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Design of *Helicobacter pylori* glutamate racemase inhibitors as selective antibacterial agents: A novel pro-drug approach to increase exposure

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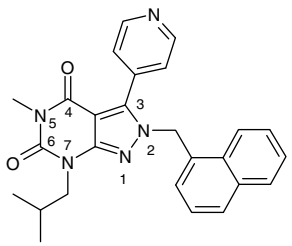
ABSTRACT

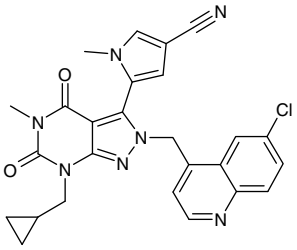
High-throughput screening uncovered a pyrazolopyrimidinedione hit as a selective, low micromolar inhibitor of *Helicobacter pylori* glutamate racemase (MurI). Variation of the substituents around the scaffold led to low nanomolar inhibitors and improved antibacterial activity. The challenge in this program was to translate excellent enzyme inhibition into potent antibacterial activity and pharmacokinetics suitable for oral therapy. Compounds were profiled for MurI inhibition, activity against *H. pylori*, microsomal stability, and pharmacokinetics in mice. Iterative cycles of analog synthesis and biological testing led to compounds with substituents optimized for both low MICs (⩽2 μg/ml) and good microsomal stability. In order to achieve high bioavailability, a novel pro-drug approach was implemented wherein a solubilizing sulfoxide moiety is oxidized in vivo to a sulfone.

The 2005 Nobel Prize in Physiology and Medicine was awarded to Barry Marshall and Robin Warren for the discovery of the bacterium *Helicobacter pylori* and for the determination that its colonization of the stomach mucosa causes gastritis and peptic ulcers.[1] An association has been made between infection by the bacterium and a predisposition to gastric cancer.[2] The diseases caused by *H. pylori* can be cured by eradication of the pathogen with various antibacterial therapies;[3] however, their shortcomings including undesired intestinal side effects due to disruption of commensal bacteria prompt the quest for a novel, selective anti-*H. pylori* therapy. The recent emergence of resistant *H. pylori* strains to the commonly used antibiotics further supports the need for novel therapies with new modes of action.[4] Selectivity versus *H. pylori* would also remove resistance pressures otherwise imposed by broad-spectrum agents on quiescent bacterial pathogens.

Phylogenetic analysis across a range of bacterial species places the *H. pylori* MurI gene encoding the enzyme glutamate racemase in a divergent niche relative to other MurI genes, and it was suggested that selective inhibition could be achieved.[5] As β-lactam antibiotics operate by blocking cell wall peptidoglycan cross-linking, inhibition of other cell wall biosynthesis targets for drug design follows.[6] Glutamate racemase (MurI) from Gram-positive and Gram-negative bacteria catalyzes the interconversion of L- and D-glutamate, the latter being a necessary component of cell wall peptidoglycan. Previous publications had described the rationale for targeting MurI for antibacterial therapy,[7] the kinetic characterization of MurI from *Escherichia coli*,[8] and the determination of a 2.3 Å resolution crystal structure of MurI from *Aquifex pyrophilus*.[9] MurI has been targeted for inhibitor design with the synthesis of glutamate analogs that bind at the active site and show a measure of antibacterial activity.[10] A selective *H. pylori* MurI inhibitor could show promise as a drug therapy if inhibition translates into antibacterial activity and pharmacokinetic (PK) properties lead to sustained blood plasma levels sufficiently high enough to eliminate the pathogen. We envisioned that an anti-*H. pylori* drug would need to be administered orally to gain widespread acceptance by the medical community.



1
MurI IC_{50} = 1400 nM
MICs: *H. pylori* (wild-type) = 8 μg/ml



2
MurI IC_{50} = 26 nM
MICs: *H. pylori* (wild-type) = 0.5 μg/ml
(*hefC*) = 0.25 μg/ml
Human microsomal Cl_{int} < 14 μl/min/mg
Mouse microsomal Cl_{int} = 50 μl/min/mg
bioavailabilty (mouse) = 7.5%

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The *H. pylori* MurI gene was cloned and overexpressed in *E. coli* and the enzyme was harvested for biochemical characterization, inhibition assay development, and protein crystallization trials.[5] High-throughput screening of the AstraZeneca compound collection identified pyrazolopyrimidinedione **1** as a low micromolar inhibitor of *H. pylori* MurI with selectivity versus isozymes from the Gram-negative pathogen *E. coli* and 2 Gram-positive pathogens, *Staphylococcus aureus* and *Enterococcus faecalis*.[5] Analog work around **1** led to **2** as a formidable low nanomolar inhibitor of MurI showing an MIC = 0.5 μg/ml against wild-type *H. pylori* and 0.25 μg/ml against an RND-efflux pump debilitated *H. pylori* strain (*hefC*$^-$).[5,11] Bacterial efflux oftentimes contributes to increased MICs, and to assess this contribution, the compounds described herein were screened against both the wild-type and pump debilitated *H. pylori* strains. Despite the encouraging anti-microbial properties of **2,** some of the properties deemed essential for an orally dosed drug are lacking. For example, low aqueous solubility (<0.4 μM) and low bioavailability (<10%) on oral dosing in mice prevent the plasma drug concentration from exceeding the MIC. Here, we attempt to synthesize more polar and water-soluble MurI inhibitors to identify anti-*H. pylori* compounds with improved bioavailability in mouse models. Ultimately, a pro-drug strategy was devised wherein an in vivo sulfoxide to sulfone conversion delivers plasma levels above the MIC on oral administration.

We set out to incorporate more polar functionality onto the fused pyrimidinedione scaffold to increase solubility anticipating a concomitant improvement in a number of PK properties (e.g. microsomal stability, plasma protein binding, and absorption) that contribute toward generating drug exposure in the blood necessary to eradicate *H. pylori*. Ideally, the polar functionality should maintain or improve inhibitory potency so as not to diminish antibacterial activity. The X-ray structure of the *H. pylori* glutamate racemase with **1** and D-glutamate bound has been determined to 1.9 Å showing that the inhibitor occupying an allosteric binding site is removed from the substrate.[5] The core scaffold accommodates variation at the 2-, 3-, 5- and 7-positions (see **1** for numbering) for analog enumeration. The 7-position isobutyl group and the 2-position naphthalene of the inhibitor are deeply buried in hydrophobic regions of the inhibitor binding pocket. The pyrazolopyrimidinedione 5-position methyl group lies against a hydrophobic surface and otherwise faces solvent. Without nearby polar residues or backbone peptide linkages to offer opportunities for an electrostatic complement, replacing the methyl group with polar functionality does not bode well for enhancing inhibitory potency.

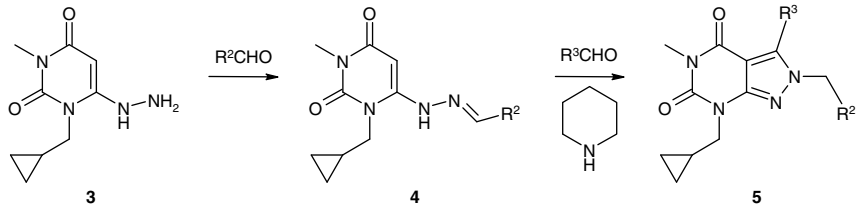
It therefore seemed best to target the region of the binding pocket surrounding the pyridine of **1** or, analogously, the pyrrole of **2** for the incorporation of polar functionality. Replacing **1** with **2** in the inhibitor binding region shows the pyrrole methyl group surrounded by a nicely complementary hydrophobic environment whereas the region surrounding the cyano substituent is lined with the hydrogen bonding atoms of a series of more polar side-chain functionalities (Ser152, Gln248, Arg247, and Trp 252) and reaches outward to solvent.[12] Here, we prepared compounds where the cyano group is replaced with the sulfur substitutions: sulfones, sulfoxides, and sulfonamides. These functionalities can be accommodated sterically by the crystallographic model and offer the potential to form hydrogen bonds with enzyme side chains or bridging water molecules. Substitution at the pyrrole 4-position can be mimicked with substitution on other aromatic 5-membered rings affording a similar 1,3-relationship to the attachment of the pyrazolopyrimidinedione scaffold. Examples of substitution on methyl pyrrole, methyl imidazole, and furan are examined here.

Pyrazolopyrimidinediones **5** can be synthesized efficiently by condensation of pyrimidinedione hydrazide **3** with aldehydes to form intermediate hydrazones **4** (Scheme 1).[13] A second condensation with a second aldehyde with heating is followed by cyclization and rearrangement to the pyrazolopyrimidinedione scaffold in a single reaction sequence. Piperidine serves as an efficient catalyst for the second condensation. Hydrazide **3** is readily prepared by methods set out in the literature.[5,14] Since the 5-position methyl and the 7-position cyclopropylmethyl afford high inhibition potency and antibacterial activity, the two substituents were maintained as constants for the purpose of this investigation. Condensations to form hydrazones **4** were investigated with three commercial aldehydes (5-chloroindole-3-carboxaldehyde, 5-chloro-1-methylindole-3-carboxaldehyde, and 6-chloroquinoline-4-carboxaldehyde) en route to compounds **5** with inhibitory potencies (IC_{50}s) consistently below 100 nM. Subsequent condensations and rearrangements to compounds **5** were carried out with a variety of furan, pyrrole and imidazole carboxaldehydes. The reaction sequence was carried out in a single reaction vessel by room temperature stoichiometric addition of the first aldehyde to form hydrazones **4** followed by addition of the second aldehyde and piperidine with heating to 80 °C to afford pyrazolopyrimidinediones **5**.

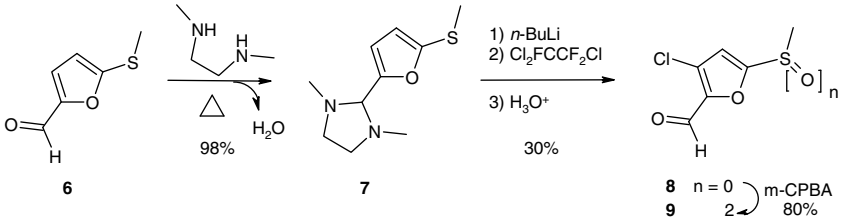
A series of furans, pyrroles and imidazole carboxaldehydes (R^3-CHO) used in Scheme 1 to affix the R^3 substituent were prepared de novo. The furan carboxaldehyde for Compound **27** is known in the literature.[15] The chlorinated furan **9** incorporated into **28** was synthesized by formation, lithiation, and chlorination of **7** followed by hydrolysis and oxidation as outlined in Scheme 2. The synthesis of the furan **12** (Scheme 3) for incorporation into **29** and **30** began by reduction of the commercial furylsulfonyl chloride **10** and alkylation with methyl iodide affording **11** via methods set out in the literature.[16,17] Under the reaction conditions some ester hydrolysis occurred requiring re-esterification to maximize recovery of **11**. The ester **11** was converted to the aldehyde **12** by reduction to the alcohol and oxidation.

An imidazole sulfonamide carboxaldehyde **14** for incorporation into **31** was prepared by silylation of the commercially available sulfonamide **13** of Scheme 4 followed by lithiation and treatment with DMF. The silyl group is removed during workup of the subsequent cyclization reaction forming **31.**

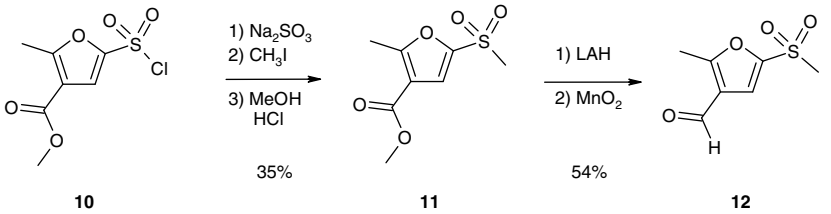
Pyrrole sulfonamides **17a–c** were derived from the sulfonyl chloride **15**[18] shown in Scheme 5 by amination followed by conversion of the ester to the aldehyde. The *t*-butyl protecting group after formation of the pyrazolopyrimidinedione was removed with TFA to give **32**. The sulfonamides otherwise were incorporated into Compounds **33–36**.



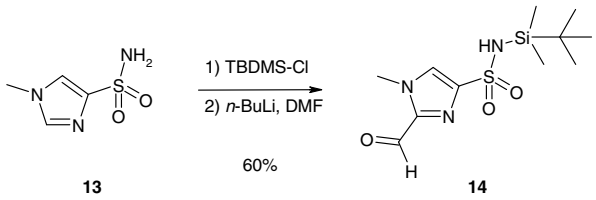
Scheme 1.



Scheme 2.



Scheme 3.



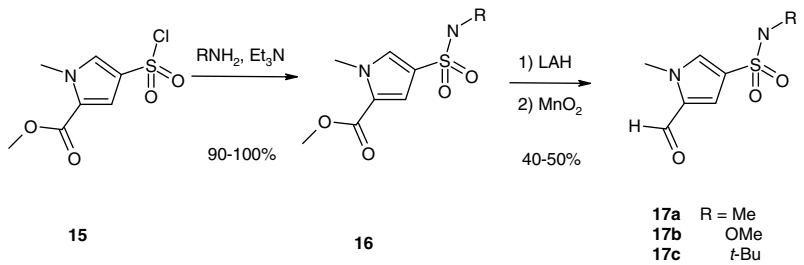
Scheme 4.

Imidazole sulfone **21** for the preparation of **37** and **38** was synthesized via the sequence outlined in Scheme 6. Imidazole carboxaldehyde **18** is protected as the acetal and doubly brominated. The bromine nearest to the imidazole methyl group is removed by lithium halogen exchange followed by proteolysis. Metal halogen exchange of the second bromine was followed by sulfenation affording **20**. Oxidation to the sulfone and hydrolysis of the ketal gave aldehyde **21**.

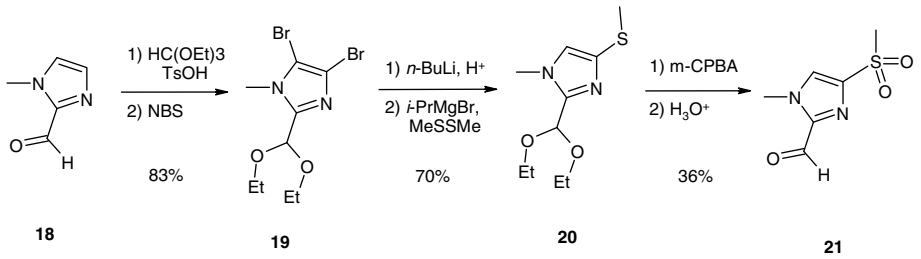
Compounds **39–44** utilized pyrrole sulfone and sulfoxide carboxaldehydes prepared by protection of 1-methyl-2-formyl-5-bromopyrrole **22**[19] as the aminal **23**, conversion to the sulfides, hydrolysis to aldehydes **24**, and oxidation as diagrammed in Scheme 7.

High inhibitory potencies (<100 nM) were recorded for the majority of compounds listed in Tables 1–3. As inhibitory potencies versus the *H. pylori* isozyme increased, selected compounds evaluated for inhibition against the MurI isozymes of *E. coli*, *S. aureus*, and *E. faecalis* showed IC_{50}s > 400 μM reinforcing a superb selectivity. The focus on 5-membered aromatic rings at the 3-position of the pyrazolopyrimidinedione scaffold over 6-membered rings reflects improved inhibitory potencies. Higher potencies with concomitant improved antimicrobial activity are further seen if a small methyl substituent is positioned on the 5-membered aromatic ring in a 1–2 relationship to the bond connecting to the scaffold as seen in **2**. This is explained by the substituent filling a hydrophobic area of the MurI inhibitor binding pocket and favoring a staggered conformation as corroborated by X-ray crystallography where the dihedral angle around the connecting bond is 63 deg. This is quite accessible energetically from the optimal unbound ground-state conformation of 49 deg. The methyl group can be incorporated onto a pyrrole ring as in **2**, imidazole as in **31,** and furan as in **29**. The chlorine atom of furan **28** serves a similar role to the methyl group increasing potency 25-fold relative to **27**, which lacks a substituent that would favor the staggered conformation (Table 1).

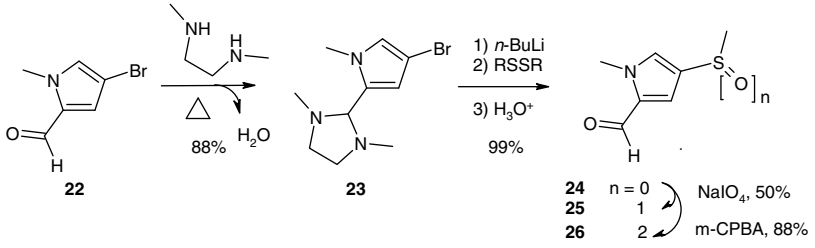
Potency is further improved when polar groups are situated in a 1–3 relationship with the linkage to the scaffold. The highest potency in the series was found for the quite polar sulfonamide substituent of **32** (Table 2). However, the polarity raises the MICs versus *H. pylori* presumably due to diminished membrane permeability. The diminished membrane permeability translates to greater susceptibility to efflux by the RND transporter demonstrated by considerably lower MICs against the *hefC*$^{-}$ strain. Adding substituents onto the sulfonamide slightly decreases inhibitory potency (compare **33** and **35** with **32**) but can improve MICs through the increase in lipophilicity. Better MICs were achieved with incorporation of less polar functionalities such as the nitrile of **2** and the methyl sulfones of compounds **28–30** (Table 1) and **41–43** (Table 3). In line with increased polarity mitigating antibacterial activity, imidazoles increased MICs relative to pyrroles (compare **37** and **38**



Scheme 5.

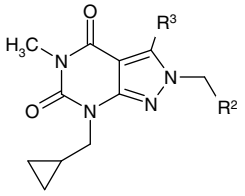


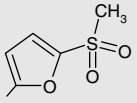
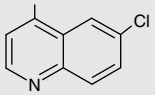
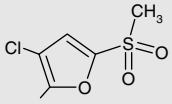
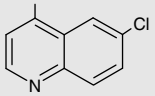
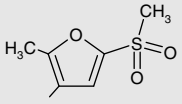
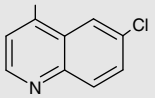
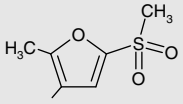
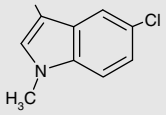
Scheme 6.



Scheme 7.

Table 1
Influence of 3-position furans on IC_{50}, MIC, and microsomal clearance (Cl_{int})



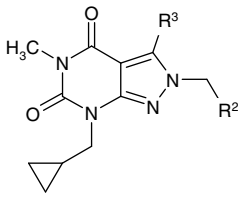
n	R^3	R^2	IC_{50}[a] (nM)	MICs[b] (μg/ml)		Cl_{int} (μl/min/mg)	
				Wild-type	*hefC*⁻	Human	Mouse
27			1500 ± 100	>64	>64	17	14
28			59 ± 4	4	4	19	22
29			51 ± 5	4	—	22	61
30			57 ± 5	1	0.5	>100	>100

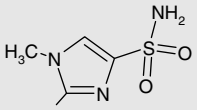
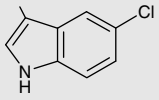
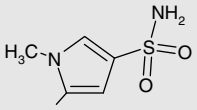
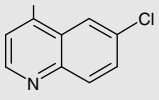
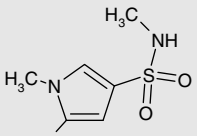
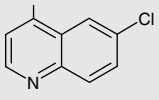
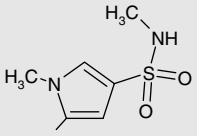
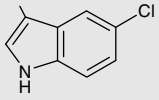
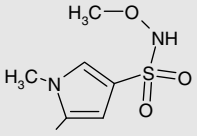
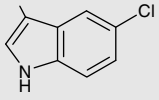
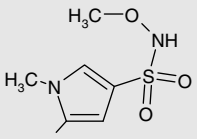
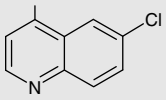
[a] IC_{50}s and standard errors from duplicate determinations.
[b] *Helicobacter pylori* MICs measured by NCCLS guidelines with a twofold variance.

with **41** and **42**, respectively, Table 3). Lower inhibitory potencies for imidazoles relative to pyrroles may also contribute to the higher MICs.

The compounds in Tables 1–3 were further assessed for potential in vivo stability by incubation in vitro with human and mouse microsomes. Many of the compounds proved unstable to microsomes eliminating their utility as drugs. All the compounds containing an indole R^2 substituent suffered from instability to human and mouse microsomes, mitigating their utility in vivo. Across the range of analogs, the chloroquinoline group slightly decreased potency; however, it was necessary (but not sufficient) for metabolic stability. Furthermore, the slight decrease in potencies generally did not diminish antimicrobial activities. Two compounds (Compounds **2** and **41**, vide infra for **41**) were evaluated for in vivo PK properties in the mouse due to the combination of lower MICs and lower intrinsic clearances. Though both compounds showed low in vivo clearances (18 and 15 ml/min/kg), respectively, both failed to achieve sufficiently high bioavailability

Table 2
Influence of 3-position pyrroles and imidazoles with sulfonamide substituents on IC_{50}, MIC, and microsomal clearance (Cl_{int})



n	R^3	R^2	IC_{50}[a] (nM)	MICs[b] (μg/ml)		Clint (μl/min/mg)	
				Wild-type	*hefC*⁻	Human	Mouse
31			38 ± 5	>32	0.5	56	>100
32			16 ± 1	32	0.25	22	<14
33			44 ± 2	16	1	>100	>100
34			27 ± 1	16	0.5	>100	>100
35			36 ± 2	8	8	ND	ND
36			55 ± 2	16	4	ND	ND

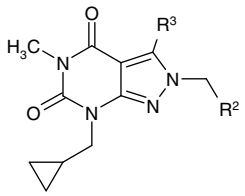
[a] IC_{50}s and standard errors from duplicate determinations.
[b] *Helicobacter pylori* MICs measured by NCCLS guidelines with a twofold variance.

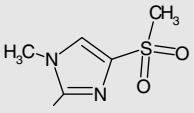
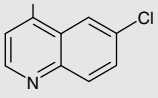
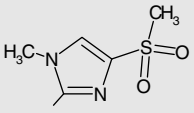
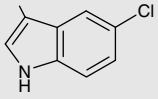
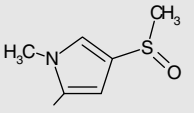
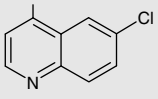
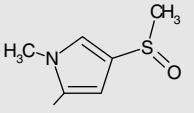
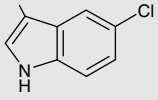
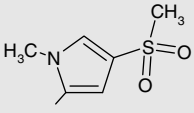
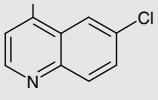
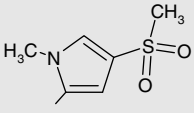
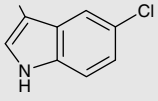
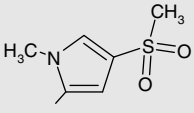
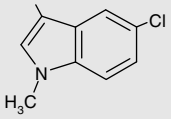
to afford concentrations above the MICs. This is perhaps due to poor solubility of the compounds preventing efficient absorption.

The data in Table 3 suggest a correlation between the clearances of **39** and **41**, direct analogs with a more polar sulfoxide and a less polar sulfone functionality. Note that the two compounds are nearly equipotent (37 and 34 nM, respectively) against the target enzyme yet show an eightfold difference in MICs. The polar sulfoxide decreases membrane permeability mitigating the expression of antibacterial activity in accordance with the discussion above. From the viewpoint of microsomal stability, the sulfoxide serves as a handle for rapid clearance, while the sulfone survives intact. It follows that the sulfoxide might be converted to the sulfone by microsomes and indeed, when **39** was incubated with both human and mouse microsomes, there was quantitative conversion to **41** (Figs. 1 and 2).

Hence, the microbiologically less potent sulfoxide was envisioned to serve as a pro-drug for the sulfone. The oral bioavailability of **41** was low (9%) when administered to mice at 40 mg/kg achieving maximum plasma levels of 1.2 μg/ml, lower than the MIC of 2 μg/ml versus *H. pylori* (Fig. 3). Oral administration of a 40 mg/kg dose of sulfoxide **39** afforded peak plasma levels of sulfone **41** of 3 μg/ml, exceeding the MIC of 2 μg/ml for 4 h. The residual sulfoxide detected in the plasma reached a peak concentration of 1.4 μg/ml. The data demonstrate that total drug levels (sulfoxide + sulfone) significantly increase on oral dosing of the former relative to the latter, affording a bioavailability of 60% for the sulfone and 10% for the sulfoxide. Hence, conversion of the sulfoxide to the sulfone is realized in vivo as predicted by in vitro incubation with microsomes. Equilibrium solubilities of the sulfoxide and sulfone (120 and 4 μM, respectively) correlate affirmatively with their abilities to achieve overall higher drug exposure. The non-steroidal anti-inflammatory drug sundilac represents another example of a more water-soluble sulfoxide compound serving as a pro-drug to improve oral bioavailability.[20] In this case, the sulfoxide is reduced in vivo to the pharmacologically active sulfide form rather than being oxidized to the sulfone.[21] The free drug concentration corrected for plasma protein binding ultimately governs the capability for eradication of disease in vivo. Despite the achievement of good

Table 3
Influence of 3-position pyrroles and imidazoles with sulfone and sulfoxide substituents on IC_{50}, MIC and microsomal clearance (Cl_{int})



n	R^3	R^2	IC_{50}[a] (nM)	MICs[b] (μg/ml)		Cl_{int} (μl/min/mg)	
				Wild-type	*hefC*⁻	Human	Mouse
37			160 ± 10	16	4	23	56
38			31 ± 4	16	2	49	>100
39			37 ± 4	16	0.5	>100	>100
40			23 ± 1	16	4	>100	>100
41			34 ± 3	2	0.5	20	<14
42			26 ± 2	4	0.5	49	64
43			33 ± 1	16	4	23	56

[a] IC_{50}s and standard errors from duplicate determinations.
[b] *Helicobacter pylori* MICs measured by NCCLS guidelines with a twofold variance.

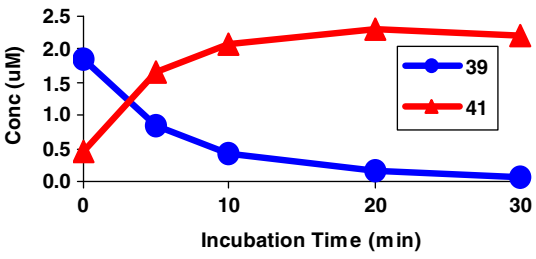


Figure. 1. Transformation of **39**–**41** in mouse microsmes.

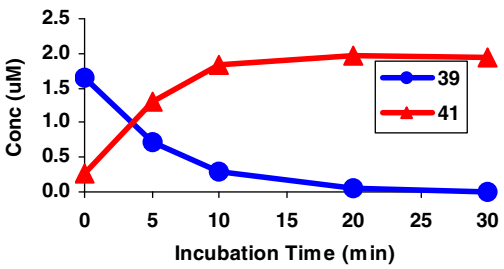


Figure 2. Transformation of **39**–**41** in human microsomes.

bioavailability through this pro-drug approach, the protein binding of **41** in mouse plasma is sufficiently high (98%) to abrogate testing of **39** for efficacy in *H. pylori* infected mice.

In summary, a series of potent inhibitors of the *H. pylori* MurI enzyme are described. In order to translate target potency to effectiveness as a drug, chemical–physical properties need to be optimized to allow sufficient membrane permeability for the expression of MICs. For oral bioavailability, chemical–physical properties also need to be optimized to achieve appropriate solu-

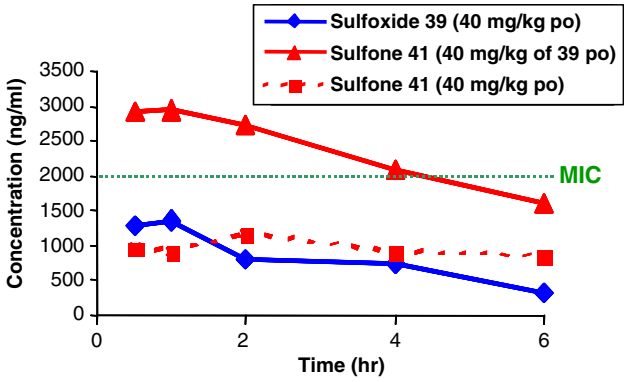


Figure 3. Mouse plasma levels of **39** and **41**.

bility and permeability for intestinal absorption. The most potent antibacterial compounds that maintained stability to microsomes failed to achieve blood plasma levels in the mouse, approaching the respective MICs. It was only when the more polar, yet less potent sulfoxide **39** was dosed orally that higher plasma levels of drug were achieved. The sulfoxide was largely converted to the more potent sulfone **41,** achieving nearly a sevenfold increase in bioavailability relative to oral administration of the sulfone. The sulfoxide thus serves as a pro-drug handle for the active sulfone metabolite as the increase in polarity and solubility enhances the absorption necessary for an oral drug.

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