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Research Paper

Liposomal Formulations of Inflammatory Bowel Disease Drugs: Local *versus* Systemic Drug Delivery in a Rat Model

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Purpose. Based on adherence to intestinal mucosa, intralumenally administered liposomal formulations of 5-aminosalicylate (5-ASA) and 6-mercaptopurine (6-MP) were studied for their potential to enhance local drug delivery to intestinal tissue for the treatment of inflammatory bowel disease.
Methods. 5-ASA was encapsulated in standard phospholipid liposomes while 6-MP required encapsulation in nonphospholipid liposomes to obtain equivalent drug loading. Encapsulation efficiency was measured by size-exclusion chromatography/high-performance liquid chromatogtaphy (HPLC). Liposomal formulations or solution of the drugs were injected into unligated jejunum to compare pharmacokinetics and into ligated loops of rat ileum and colon to evaluate local delivery. Dextran sulfate and acetic acid induced colitis were used as models of lower intestinal inflammation. Plasma, tissue and luminal drug and metabolite levels were measured by liquid scintillation counting or HPLC.
Results. Encapsulation efficiency of 6-MP was dependent on lipid content and composition. While liposomal encapsulation significantly reduced systemic absorption of 5-ASA this was not the case for 6-MP. Liposomal adherence to intestinal tissue resulted in increased tissue levels for 5-ASA; however, 6-MP local tissue levels were not improved compared to solution drug.
Conclusions. Nonphospholipid liposomes optimize encapsulation of 6-MP. While liposomal formulations show potential for local drug delivery to diseased bowel, drug physicochemical properties, absorption, and metabolic profiles dictate tissue-targeting potential. Liposomes reduce systemic availability from paracellular absorption of hydrophilic 5-ASA, but fail to improve local tissue delivery of 6-MP, a molecule absorbed by passive membrane permeation that undergoes extensive first- pass metabolism.

KEY WORDS: absorption; drug delivery; inflammatory bowel disease; intestine; liposomes.

INTRODUCTION

Local delivery of therapeutics to diseased bowel tissue through oral administration is a challenging problem but a desirable goal to treat gastrointestinal diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), or colon cancer. Contrary to most therapeutic regimens utilizing oral administration, systemic absorption is an undesirable delivery feature for these drugs. Disease localization dictates the need for maximal intestinal tissue drug exposure while systemic delivery should be minimized to avoid unwanted side effects. In the case of IBD, which primarily involves pathology in the large intestine and lower small intestine (1), delayed or controlled release oral dosage forms have proven beneficial to negate upper intestinal absorption and release the drug at the sites of disease (2–5). The potential to increase drug residence time in regions of diseased tissue would serve to further optimize this therapy.

Oral dosage forms that adhere to diseased bowel tissue could provide sustained exposure of a therapeutic agent to sites of pathology and improve its therapeutic effect. In this direction, particulate systems such as nanoparticles and microspheres have attracted attention due to their mucoadhesive properties (6–11). In a recent study (12), colonic adhesion properties of liposomal formulations were reported in studies on a potential delivery vehicle for antioxidant enzymes to treat intestinal inflammation. Liposomal dosage forms for local delivery of small drug molecules used for treatment of IBD were the focus of this research.

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ABBREVIATIONS: 5-ASA, 5-aminosalicylate; FBS, fetal bovine serum; GDS, glyceryl distearate; HPLC, high-performance liquid chromatography; IBD, inflammatory bowel disease; IBS, irritable bowel system; 6-MP, 6-mercaptopurine; NEAA, nonessential amino acids; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; POE, polyoxyethylene stearyl ether.

Two low molecular weight IBD drugs with very different physicochemical and biological delivery properties, 5-aminosalicylate (5-ASA) and 6-mercaptopurine (6-MP), were selected for study in a rat model. Marketed controlled and delayed release dosage forms of 5-ASA (3) reduce the rapid and complete upper intestinal absorption of this drug (13) to achieve delivery to the lower bowel. Systemic absorption of the more potent 6-MP, associated with bone marrow suppression, is an important concern in IBD therapy that requires close patient monitoring, can be dose limiting, and further supports the case for local delivery. Local delivery for IBD therapy is typically assessed by pharmacodynamic evaluation in animal models by utilizing biomarkers of inflammation and histological scores (8–10). In this report, systemic drug and metabolite measurements are employed to discern the drug delivery limitations for therapy. Liposomal formulations of these two drugs were tested for their capacity to reduce systemic drug levels and increase intestinal tissue levels in normal and colitic rats. While differing drug physicochemical properties dictated different liposomal formulations, biological factors such as absorption characteristics and intestinal metabolic profiles of these drugs also influence systemic versus local delivery potential.

MATERIALS AND METHODS

Materials

6-MP monohydrate and 5-ASA were purchased from ICN Biochemicals (Costa Mesa, CA) and Sigma-Aldrich (St. Louis, MO), respectively. [^{14}C]6-mercaptopurine was obtained from Moravek (Brea, CA). Saturated egg lecithin, sodium phosphatidylglycerol, and Brij-76 were from Sigma-Aldrich (St. Louis, MO); glyceryl distearate was purchased from Pfaltz and Bauer (Waterbury, CT) and cholesterol was purchased from Fisher Scientific (Pittsburgh, PA). The scintillation cocktail Ultima-Gold and tissue solubilizer Solvable were purchased from Perkin-Elmer (Boston, MA). Thiouric acid was a gift from GlaxoSmithKline. Dextran sodium sulfate was from Spectrum (Gardena, CA). *N*-Acetyl 5-ASA was synthesized by reacting 5-ASA with acetic acid anhydride under catalysis of triethylamine in ethyl ester. The *N*-acetyl 5-ASA product was extracted with ethyl ether and recrystallized. Its structure was verified by nuclear magnetic resonance (NMR) and its purity was tested using both NMR and high-performance liquid chromatography (HPLC) with fluorescence detection. Ketamine-HCl was from Fort Dodge Lab (Fort Dodge, IA), and Rompum (xylazine) from Bayer (Shawnee Mission, KS). HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animals

Pathogen-free, male Sprague-Dawley rats (Charles River Laboratories) weighing 300–400 g were used in accordance with a protocol approved by the Institutional Review Board—Committee on Use and Care of Animals (University of Michigan).

Liposomal Preparation and Characterization

Phospholipid-based 5-ASA liposomes were prepared by a solvent evaporation method. Briefly stated, a mixture of saturated egg lecithin, sodium phosphatidylglycerol, and cholesterol (2:1:2 ratio) and 5-ASA solution in phosphate-buffered saline (PBS), pH 7.4, was mixed in methyl alcohol and chloroform to form a clear homogeneous solution. The chloroform and methanol were removed together with a fraction of water under vacuum at 65–70°C, leaving a concentrated liposomal solution. Water was added to obtain a final lipid concentration of 50 mg/ml. The final 5-ASA concentration of the liposomal formulations was 7.5 mg/ml.

Nonphospholipid-based 6-MP liposomes were prepared following the procedures described by Niemiec et al. (14). Glyceryl distearate (GDS), cholesterol (CH), and polyoxyethylene (10) stearyl ether (POE) were used as the lipid components. Lipid composition was varied to maximize 6-MP loading. 6-MP solution in PBS was used as the aqueous phase. 6-MP solutions in PBS were prepared on the day of the experiment from a stock solution of 10 mM 6-MP in 0.1 N NaOH, with pH adjusted with HCl to 7.4. Appropriate amounts of the lipids were accurately weighed in small beakers. The lipids were heated with stirring to 70–75°C until the lipid melt became a clear solution. The lipid melt was then drawn into a preheated syringe. 6-MP solution, preheated to 60–65°C, was drawn to a second preheated syringe. The two syringes were connected via a three-way Teflon stopcock. The aqueous solution was then injected into the lipid phase syringe. The mixture was repeatedly mixed back and forth between the two syringes while being cooled under tap water, until the mixture reached room temperature.

For both formulations, drug encapsulation efficiency was measured by size exclusion chromatography followed by HPLC analysis of both free and liposome-encapsulated drug, using established methods (15,16). Encapsulation efficiency (E.E.) was calculated as:

$$E.E.(\%) = \frac{(liposome\ associated\ drug)}{(free\ drug) + (liposome\ associated\ drug)} \times 100$$

Liposomal size was determined using a Nicomp 380 ZLS zeta potential/particle sizer. Liposomes were used in animal studies as prepared, without separation of free drug, to maintain the original total drug concentration. Thus, liposomal formulations provide a 30–35% reduction in drug solution compared to pure drug solutions.

Animal Procedures

Male Sprague-Dawley rats weighing approximately 300 g were fasted overnight with water *ad libitum* prior to experiment. Anesthesia was induced with an intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (20 mg/kg) and maintained by an intraperitoneal injection of sodium pentobarbital (40 mg/kg). A midline longitudinal incision was made to expose the intestine for further manipulation.

For *in situ* intestinal perfusion studies, procedures were modified from a method previously reported (17). These

studies were carried out to assess regional dependence of intestinal absorption and metabolism of 5-ASA and 6-MP as limitations for treating lower bowel inflammation with standard oral dosage forms. Liposomal formulations were not evaluated in the perfusion system since large volumes of formulation would be required to obtain steady-state data which would be controlled by unencapsulated drug. Briefly stated, after the desired intestinal region (10 cm portion of upper jejunum or terminal ileum or 5 cm of proximal colon) was identified, an inlet glass cannula (0.3 cm outer diameter) was inserted and secured by ligation with silk suture. An outlet glass cannula was inserted 5 or 10 cm distal to the inlet cannulation site. The inlet tubing was connected to a 30 mL syringe that was placed in an infusion pump (Harvard Apparatus Company, South Natick, MA). All animals, perfusion solutions and the pumps were enclosed in a Plexiglas thermostatically controlled chamber set at 30°C. Intestinal effluent samples were collected into pre-weighed vials every 15 min for up to 120 min. Each effluent fraction was reweighed after collection. Steady-state water and solute transport rates were established within 30 min after initiation of perfusion at the flow rate studied. Water transport was corrected by the gravimetric method. Relative drug loss from the perfusate was measured by HPLC. The perfusion buffer consisted of MES (10 mM), NaCl (135 mM), KCl (5 mM), $CaCl_2$ (1.1 mM), glucose (5 mM), and mannitol (5 mM). The pH was adjusted to 6.5 with NaOH. All perfusion solutions were isotonic. The effective permeability (P_{eff}) measured by intestinal perfusion was based on the loss of drug from the perfusate according to the following equation:

$$P_{eff} = -\frac{Q}{2\pi rl} \times \ln \frac{C'_{out}}{C_{in}}$$

where Q is the perfusate flow rate through the segment, r is the radius of the segment (0.2 cm), L is the length of the perfused segment, and C_{in} is the drug concentration of the perfusate entering the intestinal segment. C'_{out} is the drug concentration in the exiting perfusate (C_{out}) corrected for water transport according to the gravimetric method as shown below:

$$C'_{out} = C_{out}\left(\frac{V}{Q \cdot \Delta t}\right)$$

where V is the volume of effluent and Δt is the collection interval.

The anesthesia and surgical procedures for intestinal delivery and absorption studies to compare drug solution and liposomal formulations are similar to the ones described above for the intestinal perfusion studies. After the intestine was exposed, the desired intestinal segment (terminal ileum or proximal colon) was washed with saline and air dried, prior to ligation with silk suture. From 1 to 1.5 ml of formulation were injected with a syringe into the closed compartment. In some cases, formulations were injected in the unligated upper jejunum to allow unhindered intestinal transit. Blood samples were obtained at defined time intervals for 2 h from the tail vein or by cardiac puncture. Plasma was separated from erythrocytes by centrifugation. At the end of the experiment, animals were euthanized and intestine was removed. The removed intestine was washed to collect lumenal contents and nonadherent formulation, cut open longitudinally and the mucosal layer was separated from the rest of the intestinal tissue with a glass cover slip. Mucosal or tissue samples were transferred to preweighed vials and were homogenized prior to analysis.

5-ASA and *N*-acetyl-5-ASA levels in plasma and intestinal tissue were analyzed by HPLC with fluorescent detection as previously described (15). 6-MP and metabolite plasma and tissue levels were assayed by liquid scintillation counting. 6-MP and thiouric acid in the lumenal contents was measured by HPLC using published established methods (18,19).

Partition Coefficient of 5-ASA

5-ASA was dissolved in aqueous buffers with pH ranging from 0 to 9 (0, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9). Four milliliters of these solutions were then equilibrated with an equal volume of octanol. This was done by first vortex-mixinging the mixtures vigorously and then letting the phases stand in a 37°C water bath to reform for 36 h. The 5-ASA concentrations in each equilibrated phase were then measured by HPLC.

IBD Animal Model

Colitis was induced using established protocols (20). Administration of dextran sodium sulfate (DSS) (5% w/v) in the drinking water of animals for 7 days resulted in development of colitis within 7 days, confirmed by presence of blood in the stools with a Hemoccult testing kit (Beckman Coulter, Fullerton, CA). Alternatively, a 3% v/v solution of acetic acid in normal saline was instilled in the colon of anesthetized rats, by inserting a polyethylene tube 10 cm deep through the anus. Acute colitis developed within 24 h. The surgical procedure followed is similar to the one described previously, with liposome encapsulated drug dosed simultaneously to two separate loops in distal ileum and proximal colon. For 6-MP, sample collection and preparation was similar to the described procedures for healthy animals. For 5-ASA, sampling was performed 90 min after dose administration.

Caco-2 Cell Studies

Caco-2 cells (ATCC HTB37) of passage 44 were seeded at 4×10^5 cells/cm^2 on six-well snapwell tissue culture insets (growth area = 1.13 cm^2) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids (NEAA), 45 nM vitamin E, and 100 units/ml of penicillin and streptomycin until confluent (3 days). Medium was changed to DMEM with 5% FBS, 0.1 mM NEAA, 45 nM vitamin E, 0.1 μM selenium (Na_2SeO_3), 0.003 nM zinc ($ZnSO_4 \cdot 7H_2O$), and 100 units/ml of penicillin and streptomycin, until cells were fully differentiated (2 weeks in culture). Cells were grown at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity.

On the day of the experiment, medium was removed and cells were preincubated for 30 min with HBSS transport medium pH 7.4 at 37°C. Subsequently, 6-MP solution or liposomes were added on the apical chamber and cells

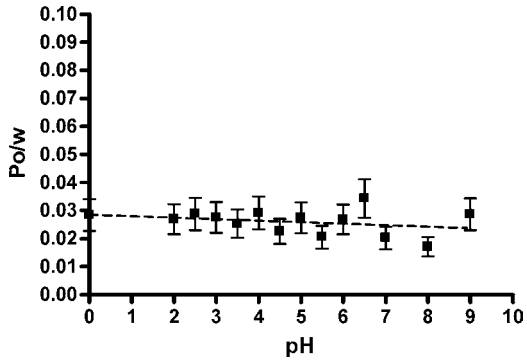


Fig. 1. Octanol/water partition coefficient of 5-ASA as a function of pH. 5-ASA was dissolved in aqueous buffers with varying pH and equilibrated with an equal volume of octanol at 37°C for 36 h. 5-ASA contents in each phase were assayed by HPLC.

returned to the incubator. After 2 h of incubation, aliquots from the donor and receiver solutions were analyzed by liquid scintillation counting. Insert membranes were excised, cells were lysed with 0.1 N NaOH–0.1% sodium dodecyl sulfate, and radioactivity assayed by liquid scintillation counting.

Statistical Analysis

Data were analyzed by Student *t*-test or one-way ANOVA where applicable. Statistical significance was set at $p < 0.05$.

RESULTS

Liposomal Encapsulation of 6-MP and 5-ASA

Encapsulation efficiency of 5-ASA phospholipid liposomes prepared by a reverse phase evaporation method was 25–35%. Lower encapsulation (10–15%) was achieved by a thin film hydration method. For the liposomes used in the experiments, the size was narrowly distributed between 0.5 and 1.5 μm.

Nonphospholipid liposomes were used to overcome the low encapsulation efficiency of 6-MP in traditional phospholipid liposomes as reported in the literature (16,21). The encapsulation efficiencies of different nonphospholipid-based formulations composed of glyceryl distearate, cholesterol and Brij 76 are shown in Table II. The two major parameters that appeared to affect encapsulation efficiency were the lipid composition and the amount of lipid incorporated into the formulation. Generally, increasing the percentage of lipid, which favors the formation of multilamellar liposomes, led to an increase in encapsulation efficiency. In most cases use of 15% lipid resulted in the highest encapsulation. Formulations with 20% or more lipid were too viscous to be used for intralumenal administration. Lipid composition also appeared to significantly affect encapsulation. Highest encapsulations were achieved with lipid compositions that consisted of about 40% GDS, 20–30% cholesterol and 30–40% POE. Of all the formulations prepared, the 40:20:40 GDS:CH:POE, 15% (v/v) lipid liposomes exhibited the highest encapsulation efficiency (31.77% ± 5.37%, mean ± SD, n = 4; similar to 5-ASA phospholipids liposomes) and were used for most of the *in vivo* studies. Increasing the aqueous phase pH to 9, which leads to ionization of 6-MP and therefore higher solubility, did not have a significant effect on encapsulation. This indicates that ionization of 6-MP does not produce a significant effect on the lipid-to-aqueous phase partition coefficient in the formulation. Particle size was measured to be between 0.5 and 2 μm in diameter. Size distribution was bimodal with the majority of the liposomes around 1–1.5 μm and a smaller population around 200 nm.

Partition Coefficients

The partition coefficients of 5-ASA between both octanol and water was measured over a nine log order acidity range (Fig. 1). The partitioning values were small and remained unchanged over the pH range of study. The partition coefficient ($K_{o/w}$) averaged about 0.03 (log $K_{o/w}$ = −1.52). 6-MP has been reported to exhibit slightly better octanol partitioning from aqueous pH 6.5 compared to pH 7.4 (22).

Intestinal Permeability of 6-MP in Different Intestinal Regions

The *in situ* intestinal perfusion studies suggest that 6-MP does not exhibit region specific intestinal absorption. At the 1 mM (170.2 μg/ml) concentration, which is close to the reported solubility of 6-MP and the intestinal concentration expected from a 50 mg oral dose in humans, the effective

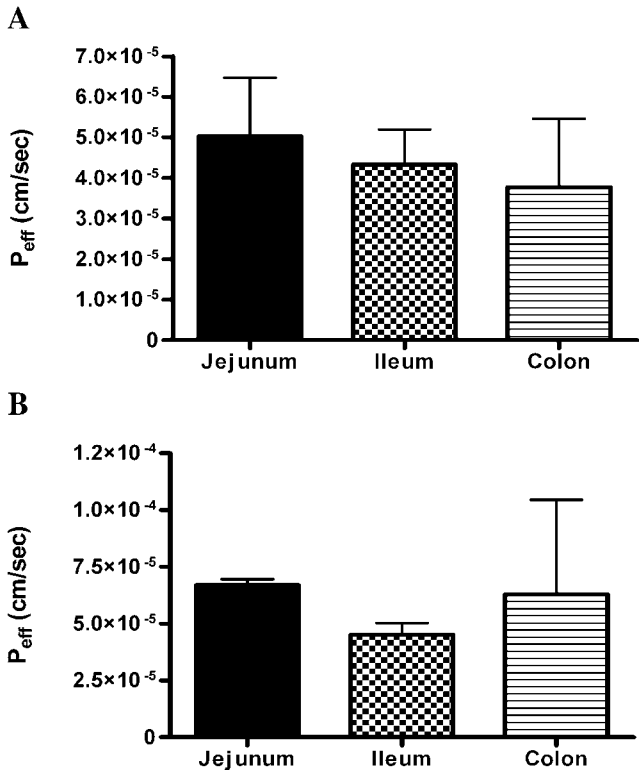


Fig. 2. Effective permeability (cm/sec) of 6-MP in healthy rat jejunum, ileum and colon at (A) 1 mM luminal concentration and (B) 0.1 mM luminal concentration (mean ± SD, n = 4–5) assessed by *in situ* rat intestinal perfusion with 6-MP solutions in MES buffer, pH 6.5.

permeability values (cm/s) for jejunum, ileum and colon were $5.02 \pm 0.65 \times 10^{-5}$, $4.33 \pm 0.86 \times 10^{-5}$, $3.77 \pm 1.68 \times 10^{-5}$ respectively (mean ± SD, n = 3–5) (Fig. 2A). At the lower concentration of 0.1 mM effective permeability exhibited a small, however not statistically significant, increase to $6.70 \pm 0.27 \times 10^{-5}$, $4.52 \pm 0.51 \times 10^{-5}$, $6.28 \pm 2.09 \times 10^{-5}$ for jejunum, ileum and colon, respectively (mean ± SD, n = 3–5) (Fig. 2B). At both concentrations, the effective permeabilities of intestinal segments for each region were not statistically significantly different. In contrast, 5-ASA permeability is strongly region dependent with higher absorption through the leakier paracellular pathway of the upper small intestine compared to the lower small intestine (15). Given the fact that liposomal formulations provide only a 30–35% reduction in drug solution and that substantial amounts of liposomal lipid are required for 120-min steady-state perfusions, regional studies with liposomal formulations were not carried out.

Intralumenal Administration Studies for 5-ASA

5-ASA formulations (11.25 mg as 7.5 mg/ml solution) were injected into the unligated upper jejunum of anesthetized rats to study systemic availability of 5-ASA and its major

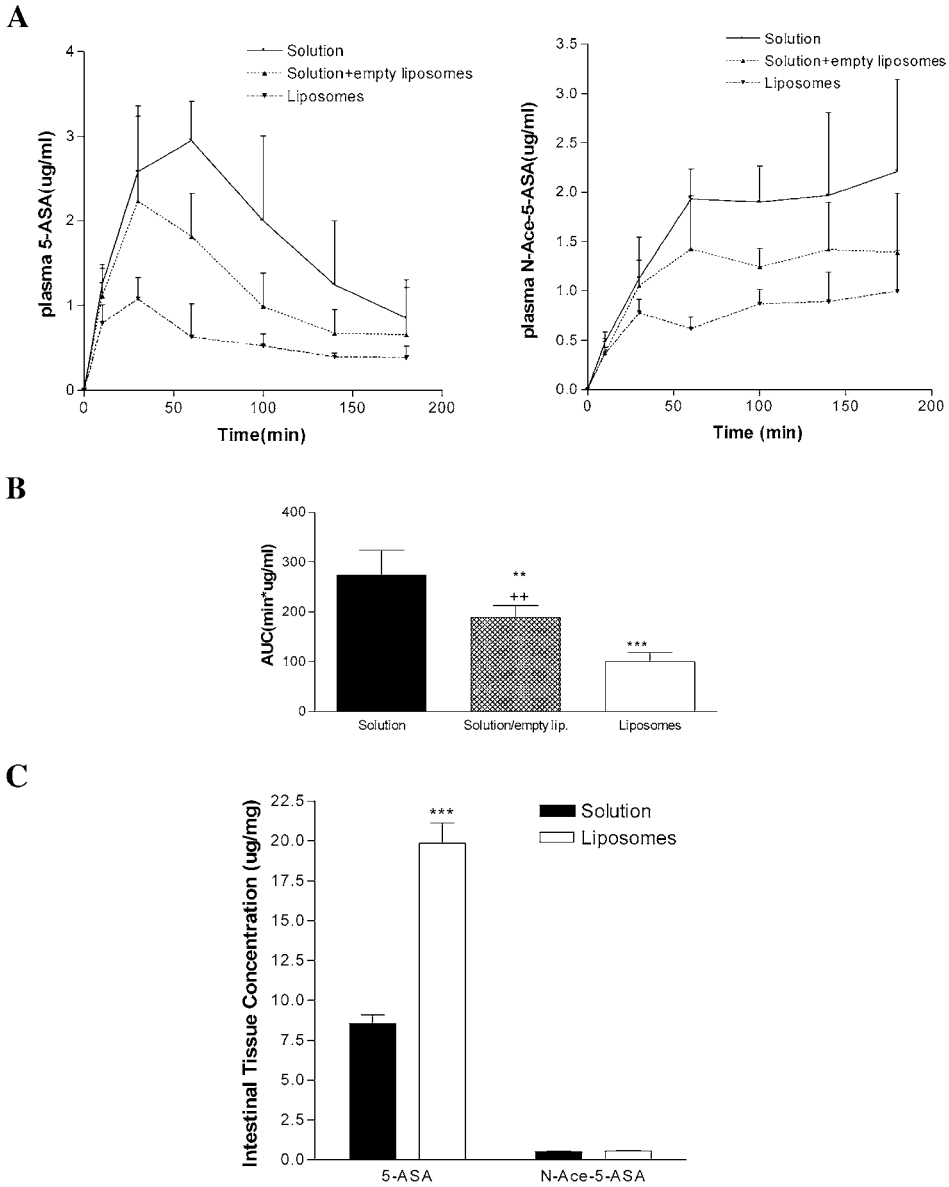


Fig. 3. Pharmacokinetics of 5-ASA and *N*-Ace-5-ASA after intralumenal administration of 5-ASA solution, liposomes or solution with empty liposomes (7.5 mg/ml 5-ASA) to healthy Sprague-Dawley rats. (A) Plasma levels of 5-ASA (left) and *N*-Ace-5-ASA (right) and (B) AUC values of 5-ASA, after direct injection of the formulations in the unligated upper rat jejunum (μg/ml of plasma, mean ± SD, n = 4, ***$p < 0.005$ compared to solution, **$p < 0.01$ compared to solution, ++$p < 0.01$ compared to liposomes). (C) Intestinal tissue levels of 5-ASA and *N*-Ace-5-ASA after injection of the formulations in ligated intestinal loops (μg/mg of tissue, mean ± SD, n = 4, ***$p < 0.005$).

metabolite, *N*-Ace-5-ASA. As shown in Fig. 3A (left graph), systemic 5-ASA levels were significantly lower after liposomal formulation administration, with the AUC value exhibiting a 63.4% reduction (273 ± 51 vs. 100 ± 18 min μg/ml, mean ± SD, n = 4, $p < 0.001$) (Fig. 3B). Plasma 5-ASA levels also decreased after administration of 5-ASA solution with empty liposomes (Fig. 3A, left graph). In that case a 30.8% reduction in AUC was observed and the difference was statistically significant from both solution and liposomal formulation administration (n = 4, $p < 0.01$). A similar trend was observed for plasma *N*-Ace-5-ASA levels (Fig. 3A, right graph). To assess tissue delivery of 5-ASA from the different formulations, 5-ASA or liposomes were injected into ligated ileal intestinal loops. Liposomal encapsulation of 5-ASA resulted in significantly higher 5-ASA levels in the intestinal tissue (19.84 ± 2.54 *vs.* 8.56 ± 1.03 μg/mg, mean ± SD, $p < 0.001$, n = 4) (Fig. 3C).

Intralumenal Administration Studies for 6-MP

6-MP solution or liposomal formulation (212.74 μg in 1 mM solution) were injected in ligated intestinal loops or

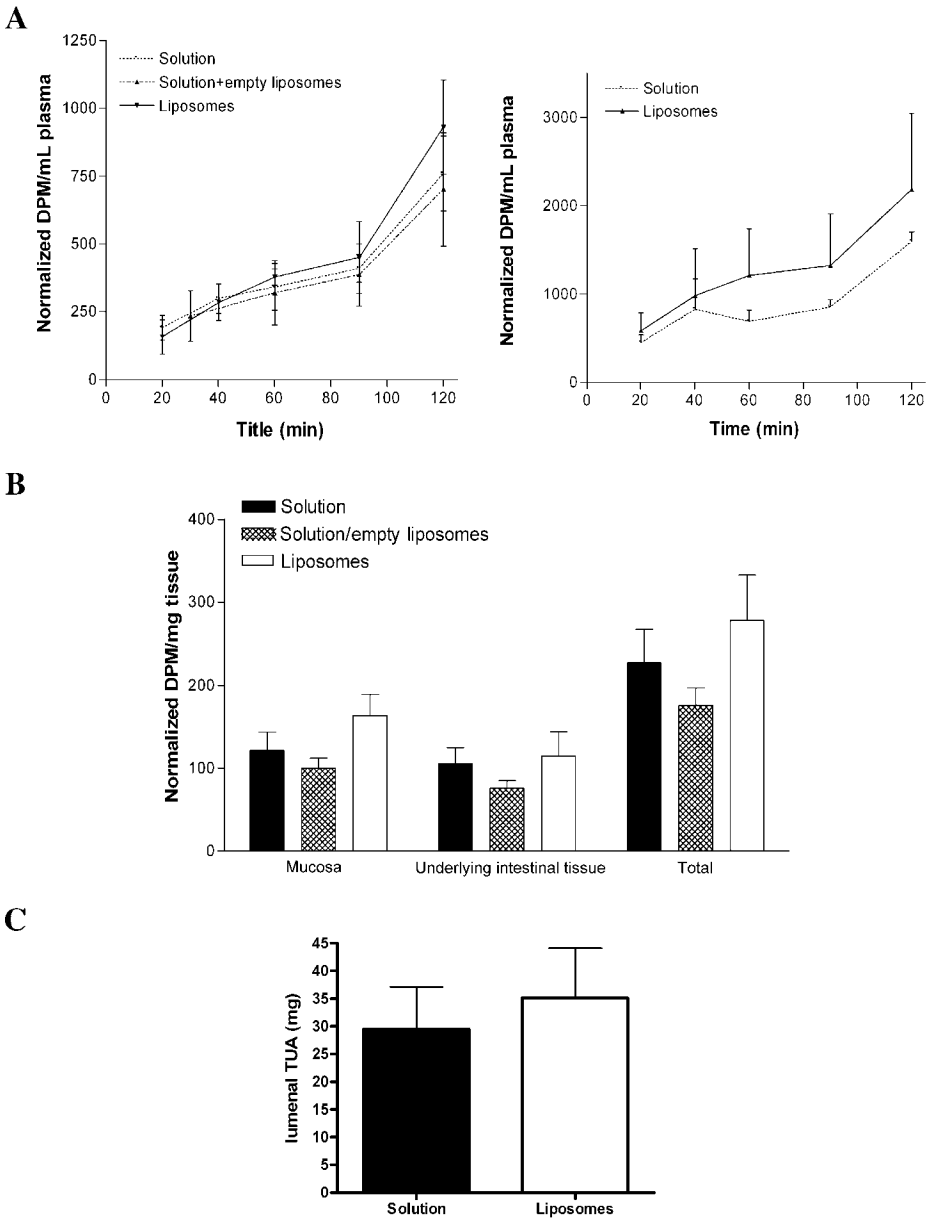


Fig. 4. Pharmacokinetics of 6-MP after intralumenal administration of 6-MP solution, liposomes or solution with empty liposomes (1 mM 6-MP traced with [^{14}C]6-MP) to healthy Sprague-Dawley rats. (A) Total radioactivity in plasma after injection of 6-MP formulations in ligated intestinal loops (right) or the unligated upper jejunum (left) (DPM/ml of plasma, mean ± SD, n = 3–5). (B) Total radioactivity in the intestinal loop tissue after administration of 6-MP formulations (DPM/mg tissue, mean ± SD, n = 4–5). (C) Lumenal thiouric acid levels after injection of 6-MP formulations in ligated intestinal loops (mg, mean ± SD, n = 4). All DPM values are normalized for initial radioactivity dose.

the unligated upper jejunum of anesthetized rats to study systemic availability and local tissue delivery of 6-MP. Prior to analysis of drug in mucus and tissue, adherence of the liposomal formulation to the mucosal border was visually apparent. Because of the complex nature of 6-MP metabolism, total radioactivity was assayed by liquid scintillation counting to assess total drug availability. Significant differences in plasma radioactivity levels between solution and liposomal formulations (Fig. 4A) were not observed for either method of intestinal administration. In contrast to observations for 5-ASA, the presence of empty liposomes, did not appear to alter absorption of 6-MP from solution (Fig. 4A, left graph). Although intestinal tissue radioactivity contents (Fig. 4B) were generally higher after liposomal 6-MP administration compared to solution, no statistically significant differences were observed between the two formulations. The "observed" difference in tissue 6-MP radioactivity was attributed to the mucus containing tissue fraction where it has been suggested that the majority of the particles are localized (23). Consistent with these findings, no statistically significant difference was observed for concentrations of luminal thiouric acid, the predominant intestinal metabolite of 6-MP (Fig. 4C).

Studies in Animal Models of IBD

Similarly to the observations made with healthy animals, encapsulation of 6-MP in nonphospholipid liposomes failed to alter either intestinal tissue levels (Fig. 5A, B) or the systemic absorption (Fig. 5C) of 6-MP dosed in DSS-colitic loops. While plasma levels of 5-ASA and its metabolite were not significantly different for lower intestinal administration, tissue associated drug levels were higher in IBD rats compared to normal rats (Fig. 6). The intestinal tissue levels from the two 6-MP liposomal formulations were identical in the ileal loops (Fig. 5A). In the colon, where more inflammation is expected after DSS treatment, liposomes resulted in higher intestinal mucosal 6-MP levels than from solution, but the levels were not statistically significantly different (Fig. 5B).

6-MP Caco-2 Transport Study

The results from the 6-MP transport studies in Caco-2 monolayers are shown in Fig. 7. No significant differences were observed in disappearance of 6-MP from the apical chamber or appearance in the basolateral chamber. Presence of liposomes does not appear to inhibit 6-MP absorption. Monolayer radioactivity content was significantly higher after application of liposomes compared to solution at the same concentration (graph C). At a concentration of 1 mM, a substantial amount of liposomal formulation adhered to the mucus-free Caco-2 surface, which most likely accounts for the increase in radioactivity within the monolayer. When a 10-fold diluted formulation was used fewer liposomes adhered to the monolayer surface resulting in smaller differences in cell monolayer radioactivity. The lack of detectable xanthine oxidase activity in this system, limited assessment of intracellular drug delivery by measuring 6-MP metabolite.

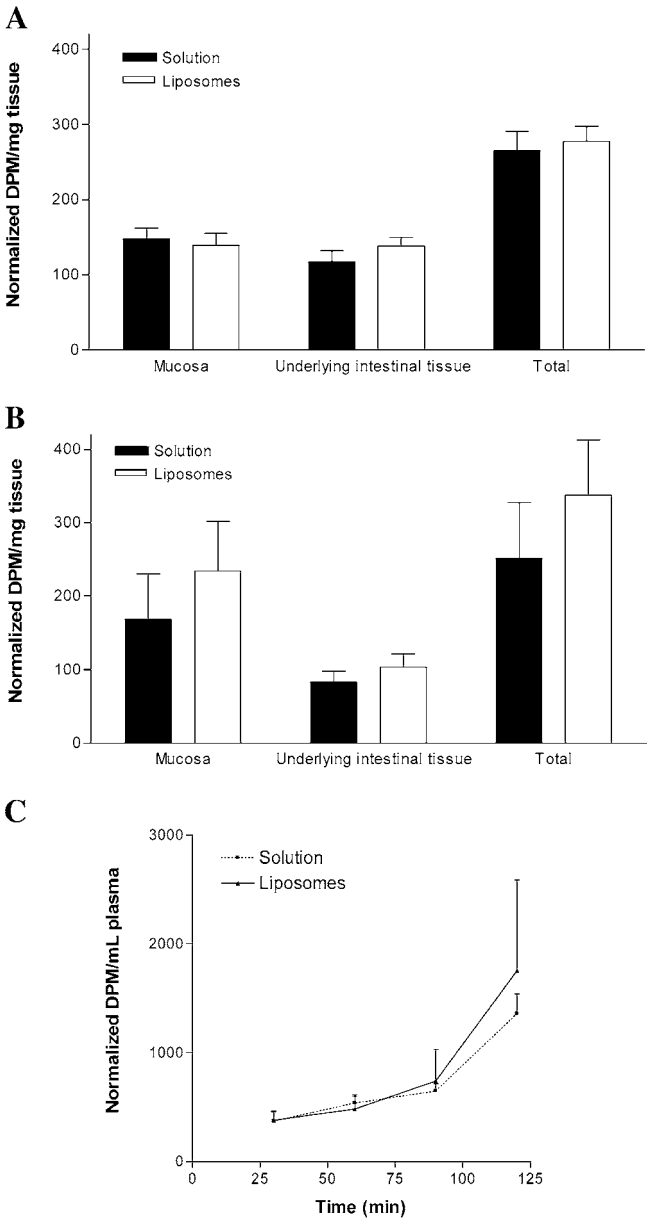


Fig. 5. Pharmacokinetics of 6-MP after intralumenal administration of 6-MP solution, or (1 mM 6-MP traced with [^{14}C]6-MP) to DSS-colitic Sprague-Dawley rats. (A) Total radioactivity in ileal intestinal loop tissue after administration of 6-MP formulations (DPM/mg tissue, mean ± SD, n = 4). (B) Total radioactivity in colonic intestinal loop tissue after administration of 6-MP formulations (DPM/mg tissue, mean ± SD, n = 4). (C) Lumenal thiouric acid levels after injection of 6-MP formulations in ligated intestinal loops (DPM/ml of plasma, mean ± SD, n = 4). All DPM values are normalized for initial radioactivity dose.

DISCUSSION

Particulate formulations, such as polymeric microspheres, nanoparticles, and liposomes, have attracted attention for oral delivery of drugs to diseased intestinal tissue for the treatment of alimentary tract disorders. These formulations were reported to exhibit increased binding to the intestinal wall in healthy intestinal tissue (6,24). Further, Lamprecht *et al.* (7) reported that microsphere and nano-

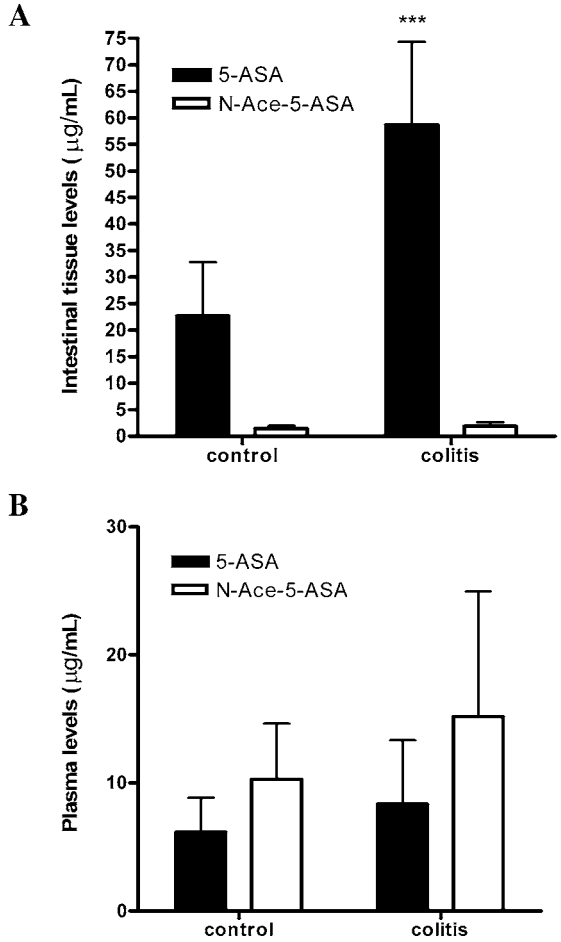


Fig. 6. Tissue (A) and plasma levels (B) of 5-ASA and *N*-Ace-5-ASA after administration of a 5-ASA liposomal formulation (7.5 mg/ml of 5-ASA) in isolated colonic loops of healthy and acetic acid-induced colitic animals.

sphere gut wall attachment significantly increases in inflamed intestinal segments of colitic rats. More recently, Jubeh *et al.* (12) reported that binding of liposomes to healthy and colitic intestinal tissue is dependent on liposomal charge and size. Attachment of the formulation to the intestinal wall could provide sustained exposure of a therapeutic agent to local sites of pathology and improve therapy.

In this study, we compared the efficiency of liposomes to locally deliver two commonly used IBD drugs, 5-ASA and 6-MP, to intestinal tissue. While 5-ASA was readily encapsulated in phospholipid liposomes, non-phospholipid based liposomes were required to achieve equivalent encapsulation of 6-MP. Based on encapsulation efficiency, liposomal formulation reduced equilibrium drug solution availability by 30–35%. Intralumenal administration of the formulation resulted in increased tissue levels and reduced systemic distribution compared to solution for 5-ASA, but no significant differences were observed for 6-MP. The differences in drug physicochemical properties not only dictate differences in liposomal formulations but also generate differences in intestinal drug absorption pathways and metabolic profiles underlying local delivery results for these two drugs.

While both 5-ASA and 6-MP are small molecules, differences in solubility (Table I), partition coefficient,

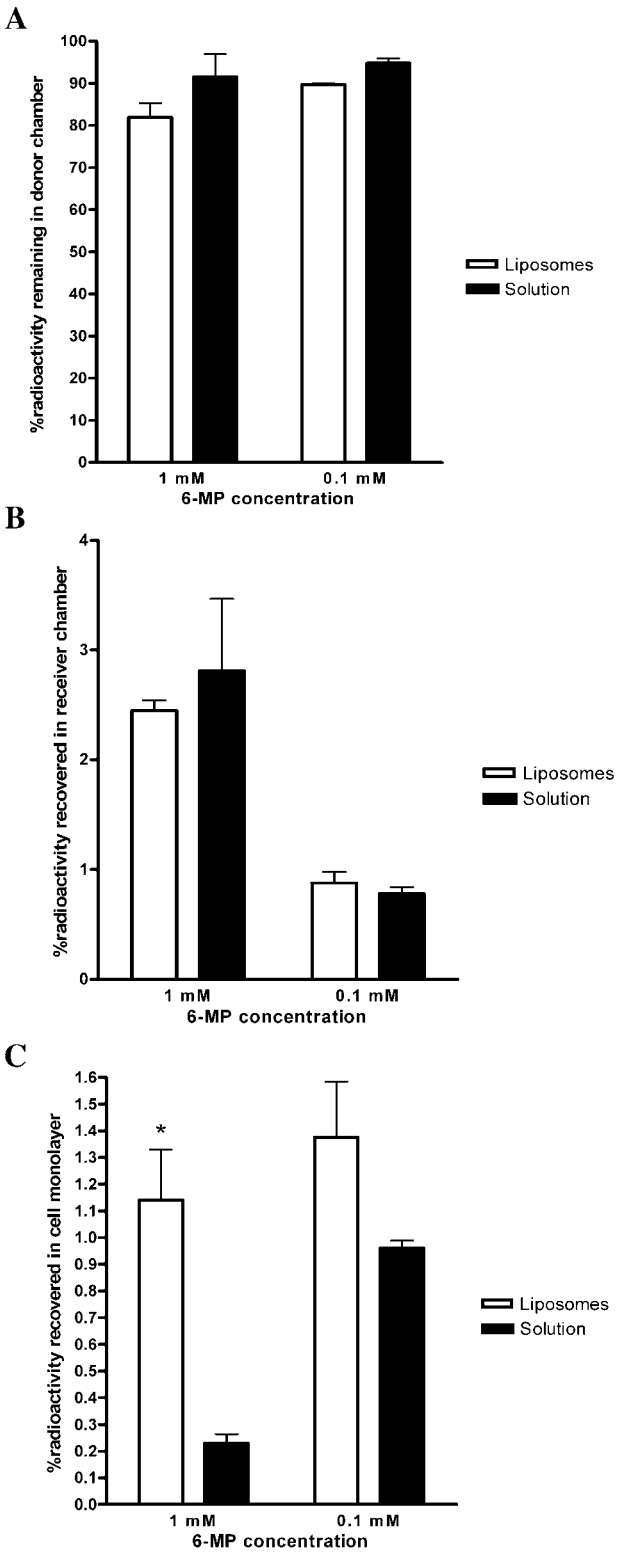
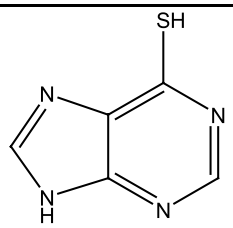
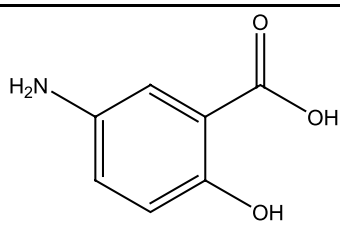


Fig. 7. 6-MP transport in Caco-2 monolayers. 1 mM or 0.1 mM 6-MP solution or liposomes (traced with [^{14}C]6-MP) were applied on the apical chamber of differentiated Caco-2 monolayers. Data are expressed as % of initial radioactivity remaining in apical chamber (A), appearance in basolateral chamber (B), and contents of Caco-2 monolayer (C) (mean ± SD, n = 3). *$p < 0.05$ compared to solution.

Table I. Physicochemical Properties and Use in IBD Therapy for 6-MP and 5-ASA

	6-MP	5-ASA
		
Physicochemical properties		
MW	152.18	153.14
pK_a	2.5, 7.77, 11.17	2.3, 5.69[a]
$P_{octanol/water}$	0.376–1.22[b]	0.03[c]
Aqueous solubility pH 7.4 (mg/ml)	0.180–0.355[d]	>18
Dose (oral, mg)	50	800–1200
Use in IBD therapy		
Disease variable	Moderate to severe	Mild
Side effects	Severe (bone marrow suppression)	Generally well tolerated

[a] Ref. (33).
[b] Refs. (22,31).
[c] Fig. 1.
[d] Refs. (22,32).

absorption characteristics and first-pass metabolism play a role in both formulation and biological delivery. The high solubility of 5-ASA permits 30–35% entrapment of the drug in the aqueous interior of a phospholipid unilammelar formulation. Similar formulations of 6-MP produce very low drug entrapment even when the pH was raised to promote drug ionization (16). Drug entrapment equivalent to that of 5-ASA was achieved for 6-MP with a non-phospholipid multilammelar formulation. Furthermore, nonphospholipid formulations offer the advantage of lower cost, better stability and ease of preparation (25). The two major factors that affected encapsulation were the lipid composition and the amount of lipid incorporated into the formulations (Table II). A cholesterol concentration of 20–30% appeared to enhance encapsulation and increasing lipid content, which promotes formation of multilamellar vesicles, resulted in higher encapsulation efficiency. The biphasic solubility of 6-MP likely results in drug association with liposomal membrane layers as well as loading in the aqueous layers.

Solubility differences for the two drugs result in different partition coefficient and permeation behavior. The low octanol/water partition coefficient of anionic/zwitterionic 5-ASA shows weak pH dependence consistent with higher octanol partitioning for the zwitterionic species (Fig. 1). With a basic and one acidic pK_a out of the range of physiologic pH, only the second acidic pK_a results in slightly better octanol partitioning from aqueous pH 6.5 compared to pH 7.4 (22).

The low partition coefficient of 5-ASA is consistent with high paracellular permeability in the small intestine. In fact, saturated solutions of 5-ASA are 100% absorbed in the human upper small intestine (13). Thus complete absorption in the upper bowel reduces the ability to deliver drug locally to inflamed lower bowel and provides a rationale for delayed release dosage forms. In previous rat intestinal perfusion

Table II. Indicative Encapsulation Efficiencies of Nonphospholipid-Based Liposomal Formulations of 6-MP

GDS:CH:POE Weight Ratio	% Lipid (v/v)	Aqueous Phase	% Encapsulation
50:15:35	5	PBS, pH 7.4	<5
	10	PBS, pH 7.4	5.73
	15	PBS, pH 7.4	11.33
45:25:30	5	PBS, pH 7.4	17.75
	10	PBS, pH 7.4	18.68
	15	PBS, pH 7.4	16.94
	20	PBS, pH 7.4	27.29
45:25:30	15	PBS, pH 9	21.42
	20	PBS, pH 9	23.00
40:30:30	5	PBS, pH 7.4	16.37
	10	PBS, pH 7.4	20.98
	15	PBS, pH 7.4	27.34
40:25:35	5	PBS, pH 7.4	13.66
	10	PBS, pH 7.4	26.02
	15	PBS, pH 7.4	33.66
40:20:40	10	PBS, pH 7.4	16.52
	15	PBS, pH 7.4	31.77 ± 5.37
	20	PBS, pH 7.4	32.29

% Encapsulation is typically the average of two different preparations. For the GDS:CH:POE 40:20:40 15% lipid (v/v) liposomes that were used in the *in vivo* studies, mean ± SD (n = 4) is provided.

experiments, significantly higher jejunal than ileal permeability for 5-ASA was observed ($6.17 \pm 1.82 \times 10^{-6}$ cm/s *vs*. $0.04 \pm 1.36 \times 10^{-6}$ cm/s) (15). This is likely due to the fact that the paracellular pathway is more available for drug transport in the leaky jejunal tissue as compared to the ileum. This suggests that delayed release dosage forms should reduce total absorption of 5-ASA although IBD is known to compromise lower gut barrier resistance to transport of hydrophilic solutes (26). The permeability of 6-MP did not show any dependence on intestinal region (Fig. 2). Combined with the better partitioning behavior of 6-MP compared to 5-ASA, this data suggests that 6-MP absorption is most likely dominated by transcellular membrane permeation as has been previously suggested (19). Given that the liposomal formulations tested in this study include a substantial drug solution component, liposomal entrapment is observed to more strongly limit systemic absorption of 5-ASA than of 6-MP. Absorption of solution 5-ASA is greater in the upper than lower intestine which is not the case for 6-MP. Although less absorbing surface area would be exposed with a delayed release 6-MP dosage form, extent of drug absorption might not necessarily be reduced (27) as observed with 5-ASA.

Given the liposomal adherence to intestinal tissue and difficulty in separating the formulation from the mucosa, attempts to monitor tissue delivery of drug utilized intestinal or luminal drug metabolite levels. The appearance of *N*-acetyl-5-ASA and thiouric acid in the tissue or perfusate would indicate that 5-ASA and 6-MP, respectively, have entered intestinal tissue from the adherent liposomal formulation. While the dominant paracellular absorption should bypass cellular metabolism for 5-ASA, there is some cellular uptake by a parallel pathway that may be partially carrier-mediated (15). Intestinal and hepatic first-pass metabolism does not have a significant impact on systemic availability of 5-ASA but significantly limits oral bioavailability of 6-MP (19,28–30). In fact, intestinal metabolism of 6-MP by xanthine oxidase is so extensive that it likely enhances absorptive 6-MP flux by maintaining a high transmucosal drug concentration gradient. This would serve to promote the complete absorption of 6-MP from a typical 50-mg oral dose within the small intestinal residence time. In addition, it would perturb drug formulation equilibrium toward rapid removal of 6-MP and promote absorption. Although 6-MP aqueous solubility is fairly low, it is sufficient to insure that dissolution rate of a 50-mg tablet would not be rate limiting to drug absorption. Thus, 6-MP is well absorbed and, while first-pass metabolism limits oral bioavailability, systemic toxicity motivates formulation for local delivery in IBD.

Subsequent to intralumenal administration of 5-ASA and 6-MP in liposomal formulations vs. equivalent drug concentration solutions in rats, plasma levels of 5-ASA and *N*-acetyl-5-ASA were monitored as a function of time by HPLC while 6-MP total drug and metabolite in the circulation were measured by radiolabel counts. In addition, intestinal lumen and tissue levels were assessed via the same analytical methods. In the case of 5-ASA, drug and metabolite plasma levels were reduced with liposomal formulation compared to solution (Fig. 3A, B). Furthermore, empty liposomes administered with solution drug resulted in plasma levels intermediate to those of formulation and solution (Fig. 3A), suggesting that liposomes interfere with 5-ASA absorption. While 5-ASA tissue levels were higher from liposomal formulation no differences in drug metabolite were observed (Fig. 3C). This indicates that 5-ASA is likely maintained at the mucosal surface by the liposomal formulation as compared to solution but tissue entry, as gauged by metabolite, is not improved. In addition, 5-ASA intestinal metabolism is easily saturable and could also account for the formulation-independent metabolite levels. Furthermore, when tissue levels following liposomal 5-ASA administration were compared between healthy and colitis animals, significant liposomal accumulation in colitic tissue was observed (Fig. 6A), while no difference was observed in systemic drug levels (Fig. 6A). In the case of 6-MP, no differences in systemic or tissue levels were observed between formulation and solution regardless of the method of intestinal administration (Fig. 4A). While there was a trend toward greater 6-MP association with the mucosa from liposomal formulation than from solution, measurements were not statistically significantly different (Fig. 4B). Lumenal thiouric acid levels were also slightly elevated after liposomal administration but the difference was not statistically significant (Fig. 4C). Although it has been previously reported that tissue pathophysiological state can affect both binding of liposomal formulations (12) as well as alter mechanisms of absorption (26), studies with DSS-colitic animals revealed a similar pattern for tissue and systemic 6-MP levels, with no clear differences between liposomes and solution (Fig. 5). While no differences were observed for 6-MP in the *in vivo* studies, application of the formulations on a "cleaner, mucus-free" Caco-2 system clearly exhibited the potential of liposomes to adhere to intestinal tissue and locally increase drug levels (Fig. 7B). As was the case with the *in vivo* studies, liposomes do not appear to significantly influence basolateral (systemic) drug appearance (Fig. 7C).

An orally administered local delivery system for 6-MP would be therapeutically beneficial for IBD to minimize dose-limiting systemic toxicity while targeting the site of pathology. However, these animal studies indicate that 6-MP is a poorer candidate than 5-ASA for such a strategy and this is at least partially based on differences in absorption pathways and intestinal metabolism. The fact that empty phospholipid liposomes reduced absorption of 5-ASA from solution suggests that this lipid formulation hinders paracellular absorption of 5-ASA. This is not the case for 6-MP where transcellular absorption is effectively coupled to intestinal metabolism which minimizes liposomal control of delivery. One possible advantage of liposomal formulation for IBD drugs over currently marketed dosage forms is an increased drug residence time at the inflamed intestinal tissue due to mucosal adherence. Nonphospholipid liposomes are worthy of consideration due to greater chemical and physical stability, as well as easier large scale manufacturing. While in these studies lumenal liposomal stability was maximized by direct administration in the intestinal site of interest, stability issues in the stomach and duodenum would certainly require additional formulation modification for oral administration of the liposomal formulations. Alternatively, liposomes could be incorporated in enema formulations. Adherent liposomes may gradually release their drug contents in the vicinity of the inflamed bowel over time, providing sustained intestinal tissue levels not achievable with

current dosage forms. However, drug physicochemical properties and intestinal absorption characteristics will dictate the potential for novel dosage forms to enhance local delivery and reduce systemic delivery of drugs developed to treat intestinal tissue pathology.

REFERENCES

1. S. F. Phillips. Pathophysiology of symptoms and clinical features of inflammatory bowel disease. In J. B. Kirsner (ed.), *Inflammatory Bowel Disease*, W.B. Saunders, Philadelphia, 2000, pp. 358–371.
2. M. Campieri, S. Adamo, D. Valpiani, A. D'Arienzo, G. D'Albasio, M. Pitzalis, P. Cesari, T. Casetti, G. N. Castiglione, F. Rizzello, F. Manguso, G. Varoli, and P. Gionchetti. Oral beclometasone dipropionate in the treatment of extensive and left-sided active ulcerative colitis: a multicentre randomised study. *Aliment. Pharmacol. Ther.* **17**:1471–1480 (2003).
3. W. J. Sandborn and S. B. Hanauer. Systematic review: the pharmacokinetic profiles of oral mesalazine formulations and mesalazine pro-drugs used in the management of ulcerative colitis. *Aliment. Pharmacol. Ther.* **17**:29–42 (2003).
4. M. Robinson. Medical therapy of inflammatory bowel disease for the 21st century. *Eur. J. Surg. Suppl.* **164**:90–98 (1998).
5. S. Edsbacker, P. Larsson, and P. Wollmer. Gut delivery of budesonide, a locally active corticosteroid, from plain and controlled-release capsules. *Eur. J. Gastroenterol. Hepatol.* **14**:1357–1362 (2002).
6. S. McClean, E. Prosser, E. Meehan, D. O'Malley, N. Clarke, Z. Ramtoola, and D. Brayden. Binding and uptake of biodegradable poly-DL-lactide micro- and nanoparticles in intestinal epithelia. *Eur. J. Pharm. Sci.* **6**:153–163 (1998).
7. A. Lamprecht, U. Schafer, and C. M. Lehr. Size-dependent bioadhesion of micro- and nanoparticulate carriers to the inflamed colonic mucosa. *Pharm. Res.* **18**:788–793 (2001).
8. A. Lamprecht, N. Ubrich, H. Yamamoto, U. Schafer, H. Takeuchi, P. Maincent, Y. Kawashima, and C. M. Lehr. Biodegradable nanoparticles for targeted drug delivery in treatment of inflammatory bowel disease. *J. Pharmacol. Exp. Ther.* **299**:775–781 (2001).
9. H. Nakase, K. Okazaki, Y. Tabata, and T. Chiba. Biodegradable microspheres targeting mucosal immune-regulating cells: new approach for treatment of inflammatory bowel disease. *J. Gastroenterol.* **38**(Suppl 15):59–62 (2003).
10. H. Nakase, K. Okazaki, Y. Tabata, S. Uose, M. Ohana, K. Uchida, T. Nishi, A. Debreceni, T. Itoh, C. Kawanami, M. Iwano, Y. Ikada, and T. Chiba. An oral drug delivery system targeting immune-regulating cells ameliorates mucosal injury in trinitrobenzene sulfonic acid-induced colitis. *J. Pharmacol. Exp. Ther.* **297**:1122–1128 (2001).
11. H. Nakase, K. Okazaki, Y. Tabata, S. Uose, M. Ohana, K. Uchida, Y. Matsushima, C. Kawanami, C. Oshima, Y. Ikada, and T. Chiba. Development of an oral drug delivery system targeting immune-regulating cells in experimental inflammatory bowel disease: a new therapeutic strategy. *J. Pharmacol. Exp. Ther.* **292**:15–21 (2000).
12. T. T. Jubeh, Y. Barenholz, and A. Rubinstein. Differential adhesion of normal and inflamed rat colonic mucosa by charged liposomes. *Pharm. Res.* **21**:447–453 (2004).
13. O. Haagen Nielsen and S. Bondesen. Kinetics of 5-aminosalicylic acid after jejunal instillation in man. *Br. J. Clin. Pharmacol.* **16**:738–740 (1983).
14. S. M. Niemiec, J. M. Latta, C. Ramachandran, N. D. Weiner, and B. J. Roessler. Perifollicular transgenic expression of human interleukin-1 receptor antagonist protein following topical application of novel liposome-plasmid DNA formulations *in vivo*. *J. Pharm. Sci.* **86**:701–708 (1997).
15. S. Y. Zhou, D. Fleisher, L. H. Pao, C. Li, B. Winward, and E. M. Zimmermann. Intestinal metabolism and transport of 5-aminosalicylate. *Drug Metab. Dispos.* **27**:479–485 (1999).
16. M. Gulati, M. Grover, M. Singh, and S. Singh. Study of azathioprine encapsulation into liposomes. *J. Microencapsul* **15**:485–494 (1998).
17. D. Fleisher, N. Sheth, H. Griffin, M. McFadden, and G. Aspacher. Nutrient influences on rat intestinal phenytoin uptake. *Pharm. Res.* **6**:332–337 (1989).
18. A. M. Pennington and J. R. Bronk. The absorption of 6-mercaptopurine from 6-mercaptopurine riboside in rat small intestine: effect of phosphate. *Cancer Chemother. Pharmacol.* **36**:136–142 (1995).
19. W. R. Ravis, J. S. Wang, and S. Feldman. Intestinal absorption and metabolism of 6-mercaptopurine in the rat small intestine. *Biochem. Pharmacol.* **33**:443–448 (1984).
20. C. O. Elson, R. B. Sartor, G. S. Tennyson, and R. H. Riddell. Experimental models of inflammatory bowel disease. *Gastroenterology* **109**:1344–1347 (1995).
21. K. Tsujii, J. Sunamoto, and J. H. Fendler. Improved entrapment of drugs in modified liposomes. *Life Sci.* **19**:1743–1749 (1976).
22. M. Chrzanowska, M. Kuehn, T. Hermann, and R. H. Neubert. Biopharmaceutical characterization of some synthetic purine drugs. *Pharmazie* **58**:504–506 (2003).
23. A. Lamprecht, H. Rodero Torres, U. Schafer, and C. M. Lehr. Biodegradable microparticles as a two-drug controlled release formulation: a potential treatment of inflammatory bowel disease. *J. Control. Release* **69**:445–454 (2000).
24. M. P. Desai, V. Labhasetwar, G. L. Amidon, and R. J. Levy. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharm. Res.* **13**:1838–1845 (1996).
25. N. Weiner, L. Lieb, S. Niemiec, C. Ramachandran, Z. Hu, and K. Egbaria. Liposomes: a novel topical delivery system for pharmaceutical and cosmetic applications. *J. Drug Target* **2**:405–410 (1994).
26. H. Mielants, M. De Vos, S. Goemaere, K. Schelstraete, C. Cuvelier, K. Goethals, M. Maertens, C. Ackerman, and E. M. Veys. Intestinal mucosal permeability in inflammatory rheumatic diseases. II. Role of disease. *J. Rheumatol.* **18**:394–400 (1991).
27. L. H. Pao, S. Y. Zhou, C. Cook, T. Kararli, C. Kirchhoff, J. Truelove, A. Karim, and D. Fleisher. Reduced systemic availability of an antiarrhythmic drug, bidisomide, with meal co-administration: relationship with region-dependent intestinal absorption. *Pharm. Res.* **15**:221–227 (1998).
28. J. R. Bronk, N. Lister, and M. I. Shaw. Transport and metabolism of 6-thioguanine and 6-mercaptopurine in mouse small intestine. *Clin. Sci. (Lond.)* **74**:629–638 (1988).
29. H. Sasaki, K. Tsuru, J. Nakamura, R. Konishi, and J. Shibasaki. Effect of allopurinol on the intestinal absorption of 6-mercaptopurine in rats. *J. Pharmacobiodyn.* **10**:697–702 (1987).
30. H. Sasaki, J. Nakamura, R. Konishi, and J. Shibasaki. Intestinal absorption characteristics of 5-fluorouracil, ftorafur and 6-mercaptopurine in rats. *Chem. Pharm. Bull (Tokyo)* **34**:4265–4272 (1986).
31. D. Taneja, A. Namdeo, P. R. Mishra, A. J. Khopade, and N. K. Jain. High-entrapment liposomes for 6-mercaptopurine—a produg approach. *Drug. Dev. Ind. Pharm.* **26**:1315–1319 (2000).
32. Y. Takeichi and T. Kimura. Improvement of aqueous solubility and rectal absorption of 6-mercaptopurine by addition of sodium benzoate. *Biol. Pharm. Bull.* **17**:1391–1394 (1994).
33. D. L. French and J. W. Mauger. Evaluation of the physiocochemical properties and dissolution characteristics of mesalamine: relevance to controlled intestinal drug delivery. *Pharm. Res.* **10**:1285–1290 (1993).