Direct *In Vivo* Evidence on the Mechanism by which Nanoparticles Facilitate the Absorption of a Water Insoluble, P-gp Substrate

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Abstract

Here we examine the mechanisms by which nanoparticles enable the oral absorption of water-insoluble, P-glycoprotein efflux pump (P-gp) substrates, without recourse to P-gp inhibitors. Both 200 nm paclitaxel *N*-(2-phenoxyacetyl)-*6*-*O*-glycolchitosan (GCPh) nanoparticles (GCPh-PTX) and a simulated Taxol formulation, facilitate drug dissolution in biorelevant media, unlike paclitaxel nanocrystals. Verapamil (40 mg kg^{-1}) increased the oral absorption from low dose Taxol (6 or 10 mg kg^{-1}) by 100%, whereas the oral absorption from high dose Taxol (20 mg kg^{-1}) or low dose GCPh-PTX (6 or 10 mg kg^{-1}) was largely unchanged by verapamil. There was virtually no absorption from control paclitaxel nanocrystals (20 mg kg^{-1}). Imaging of ex-vivo rat ileum samples showed that fluorescently labelled GCPh nanoparticles are mucoadhesive and are taken up by ileum epithelial cells. GCPh nanoparticles were also found to open Caco-2 cell tight junctions. In conclusion, mucoadhesive, drug solubilising GCPh nanoparticles enable the oral absorption of paclitaxel via the saturation of the P-gp pump (by high local drug concentrations) and by particle uptake and tight junction opening mechanisms.

List of abbreviations

ATP – Adenosine triphosphate

BCS – Biopharmaceutics classification system

FTIR – Fourier transform infrared spectroscopy

GPC-MALLS – Gel permeation chromatography – Multi-angle laser light scattering

GCPh – *N*-(2-phenoxyacetamide)-*6*-*O*-glycolchitosan

GCPQ – *N*-palmitoyl-*N*-monomethyl-*N,N*-dimethyl-*N,N,N*-trimethyl-6-O-glycolchitosan

MWCO – Molecular weight cut-off

NMR – Nuclear magnetic resonance

P-gp – P-Glycoprotein

TMC – N,N,N-trimethylchitosan

Introduction

The oral dosage form is the preferred dosage form for most conditions, due to the issues of patient convenience and relative cost. When formulating a drug for the oral route, oral absorption will only be achieved if there is sufficient drug dissolution within the gastrointestinal tract and there is sufficient permeation of the drug molecules through the gut epithelia (Amidon et al., 1995). The Biopharmaceutical Classification System (BCS) was generated based on this observation with drugs classified as: BCS Class I drugs (water soluble and gut permeable), BCS Class II drugs (water insoluble and gut permeable), BCS Class III drugs (water soluble and gut impermeable) and BCS Class IV drugs (water insoluble and gut impermeable) (Amidon et al., 1995). BCS Class IV drugs are considered the most challenging to prepare as oral immediate release solid dosage forms and only 10% of the drugs on the World Health Organisation Essential Drugs list are classed as BCS Class IV drugs, compared to over 25% of these drugs that are classed as BCS Class II drugs and 30% of these drugs that are classed as BCS Class III drugs (Dahan et al., 2009). One reason why a drug may fall into the BCS Class IV category could be because it is a substrate for the gastrointestinal P-glycoprotein efflux pump.

The P-glycoprotein (P-gp) multidrug resistance protein 1 efflux pump is an ATP dependent membrane transport protein which transports drugs across the cell membrane and out of the cell (Sharom, 1997). The P-gp efflux pump is present in a wide variety of tissues including the gut epithelium, liver, testis, brain endothelial cells, kidney, adrenal glands and the pregnant uterus (Tanigawara, 2000). The main role of this membrane protein is to limit the accumulation of exogenous compounds in these tissues by actively excreting them in the urine, bile, gastrointestinal lumen and plasma (Tanigawara, 2000). The activity of the P-gp efflux pump, within the gastrointestinal epithelium limits the absorption of drug molecules such as paclitaxel (Sparreboom et al., 1997).

The most obvious method of enabling the absorption of a water insoluble P-gp substrate has been to use solubilisers along with P-gp inhibitors such as cyclosporine A (Kruijtzer et al., 2002), HM30181 (Lee et al., 2014) or GF120918 (Malingre et al., 2001); all of which have been used clinically to enable the oral administration of paclitaxel. A number of preclinical studies abound in which various solubilisers have been examined without the inclusion of an obvious P-gp inhibitor such as the use of solubilising micelles alone (Ho et al., 2008; Yang et al., 2015; Yao et al., 2011), or the use of oil in water emulsions in the absence of P-gp inhibitors (Tiwari and Amiji, 2006). There are also reports on the use of lipid nanoparticles, such as the lipid nanoparticles used clinically in the experimental formulation DHP107 (Hong et al., 2013) and in the work of Hong and others, an exposure ceiling was identified where additional dose escalation did not result in increases in plasma drug exposure (Hong et al., 2013). It is not clear whether this exposure ceiling points to the efflux activity of the P-gp pump, limiting absorption.

Despite all of these studies, the key driver of absorption (drug solubilisation, P-gp efflux inhibition or both) of a water insoluble P-gp substrate is not clear. The current work is focused on uncovering the mechanisms underpinning the oral absorption enhancement of water insoluble P-gp drugs.

Our laboratory is engaged in developing polymeric nanoparticle delivery systems and there are a number of reports on the use of polymer nanoparticles to increase the oral absorption of a water insoluble P-gp substrate such as paclitaxel (Calleja et al., 2015; Li et al., 2010; Lv et al., 2011; Wang et al., 2014; Zabaleta et al., 2012; Zhao and Feng, 2010). However while some of these nanoparticles have been reported to inhibit the P-gp efflux pump (Mo et al., 2011), there is little direct evidence from *in vivo* experiments to support a mechanism for bioavailability enhancement.

Our aim was to *examine the mechanism* by which a chitosan based nanoparticle [prepared from N-(2-phenoxyacetamide)-6-O-glycolchitosan (GCPh)], enabled the oral bioavailability of a water insoluble, P-gp substrate, with paclitaxel serving as the model drug. Our surprise finding is that the use of active P-gp inhibitors may not actually be necessary to facilitate the oral absorption of a water insoluble P-gp substrate.

Materials and Methods

Materials

All reagents and chemicals were obtained from Sigma Aldrich Chemical Co. (Poole, UK). Solvents and acids were obtained from Fisher Scientific (Loughborough, U.K.). Visking seamless cellulose dialysis membranes were purchased from Medicell International Ltd. (London, U.K.) Deuterium oxide and methanol-d6 were obtained from Goss Scientific Instruments (Cheshire, U.K.). Paclitaxel was bought from L.C laboratories (Massachusetts, USA). All reagents and chemicals were used without further purification and were ≥98% pure.

Synthesis of *N*-palmitoyl-*N*-monomethyl-*N,N*-dimethyl-*N,N,N*-trimethyl-*6-O*-glycolchitosan (GCPQ)

The chemical synthesis of GCPQ was carried as reported earlier (Siew et al., 2012). Briefly, Glycol chitosan (GC, 2 g) was dissolved in hydrochloric acid (4 M, 150 mL) and was placed in a water bath at 50 °C with shaking (125 rpm). The reaction was stopped after 48 h and the reaction solution was exhaustively dialyzed against distilled water [molecular weight cut off (MWCO) = 3.5 kDa]. The dialysate was subsequently freeze-dried to obtain low molecular weight glycol chitosan (GC10).

To palmitoylate the GC10 (500mg), sodium bicarbonate (376 mg) was added and the mixture was dissolved in a mixture of absolute ethanol (24 mL) and water (76 mL). To this glycol chitosan solution, a solution of palmitic acid N-hydroxysuccinimide (792 mg) dissolved in absolute ethanol (150 mL) was added drop wise. The mixture was then stirred for 72 h and the product was isolated by evaporating off most of the ethanol and extracting the remaining aqueous phase with diethyl ether (3 × 100 mL). The

aqueous mixture of the polymer was exhaustively dialysed against water (MWCO 12-14 kDa) and the resultant product was freeze-dried to get PGC 10. PGC 10 (600 mg) was dispersed in *N*-methyl-2-pyrrolidone (50 mL) for 2 h at room temperature. Sodium hydroxide (80 mg), methyl iodide (2.0 g) and sodium iodide (90 mg) were added and stirred under a stream of nitrogen at 36 °C for 3 h. The product was recovered by precipitation with diethyl ether, and washed thrice with copious amounts of diethyl ether followed by copious amounts of absolute ethanol to give a brown hygroscopic solid. The solid was dissolved in water (100 mL) to give a yellow viscous solution. The resultant aqueous solution was exhaustively dialysed against water (MWCO 7 kDa). The quaternary ammonium iodide salt was then passed through a column (Amberlite IRA-96 Cl^-, 1 × 6 cm) packed with one volume of the resin (30 mL) and subsequently washed with hydrochloric acid (1 M, 90 mL) followed by distilled water until a neutral pH was obtained. The clear eluate from the column was freeze-dried to give a transparent fibrous solid (GCPQ). To deprotonate the primary amines of GCPQ, the polymer (100 mg) was dispersed in water (10 mL) and dialysed against a salt solution (5 L) containing sodium chloride (0.1 M) and sodium bicarbonate (0.01 M) for 4.5 h with three changes of the salt solution. The GCPQ suspension was then exhaustively dialysed against water (5 L) for 24 h with six changes of water and the deprotonated GCPQ was collected by freeze-drying.

Yield = 380 mg; mole % palmitoylation = 20 mole %; mole % quaternization = 9 mole %; M_w = 10,830 Da; M_n = 8710 Da; M_w/M_n = 1.24.

^{1}H NMR: $\delta_{0.90}$ = [t, C***H*****$_3$**-$(CH_2)_{14}$−CO−], $\delta_{1.30}$ = [m, CH_3−(C***H*****$_2$**$)_{12}$−CH_2−CH_2−CO−], $\delta_{1.65}$ = [m, CH_3−$(CH_2)_{12}$−C***H*****$_2$**−CH_2−CO−], $\delta_{2.05}$ = [s, -CH-NH−CO-C***H*****$_3$** - glycol chitosan], $\delta_{2.20-2.40}$ = [b, CH_3−$(CH_2)_{12}$−CH_2−C***H*****$_2$**−CO−], $\delta_{2.7-3.2}$ = [b, −CH−NH−C***H*****$_3$** and −CH−N(C***H*****$_3$**$)_2$ – glycol chitosan], $\delta_{3.30}$ = solvent, $\delta_{3.45}$ = [s −CH−N(C***H*****$_3$**$)_3$ – glycol

chitosan], $\delta_{3.50-4.40}$ = (−C***H***−O and C***H₂***-O - glycol chitosan), $\delta_{4.50-5.00}$ = (water, O−C***H***−O anomeric proton). The level of palmitoylation was calculated by comparing the ratio of palmitoyl methyl protons ($\delta_{0.89}$) to sugar methine/ methylene protons ($\delta_{3.5 - 4.4}$) and the level of quaternization calculated by comparing the ratio of quaternary ammonium methyl protons ($\delta_{3.45}$) to sugar methine/methylene protons ($\delta_{3.5 - 4.4}$).

Synthesis of *N*-(2-phenoxyacetamide)-6-*O*-glycolchitosan (GCPh):

Glycol chitosan (2 g) was dissolved in hydrochloric acid (4 M, 150 mL) and placed in a water bath at 50 °C. The reaction was stopped after 2 h and the reaction solution was exhaustively dialyzed against distilled water (5 L with six changes over 24 h). The dialysate was subsequently freeze-dried to get high molecular weight glycol chitosan (GC35). The hydrophobic pendant group phenoxy acetic acid was covalently linked to GC35 (500 mg) by dissolving GC35 in a mixture of distilled water and absolute ethanol (1:1, 50 mL). Phenoxy acetic acid (125 mg) was dissolved in absolute ethanol (100 mL) and added drop-wise at the rate of 5 mL min^{-1} to the GC35 solution, followed by 4-Methyl Morpholine (100 µL) and 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (1660.32 mg). The reaction was carried out at room temperature for 5 h in an uncapped vessel and the product was isolated by evaporating most of the ethanol and extracting the remaining aqueous phase with diethyl ether (3 x 100 ml). The aqueous mixture of the polymer was exhaustively dialyzed against water (MWCO 12-14 kDa) and the resultant product was freeze-dried to get *N*-(2-Phenoxy acetamide)-6-*O*-glycol chitosan (Figure 1) as a fibrous solid.

Yield = 0.521 g; mole % Phenoxyacetyl groups = 23 mole %; M_w = 49,070 Da; M_n = 44,280 Da; M_w/M_n = 1.108.

^{1}H NMR: $\delta_{2.05}$ = [s, -CH-NH−CO-C***H*****$_3$** - glycol chitosan], $\delta_{2.7-3.2}$ = [b, −C***H***−NH_3^+, glycol chitosan], $\delta_{3.30}$ = solvent, $\delta_{3.50-4.40}$ = (−C***H***−O and -C***H*****$_2$**O – glycol chitosan), $\delta_{4.50-5.00}$ = (solvent, O−C***H***−O anomeric carbon), $\delta_{7.05}$ = [m, aromatic -C***H***-], $\delta_{7.4}$ = [m, aromatic -C***H***-]. The level of Phenoxyl groups was calculated by comparing the ratio of phenyl protons ($\delta_{7.05}$) to sugar methine/methylene protons ($\delta_{3.5 - 4.4}$).

^{13}C NMR: δ_{50} = solvent (methanol), $\delta_{60 - 80}$ = [-***C***H in sugar unit], $\delta_{110 - 130}$ = [aromatic –***C***H- carbons], δ_{158} **=** (−***C***O−NH-C).

FTIR: (ν cm^{-1}) = 3364 (O-H stretch), 2918 and 2851 (C-H alkyl stretch), 1648 (C=O amide stretch), 1599, 1547, 1493 (C-C aromatic stretch).

Relevant NMR and FTIR spectra appear in Supplementary Information Figures S1 – S3.

Synthesis of *N,N,N*-trimethylchitosan (TMC)

Chitosan (2 g) was suspended in *N*-methyl-2-pyrrolidone (80 mL) in a three neck flask and heated in an oil bath at 60 °C under a reflux condenser. To this suspension sodium iodide (4.8 g) and sodium hydroxide (3.84 M, 11 mL) were added and stirred for 10 minutes under a stream of nitrogen. Methyl iodide (11.5 mL) was then added to this reaction mixture and after an hour the product was precipitated by adding excess ethanol (800 mL). The precipitate was isolated by carefully decanting the ethanol and washed twice with diethyl ether (300 mL). The product was dried under vacuum to remove the diethyl ether. Once dry, the product was again suspended in *N*-methyl-2-pyrrolidone (80 mL), stirred for 30 minutes at 60 °C. To this suspension sodium iodide (4.8 g), sodium hydroxide (3.84 M, 11 mL) and methyl iodide (7 mL) were added and stirred under reflux for 30 minutes. A further 5 mL of methyl iodide was added along with sodium hydroxide pellets (0.6 g) and the reaction continued for 90 minutes. The reaction was precipitated with ethanol as before and washed with diethyl ether. The

product was then dried under vacuum and suspended in a mixture of water (40 mL), sodium chloride (10 %, 40 mL), hydrochloric acid (4 M, 1 mL) and methanol (20 mL). This mixture was dialysed against water (5 L, 6 changes) using a 12 kDa MWCO Visking dialysis membrane. Trimethyl Chitosan was then isolated as a fluffy cotton wool like solid after freeze-drying.

Yield: 1.4 g; mole% trimethylation = 42 %. ^{1}H NMR: $\delta_{2.05}$ = [C***H***$_3$−CO−NH−, acetylated chitosan], $\delta_{3.1}$ = [−CH−CH−NH−C***H***$_3$− and −CH−CH−N(C***H***$_3$)$_2$, monomethylamino and dimethylamino chitosan], $\delta_{3.2}$ = [−CH−CH−N(C***H***$_3$)$_3$, trimethylamino chitosan], $\delta_{3.3\text{-}3.4}$ = [C**H**$_3$-O-CH O–methylation of chitosan, $\delta_{3.5\text{-}4.5}$ = [−C***H***(OH)− and −C***H***$_2$−OH, chitosan], $\delta_{4.8}$ = water.

The NMR spectra of TMC is shown in Supplementary Information Figure S4.

NMR Analysis

^{1}H analysis of GCPQ and GCPh were carried out by dissolving (4 mg) in CD_3OD (0.7 mL) for GCPQ and in CD_3OD (0.7 mL) with one drop of CD_3COOD for GCPh. ^{1}H analysis of TMC was carried out by dissolving (4 mg) in D_2O (0.7 mL) and adding a drop of DCl. Analysis was carried out on a Bruker Avance 400 MHz spectrometer (Bruker Instruments, Coventry, UK) at 25 °C.

^{13}C analysis of GCPh was carried out by dissolving (20 mg) in CD_3OD with one drop of CD_3COOD (0.7 mL) for GCPh. Analysis was carried out on a Bruker Avance 400 MHz spectrometer (Bruker Instruments, Coventry, UK) at 25 °C.

Molecular Weight Determination

The molecular weights of GCPQ and GCPh were determined as detailed below. Specific refractive index increments (dη/dc) of the polymers were measured in a

mixture of acetate buffer [CH3COONa (0.3 M), CH3COOH (0.2 M), pH 5]: methanol (35: 65) at 37 °C with an Optilab DSP interferometric refractometer (Wyatt Technology Corporation, USA) set at a wavelength of 690 nm. Filtered (0.2 μm) samples (0.1 to 1 mg mL^{-1}) were manually injected at a flow rate of 0.3 mL min^{-1} and the data obtained was processed using DNDC for Windows version 5.90.03 software (Wyatt Technology Corporation, USA).

The molecular weight of GCPQ was measured using Gel Permeation Chromatography − Multi-Angle Laser Light Scattering (GPC - MALLS). Measurements were performed using a DAWN EOS MALLS detector (λ = 690 nm), Optilab DSP interferometric refractometer (λ = 690 nm) and a Quasi-Elastic Light Scattering (QELS) detector (Wyatt Technology Corporation, U.S.A.). Filtered samples of GCPQ (0.2 μm, 200 μL) were injected into a POLYSEP-GFC-P 4000 column (300 × 7.8 mm, Phenomenex, U.K.) fitted with a POLYSEP-GFC-P guard column (35 × 7.8 mm, Phenomenex, U.K.) using an Agilent 1200 series auto-sampler (Agilent Technologies, USA.) at a loading concentration of 5 mg mL^{-1} with a flow rate of 0.7 mL min^{-1}. The data were analysed using ASTRA for Windows version 4.90.08 software.

CMC measurements:

The heats of demicellization of the polymers were measured using an ITC200 MicroCalorimeter (MicroCal, LLC, Northampton, MA USA). The sample cell was filled with degassed ultrapure water. Concentrated polymer samples, dissolved in ultrapure water, were loaded into a syringe (19.35 µM, 40 µL), and at 120 s intervals, polymer samples (2 µL) were injected into the sample cell and the heat flow was measured as a function of time. The syringe was rotated at 1000 rpm to enable even mixing throughout the experiment. Data analysis was carried out using the MicroCal Origin

version 7.0 Software. Each titration experiment was carried out at room temperature (25 °C).

Preparation of paclitaxel formulations

Paclitaxel nanocrystals

Deprotonated GCPQ10 (10 mg) in the case of paclitaxel fine nanocrystals or the hydrochloride salt of GCPQ10 (10 mg) in the case of paclitaxel large nanocrystals was dispersed in water (1.9 mL) and probe sonicated (MSE Soniprep 150, MSE UK Ltd, London, UK) for 10 minutes at an output that is 50% of the machine's maximum output (10 Amplitude microns). An ethanolic solution of paclitaxel (0.1 mL, 20 mg mL^{-1}) was added to the pre-sonicated polymer dispersion (final ethanol concentration = 5% v/v) while probe sonicating and the mixture was then sonicated on ice for 30 minutes in a similar manner as outlined above. To obtain the fine nanocrystal formulation from the deprotonated GCPQ formulation, the formulation was then filtered through a 0.22 μm filter.

Paclitaxel GCPh nanoparticles

GCPh35 (40 mg) was dispersed in water (1.9 mL) and probe sonicated for 10 minutes. An ethanolic solution of paclitaxel (0.1 mL, 40 mg mL^{-1}) was added to the pre-sonicated polymer dispersion (final ethanol concentration = 5% v/v) while probe sonicating and the mixture was sonicated on an ice bath for 10 minutes at 10 amplitude microns (50% of the machine's maximum capacity).

Simulated Taxol

A simulated Taxol formulation was prepared by dissolving paclitaxel (6 mg) in a mixture of ethanol and Cremophor EL (1:1, v/v, 1 mL). This solution was diluted to 2 mg mL^{-1} with water before administration.

Characterization of paclitaxel formulations:

High performance liquid chromatography (HPLC) was performed using Agilent Technologies 1200 series chromatographic system, which consisted of a vacuum degasser, a quaternary pump, a standard and preparative auto-sampler, a column compartment with a thermostat and a variable wavelength UV detector. The flow rate was set at 1.5 mL min^{-1}. Samples (10 μL) were chromatographed over a reverse phase column (Onyx Monolithic C18 column, 100 x 4.6 mm) fitted with a guard column maintained at 40 °C, and monitored for absorption at 227 nm wavelength. Samples were diluted in mobile phase (acetonitrile: water, 1: 1) and analysed using a standard curve (y = 36.79x - 0.0857, r^2 = 0.998) with a concentration range of 0.1 - 1.0 μg mL^{-1}.

The particle size and particle size distribution were measured by Photon Correlation Spectroscopy (Malvern Zetasizer 3000HSA, Malvern Instruments, UK.) at 25˚C at a wavelength of 633 nm and the data analysed using the Contin method of analysis. Measurements were performed in triplicate.

Transmission electron microscopy was performed using Philips/FEI CM120 Bio Twin (Philips, Netherlands). A drop of the formulation was dried on a copper TEM grid (300 mesh- Fomvar/ carbon coated) and stained with a drop of uranyl acetate (1% w/v, negative staining). Once dried, the samples were imaged and the representative images were photographed and documented.

Dissolution testing of oral formulations

The paclitaxel formulations were diluted with water to a final concentration of 0.01 mg mL^{-1} paclitaxel. The diluted formulation (1.5 mL) was then placed in a dialysis bag (molecular weight cut off = 7000 Da) and the whole bag was placed into 48.5 mL of simulated gastric fluid (SGF, pH 1.2) United States Pharmacopoeia 2010 or simulated

intestinal fluid (SIF, pH 6.8) United States Pharmacopoeia 2010 and shaken at 125 rpm at 37˚C. Samples (0.5 mL) were withdrawn and replaced with fresh PBS (0.5 mL) at specific time points and measured for drug content using RP-HPLC as described above.

Oral absorption studies

The paclitaxel formulations were prepared as described above. Prior to oral administration, all formulations were analysed by RP HPLC to determine the exact drug concentration for dose calculations. Male MF-1 mice weighing 22-35 g were fasted for 12 h prior to dosing and for a further 4 h thereafter. The mice had free access to water throughout the study. Paclitaxel formulations were administered at low (6.66 mg kg^{-1}, 10 mg kg^{-1}) and/ or high doses (20 mg kg^{-1}) by oral gavage either in the form of high dissolving formulations (GCPh paclitaxel nanoparticles or simulated Taxol) or as poorly dissolving formulations (paclitaxel fine or large nanocrystals). Paclitaxel was administered in the absence or presence of the P-gp efflux pump inhibitor verapamil (40 mg kg^{-1}). Blood samples were taken at various time intervals by cardiac puncture. Terminal blood samples were centrifuged for 10 minutes at 1000 g (Microcentaur, MSE UK Ltd, London, UK) and the isolated plasma (100 µL) was then mixed with internal standard solution (4-hydroxybenzoic acid n-hexyl ester 10 µg mL^{-1}, 10 µL) in 50% acetonitrile and ethyl acetate (1 mL). After vortex mixing for 1 minute, the mixture was centrifuged for 15 minutes at 10,000 g (Microcentaur, MSE UK Ltd, London, UK) and then the organic layer (900 µL) was transferred to a clean tube and evaporated until dry. The residue was dissolved in 50%v/v acetonitrile (90 µL) by vortex mixing for 1 minute, and centrifuged for 15 minutes at 10,000 g to obtain the supernatant, which was analysed for paclitaxel content using HPLC. To prepare standard curves, blank plasma samples (100 µL) were spiked with standard solutions of paclitaxel (10 µL, 0.1

– 20 µg mL^{-1}) in 50% v/v acetonitrile in water and extracted in a similar manner to the pharmacokinetics samples.

HPLC was performed using Agilent Technologies 1200 series chromatographic system, as mentioned above. The flow rate was set at 2 mL $min^{-1.}$ Plasma and standard curve extracts (50 µL) in 50%v/v acetonitrile were chromatographed over a reverse phase column (Onyx Monolithic C18 column, 200 x 4.6 mm) fitted with a guard column maintained at 40 °C, and monitored for absorption at 227 nm wavelength. Samples were analysed using a standard curve (y = 36.79x - 0.0857, r^2 = 0.998) over a concentration range of 0.02 - 10.0 µg mL^{-1}.

Ex-vivo confocal laser scanning imaging:

GCPh was labelled with Texas Red (Invitrogen, U.K.) using the protocol supplied by the manufacturer. Briefly GCPh (100 mg) was dissolved in sodium bicarbonate buffer (0.1 M, 10 mL). Texas Red-X succinimidyl ester (5 mg) was dissolved in dimethylsulfoxide (0.1 mL) and was slowly added to the GCPh solution with continuous stirring. The reaction mixture was incubated for 1 h at room temperature, and the reaction was stopped by adding freshly prepared hydroxylamine (1.5M, 0.1 mL) to the mixture. The hydroxylamine containing reaction mixture was incubated for a further 1 h at room temperature, exhaustively dialyzed (5 L with 6 changes over a period of 24 h, MWCO = 12−14 kDa) and purified by Amicon Ultra15 centrifugal filters (Millipore, UK) as indicated below. The GCPh-Texas red conjugate (50 mg) was dissolved in 60 % Methanol (50 mL). The solution was then acidified with HCl (1.5 mL, 4M) and centrifuged at 5000 g for 1 h (Hermle Z 323 K, Hermle Labortechnik, Wehingen, Germany) using Amicon spin filter columns (MWCO = 10 kDa). This step was repeated twice with the retentate and the final retentate containing purified GCPh-TR conjugate

was isolated by freeze-drying. The reaction and purification mixtures were protected from light throughout the whole process. A GPC−MALLS analysis was used to confirm the complete removal of unreacted dye from the labelled polymer (Supplementary Information Figure S5). There was no residual unreacted Texas Red in the GCPh – Texas red sample. GCPh – Texas red (20 mg mL^{-1}, 1 mL, 100 mg kg^{-1}) in distilled water was dosed to male Wistar rats (weight = 200−250 g) by oral gavage and after two hours, animals were euthanized and their small intestines harvested. The small intestines were divided into three sections, the duodenum (up to ~8 cm from the stomach), jejunum (the next ~30 cm) and ileum (the next ~20 cm). The ileum was cut along its long axis, opened flat, rolled along its long axis, embedded in optimal cutting temperature (OCT) compound and frozen in iso-pentane dry ice mixture. This procedure was carried out as quickly as possible (i.e. within 10 min) to avoid tissue deterioration. The ileum samples were sectioned into thin slices (30 μm) using a cryostat (LeicaCM1850, Leica Biosystems, Illinois, USA) set at −25 °C. The slices were placed on poly-L-lysine microscope adhesion slides and fixed with freshly prepared paraformaldehyde (4% w/v) in phosphate buffered saline (PBS, pH = 7.4). The slides were soaked in PBS for 10 min and a drop of Vectashield® Hardset™ mounting medium with DAPI stain (10 µL) was added on to tissue slices and sealed with a cover slip. Slides were imaged using a Zeiss LSM 710 laser scanning confocal microscopy imaging system, equipped with an argon ion laser and HeNe laser (LASOS Laserteknik GmbH, Carl Zeiss, Cambridge, UK) and linked to a Fujitsu Siemens computer with the Zen 2009 version 5.5.0.451 software (Carl Zeiss, Cambridge, UK). The following excitation and emission parameters were used: red fluorescence excitation wavelength = 561 nm, blue fluorescence excitation wavelength = 405 nm.

***In vitro* permeability studies:**

In order to examine the effect of the polymer nanoparticles on the integrity of the intercellular tight junctions, the transepithelial electrical resistance (TEER) and transport properties of a Caco-2 cell monolayer were monitored in the presence of GCPh nanoparticles. Caco-2 cells (passage number 38-42) were seeded on 12-well plates (Corning Costar transwells, Sigma-Aldrich, Poole, UK) with polycarbonate permeable cell culture inserts (12 mm in diameter, area = 1.2 cm^2, pore diameter 0.4 μm) at a seeding density of 64000 cells cm^{-2} . Dubelcco' s modified Eagle' s medium (DMEM, Invitrogen, Paisley, U.K.) supplemented with foetal bovine serum (FBS 10% w/v), nonessential amino acids (1% w/v), L -glutamine (1% w/v), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) were used as the culture medium and added to the basolateral compartments. A cell suspension in the same medium (0.5 mL) was added to the apical compartments, and the cells were incubated at 37 °C in an atmosphere of 95 % relative humidity and 10 % CO_2. The cells were maintained for 21 days to allow a confluent monolayer to develop. The culture media in both the apical and basolateral compartments were changed every other day. TEER measurements were performed and transport studies were conducted 21 days post-seeding, after confirming the integrity of the monolayers.

The TEER of the Caco-2 cell monolayers was measured using a Millicell-ERS meter (Millipore, Bedford, MA, USA). Polymer dispersions (1 mg mL^{-1}) were prepared in Hanks balanced salt solution (HBSS) containing Ca^{2+} and Mg^{2+} supplemented with penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). The culture medium was first aspirated from the Transwells, HBSS was added into both the apical and basolateral compartments, and the cells were incubated for an hour at 37 °C in an atmosphere of

95 % humidity and 10 % CO_2. The TEER of the cells in HBSS was then measured prior to the addition of the polymer dispersions (0.5 mL) in the apical compartments. After 120 min, the polymer dispersions were removed and replaced with fresh HBSS and the TEER was measured again after removal of the polymer dispersions. HBSS solution and trimethyl chitosan (1 mg mL^{-1}) with 42% quaternisation (TMC) solution were used as negative and positive (Kotze et al., 1999) controls respectively.

The transport kinetics of a paracellular transport marker, FITC-dextran 4000 Da (FD-4), were also measured immediately after the polymer dispersion was removed. A solution of FD-4 (1 mg ml^{-1}) was prepared in HBSS and 500 µL of this solution was added to the apical compartments of the transwells. The transwells were incubated at 37 °C, 95 % humidity and 10 % CO_2 for 120 min. Basolateral samples (250 µL) were taken every 30 min and replaced with equal volumes of fresh HBSS, and fluorescence readings (excitation wavelength = 485 nm, emission wavelength = 530 nm) were recorded using a Bio-Tek Synergy™ HT Multi detection micro plate reader (Bio Tek, Swindon, UK). FD-4 concentrations were determined with reference to a calibration curve using standard solutions (125 − 1000 µg mL^{-1}) for the fluorescence emission ($y = 45.076x + 22.514$, $r^2 = 0.9995$), and control samples (generated in the absence of polymer nanoparticles) were also analysed.

Statistical Analysis:

Statistical significance was tested with one-way and two-way analysis of variance (ANOVA) using GraphPad Prism 5 statistical software. For multiple comparisons, ANOVA plus Tukey's post-hoc test was used. In some cases, the statistical differences between two populations were compared using the Student's *t* test (Microsoft excel).

Results

Synthesis and Characterisation of GCPh

The synthesis and characterization of GCPQ has been reported previously (Chooi et al., 2014; Siew et al., 2012), whereas this is the first report for the synthesis and characterization of GCPh (Figure 1). GCPh was successfully synthesized and characterized.

CMC Determination

The CMC of the GCPh polymer was measured using Isothermal Calorimetry (ITC) and was found to be 0.3 ± 0.025 μM (Table 1, Figure 2). ITC is a probe free method used to estimate the CMC and has been previously shown to be the most reliable method for CMC measurements (Chooi et al., 2010). The low CMC value of GCPh is due to the presence of hydrophobic pendant groups, which collectively present a large hydrophobic surface to the aqueous medium. The self-assembly is driven by the entropy gain of the water molecules, once freed from the hydrophobic cavity, on self-assembly of GCPh. The GCPh $T\Delta S_{mic}$ parameter is indeed greater than that measured for GCPQ ($T\Delta S_{mic}$ = + 37 kJ mol^{-1}) (Siew et al., 2012), illustrating the greater influence of entropy on the self-assembly of GCPh, when compared to GCPQ. GCPh forms more stable micelles than GCPQ as illustrated by the lower CMC of GCPh (Table 1) compared to GCPQ (CMC = 19 μM) and indeed the more negative ΔG_{mic} of GCPh (Table 1) compared to GCPQ (ΔG_{mic} = -36.9 kJ mol^{-1}) (Siew et al., 2012). This extremely low CMC value would facilitate the formation of extremely stable self-assemblies in aqueous environments, and these stable self assemblies would enable the formulation to resist disintegration upon dilution in the gastro-intestinal tract.

Preparation of paclitaxel formulation

When paclitaxel was formulated with GCPQ it either formed fine nanocrystals (150 nm in size) or large nanocrystals (700 nm in size) depending on whether paclitaxel was formulated with the GCPQ hydrochloride salt (large nanocrystals) or the deprotonated form of GCPQ and filtered (fine nanocrystals) (Figures 3a and b).

While the GCPQ dispersions presented as 150 or 700 nm nanocrystals, formulation of paclitaxel with GCPh resulted in 200 nm nanoparticles (Figure 3c) and we hypothesise that the presentation of amorphous nanoparticles was facilitated by the π-π stacking interactions between paclitaxel and the phenoxy substituent on GCPh.

Dissolution testing of oral formulations

The dissolution rate data on the paclitaxel formulations in SGF and SIF are presented in Figure 4 and the dissolution rate followed the trend GCPh-PTX > Simulated Taxol > paclitaxel fine nanocrystals > large nanocrystals for both media. Both simulated Taxol and GCPh-PTX are nanoformulations, with simulated Taxol comprising paclitaxel loaded Chremophor EL micelles. In SGF at the two hour time point GCPh-PTX, Simulated Taxol, paclitaxel fine nanocrystals and paclitaxel large nanocrystals had 66%, 48%, 26% and 14% of the total drug dissolved respectively. At the 4 h time point in SIF, GCPh-PTX, Simulated Taxol, paclitaxel fine nanocrystals and paclitaxel large nanocrystals had 77%, 45%, 28% and 13% of the total drug dissolved respectively. It is clear that the non-crystalline, nanoformulations (GCPh-PTX, Simulated Taxol) formulations were faster dissolving formulations when compared to the crystalline formulations, although it is not clear why the dissolution from GCPh-PTX is marginally more efficient than the dissolution rate from simulated Taxol. While a reduction in particle size is known to increase dissolution rate of drug substances as summarised in the Noyes-Whitney equation (Noyes and Whitney, 1897) the GCPh-PTX and simulated Taxol formulations are essentially amorphous, i.e. non-crystalline

and such amorphous materials are known to have faster aqueous dissolution kinetics than crystalline materials due to their intrinsic higher solubility (Hancock and Parks, 2000). Dissolution from GCPh-PTX is extremely fast in both SGF and SIF, i.e. irrespective of the pH of the medium (Figure 4) and we speculate that this is most likely due to the high stability of the GCPh nanoparticles (Table 1) which are not destabilised by changes in pH, thus retaining the paclitaxel in the high surface area colloidal form irrespective of pH.

Oral Absorption of Paclitaxel

The plasma exposure data following the oral administration of paclitaxel formulations are shown in Figure 5. On oral administration, only the non-crystalline formulations (GCPh-PTX and simulated Taxol) were absorbed, with virtually no absorption observed from the crystalline formulations (Figure 5d). We observed that plasma exposure data showed a high level of variability between animals, especially when dosed at 20 mg kg^{-1} with either simulated Taxol or GCPh-PTX (Figure 5d, Table 3), with coefficient of variation levels of 0.3 – 1. The source of the variation is not clear but this variability is illustrated by the different profiles obtained from two different simulated Taxol experiments (Figure 5c and 5d). Other hydrophobic drugs are known to present highly variable absorption profiles such as all-trans-retinoic acid (Adamson et al., 1993) and methotrexate (Kuroda et al., 2001), for example.

Even though the low particle size of the nanocrystals should promote dissolution, dissolution from these nanocrystals is low (Figure 4), and the resultant plasma exposure levels are low (Figure 5d), with $AUC_{0\text{-}4h}$ levels of 67 and 4767 ng h mL^{-1} for the fine paclitaxel nanocrystals and GCPh-PTX formulation respectively, when dosed at 20 mg kg^{-1}. The amorphous nanoparticles, which have a high dissolution rate

(Figure 4), are the formulations from which paclitaxel is absorbed (Figure 5d). These formulations show good *in vitro – in vivo* correlation. On the addition of verapamil, absorption from the crystalline formulations does not change (Supplementary Information Figure S6) and we conclude that a high dissolution rate is a fundamental requirement for the absorption of paclitaxel. There was no difference in the absorption from GCPh-PTX and the simulated Taxol formulation when paclitaxel was administered at the high dose (20 mg kg^{-1}, Figure 5d).

On examination of the influence of verapamil, a P-gp inhibitor (Yusa and Tsuruo, 1989), on the oral absorption of paclitaxel from the formulations, verapamil increases the absorption of paclitaxel by 100% in the case of low dose (6.7 and 10 mg kg^{-1}) simulated Taxol, whereas there was very little influence of verapamil on the absorption of paclitaxel from low dose (6.7 and 10 mg kg^{-1}) GCPh-PTX (Figure 5a and b and Table 3). Only at the 4 h time point do we see a significant difference between absorption from GCPh-PTX in the presence and absence of verapamil. Our interpretation of this data is as follows: at the earlier time points, the high local concentrations of paclitaxel, from the high drug dissolution rate GCPh-PTX nanoparticles, which are mucoadhesive and thus in close proximity to the P-gp efflux pump, inhibit the P-gp efflux pump and it is only when the level of paclitaxel falls at the 4 h time point that we observe the activity of verapamil. The fact that the absorption seen from GCPh-PTX is lower than that seen with simulated Taxol plus verapamil further supports the fact that it is the high *local* concentrations of paclitaxel (due to particle mucoadhesion) that saturate the P-gp pump in the case of GCPh-PTX and the mucoadhesion evidence is discussed below. The high local concentration of GCPh-PTX, not withstanding, the absorption from the low dose (6 and 10 mg kg-1) GCPh-PTX does not exceed the absorption from low dose simulated Taxol in the absence of

verapamil; an indication that different mechanisms are essentially operative, with GCPh-PTX being absorbed via nanoparticle uptake as well as by diffusion of molecular drug across the absorptive cell membrane. It is conceivable that these particle uptake processes are not as efficient as the processes governing absorption of molecular paclitaxel from the simulated Taxol formulation, even in the absence of verapamil.

At the higher dose (20 mg kg^{-1}) there was no influence of verapamil on the absorption of paclitaxel from simulated Taxol (Table 3, Figure 5c) as at this higher dose, the P-gp pump appears to be saturated by paclitaxel released from the simulated Taxol formulation. *In vitro* studies have previously shown that the P-gp pump is an energy driven saturable pump (Jang et al., 2001). However, to our knowledge this is the first time that this saturation of the P-gp pump has been conclusively demonstrated in vivo for a water insoluble P-gp efflux substrate.

Our data point to the fact that the drug dissolution is a more important driver of the absorption of a water insoluble P-gp substrate than specifically inhibiting the activity of the P-gp efflux pump and as such drug developers may focus on drug dissolution to saturate the P-gp pump and thus facilitate drug absorption, without recourse to P-gp inhibitors.

Confocal Laser Scanning Microscopy

In order to probe further the mechanism by which GCPh facilitated gut permeation, GCPh was covalently conjugated to Texas red, a fluorescent dye, orally administered to rats and sections of the ileum imaged for nanoparticle localization (Figure 6). GCPh nanoparticles are mucoadhesive, seen within the villi (Figure 6a) and at the level of the basement membrane (Figure 6b), showing that GCPh nanoparticles reside in close

proximity to the absorptive epithelial cells, are absorbed and most probably absorbed via the enterocytes (Figure 6). The data in Figure 6 show that mucoadhesion would bring the GCPh-PTX nanoparticles in close proximity to the epithelial cells allowing a high local concentration of drug, within the mucus, to prevail. As stated above, this mucoadhesion would explain the lack of response to verapamil as the P-gp efflux pump is already saturated by the high local concentration of the dissolved paclitaxel. A further P-gp independent mechanism of absorption enhancement is linked to the uptake of GCPh-PTX nanoparticles (Figure 6).

***In vitro* Permeability Studies:**

Having established that the GCPh enhances the oral absorption of paclitaxel by being mucoadhesive, promoting dissolution and via the uptake of particles through enterocytes, we then decided to study the effect of the polymer on promoting the paracellular transport across the epithelium. At first, the effect of GCPh on the integrity of the tight intercellular junctions was determined using the Caco-2 cell monolayers. From the results (Figure 7a), It can be seen that GCPh reduces the TEER value, which we hypothesise is due to the opening of tight intercellular junctions. There is a trend towards reversibility with the GCPh TEER values as they increase modestly after removal of the GCPh nanoparticles. However very little (1 – 2%) of the 4 kDa paracellular marker (FD-4) is transported across the monolayer (Figure 7b) after exposure to GCPh nanoparticles, indicating that this route of transport, while present, is unlikely to have a significant effect on oral absorption. It is more likely that the nanoparticles arrived in the villi via the transcellular pathways.

Discussion

Oral formulations are the most common and preferred dosage forms and for oral absorption to take place there must be adequate dissolution of the drug substance, such that the drug is presented to the gastrointestinal mucus and epithelium in the molecular form. Additionally there must then be penetration of the drug molecules through the mucus, through the gastrointestinal absorptive cells – the enterocytes – and delivery of drug into the blood capillaries within the villi for onward transmission to the portal and systemic circulation. Two major barriers to absorption are thus a poor dissolution rate within the gastrointestinal tract and poor permeation across both the gut mucus and the gut epithelium. Strategies to improve the dissolution rate of drug substances, such as the presentation as a nanoformulation (Le et al., 2013; Siew et al., 2012), result in an increase in oral bioavailability as do the exploitation of particulate uptake mechanisms (Serrano et al., 2015) and the use of P-gp efflux pump inhibitors (Kruijtzer et al., 2003; Mo et al., 2011).

While it is clear that nanoparticles improve the oral bioavailability of paclitaxel formulations (Li et al., 2010; Mo et al., 2011) and while there has been *in vitro* evidence of particle uptake provided by some authors (Mo et al., 2011), direct *in vivo* evidence of the predominant mechanisms operating to increase oral bioavailability are not available. Our work has shown that it is first and foremost important to have a formulation which has a high dissolution rate within the gastrointestinal tract (Figures 4 and 5d) as with a high dissolution rate the $AUC_{0\text{-}4h}$ increases over 70 fold as is seen when the paclitaxel fine nanocrystals are compared to GCPh-PTX (Figures 4 and 5d). However what is interesting is that while the simulated Taxol formulation is positively influenced by verapamil at the low dose levels (Figures 5a and b, Table 3), verapamil has virtually no effect on the bioavailability from the GCPh-PTX formulations. We

conclude that the drug available from the simulated Taxol formulation is available in molecular and colloidal form within the lumen of the gastrointestinal tract and thus molecular drug interacts with the P-gp efflux pump in a more dilute form compared to the GCPh-PTX nanoparticles. The mucoadhesive GCPh-PTX nanoparticles will result in a high local drug concentration in the mucus and in close proximity to the gastrointestinal absorptive cells and this local high concentration is what actually saturates the P-gp pump fully, hence the use of verapamil has no additional effect on the P-gp efflux pump. Saturation of the P-gp efflux pump is seen at the higher simulated Taxol dose of 20 mg kg^{-1} (Figure 5c), a further indication that the drug is available in the lumen with simulated Taxol and not in the form of a mucoadhesive drug laden particle from which drug may diffuse out and provide a high local concentration, as in the case of GCPh-PTX. Furthermore the GCPh nanoparticles are taken up by the villi (Figure 6), presumably via the absorptive enterocytes of the villi, although particle uptake by other cells cannot be ruled out. Additional permeation enhancement is provided by the opening of the intercellular tight junctions (Figure 7), but this appears to provide transport of only minor quantities of a paracellular marker (1 – 2%) and so is not believed to make a significant contribution to the oral absorption processes operating *in vivo*. The possibility that high local concentrations of paclitaxel and not GCPh *per se* inhibits the P-gp efflux pump is supported by the fact that structurally similar molecules, *N*-palmitoyl-*N*-monomethyl-*N,N*-dimethyl-*N,N,N*-trimethyl-*6*-*O*-glycolchitosan (GCPQ) and *N*-palmitoyl-6-*O*-glycolchitosan do not inhibit the P-gp efflux pump *in vitro* (Siew et al., 2012).

This is the first direct evidence of particle uptake making a P-gp independent contribution to the oral absorption of a water insoluble, low gastrointestinal permeability drug such as paclitaxel. Others have reported from gut perfusion studies

of a lack of across the gut transport enhancement in the presence of verapamil with *N*-deoxycholicacid-*N,O*-hydroxyethylchitosan micelles (Li et al., 2010) or presented *in vitro* evidence from Caco-2 cells, on the uptake of *N*-octyl-*O*-sulphate chitosan micelles, concluding that an endocytotic uptake of drug loaded particles must be in evidence (Mo et al., 2011). We add to these studies by reporting direct *in vivo* evidence of particle uptake and mucoadhesion as well as direct *in vivo* evidence of a lack of susceptibility to the effects of verapamil on the absorption of paclitaxel from GCPh-PTX nanoparticles (Figure 5). We have combined the mucoadhesion data (Figure 6), high dissolution rate from GCPh-PTX nanoparticles data (Figure 4) and the *in vivo* verapamil non-susceptibility data (Figure 5) to conclude that high local concentrations of released paclitaxel from GCPh-PTX formulations act to saturate the P-gp efflux pump and promote drug absorption (Figure 8). The mechanism we report might also be applicable to other polymer nanoparticles, providing they penetrate the gastrointestinal mucus sufficiently.

In essence we have demonstrated that when faced with a water insoluble P-gp substrate, i.e. a BCS Class IV drug such as paclitaxel, increasing the dissolution rate with the use of amphiphiles and nanoparticle formulations is an approach which yields a significant increase in oral bioavailability and we question the use of P-gp inhibitors as the additional bioavailability enhancement achieved with P-gp inhibitors appears to be modest. As these P-gp inhibitors are usually drugs with pharmacological activity in their own right avoiding P-gp inhibitors seems prudent, when the data supports this avoidance.

Conclusions

Nanoparticles which promote drug dissolution, are mucoadhesive and are taken up by the gastrointestinal tract, significantly enhance the oral absorption of a water insoluble P-gp substrate.

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Figure Captions

Figure 1 Structure of *N*-(2-phenoxyacetamide)-6-*O*-glycolchitosan

Figure 2: Dilution enthalpogram for an aqueous dispersion of GCPh (19.35 μM) in water at 25 °C.

Figure 3: TEM images of Paclitaxel formulations: a) fine nanocrystals; b) large nanocrystals and c) GCPh nanoparticles. Scale bar = 500 nm.

Figure 4: Dissolution of Paclitaxel formulations (mean ± s.d., n = 3, 10 μg mL^{-1}) in a) SGF and b) SIF. □ = GCPh – PTX, ○ = Simulated Taxol, △ = Paclitaxel fine nanocrystals, ▲ = Paclitaxel large nanocrystals.. *Dissolution rates from all formulations were significantly different from each other ($p < 0.0001$).

Figure 5: Plasma paclitaxel levels following the oral administration of paclitaxel formulations (mean ± s.d., n = 3-4), open symbols = paclitaxel levels in the absence of verapamil, closed symbols = paclitaxel levels on co-administration of verapamil (40 mg kg^{-1}): GCPh-PTX = ■ □, simulated Taxol = ● ○, fine paclitaxel nanocrystals = △, large paclitaxel nanocrystals = ◇. a) = 6.7 mg kg^{-1}, b) = 10 mg kg^{-1}, c) = 20 mg kg^{-1}, d) = 20 mg kg^{-1}. * = statistically significant difference ($p < 0.05$) between paclitaxel plasma levels after administration of the simulated Taxol formulation and verapamil when compared with the simulated Taxol formulation in the absence of verapamil, # = statistically significant difference ($p < 0.05$) between plasma levels after the administration of GCPh-PTX and verapamil when compared to GCPh-PTX in the absence of verapamil, § = statistically significant difference ($p < 0.05$) in plasma paclitaxel levels when the simulated Taxol and GCPh-PTX formulations are compared to the crystal formulations. There were no significant differences observed between Taxol and GCPh-PTX formulations.

Figure 6 Confocal laser scanning micrographs of rat intestinal tissue 2 hours after dosing with GCPh-Texas red conjugate (100 mg mL^{-1}). The Texas red signal (red) can be seen lining the villi (a), inside the villi and also in the basolateral side of the villi (b,c) as indicated by the arrows. (d) Blank rat intestine for comparison. (scale bars = 10 µm).

Figure 7: GCPh permeability enhancement across Caco-2 cell monolayers (mean ± s.d., n = 3, FD-4 transport in Hank's Buffered Salt Solution – n = 2). a) The effect of GCPh (1mg mL^{-1}) on the transepithelial resistance across a Caco-2 cell monolayer. GCPh nanoparticles = ○, *N,N,N*-trimethylchitosan (1mg mL^{-1}, TMC) = □, Hanks buffered salt solution (HBSS, pH 6.8) = ●. * = statistically significant difference ($p < 0.01$) between GCPh nanoparticles and the control (HBSS). TMC was not significant different from the buffer control. b) The transport of a paracellular marker (FD-4) across a Caco-2 cell monolayer in the presence of GCPh nanoparticles. GCPh was not significantly different from TMC.

Figure 8: The mechanism of uptake of paclitaxel nanoparticles as a) Taxol and b) GCPh-PTX nanoparticles.