± 0.002
TPGS 2000	0.747 ± 0.011[b]	0.790 ± 0.001
TPGS 3500	0.754 ± 0.016	0.778 ± 0.008
TPGS 4000	0.749 ± 0.019[b]	0.793 ± 0.014
TPGS 6000	0.752 ± 0.004[b]	0.786 ± 0.002

[a] Mean ± SD, $n = 3-5$. [b] Significantly different from untreated control ($p < 0.05$).

fluidity (Figure 3). As the concentration of TPGS 1000 was increased above 1.65 mM, a progressive fluidization of the cell membrane was observed. At the highest TPGS 1000 concentration (16.5 mM), *S* was reduced from control (0.807 ± 0.005) to 0.727 ± 0.019, corresponding to a 12.9% reduction of the order parameter.

In contrast to TPGS 1000, cholesteryl PEG 1000 succinate rigidified the membrane in a dose dependent manner (Figure 3). The effect was statistically significant ($p < 0.05$) at a concentration of ≥1.65 mM. At the highest concentration, cholesteryl PEG 1000 succinate (16.5 mM) increased *S* from control (0.790 ± 0.026) to 0.902 ± 0.003, a 14.1% increase in the degree of order.

For a head to head ESR comparison of different analogues (Table 2), concentrations of 33 μM and 3.3 mM were chosen; they produced a maximal TPGS 1000 influence in the Caco-2

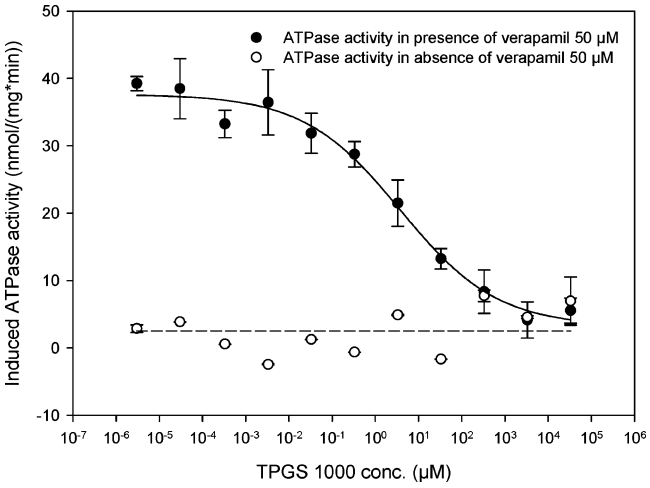

Figure 4. Influence of TPGS 1000 on P-gp ATPase activity in the absence and presence of verapamil (50 μM).

P-gp inhibition transport assay and the first significant fluidizing effects in the ESR studies, respectively. At 33 μM, all of the tested TPGS analogues had only minimal influence on rigidity. At 3.3 mM, TPGS 200 was the only analogue that slightly, although not statistically significantly ($p = 0.279$), rigidified the membrane, while *S* for TPGS 600 was comparable to control. Similar to the previous dose response curve (Figure 3), TPGS 1000 (3.3 mM) significantly ($p < 0.05$) decreased *S* from 0.780 ± 0.016 to 0.746 ± 0.004. TPGS 2000, 4000, and 6000 all showed similar extents of fluidization as TPGS 1000; the fluidizing effect appears to level off after a PEG chain length of 1000 Da. The membrane fluidization by TPGS 3500, though notable, was not statistically significant ($p = 0.17$).

ATPase Assay. To quantify surfactant (TPGS) induced changes in P-gp ATPase activity a coupled enzymatic assay using artificial human MDR1 enriched membrane vesicles was employed.[20] Varying TPGS 1000 concentrations were tested for ATPase activity modulation; experiments were conducted in the absence and presence of different P-gp substrates known to induce efflux pump action and thereby ATPase activity. TPGS 1000 did not significantly influence ATPase activity on its own, neither inducing additional ATPase activity nor inhibiting basal ATPase function (Figure 4, open circles). However, in the presence of verapamil (50 μM), which stimulated ATPase activity to a level of 39.89 ± 3.79 nmol/(mg min), a dose dependent inhibition of this activation was observed with a TPGS 1000 IC_{50} value of 3.18 ± 1.97 μM (Figure 4, closed circles, and Table 3). IC_{50} values were also determined in the presence of other P-gp substrates known to induce ATPase activity (Table 3). For progesterone (100 μM), the ATPase activating effect (44.48 ± 2.69 nmol/ (mg min)) and the IC_{50} value determined for TPGS 1000 (3.25 ± 1.29 μM) were comparable to verapamil results. If quinidine (50 μM) and nicardipine (1.0 μM) were instead used as model substrates, the IC_{50} values were about 4 to 8 times lower at 0.82 ± 0.47 μM and 0.40 ± 0.17 μM, respectively. Nicardipine induced the highest ATPase activity in the absence of TPGS 1000

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Table 3. Induction of ATPase Activity and IC_{50} Values of TPGS 1000 in the Presence of Different P-gp Substrates[a]

inducer of ATPase activity	level of induced ATPase activity (nmol/(mg min))	IC_{50} (TPGS 1000) (μM)
verapamil (50 μM)	39.89 ± 3.79	3.18 ± 1.97
quinidine (50 μM)	17.00 ± 3.96	0.82 ± 0.47
progesterone (100 μM)	44.48 ± 2.69	3.25 ± 1.29
nicardipine (1 μM)	46.72 ± 6.67	0.40 ± 0.17

[a] Mean ± SD, $n = 8$.

(46.72 ± 6.67 nmol/(mg min)), while quinidine was the weakest inducer among the tested substrates at 17.00 ± 3.96 nmol/(mg min)).

Using the same experimental setup, we expanded our findings by comparing the modulating effect of different TPGS analogues on ATPase activity. Two distinct groups of derivatives were evaluated: (i) one group consisting of homologous TPGS analogues with varying PEG chain lengths ranging between 200 and 6000 Da, analogues used in a previous TPGS SAR study;[16] and (ii) other derivatives where the hydrophobic vitamin E portion was replaced by other lipophilic moieties (γ-tocopherol, phytol, thioctic acid, or cholesterol), all containing a PEG chain length of 1000 Da (Figure 1b, A and B). The derivatives did not influence ATPase activity in the absence of P-gp substrates (data not shown), but they were able to inhibit verapamil induced ATPase activity. The head to head comparisons showed that cholesteryl PEG 1000 succinate and phytyl PEG 1000 succinate had the strongest inhibitory influence on verapamil induced ATPase activity at the tested concentrations of 33 μM (Figure 5A). Compared to the negative control in the absence of any TPGS analogue, verapamil induced ATPase activity was reduced by 30.28 ± 2.75 nmol/(mg min) and 29.61 ± 2.75 nmol/(mg min), respectively. Among the derivatives with alternative hydrophobic moieties, the thioctic acid PEG 1000 ester was the weakest ATPase activity modulator. γ-Tocopheryl PEG 1000 succinate behaved similarly to commercially available TPGS 1000. Comparing TPGS analogues that varied by PEG chain length (Figure 5B), commercially available TPGS 1000 was the most efficient ATPase activity inhibitor. Analogues with longer or shorter PEG chain lengths gradually showed weaker inhibitory potential on ATPase function. In the case of TPGS 6000, a reverse effect was observed with a slight, but not statistically significant ($p = 0.184$), induction of ATPase activity.

Furthermore, as control experiments in the ATPase assay, ATPase inducing and/or inhibiting activity was investigated using vitamin E, vitamin E succinate, and PEG1000. No significant ($p < 0.05$) influence on ATPase activity was observed over a concentration range from 0.0033 nM to 330 μM either in the presence or in the absence of P-gp substrate verapamil (data not shown).

Discussion

In recent years, interest in surfactant interaction with efflux pumps such as P-gp has steadily increased. A number of studies have attempted to evaluate inhibitory potential of different surfactants and the exact nature of their interaction with efflux transporters.[1,2,12,32] A fluidization/rigidification of the membrane environment,[2,12,33,34] steric hindrance of substrate binding,[11] and inhibition of efflux pump ATPase

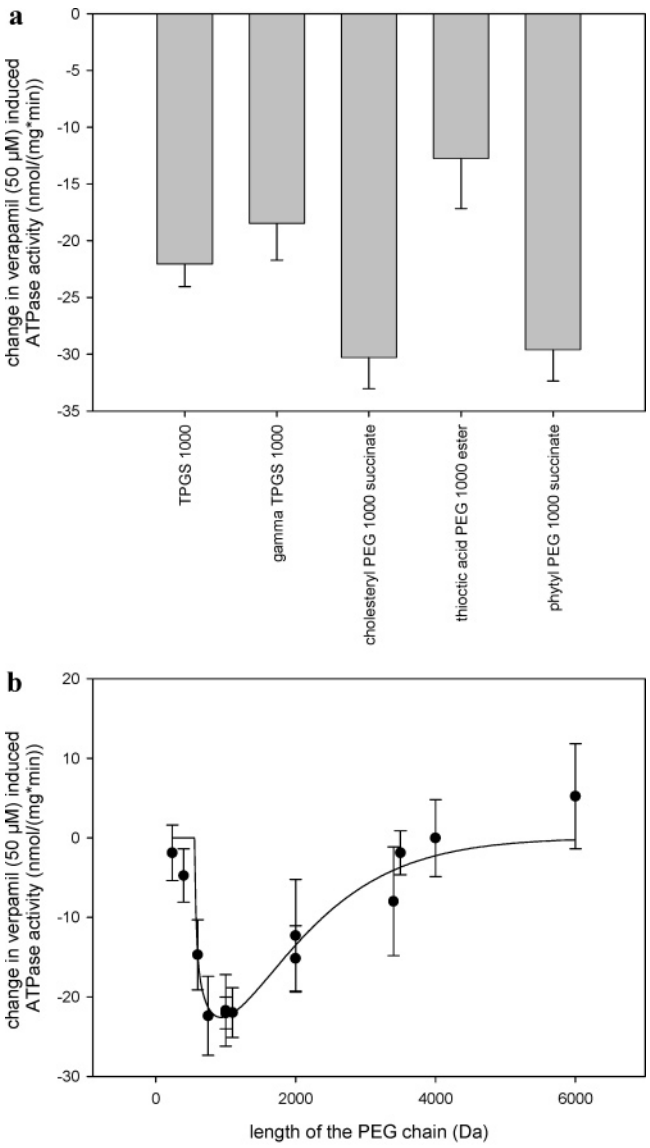

Figure 5. (A) Change of verapamil (50 μM) induced ATPase activity in the presence of different TPGS analogues (33 μM) with modified hydrophobic moieties; mean ± SD, $n = 8$. (B) Change of verapamil (50 μM) induced ATPase activity in the presence of different TPGS analogues (33 μM) with varying PEG chain lengths; mean ± SD, $n = 8$.

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activity[13−15] are among the different mechanisms that have been proposed. However, so far there is no clear answer; results from different studies are contradictory, and systematic studies with homologous groups of surfactants are lacking.

In an attempt to correlate our results with data from a previous SAR study,[16] we focused on TPGS 1000 and two groups of homologous analogues with either modified PEG chain lengths (Figure 1 A) or modified hydrophobic moieties. Using ESR as our method of choice, we investigated the influence of TPGS 1000 and its analogues on membrane fluidity. The ESR technique was already previously used to study membrane fluidizing effects of bile salts and verapamil in viable MDR1 overexpressing Chinese hamster ovary (CHO) cells.[31]

At low concentrations (up to 0.33 mM) TPGS 1000 had only negligible effects on Caco-2 membrane fluidity, not statistically significantly ($p > 0.05$) reducing the membrane microviscosity (Figure 3). Our findings contradict previous results showing TPGS 1000 (0.025 and 0.10 mM) to rigidify Caco-2 cell membranes.[2] Those measurements were performed using a fluorescence anisotropy method where the rigidifying effect of TPGS 1000 was restricted to the lipophilic fluorescent dye 1,6-diphenyl-1,3,5-hexatriene (DPH). DPH was incorporated into the nonpolar side chain regions of Caco-2 cell membranes, whereas its cationic derivative 1-(4-trimethylammoniumphenyl)-6-diphenyl-1,3,5-hexatriene (TMA-DPH) measured membrane effects in the polar head group region and was unaffected by presence of TPGS. DPH measurements often present highly variable results, as can be seen when comparing studies using other known P-gp surfactant modulators (e.g., Cremophor EL). Hugger et al.[12] reported no effect of Cremophor EL on DPH or TMA-DPH membrane fluidity in Caco-2 and MDCK-MDR1 cells; yet, other groups reported either a significant fluidization of the cell membrane in the presence of Cremophor EL[35] or a rigidifying effect of the surfactant on KB 8-5-11 (human epidermoid carcinoma) cells.[36] Besides the use of different cell lines with varying lipid compositions and protein content, the distribution of fluorescent dyes into intracellular organelles may help explain the experimental discrepancies. DPH is not plasma membrane specific; it is known to distribute to other cell organelles within the cell, such as mitochondrial membranes. Therefore, the net polarization value is a product of all these effects.[37] Furthermore, variable placement of the fluorescent dye into the lipid bilayer may also contribute to the contradictory findings. Altogether, the shortcomings of the fluorescence anisotropy method necessitate the use of alternative techniques, such as ESR. Spin probes, such as 5-SA, are derived from lipidic acids and are much more lipophilic than fluorescent dyes (e.g., DPH and TMA-DPH). Relative to fluorescent dyes, spin probes incorporated into cell membranes are more stable and have not been reported to access intracellular membranes. The spin probe position within the membrane is highly defined, and by adjusting the position of the spin active group, different regions of the cell membrane may be selectively studied.

Significant membrane fluidizing via TPGS 1000 was only observed above 3.3 mM (Figure 3). This concentration is about 100 times higher than the concentration at which full inhibition in Caco-2 transport studies was observed, and almost 1000 times higher than the IC_{50} value determined for TPGS 1000 in the transport (5.86 ± 2.17 μM, data not shown) and ATPase assays. Instead, the findings regarding membrane fluidization match quite well with results from previous cytotoxicity studies, where the release of the intracellular enzyme LDH as an indicator of cell membrane damage was increased. Increased LDH release (>20% compared to positive control Triton-X 100 1%) reflecting a destabilization of the cell membrane was observed with a TPGS 1000 concentration of ≥2.5 mM.[16] The discrepancy between membrane fluidization and effective inhibitory concentration weakens the argument that surfactant mediated modulation of P-gp efflux is caused by some unspecific membrane perturbation.

Furthermore, cholesteryl PEG 1000 succinate, which performed as well as commercial TPGS 1000 in the ATPase inhibition assay and surpassed its inhibitory potential in the Caco-2 transport assay,[17] demonstrates opposite behavior to TPGS 1000. Instead of fluidizing the cell membrane, cholesteryl PEG 1000 succinate significantly ($p < 0.05$) rigidified the cell membrane (≥3.3 mM; Figure 3). It seems highly unlikely that two opposing phenomena (e.g., membrane rigidification and fluidization) mediated by two related substances would lead to identical changes in P-gp conformational mobility, a factor believed to be responsible for efflux pump inhibition.

Previously[16] we had established that different TPGS analogues with varying PEG chain lengths afford different P-gp inhibitory potential *in vitro*. The ESR head to head comparison data also support the notion that P-gp inhibition via TPGS 1000 is not directly correlated to cell membrane fluidization (Table 2). TPGS analogues were tested at 33 μM and 3.3 mM; these concentrations produced a maximum TPGS 1000 influence in the Caco-2 P-gp inhibition transport assay and the first significant fluidizing effects in the TPGS 1000 dose−response ESR studies, respectively. At 33 μM,

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all TPGS analogues, regardless of whether they were previously shown to be P-gp active or nonactive in Caco-2 transport experiments, had only negligible influence on rigidity. These findings are consistent with the noneffect in the TPGS 1000 dose response curve at this concentration. Also, we did not observe a correlation of PEG chain length with cell membrane fluidization or rigidification. At the higher concentration of 3.3 mM, a significant ($p < 0.05$) fluidization of the cell membrane was observed for TPGS 1000 and all other derivatives with higher molecular weights. It appears that a certain PEG chain length, or rather molecular size, is required to mediate a significant fluidizing effect. However, higher molecular weight TPGS analogues, such as TPGS 4000 and TPGS 6000—nonactive in previous P-gp inhibition assays—afforded a similar decrease in *S*; intercalation of surfactant chains into the lipid bilayer of the cell membrane apparently levels off or may be reduced above a PEG molecular weight of 1000.

As an alternate hypothesis to nonspecific alterations in membrane microviscosity, inhibition of efflux pump ATPase was then addressed in an ATPase assay. In an attempt to consistently maintain a high amount of P-gp expression and selectively study a single efflux pump, artificial P-gp enriched membranes were used. Four different P-gp substrates (verapamil, quinidine, nicardipine, and progesterone) known to interact with different binding sites of the efflux pump, and/or occupy different regions of the large drug binding pocket,[38,39] were chosen as inducers of ATPase activity.

Optimal P-gp substrate concentrations were determined via standard dose response experiments. In accordance with previous studies,[20,38] verapamil, progesterone, quinidine, and nicardipine all gave bell shaped dose response curves with maximum induction of ATPase activity at 50 μM, 100 μM, 50 μM, and 1 μM, respectively (data not shown). Dose response curves for TPGS 1000 were generated in the presence and absence of P-gp substrates. When incubated on its own with artificial P-gp containing membranes (Figure 4), TPGS 1000 neither stimulated ATPase activity nor inhibited basal ATPase function over a concentration range from 0.0033 nM to 330 μM, indicating no direct interaction between the transport sites and the surfactant. TPGS 1000 therefore appears not to be a substrate of P-gp, making a competitive inhibition of substrate binding unlikely. However, a significant dose dependent inhibition of P-gp after substrate induced ATPase activation was indeed observed. The determined IC_{50} values for inhibition of verapamil and progesterone induced ATPase activity are comparable to the IC_{50} value determined in a transport assay with the fluorescent dye RHO (5.86 ± 2.17 μM); these data indicate a correlation between inhibition of substrate transport and ATPase inhibition. IC_{50} values for inhibition of quinidine and nicardipine induction of ATPase activity were about 10 times lower. Inhibition of substrate induced ATPase activity in all cases occurred at concentrations far below the CMC of TPGS 1000 (0.02 wt % corresponding to 132 μM TPGS 1000), thus ruling out an unspecific micellation of P-gp substrates as a possible cause.

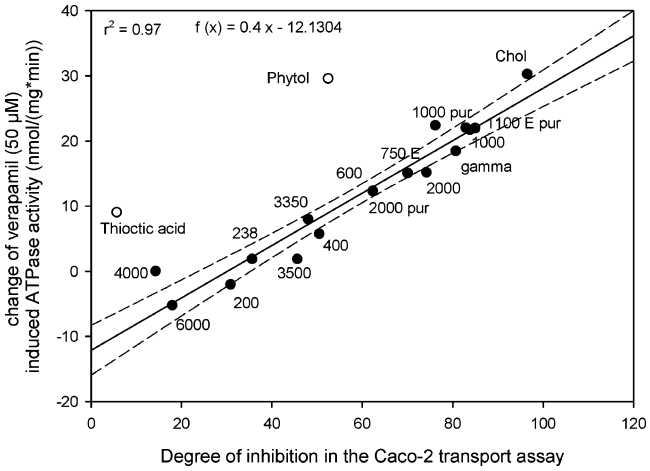

Figure 6. Correlation of change in induced ATPase activity mediated by different TPGS analogues and their degree of inhibition in the Caco-2 cell monolayer efflux assay.

As summarized in Figure 6, the change of verapamil (50 μM) induced ATPase activity could be correlated to the degree of inhibition observed in the Caco-2 transport assay. Generally, a good correlation ($r^2 = 0.97$) was found between the inhibitory potency of the various TPGS analogues and their effect on verapamil induced ATPase activity. Consistent with findings from previous transport experiments, cholesteryl PEG 1000 succinate showed the strongest inhibition of verapamil induced ATPase activity. Cholesteryl PEG 1000 succinate, as well as phytyl PEG 1000 succinate, surpassed commercially available TPGS 1000 (Figure 5A). However, phytyl PEG 1000 succinate performed considerably worse in the bidirectional Caco-2 transport assay and therefore lies outside the correlation (Figure 6). The discrepancy could be attributed to strong cytotoxic effects of phytyl PEG 1000 succinate, as shown by increased LDH leakage and a massive drop off of TEER values during the transport experiment (data not shown). Compound cytotoxicity compromises the outcome of the cellular efflux assays, making the data less reliable. Thioctic acid PEG 1000 ester is the only other derivative besides phytyl PEG 1000 succinate to lie outside the correlation. Its effect in the transport assay was markedly lower than would be expected by the extent of ATPase inhibition, likely due to an increased ester hydrolysis in the unstable compound during the course of the transport experiment. A slight ATPase activity induction was observed in the presence of TPGS 6000. This phenomenon may be

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explained by a micellar entrapment of inhibitors of unspecific ATPases (ouabain, EGTA, and sodium azide) present in the assay cocktail. A concentration of 33 μM was used, a concentration higher than its CMC of 0.02 wt % (corresponding to 30 μM TPGS 6000). Altogether, the ATPase assay data suggests that ATPase activity inhibition is a major part in the mechanism of P-gp inhibition by TPGS, and perhaps nonionic surfactants in general. Pluronic block copolymers have been shown to inhibit efflux pump ATPases.[11] Pluronic copolymers with intermediate lengths of hydrophobic PO (propylene oxide) chains and relatively short hydrophilic EO (ethylene oxide) segments, a model representative being Pluronic P85, were shown to have the highest MDR modulating potential.[40] Their modulatory influence on P-gp, and other ABC transporters, was attributed to a so-called "double punch" effect of intracellular energy depletion by disruption of mitochondria and membrane fluidization, which directly influences P-gp ATPase activity.[15]

As discussed above, we could not show relevant membrane fluidizing effects for TPGS analogues at P-gp active concentrations. Therefore, alternative hypotheses may be proposed: (i) merely incorporating large surfactant molecules into the cell membrane affords a reduction of ATPase activity; the substrate cannot become bound due to steric blocking of the binding site; and/or (ii) a direct interaction of the surfactant with allosteric sites—not with a transport site—occurs in the P-gp efflux pump. There is strong evidence for the existence of several allosteric sites in P-gp, such as the *cis*-(*Z*)-flupentixol binding site[41,42] and the SR33557 binding site.[43] Both explanations appear consistent with the varying IC_{50} values for progesterone/verapamil and quinidine/nicardipine determined in the ATPase assay. Sterically blocking substrate binding may not encompass all binding sites, or portions of the binding pocket, to the same extent. Alternatively, an allosteric modulation of P-gp may influence different binding sites to varying extents. A direct interaction of surfactant molecules with the efflux pump intracellular ATP binding domains is considered unlikely; such a modulation of ATPase activity would be expected to influence all P-gp substrates to a similar extent and perturb basal ATPase activity.

Conclusion

The ability of different TPGS analogues to inhibit verapamil induced ATPase activity was found to correlate with their inhibitory potential in the Caco-2 cell transport assay. The correlation supports the notion that efflux pump energy source depletion is a major factor in the inhibitory mechanism of TPGS. ATPase inhibition seems to be a function of sterically blocking the substrate binding and/or allosteric modulation of P-gp rather than a competitive inhibition or an unspecific rigidification or fluidization of the cell membrane; such effects were only observed at concentrations about 100-fold higher than those needed to achieve full efflux inhibition and did not correlate with the activity pattern in bidirectional transport studies.

Abbreviations Used

ESR, electron spin resonance; P-gp, P-glycoprotein; TPGS 1000, D-α-tocopheryl polyethylene glycol 1000 succinate; *S*, order parameter; 5-SA, 5-doxyl stearic acid; MDR, multidrug resistance.

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