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1 **Characterisation of three alpha-helical antimicrobial peptides**

2 **from the venom of *Scorpio maurus palmatus***

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9 **Abstract**

10 Scorpion venoms provide a rich source of anti-microbial peptides. Here we characterise three
11 from the venom of *Scorpio maurus palmatus*. Smp13 is biologically inactive, despite
12 sharing homology with other antimicrobial peptides, probably because it lacks a typically
13 charged structure. Both Smp-24 and Smp-43 have broad spectrum antimicrobial activity,
14 disrupting bacterial membranes. In addition, there is evidence that Smp24 may inhibit DNA
15 synthesis in *Bacillus subtilis*. Smp24 haemolysed red blood cells but in contrast, Smp43 was
16 non-haemolytic. The introduction of a flexible Gly-Val-Gly hinge into the middle of Smp24
17 did not alter the haemolytic activity of Smp24 (as might have been predicted from earlier
18 studies with Pandinin2 (Pin2), although C-terminal truncation of Smp-24 reduced its
19 haemolytic activity, in agreement with earlier Pin 2 studies. Smp24 and its derivatives, as
20 well as Smp-43, were all cytotoxic (ATP release assay) toward mammalian HepG2 liver
21 cells. Our results highlight the beneficial effect of helical-hinge-helical conformation on
22 promoting prokaryotic selectivity of long chain scorpion AMPs, as well as the importance of
23 examining a wide range of mammalian cell types in cytotoxicity testing.

24

25 **Introduction**

26 Over the last few decades an increasing number of pathogenic microorganisms have
27 developed resistance to conventional antibiotics posing problems in the management of
28 infection. Although treatment options for some Gram positive pathogens have undoubtedly
29 improved, our ability to successfully combat many Gram negative bacteria is becoming a
30 serious issue (Livermore., 2009). The problems in modern antibiotic drug development of
31 being able to design agents that are not easily susceptible to resistance (Jenssen *et al.*, 2006)
32 has meant that there has been a dramatic decline in the development of new antibiotics during
33 the same period.

34 Antimicrobial peptides (AMPs) represent an ancient host defence mechanism of the innate
35 immune system that transverses the evolutionary spectrum and to this day remains an
36 effective strategy against invading pathogens in the animal kingdom. AMPs are found in all
37 multicellular organisms, from molluscs to humans. They are present in vertebrate neutrophils
38 and leukocytes, invertebrate haemolymph, amphibian skin secretions and nearly all types of
39 plant cells (Bahar and Ren., 2013). The predominant mechanism of action of AMPs is to
40 disrupt the structure and function of microbial cell membranes. Because of their selectivity
41 for prokaryotes and their membrane-disruptive mechanisms for which microbes have little
42 natural resistance, the spotlight in recent years has turned towards the development of novel
43 antibiotics from these peptides (Zasloff., 2002, Hancock and Sahl., 2006)

44 AMP's are positively charged peptides of variable length (typically 12-70 amino acids) which
45 can be broadly divided into four structural classes: (i) amphipathic linear α -helix, (ii)
46 amphipathic β -sheet constrained and stabilised by disulphide bridges, (iii) extended linear
47 peptides, lacking secondary structure and enriched with one or more amino acids (e.g.
48 histidine, glycine, proline/ arginine) and (iv) loop peptides, which adopt a helical type-II

49 circular structure because of a high abundance of proline residues (Powers., 2003). Yeaman
50 and Yount (2003) have reviewed various structural parameters- amphipathicity, charge,
51 hydrophobicity, polar angle and conformation -which the authors suggest are crucial
52 molecular determinants for identifying a peptide that has selective toxicity for prokaryotic
53 membranes.

54 The key factor to membrane disruption is the initial electrostatic attraction of the peptide to
55 the negatively charged bacterial membrane surface (Huang., 2000, Shin *et al.*, 2001, Glukhov
56 *et al.*, 2005). After initial electrostatic attraction, a threshold concentration is often required
57 before membrane disruption can occur (Huang., 2000, Melo *et al.*, 2009). Following this
58 initial step, a number of mechanisms have been proposed to account for membrane
59 disruption: (i) Barrel stave mechanism - peptide oligomerization creates a central pore lumen
60 characterised by peptide-peptide interactions (Baumann and Mueller., 1974). (ii) Toroidal
61 mechanism - the pore lumen is lined by interactions between peptide and phospholipid head
62 groups (Matsuzaki *et al.*, 1996; Ludtke *et al.*, 1996). (iii) Carpet model peptides cover
63 ("carpet") the membrane surface and at a critical concentration, transient pores are formed
64 which allow the peptide access to the inner leaflet (Shai *et al.*, 1999 & Yamaguchi *et al.*,
65 2001). In this last model, it is proposed that peptides can span the transmembrane bilayer on
66 each membrane surface, causing curvature of the membrane to protect the acyl chains, which
67 then leads to the disintegration of the bilayer, due to micelle formation (Teixeria *et al.*, 2012).

68 AMPs have been found in the venoms of all venomous species examined. The physical
69 process of envenomation can variously lead to damage of fangs or telsons, resulting in
70 microbial infection. It is therefore logical to suggest that there has been evolutionary pressure
71 on venoms to produce AMPs as a protective measure to sterilize any infection (de Lima *et al.*,
72 2009). The AMP content of venoms varies widely between with a significant intra-species
73 variation, dependent on such factors as age, sex, geography and climate (Calvete., 2013).

74 Scorpion venoms provide an especially rich and diverse source of AMPs, with over 40
75 peptides having been characterised to date (see Harrison *et al.*, 2014 for a recent review); the
76 applications of proteomic and genomic based approaches are identifying new peptides at an
77 ever increasing rate. In one such study Abdel-Rahman *et al.*, (2013) examined the venom of
78 the North African scorpion *Scorpio maurus palmatus*; the study identified four potentially
79 novel AMPs, namely a 76-residue cysteine-containing peptide (Smp76), and three alpha-
80 helical peptides (Smp13, Smp24 and Smp43). Smp24 shares homology with Pandinin 2 (Pin
81 2) (54 %) an AMP from *Pandinus imperator*. Using Pin2 as a model AMP, Possani and
82 colleagues (Rodriguez *et al.*, 2011) have demonstrated the importance of a structural proline
83 "kink" in the middle of an otherwise un-interrupted alpha-helix that confers potent non-
84 specific pore-forming activity toward both eukaryotic and prokaryotic membranes;
85 substitution of this Pro residue for a more hydrophobic Gly-Val-Gly "hinge", dramatically
86 reduces the haemolytic activity of Pin2 and makes the modified peptide more selective for
87 prokaryotic membranes. The present study describes the biological characterisation of Smp
88 13, Smp24 and Smp43 and in particular examines the effects of disrupting the central Pro
89 residue of Smp24 with respect to its antimicrobial selectivity, as distinct from non-specific
90 cytotoxic properties.

91 **Materials and Methods**

92 **Materials**

93 All peptides were synthesised using Fmoc solid-phase chemistry and were purchased from
94 Think Peptides (Oxford, UK) (Purity analysis see Supplementary data). All peptide
95 sequences are listed in Table 1. All other solvents, chemicals and tissue culture reagents
96 were of the highest grade available and were obtained from Sigma (Gillingham, UK).

97

98 **Bacterial strains**

99 Microbes used throughout this study were as follows: *Escherichia coli* JM109, *Pseudomonas*
100 *aeruginosa* NCIMB 8295, *Bacillus subtilis* NCIMB 8024, *Staphylococcus aureus* SH1000,
101 *Klebsiella pneumoniae* NCTC 13439, *Staphylococcus epidermidis* ATCC12228 & *Candida*
102 *albicans* ATCC10231. Antibiotic resistant strains of *S. aureus* used were as follows:
103 methicillin-resistant *S. aureus* (MRSA) ATCC 33591, epidemic methicillin-resistant *S.*
104 *aureus* (EMRSA) 15 and EMRSA-16, clinical isolate MRSA *mecA mupA* positive, along
105 with the vancomycin intermediate resistant *S. aureus* (VISA) Mu50, and a clinical VISA
106 isolate KM126. All bacteria were from a culture collection held at Sheffield Hallam
107 University.

108 **Antimicrobial assay**

109 All antimicrobial assays were carried out by the microplate dilution method (Andrews., 2001)
110 using an automated plate reader (CENios Plus, Tecan, Switzerland). Synthetic AMPs were
111 examined at a concentration range of 0-512 µg/ml with Muller-Hinton growth medium.

112 **Haemolysis assay**

113 Haemolytic activity was determined on sheep erythrocytes as described (Corzo *et al.*, 2001).
114 Whilst differences in lipid composition are observed between sheep and human erythrocytes
115 they share significant similarities in terms of cholesterol content and neutrally charged lipid
116 composition (Nelson 1967) and therefore provide a reliable first screen for toxicity.

117 Synthetic AMPs were examined at a concentration range of 0-512µg/ml using a plate reader
118 (570nm, Infinite M200, Tecan, Switzerland) Triton X-100 (10%) was used as a positive
119 control and deionised water as the negative control. All samples were tested in triplicate.

120 **ATP release assay**

121 An ATP release assay (Sigma, UK) was used to determine the cytotoxic potential of peptides
122 against HepG2 liver cells in accordance with the manufacturer's protocol. HepG2 cells were
123 grown in DMEM (glutamate max medium) containing 1 g/l D-glucose, 10% foetal calf serum
124 and 1% penicillin-streptomycin. Cells were grown (37°C, 5% CO₂) to 80% confluence. Cells
125 were trypsinised, washed PBS and resuspended at the desired concentration to and seeded at
126 a final density of 1.1 x 10⁵ cells/well. Cells were incubated for 24 hrs, washed (PBS) and
127 peptides (0.5-512 µg/ml) added (15 mins 37°C). ATP release was quantitated using
128 luciferin/luciferase in a bioluminescent assay. All assays were performed in triplicate. Triton
129 X-100 (10%) was used as a positive control and deionised water as the negative control.
130 Luminescence was determined on a Wallac Victor2 1420 multi-label counter (Perkin Elmer,
131 Llantrisant, UK).

132 **Membrane integrity assay**

133 Bacterial membrane integrity following antimicrobial peptide attack was determined by the
134 BacLight method (adapted from Hillard *et al.*, 1999). Membrane integrity was determined for
135 both Gram positive (*S. aureus* SH100) and Gram negative (*E. coli* JM109) organisms using
136 the live/dead BacLight kit (Invitrogen UK). Overnight cultures were diluted with Muller-
137 Hinton broth to an optical density of between 0.5-0.6 at 600 nm. For each sample to be
138 tested, 500 µl of culture was pelleted (10,000 x g, 15 mins) and washed with PBS. The pellet
139 was resuspended and each peptide was added at 4x MIC. Peptides were incubated (10 mins,
140 room temp) on a rocking platform. Each sample was then centrifuged (10,000 x g, 10 mins)
141 and re-suspended in 500 µl of deionised water. The optical density was adjusted to 0.5
142 OD_{600nm} and then 1.2 µl of BacLight reagent was added according to the manufacturer's
143 instructions and incubated (15 mins, room temp) in the dark. The fluorescence excitation
144 wavelength was set at 485nm and fluorescence emission measured at 645 nm and 530 nm to
145 determine intact (green) and damaged (red) cells respectively. 100% loss of membrane

146 integrity was determined by the addition of Triton X-100 (10%) and deionised water was
147 added as a negative control. All assays were performed in triplicate.

148 **Intracellular bio-reporter gene assay**

149 To determine if any of the peptides had intracellular targets, a *Bacillus subtilis* reporter gene
150 assay (Urban *et al.*, 2007) was performed by analysing the effects of stress on specific
151 intracellular bacterial targets, using a bioluminescent luciferase gene reporter Bacteria were
152 exposed to 1/2 x MIC of each peptide for 1, 1.5, 3 & 4 hours at 37°C with luminescence
153 determined using a Wallac Victor2 1420 multi-label counter (Perkin Elmer, Llantrisant, UK).
154 All assays were performed in triplicate and responses were determined by calculating the %
155 increase from the negative control (Phosphate buffered saline solution).

156 **CD spectra**

157 CD spectra were recorded on a JASCO J-700 spectropolarimeter at 22 °C using a 0.1 cm
158 path-length cell. The spectral range was 190–250 nm and three scans were accumulated.
159 Peptides (0.2 mg/ml) were dissolved in either water or 60% (v/v) aqueous 2,2,2-
160 trifluoroethanol (TFE).

161

162

163

164

165 **Results**

166 Using a microbroth dilution assay, no antimicrobial activity was detected (up to 512 µg/ml)
167 with Smp13. In contrast, Smp24 and Smp43 displayed highest activity against Gram positive
168 organisms (Table 2). Removing the last four C-terminal residues of Smp24 (truncated
169 Smp24, Smp24T) made a negligible difference to antimicrobial activity. Similarly, increasing

170 the conformational flexibility of Smp24 by inserting a "hinge" region in the middle of the
171 peptide (replacing Pro-14 with Gly-Val-Gly, Smp24GVG) had limited effect. Because of the
172 strong activity observed with Smp24 towards *S. aureus* SH1000, this peptide was assayed
173 against a wider range of *S. aureus* strains, including methicillin-resistant strains and strains
174 with intermediate resistance towards vancomycin (Table 3). A wide range of MIC values
175 were observed between strains, particularly between EMRSA-15 (MIC = 16 µg/ml) and
176 EMRSA-16 (MIC = 512 µg/ml), highlighting the possibility of cross-resistance being
177 acquired either through plasmid acquisition, mutation or phenotypic routes. Both Smp24
178 and Smp43 showed limited activity toward the yeast, *C.albicans*.

179 The effects of the biologically active peptides upon bacterial membrane integrity determined
180 that all peptides examined were membrane active with maximum disruption against both
181 Gram-positive and Gram-negative species at a concentration of 4 x the MIC value observed
182 (Table 4).

183 Further evidence of membrane disruption was observed in *B.subtilis*, with all four active
184 peptides inducing an almost x15-fold increases in the cell envelope stress response, as
185 evidenced by the increased expression of the *ypuA* promoter after a 1 hour incubation. A
186 significant stress response was still evident after 4 hours exposure to all peptides (Smp24
187 176% ± 35.6, Smp24T 166 % ± 28.1, Smp24GVG 183% ± 21.9 & Smp43 131 ± 26.2)
188 (Fig.1). Interestingly, with the exception of Smp43, all peptides induced an up regulation of
189 the gene associated with DNA synthesis stress; Smp24GVG showed the greatest (288.4% ±
190 14.2) increase in the expression of the *yorB* promoter after 3 hours whilst Smp24 and
191 Smp24T increased expression by 228.1 % (± 12.1) and 163.3% (±6.4) respectively at the
192 same time point. A comparison of cell envelope and DNA stress responses would suggest
193 that the cell envelope has entered a recovery phase when the peptide is exerting maximum
194 damage (either directly or indirectly) on bacterial DNA synthesis. In contrast to the induction

195 of DNA stress responses, the genes associated with RNA or protein synthesis related stress
196 responses were not significantly induced by any of the peptides (Fig. 1).

197 In haemolysis assays Smp43 showed very low toxicity at the maximum concentration tested
198 (1.2 ± 0.5 % lysis at 512 $\mu\text{g/ml}$), in comparison, Smp24 caused significant erythrocyte
199 disruption (89.6 ± 5.6 %) at the same concentration (Fig.2). The effects of modifications to
200 Smp24 had contrasting outcomes (Fig 2). Truncated Smp24 showed a decrease in activity,
201 with haemolysis reduced by 43% at the highest concentration compared with the parent
202 peptide. However, inclusion of the flexible Gly-Val-Gly hinge region had little effect with no
203 significant difference in haemolytic activity. Further cytotoxic testing was carried out on
204 HepG2 cells by measuring the release of ATP (Fig 3) with cell damage occurring in a
205 concentration-dependent fashion in response to all four peptides above a concentration of
206 32 $\mu\text{g/ml}$. The consequence of Smp24 modification was similar to those observed in
207 haemolysis assays with a decrease in cytotoxicity observed with the truncated derivative
208 (11.8 % (± 4.0) at 32 $\mu\text{g/ml}$ compared with 15.9 % (± 1.9)) whilst Smp24GVG showed no
209 significant difference compared with the parent peptide (Fig 3). However, there was a
210 remarkable difference in the cytotoxicity of Smp43 when comparing erythrocyte haemolysis
211 and the release of ATP from HepG2 cells (94.8% (± 7.5) at 512 $\mu\text{g/ml}$), clearly highlighting
212 differences in eukaryotic cell membrane architecture and the need for a range of diverse
213 mammalian cell types to be assayed during cytotoxic testing (Fig. 3).

214 CD spectral analysis for both Smp24 and Smp43 revealed that both peptides are in an
215 unordered structure in aqueous solution. However, in the presence of 60 % TFE both peptides
216 adopt significant alpha helical regions characterised by two minima at 208 nm and 222 nm
217 (Fig.4). Analysis of both peptides (DichroWeb server, Whitmore and Wallace, 2008)
218 revealed Smp24 to have two regions of approximately 59 % and 22 % helical content whilst
219 Smp43 showed two regions of approximately 70 % and 25 % helical content. The remaining

220 regions of both peptides were assigned as unordered. The percentage helical content was
221 estimated using the SELCON method of analysis which determines helical content using
222 statistical algorithms that were created by analysis of 29 reference proteins. This
223 methodology was validated by analysing 4 unknown proteins where the structure was also
224 confirmed by X-ray crystallography (Sreerama *et al.*, 1999).

225

226 **Discussion**

227 A large number of scorpion AMPs have been characterised. They have a wide range of
228 biological activities and their characterisation has been facilitated because their small size
229 (<5kDa). Abdel Rahman *et al.*, (2013) identified four AMPs in the venom of *Scorpio maurus*
230 *palermatus* and this study characterises the biological activity of three of them.

231 Smp43 shares high homology (75-86%) with opistoporins and pandinin 1 (Pin 1).
232 Opistoporin 1 & Pin 1 both have been shown to have helix-hinge-helix structures (Moerman
233 *et al.*, 2002, Corzo *et al.*, 2001). CD spectral analysis of Smp43 suggested the peptide to be
234 unordered in aqueous solution whilst in the presence of 60 % TFE it adopted a di-helical
235 structure, linked by a random coil region. Smp43 showed striking similarity to Pin 1 (helical
236 regions between 3-18 and 26-37 linked by a random coil region) (Nomura *et al.*, 2005).

237 Smp24 showed a lower identity (54%) with pandinin-2 (Pin 2), although it shares
238 evolutionarily conserved motifs with the shorter (17-20 residue) chain scorpion AMPs. The
239 most notable among these is the LIPS motif which is found within the core of Pin 2 as well as
240 BmKb1, ctriporin, imcroporin, meucin-18 and mucroporin. CD spectral analysis of Smp24
241 revealed, like Smp43, an unordered structure in aqueous solution that adopted a di-helical
242 structure in 60% TFE. The structure of Smp24 however is in contrast to that of Pin 2 which

243 on the basis of NMR analysis, has only a single helical region, extending from the N-
244 terminus to residue 18 (Nomura *et al.*, 2004).

245 Despite the differences in structure the two peptides share a number of key features including
246 length (24 residues), homology (13 identical residues) and biological activity (bactericidal &
247 cytotoxic) which is highly suggestive of Smp24 belonging to the same family of mid-chain
248 helical scorpion AMPs. The shorter Smp13 belongs to the family of cytotoxic peptides such
249 as IsCT peptides (Dai *et al.*, 2002). However, unlike other peptides within this family, Smp13
250 carries no charge and therefore it is not surprising that it has no antimicrobial activity. Its role
251 within the venom remains to be elucidated.

252 The helix-hinge-helix topology of Smp43 is present in a number of longer chain AMPs, all of
253 which have high therapeutic indices, for example cecropin A (Holak *et al.*, 1988),
254 dermaseptin B2 (Galanth *et al.*, 2009), Hadrurin (Torres-Larios *et al.*, 2000), the opistoporins
255 (Moerman *et al.*, 2002), Pin1 (Corzo *et al.*, 2001) and BmKbpp (Zeng *et al.*, 2000). This
256 suggests that this class of peptides has considerable potential for the future development of
257 anti-infective agents.

258 Suggestions that substitution of a proline residue with a more flexible Gly-Val-Gly (GVG)
259 "hinge" would reduce the haemolytic effects of AMPs has been proposed by Rodriguez and
260 colleagues and, in the case of Pin2, has proved successful (Rodriguez *et al.*, 2011). However
261 there was no difference in the haemolytic activities of Smp24 and Smp24GVG.

262

263 It should be noted that the effect of proline within AMPs is ambiguous. On the one hand,
264 many proline-deficient peptides (e.g. magainin-2) show low haemolytic/cytotoxic activity
265 (Zasloff., 1987). In contrast, peptides such as melittin and pardaxin contain a proline in the

266 central region of their sequence and both have high antimicrobial and haemolytic potential.
267 Removal of this proline residue further increases toxicity (Dempsey *et al.*, 1991). Bodone *et*
268 *al.* (2013) have suggested that a proline "kink" is required for prokaryotic membrane
269 selectivity, with removal only increasing toxicity due to increased helical structure. This turn
270 increases the hydrophobic face of the peptide and its propensity to bind to neutrally charged
271 bilayers. In contrast, more compact proline-containing peptides will be less attracted to
272 neutral surfaces and retain electrostatic attraction to negatively charged bilayers.

273 Reducing the length of the C-terminal of Smp24 by the removal of the last four residues
274 increased the therapeutic index. This modification was chosen as it had been successfully
275 performed with the evolutionary related Pin 2 where the shortening of the random coil region
276 increased its therapeutic index despite a reduction in charge (Rodriguez *et al.*, 2014) although
277 this electrostatic attraction is less important towards eukaryotic membranes due to their
278 zwitterionic nature (Jiang *et al.*, 2008). The reduction in haemolysis seen in Pin 2 after
279 truncation to 14 and 17 residue peptides is of interest as CD spectra showed a change in
280 structure from a predominantly helical structure to a β -hairpin structure similar to Indolicidin
281 (Rodriguez *et al.*, 2014).

282 Although Smp43 exhibited low haemolysis it still caused significant ATP release in HepG2
283 cells suggesting disruption of eukaryotic membranes. However, it is worth noting cancer
284 derived cells have a more negatively charged membrane than normal eukaryotic cells due to
285 an increase in membrane phosphatidylserine content, differential branching and sialic acid
286 content of N-linked glycans associated with transmembrane proteins and the increase in O-
287 glycosylated mucins (Gajski and Garaj-Vrhovac., 2013). On this basis it would be useful to
288 assess any toxic effects on primary cells which exhibit normal membrane compositions both
289 to redefine peptide toxicity and determine any beneficial anticancer properties.

290 Along with their membrane disruptive mechanism the stress responses with *B. subtilis* whole
291 cell reporter assays showed a possible interaction with DNA for Smp24 and its derivatives. A
292 number of peptides have been shown to interact with intracellular targets including DNA.
293 These include buforin II (BFII) as a prototypical example (Yonezawa *et al.*, 1992, Park *et al.*,
294 1998, Zhang *et al.*, 2014), which also contains a proline in the centre of the peptide (Park *et*
295 *al.*, 1998). BFII penetrates the membrane without causing general membrane
296 permeabilisation and accumulates in the cytosol before binding to DNA. In contrast, proline
297 deficient magainin 2 (MAG 2), causes membrane permeabilisation but stays bound to the
298 phospholipid bilayer and has a low propensity for DNA binding (Kobayashi *et al.*, 2004).
299 Both peptides form transient toroidal pores although BFII pores have a significantly shorter
300 half-life (Park *et al.*, 2000). Pore collapse is electrostatically driven by repulsive forces
301 within the helical portion of the peptide (Kobayashi *et al.*, 2004). Further evidence for the
302 destabilising effect of proline is found in alamethicin where substitution of alanine for proline
303 is known to destabilise the pore (Kaduk *et al.*, 1998). Since Smp24 both disrupts membranes
304 and may have an intracellular target, we propose that the highly charged nature of the peptide
305 (+4) together with the presence of proline could create a “half-way house” between BFII and
306 MAG 2. Although Smp24 has a longer pore half-life than BFII, we suggest that, unlike MAG
307 2, Smp24 can still dissociate from the phospholipid bilayer and interact with DNA. In this
308 context however, the observation that Smp24GVG did not diminish the DNA stress response,
309 needs further investigation.

310 The fact that the intracellular stress responses of Smp43 were limited to the cell envelope is
311 perhaps not surprising, when considering that Pin1 causes complete disruption of the bilayer
312 (Nomura *et al.*, 2005). Biophysical studies in our own laboratory (unpublished observations)
313 support a carpet-like mechanism for Smp43, similar to Pin 1.

314 Antimicrobial assays of the peptides from *S. maurus palmatus* studied in our experiments
315 show wide differences in MIC values, especially between Gram-positive and Gram-negative
316 bacteria, with preferential activity towards Gram-positive bacteria, in keeping with previously
317 tested α -helical peptides from scorpion venom (Harrison *et al.*, 2014). This is perhaps not
318 surprising when considering the failure to produce Gram-negative specific insectives in
319 recent years and highlights the intrinsic difficulty in identifying suitable Gram-negative
320 specific agents.

321 In summary, three scorpion AMPs have been characterised from the venom of *S. maurus*
322 *palmatus*. Two of these peptides (Smp24 & Smp43) showed broad spectrum antimicrobial
323 activity but differed significantly in their haemolytic effects whilst the shortest peptide
324 (Smp13) had no biological activity. Different modifications of Smp24 led to both beneficial
325 and adverse biological effects and highlighted the potential for further manipulation of these
326 peptides as AMP scaffolds. Both Smp24 and Smp43 exhibited a membrane disruptive
327 mechanism of action, however Smp24 and its derivatives showed evidence of interference
328 with DNA synthesis. In accord with previous studies (Torres-Larios *et al.*, 2000, Moerman *et*
329 *al.*, 2002, Corzo *et al.*, 2001 and Zeng *et al.*, 2000), our experiments further highlight the
330 beneficial effect that a helical-hinge-helical conformation has on long chain scorpion AMPs
331 in promoting prokaryotic selectivity.

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471 **Table 1.** Sequence information of all peptides used in this study

472 **Table 2.** Minimum inhibitory activity (MIC) of AMPs

473 **Table 3.** MIC of Smp24 against a range of antibiotic sensitive and antibiotic resistant *S.*
474 *aureus* strains

475 **Table 4.** Membrane damage mediated by scorpion AMPs on Gram-positive and Gram-
476 negative membranes

477

478 **Figure 1.** Stress response of *B. subtilis* reporter constructs following exposure to Smp24
479 (blue), Smp43 (red) Smp24GVG (green) and Smp24T (purple).

480 **Figure 2.** Haemolytic activity of *Scorpio maurus palmatus* AMPs and their derivatives.

481 **Figure 3.** Cytotoxic effects of *Scorpio maurus palmatus* AMPs and their derivatives against
482 HepG2 cells measured as a function of ATP release.

483 **Figure 4.** Solvent dependent effects of Smp24 and Smp43 on peptide secondary structure.

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486 **Table 1.**

Peptide name	Sequence
<i>Smp13</i>	ILQDIWNGIKNLF-NH ₂
<i>Smp24</i>	IWSFLIKAATKLL P --SLFGGG-KKDS
<i>Pandinin-2</i>	FWGALAKGALKLIP--SLFSSFSKKD
<i>Smp24GVG</i>	IWSFLIKAATKLL GVG SLFGGG-KKDS
<i>Smp24T</i>	IWSFLIKAATKLL--PSLFGG
<i>Smp43</i>	G-VVDWIKKTAGKIWNSEPVKALKSQALNAAKNFVAEKIGATPS
<i>Pandinin-1</i>	GKVVDWIKSAAKKIWSSEPVSQLKGQVLNAAKNYVAEKIGATPT

487

488 **Table 2.**

Bacteria strain	Gram class	MIC (µg/ml)				
		Smp 13	Smp 24	Smp 24 GVG	Smp 24T	Smp 43
<i>B. subtilis</i> NCIMB 8024	+	No Activity	4	8	8	4
<i>S. epidermidis</i> sp.	+	No Activity	8	16	16	64
<i>S. aureus</i> SH100	+	No Activity	8	16	16	32
<i>E. coli</i> JM109	-	No Activity	64	64	128	128
<i>K. pneumoniae</i> NCTC 13439	-	No Activity	128	128	128	64
<i>P. aeruginosa</i>	-	No Activity	256	256	256	64
<i>C. albicans</i>	fungi	No Activity	32	128	128	128

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491 **Table 3.**

<i>S.aureus</i> strain	MIC ($\mu\text{g/ml}$)
SH1000	32
<i>S.aureus</i> ATCC 25923	32
MRSA ATCC 33591	512 (Partial)
MRSA <i>mecA</i> <i>mupA</i> +ve	16
EMRSA-15	16
EMRSA-16	512 (Partial)
VISA MU50	32
VISA KM126	64

492 **Table 4.**

Species/Strain	% Membrane damage			
	Smp24	Smp24GVG	Smp24T	Smp43
<i>E.coli</i> JM109	110.5 (± 3.9)	97.2 (± 1.2)	99.5 (± 3.5)	105.4 (± 1.8)
<i>S.aureus</i> SH1000	101.2 (± 4.8)	105.3 (± 4.5)	11.6 (± 2.9)	103.2 (± 5.1)

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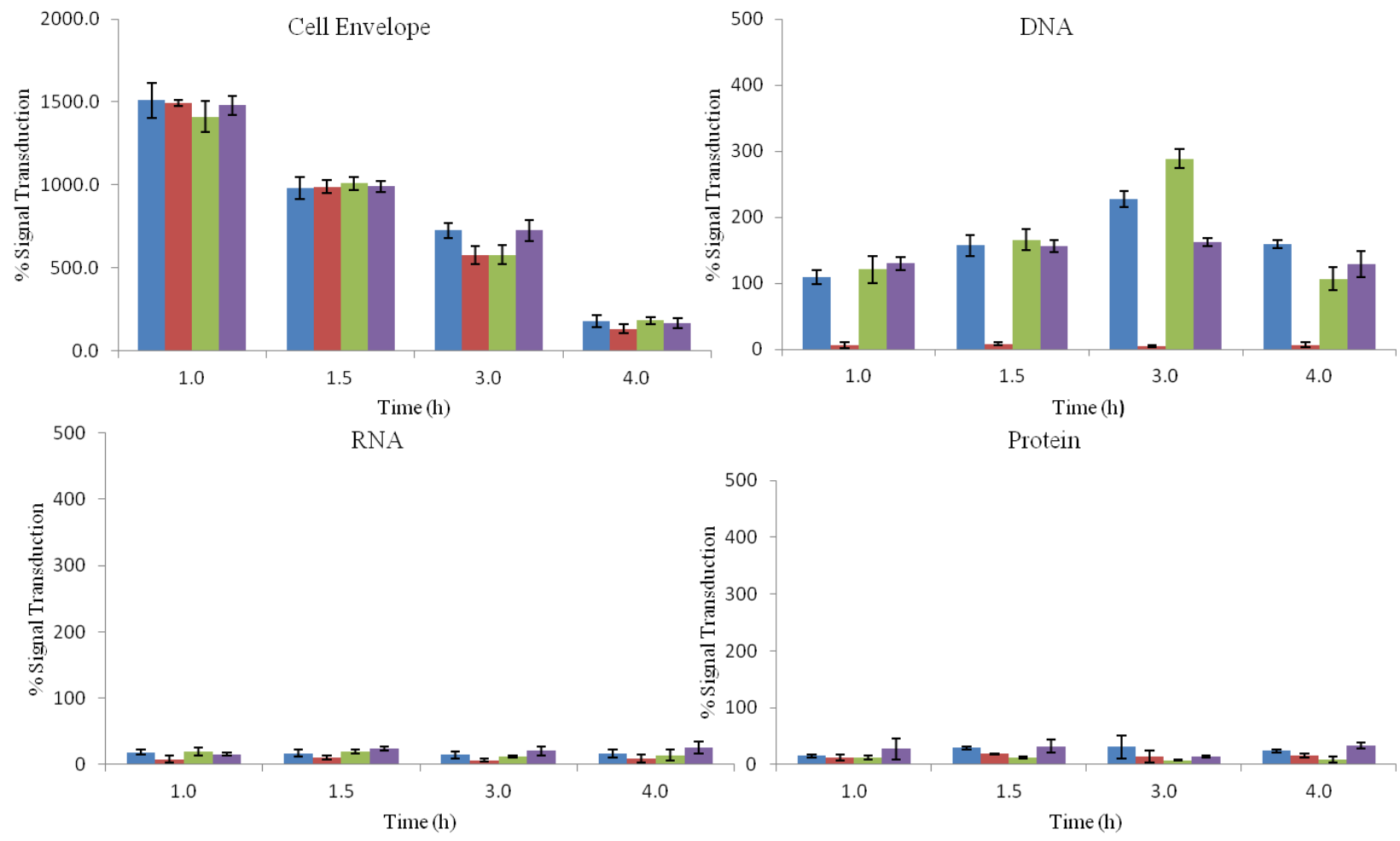


Figure 1.

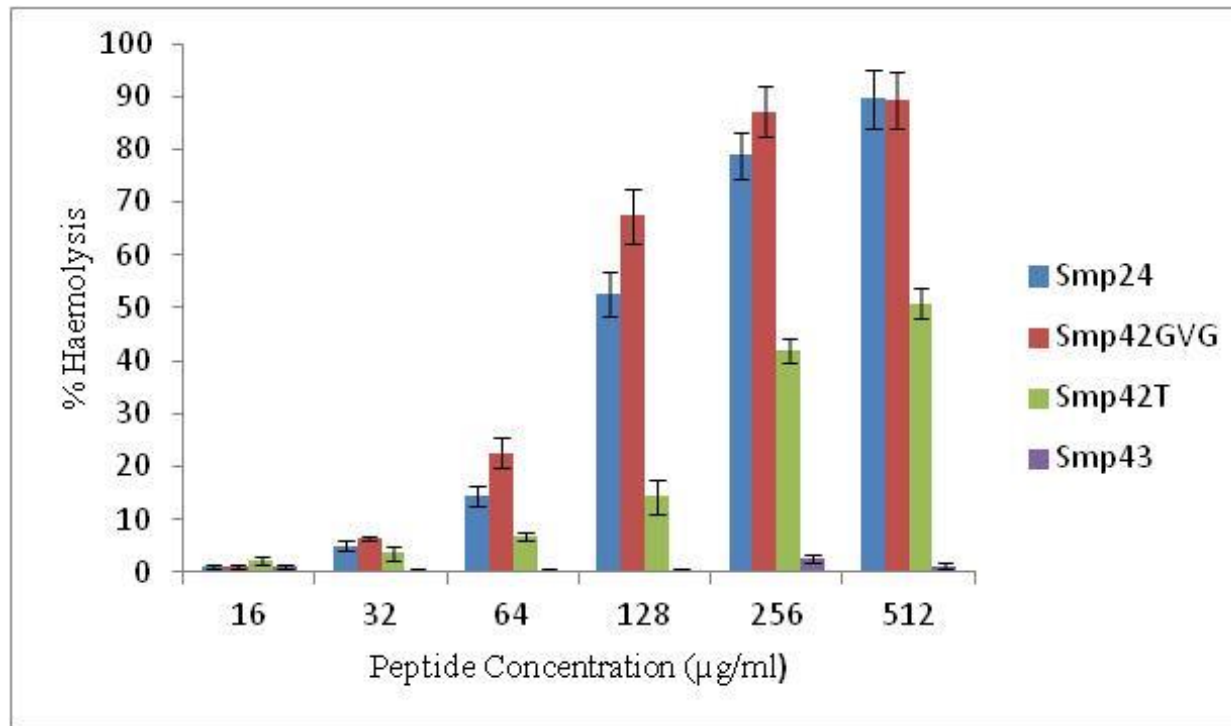


Figure 2.

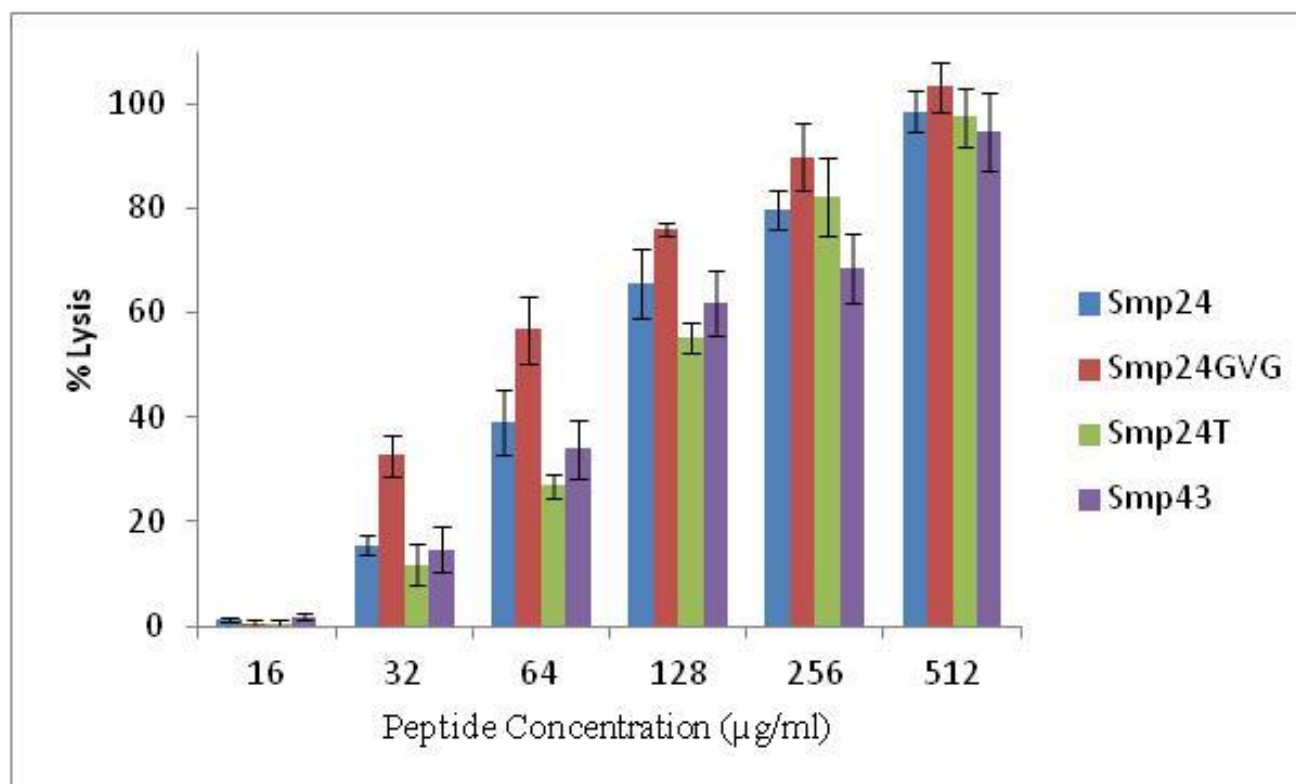


Figure 3.

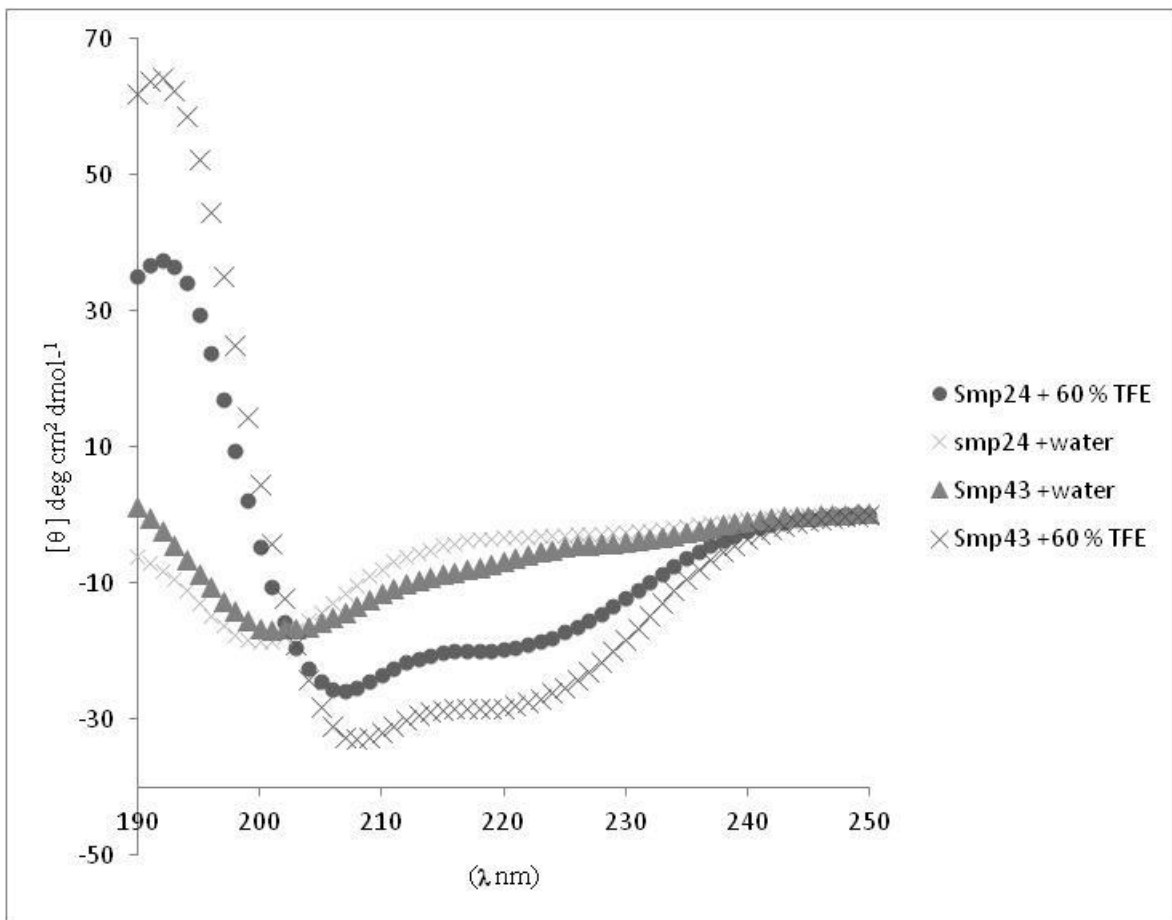


Figure 4