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Activity of Levofloxacin Alone and in Combination with a DnaK Inhibitor Against Gram-negative Rods, Including Levofloxacin Resistant Strains

Kim Credito[1], Gengrong Lin[1], Laura Koeth[2], Michael A. Sturgess[3], Peter C. Appelbaum[1]

[1] Department of Pathology, Hershey Medical Center, Hershey, PA, [2] Laboratory Specialists, Inc., Cleveland, OH and [3] Chaperone Technologies, Inc., East Stroudsburg, PA

Peter C. Appelbaum
Department of Pathology
Hershey Medical Center
Hershey, PA 17033
pappelbaum@psu.edu

ABSTRACT

Background DnaK inhibitors such as CHP-105, may potentiate effects of quinolones against Gram-negative rods. Time-kill synergy tested activity of LEV alone and in combination with CHP-105 against 28 quinolone S and 22 quinolone R Gram-neg rods (3 strains with intermediate LEV R). **Methods** Organisms were 22 *E. coli*, 22 *K. pneumoniae*, 2 *C. freundii*, 2 *E. cloacae*, 2 *P.aeruginosa*. CLSI macrodilution was used to test potency of both drugs alone. For synergy time-kills concentrations of each drug were chosen from values obtained by time-kills with single drug so that one drug was (as near as possible) inactive and the other more active. LEV was tested alone and comb. with CHP-105 in subinhibitory concentrations chosen from tests with single drugs. Synergy was ≥2 $\log_{10}$ cfu/ml between comb. and its more active constituent after 3, 6, 12, 24 h (no. of surviving organisms in presence of the comb ≥2 $\log_{10}$ below starting inoculum). At least one drug was present in a concentration which did not significantly affect organism growth when used alone. **Results** LEV and CHP-105 MICs were 0.03-64 µg/ml and 4-512 µg/ml, respectively. Against *E.coli*, 10 (6 quinolone R) showed synergy at 12 h and 15 (7 quinolone R) strains at 24 h at subinh. conc. of both drugs, respectively. Against *K.pneumoniae*, 12 (5 quinolone R) showed synergy at 12 h and 16 (6 quinolone R) strains at 24 h at subinh. conc. of both drugs, resp. One quinolone S *C.freundii* showed synergy at 12h and 24 h at CHP-105 and LEV conc. of 128 µg/ml and 0.03 µg/ml. One quinolone R *P.aeruginosa* showed synergy at 12 h and 24 h at CHP-105 and LEV concentrations of 64 µg/ml and 8 µg/ml. Other combinations were additive with no antagonism. **Conclusions** LEV + CHP-105 showed synergy against 34/50 organisms (14 quinolone R) tested at subinh. conc. after 12 h and 24h, suggesting DnaK inhibitors + fluoroquinolones may be a potential new option for R Gram-neg pathogens.

BACKGROUND

Drug-resistance has become a severe problem in members of the *Enterobacteriaceae* and Gram-negative non-fermenters, and antibiotics such as the quinolones may not be effective treatment options in the near future. Multi-resistant Gram-negative rods are increasing all over the world and there are no new drugs in development to improve therapeutic choices (4,10,11). In the absence of new drugs, combination therapy may currently be the only option to treat these resistant strains. Previous studies from our and other groups have demonstrated that time-kill is more discriminatory than checkerboard in determination of synergy *in vitro* (1-3,6,8,16,18).

CHP-105 (Fig. 1) is an example of a novel series of pyrrhocoricin derived peptide inhibitors of the bacterial chaperone DnaK. The insect-derived parent peptide has been shown to bind to the multihelical lid, and inhibit the refolding activity, of *E.coli* derived DnaK (12). Conversely pyrrhocoricin does not interact with the corresponding lid sequence derived from *S.aureus*. These peptides rapidly penetrate both Gram-negative and Gram-positive bacteria, but only show growth inhibition against Gram-negative species. However, pyrrhocoricin has limited utility due to its low proteolytic stability and its cellular membrane disrupting tendencies at high doses (14). Initial efforts to overcome these issues resulted in the identification of the dimeric analog CHP-105. This peptide has been extensively studied in an effort to explore the generic utility of DnaK inhibitors as antibacterial agents. More recent synthetic efforts have focused upon more drug-like next generation small molecule and low molecular weight DnaK inhibitors exhibiting far greater potential utility as antimicrobial agents (5,13,15).

We have recently reported that peptide inhibitors of DnaK, such as CHP-105 and pyrrhocoricin, when combined with levofloxacin acted in a synergistic manner when tested against a small panel of Gram-negative organisms (17). To further establish the utility of such combinations in the treatment of drug resistant infections, we have expanded upon these preliminary results by using time-kill synergy analysis to examine the activity of levofloxacin, with and without CHP-105, against 50 quinolone-susceptible and –resistant Gram-negative rods.

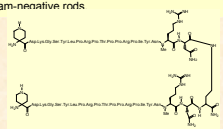


Figure 1: CHP-105; a pyrrhocoricin derived DnaK inhibitor

MATERIALS AND METHODS

Organisms tested included 22 *Escherichia coli*, 22 *Klebsiella pneumoniae*, 2 *Citrobacter freundii*, 2 *Enterobacter cloacae*, and 2 *Pseudomonas aeruginosa*. Of these, 28 were quinolone susceptible (taken as levofloxacin MICs ≤2 µg/ml) and 22 were quinolone resistant (including 3 strains with intermediate levofloxacin MICs of 4 µg/ml). Some of the organisms were provided by Ronald Jones (JMI Laboratories, Liberty City, IA) and Kenneth Thomson (Creighton University School of Medicine, Omaha, NE). Strains were frozen at -70ºC in double-strength skim milk (Difco Laboratories, Detroit, MI) before testing.

Clinical Laboratory Research Institute approved macrobroth dilution in ¼ strength cation-adjusted Mueller Hinton broth (BBL Microbiology Systems, Cockeysville, MD) was used to test MICs of all drugs alone against each of the 50 organisms (7). For time-kill, all compounds were tested alone at concentrations up to three times above and three times below the MIC. Inocula were 5 x 10^5 cfu/ml –5 x 10^6 cfu/ml. Concentrations selected for synergy testing were one to two dilutions below the MIC of each drug tested alone. Suspensions were incubated in a shaking water bath at 35ºC and viability counts for time-kill and synergy testing were performed at 0, 3, 6, 12, and 24 h. For the purposes of this study, synergy was defined as a ≥2 $\log_{10}$ decrease in cfu/ml between the combination and its more active constituent after 3, 6,12 and 24 h, with the number of surviving organisms in the presence of the combination being ≥2 $\log_{10}$ cfu/ml below the starting inoculum. At least one of the drugs was present in a concentration which did not significantly affect the growth curve of the organism when used alone. Additive was defined as effect of a second drug being similar to that of the single more effective compound and antagonism as the combination yielding higher colony counts than those seen with the more active single drug alone. The minimum countable number of cfu/ml was approximately 30 to 300 and drug carryover was addressed by dilution, as we have previously described (1,2,6,8,9,16,18).

Table 1. MIC and time-kill synergy test results - *Klebsiella pneumoniae*

Strain	MIC CHP-105	MIC Levo	CHP-105 + Levofloxacin 3h	6h	12h	24h
E363	4	0.06	Additive	Additive	Synergy 2/0.016	Synergy 2/0.03
E362	4	0.125	Additive	Additive	Synergy 2/0.03	Additive
E366	16	0.125	Additive	Additive	Synergy 4/0.03	Synergy 4/0.06
E353	4	0.25	Additive	Additive	Synergy 1/0.125	Synergy 1/0.125
E367	16	0.25	Additive	Additive	Synergy 2/0.06	Synergy 2/0.06
E372	4	0.25	Additive	Additive	Synergy 2/0.06	Synergy 2/0.06
E356	8	0.5	Additive	Additive	Additive	Additive
E418	8	1	Additive	Additive	Additive	Synergy 4/0.5
E408	16	1	Additive	Additive	Additive	Additive
E432	128	1	Additive	Additive	Additive	Synergy 64/0.5
E364	8	2	Additive	Additive	Synergy 2/1	Synergy 2/0.5
E411	8	2	Additive	Additive	Additive	Synergy 2/1
E427	8	2	Additive	Additive	Synergy 2/0.5	Synergy 4/0.5
E426	4	4	Additive	Additive	Additive	Additive
E431	4	4	Additive	Additive	Additive	Synergy 2/2
E368	8	8	Additive	Additive	2/4	2/2
E354	8	16	Additive	Additive	Additive	Synergy 16/4
E422	32	16	Additive	Additive	Synergy 8/8	Synergy 16/4
E433	8	16	Additive	Additive	Synergy 4/4	Synergy 4/4
E370	4	32	Additive	Additive	Synergy 1/16	Synergy 2/16
E371	32	32	Additive	Additive	Synergy 16/8	Synergy 8/8
E368	8	64	Additive	Additive	Additive	Synergy 4/32

Table 2. MIC and time-kill synergy test results - *Escherichia coli*

Strain	MIC CHP-105	MIC Levo	CHP-105 + Levofloxacin 3h	6h	12h	24h
E346	64	0.03	Additive	Additive	Additive	Synergy 16/0.016
E437	4	0.03	Additive	Additive	Additive	Additive
E341	16	0.06	Additive	Additive	Synergy 4/0.03	Synergy 4/0.03
E438	8	0.06	Additive	Additive	Synergy 4/0.03	Synergy 4/0.03
E430	16	0.125	Additive	Additive	Additive	Additive
E434	8	0.125	Additive	Additive	Additive	Additive
E435	8	0.125	Additive	Additive	Additive	Synergy 4/0.06
E436	8	0.125	Additive	Additive	Additive	Synergy 4/0.06
E424	64	0.25	Additive	Additive	Synergy 16/0.125	Synergy 16/0.125
E342	256	0.5	Additive	Additive	Additive	Synergy 64/0.125
E439	8	0.5	Additive	Additive	Synergy 4/0.25	Synergy 4/0.25
E425	32	2	Additive	Additive	Additive	Synergy 16/1
E410	4	4	Additive	Additive	Additive	Additive
E409	16	8	Additive	Additive	Synergy 8/2*	Synergy 4/4
E412	8	8	Additive	Additive	Additive	Additive
E413	32	8	Additive	Additive	Synergy 8/4	Synergy 8/4
E351	32	16	Additive	Additive	Additive	Additive
E393	16	16	Additive	Additive	Synergy 8/4	Additive
E395	32	16	Additive	Additive	Synergy 16/4	Synergy 16/4
E396	16	16	Additive	Additive	Synergy 8/4	Synergy 8/4
E394	32	32	Additive	Additive	Synergy 8/8	Synergy 8/8
E408	16	32	Additive	Additive	Synergy 4/16	Synergy 8/8

*Became levofloxacin susceptible in the combination.

Figure 2.

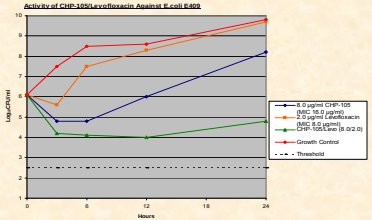
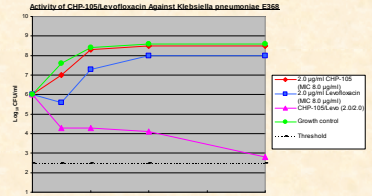


Figure 3.



RESULTS

Results are presented in Tables 1-3. As can be seen, levofloxacin and CHP-105 MICs ranged from 0.03-32 µg/ml and 4-256 µg/ml for *E. coli*; 0.06-64 µg/ml and 4-128 µg/ml for *Klebsiella pneumoniae*; 0.06, 16 µg/ml and 4, 8 µg/ml for *Enterobacter cloacae*; 0.125, 32 µg/ml and 8, 512 µg/ml for *Citrobacter freundii*; and 2, 16 µg/ml and 128, 256 µg/ml for *Pseudomonas aeruginosa*.

Levofloxacin and CHP-105 showed synergy against 34 of 50 organisms (14 quinolone resistant) when tested at sub-inhibitory concentrations after 12 and 24 h. Ten *E. coli* (6 quinolone resistant) showed synergy at 12 h and 15 (7 quinolone resistant) strains at 24 h showed synergy, all strains at sub- inhibitory concentrations of both compounds. Sub-inhibitory concentrations of CHP-105 and levofloxacin in synergistic combinations ranged between 4-128 µg/ml and 0.008-16 µg/ml, respectively. Against *Klebsiella pneumoniae*, 12 (5 quinolone resistant) strains showed synergy at 12 h and 16 (6 quinolone resistant) strains showed synergy at 24 h, all strains at sub-inhibitory concentrations of both drugs. Sub- inhibitory concentrations of CHP-105 and levofloxacin in synergistic combinations ranged between 1-64 µg/ml and 0.016-32 µg/ml, respectively. One quinolone susceptible *Citrobacter freundii* showed synergy at 12 and 24 h at CHP-105 and levofloxacin concentrations of 128 µg/ml and 0.03 µg/ml, and one quinolone resistant *Pseudomonas aeruginosa* demonstrated synergy at 12 and 24 h at CHP-105 and levofloxacin concentrations of 64 µg/ml and 8 µg/ml; synergy in both strains was found at sub-inhibitory drug concentrations. All other combinations were additive and no antagonism was observed.

Synergy between CHP-105/Levofloxacin against one *E.coli* strain and one *Klebsiella pneumoniae* strain is graphically depicted in Figure 2 and Figure 3.

Table 3. MIC and time-kill synergy test results - Other gram-negative rods

Strain	MIC CHP-105	MIC Levo	CHP-105 + Levofloxacin 3h	6h	12h	24h
E373	4	0.06	Additive	Additive	Additive	Additive
E397	8	16	Additive	Additive	Additive	Additive
E384	512	0.125	Additive	Additive	Synergy 128/0.03	Synergy 128/0.03
E400	8	32	Additive	Additive	Additive	Additive
P330	256	2	Additive	Additive	Additive	Additive
P335	128	16	Additive	Additive	Synergy 64/8.0	Synergy 64/8.0

Strains E373 and E397: *Enterobacter cloacae*
Strains E384 and E400: *Citrobacter freundii*
Strains P330 and P335: *Pseudomonas aeruginosa*

CONCLUSIONS

Our studies showed synergy in more than 2/3 of 50 Gram-negative rods after 12 h and 24 h. An additive response was observed in the remaining 1/3 of strains tested, and in no case was there antagonism between the two drugs. Although these results need to be confirmed by testing of a larger spectrum of quinolone susceptible and resistant Gram-negative rods, the initial results are encouraging. More potent DnaK inhibitors are currently in development and will be employed to further expand on the utility of this approach.

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