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NZ17074 : An Arenicin-3 variant found by HTS screening of yeast libraries

Neve,S., Raventós, D., Sandvang,D., Kruse, T. & Kristensen, H-H.
Novozymes A/S, Bagsvaerd, Denmark

Contact information:
Snev@novozymes.com
Phone: +45 44460663

Introduction

The Arenicin family consists of three members Arenicin-1 and -2 which were characterized by a Russian research group (Ovchinnikova et al., 2004) and Arenicin-3 which is a novel member of the family. Arenicin-3 was isolated from the marine lugworm *Arenicola marina* and shown to contain two disulfide bonds between Cys3, Cys20 and Cys7, Cys16. Together with 4 positively charged arginines, and 9 hydrophobic residues the peptide was shown to form a 21-residue amphipathic beta hairpin structure. Activity assays revealed that Arenicin-3 is highly active *in vitro* against a variety of Gram negative bacteria. However the Arenicin-3 molecule showed a very high protein binding to serum compopnents. Through HTS screening we managed to identify a variant (NZ17074) with decreased serum binding properties.

Here, we present a mutational campaign and high throughput screening (HTS) setup aiming to identify a Arenicin-3 variant with decreased protein binding and maintained potent *in vitro* activity compared to Arenicin-3.

Methods

Error prone PCR.
Two different strategies were used for Error Prone PCR using Taq DNA polymerase. One with different concentrations of $MnCl_2$ (0.5mM to 1mM) to stabilize noncomplementary pair and the other with different concentrations of $MnCl_2$ together with unbalanced dNTP concentrations to promote a non-biased mutation pattern. The PCR products were ligated to pYES and transformed into *S. cerevisiae* JG169. For purification, the Arenicin gene was fused to the alpha factor leader allowing export of the peptide to the culture supernatant.

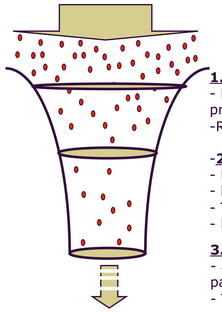
Plate screen.
The resulting libraries were plated on selective plates containing ¼ SC +gal+glu+2.5% defibrinated horse blood. After 3 days the plates were dried and over layered with *E.coli* (ATCC 10536). The plate assay was adjusted with 2.5% blood in which wild type Arenicin was unable to give rise to zones of inhibition. Yeast colonies that gave rise to inhibition zones were isolated and retested against *Pseudomonas*.

MIC.
MIC testing of clinical isolates of multi resistant *Escherichia coli* and *Pseudomonas* spp. were performed by broth micro-dilution, according to CLSI-defined methodology, M7-A5 (2003), against Arenicin-3 and NZ17074.Quality Control (QC): Results for all MIC testing were within the acceptable standards based on the CLSI recommended QC ranges for each comparator agent and the appropriate ATCC control strains.

Protein binding.
Protein binding assays were performed as follows. Peptides were mixed with 90% serum and centrifuged through a 30 kDa filter. The ultra filtrate and the non filtrated serum samples were quantified by HPLC measurements and the protein binding was subsequently calculated.

Results.

A



1. Screen – *S. cerevisiae*
- Plate screen *vs. E. coli* in the presence of 2,5% blood
-Re-test against *Pseudomonas*

-2. screen – purified peptide
- MICs against 3 bacteria +/- serum
- hemolysis
- Time kill /MBC
- Protein binding

3. screen – synthetic peptides
- a range of *in vitro* & *in vivo* parameters
- Toxicological parameters

B

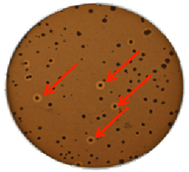


Figure A Illustrates the different levels of the yeast screen. During the first screen the yeast libraries are selected on the plates. Only colonies giving rise inhibition zones against *E.coli* are retested in radial diffusion assays against *Pseudomonas.* Only clones exhibiting activity against both *Pseudomonas* and *E.coli* are passed onto the 2'nd screen. During the second screen the individual Arenicin variants were purified and tested for protein binding and MIC assays. One variant, NZ17074, showed a lower protein binding than the wild type and good activity in MIC assays ± serum.
Figure B Shows the plate assay. Yeast colonies expressing an Arenicin variant giving rise to zones of inhibition

Name	Origin	aa-sequence
ARENICIN-3	**Sandworm, *Arenicola marina***	
NZ17074	**Arenicin-3 Variant**	
ARENICIN-2	Sandworm, *Arenicola marina*)	
TACHYPLESIN-1	Horseshoe crab, *Tachypleus tridentatus*	
TACHYPLECIN-3	Horseshoe crab, *Tachypleus tridentatus*	
POLYPHEMUSIN-1	Horseshoe crab, *Limulus polyphemus*	
POLYPHEMUSIN-2	Horseshoe crab, *Limulus polyphemus*	
ANDROCTONIN	Scorpion, *Androctonus australis*	
GOMESIN	Spider, *Acanthoscurria gomesiana*	
PROTEGRIN	Pig, *Sus scrofa*	
	50% consensus	

The alignment shows the peptides that are related to Arenicin-3 and NZ17074. Interestingly, the closest related peptide is Tachyplesin-1 identified in horseshoe crab with an identity of 57%. Tachyplesin-1 is also a beta-hairpin peptide stabilized with 2 cysteine bridges and is also highly charged with 6 positively charged residues. The positive charge of beta hairpin peptides might be involved in the mode of action where electrostatic interactions with the negatively charged bacteria membrane might be crucial. Bold residues indicates the difference between Arenicin-3 and NZ17074.

	MIC in MHB				Protein Binding (90% serum)
Variant	Clinical isolate 1	ATCC 25922	Clinical isolate 2	ATCC 27853	HPLC
	E.coli	*E.coli*	*E.coli*	*Pseudomonas*	% Binding
Arenicin-3	0,25	0,5	0,5	0,5	99
NZ17074	0,25	0,5	0,5	2	79

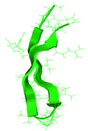
The MIC testing of both *E.coli* and *Pseudomonas* shows that NZ17074 exhibits a potent effect as Arenicin-3 but with lower protein binding properties.

Conclusions

- **NZ17074 is as new Arenicin-3 variant with lower protein binding properties and beneficial *in vitro* kinetic properties.**
- **NZ17074 exhibits the same potent activity as wild type Arenicin-3**
- **NZ17074 is a potential drug candidate.**

Reference

Ovchinnikova el al., FEBS Letters, Vol. 577 (1-2), 209-214, 2004



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