<u>Defensio</u>

PhD Thesis Marcus Seibold

Experimental and Theoretical Investigations of *Penicillium brevicompactum* Dierckx

introduction

fungus

protein

metabolite

Experimental and Theoretical Investigations of *Penicillium brevicompactum* Dierckx

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fungi	JS			
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antimicrobial peptides

- part of the innate immune system of virtually all forms of life
- appeared very early in the evolution
- common properties: small, high ratio of cationic amino acids

four groups of antimicrobial peptides:

(1) linear α-helical peptidesgenerally antibacterial activities

(2) multiple cysteine-bridge-containing peptides (**defensins**) antibacterial and antifungal activities

(3) proline-rich peptides against bacteria and filamentous fungi

(4) the glycine-rich peptides against gram-negative/positive bacteria

.. growing interest in antimicrobial peptides and defensins ...



published articles per year with key word "antimicrobial peptides"



source: NCBI

.. antimicrobial peptides are potential drug candidates in human therapy ...

Table 1 Peptides and peptide	omimetics in commercial development		
Company (location)	Drug	Stage of development	Medical use
AM-Pharma (Bilthoven, The Netherlands)	hLF-1-11 (small peptide derived from human lactoferrin)	Phase 2	Allogeneic bone marrow stem cell transplantation–associated infections
BioLineRx (Jerusalem)	BL2060 (a synthetic compound comprising fatty acid and lysine copolymers)	Lead optimization	Anti-infective
Ceragenix (Denver)	CSA-13 (cationic steroid (ceragenin) that mimics host-defense peptides)	Preclinical	Anti-infective
Helix Biomedix	HB-50 (synthetic natural peptide mimetic of cecropin)	Preclinical	Anti-infective
(Bothell, Washington, USA)	HB-107 (19-amino-acid fragment of cecropin B)	Preclinical	Wound healing
Inimex (Vancouver, BC, Canada)	IMX942 (5-amino-acid peptide)	Lead optimization	Immunomodulation; treatment of fevers and neutropenia in chemotherapy patients
Lytix Biopharma (Tromso, Norway)	Not available	Discovery	Anti-infective, antitumor
Migenix (Vancouver, BC, Canada)	Omiganan pentahydrocholoride/ CP-226/MX-226/CLS001 (12-mer analog of bactolysin)	Phase 3b/phase 2	Prevention of catheter-related infections; dermatology-related infections
Novacta Biosystems Ltd. (Hatfield, England)	Mersacidin (bacteriocin)	Preclinical	Gram-positive infections
Novobiotics (Cambridge, Massachusetts, USA)	Not available	Discovery	Nail fungus; methicillin-resistant <i>S. aureus</i>
Novozymes A/S (Bagsvaerd, Denmark)	Plectasin (fungal defensin)	Preclinical	Systemic anti-Gram positive, especially pneumococcal and streptococcal infections
Pacgen (Vancouver, BC, Canada)	PAC113 (based on the active segment of histatin 5 protein found in human saliva)	Investigational New Drug (IND) approval	Oral candidiasis

Hancock et al, Nat Biotechnol. 2006 Dec;24(12):1551-7

.. mechanisms of action of antimicrobial peptides ...



my work area: antimicrobial peptides - defensins - fungal defensins



.. fungal defensins are current topics of several research groups ..

Prof. Marx, Innsbruck P. chrysogenum, defensin PAF

- Prof. Theis, Berlin **A. gigangteus**, defensin AFP
- Prof. Nasri, Tunis *A. clavatus*, defensin AcAFP
- my contribution: *P. brevicompactum*, defensin BP

contribution of my PhD study

characterisation of a new defensin, the bubble protein

first time shown co-expression of a defensin with a clinically relevant small molecule

first time shown that *P. chrysogenum* encodes two defensins



.. once upon a time there was a fungal contamination of experimental barley cultures in a greenhouse ..

.. growing the "contamination itself" was successful and due to yellow-green fluorescence of exudate bubbles the fungus evoked some interest .. eventually leading to this defensio today ..



Experimental and Theoretical Investigations of *Penicillium brevicompactum* Dierckx



overview

fungus

identification

inhibition studies



.. after several attempts to extract genomic DNA from the fungus a protocol optimized for plant DNA was successful ..

genetic identification

- amplification of internal transcribed spacer region of 5.8 rDNA
- data base comparison at NCBI

result

- Penicillium brevicompactum Dierckx

inhibition studies

.. since the fungus is a kind of *Penicillium*, basic experiments for hints of a potential antibiotic effect were performed ..

inhibition studies

- co-cultivation with bacteria
- Enterococcus faecium
- Lactococcus lactis
- Escherichia coli
- 20 °C, 37 °C
- spores of the fungus
- bubbles of the fungus

result

- neither the spores of the fungus nor the bubbles inhibit the growth of the bacteria

.. later experiments concerning yeast told a different story .. (slide 26)

Experimental and Theoretical Investigations of *Penicillium brevicompactum* Dierckx

introduc			
fungus			
protein			
metabol	ite		

overview

protein

primary sequence

crystal structure

gDNA, exons, introns

similarity to other proteins

structure prediction of other proteins

BP homolog in *P. chrysogenum*

inhibition of yeast

Protein Data			×
<u>File H</u> ydropathy			
PROTEIN.BP			
Protein length	Molecular	weight	Isoelectric point
64 aa	6.6	6 kD	7.47
Hydrophobic	12.5%	Start	No Methionine
Hydrophillic	62.5%	End	No Stop codon
Basic	14.1%		
Acidic	10.9%		Internal Part of ORF
Neutral Hydropho 3.1% - 2 1.6% - 1 1.6% - 1 6.3% - 4 Charged, Acidic 7.8% - 5 3.1% - 2	bbic Alanine Phenylalanine Tryptophan Valine Aspartic acid Glutamic acid	Neutral, Hy 6.3% 12.5% 3.1% 20.3% 9.4% 9.4% 1.6% 6.3% 1.6% 6.3%	ydrophillic - 4 Asparagine - 8 Cysteine - 2 Glutamine - 13 Glycine - 6 Serine - 6 Threonine - 1 Tyrosine Basic - 4 Arginine - 1 Histidine - 4 Lysine
Uncertain			

primary sequence

.. due to the high content and purity of the dissolved protein in the bubbles, a significant part of the primary sequence of the bubble protein was revealed by Edman degradation straight ahead from the Petri dish and allowed primer design for subsequent PCR experiments for getting the cDNA ..

the resulting sequence shows

- high content of cysteins
- relatively basic amino acids
- relatively small size

DTCGSGYNVDQRRTNSGCKAGNGDRHFCGCDRTGVVECKGGKWTEVQDCGSSSCKGTSNGGATC

crystal structure



.. crystallization of the bubble protein was straight forward and revealed important properties:

- 4 disulfide-bridges
- for defensins typical twisted anti-parallel beta-sheet

Olsen et al. Solving the structure of the bubble protein using the anomalous sulfur signal from single-crystal in-house Cu K α diffraction data only. Acta Crystallogr D Biol Crystallogr. 2004 Feb;60 (Pt 2):250-5. Epub 2004 Jan 23. PDB Code: 1UOY

.. the architecture of the gene was revealed by genome walking using thermal asymmetric interlaced PCR ..



similarity to other proteins

signal peptide propeptide mature protein



MKVTAL+CTGFALMAATGAV+AS+VADSL+A++L+ARDEDTCGAGYGGDQRRTN++SPCK+SAQNGDRHFCGCDRTGVVECRGGKWTEIQDC+SDTCHGTNDGGARC+++

.. the alignments shows two clusters: the <u>BP cluster</u> on top and the <u>PAF cluster</u> below

similarity to other proteins

.. very high homology in the BP cluster ..



BP P. brevicompactum**chr** P.chrysogenum**the** M. thermophila**glo** C. globosum**fis** N. fischeri**fum** A.fumigatus

structure prediction of other proteins

.. successful structure prediction of hypothetical proteins from whole genome sequences of similar fungi ..



black = bubble protein colored = hypothetical proteins **fum** *A.fumigatus*

.. whole genome search revealed that *P.chrysogenum* has a BP homolog **in addition** to PAF ..

Penicillium chrysogenum Wisconsin 54-1255 hypothetical protein (Pc21g12970) mRNA, complete cds Sequence ID: <u>ref[XM_002568277.1]</u> Length: 267 Number of Matches: 1

Range 1: 1 to 264 GenBank Graphics					🔻 Next Match	🔺 Previous I	Match	
Score		Expect	Method	 ``	Identities	Positives	Gaps	Frame
125 bit	ts(315	5) 5e-35	Compositional n	natrix adjust.	68/97(70%)	77/97(79%)	9/97(9%)	+1
Query	1	MKITALFY MK+TAL +	TLMaatavsaaavaE TLMAATAVSA+	RDTLGGLEARDT L+ RDT	CGSGYNVDQRRT CG GY VDORRT	NSGCKAGNGDRE NS C+A NGDRE	IF 60 IF	
Sbjct	1	MKVTALLF	TLMAATAVSASV	LDTRDT	CGGGYGVDQRRT	NSPCQASNGDRE	IF 153	
Query	61	CGCDRTGV CGCDRTG+	VECKGGKWTEVQDCO VECKGGKWTE+ODCO	GSSSCKGTSNGGA G +SC+G S GGA	TC 97 .C			
Sbjct	154	CGCDRTGI	VECKGGKWTEIQDCO	GASCRGVSQGGA	RC 264			
Query = BP sequence								
Sbjct = translated <i>P. chrysogenum</i> gene sequence								

.. therefore the genome of *P. chrysogenum* encodes for <u>two</u> defensins <u>and</u> penicillin ..



...P. chrysogenum Wisconsin harbours the PAF gene which establishes the PAF cluster on <u>chromosome 24</u>; on <u>chromosome 21</u> also the BP analogue is present and establishes the

BP cluster ..



inhibition of yeast





.. spiking studies demonstrated a concentration dependent inhibition of yeast cultures by the bubble protein ..

viability of yeast was reduced by about 30 %

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metabolite

isolation

identification

NMR calculation

application

.. about 100 mL bubbles were collected and standard procedures yielded pure crystals ..

processing of the bubbles

- organic extraction (tert-butylmethylether)
- concentration of organic phase
- solubilisation in MeOH:H₂O
- gel chromatography
- concentration of eluate
- one week crystallisation on room temperature



picture of MPA crystals

identification



.. crystals were dissolved in $DCCI_3$ and NMR experiments identified mycophenolic acid ..



metabolite

NMR calculation



.. further verification was established by quantum chemical calculations and comparison with the SPECINFO data base ..



mycophenolic acid

- first antibiotic derived from a fungus (Cosio 1896)
- antibacterial, antifungal, antiviral, antitumor effects
- used in human therapy as an immunosuppressive



first time shown co-expression of a defensin with a clinically relevant small molecule

BP secreted together with mycophenolic acid

Chrom 24 PAF identical

AnAFI





There are many people I have to thank and especially the colleagues from the

Institute of Theoretical Chemistry

Institute of Structural Biology

Department of Pharmaceutical Technology and Biopharmaceutics