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Poly(Amido Amine) Dendrimers as Absorption Enhancers for Oral Delivery of Camptothecin

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Abstract

Oral delivery of camptothecin has a treatment advantage but is limited by low bioavailability and gastrointestinal toxicity. Poly(amido amine) or PAMAM dendrimers have shown promise as intestinal penetration enhancers, drug solubilizers and drug carriers for oral delivery *in vitro* and *in situ*. There have been very limited studies *in vivo* to evaluate PAMAM dendrimers for oral drug delivery. In this study, camptothecin (5 mg/kg) was formulated and co-delivered with cationic, amine-terminated PAMAM dendrimer generation 4.0 (G4.0) (100 and 300 mg/kg) and anionic, carboxylate-terminated PAMAM generation 3.5 (G3.5) (300 and 1000 mg/kg) in CD-1 mice. Camptothecin associated to a higher extent with G4.0 than G3.5 in the formulation, attributed to an electrostatic interaction on the surface of G4.0. Both PAMAM G4.0 and G3.5 increased camptothecin solubilization in simulated gastric fluid and caused a 2–3 fold increase in oral absorption of camptothecin when delivered at 2 hours. PAMAM G4.0 and G3.5 did not increase mannitol transport suggesting that the oral absorption of camptothecin was not due to tight junction modulation. Histologic observations of the epithelial layer of small intestinal segments of the gastrointestinal tract (GIT) at 4 hours post dosing supported no evidence of toxicity at the evaluated doses of PAMAM dendrimers. This study demonstrates that both cationic (G4) and anionic (G3.5) PAMAM dendrimers were effective in enhancing the oral absorption of camptothecin. Results suggest that drug inclusion in PAMAM interior controlled solubilization in simulated gastric and intestinal fluids, and increased oral bioavailability.

Keywords

camptothecin; poly(amido amine) dendrimer; oral drug delivery; absorption enhancer; intestinal transport

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1. Introduction

1.1 Oral delivery of camptothecin

Oral administration of chemotherapeutics has treatment advantages of patient preference, convenience of administration, cost-effectiveness and improving quality of life in palliative care (Aisner, 2007; DeMario and Ratain, 1998). In terms of treatment outcomes, oral chemotherapeutics are advantageous for protracted dosage regimens as is the case for schedule-dependent cytotoxic drugs (DeMario and Ratain, 1998; Terwogt et al., 1999). Camptothecin and its derivatives are potent topoisomerase-I inhibitors that show a schedule-dependent activity which means that they are more efficacious with low and frequent oral dosage regimens (O'Leary and Muggia, 1998). However, the oral delivery of camptothecins is limited by poor and variable bioavailability attributed to low solubility, low permeability and P-gp efflux of the drug (Stehlin et al., 1995).

Camptothecin and its derivatives have been solubilized in various micellar, liposomal, microsphere, microemulsion and cyclodextrin-based formulations (Cortesi et al., 1997; Emerson, 2000; Hatefi and Amsden, 2002; Lundberg, 1998; Sugarman et al., 1996). A number of water-soluble analogues and prodrugs have also been synthesized (Conover et al., 1998; Goldberg et al., 2011; Harada et al., 2000; Hatefi and Amsden, 2002; Kingsbury et al., 1991; Sakuma et al., 2001; Singer et al., 2000; Thiagarajan et al., 2010; Vijayalakshmi et al., 2010). However, the majority of these systems have not been evaluated for oral delivery.

1.2 Poly(amido amine) or PAMAM dendrimers as oral drug carriers

Poly(amido amine) (PAMAM) dendrimers are a class of hyperbranched polymers (Tomalia et al., 1985). They are synthesized with alternating repeating units of ethylene diamine and methyl acrylate and can be terminated to have primary amines, carboxylic acids or hydroxyl-terminated groups (Tomalia et al., 1985; Tomalia et al., 1990). As a result of the repeated branching, PAMAM dendrimers have a dense surface exterior and a relatively hollow interior, where they are known to solubilize hydrophobic moieties by encapsulation or surface interactions (Beezer et al., 2003; Chauhan et al., 2004; Cheng et al., 2008a; Cheng et al., 2007; Cheng et al., 2008b; D'Emanuele and Attwood, 2005; Devarakonda et al., 2004; Gupta et al., 2006; Ooya et al., 2004; Pistolis and Malliaris, 2002; Pistolis et al., 1997; Yiyun and Tongwen, 2005).

PAMAM dendrimers have also been extensively evaluated *in vitro* for potential oral drug delivery applications as intestinal penetration enhancers as well as carriers for transepithelial transport of small molecules (Sadekar and Ghandehari, 2012). Both cationic, amine-terminated as well as anionic, carboxylic acid-terminated PAMAM dendrimers are known to modulate tight junctions and increase paracellular transport of small molecules (El-Sayed et al., 2002; El-Sayed et al., 2003a; Kitchens et al., 2006). They are also known to be translocated across the intestinal epithelial barrier by endocytic mechanisms (El-Sayed et al., 2003b; Goldberg et al., 2010; Kitchens et al., 2007; Kitchens et al., 2008). Additionally, cationic PAMAM dendrimers are known to interact with lipid bilayers on cell membranes increasing their cellular uptake (Lin et al., 2010). Their solubilizing potential combined with their transepithelial transport make PAMAM dendrimers attractive for oral delivery of biopharmaceutics classification system (BCS) class IV drugs such as camptothecin and its derivatives: 7-ethyl-10-hydroxy-camptothecin (SN38). PAMAM-SN38 complexes and conjugates have previously been evaluated for their transepithelial transport *in vitro* across Caco-2 monolayers (Goldberg et al., 2011; Thiagarajan et al., 2010; Vijayalakshmi et al., 2010). The permeability of SN38 was seen to increase when associated with PAMAM, which was attributed to increased solubility, decreased toxicity, saturable transcellular process such as endocytic uptake and concentration-driven paracellular diffusion such as

tight junction modulation. These studies did not evaluate the *in vivo* oral absorption of SN38 when complexed or conjugated to the PAMAM dendrimer. There have been very limited studies *in vivo* to evaluate PAMAM dendrimers for oral drug delivery (Huang et al., 2011; Ke et al., 2008). In this study, camptothecin was formulated and co-delivered with cationic, amine-terminated PAMAM dendrimer generation 4 (G4.0) and anionic, carboxylate-terminated generation 3.5 (G3.5) in CD-1 mice. The hypothesis was that PAMAM dendrimers at non-toxic concentrations can increase oral absorption of drug either by drug solubilization in gastric conditions or by tight junction modulation. Such a study will provide insight into the advantages of either PAMAM complexation or co-delivery in improving the oral absorption of camptothecin. It is possible that a combination of solubilization, penetration enhancement and transepithelial translocation using PAMAM dendrimers for oral delivery can achieve efficacious levels of camptothecin when delivered orally. None of the previously reported *in vivo* studies use a carboxyl-terminated PAMAM (Huang et al., 2011; Ke et al., 2008). The novelty of this manuscript is the *in vivo* comparison between cationic (amine-terminated) and anionic (carboxyl-terminated) dendrimers.

2. Materials and Methods

2.1 Materials

Poly(amido amine) dendrimers generation 4 (G4.0-NH₂) and generation 3.5 (G3.5-COONa) were purchased from Dendritech (Midland, MI). Camptothecin was procured from A.K. Scientific (Union City, CA). Tritium (³H)-labeled camptothecin and ¹⁴C-labeled Mannitol were purchased from American Radiolabeled Chemicals (St. Louis, MO). Reagents for beta detection were purchased from Perkin Elmer (Waltham, MA).

2.2 Methods

2.2.1. Physicochemical characterization of PAMAM dendrimers—The chromatographic elution profiles of the PAMAM dendrimers under study, G4.0-NH₂ and G3.5-COONa, were obtained using a Fast Protein Liquid Chromatography (FPLC) system with an analytical Superose 6TM 10/300 GL column (GE Healthcare) and an ultraviolet detector (GE Healthcare) in order to evaluate relative elution volumes and to check for the absence of small molecular weight impurities (Figure S1, supplementary information). The mobile phase for elution was 20% (v/v) acetonitrile and 80% (v/v) phosphate buffer saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) at a flow rate of 1.0 mL/minute. Eluted peaks were detected at a wavelength of 280 nm. PAMAM dendrimers were further characterized for hydrodynamic radius (R_h) using a Dynamic Light Scattering (DLS) detector (Helleos II) attached to the FPLC system and analyzed using AstraTM 5.3.4.13 software (Wyatt Technologies Corp).

2.2.2. Camptothecin formulations with poly(amido amine) dendrimers—The camptothecin alone (0.5 mg/mL, 14.4 × 10⁻⁴ M) was formulated in a solution of DMSO: 0.05M borate buffer-pH 8.5: poly(ethylene glycol) 400 in the ratio 1:9.5:9.5. For the formulations of camptothecin co-delivered with PAMAM, a dispersion of camptothecin (0.5mg/mL, 14.4 × 10⁻⁴ M) was prepared using the solvent system DMSO: deionized water: poly(ethylene glycol) 400 in the ratio 1:9.5:9.5. Methanolic solutions of PAMAM dendrimers were evaporated up to the constant weight. The drug dispersion was stirred with PAMAM dendrimers at room temperature for 12 hours by mixing with varying concentrations and generations of the dendrimers (Table 1).

The pH values of all the formulations including the camptothecin were adjusted to be between 8.5-9.0, to facilitate head-to-head comparison of the oral absorption of

camptothecin across different treatment groups. The ratios of drug to dendrimer were chosen based on maximum tolerated oral doses of the two as well as the reported solubilization potential of PAMAM dendrimers specifically for camptothecin (Cheng et al., 2008a; Thiagarajan et al., 2012). In order to ascertain the solubility of the formulations, they were filtered through a 100 kDa centrifugal membrane filter (Nanosep 100K Omega, Pall Corporation, Ann Arbor, MI) that was pretreated with 5% (w/w) solution of Triton-X to minimize adsorption to the membrane. The filtrate obtained after spinning the centrifuge tubes at 14,000g for 20 minutes was spectrophotometrically analyzed for camptothecin content at an absorbance maximum of 370 nm (Spectramax M2, Molecular Devices, LLC., Sunnyvale, CA). The formulations of camptothecin with PAMAM were soluble at pH 8.5-9.0.

2.2.3. Mannitol oral absorption—Mannitol alone (0.5 mg/mL) was dissolved in a solution of deionized (DI) water. Methanolic solutions of PAMAM dendrimers were evaporated up to the constant weight. For the mixtures of mannitol co-delivered with PAMAM, the mannitol solution was mixed with the highest doses of PAMAMs of various generations under study as follows:

1. G4.0-NH₂ (30 mg/mL or 21.1×10^{-4} M)-300 mg/kg
2. G3.5-COONa (100 mg/mL or 77.4×10^{-4} M)-1000 mg/kg

The pH values of all the solutions were adjusted to be between 8.5-9.0, to facilitate head-to-head comparison of the paracellular transport of mannitol across different treatment groups.

2.2.4 Characterization of camptothecin – PAMAM formulations—To evaluate the percentage of camptothecin associated with the PAMAM dendrimers in the formulations, they were diluted 5x with DI water and filtered through a 3.5 kDa centrifugal membrane (Amicon® Ultra 0.5 mL, EMD Millipore, Billerica, MA) that was pretreated with 5% (w/w) solution of Triton-X to minimize adsorption to the membrane. The formulations were filtered by spinning the membrane filtration tubes at 14,000g for 20 minutes. Camptothecin associated to PAMAM was measured directly by spectrophotometric methods quantifying the residual drug that remained in the top layer upon membrane filtration. The filtrate collected upon membrane filtration was also measured for camptothecin content to account for the remaining drug not associated to PAMAM. Both filtrate and residual top layer were spectrophotometrically analyzed for camptothecin content at an absorbance maximum of 370 nm (Spectramax M2, Molecular Devices, LLC.) (U.V absorbance standard curve in Figure S2, supplementary information).

In order to assess the percentage of camptothecin in the lactone and carboxylate forms, camptothecin alone and PAMAM camptothecin formulations were eluted on a reverse phase C18 (5 μ m, 250 mm $\times$ 5 mm) high performance liquid chromatography column (XTerra, Waters®; Agilent HPLC system). The mobile phase used was acetonitrile: aqueous triethylamine acetate buffer (prepared using 0.1% v/v triethylamine, adjusted with glacial acetic acid to pH 5.5), in the ratio of 27:73 delivered at a flow rate of 1.0 ml/min with an injection volume of 20 μ l (method adapted from (Cheng et al., 2008a)). Camptothecin in the lactone form was detected at an absorbance wavelength of 370 nm and a retention time of 11.47 ± 0.12 min. Camptothecin in the carboxylate sodium salt form was detected at an absorbance wavelength of 370 nm and a retention time of 4.16 ± 0.03 min. The camptothecin calibration curve for the lactone form was $y = 75.19x - 5.50$ ($r^2 = 0.99$), when the peak area was plotted vs concentration of camptothecin in μ g/mL (Figure S3, supplementary information). The camptothecin calibration curve for the carboxylate sodium salt form was $y = 58237x + 100.88$ ($r^2 = 0.99$) when the peak area was plotted vs concentration of camptothecin in mg/mL (Figure S4, supplementary information). The

solution for the camptothecin lactone form standard curve was prepared in DMSO acidified with 0.1N HCl while the solution for the camptothecin carboxylate sodium salt form standard curve was prepared in 0.1N NaOH. The solvent system of the HPLC run (pH 5.5) did not cause inter-conversion of the lactone and carboxylate forms during the run.

In order to check the precipitation kinetics of the drug when formulated with PAMAM, camptothecin alone and PAMAM-camptothecin formulations were incubated with simulated gastric (0.2% w/v NaCl, 0.7% w/v HCl) and intestinal (6.8% w/v KH_2PO_4 , 0.9% w/v NaOH) fluids at 37°C. 500 μL of solutions were sampled at the end of 2 hours for simulated gastric fluid (SGF), and 3 hours for simulated intestinal fluid (SIF), and filtered through a 100 kDa centrifugal membrane filter (Nanosep 100K Omega, Pall Corporation, Ann Arbor, MI) that was pretreated with 5 weight % solution of Triton-X to minimize adsorption to the membrane. The formulations were filtered by spinning the membrane filtration tubes at 14,000g for 20 minutes. Camptothecin associated to PAMAM was measured directly by spectrophotometric methods quantifying the camptothecin in the residue that remained in the top layer upon membrane filtration. The filtrate collected upon membrane filtration was also measured for camptothecin content to account for the remaining drug not associated to PAMAM. Solutions were buffered back by incubation with 1:1 0.1N HCl at 37°C for 2 hours. Both filtrate and residual top layer were spectrophotometrically analyzed for camptothecin content at an absorbance maximum of 370 nm (Spectramax M2, Molecular Devices, LLC., Sunnyvale, CA).

2.2.5. In vivo oral absorption—6-8 weeks female CD-1 mice were dosed by oral gavage with 0.2 mL of camptothecin alone (5 mg/kg) and camptothecin (5 mg/kg) mixed with PAMAM G4.0- NH_2 (100 and 300 mg/kg) and G3.5-COONa (300 and 1000 mg/kg) (Dose preparation detailed in Section 2.2.2). *In vivo* dose selection of PAMAM dendrimers was based on maximum tolerated oral doses evaluated earlier in our laboratory (Thiagarajan et al., 2012). Doses were also chosen based on *in vitro* mechanistic transepithelial transport studies of dendrimers across Caco-2 monolayers that showed tight junction modulation (El-Sayed et al., 2003a; El-Sayed et al., 2003b; Kitchens et al., 2006). A similar effect of oral absorption enhancement might be observed at lower doses since the solubilizing potential of the dendrimer is known to be higher than the ratio chosen in the study (Gupta et al., 2006). However, the study was designed to test for absorption enhancement caused by dendrimer at maximum known safe doses so as to test for solubility enhancement as well as tight junction modulation. In addition, 6-8 week female CD-1 mice were dosed by oral gavage with 0.2 mL of mannitol alone (5 mg/kg) and mannitol (5 mg/kg) mixed with the highest doses of PAMAMs under study- G4.0- NH_2 (300 mg/kg) and G3.5-COONa (1000 mg/kg) (Dose preparation detailed in Section 2.2.3). Camptothecin and mannitol were tritium and ^{14}C -labeled respectively to facilitate detection. For the camptothecin formulations, animals were sacrificed at 0.5, 1, 2, 4 and 8 hours. Blood and liver samples were analyzed by ^3H -Camptothecin beta detection (procedure detailed below). For the mannitol solutions, animals were sacrificed at 2 hours, the T_{max} of mannitol absorption and blood samples were analyzed by ^{14}C -mannitol beta detection (procedure detailed below).

Tissue digestion: 50 μL of blood was digested using 0.5 mL of Solvable™ (PerkinElmer, Inc.) with incubation and shaking at 60°C for 1 hour. 50 mg of homogenized liver samples were digested using Soluene™-350 (PerkinElmer, Inc.) with incubation and shaking at 60°C for 1 hour.

Tissue bleaching: 50 μL of 0.1M EDTA solution was added to the digested samples to minimize foaming during the bleaching process. 150 μL of a 30% solution of hydrogen peroxide was added to the digested tissue samples in 3 aliquots of 50 μL each, over 30 minutes, with intermittent shaking. Samples were allowed a reaction time of 30 minutes at

room temperature and subsequently incubated at 60°C for 30 minutes. This facilitated the quenching of excess free radicals generated during the bleaching process.

Beta detection: The bleached blood and liver samples were mixed with 4.5 mL of liquid scintillation cocktails-Ultima Gold™ and Hionic Fluor™ (PerkinElmer, Inc.) respectively. The samples were light and temperature adapted for 24 hours and counted using a liquid scintillation system (Beckman LS 6000IC, Beckman Coulter, Inc.).

Parameters and efficiency of tissue sample processing: Beta detection of tissue samples is sensitive to the ratio of digestion, bleaching and detection agents. This ratio was optimized to obtain the lowest luminescence interference and the most optimal processing efficiencies (Table 2).

2.2.6. Histologic assessment of small intestinal segments—6-8 week female CD-1 mice were dosed by oral gavage with 0.2 mL of saline, PAMAM G4.0-NH₂ (100 and 300 mg/kg) and G3.5-COONa (300 and 1000 mg/kg) to evaluate changes in intestinal histology by dendrimers. In addition, 6-8 week female CD-1 mice were dosed by oral gavage with 0.2 mL of camptothecin alone (5 mg/kg) and camptothecin (5 mg/kg) mixed with PAMAM G4.0-NH₂ (100 and 300 mg/kg) and G3.5-COONa (300 and 1000 mg/kg) (Dose preparation detailed in section 2.2.2). *In vivo* dose selection of PAMAM dendrimers was based on maximum tolerated oral doses evaluated previously (Thiagarajan et al., 2012). Animals were sacrificed at 4 hours. Tissue samples were taken from different sections of the small intestine and stained by H and E staining to evaluate morphological changes in the small intestinal segments using a dark-field microscope (Olympus® BH-2, Olympus Corp., Center Valley, PA) and a digital camera for imaging (DXM1200C, Nikon Instruments Inc., Melville, NY). Small intestinal segments were also evaluated by Transmission Electron Microscopy (Hitachi I-7100 operated at 75 kV using a Leica EMUC6 ultramicrotome, South San Francisco, CA) to assess microvilli integrity.

All animal experiments were performed in accordance with the University of Utah IACUC guidelines under approved protocols.

3. Results and Discussion

3.1. Characteristics of the Formulation of PAMAM dendrimers with Camptothecin

PAMAM generations 3.5-COONa and 4.0-NH₂ are reported to have a molecular weight of 12,927.69 and 14,214.17 Da respectively, assuming perfect dendrimer synthesis (Tomalia et al., 1985). The hydrodynamic radius of G3.5 was 1.3 ± 0.1 nm and that of G4.0 was 1.7 ± 0.1 nm as measured by dynamic light scattering. The surface functionality of PAMAM G4.0 is a primary amine group (n= 64) with a reported pKa of 8-9 while that of PAMAM G3.5 is a carboxylic acid group (n=64) with a pKa of 3-4 (Tomalia et al., 1990). The interior of the PAMAM G4.0 and G3.5 has 62 tertiary nitrogen atoms (pKa of 3-6) (Tomalia et al., 1990). These functional groups on the surface and the interior of the PAMAM dendrimers are known to interact with guest molecules via electrostatic interactions, hydrogen bonding and hydrophobic interactions (D'Emanuele and Attwood, 2005; Gupta et al., 2006). This interaction depends on a number of factors, some of which are PAMAM generation and core, ratio of dendrimer to drug, physicochemical properties of drugs and pH (D'Emanuele and Attwood, 2005; Gupta et al., 2006).

Camptothecin formulated with PAMAM G4.0 showed up to 80% association with the dendrimer (Table 3). The concentration of G4.0 did not influence this association. On the other hand, camptothecin formulated with PAMAM G3.5 showed only a 20-30% association with the dendrimer (Table 3). In case of G4.0, at a 100 mg/kg dose, 1.6 moles of

camptothecin was associated with one mole of PAMAM while at 300 mg/kg, there was 0.4 moles of camptothecin associated with each mole of PAMAM. Every molecule of PAMAM also has 64 primary amine surface functionalities, 62 internal tertiary nitrogens and other internal hydrogen bonding sites contributed by the amide bonds. Camptothecin has a lactone E-ring that hydrolyzes around pH 7.0 to yield the carboxylate form of the drug (Fassberg and Stella, 1992). When camptothecin in the lactone form was dispersed with PAMAM G4.0 at pH 8-9, the primary amine group (protonated about 50%) hydrolyzed majority of the lactone of the camptothecin to yield the more soluble carboxylate form (as observed by HPLC, Figure 1A). The carboxylate group of camptothecin potentially formed an ionic bond with the partially protonated primary amine groups ($pK_a = 8-9$) on the PAMAM surface. In addition to surface electrostatic interaction, camptothecin could also have been partially encapsulated into the dendrimer interior. The association of camptothecin to the interior of PAMAM G4.0 can be attributed to hydrophobic interactions between the drug and the relatively hydrophobic interior of the dendrimer (tertiary nitrogens will not be protonated at pH 8-9) compared to the external aqueous environment. It could also be due to hydrogen bonding between the drug and the dendrimer interior. Detailed 2D-NOESY NMR spectroscopy studies on the interaction of cationic PAMAM dendrimers and anionic guest molecules have demonstrated that there exists an inclusion complex of the anionic hydrophobic drug with the cationic PAMAM dendrimer in addition to the surface electrostatic interactions (Cheng et al., 2008b; Hu et al., 2009; Markowicz et al., 2012). Camptothecin formulated with carboxylate-terminated PAMAM G3.5 showed significantly less association with the dendrimer (Table 3). This can be attributed to electrostatic repulsion between the negatively-charged PAMAM surface and the carboxylate group on the drug. Unlike PAMAM G4.0, there was no potential for surface interaction with PAMAM G3.5. The partial association observed can be attributed to encapsulation of the drug in the PAMAM interior (similar for both PAMAM G4.0 and G3.5). Concentration of the dendrimer influenced this association. A smaller ratio of G3.5 to camptothecin (1.61) showed a slightly higher drug association (29.57 ± 3.31) than a larger ratio (5.38) which showed a drug association of 23.23 ± 0.46 (Tables 1 and 3). This can be attributed to a higher extent of electrostatic repulsion in the formulation with a higher dose of G3.5, that potentially reduced encapsulation. As a consequence of lesser encapsulation, the percentage of lactone form of camptothecin in the higher dose of G3.5 (1000 mg/kg) was also slightly less than the percentage of lactone in the formulation with a smaller dose of G3.5 (300 mg/kg) (Figure 1A).

This difference in the association of drug to the cationic and anionic dendrimers has been reported for the solubilization of the hydrophobic drug sylibin (containing ionizable phenol groups) and indomethacin (containing carboxylic acid groups) (Chauhan et al., 2003; Huang et al., 2011). Both these drugs associated to a higher extent to the cationic dendrimers due to a surface electrostatic interaction that was not likely in case of the anionic dendrimer. This trend was not observed for nifedipine which does not have an ionizable group that can electrostatically interact with cationic surfaces (Devarakonda et al., 2004).

Camptothecin delivered with cationic and anionic PAMAM remained more solubilized in the gastric fluid at 2 hours than free drug alone suggesting that both cationic and anionic PAMAM dendrimers controlled release and precipitation kinetics of camptothecin at pH 1.2 (Figure 1B). It is known that the solubilization potential of cationic dendrimers for anionic molecules decreases with decrease in pH due to elimination of electrostatic interaction. (Hu et al., 2008; Hu et al., 2009; Huang et al., 2011; Pistolis and Malliaris, 2002). At pH 1.2, the tertiary nitrogen atoms in the interior of the PAMAM dendrimers G4.0 and G3.5 and the primary nitrogen atoms on the surface of G4.0 will be completely protonated but the carboxylate group will be unionized, thus not available for ionic interaction. However, there is a possibility of intermolecular hydrogen bonding between: 1) the hydrogen atom of the

carboxylic acid of camptothecin and the oxygen atom on the carbonyl group from the amide bond of the PAMAM interior, and 2) the hydrogen atom of the secondary nitrogen from the amide bond of PAMAM interior and the oxygen atom from the carbonyl group of camptothecin. This can potentially stabilize the drug as a carboxylic acid and also partially solubilize it. Such intermolecular hydrogen bonding interaction with the PAMAM interior has been postulated for other carboxylic acid-containing hydrophobes like benzoic acid (Beezer et al., 2003). Inclusion complexes of drug with PAMAM dendrimer can also potentially control release of the drug thus altering precipitation kinetics over 2 hours (Hu et al., 2008; Huang et al., 2011). In case of the camptothecin solution alone, only 20% of the drug remained solubilized in the simulated gastric fluid. However, about 60% of the drug remained solubilized in SGF over 2 hours when camptothecin was formulated with PAMAM G4.0 and G3.5.

In the simulated intestinal fluid, there was no difference in the solubilization of the drug when formulated with the PAMAM, with about 60% of the drug remaining solubilized at 3 hours. At pH 6.8, the carboxylic acid group of the drug is ionized, likely to prevent intermolecular hydrogen bonding in the dendrimer interior. Majority of the primary amine groups of the cationic dendrimer (pKa 8.0-9.0) will be unionized eliminating surface electrostatic interactions. However, the tertiary nitrogens (pKa 3-6) will be partially protonated at pH 6.8, facilitating electrostatic interaction between the ionized drug and the dendrimer interior. This internal association will be similar for both cationic and anionic dendrimers. However, there was no change in drug solubility in SIF when co-delivered with PAMAM because the drug itself is ionized and in a more water-soluble form. Inclusion complexes of the dendrimer-drug can, however, control drug release and improve oral bioavailability as seen for oral absorption studies with cationic PAMAM solubilized drugs sylibin and doxorubicin (Huang et al., 2011; Ke et al., 2008).

3.2. *In vivo* oral absorption of camptothecin co-delivered with PAMAM dendrimers

Both cationic, amine-terminated PAMAM G4.0-NH₂ and anionic, carboxylic-acid terminated PAMAM G3.5-COONa dendrimers caused an approximate 2 to 3-fold oral absorption enhancement of camptothecin *in vivo* at 2 hours (T_{max} of camptothecin oral absorption). For both G4.0-NH₂ (100-300 mg/kg) and G3.5-COOH (300-1000 range studied (Figure 2). Camptothecin levels in the blood when delivered alone or with G4.0 (300 mg/kg) and G3.5 (1000 mg/kg) increased in the first 2 hours and plateaued up to 8 hours (Figure 3). The camptothecin lactone E-ring is known to hydrolyze to the carboxylate form in the plasma and bind to human serum albumin, thus prolonging its circulation half-life to 36 hours (Scott et al., 1993).

All of the formulations tested had the same drug content but varied in the surface charge and concentration of PAMAM dendrimer and hence in the extent of drug association. The increase in absorption of camptothecin was similar for the cationic (80% camptothecin associated with PAMAM) and anionic dendrimer (20-30% camptothecin associated with PAMAM). Total amount of bioavailable drug in this formulation would be a result of multiple factors. These are drug association to PAMAM, drug solubilization in the simulated gastric and intestinal environments by PAMAM, fraction of drug retained in the unionized form by PAMAM, drug release rate from PAMAM, species being absorbed (free drug or drug associated dendrimer) and tight junction modulation by the polymer. Drug solubilization in SGF and SIF as well as fraction of drug retained in unionized lactone form by PAMAM was similar for both cationic and anionic PAMAM dendrimers at varying concentrations tested (Figure 1A, 1B). Limited reports have shown that association of drugs with PAMAM dendrimers controls drug solubilization and release, thereby increasing the oral bioavailability (Huang et al., 2011; Ke et al., 2008). It is expected therefore that the

higher the extent of association, the higher would be the bioavailability of the drug. However, electrostatic interaction on the surface between cationic PAMAM and carboxylate group of drug is likely to diminish at lower pH (below pKa of terminal primary amine groups of G4.0) (Hu et al., 2008; Hu et al., 2009; Huang et al., 2011; Pistolis and Malliaris, 2002), thus suggesting that it is only drug inclusion in PAMAM interior that controlled solubilization in SGF, SIF and oral bioavailability. It was also observed that the cationic PAMAM dendrimers bind to mucous layer restricting their interaction with the epithelial barrier *in vivo* (unpublished data). Anionic PAMAM dendrimers did not show this phenomenon. This difference in mucosal interaction and access to epithelial barrier can affect drug release and absorption *in vivo*. Tight junction modulation, which is a factor that affects drug absorption was not seen for either the cationic or the anionic PAMAM dendrimers at doses tested (discussed in Section 3.3).

In the CPT concentration time profile, differences between blood levels of CPT when codelivered with G4.0 (300 mg/Kg) and G3.5 (1000 mg/Kg) are significant only at one hour ($p < 0.05$) (only higher doses evaluated) (Figure 3). In the study evaluating effect of PAMAM dose on CPT blood concentration (Figure 2), there is a significant difference between G4.0 (100 mg/Kg) and G3.5 (1000 mg/Kg) at 2 hours ($p < 0.05$) (the only time point evaluated). Since CPT concentration in blood was not different for PAMAM G4.0 and G3.5 at comparable doses (300 mg/Kg), the results do not suggest any advantage of G3.5 over G4.0 at comparable doses.

In vitro reports have demonstrated a much higher increase in SN38 (derivative of CPT) permeability when complexed with cationic PAMAM G4.0 and anionic PAMAM G3.5 as compared to the *in vivo* oral absorption enhancement of CPT (2-3 fold) observed in this study. G4.0-SN38 complex increased SN38 flux across Caco-2 monolayers by about 5-80 times depending on concentration and extent of drug loading. A 13 to 159-fold increase in SN38 flux was observed when the drug was conjugated to PAMAM G3.5. Increase in flux depended on concentration of conjugates and choice of linker. Doses employed in the *in vivo* study reported in this paper are slightly higher than the doses tested *in vitro* across Caco-2 monolayers. However, the PAMAM dendrimers dosed in this study were subjected to dilution in the gastric and intestinal fluid which potentially reduced concentration at the intestinal surface. The treatment groups evaluated in this study had drug only partially associated to the PAMAM which was different from the *in vitro* experiments, where there was no free drug species.

Along with enhancement in blood levels, both cationic, amine-terminated PAMAM G4.0-NH₂ (300 mg/kg) and anionic, carboxylic-acid terminated PAMAM G3.5-COOH (1000 mg/kg) caused an approximate 2-fold increase in liver accumulation of camptothecin *in vivo* at 2 hours (Figure 4). Camptothecin derivative (Irinotecan) is approved for hepatic metastasis of colorectal cancer (Rothenberg, 2001). Oral delivery of camptothecin offers a route through the hepatic portal vein to access the liver directly reducing drug exposure to plasma and consequently reducing non-specific systemic toxicities. It has been reported that liver/blood exposure of camptothecin is higher when camptothecin is delivered orally (Gupta et al., 2000). However, the oral delivery of camptothecin is limited by variable and low absorption (Gupta et al., 2000). An increase in oral absorption of camptothecin will allow a therapeutic dose to reach the liver, while decreasing blood exposure of the drug as compared to a similar dose given intravenously.

3.3. *In vivo* oral absorption of paracellular marker-mannitol

PAMAM dendrimers did not cause an increase in blood levels of the paracellular marker ¹⁴C-mannitol at 2 hours. (T_{max} of Mannitol) (Figure 5). This observation suggests that at doses of 300 mg/kg for amine-terminated PAMAM G4.0-NH₂ and 1000 mg/kg for

carboxylic acid-terminated PAMAM G3.5-COONa, tight junction modulation was not observed and that the increase in absorption of camptothecin was not due to opening of tight junctions.

Previous *in vitro* transepithelial transport studies of PAMAM dendrimers across Caco-2 monolayers have reported tight junction modulation by amine and carboxylic acid-terminated PAMAM dendrimers as investigated by TEER (transepithelial electrical resistance) measurements and the permeability of ^{14}C -mannitol (El-Sayed et al., 2003a; El-Sayed et al., 2003b; Kitchens et al., 2006). Tight junction modulation was observed to be a function of PAMAM surface chemistry, concentration and incubation time (El-Sayed et al., 2003a). Both PAMAM G4.0-NH₂ (partially surface modified by FITC) and G3.5-COOH were shown to cause a sharp decline in TEER and a 5-10 fold increase in mannitol flux at concentrations of 1.0 mM, when incubated for 2 hours with Caco-2 cell monolayers (El-Sayed et al., 2003a; Kitchens et al., 2006). The highest doses of the PAMAM formulations tested in the current *in vivo* study were 2.1 mM for G4.0-NH₂ (corresponding to 300 mg/kg in animals) and 7.7 mM (corresponding to 1000 mg/kg in animals) for G3.5-COOH. These doses are slightly higher than the doses tested *in vitro* across Caco-2 monolayers. However, the PAMAM dendrimers dosed in this study were subjected to variables in the *in vivo* GIT system like dilution in the gastric and intestinal fluid, gastrointestinal transit and mucosal barrier. This potentially reduced the effective concentration and incubation time of PAMAM dendrimers at the intestinal surface at which tight junction modulation was not achieved (Figure 5).

Results presented in Section 3.3 suggest that the increase in oral absorption of camptothecin is not due to tight junction modulation. This negative result suggests that increase in oral absorption of drug by PAMAM is due to alternate reasons such as 1) increased solubilization of PAMAM associated CPT in SGF (Figure 1B) or 2) increased transcellular uptake of the PAMAM associated CPT via endocytic mechanisms over CPT alone. Results presented in Section 3.2 suggest that camptothecin association to PAMAM controlled precipitation kinetics of the drug in gastric conditions and increased oral absorption by solubilization. Previous *in vitro* studies have demonstrated the enhanced transport of PAMAM-drug complexes by endocytic uptake of the complex and bypassing of the P-gp efflux (El-Sayed et al., 2003b; Goldberg et al., 2010; Kitchens et al., 2007; Kitchens et al., 2008). It is possible that in addition to increased solubilization, camptothecin association to PAMAM increased transcellular uptake of the dendrimer-drug conjugates via endocytic mechanisms.

3.4. Histologic assessment of small intestinal segments

At the PAMAM G4.0-NH₂ and G3.5-COOH doses tested, no clinically significant histologic changes were observed. No morphological changes were noted at the villi (Figure 6; Figure S5-9, supplementary information) or the microvilli levels (Figure 7, Figure S10-14, supplementary information). Slight irregularities of microfilaments were observed for the group treated with saline (Figure 7, Figure S10, supplementary information). Previous studies *in vitro* have shown that anionic PAMAM G3.5 (1.0 mM) did not affect microvilli structure in Caco-2 cell monolayers over a 2 hour incubation period while at the same concentration, cationic PAMAM G4.0 showed disruption and loss of microvilli (Kitchens et al., 2007). The influence of surface groups on histologic morphology was not observed in this study. PAMAM G4.0 doses at concentrations of 0.7 mM (100 mg/kg) and 2.1 mM (300 mg/kg) and PAMAM G3.5 doses at 2.3 mM (300 mg/kg) and 7.7 mM (1000 mg/kg) were subjected to gastrointestinal transit time and dilution variables which reduced their effective concentration *in vivo* as compared to that on Caco-2 cell monolayers *in vitro*.

The camptothecin dosed alone or with the PAMAM dendrimers under study also did not cause any apparent morphological changes to the villi of the small intestinal segments being evaluated (Figure 8, Figure S15-19, supplementary information). However, slight irregularities in the plasma membranes and microfilaments of microvilli were observed for the camptothecin dosed alone (Figure 9, Figure S20, supplementary information). Disruption of microvilli has been previously noted in cells treated with camptothecin (Bredholt et al., 2009; Lin et al., 2008). When the camptothecin was dosed with the PAMAM dendrimers-G4.0 and G3.5, the microvilli did not show swelling and the microfilaments inside the microvilli were well organized (Figure 9, Figure S21-24, supplementary information). This suggests a protective effect of the PAMAM dendrimers to the potential histologic changes caused by camptothecin, probably due to encapsulation of the drug within the interior cavities of the dendrimers. H and E staining of small intestinal segments in animals dosed with camptothecin + G4.0 or G3.5 as well as saline showed dilated lymphatics indicative of an absorptive process (Figure 6, Figure 8, Figure S5, 16-19, supplementary information).

4. Conclusion

When formulated with cationic, amine-terminated PAMAM generation 4.0 and anionic, carboxylate-terminated G3.5, camptothecin associated to a higher extent with G4.0 than G3.5 due to an electrostatic interaction on the surface of G4.0. Despite a difference in drug association, both G4.0 and G3.5 caused a 2-3 fold increase in oral absorption of camptothecin when co-delivered with the drug at 2 hours (the T_{max} of camptothecin absorption). This was attributed to better solubilization of the drug in simulated gastric fluid which can affect drug association with PAMAM, precipitation kinetics and release in the simulated intestinal fluid that in turn affects oral bioavailability. Increased oral absorption of camptothecin was also attributed to a higher fraction of the drug in the unionized form (lactone) when formulated with PAMAM dendrimers. There was no significant difference in drug solubilization in SGF and SIF by either cationic or anionic PAMAM dendrimers. This was attributed to the absence of surface electrostatic interaction with G4.0 at lower pH. PAMAM G4.0 and G3.5 did not cause tight junction modulation at the doses tested suggesting that increase in oral absorption of camptothecin was not due to tight junction modulation. At doses tested, PAMAM dendrimers did not cause any histologic changes to the epithelial layer of the gastrointestinal tract at 4 hours post dosing. This study demonstrates that both cationic and anionic PAMAM dendrimers were equally effective in enhancing the oral absorption of camptothecin. Results suggest that drug inclusion in PAMAM interior controlled drug solubilization in SGF and SIF, and increased oral bioavailability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2D-NOESY NMR	Two-dimensional-Nuclear Overhauser effect nuclear magnetic resonance spectroscopy
BCS	Biopharmaceutical classification system
CPT	Camptothecin
DI water	Deionized water
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast protein liquid chromatography

G3.5 or G3.5-COONa	Carboxyl-terminated PAMAM generation 3.5
G4.0 or G4.0-NH₂	Amine-terminated PAMAM generation 4.0
GIT	Gastrointestinal tract
H and E stain	Hematoxylin and eosin stain
HPLC	High performance liquid chromatography
PAMAM	Poly(amido amine)
P-gp	P-glycoprotein
R_h	Hydrodynamic radius
SEC	Size exclusion chromatography
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SN38	7-ethyl-10-hydroxy-camptothecin
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy

Figure 1.

A. Percentage of camptothecin in the lactone form in its formulation alone and with PAMAM G4.0 (100 and 300 mg/kg), and G3.5 (300 and 1000 mg/kg). Significant difference between camptothecin alone and camptothecin formulation with G4.0, G3.5, * $p < 0.05$, *** $p < 0.001$ by one-way ANOVA. Camptothecin formulated with PAMAM to form a soluble formulation had a higher % of drug in the lactone (unionized form) compared to camptothecin solution alone suggesting potential encapsulation of camptothecin within the interior cavities of both cationic G4.0 and anionic G3.5. **B.** Percentage of total camptothecin solubilized in simulated gastric and intestinal fluids at 2 and 3 hours respectively; $n=3$; Significant difference between camptothecin alone and camptothecin formulation with G4.0, G3.5, *** $p < 0.001$ by one-way ANOVA.

Figure 2.

Blood concentration of orally administered ³H-Camptothecin alone and co-delivered with PAMAM G4.0 at 100 mg/kg, 300 mg/kg and G3.5 at 300 mg/kg and 1000 mg/kg, n= 3 animals at 2 hours, PAMAM dendrimers G4.0 and G3.5 significantly increased the blood concentration of ³H-Camptothecin at 2 hours, **p<0.01, ***p<0.001 by one-way ANOVA.

Figure 3. Blood concentration-time profile of orally administered ³H-Camptothecin alone and co-delivered with PAMAM G4.0 (300 mg/kg) and G3.5 (1000 mg/kg), n= 3 animals per time point, PAMAM dendrimers significantly increased the blood concentration of ³H-Camptothecin, p<0.001 by two-way ANOVA.

Figure 4.

Liver concentration of orally administered ^3H -Camptothecin alone and co-delivered with PAMAM G4.0 (300 mg/kg) and G3.5 (1000 mg/kg), $n=3$ animals at 2 hours, PAMAM dendrimers significantly increased the liver concentration of ^3H -Camptothecin at 2 hours, $*p<0.05$ by one-way ANOVA.

Figure 5. Blood concentration of orally administered ^{14}C -Mannitol alone and co-delivered with PAMAM G4.0 (300 mg/kg), G3.5 (1000 mg/kg), $n=5$ animals at 2 hours, No statistical significance as per ANOVA.

Saline

G4.0 (100 mg/kg)

G4.0 (300 mg/kg)

G3.5 (300 mg/kg)

G3.5 (1000 mg/kg)

Figure 6.

H and E staining of the small intestinal segments of CD-1 mice orally administered with saline, G4.0 (100 mg/kg), G4.0 (300 mg/kg), G3.5 (300 mg/kg), G3.5 (1000 mg/kg). Scale Bar = 25 μm. No morphological irregularities noted at the villi.

Saline

G4.0 (100 mg/kg)

G4.0 (300 mg/kg)

G3.5 (300 mg/kg)

G3.5 (1000 mg/kg)

Figure 7.

TEM images of the small intestinal segments of CD-1 mice orally administered with saline, G4.0 (100 mg/kg), G4.0 (300 mg/kg), G3.5 (300 mg/kg), G3.5 (1000 mg/kg). Scale Bar = 1 μ m. Slight microfilament irregularities observed in the saline group. No significant microfilament irregularities were noted in treatment groups.

CPT 5mg/kg

CPT + G4.0 (100 mg/kg)

CPT + G4.0 (300 mg/kg)

CPT + G3.5 (300 mg/kg)

CPT + G3.5 (1000 mg/kg)

Figure 8.

H and E staining of the small intestinal segments of CD-1 mice orally administered with CPT (5 mg/kg), CPT + G4.0 (100 mg/kg), CPT + G4.0 (300 mg/kg), CPT + G3.5 (300 mg/kg), CPT + G3.5 (1000 mg/kg). Scale Bar = 25 μ m. No morphological irregularities were noted at the villi.

CPT (5mg/kg)

CPT + G4.0 (100 mg/kg)

CPT + G4.0 (300 mg/kg)

CPT + G3.5 (300 mg/kg)

CPT + G3.5 (1000 mg/kg)

Figure 9.

TEM images of the small intestinal segments of CD-1 mice orally administered with CPT (5 mg/kg), CPT + G4.0 (100 mg/kg), CPT + G4.0 (300 mg/kg), CPT + G3.5 (300 mg/kg), CPT + G3.5 (1000 mg/kg). Scale Bar = 1 μ m. Microfilament irregularities noted in group treated with CPT alone. No irregularities were observed in groups treated with CPT formulations with PAMAM G4.0 and G3.5 suggesting a protective effect of the PAMAM in histological damage to small intestine by CPT.

Table 1
Formulation ratios of PAMAM to camptothecin (CPT)

PAMAM concentration (mg/kg)	PAMAM concentration (M)	PAMAM/camptothecin Molar ratio
CPT + G4.0-NH ₂ (100 mg/kg)	7.1×10^{-4}	0.49
CPT + G4.0-NH ₂ (300 mg/kg)	21.1×10^{-4}	1.47
CPT + G3.5-COONa (300 mg/kg)	23.2×10^{-4}	1.61
CPT + G3.5-COONa (1000 mg/kg)	77.4×10^{-4}	5.38

Table 2
Processing parameters of Tritium and ^{14}C Carbon counting from blood and liver

	^3H	^{14}C
Blood		
Lumex (%)	4.19 ± 3.19	0.17 ± 0.02
Extraction Efficiency (%)	75.48 ± 6.09	88.26 ± 0.01
Blank Blood	46.28 ± 11.35	37.17 ± 0.76
Liver		
Lumex (%)	1.14 ± 0.63	-
Extraction Efficiency (%)	77.39 ± 4.81	-
Blank Blood	41.23 ± 9.82	-

Table 3
Association of camptothecin with PAMAM dendrimers

Formulation	Percentage associated with PAMAM
CPT + G4.0-NH ₂ (100 mg/kg)	80.27 ± 0.66
CPT + G4.0-NH ₂ (300 mg/kg)	81.80 ± 0.24
CPT + G3.5-COONa (300 mg/kg)	29.57 ± 3.31
CPT + G3.5-COONa (1000 mg/kg)	23.23 ± 0.46