Resistance Selection Studies with the Myeloperoxidase Enzyme System Against *Staphylococcus aureus*, *Enterococcus faecium*, and *Pseudomonas aeruginosa*

The Interscience Conference on Antimicrobial Agents and Chemotherapy
43rd ICAAC, Sept. 14-17, 2003
Chicago, IL USA
Paper # F-353

G. A. DENYS[1], S. WOODHEAD [2], S. BECQUERELLE [3], S. VALVANI [3], O. ABRIL-HORPEL [3], W. HAAG [3];
[1]Clarian Health Partners, Inc., Indianapolis, IN, [2]Ricerca Biosciences, Concord, OH, [3]Exoxemis, Inc., Little Rock, AR.

Contact Information
www.exoxemis.com

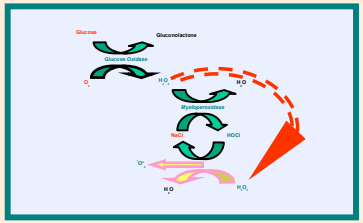
ABSTRACT

Background: Myeloperoxidase (MPO) plays an important role in the natural host defense system against pathogenic microorganisms. A cell free oxidant generating enzyme system containing MPO has been developed for local/topical use (ExOxEmis, Inc., Little Rock, AR). The MPO enzyme system shows cidal *in vitro* activity against a broad-spectrum of microorganisms. To access the potential for emergence of resistance in clinical use, the selection of resistance was investigated by *in vitro* serial passage.
Methods: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa*, ATCC 15442, and *Enterococcus faecium*, clinical strain-vancomycin resistant, were exposed to baseline concentrations of MPO system at 0.1 and 0.3 ug/ml. Late log to early stationary phase cultures were pelleted and resuspended in buffer to ~10^9 cfu/ml. A 1.0 ml volume of enzyme system with organism suspension at a final concentration of ~10^6 to 10^7 cfu/ml was tested for 60 min. The entire 1.0 ml test volume was then plated onto trypticase soy agar and incubated at 37ºC for 48 h. Growth from surviving organisms exposed to the highest tolerated concentration of MPO system was used to prepare the inoculum for the next resistance selection experiment carried out in a similar manner. A total of 30 serial passages for *S. aureus* and *E. faecium* and 25 serial passages for *P. aeruginosa* serial passages were performed.
Results: The MPO enzyme system did not select for resistant variants in *S. aureus*, *E. faecium*, and *P. aeruginosa*. The bactericidal concentrations did not increase 2-fold above the baseline. In the presence of sub-lethal concentrations of MPO system, *Staphylococcus* survivors appeared abnormal characterized by shrinkage in colony size while *Pseudomonas* colonies appeared normal.
Conclusion: These results suggest that the MPO enzyme system with its unique mechanism of action does not select for resistance.

INTRODUCTION

Myeloperoxidase (MPO) is a major neutrophil protein which is stored in azurophilic granules and released into the phagosome during phagocytosis. The enzyme uses superoxide anion and hydrogen peroxide generated by the neutrophil respiratory burst to form reactive oxidative species that cause cell damage[1]. A cell free oxidant generating enzyme system containing MPO has been developed which exploits this natural antimicrobial system (ExOxEmis, Inc., Little Rock, AR). The mechanism of activity of the MPO enzyme system (Figure 1) involves the use of hydrogen peroxide, generated by a balanced formulation *in situ*, to convert chloride into hypochlorous acid. Once generated, hypochlorous acid either participates in the direct halogenation of target cell components, or reacts with a second hydrogen peroxide molecule to yield singlet oxygen[2,3]. Singlet oxygen, a broad spectrum oxygenating agent, effects its microbicidal action via oxidative destruction of membrane integrity, oxidative inhibition of the enzymes required for metabolic function, and/or oxidative disintegration of nucleic acids required for reproduction. The half-life of signlet oxygen restricts its reactivity to within a 0.2 micron radius at the surface of the targeted bound pathogen.

Figure 1.
Myeloperoxidase enzyme system

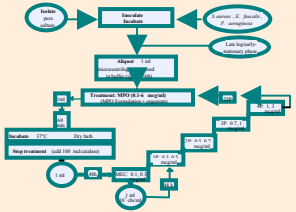


Previous studies have shown that the MPO enzyme system is rapidly bactericidal to an extensive range of microorganisms including multidrug resistant strains[4]. The potential for resistance development with the MPO enzyme system was investigated by *in vitro* mutation studies.

METHODS

Bacterial Strains and Culture Conditions: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, and a clinical strain of *Enterococcus faecium*, vancomycin resistant, were used in this study. Initial organism suspensions were prepared in a culture flask containing tryptic soy broth. A flask was placed on a rotating shaker set at 200 rpm and incubated in a programmable incubator set at 5 to 35°C for 11 hr to achieve late log to early stationary phase growth.
Mutant Selection Protocol (Figure 2): After the initial shake flask incubation, a 1ml aliquot of culture material was microcentrifuged and resuspended in buffer. This suspension in buffer was used for testing (10^7 cfu/ml). For initial treatment, a 1.0 ml volume of MPO (0.1 and 0.3 ug/ml) formulation plus organism suspension were added to the treatment vials. Treatment vials were incubated at 37°C in a dry bath. After 60 minutes, 100 ul of catalase was then added to each vial to stop the reaction, and the entire contents of each vial (1.0 ml) was cultured for 48 h on isolation media for growth. To achieve stable resistance, survivors (suspended in buffer) from the highest MPO isolation plate at 48 hours of incubation were used to prepare the inoculum (@10^7 cfu/ml) for the next series of resistance selection experiments. Each sequential experiment was performed at the highest previous MPO concentration supporting growth, and at approximately two times that concentration.

Figure 2.
Algorithm for selection for resistance to MPO enzyme system using stepwise *in vitro* exposure



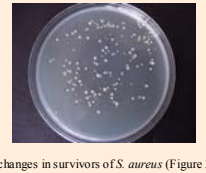
RESULTS AND DISCUSSION

Table 1. *In vitro* mutation rates obtained for the MPO enzyme system

	MPO (ug/ml)	Serial Passages	Mutation rate
S. aureus ATCC 6538	0.1, 0.3	30	$<10^{-7}$
E. faecium (VRE)	0.1, 0.3	30	$<10^{-7}$
P. aeruginosa ATCC 15442	0.1, 0.3	25	$<10^{-7}$

- Mutational studies were performed with the MPO enzyme system at baseline MPO concentrations of 0.1 and 0.3 ug/ml. Isolates of *S. aureus*, *E. faecium*, and *P. aeruginosa* were repeatedly exposed to MPO formulation (25-30 passages). After the final passage, no selection of MPO-resistant mutants occurred for all strains tested. The mutational frequencies for all three strains are $<10^{-7}$ (Table 1).
- The presence of vancomycin resistance in *E. faecium* did not appear to affect the mutational frequency.

Figure 3.
Morphology of surviving *Staphylococcus aureus* colonies after treatment with sub-lethal concentrations of MPO formulation.



- Morphological changes in survivors of *S. aureus* (Figure 3) when incubated with sub-lethal concentrations of MPO formulation resulted in abnormal growth, characterized by "shrinkage" of the bacterial cells. Upon subculture, both colony types appeared morphologically identical and retained its antimicrobial phenotype. These findings suggest that different target sites for the staphylococci may be involved when treated with MPO formulation.

CONCLUSION

- No MPO resistant variants of *S. aureus*, *E. faecium* and *P. aeruginosa* was selected after multiple serial exposures.
- No increase in baseline MPO treatment concentrations was observed for bactericidal activity.
- Low mutational frequencies observed and unique mechanism of action of the MPO enzyme system indicate a minimal potential for resistance development.
- MPO enzyme system warrants further investigation for prevention and treatment of infections.

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