A Mucoadhesive In Situ Gel Delivery System for Paclitaxel

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ABSTRACT

MUC1 gene encodes a transmembrane mucin glycoprotein that is overexpressed in human breast cancer and colon cancer. The objective of this study was to develop an in situ gel delivery system containing paclitaxel (PTX) and mucoadhesives for sustained and targeted delivery of anticancer drugs. The delivery system consisted of chitosan and glyceryl monooleate (GMO) in 0.33M citric acid containing PTX. The in vitro release of PTX from the gel was performed in presence and absence of Tween 80 at drug loads of 0.18%, 0.30%, and 0.54% (wt/wt), in Sorensen's phosphate buffer (pH 7.4) at 37°C. Different mucin-producing cell lines (Calu-3>Caco-2) were selected for PTX transport studies. Transport of PTX from solution and gel delivery system was performed in side by side diffusion chambers from apical to basal (A-B) and basal to apical (B-A) directions. In vitro release studies revealed that within 4 hours, only 7.61% ± 0.19%, 12.0% ± 0.98%, 31.7% ± 0.40% of PTX were released from 0.18%, 0.30%, and 0.54% drug-loaded gel formulation, respectively, in absence of Tween 80. However, in presence of surfactant (0.05% wt/vol) in the dissolution medium, percentages of PTX released were 28.1% ± 4.35%, 44.2% ± 6.35%, and 97.1% ± 1.22%, respectively. Paclitaxel has shown a polarized transport in all the cell monolayers with B-A transport 2 to 4 times higher than in the A-B direction. The highest mucin-producing cell line (Calu-3) has shown the lowest percentage of PTX transport from gels as compared with Caco-2 cells. Transport of PTX from mucoadhesive gels was shown to be influenced by the mucin-producing capability of cell.

KEYWORDS: paclitaxel, in situ gel, Caco-2, Calu-3, mucin.

INTRODUCTION

MUC1 is a tumor-associated antigen that is overexpressed in the majority of adenocarcinomas. It is overexpressed more than 10-fold in 90% of breast carcinoma and is being used as a target for active and passive cancer immunotherapy in many clinical trials.[1] MUC1 is a heterodimer transmembrane glycoprotein consisting of mucin-like extracellular domain and a cytoplasmic tail containing docking sites for multiple oncogenic proteins including src, erbB receptors, and β-catenin.[2,3] The overproduction of mucin in these cancerous cells could be used as a targeting strategy for mucoadhesive drug delivery system for treatment of breast and colon cancer.

Paclitaxel (PTX) is a natural plant product extracted from bark of western *Taxus brevifolia*, effective against tumors including breast and colon cancer. It blocks the G-2 M phase of the cell cycle of proliferating cell[4] and stabilizes tubulin polymer formation by promoting microtubule assembly.[5] PTX is a very potent anticancer agent, but its efficacy is limited because of low solubility and oral bioavailability.[6] Some cancer cells have a high level of efflux pump, P-glycoprotein (Pgp), which results in removal of PTX from the cell during their transport through the cell.[7] PTX is administered by IV infusion, and to enhance its solubility, cremphor EL is used as a solvent. This solvent causes severe hypersensitivity reactions and cytotoxicity and has shown incompatibility with polyvinyl chloride (PVC), commonly used in IV dosage forms.[8] Even though PTX is a highly effective anticancer agent, it cannot differentiate between cancer and normal cells, resulting in major toxicity to normal tissues. This toxicity can be fatal if not prevented. To minimize the cytotoxicity and adverse side effects associated with PTX, a localized drug delivery system needs to be developed. The underlying hypothesis of this investigation was that a mucoadhesive in situ gel delivery system containing PTX could be targeted to the cancer cells where MUC1 gene is overexpressed as compared with normal cells. This would substantially reduce toxicity to normal cells. The primary objective of this investigation was to develop a safe, sustained, and more effective novel in situ gel delivery system containing PTX for the treatment of breast and colon cancer. When injected close to the site of tumor, the ionic polymer used in the delivery system will be deprotonated and will form an instant gel at the site of injection at the biological pH (7.4). This delivery system may provide a sustained release of PTX from the in situ gel at and around the site of cancer, while the systemic drug concentration will be negligible.

The gel delivery system consisted of chitosan and glyceryl monooleate (GMO) in citric acid. Chitosan is a natural and immunogenic polymer with minimal toxicity.[9] The mucoadhesive properties of chitosan and its usefulness in drug

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delivery system design have been well documented.[10,11] Strong electrostatic interaction of positively charged chitosan with the negatively charged mucosal surface is the underlying mechanism for its mucoadhesive properties.[12] GMO, on the other hand, forms liquid crystal in the presence of water.[13] GMO also possesses bioadhesive properties that can be used to enhance the therapeutic efficacy of the dosage forms by increasing the contact time at the site of action.[14-16] The self-emulsifying property of GMO can further enhance the solubility of this highly hydrophobic compound in this formulation.

Materials and Methods

Materials

PTX (FW 853.9) with 97% purity, potassium phosphate monobasic, and sodium phosphate dibasic anhydrous were purchased from Sigma Chemical Co (St Louis, MO). Low molecular weight chitosan having formulae weight of 161 000 was obtained from Aldrich Chemical Co (Milwaukee, WI). GMO was purchased from Eastman (Memphis, TN). Anhydrous citric acid was obtained from Acros Organics (Fairlawn, NJ). Both Caco-2 and Calu-3 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and were cultured on 6-well Transwell polystrene membrane inserts (24-mm, 0.4-μm pore size; Costar Corp, Cambridge, MA). Ammonium acetate was purchased from Spectrum Chemical & Laboratory Products (Gardena, CA). Acetonitrile and methanol were purchased from Fisher Chemicals (Fairlawn, NJ). Tween 80 (polysorbate-80) was obtained from Merck (Whitehouse Station, NJ).

Analysis of Paclitaxel

A highly sensitive high-performance liquid chromatography (HPLC) method was developed and validated for the quantitation of PTX. The chromatographic separation was achieved on a ZORBAX SB C-18 column (150 × 4.6 mm, 5 μm; Agilent Technologies Inc, Palo Alto, CA) with a flow rate of 0.75 mL/min with UV detection at 227 nm. Mobile phase consisted of acetonitrile:methanol:0.1M ammonium acetate (48.5%:16.5%:35% [vol/vol/vol]). Mobile phase was filtered and degassed prior to HPLC use. Standard curve was linear over the concentration range of 0.1 to 10 μg/mL with $R^2 > 0.99$. The relative standard deviations (RSD) for day-to-day and within-day precision for this assay were less than 10%. This method was sensitive enough to detect even less than 0.1 μg/mL of PTX in solution.

Formulation of the Delivery System

Known weight of GMO was kept in an Erlenmeyer flask and heated in an oven at 45°C for 30 minutes. Chitosan powder was added to molten GMO solution and stirred with a glass rod. A known amount of PTX was added directly to 0.33 M citric acid solution followed by sonication for 30 minutes. Citric acid solution containing PTX was added to GMO and chitosan mixture. The resulting mixture was sonicated again over a period of 45 minutes. The final concentration of GMO and chitosan was kept at 3% (wt/vol).

In Vitro Release of Paclitaxel From the Delivery System

One milliliter of the delivery system containing different PTX loads (0.18%, 0.30%, and 0.54% (wt/wt) was added to 40 mL Sorensen's phosphate buffer with a syringe. The theoretical amount of PTX in this 1 mL of gel containing 0.18%, 0.30%, and 0.54% (wt/wt) of drug was 110, 180, and 330 μg, respectively. The in situ gel that formed when added to the Sorensen's buffer was shaken in a bath incubator at 80 rpm at 37°C. At predetermined time intervals (5, 15, 30, 45, 60, 90, 120, 180, and 240 minutes) 200 μL of the release medium was collected via a filtered needle and replaced by same volume of fresh buffer. The drug concentration in the release medium was analyzed using HPLC as previously described. The data were expressed as percentage of PTX released from the gel delivery system.

Cell Culturing

Caco-2 cells were obtained from ATCC and used in the transport studies from passages 25 to 39. Caco-2 cells were seeded at 25 000 cells/cm^2 density in 75-cm^2 culture flasks in Dulbecco's modified Eagle's culture media (DMEM) supplemented with 10% fetal bovine serum with Penicillin G (100 U/mL) and streptomycin (10 μg/mL). The Caco-2 cells grown on the culture flasks were passaged using a trypsin/ethylenediaminetetraacetic acid solution when ~80% confluent. The passaged cells were either further propagated in culture flasks (1:3 splitting ratio) or seeded onto Falcon polystrene membrane inserts. The density of the inserts was maintained as described earlier and used for permeability studies at 21 days. Calu-3 cells were obtained from ATCC (Rockville, MD) and maintained in a 1:1 mixture of Ham's f12 (Gibco, Grand Island, NY) and DMEM (Sigma), with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and supplemented with 100 μg/mL penicillin G (Sigma) and 100 μg/mL streptomycin sulfate (Sigma). Cells were plated in 150 cm^2 cell culture flasks and subcultured before reaching confluency using a 0.25% trypsin solution in EDTA (Sigma). The culture medium was changed every 2 days. The cells were split 1:2 during each passage. Passaged cells were seeded (5×10^5 cells/cm^2) onto Falcon polystyrene membrane inserts and used for permeability studies at 17 to 21 days.

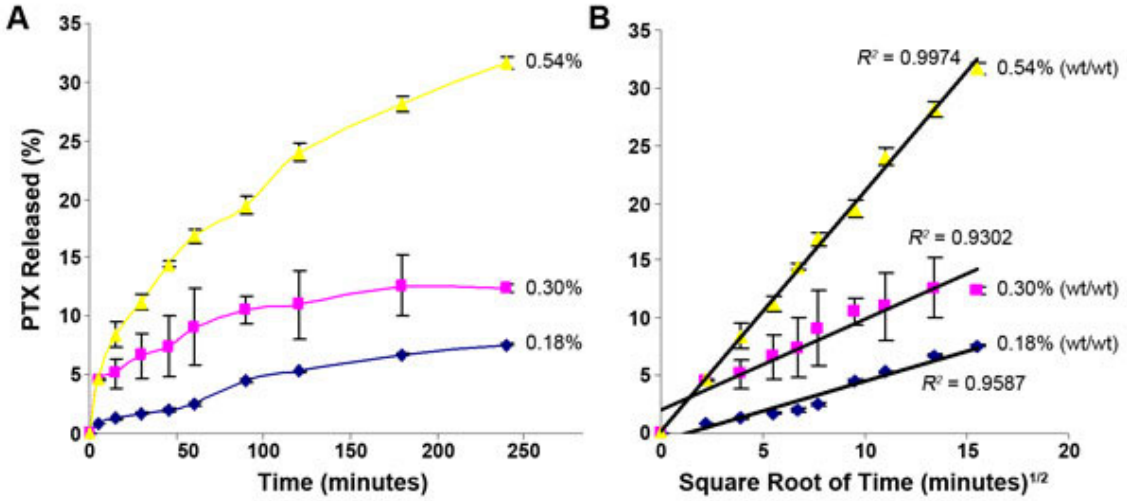

Figure 1. Effect of drug load on the in vitro release of PTX from the in situ gel. Mean ± SD (n = 4). The PTX load was 0.18%, 0.30%, and 0.54% (wt/wt). (A) Plot of percentage PTX released versus time, and (B) plot of percentage of PTX released versus square root of time.

Transport of PTX via In Vitro Cell Culture Models

Caco-2 and Calu-3 cell monolayers were used as in vitro models for evaluation of the transport of free PTX from different dosage forms (solution and in situ gel). The cell monolayer integrity was evaluated by measuring Trans Epithelial Electrical Resistance (TEER) with a Millicell-ERS volt/ohmmeter (Millipore Corp, Bedford, MA) prior to the transport studies. The cell monolayer integrity was considered ready for the transport studies when a minimum TEER value of 620 Ohm cm^2 (TEER is reported in Ohms × surface area). Resistance measurement in Ohm with cell monolayer minus the blank reading with no cells times the total surface area was obtained. This TEER value is the mean of 3 readings taken from 3 different spots of cell inserts, expressed as Ohm × cm^2 (TEER = [mean Ohms − 95 Ohms] × 4.76 cm^2). Side by side diffusion chambers were used to study the transport across the cell monolayers as previously described.[17] In brief, fresh assay II buffer (3 mL) was placed in the receiver chamber. The donor chamber contained 3 mL of either free PTX solution (25 µg/mL) or gel containing PTX (25 µg/mL). The polarity of drug transport was evaluated by investigating the differences in apical and basolateral transport. At predetermined time intervals (30, 60, 90, 120, and 180 minutes), 200 µL of the samples from the receiver side was collected and replaced with same volume of fresh buffer. The drug concentration in the sample was determined by HPLC.

Results and Discussions

Homogeneity of PTX in Gel Formulation

During the preformulation development, homogeneity of the drug in the delivery system was a major challenge. Method of incorporation of PTX in the delivery system had an important influence in the formulation of this in situ gel delivery system. PTX was incorporated in the delivery system by 2 different ways. First method included addition of PTX directly to the delivery system containing 3% (wt/vol) GMO and 3% (wt/vol) chitosan in 0.33M citric acid. The experimental drug loads determined by HPLC analysis for 3 different batches of the above formulations resulted in a high relative standard deviation (46.4%) indicating a possible nonhomogeneity in the delivery systems. To address this homogeneity issue, a second method was introduced. In this method, PTX was first dispersed into the 0.33 M citric acid followed by its addition to the mixture consisting of 3% (wt/vol) GMO and 3% (wt/vol) chitosan. The relative standard deviation for this method of incorporation of PTX to the drug delivery system was found to be reduced dramatically to 5.2% (n = 3), indicating a better homogeneity in the formulation. Therefore, for the entire investigation the second method was adopted for the fabrication of the in situ gel delivery system containing PTX.

Effect of Drug Load on the In Vitro Release of Paclitaxel From the Gel

In vitro release of PTX from the mucoadhesive gel delivery system was performed in Sorensen's phosphate buffer (pH 7.4) at 37°C. The in vitro release profiles are shown in Figure 1A. Three different PTX-loaded gels (0.18%, 0.30%, 0.54% [wt/wt]) were used to study the effect of drug load on the release of PTX from the gel. Within 4 hours, 7.61%, 12.0%, and 31.7% of PTX was released from 0.18%, 0.30%, and 0.54% (wt/wt) drug-loaded gels, respectively. The in vitro release mechanism of PTX from these gels was further evaluated. The percentage release of PTX from these 3 different drug-loaded gels was plotted against the square root of time and is shown in Figure 1B. The linear

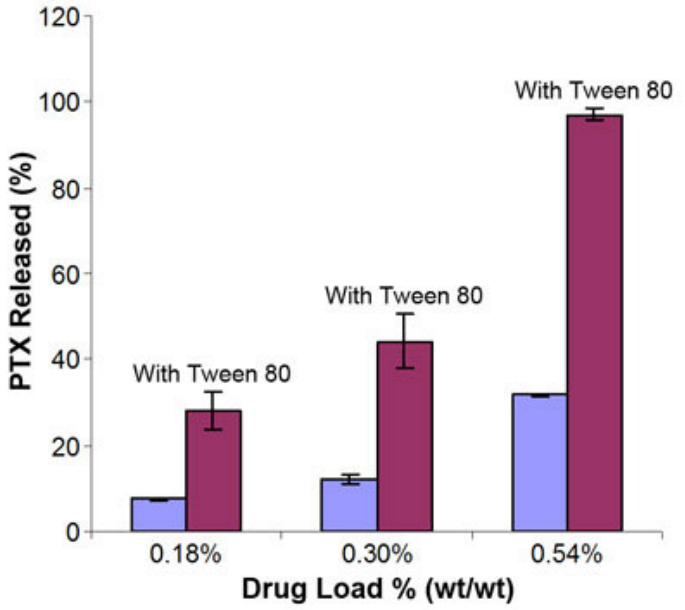

Figure 2. Effect of 0.05% (wt/vol) of Tween 80 in the release media on the PTX release. Drug load of PTX was 0.18%, 0.30%, and 0.54% (wt/wt). Mean ± SD (n = 4).

relationship between PTX released versus square root of time with $r^2 > 0.93$ indicated that the drug release from such gel delivery system followed a matrix diffusion controlled mechanism as described by Higuchi.[18] The slope of the lines in Figure 1B, which is a measure of the rate of drug release from the delivery system with 3 different drug loads, was then determined. The release rate of PTX from the gel formulation containing the highest drug load (0.54%, wt/wt) was found to be 4 times higher than the lowest drug-loaded gel (0.18%, wt/wt). However, this rate of release was only 3 times higher in the case of intermediate drug-loaded (0.30%, wt/wt) gels under similar test conditions.

Effect of Tween 80 in the Release Medium on the Paclitaxel Release

As evidenced from the in vitro release studies, the release of PTX from the in situ gel delivery system was very low, as expected. We also investigated the effect of surfactant in the release medium and its effect on the PTX release. Addition of Tween 80 will not only increase the release of PTX from the gel matrix but also make the detection of this hydrophobic drug in the release medium feasible, especially at a very low drug load. The PTX release profile from the gel in the absence and presence of Tween 80 (0.05% wt/vol) in the release medium is shown in Figure 2. Addition of Tween 80 to the release medium significantly increased the percentage of PTX released from the gel. The PTX release was 4 to 6 times higher in the presence of Tween 80 as compared with in the absence of surfactant in the release medium. The enhanced release of PTX in the presence of surfactant can be explained by the increased solubility of this hydrophobic drug in the Sorensen's phosphate buffer. Similar results have already been reported elsewhere.[19]

Transport of PTX From Solution via Caco-2 Cells

The transport and the transport polarity of PTX were evaluated in a Caco-2 cell line in this cell culture model. Transport of PTX across the monolayer was studied in both A-B and B-A directions in side-by-side diffusion chambers. The amount of free PTX transported across the Caco-2 monolayers was determined at 3 different PTX concentrations in the donor side (15.4, 27.5, and 44 μM). As the concentration of the PTX increased, there was an increase in PTX flux. However, at a higher concentration, this increase was more prominent. PTX has been reported to be affected by Pgp glycoprotein efflux systems.[20] We studied both the A-B and B-A flux of PTX at these 3 concentration levels. Results of

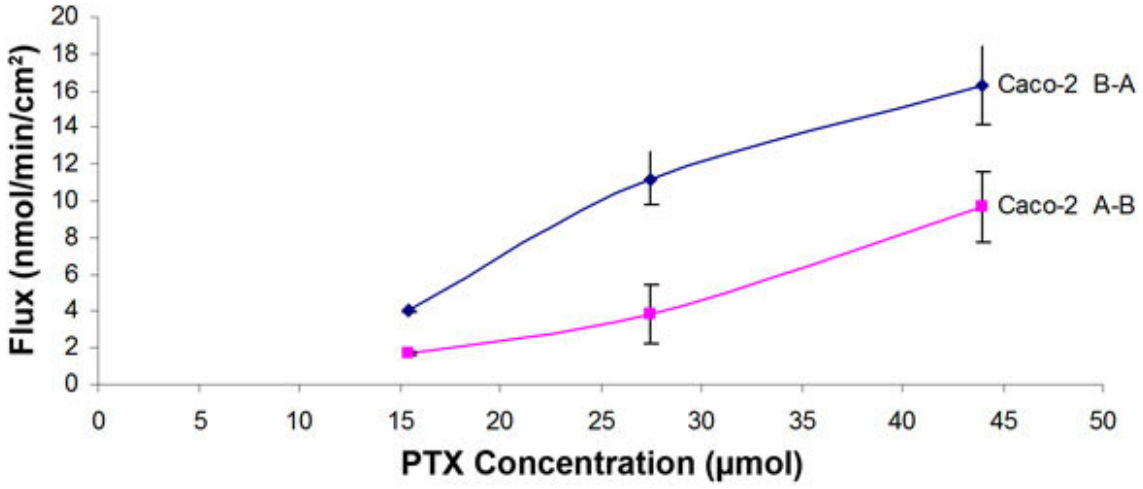

Figure 3. Effect of PTX concentration on the flux. Three concentrations, 15.4 μM, 27.5 μM, and 44 μM, of PTX were used in this study. Mean ± SD (n = 3).

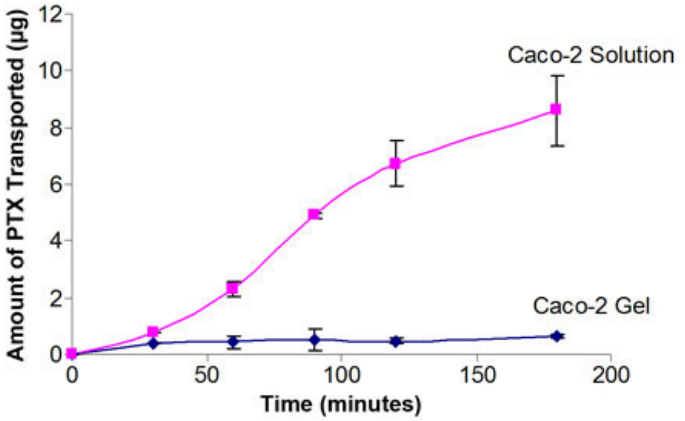

Figure 4. Apical to basolateral transport across Caco-2 monolayer. Concentration of PTX in donor side was 27.5 µM. PTX was present both in the solution and gel formulations. Mean ± SD (n = 3).

this study indicated that flux across B-A direction is 2 to 4 times higher than A-B, as shown in Figure 3. This bipolar flux can be explained by Pgp efflux mechanism as reported by other authors.

The Effects of a Gel Delivery System on the Transport of PTX

One of the objectives of this in situ gel delivery system was to provide a sustained release delivery of PTX. The PTX transport across Caco-2 monolayers was evaluated from both the free PTX in solution and the PTX-loaded gel delivery system (Figures 4 and 5). In brief, PTX (25 µg/mL) was added to the donor chamber in either free PTX in solution or loaded in the gel formulation, and samples (200 µL) were taken at various time points (30, 60, 90, 120, and 180 minutes) and replaced with fresh assay buffer. The concentration of PTX was determined by HPLC as previously described. Regardless of the cellular polarity, the transport of free PTX was significantly higher from the solution as compared with the PTX-gel delivery system (Figure 4). Certainly, the

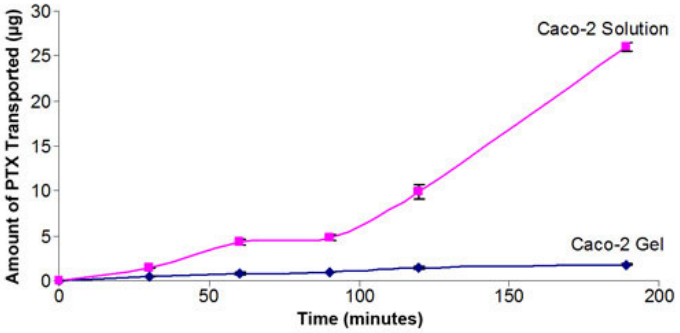

Figure 5. Basolateral to apical transport across Caco-2 monolayer of PTX from solutions and gel formulations. Concentration of PTX was 27.5 µM in both these formulations. Mean ± SD (n = 3).

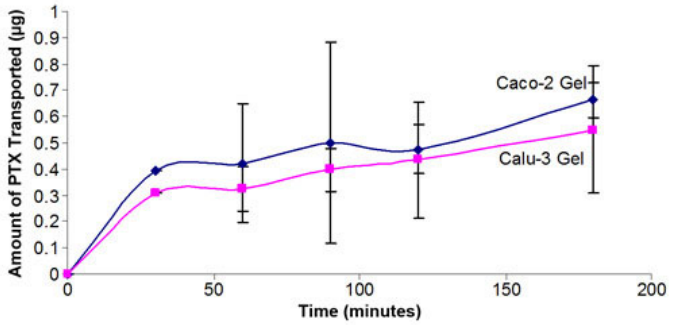

Figure 6. Apical to basolateral transport of PTX from gel formulation through Caco-2 and Calu-3 monolayers. Concentration of PTX was 27.5 µM in both of the formulations. Mean ± SD (n = 3).

most reasonable explanation for the results displayed is the reduction of the free PTX available in the gel delivery system. The effect observed with the gel delivery system could be explained by the possible attraction and interactions between the cellular mucosal secretions and the gel delivery system. The adhesion of the gel delivery system to the mucosal secretions is a reasonable explanation for the reduced transport in the basolateral direction owing to decreasing the efflux surface area. However, further investigation would be required. To further investigate the possibility of these interactions other mucosal secreting cells should be evaluated.

Caco-2 and Calu-3 cell lines were used to test the effect of mucin release on the transport of PTX from the mucoadhesive drug delivery systems. The concentration of PTX used in this transport study was kept at 25 µg/mL in the gel formulation. The results of the transport studies in both A-B and B-A directions are shown in Figures 6 and 7, respectively. The transport of PTX from both the A-B ($P < .01$) and B-A ($P < .03$) direction in case of Calu-3 cells was significantly lower than Caco-2 cells. Statistical significance was determined using 2-factor analysis of variance (ANOVA) without replication. Lower rate of transport of PTX across Calu-3 monolayer as compared with Caco-2 cells can be explained by the higher production of mucin in

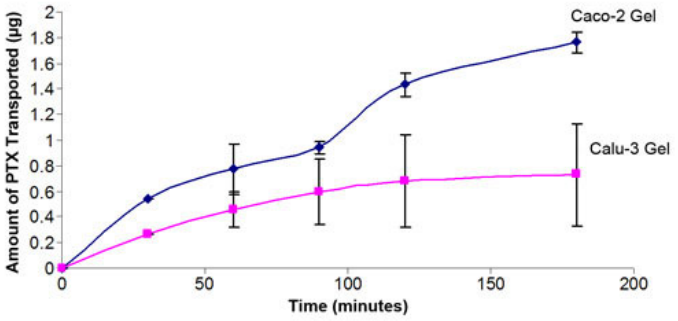

Figure 7. Basolateral to apical transport of PTX from gel formulation through Caco-2 and Calu-3 monolayers. Concentration of PTX was 27.5 µM in both of the formulations. Mean ± SD (n = 3).

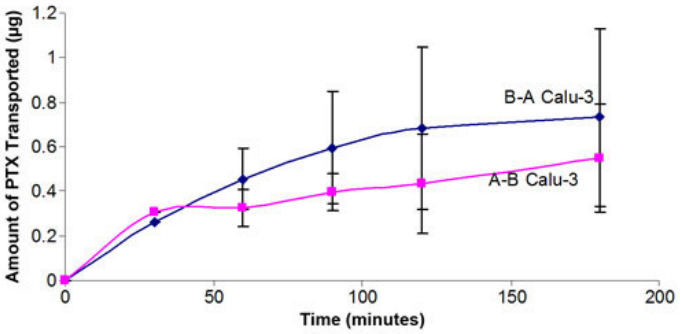

Figure 8. Comparison of apical to basolateral and basolateral to apical transport of PTX from gel formulation through Calu-3 monolayer. Concentration of PTX was 27.5 µM in the formulations. Mean ± SD (n = 3).

the Calu-3 cells. This overproduction of mucin possibly binds to the mucoadhesive delivery system, thereby providing extra barrier for diffusion of PTX and eventually affecting the transport of PTX. The transport of PTX from the gel in Calu-3 monolayers was then compared and shown in Figure 8. The A-B transport was lower than B-A transport. This difference in transport can be influenced by 2 factors. First, the production of mucin is much higher in the apical surface as compared with the basolateral surface. Second, this difference can also be explained by the presence of efflux pumps (Pgp glycoprotein) on the apical surface of the Calu-3 monolayer. However, previous studies have indicated that Calu-3 cells express lower levels of Pgp glycoprotein.[21] Therefore, these 2 in vitro cell culture models clearly indicated that overproduction of mucin is the major contributing factor for the transport of PTX and can be used as a drug-targeting strategy and for sustained delivery of anticancer drug.

Conclusions

A mucoadhesive in situ gel delivery system containing chitosan, GMO, and PTX was developed for drug targeting and sustained delivery strategy for chemotherapy to mucin-producing cancerous cells. The amount of PTX transported across cell lines was much lower in case of the gel as compared with PTX in solution indicating that this gel delivery system can be used to sustain the release of PTX. The decreased transport of PTX in case of Calu-3 cells as compared with Caco-2 cells was owing to its higher mucin-producing capability and possible strong binding with the mucoadhesive gel.

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