Acetylenic linkers in lead compounds: A study of the stability of the propargyl-linked antifolates

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Running title: Stability of the propargyl-linked antifolates

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Abbreviations: dihydrofolate reductase (DHFR); inhibition concentration 50% (IC_{50}); minimum inhibition concentration (MIC); high pressure liquid chromatography (HPLC); dimethylsulfoxide (DMSO); acetonitrile (ACN); area under the curve (AUC); mouse liver microsomes (MLM); nicotinamide adenine dinucleotide phosphate (NADPH); liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS); collision induced dissociation (CID); hydroxypropyl methylcellulose (HPMC); m-chloroperoxybenzoic acid (mCPBA).
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. As 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 stable in water as well as simulated gastric and intestinal fluids with half-lives of approximately 30 minutes 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. Based on the lessons of these metabolic studies, a more advanced inhibitor was designed, synthesized and shown to have increased (t½= 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 Figure 1) (Pelphrey et al., 2007; Bolstad et al., 2008; Liu et al., 2008; Liu et al., 2009; Paulsen et al., 2009). Crystal structures of DHFR from *Staphylococcus aureus* (Frey et al., 2009; Frey et al., 2010b; Viswanathan et al., 2012), *Candida glabrata* and *Candida albicans* (Liu et al., 2008; Liu et al., 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 Figure 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 50 % inhibition concentrations (IC$_{50}$) of 42 nM and 0.5 nM, respectively. The biphenyl-based compounds also inhibit the growth of MRSA and *C. glabrata* in culture with MIC values of 5.76 and 3 μg/mL, respectively (Liu et al., 2008; Viswanathan et al., 2012).

As 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 IC$_{50}$ values of 19 nM and MIC values of 0.09 μg/mL.

In order 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. Of interest, 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, 1004) 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 Bcr-Abl kinase inhibitor (O'Hare et al., 2009; Huang et al., 2012), where it reduces bulk near the resistance-conferring T315I mutation. Efavirenz, a non-nucleoside reverse transcriptase inhibitor (Lindberg et al., 2002) uses an alkyne linker to extend through a passage comprised of Leu 100, Tyr 181 and Tyr 188. Therefore, an investigation into the stability of compounds with a propargylic linker would not only be informative for the development of this lead series, but may also be applied more broadly.
Materials and Methods

General HPLC Analysis. A Shimadzu Prominence 20 HPLC instrument fitted with a Luna 5µm C18 (2) 100 Å column (5 µM, 4.6 mm x 250 mm, Phenomenex) and a UV diode array detection at 254 nm was used to quantify compounds.

Kinetic Solubility Assay. Compounds were initially dissolved as 20 mM or 40 mM DMSO solutions and diluted in filtered water in the presence or absence of 200 µg/mL METHOCEL A4M (Dow Chemistry, Midland, Michigan). All samples were centrifuged for 10 minutes at 15,000 RPM, incubated at room temperature for 30 minutes, and analyzed by reverse phase HPLC. The mobile phase consisted of 50% acetonitrile and 50% 50 mM potassium phosphate buffer (pH 7.0), using an isocratic flow rate of 1.5 mL/min. Solubility was determined as the maximum 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 (SGF) and simulated intestinal fluid (SIF) at 37°C for 24 hrs. All solutions contain 200 µg/mL HPMC A4M. Samples were collected before and after incubation. Assays were terminated by adding an equal volume of ice-cold acetonitrile (ACN) and centrifuging at 15,000 rpm for 10 min. One hundred µL supernatant was injected on the reverse phase column of the HPLC; solution stability was calculated using a ratio of AUC (after incubation)/ AUC (before incubation).

Metabolic Stability Assay. Compounds were incubated with mouse liver microsomes (MLMs; BD Biosciences) 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 concentration 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 MLMs 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 minutes. The samples were subjected to centrifugation at 15,000 rpm for 10 min and the supernatant phase was purified using OASIS™ HLB sample extraction cartridges (Waters) following the manufacturer’s protocol. Methanol (1 mL) was used to elute the sample, which was further diluted 2-fold with water. One hundred µL 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 to 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 (Kerns and Di, 2008).

To identify metabolites, compounds 2, 3, 7, 8 and 10 (at 10µg/mL) were incubated with MLMs. Negative controls included 1) the use of the same volume of DMSO or 2) quenching the reaction with ACN immediately after adding microsomes. Both samples and negative controls were incubated for 2h, 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 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 intra- and inter-day coefficients of variation of less than 5 %.

**LC-MS/MS Analysis and Metabolite Identification.** An Agilent 1100 HPLC system fitted with a Zorbax-C18 (3 µm, 1.0 mm x 150 mm) (Agilent Technologies, Santa Clara, CA) was used for separation. Mobile phase A, consisting of 0.01% heptafluorobutyric acid (HFBA) in water, and mobile phase B, consisting of 0.01% HFBA in ACN, were used for a linear gradient elution as
follows: 0 to 100% B in 17 min, 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 Qtof-2 mass spectrometer from Waters Corporation (Milford, MA), equipped with an electrospray ionization source. The mass spectrometer was operated in the positive ionization mode and was calibrated with Glu-1-Fibrinopeptide B on each experiment day. Capillary voltage and cone voltage was set at 3000 V 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/hr. Full scan Tof spectra were first acquired for parent compounds and metabolites in the MS mode. Subsequently, CID fragmentation spectra were obtained on the isolated protonated molecular ion of parent and metabolites in the MS/MS mod. 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 co-analyzed calibration standard Lisinopril as described (Hill et al., 2008).

The distribution of metabolites was calculated by dividing the area under the curve (AUC) of the individual metabolite by the total AUC for all metabolites within the same injection.

**Synthesis of N-oxides**

To a solution of 3 (15mg, 0.04 mmol) in THF at 0°C, m-chloroperoxybenzoic acid (7 mg, 0.04mmol) 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 reverse 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 mm x 150 mm) column was used for separation. The mobile phase consisted of 60% MeOH and 40% H\(_2\)O, 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) 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 \textit{C. glabrata} DHFR and \textit{S. aureus} DHFR bound to biphenyl-propargyl-linked antifolates (Frey et al., 2009; Liu et al., 2009; Frey et al., 2010a; Paulsen et al., 2011) guided the placement of solubility enhancing functionality. In particular, these structures reveal that while substitutions at the meta and para positions of the aryl ring interact productively with the enzyme, the region is largely solvent exposed (Figure 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 (1) with values for compounds that replace the phenyl ring with heterocycles containing endocyclic nitrogen or oxygen (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 2-6 were synthesized to evaluate this hypothesis (Viswanathan et al., 2012). Kinetic solubility data for compounds 2-6 were determined (Table 1) to experimentally gauge the impact of these substitutions. Hydroxypropyl methylcellulose (HPMC; 0.02\%) was added to the aqueous solutions in order 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 \textit{S. aureus}, with IC$_{50}$ values of 26 and 19 nM, respectively (Figure 1; Viswanathan et al., 2012). In addition to 2 and 3, pyridyl compounds 7 and 8 were also found to be potent inhibitors of \textit{S. aureus} DHFR with IC$_{50}$ values of 12 and 21 nM, respectively and MIC values of 0.04 and 0.02 µg/mL, respectively.
In order 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 Figure 1; 2 vs 3, 7-8), the propargylic position (Rₚ in Figure 1; 2, 3 vs. 7, 8) and the 2' and 3' positions (R₂ and R₃ in Figure 1; 2-3, 7 vs 8) that provide different chemical environments for the alkyne.

Compounds 2-3, 7-8 were first examined for chemical stability after 24 hours 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 mouse liver microsomes in the presence of an NADPH regenerating system for one hour. 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. Surprisingly, 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. Collectively, 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, 7-8 were identified and characterized using LC-MS/MS. Analysis of the crude microsomal incubation of compounds 2-3, 7-8 showed the presence of metabolic products at masses corresponding to M-14, M+16 and M+32 (where M-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 as 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 (Figure 2) and afforded the necessary compound (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 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 b-c bond, producing two key ions F1 and F2, respectively (Figure 3). The ion F1 at m/z=198.1 was assigned as a benzylic cation while 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 metabolites M8-1 or M8-3 was used to assign the site of oxidation to either the F1 or 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 (Figure 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 (Ramanathan, 2011) showed that two diagnostic fragments were produced by a loss of 17 and 18 mass units from the parent ion. As similar diagnostic peaks were evident in the CID spectra of M3-1 and M3-4 (Supplementary 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 shows two peaks with loss of 17 and 18 mass units, but as 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 longer retention time than the parent whereas the demethylation product M3-2 and the unassigned oxidation product M3-3 eluted with retention times shorter than 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 (mCPBA) at low temperature generated a mixture of three unique N-oxides that were assigned by NMR 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 ppm to 8.25 ppm (Figure 5). In the pyrimidine-N-oxides no significant analogous shift is 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 (Figure 4). Additionally, 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 MS/MS showing that M3-1 and M3-4 possessed the diagnostic fragments with loss of 17 and 18 mass units.

Using retention time and fragmentation patterns, the remaining metabolites from compounds 2, 7 and 8 were assigned based on analogy to the studies with compound 3. Specifically, the primary metabolite for all three compounds was assigned as the pyridine-\(N\)-oxide as it showed a longer retention time and similar fragmentation pattern as 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, 7-8 (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 previously noted, 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, either at the propargylic or C2’-positions, slow 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 since a model of its interactions with S. aureus DHFR show 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 Val 31, Leu 28, Leu 54, Ile 51 and Phe 92. The 2’ methoxy group may form van der Waals interactions with Met 42 or Thr 46. Compound 10 was synthesized according to the method shown below (Figure 6) to test these hypotheses.

Incubation of compound 10 with mouse liver microsomes for one hour shows that the half-life doubles (t ½ equal to 65 minutes) 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 (Table 3 and Figure 4). One of these metabolites M10-2 (20.3 %) was again easily determined to be the product of O-demethylation while the mass spectrum indicated that the other four products (M10-1 and M10-3-5) 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. Based on the analysis, the pyridine N-oxide remained the predominant metabolite. The chemically matched pyridine-N-oxide was present at 54.3 % while 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 10 was purified by HPLC and the weight was determined by LC-MS 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. Excitingly, C. glabrata DHFR inhibition assays with the purified pyridine-N-oxide M10-1 show that the compound retains potency (IC50= 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 to otherwise similar compounds with a hydrogen at this position. Based on these remote effects, a new active inhibitor was designed and shows improved half-life.

This new class of antifolates utilizes a somewhat uncommon acetylenic linker to bridge the pyrimidine head group and hydrophobic biaryl domains. The unique geometry and steric environment of the alkyne is central to the efficacy of the inhibitors and is 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, Wright

Conducted experiments: Zhou, Viswanathan, Hill

Performed data analysis: Zhou, Viswanathan, Anderson, Wright

Wrote or contributed to the writing of manuscript: Zhou, Viswanathan, Anderson, Wright
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Figure Legends

Figure 1. Propargyl-linked lead compounds. Panel a) propargyl-linked antifolates and their IC\textsubscript{50} and MIC values against \textit{S. aureus} DHFR and MRSA; b) compound 1 bound to \textit{Candida glabrata} DHFR (from PDB ID: 3EEJ).

Figure 2. Synthesis of compound 9. (a) Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2}, pyridine-4-boronic acid, Cs\textsubscript{2}CO\textsubscript{3}, dioxane, 80°C, 85%; (b) EthynylMgBr, THF, 0°C, 88%; (c) Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2}, Cul, Et\textsubscript{3}N, DMF, 70%.

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

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

Figure 5. Proton NMR spectra of compound 3 and the associated pyridine-N-oxide. Note the pronounced shift in the pyridyl protons (circled) upon N-oxide formation.

Figure 6. Synthesis of compound 10. a) MeI, K\textsubscript{2}CO\textsubscript{3}, DMF; (b) MeMgBr, THF; (c) MnO\textsubscript{2}, CH\textsubscript{2}Cl\textsubscript{2}, 75% over 3 steps; (d) Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2}, Cs\textsubscript{2}CO\textsubscript{3}, dioxane, 80°C, 85%; (e) Ph\textsubscript{3}P=CHOMe, THF; (f) Hg(OAc)\textsubscript{2}, KI, THF/H\textsubscript{2}O, 74% over 2 steps; (g) dimethyl(1-diazo-2 oxopropyl)phosphonate, K\textsubscript{2}CO\textsubscript{3}, MeOH, 80%; (h) Pd(PPh\textsubscript{3})\textsubscript{2}Cl\textsubscript{2}, Ethyl Pyrimidine, Cul, Et\textsubscript{3}N, DMF, 76%.
### Tables

**Table 1.** Solubility values for compounds 1-6 in 0.02% HPMC and water

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<th>Compound ID</th>
<th>cLogD&lt;sub&gt;7.4&lt;/sub&gt;</th>
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### Table 2. Half-life of compounds and distribution of metabolites

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<th>Cmpd</th>
<th>Percent Remaining After 1h</th>
<th>Half-life (min)</th>
<th>Pyridyl N-oxidation (+16)</th>
<th>Demethylation (-14)</th>
<th>Oxidation on pyrimidinyl substructure (+16)</th>
<th>Bis-oxidation (+32)</th>
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</table>

*a Percentages based on total AUC*
Table 3. Predicted mass, diagnostic ions and assignment of metabolites for compounds 3, 8 and 10

<table>
<thead>
<tr>
<th>Cmpd ID</th>
<th>Predicted M + H</th>
<th>Diagnostic Daughter Ions</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>374.1976</td>
<td>175.1; 212.1;</td>
<td>Parent</td>
</tr>
<tr>
<td>&quot;M3-1&quot;</td>
<td>390.1925</td>
<td>373.1888;</td>
<td>&quot;Pyridine N-oxide&quot;</td>
</tr>
<tr>
<td>M3-2</td>
<td>360.1819</td>
<td>175.1; 198.1;</td>
<td>Demethylation</td>
</tr>
<tr>
<td>&quot;M3-3&quot;</td>
<td>390.1925</td>
<td>213.1141; 372.1836;</td>
<td>Alternative oxidation on pyrimidinyl substructure</td>
</tr>
<tr>
<td>&quot;M3-4&quot;</td>
<td>390.1925</td>
<td>373.1914;</td>
<td>&quot;Pyrimidinyl N-oxide&quot;</td>
</tr>
<tr>
<td>8</td>
<td>360.1819</td>
<td>175.1; 198.1;</td>
<td>Parent</td>
</tr>
<tr>
<td>M8-1</td>
<td>376.1768</td>
<td>175.1; 214.1;</td>
<td>&quot;Pyridine N-oxide&quot;</td>
</tr>
<tr>
<td>M8-2</td>
<td>346.1663</td>
<td>175.1; 184.1;</td>
<td>Demethylation</td>
</tr>
<tr>
<td>M8-3</td>
<td>376.1768</td>
<td>198.1;</td>
<td>Alternative oxidation on pyrimidinyl substructure</td>
</tr>
<tr>
<td>&quot;10&quot;</td>
<td>374.1976</td>
<td>175.1008; 197.0862;</td>
<td>Parent</td>
</tr>
<tr>
<td>&quot;M10-1&quot;</td>
<td>390.1925</td>
<td>175.0974; 228.1016; 373.1907;</td>
<td>&quot;Pyridine N-oxide&quot;</td>
</tr>
<tr>
<td>&quot;M10-2&quot;</td>
<td>360.1819</td>
<td>175.1021;</td>
<td>Demethylation</td>
</tr>
<tr>
<td></td>
<td>Masses</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>----------------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>M10-3</strong></td>
<td>390.1925, 174.0863, 213.1113, 372.1768</td>
<td>Alternative oxidation on pyrimidinyl substructure</td>
<td></td>
</tr>
<tr>
<td><strong>M10-4</strong></td>
<td>390.1925, 175.0955, 228.1010, 373.1862</td>
<td>*Pyrimidinyl N-oxide</td>
<td></td>
</tr>
<tr>
<td><strong>M10-5</strong></td>
<td>390.1925, 212.1076, 372.1859</td>
<td>Alternative oxidation on pyrimidinyl substructure</td>
<td></td>
</tr>
</tbody>
</table>

*Structures confirmed by synthesized standards.

# Analyte masses were accurate to 10ppm.
<table>
<thead>
<tr>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: R₁=Me, R₂=Me, R₃=H, R₄=OMe, Ar=Ph</td>
<td>61</td>
<td>0.72</td>
</tr>
<tr>
<td>2: R₁=Me, R₂=Me, R₃=H, R₄=OMe, Ar=4-pyridyl</td>
<td>26</td>
<td>0.09</td>
</tr>
<tr>
<td>3: R₁=Et, R₂=Me, R₃=H, R₄=OMe, Ar=4-pyridyl</td>
<td>19</td>
<td>0.09</td>
</tr>
<tr>
<td>4: R₁=Me, R₂=Me, R₃=H, R₄=OMe, Ar=morpholino</td>
<td>29</td>
<td>2.94</td>
</tr>
<tr>
<td>5: R₁=Et, R₂=Me, R₃=H, R₄=OMe, Ar=morpholino</td>
<td>26</td>
<td>ND</td>
</tr>
<tr>
<td>6: R₁=Me, R₂=Me, R₃=H, R₄=OMe, Ar=piperazine</td>
<td>130</td>
<td>91.5</td>
</tr>
<tr>
<td>7: R₁=Et, R₂=H, R₃=H, R₄=OMe, Ar=4-pyridyl</td>
<td>12</td>
<td>0.04</td>
</tr>
<tr>
<td>8: R₁=Et, R₂=H, R₃=OMe, R₄=H, Ar=4-pyridyl</td>
<td>21</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Figure 1
Figure 2
Figure 3

Molecular Weight: 359.2

Collision-Induced Dissociation

Molecular Weight: 198.1

Molecular Weight: 175.1
Figure 4
Figure 6