The increasing antibiotic resistance of pathogenic bacteria calls for the development of alternative antimicrobial strategies. Possible approaches include the development of novel, broad-spectrum antibiotics as well as specific targeting of individual bacterial virulence factors. It is impossible to decide currently which strategy will prove more successful in the future since they both promise different advantages, but also introduce diverse problems. Considering both approaches, our laboratory's research focuses on the evaluation of hemocidins, broad-spectrum antibacterial peptides derived from hemoglobin and myoglobin, and staphostatins, specific inhibitors of staphopains — *Staphylococcus aureus* secreted proteases that are virulence factors regarded as possible targets for therapy. The article summarizes recent advances in both fields of study and presents perspectives for further development and possible applications.

**Keywords:** antibiotics, antimicrobial agents, hemocidins, hemoglobin, protease inhibitor, *Staphylococcus*, staphostatin, staphopain, virulence factor

The resistance of pathogenic microorganisms to currently known antibiotics is constantly increasing due to a broad use of antimicrobials in medicine, animal husbandry and agriculture. Although so far resistance of microorganisms to all approved compounds has rarely been noted, the number of such events will certainly increase with time (McGahee & Lowy, 2000). If no preventive actions are taken, this will inevitably lead to a state that preceded the discovery of antibiotics, when efficient treatment against bacterial infections was unavailable. The development of novel antibiotics and alternative therapeutic strategies is therefore a burning necessity.

Among many proposed strategies, studies on natural and artificial amphipathic peptides acting on membranes of microorganisms have yielded promising results. Currently, more than 800 such peptide antibiotics have been described and some of them have already entered clinical trials. For several years our laboratory has conducted studies on hemocidins, antibacterial peptides derived from hemoglobin and myoglobin. We have characterized several naturally occurring and artificially derived hemocidins with strong antimicrobial activity in the micromolar range (Mak et al., 2000). Both production and application of hemocidins as drugs or preservatives were claimed in a patent (PL 187999). We are presently evaluating the physiological significance of natural hemocidins in humans and trying to increase the efficiency of already developed peptides.

Alternative antimicrobial strategies assume that specific virulence factors should be addressed since bacteria are more likely to develop resistance to broad-range antibiotics. For example, *Staphylococcus aureus* is a bacteria whose virulence relies on production of a wide range of such factors (Novick, 2000). Among others, extracellular proteases constitute an important part of the bacteria’s weaponry. Recently, a novel family of very specific proteinaceous inhibitors of staphylococcal cysteine proteases has been described in our laboratory (Rzychon et al., 2003b). The achieved detailed structural characterization makes it possible to design low molecular-mass molecules that could be effective against staphylococcal infections (Dubin et al., 2003; Filipek et al., 2003; Rzychon et al., 2003a).
HEMOCIDINS — BROAD RANGE ANTIMICROBIALS

Proteolytic degradation of functional proteins can lead to generation of peptides exhibiting a variety of biological activities (Karelin et al., 1998). The peptides are released during general non-specific proteolytic processes, after the maternal protein substrates have fulfilled their primary physiological roles. The functions of the released peptides are usually distinct from those of their precursors. Additionally, in contrast to classical endocrine or paracrine peptide hormones, the bioactivity of released peptide fragments is limited to the maintenance of local tissue homeostasis.

The best characterized precursors of bioactive peptides include casein, albumin, lactoferrin, gluten, cytochrome c and, most importantly, hemoglobin (Ivanov et al., 1997). The latter can be considered as an exceptional example because the collection of hemoglobin-derived bioactive peptides is particularly rich, comprising opioid-like, analgesic, hemopoietic, coronaro-constrictory, immunomodulatory, ACTH-releasing ones and many others (Karelin et al., 1995). These peptides originate from both globin chains and the majority of them are not longer than 15 amino acids. In certain circumstances, the peptides are generated from primary fragments of about 30 amino acids already in erythrocytes.

A growing number of reports support a role of hemoglobin as a precursor of peptides able to kill microorganisms. The first communication documenting hemoglobin bactericidal properties appeared in 1958 (Hobson & Hirsh, 1958), but more convincing evidence of the activity became only available in recent years. Our laboratory contributed substantially to this development. In our early work concerning the issue (Mak et al., 2000) we demonstrated that removal of heme is essential for the hemoglobin antibacterial activity. The heme-divested and partially unfolded globin molecules were able to kill a variety of microorganisms at the half maximal lethal doses in the micromolar range. The same applies to myoglobin, whose activity is sustained after limited enzymatic or chemical digestion to approximately 50-amino acid-long fragments. Further fragmentation (e.g., by trypsin) to shorter peptides results in nearly complete extinction of its antimicrobial properties.

We proposed the term hemocidins to name the bactericidal peptides derived from heme-containing proteins. The term well describes the killing activity and points to the maternal proteins.

A representative peptide derived from horse myoglobin (amino acids 56–131) was chosen for a study on the mechanism of action of hemocidins (Mak et al., 2001). The peptide manifested pronounced activity toward a variety of microorganisms and, importantly, could be easily obtained by cyanogen-bromide digestion of myoglobin. We demonstrated that the activity of this cationic peptide (net charge +10) was, to some extent, inhibited by high salt concentrations as well as by divalent cations, altogether showing the lack of its affinity toward lipopolysaccharide. The experiments with planar bilayer membranes as well as circular dichroism spectra analysis showed that the 56–131 myoglobin peptide has a fully random structure in solution but folds rapidly upon contact with the negatively charged phospholipid membranes. The folding is associated with membrane integrity disruption. Moreover, the lack of discrete transmembrane conductance changes suggests that the peptide does not act as a channel-forming factor. These facts prompted us to hypothesize that hemocidins act in a carpet-like manner, characteristic for other α-helical antibacterial peptides (Oren & Shai, 1998). Following electrostatically driven initial interaction with the membrane, the peptides form carpet-like clusters or plaques on the phospholipid bilayer, inducing destabilization of the membrane and, finally, its disruption.

Our results concerning the high potential of hemoproteins for generation of antimicrobial peptides were recently confirmed by another research group (Parish et al., 2001). We propose that the potential is primarily associated with the abundance in hemoproteins of amphipathic α-helical domains of a characteristic bipolar distribution of charged and hydrophobic amino-acid residues. In this way the hemoprotein-derived fragments to some extent imitate important features of classical, gene-encoded peptide antibiotics.

The majority of the experiments mentioned above were performed in vitro, using either synthetic peptides or the products of enzymatic digestion of maternal proteins. However, taking into consideration the abundance of hemoproteins in animals and humans, it is of special interest to evaluate the role of hemocidins in the innate defense mechanisms against infections. The first communication that documented the presence of hemocidins in organisms referred to the bactericidal bovine hemoglobin fragment found in the gut of a tick (Fogaca et al., 1999). A similar peptide originating from rabbit hemoglobin was discovered recently (Nakajima et al., 2003), also in a tick’s gut. The authors of both papers suggested that the parasite uses the host protein as a protective antimicrobial agent in its own digestive tract. Another report on naturally occurring hemocidins concerned peptides isolated from homogenates of human placenta and from lysed erythrocytes (Liepke et al., 2003). One of those peptides was identified as a fragment of fetal γ-hemoglobin while a second one was from β-hemoglobin. Both peptides killed Gram-negative and Gram-positive bacteria in concentrations of tens µmole/l. Unfortunately, the latter work was performed on tissue homogenates.
prepared without the addition of protease inhibitors and therefore it remains unclear whether the isolated hemocidins were true peptide constituents of the analyzed tissues or rather were artifacts resulting from the purification procedure. Likewise, the physiological significance of both isolated hemocidins remains unknown.

In our recent communication we have documented that hemocidins are abundant in normal female menstrual discharge (Mak et al., 2004). Our work comprised a thorough analysis of peptide fractions isolated from the plasma of vaginal discharge of three healthy nulliparous women by high-performance liquid chromatography, automatic sequencing and mass spectrometry. Although the obtained peptide maps varied between the three subjects, the phenomenon seems natural and most probably reflects the complexity of individual vaginal ecosystem. We have identified 45 peptides altogether in all patients, 44 of which originated from hemoglobin, mainly from the N-terminal part of α-globin. All the isolated peptides demonstrated bactericidal activity toward Escherichia coli. Detailed screening of the antimicrobial action toward a variety of Gram-negative and Gram-positive bacteria was performed using two synthetic peptides identical to those found in menstrual blood. The estimated minimal inhibitory concentrations (MIC doses) for those peptides were between 27 and 293 μM. However, no killing activity toward the yeast Candida albicans was observed at the highest applied concentration (300 μM).

It is clear that specific conditions in the vagina during menstrual bleeding strongly favor the generation of hemocidins. Since the pH of the uterine cavity is neutral, hemoglobin is probably released from erythrocytes in the low pH of the vagina. Low pH is also a factor that causes dissociation of the hemoglobin molecule as well as disruption of some hydrogen bonds within the globin molecule. Partial denaturation facilitates the action of proteases. Indeed, it is noteworthy that besides the lysed functional layer of the endometrium, mucus, blood plasma and blood cells, the menstrual discharge contains also significant amounts of proteolytic enzymes, mainly matrix metalloproteinases (MMPs) and leukocyte proteases (Salamonsen & Lathbury, 2000; Salamonsen et al., 2002). We believe that these proteases, among others, are responsible for liberation of hemocidins.

From the medical point of view, one of the most important features of menstrual hemocidins is their high activity toward E. coli. Urinary tract colonization by this microorganism represents an important health problem for women. We hypothesize that the lack of hemocidins may underlie frequent vaginal colonization by E. coli in postmenopausal women (Pabich et al., 2003). Little is known about the maintenance of vaginal homeostasis during menstruation. We assume that the described vaginal hemocidins are an important part of the nonspecific bactericidal mechanisms that support human vaginal homeostasis during physiologic menstrual bleeding.

The work described above on the bactericidal menstrual hemoglobin-derived peptides clearly suggests that hemocidins constitute a new and significant mechanism supporting classical, well-known mechanisms of innate unspecific immunity against infections. However, these discoveries do not exhaust the interesting theme of vaginal homeostasis. We are presently working on genital secretions of women with infections (vaginosis) and on vaginal discharges after birth (lochia), in hope of obtaining an insight into the mechanisms of formation of vaginal hemocidins as well as on the role of the peptides in the pathophysiology of the female urogenital tract. We also plan to investigate other available clinical material that can possibly contain hemocidins. The research will comprise analysis of the peritoneal dialysis fluid obtained from patients with kidney failures, the discharge from wounds, fluids from post-surgery caves, and others. We hope that our research will allow us to obtain comprehensive answers for principal questions concerning hemocidins.

### STAPHOSTATINS — SELECTIVE TARGETING OF VIRULENCE FACTORS

As an alternative to the development of broad-range antibiotics, which the bacteria are likely sooner or later to gain resistance to, due to the great selective pressure exerted by their use, targeting of specific virulence factors has been proposed. Such strategy imposes the need of developing a great number of different agents specific against only a narrow spectrum of pathogens, limited to species or even particular strains. However, resistance is less likely to develop in such a setup and the known adverse effects of broad spectrum antibiotics on natural microflora may be avoided. Moreover, such a strategy greatly broadens the number of available targets, which, in connection with high-throughput screening techniques, may facilitate a faster development of agents suitable for therapeutic purposes against at least some bacteria.

Part of the research conducted at our laboratory is focused on Staphylococcus aureus, one of the most versatile and dangerous human pathogen. In view of the increasing antibiotic resistance of this species, screening for suitable targets and development of novel antistaphylococcal compounds is a burning necessity (McGahee & Lowy, 2000). The virulence factors of S. aureus, targets suitable for the strategy described above include, amongst others, a spectrum of secreted proteases. Staphylococci
secrete a range of serine proteases (SspA, six homologous Spl proteases, epidermolytic toxins A and B), cysteine proteases (staphopains A and B) and a metalloprotease (aureolysin) (Dubin, 2002). The importance of some of these enzymes for the virulence of the bacterium has been demonstrated in different infection models, although the evidence seems contradictory at some points and the topic still awaits thorough investigation.

Different protease and protease expressions regulating systems knock-out strains evaluated in animal infection models have produced a somewhat confusing picture as to the role of particular enzymes and the overall secreted proteolytic activity in staphylococcal virulence. Nevertheless, despite the fact that the evaluation of individual strains was done in various models and a systematic study encompassing knock-outs of all known proteases in a single model is lacking, some encouraging reports have been produced. Below, the current state of investigation on the role of staphylococcal proteases in virulence determination is briefly summarized.

A clear demonstration of a role of a particular proteolytic enzyme in staphylococcal virulence was possible only in the case of epidermolytic toxins in staphylococcal scalded-skin syndrome (Prévost et al., 1991; Redpath et al., 1991). Reportedly, the virulence of V8 serine protease (sspA<sup>−</sup>) and serine protease-like operon (spl<sup>−</sup>) knock-outs was not affected significantly in murine abscess infection model or after intraperitoneal injection, respectively (Reed et al., 2001; Rice et al., 2001). Moreover, the former knock-out showed even slightly enhanced virulence. In contrast, the virulence of the same V8 deficient strain was decreased in a Caenorhabditis elegans model (Sifri et al., 2003). Furthermore, the virulence of a strain deficient in the V8 protease and staphopain B (sspABC<sup>−</sup>) was markedly reduced in comparison to the wild type in several different mouse models (Coulter et al., 1998). The above data points at staphopain B as the main virulence determinant among secreted proteases. Further observations supporting this claim were recently published by Shaw and colleagues (2004). The virulence of four knock-out strains of main staphylococcal extracellular proteases was evaluated in a murine subcutaneous skin abscess model. The virulence of metalloprotease- (aureolysin) and one of the cysteine proteases (staphopain A)-deficient strains was not affected while knock-outs of another cysteine protease (staphopain B) and a serine protease (SspA) were significantly affected. Since the latter knock-out also lacks the coexpressed staphopain B, while the staphopain B-deficient strain expresses the SspA protease, these data again point to staphopain B as the main virulence determinant. However, unambiguous demonstration of the fact awaits studies in diverse genetic backgrounds and a comparative, over view of different animal models.

Further complicating the presented picture, the sarA regulatory locus mutant overproducing a number of proteases showed a decreased virulence in a rabbit endocarditis model (Cheung et al., 1994). However, agr null mutants, characterized by a profound defect in overall exoprotein production, showed a highly diminished virulence in a murine septic arthritis model (Abdelnour et al., 1993).

Recently, our laboratory has characterized a family of specific staphylococcal cysteine protease inhibitors (Rzychon et al., 2003b). In the light of the above presented data on staphopain B the proteins may provide suitable leading structures for the development of low molecular mass therapeutic analogs.

Staphostatins constitute a novel family of specific proteinaceous inhibitors of staphylococcal cysteine proteases — staphopains. Although the enzymes belong to clan CA of papain-like proteases, staphostatins inhibit exclusively the staphylococcal enzymes and not other clan members tested. Such specificity is of great value in the development of therapeutic molecules if it could be preserved in low molecular mass compounds, since various CA-clan peptidases act in great many physiological processes, making cross-inhibition unacceptable. The specificity of staphostatins is not only limited to staphopains but is even stricter since <i>S. aureus</i> staphostatin A inhibits only <i>S. aureus</i> staphopain A while staphostatin B inhibits only staphopain B (Rzychon et al., 2003b). In each case the inhibitor and the target enzyme are encoded in one operon, suggesting that the inhibitors were designed by nature to inhibit only their target proteases. Nevertheless, staphopains are extracellular enzymes and staphostatins reside intracellularly. Clues to the role of the latter inhibitors became available only recently with transgenesis and knock-out studies. Staphopains are toxic to <i>E. coli</i> when expressed ectopically, while co-expression with staphostatins may be easily achieved (Wladyka et al., 2005). Moreover, an <i>S. aureus</i> staphostatin B knock-out strain exhibits decreased growth rate (personal communication). The above data clearly illustrate the protective role of staphostatins towards the cytoplasm against ectopically expressed (<i>E. coli</i>) or misdirected (<i>S. aureus</i>) staphopains, and attest to the deleterious effects of those staphylococcal virulence factors.

Despite the quite recent discovery of the staphostatin family a multitude of structural data are available on the inhibitors. Structures of <i>S. aureus</i> staphostatins A and B have been solved as well as the structure of a staphopain B–staphostatin B complex (Dubin et al., 2003; Filipek et al., 2003; Rzychon et al., 2003a). The former works demonstrated that staphostatins share a fold similar to lipocalins, however, no functional similarities were demonstrated. The latter structure made it possible to pinpoint the
staphostatin active site and to infer its mode of action. Unlike the previously known cysteine protease inhibitors, the staphostatin polypeptide chain spans the active site of the protease in a manner similar to the substrate, rather resembling the “standard mechanism” of serine proteases. The inhibitor escapes cleavage due to the distinct disposition of the P1 glycine residue. The latter was confirmed by site directed mutagenesis, since replacement of P1 with other amino acids converted the inhibitor into a substrate. Despite the wealth of structural information described, the mechanism of the strict specificity demonstrated. In such a setup the resistance is less likely to develop, as in multitarget strategies, the natural microflora will not be affected and no inter-species resistance transfer will be possible. While, the development of such multicomponent strategies seems presently very unlikely, due to the enormous effort and funds necessary to develop even single-component novel drugs, the evolving high-throughput screening and evaluation techniques might prove more suitable in a setup where multiple, instead of single, targets are tested in a more randomized manner to find chemicals effective only against a small subset of those.

Whichever setup is considered, the progress in the industry will only be facilitated by basic studies. A lot remains to be done in learning the properties of antibacterial peptides, especially in vitro, and evaluating the importance of different virulence factors of bacteria. Such studies will be continued in our laboratory and by scientists around the world in the forthcoming years and some results further utilized by the industry will surely contribute to the further improvement in our struggle against microbial pathogens.

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