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RESEARCH PAPER

Predicting Intestinal Precipitation—A Case Example for a Basic BCS Class II Drug

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ABSTRACT

Purpose To investigate the prediction accuracy of *in vitro* and *in vitro/in silico* methods for *in vivo* intestinal precipitation of basic BCS class II drugs in humans.

Methods Precipitation rate of a model drug substance, AZD0865 (pKa = 6.1; log K_D = 4.2), was investigated *in vitro* using simulated intestinal media, and calculations of the crystallization rates were made with a theoretical model. Human intestinal precipitation was estimated by analysis of pharmacokinetic data from clinical studies at different doses.

Results All *in vitro* models predicted rapid drug precipitation, where the intestinal concentration of dissolved AZD0865 at the highest dose tested was expected to decrease to half after less than 20 min. However, there was no indication of precipitation *in vivo* in humans as there was a dose proportional increase in drug plasma exposure. The theoretical model predicted no significant precipitation within the range of expected *in vivo* intestinal concentrations.

Conclusions This study indicated that simple *in vitro* methods of *in vivo* precipitation of orally administered bases overpredict the intestinal crystalline precipitation *in vivo* in humans. Hydrodynamic conditions were identified as one important factor that needs to be better addressed in future *in vivo* predictive methods.

KEY WORDS absorption-biopharmaceutics classification system · gastrointestinal · *in vitro/in vivo* correlations (IVIVC) · precipitation

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INTRODUCTION

Precipitation of solid drug particles in the gastro-intestinal (GI) tract that affects both the rate and extent of intestinal drug absorption might be a factor that significantly contributes to the low and highly variable bioavailability observed for some low solubility drugs. A pre-requisite for such precipitation is that a supersaturated solution of the drug is formed within the intestinal lumen. This can, for instance, occur when using aqueous co-solvent solution formulations with exponential relationship between equilibrium solubility and concentration of co-solvent, *i.e.* the solubility decreases faster than the concentration of co-solvent when the vehicle is diluted in the GI tract. Supersaturation is also possible for solid-state forms of the drug having higher solubility than the thermodynamically most stable form, *e.g.* salts, solvates, polymorphs or amorphous drug. Intestinal precipitation could also occur for basic drugs, irrespective of drug form or formulation, as a consequence of the pH increase from acidic in the stomach (especially in the fasted state) to neutral in the small intestine.

The duration of supersaturation conditions in the GI tract will vary greatly depending on factors that directly affect the intestinal concentration, such as gastric emptying, motility, intestinal drug permeability, drug solubilisation by bile acid micelles, and drug dissolution and reabsorption of water in the intestine. The crystallization rate is dependent

on the degree of supersaturation, but can also be strongly influenced by stirring caused by the GI motility and additives in the formulation or components of the intestinal media. Examples of such effects related to hydrodynamics and additives on precipitation have been shown in other pharmaceutical and non-pharmaceutical fields (1). Thus, predictions of intestinal drug precipitation need to consider the dynamics of all these factors.

Prediction of the extent and influence of intestinal precipitation of basic drugs after oral administration has previously been described in the literature (2–8). Dai *et al.* have described 96-well plate experiments where precipitation has been measured over time in small volumes of various simulated gastrointestinal media, suitable for screening of different additives for prevention of precipitation (2). A number of different *in vitro* models using multi-compartment systems for detection of incomplete dissolution and precipitation have also been reported (3–8). Kostewicz *et al.* created a dynamic system where substance dissolved in simulated gastric fluid, SGF, was pumped into simulated intestinal media while detecting precipitation (4). Similar systems have later been developed by adding absorption chambers either by the use of absorption across CaCo-2 cells or separate absorption vessels controlled by pumping fluid out of the intestinal chamber (5–8).

There is a need to validate such *in vitro* methods aiming to capture the effects of intestinal precipitation on bioavailability by comparison to *in vivo* data. However, studies published so far have neither clearly verified precipitation *in vivo* nor distinguished this effect from other factors influencing absorption such as slow dissolution. The lack of knowledge of the relation between precipitation and human bioavailability makes it difficult to quantitatively determine the correlation to *in vitro* precipitation models (8). One empirical connection between absorbed amount of drug across a Caco-2 cell membrane in the *in vitro* model by Kobayashi *et al.* and *in vivo* human absorption ratio was established for relatively soluble substances (5), but later found to be invalid for poorly soluble compounds (7).

Crystallization theories state that crystalline precipitation rate will be faster at higher supersaturation (9). It is therefore expected that there should be a concentration or dose cut-off value where precipitation of poorly soluble bases starts to affect the *in vivo* absorption rate. Pharmacokinetic dose-linearity studies should therefore be especially useful to evaluate the occurrence of intestinal precipitation, which was the approach taken in this study.

The overall objective of this project was to apply a more fundamental approach in evaluating *in vivo* intestinal precipitation and its relation to *in silico/in vitro* prediction methods for a basic BCS class II drug (AZD0865). The model compound was selected since it could create significant supersaturation levels in the small intestine at clinical doses and has solid-state properties favoring rapid crystalline precipitation. We investigated if simple theoretical (9) and *in vitro* methods, such as the one reported by Kostewicz *et al.* (4), or other variants using simulated GI fluids, correctly predict *in vivo* precipitation by quantitatively comparing predictions to *in vivo* pharmacokinetics at increasing oral doses.

MATERIALS AND METHODS

Chemicals

AZD0865 (pKa = 6.1; log K_D = 4.2) was synthesized and purified at AstraZeneca. The purity was a minimum of 99.8%. The molecular structure of AZD0865 is shown in Fig. 1. Polyethylene glycol, PEG400 (Clariant), Ethanol, EtOH (99.5%, Kemetyl AB), hydrochloric acid, HCl, concentrated 37% (Merck), sodium hydroxide, NaOH, pellets (Scharlau and Merck), sodium chloride, NaCl, (Scharlau), sodium dihydrogenphosphate monohydrate, $NaH_2PO_4*H_2O$, (Merck), N,N-dimethylacetamide, DMA (99+%, Aldrich), the sodium salt of Aerosol OT, AOT (99%, Cytec Industries Inc), polyvinylpyrrolidone, and PVP K30 (BASF) were used as received. Sodium taurocholate, NaTC, from Biosynth AG and Prodotti Chimici E Alimentary S.p.A., was minimum 97% pure, and lecithin, Lipoid E PC S with a minimum purity of 96%, was purchased from Lipoid GmbH.

Differential Scanning Calorimetry, DSC

The melting temperature, T_m, and the enthalpy of melting, ΔH_m, of crystalline AZD0865 to be used for theoretical calculations and predictions of crystallisation were determined by differential scanning calorimetry, DSC, using a Mettler-Toledo DSC 820 in an open vial configuration and a scanning speed of 10 K/min.

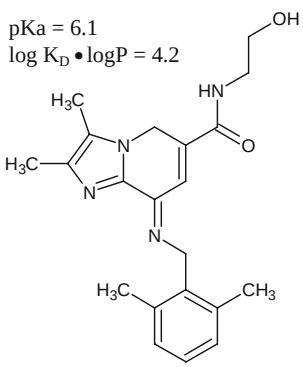

Fig. 1 Molecular structure of AZD0865. Physicochemical data from Ref (10).

Test Fluids for Solubility and *In Vitro* Precipitation Studies

Simulated gastric fluid, SGF, was prepared according to USP 32-NF 27 (11) without pepsin. Fasted State Simulated Intestinal Fluid (FaSSIF) was prepared using a slightly modified version of the fluid described by Galia *et al.* (12) using only sodium buffer ions instead of sodium and potassium ions. The FaSSIF was also used in a concentrated form in order to allow mixing with gastric fluid, providing the same pH and final concentration of taurocholate and lecithin after dilution in precipitation tests as in original FaSSIF.

Human intestinal fluid (HIF) was collected and pooled from 15 healthy volunteers in the fasted state. The HIF was collected from the proximal part of the jejunum using the Loc-I-Gut method (13,14). The HIF was then stored at −70°C until the day of experiment and was then thawed at room temperature. HIF was used for experiments within 2 years from sampling date. The sampling of HIF was approved by the Ethics Committee of Uppsala University.

Model Drug Solubility in Different Media

Solubility tests of AZD0865 were performed in pure water, SGF, FaSSIF and fasted HIF at 37°C. An excess of powder was added to 5 ml of fluid in glass vials and put into a heated shaking bath for 24 h. All tests were made in duplicates. The samples with FaSSIF and HIF were then centrifuged at 37°C, 9500 rcf for 15 min. A fraction of the supernatant was sampled and diluted for analysis. In the case of water and SGF solubility, the samples were filtered with a 0.2 μm hydrophilic PTFE filter (Dismic®-13HP; Advantec), and the filtrate was diluted for analysis using reversed phase HPLC-UV.

Formulations

Aqueous co-solvent solutions of AZD0865, which were used in the human clinical trials and in some of the *in vitro* trials, were prepared by addition of 20% PEG400 and 5% EtOH, and they were adjusted to pH 3. The solutions had concentrations of AZD0865 in the range of 0.1–8.6 mM. AZD0865 mesylate tablets 14, 45, and 100 mg, expressed as base equivalents, were also included in the human trials. The mean particle size of the mesylate salt particles was aproximately 50 μm. The tablets contained microcrystalline cellulose, mannitol, polyvinyl pyrrolidone (PVP), sodium starch glycolate and sodium stearyl fumarate, and they were manufactured by standard manufacturing processes including dry mixing of ingredients, wet granulation, drying, additional mixing and tableting. The different tablet strengths had dose-proportional compositions using the same granulate. An additional tablet was used for *in vivo* clinical studies of a single tablet including 100 mg dose of the free base of AZD0865. The mean particle size of the free base particles was 2.5 μm. The same composition and manufacturing processes were used as for the other tablets containing AZD0865 mesylate. All tablets were designed for immediate release and dissolved rapidly in the gastric environment.

A second solution vehicle that contained only 0.03 M HCl was manufactured for *in vitro* studies in order to be able to detect vehicle effects of PEG400/EtOH. The concentrations of AZD0865 in these solutions were 3.9–12.3 mM.

Theoretical Prediction of Crystallization Rate

According to classical theory of crystallization, crystalline precipitation from a solution follows a two-step process: nucleation and subsequent particle growth. We have previously presented a theoretical approach of describing and obtaining parameters for theoretical calculations of crystallization rate for BCS class II drugs (9). The nucleation rate, J, or net production of critical clusters per unit time and unit bulk volume, is a function of the steady-state concentration of clusters that overcomes the energy barrier for creating stable nuclei and the transport of monomers to these critical clusters as described in Eq. 1:

$$J = N_A \psi^* 4\pi R^* D_0 C_b C_{n^*} Z \tag{1}$$

where N_A is Avogadro's constant, D_0 is the monomer diffusion coefficient and C_b is the monomer concentration in the bulk solution. In order to correct for a non-diffusional transport of monomers to clusters, the correction term ψ^*, which is obtained from Eq. 2, was included.

$$\psi^* = \frac{R^*}{\lambda + R^*} \tag{2}$$

where the constant λ is a function of a surface integration factor that mirrors the resistance of directly attaching a molecule to the cluster surface. The radius of a critical cluster, R^*, is given by Eq. 3:

$$R^* = \frac{2\gamma_{sl}(V_m/N_A)}{k_B T \ln(C_b/S_0)} \tag{3}$$

where the interfacial tension, γ_{sl}, is the surface free energy between the solid and the liquid phase, V_m is the molar volume of the drug crystal, k_B is Boltzmann's constant, T is the absolute temperature, S_0 equals the intrinsic solubility

and T is the absolute temperature. The concentration of critical nuclei (C_{n*}) used in Eq. 1 is given by Eq. 4:

$$C_{n*} = C_{tot} \exp\left(-\Delta G^* / k_B T\right) \tag{4}$$

$$\Delta G^* = \frac{16\pi\gamma_{sl}^3 (V_m/N_A)^2}{3(k_B T \ln(C_b/S_0))^2} \tag{5}$$

where ΔG^* is the free energy of forming a critical cluster from free monomers (see Eq. 5), C_{tot} is the total concentration of substance in the system (approximately the free monomer concentration). The final factor in the expression for nucleation rate (Eq. 1) is the Zeldivich factor, $\mathcal{Z}$ (see Eq. 6), which corrects for deviations from the Boltzmann expression in Eq. 4 and dissociation of critical clusters into subcritical ones.

$$\mathcal{Z} = \frac{(k_B T)^{3/2} (\ln(C_b/S_0))^2}{8\pi\gamma_{sl}^{3/2} (V_m/N_A)} \tag{6}$$

The supercritical clusters will continue to grow according to a modified version of Fick's law of diffusion, given in Eq. 7, where R represents the radius of the growing particle. The variable ψ is similar to Eq. 2, but varies with radius of the particle formed.

$$\frac{dR}{dt} = \psi \frac{D_0 V_m}{R} (C_b - S_0) \tag{7}$$

The driving force for crystallization will depend on the concentration of free unionized monomers according to the theory (9). If monomers are distributed into micelles, the free concentration in solution is decreased, and the crystallization rate will be reduced. This will be especially important for lipophilic and poorly soluble substances (BCS class II and IV). Mithani *et al.* has shown a correlation between the logP-value of the drug and the solubilisation capacity of bile salt micelles (15). The free concentration of drug can be roughly estimated by Eq. 8, using the ratio between the solubility in intestinal media and water.

$$C_{free} = \frac{C_{\text{intestinal}} S}{S_{\text{intestinal}}} \tag{8}$$

Here, $C_{\text{intestinal}}$ is the total concentration of drug molecules in the intestinal fluid, S is the solubility of the drug in water at a given temperature, and $S_{\text{intestinal}}$ is the solubility of drug in intestinal medium at the same temperature and pH. It is here assumed that the quotient is constant over the GI pH range, which for a basic compound like AZD0865 with a pKa of 6.1 will be valid within the pH range of the intestine where micelles are present (16). When combining Eqs. 1 and 7, taking Eq. 8 into account (*i.e.* $C_b = C_{free}$), calculations of crystallization rates are possible for intestinal fluid.

A number of parameters were needed for the calculations. The crystallisation rate is strongly dependent on the interfacial tension (see Eqs. 3, 5 and 6); however, the value of γ_{sl} is difficult to measure. Bragg-Williams theory states that there should be a linear correlation between the interfacial tension and the logarithm of solubility, as can be seen in Eqs. 9–11 (9):

$$\gamma_{sl} = \frac{0.33(\chi - 5)^* k_B T}{a} \tag{9}$$

$$\chi = \frac{\ln x_s}{(1 - x_s)^2} \approx -\ln x_s \approx -\ln\left(\frac{S_0}{55.6}\right) \tag{10}$$

$$a = \left(\frac{V_m}{N_A}\right)^{2/3} \tag{11}$$

In our previous work (9), such a correlation was developed for a number of drug-like substances using literature values of the water solubility S_0 and the interfacial tension determined from contact angle measurements. The solubility of the substance in water measured in mole fraction, x_s, was calculated from the molar solubility of the drug in water, S_0, and the molar concentration of pure water, 55.6 M. The linear regression line produced was here used in order to estimate a value of γ_{sl} for AZD0865 using the water solubility at 37°C.

The molar volume, V_m (see Eqs. 3, 5–7, 11) was measured using single-crystal X-ray diffraction to 301 cm^3/mol (AstraZeneca data on file). The diffusion constant D_0 (see Eqs. 1, 7) for AZD0865 was set to 7.6×10^{-10} m^2/s, which was an estimation derived from pulse gradient NMR measurements of the diffusion coefficient of a different pharmaceutical compound (bicalutamide) with similar size and physicochemical properties as AZD0865 (9). The diffusion constant has here been adjusted to 37°C in accordance with Stokes-Einstein's law. The last unknown crystallization parameter, λ (see Eqs. 2 and 7), could be determined by using crystal growth rate experiments where small crystal particles (nanoparticles) were added to a supersaturated solution of AZD0865. The method has been described in previous articles (9,17). Nanoparticles of AZD0865 were produced and characterized according to the milling procedure described in Ref 17. The nanoparticles were grown in a supersaturated solution of AZD0865 of 30 μM containing 0.75% DMA at two different initial fractions of crystals, 0.0019 and 0.010. The particle growth was monitored over time by using the fluorescence detection of particles with excitation at 330 nm (slit 5.0 nm) and emission at 378 nm (slit 2.5 nm) in a Perkin

Elmer LS 55 Luminescence Spectrometer. The experimental growth rate could then be modelled theoretically by using λ as a fitting parameter (17).

Intestinal Precipitation in *In Vitro* Methods

Three different *in vitro* precipitation methods were used in this study: two simple two-step methods differing in fluid hydrodynamics and one method using continuous pumping of gastric fluid into an intestinal compartment, developed by Kostewicz *et al.* (4). The two *in vitro* methods using a two-step scheme consisted of a first step where the formulation was dissolved in a vehicle and diluted to an *in vivo* relevant fluid resembling the expected gastric fluid. Concentrated FaSSIF was added in the second step to instantly achieve a total concentration of bile acids and pH corresponding to FaSSIF. This procedure aimed to mimic the composition of fluids and expected substance concentration profile in the upper small intestine better than is obtained in the method developed by Kostewicz (4), where FaSSIF is gradually diluted by addition of SGF, and the intestinal concentration of drug is increased linearly. The dilution factor used in the two-step methods was based on the expected fluid volumes and secretions in the upper human GI tract, but the volumes were scaled down for convenience reasons. Normal resting volumes of fluid in the fasted stomach have been reported to be about 50 ml (18,19), and a tablet is normally swallowed together with 250 ml of water. With a gastric emptying half-life of 10 min (20–22), the complete gastric emptying would take approximately 40 min, and with an intestinal secretion rate of approximately 2 ml/min (23), the dissolved drug concentration in the stomach would be diluted 1.3 times upon entry into the duodenum.

In the first model, hereby referred to as the stirring model, a USP II mini vessel (Vankel model VK 7010) at 37°C under constant paddle stirring at 150 rpm was used with a total volume of 79 ml including 51 ml SGF, 11 ml solution formulation containing PEG400 and EtOH and 17 ml concentrated FaSSIF. The volume ratios of the respective fluids were chosen to be relevant for the volumes administered to humans in a study with solutions of AZD0865. In the second model, which will be referred to as the shaking model, similar volume ratios were used as for the stirring model, but the hydrodynamic conditions were different. The new hydrodynamic conditions were chosen to better resemble the *in vivo* situation, where intestinal motility in the fasted state is generally low (23). The experiment was made in a conical 50 ml flask containing, in total, 25 ml of fluid, including 17.8 ml SGF, 2.2 ml solution formulation and 5 ml concentrated FaSSIF. The flask was placed in a water bath (Clifton shaking bath, model NE5-10D) at 37°C and shaken at approximately 85 strokes/min with a one-way distance of 2 cm. The solution formulation of AZD0865 used in these experiments contained no PEG400 or EtOH, but a control experiment was made with the formulation containing PEG400 and EtOH in order to compare the two models.

In vitro precipitation was studied in the stirring and shaking models at three different drug concentrations, where the concentrations of AZD0865 in the resulting simulated intestinal fluids were 0.35, 0.70 and 1.1 mM, respectively.

Samples of the fluid were collected after 0 (prior to addition of concentrated FaSSIF), 2, 15, 30, 45 and 60 min by filtering the sample with a filter. In the stirring model, a 0.45 μm Millex-HV filter (Millipore Corp.) was used, but this was for practical reasons exchanged for a 0.22 μm hydrophilic PTFE filter (Advantec) in the shaking model. No difference in substance loss due to filter effects was detected. pH was recorded at different time points during 60 min with a pH meter (PHM 93 or PHM 240, Radiometer). The samples were diluted with organic solvent before analysis with HPLC-UV to avoid precipitation.

In the experiments using the same method as described by Kostewicz *et al.* (4), 100 mg of AZD0865 base, corresponding to a medium dose in the *in vivo* pharmacokinetic single dose studies in healthy volunteers, was completely dissolved in 250 ml SGF and transferred with a peristaltic pump (Ole Dich model 103) into 500 ml FaSSIF over 125 min. The acceptor fluid was maintained at 37°C and stirred with a paddle speed of 100 rpm in a USP II method (Vankel VK 7010 dissolution tester). The maximum concentration of AZD0865 in the acceptor fluid if no substance would precipitate was 0.36 mM. Samples of 5 ml were frequently withdrawn from the acceptor phase for 4 h, filtered through a 0.45 μM Millex-HV filter (Millipore Corp.), and finally diluted for analysis with HPLC-UV. The removed sample volume was replaced and compensated for when the concentration was calculated. All precipitation experiments were performed in triplicate.

Pharmacokinetic Studies in Humans

Four clinical pharmacokinetic single dose-studies were used to evaluate the possibility of reduction of bioavailability due to dissolution or precipitation issues in the intestine. All studies were randomized, single-centered studies on healthy male volunteers. The studies followed the Declarations of Helsinki for biomedical research involving human subjects and were approved by the local Ethical Committees. Informed consent was obtained prior to initiating studies. The oral doses administered in the clinical studies are summarized in Table I.

The first three studies in Table I were used to detect intestinal precipitation of AZD0865. The drug was given to

Table I Summary of Administrations in Clinical Trials

Study nr	Group nr	Dose[a]	Formulation of AZD0865	Administered volume (ml)	Number of subjects
1	I	0.08 mg/kg	Solution (PEG400/EtOH/H2O)	350	4
	II	0.22 mg/kg	Solution (PEG400/EtOH/H2O)	350	4
	III	0.5 mg/kg	Solution (PEG400/EtOH/H2O)	350	4
	IV	1.0 mg/kg	Solution (PEG400/EtOH/H2O)	350	4
	V	1.7 mg/kg	Solution (PEG400/EtOH/H2O)	350	4
	VI	2.6 mg/kg	Solution (PEG400/EtOH/H2O)	350	4
	VII	3.0 mg/kg	Solution (PEG400/EtOH/H2O)	350	4
	VIII	4.0 mg/kg	Solution (PEG400/EtOH/H2O)	350	4
2	IX	10 mg (~0.13 mg/kg)	Solution (PEG400/EtOH/H2O)	121	6
	X	20 mg (~0.26 mg/kg)	Solution (PEG400/EtOH/H2O)	121	6
	XI	40 mg (~0.51 mg/kg)	Solution (PEG400/EtOH/H2O)	121	6
	XII	65 mg (~0.83 mg/kg)	Solution (PEG400/EtOH/H2O)	121	6
3	XIII	28 mg (~0.36 mg/kg)	Tablet, mesylate salt	240	6
	XIV	45 mg (~0.58 mg/kg)	Tablet, mesylate salt	240	6
	XV	73 mg (~0.94 mg/kg)	Tablet, mesylate salt	240	6
	XVI	114 mg (~1.46 mg/kg)	Tablet, mesylate salt	240	6
	XVII	159 mg (~2.04 mg/kg)	Tablet, mesylate salt	240	6
4	XVIII	100 mg	Tablet, base	250	14
	XIX	100 mg	Tablet, base + 80 mg omeprazole	250	14

[a] Dose in equivalent amount base

fasted subjects (since 12 a.m. the night before), and the subjects were allowed standardized food intake four hours after drug administration. In the first study, AZD0865 oral solutions were given in doses of 0.08, 0.22, 0.5, 1.0, 1.7, 2.6, 3.0 and 4.0 mg/kg to 4 subjects at each dose level (Groups I-VIII). 110 ml of each drug solution with PEG400 and EtOH were rinsed down with 2×120 ml water. In total, 350 ml was given to each volunteer.

The second study setup was similar to the first study. The oral doses of the solution given were 10, 20, 40 and 65 mg, corresponding to approximately 0.13, 0.26, 0.51 and 0.83 mg/kg to 6 subjects at each dose level (Groups IX–XII). 21.3 ml of the solutions with PEG400 and EtOH were administered and rinsed down with 2×50 ml water.

The third study included 30 subjects on active treatment divided into dosing groups (XIII–XVII), where 6 subjects in each dosing group received the drug in the form of a tablet of the mesylate salt of the drug. The doses corresponded to 28, 45, 73, 114 and 159 mg of the base, or approximately 0.36, 0.58, 0.94, 1.46 and 2.04 mg/kg. The tablets were administered together with 240 ml of water.

An additional clinical trial was run in 14 healthy male volunteers that at one occasion were given a tablet of AZD0865 base 100 mg as a single dose at normal gastric pH (Group XVIII) together with 250 ml of water. At a separate occasion, the same subjects were initially treated with a bolus intravenous infusion of omeprazole 80 mg followed by a continuous intravenous infusion of 8 mg/h omeprazole for 7 h and 30 min in order to increase the gastric pH (Group XIX). The AZD0865 tablet was administered with 250 ml water 4 h after the bolus infusion of omeprazole. A median gastric pH of 5.8 was expected based on a previous study with the same omeprazole regimen (24). This treatment allowed for studying the influence of intestinal dissolution of crystalline drug on absorption, since only a minor fraction of the dose (approximately 2.5% assuming a gastric volume of 300 ml) could be dissolved in the stomach at the elevated pH.

Blood samples were collected in heparin tubes every 15 min up to 1.5 h, and subsequent samples were taken regularly up to 24, 36 or 48 h, depending on the dose given. The samples were mixed and centrifuged at approximately 1500 RCF, and plasma was transferred to a Cryovial® and immediately frozen at −18°C until analysis of the content could be made. AZD0865 was isolated with solid phase extraction on an Isolute Array C8 plate and was eluded with a formic acid solution containing acetonitrile and ammonium acetate. The analysis was performed with HPLC using a Zorbax SB-C8 column (150×4.6 mm, 3.5 μm) and an isocratic chomatographic method using a mobile phase containing 32% acetonitrile, 0.2% formic acid and 2 mM ammonium acetate. Detection was made with fluorescence detection at an excitation wavelength of 257 nm and an emission wavelength of 393 nm.

Table II Equilibrium Solubility of AZD0865 in Various Media at 37°C ($n=2$)

Media	Solubility μM	Solubility μM	pH
Water	5.2	4.9	8
FaSSIF	13.6	17.7	6.5
SGF	8100	8600	1.7
HIF	13.9	15.8	6.9

Pharmacokinetic evaluation was made by non-compartmental methods using WinNonlin Professional (Pharsight) based on individual plasma concentration-time data. The determined parameters were the maximum plasma concentration (C_{max}), the time at which it occurred (t_{max}), the terminal half-life ($t_{½}$) and AUC_{τ}, the area under the plasma concentration-time curve up to the last plasma sampling point calculated by the log-linear trapezoidal rule.

Dose proportionality of AUC_{τ} and C_{max} for pooled results from the studies including dosing of different doses was evaluated by linear regression analysis in order to elucidate the effect of any intestinal drug precipitation. The differences in C_{max} and AUC_{τ} between administrations at elevated compared to normal gastric pH in study 4 was evaluated using a Student's *t*-test considering $p<0.05$ as statistically significant.

RESULTS

Physicochemical Data for AZD0865

DSC Measurements

The enthalpy of melting, ΔH_m, was determined to be 54.0 kJ/mol, and the temperature of the onset of melting, $T_{m,\ onset}$, was 246°C.

Solubility and Intestinal Supersaturation Potential

The solubility of AZD0865 in different solutions is given in Table II. The SGF solubility shows that approximately 0.9 g of AZD0865 could be dissolved in the stomach, assuming a total fluid volume (resting and administered) of 300 ml and pH 1.7, and thus the maximum dose in the current *in vivo* studies of 4 mg/kg, or approximately 310 mg, should be freely soluble in the stomach. The solubility of AZD0865 in HIF was identical to the solubility in FaSSIF, and, hence, the use of FaSSIF in the *in vitro* precipitation test would be highly relevant from a solubility point of view. The solubility in intestinal fluids was about 500 times lower than at gastric pH, and at the highest clinical dose only 0.7% of the drug would maintain in solution at equilibrium solubility assuming an intestinal volume of about 400 ml. Thus, highly supersaturated solutions were likely to occur in the proximal small intestine, providing good pre-requisites for drug precipitation.

Theoretical Crystallization Rate of AZD0865

The particle size fractions of the milled nanoparticles described earlier (volume weighted mean 172 nm, d(0.1)= 64 nm, d(0.9)=261 nm) were used when determining the constant λ, needed for the theory of nucleation and particle growth (see Eqs. 2 and 7). By fitting the growth theory to the experimental growth by time curves with λ as the only unknown parameter, this constant was determined to be 2 μm for AZD0865. According to Eqs. 9–11, given the S_0 value in water from Table II, the value of γ_{sl} would be 25.2 N/m. The parameters were then included in Eqs. 1, 7 and 8, and the theoretical concentration drop over time in a micellar media resembling FaSSIF at different AZD0865 concentrations corresponding to the oral doses relevant for the *in vitro* and *in vivo* studies were calculated and are shown in Fig. 2. AZD0865 was not predicted to precipitate significantly at the concentrations and time frames relevant for the *in vivo* studies except for a slight effect at the highest concentration where a 30% decrease in drug concentration in solution was predicted after one hour.

In Vitro Intestinal Precipitation Model

The results of drug concentration in solution over time from the *in vitro* trials are presented in Figs. 3, 4 and 5 for the stirring model, the shaking model and the Kostewicz model, respectively. The pH in the test media was maintained within 6.3–6.5 in all precipitation experiments.

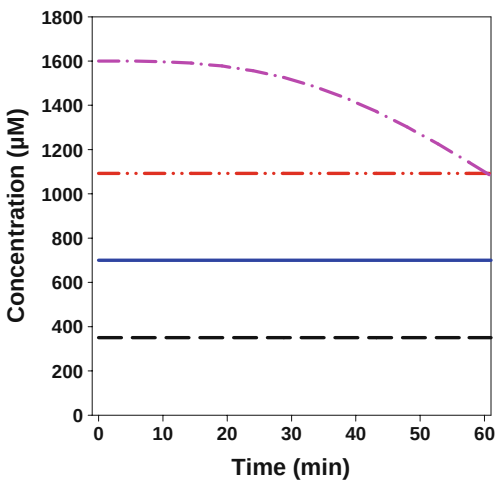

Fig. 2 Predicted concentrations of AZD0865 in solution over time at different initial supersaturation determined by Eqs. 1–11. Initial concentrations: – – – 0.35 mM, — 0.70 mM, –•–•– 1.1 mM, –•– 1.6 mM.

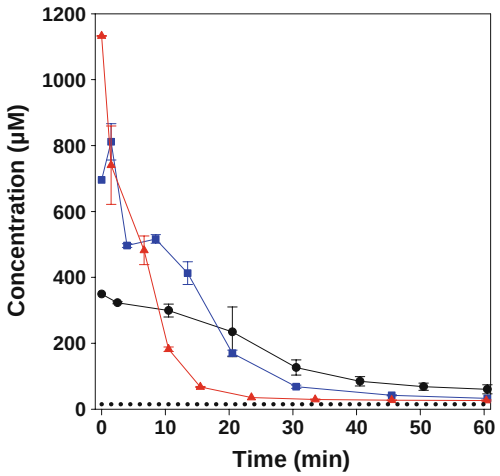

Fig. 3 Mean (±SD) concentration of drug dissolved in FaSSIF over time from supersaturated solutions using the stirring *in vitro* model ($n = 3$). Initial concentrations AZD0865: ● 0.35 mM, ■ 0.70 mM, ▲ 1.1 mM. Dotted line represents the equilibrium solubility in FaSSIF. Initial concentrations calculated from concentration in gastric fluid, before concentrated FaSSIF was added.

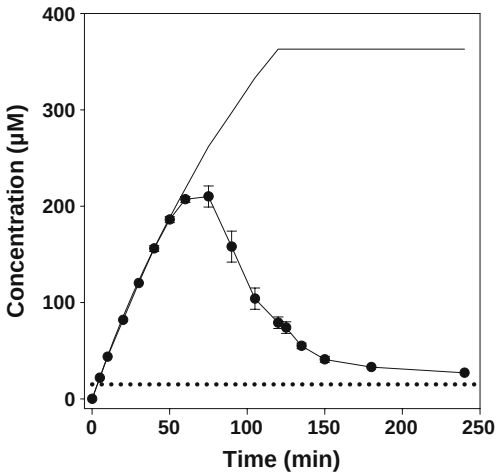

Fig. 5 Mean (±SD) concentration of AZD0865 dissolved in FaSSIF over time from supersaturated solution using the Kostewicz dynamic *in vitro* method ($n = 3$). Solid line represents the theoretical concentration of AZD0865 in solution if no precipitation occurs. Dotted line represents the equilibrium solubility in FaSSIF.

All three models indicated precipitation of AZD0865 from the solutions. A comparable experiment was made with all three models at a maximum expected concentration of AZD0865 of approximately 0.35 mM. For Kostewicz's model and the stirring model, the crystallization rate was fast enough to ensure that drug concentration in solution had been reduced to the equilibrium solubility level after one hour. The crystallization rate was slower in the shaking model compared to the models using paddle stirring, and for the lowest concentration, 0.35 mM, the precipitation was insignificant for at least one hour in the shaking bath method. Crystallization rates in the stirring and shaking models could be compared by determining the time point when the concentration in the simulated intestinal fluid was halfway down from the initial concentration to the equilibrium solubility in the fluid. In the stirring model, this time point was reached after approximately 25, 15 and 5 min for 0.35, 0.7 and 1.1 mM, respectively. In the shaking model, very little precipitation occurred for the two lower concentrations, whereas the half-time for precipitation was reached within 5–10 min at 1.1 mM. There was no difference in precipitation between the two formulations (0.7 mM of AZD0865 in solution) with and without PEG400 and EtOH. This was expected, since PEG400 and EtOH did not affect the solubility of AZD0865 significantly after dilution with relevant volumes of SGF and FaSSIF, and no other effect on precipitation could be foreseen.

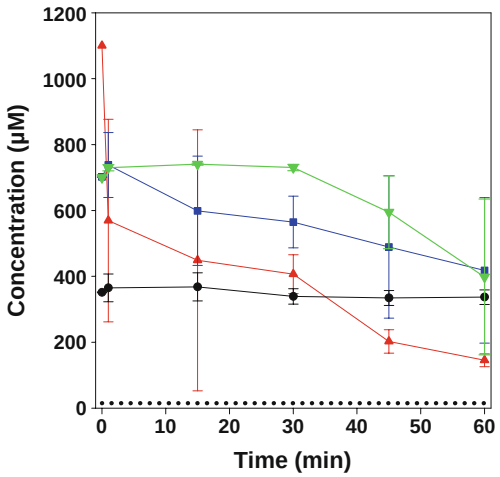

Fig. 4 Mean (±SD) concentration of drug dissolved in FaSSIF over time from supersaturated solutions using the shaking *in vitro* model ($n = 3$). Initial concentrations AZD0865: ● 0.35 mM, ■ 0.70 mM, ▲ 1.1 mM, ▼ 0.70 mM with PEG400 and EtOH. Dotted line represents the equilibrium solubility in FaSSIF. Initial concentrations calculated from concentration in gastric fluid, before concentrated FaSSIF was added.

Pharmacokinetic Data

Both AUC_τ and C_{max} increased proportionally with dose between 0.08 and 4 mg/kg, as shown by the high correlations of 0.97 and 0.96, respectively (Fig. 6). The median t_{max} was 0.6–1.6 h in the different studies, and the t_{max} seemed unaffected by the dose. The $t_{½}$ was 4.8–8.2 h in the different studies with no trend of increase or decrease with dose. The mean plasma drug concentration-time profiles were also very similar for the tablet formulation compared to the solution at similar doses, as is illustrated by Fig. 7.

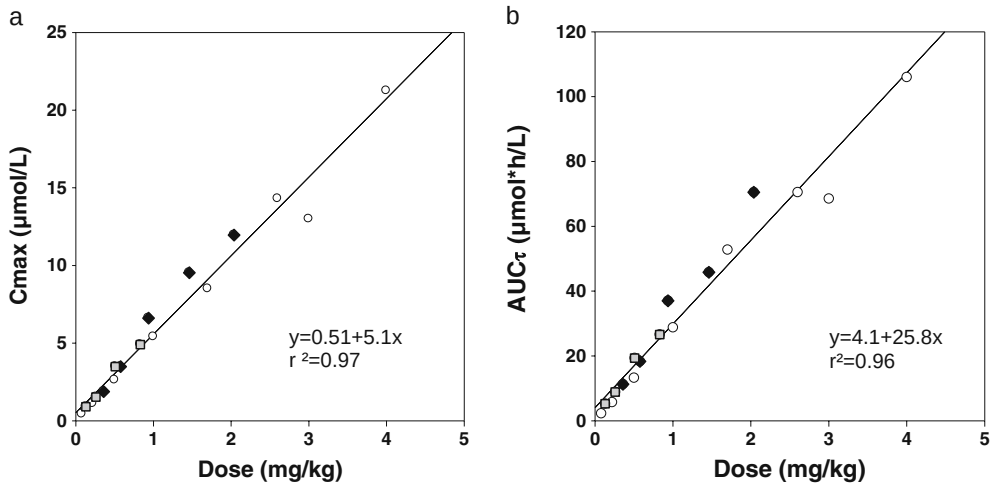

Fig. 6 Mean exposure data in humans of AZD0865 at different doses. a) C_{max} b) AUC_T including the linear regression lines. Different symbols represent different clinical trials: ○ first trial, doses 0.08–4.0 mg/kg in solution ($n=4$) ■ second trial, doses 0.13–0.83 mg/kg in solution ($n=6$) ♦ third trial, 0.36–2.0 mg/kg in mesylate salt tablet ($n=6$).

The results from the study with and without increased gastric pH are presented in Table III. There was a significant decrease in both C_{max} (−82%, $p<0.0005$) and AUC_τ (−46%, $p<0.0005$) and an increase in t_{max} ($p=0.02$) when the subjects had been treated with omeprazole prior to the AZD0865 administration, indicating a strong gastric pH dependent dissolution and absorption of AZD0865.

DISCUSSION

In these studies, precipitation rates in the intestines of the basic drug AZD0865 were investigated over a range of modes from *in vitro* precipitation models to clinical bioavailability studies *in vivo* in healthy volunteers. The precipitation rates were also modelled *in silico* by predicting crystallization rate of the substance in intestinal fluid. The clinical studies included oral administrations of AZD0865 that created intestinal drug concentrations of up to 110 times above the equilibrium solubility in the upper small intestine when the drug solution was emptied out of the stomach, *i.e.* supersaturation was achieved. The highest dose administered in the clinical trials corresponded to an estimated intestinal luminal concentration of 1.6 mM, calculated from the administered volume and the expected gastric and intestinal dilution. *In vitro* concentrations up to 1.1 mM were tested, and the results showed that *in vivo* intestinal drug precipitation at this concentration was expected to be very rapid, and the likelihood for effects of intestinal precipitation in the current study was very high.

At normal low gastric pH (about 1.7), the whole maximum dose (310 mg) given in the present study should maintain in solution, when given as a solution, or be completely dissolved in the stomach when administered as a rapidly dissolving tablet, as indicated by the solubility of 3 mg/ml, or 8.4 mM, in SGF. The complete dissolution of

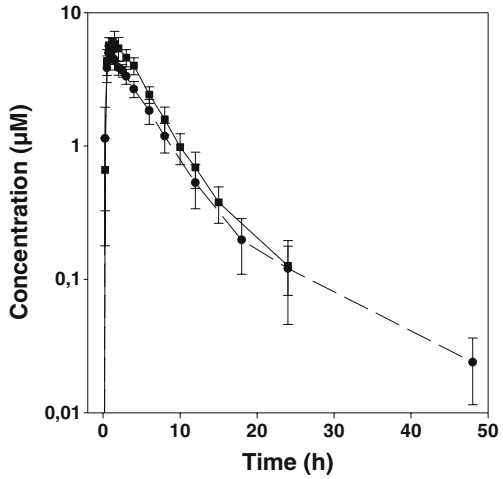

Fig. 7 Mean plasma concentration (±SD) over time for clinical *in vivo* study of AZD0865 administered as: – – ● – – 1.0 mg/kg solution Group IV ($n=4$) —■— 0.94 mg/kg mesylate tablet Group XV ($n=6$). Dose is expressed as equivalent amount base.

Table III Influence of Increased Gastric pH on the Mean (±SD) Plasma Exposure of AZD0865 (Median (Range) for t_{max}) After Oral Administration of Tablets of 100 mg With and Without Pre-treatment of Omeprazole to Increase Gastric pH

Experimental conditions	AUCt (±SD) µmol*h/l	Cmax (±SD) µmol/l	tmax (range) h
Without omeprazole	37 (9)	7.3 (1.6)	0.9 (0.8–2.0)
With omeprazole	20 (9)	1.3 (0.6)	2.5 (1.5–24[a])

[a] One outlier at 24 h due to flat plasma concentration curve

the tablets was verified by the almost identical C_{max} and AUC_τ at the highest administered tablet dose of 2 mg/kg compared to the solution after administration under normal fasting conditions. All dose-correlated plasma drug concentration curves are almost superimposable in study 1–3 (data not shown). At elevated gastric pH, the AZD0865 solubility in the gastric fluid is low, and almost all drug would be emptied out into the small intestine as undissolved drug. Both the rate and extent of absorption could therefore be limited by intestinal drug dissolution. This was confirmed in study 4, where tablets were orally administered to subjects with normal acid gastric pH and neutral pH (due to an intravenous dose of omeprazole). The total plasma exposure was reduced by 50% in the omeprazole-treated group (Group XIX) compared to the untreated group (Group XVII), which demonstrate that undissolved material of AZD0865 in the intestine significantly reduces the rate of absorption and the extent of bioavailability. Thus, if significant intraintestinal crystalline precipitation were to occur *in vivo*, this would consequently lead to slower absorption rate and a reduced bioavailability. The effect of intestinal precipitation after complete gastric dissolution should be more pronounced at higher doses, since the precipitation rate increases with increasing degree of supersaturation. This would result in a non-proportionality of C_{max} and possibly AUC_τ with increasing oral doses. The dose proportionality in Fig. 6 showed that no such effect existed at the doses administered. This conclusion was unaltered if the pharmacokinetic parameters were plotted against the expected intestinal concentrations instead of dose (data not shown). The plasma profiles and pharmacokinetic parameters of different formulations at the same dose levels were almost superimposable. Thus, these data strongly indicated that precipitation was not of importance for drug absorption of AZD0865 in the tested range including doses inevitably producing supersaturation in the upper small intestine.

Precipitation of fine amorphous particles that could instantly re-dissolve could not be excluded, but the likelihood of this event could be estimated from Eqs. 12 and 13. The apparent amorphous solubility, S_0^a, of drug substances can be approximated with the aid of the melting temperature, T_m, and the enthalpy of melting, ΔH_m (25).

$$S_0^a = S_0 e^{\frac{\Delta S_m}{R} \ln \frac{T_m}{T}} \tag{12}$$

$$\Delta S_m = \frac{\Delta H_m}{T_m} \tag{13}$$

Here, R equals the gas constant, and T is the temperature. Given the results presented under section "DSC Measurements" and the intrinsic solubility from Table II, the amorphous solubility of AZD0865 could be estimated to be 3 mM. The highest expected intestinal concentration is only 1.6 mM, and the risk of amorphous precipitation should be considered low, but it cannot be excluded that amorphous precipitation could be a factor in both *in vitro* and *in vivo* experiments since this calculation is approximative. The calculations assume that the melting enthropy, ΔS_m, equals the heat capacity, ΔC_p, and this approximation may be less correct for substances with high melting temperatures, such as AZD0865, compared to substances with lower melting temperatures. Due to the rapid crystallization of AZD0865, shown by the rapid decrease in dissolved concentration at high supersaturation, a possible formation of amorphous particles at high supersaturation does not affect the overall conclusion that precipitation does not affect the absorption of drug in the present case.

It is obvious from the results that the simple *in vitro* methods used overestimated the risk of luminal precipitation effects in the intestines for AZD0865. This observation is in contrast to previous publications where relationships between *in vitro* and *in vivo* data have been claimed (4,5). However, the majority of literature data compares different formulations of the same dose, making it very difficult to distinguish between dissolution-enhancing properties and precipitation as underlying reasons for observations of differences in rate and extent of bioavailability (2,5,7). The present study included a more mechanistically based approach to elucidate the existence of *in vivo* precipitation based on human pharmacokinetic data, and no such analysis has, to our knowledge, been published in order to evaluate the accuracy of predictions of *in vivo* intestinal precipitation of drugs.

The discrepancy between the *in vitro* predictions of intestinal precipitation and the real outcome *in vivo* is due to a number of reasons. The *in vitro* methods used did not take into account the effects of removal of drug from the intestinal fluid by absorption across the intestinal membrane (*i.e.* permeability). AZD0865 is a high permeability drug based on *in vitro* permeability data in Caco2-cells (AstraZeneca data on file), and the plasma concentration curves from clinical trials showed a fast absorption phase with an early t_{max} of 0.6–1.6 h. The rapid absorption of the substance will drastically reduce the concentration in the intestinal lumen within the first hour, and it is clear that the driving force for precipitation will be lowered if not even absent after one hour. Thus, prediction of effects of precipitation on drug absorption need to include the luminal removal of drug by the intestinal permeability either through combining *in vitro* data with *in silico* modelling of drug absorption or by using *in vitro* methods where there is continuous drug removal from the intestinal phase that mimics the *in vivo* rate. This has been previously suggested for an *in vitro* dissolution method modelling *in*

vivo dissolution of carbamazepine (26). A comparison between the opposing conclusions on possible intestinal precipitation of dipyridamole made by Kostewicz *et al.* (4) and Gu *et al.* (8) also show that the simple *in vitro* model without absorption might overpredict precipitation for other BCS class II substances. The investigations were made on different doses, but Kostewicz predicted significant precipitation at lower concentrations than was tested by Gu *et al.*

The accuracy of the *in vitro* methods could also be affected by factors in the intestinal media that are different from those in FaSSIF. The effects of differences in bile salts and lipid components is difficult to predict and will most likely be substance specific. The solubility of AZD0865 in HIF was here similar to the solubility in FaSSIF, but only pooled HIF was used in the evaluation. Different time fractions of HIF could give varying values of solubility of drug substances, as was shown by Clarysse *et al.* (27). Even if solubility of the drug compound is not affected, increased heterogeneous nucleation or increases or decreases of crystallization rates can occur due to specific interactions between the drug and the intestinal media components.

Solubility is also dependent on solid-state polymorph, and attempts were made at characterizing the solid-state form of AZD0865 *in vitro* in FaSSIF and DIF, but due to the complexity of the media and risk for solid-state transformation in sample handling, no conclusive results could be obtained. This is an area for future studies.

Another factor that may affect the relevance of the *in vitro* system is the hydrodynamics and stirring mechanism. It is well known that stirring and secondary or heterogeneous crystallization can increase the crystallization rate dramatically (28,29), and by comparing the three models used in this work, the influence of different stirring mechanisms on precipitation is further verified. At the lowest expected concentration in solution, 0.35 mM, the actual concentration of dissolved AZD0865 is only about 50 μM after 60 min for the two models using paddle stirring, but practically no precipitation was detected in the shaking model at this concentration. The precipitation rates of the two different stirring models are comparable, and considering the evidence of a complete absence of precipitation effects *in vivo* of AZD0865 and the reported low intraluminal activity in the fasted state during the main part of the time (23), it seems likely that the paddle stirring might be part of the reason for the more extensive overprediction of the crystallization rate *in vivo* by this method compared to the shaking bath approach. Thus, for *in vitro* methods used for quantitative predictions, the correct *in vivo* hydrodynamic conditions must be properly modelled.

Interestingly, the theoretical model used gave a better resemblance to the *in vivo* behaviour than the *in vitro* methods. Given the strong importance of interfacial tension on predicted precipitation rate, a more detailed investigation of this factor might be necessary for a more robust estimation of the precipitation rate and the conclusions of suitability of theoretical modelling. It should be noted that the interfacial tension was here estimated from an experimental correlation (Eq. 8) with considerable scatter (9). If the deviation for the compounds in the experimental correlation was considered, expressed as one standard error of estimate from the mean, the resulting interfacial tension value for AZD0865 would be 25.2 ± 3.8. At the lower end of this limit, theoretical precipitation would still not be detectable within one hour for 0.35 mM, but at higher concentrations precipitation would be noticeable. The used model is also only describing an unstirred system, and stirring could reduce an apparent interfacial tension due to an increase in heterogeneous nucleation and precipitation rate. The issue of apparent γ_{sl} has been addressed in articles by Sugano, where a theoretical precipitation model was built using similar crystallization theory to the one presented here, producing apparent parameters for future use in *in silico* tools of absorption modelling and prediction (16,30).

Precipitation of a poorly soluble amorphous phase of a BCS class II drug has not been addressed here, and it is possible that a simple *in vitro* model of precipitation would be more predictive for such systems, since amorphous precipitation is generally more instantaneous than crystalline precipitation. For crystallizing drug substances where precipitation is expected to be an issue, we believe that we have shown that simple *in vitro* models overpredict the precipitation rate due to the rapid crystallization rate and poor solubility of AZD0865.

CONCLUSIONS

This study suggests that simple *in vitro* methods of *in vivo* precipitation of orally administered BCS class II bases overpredict the crystalline intestinal precipitation *in vivo* in humans. Consequently, intestinal precipitation of BCS class II bases might be less of a limitation for *in vivo* drug absorption than implied from simple solubility and *in vitro* test assessments. We believe the reduction of luminal drug concentration by time because of absorption *in vivo* must be included in predictive methods. Furthermore, the importance of hydrodynamic conditions was shown by the *in vitro* experiments, and this factor also needs to be addressed in future *in vivo* predictive methods. Finally, more data relating *in silico*/*in vitro* methods with *in vivo* data is needed, and mechanistically based interpretations of *in vivo* results such as suggested in this article will contribute to further improvement in the development of such predictive tools.

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