In vivo Activity of a DnaK Inhibitor Alone and in Combination with Levofloxacin (LEV) in a Murine Model of Intraperitoneal Infection

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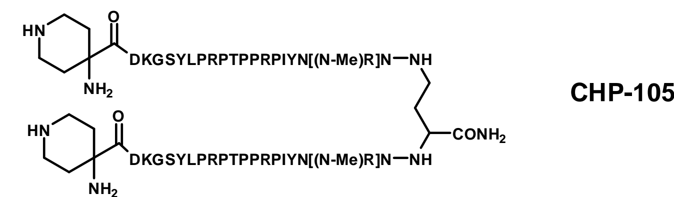
Abstract

Background. Drug-resistance has become a severe problem in members of the *Enterobacteriacae* and Gram-negative non-fermenters. One approach to this issue is the validation of novel antibacterial targets such as the bacterial HSP70 chaperone DnaK. Our recent *in vitro* results confirm DnaK inhibitors to be active as stand-alone agents, and also demonstrate synergistic activity combined with quinolones.
Methods. Synergy was shown by time-kill between CHP-105, a representative DnaK inhibitor, and LEV against two *E.coli* strains with poor susceptibility to LEV. Both strains were passaged in mice and the MIC's of CHP-105 and LEV determined, before being evaluated at escalating doses in a murine intraperitoneal infection model. *E.coli* strain EC409 (1.0 x 10^8cfu's, i.p.) was chosen to examine efficacy of the DnaK inhibitor and LEV. *Single Drug Study*. ~1 h post-challenge, mice were treated i.p. with either LEV or CHP-105 at 1, 3, 10, 30 or 100 mg/kg. *Drug Combn Study*. ~1 h post-challenge, mice were treated i.p. with combinations of LEV and CHP-105 at (3 + 3) mg/kg and (1 + 3) mg/kg, respectively. At 72 h blood, spleen and i.p. fluids were collected from all surviving mice, and bacterial loads determined.
Results. *E.coli* EC409 MIC's for CHP-105 and LEV were 32 and 8 mg/ml respectively. PD50's for CHP-105 and LEV were found to be 3.3mg/kg (R2 = 0.89) and 2.4mg/kg (R2 = 0.48) respectively. Comparable reductions in bacterial load were seen for both compounds. Treatment of mice with a combination of CHP-105 and LEV at (3 + 1) mg/kg respectively or (3 + 3) mg/kg respectively resulted in enhanced survival and greater reduction in bacterial load compared to the single agents.
Conclusions. DnaK inhibitors alone efficiently protect mice in an i.p. infection model, and are more efficacious in combination with LEV compared to single agent LEV.

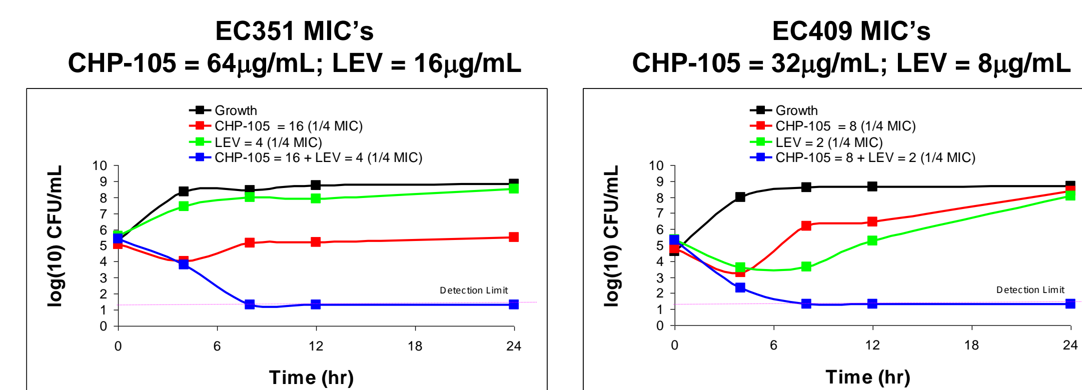


CHP-105

in vitro Synergy Study

EC351 MIC's
CHP-105 = 64μg/mL; LEV = 16μg/mL

EC409 MIC's
CHP-105 = 32μg/mL; LEV = 8μg/mL



Clinical and Laboratory Standards Institute approved macrobroth dilution in ¼ strength cation-adjusted Mueller Hinton (MH) broth was used to test MIC's of CHP-105 and LEV. Prior studies have demonstrated the reduced potency of CHP-105 in the presence of high salt containing medium. For time-kill studies, both compounds were tested alone and in combination at concentrations representing ¼ x MIC. Inocula were 5 x 10^5 cfu/ml –> 5 x 10^6 cfu/ml. Suspensions were incubated in a shaker at 35°C and viability counts for time-kill and synergy testing were performed at 0, 4, 8, 12, and 24 h. For the purposes of this study, synergy was defined as a $\geq$2 log_{10} decrease in cfu/ml between the combination and its more active constituent after 4 h, 8 h, 12 h and 24 h, with the number of surviving organisms in the presence of the combination being $\geq$2 log_{10} cfu/ml below the starting inoculum. The minimum countable number of cfu/ml was approximately 30.

A combination of ¼ x MIC concentrations of both CHP-105 and LEV demonstrated synergy at 12h and 24h with both *E.coli* strains. As a result both strains were advanced to pilot *in vivo* studies to examine which strain would be better suited to the establishment of a lethal infection in mice.

Materials and Methods

Bacterial Strain. A frozen aliquot of *E.coli* strain EC409 was thawed and used to seed 50 mL TSB. The culture was incubated statically overnight at 37°C and diluted the following morning using PBS to a targeted challenge dose of 1.60 x 10^8 CFU (MLD) based on a pre-determined optical density reading (O.D.620). Retrospective plate counting determined the concentration to be 1.47 x 10^8 CFU (Single agent studies) and 1.18 x 10^8 CFU (Combination studies) .

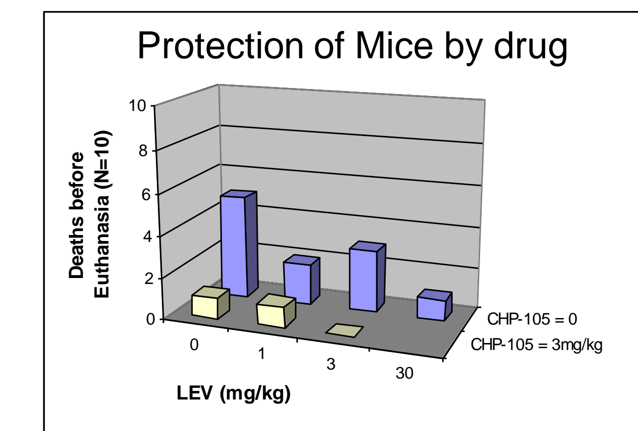
Mice. The mice used in these studies were female Crl: CD-1®(ICR)BR weighing in the range of 23-30g.

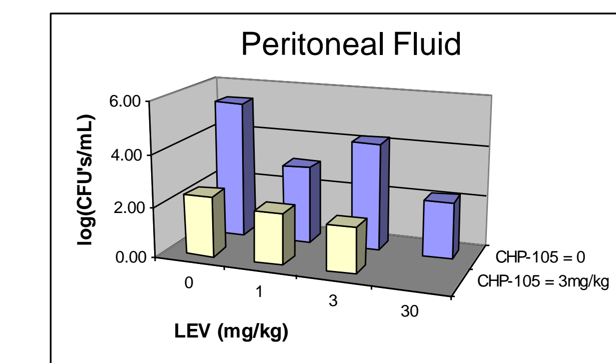
Challenge. Day 0, each mouse was challenged via the intraperitoneal (i.p.) route with EC409 in a dose volume of 0.3mL within a BioSafety Cabinet (BSC).

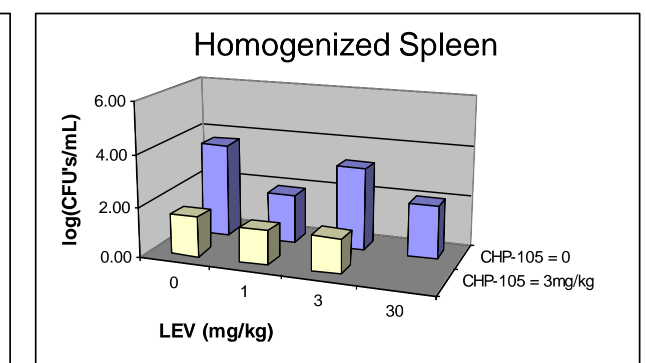
Single Drug Treatment. Day 0 at ~1 hour post-challenge, mice were treated via the i.p. route in a dose volume of 0.1mL with either levofloxacin (LEV) (1mg/kg, 3mg/kg, 10mg/kg, 30mg/kg and 100mg/kg), CHP-105-9 (1mg/kg, 3mg/kg, 10mg/kg, 30mg/kg and 100mg/kg), or a combination of the two agents. For the combination studies a CHP-105 dose of 3mg/kg was selected based on the results of the single agent studies, and this dose was combined with different doses of LEV.

Tissue Sampling and Blood Collection. Day 3, mice remaining alive were euthanized via CO_2 asphyxiation. Blood (~0.2 mL) was collected from the retro-orbital sinus into tubes containing no anticoagulant and plated onto TSA for presence or absence of bacteremia. The spleens and peritoneal fluid were collected at necropsy. For sampling of peritoneal fluid, the abdomen of each mouse was massaged for ~1 minute after injection of sterile saline (2mL), the abdomens were opened and of peritoneal fluid (1mL) was removed and plated onto TSA for recovery of challenge bacteria. Spleens were rinsed with sterile saline, blotted and immediately placed on wet ice. They were manually homogenized, serially diluted using sterile saline and plated in duplicate onto TSA for recovery of challenge bacteria.

Combination Study Results







Bacterial load after drug treatment

Discussion

Recent work from Chaperone Technologies has focused on exploration of the utility of inhibitors of bacterial Hsp70 proteins (DnaK) as antibacterial agents. Studies of *in vivo* potency of DnaK inhibitors have been largely lacking due to solubility, stability, and pharmacokinetic concerns. Pyrrhocorocin, a natural peptidic DnaK inhibitor, has a $t_{½}$ ≈ 30mins in 80% human serum. CHP-105, a somewhat more stable dimeric pyrrhocoricin analog (Cudic 2002), was found to exhibit useful antimicrobial activity against a range of Gram-negative organisms in ¼x strength MH broth. In undiluted broth this activity was greatly reduced. This phenomenon is common to many antibiotic peptide families, and has been attributed to reduced membrane-peptide interactions in the presence of high divalent cation concentrations. Anticipating that this phenomenon may be common to other DnaK inhibitors identified by Chaperone Technologies, we sought to show that this observation is not relevant to the *in vivo* potential of DnaK inhibitors in general.

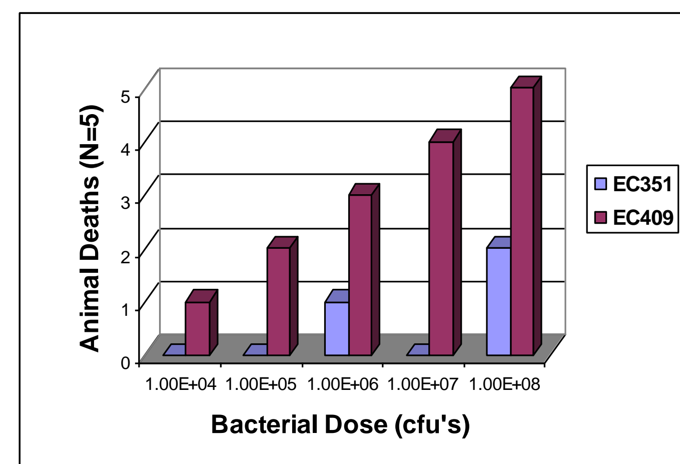
In addition to the inherent antibacterial activity of DnaK inhibitors, we have recently demonstrated that *in vitro* combination of DnaK inhibitors with existing classes of antibiotics such as aminoglycosides and fluoroquinolones leads to greatly enhanced synergistic bacterial killing (Sturgess 2006, Credito 2009). Employing time-kill methodology, we have demonstrated that ¼ x MIC concentrations of a DnaK inhibitor in combination with ¼ x MIC concentrations of a fluoroquinolone are highly efficient antibacterial combinations compared to the single agents alone against a wide selection of Gram-negative organisms including *E.coli*, *A.baumanni*, *K.pneumoniae*, *C.freundii*, and *P.aeruginosa*.

This study was designed to demonstrate the *in vivo* potency of a DnaK inhibitor in a simple animal model of infection employing LEV as a comparator agent, and to extend this result to combinations of the DnaK inhibitor and LEV. A successful outcome to this latter aspect of the study could subsequently be employed in dose-ranging and design of a considerably larger study to demonstrate statistically significant superiority of the combination over the single agents alone.

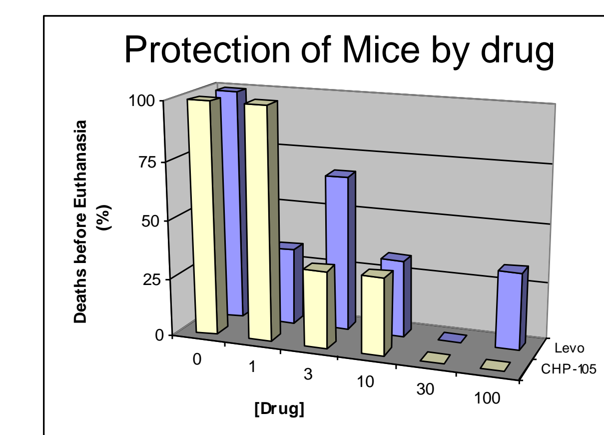
Pilot Study

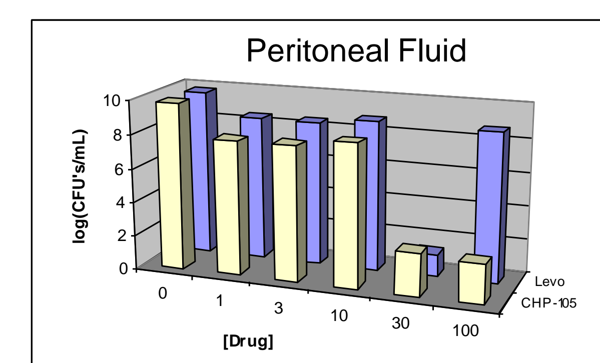
A pilot study was conducted in mice to establish the minimal lethal dose (MLD) for two strains of *E. coli*, EC409 and EC351. The strains were first passaged through mice twice to establish virulence and increase chances of inducing sepsis. The organisms recovered from the blood were cultured and used to infect female CD-1 mice in a one-log concentration range of 1.0 x 10^4 to 1.0 x 10^8 CFUs. EC351 did not establish consistent infection in mice; however, EC409 successfully established sepsis in the mice with 100% mortality in the highest concentration group, 1.0 x 10^8 CFU (actual MLD concentration of 1.60 x 10^8) and a LD50 of 6.0 x 10^5. EC409 was selected for further study.

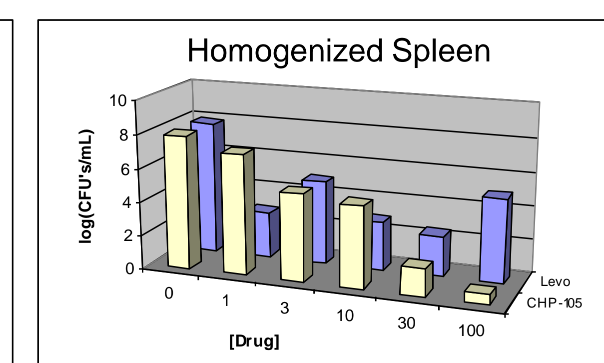
Determination of minimum Lethal Dose (MLD) and LD_{50} for two *E.coli* Strains



Single Agent Study Results







Bacterial load after drug treatment

Conclusions

- *in vitro* time-kill studies showed powerful synergy between the DnaK inhibitor and LEV.
- Single agent animal protection studies demonstrated that CHP-105 was as potent as LEV in a mouse model of intraperitoneal infection.
- The challenge was more effectively cleared from peritoneal fluid and homogenized spleen by CHP-105 than LEV in both a mole : mole and a gram : gram comparison.
- The combination of a DnaK inhibitor and LEV suggested an improved potency of the combinations over the single agents, leading to a possible clinical benefit of the combination over existing therapies against difficult to treat reduced-susceptibility and drug-resistant infections. This result strongly supports further efforts in this area to demonstrate statistical superiority of the combination treatment over the single agents alone.

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