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Quinones as antimycobacterial agents

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Abstract—*Mycobacterium tuberculosis* is a serious worldwide health threat, killing almost 3 million people per year. Other mycobacterial species, especially *Mycobacterium avium*, are emerging pathogens in the immunocompromised population, most notably AIDS patients. These nontuberculous mycobacteria (NTM) are ubiquitous in the environment, and naturally resistant to many disinfection procedures. Treatment options are limited, and no new antibiotics have been developed against mycobacteria since the 1970s. There is a desperate need for new biocides and antibiotics to prevent and treat mycobacterial infections. A small aromatic compound library has been screened for effectiveness in growth inhibition or killing of mycobacteria. Four species, representing the *M. tuberculosis* complex, the slow-growing NTM, and the rapid-growing NTM were used. Active compounds had minimal inhibitory concentrations as low as 12.5 μg/mL, with the active component being a quinone. The primarily bactericidal activity observed represents a unique mechanism of action. A fluorescent assay involving *M. smegmatis* expressing gfp was analyzed as a rapid assay for predicting inhibitory activity, but failed to predict activity well. Our compounds may have significant utility as soluble biocides against mycobacteria and other hardy nosocomial pathogens.

1. Introduction

Mycobacterium tuberculosis is a serious worldwide health threat, killing 3 million people annually, with 8 million active cases per year. The World Health Organization estimates that a staggering 1/3 of the world's population is latently infected.[1] Current therapies are long (6–9 months for fully sensitive cases, longer for drug resistant), the drugs have serious side effects, and therapy does not kill latent bacilli. There are approximately 70 other species in the genus *Mycobacterium*, many of which are opportunistic pathogens in animals and humans. Other mycobacterial species, especially *Mycobacterium avium*, are emerging pathogens in the immunocompromised population, most notably AIDS patients. These nontuberculous mycobacteria (NTM) are ubiquitous in the environment, and naturally resistant to many disinfection procedures. Treatment options are limited, the drugs have significant side effects,[2] and no new antibiotics have been developed against any mycobacteria since the 1970s. There is an urgent need for new biocides and antibiotics to prevent and treat mycobacterial infections. The caseload of the NTM is rising, and each of the approximately 20 clinically important species have different drug susceptibility patterns. Treatment again is difficult and lengthy. In certain cases, antibiotic therapy is so ineffective that surgical resection is used that is, with *M. scrofulaceum* lymph node infections in children. While clarithromycin has good activity against *M. avium*, patients with resistant infections have a poor prognosis. With such a short list of antibiotics against these emerging pathogens, there is a serious need for development of new antimycobacterials.[3–6]

There are three clinically significant groups of mycobacteria; the *M. tuberculosis* and related organisms, *M. avium* and related slow-growing NTM, and the rapidly growing NTM (RGM). RGM are a group of species that are environmental bacteria, causing a variety of opportunistic infections. The doubling time of RGM ranges from 3 to 6 h, while that of slow-growing NTM is 12–24 h, and *M. tuberculosis* is 22–24 h. Each of these

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groups is different with respect to drug susceptibilities, and we have used four strains to represent these groups. *M. tuberculosis* H37Ra is a nonvirulent variant of H37Rv, the commonly utilized virulent laboratory strain, which has been fully sequenced.[7] It represents the *M. tuberculosis* complex, including *M. bovis*. *M. avium* strain MAC104 is a clinical isolate from a human AIDS patient, also fully sequenced (but not annotated) by The Institute for Genomic Research. It represents the *M. avium* complex (MAC), the most common slow-growing NTM isolated from infected humans. The RGM are represented by two strains. *M. fortuitum* strain 1820 is a clinical isolate from a leg wound of a human patient (R. Wallace, UTHC Tyler). *M. fortuitum* was used because it is the most commonly isolated species clinically of all the RGM. *M. smegmatis* strain mc^2-155 is a laboratory strain (W. Jacobs, Albert Einstein College of Medicine), chosen also as a representative of RGM because of its common use in research and ability to be genetically transformed.

Most of the compounds we examined are quinones, which are aromatic compounds present in bacteria and eukaryotes,[8] often involved in electron transport that is, ubiquinone. They have been widely employed as antifungal agents, and are natural defensive products in plants. They have also been utilized as broad-spectrum antibacterials, and are being investigated as anticancer agents.[9] The mechanism of toxicity is still under investigation, but two theories dominate the literature,[10] with some quinones proposed to exhibit one or both mechanisms. Redox cycling is the concept that the compounds catalytically cycle and generate oxidative radicals, such as hydrogen peroxide and superoxide, which then damage the cell. Alkylation is when quinones are activated inside cells and become covalently attached to proteins, DNA, or other targets. It should be noted that virtually all published studies examine toxicity in eukaryotic systems. There are no published reports to our knowledge examining the toxicity of quinones against the notable prokaryotic genus *Mycobacterium*. Determination of native quinone structures has been utilized as a taxonomic tool in mycobacteria.[11–13]

We are using small, directed compound libraries to identify molecular targets for antimicrobial development against mycobacteria. Discovery of these targets both reveals new targets for pharmaceutical development and also new insights into mycobacterial physiology.

2. Results and discussion

Twenty-eight compounds were screened for activity against mycobacteria; 14 of these generated by parallel synthesis, and 14 purchased for comparison purposes (Fig. 1 and Table 1). The 1,4-diketone quinone motif has strong inhibitory activity, with 1,2-naphthoquinone was 8-fold less active than 1,4-naphthoquinone (**9**). Compounds without this quinone structure had much lower activity. When the diketone motif itself was retained yet moved off of the aromatic ring, activity fell, as with benzil or DEHP. Thus the ketone groups themselves were not toxic. While adding a second conjugated ring mildly improved activity (*M. smegmatis* MIC of benzoquinone 463 μM, of **9** 316 μM), adding a third ring strongly decreased activity (anthraquinone MIC of 30,700 μM).

Addition of a hydroxyl group to the second ring in position R_3 enhanced activity. Plumbagin (**7**) was the most potent synthesized compound against RGM and MAC, with an MIC of 66 μM, while juglone (**8**) had an MIC against MAC of 72 μM. For comparison, kanamycin has an MIC against *M. smegmatis* of 4 μM, and against *M. avium* of 26 μM. Not only are compounds **7** and **8** the most toxic examined, their mechanism of killing may be novel. While the other quinone compounds (**3**–**6**, **9**) are bacteriostatic (4-fold or 8-fold difference in MIC and MBC), against *M. avium* **7** and **8** are bacteriocidal (identical MIC and MBC). Eleven naphthoquinone derivatives were reported to be bacteriostatic against *Staphylococcus*,[14] making the cidal activity we observed more unique and interesting. Plumbagin has lesser activity (100–500 μg/mL) against the gram negative species *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *Pseudomonas aeruginosa*.[15] However, activity increased 10–20-fold with inclusion of multidrug pump inhibitors, indicating these proteins protect the bacilli by extruding **7**. Extrusion pumps have also been identified in mycobacteria,[16–19] and our work is currently examining the role of these in quinone susceptibility. Plumbagin also has activity against the parasites *Leishmania*,[20] *Giardia lamblia*,[21] and the malaria etiological agent *Plasmodium*.[22] Plumbagin is a natural product from the plant genus Plumbago.[23,24]

The presence of alcohols on the second ring had a major effect on potency. Naphthazarin, which has two hydroxyl groups on the second ring (5,8-hydroxy), is 2-fold more effective than **7** (5-hydroxy). Future synthetic work will focus on a diversity of oxygen-containing functional groups on this ring.

Analysis of the relationship between the chemical properties of the compounds and the activity reveals three major points. First, the role of solubility is likely minor, since **9** has a 1.5-fold lower MIC than benzoquinone yet is 16.6-fold more soluble (11100 vs 668 mg/L, respectively, in water at 25 °C). Hydroquinone is 6.5-fold more soluble than benzoquinone yet has slightly less activity. Anthraquinone is 485-fold less soluble than **9**, while only 97-fold less toxic. Two factors counter low solubility, the unusually hydrophobic cell wall of mycobacteria[25] and the long incubation time of MIC determination (>3 days). The second major point is that the one-electron reduction potential of the quinones[8] does not predict activity either, as **9** (potential −140 mV), **3** (−240 mV), and benzoquinone (+99 mV) all have comparable MIC values. Third, the mechanism of killing appears to be different from that in mammalian cells. While **3** is similar in activity to **9** against mycobacteria, **3** is 10-fold less cytotoxic to rat hepatocytes in vitro.[26,8] Compound **7** is 8-fold more effective against

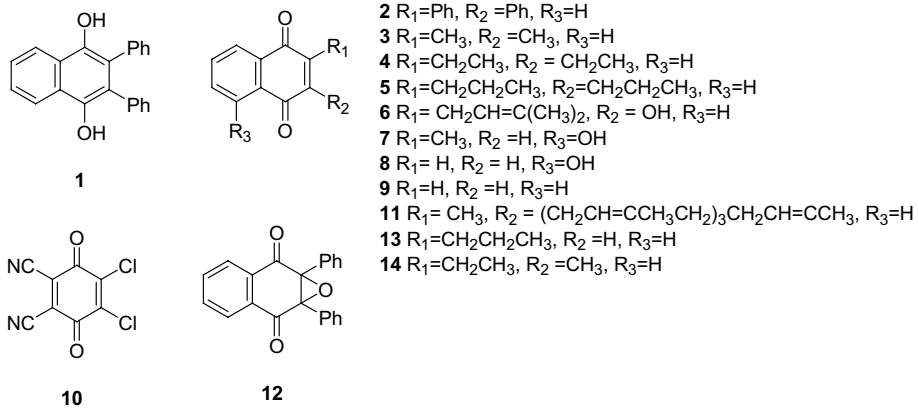

Figure 1. Structures of quinone discovery library and related compounds. All compound structures are based on the core structure (upper right), except for **1**, **10**, and **12** (shown individually).

Table 1. Toxicity measurements of compounds

Designation	Name	*M. smegmatis*	*M. avium*	*M. tuberculosis*
Benzoquinone	1,4-Benzoquinone	50/200	100/200	25/ND
Hydroquinone	1,4-Benzenediol	800/1600	1600/3200	25/ND
Benzil	1,2-Diphenylethanedione	3200/3200	800/3200	800/1600
Benzoin	2-Hydroxy-1,2-diphenylethanone	6400/6400	3200/3200	6400/6400
Benzoic acid	Benzenecarboxylic acid	800/6400	1600/3200	800/3200
Anthraquinone	9,10-Anthracenedione	6400/6400	6400/6400	6400/6400
Anthrone	9,10-Dihydro-9-oxoanthracene	>6400/>6400	ND	ND
Ethylanthraquinone	2-Ethyl-9,10-anthracenedione	3200/3200	6400/6400	6400/6400
Naphthoic acid	Naphthalenecarboxylic acid	3200/3200	3200/3200	200/400
Dioctylphthalate	DEHP	3200/3200	1600/6400	3200/6400
Dithizone	Diphenylthiocarbazone	3200/3200	400/800	400/800
Phenacetin	Acetophenetidine	>6400/>6400	800/3200	800/3200
1,2-Naphthoquinone	Beta-naphthoquinone	400/800	ND	ND
Naphthazarin	5,8-Dihydroxy-1,4-naphthoquinone	6.25/6.25	6.25/12.5	ND
1	2,3-Diphenyl-1,4-naphthalendiol	CBD/3200	CBD/3200	CBD/ND
2	2,3-Diphenyl-1,4-naphthoquinone	CBD/ND	CBD/ND	CBD/ND
3	2,3-Dimethyl-1,4-naphthoquinone	*100/200*	100/400	ND
4	2,3-Diethyl-1,4-naphthoquinone	*100/800*	200/1600	ND
5	2,3-Dipropyl-1,4-naphthoquinone	*50/400*	400/1600	ND
6	Lapachol	50/800	100/800	100/400
7	Plumbagin	12.5/50	12.5/12.5	400/800
8	Juglone	100/400	12.5/12.5	ND
9	1,4-Naphthoquinone	50/200	100/400	100/400
10	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone	100/800	800/800	400/800
11	Vitamin K3	6400/>6400	>6400/>6400	6400/6400
12	2,3-Diphenylnaphthoquinone oxide	50/200	ND	ND
13	2-Propyl-1,4-naphthoquinone	1600/6400	ND	ND
14	2-Ethyl-3-methyl-1,4-naphthoquinone	CBD/3200	ND	ND

All compounds with a numbered designation we synthesized, all others were purchased. Name is the accepted or IUPAC compound name. Under the species, the numbers given are MIC/MBC values. MIC and MBC are reported in micrograms per milliliter. >6400 means no effect observed, as this was the highest concentration tested. *M. smegmatis* strain was mc^2-155 (from William Jacobs, Jr., Albert Einstein College of Medicine), numbers in italics are for *M. fortuitum* strain 1820, a clinical isolate from Richard Wallace, University of Texas Health Center at Tyler. *M. avium* strain was MAC104, and *M. tuberculosis* strain was H37Ra. CBD is cannot be determined due to precipitation of compound in the media, and ND is not determined.

MAC than **9** but only 2.6-fold more toxic for rat hepatocytes. Thus, the toxicity levels are quite different between eukaryotes and mycobacteria. In addition, several compounds we have examined (**7**, **9**, **12**, and **13**) depolarize the mitochondrial membrane in L929 murine fibroblast cells.[27] Whether our compounds interact with the respiratory chain in mycobacteria is unknown, the natural quinone vitamin K3 (**11**) tested in our MIC assay was nontoxic (MIC > 6400 μg/mL).

There were some differences in MIC/MBC between the different species of mycobacteria. With the exception of **7** and **8**, *M. avium* was less sensitive to quinone compounds than *M. smegmatis*. This is in spite of the 3-fold

longer incubation time for MIC determination with *M. avium*. Mycobacteria are much less sensitive to hydroquinone than benzoquinone. Since hydroquinone can readily be oxidized to benzoquinone, the 16-fold difference in MIC we observed between the two compounds was surprising. Perhaps some cellular enzyme can detoxify hydroquinone via an undiscovered mechanism. Additionally, *M. tuberculosis* was more resistant to **7** than the other two species examined, presumably due to a similar yet species-specific reason. Further investigation is currently underway in our laboratories to shed light on the origin of the variances in sensitivity for these organisms.

Since mycobacteria are difficult and slow to grow (time to individual colonies of 3–14 days), we have examined a rapid fluorescence method for predicting the antibacterial activity of these compounds. A number of rapid assays have been developed recently to examine mycobacterial susceptibility to antibiotics and biocides, including luciferase, gfp, and lacZ based fluorescent assays.[28–43] The plasmid p996A461 conferring expression of green fluorescent protein (gfp) was electroporated into mc^2-155 and the fluorescence of this strain determined in the presence of compounds. A rapid (16 h as compared to 3 days for MIC growth) fluorescence assay was examined to determine the ability to predict the MIC of compounds against *M. smegmatis*. The control antibiotic kanamycin at 1/8 MIC gave 65% inhibition of fluorescence, while 1/4 MIC gave 91% inhibition. For all of the compounds (except benzoic acid and anthrone, not tested) listed in Table 1, the percent inhibition of fluorescence intensity showed a significant (p-value = 0.0047) positive linear correlation to the MIC (in micromolar). However, since the confidence limits for predicted MIC values were so large, the predictions are not useful to predict antimicrobial activity (the fluorescence assay cannot be substituted for the microbroth visual MIC determination).

Detergents are often combined with biocides to accentuate antimicrobial activity. Tween-80 (polyoxyethylene-sorbitan monooleate) improved MIC/MBC against *M. smegmatis* of benzoic acid, hydroquinone, and **7** by factors of same/8-fold, 8-fold/8-fold, and same/4-fold, respectively, (same means no improvement). Inclusion of Tween-80 presumably increases cell permeability to the compounds. Inclusion of 2% ethanol during compound screening had no effect with the same three compounds (ethanol MIC/MBC against *M. smegmatis* is 6.25%/25%), thus no synergy with this biocide was observed.

3. Conclusion

Certain naphthoquinone-based compounds, namely Plumbagin and juglone, have strong sterilizing activity against mycobacteria, potentially with a unique mechanism of action. In addition to discovery of potential molecular targets of these compounds, other uses being investigated for these compounds are as surface-attached or soluble biocides.

4. Experimental

4.1. Microbroth screening for minimal inhibitory concentration

Bacteria taken from exponential phase growth cultures were diluted to an optical density at 650 nm of 0.001 in 7H9-ADC media (Difco), to give approximately 10,000 bacteria in the 150 μL/well volume used in screening. Cells were placed in 96-well plates (Costar) and two-fold serial dilutions of compounds were made, with the first well at 6400 μg/mL, thus exposing bacilli to a 2048-fold range of concentrations. All MIC numbers came from at least two separate dilution series. Plates were incubated stationary at 37 °C until growth was visible (4 days for RGM, 2–3 weeks for MAC and MTB).

4.2. Minimal bactericidal concentration determination

The entire contents of the well read as the MIC and the three neighboring wells were plated onto 7H11 agar plates, and incubated as above. A colony count of 10 or less (99.9% lethality) was read as the MBC.

4.3. Fluorescent assay

Strain VC, a recombinant strain of *M. smegmatis* mc^2-155 carrying plasmid p996A461 (from Dr. David Carroll, UAMS) was grown in 7H9-ADC to exponential phase. This plasmid expresses gfp from the strong constitutive mycobacterial promoter rpsL. Bacteria were diluted to an optical density at 650 nm of 0.005 in 5 mL 7H9-ADC media, and compound added at a concentration of 10 μg/mL. After incubation for 16 h at 37 °C and 150 rpm, gfp fluorescence was determined (ex: 475 nm, em: 513 nm). Fluorescence inhibition was determined by comparison to controls without compound.

4.4. Compounds utilized for screening

Comparison compounds were purchased from Sigma–Aldrich and used as received. 2,3-Disubstituted naphthoquinones and naphthalendiols **1**–**5**, **12**–**14** were synthesized on solid support utilizing the Dotz reaction with solid supported Fischer carbene complexes as recently described.[44,45] Compounds were dissolved in DMSO at 20 mg/mL, stored frozen at −20 °C. DMSO itself has an MIC against *M. fortuitum*, MAC, and MTB of 12.5%.

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