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Dequalinium, a New Inhibitor of *Mycobacterium tuberculosis* Mycothiol Ligase Identified by High-Throughput Screening

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Abstract

Mycothiol ligase (MshC) is a key enzyme in the biosynthesis of mycothiol, a small molecular weight thiol that is unique to actinomycetes and whose primary role is to maintain intracellular redox balance and remove toxins. MshC catalyzes the adenosine triphosphate (ATP)-dependent condensation of cysteine and glucosamine-inositol (GI) to produce cysteine-glucosamine-inositol (CGI). MshC is essential to *Mycobacterium tuberculosis* and therefore represents an attractive target for chemotherapeutic intervention. A screening protocol was developed to identify MshC inhibitors based on quantification of residual ATP using a coupled luminescent assay. The protocol was used to screen a library of 3100 compounds in a 384-well plate format ($Z' \geq 0.65$). Fifteen hits (0.48%) were identified from the screen, and 2 hits were confirmed in a secondary assay that measures production of CGI. The structures of both hits contain N-substituted quinolinium moieties, and the more potent of the 2—namely, dequalinium chloride—inhibits MshC with an IC_{50} value of $24 \pm 1 \mu M$. Further studies showed dequalinium to be an ATP-competitive inhibitor of MshC, to bind MshC with a K_D of $0.22 \mu M$, and to inhibit the growth of *M. tuberculosis* under aerobic and anaerobic conditions with minimum inhibitory and anaerobic bactericidal concentrations of 1.2 and $0.3 \mu g/mL$, respectively. The screening protocol described is robust and has enabled the identification of new MshC inhibitors.

Keywords

competitive inhibitor; luminescent assay; mycothiol biosynthesis; screening; small molecular weight thiol

INTRODUCTION

Mycobacterium tuberculosis, the bacillus that causes tuberculosis (TB), is responsible for 1.6 million deaths per year and is the leading cause of death in HIV/AIDS patients.¹ The World Health Organization (WHO) estimates that one third of the world population is latently infected with TB, and 10% of those individuals will develop active disease in their life-time. TB can be cured in many cases. However, the current “short-course” regime is

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impractically long (6–12 months of multidrug therapy), resulting in poor patient compliance, which in turn leads to the emergence of multidrug- and extensively drug-resistant TB (MDR and XDR).² Complicating this landscape is the fact that no new TB drugs have been developed in the past 40 years.² Therefore, the urgent need for the discovery and development of new anti-TB therapeutic agents has become well appreciated.

Together with the challenge of identifying new antimycobacterials comes the difficulty of selecting a target that can potentially lead to more effective drugs with new mechanisms of action. One path to identifying such compounds is to target processes known to be essential for bacterial growth or survival during infection, or the so-called essential genes.³ Using transposon gene knockout⁴ and transposon site hybridization⁵ techniques, 2 independent groups demonstrated that the *mshC* gene (*Rv2130c*) is essential to *M. tuberculosis* Erdman, making its gene product, MshC, an attractive target for the identification of new anti-TB agents.⁴

MshC, or mycothiol ligase, catalyzes the adenosine triphosphate (ATP)-dependent ligation of cysteine to glucosamine-inositol (GI) to produce cysteine-glucosamine-inositol (CGI), adenosine monophosphate (AMP), and pyrophosphate (PPi), shown in Figure 1, which is a key step in mycothiol biosynthesis and one that occurs in *M. tuberculosis*.⁶ Mycothiol is unique to actinomycetes and is considered the functional equivalent of glutathione in these organisms.⁷ As in glutathione biochemistry, mycothiol maintains intracellular redox balance, functions as a cofactor in enzymatic reactions, and participates in the detoxification of toxins and antibiotics.^{7,8}

High-throughput screening (HTS) of libraries of chemically diverse compounds is one of the most efficient means of identifying molecules that inhibit a novel target. The development of a screening protocol is therefore a significant step toward the identification of new MshC inhibitors. Previously, Newton et al.⁹ reported a coupled spectrophotometric assay for the screening of mycothiol ligase inhibitors based on quantification of the pyrophosphate generated in the enzymatic reaction. One drawback to that assay was the high number of false positives encountered (7%), many of which were shown to inhibit pyrophosphatase itself. In addition, phosphate quantification can be affected by experimental variations such as the quantity of exogenous phosphates present in buffers and reagents, as well as precipitation of assay components at high phosphate concentrations.¹⁰ Here we describe the development of a simpler and more robust HTS protocol for the identification of mycothiol ligase inhibitors using a coupled luminescent assay. Furthermore, we demonstrate its utility by screening a small library of 3100 chemically diverse compounds that allowed us to identify a new MshC inhibitor. Studies describing the inhibitor's mechanism of inhibition, antimicrobial activity, and preliminary structure-activity relationship are also presented.

MATERIALS AND METHODS

Materials

Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of highest purity available. GI (1-*O*-(2-amino-2-deoxy- α -D-glucopyranosyl)-D-myo-inositol) was prepared by cleavage of mycothiol bimeane with enzyme mycothiol-S-conjugate amidase as described previously.¹¹ CysSA (5'-*O*-[*N*-(L-cysteinyl) sulfamoyl] adenosine) was purchased from RNA-TEC (Leuven, Belgium). 4-amino-1-quinaldinium iodide (**1a**) was obtained from the Florida Center for Heterocyclic Compounds, University of Florida (Gainesville, FL). The screening compound library was obtained from the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program at the National Cancer Institute and was composed of 3100 compounds, including 235 natural

products. In addition, 384-well white plates were purchased from PerkinElmer (Boston, MA) and Kinase-Glo[®] Plus Luminescent Kinase from Promega (Madison, WI).

Production of recombinant MBP-MshC

M. tuberculosis MshC was expressed as a maltose-binding protein (MBP) fusion protein in strain I64 *Mycobacterium smegmatis* as previously described.¹¹ To obtain sufficient amounts of enzyme for method development and screening, the published protocol was scaled up to 5 L. A 25-mL starter culture of the I64 *M. smegmatis* strain transformed with our pACE/MBP-MshC vector was grown in Middlebrook 7H9 (DIFCO) supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase, BBL), 0.05% Tween-20, and hygromycin (75 µg/mL) for 72 h at 37 °C/225 rpm and was used to inoculate a Fernbach flask containing 1 L of Middlebrook 7H9 media containing 1% glucose, 0.05% Tween-20, and hygromycin (75 µg/mL). Following 24 h incubation at 37 °C/225 rpm, cells were harvested by centrifugation (8000 g, 4 °C for 15 min) and resuspended in 5 L expression medium (Middlebrook 7H9, 0.4% acetamide, 0.05% Tween-20, and hygromycin 75 µg/mL) in a bench-top 5-L fermentor (New Brunswick Scientific, Edison, NJ) at an A_{600} of 0.3. The culture was maintained at 37 °C with stirring at 400 rpm for 24 h. Cells were harvested by centrifugation (8000 g, 4 °C for 15 min) and stored at –80 °C until further use. Recombinant MBP-MshC was purified by affinity chromatography using a column packed with amylose resin and eluting with maltose as previously described.¹¹

Determination of apparent K_m values of cysteine, ATP, and GI for MBP-MshC

Prior to determining apparent K_m and V_{max} values of Cys, ATP, and GI, initial velocity conditions for MshC were established as follows. Reaction progression curves for recombinant MshC were measured on reactions run in 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES pH 7.4, Cellgro, Manassas, VA) and 25 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris [pH 8.0], KD Medical, Columbia, MD) buffers using 10, 20, or 40 ng/µL of enzyme. All reactions were carried out in 0.2-mL microtubes at a final volume of 25 µL containing 100 µM each of GI, ATP, cysteine (Calbiochem, San Diego, CA), 1 mM bis-sulfanybutane- 2,3-diol (dithiothreitol [DTT], ICN Biomedicals, Irvine, CA), and 1 mM MgCl₂ (KD Medical). Reactions were incubated at room temperature for 60 min with aliquots taken every 10 min (see Fig. S1 at <http://jbx.sagepub.com/supplemental>), and CGI was quantified by fluorescence-detected high-performance liquid chromatography (HPLC) as described previously.⁶

Once initial velocity conditions were established, apparent K_m and V_{max} values for each substrate were determined independently using 2-fold dilutions of cysteine (concentrations ranging from 0.02 to 1.28 mM), GI (concentrations ranging from 0.40 to 1.6 mM), or ATP (concentrations ranging from 0.02 to 1.28 mM) in the presence of saturating concentrations of the other 2 substrates comprising 500 µM GI, 2 mM ATP, or 200 µM cysteine in 25 mM Tris 8.0 and 2 mM MgCl₂. Reaction mixtures also contained 1 mM DTT for determination of K_m values for GI and ATP or 3 mM DTT for determination of K_m for cysteine. Enzymatic activity was assayed at a final volume of 25 µL and measured by HPLC-detected production of CGI.⁴ Resulting curves were fit to a rectangular hyperbola by nonlinear regression analysis using the program Sigma Plot (Systat Software, Inc., Richmond, CA).

Assay optimization

Assays were optimized for luminescence detection in a 384-well plate format by systematically varying substrates or cofactors as follows. All reactions were carried out in a final volume of 25 µL in reaction buffer containing 25 mM Tris 8.0, 100 µM cysteine, 1 mM MgCl₂, and 1 mM DTT. An optimal concentration for GI was determined by varying the concentration of GI from 1.6 to 200 µM in the presence of 100 µM ATP and 20 ng/µL MBP-

MshC. An optimal ATP concentration was determined by varying the concentration of ATP from 40 to 100 μM in the presence of 100 μM GI and 20 ng/ μL MBP-MshC, and an optimal enzyme concentration was determined by varying MBP-MshC from 2.5 to 60 ng/ μL , keeping GI and ATP concentrations constant at 100 μM . Following a 1-h incubation at room temperature, 25 μL of Kinase-Glo[®] Plus (prepared as indicated by the manufacturer) was added to the mixtures and the reactions incubated for an additional 10 min at room temperature. Luminescence (relative light units, RLU) was measured using a Victor 1420 Multilabel Counter with an integration time of 1 s.

Assays designed to measure the tolerance of MBP-MshC to DMSO (0.05%–10%) were carried out in white 384-well plates at a final volume of 25 μL in 25 mM Tris 8.0, 1 mM DTT, 1 mM MgCl_2 , 100 μM ATP, 60 μM GI, 100 μM cysteine, and 20 ng/ μL MBP-MshC. The enzymatic reaction was terminated after a 1-h incubation at room temperature by addition of 25 μL of the Kinase-Glo[®] plus reagent. The reaction mix was incubated for 10 min at room temperature and the luminescence measured (Victor 1420 Multilabel Counter, 1 s integration time).

Determination of assay quality

Robustness of the assay in microplate format was assessed by determining the Z' factor, signal to background (S/B), signal to noise (S/N), and well-to-well variability (% coefficient of variation [CV]) of an assay performed in a 384-well plate at final volume of 25 μL in 25 mM Tris 8.0, 1 mM DTT, 1 mM MgCl_2 , 100 μM ATP, 60 μM GI, 100 μM cysteine, and 20 ng/ μL (225 nM) MBP-MshC using 1 μM CysSA and water ($n = 112$) as positive and negative controls, respectively. Statistical parameters were calculated as previously described.¹² To further assess suitability of the assay, we obtained a dose-response curve for inhibition of MBP-MshC by a known inhibitor CysSA^{18,19} (0.5–5000 nM) using conditions identical to those used for determining the statistical parameters.

Luminescent screen

Screening of a small library of 3100 compounds was conducted using the protocol summarized in Table 1. Briefly, 10 μL of 2.5 substrate mix (62.5 mM Tris 8, 2.5 mM MgCl_2 , 2.5 mM DTT, 250 μM ATP, and 250 μM cysteine) was added to 384-well plates using a MicroFill[™] Microplate Dispenser (BioTek, Winooski, VT), followed by sequential addition of 5 μL each of 300 μM GI, 50 μM test compound, and 100 ng/ μL MBP-MshC using a Biomek FX (Beckman Coulter, Fullerton, CA) automated liquid-handling workstation. Plates were incubated at room temperature for 1 h, after which 25 μL of Kinase-Glo[®] Plus was added to each well using a MicroFill[™] Microplate Dispenser. After incubation at room temperature for 10 min, luminescence (RLU) was measured using a Victor 1420 Multilabel Counter at 0.1 s integration time. Test compounds were evaluated at 10 μM containing 0.1% or 1% DMSO. All plates contained 16 wells each of CysSA (1 μM) and DMSO (0.1 or 1%) as positive and negative controls, respectively. In parallel with the MBP-MshC screen, a counterscreen where MBP-MshC was omitted from the reaction mixtures was carried out using conditions described above. Percentage of inhibition (PI) of test compounds was calculated using the following formula:

$$\text{PI} = 100 * [\text{NC} - \text{STC}] / [\text{NC} - \text{PC}], \quad (1)$$

where NC = average RLU signals of the negative control (0.1% or 1% DMSO), STC = RLU signal of the test compound, and PC = average of RLU signals of the positive control (1 μM CysSA).

Confirmatory assay

Compounds inhibiting MshC and/or luciferase were retested in the presence and absence of 0.1% Triton X-100 with all other conditions identical to those described for the luminescence screen. Compounds were tested in duplicate and MshC inhibitors identified by quantifying CGI production using fluorescence-detected HPLC. Dose-response curves for MshC inhibitors were in turn measured in triplicate using 3-fold serial dilutions of test compounds followed by quantifying CGI production by HPLC.⁴ The data were fit to a standard 4-parameter logistic equation by nonlinear regression analysis using the program SigmaPlot.

Mode of inhibition of dequalinium

The mode of inhibition of MshC by dequalinium was determined using the kinetic method described by Lai and Wu.¹³ In this method, changes in the inhibition profile observed upon varying the concentration of a given substrate distinguish the mode of inhibition. Optimal results are obtained when an inhibitor concentration giving ~50% inhibition is used, and the substrate concentration is varied over a range spanning 0.1 to 20 times the substrates' K_m values. Assays to determine the IC_{50} values for dequalinium at the K_m of each substrate (700 μ M GI, 200 μ M AT P, and 140 μ M cysteine) were performed in buffer containing 25 mM Tris 8.0, 1 to 2 mM DTT, 1 mM $MgCl_2$, and 20 ng/ μ L MBP-MshC, where concentrations of 2 substrates at a time were kept constant at saturating conditions (1 mM GI, 1 mM AT P, or 400 μ M cysteine). Respective IC_{50} values for dequalinium when GI, AT P, and cysteine were present at their apparent K_m values were 40 μ M, 30 μ M, and 50 μ M. To determine the mode of inhibition of dequalinium, we performed assays where dequalinium was present at concentrations equal to its IC_{50} for a given substrate. The concentration of one substrate at a time was varied (concentrations ranged from 31.3 to 4000 μ M for AT P, 15.6–500 μ M for Cys, and 34–1100 μ M for GI) while saturating conditions of the remaining 2 substrates were present. Control reactions where dequalinium was absent were performed for each condition. Reactions were monitored by quantifying production of CGI. The percent inhibition by dequalinium as a function of varying substrate concentrations was determined by comparison to control reactions. The modes of inhibition of dequalinium were determined using standard kinetic models.^{13,14}

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) measurements were performed with a Microcal VP-ITC titration calorimeter. Briefly, 35 $\times$ 7- μ L aliquots of dequalinium were added every 180 s to 1.484 mL of 14.9 μ M MBP-MshC or 15.4 μ M MBP (quantitated by A_{280}) using a 250- μ L rotating stirrer syringe, with the cell temperature set to 25 °C. Protein and ligand solutions were prepared in identical buffers containing 5% DMSO in 20 mM Tris, pH 7.4. Binding isotherms were fit to 1-site or 2-site models using Origin software.

Antimicrobial assays

Compounds were evaluated for their antimicrobial activity against a panel of clinically relevant bacteria summarized in Table 2. Seeded agar disk diffusion assays¹⁵ were performed for all strains with the exception of *M. tuberculosis*, for which the broth dilution method¹⁶ was employed. Detailed descriptions of both methods have been published recently in Plaza et al.¹⁵ and Li et al.,¹⁶ respectively.

RESULTS AND DISCUSSION

Design, optimization, and assessment of MshC luminescence assay

MshC catalyzes the ATP-dependent ligation of cysteine to GI to produce CGI, AMP, and PPi,^{6,7} as illustrated in the mycothiol biosynthetic scheme shown in Figure 1. Thus, quantification of residual ATP can be used as a direct measure of MshC activity. Expanding on the successes of high-throughput kinase screens,¹⁷ we took advantage of luciferase, an enzyme that catalyzes in an ATP-dependent manner mono-oxygenation of its substrate luciferin to produce light. MshC versus luciferase inhibitors were identified through a counter-screen where MshC was omitted.

In preparation for adapting the assay to a 384-well plate high-throughput format with luminescence detection, initial velocity conditions and apparent K_m and V_{max} values were determined. To establish initial velocity conditions (defined as 10%–15% of substrate depletion or product generated), we measured reaction progression curves for reactions carried out in 2 different buffers (HEPES 7.4 and Tris 8.0) and 3 different enzyme concentrations (10, 20, and 40 ng/ μ L) in the presence of 100 μ M each of cysteine, GI, and ATP. The desired conditions were observed in both HEPES and Tris in the presence of 10 ng/ μ L or 20 ng/ μ L MBP-MshC (Fig. S1). Because the greatest enzymatic activity was observed for reactions carried out in Tris 8.0 with 20 ng/ μ L MBP-MshC, these conditions were chosen as initial velocity conditions to be used in the screen. In a separate set of experiments, the results of which are shown in Figure 2, apparent kinetic parameters K_m and V_{max} were determined individually for each substrate by fitting to the Michaelis-Menten equation reaction curves obtained when substrate concentrations were varied (see Materials and Methods). Apparent K_m values for cysteine, ATP, and GI were calculated as 140 ± 40 μ M, 220 ± 45 μ M, and 710 ± 170 μ M, respectively, whereas apparent V_{max} values for cysteine, ATP, and GI were 85 ± 7 nmol min⁻¹ mg⁻¹, 57 ± 4 nmol min⁻¹ mg⁻¹, and 83 ± 10 nmol min⁻¹ mg⁻¹, respectively.

Once initial velocity conditions were established and K_m values determined, the concentrations of substrates and enzyme were adjusted to obtain maximum dynamic range in the luminescent assay while maintaining initial velocity conditions. For this purpose, the effect on luminescence when varying the concentration of GI (1.56–200 μ M), ATP (40–100 μ M), or MBPMshC (2.5–80 ng/ μ L) was measured in 384-well plates. The concentration of substrates and enzyme that resulted in a maximal signal while being in the linear range of the ATP calibration curve was determined to be 100 μ M ATP, 60 μ M GI, and 20 ng/ μ L MBP-MshC (see Fig. S2 at <http://jbx.sagepub.com/supplemental>). Optimal assay conditions were consequently defined as 25 mM Tris 8.0, 1 mM DTT, 1 mM MgCl₂, 100 μ M ATP, 60 μ M GI, 100 μ M cysteine, and 20 ng/ μ L (225 nM) MBP-MshC.

Because most of the compounds in any given screening library are prepared as DMSO stocks, assay optimization further included determining the effect of DMSO on MBP-MshC activity. Using the final conditions outlined above, we found that enzyme activity was not affected when DMSO was present in the reaction mixture at concentrations as high as 5% DMSO. However, an increase in enzymatic activity was observed in the presence of 10% DMSO. Thus, we set an upper limit on DMSO concentration at 5%.

To assess robustness of the optimized assay for the HTS format, we calculated the Z' factor, S/B, S/N, and well-to-well variability (CV) of signal readout for a representative assay carried out in a 384-well plate format. The assay was performed in the presence and absence of 1 μ M CysSA, a known MshC inhibitor used as a positive control.^{18,19} The statistical parameters were calculated as $Z' = 0.65$, S/B = 1.7, S/N = 23.3, and CV = 2.9%, indicative of good assay performance.¹² To further assess suitability of the assay in identifying MshC

inhibitors, we measured a dose-response curve for CysSA (Fig. 3), giving an IC_{50} value of 240 ± 1 nM. Using the fluorescence- detected HPLC assay for detection, we previously measured an IC_{50} value of 50 nM for CysSA under slightly different assay conditions.¹⁸ Together these results indicated that the assay protocol was suitable for HTS.

Screening

A library of 3100 compounds was screened in a 384-well plate format following the protocol summarized in Table 1. All plates contained 16 wells each of 1 μ M CysSA and 0.1% or 1% DMSO, used as positive and negative controls, respectively. A counterscreen was run in parallel to identify luciferase inhibitors. This counterscreen was carried out using the same conditions as those used in the main screen except that GI and MBP-MshC were omitted and the volume brought up with enzyme storage buffer. In both assays, test compounds were evaluated at 10 μ M in the presence of 0.1% or 1% DMSO. The primary screen performed well, with Z' values ranging from 0.58 to 0.79. A graphical representation of the screening results is shown in Figure 4. Compounds showing greater than 30% inhibition (calculated using formula 1) were considered preliminary hits. Analysis of the screening results obtained from the primary screen versus the counterscreen showed 15 (0.48%) compounds to be hits and 51 (1.6%) compounds to be luciferase inhibitors. Both hits and luciferase inhibitors were further evaluated in the confirmatory assay in the presence and absence of 0.1% Triton X-100 to confirm inhibition of MshC, identify aggregate-based promiscuous inhibitors,²⁰ and investigate whether any of the luciferase inhibitors also inhibited MshC. The confirmatory assay revealed that none of the luciferase inhibitors inhibited MshC. Moreover, of the 15 MshC hits identified in the screening, 2 were confirmed in the HPLC assay (NSC 166454; **1** and NSC 218439; **2**). Their structures are shown in Figure 5. Importantly, the remaining 13 hits were found to be false positives that depleted cysteine in the reaction mixture, making it unavailable for the ligase reaction.²¹ This mechanism was confirmed for all 13 compounds by quantifying residual cysteine in reactions conducted in the presence and absence of MBP-MshC by HPLC. In all cases, cysteine was depleted in the absence of MBP-MshC. Not surprisingly, most of the structures of these false positives contained a quinone moiety. Quinones are known to generate reactive oxygen species through redox cycling, whereas partially substituted quinones can undergo Michael addition, reacting with nucleophiles such as cysteinyl thiols.²² Thus, it is possible that depletion of cysteine can be occurring from oxidation to cystine in the presence of quinones or by reacting directly with electrophilic quinones during the ligase reaction. Finally, none of the luciferase inhibitors showed activity against MBP-MshC, and none of the identified hits were aggregation-prone promiscuous inhibitors.

Characterization and mode of inhibition of MshC hits

The structures of the confirmed MshC hits NSC 166454 (**1**) and NSC 218439 (**2**) are shown in Figure 5. NSC 166454 is the known compound dequalinium chloride. Dose-response curves for **1** and **2** were measured using the quantitative CGI-HPLC assay, providing respective IC_{50} values of 24 and ~ 500 μ M. The structures of **1** and **2** share some similarity in that both contain an N-substituted quinolinium moiety (Fig. 5). To begin to address the role of the quinolinium group and its substitution pattern in inhibiting MBP-MshC, we measured dose-response curves of simpler quinaldines **1a–c** using the confirmatory HPLC assay. All 3 dequalinium substructures were found to only weakly inhibit MshC, with IC_{50} values of 830, 1400, and 4200 μ M, respectively. Remarkably, even at these high concentrations, each of these compounds gave rise to sigmoidal dose-response curves whether tested in the presence or absence of detergent. These results suggest that the second quinaldinium ring and presence of an alkyl chain (here C_{10}) are required for inhibition of MshC by this class of compound. However, with respect to ligand efficiency, a parameter that provides a measure of potency in relation to molecular weight,²³ the K_D of 0.22 μ M

yields a value of 0.25 kcal per non-hydrogen atom for dequalinium, indicating that a smaller analog may be equally potent. The structures further suggest that substitution on the ring nitrogen may be required for inhibition but do not confirm whether a methyl group will suffice or a longer alkyl chain is required. It is interesting to note that although they are completely unrelated proteins, these results are similar to previous studies that analyzed structure-activity relationships for dequalinium analogs as protein kinase C (PKC) inhibitors.²⁴

The mechanism of inhibition of MBP-MshC by dequalinium was investigated using the approach described by Lai and Wu.¹³ In this method, changes in percent inhibition by a given inhibitor as a function of varying a substrate's concentration reveal the mode of inhibition. The effect on the inhibition of MBP-MshC in the presence of varying concentrations of ATP and GI is shown in Figure 6. As the concentration of ATP was increased, the extent of inhibition by dequalinium decreased, indicating dequalinium is an AT P-competitive inhibitor of MshC.^{13,14} The opposite effect was observed with increasing concentrations of GI, indicating synergy with dequalinium, whereas no significant change was observed with increasing concentrations of cysteine (data not shown), suggesting noncompetitive inhibition.^{13,14} Given the mechanism for mycothiol ligase recently established, these results may indicate that in the presence of an AT P-competitive inhibitor, early stage binding of GI may occur and facilitate binding of dequalinium.

Equilibrium association constants and stoichiometry of dequalinium binding to MBP-MshC and the fusion protein MBP were investigated using ITC. In the case of MBP-MshC, the binding isotherms could only be fit to a 2-site model, giving equilibrium dissociation constants (K_D) of $0.21 \pm 0.04 \mu\text{M}$ and $7.1 \pm 0.8 \mu\text{M}$. In contrast, binding isotherms for titration of dequalinium to the MBP tag alone could be fit to a 1-site model, only giving a K_D of $8.5 \pm 1.5 \mu\text{M}$. These results indicated that dequalinium binds MshC with high affinity (K_D 0.22 μM) and to the MBP tag with lower affinity (K_D ~7–8.5 μM). Given the IC_{50} value of ~24 μM for dequalinium, it is likely that secondary binding events, such as those to MBP, affect inhibition of MshC by this compound.²⁴

Bacteriostatic activity has been reported previously for dequalinium and related compounds.^{25–27} Given its ability to inhibit mycothiol ligase, an enzyme essential to *M. tuberculosis*, we evaluated antimicrobial activity of dequalinium against a panel of clinically significant microorganisms, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococci faecium*, *Candida albicans*, and *M. smegmatis* and *M. tuberculosis* using methods described previously.¹⁶ As summarized in Table 2, dequalinium inhibited the growth of gram-positive bacteria and *C. albicans* and displayed the highest potency against MRSA and *M. smegmatis*. As anaerobic growth of *M. tuberculosis* has been associated with persistence,^{28,29} dequalinium also was tested against *M. tuberculosis* under aerobic and anaerobic growth conditions, giving minimum inhibitory concentration (MIC) and anaerobic bactericidal concentration values of 1.2 and 0.3 $\mu\text{g/mL}$, respectively.

We have identified dequalinium as a new inhibitor of mycothiol ligase. However, dequalinium is known to display a wide range of biological activities,^{24–27,30–32} consistent with the findings reported here, and is used commercially in some countries as a general antiseptic.³³

CONCLUSIONS

We have developed a screening protocol for the identification of mycothiol ligase inhibitors that is suitable for HTS ($Z' \geq 0.65$). Application of this protocol in screening a library of

3100 compounds led to the identification of a new mycothiol ligase inhibitor, dequalinium chloride, that inhibits MshC with an IC_{50} value of 24 μ M. Dequalinium was shown to inhibit MshC in an AT P-competitive manner and to be synergistic with the substrate Gl. Dequalinium binds MshC with high affinity (K_D 0.22 μ M) and inhibits the growth of *M. tuberculosis* under both aerobic and anaerobic conditions with MIC and anaerobic bactericidal concentrations of 1.2 and 0.3 μ g/mL, respectively. Our finding that some thiol reactive compounds, most notably quinones, can deplete cysteine during this ligase reaction will facilitate identification of true hits when this luminescence assay is used for screening larger compound libraries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

Biosynthesis of mycothiol. MshC catalyzes the adenosine triphosphate (ATP)-dependent ligation of cysteine to glucosamine-inositol (GI) to yield cysteine-glucosamine-inositol (CGI), which is acetylated by MshD to produce mycothiol.

FIG. 2.

Initial velocity curves fit to the Michaelis-Menten equation. The reactions were monitored by quantifying cysteine-glucosamine-inositol (CGI) formation in 25 mM Tris 8.0 and 20 ng/ μL maltose-binding protein/mycothiol ligase (MBP-MshC) at 25 °C, as described in the Material and Methods section. Apparent K_m and V_{max} values for cysteine, adenosine triphosphate (ATP), and glucosamine-inositol (GI) were calculated as $140 \pm 40 \mu\text{M}$, $220 \pm 45 \mu\text{M}$, and $710 \pm 170 \mu\text{M}$, respectively. Apparent V_{max} values for cysteine, ATP, and GI are $85 \pm 7 \text{ nmol min}^{-1} \text{ mg}^{-1}$, $57 \pm 4 \text{ nmol min}^{-1} \text{ mg}^{-1}$, and $83 \pm 10 \text{ nmol min}^{-1} \text{ mg}^{-1}$, respectively.

FIG. 3.

Dose-response curve of CysSA (0.5–5000 nM) and maltose-binding protein/mycothiol ligase (MBP-MshC). The reaction was carried out in 25 mM Tris 8.0 in the presence of 1 mM dithiothreitol (DTT), 1 mM MgCl_2 , 100 μM adenosine triphosphate (ATP), 60 μM glucosamine-inositol (GI), 100 μM cysteine, and 20 ng/ μL (225 nM) MBP-MshC at 25 °C for 60 min.

FIG. 4.

Graphical representation of the high-throughput screening (HTS) luminescence assays. A library of 3100 compounds was screened at 10 μ M in a 384-well plate format (Z' values ranged from 0.58 to 0.79). Each plate contained 16 wells each of a positive (1 μ M CysSA) and negative control (0.1% or 1% DMSO). Positive controls are grouped in the beginning of the graph for easy visualization. Compounds that inhibited greater than 30% were considered hits. Fifteen mycothiol ligase (MshC) hits (0.48%) and 51 luciferase inhibitors (1.6%) were ultimately identified by the confirmatory assay. None of the hits were found to be MshC activators.

FIG. 5.

Structures and dose-response curves for compounds evaluated in this study. (a) Compounds 1 (dequalinium chloride) and 2 are confirmed hits for maltose-binding protein/mycothiol ligase (MBP-MshC), whereas compounds 1a (1-methyl 4-aminoquinaldinium iodide), 1b (1-methyl quinaldinium iodide), and 1c (4-aminoquinaldine) are substructures of 1. (b) Dose-response curves of dequalinium (1) and substructures 1a, 1b, and 1c obtained from the confirmatory high-performance liquid chromatography (HPLC) assay. None of the compounds showing activity were fluorescent.

FIG. 6.

Effect on the inhibition of maltose-binding protein/mycothiol ligase (MBP-MshC) by dequalinium as a function of varying adenosine triphosphate (ATP; closed circles) and glucosamine-inositol (GI; open circles) concentrations. As the concentration of ATP increases, the extent of inhibition decreases, indicating competitive inhibition. The opposite effect is observed with increasing concentrations of GI, characteristic of synergism.

Table 1

High-Throughput Screening Protocol

Step	Parameter	Value	Description
1	2.5× substrate mix	10 µL	62.5 mM Tris 8.0 2.5 mM MgCl ₂ 2.5 mM DTT 250 µM ATP 250 µM cysteine
2	Reagent	5 µL	300 µM GI
3	Test compound	5 µL	50 µM
4	Enzyme	5 µL	100 ng/µL
5	Incubation	60 min	Room temperature
6	Reagent	25 µL	Kinase-Glo [®] Plus
7	Incubation	10 min	At room temperature
8	Assay readout	0.1 s integration time	Victor 1420 Multilabel Counter

ATP, adenosine triphosphate; DTT, dithiothreitol; GI, glucosamine-inositol.

Table 2

Antimicrobial Activity of Dequalinium versus Control Antibiotics

Organism	Zone of Inhibition (mm) ^a	
	Dequalinium (50 µg)	Control ^b
<i>Escherichia coli</i>	0	21
<i>Pseudomonas aeruginosa</i>	0	14
<i>Bacillus subtilis</i>	11	21
<i>Staphylococcus aureus</i>	9	14
Methicillin-resistant <i>S. aureus</i>	16	19
Vancomycin-resistant <i>Enterococcus faecium</i>	14	17
<i>Candida albicans</i>	13	11
<i>Mycobacterium smegmatis</i>	18	14
Aerobic MIC (µg/mL) ^c		
<i>Mycobacterium tuberculosis</i>	1.2	0.4
Anaerobic bactericidal concentration (µg/mL) ^c		
<i>M. tuberculosis</i>	0.3	17.2

MIC, minimum inhibitory concentration.

^aDetermined by the disk diffusion method.¹⁵

^bControl drugs: 50 µg ampicillin (*E. coli*), 50 µg kanamycin (*P. aeruginosa*, *S. aureus*), 50 µg chloramphenicol (*B. subtilis*), 35 µg nitrofurantoin (methicillin-resistant *S. aureus*, vancomycin-resistant *E. faecium*), 35 µg amphotericin B (*C. albicans*), 15 µg rifampicin (*M. smegmatis*), rifampicin (*M. tuberculosis*, aerobic growth), metronidazole (*M. tuberculosis*, anaerobic growth)