Bioconjugate Chemistry

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Reactive Rifampicin Derivative Able to Damage Transcription Complex

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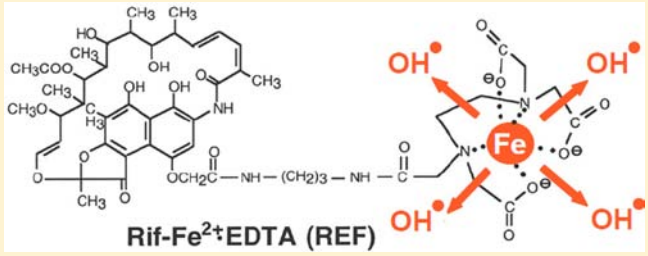
ABSTRACT: Rifampicin (Rif) is powerful broad spectrum antibiotic that targets bacterial RNA polymerase (RNAP) by blocking the transcript exit channel. The performance of the drug can be further enhanced by tagging with active chemical groups that produce collateral damage. We explored this principle by tethering Rif to Fe^{2+}-EDTA chelate. Modified drug retained high binding affinity to RNAP and caused localized cleavage of the enzyme and promoter DNA. Analysis of the degradation products revealed the cleavage of RNAP β subunit at the sites involved in the drug binding, while DNA was selectively seized in the vicinity of the transcription start site. The synthesized Rif derivative exemplifies "aggressive" types of drugs that can be especially useful for TB treatment by attacking the nongrowing dormant form of the mycobacterium, which is hardly susceptible to "passive" drugs.



INTRODUCTION

Despite availability of antibiotic treatment for over 50 years, *Mycobacterium tuberculosis* (*Mtb*), the etiological agent of tuberculosis (TB), remains one of the most successful human pathogens, accounting for up to 8 million cases of TB and nearly 2 million deaths annually. Unlike other pathogens, *Mtb* is extremely hard to eradicate, which is mostly due to its ability to penetrate human macrophages. This causes significant protection of the bacteria against common drugs. The rifamycin (Rif) antituberculosis agents—notably rifampin, rifapentin, and rifabutin—function by binding to and inhibiting *Mtb* RNA polymerase.[1−5] The rifamycin antituberculosis agents bind to a site on *Mtb* RNAP adjacent to the active center and prevent synthesis of RNA products greater than 2−3 nt in length. Rifamycins constitute the base of modern short-course chemotherapy regimens for tuberculosis.[6,7] The introduction of rifampin in the late 1960s permitted a marked reduction in the treatment duration for tuberculosis. This superior performance of rifamycin is due to the ability of the drug to diffuse freely inside the tissues and living cells and due to its extremely high affinity to RNAP (K_d ca. 2−4 nM). This explains Rif's relatively high efficiency (compared to other antituberculosis drugs) against dormant bacteria and intermittently active bacteria. Nevertheless, the treatment has to be continued for at least 6−8 months, causing side effects and in some cases developing drug resistance, which justifies the search for more efficient drugs to shorten the treatment duration. The problem can be addressed by using "aggressive" drugs able to actively damage a target in the dormant state of the bacterium, distinguished by low metabolic activity. In the present research, we explored this principle by attachment of Fe^{2+} chelate to Rif (Figure 1A). While retaining high inhibitory activity *in vitro*, this compound was able to damage the RNAP open promoter complex, causing cleavage of polypeptide chains and template DNA in close proximity to the drug binding site due to generation of reactive oxygen species following Fenton reaction.

EXPERIMENTAL SECTION

All reagents were from Sigma-Aldrich. RNAP carrying hexahistidine tag at C terminus of β subunit was obtained from RL731 *E. coli* strain. The 350 bp DNA fragment containing T7A1 promoter is described in ref 8. [α-^{32}P] labeled nucleoside-5′-triphosphates were from MP Biomedicals. Terminal labeling of DNA fragment at template or nontemplate strand was achieved through PCR of the promoter fragment whereby one of the primers was phosphorylated at the 5′ terminus. After PCR, the fragment was gel purified and incubated with polynucleotide kinase and [γ-^{32}P] ATP. All affinity cleavage and affinity labeling reactions were carried out in transcription buffer containing 40 mM Hepes pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$ at 20 °C. Limited CNBr cleavage of labeled RNAP was performed as described in refs 9,10. Adduct of Rifamycin B with diaminopropane was synthesized as described in ref 11. Adenosine-5′-trimetaphosphate (ATmP) was synthesized according to ref 12. Light absorption spectra

Received: October 16, 2012
Revised: January 29, 2013
Published: February 21, 2013

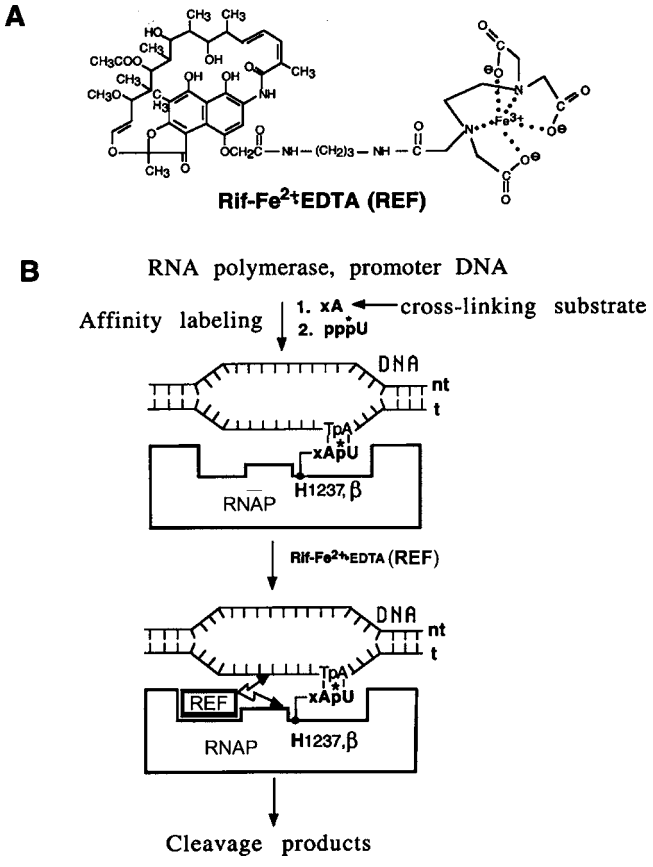


Figure 1. Scheme for affinity labeling of RNAP at β subunit His1237 and for REF-mediated cleavage. Asterisk represents radioactive phosphate. Template and nontemplate DNA strands in RNAP open promoter complex are marked as 't' and 'nt', respectively.

were recorded on a Cary 300 Bio UV–vis spectrophotometer (Varian). Mass spectra were obtained at the Center for Advanced Proteomics Research (UMDNJ) using MALDI-TOF detection mode.

Synthesis of Rif-EDTA Derivative. Aminopropyl derivative of Rif (10 μmol) and 50 μmol of EDTA dianhydride were mixed in 400 μL of DMF and 2 μL of diisopropylethylamine was added. After 10 min incubation at 20 °C, the mixture was supplemented with 400 μL of 0.1 M sodium citrate pH 5.5 and kept at 50 °C. After 30 min incubation, TLC analysis in the acetonitrile–water (4:1) developing system revealed one major product with R_f = 0.4. The product was extracted with butyl alcohol and purified by TLC in the same system. The compound was eluted with water, dried under reduced pressure, and kept at −80 °C. The compound was homogeneous as judged by reverse-phase HPLC and ion-exchange chromatography on DE-53 cellulose as well as by electrophoretic analysis using 20% PAAG. Just before the experiment, an equal amount of $Fe(NH_4)_2(SO_4)_2$ was added to a 0.14 mM solution of Rif-EDTA in 50 mM sodium acetate buffer pH 5.5 and the resulting complex immediately used for cleavage. UV–vis spectrum for Rif-EDTA compound: λ_{max1} = 304 nm (ε = 20 000 M^{-1} cm^{-1}), λ_{max2} = 430 nm (ε = 16 000 M^{-1} cm^{-1}), λ_{min1} = 281 nm (ε = 12 000 M^{-1} cm^{-1}), λ_{min2} = 361 nm (ε = 5000 M^{-1} cm^{-1}). MALDI-TOF MS for Rif-EDTA compound: $C_{52}H_{70}N_5O_{20}$ + 1 calcd. 1086.13, found 1086.46; $C_{52}H_{69}N_5O_{20}Na$ + 1 calcd. 1108.12, found 1108.37; $C_{52}H_{70}N_5O_{20}$ + 1 ($-OCH_3$) calcd. 1054.11, found 1054.45.

Affinity Labeling of RNAP at β Subunit His1237 by ATmP. This was performed as previously described.[12] Briefly, to RNAP-T7A1 promoter complex (2–5 pmol RNAP with equal amount of promoter in 10 μL of transcription buffer) ATmP (10 mM solution in DMSO) was added to final concentration 0.1 mM. After incubation at 20 °C for 20 min, the mixture was supplemented with 1 μL of [α-^{32}P] UTP and incubation continued for another 10 min. This preparation of labeled RNAP was immediately used in further experiments.

Affinity Cleavage of RNAP Open Promoter Complex by Rif-EDTA-Fe^{2+}(REF). To the solution of labeled RNAP obtained as described above, the REF compound was added at the indicated concentration (Figures 2 and 3) together with DTT (10 mM) and incubation continued at room temperature for 20 min unless specified otherwise. Cleavage products were analyzed by SDS PAGE. As sequence markers, the products of random single-hit degradation at Met residues of the same affinity-labeled subunit were used.[9,10] Briefly, the labeled enzyme was incubated with 1% SDS at 37 °C for 30 min and supplemented with 1 M HCl and 1 M CNBr to final concentration of 50 mM each. After 10 min incubation at room temperature, the reaction was terminated by addition of 1/3 (V/V) of 1 M Tris-HCl pH 8.5, 5% DTT, 30% glycerol. The protein degradation products were resolved in 6% and 10% polyacrylamide gels. For mapping of the REF-mediated cleavage sites in DNA, the reaction was performed as above, but the DNA fragment labeled at 5′ termini of either template or nontemplate strand was used to assemble RNAP open complex, while the affinity labeling stage was omitted. In this case, after incubation the reaction mixture was denatured in the presence of 1% SDS, extracted with phenol, and the products run on a DNA sequencing gel along with the standard sequencing reactions. Separated radioactive degradation products were visualized using Phosphoimager (Molecular Dynamics) and *Storm 60* software.

Molecular Modeling. was performed using the program *WebLab ViewerLight* 4.0 (Molecular Simulations Inc.). To model the RNAP initiating complex, the structure of nucleic acid scaffold of RNAP elongation complex[10] was docked to *Tth* RNAP holoenzyme.[13]

RESULTS

Synthesis of REF Derivative. Figure 1A shows the structure of REF used in this work for affinity cleavage of RNA polymerase. The synthetic strategy made use of rifamycin B carboxyl group, since modification at this position does not interfere with the activity of the drug. First, we introduced a short aminoalkyl linker by treatment of rifamycin B with DCC followed by reaction with 1,3-diaminopropane.[11] The amino group of the resulting compound was modified by EDTA dianhydride, followed by hydrolysis of the second anhydride group in mild acidic conditions. As expected, acylation greatly reduced chromatographic mobility of the compound on TLC. Due to high reactivity of EDTA, dianhydride modification of aromatic hydroxyl groups of the drug could not be excluded. However, light absorption spectrum of the product was identical to that for the original compound, which indicates the absence of chromophore modification. MS results confirmed this suggestion. The first prominent signal in the mass spectrum was detected at 1108.4, which corresponds to sodium complex of the expected product. Smaller signal (mass 1086.4) was equal to the molecular ion mass of the same compound, but lacking sodium, while the other prominent

dx.doi.org/10.1021/bc3005667 | *Bioconjugate Chem.* 2013, 24, 443–447

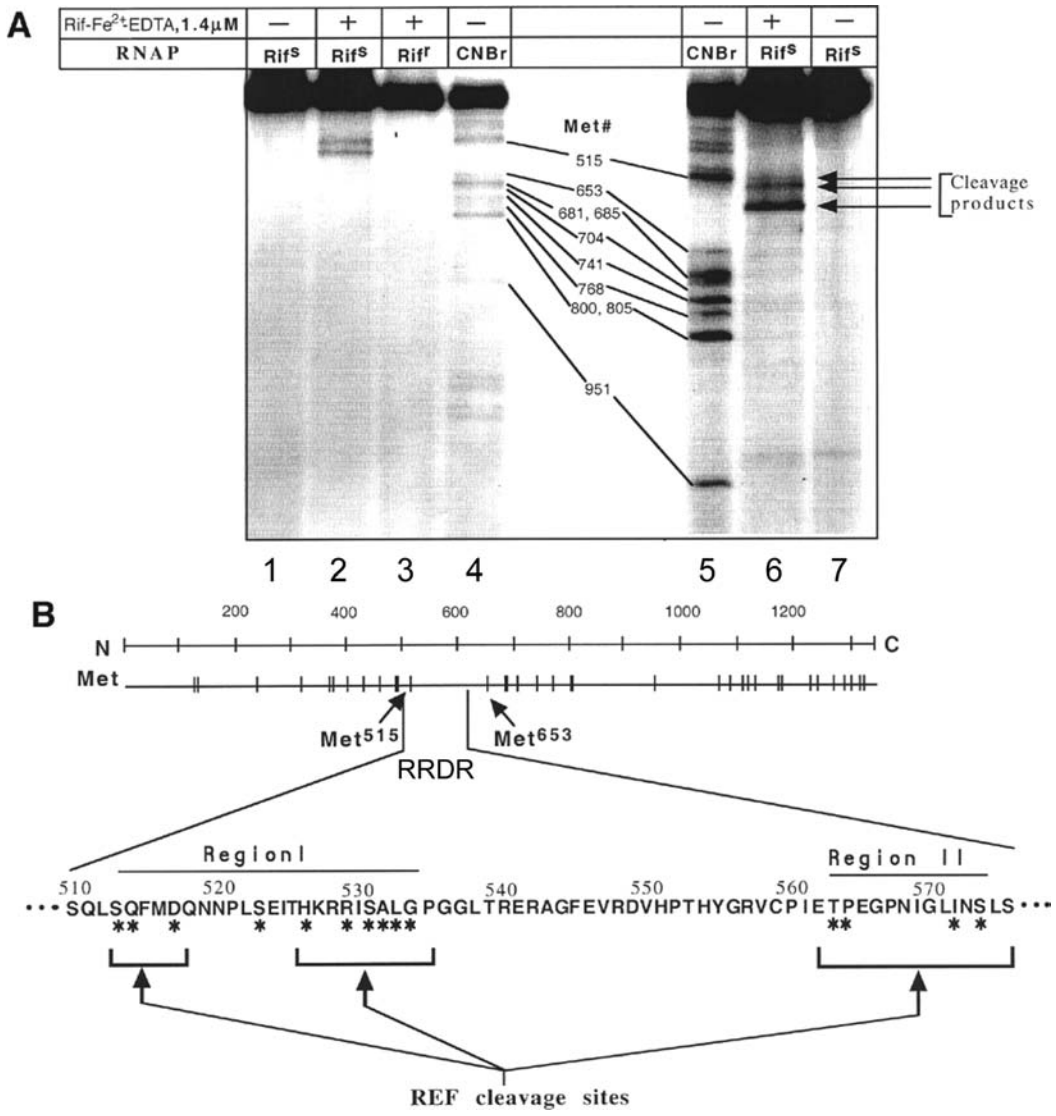


Figure 2. Mapping of the REF-mediated cleavage sites in RNAP β subunit. (A) SDS PAGE analysis of β subunit labeled Rif-sensitive (Rif^S) and Rif-resistant (Rif^r) RNAP incubated at different conditions. Left and right panels represent separation in 10% and 6% gels, respectively. The sequence markers originating due to cleavage at indicated Met residues of the β subunit are shown. (B) The position of REF cleavage sites on the linear sequence of β subunit. Arrows indicate the cleavage sites, asterisks the known Rif resistant mutations. RRDR is rifamycin resistance determining region.

signal (mass 1054.3) was consistent with the compound structure missing both sodium and oxymethyl group (at position11 of Rif ansa-bridge).

Chelation of the resulting intermediate with equivalent amount of ferrous ammonium sulfate produced the final compound, REF. Since this compound was unstable (due to oxidation of Rif core ring), the complex was obtained right before use.

Affinity Cleavage of RNAP Open Complex by REF. It is known that chelated Fe^{2+} ion generates hydroxyl radicals, which are highly reactive and able to produce a localized cleavage (within 1−1.5 nm radius) of protein and nucleic acid chains as a result of Fenton reaction. We reasoned that tethering of Fe^{2+} EDTA chelate to Rif would cause specific cleavage of the polypeptide and nucleic acid chains in the vicinity of the drug binding site. Since our attempts to map the cleavage sites in RNAP protein by conventional approaches were not successful due to modification of N-terminus in the cleaved products,[14] to aid the mapping we introduced a radioactive tag at H1237 residue of the β subunit using highly selective autocatalytic affinity labeling.[12] In this way, RNAP in binary complex with the promoter was treated by reactive derivative of the priming substrate, ATmP (Figure 1B) resulting in covalent attachment of the ATP residue to His1237 of the active center in β subunit. Subsequent treatment with [α-^{32}P] UTP (which is the next cognate nucleotide in the transcribed DNA sequence) caused elongation of the ATP residue bound to H1237 producing cross-linked pppAp*U transcript (p*-radioactive phosphate). The labeled RNAP complex was incubated with REF to allow Fenton reaction followed by separation of the cleavage products by SDS PAGE. As seen from Figure 2, incubation of REF with RNAP transcription complex caused cleavage of the subunit (lanes 2, 6). The cleavage was highly specific, since it did not occur with Rif resistant RNAP (lane 3) or when excess of Rif was added prior to REF (data not shown). REF produced three distinct cleavage sites in β subunit (lane 6). To map the cleavage sites, we used sequence markers generated by cleavage of the same β subunit-labeled RNAP promoter

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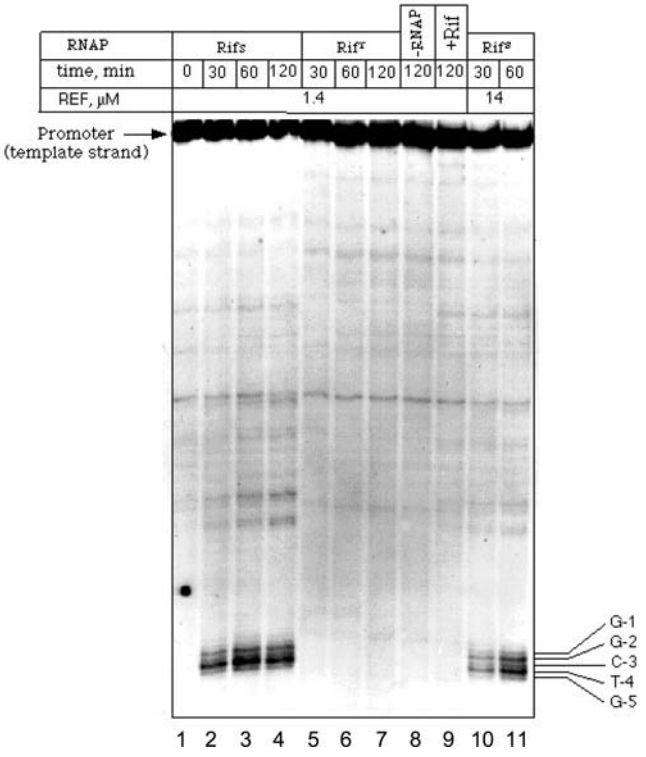

Figure 3. Electrophoretic analysis of the Rif^s and Rif^r RNAP open promoter complex (labeled at 5′ terminus of DNA template strand) incubated at various conditions: lanes 1−4, with Rif^s RNAP in the presence of 1.4 μM REF; lanes 5−7, the same as in lanes 2−4, but with Rif^r RNAP; lane 8, control labeled DNA; lane 9, the same as in lane 4, but preincubated with 2 μM Rif; lanes 10−11, the same as in lanes 2−3, but in the presence of 14 μM REF. The position of the cleave sites relative to the transcription start site is indicated.

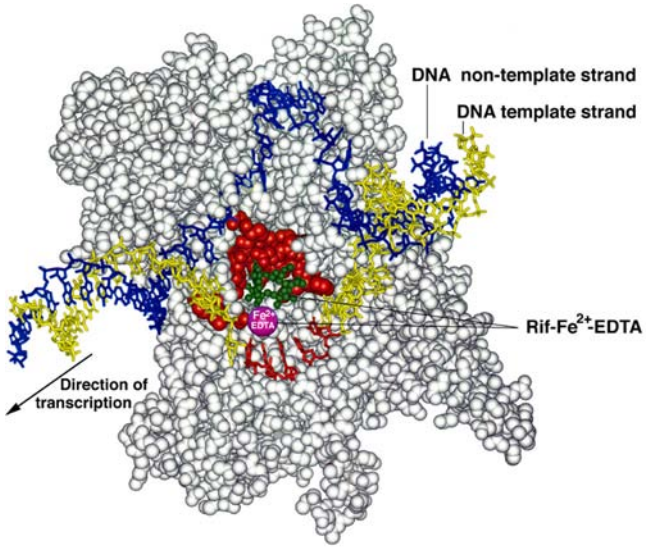

Figure 4. Projection of the REF cleavage sites on 3D structure of RNAP initiation complex model. RNAP beta subunit (gray) is shown in the space-filled representation; DNA in stick representation. Bound Rif residue of REF is highlighted in dark green. Position of the Fe^{2+}-EDTA residue of REF is shown as magenta sphere. The protein and DNA segments where the cleavage sites were mapped are highlighted in red.

complex with CNBr at Met residues under single hit degradation conditions. The positions of the cleavage sites were deduced from the relative mobility of the hydroxyl radical and CNBr cleaved products resolved by SDS PAGE. As follows from the mapping (Figure 2B), all REF cleavage sites reside in the rifamycin resistance determining region (RRDR) of β subunit where numerous Rif resistance mutations were found.[15−17]

To examine the cleavage of DNA, we performed the same experiment with end-labeled DNA promoter fragment. Subsequent electrophoretic analysis revealed a set of adjacent cleavage bands in the register (−1 to −5) relative to start point of RNA synthesis on template strand as deduced from comparison with sequence markers (Figure 3 lanes 2−4, 10−11). This cleavage pattern was expected since the DNA template strand resides in close proximity to the Rif binding site (Figure 1A). Remarkably, no cleavage occurred when the Rif resistant enzyme was used (lanes 5−7) or when excess of Rif was added prior to REF (Figure 3, lane 9). Also, no cleavage was observed in the nontemplate DNA strand (data not shown). Efficiency of cleavage was the same when 1.4 μM or 14 μM REF was used, reflecting the saturation of the binding site at low REF concentration, thus demonstrating that the modified Rif derivative retained high binding affinity to RNAP.

Figure 4 shows the position of cleavage sites in the high resolution model for RNAP initiation complex. In the absence of complete crystallographic structures, the model was constructed by pasting of nucleic acid scaffold of transcription elongation complex[10] onto cocrystal structure of RNAP holoenzyme with Rif.[13] As seen from the model, all cleavage sites are centered around Rif binding pocket and reside within 1−1.5 nm distance from the drug, which is consistent with the cleavage range for hydroxyl radicals.

CONCLUSIONS

The known "aggressive" drugs are represented by bleomycin, glycopeptide antibiotic produced by the bacterium *Streptomyces verticillus*.[18] Bleomycin, iron chelate, introduces DNA breaks by generation of hydroxyl radicals through Fenton reaction.[19] However, the use of the drug is limited due to relatively low association constant with DNA ($(4-6) \times 10^5$ M^{-1}) and low sequence specificity.[20,21] In the present research, we took advantage of this principle by attachment of a Fe^{2+} chelate to antibiotic rifampicin, which possesses high affinity to RNAP (K_d = 2−4 nM). The principal result of this study was demonstration of the ability of the resulting compound to bind to RNAP open complex and to produce highly localized specific cleavage of the protein and DNA. In the absence of active transcription in the mycobacterium dormant state, RNAP is expected to be mostly associated with the promoters, thus being more vulnerable for inhibition by Rif derivatives. Considering large number of promoters in bacterial DNA (from 4000 to 5000) and fair DNA cleavage efficiency for REF (about 30% per promoter), it is expected that REF-type compounds would produce extensive DNA damage, thereby causing genome instability. This should strongly contribute to the drug's lethal action. While displaying good performance *in vitro*, our Rif analogue has to be adjusted for therapeutic application. One of the improvements would be enhancement of the Fe^{2+} ion retention in the chelate by placing an extra group for the metal chelation. Yet, highly stable hemin-based Fe^{2+} complexes could be used for the drug design. The structure of the drug can be further optimized to adjust

dx.doi.org/10.1021/bc3005667 | *Bioconjugate Chem.* 2013, 24, 443−447

pharmacokinetic or pharmacodynamic properties as well as to provide good cellular uptake.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by NIH grant RO1 GM30717-21 to A.M.

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