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In Vitro Activity of Epetraborole, a Novel Bacterial Leucyl-tRNA Synthetase Inhibitor, in Drug Combinations Against Nontuberculous Mycobacteria Including Resistance Frequency and MIC Characterization of Mycobacterium avium ATCC 700898 Epetraborole-resistant Mutants

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ABSTRACT

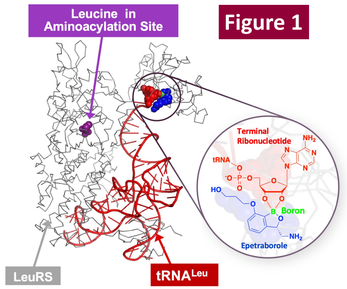
Background: Epetraborole is a boron-containing, oral inhibitor of bacterial leucyl-tRNA synthetase, an essential enzyme in protein synthesis; EBO demonstrates potent activity against nontuberculous mycobacteria (NTM). The standard of care therapy for *Mycobacterium avium* complex (MAC) lung disease consists of a combination of a macrolide, ethambutol (EMB) and a rifamycin. A total of 5 strains of MAC, *M. abscessus* ATCC 19977 and *M. peregrinum* ATCC 700686 were tested in checkerboard assays to evaluate the effects of combining EBO with other agents. The spontaneous resistance frequency (RF) was determined for EBO singly and in combination against M. avium ATCC 700898.
Methods: The effects of combining EBO with clarithromycin (CLR), rifabutin (RBT), EMB, amikacin (AMK) or bedaquiline (BDQ) were evaluated in 7 strains of NTM. Synergy, additive effects, indifference or antagonism was characterized in the checkerboard assay using EUCAST criteria. The RF of *M. avium* ATCC 700898 at 2x, 4x and 8x the MIC (8 mg/L) of EBO was determined, as was the RF of EBO combined with CLR, RBT, AMK or EMB. MICs of selected EBO mutants were determined against AMK, BDQ, CLR, RBT, EMB, and clofazimine (CFZ) and the mutants were further characterized.
Results: The RF of EBO ranged from $1.58x10^{-7}$ to $8.48x10^{-9}$ when selected on 2 - 8x agar MIC. The MIC for EBO increased 32->256-fold for the resistant mutants; however, the MICs for the other drugs tested against EBO-resistant strains did not change significantly. The activity of EBO was not antagonized, and was mainly indifferent to the addition of CLR, RBT, AMK, or BDQ for all the NTM strains tested. Synergy with EMB was observed with 2 strains and additivity with 2 additional strains of MAC. The addition of EMB (24 mg/L), CLR (32 mg/L), RFB (2 mg/L) or AMK (128 mg/L) to agar plates containing 2xMIC of EBO lowered the RF to at least < $2.13x10^{-10}$.
Conclusions: In the checkerboard studies, no evidence of antagonism was observed with any strain or EBO combination; interactions were largely indifferent. EBO combined with EMB in MAC resulted in synergy or additive effects in the checkerboard assay. The addition of EMB, CLR, RFB or AMK to EBO led to a >700-fold reduction in RF. Activity of other drugs was not impacted by EBO resistance suggesting that cross-resistance did not occur.

INTRODUCTION

There are an estimated 200,000 patients with non-tuberculosis mycobacterial (NTM) lung disease in the United States with many remaining undiagnosed. The number of cases is increasing by an estimated 8% per year. Among the approximately 55,000 patients diagnosed with NTM lung disease in the United States, approximately 44,000 patients have lung disease caused by *Mycobacterium avium* complex (MAC) and approximately 35% of these patients have treatment-refractory MAC lung disease. Treatment of these infections is difficult due to the long courses of therapy that require a multiple drug regimen. This required course of treatment poses the challenges of patient non-adherence, expense, potential drug interactions, side-effects and/or adverse events, development of drug resistance, inferior outcomes and relapse or reinfection. There are few new therapeutic agents in the drug pipeline, many of those are repurposed tuberculosis drugs or utilization of older therapeutic agents. The need for the development of new therapies to combat the growing number of patients with MAC disease is urgent.

EBO is a boron-containing, oral inhibitor of bacterial leucyl-tRNA synthetase, an essential enzyme in protein synthesis[1] (**Figure 1**). EBO demonstrates potent activity against nontuberculous mycobacteria (NTM). The standard of care therapy for MAC lung disease consists of a combination of a macrolide, EMB and a rifamycin. A total of 5 strains of MAC, *M. abscessus* ATCC 19977 and *M. peregrinum* ATCC 700686 were tested in checkerboard assays to evaluate the effects of combining EBO with other agents. The spontaneous resistance frequency was determined for EBO singly and in combination against *M. avium* ATCC 700898.

Figure 1



Methods

Minimum inhibitory concentration (MIC) determinations: MIC values for the putative EBO resistant mutants were determined by broth microdilution method (BMD) in cation-adjusted Mueller Hinton broth according to Clinical and Laboratory Standards Institute document M24-A3[2]. Agar MIC values were determined as essentially described by CLSI M24-A3 using 7H10 Middlebrook agar and 5% OADC.
Antibacterial synergy testing: The effects of combining EBO with clarithromycin (CLR), rifabutin (RBT), EMB, amikacin (AMK) or bedaquiline (BDQ) were evaluated in 7 strains of NTM. Synergy, additive effects, indifference or antagonism was characterized in the checkerboard assay using EUCAST criteria. Synergistic or antagonistic activity was determined using the sum of the fractional inhibitory concentration (ΣFIC) index. The FIC index is calculated as the sum of FIC A + FIC B, where FIC A is the MIC of drug A in the combination of drugs A and B divided by the MIC of drug A alone, plus the MIC of drug B in the combination of drugs A and B divided by the MIC of drug B alone. A combination of drugs is considered synergistic when the FIC is ≤ 0.5, additive when the FIC is > 0.5 to 1, indifferent when the FIC is >1 to 2, and antagonistic when the FIC is >2 using EUCAST criteria.
Spontaneous resistance frequency determination: The RF of *M. avium* ATCC 700898 at 2x, 4x and 8x the MIC (8 mg/L) of EBO was determined, as was the RF of EBO combined with CLR, RBT, AMK or EMB. MICs of selected EBO mutants were determined against AMK, BDQ, CLR, RBT, EMB, and clofazimine (CFZ) and the mutants were further characterized by genomic DNA analysis. Resistant colonies were confirmed by replica plating on agar plates containing antibiotic at the same concentration used to select resistance. Control plates containing no drug were prepared for inoculum determination. The RF was calculated by dividing the total CFU/mL of resistant colonies by the total CFU/mL of the inoculum.

RESULTS

The *in vitro* activity of EBO was tested in the presence of key components of the standard of care drugs for the treatment of MAC pulmonary disease, clarithromycin, ethambutol, rifabutin as well as other known active NTM drugs, amikacin and bedaquiline against 5 MAC strains and 2 rapidly-growing mycobacterial strains (**Table 1**). The activity of EBO was not antagonized by any of these drugs with any of the NTM strains we tested (**Table 2**). In most cases, especially for the two rapidly growing NTM strains, *M. abscessus* ATCC 19977 and *M. peregrinum* ATCC 700686, EBO activity was indifferent to the addition of a second drug. The sole exception was ethambutol where synergy was observed with 2 strains and additivity with an additional 2 strains out of a total of 5 MAC strains tested. Interestingly, the clarithromycin resistant strain, *M. intracellulare* 20-S-13, was the only MAC strain that showed indifference (**Table 2**) between ethambutol and EBO and this strain had the highest ethambutol MIC with a value of 64 mg/L (**Table 1**). The spontaneous resistance frequency for EBO ranged from $1.58x10^{-7}$ to $8.48x10^{-9}$ when selected on 2 - 8x agar MIC (**Table 3**), which was very similar to the resistance frequency observed for the SOC anitbacterials (**Table 4**). However, the addition of EBO to a single key SOC antibacterial significantly lowered the resistance frequency more than 700-fold to both drugs (**Table 4**). Further characterization of the EBO resistant mutants showed that the MIC value for the EBO increased 128-fold or greater, while the MIC values for amikacin, bedaquiline, clofazimine, clarithromycin and ethambutol did not change more than 4-fold (**Table 5**). The only drug tested that changed more than 4-fold from the wild-type MIC value was rifabutin but this was with a single EBO resistant mutant, 64-4A, which shifted 8-fold (**Table 5**). However, this was only with a single MIC value as its duplicate was only 4-fold different from wild-type. Since an 8-fold variance has been previously reported for this strain, *M. avium* ATCC 700898, with the related rifamycin, rifampin[3], we suggest that this 8-fold difference is within error.

Table 1. *In Vitro* Activity (mg/L)

Drug	*M. avium* ATCC 700898	*M. avium* 2285R	*M. chimaera* 20-S-05	*M. intracellulare* 20-S-13	*M. intracellulare* DNA000111	*M. abscessus* ATCC 19977	*M. peregrinum* ATCC 700686
EBO	2	0.5	2	0.25	0.5	0.06	0.06
CLR	2	0.125	4	>128	2	16	1
RFB	0.25	0.125	1	0.06	2	32	8
EMB	8	16	4	64	32	32	8
AMK	16	8	32	20	16	64	2
BDQ	0.06	0.03	0.06	>0.5	0.5	0.5	0.06

Table 2. Summary Activity of EBO Drug Combinations

Combination with EBO	*M. avium* ATCC 700898	*M. avium* 2285R	*M. chimaera* 20-S-05	*M. intracellulare* 20-S-13	*M. intracellulare* DNA000111	*M. abscessus* ATCC 19977	*M. peregrinum* ATCC 700686
CLR	I	AD	I	NE	I	I	I
RFB	I	I	I	I	AD	I	I
EMB	S	AD	AD	I	S	I	I
AMK	I	I	I	I	I	I	AD
BDQ	AD	I	I	NE	I	I	I

I = Indifferent, AD = Additive, S = Synergistic. NE = No endpoint due to MIC of second drug being out of range, CLR MIC > 128 mg/L, BDQ > 0.5 mg/L. No antagonisms were observed.

Table 3. *In Vitro* Resistance Frequency of EBO in *M. avium* ATCC 700898

Drug	Agar MIC (mg/L)	Selection Concentration (mg/L)	Resistance Frequency
EBO	8	16	1.58×10^{-7}
		32	1.21×10^{-8}
		64	8.49×10^{-9}

Table 4. *In Vitro* Resistance Frequency of SOC antibacterials alone and with EBO (2xMIC) in *M. avium* ATCC 700898

Drug	Agar MIC (mg/L)	Selection Concentration (mg/L)	Monotherapy Resistance Frequency	+EBO (2xMIC) Resistance Frequency
CLR	4	32	1.59×10^{-7}	$< 1.49 \times 10^{-10}$
RFB	0.25	2	1.03×10^{-8}	$< 1.49 \times 10^{-10}$
AMK	32	128	1.21×10^{-8}	$< 1.49 \times 10^{-10}$
EMB	8	24	3.13×10^{-7}	$< 2.13 \times 10^{-10}$

Table 5. MIC Values (mg/L) for EBO Selected Isolates of *M. avium* ATCC 700898 Compared to the Parent Strain

Drug	WT	64-3A	64-4A	32-5A	32-8A	16-3A	16-5A
EBO	0.5	>128	>128	>128	>128	>128	64
AMK	32	32	32	32	32	32	32
BDQ	0.06	0.12	0.06	0.03	0.12	0.06	0.06
CLR	2	0.5	2	1	0.5	0.5	1-0.5
CFZ	0.5	0.5	0.5	0.5	0.25	0.25	0.25
EMB	4	4	8	4	8	4	4
RFB	0.06	0.06	0.25-0.5	0.06	0.125	0.06	0.06

MIC values were determined by BMD as recommended by M24-A3[2].

CONCLUSIONS

- In the checkerboard studies, no evidence of antagonism was observed with any strain or EBO combination and interactions were largely indifferent
- EBO combined with EMB in MAC resulted in synergy or additive effects in the checkerboard assay
- EBO resistance frequency is similar to the SOC antibacterials
- The addition of EMB, CLR, RFB or AMK to EBO led to a >700-fold reduction in resistance frequency
- Activity of the key antimycobacterials tested was not impacted by EBO resistance suggesting that cross-resistance did not occur

REFERENCES AND ACKNOWLEDGMENTS

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