TARGETING CARRIER-MEDIATED TRANSPORT TO IMPROVE THE BLOOD-BRAIN BARRIER PERMEATION OF PACLITAXEL

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ABSTRACT

Purpose: Paclitaxel is a chemotherapeutic used for a variety of cancers; however, it cannot be used for cancers of the CNS. The permeation of paclitaxel across the blood brain barrier (BBB) is very poor due to its large molecular weight and its affinity for the efflux protein P-glycoprotein (P-gp). Analogues of paclitaxel with improved BBB penetration are of clinical interest. One strategy to improve penetration is to target carrier mediated transport systems expressed at the brain endothelium.
Methods: Bovine brain microvessel endothelial cells (BMECs) were grown in 24-well cell culture plates or on 0.4µm polycarbonate membranes for 10-14 days. Paclitaxel analogues containing nutrient-like substituents were first tested for potential interaction with P-gp by using the rhodamine 123 uptake assay. For those derivatives demonstrating reduced interaction with P-gp relative to paclitaxel, side-by-side diffusion studies were then performed to determine if permeability across confluent BMEC monolayers was increased.
Results: Analogues were synthesized such that nutrient-like groups were added to the C7 or C10 positions of paclitaxel. The nutrient-like groups consisted of amide esters of succinate, methyl esters of succinate, succinic acid, biotin and carboxylic acid moieties of varying alkyl chain length. In general, nutrient-like groups added to the C7 position retained or increased affinity for P-gp; however, groups added to the C10 position typically showed reduced interaction with P-gp. For some analogues showing decreased interaction with P-gp there was a slight increase in permeability across BMEC monolayers.
Conclusions: Paclitaxel analogues targeting carrier-mediated transport have been shown to have decreased interaction with P-gp and may have improved permeability across the BBB. However, for those analogues tested to date, few appear to be recognized by any uptake transporter expressed at the brain endothelium. Rather, any improvement in permeability results from the disruption of P-gp recognition through chemical modifications at specific locations on the paclitaxel molecule. However, in some cases the addition of the nutrient-like substituents increases molecular weight making the analogues less likely to permeate via passive diffusion.

INTRODUCTION

Paclitaxel (Taxol®) is a chemotherapeutic drug that binds to microtubules within the cell and causes cell cycle arrest at the G2/M phases which leads to apoptosis. Although paclitaxel is lipophilic, it does not cross the blood-brain barrier (BBB) because it undergoes active efflux by P-gp. In patients with malignant brain tumors, the BBB is often disrupted and some chemotherapeutic agents can enter the tumor, however this delivery is quite variable and unpredictable. One approach to overcoming this problem is the development of paclitaxel derivatives which due to structural modification are no longer substrates for P-gp and furthermore, are substrates for endogenous uptake transporter expressed at the BBB. However, P-gp is able to recognize and transport a wide spectrum of seemingly unrelated, structurally diverse compounds, so deciding which modifications to make is not straightforward. Moreover, rationally synthesizing compounds to be substrates for endogenous uptake systems can be challenging. *In vitro* assays, such as the rhodamine 123 uptake assay in BMECs, can direct chemistry efforts by being an initial screen for P-gp interaction. This initial screen should then be verified by performing side-by-side diffusion studies using BMEC monolayers. These studies will indicate if P-gp is being avoided and if the new derivative has improved permeability due to recognition by an endogenous uptake transporter.

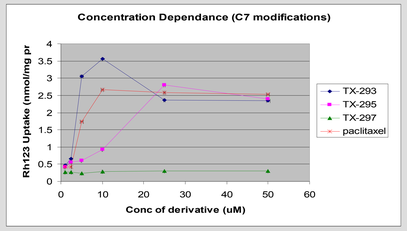
MATERIALS AND METHODS

Materials:
Rhodamine 123 was purchased from the Sigma Chemical Co. (St. Louis, MO). Paclitaxel derivatives were synthesized by the University of Kansas, Department of Medicinal Chemistry. Minimum Essential Medium, and Ham's F12 were purchased from Invitrogen (Carlsbad, CA). Media supplements including amphotericin B, polymixin B, heparin, endothelial cell growth supplement (ECGS), penicillin, streptomycin and sodium bicarbonate were also purchased from Sigma Chemical Co. (St. Louis, MO) as well as fibronectin which was used to coat plates. Plates were also coated with rat tail collagen which was prepared in house. Platelet poor horse serum was kindly provided by By-Prod/The Sirius Group (Caseyville, IL). Lysis buffer was prepared from 0.5% Triton X-100 in 0.2N NaOH. Transwell® polycarbonate 24-well systems were purchased from Corning Costar Corporation (Cambridge, MA). Acetonitrile, formic acid and TBME were all purchased from Fisher Scientific (Fairlawn, NJ).
Cell Culture:
BMECs were grown at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The cells were grown in 50% Minimum Essential Medium and 50% Ham's F12 supplemented with 100µg/mL streptomycin, 100 µg/mL penicillin G, 13mM sodium bicarbonate, 10% platelet poor horse serum, 0.5% ECGS, and adjusted to pH 7.4 with 10mM HEPES. Cells were seeded on polycarbonate Transwell® 24-well plates pre-coated with rat tail collagen and fibronectin or 0.4µm polycarbonate membranes with the same treatment. The cells were seeded at a density of approximately 25,000-50,000 cells/mL.
Rhodamine 123 Uptake:
In this assay, rhodamine 123 is used as a surrogate P-gp substrate. The effect of the test compound on rhodamine is determined by monitoring intracellular fluorescence. If the test compound is a substrate for P-gp, then addition of the compound will increase rhodamine 123 uptake relative to the negative control. Cells were rinsed with warm PBSA (PBS containing $CaCl_2$, $MgSO_4$, glucose and L-ascorbic acid) and allowed to acclimate to the warm PBSA for 10 min. The PBSA was removed and solutions of the paclitaxel derivatives were added to the wells and allowed to incubate for 30 min. at 37 degrees C in a hotbox. (Paclitaxel derivatives were run at 10µM in PBSA). Rhodamine 123 was then added to all wells at a concentration of 5µM and returned to the hotbox. Cells were incubated for an additional 2 hours. After the incubation, cells were quickly rinsed with ice cold PBSA several times. Lysis buffer was then added to all wells and allowed to solubilize cells for at least 30 min. 200µL aliquots were taken from all wells to determine rhodamine 123 concentration and 10µL aliquots were taken from all wells to determine protein concentration.

MATERIALS AND METHODS (cont.)

Sample Analysis:
Rhodamine 123 concentrations were determined on a Biotek FL600 Microplate Fluorescence Reader (excitation λ: 485, emission λ: 530). Protein determination was performed using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL) and samples were read on an EIA reader at 540nm.
Permeation Studies:
BMECs were grown on 0.4µm polycarbonate membranes in a petri dish coated with rat tail collagen and fibronectin. Once cells had formed a confluent monolayer as determined by light microscopy, the membranes were transferred to side-by-side diffusion chambers as previously described by Audus *et al*. Each chamber was filled with 3mL of PBSA and the donor chamber included 10µM of test compound. At various timepoints (5, 15, 30, 45 and 90 minutes), 200µL aliquots were removed from the receiver chamber and replaced with 200µL of blank PBSA warmed to 37 degrees C. All samples were then analyzed by LC/MS. The integrity of the cell monolayer was tested post experiment by monitoring the permeability of [^{14}C]-sucrose which should not readily cross the cell monolayer. Radioactive samples were analyzed by liquid scintillation counting.
Sample Analysis (LC/MS/MS):
Samples were extracted using tert-butylmethylether (TBME) liquid-liquid extraction, centrifuged to dryness, and reconstituted in 60% acetonitrile containing 0.1% formic acid (v/v). Samples were then chromatographed using a Zorbax Eclipse XDB-C8 column with a gradient solvent program and detected by tandem mass spectroscopy (Micromass™ triple quadropole). Electrospray ionization (positive ion mode) was used to generate product-ion spectra of the paclitaxel derivatives and optimal product-ions were selected for quantitative (MRM) analysis.

RESULTS



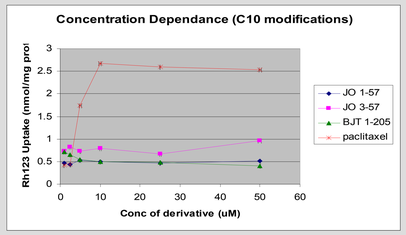


Figure 1. **Rh123 uptake in the presence of a range of concentrations of paclitaxel and several derivatives.** Studies indicate that nutrient-like substituents attached at the C7 position result in a similar interaction with P-gp compared to paclitaxel. Modifications at the C10 position however result in greatly reduced interaction with P-gp at all concentrations. This indicates that C10 may be the preferred location for attachment of nutrient like substituents.

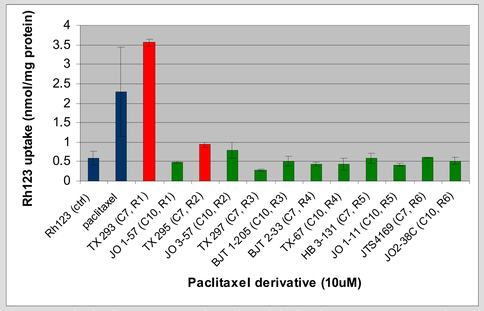


Figure 2. **Rh123 uptake in the presence of paclitaxel derivatives at a concentration of 10uM**. TX-293 with an amide ester of succinate attached at the C7 position shows a dramatic increase in interaction with P-gp as compared to JO1-57 in which the amide ester of succinate is attached at the C10 position. The difference between TX-295 and JO 3-57 is less dramatic but Figure 1 shows there is a concentration dependence involved.

RESULTS (cont.)

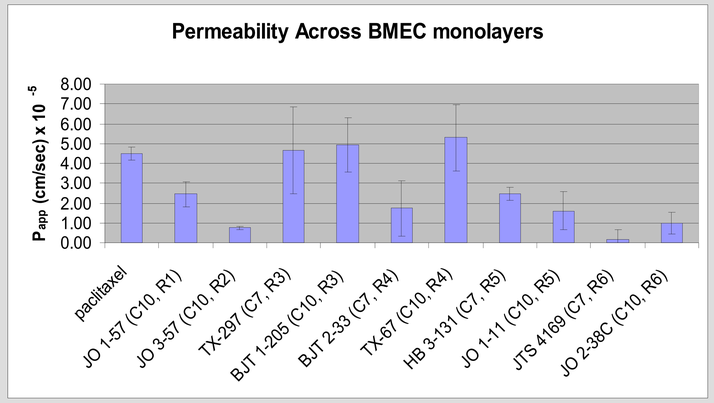


Figure 3. **Comparative permeabilities of paclitaxel derivatives across BMEC monolayers.** Although the Rh123 uptake assay indicated a reduction in P-gp interaction compared to paclitaxel, permeation across BMEC monolayers was not improved relative to paclitaxel. The increase in molecular size as well as lipophilicity may make these molecules more likely to sequester within the cells rather than permeate across.

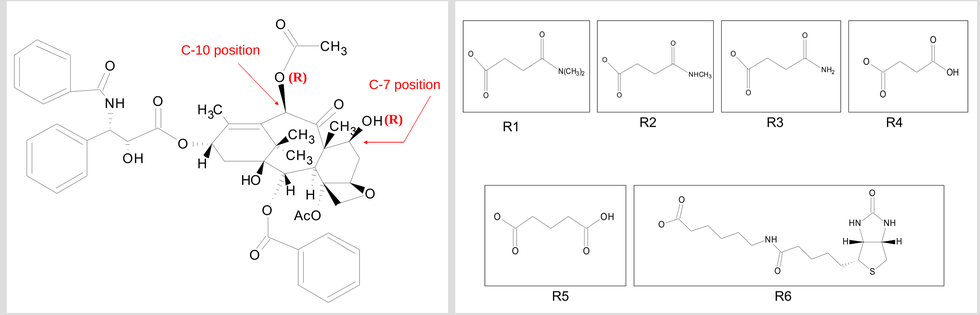


Figure 4. Structure of paclitaxel and C-7 or C-10 modifications

SUMMARY / CONCLUSIONS

- Attachment of a nutrient-like group to paclitaxel may be a strategy for circumventing P-gp and increasing BBB permeation through carrier mediated uptake. The nutrient-like groups considered in these studies were amide esters of succinate, methyl esters of succinate, succinic acid, biotin and carboxylic acid moieties of varying alkyl chain length.
- Rhodamine 123 uptake results in BMECs show that attachment of these nutrient like groups can reduce interaction with P-gp. This corresponds nicely to previous work by Seelig *et al*. in which they showed acids are not substrates nor are amides.
- Furthermore, these studies show that placement of these nutrient-like substituents may be most effective at reducing P-gp interaction in the C-10 position versus the C-7 position.
- Side-by-side diffusion studies across monolayers of BMEC indicate that although reduction in P-gp interaction has been achieved, the nutrient-like derivatives do not display a significant increase in permeability relative to paclitaxel.
- A possible hypothesis for this discrepancy may be that the compounds may enter the cell through active uptake, but due to increased lipophilicity remain trapped within, failing to permeate across the monolayer.
- Future studies will investigate cellular uptake as well as possible concentration dependence of permeability values. Concentration may have an effect based on previous studies in which the permeability of TX-67 at lower concentrations was substantially increased relative to paclitaxel.

ACKNOWLEDGMENTS

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