Inhibition of P-Glycoprotein by D-α-Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS)

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Purpose. To investigate whether d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) functions as an inhibitor of P-glycoprotein (P-gp), the multidrug resistance transporter.

Methods. Two assays were used to measure the function of TPGS on P-gp function. First, we examined the ability of TPGS to modulate the cytotoxicity of established, cytotoxic, P-glycoprotein substrates. Parental NIH 3T3 cells and NIH 3T3 cells transfected with the human MDR1 cDNA (G185) were exposed to doxorubicin, paclitaxel, colchicine, vinblastine and 5-fluorouracil (5FU) in the presence or absence of TPGS. Cytotoxicity was assessed with the MTT assay. Second, polarized transport of the P-gp substrates rhodamine 123 (R123), paclitaxel and vinblastine was measured using the human intestinal HCT-8 and Caco-2 cell lines grown in Transwell dishes. Drug flux was measured by liquid scintillation counting or fluorescence spectroscopy of the media.

Results. G185 cells were 27–135 fold more resistant to the cytotoxic drugs doxorubicin, vinblastine, colchicine and paclitaxel than the parental NIH 3T3 cells. In contrast 5FU, which is not a P-gp substrate, is equally cytotoxic to parental and G185 cells. Co-administration of TPGS enhanced the cytotoxicity of doxorubicin, vinblastine, paclitaxel, and colchicine in the G185 cells to levels comparable to the parental cells. TPGS did not increase the cytotoxicity of 5FU in the G185 cells. Using a polarized epithelial cell transport assay, TPGS blocked P-gp mediated transport of R123 and paclitaxel in a dose responsive manner.

Conclusions. These data demonstrate that TPGS acts as a reversal agent for P-glycoprotein mediated multidrug resistance and inhibits P-gp mediated drug transport. These results suggest that enhanced oral bioavailability of drugs co-administered with TPGS may, in part, be due to inhibition of P-glycoprotein in the intestine.

KEY WORDS: P-glycoprotein; TPGS; drug transport; bioavailability.

INTRODUCTION

The multidrug transporter, P-glycoprotein (P-gp), is a 170 kDa membrane protein which functions as an ATP-dependent drug efflux pump. One activity of this protein is to lower the intracellular concentration of drugs thereby reducing the cytotoxic activity of anticancer drugs. Increased expression of this protein has been observed in human tumors and is often associated with failure of chemotherapy due to drug resistance (1–5). P-gp removes a large number of chemically unrelated drugs extending over many therapeutic indications such as anticancer drugs, steroids, antihistamines, antibiotics, calcium channel blockers and anti-HIV peptidomimetics (2,4,5).

The P-gp drug transporter is encoded by one gene, MDR1, in humans whereas in rodents two genes, *mdr1a* and *mdr1b* encode highly similar drug transporters (6,7). P-gp is primarily expressed on the luminal surface of epithelial cells from several tissues including the intestine, liver, kidney, and the endothelial cells comprising the blood-brain and blood-testes barriers (8–10). The ability of this protein to export toxic compounds combined with this localization led to the hypothesis that a physiological function of the MDR1 encoded P-gp may be as a protective barrier or export mechanism for xenobiotics. Indeed, recent investigations with knockout mice in which the *mdr1a* gene was disrupted have confirmed such a protective role for P-gp (11–14). Exposure of *mdr1a* deficient mice to vinblastine or ivermectin results in significantly higher tissue and plasma levels compared to wild-type animals. Moreover, these compounds are toxic in the knockout mice at doses which are innocuous to heterozygous and wild-type mice. These experiments further suggested a role for P-gp in the blood brain barrier since the ivermectin accumulated in the brain of the *mdr1a* deficient animals but not animals with an intact *mdr1a* gene. The knockout mice displayed ivermectin toxicity at doses 50 to 100 fold less than wild-type mice.

Additional data have supported a role of P-gp in the intestine as both a barrier to absorption as well as a mechanism of disposition of drugs such as vinblastine, etoposide, paclitaxel and digoxin. For example, Su and Huang observed that inhibition of P-gp increased bioavailability of digoxin by increasing absorption as well as reducing excretion (15). A similar phenomenon was observed with etoposide (16). P-glycoprotein has recently been suggested to be critical in oral drug absorption (17–19). In concert with the drug metabolizing enzyme CYP3A, P-gp may limit oral drug bioavailability in the gut by controlling drug transport from the intestinal lumen and by affecting access to CYP3A (19).

Vitamin E TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate, is a derivative of vitamin E consisting of a hydrophilic polar head group (tocopherol succinate) and a lipophilic alkyl tail (polyethylene glycol) resulting in amphiphillic properties (Eastman Kodak, technical bulletin EFC-226). TPGS has a relatively low critical micelle concentration, 0.02 wt%, and acts to solubilize lipophilic compounds. Bordreaux et al. reported a two-fold increase in cyclosporine CsA area under the plasma concentration-time-curve (AUC) when co-administered with LiquiE, a glycerol and water solution of TPGS (20). Sokol et al. similarly observed increases up to 71% in CsA AUC in subjects who received concomitant TPGS (21). Both Sokol and Bordreaux suggested that the increased drug absorption was due to enhanced micelle formation, resulting in improved CsA solubilization. Chang *et al.* later reported a 61% increase in CsA AUC when dosed with 20–25% of the TPGS previously used in the Sokol or Bordreaux studies (22). Chang *et al.* also suggested that TPGS may interact with P-gp in the intestine to increase CsA absorption.

In the current investigation we examine the effect of TPGS on P-gp mediated drug resistance and transport of established P-gp substrates. If this agent functions as a P-gp reversal agent then perhaps its effect on drug absorption is, in part, mediated by inhibition of active drug efflux in the intestine. Our data show TPGS to be an effective inhibitor of P-gp mediated drug

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ABBREVIATIONS: P-gp, P-glycoprotein; R 123, Rhodamine 123; CsA, Cyclosporine A; TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate.

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resistance and transport at concentrations well below the reported critical micelle concentration and suggests that its reversal activity is due to an effect on transport activity.

MATERIALS AND METHODS

Cell Culture

The NIH3T3 Swiss mouse embryo cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and was grown in Dulbecco's Modified Eagles Medium (Biowhittaker, Walkersville, MD) supplemented with 4.5 g/L glucose, 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (Advanced Biotechnologies Incorporated (ABI), Columbia, MD), and 0.01 mg/ml gentamicin (ABI). The drug resistant, NIH-MDR-G185, cell line, derived by transfection of the human MDR1 gene into NIH3T3 cells (23), was obtained from M. M. Gottesman (NCI, NIH) and was maintained in similar medium supplemented with 60 ng/ml of colchicine (Sigma, St. Louis, MO). HCT-8 cells (ATCC), isolated from a human ileocecal adenocarcinoma cell line, were grown in RPMI-1640 medium (Biowhittaker) supplemented with 10% horse serum (Biowhittaker), 1 mM sodium pyruvate (Gibco BRL, Grand Island NY) and 0.01 mg/ml gentamicin. Caco-2 cells (ATCC), derived from a human colonic adenocarcinoma, were grown in Eagle's MEM (Biowhittaker) supplemented with 10% fetal bovine serum, and 0.01 mg/ml gentamicin. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Cytotoxicity Assay

Cells were plated at a density of 2.5–3.0 × 10^3 cells/well in 96-well microtiter plates (PGC, Gaithersburg, MD) and were exposed to 1–5000 nM of doxorubicin, vinblastine, colchicine, paclitaxel, 0.1–25 nM 5-fluorouracil (Sigma) and 0.001–.005% TPGS (Eastman, Kingsport, TN) for 72 hours. To ensure solubilization of the TPGS, a 1% solution of TPGS in ethanol was prepared fresh for each experiment and diluted further in cell culture medium to the indicated concentrations. Cell viability was determined with the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium, Sigma) assay as previously described (24,25) and the absorbance was measured with a Dynex MRX Microplate Reader (Chantilly, VA) at 570 nm. This assay is based on the reduction of MTT by mitochondria in viable cells to water insoluble formazan. The data presented are the mean ±SD of at least 3 independent experiments, each performed in quadruplicate.

Rhodamine 123 Transport

Rhodamine 123 (R123; Sigma) transport was examined as previously described (26,27) using both HCT-8 and Caco-2 cells. Briefly, cells were grown in 6 well Corning Transwell dishes (HCT-8) or collagen coated Transwell dishes (Caco-2) until a tight monolayer was formed as measured by transepithelial electrical resistance or lucifer yellow impermeability. The integrity of the monolayers following the transport experiments was similarly evaluated. Typical TEER values were > 300 Ohms/cm^2. R123 was added at a final concentration of 13 μM to the basal or apical compartments and 200 μl samples were taken at the indicated times from the opposite chamber. TPGS was added as an inhibitor to both compartments. Fluorescence of R123 in the media samples was measured using a Biotek FL500 Fluorescence Plate Reader (Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All experiments were performed in triplicate; the data presented are the mean ±SD and are representative of multiple experiments.

Paclitaxel, Vinblastine and Cyclosporine Transport

Inhibition of [^{3}H] paclitaxel (Moravek Biochemical, Brea, CA), [^{3}H] vinblastine (Amersham, Arlington Heights, IL), and [^{3}H] cyclosporine (CsA; Amersham) efflux by TPGS was examined in a manner similar to R123. The transported drug, 0.1 μM (0.25 μCi/ml), was added to either the basal or apical compartment and 200 μl aliquots were taken at the indicated times from the opposite chamber. Radioactivity was measured by liquid scintillation counting.

Western Blot Analysis

Western blot analysis was performed as previously described 28. Briefly, crude cell membranes were isolated by lysing the cells in 10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1 mM $MgCl_2$ supplemented with pepstatin (1.5 μg/ml), leupeptin (1.5 μg/ml) and 0.2 mM pefabloc. Cells were homogenized with 20 strokes of Dounce "B" (tight) pestle (Wheaton, Millville, NJ), nuclei and cell debris were removed by centrifugation for 10 minutes at 400 × g. The supernatants were then centrifuged at 100,000 × g for 30 minutes at 4°C and the pellets were resuspended in lysis buffer and stored at −80°C. 20 μg samples were fractionated in 8% polyacrylamide-SDS gel and transferred to 0.45 μm nitrocellulose membrane. The membranes were blocked in PBS-T (0.1% Tween-20 in PBS) containing 5% skim milk for 1 hour and then probed with 1 μg/ml of C219 antibody (Signet Laboratories, Dedham, MA) in PBS overnight. The membranes were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL).

RESULTS

Western Blot Analysis

We first measured the relative levels of P-gp expression in the NIH3T3 and G185 cell lines by western blot analysis using the C219 antibody, which recognizes all P-gp isoforms (29). Consistent with previous data, high P-gp expression was observed in the G185 cells relative to that in the parental NIH 3T3 cells (Fig. 1). We also examined P-gp expression in two human intestinal carcinoma cell lines, Caco-2 and HCT-8 which have been previously used for investigation of drug transport and to have polarized expression of P-gp (26). We observed that each of these intestinal cell lines have moderate P-gp expression albeit lower than the G185 cells (Fig. 1).

Cytotoxicity Experiments

The interaction of TPGS with P-gp was initially examined with cytotoxicity assays using parental NIH3T3 and MDR1 transfected G185 cells to cytotoxic anticancer drugs. Consistent with previous reports (23,30), G185 cells were more resistant

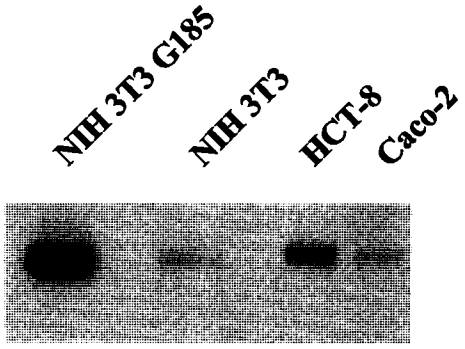

Fig. 1. Western blot analysis of P-glycoprotein expression. Twenty micrograms of total cell membrane proteins were separated by SDS-PAGE, transferred to PVDF filters which were subsequently probed with the C219 antibody and visualized using chemiluminescence as described in Materials and Methods. Lane 1, NIH-3T3 G185; lane 2, NIH-3T3; lane 3, HCT-8; lane 4, Caco-2.

Table 1. EC_{50} in NIH3T3 and NIH3T3-G185 Cells

	NIH3T3	NIH3T3-G185	NIH3T3-G185 (1 μM Verapamil)	NIH3T3-G185 (5 μM CsA)	NIH3T3-G185 (.0025% TPGS)
Doxorubicin	35	950	35	35	35
Vinblastine	2	270	20	6	40
Paclitaxel	60	>5000	100	40	1070
Colchicine	30	1000	ND	100	45

Note: NIH 3T3 and G185 cells were treated with 0–5000 nM doxorubicin, vinblastine, paclitaxel or colchicine in the absence or presence of 1 μM verapamil, 5 μM CsA or 0.0025% TPGS. The concentration of the drug that reduces cell viability by 50% (EC_{50}) was determined using the MTT cytotoxicity assay as described in Materials and Methods. Each experiment was performed in quadruplicate and repeated in at least 3 independent experiments. ND, not determined.

to doxorubicin, paclitaxel, vinblastine and colchicine compared to parental NIH3T3 cells (Fig. 2). EC_{50} values were 27 to 135 fold higher in G185 cells relative to the parental NIH3T3 cells (Table 1). Established P-gp reversal agents, such as cyclosporine A (CsA) and verapamil, reduced the resistance to doxorubicin cytotoxicity in G185 cells to levels comparable to parental NIH3T3 cells (Fig. 3). The reversal effect of CsA on doxorubicin, vinblastine, taxol and colchicine mediated toxicity in parental NIH3T3 cells was modest as previously reported. This is consistent with their low level of P-gp expression (data not shown, (27,30). Co-administration of CsA or verapamil caused a similar reversal of G185 resistance to vinblastine, paclitaxel, and colchicine (Table 1 and data not shown).

The effect of TPGS on P-gp mediated drug resistance was investigated by treating G185 cells with doxorubicin, vinblastine, paclitaxel, and colchicine concomitantly with varying doses of TPGS. The presence of TPGS increased drug sensitivity of the G185 cells to doxorubicin in a dose dependent manner

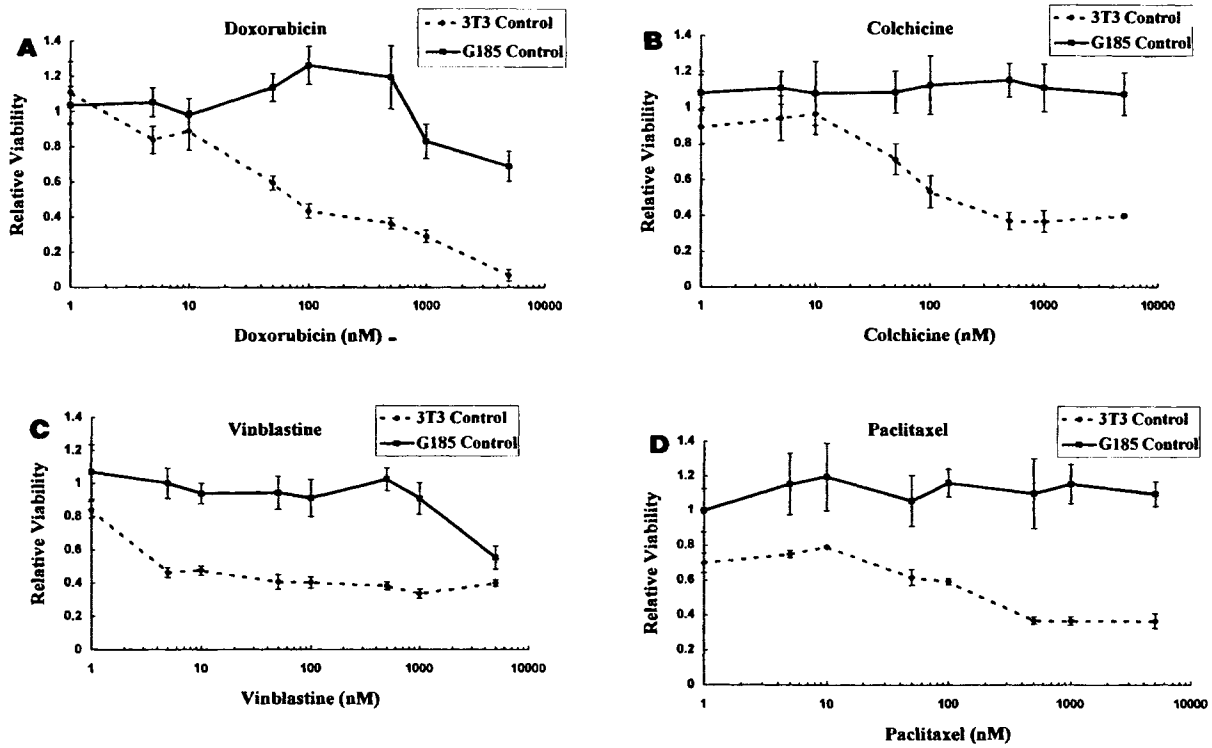

Fig. 2. Cytotoxicity of doxorubicin (A), colchicine (B), vinblastine (C), and taxol (D) in parental NIH-3T3 cells (circles), and MDR1-transfected NIH-3T3 G185 cells (squares). Cells were treated with the indicated concentrations of drugs and the viability was measured by the MTT assay as described in Materials and Methods. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ± SD of four independent experiments.

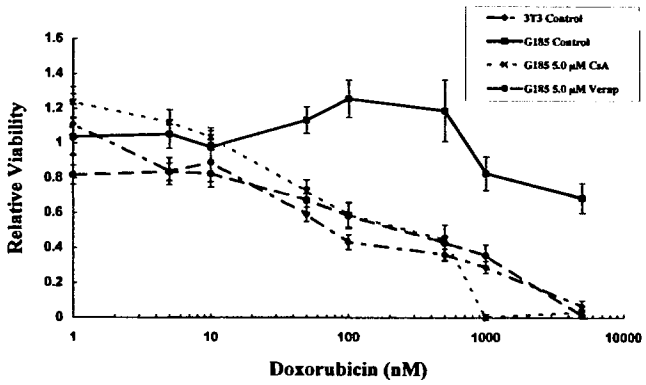

Fig. 3. Effect of CsA and Verapamil on the cytotoxicity of doxorubicin. Parental NIH-3T3 (diamonds) and G185 (squares) cells were exposed to the indicated concentrations of doxorubicin in the absence or presence of CsA, 5.0 μM, or verapamil, 5.0 μM.. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ± SD of four independent experiments.

(Fig. 4). Treatment of the drug resistant G185 cells with TPGS lowered the EC_{50} concentrations for doxorubicin, vinblastine, paclitaxel and colchicine (Table 1). TPGS, 0.0025%, sensitized the G185 cells to all four of these P-gp substrate cytotoxic drugs to levels comparable to the parental NIH 3T3 cells. The highest dose of TPGS, 0.005%, resulted in decreased viability of both NIH3T3 and G185 cells and is likely due to toxicity associated with the high concentration of TPGS. At concentrations below 0.005% TPGS itself did not affect cell viability. These data suggest that TPGS modulates drug resistance by inhibiting P-gp activity in cells which over-express the MDR1 gene.

5-Fluorouracil Cytotoxicity

Treatment of parental NIH 3T3 and G185 cells with 5-fluorouracil (5FU), a chemotherapeutic agent not transported by P-gp, results in a similar level of cytotoxicity in both cell lines (Fig. 5A) (31). Furthermore, co-incubation of 5FU with CsA had no effect on the cytotoxicity of 5FU in either G185 or NIH3T3 cells (Fig. 5B). Similarly, co-incubation of TPGS with 5FU did not increase the cytotoxicity of 5FU in either of these cell lines (Fig. 5C).

Rhodamine 123 Transport

The fluorescent dye R123, an established substrate of P-glycoprotein (32,33), was used to examine the ability of TPGS to block P-gp mediated transport. HCT-8 and Caco-2 cells have previously demonstrated directional transport of established P-gp substrates such as vinblastine, paclitaxel, CsA and R123 in the basolateral to apical direction (26,34–36). Expression of P-gp in these cells was confirmed by western blot analysis using the C219 antibody (Fig. 1). R123 was transported approximately 7 and 9 fold greater flux in the basolateral to apical direction in HCT-8 and Caco-2 cells, respectively (Fig. 6). Consistent with this transport being mediated by P-gp, R123 flux was inhibited approximately 80% by co-incubation with 5 μM CsA. Similarly, 0.001–0.0025% TPGS blocked the basolateral to apical transport of R123 in a dose responsive manner further suggesting that TPGS inhibits transport mediated by P-gp (Fig. 6).

Paclitaxel Transport

The ability of TPGS to inhibit P-gp was confirmed by measuring polarized transport of paclitaxel. [^{3}H] Paclitaxel is a good substrate for P-gp with approximately 14 and 40 fold greater transport from the basolateral to the apical compartment in HCT-8 and Caco-2 cells, respectively (Fig. 7). Addition of 5 μM CsA blocked the polarized flux of paclitaxel by 80–90%. Similarly, co-incubation with TPGS resulted in a dose dependent decrease in paclitaxel transport (Fig. 7). The IC_{50} of TPGS for inhibition of paclitaxel transport is approximately 0.001% (v/v) in HCT-8 cells and 0.005% in Caco-2 cells. Polarized

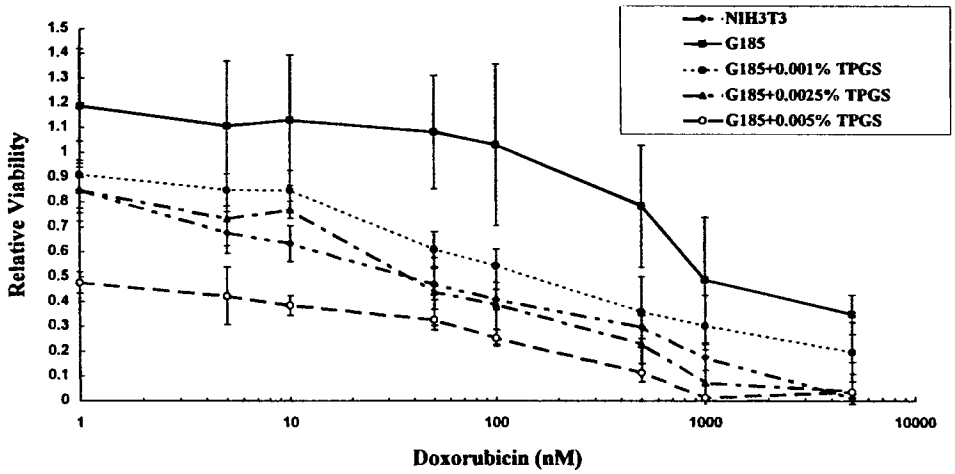

Fig. 4. TPGS reversal of P-gp mediated resistance to doxorubicin. Parental NIH-3T3 (diamonds) and G185 cells were exposed to the indicated concentrations of doxorubicin with 0% TPGS (squares), 0.001% TPGS (circles), 0.0025% TPGS (triangles) or 0.005% TPGS (open circles, ○). Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ±SD of four independent experiments.

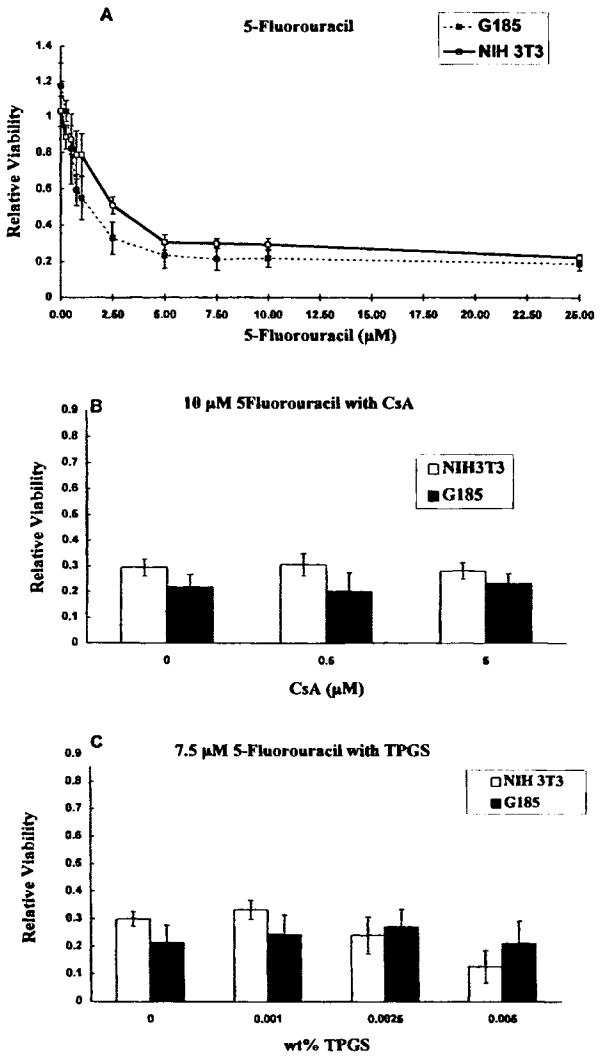

Fig. 5. Cytotoxicity of 5-fluorouracil to parental NIH-3T3, and drug resistent NIH-3T3 G185 cell lines in the presence and absence of CsA and TPGS. A. NIH-3T3 (diamonds) and NIH-3T3 G185 (squares) cells were exposed to the indicated concentrations of 5-fluorouracil. Viability was measured by the MTT assay as described in Materials and Methods. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ±SD of three independent experiments. B. Parental NIH-3T3 (open bars) and G185 (closed bars) cells were exposed to 10 μM of 5FU with the indicated concentrations of CsA. C. Parental NIH-3T3 (open bars) and G185 (closed bars) cells were exposed to 7.5 μM of 5FU and the indicated concentrations of TPGS.

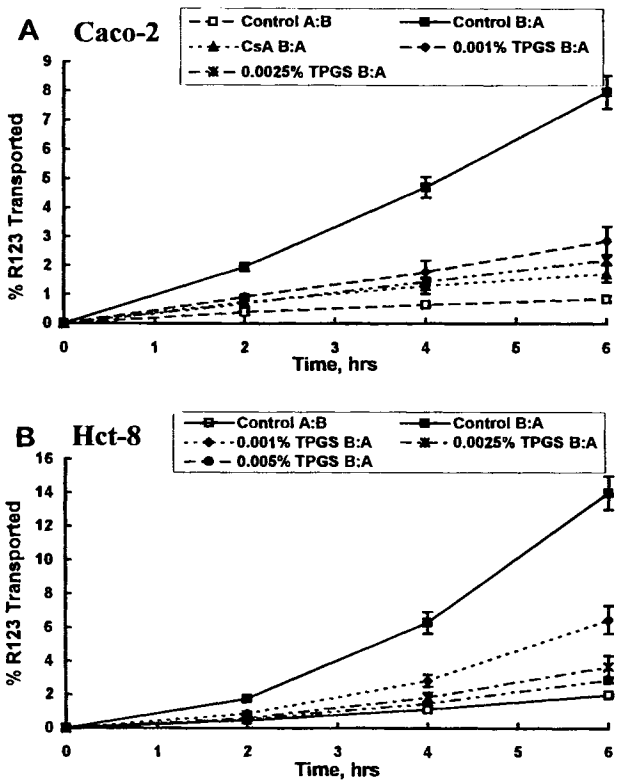

Fig. 6. Rhodamine 123 transport in Caco-2 and HCT-8 cells. Caco-2 (A) and HCT-8 (B) were grown on Transwell dishes as described in Materials and Methods. Rhodamine 123, 13 μM, was added to the apical or basolateral compartment in the absence or presence of CsA, 5 μM, or 0.0025, 0.005, 0.001% TPGS and media aliquots were taken from the opposite chamber at the indicated times. The data presented are the mean ±SD of triplicate wells and are representative of at least three independent experiments.

transport of [^{3}H] vinblastine and [^{3}H] CsA were also inhibited by addition of TPGS (data not shown). These data, combined with the cytotoxicity and R123 transport data suggest that TPGS is an effective P-gp reversal agent.

DISCUSSION

A major effort has been undertaken by many laboratories to identify inhibitors of P-glycoprotein to increase the efficacy of cancer treatment and to enhance the absorption of orally administered drugs. The data presented here support the hypothesis that TPGS functions as one such P-gp inhibitor. TPGS increased the sensitivity of P-gp expressing cells to several widely used cytotoxic drugs which are well established P-gp substrates. TPGS also effectively blocked polarized transport of R123 and paclitaxel in an epithelial cell transport assay. The reduction of directional transport provides strong evidence for TPGS functioning as an inhibitor of P-gp. Conversely, no effect was observed with 5FU, a cytotoxic drug not associated with P-gp mediated drug resistance or transport. 5FU is not transported by the P-gp pump thus, its cytotoxicity is unaffected by the addition of established P-gp inhibitors such as quinine, quinidine or verapamil (31,37). In the experiments presented here neither TPGS nor CsA impacted the cytotoxicity of 5FU in either the NIH 3T3 or G185 cells.

Previously it has been suggested that co-administration of TPGS with CsA enhanced absorption of the immunosuppressant due to micelle formation (21). Concentrations of TPGS administered in the current work are well below the critical micelle concentration, 0.02 wt% in water at 37°C, therefore it is unlikely that micelle formation is responsible for the observed effects. In fact, the IC_{50} required to inhibit R123 and paclitaxel transport across HCT-8 or Caco-2 cell monolayers is 20 fold less than the critical micelle concentration. Further, 0.001 wt% TPGS also significantly reversed the multidrug resistant phenotype of the NIH3T3-G185 cell line to doxorubicin, vinblastine, taxol

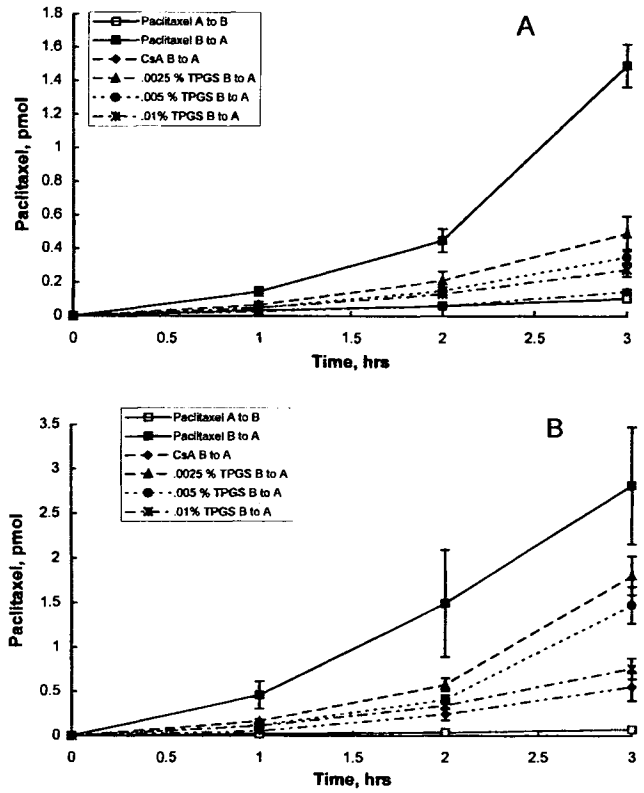

Fig. 7. Paclitaxel transport in HCT-8 and Caco-2 cells. Cells were grown on in Transwell dishes as described in Materials and Methods. [^{3}H]Paclitaxel, 0.1 μM (0.2 μCi/ml) was placed in the basolateral (B) or apical (A) chamber; media aliquots were taken at the indicated times and radioactivity was measured by liquid scintillation counting. Control Caco-2 (A) or HCT-8 (B) cells B to A (closed squares), control A to B (open squares), 5 μM CsA B to A (diamonds), 0.0025% TPGS B to A (triangles), 0.005% TPGS B to A (circles).

and colchicine, all established P-gp substrates. These data suggest that TPGS micelle formation in the intestinal lumen may not be the sole factor behind the increase in CsA absorption previously observed (20–22).

Several other surfactants, e.g. polysorbates, Cremophor EL, and Solutol 15, have been observed to be inhibitors of P-gp (38–41). These compounds are frequently added to pharmaceutical formulations to enhance solubility. These agents may also function to inhibit P-gp to add to their effect of enhancing drug absorption. Indeed the plasma concentrations of Cremophor EL in patients administered paclitaxel, which is formulated with this surfactant, reach levels sufficient to inhibit P-gp *in vitro* (42). The efficacy of this drug may, in part, be due to the activity of the Cremophor EL. Pluronic P85 has also recently been observed to block P-gp mediated rhodamine 123 efflux in Caco-2 and bovine brain microvessel endothelial cells (43). These data suggested that this agent may be useful for formulations to enhance brain and oral absorption.

TPGS has been used to enhance the bioavailability of CsA in liver transplant patients with the effects of significantly improving absorption and reducing daily drug cost. Sokol *et al.* and Boudreaux et al. reported increases in CsA absorption in pediatric transplant recipients treated with oral TPGS, 12.5 IU/kg and 10 IU/kg, respectively. The majority of patients receiving TPGS had previously experienced chronic cholestasis resulting in decreased bile flow suggesting poor solubilization of the lipophilic CsA. It was hypothesized that TPGS functioned as a bile substitute and solubilized the CsA through micelle formation, thus facilitating the absorption of the drug through the intestinal lumen. Similarly, Pan *et al.* reported a 28 and 32% decrease in CsA daily dose when co-administered with Liqui-E, a water soluble form of TPGS and a 26% decrease in daily CsA cost (44). Using normal healthy volunteers, Chang *et al.* observed a 60% rise in CsA area under the curve (AUC) in subjects receiving a TPGS-CsA cocktail. Decreased oral clearance and volume of distribution were also observed in those subjects. These authors proposed that the large, amphipathic TPGS may also be acting as an inhibitor of P-glycoprotein to enhance absorption and decrease transport back into the intestinal lumen. The current data support the hypothesis that one mechanism through which TPGS may enhance oral bioavailability is via inhibition of P-gp. Clearly further study on the effect of TPGS on oral drug delivery is required to confirm such a role.

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