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Synergistic *in vitro* activity of DnaK inhibitors and levofloxacin against Gram-negative organisms

M. A. Sturgess[1], P. C. Appelbaum[2], J. M. DiFranco[3], L. M. Koeth[3]

[1]Chaperone Technologies, Malvern, PA, [2]Hershey Med. Ctr., Hershey, PA, [3]Laboratory Specialists, Inc., Westlake, OH

Corresponding Author:
Michael A. Sturgess
Chaperone Technologies
508 Lapp Road, Great Valley Corp. Center
Malvern, PA 19355
Phone: 570-504-7903
Email: msturgess@chaperonetechnologies.com

Abstract (Revised)

Background: Pyrrhocoricin and CHP-105, are novel peptide antimicrobial agents that exhibit antimicrobial activity through inhibition of the bacterial heat shock protein DnaK. This study evaluates the *in vitro* activity of two DnaK inhibitors, in combination with levofloxacin, a representative fluoroquinolone, against Gram-negative organisms.
Methods: The interaction of these agents was determined using time-kill methods against *K. pneumoniae* (KP), *E. coli* (EC), *E .cloacae* (EL), *C. freundii* (CF), and *P. aeruginosa* (PA). Peptides were tested at concentrations of ¼ and ½ MICs and levofloxacin at ¼ MIC. Synergy is defined as >2 log10 decrease in CFU/mL between the combination and its most active constituent after 24 h and the number of surviving organisms in the presence of the combination must be ≥2 log10 CFU/mL below the starting inoculum. At least one of the drugs must be present in a concentration which does not affect the growth curve of the test organism when used alone. Antagonism is defined as >2 log increase in CFU/mL between the combination and its most active constituent after 24 h.
Results: Significant synergy was observed in strains of *E. coli*, *K. pneumoniae*, *E.cloacae* and *C.freundii*. No antagonism was detected.

DnaK inhibitor	Strain	Reduction in CFU [log(10)] compared to most active agent					
		8 hr		12 hr		24 hr	
		¼ MIC*	½ MIC*	¼ MIC*	½ MIC*	¼ MIC*	½ MIC*
Pyrrhocoricin	EC2	**4.03**	0.74	**6.04**	**5.55**	**5.42**	**6.57**
CHP-105	EC2	**3.70**	1.49	**2.07**	**2.04**	**5.08**	**4.49**
CHP-105	CF2	**2.81**	1.92	**4.65**	**3.78**	**4.15**	**2.57**
CHP-105	EL1	**2.70**	**2.70**	**3.90**	**3.71**	2.99	**4.35**
CHP-105	KP2	**2.60**	0.59	2.59	1.82	2.15	**6.48**

*Dose of DnaK inhibitor combined with ¼ MIC x levofloxacin
Figures in bold type face indicate synergy

Conclusion: The combination of sub-MIC levels of both DnaK inhibitors and levofloxacin showed enhanced killing compared to each of the agents alone. This initial study demonstrates a unique potential for an "enhanced quinolone" through combination with DnaK inhibitors.

Introduction

Pyrrhocoricin is a glycosylated 20-mer peptide originally isolated from the European sap sucking bug. This peptide is a member of a larger family of antimicrobial peptides, the "proline-rich oligopeptides" (PRO's). Of these, the insect derived species highlighted by pyrrhocoricin and drosocin have been shown to target the bacterial DnaK chaperone system. Natural pyrrhocoricin was originally isolated as the Thr10 glycosylated peptide with a free carboxy terminus; however most of the investigative work focused on this peptide has employed the non-glycosylated C-terminal primary amide which has been shown to exhibit a similar antibacterial profile and potency compared to the natural product. In the literature, and this presentation, this construct is commonly referred to as "pyrrhocoricin".

Structural studies with pyrrhocoricin have shown that the N-terminal portion of this peptide specifically interacts with the multihelical "lid" domain of bacterial DnaK, while the C-terminal region facilitates the entry of the complete peptide into the bacterial cell (1). More interestingly, pyrrhocoricin was shown to specifically interact with the multihelical "lid" domain of *E.coli* DnaK, but not that of other organisms such as *S.aureus*. This pattern is reflected in the antibacterial profile of the peptide.

While pyrrhocoricin displays inhibition of bacterial DnaK, it also increases membrane permeability leading to membrane disruption at concentrations significantly above the MIC for *E.coli* (4-16mg/mL). As a modification of the natural peptide it has been shown that the diaminocarboxylic acid (e.g. 2,4-diaminobutyric acid) linked dimeric structures such as CHP-105 have vastly improved properties, including negligible generic cellular toxicity and improved serum stability (2). This construct has subsequently been employed in a range of efficacy studies confirming the utility of DnaK inhibitors as antimicrobial agents (3).

While the bacterial DnaK chaperone system is not a classical antimicrobial target, standard deletion studies have confirmed its role in facilitating robust bacterial growth and survival under adverse conditions. Similar studies have suggested that *E.coli* DnaK deletion mutants are more sensitive to fluoroquinolone antibiotics than the parent wild-type strain (4).

While earlier studies with these DnaK inhibiting peptides have confirmed the utility of DnaK inhibitors as stand-alone antibiotic agents, the potency of these peptide species coupled with their molecular size, cost of manufacture, and pharmacokinetic profiles would suggest a reduced market potential for such species.

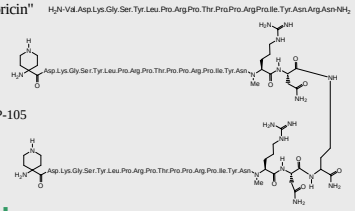
Alternatively, the increased sensitivity of *E.coli* DnaK deletion mutants to fluoroquinolones suggested to us a novel and more lucrative indication for DnaK inhibitors. Our expectation was that use of a DnaK inhibitor in combination with a fluoroquinolone would mirror the effect observed by Yamaguchi, leading to both increased potency of the fluoroquinolone species and the DnaK inhibitor. At a time when fluoroquinolone resistance is emerging as a significant health issue within Gram-negative infections such a scenario would have tremendous benefits for both extending the useful lifetime of existing fluoroquinolone agents, and in the development of new agents.

The potential for combination antimicrobial activity is assessed utilizing the *in vitro* method of time kill assays. In this study, the time kill assay was performed to test the two compounds in combination with a fluoroquinolone agent, levofloxacin against *Escherichiae coli, Citrobacter freindii, Enterobacter cloacae, Klebsiella pneumoniae* and *Acinetobacter baumanii*.

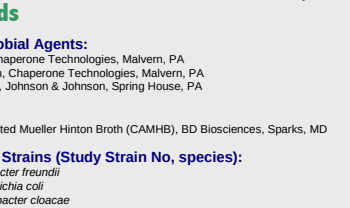
Chemical Structures:

Natural Pyrrhocoricin: H_2N-Val-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Asn-OH (Sugar)

"Pyrrhocoricin": H_2N-Val-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Asn-NH_2



CHP-105



Methods

Antimicrobial Agents:
CHP-105, Chaperone Technologies, Malvern, PA
Pyrrhocoricin, Chaperone Technologies, Malvern, PA
Levofloxacin, Johnson & Johnson, Spring House, PA

Media:
Cation Adjusted Mueller Hinton Broth (CAMHB), BD Biosciences, Sparks, MD

Bacterial Strains (Study Strain No, species):
CF2, *Citrobacter freundii*
EC2, *Escherichia coli*
EL1, *Enterobacter cloacae*
KP2, *Klebsiella pneumoniae*
PA1, *Pseudomonas aeruginosa*
AC1, Acinetobacter baumanii

Testing Site:
Laboratory Specialists, Inc., Westlake, OH

MIC Method:
CHP-105, pyrrhocoricin and levofloxacin MICs were determined in duplicate prior to performing the time kill assays according to current CLSI microbroth dilution guidelines with modification of ¼ strength CAMHB for peptide solution preparation. (5) Colony counts were determined for each inoculum. CLSI recommended QC strain E. coli (ATCC 25922) was also tested.

Synergy Method:
Synergy testing was performed according to time kill methods utilizing CLSI bactericidal guidelines with exception of ¼ strength CAMHB.(6) Colony counts were performed at 0, 4, 8, 12 and 24 hour intervals. Synergy is defined as >2 log10 decrease in CFU/mL between the combination and its most active constituent after 24 h and the number of surviving organisms in the presence of the combination must be ≥2 log10 CFU/mL below the starting inoculum. At least one of the drugs must be present in a concentration which does not affect the growth curve of the test organism when used alone. Antagonism is defined as >2 log increase in CFU/mL between the combination and its most active constituent after 24 h.

Results:

Time kill graphs and MICs for CHP-105 + levofloxacin are shown in Figure 1 and summarized in Table 1. The time kill graph and MICs for pyrrhocoricin and *E. coli* is shown in Figure 2.

- **Significant synergy of CHP-105 and levofloxacin was detected with *C. freundii, E. coli, E. cloacae, K. pneumoniae* and *A. baumanii*.**
- **Pyrrhocoricin, which was tested only against *E. coli*, also demonstrated synergy.**
- **No synergy was detected with CHP-105 and levofloxacin against *P. aeruginosa*.**
- **No antagonism was detected for either peptide in combination with levofloxacin against any of the strains tested.**

Figure 1: Synergy Results by time kill for CHP-105 and levofloxacin

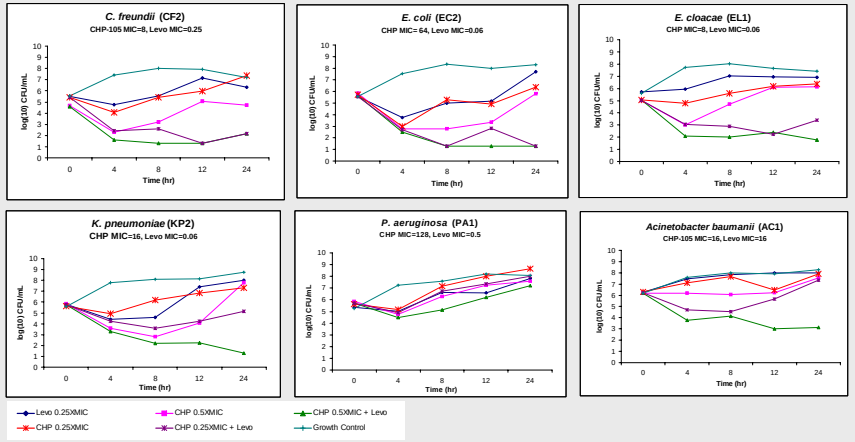


Table 1: Difference in CFU [log(10)] of CHP-105 + levofloxacin compared to most active single agent

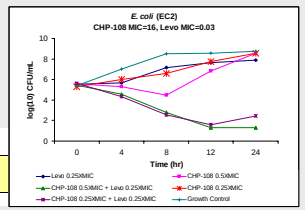
CHP-105 concentration + levofloxacin (1/4X MIC)	*C. freundii*			*E. coli*			*E. cloacae*			*K. pneumoniae*			*P. aeruginosa*			*A. baumanii*		
	8 h	12 h	24 h	8 h	12 h	24 h	8 h	12 h	24 h	8 h	12 h	24 h	8 h	12 h	24 h	8 h	12 h	24 h
1/4X MIC	2.81	4.65	4.15	3.70	2.07	5.08	2.70	3.91	2.99	2.60	2.59	2.15	-0.11	-0.74	-0.19	3.11	0.80	0.56
1/2X MIC	1.92	3.78	2.57	1.49	2.04	4.49	2.70	3.71	4.36	0.59	1.82	6.48	1.16	0.40	0.40	1.93	3.21	4.40

Synergy Detected

Figure 2: Synergy results by time kill for pyrrhocoricin (CHP-108) and Levofloxacin against *Escherichiae coli*

Difference in CFU [log(10)] of pyrrhocoricin + levofloxacin compared to most active single agent				
	4 Hr	8 Hr	12 Hr	24 Hr
CHP-108 0.25 + levofloxacin	1.34	4.05	6.04	5.42
CHP-108 0.5 + levofloxacin	0.74	1.65	5.54	6.57

Synergy Detected



Conclusions:

- **The results of these *in vitro* synergy tests suggests the potential for combination DnaK inhibitor and fluoroquinolone therapy against certain *Enterobacteriaciae* and *Acinetobacter baumanii* strains.**
- **Additional *in vitro* synergy testing with larger numbers of isolates and clinically efficacy in animal models is suggested for further validation.**

References

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2. Cudic M, Condie BA, Weiner DJ, Lysenko ES, Xiang ZQ, Insug O, Bulet P. Otvos L *Peptides* 2002, 23, 2071.
3. Cudic M, Lockatell V, Johnson DE, Otvos L *Peptides* 2003, 24, 807. Yamaguchi Y, Tomoyasu T, Takaya A, Morioka M, Yamamoto T *BMC Microbiol.* 2003 3, 16
4. National Committee for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard M7-A6. National Committee for Clinical Laboratory Standards, 2003.
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