The First Biologically Active Small Molecule Bacterial HSP70 (DnaK) Inhibitors

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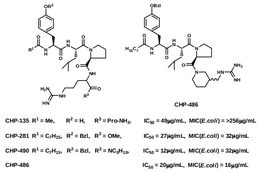
Abstract

Background. Antibacterial peptide Pyrrhocoricin (Pyrr) inhibits the bacterial HSP70 chaperone protein DnaK. Earlier studies identified residues in the N-terminal half of Pyrr that influence this activity, however attempts to truncate the peptide to include only these residues resulted in loss of the antibacterial activity. Similarly a fatty acid conjugated dipeptide showed good DnaK inhibitory activity *in vitro*, but did not elicit an antibacterial response at physiological temperatures.
Methods. Fragments of Pyrr were prepared and screened in a DnaK–induced luciferase refolding assay. Active fragments were evaluated for antimicrobial activity against *E.coli* (ATCC 25922) (CLSI MIC). Modifications of the most active fragment were designed and tested in an iterative manner. The most active DnaK inhibitors were screened for mammalian cell toxicity against human red blood cells and COS-7 kidney cells.
Results. Small molecule DnaK inhibitors, identified by *in vitro* screening assays, show DnaK inhibition and *in vitro* antibacterial activity in the same low mg/mL concentration range, are stable to serum proteases and non-toxic to mammalian cells.



CHP-486

CHP-135 R^1 = Me, R^2 = H, R^3 = Pro-NH_2, IC_{50} = 49μg/mL, MIC(*E.coli*) = >256μg/mL
CHP-281 R^1 = C_6H_{13}, R^2 = Bzl, R^3 = OMe, IC_{50} = 27μg/mL, MIC(*E.coli*) = 32μg/mL
CHP-490 R^1 = C_6H_{13}, R^2 = Bzl, R^3 = NC_4H_{10}, IC_{50} = 32μg/mL, MIC(*E.coli*) = 32μg/mL
CHP-486 IC_{50} = 20μg/mL, MIC(*E.coli*) = 16μg/mL

Conclusions. The first small molecule inhibitors of the bacterial HSP70 chaperone DnaK that show efficient antibacterial activity at physiological temperatures have been identified.

Background

The bacterial HSP70 proteins are best characterized by the *E.coli* derived protein DnaK. The biological relevance of DnaK inhibition to the discovery of antibacterial agents can be illustrated by the characteristics of DnaK deletion mutants of human pathogens. *E.coli* mutants expressing an inactive DnaK protein are highly sensitive to non-optimal temperature and nutrient levels (Paek 1987), osmotic stress (Meury 1991), and other stresses (Hanawa 1999), and exhibit reduced growth and viability (Bukau 1989) at physiological temperatures. *Brucella suis* DnaK null mutants were marginally viable in culture media and unable to sustain growth in macrophages (Kohler 1996). Similarly a *Salmonella enterica serovar Typhimurium* DnaK/DnaJ depleted-mutant was shown to be deficient in both intracellular growth and invasion of epithelial cells (Takaya 2004).

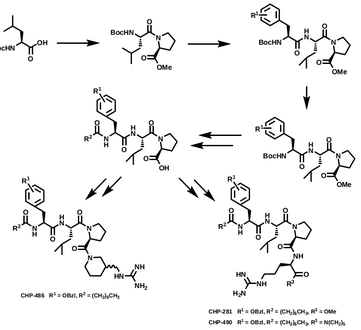
Interestingly, a similar trend has also been observed regarding DnaK null mutant strains of Gram-positive organisms. Partial deletion and disruption of the dnaK gene of *S.aureus* strain COL resulted in moderately reduced growth capacity under standard conditions, and enhanced susceptibility to oxidative and antibiotic induced stress (Singh 2007). Furthermore, the mutant strain displayed a reduced ability to survive in a mouse host compared to the parent strain.

Among the earliest examples of DnaK inhibitors are ~20-mer peptides of the cationic polyproline series (CPP's) such as Pyrrhocoricin (Pyrr). This peptide, and related analogs (e.g. CHP-105 (Cudic 2002), have been shown to inhibit bacterial HSP70 (DnaK) both *in vitro* and intracellularly leading to good antibacterial activity across a spectrum of Gram-negative organisms in 1/4x strength M-D broth. Unfortunately, neither Pyrr, or CHP-105, is active against either Gram-positive organisms in diluted broth or Gram-negative organisms in full strength broth. Early examples of small molecule inhibitors of DnaK have been documented (Wisen 2008, Liebscher 2007), but have failed to demonstrate antibacterial activity under standard conditions of growth medium and temperature.

To examine the role of sequence elements within the Pyrr sequence we have constructed overlapping pentapeptide fragments of the parent peptide and evaluated the fragments for both *in vitro* DnaK inhibition and antibacterial activity. The fragment Ac-YLPRP-NH2 was found to retain most of the biochemical inhibition of DnaK, but was microbiologically inactive against both *E.coli* (ATCC 25922) and *E.coli* (D22) - a cell wall defective strain.

Modified Tetrapeptides

Based on the biochemical activity of the Pyrr fragments attention was focused on the YLPRP fragment of Pyrr. Preliminary results indicated that the C-terminal proline residue was not essential for activity, reducing the active construct to a tetrapeptide core. Synthesis of analogs was completed employing standard amide coupling protocols. Simple analogs of the C-terminal arginine residue were designed and constructed employing a range of cyclic and acyclic mono-protected diamines. All samples were purified by preparative HPLC to give the TFA salts.



	DnaK IC_{50} (μg/mL)
Pyrrhocoricin-NH_2	23
CHP-105	40
NRLLLTG-NH_2	40
$Me(CH_2)_6CO$-LYChaLPRP-NH_2	3
CHP-135	49
CHP-281	27
CHP-486	20
CHP-490	12

CHP-486 R^1 = OBzl, R^2 = $(CH_2)_3CH_3$

CHP-281 R^1 = OBzl, R^2 = $(CH_2)_5CH_3$, R^3 = OMe
CHP-490 R^1 = OBzl, R^2 = $(CH_2)_5CH_3$, R^3 = $N(CH_2)_5$

Growth Media Effects

Previous examples of DnaK inhibitors were very sensitive to the chosen growth media. Pyrr and CHP-105 were only marginally active in full strength M-H broth compared to 1/4x strength broth. This phenomenon was "corrected" by later generation dimeric peptide analogs (Cassone 2008). However, doubt have been raised regarding the mechanism of killing of these later generation dimeric peptides since these large highly cationic peptides have been demonstrated to disrupt bacterial membranes.

METHODS:

Minimum Inhibitory Concentration (MIC) determinations were performed according to the CLSI broth microdilution standard method using ¼, ½ and full strength cation adjusted Mueller Hinton broth (CAMHB) against 4 ATCC organisms

RESULTS:

The effect of the broth strength was shown to be marginal for CHP-486 compared to the dramatic effects observed previously for Pyrr-derived peptide inhibitors of the bacterial Hsp70 system.

	¼ Strength CAMHB		½ Strength CAMHB		Full Strength CAMHB	
	CHP-105	CHP-486	CHP-105	CHP-486	CHP-105	CHP-486
E.Coli (ATCC25922	32	16-64*	NT	32	**>128**	32
S.aureus (ATCC 29213)	>128	16	NT	16	NT	16
E.faecalis (ATCC 29212)	NT	16	NT	16	NT	32
P.aeruginosa (ATCC27853)	NT	128	NT	64	NT	128

***** n=4, MICs obtained on 3 different days with two lots of powder

Gram-Negative Pathogens

METHODS:

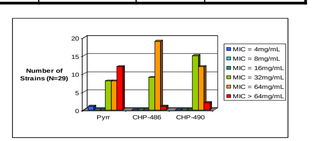
Minimum Inhibitory Concentration (MIC) determinations were performed according to the CLSI broth microdilution standard method against 4 stock organisms.

RESULTS:

	DnaK Inhibition (μg/mL)	MIC (μg/ml)			
		E. coli (ATCC 25922)	*A. baumannii* (F010)	*Y. pestis* (Col92)	*M. catarrhalis* (ATCC 203026)
Pyrr	23	16	32	32	NT
CHP-105	40	32	32	32	NT
CHP-281	**27**	**32**	**64**	**>64**	**NT**
CHP-486	**20**	**32**	**32**	**32**	**16**
CHP-490	**12**	**32**	**32**	**64**	**NT**

Activity of CHP-486 and CHP-490 against *Y. pestis*

(Dr. Henry S. Heine, USAMRIID, Ft. Detrick, personal communication)



Gram-Positive Pathogens

METHODS:

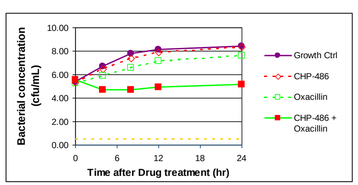
Minimum Inhibitory Concentration (MIC) determinations were performed according to the CLSI broth microdilution standard method against 4 ATCC and 3 clinical strains. Kill curve was performed according to time kill method based on the CLSI bactericidal procedure against a MRSA at ¼ MIC CHP-486, oxacillin at 5 μg/mL and combination of CHP-486 and oxacillin at similar concentrations.

RESULTS:

	MIC (μg/ml)						
	S.aureus (ATCC 29213)	MRSA	*S.pyogenes* (ATCC 12384)	*S.pneumoniae* (ATCC 49619)	*E.Faecalis* (ATCC 29212)	VRE (*E.faecalis)*	VRE (*E.faecium*)
CHP-105	>128	NT	NT	NT	NT	NT	NT
CHP-281	**16**	**NT**	**NT**	**NT**	**NT**	**NT**	**NT**
CHP-486	**16**	**16**	**16**	**32**	**16**	**8**	**16**
CHP-490	**8**	**16**	**NT**	**NT**	**16**	**8**	**8**

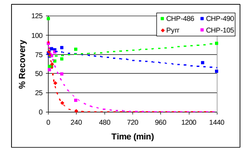
Effect of Combination of CHP-486 with oxacillin against MRSA strain:

CHP-486 MIC = 16μg/mL
Oxacillin MIC >32μg/mL

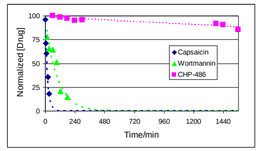


Stability/Metabolism and Mammalian Cell Toxicity

A major drawback to the Pyrr-derived DnaK inhibitors is their degradation by cellular and serum proteases. CHP-486 and CHP-490 are extremely stable in either 80% rat or human serum. Also, CHP-486 is NOT metabolized by rat liver S9 extracts in a standard *in vitro* metabolism assay.

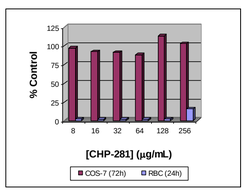


Stability in Human Serum (80%)



Stability against Rat Liver S9 Extract

COS-7 Assay Method
Plates were seeded with COS-7 cells (3,000 cells per well in a total volume of 50μL medium) and incubated overnight in a humidified 37°C cell culture CO2 incubator. Test articles were added (50μL 2X stocks). After 72hr, plates were read by CellTiter-Glo assay.
Red Blood Cell Hemolysis Assay Method
Human RBC's were washed 3X with PBS and suspend to 10% with PBS. To cells (50μL per well) was added test articles (50μL 2X stocks). Plates were mixed gently and incubated for 1hr at r.t. then spun at 1500 rpm for 15min to pellet RBCs. Supernatant (75μL) was transfered to a FLAT bottom plate and the OD read at 570 nm.



Assays limited by compound insolubility above 256μg/mL

Conclusions

- **Simple small molecule constructs efficiently inhibit the bacterial Hsp70 (DnaK) chaperone system.**
- **Unlike previously documented bacterial Hsp70 (DnaK) inhibitors, these constructs demonstrate potent antibacterial efficacy under standard conditions of temperature and growth medium.**
- **CHP-486/CHP-490 are equally active against a broad spectrum of standard and drug resistant clinical isolates of Gram-negative AND Gram-positive organisms.**
- **Examples of this series are non-toxic to mammalian cells.**
- **CHP-486 is STABLE ($t_{½}$>24h) in serum and NOT metabolized by rat S9 liver extracts**

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