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Review

ParabutoPORIN, a cationic amphipathic peptide from scorpion venom: Much more than an antibiotic

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ABSTRACT

ParabutoPORIN (PP) from the South African scorpion *Parabuthus schlechteri* is a 45-mer lysine-rich and cysteine-free peptide. At micromolar concentrations it has antimicrobial effects against G+ and G– bacteria and is antifungal as well.

However, at submicromolar concentrations, parabutoPORIN also directly interferes with cellular functions of the human innate immune system, especially polymorphonuclear neutrophils (PMN): parabutoPORIN acts as a chemoattractant for neutrophils, induces their degranulation, while delaying constitutive neutrophil apoptosis. In addition, it potently inhibits induced superoxide production.

Different signalling pathways regulating these biochemical processes were identified as targets of parabutoPORIN. Therefore, parabutoPORIN is a well documented scorpion venom peptide with immuno-regulatory properties beyond its antibiotic effects.

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1. Introduction

Scorpion venom is a complex mixture of low molecular weight bioactive molecules, small peptides and enzymes (Tytgat et al., 1999; Rodriguez de la Vega and Possani, 2005; du Plessis et al., 2008). Together, they play an essential role in (i) prey capture (ii) defence against predators and (iii) protection against microorganisms (Kuhn-Nentwig, 2003).

Abbreviations: CGD, Chronic Granulomatous Disease; CRAMP, Cathelicidin related antimicrobial peptide; fMLP, formyl-methionyl-leucyl-phenylalanine; FPRL1, Formyl peptide receptor like 1; GST, Glutathion S Transferase; LL37, 37 amino acid peptide derived from cathelicidin; LPS, lipopolysaccharide; Mcl-1, myeloid cell leukemia sequence 1; MIC, minimum inhibitory concentration; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; PI3 K, Phosphatidylinositol-3-kinase; PKC, Protein Kinase C; PMA, phorbol 12-myristate 13-acetate; PMN, Polymorphonuclear neutrophil; PP, parabutoPORIN; PR39, 39 amino acid peptide rich in proline and arginine; PTX, pertussis toxin; ROS, reactive oxygen species; SH3, Src Homology domain 3.

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In addition to its complex composition, scorpion venom does not always occur as one homogenous liquid (Dyason et al., 2002). Indeed, it was demonstrated that *Parabuthus* scorpions can make use of a 'prevenom' – consisting of a clear liquid enriched in potassium ions and some small peptides – to deter predators, immobilize small preys, and possibly also during mating. If the treat persists or more powerful tools are required, a more viscous, more lethal and protein rich venom is secreted (Inceoglu et al., 2003).

The abundance of peptides found in scorpion venom can be roughly divided into two broad classes, depending on whether they interfere with ion channels or whether they are antimicrobial.

The first class comprises disulfide-bridged peptides which interact with excitable cells and can be further subdivided according to the specific ion channel they interact with, and whether they stimulate or inhibit these channels. These peptides are responsible for most of the envenomation symptoms (du Plessis et al., 2008; Chippaux and Goyffon, 2008). In general, most of these cysteine containing peptides **not only** interfere with sodium or

potassium channels (Rodriguez de la Vega and Possani, 2004; du Plessis et al., 2008), but also calcium and chlorine channels have been reported to be affected (Zhijian et al., 2006).

In addition to these ion-channel interacting peptides, whose main function is to induce pain, immobilize or paralyze prey, scorpions also have an arsenal of antimicrobial and cytolytic peptides (Kuhn-Nentwig, 2003).

These latter peptides are found not only in their venom but also in the hemolymph, and form a crucial part of their innate immune system, enabling them to fight microorganisms. Many different and diverse antimicrobial scorpion peptides have been described. In general, they are small (12–50 AA), amphipathic and positively charged (+2 → +9) peptides, which all share the ability to disturb membranes and to insert themselves into phospholipid bilayers by mechanisms described as 'barrel-stave', 'carpet', 'toroidal-pore', 'detergent' and 'aggregate models' (Jenssen et al., 2006).

A first class consists of homologues of the well-known family of defensins. They are cationic, disulfide-bridge stabilized and are quite homologous with insect defensins (Cociancich et al., 1993; Zhu et al., 2000; Kuhn-Nentwig, 2003).

A second class is cysteine-free and most members are cationic and α -helical (Zeng et al., 2005). Parabutoprin belongs to the this class and was originally isolated from the venom of *Parabuthus schlechteri* (Verdonck et al., 2000; Willems et al., 2002). Other members are pandinins 1 and 2 from *Pandinus imperator* (Corzo et al., 2001), hadrurin from *Hadrurus aztecus* (Torres-Larios et al., 2000), Bmkbpp3 from *Buthus martensii* (Zeng et al., 2000), and opistoprin 1 and 2 from the venom of *Opisthophthalmus carinatus* scorpions (Willems et al., 2002). These peptides belong to a greater family of peptides with pore-forming properties found in many scorpions, and they are characterized by a conserved S(x)3KxWxS(x)5L sequence (Moerman et al., 2002).

A third group consists of unusual peptides, because they combine the properties of both classes. A well described example of this group is scorpine, isolated from *Pandinus imperator* (Conde et al., 2000). Finally, much smaller α -helical non-cationic antimicrobial peptides like IsCT were also described (Dai et al., 2001; Lee et al., 2004).

As yet, most attention was originally given to the microbicidal activity of these membrane perturbing peptides and they are still considered as promising therapeutic tools against pathogens which are increasingly resistant to conventional antibiotics. Indeed, most of them seem to show broad-spectrum lethal activity against bacteria, fungi, enveloped viruses and protozoa (Hancock and Diamond, 2000). However, the clinical value of their microbicidal effects is challenged with other disadvantages, such as (i) high manufacturing costs (ii) loss of microbicidal activity under physiological conditions (as a result of increased salt concentrations, proteolytic degradation and glycosaminoglycan binding) and (iii) unwanted systemic effects (Hancock and Diamond, 2000). These latter effects comprise aspecific toxicity against mammalian cells and unknown immuno-modulating effects.

Nevertheless, these obstacles might be overcome, as exemplified by the follow up of many antimicrobial peptides and their derivatives in final phases of clinical studies (Zaiou, 2007).

2. Parabutoprin as an antimicrobial peptide

Initial studies on the pore-forming activity of parabutoprin demonstrated that growth of Gram-negative bacteria was inhibited at minimum inhibitory concentrations (MIC) ranging from 1.6 μ M (*Klebsiella pneumoniae*) and 3.1 μ M (*Escherichia coli* and *Haemophilus influenzae*) to over 50 μ M (*Serratia marcescens*) in optimal conditions. However, higher salt concentrations lowered their effectiveness 5–10 fold (Moerman et al., 2002).

Furthermore, parabutoprin was shown to be hemolytic with an LD₅₀ of red blood cell lysis at 38 μ M. Homologous scorpion peptides like pandinin 1 and pandinin 2 also exert haemolytic activities (Corzo et al., 2001). Of note, parabutoprin is more lytic for other eukaryotic cells: it has an LD₅₀ at 1.3 μ M for human neutrophils, and similar sensitivities to pore formation by parabutoprin have been demonstrated for rat cardiac myocytes (Elgar et al., 2006). The observation of both the antimicrobial and the lytic activities against mammalian cells of parabutoprin in the micromolar concentration range is thus an obvious limitation for potential clinical applications. However, at submicromolar concentrations, parabutoprin is nonlytic against any known human cell line.

Although all cationic peptides are able to interact with membranes, which can result in membrane destabilization at a given concentration, there is no absolute correlation between their antimicrobial effects and pore-forming properties (Mookherjee and Hancock, 2007). With regard to parabutoprin, potentiating and synergistic effects have been reported at nonlytic concentrations when applied in combination with classical antibiotics like cefuroxime or erythromycin, as well as for the structurally related α -helical peptide opistoprin 1 (Moerman et al., 2002).

3. Nonmicrobicidal effects of antimicrobial peptides

Most cationic peptides which have been demonstrated to exert microbicidal effects (Hancock, 2001), appear to do so only to a limited extent under physiological conditions. Inactivation by physiological salt concentrations and scavenging by negatively charged proteoglycans have been demonstrated to antagonize their direct antimicrobial effects. In contrast, a wide range of other functions have been demonstrated under physiological conditions for many cationic peptides, including α - and β -defensins, indolicidins and the cathelicidins LL37, CRAMP, and PR39 (Mookherjee and Hancock, 2007). These alternative functions affect wound healing (Gallo et al., 1994; Hancock and Diamond, 2000), angiogenesis (Koczulla et al., 2003) and LPS neutralization (Scott et al., 2002; Giacometti et al., 2002). In addition, they can act as immuno-modulating agents, regulating polarization of adaptive immunity (Yang et al., 2002), induction of inflammatory gene expression (Bowdish et al., 2005) and stimulation of chemotaxis. For example, the human cathelicidin LL37 has been reported to

recruit immune cells both directly, through direct signalling via FPRL1 (De et al., 2000) and indirectly by inducing the production of the chemoattractant MCP-1 in macrophages (Scott et al., 2002).

In view of these recent observations, it has been proposed by Hancock and co-workers that these additional functions of cationic peptides, which are effective at lower peptide and higher salt concentrations, are as important, or even more important, for their generally accepted protective effects, as compared to their reported direct microbicidal effects (Mookherjee and Hancock, 2007).

Since most attention was originally given to the direct antimicrobial effects of cationic peptides, little has been reported concerning potentially additional immunomodulating effects, especially regarding non mammalian cationic peptides.

Initially, parabutoporin was not an exception in this context. However, parabutoporin has recently been shown to affect the cellular functions of human neutrophils in many ways and by different mechanisms (see Table 1). These neutrophil cells are short lived but nevertheless very abundant phagocytes which constitute an important first line defence against invading pathogens, containing a diverse load of antimicrobial components. Both oxygen dependent and independent killing strategies of neutrophils are known to be potentially harmful to the host itself. Consequently, the release and activation of the biochemical arsenal in neutrophils is highly controlled, by strict regulation of degranulation and superoxide production, as well as by regulating the neutrophil lifespan and their recruitment into tissues.

4. Parabutoporin stimulates neutrophil motility and recruitment

At nonlytic submicromolar concentrations, parabutoporin has been shown to attract neutrophils through activation of pertussis toxin sensitive signalling pathways (see Table 1). In this regard, parabutoporin was also shown to upregulate levels of GTP bound Rac2 (Willems et al., 2004). The importance of this finding is illustrated by the observation that neutrophils from Rac2 KO mice show little or no chemotaxis in response to agonists signalling through G protein coupled receptors (Li et al., 2002). The receptor responsible for the increased neutrophil motility induced by parabutoporin remains to be identified. However, a receptor independent mechanism leading to the

activation of G_i-proteins by parabutoporin has already been proposed (Moerman et al., 2003). The amphipathic α -helical structure might allow parabutoporin to cross or insert in the phospholipid bilayer, without membrane destabilization. This has been shown to be the case for mastoparan, which shares many physico-chemical properties with parabutoporin (Tisch-Idelson et al., 2001) and also attracts human neutrophils via a pertussis toxin sensitive mechanism (Norgauer et al., 1992). In further support of this hypothesis, cationic α -helical peptides have been shown to traverse the plasma membrane at concentrations well below those that cause rupture of plasma membrane (Zhang et al., 2001).

5. Parabutoporin stimulates neutrophil degranulation

In addition to the stimulation of neutrophil motility, parabutoporin has also been shown to induce neutrophil degranulation (see Table 1). Although the mechanisms mediating neutrophil degranulation are poorly understood, increases in cytosolic calcium are implicated in this process. In this regard, parabutoporin has been shown to induce calcium release from intracellular stores of differentiated HL-60 cells (see Table 1). This was evidenced by parabutoporin induced increases in cytosolic calcium levels in the presence and absence of extracellular calcium (Moerman et al., 2003).

6. Parabutoporin inhibits NADPH oxidase dependent superoxide production

Another important function of neutrophils is the capability to mount an 'oxidative burst' upon microbial challenge; i.e. the capability to produce large amounts of superoxide which results in the additional generation of many derivatives of superoxide, such as hydrogen peroxide, hydroxyl radicals, and (myeloperoxidase catalyzed generation of) hypochlorous acid. Collectively, these reactive oxygen species (ROS) play a crucial antimicrobial role by various mechanisms (Clark and Klebanoff, 1979; Reeves et al., 1999; Segal, 2005). Superoxide production is generated by NADPH oxidase, a multiprotein complex, which is kept inactive by separation of essential cytosolic subunits (p47^{phox}, p40^{phox}, p67^{phox} and Rac2) from the catalytic transmembrane component, cytochrome b₅₅₈ (a heterodimer of gp91^{phox} and p22^{phox}). Upon activation with bacterial peptides (such as fMLP) or opsonized particles, the NADPH oxidase assembles at the phagosome or plasma membrane, leading to the generation of superoxide in the lumen or extracellular environment, respectively. The vital role of NADPH oxidase is well exemplified by patients with Chronic Granulomatous Disease (CGD), who's NADPH oxidase function is impaired and who suffer from recurrent and life threatening infections (Winkelstein et al., 2000; van den Berg et al., 2009). This disease is most commonly associated with mutations in the catalytic subunit gp91^{phox} (~60%), and in p47^{phox} (~30%). The latter is responsible for the translocation of the cytosolic complex p40^{phox}/p47^{phox}/p67^{phox} to cytochrome b₅₅₈. Extensive Protein Kinase C (PKC) driven phosphorylation of the C-terminus of p47^{phox}

Table 1

Multifunctional effects of scorpion venom peptide parabutoporin

Effect	Interference	Ref.
Ca ²⁺ release from intracellular stores	+	Moerman et al. (2003)
Antimicrobial	+	Moerman et al. (2002)
Neutrophil chemotaxis	+	Willems et al. (2002), Willems et al. (2004)
Neutrophil degranulation	+	Willems et al. (2002)
Neutrophil apoptosis	–	Remijsen et al. (2009)
PKC activity	–	Remijsen et al. (2006)
NADPH oxidase activation	–	Willems et al. (2002, 2004), Remijsen et al. (2006)

induces conformational changes leading to the exposure of an N-terminal SH3 domain (Babior, 1999), which subsequently allows association of p47^{phox} with a Pro rich SH3 binding domain of p22^{phox}.

Despite the capability of parabutoporin to stimulate Rac2, which is an essential subunit of NADPH oxidase, parabutoporin was unexpectedly found to strongly inhibit fMLP triggered NADPH oxidase activity (Willems et al., 2004). In addition, superoxide production triggered by the direct PKC activator phorbol myristate acetate (PMA) is also potently inhibited by parabutoporin (see Table 1), with an IC₅₀ of 0.3 μM (Willems et al., 2002; Willems et al., 2004).

Analysis of the primary structure of parabutoporin reveals that two of its three serine residues are located in a PKC target sequence, suggesting that parabutoporin might be a direct substrate for PKC. This was confirmed by mass spectrometric analysis, and parabutoporin seems to be a good in vitro substrate for all PKC isotypes expressed by neutrophils, as compared to p47^{phox} (Remijnsen et al., 2006). In vitro experiments further demonstrated that parabutoporin can operate as an effective competitive inhibitor of GST-p47^{phox}-phosphorylation. Finally, it was shown that both the PKC-driven phosphorylation of p47^{phox} as well as its subsequent translocation to the membrane is effectively inhibited by parabutoporin in neutrophil cells, ex vivo (see Table 1). The inhibition of these initial steps by parabutoporin are not counteracted by its ability to activate Rac2. This can be explained by the observation that p47^{phox} translocation precedes Rac2 recruitment to cytochrome b₅₅₈ (Kleinberg et al., 1994). The recruitment of Rac2 probably depends on interaction with previously recruited p67^{phox}, by p47^{phox}. This is in agreement with results showing that Rac2 binds to p67^{phox}, not to p47^{phox} (Kwong et al., 1993).

The two serines located in an apparent PKC target sequence are present in several other antimicrobial scorpion venom peptides, such as opistoporin 1 and 2, hadrurin, padinin 1 and 2 Bmkbbp3, suggesting a conserved importance for these residues (see Fig. 1). Regarding parabutoporin, substitution of these two N-terminal serine residues for alanines results in a peptide

with the same affinity for membranes and pore-forming properties, as compared to the wild type peptide. Nevertheless, the double mutant parabutoporin S5/13A is a significantly weaker inhibitor of both stimulated PKC and NADPH oxidase activity (Remijnsen et al., 2006). Furthermore, the third serine C-terminal residue of parabutoporin, which is not conserved in the above-mentioned peptides, is not phosphorylated during incubation with PKC, as evidenced by the lack of phosphate incorporation in the double mutant parabutoporin S5/13A (Remijnsen et al., 2006).

Although this PKC-dependent mechanism of NADPH oxidase interference has thus far only been reported for parabutoporin, other natural peptides have also been reported to influence NADPH oxidase activity, demonstrating that NADPH oxidase interference is affected by many antimicrobial peptides. A Pro rich porcine peptide, PR39, was shown to inhibit NADPH oxidase activity with an IC₅₀ of 5 μM, as a result of its affinity for SH3 domains of p47^{phox}, preventing its association with cytochrome b₅₅₈. Mastoparan, a 14-mer peptide from wasp venom, was also shown to inhibit NADPH oxidase through interference with p67^{phox}, with an IC₅₀ of about 2 μM (Tisch et al., 1995; Tisch-Idelson et al., 2001).

In contrast, the cationic cathelicidin LL37 has been shown to stimulate NADPH oxidase activity through FPRL1 signalling (Zheng et al., 2007). This illustrates that cationic peptides can exert very diverse and even opposite functions, despite their common physico-chemical properties such as amphipathicity and positive charge.

7. Parabutoporin delays spontaneous neutrophil apoptosis

Neutrophils have a short lifespan of 24–48 h which provides a sensitive balance between their role as effector cells and their potential to inflict damage to host tissues. Upon inflammation, many pro-inflammatory components prolong the neutrophil lifespan, by delaying the caspase dependent apoptotic cell death programs. These observations have been attributed to activation of various

Hadrurin	-----G-ILDTIKSIASKVWNSKTVQDLKRKGIN-----WVANKLGVSPQAA-----	NDBP-3.1
parabutoporin	-----FKLGSFLKKAWKSKLAKKLRAKGKEMLKDYAKGLLEGGSEE---VPGQ-	NDBP-3.2
Bmkbbp-3	MLIVDEVNSFRFGSLKKVWKS LAKKLRSKGKQLLKDYANKVLNGPEEEA-AAPAE	NDBP-3.3
Pandidin-1	-----GKVWDWIKSAAKKIWSSEPVSQLKGQVLNAAKNYVAEKIGATPT-----	NDBP-3.4
opistoporin-1	-----GKVWDWIKSTAKKLWNSEPVKELKNTALNAAKNLVAEKIGATPS-----	NDBP-3.5
opistoporin-2	-----GKVWDWIKSTAKKLWNSEPVKELKNTALNAAKNFVAEKIGATPS-----	NDBP-3.6
Tx297	MLIVDEVNSFRFGSLKKMWKS LAKKLRSKGKELLKDYANKVLNGPEEEA-AVPAE	

Fig. 1. Primary sequences of cationic α-helical scorpion venom peptides with conserved Serine residues (indicated by ***) in a consensus sequence S(x)3KxWxS(x)5L. Hadrurin from *Hadrurus aztecus*, with conserved Ser 8 and 16. Parabutoporin from *Parabuthus schlechteri*, with conserved Ser 5 and 13. Bmkbbp3 from *Buthus martensii*, with conserved Ser 27 and 35. Pandinin 1 from *Pandinus imperator*, with conserved Ser 9 and 17. Opistoporin 1 and 2 from the venom of *Opistophthalmus carinatus*, both with conserved Ser 9 and 17. The putative Toxin Tx297 from *Buthus occitanus* Israelis, with conserved Ser 27 and 35. Although the role of these conserved Ser residues is unclear, experiments with parabutoporin do provide some relevant data. Both Ser 5 and 13 have been shown to be targets for phosphorylation by Protein Kinase C (PKC). Substitution of these Ser for Ala residues provides a double mutant parabutoporin which no longer incorporates phosphate upon incubation with PKC, but still exerts similar lytic effects on mammalian cells, as compared to wild type parabutoporin. This suggests that plasma membrane affinity is not affected as the result of the substitution itself. However, the capability of the double mutant parabutoporin to inhibit PMA induced NADPH oxidase activity is significantly reduced. This demonstrates that Ser 5 and 13 of parabutoporin serve a functional role on isolated neutrophil cells, ex vivo.

Fig. 2. Effect of parabutopporin on neutrophil survival. At submicromolar nonlytic concentrations, parabutopporin exerts a protective effect. Parabutopporin (PP) delays the constitutive neutrophil apoptosis through activation of prosurvival signalling via Akt. In addition, parabutopporin inhibits PMA and fMLP stimulated NADPH oxidase activity, by acting as a competitive inhibitor of PKC isotypes. This inhibition is observed, despite the fact that parabutopporin also activates Rac2, which is a positive regulator of NADPH oxidase.

prosurvival pathways, involving NF- κ B, STATs, ERK or Akt (Maiani et al., 2004; Luo and Loison, 2008).

Parabutopporin has the capability to delay the spontaneous neutrophil apoptosis (see Table 1). This is evidenced by parabutopporin dependent inhibition of apoptotic hallmarks such as caspase activity, DNA fragmentation, Mcl-1 degradation and the exposure of the 'eat me' signal phosphatidyl serine (Remijns et al., 2009). The latter is responsible for the recognition and subsequent clearance of apoptotic neutrophils by macrophages.

The parabutopporin dependent delay of neutrophil apoptosis has been shown to depend on stimulation of PI3K and Akt, and is not the result of its capability to inhibit stimulated NADPH oxidase activity. Of interest, the cationic peptide LL37 has also been reported to exert prosurvival effects on neutrophils which depend on PI3K activity, as suggested by the abrogation of its anti-apoptotic effects by wortmannin (Barlow et al., 2006).

Importantly, parabutopporin requires intact lipid rafts to activate these prosurvival pathways (Remijns et al., 2009). In contrast, both the activation of NADPH oxidase by fMLP or PMA, as well as the inhibition of superoxide production by parabutopporin do not depend on intact lipid rafts. In addition, the double mutant parabutopporin S5/13A, which is a weaker PKC and NADPH oxidase inhibitor, exerts the same protective effect on neutrophil survival, *ex vivo* (Remijns et al., 2009). Therefore, the prosurvival effect of parabutopporin on spontaneous neutrophil apoptosis does not depend on its inhibition of (unstimulated) NADPH oxidase (see Fig. 2). These results are in agreement with the observation that neutrophils from healthy volunteers and CGD patients, lacking NADPH oxidase activity, undergo spontaneous apoptosis at the same rate (Remijns et al., 2009).

8. Conclusion

Taken together, the multifunctional cationic peptide parabutopporin clearly exerts antimicrobial and lytic effects in the micromolar concentration range. It is likely that these properties of parabutopporin contribute to pathogen protection of the venom, where parabutopporin is present at relatively higher peptide and lower salt concentrations.

At nonlytic submicromolar concentrations, parabutopporin can also interfere with cellular signalling pathways of prominent immune cells, such as the abundant

neutrophil cells (see Table 1). Parabutopporin has the capability to cause neutrophil accumulation by prolonging their lifespan and by stimulating their motility, while inducing degranulation. All these pro-inflammatory processes are stimulated. In contrast, activation of NADPH oxidase is potentially inhibited by parabutopporin, and it is expected that many downstream signalling pathways depending on superoxide production are also affected, such as indol amino dioxygenase (IDO). IDO requires superoxide as a cofactor for its enzymatic activity, and has recently been demonstrated to be crucial for the resolution of inflammation of *Aspergillus* infections in mice (Romani et al., 2008). As such, the inhibition of NADPH oxidase by parabutopporin might contribute to increased or prolonged inflammation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Uncited reference

Fontayne et al., 2002.

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