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# Extended Spectrum Pleuromutilins: Mode-of-Action Studies

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## AMENDED ABSTRACT

**Objectives:** Pleuromutilin antibiotics are semi-synthetic inhibitors of bacterial protein synthesis which act by binding to the peptidyl transferase center. Conventional pleuromutilins exhibit potent activity against Gram-positive pathogens including multi-drug resistant staphylococci, streptococci and *Enterococcus faecium*, atypical respiratory pathogens and some fastidious Gram-negative organisms such as *Haemophilus influenzae* and *Moraxella catarrhalis*, but are inactive against *Escherichia coli* and other *Enterobacteriaceae*.
Nabriva discovered a new generation of pleuromutilins, the extended spectrum pleuromutilins (ESP) with an antibacterial profile covering the *Enterobacteriaceae* in addition to the conventional pleuromutilin spectrum. This study presents results on transcription and translation (TT), intracellular concentration and MICs of a series of novel ESP and comparators.
**Methods:** Inhibition of ribosomal protein synthesis was measured by coupled *in vitro* TT-assays using *E. coli* S30 Extract System (Promega) or S30 extract from *Staphylococcus aureus* RN4220 and plasmids encoding the luciferase reporter gene with suitable promoters. The $IC_{50}$ was calculated from ten serial drug dilutions in duplicate. The intracellular concentrations of ten ESP were determined by HPLC-MS/MS after incubation of mid-log phase *E. coli* with the ESP. The MICs of the ESP and comparators were determined by broth microdilution according to CLSI (M7-A9).
**Results:** TT-assay results for conventional pleuromutilins and a series of novel ESP revealed potent inhibition of transcription translation for both, *E. coli* and *S. aureus* ribosomes. The $IC_{50}$ of the compounds with best activity against *S. aureus* ranged from 0.33-0.59 µM in *S. aureus* and 0.53-0.68 µM in *E. coli*. This was similar to $IC_{50}$ measured for erythromycin ($IC_{50}$ *S. aureus*/*E. coli*, 0.49/0.55 µM) and azithromycin ($IC_{50}$ *S. aureus*/*E. coli*, 0.58/0.59 µM), while linezolid and doxycycline showed weaker inhibition of TT in *E. coli* (LZD, 5.08 µM; DOX, 4.19 µM) than in *S. aureus* (LZD, 1.62 µM; DOX, 0.54 µM).
While for *S. aureus* the $IC_{50}$ and MIC correlated well, this was not the case for *E. coli*. In *E. coli* efflux and permeation into the cell are additional factors with impact on the antibacterial activity. The ESP with potent activity against *E. coli* (MIC 0.25-2 µg/ml) showed the highest intracellular concentrations (up to 12.6 µg/$10^9$ CFU) in wt and Δ*tolC E. coli*.
**Conclusion:** The presented ESP demonstrated potent inhibition of prokaryotic protein synthesis of both Gram-positive and Gram-negative organisms in addition to high intracellular concentrations mediated by good permeation into and reduced efflux out of the Gram-negative cell thereby extending the potent antibacterial activity of pleuromutilins to *Enterobacteriaceae*.

## 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).[1-5] Though conventional pleuromutilins such as 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).

Extended spectrum pleuromutilins (ESP), the new generation of pleuromutilins with an extended antibacterial profile covering also multi-drug resistant *Enterobacteriaceae* in addition to the conventional spectrum, show potent inhibition of bacterial transcription-translation, partially overcome efflux and show high permeation into the target cells (data presented in this study).

## METHODS

ESP were synthesized at Nabriva Therapeutics AG as semi-synthetic derivatives of pleuromutilin, a homochiral natural fermentation product.
MIC were determined by broth microdilution using CA-MHB according to CLSI M7-A9 (2012).
**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.
**Whole cell drug accumulation (uptake) assay:** The uptake of ESP and comparators in *E. coli* was determined in duplicate as described earlier with minor modifications.[6] Briefly, *E. coli* (approx. $10^9$ CFU/mL) in mid-log phase were washed in 50 mM sodium phosphate buffer (pH7.4), subsequently incubated with test compound at a concentration of 0.06 µmol/mL at 37 ºC for 20 min and finally centrifuged through cold silicone oil to remove free test compound. Bacteria were then lysed in glycine hydrochloride (pH 3.0). After removal of cellular debris, the intracellular concentration was determined by LC/MS ion trap.

## RESULTS

**Table 1. Inhibition of *in vitro* transcription/translation and MIC for *E. coli* and *S. aureus***

| BC-Code | $IC_{50}$ [µM] | | MIC [µg/mL] | |
|---|---|---|---|---|
| | *E. coli* | *S. aureus* | *E. coli* (*n*=4) | *S. aureus* (*n*=1) |
| Linezolid | 5.08 | 1.62 | >32 | 4 |
| Doxycycline | 4.19 | 0.54 | 1-32 | 0.06 |
| Chloramphenicol | 4.95 | 8.26 | 4-8 | 8 |
| Tigecycline | 0.92 | 0.53 | 0.125-0.5 | 0.25 |
| Erythromycin | 0.55 | 0.49 | 32->32 | 0.25 |
| Azithromycin (AZI) | 0.59 | 0.58 | 4->32 | 1 |
| BC-3781 | 0.51±0.03[a] | 0.36±0.06 [a] | 16->32 | 0.03 |
| BC-7013 | 0.74 | 0.64 | 32->32 | ≤0.03 |
| BC-7640 | 0.80 | 0.57 | 32->32 | ≤0.03 |
| Pleuromutilin (PLE) | 0.76 | 1.73 | >32 | 1 |
| BC-9522 | 0.79 | 0.63 | >32 | 16 |
| BC-9027 | 49.0 | ≥89.8 | >32 | 16 |
| **ESP** | | | | |
| BC-7634 | 0.58 | 0.39 | 0.25-0.5 | ≤0.03 |
| BC-7641 | 0.68 | 0.38 | 1-2 | ≤0.03 |
| BC-9520 | 0.53 | 0.59 | 0.5-1 | ≤0.03 |
| BC-9529 | 0.55 | 0.33 | 0.25-1 | ≤0.03 |
| BC-9539 | 0.59 | 0.36 | 0.5-1 | ≤0.03 |
| BC-9543 | 0.58 | 0.35 | 0.25-0.5 | ≤0.03 |
| BC-9545 | 0.61 | 0.36 | 0.5-1 | ≤0.03 |

[a], Mean±STD; *n*=66 separate experiments

## RESULTS continued

- The novel ESP as well as conventional pleuromutilins inhibit bacterial transcription/translation (TT) by binding to the A- and P-site of the peptidyl transferase center of the large ribosomal subunit thereby inhibiting the correct positioning of the tRNA (Figure 1).
- ESP and conventional pleuromutilins (e.g. BC-3781, BC-7013 and BC-7640) demonstrated potent inhibition of the bacterial TT *in vitro* of both *E. coli* and *S. aureus* (Table 1).

**Figure 1. ESP in the peptidyl transferase center (PyMol)**

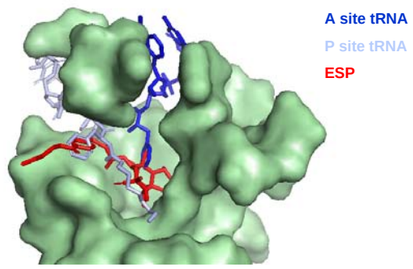
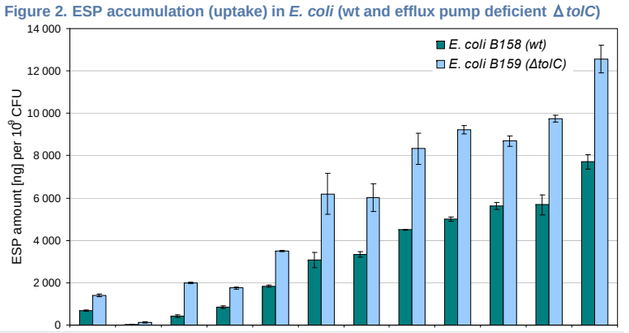



**Figure 2. ESP accumulation (uptake) in *E. coli* (wt and efflux pump deficient Δ*tolC*)**




| MIC [µg/mL] | AZI | PLE | BC-7378 | BC-3781 | BC-9522 | BC-7634 | BC-9529 | BC-9543 | BC-7641 | BC-9539 | BC-9520 | BC-9545 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *E. coli* wt | 8 | >32 | 32 | >32 | >32 | 0.5 | 1 | 0.5 | 2 | 1 | 1 | 1 |
| *E. coli* Δ tolC | 1 | 1 | 0.12 | 0.12 | 16 | 0.06 | 0.06 | ≤0.03 | 0.25 | 0.12 | 0.12 | 0.06 |
| **TT $IC_{50}$ [µM]** | | | | | | | | | | | | |
| *E. coli* | 0.59 | 0.76 | 0.60 | 0.51 | 0.79 | 0.58 | 0.55 | 0.58 | 0.68 | 0.59 | 0.53 | 0.61 |

## RESULTS continued

- $IC_{50}$ of ESP were in the range of erythromycin or azithromycin. Though conventional pleuromutilins and macrolides showed inhibition of TT in *E. coli*, this was not translated into antibacterial activity likely due to AcrAB-TolC mediated efflux (Table 1, Figure 2).
- ESP displayed potent inhibition of *E. coli* (MIC range 0.25-2 µg/mL) in addition to that of *S. aureus* (MICs ≤0.03 µg/mL).
- Measurement of the ESP uptake (accumulation) of ESP in comparison to conventional pleuromutilins and azithromycin in *E. coli* (wt and Δ*tolC*) showed a correlation of intracellular concentration and antibacterial activity. Additionally, ESP appear to be less subject to efflux by AcrAB-TolC efflux pumps than conventional pleuromutilins (Figure 2).

## CONCLUSIONS

- The novel ESP translated potent inhibition of TT in Gram-negative and Gram-positive organisms into potent antibacterial activity in both groups, which is differentiating them from conventional pleuromutilins.
- ESP displayed high uptake by the target cells and appear to be less subject to AcrAB-TolC mediated efflux than conventional pleuromutilins.
- Thus, ESP are considered as a new generation of pleuromutilins with a broad antimicrobial profile covering the most prevalent bacterial pathogens (e.g. staphylococci, streptococci, atypical respiratory pathogens, etc.) significantly extended by the activity against *Enterobacteriaceae*.

## REFERENCES

(1) Davidovich, C., Bashan, A., uerbach-Nevo, T., Yaggie, R. D., Gontarek, R. R., Yonath, *A. Proc. Natl. Acad. Sci. U. S. A* 104(11), 4291 (2007)
(2) Long, K. S., Hansen, L. H., Jakobsen, L., Vester, B. *Antimicrob. Agents Chemother.* 50(4), 1458 (2006)
(3) Poulsen, S. M., Karlsson, M., Johansson, L. B., Vester, B. *Mol. Microbiol.* 41(5), 1091 (2001)
(4) Schlunzen, F., Pyetan, E., Fucini, P., Yonath, A., Harms, *J. M. Mol. Microbiol.* 54(5), 1287 (2004)
(5) Yan, K., Madden, L., Choudhry, A. E., Voigt, C. S., Copeland, R. A., Gontarek, R. R. *Antimicrob. Agents Chemother.* 50(11), 3875 (2006)
(6) Schumacher, A., Trittler, R., Bohnert, J. A., Kummerer, K., Pages, J. M., Kern, W. V. J. *Antimicrob. Chemother.* 59(6), 1261 (2007)

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