Poster # 139

Use of 3F technology to inhibit *Mycobacterium tuberculosis*

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Abstract

The development of novel and effective countermeasures for pandemic containment and bioweapons are among the current priorities of applied research in Biodefense. To support this area of research, the 3F antimicrobial technology (Functional Fragrance Formulation) was developed for potential use in the decontamination of areas where pathogenic organisms are aerosolized or dispersed, by inhibition of known bacterial targets. This proprietary technology comprises a combination of 13 volatile compounds (VC) with known antimicrobial effects, including several terpenes and phenols known to inhibit quorum sensing, bacterial replication, and/or biofilm formation. Further, each component in the mixture is considered safe and has been approved for use in food and cosmetics. In this study, we tested the 3F antimicrobial for the ability to inhibit virulent and avirulent strains of *Mycobacterium tuberculosis* (MTB) when used in solution. Our results demonstrated a minimal inhibitory concentration (MIC) of 0.75% over 10 days *in vitro* against the two strains of MTB, and the assay was validated using isoniazid (IZ) and ethambutol (EB) antimicrobial standards. The low MIC provides evidence supporting further testing of the 3F antimicrobial technology to demonstrate activity on contaminated surfaces and against aerosolized MTB. The development of a non-toxic aerosol against MTB and other pathogens would provide an important resource for preventing and controlling the spread of tuberculosis and other infections, particularly in confined public spaces, in clinical settings and for defense agency use in deployments. Further testing of the 3F antimicrobial should be performed to explore these critical potential applications.

Introduction

Fragrances have been used since ancient times for cosmetic and medical purposes. Their properties have been exploited throughout historyas aromatic, antiseptic and preservation agents. Numerous studies have confirmed the medical properties of these compounds, and their chemical structures have been defined. Most of these compounds can be artificially synthetized, and derivatives have been studied. As summarized in **Table 1**, many of these compounds have demonstrated antimicrobial activities against bacteria and fungi (1-13). These compounds inhibit bacteria and fungi by blocking different targets of the quorum sensing system, cellular replication and/or preventing biofilm formation.

Both native (plant extracts and aromatic oils) and chemically produced fragrances and their derivatives have been widely tested and are approved to be used as food additives and/or cosmetics. Because of their volatile nature, these compounds aerosolize and spread easily covering large areas.

The combination of antimicrobial properties and easy aerosolization make these compounds obvious candidates for testing as countermeasures for pandemic and bioweapons containment. With this in mind, the 3F antimicrobial technology (Functional Fragrance Formulation) was developed. This proprietary technology is composed of a combination of 13 volatile compounds. Each compound is known to have antimicrobial effects against different microbes using different molecular mechanisms of action. 3F is anticipated to have inhibitory action against a wide range of microbes. In this study, 3F antimicrobial was tested for antimicrobial activity against MTB.

Table 1. Mechanism of action of some volatile compounds against microbial agents.

		Antimicrobial mechanism anti-				
Type	Base compound	Quorum sensing	Replication	Biofilm formation	Microbes inhibited	References
Terpenes	β-pinene	X			*Chromobacterium violaceum*	8
	Citral	X	X		*Cronobacter sakazakii, Staphylococcus aureus*	4
	Limonene	X		X	*Streptococcus pyogenes, S. mutans and S. mitis*	9
	Farrnesol	X		X	*Candida albicans*	7,2
	Linalool	X		X	*C. albicans; Shigella Flexneri, C. violaceum*	3,5,6
Phenols	Vanillin	X	X	X	*Escherichia coli, Pseudomonas putida, S. aureus*	10
	Cinnamal	X		X	*P. fluorescens, Vibrio Harveyi, P. aeruginosa*	3,11
	Eugenol	X		X	*E. coli, P. aeruginosa, C. violaceum*	3
	Thymol	X		X	*C. violaceum*	3

Methods

We prepared a broth microdilution (BMD) MIC assay using 3F antimicrobial against virulent and avirulent strains of *M. tuberculosis*. As controls, we included anti-mycobacterial standard drugs IZ and EB. *M. tuberculosis* avirulent strain H37Ra (ATCC® 25177™) and virulent strain H37Rv (ATCC® 27294™) were grown for ~21 days in Middlebrook 7H10 agar plates following routine procedures under biosafety containment. Isolated colonies were used to prepare a bacterial cell solution in Middlebrook 7H9 broth supplemented with OADC. The solution was adjusted to a turbidity equivalent to a 0.5 McFarland standard using a calibrated nephelometer. For each bacterial strain, 100 µL cell solution was transferred to a tube of 11 mL Middlebrook 7H9 supplemented with OADC broth, and used to prepare 96 well master plates. 3F antimicrobial stock was provided as a solution, and was considered as “100%” stock. For IZ and EB, 100 µg/mL stock solutions were prepared in sterile water.

Stock solutions were used to prepare serial dilutions in 96 well plates, using sterile Middlebrook 7H9 supplemented with OADC broth as diluent. The antimicrobial dilutions were aliquoted at 50 µL per well. Three test plates were prepared, one per antimicrobial (**Figure 1**). Each plate included negative and positive controls. Serial dilutions in each row were performed independently, representing replicates of the drug dilutions. Fifty microliter (50 µl) aliquots of bacterial solution was added per well to a final volume of 100 µL per well (**Figure 2**).

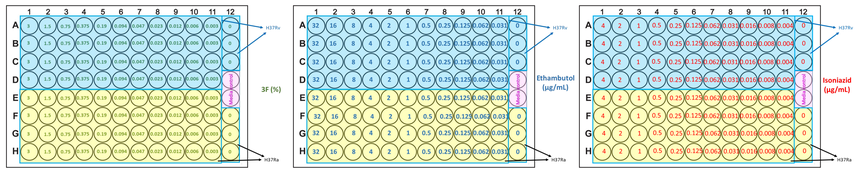


Figure 1: Map of 96-well test plates for 3F, IZ and Eb. Numbers correspond to the final concentration of antibiotic in each well: 3F is indicated in %, while IZ and EB are shown in µg/mL. Wells with a “0” correspond to positive growth control wells, and negative controls (non-inoculated media controls) are shown in pink. H37RV and H37Ra strains were inoculated in the well shaded in blue and yellow respectively.

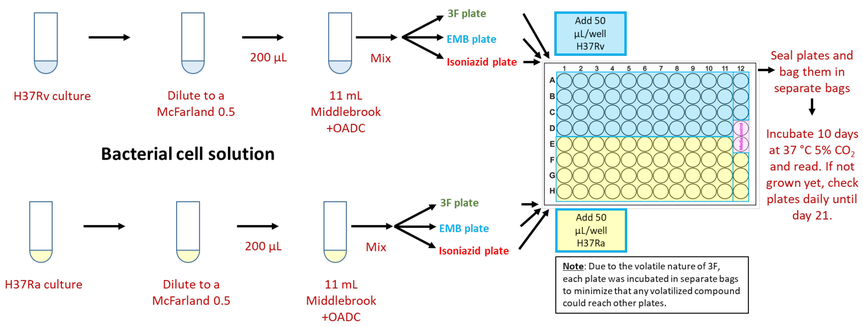
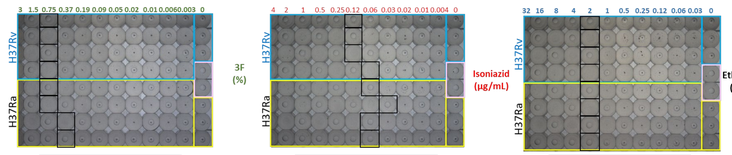


Figure 2: Preparation of bacterial cell solutions and inoculation of plates for BMD antimicrobial susceptibility testing.

Results

After 10 days of incubation plates were read. Negative controls (D12 and E12) were confirmed negative in all three plates, while positive controls (wells A12, B12, C12, F12, G12 and H12) were each positive for growth in all plates. Reading of growth in the wells containing each compound was then performed and interpretation of the MIC for each control antibiotic and test compound was determined (**Figure 3**).



3F (%)		
MTB	H37Rv	H37Ra
Replicate	0.75	0.75
	0.75	0.75
	0.75	0.375
	0.75	0.375
Final MIC	0.75	0.75*

Isoniazid µg/mL		
MTB	H37Rv	H37Ra
Replicate	0.125	0.063
	0.125	0.031
	0.125	0.063
	0.063	0.063
Final MIC	0.125*	0.063*

Ethambutol µg/mL		
MTB	H37Rv	H37Ra
Replicate	2	2
	2	2
	2	2
	2	2
Final MIC	2	2

Figure 3: Photodocumentation of plates after 10 days incubation. Numbers above each vertical column correspond to the concentration of antimicrobial. The inoculated MTB strain is indicated on the left of each plate, and black squares indicate wells with the lowest antimicrobial concentration in which no growth was observed for that particular serial dilution. This concentration corresponds to the MIC.
*__Note__: Not all results from replicates were identical, but all minimum positive growth results were within one log dilution, within BMD assay acceptance criteria. To confirm positive growth results, the plates were incubated for an additional week and were read again. Results showed no variation from initial reading.

Conclusions

- Virulent and avirulent stains of MTB were successfully tested for antimicrobial activity using the 3F antimicrobial fragrance test compound and IZ and EB standards. Validity of the BMD antimicrobial assay was confirmed by growth of the two MTB strains in inoculated positive control wells, and no growth in the negative control wells. The MICs determined for the antimicrobial standards IZ and EB were within the anticipated susceptibility range for the concentrations tested.
- The 3F antimicrobial fragrance test compound inhibited MTB growth at and above 0.75% of stock for both strains of MTB. Lower concentrations did not inhibit growth. The replicates produced consistent results and minor variations observed between replicates were within expected variance for BMD growth susceptibility test assays.
- The results provide evidence to support further testing of the 3F antimicrobial fragrance technology for potential applications such as antimycobacterial activity when applied to contaminated surfaces, and as an active volatile against aerosolized MTB.

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