Discovery of bicyclic inhibitors against menaquinone biosynthesis

Introduction: Menaquinone is used for transporting electrons and is essential for the aerobic and anaerobic respiratory systems of all pathogens and prokaryotes. Many Gram-positive bacteria use only menaquinone in the electron transport system. Thus, menaquinone biosynthesis is a potential target for the development of inhibitors against bacteria including drug-resistant pathogens. **Results:** After modeling, synthesis and *in vitro* testing, we determined that 7-methoxy-2-naphthol-based inhibitors targeted the MenA enzyme of the menaquinone biosynthesis pathway. The developmental compounds **1** and **2** were active against *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus* with a minimal inhibitory concentration of 3–5 µg/ml. **Conclusion:** Nontraditional bicyclic inhibitors, compounds **1** and **2** could serve as lead compounds for the development of an antimicrobial agent, with activities against *M. tuberculosis* and methicillin-resistant *S. aureus*.

Traditional antibiotics generally act upon cell wall synthesis (penicillins, β -lactams and glycopeptides), protein synthesis (tetracyclines, aminoglycosides and macrolides) or nucleic acid synthesis (fluoroquinolones and rifamycins). Bacteria are capable of using multiple different drug-resistant mechanisms to bypass the effect of antibiotics. The increasing frequency of **drug-resistant pathogens** has tremendous impact in the field of drug development and has inspired scientists to find new targets to fight the emerging threats around the world. The emergence and spread of the drug-resistant infectious diseases is a major obstacle for prevention and control efforts. For example, even though the death rate from *Mycobacterium tuberculosis* (*M. tb*) infection decreased 45% between 1990 and 2013, out of the 9 million *M. tb* in 2013 cases, 1.5 million still resulted in death [1]. Extensively drug-resistant tuberculosis (XDR-TB), multidrug-resistant TB (MDR-TB) and HIV-TB coinfection have all contributed to the observed high death rate.

The appearance of new drug-resistant mechanisms increases the importance of developing nontraditional inhibitors to treat

individuals infected with drug resistant organisms. Recently, the electron transport system has been identified as a potential target for the development of antibacterial drugs. The lipid-soluble electron carriers, ubiquinone and menaquinone (MK), play essential roles in electron transport coupled adenosine triphosphate (ATP) synthesis [2]. MK transfers electrons under aerobic or anaerobic respiration in most Gram-positive bacteria while ubiquinone is responsible for electron transfer under aerobic conditions and MK under anaerobic condition in Gram-negative bacteria [3]. Since MK biosynthesis does not occur in human cells, this pathway is an excellent target for the development of new antimicrobials to overcome multidrug resistance, particularly against Gram-positive bacteria.

There are two known biosynthetic pathways for MK biosynthesis, the classical MK pathway and the futasine pathway (Figure 1) [4]. Both routes begin with chorismate; however, the precursors for MK biosynthesis are different in both pathways. In the classical MK pathway, 1,4-dihydroxy-2-naphthoate is derived from chorismate by Men FDHCEB enzymes and MenI [5] is

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Key terms

Drug-resistant bacteria: Pathogens with ability to grow in presence of antibiotics.

Bicyclic: A chemical structure that features two fused rings.

Minimal inhibitory concentration: Value represents inhibition of pathogen growth by >90%.

converted to an unmethylated MK by 1,4-dihydroxy-2-naphthoate prenyltransferase (MenA), followed by methylation by MenG/UbiE to produce the final product MK. In the alternative futasoline pathway, MK is biosynthesized from 1,4-dihydroxy-6-naphthoate, although unknown enzymes catalyze the final reaction [6]. Futasoline pathway is mainly used by some Gram-negative bacteria and has not been well studied to date.

Recent reports have reinforced the importance of enzymes involved in the MK biosynthesis pathway as potential targets for novel molecules to fight multidrug resistant Gram-positive pathogens [7,8]. Lately, inhibitors of *M. tb* 1,4-dihydroxy-2-naphthoyl-CoA synthase (MenB) inhibitors have been reported [7]. In a subsequent mechanism-based narration, o-succinylbenzoate-AMP-based analogs were identified as inhibitors of acyl-CoA synthetase (MenE) [9]. Earlier, benzophenone-based compounds were also reported to show MenA inhibitory activities against Gram-positive

bacteria including *Mycobacterium* species [10,11]. These findings indicated that MenA is a valid target for developing agents against Gram-positive bacteria and that this MenA inhibitor was active against the related *Mycobacterium* species [10]. However, the reported lead compound was toxic due to the presence of a benzophenone moiety [12,13]. A subsequent report described that the modification of benzophenone also has the potential to degrade and generate a toxic compound that results in its loss of activity [11].

Results & discussion

In an attempt at finding and designing safe and potential MenA inhibitors, we screened commercially available compounds for compounds with a structural match to dihydroxy naphthoate (DHNA). DHNA is a substrate for the MenA enzyme and a compound similar to its structure will be a potential competitor, engaging with the MenA enzyme active site and reducing its activity. Interestingly, after the overlay experiment in ChemBio3D (see [Supplementary Material](#)), we found 7-methoxy-2-naphthol to be highly similar to DHNA. Also, 7-methoxy-2-naphthol is non-toxic compared with quinone. In the current study, we used 7-methoxy-2-naphthol as a core structure to develop antimicrobials against Gram-positive bacteria. The transmembrane protein MenA is predicted to be Mg^{2+} dependent and have two characteristic conserved aspartate-rich motifs. Mutations of residues in

Figure 1. Classical menaquinone and alternative futasoline pathways from chorismate.

MenC: o-succinylbenzoate synthase; MenD: 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase; MenF: Isochorismate synthase; MenH: (1R, 6R)-6-hydroxy-2-succinylcyclohexa-2, 4-diene-1-carboxylate synthase; MqnA: Futasoline synthase; MqnB: Futasoline hydrolase; MqnC: dehypoxanthinyl futasoline cyclase; MqnD: 1,4-dihydroxy-6-naphthoate synthase.

Figure 2. Synthesis of 7-methoxy-2-naphthol derivatives by alkylating with dibromo alkane followed by amination. Reagents and conditions: (A) K_2CO_3 , DMF, dibromooctane, (B) K_2CO_3 , MeCN, N-methylallylamine or piperidine.

these two conserved motifs of *Escherichia coli* MenA resulted in loss of enzyme function, confirming that aspartates are crucial for activity [14]. Thus, an amine group was introduced at the end of the carbon chain of an inhibitor to interact with aspartates in the active site and with the **bicyclic** backbone. First, we synthesized approximately 25 compounds containing a 7-methoxy-2-naphthol moiety as described in Figure 2. This class of compounds possesses a long saturated carbon chain that mimics the prenyl group of demethylmenaquinone. The 7-methoxy-2-naphthol-based compounds were prepared in two easy steps and provided >50% yield. In contrast, other reports describing the generation of benzophenone-based inhibitors required many complex steps and only provided <5% yields [10]. Briefly, our lead compound was synthesized from commercially available 7-methoxy-2-naphthol by two consecutive substitution reactions. Initially, the naphthol was alkylated with dibromooctane using

K_2CO_3 in dimethylformamide (DMF), which provided the 2-*o*-alkylated naphthol in high yield. The alkylated naphthol was then aminated with N-methylallylamine or piperidine in the presence of K_2CO_3 to generate the final product **1** or **2** in high yield, respectively (Figure 2). The synthesized compounds **1** and **2** were characterized and confirmed by both nuclear magnetic resonance (NMR) and mass spectrometry (see **Supplementary Material**).

The compounds **1** and **2** showed better microbial growth inhibitory activities against *M.tb*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus anthracis* (Table 1). As expected, these 7-methoxy-2-naphthol derivatives showed good inhibition against most of the bacteria evaluated. Both compounds **1** and **2** exhibited considerable inhibitory activity against *M.tb* with a **minimal inhibitory concentration** (MIC) value of 3 $\mu\text{g/ml}$. Compounds **1** and **2** also substantially inhibited MRSA with MIC values of 5 and 3 $\mu\text{g/ml}$.

Table 1. Antimicrobial activities of compounds 1 and 2 with different bacterial strains.

Species and strains	MIC ($\mu\text{g/ml}$)	
	Compound 1	Compound 2
<i>Mycobacterium tuberculosis</i> H37Rv	3	3
MRSA high resistance [†]	5	3
<i>Bacillus anthracis</i> Sterne	25	7.5
<i>Yersinia pestis</i> A1122	50	75
<i>Burkholderia cepacia</i> ATCC 25608	>150	100
<i>Burkholderia thailandensis</i> ATCC 700388	>150	50
<i>Francisella tularensis</i> subspecies holarctica ATCC 29684 (live vaccine strain)	>150	12.5
<i>Klebsiella pneumonia</i> ATCC BAA-1705	>150	100

The compounds 1 and 2 showed good MIC against *M. tb* and MRSA.
MIC: Minimal inhibitory concentration.

Table 2. *Mycobacterium tuberculosis* MenA enzyme inhibitory activities of compounds 1 and 2.

Compound	IC ₅₀ (μg/ml) [†]	Positive control (μg/ml)
6	6
5	6

The compounds 1 and 2 are tested against *Mycobacterium tuberculosis* MenA and also compared with the reported compound.
[†]IC₅₀ values were measured as reported before and the same reported compound, 4-(8-(Allyl(methyl)amino)octyloxy)-2-hydroxyphenyl) (4-chlorophenyl)methanone was used as positive control [10].

ml, respectively. MRSA is a Gram-positive agent causing severe infections in hospital and community environment [15]. Community-associated MRSA infection is emerging in recent times around the world, making it critical to develop safe, efficacious and cost-effective inhibitors against MRSA. Our results indicate that the 7-methoxy-2-naphthol-based inhibitors may be lead candidates for further development of antibiotics to treat MRSA infections. However, the compounds **1** and **2** did not have strong inhibitory activities against Gram-negative bacteria such as *Burkholderia cepacia* and *Klebsiella pneumonia* (Table 1). This outcome may be due to the presence of the ubiquinone or futasoline pathway in the process of respiration in these pathogens.

MenA enzymatic assays were performed in order to verify 7-methoxy-2-naphthol-based compounds as a MenA inhibitor in MK biosynthesis [10,11]. Promisingly, the compounds **1** and **2** reduced the activity of MenA with IC₅₀ values ranging from 5 to 6 μg/ml, respectively (Table 2). This enzymatic inhibition indicates that the reported compounds are indeed MenA inhibitors of MK biosynthesis. We also synthesized compound **2** with various chain lengths (n = 2–10). When n = 2, the compound showed poor IC₅₀ >12 μg/ml. Whereas, when we increased the chain length to n = 4, we noticed improvement in the IC₅₀ and when n = 6, we obtained the best IC₅₀ = 5 μg/ml. The eight carbon chain length (n = 6) also showed the best MIC (Table 3). However, when we tried to increase the chain length beyond eight carbons in the linker, we observed a decrease in IC₅₀, indicating that the compound is unable to bind completely to MenA. Also, it appears that the substitution on the naphthol backbone structure may influence the inhibitory effect on MenA activity. Those substitutions might play an

important role in increasing the MIC effect. We also synthesized and tested compounds having nitrogen exchanged with oxygen and secondary amine instead of tertiary amine at the terminal end. However, the modified compounds did not show MenA inhibition at a low concentration. Nevertheless, the lead optimization of these MenA inhibitors is ongoing.

In a recent study, the MIC and IC₅₀ results correlated mutually with an *in vitro* rescue study, where MK-deficient *M. tb* was unable to reach a MK concentration to a level that was required for survival, indicating the importance of MK [11]. In addition, a good correlation between MenA enzyme inhibition and *M. tb* growth inhibition by compounds **1** and **2** suggests that the electron transport chain and **menaquinone biosynthesis pathway** are critical for the survival of Gram-positive pathogens.

Since the MIC and IC₅₀ results for the MenA inhibitory compounds **1** and **2** were promising, we tested the toxicity of these inhibitors using peripheral blood mononucleated cells in a MTT assay [16]. Briefly, human monocyte-derived macrophages were cultured at a concentration of 1 × 10⁶ cells/ml and treated with inhibitory compounds (**1** and **2**) for 12 h at 37°C in 5% carbon dioxide. After loading the cell with inhibitor (300 μM), cells were washed with serum-free culture medium to remove excess compounds. Cytotoxicity over the subsequent 24 h was assessed using standard MTT assay procedures. Importantly, the inhibitors were nontoxic to peripheral blood mononucleated cells (Figure 3). Therefore, these compounds are lead candidates for development of nontraditional antibiotics that can be used to treat *M. tb* and MRSA infections. The solubility of compound **2** was found to be 0.33 mg/ml and its protein bounding was 96%. It also showed high permeability in Caco-2 cells with Pe = 7 × 10⁻⁶ cm/s by PercaptaTM (see SI).

Conclusion

In summary, novel 7-methoxy-2-naphthol-based MenA inhibitors were designed, synthesized and tested for microbial inhibitory activities. Exposure to com-

Key terms

Enzymatic inhibition: Targeting of key enzyme function as means to inhibit growth.

Menaquinone biosynthesis pathway: Biochemical process used in organisms to synthesize menaquinone.

Table 3. Minimal inhibitory concentration and IC₅₀ values of 7-methoxy-2-naphthol derivatives containing different chain lengths.

Compound	n	IC ₅₀ µg/ml	MIC µg/ml
3	2	>12	ND
4	4	12	ND
2	6	5	3
5	8	8	3
6	10	12	ND

MIC: Minimal inhibitory concentration. ND: Not determined.

pounds **1** and **2** yielded a low IC₅₀ against MenA and a low MIC against *M.tb* and MRSA. These findings demonstrate that 7-methoxy-2-naphthol-based molecules could serve as lead structures for the development of non-traditional inhibitors of *M.tb* and drug-resistant pathogens, such as MRSA. To improve inhibitory activities, various naphthalene-based molecules are currently being synthesized and tested for lead optimization against numerous drug-resistant pathogens.

Future perspective

Recently, the numbers of drug-resistant pathogens are rapidly increasing and will most likely continue to increase in the future. Therefore, it is very important to target drug-resistant pathogens by a novel methodology and novel antibiotic(s). Our drug, which is a nontraditional inhibitor, can be a potential novel antibiotic to treat drug-resistant pathogens. In addition, our MenA inhibitor is more specific to Gram-positive bacteria and is nontoxic. Our novel inhibitor has many possibilities for structure–activity relationship studies; for example, the bicyclic backbone can be substituted with many functional groups and terminal piperidine group activity can be easily enhanced with various substitutions.

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Ethical conduct of research

We received the human samples from an already existing collection and all samples were anonymized. Experiments with human samples are performed in full compliance with the NIH. The methods were carried out in accordance with the approved University of Nebraska Medical Center ethical guidelines. All experimental protocols were approved by Institutional Review Board # 162–93-FB with the title 'Leukapheresis of normal donors for use in studies of disease pathogenesis and therapy'. We have the informed written consent from all participants involved in this study. The authors state that they have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Figure 3. Cytotoxicity test for compounds **1** and **2**. The compounds **1** and **2** are not toxic and are comparable with the positive control without drug treatment. Data are shown as mean ± SEM for n = 3 replicate wells. SEM: Standard error of the mean.

Executive summary

- The manuscript introduces a novel and nontoxic bicyclic MenA inhibitor.
- The study indicates a nontraditional inhibitor.
- The lead compounds are active against *Mycobacterium tuberculosis*.
- The lead compounds are also active against methicillin-resistant *Staphylococcus aureus*.
- In future, it can be used to treat drug-resistant strains.

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