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RESEARCH PAPER

Non-linear Increases in Danazol Exposure with Dose in Older *vs*. Younger Beagle Dogs: The Potential Role of Differences in Bile Salt Concentration, Thermodynamic Activity, and Formulation Digestion

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ABSTRACT

Purpose To explore the possibility that age-related changes in physiology may result in differences in drug bioavailability after oral administration of lipid based formulations of danazol.

Methods Danazol absorption from lipid formulations with increasing drug load was examined in younger (9 months) and older (8 years) beagles. Age related changes to hepatic function were assessed via changes to systemic clearance and serum bile acid concentrations. Changes to lipolytic enzyme activity and intestinal bile salt concentration were evaluated using *in vitro* lipolysis.

Results Drug exposure increased linearly with dose in younger animals. In older animals, bioavailability increased with increasing dose to a tipping point, beyond which bioavailability reduced (consistent with initiation of precipitation). No differences in hepatic function were apparent across cohorts. Changes to enzyme concentrations in lipolysis studies had little impact on drug precipitation/solubilisation. In contrast, higher bile salt concentrations better supported supersaturation at higher drug loads.

Conclusions Differences in animal cohort can have a significant impact on drug absorption from lipid based formulation. For danazol, bioavailability was enhanced under some circumstances in older animals. *In vitro* experiments suggest that this was unlikely to reflect changes to metabolism or lipolysis, but might be explained by increases in luminal bile salt/phospholipid concentrations in older animals.

KEY WORDS absorption · bile salt · bioavailability · danazol · lipid-based drug delivery systems · non-linear bioavailability · solubility · supersaturation

ABBREVIATIONS

4-BPB	Bromophenyl boronic acid
AP_{DISP}	Colloidal aqueous phase formed on dispersion of a SEDDS formulation
AP_{DIGEST}	Colloidal aqueous phase formed on digestion of a SEDDS formulation

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AUC	Area under the curve
BA	Bioavailability
BS	Bile salt
C_{max}	Peak plasma concentration
CrEL	Cremophor EL
CYP	Cytochrome P450
F	Absolute bioavailability
GI	Gastrointestinal
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
IV	Intravenous
LBDDS	Lipid-based drug delivery system
LCMS	Liquid chromatography mass spectrometry
MC	Medium-chain
NaTDC	Sodium taurodeoxycholate
PL	Phospholipid
PPI	Polymeric precipitation inhibitors
S	Supersaturation ratio
SBA	Serum bile acid
SEDDS	Self-emulsifying drug delivery system
$t_{1/2}$	Half life
TBU	Tributyrin units
T_{max}	Time of occurrence of peak plasma concentration
Vd_{β}	Volume of distribution

INTRODUCTION

After oral administration, the absorption of drugs with intrinsically poor water-solubility is often low, reflecting slow drug dissolution and incomplete solubilisation in the gastrointestinal (GI) fluids (1). Several formulation approaches have been explored as a means to overcome these limitations (2), and lipid-based drug delivery systems (LBDDS) represent one method that has proven highly effective in enhancing the oral bioavailability of poorly water-soluble, lipophilic drugs (3–6).

In most cases, LBDDS present drug to the GI tract in a molecularly dispersed, solubilised state thereby circumventing traditional dissolution. LBDDS also enhance drug solubilisation via stimulation of bile salt secretion and the formation of mixed colloidal species in the GI tract comprising exogenous (i.e. formulation derived) and endogenous lipids (bile salts, phospholipids) (7–10). The majority of LBDDS formulations are digested after oral administration, and digestion of the lipids and surfactants present in the formulation commonly results in a reduction in solubilisation capacity (8,10–13). Where digestion results in a loss in solubilisation capacity, supersaturation usually eventuates. The degree of induced supersaturation is dose-dependent and expected to increase with increasing drug load in the formulation (14). Supersaturation may promote absorption via an increase in the thermodynamic activity of solubilised drug, however, the metastable supersaturated state also increases the likelihood of drug precipitation, which may reduce drug absorption (by re-introducing the need for dissolution from precipitated drug particles). The potential for a reduction in drug absorption on precipitation may be attenuated in situations where drug phase-separates in the amorphous form, but in most cases, the performance of LBDDS is expected to be dictated by the solubilisation capacity, the degree of supersaturation and the ability of the formulation to maintain supersaturation for sufficient time to allow for drug absorption (14,15).

In a previous study (14), we examined the impact of the addition of a polymeric precipitation inhibitor (PPI), hydroxypropyl methylcellulose (HPMC), to a danazol-containing self-emulsifying LBDDS formulation, as a means of stabilising supersaturation, reducing precipitation and promoting absorption. The PPI had a marked impact on supersaturation stabilisation *in vitro*, however, *in vivo* effects were more moderate. As part of the same study, it was observed that increasing the dose of drug in the formulation resulted in an increase in bioavailability on oral administration. Interestingly, this occurred in contrast to traditional drug absorption paradigms that suggest that increases in dose for poorly water-soluble drug are expected to reduce absorption (since the mass of drug that must be dissolved increases) (14).

The current study was therefore initiated to explore in more detail the mechanism(s) by which increasing drug load resulted in increased danazol bioavailability following oral administration to beagle dogs. The working hypothesis that underpinned these investigations was that the increase in bioavailability with dose was a result of either an increase in thermodynamic activity in the colloidal species formed by formulation digestion, or a decrease in first pass metabolism. As part of this investigation, it became evident that the non-linearity in danazol bioavailability with dose (14), was dependent on the animal cohort in which the study was conducted and was only apparent after administration of higher doses to an older group of animals. The possibility that age-related changes in physiology may have resulted in differences in bioavailability across the two dog cohorts was therefore explored. In particular, the potential for differences in hepatic function, lipolytic enzyme activity and intestinal bile salt concentration was addressed in an attempt to explain the *in vivo* data obtained.

MATERIALS AND METHODS

Materials

Danazol (pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol) was supplied by Sterling Pharmaceuticals (Sydney, Australia) and progesterone was from Sigma-Aldrich (St Louis, MO, USA). Captex 300, a medium-chain (MC) triglyceride, and Capmul MCM, a blend of medium-chain mono-, di-, and triglycerides, were donated by Abitec Corporation (Janesville, WI, USA). Cremophor EL (polyoxyl 35 castor oil), sodium

taurodeoxycholate 97% (NaTDC) and porcine pancreatin (8×USP specification activity) were from Sigma Aldrich (St Louis, MO, USA). Lipoid E PC S, (Lecithin from egg, approximately 99% pure phosphatidylcholine) was from Lipoid GmbH (Ludwigshafen, Germany), 4-bromophenylboronic acid (4-BPB) was obtained from Sigma Aldrich (St Louis, MO, USA) and 1 M sodium hydroxide, which was diluted to obtain 0.6 M NaOH titration solution, was purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) purification system. All other chemicals and solvents were of analytical purity or high performance liquid chromatography (HPLC) grade.

Preparation of SEDDS Formulations Containing Danazol

A type IIIA SEDDS ('SEDDS-III') was used in all studies. The formulation was based on medium-chain (MC) lipids and comprised 30% (w/w) Captex 300, 30% Capmul MCM, 30% Cremophor EL (as surfactant) and 10% ethanol (as cosolvent). All formulations were prepared as previously described (16) and contained danazol as a model poorly water-soluble drug. Danazol is a synthetic steroid originally developed to treat endometriosis and has an aqueous solubility of 0.59 μg/ml (17) and a log *P* of 4.53 (18). The drug was incorporated into SEDDS-III at various drug loadings (mg/g) (tabulated in Table I), representing different proportions of saturated solubility in the formulation (based on the measured equilibrium solubility determined at 37°C) as previously described (14).

Oral Bioavailability Studies in Beagle Dogs

All surgical and experimental procedures were approved by the Melbourne University animal ethics committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Table I Danazol Solubility In SEDDS-III and the Corresponding Drug Load and Treatment Doses Utilized in *in vivo* And *in vitro* Studies

Saturation level [%]	15	30	40	60	80	90	100[a]
Danazol [mg/g][b]	4.5	9.1	12.1	18.2	24.3	27.3	30.3
Treatment dose [mg][c]	7.3	14.5	19.4	29.1	38.8	43.6	–

[a] Saturated solubility of danazol in the MC SEDDS-III formulation comprising 60% lipid, 30% Cremophor EL and 10% ethanol Anby *et al*. (14)

[b] Quantity of danazol dissolved in the SEDDS-III formulation

[c] Total dose administered in two gelatine capsules containing 800 mg SEDDS-III formulation in each capsule

Study Design

A previously published study (14) (referred to here as the 'pre-study' for clarity) showed evidence of non-linear increases in danazol exposure after oral administration to beagle dogs of a SEDDS formulation comprising 30% (w/w) Captex 300, 30% Capmul MCM, 30% Cremophor EL and 10% ethanol (SEDDS-III) and increasing quantities of danazol. In this pre-study, danazol was administered in two capsules each containing 800 mg of the SEDDS-III formulation and with danazol dissolved at either 40% or 80% of saturated solubility in the formulation (equivalent to 12 mg/g and 24 mg/g, respectively) (14). Bearing in mind the non-linearity in exposure in the pre-study, but realising that data was only obtained at two dose levels, the current study sought initially to expand the pre-study and to evaluate the potential for dose linearity/non-linearity across a wider dose range. In the first dog study described herein (study I), animals were therefore administered the same SEDDS-III formulation that was used in the pre-study, but with danazol incorporated (nominally) at 5 mg/g, 9 mg/g, 18 mg/g and 27 mg/g (equivalent to 15%, 30%, 60% and 80% of the saturated solubility in the formulation, respectively). Surprisingly, in this repeat study no evidence of non-linearity with dose was apparent. Since study I was conducted in a separate (and younger) cohort of animals (average age 9 months) than that employed in the pre-study (14), a second study (study II) was conducted in the original (older) beagle cohort (approximate age 8 years) to explore whether the non-linearity observed in the pre-study was specific to the older dog cohort. In study II, the older animal cohort was therefore administered the same SEDDS-III formulation, with danazol dissolved (nominally) at 5 mg/g, 9 mg/g, 18 mg/g and 27 mg/g (equivalent to 15%, 30%, 60% and 90% saturation, respectively).

Administration, Sampling and Analysis

Both studies (Study I and Study II) were conducted as four-way crossovers in male beagle dogs (13–23 kg) (with a 7-day washout period). Treatments were hand filled into gelatine capsules 2 h prior to dosing as previously described (19). Each treatment was administered in two capsules (2×800 mg formulation) with approximately 50 mL water. Treatments were based on SEDDS-III as above and danazol was incorporated at the drug loads tabulated in Tables II and III. Dogs were fasted for at least 20 h prior to dosing and remained fasted until 10 h post-dose after which they were fed on a daily basis. Water was available *ad libitum*.

Blood samples (3 ml) were collected pre-dose and at 0, 15, 30, 45, 60, and 90 min, then at 2, 3, 4, 6, 8 and 10 h post-dose. Samples were collected via an indwelling catheter inserted in the cephalic vein and additional samples were obtained by individual venepuncture at 24, 32 and 48 h post-dose. Blood

samples were collected into 4 mL tubes containing dipotassium EDTA. Plasma was separated within 2 h by centrifugation for 10 min at 1,328×*g* (Eppendorf 5702 R/A-4-38 centrifuge, Eppendorf AG, Hamburg, Germany) and stored at −80°C until sample analysis. Danazol concentrations in plasma were quantified by LC-MS as described previously (14).

Systemic Clearance and Absolute Bioavailability Determination

To evaluate the possibility of differences in systemic clearance across the older and younger dog cohorts and to provide an indication of absolute bioavailability, a subsequent study sought to examine danazol intravenous pharmacokinetics in both dog cohorts. An intravenous formulation of danazol (1.3 mg/mL) was prepared using 20% (w/v) sulphobutyl ether β-cyclodextrin (Captisol®). Danazol and Captisol® were dissolved in 0.9% saline using a magnetic stirrer (Teflon coated stirrer bar, 10×6 mm) at ambient temperature and filtered through a 0.22 μm filter (Millix®-GV) before use.

Study conditions were similar to that described for the oral bioavailability studies, and the intravenous formulation (10 mL to provide an administered dose of approximately 0.85 mg/kg) was administered to fasted dogs by infusion pump (2 mL/min over 5 mins) into a cephalic catheter. Blood samples (3 mL) were taken pre-dose, at −2 min (2 min after start of infusion), 0 (at the conclusion of the infusion), 2, 5, 15, 30, 45, 60, 90, min, then at 2, 3, 4, 6, 8, 10 and 24 h into vaccutainer tubes and samples treated and analyzed as above.

Pharmacokinetic Data Analysis

The peak plasma concentrations (C_{max}) and the time for their occurrence (T_{max}) were noted directly from the individual plasma concentration *vs*. time profiles. The area under the plasma concentration *vs*. time profiles ($AUC_{0\text{-}10}$) was calculated using the linear trapezoidal method. Because the danazol plasma concentrations were typically below the limit of quantification at 24, 32 and 48 h post-dose, accurate determination of the terminal elimination rate constant and ($AUC_{0\text{-}\infty}$) was not possible. However, the danazol plasma concentrations at 10 h were low and the extrapolated AUC ($AUC_{10\text{-}\infty}$) was therefore expected to contribute only a minor proportion of the total AUC ($AUC_{0\text{-}\infty}$). Relative bioavailability comparisons were therefore performed using ($AUC_{0\text{-}10}$). Clearance (Cl) and volume of distribution (Vd_β) were calculated from the IV data ($AUC_{0\text{-}\infty}$) using standard methods. Statistically significant differences were determined by ANOVA followed by a Tukey test for multiple comparisons at a significance level of $\alpha = 0.05$. All statistical analysis was performed using SigmaPlot Statistics for Windows version 11.0.

Liver Function Assessment in Beagle Dogs

To assess whether the differences in dose linearity in the two animal cohorts was a result of differences in liver function (that in turn resulted in a difference in hepatic clearance and first pass metabolism), a measure of liver function was obtained via quantification of serum bile acid levels pre- and post-prandially. Serum bile acid levels are routinely utilised as a liver function test in dogs (20,21). The test is based on the realisation that the presence of food in the GI tract stimulates the release of bile salts from the gall bladder into the intestine and that these bile salts are then absorbed (largely via active transport) in the lower small intestine. This stimulates a transient increases in serum bile acid levels (20–22). Where liver function is normal, serum bile salt levels remain relatively constant post-prandially (regardless of differences in bile salt secretion) due to rapid and efficient bile salt uptake into the liver. In contrast, in animals with reduced hepatic function, hepatic uptake of serum bile acids is reduced leading to increased serum bile acid levels (23–25).

To test liver function, animals in both cohorts were fasted for at least 20 h with free access to drinking water prior to the test. Blood samples were collected via an indwelling catheter inserted in the cephalic vein pre-prandially (just prior to feeding) and post-prandially (2 h after being fed a small meal (~85 g) of standard canned dog food). Blood samples (approximately 2 ml) were collected into 3 mL vacutainer tubes (no clotting agent). Serum was analysed using an enzymatic, colorimetric test for total serum bile acids (Randox Laboratories Limited, Crumlin, UK).

GI and Gall Bladder Bile Salt Concentrations in Young and Old Beagle Dogs

To provide an indication of potential differences in bile salt concentrations in the GI tract of the two beagle cohorts, samples of bile were obtained directly from the gall bladder in one young beagle dog and two older beagles pre- and post-mortem. Data was obtained in a limited subset of animals since euthanasia of the majority of the younger cohort was not justified (the animal that was used developed behavioural problems requiring euthanasia), and one of the older dogs had to be euthanized (tumour growth) prior to the conduct of the bile collection study.

The dogs were fasted for at least 12 h and pre-medicated with a combination of acepromazine (0.03 mg/kg) and methadone (1 mg/kg) and anaesthetised with propofol (4 mg/kg). Animals were subsequently maintained on isoflurane in oxygen for the period of the procedure and did not recover from the anaesthetic. An incision was made to the abdominal wall revealing the gastrointestinal tract and a 23G syringe was used to collect bile directly from the gall bladder. An overdose of sodium pentobarbitone was subsequently administered to

euthanise the animal. Post-mortem, tissue samples of the duodenum, gastric duodenal junction, stomach, liver, pancreas and other tissues were collected for pathological and histopathological analysis. Attempts were made to collect GI fluids prior to sampling gall bladder bile, but were unreliable, presumably due to slowed gastric emptying and reduced gall bladder contraction in anaesthetised animals.

Bile salt concentrations were analysed via a kinetic enzyme cycling reaction in a 96-well plate (Total bile acid kit from Diazyme Laboratories, Poway, USA). Samples were measured at 410 nm via a microplate reader (Fluostar Optima, BMG Labtech GmbH, Ortenberg, Germany) following significant (2000 fold) dilution in milliQ water. The method was validated in the range 5.25–100.5 μM by analysis of 5 replicate standards made up at three different concentrations (5.25, 25.125 and 100.5 μM sodium glycodeoxycholate). Intra-assay variability was accurate to 114.7, 92.1 and 98.7% and precise to ±2.7, 5.1 and 3.1% of 5.25, 25.125 and 100.5 μM. Inter-assay variability was assessed over 2 separate days and was accurate to 111.6, 90.6 and 97.9% and precise to ±1.6, 4.1 and 3.8% of 5.25, 25.125 and 100.5 μM.

In Vitro Evaluation

Drug Solubilisation During Formulation Dispersion and Digestion

To explore the impact of increased drug loading on the potential for drug supersaturation and/or precipitation during processing of the formulations in the GI tract, drug solubilisation patterns *in vitro* were assessed using a previously described *in vitro* model of lipid digestion (14). Formulations contained danazol at differing levels of saturated solubility in the formulation as described in Table I, and the *in vitro* solubilisation/precipitation behaviour was examined as described previously (14). Briefly, 1 g of each formulation was first dispersed in 36 g of digestion medium (50 mM TRIS maleate, 150 mM NaCl, 5 mM $CaCl_2.2H_2O$, pH 7.5) containing bile salt (BS) and phospholipids (PL) (as phosphatidylcholine) at either low (BS: 5 mM NaTDC, PL: 1.25 mM) or high (BS: 20 mM NaTDC, PL: 5 mM) concentrations ([BS/PL]), at 37°C for 30 min. After 30 min, digestion was initiated by addition of 4 mL of pancreatin extract containing 40,000 TBU (to provide approximately 10,000 TBU per mL of extract and approximately 1000 TBU per mL of digest) of pancreatic lipase. Digestion was followed for a subsequent 60 min period using a pH-stat titration unit (Radiometer, Copenhagen, Denmark) that maintained the pH at 7.5 via titration of liberated fatty acids with 0.6 M NaOH. Aliquots (4.2 mL) were taken from the dispersion/digestion media throughout the 90 min experimental period at $t = 10$, 30, 40, 50, 60 and 90 min. Lipid digestion inhibitor (4-BPB, 9 μL of a 0.5 M solution in methanol per mL of dispersion/digestion medium) was added to each sample immediately after sampling to prevent further lipolysis (9,26). Samples were centrifuged and phase separated as previously described (14) and each phase was assayed for danazol content by HPLC. Phase separation led to the generation of an 'aqueous phase' (AP) containing dispersed colloidal structures (a combination of bile salt/phospholipid micelles and surfactant micelles swollen with lipid digestion products) and a pellet phase (containing insoluble calcium soaps and any precipitated drug).

Solubility in the Aqueous Phase Pre-Digestion (Dispersion Phase) (AP_{DISP}) and During Digestion (AP_{DIGEST})

The solubility of danazol in dispersed emulsified blank (i.e. drug-free) formulation was evaluated as described previously (14). The solubility of danazol in the aqueous colloidal phase generated by digestion of blank (i.e. drug-free) formulation was evaluated after 5 and 60 min digestion as described previously (14). Conditions for digestion of the blank formulation were as described above and digestion samples were ultracentrifuged and the aqueous phase separated prior to measurement of drug solubility.

Quantification of Danazol in In Vitro Experiments by HPLC

Aqueous phase samples obtained during dispersion experiments and after initiation of digestion were diluted 1:10 (v/v) with acetonitrile before HPLC analysis. Samples of danazol in the pellets (precipitate) from the digestion studies were first dissolved in 5 mL of chloroform/methanol (2:1 v/v) and subsequently diluted 1:10 (v/v) in acetonitrile prior to analysis by HPLC as described previously (14).

Data Analysis for *In Vitro* Experiments

Solubilisation and Supersaturation During In Vitro Experiments

The impact of drug loading on the ability of the formulation to maintain danazol in a metastable, supersaturated state during dispersion and digestion experiments was assessed using solubilisation/precipitation profiles as described previously (14). The ability of the formulation to maintain supersaturation during dispersion and digestion was expressed as a supersaturation ratio, S (Eq. 1). S was determined as the solubilised drug concentration in the aqueous phase (AP) obtained after centrifugation of samples collected during dispersion (AP_{DISP}) and

digestion (AP_{DIGEST}) divided by the solubility of danazol in AP_{DISP} and AP_{DIGEST}.

$$S = \frac{\text{Solubilised drug conc. in } AP_{DISP \text{ or } DIGEST}}{\text{Drug solubility in } AP_{DISP \text{ or } DIGEST}} \quad (1)$$

Note that in a previous publication (14), AP_{DISP} or AP_{DIGEST} was used to describe the solubility in the aqueous phase during dispersion or digestion. We have since progressed to use this term more broadly to simply indicate the aqueous phase in general. Thus 'AP_{DISP}' as used in Anby *et al*. (14) becomes 'drug solubility in AP_{DISP}' here. 'Solubilised drug conc. in AP_{DISP}' is used to make a distinction between the drug concentration measured kinetically during the dispersion experiment and the equilibrium solubility of drug in the equivalent phase ('drug solubility in AP_{DISP}'). Analogous changes have been made to the terminology used to describe drug concentrations measured in the AP during digestion and the equivalent solubility measurements. To allow representation of drug solubility in the AP_{DIGEST} in the current plots, the change in solubility (during the course of digestion) was assumed to be linear between 5 min and 60 min post digestion.

RESULTS

In Vivo Evaluation

Effect of Increasing Dose on Danazol Exposure In Vivo

The mean plasma concentration *versus* time profiles for danazol following oral administration of the SEDDS formulation (SEDDS-III) containing danazol at 5–27 mg/g (15–90% of saturation in the formulation) administered to a relatively young (mean age; 9 months) cohort of fasted beagles (Study I) is shown in Fig. 1a. The corresponding mean pharmacokinetic parameters are summarized in Table II and show a dose-proportional increase in maximum danazol plasma concentration (C_{max}) and area under the plasma level time curve (AUC). The mean T_{max} was 1.2±0.2 h and the danazol half-life was 5.9±1.2 h. The relationship between exposure (AUC) and administered dose is presented in Fig. 2. The non-linearity with dose observed previously (14) was not evident in this younger cohort (Study I). The study was therefore repeated in the original cohort used in the pre-study (14). This group of animals was considerably older (mean age 8± 1.0 years.) The mean plasma concentration *versus* time profiles for danazol following oral administration to this older cohort (Study II) is shown in Fig. 1b (along with the data obtained in the previously published study). The corresponding mean pharmacokinetic parameters are summarized in Table III showing non-linear increases in C_{max} and AUC, a mean T_{max} of 1.6±0.3 h and a danazol half-life of 3.8±0.5 h across the different doses.

Table II Summary Pharmacokinetic Parameters for Danazol After Administration in the SEDDS-III Formulation Comprising Increasing Drug Loadings to the Young Beagle Cohort (Study I) [mean ± SEM ($n = 4$)] (Corresponding Plasma Profiles Are Presented In Fig. 1a)

	SEDDS-III formulation treatments in younger beagle dogs			
Danazol loading [mg/g]	5	9	20	27
Treatment dose [mg][a]	8.5	14.5	32.1	43.2
AUC_{0-10h} (ng.h/mL)	59±12	132±46	321±69	454±128
Relative BA (%)[b]	70±14	97±36	102±22	106±21
C_{max} (ng/mL)	24±4	53±20	107±21	168±33
T_{max} (h)	1.3±0.3	1.1±0.2	1.4±0.1	1.1±0.2
$t_{1/2}$ (h)	5.4±1.8	4.9±0.5	6.8±0.8	6.6±1.7

[a] Each treatment was administered in two capsules (2 × 800 mg formulation)

[b] Relative BA was the relative bioavailability (%) expressed in comparison to the danazol AUC_{0-10} obtained after oral administration of SEDDS-III at the lowest absolute dose (5 mg/g) in the older beagle cohort as determined by the ratio of the dose-normalized AUC_{0-10}

A comparison of the dose–response relationships is shown in Fig. 2a where the areas under the plasma concentration *versus* time curves in Fig. 1a and b are plotted against dose administered. For the younger cohort (closed symbols), a linear relationship was obtained indicative of dose independent pharmacokinetics, and is consistent with a previous study (27) in humans where danazol was administered in a lipid emulsion formulation in the dose range 50–200 mg (equivalent to ~0.7–3 mg/kg). The dose–response curve for the older cohort, however, displayed marked deviations from linearity where drug was incorporated in the formulation at concentrations higher than 11 mg/g (equivalent to 40% of the saturated solubility in the formulation).

Figure 2b shows the relative bioavailability for the two cohorts based on the lowest absolute dose in the older beagle cohort. In the young cohort, the relative bioavailability was constant across all doses. In contrast, in the old cohort the relative bioavailability increased with increasing dose leading to a more than 2-fold increase in the relative bioavailability at a 21 mg/g dose (70% saturation in the formulation). Above this dose, however, exposure reduced significantly and approached that obtained in the younger cohort at the same dose. This trend in exposure was consistent across individual animals in the older beagle cohort (the relative bioavailability in individual dogs (older cohort) compared to the younger cohort following administration of increasing danazol doses can be found in the Supplementary Material, S1).

Table III Summary Pharmacokinetic Parameters for Danazol After Administration in the SEDDS-III Formulation Comprising Increasing Drug Loadings to the Older Beagle Cohort (Pre-study and Study II) [mean ± SEM ($n = 3$)] (Corresponding Plasma Profiles Are Presented in Fig. 1b)

	SEDDS-III formulation treatments in older beagle dogs					
Danazol loading [mg/g]	5	10	11[a]	18	21[a]	27
Treatment dose [mg][b]	7.6	15.6	17.3	28.1	34.2	43.4
AUC_{0-10h} (ng.h/mL)	75 ± 9	133 ± 12	194 ± 41	421 ± 51	762 ± 46	617 ± 15
Relative BA (%)[c]	100 ± 12	86 ± 8	114 ± 24	152 ± 18	226 ± 14	144 ± 4
C_{max} (ng/mL)	45 ± 13	42 ± 3	104 ± 30	150 ± 38	227 ± 10	211 ± 23
T_{max} (h)	1.1 ± 0.2	2.5 ± 0.5	0.8 ± 0.3	1.5 ± 0.5	2.0 ± 0.0	1.5 ± 0.5
$t_{1/2}$ (h)	2.7 ± 0.4	2.9 ± 0.7	4.4 ± 0.7	4.7 ± 0.5	3.3 ± 0.2	4.8 ± 0.4

[a] Data reproduced from Anby *et al*. (14)

[b] Each treatment was administered in two capsules (2 × 800 mg formulation)

[c] Relative BA was the relative bioavailability (%) expressed in comparison to the danazol AUC_{0-10} obtained after oral administration of SEDDS-III at the lowest absolute dose (5 mg/g in the older beagle cohort) as determined by the ratio of the dose-normalized AUC_{0-10}.

The appearance of dose dependency in danazol bioavailability after oral administration in the older beagle cohort, but not the younger cohort, prompted a closer examination of the possible explanations for these effects, and in particular, the potential for cohort (or age) dependent drivers of non-linearity. Two working hypotheses were suggested to explain these potential trends. Firstly, that the increases in exposure with increasing dose resulted from saturation of first pass metabolism (and that differences in metabolic capabilities were evident in the two cohorts). Secondly, that the increase in exposure at higher dose resulted from increases in thermodynamic activity in the colloids formed during digestion of formulations containing higher drug loads (and that more robust solubilising conditions were prevalent in the intestinal tract of the older animals allowing ongoing solubilisation of the supersaturated solutions).

Intravenous Pharmacokinetics and Absolute Bioavailability of Danazol in Both Animal Cohorts

To provide an indication of possible differences in systemic pharmacokinetic behaviour in both animal cohorts, systemic clearance and volume of distribution were evaluated via the conduct of intravenous pharmacokinetic studies. Plasma profiles in both cohorts are shown in S2 in the Supplementary Material and pharmacokinetic parameters are tabulated below in Table IV. No significant differences in either pharmacokinetic parameter were evident in the two groups. The generation of intravenous pharmacokinetic data also allowed for estimation of danazol absolute bioavailability in the previous oral studies. These data are given in S3 and S4 in the Supplementary Material. Absolute bioavailability ranged

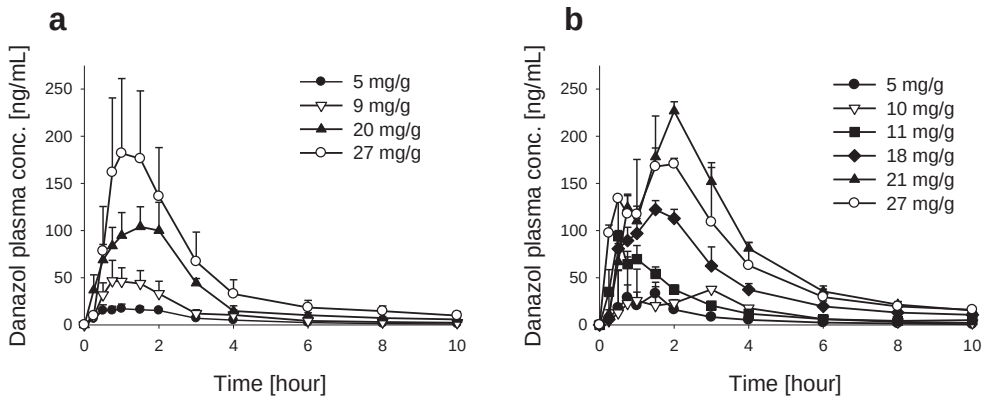

Fig. 1 Mean plasma conc. *vs* time profiles for danazol after oral administration of a SEDDS-III containing [60/30/10% w/w] of [lipid/CrEL/ethanol]. SEDDS-III administered to (**a**) a young beagle cohort (study I) with danazol loading of 5 mg/g (*black circle*), 9 mg/g (*inverted white triangle*), 20 mg/g (*black triangle*) and 27 mg/g (*white circle*) (doses are tabulated in Table II) [mean ± SEM ($n = 4$)], and (**b**) to an older cohort (pre-study and study II) with danazol doses of 5 mg/g (*black circle*), 10 mg/g (*inverted white triangle*), 11 mg/g (*black square*)[a], 18 mg/g (*black diamond*), 21 mg/g (*black triangle*)[a] and 27 mg/g (*white circle*), respectively (doses and equivalent saturation levels are tabulated in Table III [mean ± SEM ($n = 3$)]. [a]Data reproduced from Anby *et al*. (14).

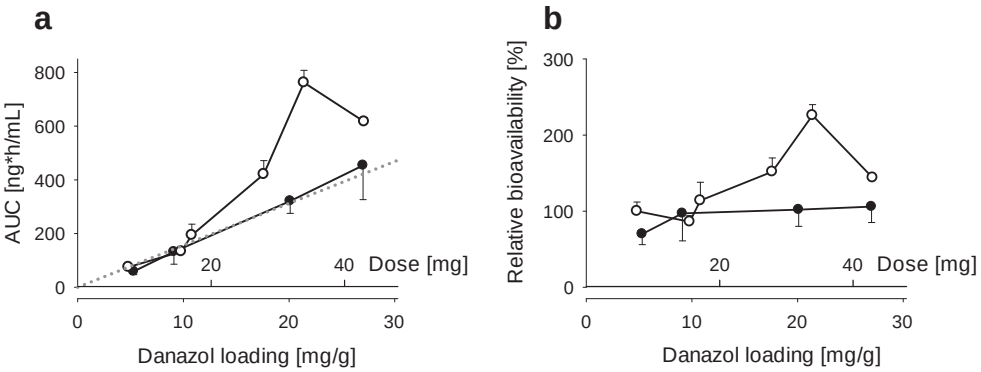

Fig. 2 Dose linearity of (**a**) danazol exposure (AUC) and (**b**) relative bioavailability after oral administration of increasing danazol doses (expressed both as absolute dose (mg) and drug concentration in the formulation (mg/g) in a type III SEDDS formulation to both a younger beagle cohort (*black circle*) [mean ± SEM ($n = 4$)] and an older beagle cohort (*white circle*)[mean ± SEM ($n = 3$)]).

from 8 to 13% in the younger animals and 10 to 26% in the older animals.

Evaluation of Liver Function in Young *Versus* Old Beagle Cohorts

To provide an additional indication of potential differences in metabolic function in the two cohorts, liver function tests were performed in both sets of animals. To assess liver function, the serum bile acid (SBA) test was conducted under pre-prandial and post-prandial conditions. Figure 3 illustrates the SBA levels for the two cohorts. Pre-prandial SBA levels of 4.9 ± 0.9 μmol/L and 7.5 ± 4.2 μmol/L and post-prandial levels of 4.8 ± 2.8 μmol/L and 10.3 ± 4.5 μmol/L were obtained for the young and old cohort, respectively. The data are consistent with previously reported SBA values for dogs under similar conditions (20,25,28). No significant differences in liver function were evident between the two cohorts, although SBA levels were, in general, slightly higher in the older dogs and showed higher variability than the equivalent data for the younger animals.

Table 4 Pharmacokinetic Parameters After Intravenous Administration of A 20% Captisol® Solution Containing Danazol (1.3 mg/mL) to the Younger Beagle Cohort [mean ± SEM ($n = 4$)] and Individual Data for Two Older Beagle Dogs

Treatment	$AUC_{0-\infty}$ [ng h mL^{-1}]	Cl [mL min^{-1} kg^{-1}]	Vd [L kg^{-1}]	$t_{1/2}$ [h]
Younger cohort	1046 ± 70	823.4 ± 54	7.9 ± 0.4	6.6 ± 0.3
Older cohort	1173; 1033	725.5; 823.1	6.1; 7.0	5.9; 5.9
Average[a]	1075 ± 70	799 ± 51	7.2 ± 0.4	6.3 ± 0.2

Data are dose normalized to 0.85 mg/kg

[a] Data from both cohorts was used to calculate absolute bioavailability in S3 and S4 [mean ± SEM ($n = 6$)]

Gall Bladder Bile Salt Concentrations in Young *Versus* Old Beagle Cohorts

Unfortunately, the advanced age of the older animals dictated that ethics approval to conduct further detailed studies could not be obtained. However, approval was obtained to collect bile immediately prior to euthanasia in two of the older animals. Similar data was also obtained in one of the younger animal cohort who was euthanized due to a deteriorating behavioural condition. The data are given in S5 in the Supplementary Material and reveal gall bladder bile salt concentrations in the young animal of 162 mM pre-mortem and 187 mM post-mortem and in the older two dogs of 194 mM and 208 mM pre-mortem and 208 mM and 240 mM post-mortem. The data are broadly consistent with previous data (253 ± 6 mM, $n = 15$ (29), 247 ± 40 mM, $n = 15$ (30) and with the SBA data (Fig. 3) that suggest slightly (but insignificantly) higher BS levels in the older animals.

Since further *in vivo* studies could not be undertaken in the older cohort, experiments were conducted *in vitro* to explore possible differences in drug solubilisation/precipitation (and accompanying differences in supersaturation and thermodynamic

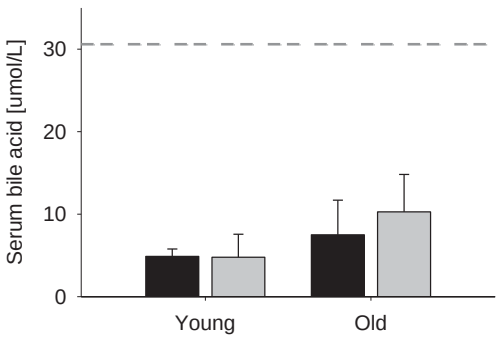

Fig. 3 Serum bile acid (SBA) concentrations measured pre-prandially (*black bars*) and post-prandially (2 h) (*grey bars*) in two beagle dog cohorts. Mean SBA levels are shown for the young [mean ± SD ($n = 4$)] and old cohort [mean ± SD ($n = 3$)]. The *upper dashed line* represents the reference value for normal liver function post-prandial conditions. No statistical significant difference was evident between old and young beagle cohort.

activity) as a function of (i) drug dose, and (ii) differing intestinal conditions (that might be expected to vary in differing cohorts) such as differences in endogenous bile salt concentrations or lipase levels in the GI tract (the latter might be expected to impact solubilisation capacity due to differential hydrolysis of digestible excipients).

In Vitro Evaluation

Impact of Increasing Drug Loading on Solubilisation During In Vitro Dispersion and Digestion at Low and High Bile Salt Levels

To evaluate the impact of drug saturation in the SEDDS-III formulation on danazol solubilisation patterns following dispersion and digestion of the formulation, a series of studies were conducted using a previous described *in vitro* lipid digestion model (14). These experiments sought to explore the impact of increasing drug load in the formulation on drug precipitation during dispersion and digestion and in particular, the potential for supersaturation stabilization. Experiments were conducted in biorelevant digestion media containing low [BS/PL] (i.e. 5 mM BS: 1.25 mM PC) and high [BS/PL] (i.e. 20 mM BS: 5 mM PC) to explore the potential for increased bile salt levels in either dog cohort to explain the *in vivo* data obtained. Data were obtained using SEDDS-III formulations at a danazol loading of 5–27 mg/g (equivalent to 15%–90% of danazol saturation in the formulation).

To gain a better understanding of the drivers of drug precipitation during dispersion and digestion and to quantify the degree of supersaturation during formulation processing, the apparent solubility of danazol in the colloidal aqueous phase (AP) generated by dispersion (AP_{DISP}) and after 5 min and 60 min digestion (AP_{DIGEST}) of a blank (drug free) formulation was also measured. The data are tabulated in Table V and show that initiation of formulation digestion led to a significant decrease in solubilisation capacity, regardless of BS/PL concentration in the digestion medium. Within 5 min of digestion initiation, danazol solubility in the AP_{DIGEST} decreased by >60% when compared to the solubility in the AP_{DISP}. As digestion continued to 60 min, danazol solubility in AP_{DIGEST} under low [BS/PL] decreased a further 2-fold compared to the solubility at 5 min. In contrast, under high [BS/PL], continued digestion (to 60 min) led to only a further ~10% decrease in danazol solubility in AP_{DIGEST}. Significant differences in danazol solubility in the AP at low and high [BS/PL] were evident at each time point, but were most obvious at 60 min post digestion.

Table V Danazol Solubility in the Aqueous Colloidal Phase Post Dispersion or During Digestion of the Drug-free SEDDS-III Formulation [mean ± SD ($n = 3$)]

	Low bile salt [5 mM][a]	High bile salt [20 mM]
Solubility in AP_{DISP} [ug/mL][b]	301 ± 3.9	331 ± 6.0[f]
Solubility in AP_{DIGEST} (5 min) [ug/mL][c]	106 ± 3.6[d]	120 ± 3.2[d,f]
Solubility in AP_{DIGEST} (60 min) [ug/mL][c]	56 ± 0.9[d,e]	109 ± 7.9[d,f]

[a] Data reproduced from Anby *et al*. (14)

[b] The solubility of danazol in the AP formed by dispersion of blank (drug-free) formulation for 10 min

[c] The solubility of danazol in the AP formed by digestion of blank (drug-free) formulation for 5 or 60 min

[d] Statistically significant difference compared to AP_{DISP} ($P < 0.050$)

[e] Statistically significant difference compared to AP_{DIGEST} (5 min) ($P < 0.050$)

[f] Statistically significant difference compared to low bile salt [5 mM] ($P < 0.050$)

The impact of drug loading in the SEDDS-III formulation on kinetic changes to danazol solubilisation during dispersion and digestion (rather than solubility in the phases formed) under low [BS/PL] are presented in Fig. 4a with the dotted line illustrating the solubility in the AP over time.

Dispersion of SEDDS-III formulations containing danazol at 5 mg/g and 9 mg/g resulted in AP concentrations below the danazol solubility limit in AP_{DISP} (time −20 to 0 min in Fig. 4a) and therefore, no drug precipitation was evident on dispersion. Initiation of digestion led to rapid changes to the nature of the colloids present in the aqueous phase as illustrated by the decrease in danazol solubility in AP_{DIGEST} (Table V). However, the moderate drug load of 5–9 mg/g in the SEDDS-III formulations resulted in relatively low drug concentrations in AP_{DIGEST}, and therefore limited precipitation on digestion. In the case of SEDDS-III containing 5 mg/g, the concentrations attained in the digest were approximately equivalent to the danazol solubility (hence the alignment of the dotted line and the black line in Fig. 4a). The concentrations attained in the AP_{DIGEST} for SEDDS-III at 9 mg/g were approximately 2-fold higher than the danazol solubility in AP_{DIGEST}, however, supersaturation was well maintained over the 60 min digestion period and precipitation was limited. Increasing the drug loading to 12 mg/g and 18 mg/g in SEDDS-III initially led to danazol concentrations above solubility in AP_{DISP}, but the degree of supersaturation generated did not lead to drug precipitation. On digestion, however, the drop in solubilisation capacity of the AP (Table V) resulted in more significant initial supersaturation and more rapid drug precipitation. This was faster at the higher drug loading (18 mg/g) compared to 12 mg/g and as a result, supersaturation was maintained for a shorter time period. Increasing the drug loading to 80% and 90% of solubility in the formulation resulted in greater drug precipitation on dispersion and supersaturation was not maintained on initiation of digestion.

Increasing the [BS/PL] in the digestion media (Fig. 4b), resulted in moderate (but significant) differences in danazol solubility in the aqueous colloidal phase on dispersion and digestion (dotted line in Fig. 4, Table V). The increase in [BS/PL], therefore, promoted maintenance of supersaturation and reduced the extent of precipitation. Thus, at raised [BS/PL], no drug precipitation was evident on dispersion regardless

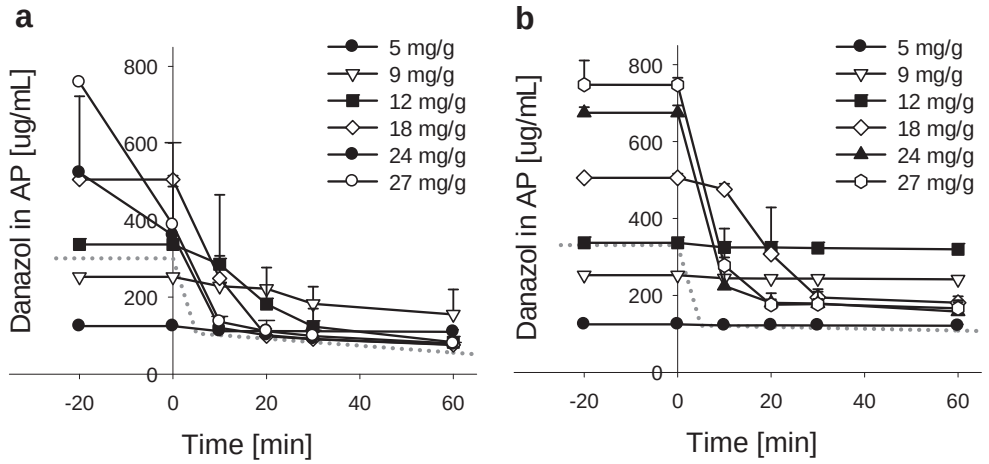

Fig. 4 Drug solubilisation profiles during dispersion (−30 to 0 min) and digestion (0 to 60 min) of SEDDS formulations with danazol loading of; 5 mg/g (*black circle*), 9 mg/g (*inverted white triangle*), 12 mg/g (*black square*)[a], 18 mg/g (*white diamond*), 24 mg/g (*black triangle*)[a] and 27 mg/g (*white circle*) under (**a**) low [BS/PL] (i.e. 5 mM BS: 1.25 mM PL), (**b**) high [BS/PL] conditions (20 mM BS: 5 mM PL). The *dotted line* indicates the drug solubility in the aqueous colloidal phase produced on dispersion (AP_{DISP}) and digestion (AP_{DIGEST}) of drug-free SEDDS-III. All data presented as mean ± SD ($n = 3$). [a]Data reproduced from Anby *et al*. (14).

of drug loading in SEDDS-III in contrast to the data at low [BS/PL]. In addition, no drug precipitation was evident and supersaturation was maintained during the 60 min digestion period for SEDDS-III containing 12 mg/g and precipitation was reduced and delayed for SEDDS-III containing danazol at up to 18 mg/g. The significant decrease in solubilisation capacity on initiation of digestion did lead to rapid precipitation for SEDDS-III when drug loading was high (24–27 mg/g), although some degree of supersaturation was maintained up to and beyond 20 min post digestion. In general, therefore, the significantly higher danazol solubility in the colloids formed under high [BS/PL] (Table V), reduced the extent of supersaturation and resulted in the maintenance of higher danazol concentrations in AP_{DIGEST} when compared to low [BS/PL].

Impact of Pancreatic Lipase Activity on Drug Solubilisation During In Vitro Digestion

To simulate the potential for differences in intestinal lipid digestion in the two dog cohorts (as a result of differences in pancreatic lipase secretion) to affect drug precipitation from the SEDDS formulation, the impact of changes to the volume of pancreatic lipase added to the digestion vessel was explored. A SEDDS-III formulation containing 12 mg/g danazol was utilized during *in vitro* digestion experiments and the impact of decreasing pancreatic lipase extract concentrations on danazol solubilisation profiles is shown in Fig. 5.

Dispersion of SEDDS-III containing danazol at 12 mg/g did not result in drug precipitation as shown in Fig. 4a, however, initiation of digestion with 4 mL of pancreatic enzyme extract led to significant precipitation and only transient supersaturation.

Figure 5 shows that decreasing the volume of pancreatic lipase extract added to the digest reduced drug precipitation during digestion, but that significant changes were only evident when the volume of lipase extract was reduced considerably (from 4000 μL to 100 μL). After 60 min digestion, drug precipitation was still evident after addition of only 100 μL lipase extract (equivalent to 10% of the original quantity), however, the degree of precipitation was much lower than that seen at higher enzyme levels.

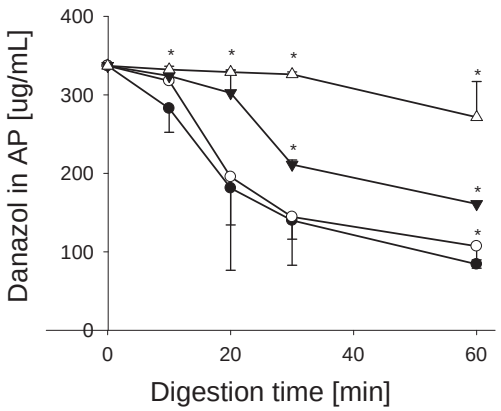

Fig. 5 Drug solubilisation during 60 min *in vitro* digestion of a SEDDS-III formulation as a function of pancreatic enzyme concentration [mean ± SD ($n = 3$)]. Danazol loading was 12 mg/g (equivalent to 40% of the equilibrium solubility in SEDDS-III). The quantities of enzyme employed were 4000 μL (*black circle*)[a], 1000 μL (*white circle*), 400 μL (*inverted black triangle*) and 100 μL (*white triangle*) of pancreatic lipase extract, where 4 mL is the volume added under normal conditions. [a] Data reproduced from Anby *et al*. (14). *Statistically significant different compared to data obtained using 4000 μL lipase extract ($P < 0.050$).

DISCUSSION

Following oral administration, the properties of the colloidal species that are formed on dispersion of SEDDS formulations typically change as digestive enzymes hydrolyse included glyceride lipids and fatty acid ester surfactants. In most cases, these chemical changes lead to decreases in solubilisation capacity for co-formulated poorly water-soluble drugs (11,31–33). Depending on the drug load in the formulation, this may result in the generation of transiently supersaturated conditions in the GI tract (14). Supersaturation has the potential to enhance absorption via increases in thermodynamic activity, but may also reduce absorption by increasing the likelihood of drug precipitation. SEDDS performance under digesting conditions is therefore a balance between an increase in supersaturation (and thermodynamic activity) and absorption promotion, and the potential for highly metastable conditions to increase drug precipitation and reduce absorption. This is further complicated where drugs are substrates for first pass metabolism.

Increases in drug dose have an inherent impact on these processes since an increase in drug dose is expected to increase the degree of supersaturation generated on formulation digestion and also to increase the likelihood of inhibition of first pass metabolism. In this regard, previous studies of danazol absorption from a LFCS type III SEDDS (termed SEDDS-III here) comprising 60% MC lipid, 30% Cremophor EL and 10% ethanol revealed non-linear increases in exposure (bioavailability) with increasing drug dose in beagle dogs (14). This prompted the current, more detailed, investigation of the impact of drug dose on danazol absorption using the same formulation. Initial studies revealed that these effects were highly dependent on the dog cohort, and the age difference in the two groups of animals employed suggested the potential for age-related differences in absorption or bioavailability. As such further studies were performed to probe the potential for age-related changes to physiology to lead to differences in danazol exposure.

Effect of Drug Dose on *In Vivo* Exposure in Older *vs*. Younger Beagles

The plasma profiles in Fig. 1 and the pharmacokinetic parameters presented in Tables II and III reveal lower exposure and a dose-dependent relationship with AUC for the younger beagle cohort with constant relative bioavailability evident across a range of drug doses (Fig. 2). This is in contrast to the previous studies conducted using an identical formulation in an older group of animals where increases in bioavailability were seen with a two-fold increase in dose (14). This prompted an extended evaluation of the dose–response in the first (older) cohort (Fig. 2). In the older cohort, deviations from linearity in dose *versus* exposure relationships were apparent after administration of formulations containing danazol at concentrations above 12 mg/g in the formulation (the mass of SEDDS-III formulation was constant across all studies so increases in saturation level in the formulation resulted in increases in drug dose) (Fig. 2a). Relative bioavailability increased with dose until a 'tipping point' was reached at doses equivalent to concentrations of 21 mg/g danazol in the SEDDS-III formulation (71% of drug solubility in the formulation). Above this, relative exposure dropped significantly and approached that obtained in the younger cohort (Fig. 2b).

The variation in exposure as a function of dose and animal cohort, and the fact that the increase in exposure in the older cohort was evident only to a critical point suggests the potential for multiple controlling factors. Increasing age is known to alter various physiological parameters and may change absorption and bioavailability. In particular, gastrointestinal changes are evident in older animals including reduced gastric acidity (although this might not occur in dogs (34) and gastric pH is often higher in dogs when compared to humans (35)), gastric emptying (36) and intestinal motility (37,38). Changes in permeability of the intestinal tissue (39) and a reduction in renal and/or hepatic drug elimination have also been described with increases in age (40,41). Several studies have addressed permeability related issues from solubilising formulations, and have shown that permeability is critically dependent on colloid properties (42–45). Age-related changes might therefore be expected to influence permeability directly, via physiological changes to the absorptive membrane, and indirectly via changes to GI luminal conditions, thereby altering colloidal structure.

Previous studies have also shown that oral administration of (^{14}C) labelled danazol to rats leads to significant (~70%) biliary excretion of danazol metabolites and extensive enterohepatic recycling (46). *In vitro* studies utilizing pooled human liver microsomes and cytochrome P450 inhibitors further suggest that danazol is a substrate for CYP-enzymes, in particular CYP3A4 (47). A significant role for CYP-mediated metabolism in danazol clearance in dogs is therefore likely and is consistent with the potential for increases in danazol exposure with dose to result from saturation of first pass metabolism. It is less clear, however, why this might only be evident in the older animals (unless there are significant differences in hepatic function across the two cohorts), or why these effects are only evident up to a certain dose in the older animals.

An alternate explanation for the increase in absorption lies in the potential for increases in dose to increase thermodynamic activity in the colloidal species produced post digestion of the SEDDS-III formulation (through induction of supersaturation). The degree of supersaturation generated by dose escalation might also be expected to ultimately lead to spontaneous nucleation in the supersaturated solution resulting in precipitation and reduced absorption (and bioavailability) at the highest doses. This suggestion is consistent with the trends in absorption with dose observed *in vivo* in the older cohort.

In this latter scenario, the differences in exposure in the two beagle cohorts might be explained by differences in intestinal conditions, such as differences in digestive enzyme levels or bile salt secretion, that lead to differences in solubilisation capacity and/or an altered capability to support supersaturation. In an attempt to clarify these possibilities, a series of *in vitro* and *in vivo* studies were therefore undertaken to explore the potential for differences in metabolism or intestinal solubilisation to explain the differences in exposure observed in the dog studies.

Differences in Serum Bile Acid Levels and Systemic Clearance as an Indicator of Age-Related Changes to Danazol Metabolism

The potential for changes in liver function to lead to different degrees of pre-systemic hepatic metabolism in the two beagle cohorts was assessed via comparison of pre- and postprandial serum bile acid (SBA) levels and by monitoring for changes in systemic clearance after intravenous administration. Many aspects of hepatobiliary function are involved in bile acid metabolism, including bile acid synthesis by cytochrome P450 hydroxylation of cholesterol in the pericentral hepatocytes, bile acid conjugation with e.g. amino acids (i.e. glycine or taurine), and ultimately active secretion into bile (22,48,49). Due to the structural similarities between danazol and the endogenous steroids involved in bile acid metabolism, differences in serum bile acid levels were utilised to provide an indicator of potential differences in microsomal hydroxylation and conjugation of danazol across the two cohorts.

The SBA test showed no significant differences in SBA levels pre- and post-prandially for either cohort, and SBA levels were within the range of previously published reference values (20,25,28). Comparison between cohorts revealed an insignificant increase in SBA with age, and in particular identified a single dog in the older cohort that had a higher SBA level (albeit within normal levels) compared to the other animals. However, this difference was not replicated across other animals, and although differences in serum bile acid levels have been reported in older *versus* younger rats, this was previously suggested to reflect differences in bacterial growth in the GI tract (and therefore changes to enterohepatic recycling) rather than differences in bile acid secretion (50). Differences in GI bacterial growth have also been reported in aged dogs (51), however, it is unclear whether this has any impact on systemic danazol levels via differences in enterohepatic recycling, and little evidence of recycling was apparent in the oral profiles obtained here (Fig. 2). Note that double peaks were seen in the early time periods of the mean plasma profiles in the older animals (Fig. 2b), however, this reflected differences in T_{max} in individual dogs rather than the occurrence of double peaks in individual animals, and thus was likely to be unrelated to enterohepatic recycling.

The SBA data therefore suggest limited differences in liver function across the two cohorts and do not support the suggestion that the differences in dose linearity and bioavailability across the groups reflect differences in hepatic first pass metabolism. The data obtained following intravenous administration of danazol (Table IV and S2 in Supplementary Material) further supports this suggestion and failed to show differences in systemic clearance across the two cohorts. This is also consistent with the fact that danazol bioavailability at the highest dose was similar in both cohorts, a situation that would seem at odds with significant differences in first pass metabolic function. Differences in pre-hepatic, pre-systemic metabolism may also play a role in differences in danazol disposition however, the lack of readily accessible biomarkers for enterocyte-based metabolic function precluded further assessment here.

Age-Related Changes to Drug Absorption from the Gastrointestinal Tract

Several studies have addressed the potential for age related physiological changes to alter drug disposition. These are well reviewed in Cusack (52), Fahey *et al*. (53) and McLean & Le Couteur (54). Physiological changes include changes to body weight, intestinal permeability, gastric pH, gastric emptying and gastric motility. Increases in body fat, for example, can lead to changes in volume of distribution due to accumulation in the adipose tissue, thereby prolonging elimination half-life and exposure for lipid-soluble drugs (52,54). In the current study, the older animal cohort was 5–10 kg heavier than the younger cohort and might therefore be expected to carry a higher proportion of body fat. The apparent half-life of danazol was, however, not significantly different in either group suggesting that differences in body weight were unlikely to explain the observed exposure differences. The IV study (Table IV and S2 in Supplementary Material) further supports the lack of significant differences in volume of distribution (Vd) between the two cohorts.

Intestinal permeability has also been reported to increase for some drugs in older animals (rats), due to age-related changes to membrane permeability in the enterocyte (39). However, for a typical class II BCS drug like danazol, intestinal permeability is expected to be relatively high and for most substrates, passive intestinal permeability appears to be unchanged with age (55,56). Increases in gastric pH, delays in gastric emptying and decreases in gastrointestinal motility are also commonly associated with increased age (40). These changes might all be expected to alter absorption and potentially increase absorption (although pH is unlikely to alter the absorption of an non-ionizable drug such as danazol), but are rarely clinically significant (52) and seem unlikely to explain

the increase in bioavailability seen here at higher doses when compared to lower doses.

For poorly water-soluble drugs such as danazol, drug solubility in the GI fluids is expected to be the most significant determinant of drug absorption. Changes in *in situ* solubilisation are therefore likely to have the most significant impact on absorption. Indeed recent studies have reported age-related increases in danazol solubility in human GI fluid and coincident increases in intestinal bile salt concentrations (although the changes observed were not statistically significant due to high variability within groups (39)). The authors reported a ~2-fold increase in danazol solubility in human intestinal fluid with age, and concluded that this may have an impact on drug absorption profiles and bioavailability in older subjects. In light of the importance of intestinal solubility in danazol absorption and the potential for increasing solubilisation properties and bile salt concentrations with age, a more detailed evaluation of the potential impact of changes to GI solubilisation capacity on danazol solubilisation during the digestion of SEDDS was undertaken. These studies focussed on the impact of potential changes to lipolysis and bile salt secretion in older animals since both are critical to patterns of drug solubilisation during the intestinal processing of a lipid based SEDDS.

Impact of Lipase Activity on Drug Solubilisation during SEDDS Digestion in the GI Tract

Following oral administration of SEDDS, the solubilisation capacity of the formulation (or the colloidal species generated by intercalation of the formulation components into the lipid digestion cascade) typically decreases as a result of lipid digestion. The degree of digestion (and potentially, therefore, the extent of solubilisation) is expected to be dependent on the quantity and activity of secreted pancreatic lipase (the main lipase responsible for lipid digestion in the GI tract) (57). To evaluate the impact of pancreatic lipase levels on digestion and subsequent drug solubilisation, the effect of changes to the quantity of pancreatic enzyme employed in an *in vitro* digestion model of SEDDS processing in the GI tract, was therefore explored. The data are summarized in Fig. 5.

Under normal conditions, the specific activity of pancreatic lipase in the dog is high (58) and similar to that in humans (59). In the current *in vitro* experimental protocol, replication of the lipase activities expected in the human (or canine) GI tract resulted in significant formulation digestion and danazol precipitation. In order to probe the potential impact of physiological changes in enzyme activity or in the quantity of enzyme secreted in the different animal cohorts, these studies were also conducted using decreasing quantities of enzyme extract. Decreasing the quantity of enzyme extract employed, however, had little impact on digestion or drug precipitation and very large decreases were required before appreciable differences in drug precipitation were evident. Lower lipase secretion in the older cohort, resulting in reduced lipid digestion and increased solubilisation capacity, is therefore unlikely to explain the higher danazol absorption seen in the older cohort. Indeed, the digestibility of protein and fat in beagle dogs has previously been reported to be higher in older dogs when compared to weanlings (60).

Impact of Bile Salt Concentration on Drug Solubilisation in the GI Tract

A subsequent study evaluated the bile salt concentration recovered in the gall bladder from one young and two older beagles from the two cohorts. Acknowledging the limited number of animals, the data supports the suggestion that BS levels in the older dogs may be slightly higher when compared to the younger dogs. These trends were evident both pre- and post-mortem (data are presented in S5 in Supplementary Material) and are consistent with previous studies in humans (39) where a tendency towards elevated bile concentrations was noted with age (7 ± 4 mM *vs* 5 ± 3 mM), although the differences in this case were not statistically significant. It is difficult to predict with accuracy how the differences in gallbladder bile concentrations might be manifest in changes to luminal BS concentrations, since the degree of dilution is unknown. It is also apparent that local concentrations in areas of the GI tract may vary significantly during the dilution process. Nonetheless, the available data support the possibility that in some areas of the GI tract BS levels may be higher in older subjects.

The influence of [BS/PL] on drug solubilisation and the kinetics of drug precipitation during formulation dispersion and digestion were evaluated *in vitro* and the data are shown in Fig. 4. Two BS/PL conditions (low (5 mM BS: 1.25 mM PL) and high (20 mM BS: 5 mM PL)) were employed to provide a proof-of-concept indication of whether changes in intestinal bile salt concentrations (should they be evident across the two animal cohorts) might explain the patterns of absorption seen in the *in vivo* studies.

After dispersion and initiation of digestion, the patterns of drug solubilisation varied significantly over time revealing simultaneous effects of both drug loading in the formulation and the [BS/PL] in the digestion medium. To facilitate better comparison of the results over time, the solubilised drug concentration as a function of the quantity of drug in the formulation under high and low [BS/PL] at different digestion times are presented in Fig. 6. The figure also illustrates the impact of digestion time on drug solubilisation in an attempt to present relative *in vitro* 'exposure' at different time points.

Following 10 min dispersion (Fig. 6a), linear increases in *in vitro* solubilisation/exposure were apparent with increases in drug concentration in the formulation under most conditions. However after 30 min of dispersion, some precipitation was

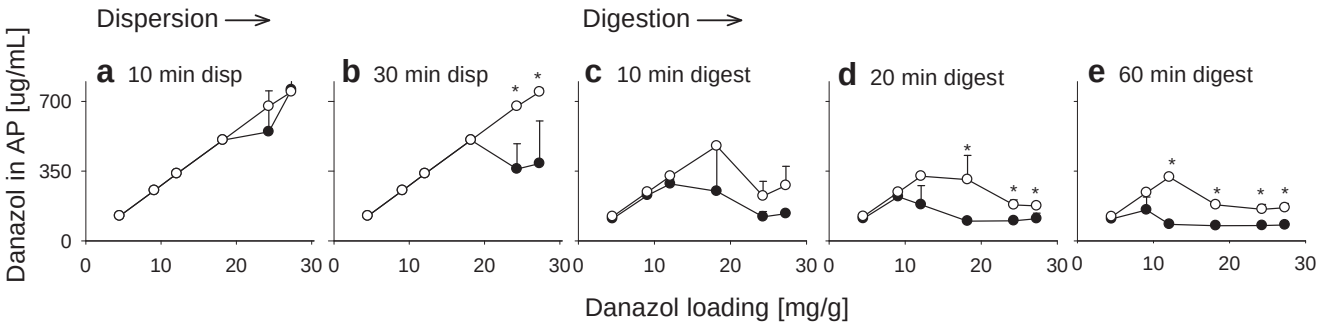

Fig. 6 Drug solubilisation *versus* initial danazol saturation level in the formulation during *in vitro* digestion at low [BS/PL] (*black circle*) and high [BS/PL] (*white circle*) [mean ± SD ($n = 3$)]. Individual figures represent time points in Fig. 4; (**a**) early dispersion (−20 min, ie after 10 min dispersion), (**b**) the end of the dispersion period (time zero, ie after 30 min dispersion); (**c**) 10 min post digestion initiation (10 min), (**d**) 20 min post digestion initiation (20 min) and (**e**) 60 min post digestion initiation (60 min). *Statistically significant different compared to danazol solubilisation under low [BS/PL] ($P < 0.050$).

evident at low [BS/PL] and higher drug loads (Fig. 6b). Initiation of digestion resulted in a rapid drop in the solubilisation capacity of mixed colloidal species present in the digest (Fig. 4). For example, at low [BS/PL], drug solubility dropped from 301 μg/mL in the dispersed formulation to 106 μg/mL after only 5 min of digestion (Table V). However, at low drug loads (5 mg/g) the quantity of drug in the formulation was sufficiently low that it remained below the solubility limit throughout the digestion period and no precipitation occurred at either [BS/PL]. At drug loads of 9 mg/g, precipitation was evident, but minimal, and only observed under low [BS/PL] after 30 min digestion.

As such, linear increases in *in vitro* exposure with increases in drug dose up to 9 mg/g were evident on digestion in almost all cases (Fig. 6). Higher drug loading, however, did result in precipitation, and this was more dependent on [BS/PL] concentration. Thus, at low [BS/PL], linear increases in *in vitro* 'exposure' were evident only up to drug loads of 12 mg/g (representing ~40% of drug solubility in the formulation) and then only up to 10 min post digestion (Fig. 6c). At later time points and at higher drug loads, precipitation was more significant and further increases in drug load led to little additional solubilisation benefit (Fig. 6c–e, filled symbols). In contrast, at the higher [BS/PL] (Fig. 6, open symbols), solubilisation increases with dose were more apparent and total quantities of solubilised drug were significantly greater, especially at later time points (Fig. 6c–e, open symbols).

The data in Fig. 6 therefore provide a possible explanation for more robust absorption in the older animal cohort in the event that intestinal BS concentrations were elevated as described previously (39). However, the *in vivo* data shows that bioavailability (not just exposure) increased with increasing dose in the older animals. This suggests the presence of a mechanism by which increasing dose increases bioavailability rather than simply maintaining linear increases in exposure. Closer examination of Fig. 4 provides some indication of a possible means by which bioavailability increases with dose may occur, and also a rationale for how this might be more prevalent under increased [BS/PL]. Thus, initiation of digestion resulted in a very significant reduction in equilibrium solubility of drug in the digested formulation (dotted line in Fig. 4, Table V) and yet drug precipitation, was either avoided or delayed in many cases. This results in supersaturation (14), an increase in thermodynamic activity, and therefore a rationale for increases in membrane flux and (potentially) bioavailability. Importantly, the degree of supersaturation produced was highly dependent on dose and also sensitive to [BS/PL]. This is well illustrated in Fig. 7 where the data have been

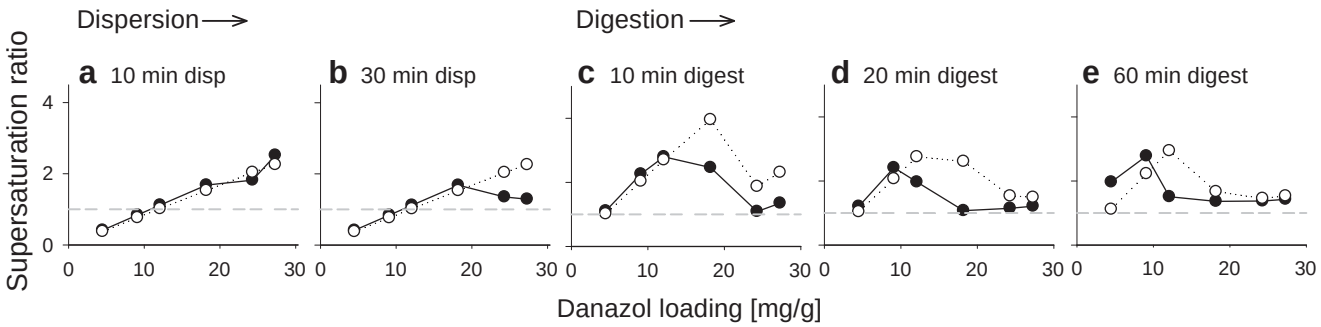

Fig. 7 Supersaturation ratios *versus* danazol load in the formulation during *in vitro* digestion at 5 mM (*black circle*) and 20 mM (*white circle*) bile salt concentration [mean ± SD ($n = 3$)]. Individual figures represent time points in Fig. 4; (**a**) early dispersion (−20 min, ie after 10 min dispersion), (**b**) the end of the dispersion period (time zero, ie after 30 min dispersion); (**c**) 10 min post digestion initiation (10 min), (**d**) 20 min post digestion initiation (20 min) and (**e**) 60 min post digestion initiation (60 min). The *dashed grey line* illustrates a supersaturation ratio of 1 with ratios above this line indicating supersaturated conditions.

presented as the supersaturation ratio (i.e. the ratio of the solubilised drug concentration in the digest to the solubility of drug in the same colloids).

A supersaturation ratio of 1 suggests no increase in thermodynamic activity and in the absence of solubility limitations might be expected to translate into linear increases in exposure with dose. In contrast, supersaturation ratios above 1 dictate an increase in thermodynamic activity and the potential for non-linear increases in exposure or increases in bioavailability. On dispersion, it is apparent that increasing dose resulted in an increase in supersaturation ratio under most conditions. After initiation of digestion, however, the degree of supersaturation increased at lower drug loads and decreased at higher drug loads (due to precipitation), resulting in a parabolic relationship between drug load and supersaturation (similar to the parabolic relationship between bioavailability and dose seen in the older cohort (Fig. 2)). This was most marked under higher [BS/PL], consistent with the hypothesis that elevated bile salt levels in the older cohort may provide an explanation for non-linear increases in bioavailability at moderate drug loads, whereas at the highest drug loads, increases in precipitation ultimately limit bioavailability enhancement.

CONCLUSION

In the current study, danazol absorption from SEDDS formulations containing drug at increasing drug loads was examined in two cohorts of beagle dogs, one younger (9 months) and one older (8 years). In the younger animals, linear increases in exposure were evident and bioavailability remained constant, even after administration of formulations where drug was dissolved in the formulation at up to 27 mg/g (90% of saturated solubility in the formulation). This occurred in spite of *in vitro* studies that suggested significant drug precipitation as the formulations were digested in the GI tract. Even more surprisingly, in the older cohort, not only was bioavailability maintained with increasing dose, bioavailability increased with increasing dose up to a tipping point (drug dissolved at 21 mg/g in the formulation and equivalent to 70% saturation in the formulation), beyond which bioavailability returned back towards that observed in the younger cohort.

Unfortunately, definitive data to explain the results obtained remain elusive. Danazol has been reported to be a substrate of CYP3A (47) and therefore saturation of pre-systemic metabolism at increasing dose might be expected to provide an explanation for increases in exposure with dose. Similarly, increases in thermodynamic activity with increases in dose might also promote exposure. In contrast, increases in drug dose are also expected to encourage drug precipitation from the digesting formulation and to therefore reduce exposure with increasing dose. The net effect of these three competing forces is likely to dictate the ultimate profile of exposure with dose. This is illustrated in Fig. 8, where the potential relationship between dose and exposure for solubility-limited exposure (dotted line) and first pass/thermodynamic activity enhanced exposure are shown (dashed line).

In the current studies, it seems likely that in the younger cohort as dose increased, negative effects on exposure mediated by increased precipitation with increasing dose were attenuated by positive effects on exposure mediated by saturation of first pass metabolism or increases in thermodynamic activity. The net result was therefore the observed linear relationship between dose and exposure (Fig. 2).

In contrast, in the older animals, the increase in bioavailability observed up to a critical drug load (21 mg/g) was consistent with the maintenance of more robust solubilisation conditions in the GI tract such that the benefits obtained by an increase in thermodynamic activity or saturation of first pass metabolism outweighed the decreases in exposure due to precipitation. Under these conditions exposure is expected to increase to a critical point or critical supersaturation ratio, beyond which precipitation pressures start to outweigh solubilisation and supersaturation capacity and exposure is reduced.

These suggestions are consistent with the available *in vivo* data, but require a physiological change in the older animal cohort to drive the differences seen. One explanation would be a notable difference in metabolic behaviour in the two cohorts such that first pass metabolism is more readily saturated in the older animals. However, the serum bile salt assay data and clearance data suggest limited differences in hepatic function between the two animal cohorts (at least in steroid

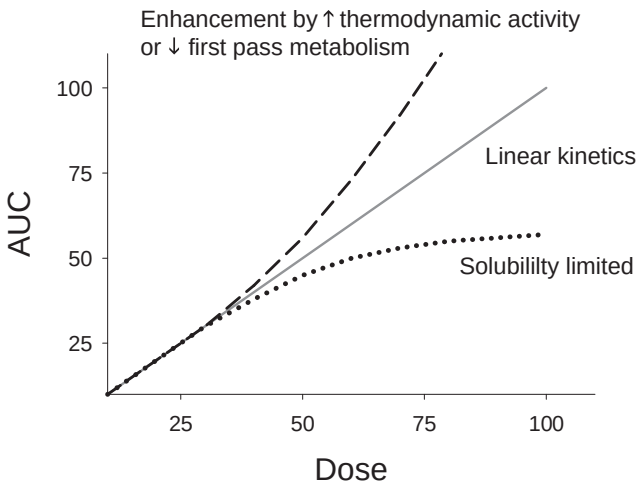

Fig. 8 Theoretical relationship between exposure and dose following oral administration in a solubilized formulation of a poorly water-soluble drug that undergoes first pass metabolism. The plot illustrates the exposure following linear kinetics (*solid line*) and the potential beneficial effects of saturation of first pass metabolism or promotion of supersaturation and thermodynamic activity (*dashed line*) and the unfavourable effect of solubility-limited absorption (*dotted line*)

processing), and the literature provides little evidence to support significant reductions in hepatic enzymatic activity (as an indicator of pre-systemic (hepatic) metabolic activity) with age (52,54,61,62). The latter suggestion, however, should be viewed with the caveat that hepatic function is notoriously difficult to capture with a single biomarker, and that differences in enterocyte-based metabolism may also be responsible for differences in metabolic activity.

Alternatively, differences in first pass metabolism may be limited and instead differences across the two cohorts may be driven by differences in intestinal conditions and therefore absorption. This is consistent with recent reports describing increases (albeit non-significant increases) in danazol luminal solubility in the elderly (39) and supported by subsequent analysis of gallbladder BS levels in the two cohorts here, although the data set (and the magnitude of the differences) is limited. To explore the potential impact of changes in intestinal conditions on exposure, we examined the impact of changes to drug solubilisation with increasing drug dose under differing [BS/PL] and these data provide conditions and data consistent with the scenario described above. Thus, *in vitro* digestion data obtained at higher [BS/PL] resulted in more sustained drug solubilisation and greater supersaturation than was evident at lower [BS/PL]. Increased luminal [BS/PL], resulting in prolonged solubilisation and increased thermodynamic activity, is therefore a plausible explanation for the non-linear increases in bioavailability observed in the older *versus* younger animals. The increase in absorption stemming from an increase in thermodynamic activity might also be expected to lead to more effective saturation of first pass metabolism, leading to even greater increases in exposure.

In summary, the current studies illustrate the complexity of interpretation of dose-linearity studies from solubilised formulations, especially where first pass metabolism provides a limitation to bioavailability. The data also show that differences in animal cohorts can have a significant impact on absorption, and at least in the case of danazol, that under some circumstances bioavailability appears to be enhanced in older animals. *In vitro* experiments suggest that this could be explained by an increase in luminal [BS/PL] in these animals resulting in more robust solubilisation, increased supersaturation and enhanced exposure. These trends are likely to be amplified in the event of significant first pass metabolism, although the latter has not been directly studied here.

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