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Impact of excipients on the absorption of P-glycoprotein substrates in vitro and in vivo

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Abstract

The efflux transporter, P-glycoprotein (P-gp), located in the apical membranes of intestinal absorptive cells, can reduce the bioavailability of a wide range of orally administered drugs. A number of surfactants/excipients have been shown to inhibit P-gp, and thus potentially enhance drug absorption. In this study, the improved everted gut sac technique was used to screen excipients for their ability to enhance the absorption of digoxin and celiprolol in vitro. The most effective excipients with digoxin were (at 0.5%, w/v): Labrasol > Imwitor 742 > Acconon E = Softigen 767 > Cremophor EL > Miglyol > Solutol HS 15 > Sucrose monolaurate > Polysorbate 20 > TPGS > Polysorbate 80. With celiprolol, Cremophor EL and Acconon E had no effect, but transport was enhanced by Softigen 767 > TPGS > Imwitor 742. In vivo, the excipients changed the pharmacokinetic profile of orally administered digoxin or celiprolol, but without increasing the overall AUC. The most consistent change was an early peak of absorption, probably due to the higher concentration of excipient in the proximal intestine where the expression of P-gp is lower. These studies show that many excipients/surfactants can modify the pharmacokinetics of orally administered drugs that are P-gp substrates.

Keywords: Digoxin; Celiprolol; Everted sacs; P-glycoprotein; Excipients; Surfactants

1. Introduction

P-glycoprotein (P-gp) is a membrane transporter that actively "pumps" xenobiotics out of cells. It is expressed in the apical membranes of the epithelial cells of the intestine and, due to its action, drugs that have been absorbed by these cells can be pumped back into the lumen of the intestine. Given the very broad specificity of P-gp, then its activity in the intestine may reduce the oral bioavailability of a wide range of drugs. However, there is not agreement on the significance of P-gp in modulating the absorption of orally administered drugs. For example, based largely on human pharmacokinetic data, Chiou and colleagues claim that for a number of common drugs that are known P-gp substrates, the role of P-gp is not important (Chiou et al., 2001). It is clear that there can be drug interactions centered on intestinal P-gp. Thus, in human volunteers, the oral bioavailability of digoxin was significantly increased when

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talinolol was co-administered orally, but not when it was administered intravenously, suggesting an interaction at the intestinal level (Westphal et al., 2000). Many NCEs are poorly absorbed and are substrates of P-gp, and there is considerable interest in trying to increase the bioavailability of oral drugs that are P-gp substrates particularly anticancer and anti-HIV drugs. The oral administration of competing low molecular weight compounds, such as verapamil or cyclosporine, can enhance the oral bioavailability of P-gp substrate drugs, but such compounds have themselves pharmacological activity. Thus, they may also interact with P-gp everywhere that it is present, as is also the case with the new generation of low molecular weight P-gp inhibitors currently appearing and in development. So the challenge for oral drug administration is to find inhibitors of P-gp that do not have such drawbacks. A number of excipients, which are commonly added to pharmaceutical formulations, may disrupt the function of intestinal P-gp and thus enhance the intestinal permeability of a substrate drug. Vitamin E-TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate) is a good example, as it increases the absorption flux of amprenavir (Yu et al., 1999), and has been characterized as an inhibitor of P-gp-mediated drug transport in the human intestinal Caco-2 cell monolayers and other cell lines (Dintaman and Silverman, 1999; Bogman et al., 2003). It has been shown to enhance the bioavailability of cyclosporine in human volunteers (Chang et al., 1996) and of colchicine in rats when the drugs were administered orally (Bittner et al., 2002). Many of the in vitro studies on the intestinal absorption of P-gp substrates have been carried out using the Caco-2 cell monolayer, but these cells tend to show variations in P-gp expression depending upon the culture conditions (Anderle et al., 1998) and it is suggested that they overexpress P-gp (Collett et al., 1999). In our study, we have used the improved rat everted gut sac for the in vitro studies. This model uses tissue culture medium (TC 199) as the incubating medium, ensuring high viability and maximal functionality of the tissue as previously demonstrated by various criteria (Barthe et al., 1998a). There is a large surface available for absorption, and good mixing of substrates and excipients and the technique has already proved to be a very useful in vitro tool to evaluate the role of P-gp in drug absorption (Barthe et al., 1998b; Carreno-Gomez and Duncan, 2000). Initial studies in our laboratory using this technique have shown that the excipients/surfactants Cremophor EL and Polysorbate 80 (Tween 80) could enhance net digoxin absorption (Cornaire et al., 2000). In the current study, we have greatly extended the range of surfactants/excipients tested as well as using a second P-gp substrate drug (celiprolol) and carrying out studies in vivo in rats. Excipients were selected because they are currently used in pharmaceutical formulations and/or had been demonstrated to modulate P-gp activity in other systems. Some were selected because they were amphiphilic or lipophilic but soluble or dispersible in water. We first used digoxin as a model drug because it has clearly been shown to be a P-gp substrate and there is also a considerable amount of data available on its pharmacokinetics. However, digoxin is metabolized by the enzyme CYP3A from rat liver (Salphati and Benet, 1999), and as this enzyme is present in the intestinal epithelium, there is the possibility of first-pass metabolism during absorption by the GI tract. Hence, we decided to use celiprolol as a second model drug because it is a P-gp substrate that is not metabolized in the rat intestine, and P-gp has been implicated in its absorption in man (Karlsson et al., 1993; Engman et al., 2001). The rationale of this work was to screen the excipients in vitro for absorption enhancement with the model drugs digoxin and celiprolol. The most promising excipients were subsequently taken forward for in vivo studies.

2. Materials and methods

2.1. Materials

[^{3}H]Digoxin (250 μCi/mmol) and Ready Safe™ scintillation fluid were obtained from NEN-Life Sciences (Le Blanc-Mesnil, France) and from Beckman Instruments (Gagny, France), respectively. D-[1-^{14}C]Mannitol (55 mCi/mmol) was from American Radiolabelled Chemicals (St Louis, MO, USA). The excipients, purchased by the Galenic Research and Preformulation Department of UPSA Laboratories (Rueil-Malmaison, France), were: Cremophor EL, Solutol HS 15 (BASF), Softigen 767, Imwitor 742, Imwitor 370 (Hüls); Labrasol, Labrafil M1944CS, Sucroester 7, Sucroester 15, Gelucire 44/14, Plurol oleic (Gattefossé); D-α-tocopheryl polyethylene gly-

col 1000 succinate, TPGS (Eastman); Sucrose monolaurate (Fluka); Polyethylene glycol 400 (PEG 400) (Merck); Propylene glycol, Polysorbate 80 (Labosi); Polysorbate 20 (Sigma); Acconon E (Abitec) and Miglyol 840 (Condea). The lactate dehydrogenase kit (LDH SFBC Unimate 3) and the Digoxin Online kit were supplied by ABX (Montpellier, France). All other reagents were analytical grade and from Sigma Aldrich Chimie (St. Quentin Fallavier, France).

2.2. Analysis of digoxin and celiprolol

For the everted sac experiments radiolabeled digoxin was used and so it was measured directly by scintillation counting. In the in vivo experiments, plasma digoxin was measured by an automated immunological method using Cobas Mira® (Roche, Paris, France). Celiprolol was measured using a published HPLC method, using a C18 reverse phase column with spectrophotometric detection at 232 nm (Braza et al., 1998). The mobile phase was 1.2% triethylamine (w/v) in acetonitrile:water (29:71, v/v), adjusted to pH 3.0 with 85% orthophosphoric acid. The method was validated according the strict criteria of the laboratory: the Arlington criteria (Shah et al., 2000). For the analysis of celiprolol in the serosal fluid of the gut sacs, direct measurement was carried out following a brief centrifugation, but for the plasma analysis an extraction step was required. 1 ml of plasma was alkalinized with 0.1 ml of 1 M NaOH and then extracted with 6 ml of dichloromethane. This was evaporated under a stream of nitrogen and the residue dissolved in the mobile phase for injection into the HPLC.

2.3. Everted gut sac method

The everted sac method was used as previously described (Barthe et al., 1998a,b; Cornaire et al., 2000) with the sacs incubated in tissue culture medium TC 199 containing the drugs and the excipients. In the case of digoxin the concentration was 10 μM plus a spike of [^{3}H]digoxin, and for celiprolol the concentration was 100 μM. Mannitol transport was also measured using mannitol at 1 mM spiked with [^{14}C]mannitol. The excipients were tested at 0.05, 0.1, and 0.5% (w/v) and the incubation time was 90 min. This time was chosen to ensure maximum absorption and effects of excipients, while at the same time maintaining tissue at full viability and function. After incubation the sacs were washed three times in 0.9% (w/v) saline, blotted dry, and weighed. The sacs were cut open, the contents collected for analysis and the sacs reweighed to enable the precise volume of the contents to be obtained. The sacs were then dissolved in 1 M NaOH (2 h at 37 °C) and the protein content measured by a modification of the Lowry method (Peterson, 1986). From the radioactivity measured in the sacs or the direct analysis of celiprolol, drug transport was expressed as nanomoles transported per milligram of tissue protein. Tests for significant differences between conditions were carried out using two-way (time and concentrations of excipients) ANOVA with multiple comparisons (Fischer's pairwise comparisons).

2.4. Measurement of LDH

As an indicator of cell damage, the liberation of the cytosolic enzyme LDH was determined in the incubation media in the absence or presence of the excipients using Cobas Mira® (Roche). The results were calculated as units/mg of sac protein (1 unit reduces 10^{-6} mol pyruvate per minute at pH 7.5) and expressed as % released relative to the control without excipients (100%).

2.5. In vivo experiments and pharmacokinetic data

All the experiments were performed with male Sprague–Dawley rats (Dépré, St-Doulchard, France) weighing around 300 g. The animals were housed and handled according to the "Principles of Laboratory Animal Care" (NIH Publication 85-23) and food and water were given ab libitum. At time zero, rats were dosed by oral gavage with digoxin (0.25 mg/kg), dissolved at 0.025% (w/v) in water, propylene glycol, and ethanol (50:40:10, v/v/v) or celiprolol (30 mg/kg), dissolved at 1% (w/v) in water. The co-administered excipients were added to the drug solutions at the concentrations of 1 and 120 mg/kg for digoxin and celiprolol, respectively, to give the same drug excipient ratio for each. Three rats were used for each time point, which were 0, 20, 40, 60, 90, 120 180, and 360 min (for celiprolol, the earlier times were 15, 30, 45, and 60 min). At each time point the rats were anaesthetized and blood was collected from the

carotid artery into heparinized tubes for plasma analysis. The pharmacokinetic parameters were obtained according to a non-compartmental model, using Kinetica® software (Innaphase, PA 1903, USA).

3. Results

3.1. Effect of excipients on digoxin transport and LDH release by everted gut sacs

Among the selected excipients, propylene glycol, PEG 400, Plurol oleic, Labrafil M1944CS, Gelucire 44/14, Imwitor 370, Sucroester 7, and Sucroester 15 had no significant effect on digoxin uptake into the sac contents at the three tested concentrations of excipient (data not shown).

On the other hand, as shown in Table 1, Cremophor EL, TPGS, Solutol HS 15, Sucrose monolaurate, Acconon E, Polysorbate 20, Polysorbate 80, Labrasol, and Softigen 767 all showed enhancement of the digoxin uptake into the sac contents. In general, the higher concentrations of excipients gave the greatest enhancement of digoxin transport, but this was not always the case. It can be seen that with Miglyol, the lowest concentration (0.05%, w/v) gave the greatest enhancement, and with TPGS, each concentration gave similar results. The greatest enhancements, 4.3- and 5.1-fold, were given by the highest concentrations (0.5%, w/v) of Imwitor 742 and Labrasol, respectively.

In order to evaluate their potential cell toxicity, the effect of these excipients on the release of the cytosolic enzyme LDH was also examined. In a preliminary study (data not shown), there was no difference in LDH release by everted gut sacs incubated in TC 199 medium with or without digoxin. The results obtained in the presence of the excipients are shown in Table 2, and it can be seen that LDH release was not modified by TPGS, Acconon E or Polysorbate 80. A number of the excipients: Miglyol M840, Solutol HS 15, Imwitor 742, Labrasol, and Softigen, at the maximum concentration of 0.5% (w/v), caused a release of LDH, but the maximum increase over the control was around twofold. This level of release can probably be considered as non-toxic, whereas the 3.8-fold increase in release caused by sucrose monolaurate most likely indicates toxicity.

LDH release may not reflect subtle changes in the epithelial barrier, for example, any breakdown of the tight junctions (TJs) between cells. Mannitol is the standard marker of paracellular transport via the tight junctions and so the excipients that were being taken forward for the in vivo study were tested for their influence on mannitol transport. At 0.5% concentration

Table 1
The effect of excipients on digoxin transfer across the intestinal mucosa in the rat everted gut sac model

	Excipient concentration (%, w/v)		
	0.05	0.1	0.5
Control (no excipient)	2.8 ± 0.8	2.8 ± 0.8	2.8 ± 0.8
Miglyol	12.7 ± 2.7 (4.5×)**	4.3 ± 0.7 (1.5×) NS	7.7 ± 5.1 (2.8×) NS
Cremophor EL	4.8 ± 1.5 (1.7×)*	5.9 ± 0.4 (2.1×)*	8.2 ± 0.2 (2.9×)**
TPGS	7.0 ± 1.1 (2.5×)**	6.6 ± 0.3 (2.4×)**	5.4 ± 0.2 (1.9×)**
Solutol HS 15	6.2 ± 1.8 (2.2×)**	4.9 ± 0.8 (1.75×)*	6.8 ± 0.7 (2.4×)**
Sucrose monolaurate	4.8 ± 1.5 (1.7×)*	6.2 ± 1.7 (2.2×)**	6.5 ± 0.6 (2.3×)**
Acconon E	5.1 ± 1.4 (1.8×)*	8.3 ± 2.0 (3.0×)**	10.5 ± 0.3 (3.8×)**
Imwitor 742	3.1 ± 0.7 (1.1×) NS	7.1 ± 4.0 (2.5×)*	12.1 ± 3.5 (4.3×)**
Polysorbate 20	4.2 ± 1.8 (1.5×) NS	3.6 ± 0.5 (1.3×) NS	6.0 ± 2.8 (2.1×)*
Polysorbate 80	3.3 ± 2.2 (1.2×) NS	3.0 ± 0.3 (1.1×) NS	5.0 ± 0.6 (1.8×)*
Labrasol	3.3 ± 0.6 (1.2×) NS	3.4 ± 0.6 (1.2×) NS	14.2 ± 0.5 (5.1×)**
Softigen 767	4.5 ± 1.4 (1.6×) NS	5.0 ± 2.1 (1.8×) NS	10.7 ± 3.3 (3.8×)**

Uptake is expressed as $\times 10^2$ nmol/mg protein in the serosal contents of everted gut sacs. Mean of three sacs ± S.E. The figure in brackets shows the change compared with the control without test excipient.
NS: no significant difference in comparison to control.
* $P < 0.05$ (ANOVA).
** $P < 0.01$.

Table 2
LDH release from intestinal mucosa into the incubation medium in the absence and presence of tested excipients

	Excipient concentration (%, w/v)		
	0.05	0.1	0.5
Control (no excipient)	100%	100%	100%
Miglyol M840	59% ± 45% NS	97% ± 12% NS	154% ± 35%*
Cremophor EL	71% ± 31% NS	108% ± 11% NS	112% ± 1% NS
TPGS	86% ± 39% NS	124% ± 64% NS	120% ± 39% NS
Solutol HS 15	73% ± 6% NS	102% ± 64% NS	158% ± 17%*
Sucrose monolaurate	134% ± 13% NS	116% ± 54% NS	378% ± 19%**
Acconon E	88% ± 18% NS	97% ± 22% NS	78% ± 30% NS
Imwitor 742	n.d.	92% ± 49% NS	179% ± 15%**
Polysorbate 20	n.d.	133% ± 38% NS	171% ± 59% NS
Polysorbate 80	n.d.	58% ± 58% NS	109% ± 26% NS
Labrasol	n.d.	n.d.	239% ± 11%**
Softigen 767	n.d.	n.d.	168% ± 57%**

Data are expressed as a percentage of the control value (no added excipient) and are the means of three sacs ± S.E.
NS: no significant difference compared to control. n.d.: not determined.
* $P < 0.05$ (ANOVA).
** $P < 0.01$.

(w/v) and after 90-min incubation, Cremophor EL, Imwitor 742, TPGS, and Acconon E had no significant effect on mannitol transport. However, Softigen 767 significantly increased mannitol transport by a factor of 2 (data not shown). This suggests that this excipient may modify the paracellular passage by opening the tight junctions, as seen with chelating agents (Barthe et al., 1998a).

3.2. In vivo studies with digoxin

For the in vivo study, five excipients were selected among those that had enhanced digoxin uptake in vitro. The plasma profiles are shown in Fig. 1(a)–(f) for digoxin alone and administered with the excipients, where each point is the mean of three different rats. Fig. 2 shows the profiles plotted on the same graph, but for clarity, without the error bars. From the curves of the means for each point, the pharmacokinetic parameters calculated are shown in Table 3, where the parameters are given for the total time as well as for the early part of the kinetics, as this is where the major changes occurred. It can be seen that the presence of excipients modified the pharmacokinetic profile but in different ways. Only Softigen 767 produced a major increase in the AUC for the total time course (0–480 min), and Cremophor EL a reduction, but in doing so it also shifted the t_{max} to the right. Imwitor 742 also increased the t_{max} and slightly increased the AUC. However, between 0 and 40 min, certain excipients produced an early absorption of digoxin, notably TPGS, Imwitor 742,

Table 3
Pharmacokinetic parameters of digoxin after oral administration in the absence or the presence of excipients

Administered compound	C_{max} (μg/L)	t_{max} (min)	$AUC_{[0–480]}$ (μg min/L)	$t_{1/2}$ (min)	$C_{max[0–40]}$ (μg/L)	$t_{max[0–40]}$ (min)	$AUC_{[0–40]}$ (μg min/L)
Digoxin alone	20.0	90	4011.2	165.1	9.3	20	252.2
Digoxin + Cremophor EL	8.7	120	2050.4	554.8	6.5	40	145.1
Digoxin + TPGS	18.1	20	3688.7	149.4	18.1	20	433.7
Digoxin + Acconon E	13.7	20	2338.1	123.9	13.7	20	364.1
Digoxin + Imwitor 742	20.3	120	4530.8	338.3	16.4	20	437.8
Digoxin + Softigen 767	17.0	20	5532.8	179.7	17.0	20	452.6

The dose of digoxin administered by oral gavage was 0.25 mg/kg and that of the excipients was 1.0 mg/kg. Data were calculated from the means of three rats.

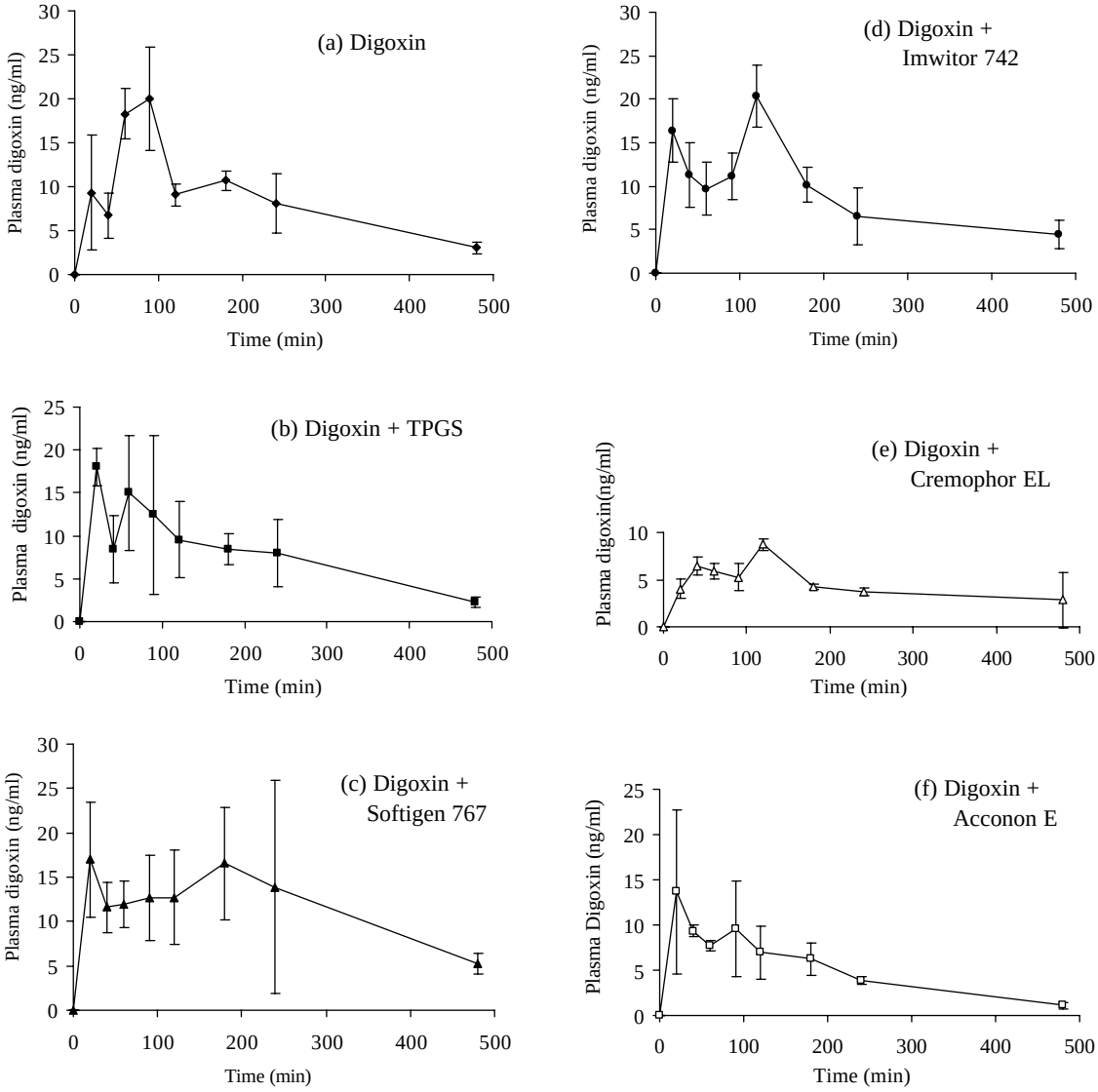

Fig. 1. Plasma digoxin concentration in rats after oral administration in the absence (a) or the presence of excipients (b)–(f). Each time point is the mean plasma concentration of three rats ± S.D.

and Softigen 767. In the presence of these excipients, the AUC for the time period 0–40 min of digoxin absorption was increased from 252.2 μg min/L for the control value to about 440 μg min/L. With Acconon E, the same phenomenon was observed but was less marked. A second plasma peak of digoxin was also seen with TPGS, Softigen 767, and Imwitor 742.

3.3. Celiprolol transport across everted gut sacs

Celiprolol has been shown to be a P-gp substrate in the Caco-2 cell model (Karlsson et al., 1993). In order to demonstrate that the effect of P-gp on celiprolol absorption could be seen in the gut sac model, two criteria were considered. Firstly, non-everted sacs were used to show that the transport of celiprolol was

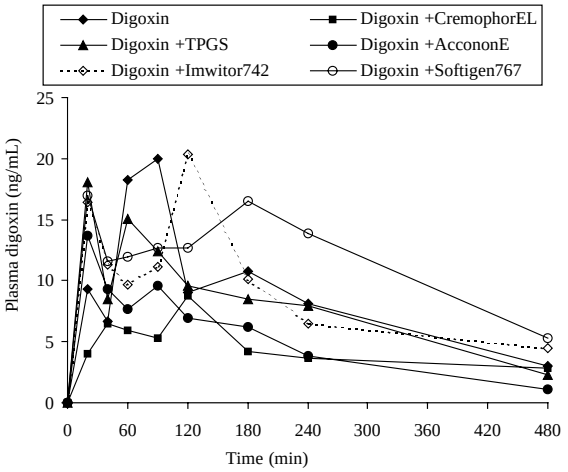

Fig. 2. Plasma concentrations of digoxin administered with and without excipients, plotted together. For clarity the error bars have been omitted.

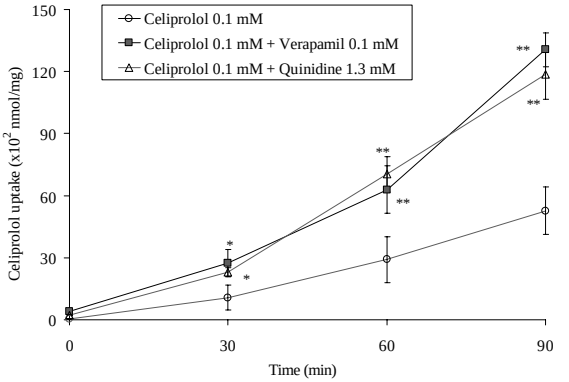

Fig. 3. Celiprolol uptake (100 μM in the incubation medium) into the serosal contents of everted gut sacs in the absence or the presence of verapamil (100 μM) or quinidine (1.3 mM). Data are the means of three sacs ± S.D. Statistically significant differences were calculated using ANOVA: $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control.

higher in the serosal to mucosa direction than in the mucosal to serosal, by a factor of about eightfold (data not shown). Given that celiprolol is absorbed by passive diffusion, the observed difference was certainly the consequence of a secretory transport system. Secondly, using everted sacs, celiprolol transport across the sacs was shown to be augmented in the presence of the "classic" P-gp inhibitors verapamil and quinidine, as had previously been shown with digoxin (Barthe et al., 1998b). These results are shown in Fig. 3. It can be clearly seen that the two P-gp inhibitors enhanced the net transport of celiprolol across the everted sacs by 2.2- to 2.5-fold, confirming the role of P-gp in celiprolol absorption. Five of the excipients that had enhanced digoxin absorption by everted sacs were tested for their effects on celiprolol uptake, and the results are shown in Table 4. At the highest excipient concentration (0.5%, w/v), TPGS, Imwitor 742, and Softigen 767 all enhanced celiprolol transport across the intestinal mucosa by two- to threefold, but in the case of the latter two, they were less effective than with digoxin (approximately fourfold enhancement). Interestingly, Acconon E and Cremophor EL, both of which significantly enhanced digoxin transport across everted sacs, had no effect on the transport of celiprolol.

Table 4
Effect of excipients on celiprolol absorption in the everted gut sac

	Excipient concentration (%, w/v)		
	0.05	0.1	0.5
Control (no excipient)	100%	100%	100%
Cremophor EL	91% ± 58% NS	87% ± 68% NS	66% ± 18% NS
TPGS	52% ± 11%*	53% ± 15% NS	215% ± 20%**
Acconon E	92% ± 17% NS	50% ± 12% NS	96% ± 34% NS
Imwitor 742	141% ± 22% NS	123% ± 20% NS	189% ± 24%**
Softigen 767	120% ± 22% NS	96% ± 13% NS	282% ± 32%**

Uptake is expressed as percentage of celiprolol uptake into the serosal contents of everted gut sacs in the presence of excipient compared with the control (100%) for each experiment. Data are means of three sacs ± S.E.
NS: not significant, by comparison with the control.
* $P < 0.05$ (ANOVA).
** $P < 0.01$.

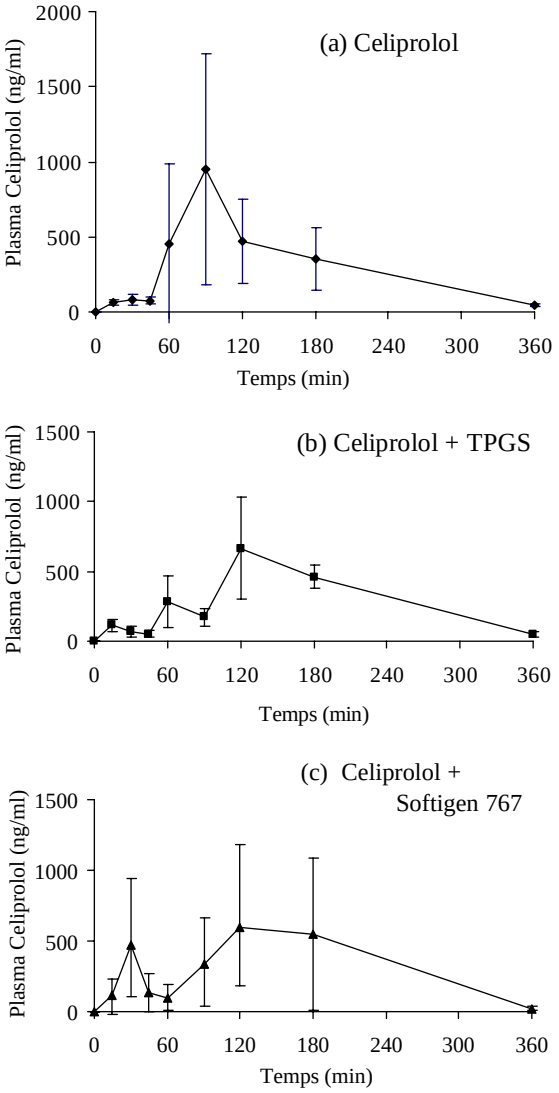

Fig. 4. Plasma celiprolol concentration in rats after oral administration in the absence (a) or the presence (b) and (c) of excipients. Each time point is the mean plasma concentration of three rats ± S.D.

3.4. *In vivo studies with celiprolol*

Softigen 767 and TPGS were tested with celiprolol in vivo. The pharmacokinetic profiles are shown in Figs. 4 and 5 and the pharmacokinetic data calculated from the mean values are shown in Table 5. While the Softigen and TPGS did not increase the AUC or the C_{max} of the celiprolol between 0 and 360 min

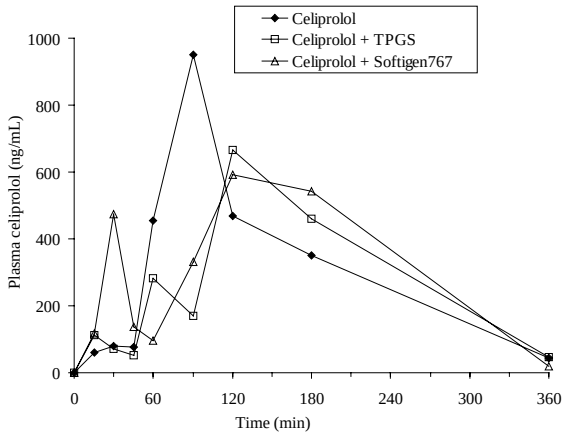

Fig. 5. Plasma concentrations of celiprolol administered with and without excipients, plotted together. For clarity, the error bars have been omitted.

(Table 5), their co-administrations modified the profile of the plasma concentrations of celiprolol. TPGS produced a first peak at 60 min and shifted the t_{max} to 120 min compared with the control value of 90 min. With Softigen 767, a sharp first peak of concentration appeared at 30 min, and again the t_{max} was shifted to the right. The residual concentrations of celiprolol at the end of 6 h were equivalent in the absence or presence of the two excipients.

4. Discussion

Within recent years a number of studies have suggested that several common pharmaceutical surfactants/excipients can modulate the activity of the efflux transporter P-gp, and possibly other transporters. Thus, the concept of all excipients being “inactive” has been challenged, and the idea that they were largely inert molecules used to control the stability and solubility of drugs has had to be reassessed (Wandel et al., 2003). In trying to evaluate the potential of such excipients to enhance the oral bioavailability of drugs, studies have been carried with many different in vitro systems, different drugs, and wide ranges of excipient/surfactant concentrations and it is proving difficult to build up a coherent picture. Our study used the everted gut sac method that has been improved and perfected in the Toulouse laboratory. It is appropriate because although being rat tissue it measures drug

Table 5
Pharmacokinetic parameters of celiprolol after oral administration in the absence or presence of excipients

Administered compound	C_{max} (μg/L)	t_{max} (min)	$AUC_{[0–360]}$ (mg min/L)	$t_{1/2}$ (min)	$C_{max[0–45]}$ (μg/L)	$t_{max[0–45]}$ (min)	$AUC_{[0-45]}$ (mg min/L)
Celiprolol alone	951.5	90	109.3	148.0	78.6	30	2.67
Celiprolol + TPGS	664.4	120	104.3	164.4	113.2	15	3.12
Celiprolol + Softigen 767	592.4	120	116.6	148.0	473.3	30	9.35

The dose of celiprolol administered by oral gavage was 30 mg/kg and of the excipients was 120 mg/kg. Data were calculated from the means of three rats.

transport in the small intestine, which is the major absorption site in vivo, as opposed to colon, from where the commonly used Caco-2 cell line originates. The method had already been shown to be a useful system for studying the effect on drug absorption of modulating P-gp activity (Barthe et al., 1998b; Carreno-Gomez and Duncan, 2000; Cornaire et al., 2000). The first drug chosen as a test drug was digoxin, which is well established from numerous in vitro and in vivo studies as P-gp substrate drug, though it has been suggested recently that there may another as yet unidentified efflux system for digoxin as well as P-gp (Lowes et al., 2003). Given that there was a possibility that digoxin might be metabolized in rat intestinal tissue, a metabolically stable P-gp substrate drug, celiprolol, was also tested. With log *P* values of 1.51 and 0.31, respectively, both these drugs will enter cells by passive diffusion before being subject to efflux transport by P-gp. The rationale of the study was to use the everted gut sac method as a screen to determine which excipients gave enhanced drug transport across the intestinal mucosa, and then to test selected ones in vivo to ascertain whether there was an in vitro:in vivo correlation and increased drug absorption in whole animals. An additional objective was to try and identify chemical characteristics of excipients that made them inhibitory molecules.

Amongst the molecules tested with digoxin, the non-soluble but dispersible excipients (emulsifying agents), such as Labrafil M1944CS, Plurol oleic, and Imwitor 370, were not active as transport enhancers (data not shown). The agents that were active were all perfectly soluble in aqueous medium. However, the highly soluble PEG 400 was not active in enhancing digoxin transport. Recently, a series of reports have been published showing that PEG 300 can modulate the transport of the P-gp substrate anticancer drugs taxol and doxorubicin across Caco-2 cells (Hugger et al., 2002a,b, 2003). It should be noted, however, that those studies were using high concentrations of PEG: from 2.5 to 20%. Thus, the lowest concentration used was five times higher than that used in our current study. In a similar study using Caco-2 cells, Rege et al. (2001) observed no effect of PEG 400 at 1.5% on the transport of cimetidine and furosemide, drugs subject to the action of efflux transporters. Using rat intestine mounted in Ussing chambers, and digoxin as the P-gp substrate, Johnson et al. (2002) also observed significant inhibition of efflux with PEG 400 at 1, 5, and 20%, again concentrations higher than in the current study. The same investigators, however, failed to observe any inhibition with TPGS (unlike the modest effect in the current study), but in this case the concentration used was only 0.01%, well below that used by us and most other researchers. These differences in drugs and tissues used, and the varying surfactant concentrations, highlight the difficulties in comparing the data from different laboratories. The concentrations used in our gut sac experiments are probably realistic if one is thinking in terms of the actual concentrations that might be attained in the intestinal lumen with real potential pharmaceutical formulations.

As can be seen from Table 1, the rank order of excipient effectiveness (at the maximum concentration of 0.5%, w/v) was Labrasol > Imwitor 742 > Acconon E = Softigen 767 > Cremophor EL > Miglyol > Solutol HS 15 > Sucrose monolaurate > Polysorbate 20 > TPGS > Polysorbate 80. Table 2 shows the release of the cytosolic enzyme LDH in the presence of the test substances. It is a matter of some judgement as to what level of LDH release constitutes an indication of toxicity. In this study, we consider a release below a 200% increase (relative to the control at 100%)

is probably non-toxicity. On this basis only Sucrose monolaurate (and possibly) Labrasol were toxic to the intestinal cells under the experimental conditions described (378 and 239% LDH release, respectively).

Celiprolol was then used as a target drug in the everted gut sac system with some of the more active excipients. It was clear, as shown in Fig. 3, that celiprolol behaved as a P-gp substrate in the system in that its transport was inhibited by the classical P-gp inhibitors verapamil and quinidine. Table 4 shows that of the five excipients tested only three, Softigen 767 > TPGS > Imwitor 742, caused an increase in celiprolol transport at the highest concentrations. These effects were less marked than with digoxin as a substrate, which was also the case for the inhibition by quinidine, which had a more inhibitory effect on digoxin transport (Barthe et al., 1998b). Surprisingly, Cremophor EL and Acconon E, which increased digoxin transport by 300 and 400% had no significant effect on celiprolol transport. There may be a number of reasons, including the affinity for P-gp and the mechanism of transport of each drug, and it raises the question as to whether the effect of excipients on drug transport by P-gp may be different for different classes of drugs. P-gp has multiple binding sites and a very broad specificity for drugs of widely varying structure and many studies (beyond the scope of this discussion) are attempting to define the structure activity relationships of P-gp and how inhibitors may interact (Seelig and Landwojtowicz, 2000; Stouch and Gudmundsson, 2002; Kaur, 2002). At a basic level it can be noted that digoxin is a more lipophilic drug than celiprolol and that this may play a role in susceptibility to P-gp efflux, and possibly to the action of the excipients.

The mechanisms of action of the excipients/surfactants on efflux transport are clearly complex and may be multiple. One feature that some authors consider important for the ability of molecules to inhibit P-gp is the hydrophilic-lipophilic balance (HLB). Buckingham et al. (1995) tested Solutol HS 15 and Cremophor EL and subsequently synthesized a series of ethylene oxide oleic esters which were more active than the original surfactants. They considered that the structure of the hydrophobic domain as well as the HLB were important in determining the inhibitory potency. A recent study of epirubicin transport with both Caco-2 cells and everted gut sacs, and a range of excipients, demonstrated that an HLB of between 10 and 17 was optimal in inhibiting drug efflux and enhancing transport (Lo, 2003). One of the surfactants tested (Span 80) had a low HLB (4.3) and only slight activity against efflux. Here again the problem of the concentrations used makes comparisons difficult as the concentrations in Lo's study are given in molar terms. Thus, the *maximum* concentration used was 200 μM which in mass terms is only 0.0086% (w/v), which is lower than the lowest concentration used in our studies. Batrakova et al. (2003) have used a series of pluronic block copolymers, many of which are effective inhibitors of P-gp-mediated efflux, to try and unravel the physico-chemical properties required. Moderate lipophilicity with an HLB < 20 appeared to be a factor. In our study effective compounds had a wide range of HLB values, for example, Imwitor 370, 3–4; Cremophor EL, 14; Softigen 767, 19; and so there was no obvious correlation between inhibitory activity and HLB.

While there seems to be a consensus view that the excipient/surfactant effects on P-gp transport are associated with membrane disruption and/or that fluidization is a likely factor, there may well be other parameters, such as the target drug, direct effects on different transporters, and different cell types. While Cremophor EL and Tween 80 (Polysorbate 80) have been reported to be fluidizers of lipid bilayers, TPGS rigidizes them (Rege et al., 2002). It has also been reported that at low concentrations, Cremophor EL and Tween 80 (Polysorbate 80) inhibit anticancer drug efflux from cells by directly binding to the P-gp (Friche et al., 1990). As noted above, our observed difference in results between digoxin and celiprolol, suggests that in some cases the response of the transport system may depend on the drug being transported. Other studies indicate that the excipients can have different effects on different transporters. Rege et al. (2002) showed that Cremophor EL, Tween 80 (Polysorbate 80), and TPGS inhibited P-gp driven basolateral to apical rhodamine 123 transport in Caco-2 cells, and yet in the case of TPGS, net apical to basolateral transport was not enhanced. Of the three surfactants tested, Tween 80 also uniquely inhibited the Pept 1 (influx) transporter and Cremophor EL the monocarboxylic acid (influx) transporter. Bogman et al. (2003) recently showed that P-gp was inhibited by TPGS > Cremophor EL > Tween 80, but that MRP2, another important efflux transporter responsible for multidrug resistance and also present

in the apical membranes of intestinal cells, was not inhibited. Thus, there is evidence of transporter specificity, although the difference may also have been due to the different cell types being used in the studies, a leukemia line for the P-gp transport and a transfected kidney cell line for the MRP2. A difference between cell types has been reported by Hugger et al. (2002a) who compared the effects of PEG 300, Cremophor EL, and Tween 80 on the transport of the P-gp substrate taxol across Caco-2 cells and a polarized canine kidney cell line which had been transfected with the human P-gp gene (*MDR1*-MDCK cells). While the PEG 300 (at high concentrations: up to 20%) inhibited P-gp in both cell types, the Cremophor and the Tween 80 only inhibited P-gp in the Caco-2 cells and not in the transformed cells. Studies on the membrane fluidity revealed that for these latter excipients "no clear relationship between fluidity changes and P-gp inhibition could be seen". Thus, the whole question of how excipients may modulate efflux transporter activity is far from clear. While some modification of the membrane structure/properties in the immediate vicinity of the transporter protein is most likely, there may well be other factors superimposed, such as the nature of the drug substrate and the type of cell membrane. It seems likely that there is not a universal mechanism for all the excipients/surfactants tested.

The objective of the in vitro studies with the excipients was to identify compounds that might increase the bioavailability of orally administered drugs by co-administration with the drugs. From the results obtained with the everted sacs, five of the excipients were co-administered to rats with digoxin and the plasma levels measured and pharmacokinetic parameters calculated as seen in Table 3, with the plasma profiles shown in Figs. 1 and 2. Table 3 has been presented in two parts, with the pharmacokinetic data calculated over the entire time span of the experiment (6 h) but also over the time span of the early part of the experiment, that is from 0 to 40 min. This was done to highlight the major changes in the absorption profiles. It can be seen that all the excipients tested modified the profiles in some way. The most consistent modification, occurring with TPGS, Acconon E, Imwitor 742, and Softigen 767, was the enhancement of an early peak (at 20 min) of absorption. Consequently, the $AUC_{[0–40]}$ was increased compared with the digoxin alone. Except in the case of Softigen 767, the AUC for the whole time period was not significantly altered. As can be seen in Figs. 4 and 5 and Table 5, TPGS and Softigen 767 also modified the plasma profile of celiprolol. In particular, Softigen 767 produced the rapid early absorption peak, and both excipients moved the t_{max} to the right. These observations are important in that they show that excipients can significantly change the pharmacokinetic profiles of P-gp substrates. The data are not absolute proof that it is because of effects on P-gp, but it is the most likely explanation. Both drugs were administered in solution and so there is unlikely to have been any role of the excipients on drug dissolution. However, as the drugs and the excipients were administered together, as would be the case in actual oral formulations, some direct interaction between the drugs and the excipients cannot be completely ruled out.

In the case of Softigen 767 it had been shown to increase mannitol transport with the everted sacs, indicating that it acted on tight junctions and increased paracellular transport. Thus, some of the increased absorption caused by this excipient may have been because of increased paracellular passage, but it must be noted that this absorption route is normally only relevant to very hydrophilic drugs and both digoxin and celiprolol are lipophilic.

While precise quantitative data on P-gp expression and activity along the whole length of the GI tract are not yet available, there are number of studies to show that the P-gp activity gradually increases down the gut. For example, Stephens and colleagues have studied the transport in vitro of a number of drugs, including digoxin, using intestines from mice in which the P-gp gene has been deleted. With digoxin it was clear that the influence of P-gp on limiting its absorption was greater in the lower parts of the GI tract (Stephens et al., 2002). Studying digoxin absorption in rats, but using an in situ perfusion technique, Sababi et al. (2001) clearly showed that when they perfused segments of the gut with digoxin and the P-gp inhibitor verapamil, the absorption enhancement caused by the inhibitor was more pronounced in the duodenum than in the jejunum. A study (submitted) has recently been carried out in our laboratory in which digoxin permeability was measured every few centimeters down the intestine and the results showed that its permeability across the intestinal mucosa showed a gradual decrease as one moves down the small intestine. The

difference was not large, about 2.4-fold between the maximum permeability in the proximal jejunum and that in the terminal ileum. This figure is very similar to the increase seen in P-gp expression down the length of the human intestine as recently published (Mouly and Paine, 2003).

Thus, an explanation for the early absorption peaks seen in the current study would be that in the upper part of the intestine (duodenum, proximal jejunum) the administered drug would be at its highest concentration and as it is absorbed by transcellular diffusion, which is concentration dependent, the absorption rate would be at its maximum. At the same time the concentration of the P-gp inhibitor would also be at its highest concentration, and as the P-gp level is lower than further down the gut, the effect of the inhibitor would be more pronounced. As the drug and excipient move down the intestine, they would both be diluted by the intestinal fluids and the action of peristalsis. This would thus reduce the effectiveness of the inhibitor especially in the face of increased levels of P-gp. Ideally, a formulation should release both the drug and inhibitor together. This concept has been elegantly demonstrated by Baluom et al. (2001). Using a specially designed tablet system, they showed that the release of P-gp inhibitors (verapamil or quinidine) in synchrony with the release of sulpiride could increase its relative bioavailability.

In conclusion, this study has shown that the everted gut sac system is a useful method to screen pharmaceutical excipients/surfactants for their ability to enhance the intestinal uptake of P-gp substrate drugs. To achieve an increase in drug transport by P-gp inhibition requires that the modulator compounds have various properties including: water solubility, the presence of ester bonds, a saturated carbon chain of about C10, and polyoxyethylene groups. However, the mechanisms of action with individual drugs and transporters are far from clear. When the most effective excipients were tested in vivo, they all changed the pharmacokinetic profile of drug absorption, notably in several cases, causing early peaks of absorption. In vivo, the situation is complicated by the dilution factors changing the concentrations of the drugs and excipients. To improve oral bioavailability, much work remains to be carried out with different concentrations of excipients and drugs and with the development of formulations that give the synchronous release of drugs and excipients.

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