

The biochemical diversity of aquatic life, which is only beginning to be recognized, could make a significant contribution to the future progress of the never-ending battle against infectious diseases.

Piscidins: A Novel Family of Peptide Antibiotics from Fish

by Edward J. Noga
and Umaphorn Silphaduang

Even before our heightened awareness of threats from bioterrorism, infectious disease was recognized as a leading cause of morbidity and mortality worldwide. In addition, more than 30 new disease agents have been identified since 1973.¹ Another very troubling aspect of this sobering picture is the increasing development of antibiotic-resistant bacteria, which is creating a serious worldwide crisis in both human and veterinary medicine. Epidemics of various types of multidrug-resistant pathogens are being increasingly reported, including both Gram-positive (e.g., methicillin-resistant *Staphylococcus aureus* [MRSA] and vancomycin-resistant enterococci [VRE]) and Gram-negative (e.g., *Salmonella*, *Pseudomonas*) pathogens.²⁻⁴ At the same time, resistance to even the latest versions of antibiotics is rapidly developing. Resistance to the semisynthetic streptogramin antibiotic quinupristin-dalfopristin (Synercid, Aventis) ap-

Summary

The global emergence of many new infectious diseases, as well as concerns about the antibiotic resistance of an increasing number of microbial pathogens, necessitates that new approaches be sought in combating these serious infections. Peptide antibiotics, host-produced antimicrobial defenses that have been isolated from all types of organisms, from plants to mammals, possess a number of characteristics that make them attractive drug candidates. An example of the diversity and potential for new discoveries in this area is a novel family of peptide antibiotics named "piscidins," which have been recently isolated from fish. Piscidins have potent, broad-spectrum *in vitro* activity against many pathogens, including multidrug-resistant bacteria. Interestingly, piscidins reside in mast cells, a highly common tissue granulocyte of uncertain function that is ubiquitous in all vertebrate classes. The discovery of peptide antibiotics in mast cells may be a previously unappreciated, yet crucial, function for this highly common yet enigmatic immune cell. © 2003 Prous Science. All rights reserved.

peared after its introduction in late 1999 to treat vancomycin-resistant *Enterococcus faecium*. Linezolid, a member of the new oxazolidinone class of antibiotics (the only new class of antibiotic introduced in the last 30 years), was approved by the U.S. Food and Drug Administration in April 2000; resistance of MRSA to this antibiotic has already been detected.⁵ In hospitals, where bacterial resistance poses the most serious threat, an estimated 2 million nosocomial (hospital-acquired) infections occur annually in the United States, accounting for

more than 25,000 deaths and over \$4.5 billion in additional healthcare costs.^{6,7}

While more judicious use of antimicrobials, as well as improved methods for preventing the spread of pathogens, are important strategies for fighting both new and reemerging infectious diseases, there is also clearly a need for new modes of antimicrobial action. Consequently, novel, effective antibiotics represent an important means to address our growing problems with infectious diseases, as well as being a significant potential world-

wide market. Antibiotics are the second most commonly prescribed category of drug. And they are now the third-largest pharmaceutical market sector, trailing only cancer and cardiovascular products. The antibiotic sector comprises well over 100 drugs, with total worldwide sales of over \$30 billion. Over 30 antibiotics exceed \$250 million in annual sales—at least two exceed \$2 billion in sales.

Peptide antibiotics from plants and animals

With increasing problems from infectious disease, one group of antimicrobials that has received considerable attention in recent years are peptide antibiotics. Peptide antibiotics isolated from microbes have been known for many years and some have been used in clinical medicine (e.g., gramicidins, polymyxins). However, the discovery within the last 20 years of an increasing number of antimicrobial peptides in plants and animals (both invertebrates and vertebrates) has led to a surge in interest to understand the function of these molecules and explore their use as therapeutics. Produced by the host organism as an innate defense, peptide antibiotics probably occur in most, if not all, animals, and there is increasing evidence that they play a key role in innate immunity.⁸ These peptide antibiotics typically possess broad-spectrum activity and are rapidly lethal to target pathogens; most cause cell death via rapid membrane lysis, although some appear to act via other mechanisms. A very important consideration for commercial development is that, because of their nonspecific, detergent-like mechanism(s) of action, resistance is slow to develop (and some have argued that resistance is unlikely to develop at all,⁹ providing a potentially long product life.

Peptide antibiotics are typically 10–50 amino acids long and have at least two excess positive charges due to basic amino acids. Usually, about half of their amino acids are hydrophobic. This basic structure (cationic and hydrophobic) is needed to impart the

amphipathic nature essential for their functioning (see below). Peptide antibiotics usually display one of four major structural motifs: α -helical, β -sheet, extended coil or loop structures.¹⁰ While literally hundreds of natural structures have been isolated, many, if not most, do not have all the desirable features needed for possible clinical application (see below).

Fish as a source of novel peptide antibiotics

With over 40,000 known species, fish are by far the most abundant vertebrates. They combat infections caused by viruses, bacteria, fungi and parasites that are similar to those of humans and other higher vertebrates. Their primitive immune systems rely heavily on rapidly responding innate immunity. They also inhabit a number of highly diverse ecological niches and thus are exposed to a very wide array of pathogens. These features suggest that they might have devised various, alternative (and in some cases undiscovered) strategies for protecting against infectious agents, further suggesting that these animals may be a rich source of nonspecific defenses, such as antimicrobial peptides.

Fish have been largely ignored as a potential source of novel antimicrobial peptides. Of approximately 600 peptide antibiotics that have been isolated

from various animals, relatively few have been identified from fish (Tables I and II), suggesting that fish may represent a vast, untapped chemical library.

Piscidins, a novel group of peptide antibiotics

In searching for important mechanisms of innate immunity in hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female), an important aquacultured fish, we discovered several types of polypeptide antibiotics in the skin, gill and gastrointestinal tract¹¹ (Silphaduang and Noga, unpublished data). One of the most potent of these antibiotics was a novel family of three, broad-spectrum antibacterial peptides, named “piscidins” (from *pisces* meaning fish).¹² Piscidins are 22-amino acid peptides having a highly conserved amino terminus that defines the group (Fig. 1). Piscidins adopt an amphipathic α -helical conformation upon interaction with hydrophobic cell membranes (Park et al., unpublished data). In this conformation, the charged and polar residues align on a portion of the helical cylinder, while the hydrophobic residues occupy the remaining surface. This orientation is typical of most linear antimicrobial peptides.¹³

Piscidins are especially rich in the basic amino acid histidine (19–24 mole

TABLE I: THE NUMBER OF DIFFERENT MULTICELLULAR ORGANISMS FROM WHICH PEPTIDE ANTIBIOTICS HAVE BEEN REPORTED .

MAJOR TAXONOMIC GROUP	NUMBER OF SPECIES
Mammals	27
Birds	2
Amphibians (frogs, toads)	33
Fish	11
Tunicates	2
Insects	46
Crustaceans (crabs, shrimp)	3
Arachnids (spiders, scorpions, ticks)	10
Merostomes (horseshoe crabs)	2
Worms (nematodes, annelids)	3
Plants	37
Total	177

Data provided are courtesy of Prof. Alessandro Tossi from the AMSDbase (www.bbcm.univ.trieste.it/~tossi/antimic.html).

TABLE II: PEPTIDE ANTIBIOTICS ISOLATED FROM FISH.

NAME	FISH	APPROXIMATE MW AND NUMBER OF AMINO ACIDS	LOCATION	REFERENCE
HFIAP (hagfish intestinal antimicrobial peptides)	Atlantic hagfish	3.5-4.6 kDa (30-37 AAs)	Intestine	27
HFS-1	Pacific hagfish	NR (10 AAs)	Skin	28
Pardaxins	Red Sea Moses sole, peacock sole	3.3 kDa (33 AAs)	Skin (mucus glands)	29
Pleurocidins	Winter flounder, probably others	2.7 kDa (25 AAs)	Skin, intestine	30, 31
Piscidins	Hybrid striped bass, white bass, striped bass, probably many others	2.5 kDa (22 AAs)	Many organs, including skin, gill, gastrointestinal tract	12, 15
Misgurin	Loach	2.5 kDa (21 AAs)	Whole fish	32
Hepcidins	Hybrid striped bass, white bass	2.3 kDa (21 AAs)	Liver, low expression in other tissues	33, 34
LCRP (lamprey corticostatin-related peptide)	Sea lamprey	2.2 kDa (19 AAs)	Skin	35
Parasin I (N-terminal fragment of histone H2A)	Asian catfish	2.0 kDa (19 AAs)	Skin	36, 37
HSDF (N-terminal fragment of histone H1)	Coho salmon	NR (26 AAs)	Mucus, blood	38

NR: not reported; AA: amino acids.

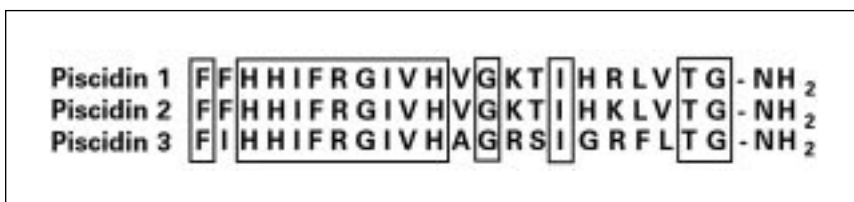


Fig. 1. Amino acid sequences of piscidins. Conserved residues are in the blocks. Piscidin 1 and piscidin 2 have 95% homology. Piscidin 3 is 77% and 73% homologous to piscidins 1 and 2, respectively. Mass spectrometry suggests that natural piscidins may occur with either an amidated or a free carboxyl terminus.

percent of the amino acid composition). Interestingly, the presence of large amounts of histidine would be expected to make these peptides relatively inactive at physiological pH. For example, clavanins, a group of histidine-rich peptide antibiotics from tunicates (a marine invertebrate), are only weakly active at pH 7.4.¹⁴ However, piscidins are highly active at neutral pH,^{12,15} possibly owing to their greater net positive charge.

Piscidins have potent, broad-spectrum antibacterial, antifungal and antiparasitic activity¹² (Silphaduang and Noga, unpublished data), which is usually evident well below the concentration that damages vertebrate cells.¹² Both important fish and human

pathogens are susceptible to piscidins, including MRSA and VRE^{12,15} (Silphaduang and Noga, unpublished data).

Piscidins, like most other naturally occurring peptide antibiotics, can probably be improved in their therapeutic efficacy via rational drug design (truncation, amino acid substitution, etc.).¹⁶ Piscidins possess a number of features that make them attractive as possible templates for the design of new antimicrobials. Among these are their novel sequences, relatively small size, uncomplicated structure, potent, broad-spectrum activity, potentially low toxicity (as suggested from preliminary *in vitro* data) and strong activity in physiological salt concentrations.

In fact, piscidins appear to be some of the most salt-tolerant antimicrobial peptides identified to date. We have demonstrated that piscidins are highly active in the presence of sodium chloride,¹² and this observation was further substantiated by others who demonstrated that piscidins were relatively unaffected by both monovalent and divalent cations (Table III).

Piscidins reside in mast cells

One of the most common cell types involved in innate immunity is the mast cell, the major tissue granulocyte of vertebrates.¹⁷ Fish mast cells (also known as eosinophilic granule cells) are similar to their mammalian counterpart in being cells filled with large granules that often stain metachromatically. These granules contain a large array of bioactive compounds that are actuated by degranulation (the release of granule contents extracellularly). Degranulation can occur with various stimuli, although in fish it is not mediated via IgE as with mammalian mast cells.^{17,18}

The mast cells of hybrid striped bass express piscidins (Fig. 2). We do not yet know whether piscidins are released into the extracellular environ-

TABLE III: SENSITIVITY OF SELECTED ANTIMICROBIAL PEPTIDES TO MONOVALENT AND DIVALENT CATIONS. CONCENTRATIONS GIVEN ARE THE CATION LEVELS AT WHICH SIGNIFICANT INHIBITION WAS DETECTED. NOTE THAT INHIBITORY LEVELS ARE ONLY PRESENTED AS ROUGH COMPARISONS SINCE DIFFERENT METHODS WERE USED TO ASSESS CATION TOLERANCE BY VARIOUS INVESTIGATORS.

PEPTIDE	PEPTIDE CLASS	INHIBITORY CONCENTRATION (μ M)			PATHOGEN TESTED	REFERENCE
		Na	Ca	Mg		
Human β -defensin 4	β -Sheet	25			G+ bacteria	39
Cryptdin 2	β -Sheet	10	1	1	protozoa	40
Hepcidin	β -Sheet?	100			G \pm bacteria	33
Cecropin P1	α -Helical	50			G+ bacteria	14
Cecropin P1	α -Helical	200			G- bacteria	41
Pleurocidin	α -Helical	625	1	5	G- bacteria	30, 42
Styelin A	α -Helical	>400			G \pm bacteria	43
Piscidin 2	α -Helical	>1280	10	40	G+ bacteria	15
Clavanin A	α -Helical	300			G \pm bacteria	14
Magainin 1	α -Helical	50			G \pm bacteria	14
Indolicin	Extended coil		1		Protozoa	40

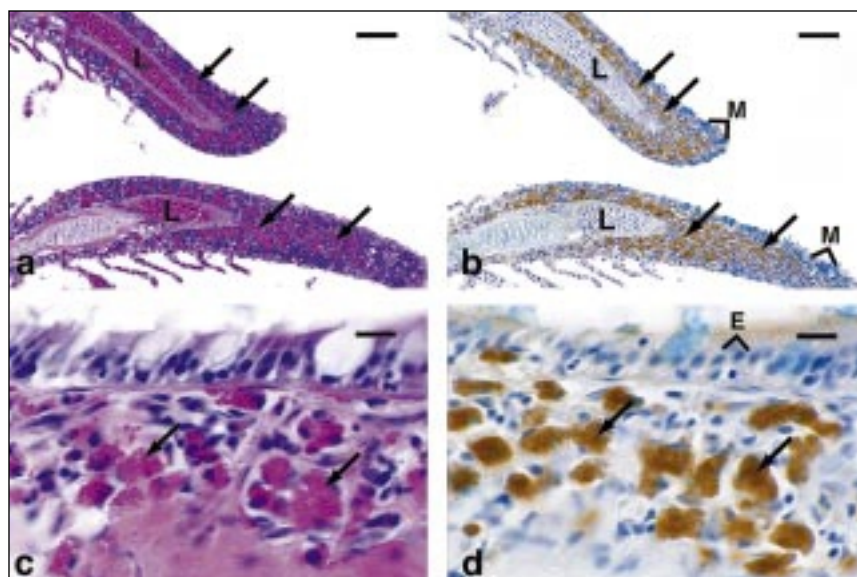


Fig. 2. Immunohistochemical localization of piscidins in mast cells (MC) of hybrid striped bass. **A.** Low-power view of gill lamella (L) stained with hematoxylin and eosin (HE), demonstrating the highly abundant, eosinophilic (bright red-staining) MC in this tissue (arrows). Bar = 50 μ m. **B.** Same gill section as (A) treated with antipiscidin antibody. Arrows, MC. M: mucus cells. Bar = 50 μ m. **C.** High power view of MC in the submucosa of the gut (arrows). HE stain. Note the abundant eosinophilic granules filling the cells. Bar = 10 μ m. **D.** Same gut section as (C) treated with antipiscidin antibody. Arrows, MC. E: gut epithelium. Bar = 10 μ m.

ment, such as during degranulation, although the proximity of many mast cells to body surfaces (skin, gills, gut) makes this a good possibility. However, mammalian mast cells are also phagocytic,¹⁹ and if this is also true for piscine mast cells, piscidins might function intracellularly. Not all hybrid striped bass mast cells are positive for piscidins, suggesting that piscidin-negative mast cells are either at a different stage of development or that

they have differentiated independently of the piscidin-positive mast cell lineage. Phenotypic heterogeneity in mast cells is well recognized in fish^{18,20} and mammals.^{21,22} We have evidence for differential expression of various piscidins in different mast cells (Silphaduang and Noga, unpublished data).

The mast cells of several types of other fish also express piscidins, including the parental stocks of hybrid

striped bass (white bass [*M. chrysops*] and striped bass [*M. saxatilis*], family Moronidae), where the genes for piscidins 1 and 2 have been cloned.¹⁵ Members of the Sciaenidae family (spot, *Leiostomus xanthurus*; croaker, *Micropogonias undulatus*),¹² as well as a number of other fish species (Silphaduang et al., unpublished data) also express piscidin-like peptides. The Moronidae and the Sciaenidae are in the suborder Percoidei, order Perciformes. This suggests that piscidins may be evolutionarily conserved in this group. The Percoidei is the largest suborder in the Perciformes, which is the largest order of living vertebrates. The widespread occurrence of piscidin-like peptides in fish raises the question as to whether other vertebrates might also express piscidins. It is interesting to note that some types of peptide antibiotics are found in highly divergent groups (e.g., cecropins in insects and swine).

Do antibiotics occur in mast cells of other vertebrates?

Mast cells are found in all classes of vertebrates including fish, frogs, reptiles, birds and mammals. Since peptide antibiotics occur in mast cells of fish, it will be interesting to see whether this innate defense occurs in mast cells of other vertebrates. If so, they might define a totally new role for the mast cell in vertebrate host defense. Mast cells have been most commonly

associated with allergic reactions/disorders. Of course, this is a maladaptive response that does not appear to confer any benefit to the host. Elie Metchnikoff²³ was probably the first to suggest that mast cells might defend against disease, and mast cells have been associated with defense against certain parasitic infections for some time.^{17,22} Recent evidence suggests that mast cells also play an important role in fighting bacterial infections.^{19,24} However, in such cases, they have been considered to be primarily involved in orchestrating the activation of other effector cells, to kill invading pathogens. For example, mast cells play a crucial role in secreting tumor necrosis factor α (TNF- α), which recruits and activates neutrophils for protecting mice against septic peritonitis.²⁵ Whether mast cells play a more direct role in killing microbes and parasites by release of antimicrobial peptides should be more closely investigated.

Concluding remarks

We are precipitously approaching a postantibiotic era. Current methodologies to search for new antibiotics, such as high-throughput screening, have focused on searches of synthetic combinatorial libraries rather than natural sources to discover new antibiotics. Unfortunately, this emphasis has mainly yielded minor modifications of present-day drugs.⁵ The movement away from exploring natural sources, which still constitute over half of all the medicines currently used, is considered by some to be a major reason why the pharmaceutical industry's drug pipelines are currently lacking in new candidates.²⁶ The discovery of piscidins and their subsequent localization to the enigmatic mast cell is an example of the information richness in comparative medicine and, in particular, the value of exploring the resistance mechanisms of lower animals. The biochemical diversity of aquatic life, which is only beginning to be recognized, could make a significant contribution to the future progress of the never-ending battle against infectious diseases.

Note added in proof

Ijima et al. have recently isolated peptides with some sequence similarity to piscidins from a marine fish. These peptides were localized to what appeared to be mast cells.⁴⁴ Di Nardo et al. have recently reported the presence of cathelicidin peptide antibiotics in both murine and human mast cells and have provided evidence that these antibiotics might play a major role in the antimicrobial function of mast cells.⁴⁵

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Edward J. Noga*, D.V.M., is a Professor, and Umaporn Silphaduang, D.V.M., Ph.D., is a Research Associate in the Department of Clinical Sciences at North Carolina State University, 4700 Hillsborough Street, Raleigh, North Carolina 27606, U.S.A. Edward Noga is also Chief Scientific Officer and Vice-President for Research and Development at Norcarex Bio Corporation, 2501 Blue Ridge Road, Suite 150, Raleigh, North Carolina 27607, U.S.A. E-mail: ed_noga@ncsu.edu; Tel: +1-919-513-6236; Fax: +1-919-513-6236. *Correspondence.

AVI BIOPHARMA'S NEUGENE PROGRAM ADVANCES

AVI BioPharma, Inc. announced March 26, 2003, that it has made further progress with its *NeuGene*[®] anti-sense program targeting the oncogene c-myc. Phase I studies have now been completed with **AVI-4126**, a *NeuGene* compound targeting the oncogene. AVI-4126 has been evaluated in numerous trials for various

indications, including phase Ib studies in patients with cancer and polycystic kidney disease, and a phase II study in cardiovascular restenosis. Interim data from this latter study indicated that AVI-4126 was effective in preventing restenosis, consistent with results from preclinical studies.

Drug-related adverse events have not been observed to date in trials of

the drug. In preclinical studies, AVI-4126 specifically inhibited the growth of prostate cancer cells. In combination with other agents, AVI-4126 inhibited tumor growth and produced complete tumor regression. AVI-4126 blocked the expression of the target gene, resulting in a decrease in c-myc protein.