

# Structure-Guided In Vitro to In Vivo Pharmacokinetic Optimization of Propargyl-Linked Antifolates<sup>§</sup>

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## ABSTRACT

Pharmacokinetic/pharmacodynamic properties are strongly correlated with the in vivo efficacy of antibiotics. Propargyl-linked antifolates, a novel class of antibiotics, demonstrate potent antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria, including multidrug-resistant *Staphylococcus aureus*. Here, we report our efforts to optimize the pharmacokinetic profile of this class to best match the established pharmacodynamic properties. High-resolution crystal structures were used in combination with in vitro pharmacokinetic models to design compounds that not only are metabolically stable in vivo but also retain potent antibacterial activity. The initial lead compound was prone to both *N*-oxidation and demethylation, which resulted in an abbreviated in vivo half-life (~20 minutes) in mice. Stability of leads toward mouse

liver microsomes was primarily used to guide medicinal chemistry efforts so robust efficacy could be demonstrated in a mouse disease model. Structure-based drug design guided mitigation of *N*-oxide formation through substitutions of sterically demanding groups adjacent to the pyridyl nitrogen. Additionally, deuterium and fluorine substitutions were evaluated for their effect on the rate of oxidative demethylation. The resulting compound was characterized and demonstrated to have a low projected clearance in humans with limited potential for drug-drug interactions as predicted by cytochrome P450 inhibition as well as an in vivo exposure profile that optimizes the potential for bactericidal activity, highlighting how structural data, merged with substitutions to introduce metabolic stability, are a powerful approach to drug design.

## Introduction

Antimicrobial drug resistance remains a top-of-mind public health concern and is often highlighted as an area of critical need for drug development. The difficulty of delivering effective antibacterial agents begins with developing lead compounds with potency against clinically relevant pathogens that also maintain optimal pharmacokinetic profiles. The importance of pharmacokinetic properties in antibiotic development lies in how crucial pharmacodynamics are to clinical effect (Jacobs, 2001). In this sense, antimicrobials are a somewhat unique therapeutic class; it is possible to directly link drug exposure to clinical efficacy through in vitro measurements of activity (Levison and Levison, 2009). The relationship between the efficacy of antibiotics and their pharmacokinetic properties highlights the need to incorporate such work early in the drug development process.

Understanding pharmacodynamics can be extremely useful for predicting the efficacy of preclinical leads and, ultimately, in developing dosing strategies. Time-dependent antibiotics rely on maintaining an

effective concentration throughout the duration of treatment. The rate and extent of antimicrobial activity are independent of drug concentrations above the minimum inhibitory concentration (MIC). Rather, it is the amount of time the plasma levels are above the MIC that is most predictive of efficacy. Concentration-dependent antibiotics, on the other hand, exert their optimal effect through achieving the highest tolerable concentration above the MIC, meaning that  $C_{\max}/MIC$  and area under the curve (AUC)/MIC are more predictive of efficacy (Craig, 1993).

We have been developing a novel class of antimicrobials, propargyl-linked antifolates (PLAs), as effective inhibitors of the folate biosynthetic pathway. This class of agents targets the essential enzyme dihydrofolate reductase (DHFR), displaying nanomolar enzyme inhibition and exhibiting potent antibacterial activity (Scocchera et al., 2016). Structurally, PLAs are characterized by a distinct scaffold consisting of a conserved diaminopyrimidine ring connected to a biaryl system through an acetylenic linker. While the diaminopyrimidine moiety is essential for target binding to a conserved Asp/Glu residue, the acetylene-linked biaryl system allows PLAs to access key hydrophobic regions in the active site, expanding the spectrum of activity to drug-resistant enzymes (Keshipeddy et al., 2015; Lombardo et al., 2016). Crystal structures of trimethoprim-insensitive DHFRs reveal that the biphenyl system is essential for potent binding. That same biaryl system, however, diminishes the aqueous solubility of the PLAs. This issue prompted

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**ABBREVIATIONS:** ACN, acetonitrile; AUC, area under the curve; CL, clearance;  $CL_{int}$ , intrinsic clearance; DHFR, dihydrofolate reductase; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; HPMC, hydroxypropyl methylcellulose; IS, internal standard; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MIC, minimum inhibitory concentration; MS, mass spectrometry; P450, cytochrome P450; PLA, propargyl-linked antifolate.

the exploration of nitrogen-containing heterocyclic derivatives such as compound **10**, reported by Zhou et al. (2012). Compound **10** not only had significantly improved physicochemical properties compared with earlier PLA derivatives but also showed potent activity against *Staphylococcus aureus* with MIC values  $\leq 0.625 \mu\text{g/ml}$  (Zhou et al., 2012). It was also found that manipulating stereochemistry at the propargyl position can improve efficacy against common clinical resistance mechanisms, leading to the discovery of compound **11**, an enantiomer of compound **10** (Keshipeddy et al., 2015).

With broad anti-Staphylococcal activity achieved, we turned our focus to characterizing and optimizing the pharmacokinetic profile of compound **11**, specifically increasing AUC levels by extending the half-life and overall drug exposure. Initial in vitro pharmacokinetic profiling of the lead indicated that, while the acetylenic linker was metabolically stable, the compound was vulnerable to metabolism at multiple sites on the biphenyl system. In mouse liver microsomes, the racemic compound undergoes arene hydroxylation, demethylation (2'-methoxy), and *N*-oxidation (pyridine) with a corresponding intrinsic clearance ( $CL_{\text{int}}$ ) of approximately  $21 \mu\text{l/min/mg}$  mouse microsomal protein (Zhou et al., 2012).

The  $CL_{\text{int}}$  of the racemate forecasted a positive pharmacokinetic profile for the single enantiomer **11** in vitro, so we moved to determine the in vivo pharmacokinetics of the enantiopure compound in mice. Surprisingly, the in vivo pharmacokinetic profile diverged significantly from the in vitro data, revealing a short half-life and overall poor exposure. As preliminary data indicated that intrinsic clearance was significantly lower in human liver microsomes compared with mouse liver microsomes, we used in vitro mouse data to guide medicinal chemistry efforts in anticipation of in vivo murine models of bacterial infection. To determine what modifications would be needed to enhance drug exposure without compromising the strong antibacterial activity, it was first necessary to understand the source of the discrepancies between the in vitro and in vivo data sets.

In this report, we evaluate the pharmacokinetic liabilities of compound **11** and investigate strategies to decrease cytochrome P450 (P450) inhibition and block the major routes of metabolism. We determined a high-resolution crystal structure of the lead in complex with the bacterial reductase and used these data to guide the synthesis of compounds with improved physicochemical properties and potent antibacterial activity. An efficient synthesis of the PLA scaffold allowed for the rapid generation of more than a dozen new analogs, which enabled us to generate new, superior compounds.

## Materials and Methods

### Materials

For all stock solutions, test compounds were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). Buffers required for purification of recombinant DHFR were prepared with dithiothreitol, ethylenediaminetetraacetic acid (EDTA), glycerol, and Tris base purchased from Fisher Scientific (Hampton, NH). Crystallization solution was prepared with  $\gamma$ -butyrolactone; 4-morpholineethanesulfonic acid sodium salt; nicotinamide adenine dinucleotide phosphate (NADPH); polyethylene glycol 10,000; and sodium acetate purchased from Sigma-Aldrich. Hydroxypropyl methylcellulose (HPMC; Sigma-Aldrich) and potassium phosphate buffer (BD Biosciences, San Jose, CA) were used throughout microsomal experiments. Acetonitrile [high-performance liquid chromatography (HPLC) grade] was purchased from Fisher Scientific. Ethyl acetate (HPLC grade) was purchased from MilliporeSigma (Burlington, MA). Heptafluorobutyric acid was purchased from Sigma-Aldrich. All mobile phases were filtered using  $2.0\text{-}\mu\text{m}$  filtration discs from MilliporeSigma.

### Synthesis of Propargyl-Linked Antifolates

Compounds used in this study were synthesized through a previously reported conjugate addition/Suzuki coupling protocol that allowed for efficient synthesis of

racemic compounds and their corresponding enantiomeric pairs (Keshipeddy et al., 2015). Experimental and NMR spectra are provided in the Supplemental Methods.

### In Vitro Antibacterial Activity

MICs were determined according to the Clinical and Laboratory Standards Institute's Performance Standards for Antimicrobial Susceptibility Testing (www.clsi.org; CLSI, 2015) and performed in triplicate. The microdilution broth assay was performed using the American Type Culture Collection *S. aureus* quality control strain 43300 at an inoculum of  $5 \times 10^5$  colony-forming units/ml in Oxoid Iso-Sensitest Broth (Thermo Fisher Scientific, Waltham, MA). The MIC was defined as the lowest concentration of inhibitor to visually inhibit growth following an 18-hour incubation at  $37^\circ\text{C}$ .

### Time-Kill Assays with Compound 19.

Time-kill curves for compound **19** were performed in triplicate using *S. aureus* American Type Culture Collection 43300. BD Difco Mueller-Hinton Broth (Thermo Fisher Scientific) was used throughout the experiments. Single colonies from overnight cultures grown on agar plates were added to Mueller-Hinton Broth (100 ml) and incubated at  $37^\circ\text{C}$  until confluent. The bacterial suspension was adjusted to  $\sim 5.5 \times 10^5$  colony-forming units/ml (10 ml) and compound in DMSO was added to give concentrations  $1 \times (0.156 \mu\text{g/ml})$ ,  $5 \times (0.78 \mu\text{g/ml})$ ,  $10 \times (1.56 \mu\text{g/ml})$ , and  $100 \times (15.6 \mu\text{g/ml})$  the MIC. Samples (100  $\mu\text{l}$ ) taken at 0, 1, 2, 4, 6, 8, 10, 12, and 24 hours were serially diluted in chilled normal saline and plated on Mueller-Hinton agar plates for quantification of viable colony-forming units.

### Cloning, Expression, and Enzyme Purification

The gene encoding *S. aureus* DHFR was synthesized and cloned in the pET41a(+) expression vector containing a C-terminal His-tag (GenScript, Nanjing, China) as previously reported (Frey et al., 2009). Recombinant *S. aureus* DHFR was then overexpressed in One Shot BL21(DE3) Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA), purified via nickel affinity chromatography, and desalted using a PD-10 column (GE Healthcare, Chicago, IL) into buffer containing 20 mM Tris, pH 7.0; 20% glycerol; 0.1 mM EDTA; and 2 mM dithiothreitol. Recombinant protein was concentrated to  $\sim 10 \text{ mg/ml}$ , flash frozen with liquid nitrogen, and stored at  $-80^\circ\text{C}$ .

### Crystal Structure Determination

*S. aureus* DHFR was crystallized with NADPH and compound **11** using the hanging-drop vapor diffusion method. Purified protein (13 mg/ml) was incubated with compound **11** (2 mM) and NADPH (4 mM) for 3 hours on ice. An equal volume of protein/ligand/NADPH complex was mixed with the optimized crystallization solution consisting of 0.1 mM 4-morpholineethanesulfonic acid, pH 5.0; 0.1 mM sodium acetate; 13% polyethylene glycol 10,000; and 20%  $\gamma$ -butyrolactone additive. Crystals were observed within 14 days when stored at  $4^\circ\text{C}$ . Crystals were frozen in cryoprotectant buffer containing 20% glycerol and stored in liquid nitrogen.

High-resolution diffraction data for *S. aureus* DHFR:NADPH:**11** were collected at the Stanford Synchrotron Radiation Lightsources on beamline 14-1. Data were indexed and scaled using HKL-2000 (HKL Research, Inc.) (Otwinowski and Minor, 1997). The structure was refined and validated using noncrystallographic symmetry and structure restraints with the PHENIX suite, whereas COOT was used throughout the model-building process (Adams et al., 2010; Emsley et al., 2010). Phaser was used for molecular replacement using the *S. aureus* DHFR model Protein Data Bank 3FOQ as a probe (McCoy et al., 2007). Inhibitor Protein Data Bank and Crystallographic Information File files were generated with PRODRUG (Schüttelkopf and van Aalten, 2004). Data collection and refinement statistics are reported in Supplemental Table 1.

### Cytochrome P450 Inhibition

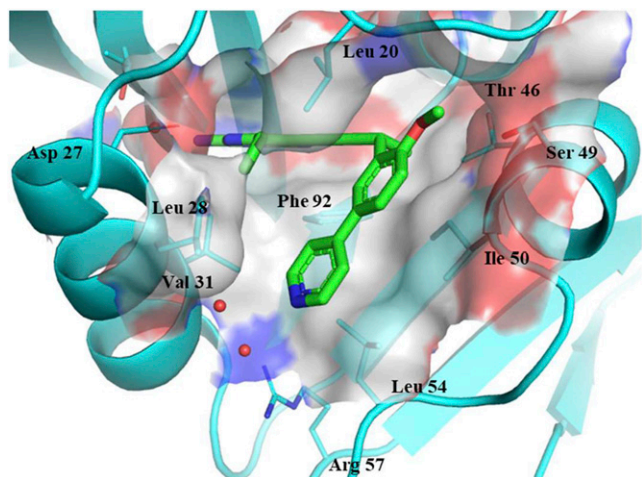
$IC_{50}$  values for recombinant human CYP3A4 were determined using a fluorescence-based high-throughput inhibition assay (GENTEST; BD Biosciences). Compounds were tested for inhibition activity by monitoring the conversion of 7-benzyloxy-quinoline to the fluorescent 7-hydroxy-quinoline

metabolite at 409-nm excitation and 530-nm emission. The reaction was carried out in 96-well black microtiter plates (BD Biosciences). Test compounds (0.6  $\mu$ l, 50  $\mu$ M final concentration) were mixed with NADPH–glucose 6–phosphate dehydrogenase mix (149.4  $\mu$ l), and one-third serial dilutions were performed to a low concentration of 0.008  $\mu$ M. The plate was preincubated for 10 minutes at 37°C prior to initiation with enzyme/substrate mix (100  $\mu$ l). Following a 30-minute incubation at 37°C, the reaction was terminated with Tris base (75  $\mu$ l, 0.5 M). The concentration of test compound that corresponds to 50% inhibition was calculated by linear interpolation using the calculated percentage inhibition of each compound concentration.

IC<sub>50</sub> values for recombinant human CYP2D6 were determined using a fluorescence-based high-throughput inhibition assay (GENTEST; BD Biosciences) where inhibition was monitored through metabolic conversion of 3-[2-(*N,N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin to the fluorescent metabolite 3-[2-(*N,N*-diethylaminoethyl)-7-hydroxy-4-methylcoumarin hydrochloride at 390-nm excitation and 460-nm emission. The reaction was carried out in 96-well black microtiter plates (BD Biosciences) following the protocol as described for CYP3A4 IC<sub>50</sub> determination.

### Microsomal Stability

Microsomal CL<sub>int</sub> was determined via incubation with male mouse microsomes (CD-1; BD Biosciences). Test compound (2  $\mu$ l) was added to reaction buffer (973  $\mu$ l) containing 200  $\mu$ g/ml HPMC; 100 mM potassium phosphate, pH 7.4; and NADPH Regenerating System Solution (1.3 mM NADPH, 8 mM MgCl<sub>2</sub>, 3.3 mM glucose-6-phosphate, 0.5 U/ml Glucose-6-phosphate dehydrogenase; BD Biosciences) to give a final test compound concentration of either 5 or 0.5  $\mu$ g/ml. After the mixture was prewarmed to 37°C, the reaction was initiated with the addition of liver microsomes (25  $\mu$ l), resulting in a final protein concentration of 0.5 mg/ml. After 0, 10, 20, 30, 40, and 60 minutes, 100  $\mu$ l of sample was removed, and the reaction was terminated with equal parts ice-cold acetonitrile (ACN). Samples were subsequently centrifuged at 10,000g for 10 minutes, and the supernatant was withdrawn. Samples (5  $\mu$ l) were injected onto a Shimadzu Nexera UHPLC system equipped with a DGU-20A5 Prominence degasser, SIL-30AC Nexera autosampler, CTD-30A Nexera column oven, two LC-30AD pumps (Shimadzu, Kyoto, Japan), LCMS-2020 mass spectrometer, and Kinetex C18 column (1.7  $\mu$ m, 100 Å, 2.1 × 50 mm; Phenomenex, Torrance, CA). The following chromatographic methods were developed to optimize resolution and detection of PLAs. Mobile phase A [0.01% heptafluorobutyric acid (HFBA) in water] and mobile phase B (0.01% HFBA in ACN) were used for a binary gradient elution that began at 95% A and 5% B, then increased to 95% B over 7 minutes, held at 95% B for 2 minutes, then decreased to 5% B over 1 minute, and held at 5% B for 2 minutes.



**Fig. 1.** Crystal structure of *S. aureus* DHFR (cyan) in complex with compound **11** (green). Compound **11** makes conserved hydrogen bonds with Leu 5 and Asp 27 through the diaminopyrimidine ring and hydrophobic residues as well as significant van der Waals contacts with Leu 20, Ser 49, Ile 50, and Phe 92. Protein Data Bank ID: 6ND2.

The mass spectrometer was set to a nebulizing gas flow of 1.5 l/min, a drying gas flow of 15 l/min, the desolvation line at 250°C, the heat block at 400°C, and the interface temperature at 350°C. Mass spectrometry (MS) was performed in the positive ion mode with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) dual ionization mode using diltiazem (0.5  $\mu$ g/ml) as an internal control. The half-life, calculated via quantification based on MS area under the curve responses and comparison with authentic standards, was converted to give CL<sub>int</sub>.

### Metabolite Identification for Compound 12

Phase I and phase II metabolites were identified via mouse liver S9 fractions (BD Biosciences) as the source of membrane-bound drug-metabolizing enzymes. For generation of P450 and uridine glucuronosyl transferase metabolites, test compound (2  $\mu$ g/ml) was supplemented with NADPH Regenerating System Solution (1.3 mM NADPH, 8 mM MgCl<sub>2</sub>, 3.3 mM glucose-6-phosphate, 0.5 U/ml Glucose-6-phosphate dehydrogenase; BD Biosciences) and uridine glucuronosyl transferase Reaction Mix (25  $\mu$ g/ml almethicin, 2 mM UDP glucuronic acid; BD Biosciences) in 100 mM potassium phosphate (pH 7.4) and HPMC (200  $\mu$ g/ml) to aid in compound solubility. After the mixture was prewarmed to 37°C, the reaction was initiated with the addition liver S9 (0.5 mg/ml). Time points were collected at 0 and 2 hours, the reaction was quenched with equal parts of ice-cold ACN. Metabolites were reconstituted in 50% methanol via Solid-phase extraction isolation using Oasis HLB Cartridges (Waters, Milford, MA).

Samples were analyzed at the Yale School of Medicine Proteomics Center with the help of Dr. Tukiet Lam using liquid chromatography–tandem mass spectrometry (LC-MS/MS) on a Thermo Scientific LTQ Orbitrap XL (Thermo Fisher Scientific) fitted with an ACQUITY UPLC System (Waters) and Ultra AQ C18 column (3  $\mu$ m, 10 × 1.0 mm, particle size: 3  $\mu$ m; Restek, Bellefonte, PA). Metabolites were resolved using a gradient of 0.01% HFBA in water (A) and ACN (B). The gradient started with 0% B and increased to 100% B over 17 minutes followed by an isocratic hold at 100% B for 5 minutes at a flow rate of 75  $\mu$ l/min and the injection volume set to 8  $\mu$ l. Resolution was set to 30,000. Collision energy was set to 35 eV. The collision gas was helium.

### Stability of Compound 17 in Human Hepatocytes

The intrinsic clearance of compound **17** was measured in mixed-sex pooled cryopreserved human hepatocytes. Aliquots (45  $\mu$ l) of human hepatocytes at a cell density of 500,000 cells/ml were suspended in Williams' E medium to a final volume of 200  $\mu$ l in a 96-well plate with a final substrate concentration of 1  $\mu$ M. Incubations were carried out at 37°C under an atmosphere of 95% O<sub>2</sub>, 5% CO<sub>2</sub>, 75% humidity on a rotating shaker. Sample aliquots (25  $\mu$ l) were taken at 0, 0.5, 1, 2, 3, and 4 hours and added to 75  $\mu$ l of chilled ACN plus internal standard. Samples were centrifuged at 10,000g at 4°C for 10 minutes, and supernatant was collected, loaded on to a Kinetex C18 column (1.7  $\mu$ m, 100 Å, 2.1 × 50 mm), and analyzed via LC-MS as previously described. The percentage of parent remaining (log-transformed) over time was used in a linear regression analysis to estimate half-life (first-order), which was used in subsequent calculations of intrinsic clearance.

### Metabolite Identification for Compound 17





















The in vitro metabolites of compound **17** were determined by LC-MS/MS following incubation with mixed-sex pooled cryopreserved mouse and human hepatocytes (Bioreclamation IVT, Westbury, NY). Thawed hepatocytes were suspended in Williams' E medium (Thermo Fisher Scientific) at a final concentration of 750,000 viable cells/ml as determined by trypan blue staining. Incubations were initiated with the addition of compound in DMSO (10  $\mu$ M) to an aliquot of cell suspension (1 ml). Samples were incubated at 37°C under an atmosphere of 95% O<sub>2</sub>, 5% CO<sub>2</sub>, 75% humidity with shaking (50 rpm) in an HERAccl 240i incubator (Thermo Fisher Scientific). Following 45 minutes, an aliquot (500  $\mu$ l) was added to chilled ACN (2 ml) to capture early primary metabolites. At 4 hours, the remaining sample (500  $\mu$ l) was crashed with chilled ACN (2 ml) and combined with the early time point. Samples were centrifuged at 10,000g at 4°C for 10 minutes and supernatant was collected. Samples were dried overnight using a GeneVac EZ 2 Plus Personal Solvent Concentrator (Genevac Ltd., Valley Cottage, NY).

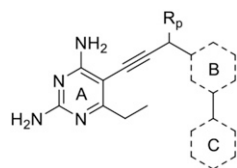
Samples were reconstituted in 0.1% formic acid in water (200  $\mu$ l) and analyzed by LC-MS/MS using a Thermo Orbitrap Elite mass spectrometer (Thermo Fisher

TABLE 1

Propargyl-linked antifolates synthesized to reduce P450 inhibition imparted through the unsubstituted pyridine of compound **11**

The intrinsic clearance is reported for the two substrate concentrations tested, 5 and 0.5  $\mu\text{g/ml}$ .

Scaffold	Compound	Stereochemistry	B-Ring	C-Ring	MIC	MLM CL <sub>int</sub>		CYP3A4	CYP2D6
						5 $\mu\text{g/ml}$	0.5 $\mu\text{g/ml}$		
					$\mu\text{g/ml}$	$\mu\text{l/min/mg}$	$\mu\text{l/min/mg}$	$\mu\text{M}$	$\mu\text{M}$
	<b>10</b>	Racemic			0.625	21	—	—	—
	<b>11</b>	S			0.078	10	99	0.05	1.6
	<b>12</b>	Racemic			0.156	40	173	3.6	9.8
	<b>13</b>	Racemic			0.156	69	—	2.6	13.2
	<b>14</b>	Racemic			0.312	—	154	11	12.9
	<b>15</b>	Racemic			0.625	—	22	>50	>50
	<b>16</b>	Racemic			0.156	—	69	0.5	27.4
	<b>17</b>	Racemic			0.312	—	25	43	>50
	<b>18</b>	Racemic			0.625	—	28	5.4	>50
	<b>19</b>	S			0.156	—	18	41	>50



Scientific) coupled to a Waters ACQUITY UPLC System with a Restek Ultra AQ C18 column (3  $\mu\text{m}$ ,  $10 \times 1.0$  mm, particle size: 3  $\mu\text{m}$ ). Electrospray ionization in positive mode was used, recording full scans ( $m/z$  100–1000), targeted, and data-dependent MS<sup>n</sup> at high resolution (30,000) as needed. Metabolites were resolved using a gradient of 0.1% formic acid in water (A) and ACN (B). Samples (10  $\mu\text{l}$ ) were injected, and a linear gradient was applied to 90% B over 35 minutes followed by a hold at 90% B for 5 minutes before returning to initial conditions and re-equilibrating for 5 minutes.

#### In Vivo Pharmacokinetic Characterization of Compound **19**

**Animals.** Experimental procedures were performed according to Institutional Animal Care and Use Committee protocol AUP022-14, which was approved by the University of Montana and its Institutional Animal Care and Use Committee. National Institutes of Health Guidelines for the Care and Use of Laboratory

Animals were followed for all experiments. Thirteen- to 15-week-old female, CD1 mice (Envigo, Huntingdon, UK) were housed in static micro isolated cages under pathogen-free, high-efficiency particulate air-filtered conditions. Other housing conditions were as follows: 12-hour light/dark cycles, controlled temperature (20.5–22.5°C) and humidity (25%–45%), with weekly cage changes of bedding. Food and water were provided ad libitum. After animal delivery, animals were given at least 3 days to acclimate to their new environment prior to the start of any experiments.

Compound **19** was formulated for intraperitoneal delivery at a concentration of 0.2 mg/ml as follows: 1.8 mg compound **19** was dissolved in 0.180 ml of *N*-methylpyrrolidone, 2.0 ml of 45% (w/v) (2-hydroxypropyl)- $\beta$ -cyclodextrin in phosphate-buffered saline (PBS), and 6.820 ml of PBS. Five mice were used in the study, and the average mouse weight was 34 g. Blood samples were collected via the saphenous vein into heparinized capillary tubes followed by transfer to 1.5-ml Eppendorf tubes with EDTA (30  $\mu\text{l}$  of 100 mM EDTA at pH 8) and mixed. Blood

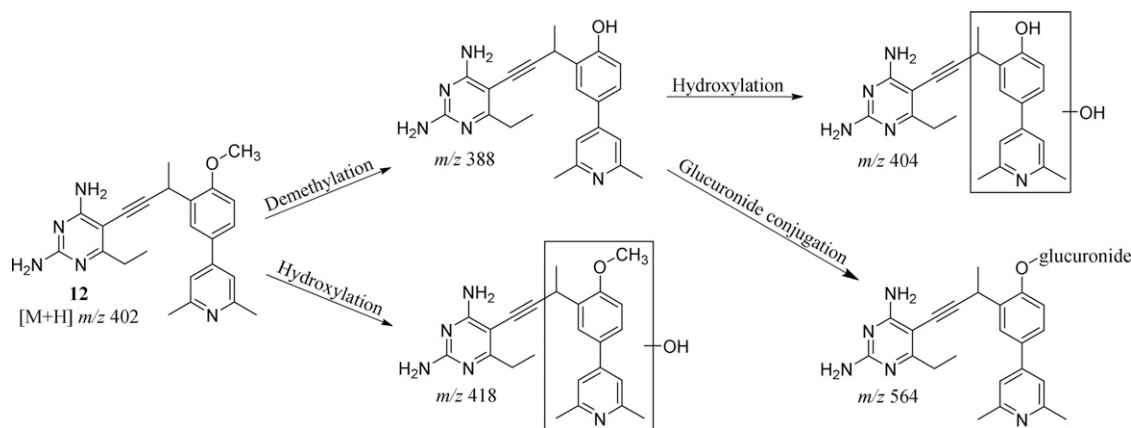


Fig. 2. The proposed metabolic scheme for compound **12** following incubation with mouse liver microsomes.

was not taken more than three times from any one mouse. Samples were stored at 0°C until centrifuging later that same day. Following the last time point, all blood samples were centrifuged at 13,500 rpm for 15 minutes at 4°C. The supernatant was then transferred to a new 1.5-ml Eppendorf tube and stored at -80°C until the sample extraction step.

**Sample Extraction Procedure.** For each plasma sample, a small volume (20  $\mu$ l), held in an Eppendorf tube, was mixed briefly by vortexing with internal standard (IS) spiking solution (10  $\mu$ l); the IS spiking solution consisted of 500 ng/ml 6-ethyl-5-[5-(4-pyridyl)pent-1-ynyl]pyrimidine-2,4-diamine. The samples were vortexed for 3 minutes. Ethyl acetate (500  $\mu$ l) was added to each sample, mixed by vortexing, shaken for 10 minutes, vortexed briefly again, and then the phases were separated by centrifugation at 10,000 rpm for 10 minutes at room temperature. The ethyl acetate supernatant (450  $\mu$ l) was transferred to a new Eppendorf tube and concentrated to dryness in vacuo using a Savant SpeedVac (Thermo Fisher Scientific). The dried samples were reconstituted in HPLC mobile phase (50  $\mu$ l) for analysis.

**Preparation of Calibration Standards and Quality Control Standards.** The primary, standard stock solution of compound **19** was prepared at a concentration of 6.4 mg/ml in DMSO. The standard spiking solutions were prepared in 50:50 (v/v) acetonitrile:water at concentrations of 500; 1000; 2500; 5000; 10,000; 20,000; 40,000; and 80,000 ng/ml. The IS stock solution was prepared at a concentration of 2.5 mg/ml in 50:50 (v/v) acetonitrile:water, from which the IS spiking solution was prepared at a concentration of 500 ng/ml in 50:50 (v/v) acetonitrile:water. Standard spiking solutions and IS spiking solution were stored at -20°C or 4°C until use. Plasma standards were prepared by adding 11  $\mu$ l of respective standard spiking solution to 99  $\mu$ l of drug-free mouse plasma. Thus, the working standard concentrations for plasma standards were 50, 100, 250, 500, 1000, 2000, 4000, and 8000 ng/ml.

**Instrumentation.** Analysis was performed on an Agilent HPLC system (Agilent Technologies, Santa Clara, CA) equipped with an autosampler (1260 Infinity, Model G1329B; Agilent), a degasser (1200, Model G1379B; Agilent), and a binary pump (1200, Model G1312B; Agilent) coupled to a Bruker Amazon SL ion trap mass spectrometer (Bruker, Billerica, MA). Data were analyzed using the Bruker Compass DataAnalysis software suite (version 4.2, build 383.1). A Phenomenex Gemini column (3.0  $\times$  150 mm, 100  $\text{\AA}$ , 5  $\mu$ m) with an attached Phenomenex SecurityGuard (C18, 4  $\times$  2.0 mm, part number AJ0-4286) guard column was used for analysis. The analysis was performed under isocratic conditions at ambient temperature. The mobile phase was premixed at a ratio of 30:70 (v/v) 10 mM  $\text{NH}_4\text{HCO}_3$ :acetonitrile. The mobile phase was filtered through 2.0- $\mu$ m filtration discs (catalog number AP2504700; Millipore). The flow rate used was 0.320 ml/min with a resultant backpressure of approximately 35 bar. The total run time was 6 minutes with an injection volume of 10  $\mu$ l.

Detection was performed with positive-mode electrospray ionization and selected ion monitoring. The  $[\text{M}+\text{H}]^+$  ions at  $m/z$  406.2 and 282.2 were chosen for selected ion monitoring data collection for quantitation of **19** and the internal standard 6-ethyl-5-[5-(4-pyridyl)pent-1-ynyl]pyrimidine-2,4-diamine, respectively. The following MS parameters were used during data

collection: electrospray voltage, +4500 V; source temperature, 250°C; nebulizer gas (nitrogen), 29 psi; and dry gas flow rate (nitrogen), 12 l/min.

## Results

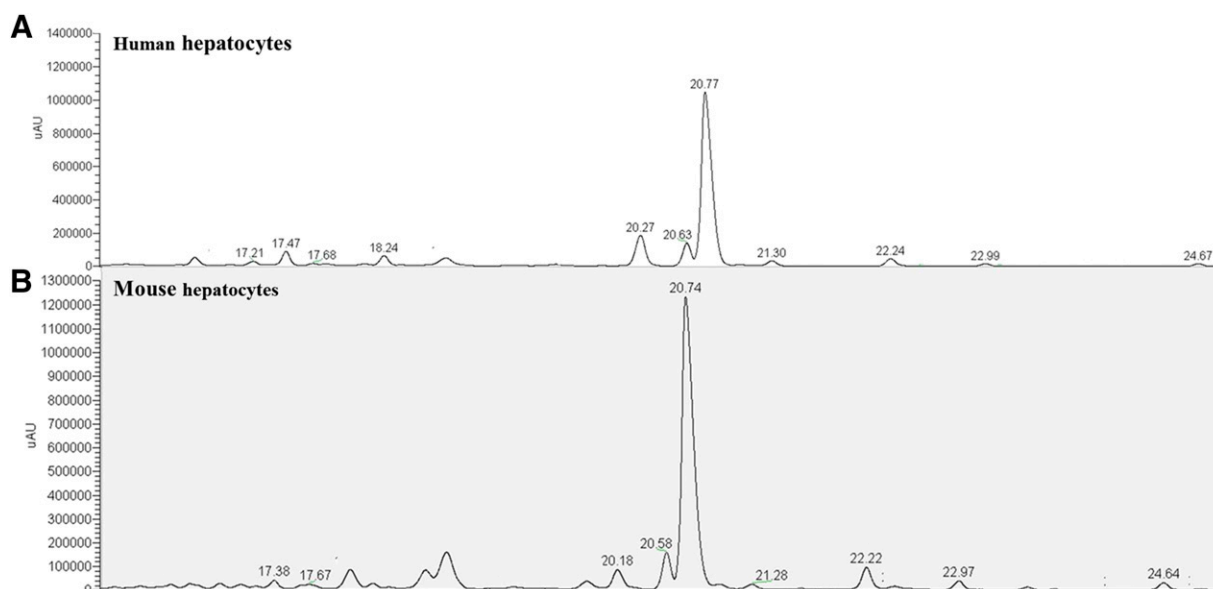
**In Vivo Pharmacokinetics for Compound 11.** Plasma profiles of compound **11**, through both intraperitoneal injection and oral gavage, were determined in mice using a single dose of 16 mg/kg. Following i.p. injection at 16 mg/kg, the  $C_{\text{max}}$  reached 10.2  $\mu\text{g/ml}$  and the AUC, 294 minutes $\cdot\mu\text{g/ml}$ . Clearance (CL) and half-life ( $t_{1/2}$ ) were 54 ml/min/kg and 20 minutes, respectively. When administered orally at 16 mg/kg, the  $C_{\text{max}}$  and AUC were 1.44  $\mu\text{g/ml}$  and 24.6 minutes $\cdot\mu\text{g/ml}$ , giving a bioavailability of 8.4%.

**Crystal Structure of *S. aureus* DHFR in Complex with NADPH and Compound 11 and Design of New Analogs.** Diffraction data yielded a 2.24- $\text{\AA}$ -resolution structure of *S. aureus* DHFR:NADPH:**11**. As expected, the diaminopyrimidine ring formed conserved hydrogen bonds with the carboxyl group of Asp 27 and the carbonyl groups of Leu 5 and Phe 92 (Fig. 1). The methoxyphenyl moiety is positioned in a predominantly hydrophobic pocket consisting of Leu 20; Ser 49; Ile 50; and the cofactor, NADPH. The pyridine ring is surrounded by four hydrophobic residues (Leu 20, Leu 28, Ile 50, Leu 54) while forming a water network of hydrogen bonds with Arg 57. The 1-, 2-, and 6-positions of the pyridyl group are solvent exposed, suggesting that modifications in this domain would be well tolerated by the enzyme.

**In Vitro Optimization of Compound 11 Leads to the Development of Compound 19.** Microsomal stability was determined for compounds **11**, **12**, and **13** by monitoring the presence of compound after incubation in mouse liver microsomes (Table 1). The experiments were carried out with a substrate concentration of 0.5  $\mu\text{g/ml}$ , giving an observed  $\text{CL}_{\text{int}}$  of 10  $\mu\text{l/min/mg}$  mouse microsomal protein for compound **11**. Surprisingly, both compounds **12** and **13** had elevated clearances relative to **11** (40 and 173  $\mu\text{l/min/mg}$ , respectively) despite the presence of bulky ortho substitutions designed to reduce the rate of formation of the *N*-oxide metabolite.

Of the CYP450 isoforms, CYP3A4 and CYP2D6 account for 50% and 30% of all xenobiotic metabolism and play an important role in monitoring the potential for drug-drug interactions throughout the drug discovery process (Sun et al., 2011). As such, CYP3A4 and CYP2D6  $\text{IC}_{50}$  values were determined in parallel by monitoring conversion of a nonfluorescent substrate to a fluorescent metabolite and calculated from the net fluorescent signal. Compound **11** was determined to have potent P450 inhibition capability (CYP3A4  $\text{IC}_{50}$ : 0.05  $\mu\text{M}$ /CYP2D6  $\text{IC}_{50}$ : 1.6  $\mu\text{M}$ ). The dimethyl substitution of compound **12** reduced CYP3A4 and CYP2D6 inhibition 370- and





**Fig. 3.** The UV trace at 284 nm for compound **17** metabolite identification in mouse and human hepatocytes. (A) Incubation with human hepatocytes indicates there are 10 distinct metabolites: four  $m/z$  438 (double hydroxylation products); three  $m/z$  with MS2 data, indicating a hydroxylation product; two  $m/z$  422 with MS-2 data indicating  $N$ -oxide products; one  $m/z$  436 with MS-2 data indicative of methyl conversion to a carboxylic acid. MS-2 spectra are provided in the Supplemental Materials. (B) Similarly, mouse hepatocyte incubation indicates there are eight distinct metabolites: three  $m/z$  438 (double hydroxylation products); two  $m/z$  422 with MS-2 data indicating  $N$ -oxide products; one  $m/z$  436 with MS-2 data indicative of methyl conversion to a carboxylic acid.

6-fold, while the methoxy substitution of compound **13** reduced CYP3A4 and CYP2D6 inhibition 270- and 8-fold (see Table 1).

Microsomal stability was then redetermined using a 10-fold lower concentration of substrate than initially used. It is noteworthy that the reduced concentration resulted in a  $CL_{int}$  of  $\sim 99 \mu\text{l}/\text{min}/\text{mg}$  for compound **11**, which was 10-fold higher than the  $CL_{int}$  estimated in experiments with the  $0.5 \mu\text{g}/\text{ml}$  substrate concentration. Similarly, the reduced concentration of compound **12** resulted in an estimated  $CL_{int}$  of  $\sim 173 \mu\text{l}/\text{min}/\text{mg}$ , which was 4-fold faster than in the initial microsome incubations. To improve the metabolic stability of the leads while simultaneously reducing the potency of P450 inhibition, it was necessary to first identify the preferred routes of biotransformation for these new derivatives.

A demethylation product metabolite of compound **12** was identified following incubation with mouse liver microsomes. In addition, monohydroxylation metabolites were also detected. When compound **12** was incubated in the presence of both phase I and phase II cofactors, the phenolic metabolite undergoes subsequent glucuronide conjugation (Fig. 2).

Once it was determined that the methoxy group was a major site of metabolic vulnerability, additional derivatives of compound **12** were synthesized bearing less reactive substituents at that position to diminish the formation of these demethylated metabolites. The crystal structure data suggest that the 2-methoxy-phenyl occupies a tight, predominantly hydrophobic pocket consisting of Leu 20; Ser 49; Ile 50; and the cofactor, NADPH. With limited physical space to modify the compound, two strategies were envisioned to reduce or prevent the formation of the demethylation product: 1) modification of the methoxy group through deuteration or fluorination and 2) replacement of the methoxy group altogether.

Compound **14**, a deuterated derivative of **12**, and compound **15**, a fluorinated derivative of **12**, were designed to potentially slow the demethylation route and gave  $CL_{int}$  values of  $\sim 154$  and  $\sim 22 \mu\text{l}/\text{min}/\text{mg}$ , respectively. The pharmacokinetic effects of removing the metabolic liabilities present in compound **11** were investigated through the synthesis of three analogs: hydro-substituted

(compound **16**), chlorine-substituted (compound **17**), and fluorine-substituted (compound **18**) phenyl derivatives. Where the  $CL_{int}$  for compound **16** was calculated to be  $\sim 70 \mu\text{l}/\text{min}/\text{mg}$ , a modest 2.5-fold decrease from compound **12**, the chloro- and fluoro-derivatives recorded clearances of  $\sim 25$  and  $\sim 28 \mu\text{l}/\text{min}/\text{mg}$ , respectively (see Table 1). The 2-chlorophenyl derivative, compound **17**, demonstrated virtually no P450 inhibition, a significantly extended half-life, maintained potent antibacterial activity, and was selected for further testing.

**Human Hepatocyte Stability for Compound 17.** The metabolic stability of our most promising compound, **17**, was subsequently determined in human hepatocytes to provide context for data collected in *in vitro* mouse models and predicted human clearance. The resulting half-life was 180 minutes and corresponded to a  $CL_{int}$  of  $\sim 8 \mu\text{l}/\text{min}$  per  $10^6$  cells, a  $CL_{int,app,scaled}$  of  $\sim 19 \text{ ml}/\text{min}/\text{kg}$ , and subsequent extrapolation to a hepatic clearance of  $\sim 10 \text{ ml}/\text{min}/\text{kg}$ .

**Metabolite Identification for Compound 17 in Human and Mouse Hepatocytes.** Prior to *in vivo* evaluation, we identified the major metabolites of compound **17** in mouse and human hepatocytes to confirm that we did not introduce unexpected metabolites. Based on the UV trace of the metabolites generated, the major metabolic pathway for compound **17** in mouse and human hepatocytes was oxidation, either hydroxylation or  $N$ -oxidation (Fig. 3). In the high-resolution full scan, the parent  $[M+H]^+$  was observed at  $m/z$  406.1746, which is identical to the calculated value for compound **17**. Results from incubation with mouse hepatocytes mirrored those from human hepatocyte incubation. For both experiments, metabolite ions were observed at  $m/z$  438 (bis-hydroxylation),  $m/z$  422 (monohydroxylation or  $N$ -oxidation), and  $m/z$  436 (methyl conversion to carboxylic acid). The fragmentation patterns of the metabolites were distinct from that of the parent, and either the fragment ion at  $m/z$  189, corresponding to the diaminopyrimidine ring and propargyl linker, or the fragment ion at  $m/z$  244, corresponding to the propargyl methyl and biphenyl system, was used to identify and assign the masses for metabolites and the parent compound. The  $N$ -oxide and hydroxylation metabolites were assigned by either the loss of 16 mass units, consistent with  $N$ -oxide fragmentation by loss of oxygen, or

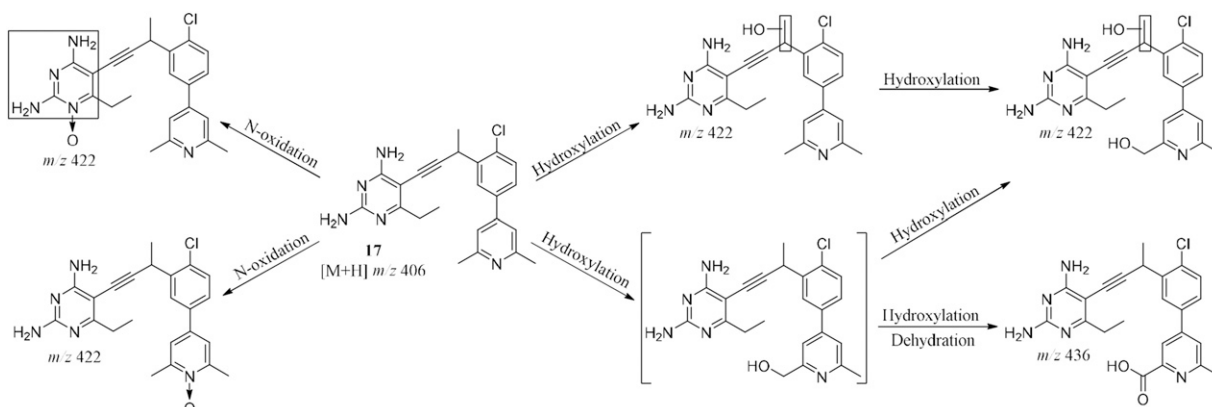


Fig. 4. The proposed metabolic pathway for compound 17.

the loss of 18 mass units indicative of hydroxyl release. We were able to discern that hydroxylation occurs at the propargyl methyl in mouse hepatocytes. Likewise, it was possible to observe conversion of a methyl to a carboxylic acid ortho to the pyridine nitrogen in human hepatocytes. There were no diagnostic fragments that could aid in identifying the location of the other hydroxylation products even though each had distinct retention times. The proposed metabolic pathway is summarized in Fig. 4, and the MS<sup>2</sup> and MS<sup>3</sup> spectra are available in Supplemental Figs. 1–11.

**In Vivo Pharmacokinetics for Compound 19.** For comparison with compound 11, compound 19, the single S-isomer of racemate 17, was synthesized for in vivo analysis. The plasma profiles of compound 19 were analyzed following intraperitoneal and oral doses of 5 mg/kg via noncompartmental analysis using a linear trapezoidal method (see Supplemental Figs. 15 and 16). Intraperitoneal administration yielded a  $C_{\max}$  of 4.1  $\mu\text{g/ml}$ , AUC of 838 minutes $\cdot\mu\text{g/ml}$ , CL of 6.0 ml/min/kg, and half-life of 69 minutes. When administered orally at 16 mg/kg, the  $C_{\max}$ , AUC, and half-life were 0.4  $\mu\text{g/ml}$ , 156 minutes $\cdot\mu\text{g/ml}$ , and 4.5 hours, respectively, giving a bioavailability of 18.6%.

**Time-Kill Assays with Compound 19.** Time-kill curve profiles were determined for compound 19 at 1 $\times$ , 5 $\times$ , 10 $\times$ , and 100 $\times$  MIC, and the results are summarized in Fig. 5. Overall, all concentrations above the MIC demonstrated similar bactericidal activity at 12 hours; however, there was significantly faster bacterial killing at concentrations  $>10\times$  MIC. When exposed to 100 $\times$  the MIC, the bacterial population was reduced  $\sim 40\%$  within 4 hours, whereas it took 8 hours to reach the same level at lower concentrations.

## Discussion

In this report, we describe some of the challenges faced in the development of new antifolate antibiotics. We determined that our in vitro pharmacokinetic model was biased to select for strong P450 inhibitors and suggested artificially long half-lives. After successfully adjusting the assay to better represent in vivo conditions, we were able to design compounds with minimal P450 inhibition and ideal pharmacokinetic profiles. Use of high-resolution crystal structures allowed us to do this in an extremely efficient manner without compromising antibacterial activity, demonstrating that structure-based design leveraged with metabolism data (sites and rates) can be combined in the design of superior analogs.

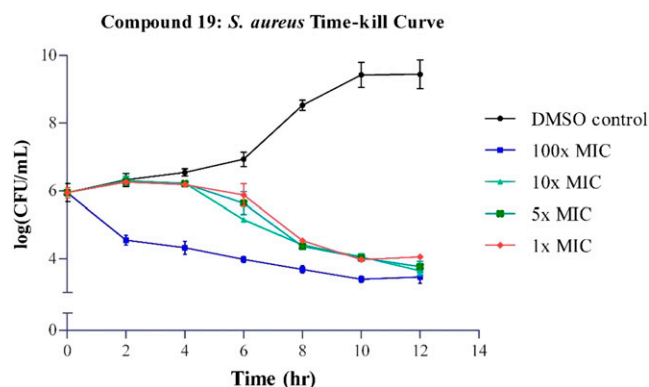
Previous work proved compound 11 had exceptional antibacterial activity against multidrug-resistant *S. aureus*, and initial in vitro pharmacokinetic data indicated potential for successful in vivo studies with a clearance of  $\sim 10$   $\mu\text{l/min/mg}$  mouse microsomal

protein (Keshipeddy et al., 2015). However, the resulting in vivo clearance was  $\sim 54$  ml/min/kg, higher than expected and not practicable for in vivo infection models. To explain the discrepancies between the in vitro and in vivo data sets, it was hypothesized that the pyridine moiety of compound 11 (or, potentially, the corresponding *N*-oxide metabolite) could be inhibiting P450 enzymes in vitro and producing an artificially long in vitro half-life due to saturation of metabolism. Thus, it is possible that the off-target activity of compound 11 was, itself, responsible for the divergence between the in vitro and in vivo clearance measurements. Crystal structure data of *S. aureus* DHFR with compound 11 guided the synthesis of compounds 12 and 13 to test this hypothesis.

The metabolite profile of compound 10 indicated there are four major metabolites: *N*-oxidation and hydroxylation of the diaminopyrimidine ring, demethylation of the 2'-methoxy group, and *N*-oxidation of the pyridine ring (Zhou et al., 2012). Sites of biotransformation on the diaminopyrimidine ring cannot be modified, as this functionality is responsible for key hydrogen bonds deep within the active site; however, as the pyridine ring and 2'-methoxy group do not make crucial contacts with the enzyme, they are ideal for chemical modification to modulate pharmacokinetic parameters.

Crystal structure analysis of *S. aureus* DHFR complexed with compound 11 indicated that four hydrophobic residues (Leu 20, Leu 28, Ile 50, and Leu 54) could accommodate substitutions adjacent to the nitrogen of the pyridine ring. Two new analogs, 12 and 13, which contain a 2,6-dimethylpyridyl or 2-methoxypyridyl ring, respectively, were synthesized. Computationally, the dimethyl and methoxy substitutions were predicted to increase ligand-target interactions through enhanced van der Waals contacts. Additionally, these modifications were not expected to disrupt the hydrogen bonding network with Arg 57 through an active site water. As anticipated, these substitutions were well tolerated with regard to antibacterial activity, yielding potent MIC values of 0.156  $\mu\text{g/ml}$ .

Evaluation of structure-activity relationships and metabolite identification data for compound 12 confirmed that adding bulky substituents adjacent to the pyridyl nitrogen not only eliminated P450 inhibition but also completely abrogated the *N*-oxide metabolite formation. Interestingly, as P450 inhibition was reduced, the in vitro clearance of lead compound 12 remained unchanged, if not enhanced, as compared with compound 11. Metabolite identification studies indicated that 2-methoxyphenyl demethylation was the primary metabolite. A small but focused series of five compounds was synthesized predicated on the structural analysis of metabolites and the modes of binding to *S. aureus* DHFR to decrease clearance rates.



**Fig. 5.** Time-kill curve determination of *S. aureus* American Type Culture Collection 43300 exposed to compound **19** at 1 $\times$ , 5 $\times$ , 10 $\times$ , and 100 $\times$  the MIC. Data suggest that compound **19** demonstrates concentration-dependent bactericidal activity at concentrations  $>10\times$  the MIC. CFU, colony-forming unit.

Deuterated and fluorinated derivatives, **14** and **15**, were designed to slow or prevent formation of the demethylated metabolites. Deuteration was an attractive approach, as there are minimal differences between the physicochemical chemical properties of hydrogen, and behavior in the DHFR active site is expected to be the same. The effects of substituting hydrogens for deuterium isotopes has gained interest as an approach to reduce the reactivity and slow the biotransformation of drugs (Guengerich, 2017). In the case of deutetrabenazine, approved in 2017 as the first deuterated therapeutic, two deuterated methoxy groups are proposed to slow the generation of the hydroxyl metabolite to a useful degree (Schmidt, 2017). However, the deuteration of compound **12** showed minimal benefit, imparting little to no effect on the in vitro clearance (compound **12**  $CL_{int}$ : 8 minutes; compound **14**, the deuterated derivative,  $CL_{int}$ : 9 minutes). The trifluoromethyl substitution has become fairly commonplace in therapeutics, as it is known to impart metabolic stability when replacing a methyl group, setting precedent for the synthesis of compound **15** (Wang et al., 2014; Feng et al., 2016). The strong electronegativity of the trifluoromethoxy had a profound effect on metabolic stability, increasing half-life nearly 8-fold and eliminating P450 inhibition (CYP3A4  $IC_{50}$ :  $>50\ \mu M$ ; CYP2D6  $IC_{50}$ :  $>50\ \mu M$ ).

Three compounds were designed to test the effects of replacing the methoxy group of compound **12** on the observed in vitro clearance. An unsubstituted phenyl derivative resulted in a modest 2.5-fold decrease in  $CL_{int}$ , likely due to the propensity for exposed phenyl rings to be P450 substrates. Chlorinated (compound **17**) and fluorinated (compound **18**) derivatives were synthesized, as substituting aromatic rings with electronegative atoms is known to slow oxidative metabolism (Wang et al., 2014). Crystal structure data indicate that Ser 49 participates in a hydrogen bond with the oxygen of compound **11**, and with evidence suggesting that halogens may act as weak hydrogen bond acceptors, it was anticipated that chlorine and fluorine substitutions would have minimal impact on ligand binding (Lin and MacKerell, 2017).

This had a marked effect on the in vitro clearance, decreasing it  $\sim 4$ -fold for compounds **17** and **18**, respectively. In vitro estimations in mouse liver microsomes correspond with intrinsic clearance calculated in human hepatocytes (compound **17**  $CL_{int,app,scaled}$ :  $\sim 19\ \mu L/min/kg$ ). Compound **19**, the corresponding enantiomer of **17**, was synthesized not only for direct in vivo comparison with compound **11**, but also because crystal structure data suggest this enantiomer predominantly occupies the active site of DHFR. Our conclusions from in vitro experiments translated to improvements in vivo, as half-life, AUC, and CL improved

**TABLE 2**  
In vivo comparison of compound **11** and compound **19**

	Compound <b>11</b> (16 mg/kg)		Compound <b>19</b> (5 mg/kg)	
	i.p.	PO	i.p.	PO
$t_{1/2}$ (min)	19.8	11.4	68.6	272.8
AUC (min $\cdot\mu g/ml$ )	294	24.6	838.1	155.8
$C_{max}$ ( $\mu g/ml$ )	10.16	1.44	4.1	0.4
CL (ml/min/kg)	54.37	—	5.96	—
F (%)	—	8.4	—	18.6

PO, by mouth.

3-, 3-, and 9-fold, respectively, when comparing intraperitoneal data (Table 2).

While an 8-fold loss in potency, as assessed by MIC, was observed for compound **19** compared with compound **11**, the pharmacodynamic profile of compound **19** provides powerful insight into predicting efficacy. Time-kill curve analysis indicates that compound **19** demonstrates concentration-dependent antibacterial activity at concentrations  $>10\times$  MIC. In this case, peak/MIC and AUC/MIC are the important pharmacodynamic descriptors. If given by the intraperitoneal route, the peak/MIC is 26 and the AUC/MIC is over 5000. For context, aminoglycosides, a staple in several antibiotic regimens, demonstrate concentration-dependent antibacterial activity, and a  $>90\%$  rate is associated with a peak/MIC ratio of  $\geq 12$  (Moore et al., 1987). Likewise, fluoroquinolones, another class of powerful concentration-dependent antibiotics, are associated with a 100% microbiological response if they achieve an AUC/MIC ratio  $>33$  (Ambrose et al., 2001). In the case in which plasma levels are  $\leq 10\times$  MIC, time-dependent antibacterial activity and time  $> MIC$  are the pharmacodynamic properties that correlate with efficacy. With the significantly improved half-life, plasma levels are maintained for nearly 10 hours above the MIC, indicating effective concentrations can be maintained.

In this report, we describe the further optimization of propargyl-linked antifolates as potential new antibiotic agents targeting several of the most important pathogenic bacteria. Key to the success of these efforts was the availability of a high-resolution crystal structure of the lead agent in complex with the bacterial target. Analysis of the metabolic profile in the context of this structure allowed us to design and evaluate a small number of new analogs constructed to limit major metabolic liabilities while maintaining strong interactions with the target, this maintaining strong antibacterial activity while extending exposure to the agent.

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## Authorship Contributions

*Participated in research design:* Lombardo, G-Dayananadan, Obach, Priestley, Wright.

*Conducted experiments:* Lombardo, G-Dayananadan, Zhou, Reeve, Alverson, Barney, Walker, Hoody, Priestley.

*Contributed new reagents or analytic tools:* G-Dayananadan, Keshipetty, Si, Obach.



*Performed data analysis:* Lombardo, G-Dayananadan, Obach, Wright.

*Wrote or contributed to the writing of the manuscript:* Lombardo, G-Dayananadan, Wright.

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