Characterization of a novel potassium-competitive acid blocker of the gastric
H,K-ATPase, 1-[5-(2-fluorophenyl)-1-(pyridin-3-ylsulfonyl)-1H-pyrrol-3-yl]-N-
methylmethanamine monofumarate (TAK-438)

Jai Moo Shin, Nobuhiro Inatomi, Keith Munson, David Strugatsky, Elmira
Tokhtaeva, Olga Vagin, and George Sachs

Department of Physiology and Medicine, David Geffen School of Medicine,
University of California at Los Angeles, and VA Greater Los Angeles Healthcare System,
Los Angeles, California, CA90073, USA (J.S., K.M., D.S., E.T., O.V., G.S.); and
Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Japan
(N.I.)
Running title: Potassium-competitive inhibition of TAK-438

Corresponding Author:
Dr. Jai M. Shin, Membrane Biology Laboratory, VA Greater Los Angeles Healthcare System, 11301 Wilshire Blvd., Bldg. 113, Rm 324, Los Angeles, CA 90073, Tel.: 310-268-4672; FAX: 310-312-9478. E-mail address: jaishin@ucla.edu (J. Shin)

Number

Number of text pages: 37

Number of Tables: 1

Number of Figures: 9

Number of References: 30

Number of words in Abstract: 231

Number of word in Introduction: 720
List of nonstandard abbreviations

GERD, gastroesophageal reflux disease; PPI, proton pump inhibitor; P-CAB, potassium-competitive acid blocker; SCH28080, (8-benzyloxy-2-methyl-imidazo(1,2-\(a\))pyridine-3-yl)acetonitrile; TAK-438, 1-[5-(2-fluorophenyl)-1-(pyridin-3-ylsulfonyl)-1\(H\)-pyrrol-3-yl]-\(N\)-methylmethanamine monofumarate

Recommended section assignment

Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

Inhibition of the gastric H,K-ATPase by the potassium-competitive acid blocker (P-CAB) 1-[5-(2-fluorophenyl)-1-(pyridin-3-ylsulfonyl)-1H-pyrrol-3-yl]-N-methylmethanamine (TAK-438), is strictly K⁺-competitive with a Ki of 10nM at pH 7. In contrast to previous P-CABs, this structure has a point positive charge (pKa 9.06) allowing for greater accumulation in parietal cell compared to previous P-CABs (e.g. SCH28080, pKa 5.6). The dissociation rate of the compound from the isolated ATPase is slower than other P-CABs, with the t1/2 being 7.5h in 20 mM KCl at pH 7. The stoichiometry of binding of TAK-438 to the H,K-ATPase is 2.2 nmol/mg in the presence of Mg-ATP, Mg-vanadate, or Mg-Pi. However, TAK-438 also binds enzyme at 1.3 nmol/mg in the absence of Mg²⁺. Modeling of the H,K-ATPase to the homologous Na,K-ATPase predicts a close approach and hydrogen bonding between the positively charged N-methylamino group and the negatively charged Glu795 in the K⁺-binding site in contrast to the planar diffuse positive charge of previous P-CABs. This likely accounts for the slow dissociation and high affinity. The model also predicts hydrogen bonding between the hydroxyl of Tyr799 and the oxygens of the sulfonyl group of TAK-438. A Tyr799Phe mutation resulted in a threefold increase of the dissociation rate, showing that this hydrogen bonding also contributes to the slow dissociation rate. Hence, this K⁺-competitive inhibitor of the gastric H,K-ATPase should provide longer lasting inhibition of gastric acid secretion as compared to previous drugs of this class.
Introduction

Acid related diseases, especially gastroesophageal reflux disease (GERD), are still often a challenge for treatment. The introduction and use of proton pump inhibitors (PPIs, e.g. omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole), drugs targeting the gastric acid pump, improved acid control and improved healing of erosive esophagitis compared to the H₂ receptor antagonists but healing still requires 8 weeks of treatment. Further, over 25% of GERD patients continue to have symptoms particularly at night (Jones and Patrikios, 2008; Gisbert et al., 2009). There is therefore room for improvement of therapy by better acid control.

PPIs are acid-activated prodrugs that require the presence of acid secretion for conversion to the active form that binds covalently to certain cysteines in the luminal domain of the gastric H,K-ATPase (Besançon et al., 1997; Shin et al., 2004). Since not all H,K-ATPases are active at any one time, and the drugs are not present at effective concentrations after a single administration, several doses are required to capture newly active pumps and to achieve steady state inhibition of the ATPase. To ensure the exposure of as many active pumps as possible to each dose of the PPI, it is also important to administer these drugs about 30 minutes before breakfast and also before dinner if they are given twice a day. It therefore requires about 3-5 days of oral treatment for steady state inhibition of acid secretion. The ATPase has a half-life of about 50 hours and therefore about 25% of pumps are synthesized de novo each day and those made at night are not exposed to the PPI whether on once or twice a day regimens due to the short plasma half-life of all the PPIs, namely, 60-90 min. Hence, there is continuing acid secretion especially at night, of low volume but high acidity, resulting in continuing symptoms and damage to the esophagus or stomach. Further, since acid secretion is required for the effect of PPIs, there are multiple acidic excursions following the initial
oral dose. Continuous once a day treatment with the PPIs results in about 70% inhibition of maximal acid output on morning dosage and 80% on twice a day treatment (Katz et al., 2004; Spechler et al., 2009).

These shortcomings highlight the need for anti-secretory drugs with faster onset and better inhibition than the PPIs. The potassium-competitive inhibitors of the H,K-ATPase (P-CABs), which block the access of potassium ion to its binding site on the gastric H,K-ATPase resulting in immediate inhibition of acid secretion, are an alternative that lack the deficiencies of the PPIs. The discovery that tertiary amines were K+-competitive inhibitors of the ATPase (Im et al., 1984) led to the elucidation of the mechanism of action of an imidazo[1,2-a]pyridine, (8-benzyloxy-2-methyl-imidazo(1,2-a)pyridine-3-yl)acetonitrile (SCH28080). SCH28080 binds to the E₂ or E₂P form of the enzyme and is a strictly K⁺-competitive inhibitor (Wallmark et al., 1987; 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 without a requirement for pump activity, thus preventing transient acidic excursions (Wilder-Smith et al., 1995). However, since inhibition is reversible, with either a relatively short plasma half-life or rapid dissociation of the drug, acid secretion rapidly returns to pretreatment levels, reducing efficacy of once a day dosing. After the discovery of SCH28080, many P-CABs were developed based on a similar planar core structure, but were unsatisfactory for clinical usage either due to their short duration of action or due to side effects such as hepatotoxicity or inhibition of the Herg K⁺-channel (Berg et al., 2008; Dent et al., 2008).

Here, we describe the properties of a new reversible inhibitor of the H,K-ATPase, 1-[5-(2-fluorophenyl)-1-(pyridin-3-ylsulfonyl)-1H-pyrrol-3-yl]-N-methylmethanamine monofumarate, TAK-438 (Figure 1). The aim of this study is to elucidate, the mechanism...
of inhibition by TAK-438 and to evaluate the potency of this compound. This work demonstrates that TAK-438 is a selective K\textsuperscript{+}-competitive inhibitor of the H,K-ATPase with very slow reversibility, hence a long duration of action on the H,K-ATPase. Its structure when docked to a novel homology model of the gastric H,K-ATPase based on a recent 3D structure of the Na,K-ATPase explains the very slow off-rate of the inhibitor as compared to the imidazopyridine class of P-CABs due to the close approach of the charged methylamino group of TAK-438 to the K\textsuperscript{+} site at Glu795.
Methods

_Hog Gastric H,K-ATPase Enzyme Preparation_

Gastric H,K-ATPase was prepared from hog gastric mucosa as previously described (Sachs et al., 1976; Chang et al., 1977; Hall et al., 1990). All operations were carried out at 1-4°C. The crude gastric mucosal membranes were collected from the stomach and homogenized in a solution of 0.25M sucrose, 5mM PIPES/Tris, pH 6.8, 1mM EDTA, and 1mM EGTA. The homogenate was centrifuged at 20,000 x g in a Sorvall GSA rotor for 45 min. The pellet was discarded and the supernatant was centrifuged at 134,000 x g in a Beckman type 35 rotor for 1h. The microsomal membrane pellet was resuspended in a solution of 0.25M sucrose, 5mM PIPES/Tris, pH 6.8, 1mM EDTA, and 1mM EGTA, and was purified on a step gradient sucrose solution; composed of 34% (w/v) sucrose, 5mM PIPES/Tris, pH 6.8, 1mM EDTA, and 1mM EGTA overlaid by a solution composed of 7.5% Ficoll, 0.25M sucrose, 5mM PIPES/Tris, pH 6.8, 1mM EDTA, and 1mM EGTA, using a SW 28 rotor at 130,000 x g for 2h. The vesicles above the 7.5% Ficoll gradient step were collected and diluted by adding three volumes excess of a solution of 5mM PIPES/Tris, pH 6.8, 1mM EDTA, and 1mM EGTA. The suspension was centrifuged at 130,000 x g for 1h and the pellet was resuspended in a solution of 0.25M sucrose, 5mM PIPES/Tris, pH 6.8.

The membrane vesicles were over 90% cytoplasmic side-out. Mg$^{2+}$-dependent activity was about 7 µmol•mg$^{-1}$•h$^{-1}$. The activity in the presence of nigericin was 95 µmol Pi•mg$^{-1}$•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 with the K$^+$ site facing the lumen.
Measurement of Acid-stable Phosphoenzyme Levels

Gastric vesicles (100-200 µg/mL) were incubated for 10 seconds at 25°C in a buffer composed of 20mM Tris/HCl, pH 7.0, 2mM MgCl₂, and [γ-32P]ATP (10µM to 0.1mM). An aliquot (0.4mL) was taken and added to an ice-cold stop solution (1mL) composed of 40% trichloroacetic acid, 10mM phosphoric acid, and 1mM ATP. This was immediately filtered through a nitrocellulose membrane filter (HAWP Millipore filter, 0.45µm) prewetted with an ice-cold solution composed of 10% trichloroacetic acid and 20mM phosphoric acid placed on top of a glass fiber filter. After washing four times with 2.5mL of an ice-cold solution composed of 10% trichloroacetic acid and 20mM phosphoric acid, the membrane was placed in a 20 mL scintillation vial, dimethylacetamide (0.5 mL) was added to dissolve the membrane, and 10 mL of scintillation solvent was added and counted. Non-specific Pi binding was measured using a gastric vesicle suspension in a buffer composed of 20mM Tris/HCl, pH 7.0, [γ-32P]ATP (10µM to 0.1mM) and 5mM CDTA with no Mg²⁺, since Mg²⁺ is essential for phosphoenzyme formation. The acid-stable phosphoenzyme level was determined by subtracting the non-specific binding obtained in the presence of CDTA-ATP from the total binding obtained in the presence of MgATP.

Inhibition of ATPase activity

ATPase activity was measured over a range of 0-100mM KCl in the presence of different concentrations of TAK-438 (0-0.1µM) and nigericin. The gastric vesicles (2-3 µg/mL) were resuspended in a buffer composed of 20mM Tris/HCl, pH 7.0, 2mM MgCl₂, KCl (0-10mM), nigericin (1 µg/mL), and TAK-438 (0-0.1µM). Background phosphate
release was measured using an enzyme suspension as described above but in the absence of MgCl₂. Activity was initiated by adding a final concentration of 2mM ATP at 37°C and incubation for 30 min. Released inorganic phosphate was measured and the ATPase activity was calculated. The results obtained for the K⁺-stimulated ATPase activity were fitted to equations describing patterns of competitive, non-competitive, and mixed inhibition by least squares fitting using the computer program GraphPad Prism 4 (GraphPad Software Inc., San Diego, California, USA).

The IC₅₀ was determined in the presence of 10mM KCl over a range of TAK-438 concentrations (0-0.1μM).

Inhibition of acridine orange uptake

Gastric vesicles (20 µg/ml) were suspended in a buffer composed of 3mM Pipes/Tris (pH 7), 2mM MgCl₂, 150mM KCl, 5 µg/ml of valinomycin, in the presence of 1µM acridine orange. Acridine orange accumulation reflecting intravesicular acidification was measured using a spectrofluorimeter with the excitation wavelength at 490 nm and the emission wavelength at 530 nm. After obtaining a steady baseline, ATP was added at 2mM using 0.1M ATP stock solution (pH 7). After adding ATP, the enzyme acidified the lumen of the vesicles resulting in acridine orange uptake with quenching of the fluorescence. When maximal fluorescence quenching was obtained, either SCH28080 or TAK-438 was added and the rate of return of fluorescence measured to estimate the relative rates of binding of the imidazo-pyridine and the pyrrolo-pyridine.
**TAK-438 binding to the gastric H,K-ATPase**

[^14C]TAK-438 binding studies were carried out at 25°C or 37°C. All experiments were performed at least in triplicate and the average of the results was used for analysis. In saturation experiments to determine the binding stoichiometry of TAK-438, the gastric vesicles (0.01-0.02 mg/mL) were resuspended in a buffer composed of 20mM Tris/HCl, pH 7.0, 2mM MgCl₂, 2mM ATP (pH 7.0 by Tris), and in the presence of increasing concentrations of[^14C]TAK-438 (0.1nM to 0.5µM). The enzyme suspension (1 mL) was incubated at 25°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 20mM Tris/HCl, pH 7.0, 10% PEG3350 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 20mM Tris/HCl, pH 7.0, 10% PEG3350 to remove unbound inhibitor. The membrane was placed in a 20 mL scintillation vial, dimethylacetamide (0.5 mL) was added to dissolve the membrane, and 14 mL of scintillation solvent was added and the radioactivity counted.

Non-specific binding of TAK-438 was determined as follows: the enzyme was pre-incubated with 200 fold excess of unlabeled TAK-438 over the above concentration range of[^14C]TAK-438 for 30 min, then, treated with[^14C]TAK-438. After filtration and washing as described above, non-specific binding was measured. The specific binding of[^14C]TAK-438 was determined by subtracting the non-specific binding of[^14C]TAK-438 from the amounts of[^14C]TAK-438 bound to the membrane in the absence of the cold inhibitor.

In order to find out whether the binding is covalent or not, an aliquot of[^14C]TAK-438 bound enzyme was precipitated by methanol. In a typical run, an aliquot of[^14C]TAK-438 bound enzyme (0.1 mL) prepared as described above was treated with 1
mL of ice-cold methanol, and the mixture was incubated on ice for 30 min. The mixture was centrifuged and the protein was separated. [$^{14}$C]TAK-438 in the protein precipitate was counted.

In KCl competition experiments, a fixed concentration of [$^{14}$C]TAK-438 (10nM - 100nM) was incubated in the presence of varying concentrations of KCl (0.001 to 330mM) at 25°C or 37°C for 0.1 - 7h as indicated, in the presence and absence of nigericin (5 µg/ml). An aliquot at a given concentration of KCl was taken out at timed intervals and the radioactivity bound to the enzyme was measured as described above.

The exchange rate of [$^{14}$C]TAK-438/TAK-438 was measured as follows: enzyme (0.01 mg/mL) was pre-incubated with 100nM [$^{14}$C]TAK-438 at room temperature for 60 min. The enzyme suspension was then treated with 500-fold excess of non-labeled TAK-438 and an aliquot was incubated at 37°C, and taken out at timed intervals. The radioactivity bound to the enzyme was measured as described above.

To investigate effects of various ligands on inhibitor binding, the gastric vesicles (0.01mg/mL) were incubated at 37°C for 60 min in a buffer composed of 20mM Tris/HCl, pH 7.0, and different ligands such as ± 2mM MgCl₂, ± 5 (or 10) mM CDTA, ± 2mM ATP (pH 7.0 adjusted by Tris), ± 0.2mM vanadate (pH 7.0), ± 5mM inorganic phosphate/Tris (pH 7.0) in the presence of 100nM [$^{14}$C]TAK-438. The radioactivity bound to the enzyme was measured as described above.

**Binding stoichiometry of TAK-438 with phosphoenzyme (EP)**

To determine the stoichiometry of the inhibitor binding to the acid-stable phosphoenzyme intermediate (EP), first, EP was measured in the presence of non-
labeled TAK-438. Intact gastric vesicles were incubated at 25°C for 1h in a buffer composed of 20mM Tris/HCl, pH 7, 2mM MgCl₂, 10 µg nigericin/ml, 0.1µM non-labeled TAK-438 at a 100 µg/mL protein concentration. Using this TAK-438-bound enzyme, [γ-³²P]ATP was then added at a final concentration of 0.1mM and incubated at 25°C for 10s, 20s, 60s and 120s. Acid-stable EP was measured as described above. TAK-438 binding was measured as follows: intact gastric vesicles were incubated at 25°C for 1h in a buffer composed of 20mM Tris/HCl, pH 7, ± 2mM MgCl₂, ± 10mM CDTA, 10 µg nigericin/ml, 0.1µM [¹⁴C]TAK-438 at a 100 µg/mL protein concentration. TAK-438 binding was measured as described above.

**Dissociation rate of TAK-438 from wild type and mutants of the H,K-ATPase expressed in HEK293 cells**

Wild type and mutants of the rabbit gastric H,K-ATPase expressed in HEK293 cells were prepared as described by Vagin (Vagin et al., 2002; Vagin et al., 2003).

Three groups of membranes containing the various forms of the H,K-ATPase were prepared as follows. Group A) Expressed enzyme was resuspended in a buffer composed of 50mM Tris/HCl, pH 7, 1mM Mg-ATP, 20mM KCl, 2 µg/ml of nigericin, and 50nM [¹⁴C]TAK-438 at a concentration of 2.5 µg/mL of the H,K-ATPase. Group A was used for measuring TAK-438 dissociation by KCl. Group B) Expressed enzyme was resuspended in a buffer composed of 50mM Tris/HCl, pH 7, 1mM Mg-ATP, 2 µg/ml of nigericin, and 50nM [¹⁴C]TAK-438 at a concentration of 2.5 µg/mL of the H,K-ATPase. This was used for measurement of the full binding of TAK-438. Group C) Expressed enzyme was resuspended in a buffer composed of 50mM Tris/HCl, pH 7, 1mM Mg-ATP, 2 µg/ml of nigericin, and 50µM cold TAK-438 at a concentration of 2.5 µg/mL of the H,K-
ATPase. This mixture was incubated at 37°C for 30 min and the isotope was added at a concentration of 50nM [14C]TAK-438. Group C determined the non-selective binding of TAK-438.

The enzyme suspension (1 mL) was incubated at 37°C for 0.1 - 7h as indicated and rapidly filtered through a nitrocellulose membrane filter (HAWP Millipore filter, 0.45 µm) pre-wet with a solution composed of 20mM Tris/HCl, pH 7.0, 10% PEG3350 that was placed on top of a glass fiber filter. The nitrocellulose membrane was washed five times with 2.5 mL of a buffer composed of 20mM Tris/HCl, pH 7.0, 10% PEG3350 to remove unbound inhibitor. The membrane was then placed in 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. The quantity of TAK-438 bound to the mutant was calculated by subtracting group C) from group A). Group B) showed the stability of TAK-bound enzyme during the incubation period. When a significant loss of TAK-binding was observed in group B), the data of group A) obtained at the time of the loss were not used for analysis.

*Modeling of TAK-438 binding*

A homology model of the H,K-ATPase was constructed based on the dogfish Na,K-ATPase structure, *pdb.2zxe*, that contains both α and β subunits to provide a framework for predicting TAK-438 interaction with the gastric enzyme (Ogawa et al., 2009). This form of the Na,K-ATPase represents the E2·2K·Pi conformation to which TAK-438 binds. The peptide backbone from the *pdb.2zxe* structure file was copied and side chains were replaced with those of the H,K-ATPase based on BLASTP alignments of the rabbit H,K-ATPase α and β sequences to those of the dogfish.
Model building was performed with Insight II and Discover software from Accelrys Inc., San Diego utilizing the consistent valence force field. Initial side chain dihedral angles before energy minimization were assigned to nonconserved residues based on the allowed ranges found in high resolution structures such that no van der Waals overlap was given with neighboring conserved side chains in either the α or β subunits. Nonconserved loop replacements were made by searching a data base of loop structures contained in the Searchloop module of the software for loops with the desired number of amino acids that also matched the secondary structure in the conserved regions before and after the loop. Loop replacements containing the additional, nonconserved carbohydrate sites in the H,K-ATPase β-subunit (total of seven sites as opposed to three in Na,K-ATPase β-subunit) were selected to allow the modified asparagines to be exposed to the exterior of the protein. After nonconserved loop and side chain replacement, the model was energy minimized with only conserved side chains and internal secondary structures, including disulfide bonds, held fixed. Energy minimization was carried out to an average absolute derivative of less than 0.01 kcal/mole Å with a fixed dielectric constant of 15, and a 15 Å non-bonded cutoff.

Data analysis and statistics

All experiments were performed in triplicate or more and the average of the results was used for analysis. Mean values are expressed ± S.E. of n individual experiment performed.
Materials

\(^{[14]C}\)TAK-438 (specific activity, 5.17 MBq/mg) was a generous gift from Dr. Nobuhiro Inatomi (Takeda Pharmaceutical Company Limited, Osaka, Japan). \(^{\gamma\cdot32}P\)ATP (specific activity, 6000 Ci/mmole) was purchased from Amersham Biosciences. \(^{\gamma\cdot32}P\)ATP (0.25 mCi) was diluted to 2 mL in 2mM ATP/Tris (pH 7.0) to give a radioactivity of 0.125 mCi/mL and used within 5 days. All other reagents were analytical grade or higher.
Results

H,K-ATPase inhibition by TAK-438

In this experiment, TAK-438 inhibited the gastric H,K-ATPase with an IC$_{50}$ of 17 nM and inhibited the enzyme K-competitively. The K$_{i}$ was 10 nM when measured at pH 7 (Figure 2). A lower calculated Ki of 3nM was reported when the Ki was measured at pH 6.5 (Hori et al., 2010). TAK-438 selectively inhibited the gastric H,K-ATPase as compared to the homologous Na,K-ATPase. TAK-438 inhibits the dog and pig Na,K-ATPases with an IC$_{50}$ of 44µM and 95µM, respectively. Given an IC$_{50}$ of 17nM on the gastric H,K-ATPase, this compound has a greater than 1000 fold selectivity for the gastric H,K-ATPase.

To distinguish between covalent or reversible inhibition, reversibility of binding was determined. To do this, the gastric enzyme was first inhibited by TAK-438 and [¹⁴C]TAK-438 binding was measured as described in methods. Then, [¹⁴C]TAK-438 bound enzyme was precipitated by methanol. If TAK-438 inhibited the enzyme activity via covalent binding, the protein precipitate should contain the labeled TAK-438. After methanol precipitation, only about 1% of TAK-438 was found in the precipitate and ~ 99% of the labeled TAK-438 was in the supernatant after methanol precipitation. Thus TAK-438 binding to the gastric H,K-ATPase is non-covalent.

The TAK-438 binding rate was measured using inhibition of acridine orange uptake in acid transporting H,K-ATPase vesicles and compared to SCH 28080. The IC$_{50}$ of SCH28080 is 150 nM at pH 6.5 (Wallmark et al., 1987), while the IC$_{50}$ of TAK-438 is 17 nM, thus TAK-438 inhibition is about 10 times more potent than SCH28080, therefore, the effect of TAK-438 at 10 nM was compared to that of SCH28080 at 100 nM. When the inhibitors were added at these concentrations, acridine orange uptake
due to the proton gradient reversed due to inhibition of acid secretion. The slope reflects the rate of inhibition and when calculated from the Boltzmann sigmoidal equation for inhibition of vesicle acidification was 9.6 and 42 for for SCH28080 and TAK-438 respectively (Figure 3). These data show that the rate of binding of TAK-438 is slower than SCH28080 as expected from the slow dissociation. However, given that the plateau of inhibition is reached within 30 sec with SCH28080 and 200 sec with TAK-438, this will not affect the efficacy of the latter on diurnal pH in vivo.

Potassium ion competition of TAK-438 binding

Reversal of TAK-438 binding was investigated at various concentrations of KCl. As shown in figure 4, the half-time of TAK-438 dissociation by KCl was 12.5 hours in the presence of 10mM KCl, and 7.5 hours in the presence of 20mM KCl, and 3 hours in the presence of 300mM KCl. The dissociation rate of TAK-438 from the isolated ATPase is slower than other P-CABs, For example, 60% of SCH28080 dissociated in the presence of 10 mM KCl in 2 min (Keeling et al., 1989). Physiologically the H,K-ATPase is likely exposed to a concentration of about 15 mM KCl in stimulated gastric juice, hence the in vivo dissociation rate is expected to be > 7.5h, slow enough to result in stable inhibition of acid secretion following single dose administration.

The rate of dissociation of TAK-438 binding increased as the concentration of K ion increased. As shown in figure 5A, about half of TAK-438 binding dissociated in 150mM KCl after 3 hours incubation after 50nM \(^{14}\text{C}\)TAK-438 treatment. When a higher concentration of TAK-438 was used, a higher KCl concentration was required to get the same degree of TAK-438 dissociation.
The dissociation rate was affected by medium pH. TAK-438 is a basic amine with the methylamino group having a calculated $pK_a$ of 9.06. The $pK_a$ of the pyridine is likely very low (calculated $pK_a = 0.38$) due to electron withdrawal by the sulfonyl substituent and unlikely to contribute to the pH dependency of binding. Thus, in acidic media, the effect of TAK-438 on the enzyme is somewhat less sensitive to KCl at pH 6.1 than that at pH 8 due to increased protonation of the methylamino group and therefore stronger binding (figure 5B). This implies that the protonated form of TAK-438 has a higher affinity for its binding site on the enzyme, consistent with the model described below.

**Displacement of TAK-438 binding**

The reversibility of TAK-438 bound to the enzyme was measured by displacement of isotope-labeled TAK-438 by unlabeled TAK-438. TAK-438 binding to the enzyme was carried out using radio-labeled TAK-438 as described in Methods and then, non-labeled TAK-438 was added at 500 fold higher concentration compared to labeled TAK-438, and an aliquot was taken out at timed intervals to measure the isotope binding. Half of the labeled TAK-438 was exchanged after 3.5 hours of incubation with 500 fold excess of non-labeled TAK-438 (Figure 6). This prolonged dwell time is similar to that seen with KCl dependent reversal.

This slow dissociation of enzyme-bound TAK-438 both with extremely high concentrations of KCl or non-labeled TAK-438 shows that TAK-438 binding is remarkably stable as compared to the rapid reversal of the effects of the imidazopyridine class of compound.
Stoichiometry and ligand effects on TAK-438 binding

TAK-438 binding studies were carried out at 25°C or at 37°C. Since TAK-438 binding was slow compared to other P-CABs such as SCH28080 or BYK compounds, we incubated TAK-438 for longer times. TAK-438 binding stoichiometry was determined using [14C]TAK-438 (0.1µM) in the presence of various ligands (Figure 7). The stoichiometry of binding of TAK-438 to the H,K-ATPase was 2.2 nmol/mg in the presence of Mg-ATP, Mg-vanadate, or Mg-Pi. Given the purity of G1 as 85%, TAK-438 binding to the H,K-ATPase was same as other P-CABs, namely about 2.6 nmol/mg (Shin et al., 2005) with therefore a 1:1 stoichiometry with respect to EP. TAK-438 binds to the enzyme with reduction of stoichiometry to about 1.3 – 1.4 nmol/mg in the presence of the Mg ion chelator (CDTA) i.e. in the absence of Mg2+. This result is different from other P-CABs such as SCH28080 that do not bind in the absence of Mg ion. Hence, TAK-438 binding does not require the Mg2+ induced conformational changes of the H,K-ATPase compared to other P-CABs. Since the pump is a dimeric oligomer (Shin et al., 2005), it seems possible that the resting conformation in the absence of Mg2+ allows docking of TAK-438 because of the close approach of the protonated methylamino group in contrast to the more distant binding of the imidazopyridines.

Dissociation rate of TAK-438 from mutants of the H,K-ATPase

The wild type rabbit H,K-ATPase expressed in HEK293 cells gave a similar stoichiometry of TAK-438 binding to the pig H,K-ATPase. Dissociation of TAK-438 binding from the rabbit H,K-ATPase expressed in HEK293 cells was a little faster than that from the pig H,K-ATPase prepared from the stomach. The t1/2 of dissociation of TAK-438 binding was 4.7 hours. The mutants M334A and M334I had an unchanged t1/2.
of binding at 4.1 hours and 4.9 hours, respectively. However the mutant Y799F had a $t_{1/2}$ of 1.5h (Table 1) demonstrating a minor role of the -OH group of the tyrosine at position 799 compared to hydrogen bonding with methylamino group of TAK-438. The major determinant of the slow dissociation rate is likely the hydrogen bonding interaction between the K$^+$ site at E795 and the methylamino group on TAK-438.

**Molecular modeling of the gastric H,K-ATPase based on the crystal structure of the dogfish Na,K-ATPase.**

The gastric H,K-ATPase belongs to the class of P$_2$-type membrane ATPases. Two members of the class have been crystallized and their structures determined, the srCa-ATPase in several on its conformations and the Na,K-ATPase in the conformation that has the K$^+$ counter ion occluded (E$_2$.2K.Pi). This state of the gastric H,K-ATPase is generated after K$^+$ binding to the E$_2$-P conformation which also binds the K$^+$-competitive inhibitors. Previously, the binding site location was identified with the aid of a large array of point mutations of the H,K-ATPase whose effects on activity and inhibitor affinity were evaluated. The results were used to assign the placement of the inhibitors in the models for energy minimization (Vagin et al., 2002; Munson et al., 2005; Munson et al., 2007). The predicted orientation of SCH28080 was further restricted by biochemical results which included photoaffinity labeling of the TM1/TM2 pair of membrane helices with a derivative modified with azide in the para position of the phenyl ring and by pharmacological studies that defined the active conformation for this inhibitor (Munson et al., 1991). The binding site was localized to a cavity next to the TM5/TM6 loop within the membrane domain. This assumption is now supported in the recent crystal structure of the Na,K-ATPase, pdb.2zxe. which shows a vestibule in the same location that provides
the site of ouabain binding in the Na,K-ATPase and for omeprazole binding to cysteine 813 in the H,K-ATPase. This demonstrates the substantial accuracy of the homology modeling approach. The H,K- and Na,K-ATPases both possess a β subunit. We therefore generated a homology model of the rabbit H,K-ATPase to include both the α and β subunits from the Na,K-ATPase pdb.2zxe structure (Ogawa et al., 2009). The high homology between Na,K- and H,K-ATPases is expected to give this model improved accuracy over the one based on the srCa-ATPase. SCH28080 and TAK-438 were each docked to the new model in the vestibular space next to TM5/TM6 and then energy minimized. The vertical view of the H,K-ATPase model with TAK-438 bound (Figure 8) shows the arrangement of the nucleotide binding (N), phosphorylation (P), actuator (A), membrane associated, and β subunit domains in the E₂ configuration. The inhibitor gains access to its binding site from the lumen through a wide entry space bounded by the TM1/TM2 and TM5/TM6 loops and the extracytoplasmic ends of TM4, TM8, and TM9. There is no apparent contribution from the beta subunit for either entry or binding. After entry of the inhibitor, the space closes and the inhibitor is trapped in the vestibule. The location of the positively charged N-methylamino side chain of TAK-438 is within 2.4 Å of Glu795 producing strong hydrogen bonding and charge interaction with the K⁺ site at Glu795 in contrast to SCH28080 and other P-CABs. Presumably Mg²⁺ is required to generate a form of the luminal vestibule that is essential for binding of the imidazopyridines but not the pyrrolo-pyridines to explain binding of the latter in the absence of Mg²⁺. Another difference in predicted binding of TAK-438 to the vestibule in the H,K-ATPase is the suggested hydrogen bonding between Tyr799 and the sulfone of the inhibitor as shown in Figure 9 where we compare the predicted binding of SCH28080 and TAK-438 on the Na,K-ATPase based model of the H,K-ATPase showing H bonding between the sulfone and Tyr799.
Discussion

Rapid and complete inhibition of the gastric H,K-ATPase is the goal for controlling acid secretion in the stomach. Here we have described properties of TAK-438 that make it superior to all other known inhibitors of the gastric acid pump. *In vitro* assays using isolated hog H,K-ATPase gave an IC₅₀ of 17nM. Thus, TAK-438 inhibits the gastric acid pump more effectively than other P-CAB inhibitors. For example, imidazopyridine types of P-CABs such as AZD0865, revaprazan and PF-03716556 have an IC₅₀ of about 1µM (Gedda et al., 2007; Mori et al., 2009). Until TAK-438 was discovered, the best inhibition by a P-CAB was achieved by an imidazo-naphthyridine type of P-CAB, soraprazan, which has IC₅₀ of 100nM (Shin et al., 2005; Simon et al., 2007). TAK-438 is at least 5 times more potent than soraprazan. Given that omeprazole and many other PPIs also have IC₅₀ about 1µM, TAK-438 is most potent anti-secretory agent among all these inhibitors, PPIs and P-CABs, based on its affinity.

The effectiveness of TAK-438 inhibition is due to binding of the inhibitor close to the K⁺ ion binding site located in the luminal domain of the H,K-ATPase. TAK-438 competes with K⁺ and the inhibition of TAK-438 is reversible. TAK-438 binding is remarkably stable, with very slow dissociation in the presence of even very high K⁺ concentrations. A possible explanation is given by the homology model which suggests that TAK-438 is bound closer to the K⁺ binding site of the enzyme at Glu795 enabling strong hydrogen bonding with the carboxylates of the ion binding site.

The TAK-438 binding stoichiometry was 2.2-2.3 nmol/mg in the presence of Mg-ATP and a 1:1 ratio of stoichiometry of P-CAB binding relative to the phosphoenzyme. Like other P-CABs, one molecule of TAK-438 was enough to inhibit one dimeric oligomeric form of the gastric enzyme (Shin et al., 2005). Saturation of binding by TAK-
438 was achieved in the presence of Mg-ATP, Mg-Pi, or Mg-vanadate. Even though TAK-438 binding was maximal in the presence of Mg-ATP, Mg-Pi, or Mg-vanadate, approximately 50% of TAK-438 bound to the enzyme in the presence of CDTA, a chelator of Mg ion, as shown in Figure 6. The binding rate of TAK-438 in the absence of Mg ion was slow (data not shown), compared to binding rate in the presence of Mg ion. However, this TAK-438 binding in the absence of Mg$^{2+}$ ion differs from other P-CABs such as SCH28080, AZD0865, revaprazan, and soraprazan, since these P-CABs do not bind to the gastric enzyme at all in the absence of Mg (Mendlein and Sachs, 1990; Shin et al., 2005; Simon et al., 2007). This implies that the details of binding of TAK-438 differ from these other P-CABs, perhaps due to its hydrogen bonding ability allowing binding even in the absence of Mg$^{2+}$. This result might be explained by the possible dimeric form of the H,K-ATPase oligomer (Shin et al., 2005) where TAK-438 may be able to bind to half of the dimer in the absence of magnesium.

Molecular modeling of TAK-438 binding in comparison with SCH28080 binding explains the differences in kinetic properties of the two inhibitors. SCH28080 binding in the model occurs in a cleft between Cys813 and Ala335 which is bounded on one end by Pro798 and Leu809-leu811 and on the other by Met334, Leu141, Cys120, Asp137, and Asn138 (Figure 9, panel A). Tyr799 and Phe332 contact the inhibitor on the luminal side. Genetic engineering has shown that mutation of these residues reduces SCH28080 binding (Asano et al., 2004). The pdb.2zxe Na,K-ATPase structure however, has only ~6 Å between the positions equivalent to Cys813 and Ala335. This is at least 3 Å too narrow to accommodate SCH28080 and explains the competitive nature of this inhibitor where binding cannot occur to the K$^+$ occluded conformation. Docking and energy minimization of SCH28080 in this narrow space expanded the cavity with displacement of the TM4 and TM1 helices. The same type of expansion is observed
when ouabain diffuses into crystals of the Na,K-ATPase giving low affinity binding (Ogawa et al., 2009). Furthermore, the orientation of these helices with respect to the TM5/TM6 loop is different in the various known conformational states of the srCa-ATPase demonstrating their positional flexibility. The closer proximity of the TM1/TM2 segments in the new model predicts that the side chains of Cys120, Asp137, Asn138, and Leu141 enclose the phenyl ring of SCH28080. This accounts for the photo-activated insertion of the para-azido derivative into this region of the protein (Munson et al., 1991). It is important to note that the distance of SCH28080 from the K binding site does not allow H bonding between the inhibitor and the pump.

The modeled binding (Figure 9, panel B) of TAK-438 (surfaced stick in blue) to the H,K-ATPase shares many similarities with SCH28080 but also important differences. Docking is again in a cleft between A335 and C813 and is enclosed on one end by the TM5TM6 loop (Pro798 and Leu809 to Leu811) and TM1 and TM2 with residues Leu141, Cys120, Asp137, and Asn138 (some residues not shown for clarity in Figure 9, compare Figure 8). Unlike SCH28080 however, TAK-438 shows additional interactions which contribute to the extremely slow off rate of this inhibitor. Most important is the proximity of its positively charged N-methylamino group of TAK-438 to the negatively charged group of side chains in the ion binding site, especially Glu795, that cannot happen with the imidazopyridines. A less important interaction is the hydrogen bonding between the hydroxyl of Y799 and the oxygens of the sulfonyl group of TAK-438. The faster dissociation of TAK-438 from the Tyr799Phe mutant of the H,K-ATPase supports this detail of the model.

*In vivo* in the rat, TAK-438 exerts a more potent and longer-lasting antisecretory effect than previous PCABs and even PPIs, due to various factors. One is the greater accumulation of TAK-438 due to its higher pKₐ resulting in slow clearance from gastric
tissue (Hori et al., 2011; Matsukawa et al. 2011) lasting for the full 24 hr time span following a single dose in the rat. Second is the slow dissociation of TAK-438 once bound giving a long duration of action. An additional benefit of acid inhibition by this compound is that it is independent of acid secretion hence it is mealtime independent again in contrast to PPIs. The slow dissociation rate of TAK-438, as discussed above, is accompanied by a slow on-rate and this kinetic property helps explain the effectiveness of acid secretory inhibition by this novel K⁺ competitive antagonist.

Acknowledgements

We thank Drs. Jeffry Kraut and David Scott for their helpful suggestions and Dr. Mark Holoboski for the pKₐ calculations.

Authorship contributions

Participated in research design: Shin, Sachs

Conducted experiments: Shin, Munson, Strugatsky, Tokhtaeva, Vagin

Contributed new reagents or analytical tools: Shin, Inatomi

Performed data analysis: Shin, Munson

Wrote or contributed to the writing of the manuscript: Shin, Inatomi, Munson, Sachs
Reference


Footnotes

This work was supported by Takeda Pharmaceutical Company Limited; the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant number’s DK053642, DK058333, and DK077149]; and US VA Merit Grant Award [Number I01BX001006-01].
Legends for figures

Figure 1. Structure of TAK-438

Figure 2. Inhibition by TAK-438

Panel A) An aliquot of the gastric H,K-ATPase enzyme suspension (3 µg/ml) was pre-incubated in a buffer composed of 10 mM KCl, 2 mM MgCl₂, ± 2 µg/mL of nigericin, 20 mM Tris/HCl, pH 7.4, for 2 hr, and the enzyme activity was measured by adding 2 mM ATP for 30 min at 37°C. Panel B) 1/V versus 1/[KCl] plot in the presence of different fixed concentrations of TAK-438. V represents the enzyme activity (µmol Pi•mg⁻¹•h⁻¹). [KCl] represents a concentration of KCl (mM). Each point represents mean ±S.E. of three experiments.

Figure 3. The enzyme suspension (20 µg/ml) in a buffer composed of 3 mM Pipes/Tris (pH 7), 2 mM MgCl₂, 150 mM KCl, 5 µg/ml of valinomycin, and 1µM acridine orange, was preincubated at 37°C in a cell of a spectrofluorimeter for 5 min. After 60 seconds, ATP (2 mM) was added to initiate acridine orange uptake. The inhibitor was added at the time as indicated by P-CAB. SCH and TAK represent SCH28080 and TAK-438 respectively.

Figure 4. Dissociation of TAK-438 binding by KCl at different incubation times

Panel A) Enzyme (10 µg/mL) was resuspended in a buffer composed of 50mM Tris/HCl, pH 7.0, 2mM MgCl₂, 2mM ATP, and 50nM [¹⁴C]TAK-438, and incubated at room
temperature (25°C) for 60 min. Under this condition, TAK-438 binds to the enzyme with full inhibition. To this enzyme suspension, KCl was added at 10mM or 20mM, and incubated at 37°C. At timed intervals, an aliquot was taken to measure the binding.

Panel B) Enzyme (10 µg/mL) is resuspended in a buffer composed of 50mM Tris/HCl, pH 7.0, 2mM MgCl₂, 2mM ATP, and 100nM [¹⁴C]TAK-438, and incubated at room temperature (25°C) for 60 min. Under these conditions, TAK-438 binds to the enzyme with full inhibition. To this enzyme suspension, KCl is added up to 0.3M final concentration, and incubated at 37°C. At timed intervals, an aliquot is taken to measure the binding as described in Methods.

Figure 5. Dissociation of TAK-438 binding as a function of KCl concentration and medium pH.

Panel A) Enzyme (5 µg/mL) was resuspended in a buffer composed of 2mM MgCl₂, 2mM ATP, 2 µg nigericin/mL, 50nM [¹⁴C]TAK-438, and 50mM Tris/HCl (pH 7.0). The enzyme suspension at pH 7 was incubated at 37°C for 3h in the presence of various concentrations of KCl. Panel B) The enzyme suspensions at pH 6.1 and pH 8 were treated identically except for incubation time and temperature. These enzyme suspensions were incubated at 25°C for 2h. TAK-binding was measured as described in Methods. In this experiment, TAK-438 binding measured in the absence of KCl was 2.15 nmol/mg at 50nM TAK-438. This was taken as representing 100% binding allowing calculation of the %TAK-438 binding at given KCl concentration.
Figure 6. Exchange of bound $[^{14}\text{C}]$TAK-438 by unbound unlabeled TAK-438 after different incubation times. Enzyme (10 µg/mL) was resuspended in a buffer composed of 50mM Tris/HCl, pH 7.0, 2mM MgCl$_2$, 2mM ATP, and 100nM $[^{14}\text{C}]$TAK-438, and incubated at room temperature (25°C) for 60 min. Under these conditions, TAK-438 binds to the enzyme with full inhibition. To this enzyme suspension, non-labeled TAK-438 was added up to 50µM final concentration, and incubated at 37°C. At timed intervals, an aliquot was taken to measure the exchange as described in Methods.

Figure 7. Effects of various ligands on TAK-438 binding. The gastric vesicles (0.01 mg/mL) were incubated at 37°C for 60 min in a buffer composed of 50mM Tris/HCl, pH 7.0, and different ligands such as ± 2mM MgCl$_2$, ± 5mM CDTA, ± 2mM ATP, ± 0.2mM vanadate, ± 5mM inorganic phosphate/Tris (pH 7.0) in the presence of $[^{14}\text{C}]$TAK-438 (100nM). Binding stoichiometry was determined as described in Methods.

Figure 8. Ribbon representation of the H,K-ATPase model based on the crystallographic structure of the Na,K-ATPase, 2zxe.pdb. The major domains are noted and the position of the membrane is indicated with yellow lines and TM1 dark blue, TM2 cyan, TM3 light green, TM4 green-blue, TM5 light yellow, TM6 dark yellow, TM7 light brown, TM8 dark brown, TM9 light red, TM10 dark red. This color scheme is maintained in all figures. The position of the ion binding site is close to the middle of the membrane (ball and stick). The inhibitor, TAK-438 (space filling in light blue), binds ~10 Å from the ion binding sight in a cavity bounded by TM1, TM2, TM4, and the TM5TM6 loop. The N-terminal 27 residues of the beta subunit (pink ribbon) are not present in the Na,K-ATPase structure 2zxe.pdb and therefore not in the HKzxe model.
Figure 9. Panel A) SCH28080 binding to the H,K-ATPase model based on pdb.2zxe. Binding is in the space between A335 and C813 (green and yellow spheres, respectively). TM1 and TM2 helices (light blue and blue) enclose the site to the right providing interaction between the inhibitor and C120, N138, L141, and D137 (stick). There is also a closer approach by the inhibitor to P798 (TM8) and M334 (TM4) than in previously published models. The binding site is stabilized from below by aromatic interactions between Y799 and F332 (below A335 in TM4, not shown for clarity). Panel B) Binding of TAK-438 to the H,K-ATPase. The inhibitor is predicted to bind in the same cleft as SCH28080. Hydrogen bonding between Y799 and the sulfonyl oxygens of the inhibitor and the proximity of its amino group to the ion binding site are predicted to contribute to the slow off rate for TAK-438.
Table 1. TAK-438 dissociation of the mutants expressed in HEK293 cells

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Half-time (h) of TAK-438 dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild</td>
<td>4.7</td>
</tr>
<tr>
<td>M334A</td>
<td>4.1</td>
</tr>
<tr>
<td>M334I</td>
<td>4.9</td>
</tr>
<tr>
<td>Y799F</td>
<td>1.5</td>
</tr>
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</table>

Wild type and mutants of the rabbit gastric H,K-ATPase expressed in HEK293 cells were prepared at a concentration of 2.5 µg/mL of the H,K-ATPase. Each mutant was incubated with 50nM [14C]TAK-438 and the dissociation of TAK-438 was measured as described in Methods.
Figure 1
Figure 3

- **AO Fluorescence**
- **Time (sec)**
- **SCH (100nM)**
- **TAK (10nM)**

Key Events:
- ATP
- Inhibitor
- nigericin

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Figure 4

(A) % TAK-438 Binding vs. Incubation Time (h) for 10 mM KCl and 20 mM KCl.

(B) TAK-438 (nmol/mg) vs. Incubation Time (h).
Figure 5

A

B

% TAK-438 binding vs log [M, KCl]

% TAK-438 binding vs log [M, KCl]

- pH 6.1
- pH 8
Figure 6

![Graph showing time course of TAK-438 bound (nmol/mg) over incubation time (h).]
Figure 8

- N domain
- A domain
- P domain
- Membrane domain
- Ion binding site
- TAK-438
- M5/M6 loop
- Beta subunit (pink)