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RESEARCH PAPER

Early development evaluation of AZD8081: a substrate for the NK receptors

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Abstract

The purpose of the present study was to find out if AZD8081, a dual neurokinin (NK)1/2 receptor antagonist, was suitable for development of an oral, solid immediate release (IR) formulation and in a further perspective also as an oral extended release (ER) formulation. AZD8081 is a base with pK_a values <2.5 and about 8.5. The measured intrinsic solubility is about 0.1 mg/mL and the solubility in FaSSIF (fasted simulated small intestinal fluid) is about 3.2 mg/mL. Aqueous buffer solutions are stable for at least 1 month between pH 1–7 up to 37°C. In the solid-state, a mixture of amorphous and crystalline substance showed significant chemical instability in the initial stress testing studies. No degradation was, however, observed for highly crystalline material at similar conditions. It is concluded that the impurity profile and/or the present solid-state of the batches affect the stability of the substance. The amorphous contribution of the substance is the main cause to the observed degradation in solid-state. Crystalline AZD8081 is polymorphic with two known monotropic forms, form A and form B. Both forms are only slightly hygroscopic ansolvates with melting points of approximately 108°C and 117°C, respectively. Form B is the more stable of the two forms and is therefore most suited for further development. The candidate is suitable for development of standard IR formulations since no specific limitations of significance for formulation development were identified. In addition, the good stability in human intestinal fluid and in colon slurry makes AZD8081 a suitable candidate for ER formulation.

Keywords: Drug development; drug delivery; IBS; NK receptor; preformulation; stability

Introduction

Almost all new drugs are marketed as tablet or capsules. Although only a few are marketed as an injection device, the intravenous (i.v.) route is always required during early discovery evaluation and for early toxicity studies. Prior to the development of these dosage forms, it is essential that certain fundamental physical and chemical properties of the drug molecule are determined. This information dictates many of the subsequent events and approaches in the formulation development. This first learning phase is known as preformulation or early development. These early studies have a significant part to play in anticipating formulation problems and identifying logical paths in both liquid and solid dosage form development. One of the first steps in preformulation is to establish simple analytical methods to be able to measure solubility and follow stability of the substance. In the development of pharmaceutical dosage forms, one of the persistent challenges is assuring acceptable stability, i.e., the storage time allowed before the content is too low or a degradation product in the dosage form achieves a sufficient level to represent a risk to the patient. Based on this time, the expiration date (shelf-life) of a product is determined. It is of high importance to identify any instability in pharmaceutical formulations as early as possible in the development process. It is also important to investigate, and in some cases, improve, the solid-state properties of the substance to find a suitable, preferable crystalline material, for further development. Another important area to consider is the biopharmaceutical aspects i.e., properties that affect drug delivery. These properties can be summarized as the properties of the drug (e.g., physicochemical properties, solubility and stability in *in vivo* media), the used formulation (e.g., immediate or

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modified release) and the route of administration (e.g., oral or i.v.), which all affect the rate and/or the extent of substance absorbed. Important primary properties for efficient absorption are solubility, stability and permeability in the gastrointestinal tract. In the present work, we have focused on the solubility and stability in *in vivo* relevant media. The permeability was measured by our Discovery organization and the measurement is out of the scope of the present work, while the results of the experiments are of central importance and used in our discussion. In the present report AZD8081 (Figure 1), a neurokinin (NK)1/2 receptor antagonist, is investigated.

The peptides of the tachykinin family are widely distributed within the mammalian peripheral and central nervous system (CNS) and play a well-recognized role as neurotransmitters[1,2]. The best known and characterized substances in the family are the structurally related NKs substance P, NKA, and NKB[3]. The NKs mediate their effects via binding to G-protein-coupled NK receptors[1,4,5]. There are at least three types of tachykinin receptors described in the literature. These are denoted NK1, NK2, and NK3 receptors and are heterogeneously distributed *in vivo*. The NK1 receptor is widely expressed at both the central and the peripheral level and are present in e.g., neurons, vascular endothelial cells, muscle, and different types of immune cells[6–10]. The NK2 receptor is primarily detected in the periphery and its expression in the CNS appears to be minor[7,11–15]. The NK3 receptor is mainly expressed in the CNS and has only been detected in certain peripheral tissues[7,16–20].

Historically, the NK1 receptor was the first to be described. About 80% of the existing patents are related to NK1 antagonists and non-peptide substances[2]. Many companies have found compounds suitable for clinical development for different therapeutic uses. Examples of the wide-variety of indications are asthma, pain, psychiatric disorders, emesis, gastrointestinal disorders, and urine incontinence[21–24]. In 2003, Merck launched the first NK1 binding substance in the US, and in 2004 in Europe with the name Emend (the active ingredient is aprepitant). Emend is approved for the prevention of chemotherapy-induced nausea and vomiting[25].

In the present report is the early development/preformulation evaluation performed on AZD8081, a substance that is active in the NK field. The project objective is to develop a dual NK1/2 receptor antagonist which is potent, centrally available, safe and orally active at once or twice daily dosing aimed for the treatment of irritable bowel syndrome. The project was aimed for the development of an oral immediate release (IR) dosage form and the preliminary predicted dose in man is in the order of 50–100 mg twice a day. The evaluation was also performed with an eye towards line extension and a possible future oral extended release (ER) formulation.

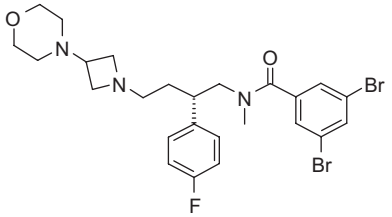

Figure 1. The chemical structure of AZD8081. The molecule is based on a four carbon chain with a chirality (*S*-enantiomer) at the carbon where the fluorobenzene ring is attached, with a lipophilic 3,5-dibromophenyl amide on the right side of the molecule, and a more polar azetidine-morpholine moiety on the left hand-side.

Material and methods

The test compound

AZD8081 was synthesized at AstraZeneca R&D Mölndal, Sweden, as neutral form. The compound is owned by AlbireoPharma, Göteborg, Sweden. The molecule is based on a four-carbon chain with a chirality (*S*-enantiomer, verified by single crystal X-ray diffraction – data not shown) at the carbon where the fluorobenzene ring is attached, with a lipophilic 3,5-dibromophenyl amide on the right side of the molecule, and a more polar azetidine-morpholine moiety on the left hand-side. The substance (583.3 g/mol) is a base with pK_as of <2.5 and 8.2 (determined by capillary electrophoresis[26]). Estimated log D at pH 6.8 [obtained by liquid chromatography-mass spectrometry (LC-MS)] is 3.1.

X-ray powder diffractometry

X-ray powder diffractometry experiments were performed on a D8 Advance diffractometer (Bruxer AXS GmbH, Karlsruhe, Germany) with Bragg-Brentano geometry, equipped with a VÅNTEC-1 position sensitive detector. Nickel-filtered Cu K_α radiation was used. The samples, approximately 10 mg, were mounted on a zero-background holder (silicon crystal). Data were collected using continuous scan mode in the range 1–50°2θ, with a step size of 0.017° and a step time of 0.5 s. A variable (V20) divergence slit and a detector slit of 12 mm, corresponding to a 3.47° wide detector window, were applied.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed using a DSC Q1000 (TA Instruments, New Castle, USA). The temperature and heat flow were calibrated using indium. Experiments were run between 25 and 200 or 250°C with a heating rate of 10°C/min in a dry purge of nitrogen gas (50 mL/min). The sample was analyzed in an aluminum pan with closed lid; however, an approximately 1 mm-wide hole had been made in the lid with a needle.

Thermogravimetrical analysis

Thermogravimetrical analysis (TGA) analysis was performed using a TGA Q500 (TA Instruments, New Castle, USA). The temperature was calibrated using the Curie points of alumel alloy and nickel. The balance was calibrated using 100 and 1000 mg standard weights. The samples were heated from room temperature to 220°C

with a heating rate of 10°C/min in a dry purge of nitrogen gas (90 mL/min).

Dynamic vapor sorption

Water sorption measurements were carried out using a commercial instrument: dynamic vapor sorption (DVS-1; Scientific & Medical Products Ltd, Manchester, UK). The automated instrument measures the uptake or loss of water vapor gravimetrically using a Cahn D200 recording ultramicrobalance, in a symmetric arrangement of sample and reference weighing pan, with a mass resolution of 0.1 μg. The relative humidity (RH) around the sample is controlled by mixing saturated (100% RH) and dry (0% RH) nitrogen carrier gas streams to a target-RH using electronic mass flow controllers. In addition, the target-RH and temperature are measured/verified with solid-state transducers near the sample and reference pan. The whole system is enclosed in a research incubator maintaining constant temperature to within 0.1°C. The target-RH and the transducers-RH were calibrated/checked to be within 0.05% RH-units by measuring the RH in equilibrium with saturated aqueous salt solutions (NaCl, LiCl and $MgCl_2$) and comparing with literature RH values.

The water sorption isotherm of a sample (typically 5–30 mg) was obtained using a predetermined analysis program (time vs. % RH) which exposed the sample to a constant % RH for sufficiently long time to allow equilibrium sorption to be attained, and thereafter the % RH was changed in order to determine a next equilibrium allowing a complete sorption/desorption curve (e.g., % weight increase vs. % RH and time) to be obtained in one extended run (typically 24 h). Usually the sample was subjected to two consecutive sorption/desorption cycles.

Stability in HIF (and FaSSIF)

The *in vitro* stability of AZD8081 in human intestinal fluid (HIF) was investigated by incubation at a concentration of 7 μM and at 37°C. HIF was obtained from Biomedical Centre at Uppsala University. The Ethics Committee of the Medical Faculty at Uppsala University, Sweden, approved collection. The fluid was sampled from 15 healthy individuals, aged 18–40 years. All the subjects had normal clinical and laboratory values and none of the subjects received any medication before or on the day of fluid collection. Before the collection, the subjects had fasted for at least 10 h. An intestinal intubations device (Loc-I-Gut®) was used when collecting intestinal fluid from upper jejunum. The perfusate (intestinal fluid) leaving the jejunal segment was collected on ice, pooled, and immediately frozen at –70°C[27].

Fasted state simulated intestinal fluid (FaSSIF) was prepared from a phosphate buffer, pH 6.5, and contained 3 mmol/L sodium taurocholate (purchased from Prodotti Chimini E Alimentari S.P.A, Italy) and 0.75 mmol/L lecithin (purchased from Lipoid GmbH Ludwigshafen, Germany). The phosphate buffer was prepared by dissolving 1.7 g NaOH pellets, 19.8 g $NaH_2PO_4 \times H_2O$ and 30.9 g NaCl (purchased from Sharlau Chemie S.A, Barcelona, Spain; Merck KgaA Darmstadt, Germany and Merck KgaA Darmstadt, Germany, respectively) in 5 L milli-Q-purified water. pH was adjusted with 1 M sodium hydroxide solution or 1 M hydrochloric acid solution[28].

900 μl of HIF or FaSSIF, as a control experiment ($n = 2$ for each media), was taken from the freezer and preincubated in 2 mL glass vials in an Eppendorf thermomixer comfort (Eppendorf AG, Hamburg, Germany) at 37°C for 15 min. By adding 100 μl of a 70 μM preheated FaSSIF solution AZD8081 to each vial, the incubation was started. Samples of 100 μl were taken out after 0, 5, 10, 15, 20, 30, and 60 min. 300 μl ice-cold acetonitrile (purchased from Merck KgaA Darmstadt, Germany), containing 0.5 vol-% formic acid (also from Merck KgaA), was added to each sample to stop the enzymatic activity. The samples were vortexed and centrifuged (Rotina 46R, Hettich centrifuge, Tuttlingen, Germany) at 2°C, for 15 min, and 10 500 g. The supernatant was analyzed by high-performance liquid chromatography (HPLC) to measure the content of AZD8081. The pH in HIF was 6.8 during the experiment (Radiometer PHM240, Radiometer Analytical S.A., France, with a Hamilton Biotrode). All parts of the experiment were carried out under aerobic conditions.

The HPLC system used consisted of a Waters 2690 Alliance separations module and a 2487 dual band absorbance detector (both from Waters, Milford, MA, USA). The detector wavelength was set to 230 nm and 40 μl was injected. The wavelength was chosen to give a better signal to noise ratio compared with 210 nm (see below), where components from the intestinal fluids influence the overall absorbance. The isocratic mobile phase used was a mixture of acetonitrile-acetatebuffer, pH 5.5 and ionic strength of 0.02 (39:61 v/v). A reversed phase column Waters XTerra MSC18, 5 μm, 150 mm × 4.6 mm (Waters) with a precolumn in the same material, was used at a flow rate of 1.0 mL per min. All samples were diluted with one part of milli-Q-purified water prior analysis. Chromatographic data was collected by the computerized data management system Millennium[29] software, 4th version (Waters). No external standards were used; only area units have been used. All samples analyzed had a concentration above limit of detection (LOQ), and LOQ was 0.5 μM.

Stability in colon

To evaluate the stability of AZD8081 in a colonic environment, an *in vitro* model for the colonic lumen was used. 3.0 mL stock solution in physiological saline at 25 μM AZD8081 was added to 12.0 mL of a human feces slurry (pooled feces from three men, aged 30–40 years), and 12.0 mL of physiological saline (as a control experiment, $n = 2$ for each media). The Ethics Committee in Göteborg, Sweden, approved the study. The incubation at 37°C took place under anaerobical conditions (Anaerobical Workstation, AW 800 TG, AddVise, Malmö, Sweden). Samples of 200 μl were taken out after 0, 5, 10, 15, 30,

and 60 min. 600 µl ice-cold acetonitrile (purchased from Merck KgaA Darmstadt, Germany), with 0.5 vol-% formic acid (also from Merck KgaA), was added to each sample to stop the enzymatic activity. The samples were vortexed and centrifuged (Rotina 46R, Hettich centrifuge, Tuttlingen, Germany) at 2°C, for 15 min, and 10 500 g. The content of AZD8081 in the supernatant was analyzed by HPLC according to the method above (stability in HIF). The pH in the slurry was 6.2 under the experiment (Radiometer PHM240, Radiometer Anlytical S.A., France, with a Hamilton Biotrode)[29].

Solubility in FaSSIF and DIF

Solubility of AZD8081 was measured in FaSSIF and dog intestinal fluid (DIF).

Fasting DIF was collected from four male Labradors dogs having a chronic nipple valve fistula at mid-jejunum (approximately 76 cm below pylorus). The fluid was collected on ice after administration of 75 mL physiological saline, pooled, centrifuged (Rotina 46R, Hettich centrifuge, Tuttlingen, Germany) for 10 min and 1700 g at 4°C and immediately frozen at −70°C. The Animal Ethics Committee in Göteborg, Sweden approved the study.

Around 10 mg (FaSSIF) and 4 mg (DIF) of AZD8081 was placed in a 2 mL vial and 1000 µl FaSSIF and DIF, respectively, was added ($n=2$ for each media). The vials were shaked in an Eppendorf thermomixer comfort (Eppendorf AG, Hamburg, Germany) at 37°C and 200 µl samples were withdrawn at 1, 5, and 24 h. After centrifugation (Rotina 46R, Hettich centrifuge, Tuttlingen, Germany) at 37°C, for 15 min and 10 500 g, 100 µl supernatant was diluted to 25.0 mL with a mixture of acetonitrile-acetatebuffer, pH 5.5 and ionic strength of 0.02 (39:61 v/v). 10 µl of each sample was injected on the same HPLC system as the stability experiment in HIF, but here external standards were used. pH (Radiometer PHM240, Radiometer Anlytical S.A., France, with a Hamilton Biotrode) in DIF had increased from 6.9 (start) to 7.6 (24 h) and in FaSSIF from 6.5 (start) to 6.9 (24 h).

Measurement of critical micelle concentration or aggregates

The ability of the ionized form of AZD8081 to form aggregates was studied by surface tension measurements and fluorescence probe studies. The surface tension of solutions with varying concentrations of AZD8081 was determined with the Wilhelmy plate method (RM6 Lauda) in water at 23°C. The second method used was the pyrene binding method[30] (and references therein). The pyrene spectrum shows several vibronic peaks and the fluorescence emission intensity ratio of the first (at 372.5 nm, Py1) and third (at 383 nm, Py3) vibronic peaks, Py1/Py3, is a sensitive indicator of the polarity of the pyrene microenvironment. For the cmc determination, a series of AZD8081 solutions were prepared, and the fluorescence probe was added. The concentration of pyrene was fixed at a concentration of 1 µM. The samples were incubated for 30 min in the dark at room temperature before measuring the fluorescence (Perkin Elmer LS 55 Luminescence Spectrometer). The excitation wavelength was 317 nm, and spectra were recorded at 23°C (water) or 37°C (150 mM sodiumchloride solution). The ratio Py1/Py3 was plotted against the concentration of AZD8081, and the critical micelle concentration (cmc) was determined from the center of the sigmoid.

Reverse phase chromatography

Organic impurities Degradation of AZD8081 in solution and solid phase were analyzed at an Agilent Technologies 1100 HPLC system equipped with a quaternary pump, an autoinjector, a diode-array detector and an 1100 MS, (Agilent Technologies, Wilmington, DE, USA). The analytical column (Thermo Electron Corporation, UK) was a Hypersil™ GOLD 4.0 × 150 mm, particle size 3 µm. The mobile phases were formed from mixing of phosphate buffer 0.025 M pH 2.2 (100% A) and acetonitrile (100% B). The chromatographic method uses a gradient consisted of 0–10 min at 20–36% B, 10–12 min at 36% B, 12–27 min at 36–80% B, 27–30 min at 80% B, 30–31 min at 20% B and 31–40 min at 20% B. Ultraviolet (UV) detection were 210 nm, cell path length 10 mm, and the flow rate were 1 mL/min (Figure 2 shows the UV/visible (VIS) spectrum of AZD8081 with a maximum peak at 210 nm. The spectrum was collected by a Cintra 40 UV/VIS spectrometer from GBC Scientific Equipment Ltd, Dandenong, Australia). A sample concentration of 0.25 mg/mL with an injection volume of 15 µl was used. The amount of impurity/degradation product was estimated by comparing the area of one peak with the total area of all eluted peaks in the chromatogram (Figure 3).

The degradation products in solution were identified by an 1100 MS (Agilent Technologies, Wilmington, DE, USA). The mobile phase for MS analysis was 0.025% (volume) Trifluoric acid (Uvasol® for spectroscopy, Merck, Germany) in water and acetonitrile, respectively. The gradient and analytical column was the same as used for the phosphatebuffer/acetonitrile mobile phase.

A simple validation, linearity and LOQ, of the reverse phase chromatographic method for analyzing degradation were performed. The linearity of the impurity method was performed by diluting a sample of 0.25 mg/mL AZD8081 down to 0.05% of the sample. The diluted sample was analyzed and the method is considered linear if the achieved area is within ± 20% of the theoretical value. The method was found linear with a deviation of 2% from the expected area of the diluted sample. The LOQ at S/N = 10, for the method was estimated to 0.01%.

Enantiomeric purity AZD8081 is the *S*-enantiomer of a racemate substance. The chiral center is situated at the carbon to which the fluorobenzene ring is attached. All investigated batches contained 0.1–1.7% of the *R*-enantiomer (Figure 4).

Different intermediates, which contain the chiral center, were also analyzed. The results indicate that enantiomeric pure intermediates result in equally pure product, i.e., no racemization occurs during the last steps of the synthesis.

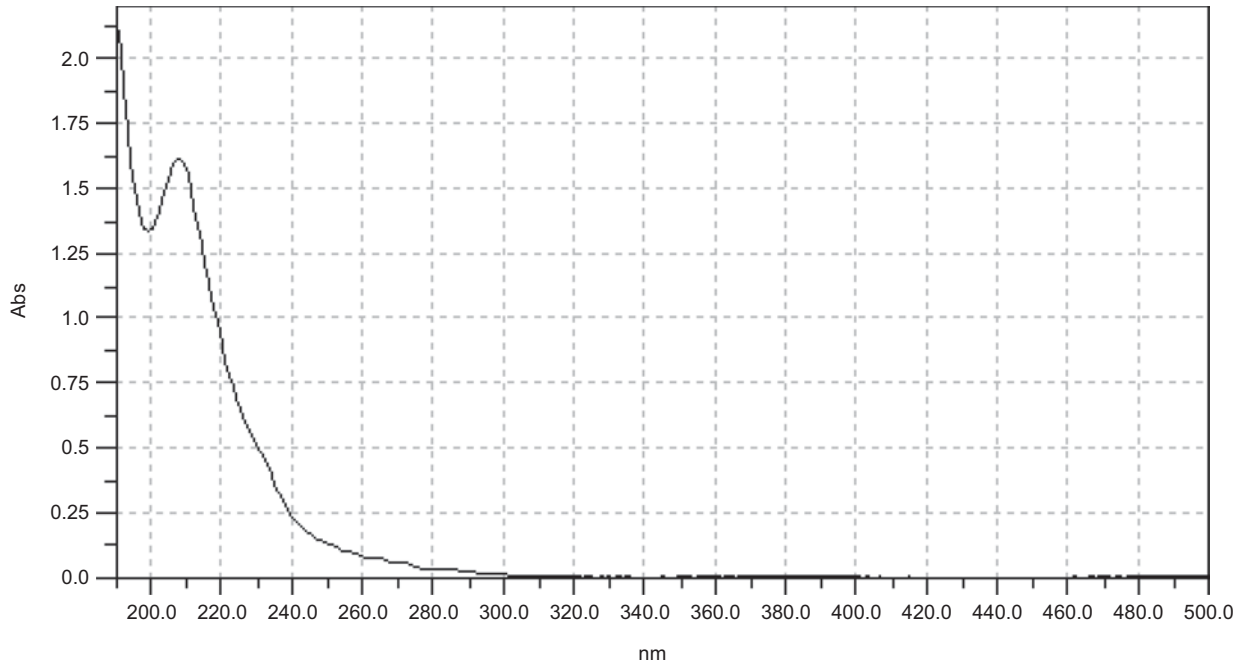

Figure 2. Ultraviolet/visible spectrum of AZD8081.

The same instrumentation as in the organic impurities method was used. The chiral column was a Chiral AGP 100 × 4.0 mm, particle size 5 µm, from Chromtech AB Sweden. The mobile phase was 25% methanol, 2% acetonitrile, and 73% phosphate buffer 0.1 M at pH 7.3. The chiral method used isocratic elution with a flow rate of 1 mL/min, injection volume 20 µl and UV detection at 220 nm (a commonly used wavelength for enantiomeric separation in early phase at AstraZeneca R&D Mölndal, Sweden). The sample concentration used was 0.1 mg/mL AZD8081. The enantiomeric impurity AZ12379060 was eluted before AZD8081. The amount of AZ12379060 was estimated by comparing its peak area with the total peak area of AZD8081 and AZ12379060.

The linearity of the enantiomeric purity method was measured by dilution of 0.1 mg/mL AZD8081. A deviation of 12% from the expected area of the diluted sample showed that the method is linear between 100% (0.1 mg/mL) and 0.4% (0.4 µg/mL). The LOQ (S/N = 10) of the method were found to be 0.4% (area) of 0.1 mg/mL AZD8081.

Results

Solubility

The solubility of AZD8081 was determined in buffered aqueous solutions at pH 5, 6, 7, and 12. The results show that the solubility is pH-dependent with increasing solubility at more acidic pH (Table 1 and Figure 5). The solubility at the lowest pH was not saturated. The results also confirmed the measured pK_a value (see Material and Methods). To verify that there were no ionic effects on the solubility, HCl was used to adjust to some pHs that correlates well with the curve in Figure 5 (data not shown). No differences in solubility were observed between the two crystalline forms (form A and form B, see below). Both forms showed an intrinsic solubility of 0.1 mg/mL (which also is the obtained solubility of pure substance in water, pH 8.5). There were no differences between the two crystalline forms in *in vivo* exposures (in rats and dogs, data not shown) or when comparing with experiments performed with pure amorphous material. No change in solid-state was observed after the solubility experiments described above. Notably is that the substance has a buffering capacity (Table 1).

Stability in solution

The stability in solution was investigated under 16 different conditions, including variation of pH, temperature and absence or presence of light (Table 2). All solutions were stable between pH 1–7 at room temperature and up to 37°C during at least 1 month. Substance stored in a pH 1 solution at 50°C showed a degradation of 2% after 2 weeks (Figure 3B). To produce more of the degradation product, an extreme, unphysiological pH was used (pH 13, Figure 3C). The degradation product was identified as a dibromobensoic acid (LC-MS and by analyzing spiked samples) as a consequence of that the amide bond is the weakest part of the molecule (Figure 1). The left side of the amide bond in AZD8081 was eluated in the void. However, no increase in the amount of R-enantiomer was found after 1 month at pH 1 and at 50°C. The results in Table 2 show that the solutions are not sensitive to light.

Solid-state

The crystallinity was analyzed for several small batches of AZD8081. The crystallinity varied from amorphous to mostly crystalline. Two different polymorphs (form A and form B) were identified (Table 3, Figures 6 and 7). Form

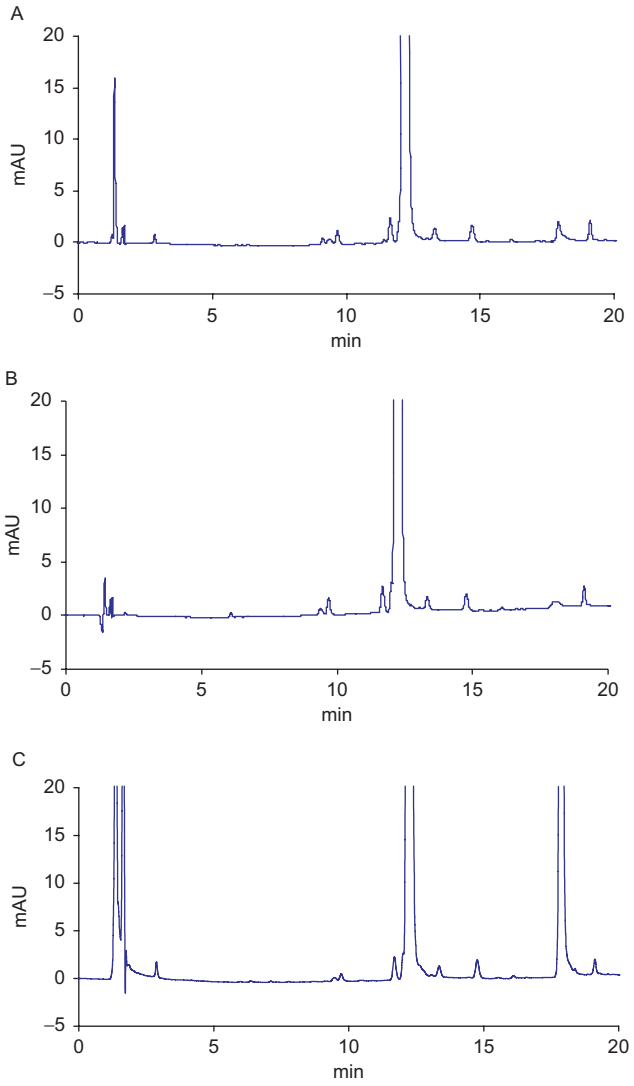

Figure 3. Chromatograms of AZD8081; 0-analyze (A), after 1 month in 0.1 M HCl (pH about 1–2) at 50°C showing the appearance of dibromobensoic acid after 18 min. (B), and after 9 days in 0.1 M NaOH (pH about 13) showing the appearance of dibromobensoic acid after 18 min. (C). During the basic conditions, about 20% of the parent compound formed the degradation product.

A is only slightly hygroscopic, it does not deliquesce (at RH up to 95%) and the weight loss on heating shows that it is not a solvate and that it contains only small amounts of residual solvents. However, re-crystallization to a form with higher melting point (form B) occurs when form A is heated to its melting point. The melting point of form A is 108°C (onset) whereas form B melts at 117°C (onset) (Figure 8). All initial batches were either of the low melting point form (form A) or mixtures of form A and form B. Slurry experiments, performed in ethylacetate at 4°C and room temperature, starting with a 50/50-mixture of form A and form B, resulted in pure form B after 24 h. The slurry experiments and the DSC measurements showed that form B is more stable than form A over the temperature range 4°C to the melting point (i.e., form A and B are monotropic in that range). This means that form A can

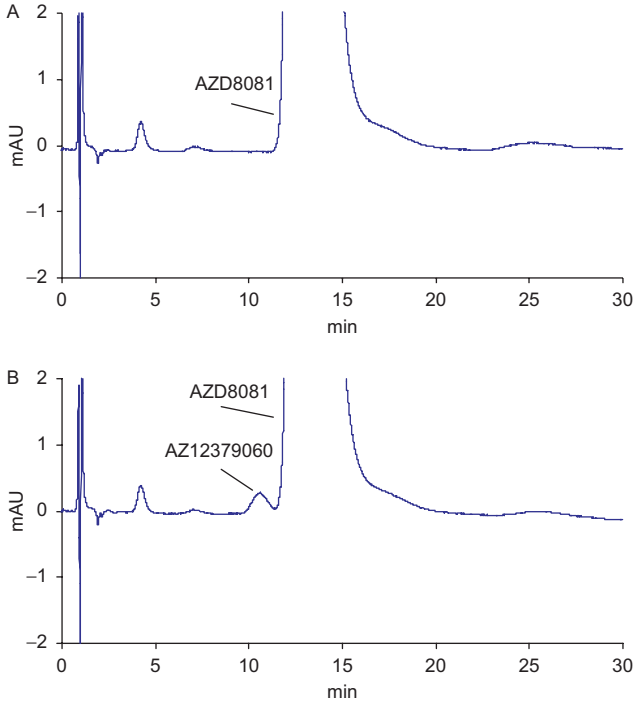

Figure 4. Chiral separation of AZD8081, showing AZD8081 (A) and spiked with 0.3% of its enantiomer AZ12379060 (B).

Table 1. Solubility in solution of AZD8081.

Solutions/pH[a]	pH[b]	Concentration/mg/mL (mM)
0.1 M acetate/5.1[c]	5.1	>91 (>156)
0.1 M HCl/1	6.2	68 (116)
0.1 M phosphate/3.0	6.5	33 (56)
0.1 M phosphate/7.0	7.2	2 (3.3)
0.1 M NaOH/ 13	12	0.1 (0.2)

[a]pH of buffer media before addition of substance.
[b]pH value after 24 h equilibration.
[c]0.1 M acetate buffer pH 5.0 saturated with substance and adjusted to pH 5.1 with 1 M acetic acid before 24 h equilibration.

spontaneously convert to form B. For this reason form, B should be used for future development.

Chemical stability in solid-state

The chemical stability of partly crystalline AZD8081 (form A + amorphous substance, roughly 70/30) was investigated. Already after 1 week at 40°C/75%RH the compound showed a degradation of about 4% (Table 4). The same degradation pattern was observed at 25°C/60%RH although under this condition the degradation was slower. The results showed no difference between closed and open container at 25°C/60%RH, indicating that elevated temperature was the major cause for degradation and not the humidity. In fridge, the degradation process slowed down further and only 1% degradation of the compound was observed after 3 months. No increase in the amount of *R*-enantiomer was found after 3 months storage at 40°C/75%RH. No conversion of

form A or amorphous material to form B were observed in the X-ray powder diffractogram.

To find out if the poor chemical stability (presented in Table 4) is specific to the partly crystalline substance, to the crystalline form, to the impurity profile and/or if the compound is sensitive to the presence of oxygen, complementary studies were initiated with crystalline materials of both forms. The conditions studied were 25°C/60%RH and 40°C/75%RH with and without nitrogen gas over the

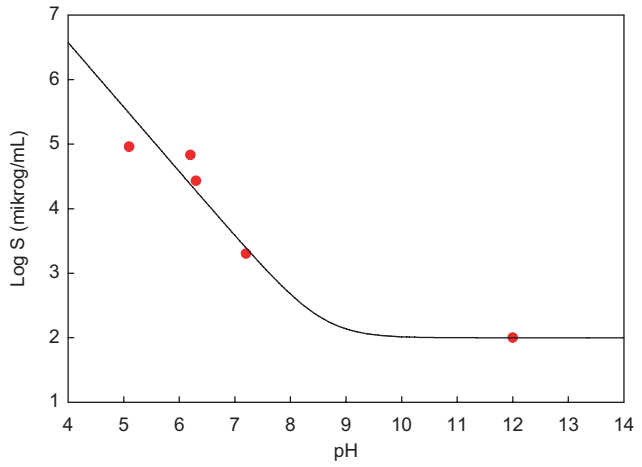

Figure 5. Solubility vs. pH of AZD8081. Note that saturation was not reached at pH 5.1 (all substance was dissolved, solubility >90 mg/mL). The solid line represents the Henderson–Hasselbach equation, with the fit parameters pK_a 8.6 (in close agreement with the value obtained with capillary electrophoresis, see Material and Methods) and an intrinsic solubility of 100 µg/mL. For further details, see Material and Methods as well as Table 1.

Table 2. Stability of AZD8081 (0.1 mg/mL) in 0.1 M buffer solutions.

Conditions	Day 1	Day 3	Day 7	Day 14	Day 28
RT (light)					
pH 1	92.8	92.7	92.3	92.3	93.2
pH 3	93.2	92.9	92.4	92.4	93.5
pH 5	94.3	94.1	93.8	93.8	94.5
pH 7	95.2	95.0	94.8	95.0	95.2
RT (dark)					
pH 1	92.7	92.7	92.3	92.4	93.3
pH 3	93.1	93.0	92.6	92.9	93.3
pH 5	93.9	93.8	93.3	93.3	94.0
pH 7	96.0	95.8	95.2	95.3	95.2
37°C (dark)					
pH 1	92.9	92.8	93.6	93.6	93.2
pH 3	93.2	93.2	93.7	93.8	94.1
pH 5	94.1	94.1	94.4	93.9	93.6
pH 7	95.8	95.6	95.7	95.6	95.6
50°C (dark)					
pH 1	93.5	93.7	92.9	91.4	88.6
pH 3	93.5	94.0	94.4	94.2	94.0
pH 5	94.2	94.4	94.4	94.4	94.6
pH 7	96.1	96.0	96.4	96.5	96.6

Listed as area% remaining in LC-purity.
Note: The difference in area% day 1 between pH 1 and pH 7 is not a difference in stability but a difference in solubility of impurities at different pH. Therefore should these figures only be compared at respective pH and not between pH values. There was no degradation of AZD8081 at a specific pH, except at pH 1 at 50°C.
LC, liquid chromatography.

Table 3. Solid-state properties of AZD8081.

Substance	Forms	DVS-water uptake in % at 80% RH	DSC-thermal events on heating.	TGA-weight loss (%)
AZD8081	A	0.7%	Melting: Tm (onset) = 108°C followed by re-crystallization to form B.	0.7% up to 120°C
AZD8081	B	<0.2%	Melting: Tm (onset) = 117°C	0.2% up to 125°C

DSC, differential scanning calorimetry; DVS, dynamic vapor sorption; RH, relative humidity; TGA, thermogravimetrical analysis.

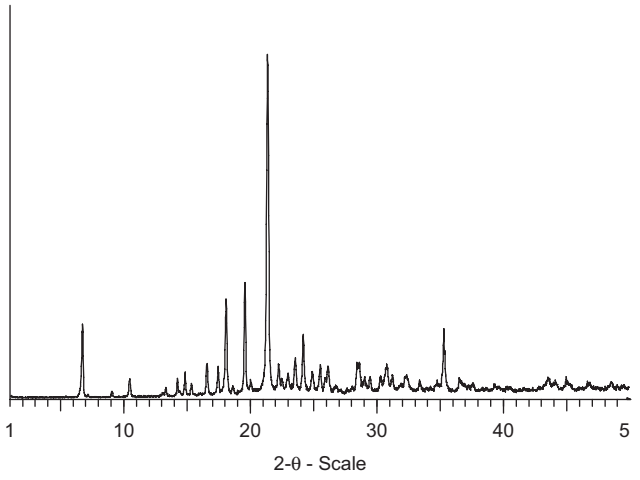

Figure 6. Powder X-ray diffraction pattern of form A of AZD8081. The characteristic X-ray powder diffractometry peaks of form A were observed at 6.7, 18.1, 19.2, 21.3, 22.2, 23.5, 24.2, and 30.8 (2θ).

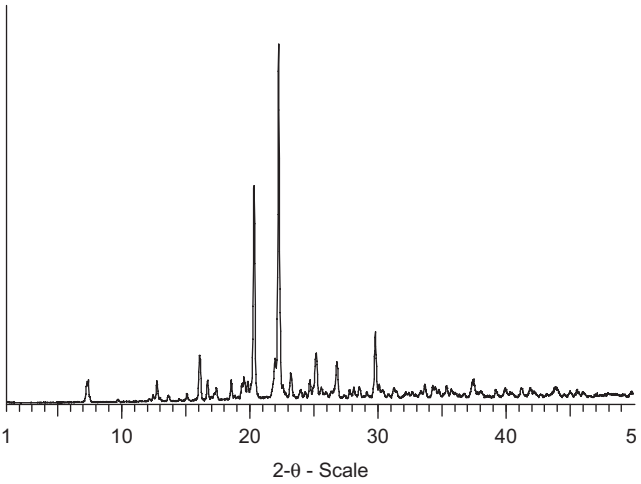

Figure 7. Powder X-ray diffraction pattern of form B of AZD8081. The characteristic X-ray powder diffractometry peaks of form B were observed at 16.1, 20.3, 22.0, 22.2, 25.2, 26.8, and 29.9 (2θ).

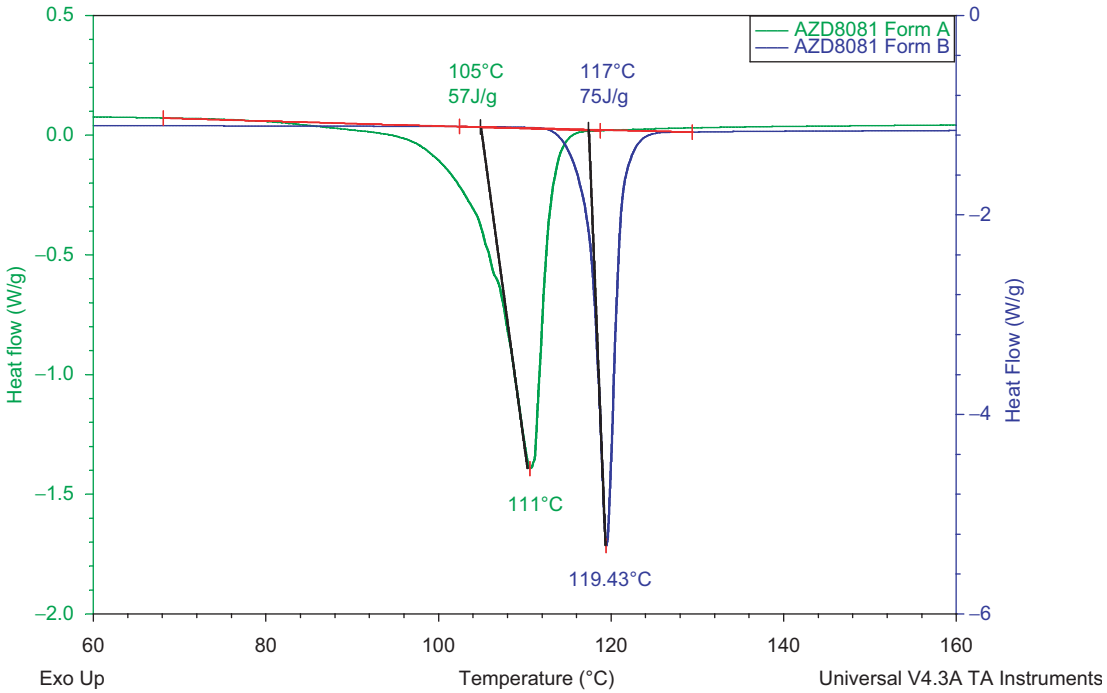

Figure 8. The differential scanning calorimetry profiles of form A and form B of AZD8081 showed one endothermic peak at 108°C and 117°C, respectively, due to the melting of respective form.

Table 4. Solid-state chemical stability of AZD8081 (mixture of form A and amorphous compound) at 25°C/60%RH (closed and open container), 40°C/75%RH (open container), 80°C (closed container) and 4°C (closed container).

Conditions	Day 0	1 Week	1 Month	2 Months	3 Months
25°C/60%RH (closed container)	92.4	92.1	88.5	ND	83.0
25°C/60%RH (open container)	92.4	93.3	89.7	ND	83.7
40°C/75%RH (open container)	92.4	88.1*	73.2	ND	51.0
80°C (closed container)	92.4	34.5	21.7	ND	ND
4°C (closed container)	92.4	91.7	91.6	91.3	91.4

Listed as % remaining in LC-purity. Note that closed container means that the substance was not exposed to RH, even if the vial was placed in that climate.
*>16 Peaks increased and/or appeared.
LC, liquid chromatography; ND, not determined; RH, relative humidity.

compound, as well as 40°C/75%RH (and some analysis were also performed at 80°C). After 3 months (just 1 month followed for form A, 40°C/75%RH, open container, Table 5), no degradation was observed for any of the samples (Tables 5 and 6, form A and form B, respectively). The chemical degradation is not related to the crystalline form of the substance. Neither is the compound sensitive to the presence of oxygen. A major difference between the batches is the contribution of related substances, which is higher (6–7%) in the partly crystalline material compared to the crystalline batches. Even the amorphous contribution was significantly higher in the former. Since impurities in solution are supposed to influence stability more than in solid-state, and AZD8081 in solution is stable (see above, same batch used in the experiments presented in Tables 2 and 4), the degradation in solid-state is mainly caused by the amorphous contribution. No increase in the amount of R-enantiomer was found after 3 months storage at 40°C/75%RH for the crystalline batch. No conversion of forms could be observed after the experiments.

Since form B is the thermodynamically stable form, and the likely choice for further development, the stability study was extended to cover 6 months at 25°C/60%RH and 40°C/75%RH (Table 6). The substance was chemically stable during all conditions. No increase in the amount of R-enantiomer (<0.3%) was found. No changes were observed in the X-ray powder diffractogram.

The photostability of AZD8081 was investigated (Table 7). The testing conditions used were 250 W/m^2 during 24 h under glass filter and an illumination of more than 1.2 million lux hours. The batch above, containing partly crystalline material, was used as well as a batch containing about 50/50 form A and form B. While the latter batch was unaffected, the former had a 3% increase of related substances. The degradation was evident by the increase of two unidentified peaks. The increased degradation reflects the higher molecular mobility in the amorphous material compared to the crystalline substance. No enantiomeric inversion occurred when the substance was exposed to light. No

Table 5. Solid-state chemical stability of AZD8081 (form A) at 25°C/60%RH and 40°C/75%RH under aerobic and anaerobic conditions and 40°C/75%RH and 80°C.

Conditions	Day 0	1 Week	1 Month	2 Months	3 Months
25°C/60%RH (closed container)	98.9	98.5	98.9	98.7	98.5
25°C/60%RH (closed container, anaerobic)	98.9	98.4	98.8	98.7	99.0
40°C/75%RH (closed container)	98.9	98.5	98.5	98.8	99.0
40°C/75%RH (closed container, anaerobic)	98.9	98.6	98.7	98.9	99.3
40°C/75%RH (open container)	98.9	98.8	98.8	ND	ND
80°C (closed container)	98.9	99.0	ND	ND	ND

Listed as % remaining in LC-purity. Note that closed container means that the substance was not exposed to RH, even if the vial was placed in that climate.
LC, liquid chromatography; ND, not determined; RH, relative humidity.

Table 6. Solid-state chemical stability of AZD8081 (form B) at 25°C/60%RH (closed container), 40°C/75%RH (closed and open container) and 80°C (closed container).

Conditions	Day 0	1 Week	1 Month	3 Months	6 Months
25°C/60%RH (closed container)	98.7	98.7	98.7	98.7	98.7
40°C/75%RH (closed container)	98.7	98.7	98.7	98.7	98.7
40°C/75%RH (open container)	98.7	98.7	98.7	98.7	98.7
80°C (closed container)	98.7	98.6	98.7	98.7	ND

Listed as % remaining in LC-purity. Note that closed container means that the substance was not exposed to RH, even if the vial was placed in that climate.
LC, liquid chromatography; ND, not determined; RH, relative humidity.

Table 7. Photostability of AZD8081. Listed as % related substance in LC-UV purity.

AZD8081	Increase in related substance (area%)
form A + B	<0.1%
amorphous + form A	3.2%

LC, liquid chromatography; UV, ultraviolet.

conversion of form A or amorphous material to form B could be observed after the experiments.

Solubility and stability in vehicles

Solubility in vehicles

The initial request from Safety Assessment was to develop a well-tolerated and stable formulation, which could be administrated at doses up to 100 mg/mL. There is an obvious pH dependency for the solubility. Using equimolar amounts of HCl (or a small excess of HCl), it was possible to dissolve >100 mg/mL of crystalline AZD8081 (about pH 5) in water.

Oral administration of AZD8081

For studies where high p.o. doses were required, equimolar HCl solutions (or a small excess of HCl) were the best choice with or without mannitol present.

I.v. administration of AZD8081

For i.v. studies the tonicity of the solution was modified to be as close as possible to physiological tonicity, in order to minimize hemolysis and vessel damage. During early *in vivo* studies, 5% mannitol was used (saline was used in some experiments). A liquid formulation of AZD8081, with mannitol present, can be frozen and thawed at least three times.

Stability in vehicles

Some initial stability studies were performed (Table 8). The formulations were manufactured, diluted to suitable concentrations (1 mg/mL and 12 mg/mL) and analyzed by HPLC. The formulations of AZD8081 were kept in freezer (–20°C) as well as at room temperature (22°C). After 1 month, the formulations (in equimolar HCl solutions with 5% mannitol, pH 6) have sufficient chemical stability for the performed as well as the planned studies.

Biopharmaceutical properties

Solubility and stability in the gastrointestinal tract

AZD8081 is a base with a primary pK_a of about 8.5, which results in a pH-dependent solubility (Table 1 and Figure 5). The compound also has a strong buffering capacity, which could potentially increase the gastric pH (Table 1). However, dissolving AZD8081 in simulated gastric fluid, using a dose of 400 mg (predicted therapeutic dose 50–100 mg twice a day) and an *in vivo* relevant volume (about 250 mL), gives a pH of 1.17 (initial pH was 1.13). This suggests that the buffering capacity is of no relevance in the human *in vivo* situation. In FaSSIF at pH 6.9, the solubility of crystalline AZD8081 is 3.2 mg/mL after 24 h (the solubility was reached already after the first measurement after 1 h). In this experiment pH increased from 6.5 to 6.9. This value correlates well with the measured solubility in pH-adjusted water (5 mg/mL at pH 6.8) and in buffered solutions (Table 1 and Figure 5). In pooled intestinal fluid from dogs, the solubility of AZD8081 is at least 4 mg/mL (All substance was dissolved, but saturation was not reached. In this experiment, the pH increased from 6.9 to 7.6 in 24 h). The available solubility data

indicate that the predicted doses (50–100 mg) are soluble in less than 250 mL of intestinal fluid in the pH range of 1–7, which is sufficiently high to conclude that solubility is not expected to limit the bioavailability of the neutral form. The results also suggest that AZD8081 has potential to be classified as a high solubility compound according to the Biopharmaceutics Classification System (BCS)[31].

Incubations in HIF (7 μM, 37°C) and pH stability studies (pH range 1–7, Table 2) indicate that dissolved AZD8081 is stable in the gastric and small intestinal environment (Figure 9). AZD8081 is also stable in human colonic fluid (<10% degradation after 1 h incubation, Figure 10), a region that may be important for absorption independent of formulation approach..

Surface tension

During vehicle preparation at high concentrations, it was observed that the formulations formed frothing, an initial sign for a surface-active component. The ability of the ionized form of AZD8081 to form aggregates was studied by surface tension measurements and fluorescence probe studies. The surface tension of solutions with varying concentrations of AZD8081 was determined with the Wilhelmy plate method in water. The surface tension curve showed a marked decrease in surface tension with increasing concentration of AZD8081 up to a concentration of 30 mM where a minimum in surface tension is observed (data not shown). Aggregate formation was also studied with a fluorescence probe technique using pyrene as hydrophobic probe. Aggregates starts to form at about 4 mM (Figure 11) both in water at 23°C and in 150 mM NaCl at 37°C. This is in the same range as the cmc for several bile salts and it is likely to expect

Table 8. AZD8081 in equimolar HCl solution with 5% mannitol, pH about 6 (for both concentrations).

Conditions	Day 0	Day 7	Day 30
1 mg/mL, 22°C	98.4	98.7	98.1
1 mg/mL, –20°C	98.4	98.5	98.4
1 mg/mL, 22°C, dark	98.4	98.6	98.4
12 mg/mL, 22°C	98.0	98.6	98.4
12 mg/mL, –20°C	98.0	ND	98.5

Listed as % remaining in LC purity.
LC, liquid chromatography; ND, not determined.

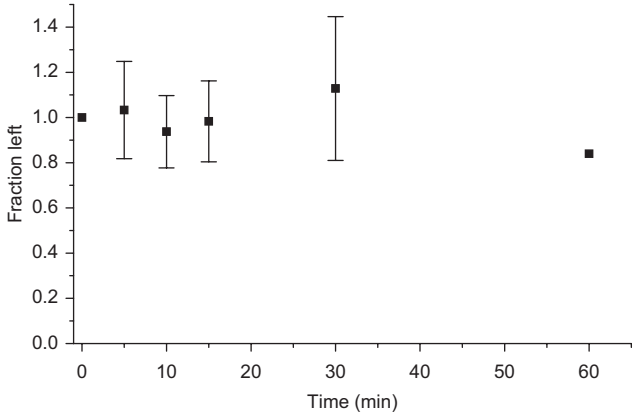

Figure 9. The stability of AZD8081 in human intestinal fluid was investigated by incubation at a concentration of 7 μM and at 37°C ($n=2$). Samples of 100 μl were taken out after 0, 5, 10, 15, 20, 30, and 60 min. The samples were analyzed by high-performance liquid chromatography to measure the content of AZD8081. The pH in HIF was 6.8 during the experiment. All parts of the experiment were carried out under aerobic conditions. One point (after 60 min) in the second serie was excluded from the study due to technical circumstances. The standard deviation (%relative standard deviation) for each serie was 14% and 7%, respectively [for the FaSSIF (fasted simulated small intestinal fluid) study, the figures were 4% and 7%, data not shown]. For further details, see Material and Methods.

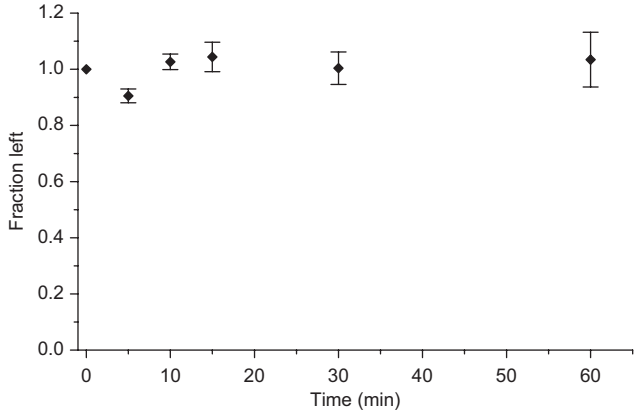

Figure 10. Stability of AZD8081 in fecal fluid at 37°C ($n=2$). The incubation at 37°C took place under anaerobical conditions. Samples of 200 μl were taken out after 0, 5, 10, 15, 30, and 60 min and were then analyzed by high-performance liquid chromatography. The pH in the slurry was 6.2 under the experiment. The standard deviation (%relative standard deviation) for each serie was 3% and 7%, respectively (in the control study, the figures were 3% and 6%, data not shown). For further details, see Material and Methods.

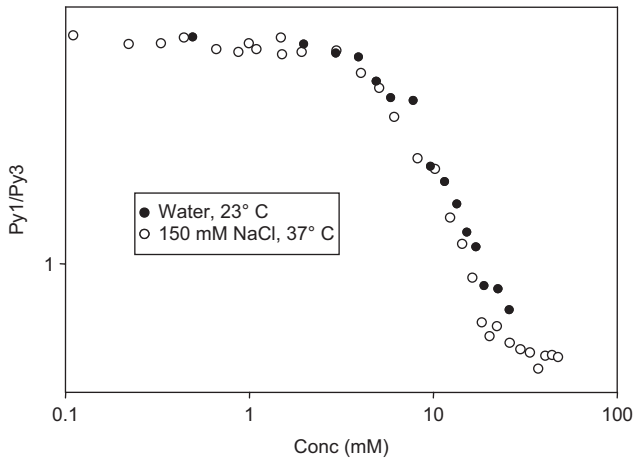

Figure 11. Aggregation behavior of AZD8081 as studied by the pyrene bindning method. Py1/Py3 is the ratio of fluorescence emission intensities for pyrene at 372.5 nm (Py1) and 383 nm (Py3). The excitation wavelength was 317 nm and the experiments were performed in either water at 23°C or in 150 mM sodiumchloride solution at 37°C.

significant interaction with bile salts in the intestine. as well as different membranes in the gastrointestinal.

Discussion

For Safety Assessment- and phase I clinical studies, vehicles will be required that can deliver high doses of test compound for oral as well as i.v. administration. For oral administration this can be achieved by using an aqueous based solution of the test compound (i.e., the neutral form or a salt).

Although the aim of the project is to deliver an oral solid dosage form, at least one study will be performed in man by i.v. administration. Therefore an i.v. dosage form needs to be developed. For i.v. studies the tonicity of the solution should be modified to be as close as possible to physiological tonicity, in order to minimize hemolysis and vessel damage. Mannitol was used as tonicity modifier in the i.v. studies in animals, and can be a possible choice in the future. Also saline, glucose, glycerol and sorbitol may be considered for AZD8081 and/or its future salt.

Some initial stability studies were performed on the substances in vehicles. It was shown that formulations of AZD8081 are stable for at least 4 weeks in freezer. Since there is a risk for affecting the microbiological quality of the solutions, the formulations should preferably be stored frozen until administration.

The most physically stable polymorph of AZD8081 known today, the form B, should be used for formulation work in order to make sure that no solid-state transitions occur during pharmaceutical processing.

The crystallinity of AZD8081 (form A and form B) is high and the substance is classified as just slightly hygroscopic, which is an advantage for solid oral formulations. However, the solid-state stability during pharmaceutical processes such as wet-granulation and compression should be studied in order to investigate the drugability of AZD8081 (e.g., risk of formation of amorphous compound in the solid formulation or possible polymorphic changes). Both chemical- and photostability are sensitive to the presence of amorphous substance.

The available permeability (high permeability in Caco-2 and human colon, data not shown) and solubility data, as well as the data on intestinal stability, suggest that neither of these processes would limit the bioavailability of AZD8081 *in vivo* in humans at the suggested dose range (50–100 mg). Thus, from a biopharmaceutical point of view, development of a solid IR formulation could follow standard approaches without special emphasis on biopharmaceutical aspects. Moreover, *in silico* simulations have shown that particle size does not affect the absorption of AZD8081, irrespective whether the pH in the stomach is high or low (data not shown). This suggests that there will be no need for micronization to ensure rapid dissolution of the compound.

The available solubility data also suggest that dissolution is sufficiently rapid for the neutral form (in FaSSIF the solubility is reached within an hour). Consequently, there is no need for a salt in order to improve dissolution rate. Overall, no biopharmaceutical issues could be identified that would hamper the development of an IR formulation and in a further (possible) perspective, an ER formulation.

AZD8081 is surface active. Such compounds could be irritating for tissues and signs of such effects should be further explored. If relevant, the risk of getting high local concentrations by sticking to the mucosa in esophagus or gastrointestinal tract should be minimized by formulation design and selection of the most suitable solid-state form. Furthermore, the substance may form micelles and potentially other colloidal particles in the gastrointestinal tract, which potentially could lead to unpredictable absorption phenomena, not yet identified. These properties will be most pronounced for solid controlled release formulations. On the other hand, the fact that the substance acts as a surfactant, improves the wettability of the drug substance in wet-granulation process and in dissolution, which facilitates the development of an IR tablet.

The short-half life of AZD8081 (1–3 h in rat and dog, data not shown) is an indicator that ER might improve clinical utility in the future. The good solubility and the high permeability data in Caco-2 and human colon, as well as the fact that it is stable in the colonic fluid, suggest that AZD8081 has sufficient colonic drug absorption and stability for development of an ER formulation. An ER formulation may also reduce the risk of undesired side-effects related to the initial plasma peak.

For highly soluble and permeable compounds like AZD8081, which are well absorbed, the rate-limiting step in the absorption process will be dissolution or gastric emptying, if dissolution is rapid enough. Any process affecting the gastric emptying rate and/or intestinal transit time may therefore potentially change the fraction absorbed or the absorption rate. The effect of NK1/2 receptor antagonists on gastric emptying and/or intestinal motility is not fully evaluated. To rule out any such effect of AZD8081 *in vivo*, an intestinal transit study should be considered. The potential for classifying this drug as a class I compound according to BCS[31] should be further explored in development to reduce risk and simplify documentation by use of biowaivers (permission from the regulatory authority to use *in vitro* dissolution tests as a surrogate for *in vivo* pharmacokinetic data when assessing bioequivalence) in changes between different clinical trial formulations and/or any future marketed product[32–34].

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA. (2004). Tachykinins and tachykinin receptors: a growing family. Life Sci, 74:1445–1463.
2. Quartara L, Altamura M. (2006). Tachykinin receptors antagonists: from research to clinic. Curr Drug Targets, 7:975–992.
3. Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, Erspamer V. (2002). The tachykinin peptide family. Pharmacol Rev, 54:285–322.
4. Maggi CA, Patacchini R, Rovero P, Giachetti A. (1993). Tachykinin receptors and tachykinin receptor antagonists. J Auton Pharmacol, 13:23–93.
5. Maggi CA. (1996). Tachykinins in the autonomic nervous system. Pharmacol Res, 33:161–170.
6. Stewart-Lee A, Burnstock G. (1989). Actions of tachykinins on the rabbit mesenteric artery: substance P and [Glp6,L-Pro9]SP6-11 are potent agonists for endothelial neurokinin-1 receptors. Br J Pharmacol, 97:1218–1224.
7. Tsuchida K, Shigemoto R, Yokota Y, Nakanishi S. (1990). Tissue distribution and quantitation of the mRNAs for three rat tachykinin receptors. Eur J Biochem, 193:751–757.
8. Ho WZ, Lai JP, Zhu XH, Uvaydova M, Douglas SD. (1997). Human monocytes and macrophages express substance P and neurokinin-1 receptor. J Immunol, 159:5654–5660.
9. Lai JP, Douglas SD, Ho WZ. (1998). Human lymphocytes express substance P and its receptor. J Neuroimmunol, 86: 80–86.
10. Patacchini R, Maggi CA. (2001). Peripheral tachykinin receptors as targets for new drugs. Eur J Pharmacol, 429:13–21.
11. Naline E, Devillier P, Drapeau G, Toty L, Bakdach H, Regoli D et al. (1989). Characterization of neurokinin effects and receptor selectivity in human isolated bronchi. Am Rev Respir Dis, 140:679–686.
12. Pennefather JN, Zeng XP, Gould D, Hall S, Burcher E. (1993). Mammalian tachykinins stimulate rat uterus by activating NK-2 receptors. Peptides, 14:169–174.
13. Croci T, Aureggi G, Manara L, Emonds-Alt X, Le Fuhr G, Maffrand JP, Mukenge S, Ferla G. (1998). *In vitro* characterization of tachykinin NK2-receptors modulating motor responses of human colonic muscle stripes. Br J Pharmacol, 124:1321–1327.
14. Saffroy M, Torrens Y, Glowinski J, Beaujouan JC. (2001). Presence of NK2 binding sites in the rat brain. J Neurochem, 79: 985–996.
15. Saffroy M, Torrens Y, Glowinski J, Beaujouan JC. (2003). Autoradiographic distribution of tachykinin NK2 binding sites in the rat brain: comparison with NK1 and NK3 binding sites. Neuroscience, 116:761–773.
16. Massi M, Panocka I, de Caro G. (2000). The psychopharmacology of tachykinin NK-3 receptors in laboratory animals. Peptides, 21:1597–1609.
17. Page NM, Bell NJ. (2002). The human tachykinin NK1 (short form) and tachykinin NK4 receptor: a reappraisal. Eur J Pharmacol, 437:27–30.
18. Patak E, Candenas ML, Pennefather JN, Ziccone S, Lilley A, Martín JD et al. (2003). Tachykinins and tachykinin receptors in human uterus. Br J Pharmacol, 139:523–532.
19. Fioramonti J, Gaultier E, Toulouse M, Sanger GJ, Bueno L. (2003). Intestinal anti-nociceptive behaviour of NK3 receptor antagonism in conscious rats: evidence to support a pheripheral mechanism of action. Neurogastroenterology and Motility, 15:363–369.
20. Lecci A, Maggi CA. (2003). Peripheral tachykinin receptors as potential therapeutic targets in visceral diseases. Expert Opin Ther Targets, 7:343–362.
21. Kramer MS, Cutler N, Feighner J, Shrivastava R, Carman J, Sramek JJ et al. (1998). Distinct mechanism for antidepressant activity by blockade of central substance P receptors. Science, 281:1640–1645.
22. Leroy V, Mauser P, Gao Z, Peet NP. (2000). Neurokinin receptor antagonists. Expert Opin Investig Drugs, 9:735–746.
23. Holzer P, Holzer-Petsche U. (2001). Tachykinin receptors in the gut: physiological and pathological implications. Curr Opin Pharmacol, 1:583–590.
24. Lecci A, Valenti C, Maggi CA. (2002). Tachykinin receptor antagonists in irritable bowel syndrome. Curr Opin Investig Drugs, 3:589–601.
25. Wu Y, Loper A, Landis E, Hettrick L, Novak L, Lynn K et al. (2004). The role of biopharmaceutics in the development of a clinical nanoparticle formulation of MK-0869: a Beagle dog model predicts improved bioavailability and diminished food effect on absorption in human. Int J Pharm, 285:135–146.
26. Wan H, Holmén AG, Wang Y, Lindberg W, Englund M, Någård MB et al. (2003). High-throughput screening of pKa values of pharmaceuticals by pressure-assisted capillary electrophoresis and mass spectrometry. Rapid Commun Mass Spectrom, 17:2639–2648.
27. Tannergren C, Petri N, Knutson L, Hedeland M, Bondesson U, Lennernäs H. (2003). Multiple transport mechanism involved in the intestinal absorption and first-pass extraction of fexofenadine. Clinical Pharmacol Ther, 74:423–436.
28. Dressman JB, Amidon GL, Reppas C, Shah VP. (1998). Dissolution testing as a prognostic tool for oral drug absorption: immediate release dosage forms. Pharm Res, 15:11–22.
29. Mortensen PB, Clausen MR, Bonnén H, Hove H, Holtug K. (1992). Colonic fermentation of ispaghula, wheat bran, glucose, and albumin to short-chain fatty acids and ammonia evaluated *in vitro* in 50 subjects. J Parenter Enteral Nutr, 16:433–439.
30. Nakahara Y, Kida T, Nakatsuji Y, Akashi M. (2005). New fluorescence method for the determination of the critical micelle concentration by photosensitive monoazacryptand derivatives. Langmuir, 21:6688–6695.
31. Amidon GL, Lennernäs H, Shah VP, Crison JR. (1995). A theoretical basis for a biopharmaceutic drg classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. Pharm Res, 12:413–420.
32. Lennernäs H, Abrahamsson B. (2005). The use of biopharmaceutic classification of drugs in drug discovery and development: current status and future extension. J Pharm Pharmacol, 57:273–285.
33. Gupta E, Barends DM, Yamashita E, Lentz KA, Harmsze AM, Shah VP et al. (2006). Review of global regulations concerning biowaivers for immediate release solid oral dosage forms. Eur J Pharm Sci, 29:315–324.
34. Kortejärvi H, Urtti A, Yliperttula M. (2007). Pharmacokinetic simulation of biowaiver criteria: the effects of gastric emptying, dissolution, absorption and elimination rates. Eur J Pharm Sci, 30:155–166.