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Design, Synthesis, and Study of a Mycobactin−Artemisinin Conjugate That Has Selective and Potent Activity against Tuberculosis and Malaria

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Supporting Information

ABSTRACT: Although the antimalarial agent artemisinin itself is not active against tuberculosis, conjugation to a mycobacterial-specific siderophore (microbial iron chelator) analogue induces significant and selective antituberculosis activity, including activity against multi- and extensively drug-resistant strains of *Mycobacterium tuberculosis*. The conjugate also retains potent antimalarial activity. Physicochemical and whole-cell studies indicated that ferric-to-ferrous reduction of the iron complex of the conjugate initiates the expected bactericidal Fenton-type radical chemistry on the artemisinin component. Thus, this "Trojan horse" approach demonstrates that new pathogen-selective therapeutic agents in which the iron component of the delivery vehicle also participates in triggering the antibiotic activity can be generated. The result is that one appropriate conjugate has potent and selective activity against two of the most deadly diseases in the world.

Tuberculosis (TB) and malaria are the two most widespread and lethal infectious diseases in the world.[1] Approximately one-third of the world's population is presently infected with *Mycobacterium tuberculosis* (*Mtb*), the causative pathogen of TB, and 40% is affected by malaria. Each of these diseases causes about 2 million deaths worldwide every year.[2] The development and spread of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mtb* have stimulated research efforts globally.[3] As a consequence, the pipeline of potential new drugs has expanded,[4−6] but no effective new anti-TB drug has been marketed in decades.[7] *Plasmodium falciparum* parasites that cause malaria also have become resistant to standard antimalarial drugs such as quinine and chloroquine.[8] Current chemotherapeutic treatments of both malaria and tuberculosis are inadequate. New leads for drug development are urgently needed. Herein we report that an artemisinin conjugate of a mycobactin T analogue (**3**; Figure 1) not only retains antimalarial activity similar to that of artemisinin itself but is exquisitely microbe-selective and has remarkably potent activity against *Mtb*.

Artemisinin (**1**), also called qinghaosu, is a natural peroxide-containing sesquiterpene based on 1,2,4-trioxane, which is a highly active and relatively nontoxic antimalarial agent.[9] The trioxane derivatives dihydroartemisinin, artemether, and water-soluble sodium artesunate have been studied extensively and are widely used, and efforts toward the development of efficient methods for their preparation continue.[10] Mode-of-action studies indicate that ferrous iron [in the form of heme or iron(II) salts] triggers reductive cleavage of the peroxide bond in artemisinin to form oxygen-centered radicals. The oxy radicals then form carbon-centered radicals that ultimately lead to death of the malaria parasites. Related mechanistic work has been used to rationally design new antimalarial peroxides,[11] including dimeric versions.[12] Similarly, in cancer-related therapeutic applications, iron released from transferrin can react with artemisinin bound to a transferrin-receptor-targeting peptide to generate free radicals that can kill cells.[13] Since cancer cells overexpress transferrin receptors for iron uptake, the artemisinin−peptide conjugates and transferrin−artemisinin conjugates are selective and potent anticancer agents.[14]

The virulence of *Mtb* depends on its ability to assimilate iron, and like most microbes, it synthesizes and utilizes siderophores (low-molecular-weight iron chelators) to sequester iron.[15] On the basis of natural and synthetic examples of siderophore−antibiotic conjugates that enable active transport of drugs into specifically targeted bacteria,[16] we anticipated that while

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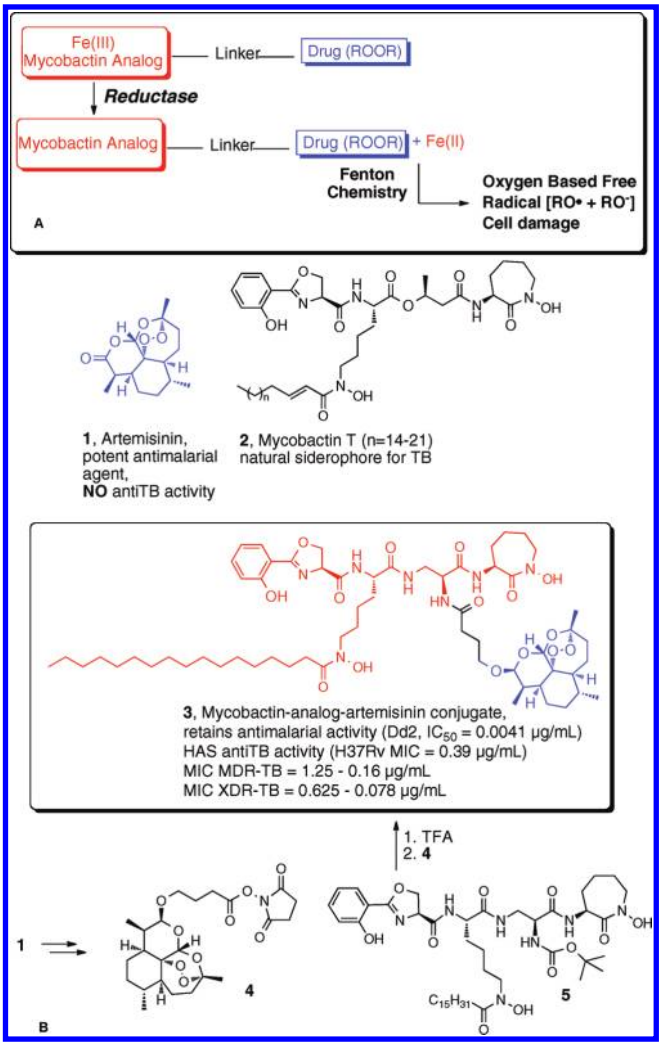

Figure 1. (A) Conjugate design. (B) Synthesis, anti-*Mtb* activity, and antimalaria activity of mycobactin−artemisinin conjugate **3**.

artemisinin itself has no antituberculosis activity, attachment of an artemisinin derivative to an analogue of mycobactin T (**2**),[17] an *Mtb* siderophore critical for growth under iron restriction, might allow the peroxide drug to be actively assimilated by the pathogen. Subsequent reduction of the bound ferric ion to its lower-affinity ferrous form might then induce Fenton-like chemistry from the interaction of the ferrous iron with the nearby peroxide. The consequent generation of free radicals would then be expected to cause intracellular damage selectively to the assimilating bacteria.[18] To test this hypothesis, we synthesized **3**, an artemisinin conjugate of a mycobactin T analogue (Figure 1, and found that it not only retains antimalarial activity similar to that of artemisinin itself (Table 1) but is exquisitely microbe-selective and has remarkably potent activity against *Mtb* (Table 2). The mycobactin−arteminisin conjugate is very active against not only H37Rv *Mtb* [minimum inhibitory concentration (MIC) = 0.39 μg/mL] but also both MDR *Mtb* (MIC = 0.16−1.25 μg/mL) and XDR *Mtb* (MIC = 0.078−0.625 μg/mL) (Tables 2 and 3).[19] Moreover, as described below, it is selectively active against *Mtb* relative to fast-growing species of mycobacteria and a broad set of other Gram-positive and Gram-negative bacteria. Physicochemical and whole-cell studies indicated that ferric-to-ferrous reduction of the iron

Table 1. Activities of Conjugate 3 against *P. falciparum* Strains (IC_{50} in μg/mL)[a]

	HB3	7G8	3D7	Dd2
conjugate **3**	0.0047	0.0051	0.0040	0.0041
artemisinin	0.0013	0.0004	0.0036	0.0022

[a] Abbreviations: HB3, Honduras (chloroquine-sensitive, low-level pyrimethamine-resistant); 7G8, Brazil (low-level chloroquine-resistant); 3D7, Airport (sensitive to sulfadoxine); Dd2, Indochina (multi-drug-resistant).

complex of **3** does initiate the expected lethal radical-producing Fenton chemistry on the artemisinin component.

The synthesis of mycobactin−artemisinin conjugate **3** is summarized in Figure 1. Since mycobactin T has no functional group for use in linking to the artemisinin component, we resynthesized our previously reported mycobactin analogue **5**,[20] which incorporates a diaminopropionic acid as a replacement for the natural β-hydroxybutyrate. Artemisinin is remarkably amenable to chemical modification despite containing inherently sensitive functionality. Thus, a sequence analogous to previous reports[21] gave *N*-hydroxysuccinimide (NHS)-active ester **4**. Treatment of mycobactin analogue **5** with trifluoroacetic acid to remove the Boc group followed by neutralization and direct treatment with **4** gave the desired conjugate **3** (see the Supporting Information).

As indicated, conjugate **3** not only retained efficacy against *P. falciparum* (Table 1) but also was remarkably potent against *Mtb* H37Rv [MIC (0.338 μM) = 0.39 μg/mL], while artemisinin (**1**) itself and all synthetic intermediates to active ester **4** (see the Supporting Information) had no activity against *Mtb*. Further studies revealed that conjugate **3** was similarly potent against MDR and XDR strains of *Mtb* (Tables 2 and 3). Thus, by using a mycobacterial siderophore to deliver an artemisinin derivative and provide a source of reactive iron to *Mtb*, we retained its antimalarial activity and enabled it to be a potent antitubercularculosis agent.

Conjugate **3** was not active against a broad set of bacteria at the highest levels tested (2 mM), including Gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* SG 511) and Gram-negative bacteria [*Serratia marcescens* SG 621, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 25922, *E. coli* DC0, *E. coli* DC2 (a permeability mutant), *Pseudomonas aeruginosa* KW 799/WT, *P. aeruginosa* KW 799/61 (a permeability mutant), *P. aeruginosa* SG 137, *P. aeruginosa* ATCC 27853, and *Stenotrophomonas maltophilia* GN 12873] (see the Supporting Information). As a further indication of the unique antimalarial and anti-TB selectivity of **3**, it was tested and found to have negligible activity against a number of fast-growing strains of mycobacteria [*Mycobacterium vaccae* IMET 10670, MIC = 50 μg/mL (43 μM); *Mycobacterium smegmatis* SG987, MIC = 200 μg/mL (173 μM); *Mycobacterium aurum* SB66, MIC = 12.5 μg/mL (43.3 μM); *Mycobacterium fortuitum* B, MIC = 100 μg/mL (87 μM); see the Supporting Information]. Thus, the antibiotic activity of conjugate **3** is microbe-selective, as anticipated.

In order to determine whether the remarkable activity of **3** depends on its ability to bind iron, we synthesized and tested the activity of derivative **6**, in which the iron-binding hydroxamates were protected (Figure 2). This modification reduced the antimalarial activity by >20- to 100-fold. The activity against all strains of *Mtb* was completely lost (>12.5 μM). To determine the effect of conjugation to a different siderophore, we prepared **7**, an

Table 2. Activities of Conjugate 3 against Eight Different MDR *Mtb* Clinical Strains[19] (MIC in μg/mL)[a]

strain 1 HRESP	strain 2 HREZSP	strain 3 HCPTTh	strain 4 HREKP	strain 5 HRERb	strain 6 HREZSKPTh	strain 7 HREZRbTh
1.25	0.625	0.625	0.625	0.3125, 0.156,[b] 1.25[b]	0.625	0.3125

[a] Abbreviations denote resistance to the following: H, isoniazid; R, rifampicin; E, ethambutol; Z, pyrazinamide; S, streptomycin; C, cycloserine; T, ethionamide; K, kanamycin; P, *p*-aminosalicylic acid; Rb, rifabutin; Th, thioacetazone; O, ofloxacin. [b] The *Mtb* strains are subsets of those reported previously.[19]

Table 3. Activities of Conjugate 3 against XDR *Mtb* Strains[19] and *M. smegmatis* (MIC in μg/mL)[a]

strain 8 HRESPOCTh	strain 9 HREPKOTh	strain 10 HRESPO	strain 11 HRESPOCTh	*M. smegmatis*
0.31	0.078	0.625	0.31	>5

[a] Abbreviations are the same as in Table 2.

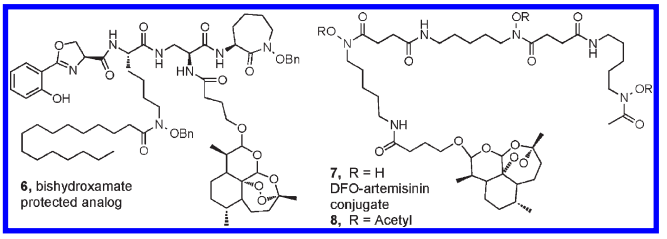

Figure 2. Protected mycobactin conjugate **6**, DFO conjugate **7**, and triacetyl-protected DFO conjugate **8**.

artemisinin conjugate of desferrioxiamine (DFO), a natural siderophore derived from *Streptomyces pilosus* that is therapeutically used for treatment of iron overload and also has efficacy for revival of patients in malaria-induced comas.[22] We also synthesized DFO derivative **8** in which all three of the hydroxamates were acetylated to negate iron binding. With half-maximal inhibitory concentration (IC_{50}) values ranging from 0.16 to >2.2 μM, both of the DFO-derived conjugates were more active against *P. falciparum* than DFO itself but much less active than conjugate **3** or artemisinin, and they were completely inactive against *Mtb*, since *Mtb* probably has no uptake route for the siderophore (DFO) on the basis of studies with *M. smegmatis*.[23]

Since our intent was to use the mycobactin to deliver iron and the redox-sensitive agent artemisinin to *Mtb* and induce damaging Fenton chemistry during the attempt of the pathogen to reductively assimilate the iron, we studied the related physicochemical properties of conjugate **3**. A cyclic voltammogram for commercially available iron-loaded mycobactin J (used as a control) is shown in Figure 3. The strongly negative cathodic peak current is consistent with that observed for other high-iron-affinity siderophore complexes.[24] The absence of a return oxidation wave is indicative of an irreversible Fe^{3+}/Fe^{2+} couple, with dissociation of the labile ferrous ion subsequent to reduction. A cyclic voltammogram for iron-loaded mycobactin−artemisinin conjugate **3** is also shown in Figure 3, along with that of artemisinin alone. Both voltammograms show irreversible behavior, consistent with the literature report for artemisinin.[25] The iron-loaded conjugate shows a shoulder and a main peak, which we assign to the Fe-mycobactin and artemisinin components, respectively. The Fe-mycobactin component in the conjugate is shifted negatively by ~100 mV relative to the control Fe-mycobactin J, and the peak current is increased from 7 to 18 μA/mM. The main peak attributable to the artemisinin component

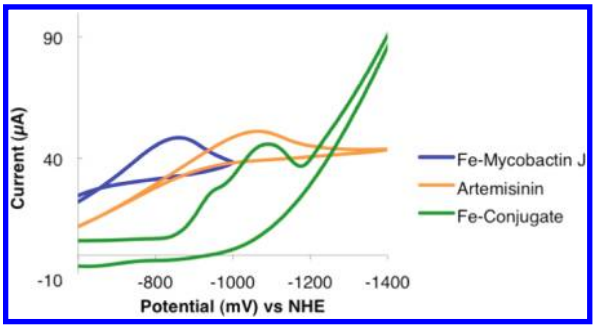

Figure 3. Cyclic voltammograms of Fe-loaded mycobactin J, artemisinin, and the Fe-loaded mycobactin−artemisinin conjugate. Conditions: glassy carbon working electrode; Pt wire auxiliary electrode; Ag/AgCl reference electrode; scan rate =10 mV/s; $[NaClO_4]$ = 100 mM as the background electrolyte in 95% EtOH. [Fe-mycobactin J] = 3 mM, E_{cp} = −856 mV vs NHE (7 μA/mM); [artemisinin] = 3 mM, E_{cp} = −1063 mV vs NHE (9 μA/mM); [Fe-mycobactin−artemisinin conjugate] = 1.625 mM, E_{cp} = −955 mV vs NHE (18 μA/mM), E_{cp} = −1090 mV vs NHE (19 μA/mM).

of the conjugate is shifted to a slightly more negative value, and the peak current is increased from 9 to 19 μA/mM. These results suggest that the combination of Fe-loaded mycobactin and artemisinin within the conjugate results in a chemical interaction between the two redox-active components, apparently subsequent to the reduction of Fe^{3+} to Fe^{2+} in the mycobactin binding site. This is consistent with the increased currents, suggesting a chemical event subsequent to the electrochemical (EC) reduction event (i.e., a modified EC process). We hypothesize that reduction of the bound ferric iron to its ferrous form induces Fenton-like chemistry from the interaction of the ferrous iron with the peroxide linkage of the artemisinin, leading to the reduction and cleavage of the peroxo bridge. This correlates with the biological activity observed for this complex, since this latter reduction would generate free radicals that would be expected to cause intracellular damage. We propose the coupled electrochemical/chemical processes shown in Scheme 1 (where art_O and art_R represent the artemisinin component of the conjugate in the oxidized and reduced forms, respectively), which are consistent with our observations. This scheme illustrates an initial reduction of conjugate-bound Fe^{3+} followed by dissociation of the labile Fe^{2+} and/or intramolecular redox reaction with the artemisinin component and regeneration of Fe^{3+}, with subsequent electrochemical reduction and dissociation of Fe^{2+}. The free $Fe^{2+}(aq)$ can generate toxic reactive oxygen species by Fenton-type reactions, as discussed earlier.

To verify that conjugate **3** fueled the formation of hydroxyl radicals in *Mtb*, we exposed washed drug-treated cells to the dye hydroxyphenylfluorescein, which is oxidized by hydroxyl radicals to yield a fluorescent derivative.[26] Conjugate **3** resulted in hydroxyl radical formation within 3 h of treatment at 1× and 10× MIC (Figure 4). Rifampicin, a transcriptional inhibitor and

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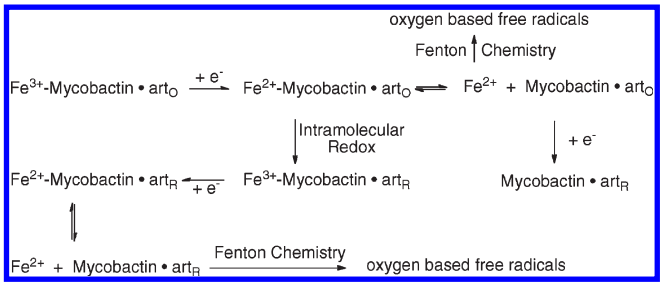
Scheme 1. Proposed Reaction for Mycobactin−Artemisinin Conjugate 3



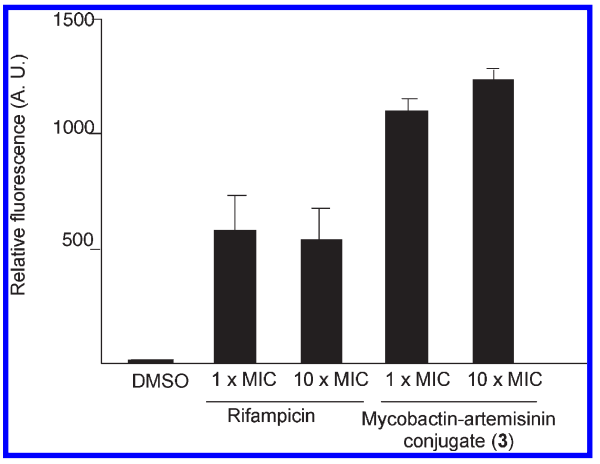


Figure 4. Treatment of *M. tuberculosis* with mycobactin−artemisinin conjugate **3** generates reactive oxygen species. H37Rv was treated with rifampicin (0.1 μg/mL and 1 μg/mL) or mycobactin−artemisinin conjugate **3** (0.5 μg/mL and 5 μg/mL) at 1× and 10× MIC. Following 3 h incubation, reactive oxygen species were measured with HPF. *M. tuberculosis* treated with DMSO was used as a vehicle control. Shown is a representative graph from at least three independent experiments performed in triplicate; means ± standard deviations are displayed.

bactericidal anti-TB drug,[27] showed more than 2-fold lower levels of hydroxyl radical release (Figure 4).

In conclusion, we have demonstrated that a conjugate of a antimalarial agent and a mycobacterial siderophore analogue retains the antimalarial activity while also inducing selective potent activity against *Mtb*. In contrast to other siderophore-based Trojan horse antibiotics,[16,28,29] the unique activity of **3** depends on active participation of the microbe-selective siderophore and the chemistry of both the iron and the drug (artemisinin) being transported.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Experimental procedures, syntheses of conjugate **3** and control compounds, details of all microbiological and biological studies, and complete refs 6 and 19. This material is available free of charge via the Internet at http://pubs.acs.org.

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