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Extended Spectrum Pleuromutilins: Potent Translation Inhibitors with Broad-Spectrum Antibacterial Activity *In Vitro* and *In Vivo*

S. Paukner, W.W. Wicha, W. Heilmayer, K. Thirring, R. Riedl [1]
[1] Nabriva Therapeutics AG, Vienna, Austria;

Nabriva Therapeutics AG
Lebersstrasse 20
1110 Vienna
Austria
www.nabriva.com
+43-1-74093-0
susanne.paukner@nabriva.com

ABSTRACT (amended)

Background:
Extended spectrum pleuromutilins (ESP) are a new generation of pleuromutilin (PM) antibiotics with chemical modifications at the C-14 side chain and the tricyclic PM core resulting in extending the antibacterial spectrum of conventional PMs to include a wide range of Gram-negative bacterial pathogens including *Enterobacteriaceae* in addition to staphylococci, streptococci, and fastidious Gram-negative bacteria.
A series of ESP (BC-7634, BC-9539, BC-9545 and BC-9543) were investigated for their 1) ability to inhibit bacterial transcription/translation (TT) of *E. coli* and *S. aureus*; 2) *in vitro* antibacterial activity; and 3) *in vivo* efficacy in a murine sepsis model induced by *E. coli* and *S. aureus*.

Methods: Inhibition of protein synthesis was measured by coupled *in vitro* TT using *E. coli* or *S. aureus* S30 extracts and a luciferase reporter gene. MICs were determined by broth microdilution according to CLSI (M7/A9). The therapeutic *in vivo* potency (ED_{50}) was evaluated in mice infected with *E. coli* or methicillin-susceptible *S. aureus* via i.p. injection causing lethal sepsis within 24 h.

Results: All tested ESP were potent inhibitors of *in vitro* TT with IC_{50} of 0.58-0.61 µM for *E. coli* and 0.35-0.39 µM for *S. aureus*. ESP derivatives demonstrated *in vitro* activity against a broad spectrum of organisms (see Table 1). The corresponding derivative without PM core substitution showed activity against *S. aureus*, *S. pneumoniae*, and *H. influenzae* but not against *Enterobacteriaceae* (MIC ≥ 32 µg/mL).
In the sepsis model all tested ESP displayed potent *in vivo* efficacy against both *S. aureus* and *E. coli* with ED_{50} of 0.09-1.43 mg/kg/day and 3.46-8.98 mg/kg, respectively. Against *S. aureus* the *in vivo* efficacy was comparable to tigecycline (ED_{50} of 0.99 mg/kg) and superior to that of linezolid (10.3 mg/kg).

Conclusions: ESP are a new generation of PM with broadened Gram-negative antibacterial activity compared to conventional PM and warrant further evaluation as broad spectrum antibiotics.

Table 1. Antibacterial *in vitro* activity of ESP

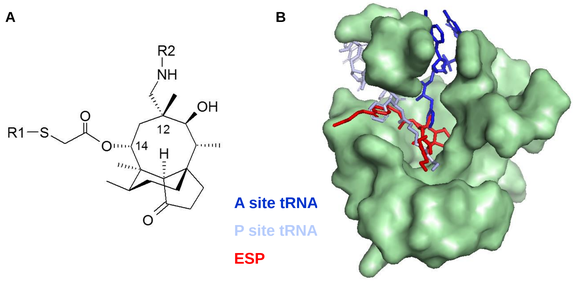
Species (no of isolates)		MIC$_{90}$ / Range [µg/mL] Comparator BC-7640	BC-7634	BC-9539	BC-9545	BC-9543
	R1	Aromatic	Aromatic	Aromatic	Aromatic	Aromatic
	R2	-	C3	C6	C8	C6 mod
***E. coli* (32)** [a]		32->32 [d]	1	2	1	0.5
***K. pneumoniae* (24)**		>32 [f]	>32	2	4	2
***C. freundii* (2)**		32->32 [e]	0.5-1	1	1	0.5
***E. cloacae* (2)**		>32 [e]	1-2	0.5-1	0.5-1	0.5
***H. influenzae* (32)**		0.5-1 [e]	8	16	8	8
***S. aureus*, CA-MRSA (20)** [b]		≤0.03 [g]	0.12	0.06	0.06	0.12
***S. pneumoniae* (30)** [c]		≤0.03-0.25 [e]	0.25	0.25	0.12	0.5

[a], *E. coli*: 28.1 % CTX-M ß-lactamase producers, 50 % TEM-type ß-lactamase producers; [b], CA-MRSA: 75 % USA300, 25 % USA400; [c], *S. pneumoniae*: 76.7 % macrolide-resistant; [d], *n* = 4; [e], *n* = 2; [f], *n* = 3; [g], *n* = 1;

INTRODUCTION

Pleuromutilins bind to the eubacterial peptidyl transferase center with high target specificity, hampering A- and P-site tRNA accommodation thereby inhibiting bacterial protein synthesis (Figure 1). Though conventional pleuromutilins such as lefamulin (BC-3781, Nabriva's lead product), retapamulin, or tiamulin, show comparable affinity to the ribosomal target in *E. coli* and *S. aureus*, they lack activity against *Enterobacteriaceae*. This intrinsic resistance is mediated by the AcrAB-TolC efflux pump (Nabriva, unpublished data).
By the targeted modification of side chains (R1, R2 in Figure 1) Nabriva discovered the ESP showing potent *in vitro* activity against *Enterobacteriaceae* in addition to the bacterial spectrum covered by conventional pleuromutilins.[1-5]
This study investigated a series of ESP derivatives having the same R1 side chain but distinct R2 side chains for their ability to inhibit bacterial translation and for their antibacterial *in vitro* and *in vivo* activity.

Figure 1. (A) Structures of tested ESP and (B) location in the peptidyl transferase center (PyMol)



METHODS

Inhibition of coupled *in vitro* transcription/translation (TT) was tested by measurement of functional luciferase produced in presence of test compound (at 10 serially diluted concentrations) in duplicate using the Steady-Glo Luciferase Assay System (Promega), ribosomal extracts of *E. coli* (Promega) and *S. aureus* (Nabriva) and the plasmids encoding luciferase under the control of promoters for *E. coli* (pBEST-Luc) and *S. aureus* (pEC270). IC_{50} were calculated using GraphPad Prism 5.02.

The **minimal inhibitory concentration (MIC)** was determined by broth microdilution using CA-MHB according to CLSI M7-A9 (2012).

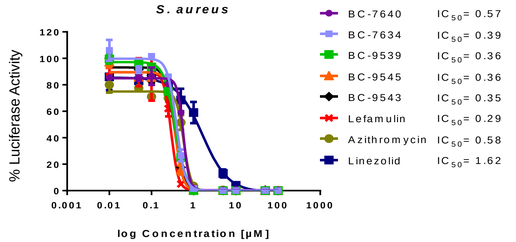
Bacterial strains were kindly provided by various sources: MSSA isolates by ATCC and the general hospital (AKH) Vienna, Austria; CA-MRSA and *S. pneumoniae* by JMI Laboratories (North Liberty, IA, USA); *E. coli* isolates by D. Livermore (Health Protection Agency, UK) and F.J. Schmitz (Klinikum Minden, D).

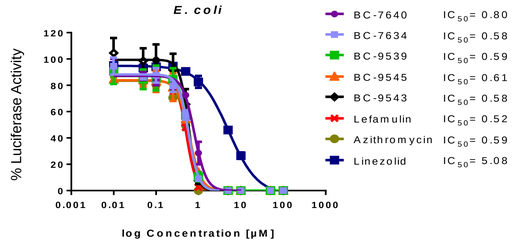
The ***in vivo* antibacterial activity** of the ESP and tigecycline was determined in a sepsis model in immuno-competent NMRI mice. Mice were infected intraperitoneally with *S. aureus* ATCC49951 using an inoculum of approximately 4 x 10^7 CFU per mouse. The drugs were administered s.c. as single dose 1 h post infection and survival was recorded for 96 h. The total daily dose required for survival of 50 % of mice (ED_{50}) and 95 % confidence limits were calculated by binary probit analysis.

RESULTS

- All tested ESP showed potent inhibition of bacterial transcription/translation in a cell-free *in vitro* system in both, *S. aureus* and *E. coli* (Figure 2).

Figure 2. Inhibition of coupled *in vitro* transcription/translation by ESP for *S. aureus* and *E. coli*





- All tested ESP having distinct substitutions at R2 (Figure 1A, Table 1) exhibited potent antibacterial activity against the tested clinical *S. aureus* isolates including both MSSA and CA-MRSA and resistant *S. pneumoniae* (Table 1).
- The MIC_{90} of ESP against CA-MRSA including isolates resistant to macrolides, tetracyclines and fluoroquinolones ranged between 0.06 and 0.12 µg/mL, which was as potent as tigecycline (MIC_{90}, 0.06 µg/mL) and significantly more active than vancomycin (MIC_{90}, 1 µg/mL). Against *S. pneumoniae* the ESP derivatives were with MIC_{90} of 0.25 - 0.5 µg/mL similarly potent as ceftriaxone (MIC_{90}, 0.25 µg/mL) and vancomycin (MIC_{90}, 0.25 µg/L; Table 1).
- By the substitution at R2 ESP gained activity against *Enterobacteriaceae* including resistant *E. coli*, *K. pneumoniae*, *C. freundii* and *E. cloacae*. For comparison, BC-7640 without R2 substitution displayed no relevant activity against these isolates (Table 1).
- Particularly the activity against *K. pneumoniae* was dependent on the length of R2 substitution: longer R2 substituents improved the antibacterial activity.
- Thus, albeit convential pleuromutilins with just R1 substitutions are effective translational inhibitors in *E. coli*, substitution at R2 seems to be essential to enter the bacterial cell and to overcome efflux, respectively, and hence for an effective antibacterial activity in *Enterobacteriaceae.*

RESULTS continued

- In the murine bacteremia model all tested ESP showed good *in vivo* efficacy with ED_{50} of 0.09-1.43 mg/kg against *S. aureus* induced sepsis and ED_{50} of 3.46-8.98 mg/kg against *E. coli* (Table 2). Overall, the ED_{50} correlated well with the *in vitro* activity.
- The ED_{50} of the tested ESP were comparable to that of tigecycline (ED_{50} of 0.45 mg/kg) against *E. coli* and significantly more active than linezolid (ED_{50} of 10.3 mg/kg) against *S. aureus*.

Table 2. *In vivo* efficacy of ESP and comparators against *S. aureus* and *E. coli*

Compound	*S. aureus* ATCC 49951 MIC [mg/L]	*S. aureus* ATCC 49951 ED_{50} [mg/kg/day]	*E. coli* ATCC 25922 MIC [mg/L]	*E. coli* ATCC 25922 ED_{50} [mg/kg/day]
BC-7640	≤0.03	**1.37**	32	ND
BC-7634	≤0.03	**0.26**	0.25	**3.46**
BC-9539	≤0.03	**0.09**	0.5	**8.98**
BC-9545	≤0.03	**1.43**	0.5	**6.14**
BC-9543	≤0.03	**0.39**	0.25	**4.26**
Linezolid	2	**10.3**	>16	ND
Tigecycline	0.25	**0.99**	0.25	**0.45**

CONCLUSIONS

- **ESP are potent inhibitors of Gram-positive and Gram-negative bacterial translation and display potent activity against resistant staphylococci and streptococci.**
- **Targeted substitution at R2 of the pleuromutilin core significantly extended the antibacterial profile of pleuromutilins to additionally cover *Enterobacteriaceae*.**
- **The potent antibacterial *in vitro* activity could be fully translated into good *in vivo activity* in the bacteremia model in mice demonstrating good drug disposition.**
- **These proof-of-concept studies warrant the further exploration of ESP as potent broad-spectrum antibiotics.**

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