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Inhibition of Bacterial Mevalonate Diphosphate Decarboxylase by Eriochrome Compounds

D. Andrew Skaff[†], William J. McWhorter[‡], Brian V. Geisbrecht^{‡,1}, Gerald J. Wyckoff[†], and Henry M. Miziorko^{†,*}

[†] Division of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, Missouri 64110, United States

[‡] Division of Cell Biology and Biophysics, University of Missouri—Kansas City, Kansas City, Missouri 64110, United States

Abstract

Mevalonate diphosphate decarboxylase (MDD; EC 4.1.1.33) catalyzes the irreversible decarboxylation of mevalonate diphosphate in the mevalonate pathway to form isopentenyl diphosphate, which is a precursor in the biosynthesis of many essential polyisoprenoid natural products, including sterols. In low G/C Gram-positive bacteria, which utilize the mevalonate pathway, MDD is required for cell viability and thus is a potential target for development of antibiotic drugs. To identify potential inhibitors of the enzyme, the National Cancer Institute's Mechanistic Diversity Set library of compounds was screened for inhibitors of *Staphylococcus epidermidis* MDD. From this screen, the compound Eriochrome Black A (EBA), an azo dye, was found to inhibit the enzyme with an IC_{50} value $< 5 \mu M$. Molecular docking of EBA into a crystal structure of *S. epidermidis* MDD suggested binding at the active site. EBA, along with the related Eriochrome B and T compounds, was evaluated for its ability to not only inhibit enzymatic activity but to inhibit bacterial growth as well. These compounds exhibited competitive inhibition towards the substrate mevalonate diphosphate, with K_i values ranging from 0.6 to $2.7 \mu M$. Non-competitive inhibition was observed versus ATP indicating binding of the inhibitor in the mevalonate diphosphate binding site, consistent with molecular docking predictions. Fluorescence quenching analyses also supported active site binding of EBA. These eriochrome compounds are effective at inhibiting *S. epidermidis* cell growth on both solid media and in liquid culture (MIC_{50} from 31–350 μM) raising the possibility that they could be developed into antibiotic leads targeting pathogenic low-G/C Gram-positive cocci.

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*Corresponding Author miziorkoh@umkc.edu Phone: 816-235-2246 Fax: 816-235-5595 Address: School of Biological Sciences University of Missouri-Kansas City, 5100 Rockhill Rd., Kansas City, Missouri 64110.

¹Current address: Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, KS 66506 USA

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Keywords

mevalonate diphosphate decarboxylase; mevalonate pathway; isoprenoid biosynthesis; enzyme inhibitors

INTRODUCTION

The ever-growing trend among many bacterial pathogens toward antibiotic resistance represents a great threat to public health in both developing and modern nations. In particular, a growing body of literature from the last decade has demonstrated that many strains of the widespread Gram-positive coccal organisms *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Streptococcus pyogenes* are now insensitive toward antibiotics that were once considered front-line therapeutics (1, 2). Given the diminution in effective therapeutic tools to combat these diseases, there is now renewed interest in novel classes of antimicrobials that are effective against sensitive and resistant strains alike, and which may diversify the currently available therapeutic strategies.

Many Gram-positive pathogens (including all of those mentioned above) rely on the mevalonate (MVA) pathway (3) for synthesis of isopentenyl 5- diphosphate (IPP), a precursor to many essential isoprenoid intermediates (e.g. undecaprenyl phosphate required for their cell wall synthesis) and knockout of the genes (including MDD) for these enzymes has bacteriostatic or bacteriocidal effects. The MVA pathway produces one molecule of IPP from three acetyl-CoAs. The decarboxylation of the C6 intermediate mevalonate 5-diphosphate is catalyzed by mevalonate diphosphate decarboxylase (MDD) accounting for formation of this C5 branched chain isoprenoid (4; reaction shown below).

MDD has been shown to be crucial to growth of these low-G/C Gram positive organisms (3) and thus appears to be an attractive target for antibiotic development. Recently we have published the first crystal structures of MDD liganded to metabolites or to the potent inhibitory substrate analogs fluoromevalonate diphosphate and diphosphoglycolyl proline (5, 6). These accomplishments provided considerable insight into the active site and confirmed many of our earlier functional assignments for active site residues. Substantial heterology is observed between the various proteins encoded by eukaryotic versus prokaryotic MDD genes. This has prompted the suggestion that MDD could be targeted for development of antimicrobial agents (7). With the perspective afforded us by these observations, it seemed reasonable to initiate work on identification of small drug-like compounds that inhibit bacterial MDD. The results of these experiments are presented in this publication.

A preliminary report of the results presented in this account has appeared (8).

EXPERIMENTAL PROCEDURES

Mevalonate diphosphate (MVAPP) was synthesized and purified by the method of Reardon and Abeles (9). Compounds in the Mechanistic Diversity Set were acquired from the National Cancer Institute. For post-screening experiments, Eriochrome Black A, B, and T were purchased from Fisher. All other reagents were purchased from Sigma-Aldrich or Fisher.

Cloning, overexpression and purification of recombinant forms of MDD

The wild-type and mutant *S. epidermidis* mevalonate diphosphate decarboxylase enzymes were cloned, expressed and purified as described by Barta *et al.* (5). The mutant MDD enzymes, Arg¹⁹³→Thr, Trp¹⁹→Phe and Trp¹⁵³→Phe were prepared using site directed mutagenesis and the resulting genes were sequenced confirm accuracy. All enzymes were stored in 10 mM Tris (pH 7.5), 50 mM NaCl, and stored at –80 °C for further use.

Kinetic characterization of wild-type and mutant *S. epidermidis* MDD

Enzymatic activity was determined using a spectrophotometric assay (10) coupling product ADP formation to pyruvate kinase/lactate dehydrogenase oxidation of NADH at 340 nm using an Agilent Technologies Cary 60 UV-Vis spectrophotometer. Assays were performed at 30 °C in 100 mM HEPES (pH 7.0) containing 100 mM KCl, 10 mM MgCl₂, 0.2 mM NADH, 0.4 mM phosphoenolpyruvate and 4 U each of pyruvate kinase and lactate dehydrogenase in a total volume of 1 mL. All assays were initiated by addition of MVAPP. V_{max} , K_m , and values were determined by fitting data to the Michaelis-Menten equation. For inhibition kinetics, substrate (MVAPP or ATP) was varied versus inhibitor concentrations for each of the eriochrome compounds. For inhibition versus MVAPP, the concentration of ATP was held constant at 250 μ M, while MVAPP concentrations were varied from 2.5-40 μ M. For inhibition versus ATP, the concentration of MVAPP was held constant at 100 μ M, while ATP concentrations varied from 7.5-120 μ M. Eriochrome Black A concentrations varied from 0.5-2 μ M versus MVAPP and 0.5-3 μ M versus ATP. Eriochrome Black T concentrations varied from 0.25-2 μ M versus both MVAPP and ATP. Eriochrome Blue Black B concentrations varied from 1-8 μ M versus MVAPP and 2-16 μ M versus ATP. Optimal data fits ($R^2 > 0.96$) were obtained using either competitive (inhibitor versus MVAPP) or non-competitive (inhibitor versus ATP) inhibition models. All kinetic data analysis was performed using SigmaPlot 10.0/Enzyme Kinetics Module 1.3 (Systat Software, Inc.).

Compound library screening

The Mechanistic Diversity Set was acquired from the National Cancer Institute via Fisher Scientific. Each compound was tested against the activity of the *S. epidermidis* mevalonate diphosphate decarboxylase in a microplate version (scaled to 120 μ L) of the assay described above, using K_m concentrations of both substrates. Compounds showing inhibition level of 50% were tested a second time to rule out false positives. IC₅₀ values were then determined for successful compounds by using two-fold dilutions of compound in the same microplate-based assay using a Molecular Devices SpectraMax 250 plate reader. Data for IC₅₀s were fit to a sigmoidal dose-response model using GraphPad Prism 4.

Molecular docking

The B chain from the PDB file, 3QT5, representing the apo form of the enzyme, was used as the model for docking. Compounds from the NCI Mechanistic Diversity Set were docked using Autodock Vina (11) implemented on an Amazon Web Services cluster. In order to provide additional metrics for evaluating the results of virtual screening NNSCORE 2.01 (12) was employed to re-rank these results. NNScore utilizes a neural network to enable a re-examination of binding factors that can be missed in the composite binding energy score normally reported for Autodock Vina; as such, it is valuable for identifying binders that would otherwise be ranked poorly within Vina results. The top hits (pose and binding energy) were modeled in PyMol. The ChemVassa program (13) also listed eriochrome as a potential lead compound when a screen of the combined NCI Mechanistic and the ZINC “Goldilocks” libraries was performed.

MIC₅₀ determinations

Determination of the minimum inhibitory concentration (MIC) against *S. epidermidis* cell culture was conducted by a two-fold serial dilution method in Mueller-Hinton broth (14). The MIC₅₀ was considered as the lowest concentration of the agents that yielded less than fifty percent growth of microorganisms according to the absorbance at 600 nm relative to a culture containing no inhibitor after 24 h of incubation at 37°C. Results were measured in 24-well microplates using a BioTek Synergy H1 microplate reader.

K_d determinations

The binding constant (K_d) was determined by addition of inhibitor to a solution of 150 nM enzyme in 50 mM NaH₂BO₃, pH 8.3 with 150 mM NaCl, at 25°C in a PTI Quantamaster fluorometer with slit widths set to 3nm. The excitation wavelength was set to 280nm and the values at the emission wavelength peak of 330nm were recorded. Data were fit to a one-site binding model using GraphPad Prism 4. R² values for all data were >0.93.

RESULTS

Compound library screening

The Mechanistic Diversity Set was acquired from the National Cancer Institute. This library consists of 879 compounds which represents a subset of chemicals from structurally based clusters from a collection of nearly 40,000 compounds tested against 60 different human tumor cell lines. Each compound (at a concentration of ~ 34 μM) was tested for inhibition of the *S. epidermidis* mevalonate diphosphate decarboxylase. From the screen, one compound, Eriochrome Black A, was found to successfully inhibit the activity of *S. epidermidis* MDD with an IC₅₀ of <5 μM (Figure 1). Based on this observation, additional supplies of Eriochrome Black A as well as the structurally related Eriochrome Black T and Blue Black B dyes (Fig. 2) were acquired for further experimentation.

Molecular docking predictions for Eriochrome dye binding to MDD

Eriochrome Black A likewise proved to be a top hit based on binding energy predictions from a virtual screening of the NCI Mechanistic diversity set compound library. When a

larger compound library was screened with ChemVassa (13), Eriochrome Black A was also listed as a top lead compound. The best scoring pose places the inhibitor in the active site of MDD. The energy minimized molecule was docked into the active site of the PDB file 3QT5 (B chain), which is the apo form of the *S. epidermidis* MDD, with the AutoDock Vina program and NNScore. NNScore proved to rank eriochrome dyes more highly than the native Vina scores in all cases, and this was true when MDD structures from different organisms were used for docking as well. Moreover, an overlay of the docking model with the experimental structure of the binary enzyme complex with the potent inhibitor fluoromevalonate diphosphate (5) suggests (Fig. 3A) a substantial overlap of Eriochrome Black A with the mevalonate portion of this substrate analog. This observation leads to the prediction of competitive binding with respect to the substrate mevalonate diphosphate by the eriochrome compounds, which could correlate with high efficacy of inhibition.

Kinetic studies on MDD inhibition by Eriochrome Black dyes

All three eriochrome compounds (isomers A, T, and B) were investigated by steady state kinetic analyses and each was observed to strongly inhibit *S. epidermidis* MDD (Table 1). The results for Eriochrome Black A are presented in Figure 4. Competitive inhibition with respect to mevalonate diphosphate is apparent, in accord with the docking prediction. Noncompetitive inhibition with respect to ATP is also observed. Since ATP binds with its gamma phosphoryl juxtaposed to the mevalonate C3 hydroxyl (6), the nucleoside portion of the compound does not substantially overlap with the mevalonate moiety with which eriochrome compounds are predicted to overlap. This would account for the observation of noncompetitive inhibition by such compounds. Similar inhibition pattern results were obtained for Eriochrome Black T and Blue Black B (Table 1), with K_i values for competitive inhibition (with respect to mevalonate diphosphate) ranging from 0.6 to 2.7 μM and for noncompetitive inhibition (with respect to ATP) ranging from 0.6 to 6.8 μM .

Investigation of the influence of active site tryptophan residues on substrate and inhibitor binding

Both tryptophan 19 and tryptophan 153 are located in the MDD active site pocket (Fig. 3B) and intrinsic MDD fluorescence is influenced by substrate site (McWhorter and Geisbrecht, unpublished observations) or inhibitor site occupancy (Figure 5). Upon substitution of phenylalanine for tryptophan, the purified W19F and W153F proteins were studied by steady state kinetics and fluorescence quenching (Table 2). Neither mutation significantly affects MDD catalytic rate. The W19F mutant exhibits substantial (>130-fold) inflation of K_m for mevalonate diphosphate and modest inflation of K_m for ATP (>10-fold). Only modest effects on kinetic parameters are observed with W153F. Since K_m is often not a reliable reporter of substrate binding, physical binding (K_d) of Eriochrome Black T was determined using fluorescence quenching titrations and K_d values compared to evaluate effects of these mutations. Again, W19F exhibited a larger effect (diminution) on K_d (~80-fold) than measured for W153F (9-fold). R193 interacts with the beta phosphoryl group of mevalonate diphosphate and is well removed from the predicted Eriochrome Black T binding site. In *control* kinetic and binding experiments with R193T, a substitution that replaces the arginine residue found in prokaryotic MDDs with the threonine found in all eukaryotic MDDs, the influence of this residue is modest. The very modest R193T mutation

effects on inflation in K_m for mevalonate diphosphate is consistent with the negligible change in K_d for Eriochrome Black T. However, the tryptophan mutations correlate with tighter Eriochrome Black T binding in contrast to K_m inflations (most notably for mevalonate diphosphate), complicating any definitive explanation for these tryptophan mutagenesis effects. However one possible explanation is that loss of this tryptophan residue may provide additional “room” in the active site for the EBT, which is larger than FMVAPP. In any event, both K_m and K_d effects do demonstrate the influence of W19 on the active site pocket, as predicted by molecular modeling (Fig. 3B) and demonstrated by X-ray structural results (5,6).

In vivo* effects of Eriochrome compounds on *S. epidermidis

In contrast to the physiological barriers anticipated for entry of mevalonate diphosphate into bacterial cells, the chemical properties of eriochrome dyes, which lack phosphoryl substituents while containing substituted naphthalene components with hydrophobic traits, suggest that permeability to cells would be more favorable. In studies of the effect of these inhibitors, Eriochrome Black A (at a concentration of 50 μ M) delayed 50% bacterial growth (Figure 6) by 1.6 hours. At a level of 100 μ M, the measured delay was 6.8 hours. The correlation between *in vivo* and *in vitro* effects of eriochromes is not precise, possibly reflecting cell permeability differences between these compounds. While there are only modest differences between IC_{50} values for these eriochromes (Table 3), MIC_{50} values indicate that Eriochrome Black T or Eriochrome Blue Black B are more potent than Eriochrome Black A in exerting *in vivo* inhibition in bacterial cultures. Nevertheless, the results do suggest that development or optimization of structural analogs of the eriochromes may lead to improved potency of MDD inhibition in pathogenic Gram-positive bacteria.

Discussion

Inhibitors of MDD have been investigated by various approaches. The tight binding mevalonate diphosphate analogs fluoromevalonate diphosphate (9) and diphosphoglycerol diphosphate (15) inhibit MDD by competing with mevalonate diphosphate binding in the MDD active site (5) but their cellular permeability is questionable. Another approach to MDD inhibition involved attempts to develop bifunctional inhibitors that would interact with both mevalonate diphosphate and ATP sites (16). Other attempts involved synthesis of mevalonate analogs that were evaluated as inhibitors of mevalonate kinase as well as MDD (17). In this case, the compounds showed more promise as negative allosteric effectors of bacterial mevalonate kinase rather than as MDD inhibitors.

Our approach, which has involved experimental screening of the modestly sized NCI Mechanistic Diversity Set library, has led to successful detection of Eriochrome Black A, a “drug-like” dye compound that exhibits a sub-micromolar inhibitor constant for *S. epidermidis* MDD. Recent precedent for dye compounds as enzyme inhibitors has been reported. Methylene blue, meldola blue, and Nile blue dyes bind to human butyrylcholinesterase but the observation of nonlinear inhibition kinetics suggested multisite binding (18). Our data indicate noteworthy inhibitory efficacy with each of three related eriochrome compound dyes. The agreement between the inhibitor binding site predicted by

molecular docking for a tight binding eriochrome dye with the experimental demonstration that all of the related eriochromes are competitive inhibitors with respect to mevalonate diphosphate is an interesting and useful result. While we have only solved liganded MDD crystal structures involving ligands containing a phosphoryl group, these solution data indicate that an unphosphorylated compound can quite effectively bind with good specificity within the site normally occupied by the mevalonate moiety of the substrate.

Regarding specificity (versus promiscuity) in binding of drug library compounds as enzyme inhibitors, Shoichet (19) points out that a classical single site binding inhibitor results in inhibition that ranges from 10-90% over a 81-fold change in inhibitor concentration. Figure 1's IC₅₀ curve shows that a concentration range of Eriochrome Black A 100 fold is required for such an effect, suggesting classical binding. As also indicated by Feng & Shoichet (20), observation of competitive inhibition of enzyme activity makes an aggregation effect unlikely. Figure 4 demonstrates that Eriochrome Black A inhibits MDD as a linear competitive inhibitor with respect to ATP, suggesting classical active site binding of an inhibitor. Moreover, Feng & Shoichet (20) suggest that observation of comparable inhibition of activity in the absence/presence of 0.01% Triton discounts an aggregation effect. In this account, all reported parameters based on activity assays (e.g. IC₅₀, kinetics, fluorescence binding experiments, etc.) do not substantially change when 0.01% Triton is either present/absent in the assay mix. This further suggests that inhibition of MDD by eriochromes is not due to a nonspecific aggregation effect.

Previous structure/function investigations have implicated at the catalytic site: Asp-283 (active site base; 21), Ser-141 (ATP binding motif residue; 22), Arg-144 (ligand to mevalonate C1 carboxyl; 23), and Ser-192 (ligand to phosphoryl groups of mevalonate diphosphate; 5). It seems quite possible that derivatization of the eriochrome molecules to incorporate functional groups that would interact with these polar active site side chains could produce an MDD inhibitor with much improved binding affinity. Alternatively, since the binding components of the ATP substrate site have also been experimentally determined (6), there may be adequate structural background to support future design of more effective bifunctional inhibitors. In planning any extension of synthetic work in such directions, it will be important to consider the consequences of eriochrome derivatization on permeability of such compounds to the bacterial membrane. Our results in "in vivo" tests of eriochrome effects on *S. epidermidis* inhibition have provided a useful reference point against which any future inhibitory eriochrome derivatives can be compared.

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Abbreviations

MDD	mevalonate diphosphate decarboxylase
IPP	isopentenyl 5-diphosphate

EBA	Eriochrome Black A
EBT	Eriochrome Black T
MVA	mevalonate
MVAPP	mevalonate diphosphate
FMVAPP	fluoromevalonate diphosphate
MIC	minimum inhibitory concentration

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- Mevalonate pathway enzymes as drug targets in Gram-positive bacteria
- Mevalonate diphosphate decarboxylase (MDD) inhibition by drug-like compounds
- NCI Mechanistic Diversity Set compound library screening
- Eriochromes A, B, and T as *S. epidermidis* MDD inhibitors

Figure 1.

IC₅₀ determination for MDD inhibition by Eriochrome A. IC₅₀ value was determined by using two-fold dilutions of compound (18 μ M to 2 nM) in a microplate-based assay using a Molecular Devices SpectraMax 250 plate reader. Data from IC₅₀ titrations were fit to a sigmoidal dose-response model using GraphPad Prism 4.

Figure 2.
Structures of three eriochrome compounds used in these experiments.

Figure 3.

Orientations of MDD-bound eriochrome and active site tryptophans. Panel A shows the overlay of docked Eriochrome Black T (carbons in green) with the empirically determined binding orientation of the potent inhibitor, FMVAPP (carbons in yellow). Panel B depicts the experimentally determined (5) orientations of the active site tryptophan residues predicted to contribute to protein fluorescence quenching upon binding of a ligand in the site depicted for the potent inhibitor FMVAPP. The protein (Chain A) is shown in purple, while the sidechains of W19 and W153 are highlighted in blue. Inhibitor FMVAPP is shown with carbon as yellow, oxygen red, phosphorous orange, and fluorine cyan. The mainchain position of catalytic residue D283 is colored light orange for the purposes of orientation.

Figure 4.

Inhibition of *S. epidermidis* MDD by Eriochrome Black A. For inhibition versus MVAPP (panel A), concentrations of inhibitor were: no inhibitor (■), 0.5 μ M (▲), 1 μ M (○), 1.5 μ M (□) and 2 μ M (△). For inhibition versus ATP (Panel B), concentrations of inhibitor were: no inhibitor (■), 0.5 μ M (▲), 1 μ M (△), 2 μ M (□) and 3 μ M (○).

Figure 5.

Binding of Eriochrome Black T to wild-type *S. epidermidis* MDD. Eriochrome Black T was titrated to a final concentration of 50 μ M into a solution containing 50 nM wild-type enzyme. Quenching of tryptophan residue fluorescence was measured at each concentration of inhibitor by recording the fluorescence intensity at the emission wavelength of 335 nm after excitation at 280nm.

Figure 6.

S. epidermidis cell growth inhibition by Eriochrome Black A. *S. epidermidis* cells were grown in LB media in the presence of no Eriochrome Black A (solid line), 50 μ M EBA (dashed line) or 100 μ M EBA (dotted line). In liquid culture, Eriochrome Black A at the concentration of 50 μ M, delays the ability of the culture to achieve 50% growth by 1.6 hours. At 100 μ M, Eriochrome Black A hinders the growth by 6.8 hours.

Table 1Kinetic constants for *S. epidermidis* MDD with eriochrome compounds^a

Compound	K _i vs MVAPP (μM)	Inhibition Type vs MVAPP	K _i vs ATP (μM)	Inhibition Type vs ATP
Eriochrome Black A	0.58 ± 0.08	Competitive	1.8 ± 0.1	Non-Competitive
Eriochrome Black T	1.2 ± 0.1	Competitive	0.59 ± 0.05	Non-Competitive
Eriochrome Blue Black B	2.7 ± 0.5	Competitive	6.8 ± 0.8	Non-Competitive

^aR² values for all data are 0.95.

Table 2Kinetic and binding constants for *S. epidermidis* MDD mutants^a

Enzyme	Specific Activity (U/mg)	k_{cat} (s ⁻¹)	$K_{\text{m,MVAPP}}$ (μM)	$K_{\text{m,ATP}}$ (μM)	$K_{\text{d,EBT}}$ (μM)
Wild-type	8.6 ± 0.1	5.3 ± 0.1	9.3 ± 0.5	26.7 ± 0.3	1.8 ± 0.2
W19F	3.0 ± 0.1	1.8 ± 0.1	1300 ± 300	300 ± 50	0.023 ± 0.013
W153F	1.85 ± 0.09	1.14 ± 0.06	27 ± 5	44 ± 6	0.2 ± 0.015
R193T	14.4 ± 0.2	8.8 ± 0.1	38 ± 2	15 ± 1	1.3 ± 0.3

^aR² values for all data are 0.95.

Table 3

Comparison of concentrations at which eriochrome compounds interact with MDD *in vitro* with isolated enzyme (IC₅₀) or *in vivo* with *S. epidermidis* cell culture (MIC₅₀)

Compound	IC ₅₀ (μM) ^a	MIC ₅₀ (μM) ^b
Eriochrome Black A	5 ± 2	250
Eriochrome Black T	3.5 ± 0.8	31
Eriochrome Blue Black B	3.5 ± 0.7	63

^a Calculated from a sigmoidal curve fit with R² value 0.95.

^b Estimated from the first data point below 50% of saturated cell culture from the average of three biological replicates.