Acetylenic Linkers in Lead Compounds: A Study of the Stability of the Propargyl-Linked Antifolates^S

Wangda Zhou, Kishore Viswanathan, Dennis Hill, Amy C. Anderson, and Dennis L. Wright

Department of Pharmaceutical Sciences, University of Connecticut, Storrs, Connecticut

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ABSTRACT:

Propargyl-linked antifolates that target dihydrofolate reductase are potent inhibitors of several species of pathogenic bacteria and fungi. This novel class of antifolates possesses a relatively uncommon acetylenic linker designed to span a narrow passage in the enzyme active site and join two larger functional domains. Because the use of alkyne functionality in drug molecules is limited, it was important to evaluate some key physicochemical properties of these molecules and specifically to assess the overall stability of the acetylene. Herein, we report studies on four compounds from our lead series that vary specifically in the environment of the alkyne. We show that the compounds are soluble, chemically sta-

ble in water, as well as simulated gastric and intestinal fluids with half-lives of approximately 30 min after incubation with mouse liver microsomes. Their primary in vitro route of metabolism involves oxidative transformations of pendant functionality with little direct alteration of the alkyne. Identification of several major metabolites indicated the formation of *N*-oxides; the rate of formation of these oxides was highly influenced by branching substitutions around the propargyl linker. On the basis of the lessons of these metabolic studies, a more advanced inhibitor was designed, synthesized, and shown to have increased ($t_{1/2} = 65$ min) metabolic stability while maintaining potent enzyme inhibition.

Introduction

Over the past several years, we have focused on the development of a novel class of antifolates designed to inhibit dihydrofolate reductase (DHFR) from pathogenic organisms. These compounds are characterized by a conserved diaminopyrimidine moiety linked through a propargylic spacer to a variable hydrophobic domain (see Fig. 1) (Pelphrey et al., 2007; Bolstad et al., 2008; Liu et al., 2008, 2009; Paulsen et al., 2009). Crystal structures of DHFR from *Staphylococcus aureus* (Frey et al., 2009, 2010b; Viswanathan et al., 2012), *Candida glabrata*, *Candida albicans* (Liu et al., 2008, 2009; Paulsen et al., 2011), and *Bacillus anthracis* (Beierlein et al., 2008) reveal that the propargyl linker occupies a narrow space bridging two critical pockets in the enzyme, one of which binds the diaminopyrimidine group and the other that is primarily hydrophobic. Using these crystal structures, several compounds with this generalized scaffold and a biphenyl moiety (example compound 1 in Fig. 1) were designed, synthesized, and shown to be potent leads against a variety of prokaryotic and eukaryotic pathogens. For example, a 3,5-dimethyl derivative of compound 1 inhibits *S. aureus* and *C. glabrata* DHFR with

IC₅₀ values of 42 and 0.5 nM, respectively. The biphenyl-based compounds also inhibit the growth of methicillin-resistant *S. aureus* as well as *C. glabrata* in culture with minimal inhibitory concentration (MIC) values of 5.76 and 3 μg/ml, respectively (Liu et al., 2008; Viswanathan et al., 2012).

Because the lead series incorporating a biphenyl substructure substantially increased the hydrophobicity of the compounds, we designed and synthesized a series of propargyl-linked antifolates with nitrogenous heterocyclic moieties (compounds 2–8) predicted to increase solubility (Viswanathan et al., 2012). Several of these heterocyclic-bearing analogs show superior activity against *S. aureus* DHFR with IC₅₀ values of 19 nM and MIC values of 0.09 μg/ml.

To further investigate the potential of the propargyl-linked antifolates as drug leads, we present here an investigation into the physicochemical properties of key propargyl-linked antifolates. The acetylenic linker in these antifolates is relatively rare in drug molecules. However, the appearance of acetylenic groups in drugs is increasing, given the frequency of use and ease of the Sonogashira coupling reaction (King and Yasuda, 2004) that provides for a facile coupling of terminal alkynes to various aromatic systems. In fact, an ethynyl group is an important feature of ponatinib, a new breakpoint cluster region-Abelson kinase protein kinase inhibitor (O'Hare et al., 2009; Huang et al., 2010), where it reduces bulk near the resistance-conferring T315I mutation. Efavirenz, a nonnucleoside reverse transcriptase inhibitor (Lindberg et al., 2002), uses an alkyne linker to extend through a passage composed of Leu100, Tyr181, and Tyr188. Therefore, an investigation into the stability of compounds with a propar-

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ABBREVIATIONS: DHFR, dihydrofolate reductase; MIC, minimal inhibition concentration; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; ACN, acetonitrile; HPMC, hydroxypropylmethylcellulose; AUC, area under the curve; MLM, mouse liver microsomes; LC-MS/MS, liquid chromatography/tandem mass spectrometry; CID, collision-induced dissociation; THF, tetrahydrofuran.

	IC ₅₀ (nM)	MIC (μg/mL)
1: R ₁ =Me, R _P =Me, R ₂ =H, R ₃ =OMe, Ar=Ph	61	0.72
2: R ₁ =Me, R _P =Me, R ₂ =H, R ₃ =OMe, Ar=4-pyridyl	26	0.09
3: R ₁ =Et, R _P =Me, R ₂ =H, R ₃ =OMe, Ar=4-pyridyl	19	0.09
4: R ₁ =Me, R _P =Me, R ₂ =H, R ₃ =OMe, Ar=morpholino	29	2.94
5: R ₁ =Et, R _P =Me, R ₂ =H, R ₃ =OMe, Ar=morpholino	26	ND
6: R ₁ =Me, R _P =Me, R ₂ =H, R ₃ =OMe, Ar=piperazine	130	91.5
7: R ₁ =Et, R _P =H, R ₂ =H, R ₃ =OMe, Ar=4-pyridyl	12	0.04
8: R ₁ =Et, R _P =H, R ₂ =OMe, R ₃ =H, Ar=4-pyridyl	21	0.02

FIG. 1. Propargyl-linked lead compounds. Top, propargyl-linked antifolates and their IC₅₀ and MIC values against *S. aureus* DHFR and methicillin-resistant *S. aureus*. Bottom, compound 1 bound to *C. glabrata* DHFR (from Protein Data Bank identifier 3EEJ).

glycyl linker would not only be informative for the development of this lead series but may also be applied more broadly.

Materials and Methods

General High-Performance Liquid Chromatography Analysis. A Shimadzu Prominence 20 high-performance liquid chromatography (HPLC) instrument (Shimadzu, Kyoto, Japan) fitted with a Luna 5 μm C18(2) 100 Å column (5 μm, 4.6 × 250 mm; Phenomenex, Torrance, CA) and a UV diode array detection at 254 nm was used to quantify compounds.

Kinetic Solubility Assay. Compounds were initially dissolved as 20 or 40 mM dimethyl sulfoxide (DMSO) solutions and diluted in filtered water in the presence or absence of 200 μg/ml methylcellulose (METHOCEL A4M; Dow Corning, Midland, MI). All samples were centrifuged for 10 min at 15,000 rpm, incubated at room temperature for 30 min, and analyzed by reversed-phase HPLC. The mobile phase consisted of 50% acetonitrile (ACN) and 50% potassium phosphate buffer (50 mM, pH 7.0), using an isocratic flow rate of 1.5 ml/min. Solubility was determined as the maximal concentration for which absorption is linearly related to the log of the concentration (Kerns and Di, 2008).

Solution Stability Assay. Compounds at 5 μg/ml were incubated with water, simulated gastric fluid and simulated intestinal fluid at 37°C for 24 h. All solutions contained 200 μg/ml hydroxypropylmethylcellulose (HPMC) A4M. Samples were collected before and after incubation. Assays were terminated by adding an equal volume of ice-cold ACN and centrifuging at 15,000 rpm for 10 min. One hundred microliters of supernatant was injected on the reversed-phase column of the HPLC; solution stability was calculated using a ratio of area under the curve (AUC) (after incubation)/AUC (before incubation).

Metabolic Stability Assay. Compounds were incubated with mouse liver microsomes (MLM; BD Biosciences, San Jose, CA) in 0.1 M potassium phosphate buffer (pH 7.4) and 200 μg/ml HPMC A4M in the presence of an

NADPH regenerating system at 37°C. The final concentrations of inhibitor, microsomes, NADP⁺, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and MgCl₂ were 5 μg/ml, 0.5 mg/ml, 1.3 mM, 3.3 mM, 0.5 U/ml, and 3.3 mM, respectively. DMSO and compounds without MLM were used as negative controls. The reactions were initiated by the addition of microsomes and quenched by the addition of an equal volume of ice-cold ACN after 60 or 120 min. The samples were subjected to centrifugation at 15,000 rpm for 10 min, and the supernatant phase was purified using Oasis hydrophilic-lipophilic-balanced sample extraction cartridges (Waters, Milford, MA) following the manufacturer's protocol. Methanol (1 ml) was used to elute the sample, which was further diluted 2-fold with water. One hundred microliters of the diluted sample was injected on to the HPLC reversed-phase column. To compare the metabolic stability of different compounds, the percentage of the parent compound remaining after incubation as compared with the amount before incubation was calculated. The in vitro metabolic half-life was calculated using a single time point approach following first-order kinetics as described previously (Kerns and Di, 2008).

To identify metabolites, compounds 2, 3, 7, 8, and 10 (at 10 μg/ml) were incubated with MLM. Negative controls included 1) using the same volume of DMSO or 2) quenching the reaction with ACN immediately after adding microsomes. Both samples and negative controls were incubated for 2 h, and the reaction was terminated with an equal volume of ice-cold ACN. After removal of protein precipitates by centrifugation, samples and negative controls were purified as described in the paragraph above. The concentration of compounds before and after metabolic incubation was calculated by standard curves obtained with each compound. Standard curves were performed multiple times and showed intraday and interday CV of less than 5%.

Liquid Chromatography/Tandem Mass Spectrometry Analysis and Metabolite Identification. An Agilent 1100 HPLC system fitted with a Zorbax-C18 (3 μm, 1.0 × 150 mm) (Agilent Technologies, Santa Clara, CA) was used for separation. Mobile phase A, consisting of 0.01% heptafluorobutyric acid in water, and mobile phase B, consisting of 0.01% heptafluorobutyric acid in ACN, were used for a linear gradient elution as follows: 0 to 100% B in 17 min and isocratic hold at 100% B for 5 min. The flow rate was 75 μl/min, and the injection volume was 8 μl. Mass spectrometric detection was performed on a Micromass Q-ToF-2 mass spectrometer from Waters, equipped with an electrospray ionization source. The mass spectrometer was operated in the positive ionization mode and was calibrated with Glu1-fibrinopeptide B on each experiment day. Capillary voltage and cone voltage were set at 3000 and 20 V, respectively. Source and desolvation temperatures were 100 and 150°C. Nitrogen was used as a desolvation gas at a flow rate of 450 ml/h. Full-scan time-of-flight spectra were first acquired for parent compounds and metabolites in the mass spectrometry mode. Subsequently, collision-induced dissociation (CID) fragmentation spectra were obtained on the isolated protonated molecular ion of parent and metabolites in the tandem mass spectrometry mode. Argon was used as the collision gas, and the collision energy was optimized for each compound in the range of 20 to 40 eV. Accurate mass was calculated using fragments of the coanalyzed calibration standard lisinopril as described previously (Hill et al., 2008). The distribution of metabolites was calculated by dividing the AUC of the individual metabolite by the total AUC for all metabolites within the same injection.

Synthesis of N-Oxides. To a solution of compound 3 (15 mg, 0.04 mmol) in tetrahydrofuran (THF) at 0°C, *m*-chloroperoxybenzoic acid (7 mg, 0.04 mmol) was added in one portion. The reaction mixture was stirred for 30 min, and the reaction was quenched by adding sodium metabisulfite. The reaction mixture was filtered, and potassium carbonate was added in excess to quench the acid. The organic layer was then dried over MgSO₄, filtered, and concentrated. The mixture of *N*-oxides was then separated from starting material by reversed-phase chromatography using amino-capped silica and dichloromethane as an eluent.

Isolation of Pyridine-*N*-Oxide. An Agilent 1200 HPLC system fitted with an Eclipse-C18 (5 μm, 4.6 × 150 mm; Agilent Technologies) column was used for separation. The mobile phase consisted of 60% MeOH and 40% H₂O, using an isocratic flow rate of 1.0 ml/min. The required pyridine-*N*-oxide separated from the mixture with a retention time of 5.01 min.

Enzyme Inhibition. Enzyme activity assays were performed by monitoring the rate of NADPH consumption at 340 nm over 5 min. Reactions were performed with 50 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, and 1 mg/ml bovine serum albumin. Concentrations of cofactor (100 μM NADPH)

TABLE 1

Solubility values for compounds 1 through 6 in 0.02% HPMC and water

Compound Identifier	cLogD _{7.4}	Solubility
		μg/ml
1	3.89	20
2	2.39	40
3	3.44	40
4	1.85	>80
5	2.90	>80
6	1.11	60

and substrate (1 mM dihydrofolate) were used with limiting concentrations of pure enzyme. The assay was performed in triplicate.

Results

Design of the Heterocyclic Propargyl-Linked Antifolates. The crystal structures of *C. glabrata* DHFR and *S. aureus* DHFR bound to biphenyl-propargyl-linked antifolates (Frey et al., 2009, 2010a; Liu et al., 2009; Paulsen et al., 2011) guided the placement of solubility-enhancing functionality. In particular, these structures reveal that although substitutions at the meta and para positions of the aryl ring interact productively with the enzyme, the region is largely solvent exposed (Fig. 1) and could tolerate the substitution of more polar atoms.

A comparison of cLogD values for compounds with a phenyl ring at the distal position (compound 1) with values for compounds that replace the phenyl ring with heterocycles containing endocyclic nitrogen or oxygen (compounds 2–6) (Table 1) predicts that the presence of heteroatoms in the distal ring will lower the hydrophobicity of the compounds. Several analogs, including compounds 2 to 6, were synthesized to evaluate this hypothesis (Viswanathan et al., 2012). Kinetic solubility data for compounds 2 to 6 were determined (Table 1) to experimentally gauge the impact of these substitutions. HPMC (0.02%) was added to the aqueous solutions to prevent precipitates from forming after standing overnight.

All of the heterocyclic substitutions correlated with increased solubility of the compounds. Compounds with a pyridyl ring system such as 2 and 3 are very potent inhibitors of DHFR, specifically from *S. aureus*, with IC₅₀ values of 26 and 19 nM, respectively (Fig. 1) (Viswanathan et al., 2012). In addition to 2 and 3, pyridyl compounds 7 and 8 were also found to be potent inhibitors of *S. aureus* DHFR, with IC₅₀ values of 12 and 21 nM, respectively, and MIC values of 0.04 and 0.02 μg/ml, respectively.

To investigate the chemical and metabolic stability of the propargyl-linked antifolates, compounds 2, 3, 7, and 8 were chosen for analysis. These four compounds have varying substitution patterns at the C6 position of the pyrimidine (R₁ in Fig. 1; 2 versus 3, 7–8), the propargylic position (R_p in Fig. 1; 2, 3 versus 7, 8), and the 2' and 3' positions (R_{2'} and R_{3'} in Fig. 1; 2–3, 7 versus 8) that provide different chemical environments for the alkyne.

Compounds 2, 3, 7, and 8 were first examined for chemical stability after 24 h of incubation at 37°C in simulated intestinal fluid (pH 7.4),

simulated gastric fluid (pH 1.2), and water. HPLC analysis reveals that there is no change in AUC or peak shape after 24 h in any of the fluids, suggesting that the compounds are stable under all tested conditions.

All four compounds were evaluated for phase I metabolic stability by incubating with MLM in the presence of an NADPH-regenerating system for 1 h. The percentage of compound remaining after incubation relative to the initial concentration was calculated (Table 2). Compounds 2 and 3 that differ in substitution at C6 both show moderate and similar stability, with values of 33.7 and 29.2% remaining, respectively, after 1 h. However, substituting the methyl group at the propargyl position with a hydrogen (compound 7) significantly reduces the metabolic stability whereby only a trace of the parent compound remains after 1 h. Compound 8, with a hydrogen at the propargylic position and a methoxy group at the 2' position instead of the 3' position, exhibits metabolic stability similar to that of compounds 2 and 3. Taken together, it is clear that the metabolic stability of these compounds is highly sensitive to substitution at propargylic position as well as the 2' and 3' positions on the adjacent phenyl ring.

The metabolites of compounds 2, 3, 7 and 8 were identified and characterized using LC-MS/MS. Analysis of the crude microsomal incubation of compounds 2, 3, 7, and 8 showed the presence of metabolic products at masses corresponding to M-14, M + 16, and M + 32 (where M is monoisotopic molecular weight). In all four metabolites, the M-14 product was easily assigned as the result of oxidative O-demethylation. Assignment of the M + 16 products was more complicated because a variety of potential oxidation sites exist in these molecules including unsubstituted aromatic systems, the activated propargylic position, C6 benzylic positions, the acetylene linker, and the endocyclic basic nitrogens of the pyrimidine and pyridine systems. Using both analysis of the mass spectrum and chemical synthesis, attempts were made to assign the structures of the major products of mono-oxidation.

One of the more interesting features of the metabolic studies was the greatly reduced half-life for compound 7 relative to compound 3 that differs only by branching at the activated propargylic position. It appeared likely that a facile oxidation of the unsubstituted propargylic position in 7 to the secondary alcohol would occur more rapidly than the corresponding oxidation of 3 to the tertiary alcohol and would rationalize the difference in half-lives for these compounds. Synthesis of an authentic sample of the racemic alcohol was straightforward (Fig. 2) and afforded the necessary compound (i.e., 9) for comparison. However, compound 9 did not match any of the mono-oxidation products observed in the microsomal incubation, thus indicating that the direct oxidation of the propargylic position does not appear to be a primary route of metabolism for these compounds.

Metabolites produced from compound 8 proved especially useful in assigning sites of transformation (Table 3). Three principle metabolites arise from compound 8, one of which is the product of O-demethylation (M8-2, 19.7%), two arising from mono-oxidation [74.2 (M8-1) and 6.3% (M8-3)] and a minor amount of a product arising

TABLE 2

Half-life of compounds and distribution of metabolites

Compound	Percentage Remaining after 1 h	Half-Life	Pyridyl N-Oxidation (+16)	Demethylation (−14)	Oxidation on Pyrimidinyl Substructure (+16)	Bis-Oxidation (+32)
		min				%
2	33.7	38	59.9% (M2-1)	15.9% ^a (M2-2)	13.8% (M2-3)	10.3
3	29.2	34	45.8% (M3-1)	28.0% (M3-2)	25.8% (M3-3) <5% (M3-4)	10.1
7	0	<10	70.5% (M7-1)	6.3% (M7-2)	5.3% (M7-3)	15.1
8	26.5	31	74.2% (M8-1)	19.7% (M8-2)	6.3% (M8-3)	1.7

^a Percentages based on total AUC.

FIG. 2. Synthesis of compound 9. a, Pd(PPh₃)₂Cl₂, pyridine-4-boronic acid, Cs₂CO₃, dioxane, 80°C, 85%. b, ethynyl-MgBr, THF, 0°C, 88%. c, Pd(PPh₃)₂Cl₂, CuI, Et₃N, dimethylformamide, 70%.

from two consecutive oxidations (1.7%). The CID spectrum of the parent compound 8 showed that major fragments were formed through cleavage of either the a–b bond or the b–c bond, producing two key ions F1 and F2, respectively (Fig. 3). The ion F1 at m/z = 198.1 was assigned as a benzylic cation, whereas ion F2 at m/z = 175.1 was assigned as the propargylic cation. The appearance of either the m/z = 198.1 fragment or the m/z = 175.1 fragment in the CID spectrum of metabolite M8-1 or M8-3 was used to assign the site of oxidation to either the F1 or the F2 domains of the inhibitor. The major metabolite M8-1 shows the presence of the pyrimidinyl F2 fragment, thus indicating that oxidation had occurred on the 4-phenylpyridyl subunit (Fig. 4). The spectrum of the minor metabolite M8-3 showed a strong fragment at m/z = 198.1, signifying that the biaryl domain was unchanged in the metabolite and that oxidation had occurred on the pyrimidine heterocycle. Although this mode of fragmentation was observed for the parent compounds (2, 3, or 7), these diagnostic ions did not appear in the CID spectra of the metabolites.

For oxidation occurring on both the pyrimidine or 4-phenylpyridine substructures of the molecule, both C- and N-oxidation products are possible, leading to pyrimidine-*N*-oxides [as observed with trimethoprim (Sigel et al., 1973)], pyridine-*N*-oxides, as well as various phenols and alcohols. A reported analysis of pyridine-*N*-oxides derived from the metabolic transformation of desloratadine (Ramathan, 2011) showed that two diagnostic fragments were produced by a loss of 17 and 18 mass units from the parent ion. Because similar diagnostic peaks were evident in the CID spectra of M3-1 and M3-4 (see supplemental data) and are clearly not related by isotopic effects (estimated to be 22.8%), as assessed by integration of the two peaks, the formation of *N*-oxides as a major metabolic pathway seemed probable. In comparison, the spectra of M3-3 show two peaks with loss of 17 and 18 mass units, but because the integration is close to the expected isotopic effect (23%), the presence of an *N*-oxide cannot be unambiguously assigned. Moreover, the peaks associated with the *N*-oxides M3-1 and M3-4 eluted with retention time longer than that of the parent, whereas the demethylation product

M3-2 and the unassigned oxidation product M3-3 eluted with retention times shorter than those of the parent. This difference in retention time is likely due to the loss of one of the basic nitrogens, an effect that would be expected to be more pronounced at the low pH of the mobile phase.

To further validate the identity of these types of metabolites, we attempted to prepare authentic standards of the *N*-oxidation products through chemical oxidation. It was found that controlled oxidation of 3 with *m*-chloroperoxybenzoic acid at low temperature generated a mixture of three unique *N*-oxides that were assigned by nuclear magnetic resonance analysis as the two regioisomeric pyrimidine-*N*-oxides and the pyridine-*N*-oxide. Assignment of the pyridine-*N*-oxide was straightforward because of a pronounced change in the chemical shifts of the protons adjacent to the endocyclic nitrogen from 8.65 to 8.25 ppm (Fig. 5). In the pyrimidine-*N*-oxides, no significant analogous shift was observed. Careful separation of the pyridine-*N*-oxide from the two pyrimidine-*N*-oxides was possible using HPLC; LC-MS/MS analysis of the mixture of *N*-oxides indicated that two of the three chemical oxidation products exactly matched two of the metabolites formed during microsomal incubation. This analysis allowed us to unambiguously assign the structure of M3-1 as the pyridine-*N*-oxide using both retention time and CID spectra (Fig. 4). In addition, one of the two isomeric pyrimidine *N*-oxides was also matched to the minor metabolite M3-4 (less than 5%). The remaining mono-oxidation product (M3-3; 25.8%) has not been unambiguously assigned, although the fragmentation pattern clearly indicates that oxidation had occurred on the fragment containing the pyrimidine and is likely the product of benzylic oxidation at C6 of the pyrimidine. The chemical validation experiments provided a valuable correlation to the assignments based on tandem mass spectrometry showing that M3-1 and M3-4 possessed the diagnostic fragments with loss of 17 and 18 mass units, respectively.

Using retention time and fragmentation patterns, the remaining metabolites from compounds 2, 7, and 8 were assigned on the basis of analogy to the studies with compound 3. Specifically, the primary

TABLE 3
Predicted mass, diagnostic ions and assignment of metabolites for compounds 3, 8, and 10

Compound Identifier	Predicted M + H	Diagnostic Daughter Ions	Notes
3	374.1976	175.1; 212.1;	Parent
M3-1 ^b	390.1925	373.1888;	Pyridine- <i>N</i> -oxide ^a
M3-2	360.1819	175.1; 198.1;	Demethylation
M3-3 ^b	390.1925	213.1141; 372.1836;	Alternative oxidation on pyrimidinyl substructure
M3-4 ^b	390.1925	373.1914;	Pyrimidinyl <i>N</i> -oxide ^a
8	360.1819	175.1; 198.1;	Parent
M8-1	376.1768	175.1; 214.1;	Pyridine- <i>N</i> -oxide
M8-2	346.1663	175.1; 184.1;	Demethylation
M8-3	376.1768	198.1;	Alternative oxidation on pyrimidinyl substructure
10 ^b	374.1976	175.1008; 197.0862;	Parent
M10-1 ^b	390.1925	175.0974 228.1016; 373.1907;	Pyridine- <i>N</i> -oxide ^a
M10-2 ^b	360.1819	175.1021; 184.0792;	Demethylation
M10-3 ^b	390.1925	174.0863; 213.1113; 372.1768;	Alternative oxidation on pyrimidinyl substructure
M10-4 ^b	390.1925	175.0955; 228.1010; 373.1862;	Pyrimidinyl <i>N</i> -oxide ^a
M10-5 ^b	390.1925	212.1076; 372.1859;	Alternative oxidation on pyrimidinyl substructure

^a Structures confirmed by synthesized standards.

^b Analyte masses were accurate to 10 ppm.

FIG. 3. Diagnostic fragments derived from ionization of compound 8.

metabolite for all three compounds was assigned as the pyridine-*N*-oxide because it showed a longer retention time than and fragmentation pattern similar to that of M3-1. For all three compounds, the percentage of pyrimidine-*N*-oxide appeared to be minimal. Metabolites M2-3, M7-3, and M8-3 are likely to be products of oxidation on the pyrimidine substructure, such as C6 side-chain oxidation or hydroxylamine formation.

Half-life and product distribution analysis of compounds 2, 3, 7, and 8 (Table 2) suggested that remote substituents both at the propargylic position and the aromatic phenyl ring could influence metabolism at more distal sites in the molecule. For example, comparison of the metabolic profiles of compounds 3 and 7 shows that incorporation of branching at the propargyl position (3) increases the half-life and decreases the relative abundance of the pyridyl-*N*-oxide while increasing the degree of O-demethylation. Likewise, as noted previously, comparison of compounds 7 and 8 suggests that the placement of a 2'-methoxy substituent increases the half-life, although product distribution is largely unaffected. These effects suggest that substitution near

the acetylene bridge, at either the propargylic or the C2' position, slows the rate of metabolism at the distal pyridine nitrogen likely by altering the presentation of the nitrogen to the cytochrome active site. This hypothesis is supported by the observation of significant conformational differences of compounds with varying substitutions at the propargylic and 2'-phenyl positions in crystal structures with the target DHFR enzyme (Viswanathan et al., 2012).

It was anticipated that incorporation of both of these design elements may be useful in extending the overall half-life of the lead series assuming that key biological activity could be retained. A hybrid inhibitor, compound 10, was predicted to bind the active site of DHFR because a model of its interactions with *S. aureus* DHFR shows conservation of the hydrogen bonds between the pyrimidine and active site residues as well as hydrophobic interactions between the C6 ethyl and propargyl group with Val31, Leu28, Leu54, Ile51, and Phe92. The 2' methoxy group may form van der Waals interactions with Met42 or Thr46. Compound 10 was synthesized according to the method described below (Fig. 6) to test these hypotheses.

FIG. 4. Major metabolites derived from compounds 2, 3, 7, 8, and 10.

FIG. 5. Proton nuclear magnetic resonance spectra of compound 3 and the associated pyridine-*N*-oxide. Note the pronounced shift in the pyridyl protons (circled) upon *N*-oxide formation.

Incubation of compound 10 with MLM for 1 h shows that the half-life doubles ($t_{1/2} = 65$ min) relative to the initial lead series. LC-MS/MS analysis of the crude reaction mixture revealed that five major metabolites were formed during microsomal incubation (Fig. 4; Table 3). One of these metabolites M10-2 (20.3%) was again easily determined to be the product of O-demethylation, whereas the mass spectrum indicated that the other four products (M10-1, M10-2,

M10-3, and M10-5) resulted from oxidation. Once again, the presence of a 2'-methoxy donor promoted fragmentation along the central propargylic linker to generate diagnostic fragments that were useful in assigning sites of oxidation to the pyrimidinyl or 4-phenylpyridinyl portions of the molecule. Likewise, chemical oxidation of compound 10 was instrumental in assigning N-oxidation products. On the basis of the analysis, the pyridine *N*-oxide remained the predominant me-

FIG. 6. Synthesis of compound 10. a, MeI, K_2CO_3 , dimethylformamide. b, MeMgBr, THF. c, MnO_2 , CH_2Cl_2 , 75% over three steps. d, $Pd(PPh_3)_2Cl_2$, CS_2CO_3 , dioxane, 80°C, 85%. e, $Ph_3P = CHOMe$, THF. f, $Hg(OAc)_2$, KI, THF/ H_2O , 74% over two steps. g, dimethyl(1-diazo-2-oxopropyl) phosphonate, K_2CO_3 , MeOH, 80%. h, $Pd(PPh_3)_2Cl_2$, ethyl pyrimidine, CuI, Et_3N , dimethylformamide, 76%.

tabolite. The chemically matched pyridine-*N*-oxide was present at 54.3%, whereas a matched pyrimidine-*N*-oxide was present at 7.2%. There were two additional mono-oxidation products localized to the pyrimidinyl fragment (8.7 and 9.4%) that have been tentatively assigned to the C6 position of the pyrimidine.

Structural analysis of the DHFR protein bound to compound 3 clearly shows that the pyridyl nitrogen does not directly contact the enzyme and in fact is extended toward the solvent interface, suggesting that the *N*-oxide product may still retain enzyme inhibition activity. A sample from the chemical oxidation of compound 10 was purified by HPLC, and the weight was determined by liquid chromatography/mass spectrometry using a known quantity of compound 10 as a standard. To evaluate residual activity in the primary metabolite, the purified sample was included in enzyme inhibition assays with *C. glabrata* DHFR. We were excited to find that *C. glabrata* DHFR inhibition assays with the purified pyridine-*N*-oxide M10-1 show that the compound retains potency ($IC_{50} = 45$ nM), suggesting the formation of an active metabolite.

Discussion

As we have improved the potency of the propargyl-linked antifolates against several bacterial and fungal species, it has become increasingly important to consider their properties as drug leads. In this study, we show that compounds with heterocyclic functionality intended to bind the hydrophobic pocket have favorable solubility for in vivo studies and are more soluble than the previous biphenyl-based leads. Three propargyl-linked antifolates with pyridyl ring systems were found to be chemically stable in water as well as simulated intestinal and gastric fluids. Four promising propargyl-linked antifolates that differ in the chemical environment of the alkyne were evaluated for metabolic stability. Fragmentation analysis and the use of chemically matched compounds provide confirmation that the primary route of metabolism involves the formation of *N*-oxide products and that the propargyl linker appears to be stable. Results show that branching at the propargylic position and substitution of the aromatic ring affect metabolic stability; compounds that possess a methyl at the propargylic position show longer half-lives compared with otherwise similar compounds with a hydrogen at this position. On the basis of these remote effects, a new active inhibitor was designed and shows improved half-life.

This new class of antifolates uses a somewhat uncommon acetylenic linker to bridge the pyrimidine head group and hydrophobic biaryl domains. The unique geometry and steric environment of the alkyne are central to the efficacy of the inhibitors and are not easily mimicked by other functionality. As such, determining the stability of this unsaturated unit is critical for further development of these compounds. These studies indicate that the linkage is chemically stable and is not a primary site of metabolic transformation.

Authorship Contributions

Participated in research design: Zhou, Viswanathan, Anderson, and Wright.

Conducted experiments: Zhou, Viswanathan, and Hill.

Performed data analysis: Zhou, Viswanathan, Anderson, and Wright.

Wrote or contributed to the writing of the manuscript: Zhou, Viswanathan, Anderson, and Wright.

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Address correspondence to: Dennis L. Wright, Department of Pharmaceutical Sciences, 69 N. Eagleville Rd., University of Connecticut, Storrs, CT 06269. E-mail: dennis.wright@uconn.edu