Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 336 (2007) 183–190
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

In vitro evaluation of dendrimer prodrugs for oral drug delivery

Mohammad Najlah [1], Sally Freeman, David Attwood, Antony D'Emanuele *

School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester M13 9PL, UK

Received 30 September 2006; received in revised form 19 November 2006; accepted 22 November 2006
Available online 28 November 2006

Abstract

Dendrimer-based prodrugs were used to enhance the transepithelial permeability of naproxen, a low solubility model drug. The stability of the dendrimer–naproxen link was assessed. Naproxen was conjugated to G0 polyamidoamine (PAMAM) dendrimers either by an amide bond or an ester bond. The stability of G0 prodrugs was evaluated in 80% human plasma and 50% rat liver homogenate. The cytotoxicity of conjugates towards Caco-2 cells was determined and the transport of the conjugates across Caco-2 monolayers (37 °C) was reported. In addition, one lauroyl chain (L) was attached to the surface group of G0 PAMAM dendrimer of the diethylene glycol ester conjugate (G0-deg-NAP) to enhance permeability. The lactic ester conjugate, G0-lact-NAP, hydrolyzed slowly in 80% human plasma and in 50% rat liver homogenate ($t_{1/2}$ = 180 min). G0-deg-NAP was hydrolyzed more rapidly in 80% human plasma ($t_{1/2}$ = 51 min) and was rapidly cleaved in 50% liver homogenate ($t_{1/2}$ = 4.7 min). The conjugates were non-toxic when exposed to Caco-2 cells for 3 h. Permeability studies showed a significant enhancement in the transport of naproxen when conjugated to dendrimers; L-G0-deg-NAP yielding the highest permeability. Dendrimer-based prodrugs with appropriate linkers have potential as carriers for the oral delivery of low solubility drugs such as naproxen.

Keywords: PAMAM dendrimers; Enzymatic stability; Transepithelial transport; Caco-2 cells; Prodrugs

1. Introduction

Dendrimers represent a relatively new class of highly branched polymers with a well-defined structure that allows precise control of size, shape and terminal group functionality (Tomalia et al., 1985). Dendrimers have several pharmaceutical applications (D'Emanuele and Attwood, 2005; D'Emanuele et al., 2003) including, for example, the enhancement of drug solubility (Milhem et al., 2000) and permeability (D'Emanuele et al., 2004).

Dendrimers show potential as carrier/delivery systems that can cross cell barriers (Florence et al., 2000; Wiwattanapatapee et al., 2000) by both paracellular and transcellular pathways (El-Sayed et al., 2003; Jevprasesphant et al., 2003a). The cytotoxicity and permeability of PAMAM dendrimers was found to be concentration, generation, and charge dependent. Anionic PAMAM dendrimers were less cytotoxic and permeated cells at lower rates than cationic PAMAM dendrimers (Jevprasesphant et al., 2003a). Low generation PAMAM dendrimers (G0 and G1) exhibit significantly less cytotoxicity and higher permeability than higher generations (G2, G3 and G4) (El-Sayed et al., 2002). However, a significant reduction in cytotoxicity and enhancement in permeability can be achieved by surface engineered cationic PAMAM dendrimers with lauroyl chains (Jevprasesphant et al., 2003a,b).

The utilization of dendrimers as drug delivery carriers is of great interest due to their highly controllable structure and size. The terminal functional groups of dendrimers show higher chemical reactivity compared to that when present in other classes of polymers (Fréchet, 1994). The functional groups of dendrimers have been conjugated to various biologically active molecules such as drugs, antibodies (Roberts et al., 1990), sugar moieties (Aoi et al., 1995), and lipids (Jevprasesphant et al., 2003b). In dendrimer–drug conjugates (prodrugs) the drug is combined through a covalent bond either directly or via a linker/spacer to the dendrimer. The release of drug from a prodrug occurs *via* chemical or enzymatic cleavage of a hydrolytically labile bond. Several reports have investigated the

* Corresponding author at: School of Pharmacy and Pharmaceutical Sciences, University of Central Lancashire, Preston PR1 2HE, UK. Tel.: +44 1772 895801; fax: +44 7092 030763.
E-mail address: Antony@DEmanuele.net (A. D'Emanuele).
URL: http://www.dendrimerweb.com (A. D'Emanuele).
[1] Present address: School of Pharmacy and Pharmaceutical Sciences, University of Central Lancashire, Preston PR1 2HE, UK.

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.11.047

conjugation of drugs to PAMAM dendrimers as drug delivery systems. For example, 5-fluorouracil (5FU)-PAMAM dendrimer conjugates (G4 and G5) gave a slow release of 5FU (Zhuo et al., 1999). 5-Aminosalicylic acid (5-ASA) was conjugated using two different spacers, both containing an azo-bond, to G3 PAMAM dendrimer for use as a carrier for colonic delivery. Colon specificity and prolonged release of 5-ASA from the conjugates were reported suggesting that PAMAM dendrimers have potential for use as colon-specific drug carriers (Wiwattanapatapee et al., 2003). Propranolol, an insoluble drug and a substrate for the P-glycoprotein (P-gp) efflux transporter, was conjugated to surface modified G3 PAMAM dendrimer. The conjugate was shown to bypass the efflux of P-gp transporters in Caco-2 cells, thus dendrimer nanocarriers may enhance the bioavailability of drugs that are poorly soluble and/or substrates of efflux transporters (D'Emanuele et al., 2004).

Our previous work described the design, synthesis, and characterization of a series of zero generation (G0) PAMAM dendrimer-based prodrugs for the potential enhancement of drug solubility and bioavailability (Najlah et al., 2006). Naproxen was conjugated to the dendrimer either directly or via a linker (lactic acid or diethylene glycol) to give prodrugs (Fig. 1). Conjugates in which naproxen was linked to the G0 dendrimer through a diethylene glycol linker showed high chemical stability in buffers, but readily released naproxen in plasma. Such conjugates have potential as carriers for low solubility drugs such as naproxen.

In the present study, the stability of these G0 PAMAM conjugates in 50% liver homogenate is compared to that in 80% human plasma (Najlah et al., 2006). The biological evaluation of the conjugates including their cytotoxicity (MTT assay) and transport across Caco-2 monolayers is described. The influence of attaching a surface modifier (lauroyl chain) on the transport properties is also evaluated.

2. Materials and methods

2.1. Materials

Zero generation PAMAM dendrimers (G0) with ethylenediamine cores were purchased from Dendritech Inc. (Michigan, USA). Trifluoroacetic acid (TFA), triethylamine (TEA), (*S*)-

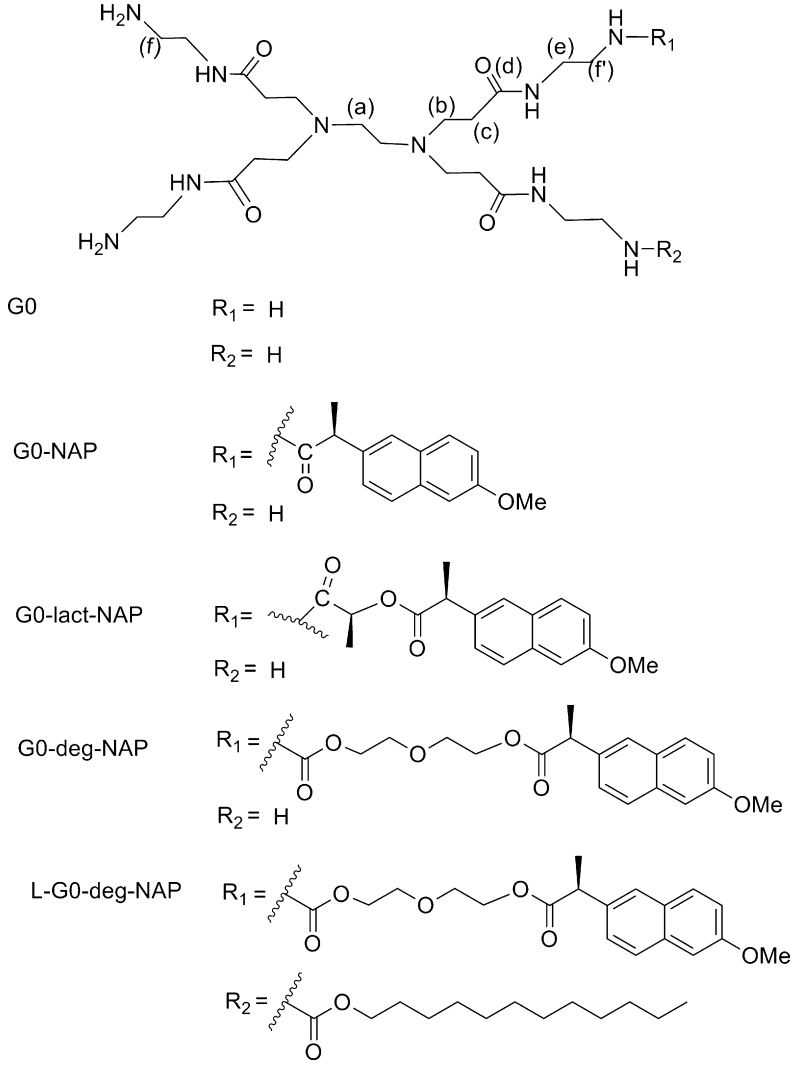


Fig. 1. Structures of dendrimer and dendrimer–naproxen conjugates. Letters on G0 PAMAM dendrimer structure are included to aid NMR assignments.

(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid (naproxen), 4-nitrophenyl chloroformate, 1-dodecanol (lauroyl alcohol), trypan blue, 3-(4,5-dimethythiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Sephadex LH-20 were purchased from Sigma–Aldrich Co. Ltd. (Poole, Dorset, UK). Cell culture materials were from Gibco BRL Life Technologies (Paisley, Scotland). Polycarbonate cell culture inserts (Transwell® 12 mm diameter) and cluster plates (96 well) were purchased from Corning Costar UK (High Wycombe, Bucks, UK). Rat livers were provided by the Animal Support Unit (University of Manchester).

G0 dendrimer–naproxen conjugates including G0-naproxen (G0-NAP), G0-lactic-naproxen (G0-lact-NAP), and G0-diethylene glycol-naproxen (G0-deg-NAP) were synthesized and characterized as described previously (Najlah et al., 2006). The lauroyl (L) conjugate (L-G0-deg-NAP) and its synthetic intermediates were characterized using ^{13}C and ^{1}H NMR spectroscopy (Bruker Avance 300, Bruker, Coventry, UK). ^{13}C NMR spectra were assigned with the aid of DEPT-135. HPLC analyses were carried out using a Hewlett-Packard Series II 1090 (Germany) instrument equipped with a Luna 5 μm, C18 column (250 mm × 4.6 mm, Phenomenex, Cheshire, UK).The solvent system used to characterize the conjugates was methanol:aq H_3PO_4 (0.05%, w/v), ratio 5:95 for 4 min then 80:20 for the remaining elution time, the flow rate was 1.2 ml/min and UV detection was at λ = 230 nm.

2.2. Synthesis of lauroyl (4-nitrophenyl carbonate)

Lauroyl alcohol (0.93 g, 5 mmol) was dissolved in THF (10 ml) and TEA (1.02 g, 10 mmol) was added. The mixture was stirred for 10 min after which 4-nitrophenyl chloroformate (2.01 g, 10 mmol) was added portionwise and stirred for 24 h at room temperature. THF was evaporated under vacuum and the residue was dissolved in hexane:EtOAc (85:15), filtered and purified by silica gel column chromatography, eluting with the same solvent system to give lauroyl (4-nitrophenyl carbonate) (Rf = 0.4). The yield was 84%. ^{1}H NMR ($CDCl_3$): 0.80 (3H, t, $J = 6.9$, CH_3), 1.35–1.15 (18H, m, 9× $-CH_2-$), 1.68 (2H, pentet, $J = 6.8$, $-CH_2-$), 4.20 (2H, t, $J = 6.8$, $-CH_2-O-CO-$), 7.35–7.25 (2H, m, Ar), 8.2–8.15 (2H, m, Ar). ^{13}C NMR ($CDCl_3$): 14.5 ($-CH_3$), 23.1 ($-CH_2-$), 26.0 ($-CH_2-$), 30.0–28.9 (7× $-CH_2-$), 32.3 ($-CH_2-$), 70.0 ($-CH_2-O-CO-$), 122.1 (2× CH, Ar), 125.6 (2× CH, Ar), 145.7 (C, Ar), 152.9 (C, Ar), 156.0 (–CO–).

2.3. Synthesis of lauroyl-G0-deg-NAP (L-G0-deg-NAP)

Lauroyl (4-nitrophenyl carbonate) (22.5 mg, 0.064 mmol) in DMF (1 ml) was added dropwise to a stirred solution of G0-deg-NAP (50 mg, 0.058 mmol) in DMF (2 ml). The reaction mixture was stirred for 5 days. DMF was evaporated under vacuum and the residue was purified by size exclusion (gel filtration) chromatography using Sephadex LH 20 with methanol:water (5:1). The resulting product was dissolved in water and filtered; the filtrate was concentrated under vacuum and purified again by size exclusion chromatography (same conditions). The yield of L-G0-deg-NAP was 57%. ^{1}H NMR (d_4-MeOD): 0.89 (3H, t, $J = 6.8$, CH_3, L), 1.28–1.21 (18H, m, 9× $-CH_2-$, L), 155–1.52 (5H, m, CH_3, NAP and $-CH_2-$, L), 2.40–2.32 (8H, m, 4× c-G0), 2.57 (4H, s, 2× a-G0), 2.91–2.69 (8H, m, 4× b-G0), 3.09–2.95 (4H, m, 2× f-G0), 3.39–3.27 (12H, m, 2× f′-G0 and 4× e-G0), 3.54–3.44 (2H, m, $-CH_2O-$, deg), 3.64–3.58 (2H, m, $-OCH_2-$, deg), 3.92–3.88 (4H, m, CHCOO– and $O-CH_3$, NAP), 4.04–3.94 (4H, m, $-CH_2-O-CO-$, L and $-CH_2-O-CO-$, deg), 4.20–4.16 (2H, m, $-COOCH_2-$, deg), 7.72–6.77 (6H, Ar, NAP). ^{13}C NMR (d_4-MeOD): 14.5 ($-CH_3$, L), 19.0 ($-CH_3$, NAP), 23.8 ($-CH_2-$, L), 27.0 ($-CH_2-$, L), 30.8–30.3 (7× $-CH_2-$, L), 33.1 ($-CH_2-$, L), 34.2 (4× c-G0), 38.6 (2× f-G0), 39.9 (2× f′-G0), 41.3 (4× e-G0), 46.6 (–CH–, NAP), 50.9 (4× b-G0), 51.9 (2× a-G0), 53.5 ($-OCH_3$, NAP), 65.0 ($-CH_2OCONH-$, deg), 65.2 ($-CH_2OCONH-$, L), 66.0 ($-COOCH_2-$, deg), 70.0 ($-CH_2O-$ deg), 70.5 ($-OCH_2-$ deg), 106.6 (CH–Ar, NAP), 120.1 (CH–Ar, NAP), 127.0 (CH–Ar, NAP), 127.2 (CH–Ar, NAP), 128.3 (CH–Ar, NAP), 130.3 (CH–Ar, NAP), 130.4 (C–Ar, NAP), 135.3 (C–Ar, NAP), 137.0 (C–Ar, NAP), 158.8 (NHCOO, deg), 159.2 (–CO–, L), 164.2 (C–Ar, NAP), 175.1 (–COO–, NAP), 176.2 (4× d-G0). (+)-ESI-MS: 1074 [$M^+ + H$].

2.4. Enzymatic hydrolysis of naproxen prodrugs

2.4.1. Hydrolysis of the prodrugs in 80% human plasma

Human plasma was diluted to 80% with 0.05 M phosphate buffer. The reaction was initiated by adding an appropriate amount of conjugate to 5 ml of preheated plasma solution to give a final concentration of 2×10^{-4} M. The solution was maintained at 37 °C, and at appropriate intervals, samples (50 μl) were withdrawn and 200 μl of a methanolic solution of sodium salicylate (1.25 mM) (internal standard) was added in order to deproteinize the plasma and quench the reaction. After immediate centrifugation for 5 min at 9000 × *g*, the clear supernatant was stored at −20 °C awaiting analysis by HPLC (solvent systems: ACN:aq H_3PO_4 (0.05%, w/v) (40:60)) for NAP-lact and NAP-Me, and methanol:ACN:aq H_3PO_4 (0.05%, w/v) (17.5:30:52.5) for NAP-deg.

2.4.2. Hydrolysis of the prodrugs in 50% rat liver homogenate (RLH)

Male Sprague–Dawley rat livers were blotted dry, weighed, cut into small pieces and homogenized in ice-cooled PBS (pH 7.4) at a ratio of 1 g tissue to 1 ml buffer. The liver homogenate was centrifuged at 9000 × *g* for 45 min. The supernatant was used to perform the hydrolysis study. The reaction was initiated by adding an appropriate amount of conjugate to 2 ml of preheated liver supernatant to give a final concentration of 2×10^{-4} M. The solution was maintained at 37 °C, and at appropriate intervals, samples (50 μl) were withdrawn and 200 μl of a methanolic solution of sodium salicylate (1.25 mM) (internal standard) was added in order to deproteinize the sample and quench the reaction. After immediate centrifugation for 5 min at 9000 × *g*, the clear supernatant was stored at −20 °C and subsequently analyzed by HPLC using the same solvent systems as that used for plasma studies.

2.5. Cytotoxity studies

Human intestinal adenocarcinoma cells (Caco-2) (passage 80–85) were seeded at 30,000 cells/well in 96-well plates and maintained at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 50 IU/ml penicillin and 50 mg/ml streptomycin. The medium was changed on alternate days, and after 3 days DMEM was removed, the cells were washed in phosphate buffered saline, and 100 μl Hanks Balanced Salt Solution (HBSS) containing G0 PAMAM dendrimer or conjugates was added. After 3 h of incubation at 37 °C, 20 μl of 3-(4,5-dimethythiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added and cells incubated for a further 4 h. HBSS was removed, 100 μl dimethylsulfoxide (DMSO) was added and the optical density at 550 nm was measured. The level of colour development in the control wells (containing only HBSS) was assumed to indicate 100% viability.

2.6. Transport studies of G0 PAMAM dendrimer and conjugates

Caco-2 cells (passage 83–92) were seeded onto polycarbonate 12-well transwell filters (pore size 3.0 μm) at a density of 1.2×10^5 cells/cm^2. Cells were grown at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity in DMEM supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 50 IU/ml penicillin and 50 mg/ml streptomycin. The medium was changed every other day for 21–22 days. The integrity of each batch of cells was tested by measuring the transepithelial electrical resistance (TEER) using a voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL, USA) before and after the experiments. Before the experiments, cells were equilibrated with HBSS for 20 min at 37 °C, and the TEER was determined. The TEER value, corrected for the blank filter resistance, was in the range of 800–950 Ω cm^2. Only confluent monolayers were used for the transepithelial transport studies. Transport was determined in both apical-to-basolateral (A → B) and basolateral-to-apical (B → A) directions. The transport medium (TM) was HBSS with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and was placed in the donor and receiver compartments. Naproxen, G0 PAMAM dendrimer and conjugates (each 100 μM) were placed in the donor compartment, and cells were incubated in a humidified atmosphere at 37 °C. TEER was measured every 30 min during the experiment, samples (50 μl) were removed from the receiver compartment at time zero and after 60, 120, and 180 min, and from the donor compartment after 180 min. In order to calculate the percentage of naproxen released from the conjugate during transport studies, an additional sample was taken from the receiver after 180 min and TFA was added to release all conjugated naproxen. The samples were analyzed by HPLC using phenanthrene as an internal standard. The solvent system used was methanol:aq H_3PO_3 (0.05%, w/v) (80:20), the flow rate was 1 ml/min and UV detection was at λ = 230 nm.

3. Results and discussion

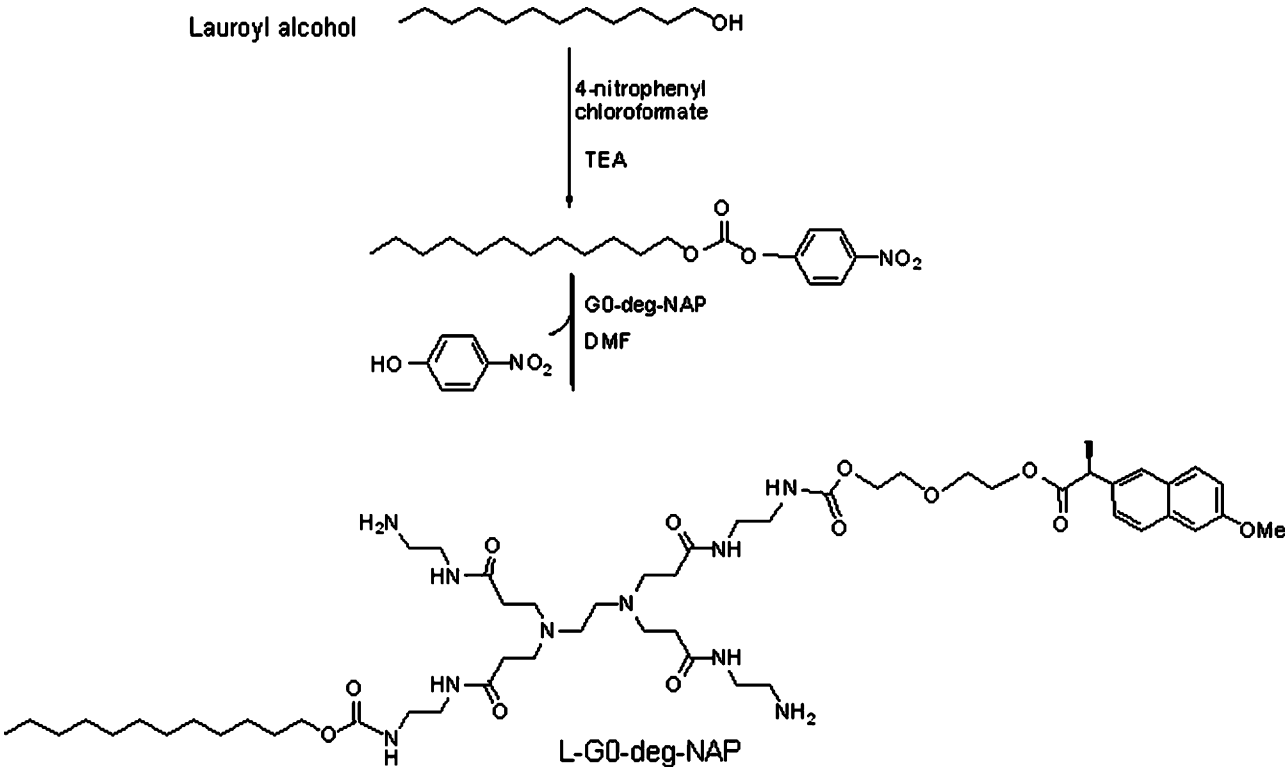
3.1. Synthesis and characterization of L-G0-deg-NAP

Lauroyl alcohol was attached to a primary amine of G0-deg-NAP through a carbamate bond (Scheme 1). Lauroyl alcohol was initially reacted with 4-nitrophenyl chloroformate to obtain lauroyl (4-nitrophenyl carbonate) which was characterized by ^{1}H NMR (Fig. 2) and ^{13}C NMR spectroscopy. G0-deg-NAP and lauroyl (4-nitrophenyl carbonate) were reacted in an equimolar ratio to form a carbamate bond between the lauroyl chain and the dendrimer. A pure sample, with one lauroyl chain attached to the conjugate (L-G0-deg-NAP), was obtained after several purification steps (size exclusion chromatography and solubilization in water). Conjugates with two or three lauroyl chains were insoluble in water and were not studied further. The relative intensities of the peaks in the ^{1}H NMR spectrum originating from the lauroyl chain compared to those of G0-deg-NAP confirmed the 1:1 ratio of lauroyl-dendrimer (Fig. 2). In the ^{13}C NMR spectrum of L-G0-deg-NAP, the appearance of a peak at 157.8 ppm confirmed the formation of a carbamate bond between the lauroyl chain and the amine surface group. It should be noted that the conjugate may be a mixture of the "*trans*" and "*cis*" compounds, with the naproxen and the lauroyl chain being on opposite or the same dendrimer arms (Fig. 1). The appearance of a single peak (Rt = 10.6) in the RP-HPLC chromatogram (Fig. 3) confirmed the purity of L-G0-deg-NAP.

3.2. Enzymatic stability

As previously reported, G0 PAMAM dendrimer conjugates (G0-NAP, G0-lact-NAP and G0-deg-NAP) were found to be stable under neutral and acidic conditions, but varied in stability in the presence of 80% human plasma (Najlah et al., 2006). Stability under neutral and acidic conditions is required to enable the prodrug to pass through the gastrointestinal tract intact or with minimal degradation. The stability of naproxen prodrugs in enzymatic rich media (80% human plasma and 50% rat liver homogenate) varied according to the medium, and the linker between the dendrimer. The direct linkage of naproxen to the G0 dendrimer (G0-NAP) resulted in a very stable amide prodrug; no traces of naproxen could be detected in either plasma or liver homogenate (data not shown). These results confirm previous reports that amide bonds are poor substrates for hydrolyzing enzymes (Franssen et al., 1992).

As shown in Fig. 4, the ester conjugate G0-lact-NAP was slowly hydrolyzed in 80% human plasma, with 20% release of naproxen after 16 h. There was no significant difference in drug release between G0-lact-NAP and NAP-lact. However, in 50% rat liver homogenate, both esters hydrolyzed with pseudo-first-order kinetics, with NAP-lact ($t_{1/2}$ = 44.0 min) reacting faster than G0-lact-NAP ($t_{1/2}$ = 180.1 min), indicating that attaching the lactic acid linker to PAMAM dendrimer decreases the rate of hydrolysis. The rate of degradation of the lactic ester prodrugs increases with increase of the esterase activity of the medium (liver > plasma). The stability of G0-lact-NAP in plasma suggests that L-lactic acid may be a suitable linker for prodrugs



Scheme 1. Synthesis of L-G0-deg-NAP (shown as "*trans*" isomer).

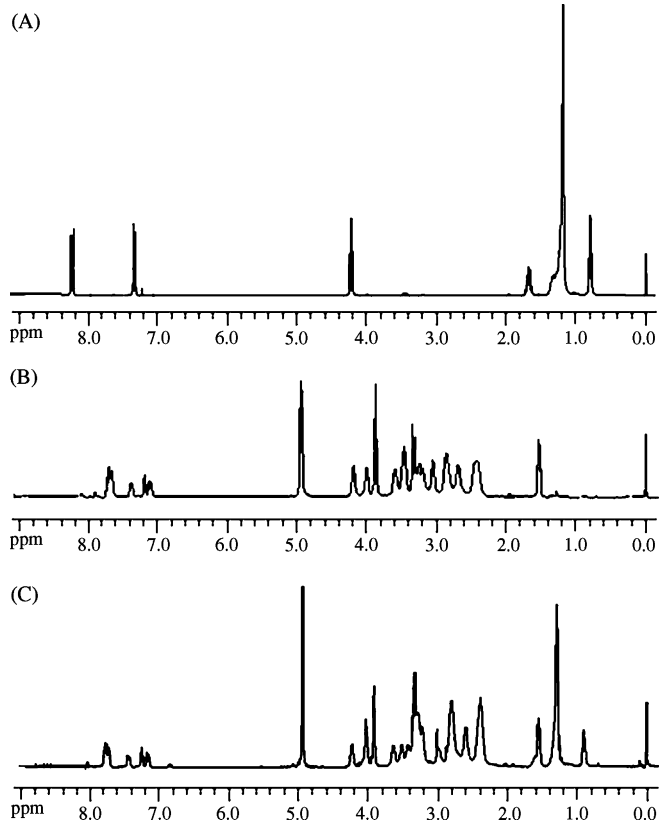


Fig. 2. ^{1}H NMR spectra (300 MHz) of (A) lauroyl (4-nitrophenyl carbonate) ($CDCl_3$), (B) G0-deg-NAP (d_4-MeOD), and (C) L-G0-deg-NAP (d_4-MeOD).

intended for use in controlled release delivery or for drug targeting applications.

G0-deg-NAP hydrolyzed following pseudo-first-order kinetics in both 80% human plasma ($t_{1/2}$ = 51.0 min) (Najlah et al., 2006) and 50% rat liver homogenates ($t_{1/2}$ = 4.7 min) (Table 1). The degradation of G0-deg-NAP was significantly faster than G0-lact-NAP in both human plasma and rat liver. This suggests that the diethylene glycol linker produces esters that are more susceptible to enzymatic hydrolysis than lactate esters because of the increased length and lower steric hindrance of this primary alcohol linker compared to lactic acid. In addition, its oxyethylene structure should produce prodrugs with good water solubility and rapid enzymatic hydrolysis (Bonina et al., 2001, 2002). In contrast to the lactate linker, attaching the diethy-

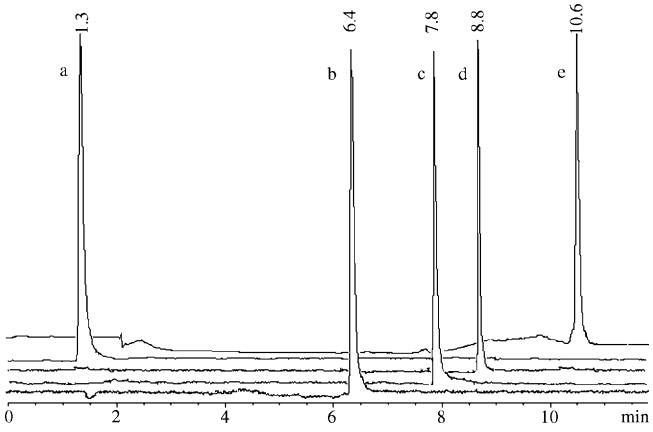


Fig. 3. RP-HPLC Chromatograms of (a) PAMAM G0 dendrimer, (b) G0-deg-NAP, (c) G0-lact-NAP, (d) G0-NAP, and (e) L-G0-deg-NAP.

Table 1
Enzymatic hydrolyses ($t_{1/2}$) of naproxen esters in 80% human plasma and 50% rat liver homogenate

$t_{1/2}$	G0-lact-NAP	NAP-lact	NAP-Me	G0-deg-NAP	NAP-deg
50% rat liver homogenate	180 min	44.0 min	10.3 min	4.7 min	3.3 min
80% human plasma	More than 24 h	More than 24 h	316 min	51.0 min	38.6 min

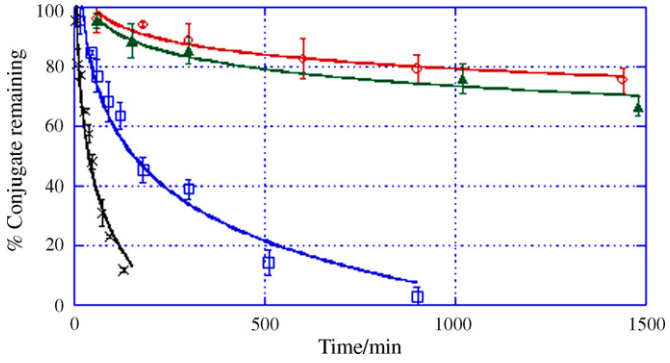


Fig. 4. Stability of G0-lact-NAP (◯) and NAP-lact (▲) esters in 80% human plasma, and G0-lact-NAP (□) and NAP-lact (×) in 50% rat liver homogenate (mean ± S.D.).

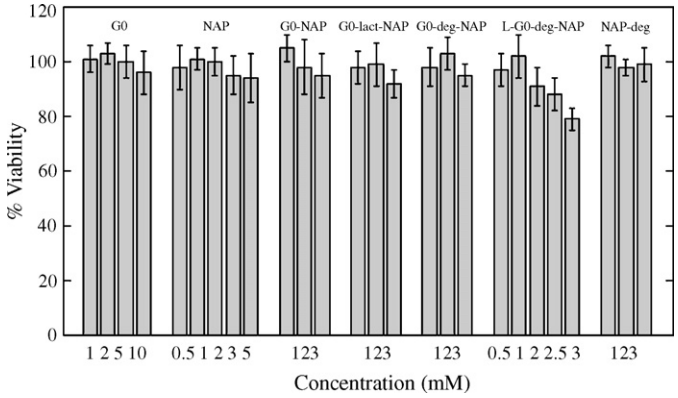


Fig. 5. The effect of G0 PAMAM dendrimer, naproxen and conjugates on the viability of Caco-2 cells (mean ± S.D.).

lene glycol linker to G0 PAMAM dendrimers had no significant impact on the rate of degradation of the drug-linker ester NAP-deg indicating that the diethylene glycol linker gives the required space between the drug and the carrier for esterases to attack the ester bond. Both G0-deg-NAP and NAP-deg showed significantly faster hydrolysis and release of naproxen, compared with the methyl ester of naproxen (NAP-Me, 80% human plasma ($t_{1/2}$ = 316 min) and 50% rat liver homogenate ($t_{1/2}$ = 10.3 min)). The results indicate that dendrimer prodrugs with diethylene glycol linkers have the ability to release the parent drug in plasma once absorbed.

3.3. Cytotoxicity of G0 PAMAM dendrimer–naproxen conjugates

The influence of PAMAM dendrimers on the viability of Caco-2 cells is reported using the MTT assay. MTT is a tetrazolium salt oxidized by mitochondrial dehydrogenase in living cells to produce a dark blue formazan product (Mosmann, 2003). Damaged or dead cells show reduced or no dehydrogenase activity. As shown in Fig. 5, G0 PAMAM dendrimer was not toxic to Caco-2 cells at concentrations of up to 10.0 mM with an incubation time of 180 min. This result is in agreement with previous observations based on the lactate dehydrogenase (LDH) assay (El-Sayed et al., 2002). Attaching naproxen directly or via a linker to the G0 PAMAM dendrimer had no influence on the viability of Caco-2 cells at concentrations up to 3.0 mM for an incubation time of up to 180 min. However, a decrease of approximately 20% in viability was noted with the lauroyl conjugate L-G0-deg-NAP at a concentration of 3.0 mM.

3.4. Transport of naproxen, G0 PAMAM dendrimer and conjugates across Caco-2 monolayers

The transport of naproxen and conjugates across Caco-2 monolayers was investigated in both A → B and B → A directions at non-toxic concentrations (as determined by the MTT assay). G0-NAP and G0-lact-NAP permeated across Caco-2 monolayers without any degradation (no traces of free naproxen could be detected in the receiver compartment). However, 8% of the permeated G0-deg-NAP was hydrolyzed to naproxen, which was detected in the receiver compartment. Therefore, in order to compare transport data for the range of conjugates, permeability was expressed as the percentage of equivalent naproxen passed across Caco-2 monolayers after 180 min (Fig. 6) rather than P_{app}.

The permeability of G0-NAP and G0-lact-NAP across Caco-2 monolayers, especially in the A → B direction, was significantly higher than that of naproxen itself and reflected an obvious pro-moiety dependency. No significant difference can be observed between the permeability of the direct amide linked

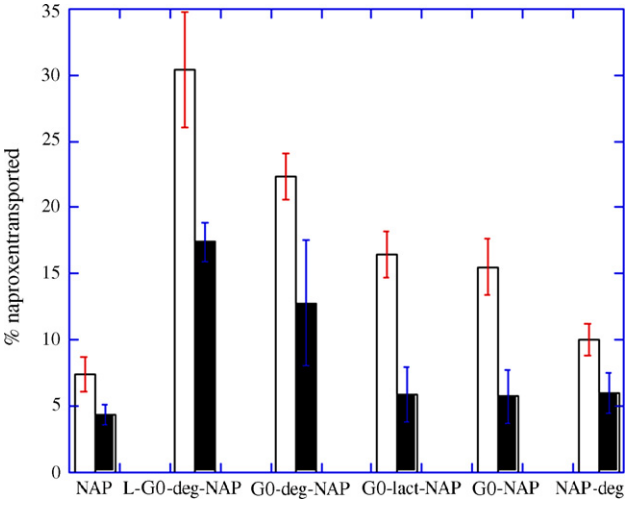


Fig. 6. The A-B (□) and B-A (■) transport of naproxen across Caco-2 cell monolayers for naproxen, and conjugates (equivalent naproxen) after 3 h of incubation (mean ± S.D.).

conjugate (G0-NAP) and the lactic ester prodrug (G0-lact-NAP). This suggests that using lactate as a linker had no impact on the permeability of the resulting conjugate. In contrast, using the diethylene glycol linker in G0-deg-NAP resulted in an increase in permeability compared with G0-NAP. However, a comparison between the NAP-deg and NAP transport results confirms that most of the enhanced permeability of the conjugate arises from the attachment of the drug to the G0 PAMAM dendrimer.

The high permeability of these conjugates may be attributed to the positively charged amine groups of the G0 PAMAM dendrimers. Knipp et al. (1997) reported that because of the favorable electrostatic interaction with the negatively charged epithelial surfaces, positively charged molecules permeate at a higher rate across Caco-2 monolayers compared to neutral or anionic molecules. Similarly, Jevprasesphant et al. have found that the permeability of positively charged dendrimers (G2, G3, and G4) was higher than that of anionic dendrimers (G2.5 and G3.5) (Jevprasesphant et al., 2003a). However, in this study, the G0 PAMAM dendrimer was used to minimize the impact of cytotoxicity exhibited by higher generations. In addition, the A → B permeability of G0 PAMAM dendrimers was found to be several fold higher than the A → B permeability of higher generations (G3 and G4) (El-Sayed et al., 2002).

G0-deg-NAP showed high chemical stability at pHs 1.2, 7.4, and 8.5, whereas it showed rapid release of naproxen in plasma (Najlah et al., 2006). In addition, G0-deg-NAP exhibited high permeability across Caco-2 monolayers, demonstrating the potential of dendrimers as carriers for low solubility drugs such as naproxen. To enhance the permeability further, one lauroyl chain was attached to the surface group (amine) of G0-deg-NAP to give the conjugate (L-G0-deg-NAP) (Scheme 1). It has been reported that conjugation of cationic G3 PAMAM dendrimer with six lauroyl molecules decreased its cytotoxicity and increased its permeation through Caco-2 cell monolayers (Jevprasesphant et al., 2003a).

Here, lauroyl alcohol was attached through a carbamate bond. As expected, L-G0-deg-NAP showed the highest permeability in both directions across Caco-2 cell monolayers of all the G0 conjugates evaluated. The A → B permeability of L-G0-deg-NAP was over four-fold higher than that of naproxen. However, attaching the lauroyl chain to the G0-deg-NAP increased its cytotoxicity at high concentrations (higher than 2.0 mM) (Fig. 5). The difference in cytotoxicity profile between surface modified G0 and G3 PAMAM dendrimers (Jevprasesphant et al., 2003a) may be due to the fact that a single lauroyl chain represents a much higher proportion of the molecular weight of a surface modified G0 than a G3 PAMAM dendrimer. It has been shown that significantly increasing the number of lauroyl chains (from 6 to 9) on a G3 dendrimer increases cytotoxicity (Jevprasesphant et al., 2003a).

Measurements of TEER values following apical incubation of NAP, NAP-deg, and G0-naproxen conjugates (each100 μM) showed that NAP and NAP-deg had no significant impact on TEER values compared to the control (HBSS with 25 mM HEPES) (Fig. 7), However, TEER values decreased by approximately 30% when exposed to G0-NAP, G0-lact-NAP, G0-deg-NAP or L-G0-deg-NAP. This suggests that the enhancement of permeability may be partly due to the modulation of the tight junction. However, previous work has demonstrated that transepithelial transport of dendrimer conjugates involves both paracellular and transcellular pathways (Najlah and D'Emanuele, 2006).

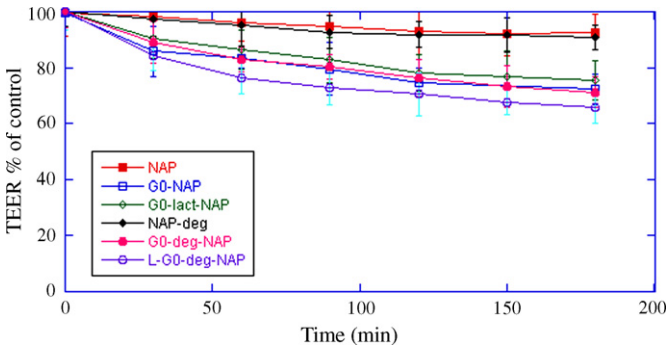


Fig. 7. Effect of naproxen and naproxen prodrugs (all at 100 μM) on the TEER of Caco-2 cell monlayers after apical incubation (mean ± S.D.).

4. Conclusions

The use of G0 PAMAM dendrimers as drug carriers was investigated *in vitro*. Direct amide linkage of naproxen to the G0 dendrimer produced prodrugs of high stability in plasma and liver homogenate. The use of the lactate ester linker gave prodrugs of high stability in plasma with slow hydrolysis in liver homogenate; such conjugates may have potential in controlled release systems or as prodrugs for drug targeting. In contrast, using diethylene glycol as a linker yielded an ester conjugate that showed high chemical stability, but readily released drug in plasma and liver homogenate. Cytotoxicity studies indicated non-toxic effects of G0 dendrimer and conjugates on Caco-2 monolayers. Conjugation of naproxen to G0 PAMAM dendrimer appreciably increased its permeability in both directions. A more pronounced increase of naproxen transport was observed when a lauroyl chain was attached to the surface of G0 PAMAM dendrimers. Our results suggest that G0 PAMAM dendrimers demonstrate potential as nanocarriers for the enhancement of oral bioavailability.

Acknowledgements

Thank you to Dr. Jeff Penny for Caco-2 facilities and Al-Baath University (Syria) for financial support.

References

Aoi, K., Itoh, K., Okada, M., 1995. Globular carbohydrate macromolecule "sugar balls". 1. Synthesis of novel sugar-persubstituted poly (amido amine) dendrimers. Macromolecules 28, 5391–5393.

Bonina, F., Puglia, C., Santagati, N.A., Saija, A., Tomaino, A., Tita, B., 2002. Oligoethylene ester derivatives of ketoprofen, naproxen and diclofenac as oral prodrugs: a pharmacological evaluation. Pharmazie 57, 552–555.

Bonina, F.P., Puglia, C., Barbuzzi, T., de Caprariis, P., Palagiano, F., Rimoli, M.G., Saija, A., 2001. In vitro and in vivo evaluation of polyoxyethylene esters as dermal prodrugs of ketoprofen, naproxen and diclofenac. Eur. J. Pharm. Sci. 14, 123–134.

D'Emanuele, A., Attwood, D., 2005. Dendrimer–drug interactions. Adv. Drug Del. Rev. 15, 2147–2162.

D'Emanuele, A., Attwood, D., Abu-Rmaileh, R., 2003. Dendrimers. Encyclopedia Pharmaceut. Technol., 1–21.

D'Emanuele, A., Jevprasesphant, R., Penny, J., Attwood, D., 2004. The use of a dendrimer-propranolol prodrug to bypass efflux transporters and enhance oral bioavailability. J. Control. Rel. 95, 447–453.

El-Sayed, M., Ginski, M., Rhodes, C., Ghandehari, H., 2002. Transepithelial transport of poly(amidoamine) dendrimers across caco-2 cell monolayers. J. Control. Rel. 81, 355–365.

El-Sayed, M., Rhodes, C.A., Ginski, M., Ghandehari, H., 2003. Transport mechanism(s) of poly (amidoamine) dendrimers across caco-2 cell monolayers. Int. J. Pharm. 265, 151–157.

Florence, A.T., Sakthivel, T., Toth, I., 2000. Oral uptake and translocation of a polylysine dendrimer with a lipid surface. J. Control. Rel. 65, 253–259.

Franssen, E.J.F., Koiter, J., Kuipers, C.A.M., Bruins, A.P., Moolenaar, F., Zeeuw, D., Kruizinga, W.H., Kellogg, R.M., Meijer, D.K.F., 1992. Low molecular weight proteins as carriers for renal drug targeting. Preparation of drug-protein conjugates and drug-spacer derivatives and their catabolism in renal cortex homogenates and lysosomal lysates. J. Med. Chem. 35, 1246–1259.

Fréchet, J.M.J., 1994. Functional polymers and dendrimers: Reactivity, molecular architecture and interfacial energy. Science 263, 1710–1715.

Jevprasesphant, R., Penny, J., Attwood, D., McKeown, N.B., D'Emanuele, A., 2003a. Engineering of dendrimer surfaces to enhance transepithelial transport and reduce cytotoxicity. Pharm. Res. 20, 1543–1550.

Jevprasesphant, R., Penny, J., Jalal, R., Attwood, D., McKeown, N.B., D'Emanuele, A., 2003b. The influence of surface modification on the cytotoxicity of PAMAM dendrimers. Int. J. Pharm. 252, 263–266.

Knipp, G.T., Ho, N.F.H., Barsuhn, C.L., Borchardt, R.R., 1997. Paracellular diffusion in caco-2 cell monolayers: effect of perturbation on the transport of hydrophilic compounds that vary in charge and size. J. Pharm. Sci. 86, 1105–1110.

Milhem, O.M., Myles, C., McKeown, N.B., Attwood, D., D'Emanuele, A., 2000. Polyamidoamine Starburst® dendrimers as solubility enhancers. Int. J. Pharm. 197, 239–241.

Mosmann, T., 2003. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immun. Meth. 65, 55–63.

Najlah, M., D'Emanuele, A., 2006. Crossing cellular barriers using dendrimer nanotechnologies. Curr. Opin. Pharmacol. 6, 522–527.

Najlah, M., Freeman, S., Attwood, D., D'Emanuele, A., 2006. Synthesis, characterization and stability of dendrimer prodrugs. Int. J. Pharm. 308, 175–182.

Roberts, J., Adams, Y.E., Tomalia, D., Mercer-Smith, J.A., Lavallee, D.K., 1990. Using Starburst® dendrimers as linker molecules to radiolabel antibodies. Bioconjug. Chem. 1, 305–308.

Tomalia, D.A., Baker, H., Dewald, J., Hall, M., Kallos, G., Martin, S., Roeck, J., Ryder, J., Smith, P., 1985. A new class of polymers: starburst-dendritic macromolecules. Polym. J. (Tokyo, Japan) 17, 117–132.

Wiwattanapatapee, R., Carreno-Gomez, B., Malik, N., Duncan, R., 2000. Anionic PAMAM dendrimers rapidly cross adult rat intestine in vitro: A potential oral delivery system? Pharm. Res. 17, 991–998.

Wiwattanapatapee, R., Lomlim, L., Saramunee, K., 2003. Dendrimers conjugates for colonic delivery of 5-aminosalicylic acid. J. Control. Rel. 88, 1–9.

Zhuo, R.X., Du, B., Lu, Z.R., 1999. In vitro release of 5-fluorouacil with cyclic core dendritic polymer. J. Control. Rel. 57, 249–257.