Soraprazan: setting new standards in inhibition of gastric acid secretion

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Non-standard abbreviations:
APA acid pump antagonist
$^{14}$C-AP [dimethyl-amine-$^{14}$C-] aminopyrine
GERD gastroesophageal reflux disease
P-CAB potassium-competitive acid blocker
PPI proton pump inhibitor

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Abstract

Background and aims: After treatment of millions of patients suffering from GERD and other acid-related ailments with proton pump inhibitors (PPIs), there are still unmet medical needs such as rapid and reliable pain relief especially due to nocturnal acid breakthrough. In this work we introduce and characterize the biochemistry and pharmacology of the potassium-competitive acid blocker (P-CAB) soraprazan, a novel, reversible and fast-acting inhibitor of gastric H,K ATPase. Methods: Inhibitory and binding properties of soraprazan were analyzed together with its mode of action, its selectivity and in vivo potency. Results: This P-CAB has an IC$_{50}$ of 0.1 µM if measured with ion leaky vesicles and of 0.19 µM in isolated gastric glands. With a K$_{i}$ of 6.4 nM, a K$_{d}$ of 26.4 nM and a B$_{max}$ of 2.89 nmole/mg this compound is a highly potent and reversible inhibitor of the H,K ATPase. Soraprazan shows immediate inhibition of acid secretion in various in vitro models and in vivo and was found to be more than 2000 fold selective for H,K ATPase over Na,K- and Ca-ATPases. Soraprazan is superior to esomeprazole in terms of onset of action and the extent and duration of pH elevation in vivo in the dog. Conclusion: Rapid and consistent inhibition of acid secretion by soraprazan render the P-CABs a promising group of compounds for therapy of gastroesophageal reflux disease (GERD).
Introduction

Acid related diseases of the upper gastrointestinal tract, especially gastroesophageal reflux disease (GERD), continue to be a widespread problem worldwide (Bytzer and Blum, 2004). With the introduction of the histamine H2 receptor antagonists the first effective, acceptable medical treatment became available, which revolutionized therapy of these ailments. However, although this class of drugs was effective in healing peptic ulcers, it was less effective in treatment of erosive esophagitis. Also, all the H2 receptor antagonists show about 50% tachyphylaxis after administration for several days and do not inhibit the cholinergic stimulation of acid secretion (Teyssen et al., 2001). The introduction and use of Proton Pump Inhibitors (PPIs), drugs targeted against the gastric acid pump, improved acid control and were able to heal erosive esophagitis after 8 weeks treatment (Klinkenberg-Knoll et al., 2000; Stolte et al., 2000) with significant superiority compared to the H2 receptor antagonists (DiPalma, 2001). However, these are acid-activated prodrugs and their mechanism of action requires activity of the ATPase and therefore acid secretion, to allow conversion to the active thiophilic derivative that binds covalently to the pump. Therefore the effectiveness of PPIs is dependent on food intake or other means of stimulation of acid secretion. There is a lag phase before secretory inhibition is achieved and there is a delay in, and less than full steady state inhibition (Sachs, 2003; Sachs et al., 2006). This is because not all pumps are active during the residence time of effective drug levels in the blood and turnover of the pump is about 25% per day (Gedda et al., 1995). The circadian rhythm of acid secretion shows strong activity in the early morning hours. However, after morning
dosing, generally only a variable fraction of the available pumps are stimulated and, therefore, inhibited. This results in about 70% inhibition of maximal acid output on morning dosage and relatively poor performance at night (Ang and Fock, 2006). To achieve optimal acid suppression and successful therapy, administration of the PPIs for at least three days is necessary (Bytzer and Blum, 2004; Sachs et al., 2006; Sachs, 2001).

The gastric H,K ATPase is a P2 type ATPase. This enzyme is responsible for secretion of H⁺ into the secretory canaliculus of the parietal cell by electroneutral exchange of H⁺ for K⁺ (Ganser and Forte, 1973; Sachs et al., 1976). Inhibition of the enzyme is accepted currently as being the best target for medical treatment of acid-related diseases as it is the final step of acid secretion.

As dephosphorylation of the enzyme is dependent on the presence of potassium (Stewart et al., 1981), an alternative means of inhibition of the H,K ATPase is to block the access of potassium to the ion binding site, competitively or non-competitively (Vagin et al., 2002; Vagin et al., 2003; Wallmark et al., 1987). The discovery that tertiary amines were K⁺-competitive inhibitors of the ATPase (Im et al., 1984) led to the elucidation of the mechanism of an imidazo-[1,2α] pyridine, SCH28080 that was a potent inhibitor of this type (Beil et al., 1986; Wallmark et al., 1987). Other imidazo-[1,2α] pyridines, like pumaprazole (von Büdingen et al., 1996) were synthesized. These structures provide an alternative means of pump inhibition. These particular imidazopyridines bind to the E₂P form of the enzyme and are strictly K⁺-competitive (Mendlein and Sachs, 1990). This mechanism allows rapid inhibition of the pump without the need for acidity at its luminal surface since the pump is blocked in mid-cycle.
Several structural derivatives were tested as inhibitors of the H,K ATPase allowing development of a model of the active conformation of SCH28080 where the benzene ring was orthogonal to the imidazopyridine. The generation of a bridged structure such as a napthyridine promised greater efficacy than the more flexible SCH28080. This was achieved with the synthesis of soraprazan (BYK61359, (7R,8R,9R)-2,3-Dimethyl-8-hydroxy-7(2-methoxyethoxy)-9-phenyl-7,8,9,10-tetrahydro-imidazo-[1,2-h][1,7]-napthyridine).

Here, we describe the properties of this reversible inhibitor of the H,K ATPase. Soraprazan is purely K⁺-competitive and therefore has been named a Potassium-Competitive Acid Blocker (P-CAB) (Senn-Bilfinger et al., 2006). A more general term for reversible H,K ATPase inhibitors would be Acid Pump Antagonists (APAs) since not all of these will be strictly K⁺-competitive. This nomenclature has been used previously for the napthyridine discussed here (Shin et al., 2005).

The aim of this study is to characterize potency, affinity and mode of action of this napthyridine inhibitor. This work demonstrates that soraprazan is a selective K⁺-competitive inhibitor of the H,K ATPase with convincing in vitro and in vivo potency.
Methods and Materials

Hog Gastric H,K ATPase Enzyme Preparation

The gastric H,K ATPase was derived from hog gastric mucosa by a previously published method, which involves differential and density gradient centrifugation (Rabon et al., 1988). Briefly, all operations were carried out at 1-4 °C. The gastric mucosa was stripped from the stomach fundus and homogenized in a solution of 0.25M sucrose, 5 mM PIPES/Tris, pH 6.8, 1 mM EDTA, and 1 mM EGTA. The homogenate was centrifuged at 11,000 rpm in a Sorvall GSA rotor for 45 min. The pellet was discarded and the supernatant was centrifuged at 34,000 rpm in a Beckman type 35 rotor for 1 h. The microsomal membrane pellet was resuspended in a solution of 0.25M sucrose, 5 mM PIPES/Tris, pH 6.8, 1 mM EDTA, and 1 mM EGTA, and was purified on a ficoll/sucrose step gradient; composed of 34% (w/v) sucrose, 5 mM PIPES/Tris, pH 6.8, 1 mM EDTA, and 1 mM EGTA overlaid by a solution composed of 7.5% Ficoll, 0.25M sucrose, 5 mM PIPES/Tris, pH 6.8, 1 mM EDTA, and 1 mM EGTA, using a SW 28 rotor at 27,000 rpm for 2 h. The ion-tight vesicle fraction above the 7.5% Ficoll gradient was collected and diluted by adding three volumes excess of a solution of 5 mM PIPES/Tris, pH 6.8, 1 mM EDTA, and 1 mM EGTA. The suspension was centrifuged at 100,000 g for 1 h and the pellet was resuspended in a solution of 0.25M sucrose, 5 mM PIPES/Tris, pH 6.8.

The vesicles obtained have been shown to be over 90% cytoplasmic side out (Shin et al., 2005). The Mg²⁺-dependent activity was about 5.7 µmol mg⁻¹ h⁻¹. The ion impermeability of the vesicles was determined by the difference in K⁺ stimulation of
ATPase activity in the presence of KCl alone and in the presence of KCl and the ionophore, nigericin that allows K⁺ penetration to the luminal surface of the pump. The activity in the presence of nigericin was 105 µmol ATP hydrolyzed mg⁻¹ protein h⁻¹, and in the absence of nigericin only 7.6 µmol mg⁻¹ h⁻¹. Thus, greater than 90% of the K⁺-stimulated ATPase activity was dependent on the addition of the K⁺ ionophore, nigericin, showing that this fraction of the vesicles was K⁺ impermeant. Pi released was measured by the method of Yoda and Hokin (Yoda and Hokin, 1970) and protein concentration was determined by a modified Lowry method (Lowry et al., 1951) with 0.1% SDS.

**H,K ATPase activity in ion-leaky gastric vesicles.**

1µg of protein was incubated in the presence of 1 mM Mg·ATP, in 100mM Pipes/200mM Tris HCl buffer pH 7.4, 250mM Sucrose and 0.5 - 5.0 mM KCl. Soraprazan was used in nanomolar concentrations for Kᵢ calculation and at 10⁻⁴–10⁻⁹ M for IC₅₀ determination. The reaction was terminated by addition of a 2:1 mixture of 4.5% (w/v) malachite green and 42g/L ammonium molybdate. The phosphomolybdate complex was measured at 690 nm in a multiwell spectrophotometer, as previously described (Vagin et al., 2002). Graphic determination of Kᵢ was performed according to Dixon (Dixon, 1953). IC₅₀ values were calculated with the help of GraphPad Prism software (version 4.02).

**Proton transport activity:**

Acidification of the gastric vesicles was measured by the quenching of acridine orange. The vesicles at 10 µg/ml were suspended in a medium containing 250 mM sucrose, 150 mM KCl, 3 mM MgCl₂, 1 µM acridine orange, 4 mM Pipes/8 mM Tris buffer pH 7.4 and 10 µg valinomycin to allow K⁺ access to the interior of the vesicles. Transport was
initiated by the addition of 2 mM ATP (pH 7.4) and the fluorescence of acridine orange was measured over time at an excitation wavelength of 480 nm and emission at 530 nm (Rabon et al., 1978; Wolosin and Forte, 1981) in a Victor³ multilabel counter from Perkin Elmer. The inhibitors were added at the indicated concentrations after maximal acidification of the vesicles 30 min after start of the reaction by ATP.

Reversibility:

Reversibility experiments were carried out with ion-leaky gastric vesicles as described above. To measure the reversibility of inhibition by soraprazan in the presence of 1 mM KCl, recovery of H,K ATPase activity after dilution of the incubation mixture was measured. 2.5 µM soraprazan was the starting concentration in two independent experiments triplicate and the dilutions were 2, 5, 10, 20, 50- and 100-fold at constant KCl. H,K ATPase activity was set to 100% at any dilution without inhibitor.

14C-aminopyrine accumulation in intact gastric glands:

Gastric acid secretion is stimulated by gastrin, histamine and acetylcholine via the receptors on the parietal or the enterochromaffin-like cell. These physiologic stimuli influence the intracellular cyclic AMP and Ca²⁺ levels, thus leading to relocation and activation of H⁺,K⁺-ATPase. Instead of the physiologic agonists, the membrane-permeant dibutyryl-cyclic AMP was used to stimulate receptor-independent acid secretion in isolated gastric glands. Accumulation of the weak base [dimethyl-amine-14C-] aminopyrine (14C-AP) in the acidic compartment of the canaliculi serves as an indirect measure of acid secretion and forms the basis of measurement of acid secretion in this in vitro model of the mammalian stomach. Intact gastric glands were prepared from
anesthetized New Zealand rabbits (weight 2-3 kg) by high-pressure perfusion of the stomach, separation of the fundic mucosa and subsequent collagenase digestion of fragments of the mucosa (Berglindh et al., 1976; Berglindh and Obrink, 1976). After the gastric glands were washed several times, they were suspended in Krebs-Henseleit solution containing 2 mg/mL rabbit serum albumin and 2 mg/mL glucose. Glands were incubated for 30 min at 37°C in a shaker bath (200 osc/min) in the presence of 0.125 µM $^{14}$C-AP (113 µCi/µmol) at pH 7.4. Glands were stimulated with 1 mM dibutyryl cAMP in absence or presence of soraprazan (concentration range 3 nM-100 µM). The reaction was stopped by centrifugation (10 sec at 20,000 g). After centrifugation, the accumulation of $^{14}$C-AP in the glands was calculated as follows: radioactivity was measured in an aliquot of the supernatant (200 µl) and in the precipitate after dissolution in 1 ml of 1 N NaOH. In order to calculate the amount of protein, the Eppendorf tubes were weighed empty, with protein (wet weight) and with freeze-dried protein (dry weight). This ratio of supernatant and pellet protein radioactivity was used to calculate the accumulation of $^{14}$C-AP in the glands. The inhibitor concentration required to achieve 50 % inhibition (IC$_{50}$) of $^{14}$C-AP accumulation was determined by fitting the equation for the expected inhibition pattern to the data points.

**Soraprazan binding to ion-leaky gastric H,K ATPase**

$[^3]$H]Soraprazan binding studies were carried out at 20°C. In saturation experiments to determine the K$_d$ value, ion-leaky gastric vesicles (0.01-0.02 mg/mL) were resuspended in a buffer composed of 20 mM Tris/HCl, pH 7.0, 2 mM MgCl$_2$, 2 mM ATP (pH 7.0 by Tris), and in the presence of increasing concentrations of $[^3]$H)soraprazan (0.1 nM to 1
µM). Non-specific binding was determined in the presence of a 100 fold excess of unlabeled soraprazan over the concentration range of \([^{3}H]\)soraprazan used. The enzyme suspension (1 mL) was incubated at 20°C for 30 min and rapidly filtered through a nitrocellulose membrane filter (HAWP Millipore filter, 0.45 µm) pre-wet with a solution composed of 20 mM Tris/HCl, pH 7.0, 10% PEG 3350 that was placed on top of a glass fiber filter. The membrane was washed five times with 2.5 mL of a buffer composed of 20 mM Tris/HCl, pH 7.0, 10% PEG 3350 to remove unbound inhibitor. The membrane put into a 20 mL scintillation vial, dimethylacetamide (0.5 mL) was added to dissolve the membrane, and 14 mL of scintillation solvent was added and counted. Binding of \([^{3}H]\)soraprazan was determined by subtracting the non-specific binding of \([^{3}H]\)soraprazan, obtained in the presence of the 100 fold excess of non-radioactive soraprazan, from the amounts of \([^{3}H]\)soraprazan bound to the membrane in the absence of the cold inhibitor.

In KCl competition experiments, a fixed concentration of \([^{3}H]\)soraprazan (18 nM) was incubated in the presence of varying concentrations of KCl (0.001 to 300 mM) at 20 °C for 30 min. An aliquot at given concentration of KCl was taken out and the radioactivity bound to the enzyme was measured as described above. All experiments were performed in triplicate or more and the average of the results was used for analysis.

**Na,K-ATPase measurement:**

The Na,K-ATPase purified from rabbit kidney was purchased from Prof. HJ Apell (University of Konstanz, Germany). Inhibitor activity on the Na,K-ATPase was measured
in the same way as the H,K ATPase activity in ion-leaky gastric vesicles with the exception that NaCl was added at 12 mM final concentration.

**pH-metry in the gastric fistula dog:**

Male Beagle dogs (Boehringer Ingelheim and Harlan, Germany) were used. At an age of 1-2 years, a metallic cannula (V4a or titan) was placed in an artificial fistula at the lowest part of the gastric corpus near the greater curvature. At the beginning of the present study, the animals were aged 2 - 4 years. Their body weight was between 12 and 19 kg (mean ± sem: 14.3 ± 2.3 kg). They were kept in groups of 2-4 animals and housed at 20-23°C, 55-65% relative humidity under seasonally varying light-/dark-rhythm. They received water-presoaked standard dog diet (Provimi Kliba, Switzerland) once daily at 10 a.m. with tap water ad libitum. 20-22 hours prior to and during the day of the experiment, the animals were fasted.

The experimental procedure has been described in detail elsewhere (Postius et al., 1991). Briefly, on the experimental day, the animals were supplied with an ambulatory pH-meter containing a solid state storage unit (Digitrapper pH100, Medtronic, Germany) and a programmable infusion pump (Panomat P, Disetronic, Switzerland). Intragastric pH was measured by means of a combined pH-glass electrode (type 440-M3, Ingold, Switzerland) inserted into the gastric cannula. Gastric acid secretion was stimulated by continuous subcutaneous infusion of pentagastrin (6 µg/kg/h). Recording of intragastric pH started at 8 a.m., pentagastrin infusion at 9 a.m. At 10:30 a.m., the animals received the test substance orally. The experiment was terminated at 7:30 a.m. next day, and pH readings were transformed by the Polygram98 program (Medtronics, Düsseldorf,
Germany) to yield individual 24 hour pH profiles. Applying a second program (StatpHac2000, Leif Fransson, Karlskrona, Schweden), the individual pH profiles of one treatment group were processed to establish median pH for intervals of 10 minutes each. Calculation of the significance of achieved pH-levels was performed by use of non-parametric analysis according to Kruskal-Wallis. The statistical tools are included in StatpHac2000.

Drugs for dog studies were soraprazan granulate ((7R,8R,9R)-2,3-Dimethyl-8-hydroxy-7(2-methoxyethoxy)-9-phenyl-7,8,9,10-tetrahydro-imidazo-[1,2-h][1,7]-naphthyridine); ALTANA Pharma AG) and esomeprazole (Nexium MUPS, AstraZeneca). Oral administration of the calculated amounts/body weight was performed comparably for both drugs in hard gelatine capsules. Encapsulation has been demonstrated not to affect the release and absorption characteristics of enteric coated omeprazol MUPS in man (Schaltenbrand et al., 2001). With each animal, a drug-free control run was done. The comparison of both drugs was done as a randomized intraindividual dose-response study with 6 animals. Oral doses were 1, 3, 9 and 27 µmol/kg.

**Primers and conditions for TaqMan™ PCR:**

Human RNAs were obtained from several sources: ABS (Basel, Switzerland), Ambion (Huntingdon, UK), Ardais Corp. (Lexington, MA, USA), Biocat (Heidelberg, Germany), Stratagene (Amsterdam, The Netherlands) and AXXAM srl (Milan, Italy). The purity and integrity of all RNAs was assessed on the Agilent 2100 bioanalyzer with the RNA 6000 NanoChip reagent set (Agilent Technologies, Böblingen, Germany). Samples were treated with DNase in order to remove traces of contaminating genomic DNA. RNAs
were quantified with the Nanodrop ND-1000 spectrophotometer (PEQLAB Biotechnologie GMBH, Erlangen, Germany) and then stored at -80°C. Additionally, the human tissue RNA collection from AXXAM srl in combination with AXXAM srl’s TaqMan™ analysis service was used. RNA samples were exchanged and TaqMan™ PCR procedures were cross-validated between ALTANA Pharma AG and AXXAM srl. RNAs for every tissue came from several independent donors from both sexes. 1 µg of RNA was reverse-transcribed using random hexanucleotide primers (Roche Applied Science, Mannheim, Germany), dNTPs (PCR 3 Mix, Larova, Teltow, Germany) and avian myeloblastosis virus (AMV) reverse transcriptase (Roche Molecular Biochemicals) at 42°C for 1 hour. All cDNAs were diluted with Tris-HCl buffer (1mM Tris, 0.1mM EDTA pH 8.0, Ambion) to a final concentration of 2 ng/µl and stored at -20°C until further use. As a control for genomic DNA contamination each RNA sample was incubated also in absence of AMV reverse transcriptase and this sample was run along in the TaqMan™ PCR runs. All primers VIC™-labeled probes were obtained from Applied Biosystems (Darmstadt, Germany). Assay on demand HS00167575_m1 was used for specific detection of the gastric H,K ATPase alpha subunit. The following endogenous 18S rRNA control primers and probe were used:

sense 5’-CGGCTACCACATCCAAGGAA-3’,
antisense 5’-GCTGGAATTACCGCGGCT-3’,
probe 5’-VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA-3’.

TaqMan™ PCRs were run on ABI 7900 HT and ABI 7700 Sequence Detection Systems (Applied Biosystems). Each PCR reaction was performed in a total volume of 25 µl in 96-well plate format, containing 2.5 µl cDNA, 12.5 µl qPCR Mastermix Plus (Eurogentec,
Seraing, Belgium), 1.25 µl of the commercial primer/probe set, and nuclease-free water (Ambion). 18S rRNA primers and probe were used at 50 nM each. The TaqMan™ PCR parameters were 2 min 50°C, 10 min 95°C, followed by 40 cycles of amplification (95°C denaturation for 20 sec and 60°C annealing/extension for 1 min). Every run included a water control to check for DNA contamination and probe degradation. All PCRs were performed in triplicate for each sample. Expression levels were calculated from ∆Ct with expression in stomach fundus being normalized to 100%.

**Immunohistochemistry:**

Paraffin-embedded human normal tissue slides were supplied by Biocat and DCS (Hamburg, Germany) and were stained as follows in brief. After deparaffination and rehydration to DCS LabLine buffer (AL120R500, DCS), tissue slides were treated with protease (Sigma, 8038). Slides were blocked with dual endogenous enzyme block (S2003, DAKO, Hamburg, Germany) followed by a biotin blocking system (X0590, DAKO). Afterwards samples were blocked with 10% normal donkey serum (017-000-001, Dianova) in TBS. Monoclonal antibody 1H9 (D031-3, MoBiTec, Göttingen, Germany), directed against gastric H,K ATPase alpha subunit was used 1:2000 in common antibody diluent (HK156-5K) from DCS with 5% donkey serum. An isotype control antibody was used as negative control (N1698, DAKO). Secondary Biotin-SP-conjugated Affini Pure Donkey Anti Mouse IgG (715-065-150, Dianova) was used at 1:1000. Alkaline phosphatase was introduced by use of the StreptABC complex system from DAKO (K0391). Signal was visualized with Sigma Fast Red (F4648) and samples were counterstained with ChemMate Hematoxylin (S2020, DAKO). Tissue slides were
mounted on Kaiser glycerin gelatine (109242, Merck, Darmstadt, Germany). Microscopic pictures were taken with an Axiovert 200 microscope from Zeiss in combination with Zeiss’ AxioVision software (version 4.5).

Protein determination:

The protein contents of the membrane vesicle preparations were determined according to Lowry (Lowry et al., 1951).

Chemicals:

Soraprazan: (7R,8R,9R)-2,3-Dimethyl-8-hydroxy-7(2-methoxyethoxy)-9-phenyl-7,8,9,10-tetrahydro-imidazo-[1,2-h][1,7]-naphthyridine and esomeprazole: 6-Methoxy-2-(((S)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfinyl)-1H-benzimidazole were obtained from Altana chemical research. [3H]soraprazan (specific activity 30 Ci/mmol) was obtained from Amersham. Ionophores were dissolved in methanol. Soraprazan and esomeprazole were dissolved in dimethylsulphoxide. These solvents were shown not to affect ATPase activity in any assay at the concentrations used. All other reagents were analytical grade or better.
Results

Structure-activity relationship of naphthyridines versus imidazopyridine

Soraprazan, (7R,8R,9R)-2,3-Dimethyl-8-hydroxy-7(2-methoxyethoxy)-9-phenyl-7,8,9,10-tetrahydro-imidazo-[1,2-h][1,7]-naphthyridine, with the benzene ring orthogonal to the imidazopyridine (fig 1), was synthesized as described (Senn-Bilfinger et al., 2006). The generation of a relatively rigid bridged structure such as a napthyridine, promised greater efficacy and more metabolic stability than the more flexible SCH28080.

Soraprazan is highly potent in vitro

Soraprazan is a potent inhibitor of gastric H,K ATPase. It has an IC\textsubscript{50} of 0.1 \textmu M when measured in ion leaky vesicles in the presence of 1 mM potassium. Soraprazan also effectively inhibits dibutryl cAMP-stimulated \textsuperscript{14}C-AP accumulation in isolated gastric glands with an IC\textsubscript{50} of 0.19 \mu M (0.09 – 0.40 \mu M geometric mean from n=6 with 95% confidence limits), which is similar to the IC\textsubscript{50} (0.2 \mu M) found for SCH28080 - the first of the reversible proton pump inhibitors with an imidazo[1,2\alpha] pyridine structure (Wallmark et al., 1987).

Kinetics of soraprazan on the gastric H,K ATPase

The Lineweaver-Burk plot shown in figure 2 demonstrates, that in ion leaky vesicles, soraprazan is a potent, K\textsuperscript{+}-competitive inhibitor of the H,K ATPase. The K\textsubscript{i} of soraprazan for inhibition of the H,K ATPase is 6.4 nM (arithmetic mean from 4 separate experiments). From one of these experiments, the K\textsuperscript{+}-competitive characteristic of inhibition was derived (fig 2). The validity of this experiment was demonstrated by K\textsubscript{m}
determination for potassium from the same data of 0.57 mM. Soraprazan binds to the H,K ATPase in ion-leaky vesicles with a K_d of 26.4 ± 3.4 nM and a B_{max} of 2.89 nmole/mg. This result was very similar to that found for intact vesicles (K_i 47 nM; K_d 30.9 nM) (Shin et al., 2005). The binding of soraprazan in the presence of 2 mM Mg.ATP (K_D 28.45) was in agreement with the K_i of 6.4 nM. (K_i 47 nM; K_d 30.9 nM) (Shin et al., 2005). Comparable data for SCH28080 have been published (K_d 45 nM and K_i 24 nM) (Keeling et al., 1988; Keeling et al., 1989; Wallmark et al., 1987).

Inhibition by soraprazan is fully reversible

Consistent with the Lineweaver-Burk plot (fig 2), data from dilution inhibition experiments (fig 3) reveal that the inhibition of H,K ATPase by soraprazan is fully reversible. The inhibition of the H,K ATPase after 2, 5, 10, 20, 50 and 100-fold dilution subsequently reached the activity seen in the absence of inhibitor (100%). Additionally, the radioactive binding experiments demonstrate that potassium can displace the radiolabelled soraprazan from its binding site (fig 4). There was a concentration-dependent displacement of drug binding by K^+ in the presence of nigericin. KCl did not displace drug binding in intact vesicles since the cation does not access the luminal drug binding site. However, in the presence of nigericin, KCl displaced the drug, since in the presence of this ionophore K^+ was able to reach the luminal surface of the enzyme, the location of the inhibitor binding site. There was a concentration-dependent displacement of drug binding by K^+ in the presence of nigericin. The K_{m(app)} was 5.2 mM at 18 nM soraprazan. This shows that binding of
soraprazan interferes with binding of K\(^+\) to the luminal binding site as previously found for SCH28080 (Keeling et al., 1988; Keeling et al., 1989; Shin et al., 2005).

**Soraprazan is highly selective for H,K ATPase**

Soraprazan has a high selectivity for the H,K ATPase versus the Na,K-ATPase. The \(K_i\) for inhibition of the Na,K ATPase was found to be 14.6 \(\mu\)M (arithmetic mean from 3 separate experiments). The geometric mean, -log \(K_i\) ± SD, was 4.83 ± 0.06 M. This compares with a \(K_i\) of 6.4 nM for the H,K ATPase demonstrating a selectivity for the latter by a factor of > 2000.

**Rate of inhibition by soraprazan**

As demonstrated in figure 5 soraprazan inhibits H,K ATPase immediately without a lag phase. This is the expected result for inhibitors of an enzyme that do not require activation. Upon addition of the compound (arrow) soraprazan immediately raised the intravesicular pH. This rapid inhibition is independent of intravesicular pH. Esomeprazole, in contrast, showed the typical delayed inhibition of a pH activated PPI with approximately 3 h between compound addition and half-maximal inhibition in this assay system (Shin et al., 2004).

**Gastric H,K ATPase is a stomach-specific target**

The following human organs, tissues and cells were tested for expression of gastric H,K ATPase alpha and beta subunits namely: parts of the GI tract from esophagus to colon, kidney, eye, retinal pigment epithelium, pancreas, adrenal gland, skeletal muscle, breast, ovary, placenta, uterus, fallopian tube, lymph node, spleen, heart, tongue, skin,
liver, bladder, trachea, bronchus, lung, different parts of the central and peripheral
nervous system, testis, prostate, different blood vessels, various adipose tissues, parotid
and thyroid gland, white blood cells, thymus, tonsil, bone marrow and larynx. Figure 6
shows the results for only these organs and tissues, in which the TaqMan™ PCR was
positive for the alpha subunit of H,K ATPase. Displayed are all organs/tissues with more
than 0.2% of the H,K ATPase alpha subunit expression found in stomach fundus. It can
be seen that alpha subunit expression is maximal in stomach and marginally present in
some other tissues. Not shown is the respective expression of the beta subunit but the
data were negative for adrenal gland and cerebellum. Since, without beta subunit the
alpha subunit is unstable and degraded (Geering, 2001; Vagin et al., 2005), these
results show that no significant expression of functional heterodimeric H,K ATPase is
present in organs other than gastric mucosa. These findings were confirmed on the
protein level by immunohistochemistry with human tissue slides (fig 7). Whereas
stomach fundus displayed a strong and very specific staining of H,K ATPase alpha
subunit in parietal cells, adrenal gland was completely negative as was the concomitant
isotype control.

Superiority of soraprazan over esomeprazole in the gastric fistula dog

In the gastric fistula dog in vivo model the efficacy of compounds on acid secretion and
thus on intragastric pH can be determined. Figure 8 shows the intragastric 24 h pH
profiles of both compounds upon oral administration of 1, 3, 9 and 27 µmol/kg. Although
both compounds elevate the intragastric pH in a dose-dependent manner, the very
straight and clear pH profile of soraprazan differs strikingly from the inconsistent pH-
Elevating effect of esomeprazole. Statistics of the 24 h profiles are compiled in Table 1. Accordingly, mean times to pH 4 and 6 are fast with all doses of soraprazan (fig 7 a-d) but significantly later and highly variable with all doses of esomeprazole (fig 7 e-h). At 1 µmol/kg only 1 animal reaches pH 4 but none pH 6 with esomeprazole in contrast to three animals for soraprazan for both pH levels. The lowest dose of soraprazan reaches pH 4 and 6 faster than any dose of esomeprazole used. The 22 h pH median achieves values of 3.7 and 6.7 at 9 and 27 µmol/kg of soraprazan, whereas same doses of esomeprazole yielded pH medians of 1.9 and 2.2. Neutral pH was reached with 3 µmol/kg and 27 µmol/kg with soraprazan and esomeprazole, respectively. 3 µmol/kg soraprazan achieve a very consistent intragastric pH-plateau (fig 7, curve b), that is statistically significant as compared to controls (p<0.01). In contrast, even 27 µmol/kg esomeprazole do not achieve consistent and reliable intragastric neutrality (fig 7, curve h). Both onset of action and maximum pH reached are much more variable with esomeprazole, which here is clearly inferior to soraprazan (fig 7, curves e-h). No statistical significance against control (fig 7, curve A) could be detected for esomeprazole, whereas 6 hours of significant pH-elevation was found with soraprazan. A very common measure from clinical studies, the period of time at a pH greater than 4.0 (Bell et al., 1992), is 24.4, 49.6, 87.4 % versus 0, 15 and 35.2 % for 3, 9 and 27 µmol/kg of soraprazan and esomeprazole, respectively. In this model, soraprazan showed clear superiority over esomeprazole with respect to the extent and duration of pH elevation, speed of rise of pH, and interindividual variation.
Discussion

Full control of gastric acid secretion has not yet been obtained in clinical practice. It is believed that full inhibition of secretion would alleviate, in particular, nocturnal acid breakthrough that may result in nocturnal symptoms or nocturnal GERD (Ang and Fock, 2006).

Although the proton pump inhibitors (PPIs) of the substituted pyridyl methyl sulphinyl benzimidazole class treat most of the acid-related complications in the upper gastrointestinal tract, there is still a need to develop an improvement in suppression of gastric acid secretion to obtain better symptom relief. The drawbacks of the PPIs relate to their mechanism of inhibition and pharmacokinetics of this class of drugs. The mechanism of inhibition by PPIs requires food intake not less than half an hour after administration of the drug. This is because acid secretion in the secretory canaliculus of the parietal cell has to be induced by food via cholinergic and histaminergic pathways. Acidification on the luminal side of the pump in the membrane of the secretory canaliculus is necessary for firstly the accumulation of the PPI and then for the acid-catalyzed conversion of the PPI prodrugs into the active, thiophilic sulfenic acid form that is able to bind to various luminal cysteines of the H,K ATPase forming a covalent disulfide, thereby inhibiting the enzyme and, in consequence, acid secretion. Food stimulation of acid secretion and a plasma half-life of about one hour make the window for inhibition of active proton pumps very narrow. Acid secretion during day and night therefore cannot be controlled by once daily dosage of PPI in the morning. The mechanism of acid control by the PPIs involves also the dynamics of partial inhibition of the H,K ATPase after the first dosage only, the
recovery of acid secretion during 24 hours due to food activation of inactive H,K ATPase, the reversal of inactivated enzyme (Huber et al., 1995) and the daily 25 % de novo biosynthesis of new enzyme (Gedda et al., 1995; Shin and Sachs, 2002). Therefore, a drug has been developed, which can immediately and more completely inhibit the H,K ATPase independent of food and time of administration, since at adequate dose, no pump would escape inhibition. Soraprazan showed in vitro potency with an IC$_{50}$ of 0.19 µM in gastric glands and bound to the H,K ATPase with a K$_d$ of 28.27 nM. This value is close to the K$_i$ for the enzyme. K$^+$-competitive inhibition and full reversibility was shown, the latter by three independent methods (Lineweaver Burk plot, dilution experiments and reversal of radiolabelled compound binding). One main characteristic of these reversible inhibitors, the fast onset of action, could impressively been shown in vitro in comparison with esomeprazole (fig 5). Vesicles in this in vitro system do not acidify as much as do the canaliculi in vivo, and therefore esomeprazole is not activated at an adequate rate. However, even with faster activation of esomeprazole in vivo, soraprazan is expected to be faster in any system due to its mode of action independent from low pH. Due to this incomplete acidification of vesicles in this in vitro setting, this system emphasizes the advantage of acid-independent inhibition of P-CABs over PPIs. Selectivity against Na,K- and other ATPases by a factor of more than 2000 along with the demonstrated, exclusive expression of gastric H,K ATPase in human stomach suggests that no effects on ATPase-mediated H$^+$ or K$^+$ homeostasis in other organs should be found.

Soraprazan demonstrated an impressive in vivo efficacy and superiority to esomeprazole in the fistula dog model. Standard deviations for esomeprazole were
always much higher, which translates into higher interindividual variations in dogs and probably also in patients. The in vivo results in the dog clearly show the dose-independent duration of action of esomeprazole. With PPIs the time of acid inhibition is achieved by blockade of stimulated parietal cells. Due to the short half-life of 0.5 h in dog, increase of dosing does not lead to prolonged duration of action. In contrast, inhibition of acid secretion by soraprazan is not dependent on active parietal cells, but rather on the availability of effective plasma concentrations. Therefore an increasing dose of soraprazan leads to prolongation of pH elevation as seen in classical receptor pharmacology. It is of note that the relation of the half-lifes of soraprazan and esomeprazole (2.7 and 1.1 h, unpublished) is comparable in humans. Therefore, it is likely that the relative effects of both compounds in man are comparable to those seen in dog on day 1.

Soraprazan is a potent and reversible inhibitor of the H,K ATPase and introduces a new class for therapy of acid related diseases. There is an increasing awareness of unmet medical needs in GERD therapy today, especially complete relief from heartburn, day and night. P-CABs may fulfill this goal for acid-related pain in GERD by rising intragastric pH to a reliable plateau of pH 6 to 7 and therefore should be able to quickly eliminate pain caused by acid reflux into the esophagus. On top of bringing fast symptom relief to GERD patients, this is expected to be of great value for gastrointestinal bleeding and pain relief in intensive care patients. The pharmacology with a fast onset of action favours compounds of this class for on-demand use in clinical practice. Soraprazan therefore provides the possibility to effectively ablate gastric acid secretion for any period of time. This would eliminate the problems associated with
PPIs, such as nocturnal acid breakthrough, and also allow rational on-demand therapy (Ang and Fock, 2006; Sachs et al., 2002).

The immediate and profound inhibition of acid secretion, as shown for soraprazan, promises significant improvement in therapeutic efficacy over PPIs in acid related diseases.
References


Footnotes

* W.A.S. and M.H. contributed equally to this work
Legends for figures

Fig 1
Chemical structure of SCH28080 and of soraprazan ((7R,8R,9R)-2,3-Dimethyl-8-hydroxy-7(2-methoxyethoxy)-9-phenyl-7,8,9,10-tetrahydro-imidazo-[1,2-h][1,7]-naphthyridine)

Fig 2
Lineweaver-Burk plot, showing the data used for $K_i$ determination with ion-leaky gastric vesicles. The control line without inhibitor indicates a $K_m$ for potassium of 0.57 mM, which is in accord with the literature.

Fig 3
Reversibility of inhibition by soraprazan. Activity of H,K ATPase was measured in presence and absence of 2.5 µM soraprazan as starting concentration. Upon dilution by 2, 5, 10, 20, 50 or a 100-fold in assay buffer with constant potassium at 1 mM, H,K ATPase activity in presence of soraprazan returned to almost 100% of control due to dissociation of soraprazan. For each dilution activity without inhibitor was set as 100% control. Activity in the undiluted reaction was 2.8 nmol phosphate/h*µg protein with 2.5 µM soraprazan.

Fig 4
Panel A: The gastric vesicles (11 µg/mL) were incubated in a buffer composed of 20 mM Tris/HCl, pH 7.0, 2 mM MgCl₂, 2 mM ATP (pH 7.0 by Tris), nigericin (2 µg/mL), and in the presence of soraprazan. Soraprazan bound to the enzyme was determined as described in Materials and Methods. Bₘₐₓ of 2.89 ± 0.1 nmole/mg of protein was obtained at 20°C.

Panel B: Soraprazan binding was determined at 20°C in the presence of 2 mM Mg.ATP. The intact gastric vesicles curve represents the incubation in the absence of nigericin and the leaky vesicle curve represents the incubation in the presence of nigericin. The enzyme (10 µg/mL) was incubated in a buffer composed of 20 mM Tris/HCl, pH 7.0, 2 mM MgCl₂, 2 mM ATP, ± 5 µg nigericin/mL, and 18 nM [³H]soraprazan, and KCl (0.001 to 300 mM). No effect of KCl addition was seen in this experiment in the absence of nigericin. In contrast, in the presence of nigericin, a Kₘ(app) of 5.2 mM KCl in the presence of 18 nM soraprazan was observed. Each measurement was average of three experiments.

Fig 5

A proton gradient was created in intact vesicles by gastric H,K ATPase as soon as the enzymatic reaction was started by ATP. Upon addition of the inhibitors (indicated by arrow), inhibition by soraprazan occurred instantaneously, whereas esomeprazole showed the typical delay of acid-dependent activation of a PPI. The inhibition by soraprazan reached its half-maximal level in less than 5 min after addition of the inhibitor. This immediate and acid-independent mechanism of inhibition is observed with reversible interaction of a substrate with an enzyme. Esomeprazole reached half
maximal inhibition only within hours in this leaky vesicle system. Furthermore soraprazan displayed a higher pH elevation in this system. Diagrams show arbitrary units (a.u.) over time, which represent relative intravesicular pH values.

Fig 6
Relative expression of gastric H,K ATPase alpha subunit in human organs/tissues with significantly detectable expression. Expression was detected by TaqMan™ PCR and mean values and standard deviations were calculated from ∆Ct. The mean of stomach fundus was set as 100%. Number of independent donors measured: stomach cardia N=3, fundus N=12, corpus N=4, antrum N=3, pyloric sphincter N=3, pancreas N=7, adrenal gland N=4, adrenal gland cortex N=2, adrenal gland medulla N=2, cerebellum N=4.

Fig 7
Immunohistological staining of gastric H,K ATPase alpha subunit in human paraffin tissue slides from stomach fundus (A) and adrenal gland (C) together with the respective isotype negative controls (B and D). The pictures clearly show that no specific staining and no unspecific background was observed in B, C and D. 100 µm bar annotations are indicated as scale.

Fig 8
Dose dependent effects of soraprazan and esomeprazole on intragastric 24 h pH profiles in the pentagastrin-stimulated gastric fistula dog.
Abscissa: time of day; ordinate: intragastric pH. Intraindividual comparison with N = 6 dogs. The diagrams show medians (solid curves) and 25 and 75% quartiles (shaded areas). Continuous stimulation of acid secretion is done by s.c. infusion of pentagastrin from 9:00 a.m. to 7:30 a.m., next day (open frame in each diagram). Administration of drugs at 10:30 (black triangle); soraprazan a-d, esomeprazole e-h. Oral doses were 1, 3, 9 and 27 µmol/kg. For statistical description of the 24h pH profiles, see table 1.
### Tab 1

**Statistical description of the 24h pH-metry profiles shown in fig 8**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose p.o. (μmol/kg)</th>
<th>Mean time to pH 4 (min)</th>
<th>Mean time to pH 6 (min)</th>
<th>22h pH 25 / 75% quartiles</th>
<th>Mean % time pH above 4</th>
<th>25 / 75% quartiles</th>
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<tr>
<td></td>
<td></td>
<td>Mean time</td>
<td></td>
<td>Median</td>
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<tr>
<td></td>
<td></td>
<td>to pH 4</td>
<td>SD</td>
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<tr>
<td></td>
<td></td>
<td>to pH 6</td>
<td>SD</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Controls</td>
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<td>n.v.</td>
<td>1.3</td>
<td>1.3 / 1.3</td>
<td>0</td>
<td>1.5</td>
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<tr>
<td>Soraprazan</td>
<td>1</td>
<td>150&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33</td>
<td>37</td>
<td>1.3</td>
<td>1.3 / 1.4</td>
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<tr>
<td>(1.4h)</td>
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<td></td>
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<tr>
<td></td>
<td>3</td>
<td>118</td>
<td>35</td>
<td>53</td>
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<td></td>
<td>9</td>
<td>115</td>
<td>41</td>
<td>45</td>
<td>3.7</td>
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<tr>
<td></td>
<td>27</td>
<td>108</td>
<td>51</td>
<td>123</td>
<td>5.0</td>
<td>5.3 / 6.9</td>
</tr>
<tr>
<td>Esomeprazole</td>
<td>1</td>
<td>290&lt;sup&gt;c&lt;/sup&gt;</td>
<td>n.v.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.2</td>
<td>1.2 / 1.3</td>
<td>0</td>
</tr>
<tr>
<td>(0.5h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>447&lt;sup&gt;e&lt;/sup&gt;</td>
<td>247</td>
<td>445&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.3</td>
<td>1.3 / 1.3</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>365</td>
<td>213</td>
<td>302&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>27</td>
<td>245</td>
<td>112</td>
<td>290</td>
<td>2.2</td>
<td>1.7 / 5.7</td>
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</table>

In indicated experiments only N animals reached the respective threshold: N=3 (a,b), N=1 (c), N=0 (d), N=3 (e), N=2 (f), N=5 (g). n.v.: no value

24h pH profiles were recorded for 6 gastric fistula dogs treated with soraprazan and esomeprazole respectively. Profiles were compared with respect to speed and extent of pH rise as well as stability of the pH plateau reached. Values are calculated on basis of
6 intraindividually and randomly performed experiments. $T_{1/2}$ of both compounds has been determined in separate dog studies.
Figure 1

SCH28080

Soraprazan
Figure 2

\begin{align*}
1/\Delta E &= \text{control} \\
1/\Delta E &= 2.5\text{nM} \\
1/\Delta E &= 10\text{nM} \\
1/\Delta E &= 20\text{nM}
\end{align*}

- \( k_i = 6.46\text{nM} \)
- \( K_m = 0.57\text{mM} \)
**Figure 4**
Figure 5
<table>
<thead>
<tr>
<th>Dose</th>
<th>Soraprazan</th>
<th>Esomeprazole</th>
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<tbody>
<tr>
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<td></td>
<td></td>
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<tr>
<td>3</td>
<td></td>
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<tr>
<td>9</td>
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**Figure 8**