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Synthetic Mimics of Antimicrobial Peptides with Immunomodulatory Responses

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Abstract

A new series of aryl-based SMAMPs with antimicrobial activity and selectivity have been developed via systematic tuning of aromatic groups and charge. The addition of a pendant aromatic group improved the antimicrobial activity against gram-negative bacteria, while the addition of charge improved the selectivity. SMAMP **4**, with six charges and a naphthalene central ring, demonstrated a selectivity of 200 against both *S. aureus* and *E. coli*, compared to a selectivity of 8 for the peptide **MSI-78**. In addition to the direct antimicrobial activity, SMAMP **4** exhibited specific immunomodulatory activities in macrophages both in the presence and absence of LPS, a TLR agonist. SMAMP **4** also induced the production of the neutrophil chemo-attractant, murine KC, in mouse primary cells. This is the first, non-peptidic SMAMP demonstrating both good antimicrobial and immunomodulatory activity.

The rise in bacterial resistance and the declining approval rate of novel anti-infective drugs are a major threat to global public health, especially in hospitals and other health care settings.¹ Antimicrobial peptides (AMPs), found in almost every multicellular organism, have attracted considerable attention as models for the design of new therapeutic agents due to their broad spectrum activity and reduced bacterial resistance development.² AMPs form the core of the innate immune system that effectively deals with microbial invasion, and most AMPs show direct antimicrobial activity against a variety of pathogens. Only recently has it been recognized that they play a key role in immunomodulation and hence have also been termed host defense peptides (HDPs).³ For example, LL-37 and defensins affect innate immune cell functions, including the induction and modulation of chemokine and cytokine production, direct chemo-attraction of immune cells, angiogenesis promotion and wound healing. Despite the many interesting properties of these natural peptides, their development into therapeutic agents has been limited due to their toxicity, high manufacturing cost and poor *in vivo* efficacy.³ This has driven research towards the development of non-natural or synthetic mimics of antimicrobial peptides (SMAMPs), including non-natural peptide mimics⁴⁻⁷ as well as synthetic polymers⁸⁻¹¹ and oligomers,¹²⁻¹⁴ which aim to reproduce critical AMP biophysical characteristics such as cationic charge and amphiphilic structure.

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ASSOCIATED CONTENT

Supporting Information. Experimental details, HPLC data, broad spectrum antimicrobial activity, and additional figures are included in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Systematic structure-activity relationship (SAR) studies on SMAMPs have led to the development of several compounds that exhibit direct antimicrobial activity and reduced cytotoxicity, some of which are already in clinical development.¹⁵ However, a greater potential may lie in harnessing the innate immune system to combat bacterial infections.¹⁶ Towards this, a number of immunomodulatory molecules, including synthetic HDP mimics and innate defense regulators (IDRs) that do not show direct antimicrobial response, are being developed into therapeutic candidates and adjuvants.¹⁷ The combination of direct antimicrobial activity and controlled immunomodulation in an antimicrobial system would present a novel strategy to treat infections with multiple mechanisms of action against pathogens, thus minimizing pathogen-antimicrobial responses. Herein, we report a new series of aryl-based SMAMPs with improved gram-negative and gram-positive antimicrobial activity, some of which have significantly enhanced selectivity and reduced toxicity compared with the well-known magainin analog **MSI-78**, which is in phase III clinical trials as a topical antibiotic.¹⁵ One of these new SMAMPs also showed unique immunomodulatory responses.

We previously reported a new series of SMAMPs from simple aryl scaffolds synthesized via Suzuki coupling.¹⁸ The use of Suzuki coupling was advantageous because of its mild reaction conditions, high functional group tolerance, and easy scalability. However, the design of these SMAMPs, with only three aryl rings and two positive charges, proved to be insufficient for achieving the antimicrobial activity and selectivity comparable to the previously described oligomeric SMAMPs.

In this report, a new series of aryl SMAMPs were designed in order to evaluate the effect of charge and aromatic group hydrophobicity on the biological activity, while still maintaining an amphiphilic topology. Cationic charges of four and six were investigated, and three different central rings were used to tune the overall hydrophobicity: benzene, naphthalene, and phenyl- benzene (See the Supporting Information for the synthesis and characterization of the SMAMPs). The hydrophobicity of the aryl SMAMPs was quantified using reversed-phase HPLC retention time (R_t). The antimicrobial activity of these SMAMPs was tested against four pathogens (SI, Tables S1, S2) including both gram-negative and gram-positive bacteria in terms of the minimum inhibitory concentration (MIC), and the hemolysis was evaluated in terms of HC_{50} (the lowest concentration that causes 50% hemolysis of the red blood cells, RBCs).

Table 1 summarizes the biological activity of the SMAMPs in comparison to **MSI-78**. Compared to the previously studied triaryl benzene oligomers,¹⁸ SMAMP **1**, containing a benzene central ring, showed a significant decrease in hemolytic activity ($HC_{50} > 1000 \mu\text{g/ml}$ vs. $36 \mu\text{g/ml}$) although the antimicrobial activity was still low. The reduced toxicity was attributed to the increase in hydrophilicity due to more cationic charge (4 vs. 2).¹⁸ Changing the central ring from benzene (SMAMP **1**) to naphthalene (SMAMP **2**) increased the hydrophobicity but did not alter the antimicrobial activity. SMAMP **2** was more hemolytic and thus had a lower selectivity compared to SMAMP **1**. SMAMP **3**, with a pendant phenyl group, was the most hydrophobic among the three compounds in the series containing four charges, with an R_t of 28.8 min. Compared to SMAMP **2**, SMAMP **3** showed an 8-fold increase in antimicrobial activity against *E. coli* (MIC = $3.13 \mu\text{g/ml}$) as well as a higher HC_{50} , thus improved selectivity of 172. The increase in activity against *E. coli* for SMAMP **3** was attributed to the arrangement of the hydrophobic pendant aromatic ring, which is known to insert into the membrane interface.¹⁹

To elucidate the role of charge, SMAMPs **4** and **5**, each with six charges, were designed and synthesized for comparison to their 4-charge analogues (SMAMPs **2** and **3**). SMAMPs **4** and

5, containing naphthalene and phenylbenzene central rings, respectively, had higher HC_{50} values (thus were less toxic against RBCs) compared to SMAMPs **2** and **3**. SMAMP **4** showed improved antimicrobial activity against both *S. aureus* and *E. coli* compared to SMAMP **2**, which resulted in a very high selectivity of 200. This was almost 20 times higher than the selectivity of **MSI-78**, while simultaneously increasing potency (MIC ~3 Kg/ml vs. 16 Kg/ml). SMAMP **5** did not show a significant improvement in the antimicrobial activity over SMAMP **3**, which was already active, but it had better selectivity for *S. aureus*. Similar results have been observed for antimicrobial polynorbornenes, where increasing the charge density led to non-hemolytic and active polymers.²⁰ These data confirm that increasing the charge improved the selectivity.

In addition to the direct antimicrobial activity demonstrated by the SMAMPs **2-5**, their ability to modulate the immune response was measured in terms of the production of the pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin-6 (IL-6) and the anti-inflammatory cytokine interleukin-10 (IL-10) in the murine macrophage cell line RAW 264.7. Among the antimicrobial SMAMPs reported here, only SMAMP **4** induced TNF production in RAW 264.7 cells (Figure 1A). Natural peptides like human neutrophil α -defensins are known to stimulate TNF production in monocytes.²¹ Lipopolysaccharide (LPS),²² a bacterial cell wall component, and bacterial DNA (CpG-ODN)²³ are also known for their direct stimulation of TNF in macrophages, and some synthetic analogues like polysaccharides (branched chitins)²⁴ and monophosphoryl lipid A (MLA) have been reported to induce TNF production.²⁵ However, to the best of our knowledge, no SMAMP has been reported to have agonistic (immunostimulatory) activity. LPS contamination during SMAMP **4** preparation was ruled out, since pre-treatment of RAW 264.7 cells with Polymyxin B, known for its high LPS binding affinity,²² did not affect the ability of SMAMP **4** to increase TNF production (SI, Figure S6). In addition, the TNF production varied with SMAMP concentration, which indicated the controlled stimulation by SMAMP **4**. To evaluate the immunomodulatory effect of these SMAMPs in the presence of LPS, RAW 264.7 cells were pre-incubated with the SMAMPs and then stimulated with LPS (SI, Figure S7). SMAMP **4** increased the LPS-induced production of the pro-inflammatory cytokines TNF and IL-6 (Figure 1B, 1C). However, LPS-induced anti-inflammatory cytokine (IL-10) production was inhibited in the presence of SMAMP **4** to background levels (Figure 1D). This unique ability of SMAMP **4** is different from the peptides like LL-37 and IDR peptides, which do not directly stimulate TNF production but suppress LPS-induced TNF production by upregulation of IL-10 and, in the case of LL-37 also binding to LPS.^{26, 27} Based on the results shown in Figure 1A, it was not surprising that SMAMPs **2**, **3**, and **5** did not cause any additional increase in TNF production upon LPS stimulation, although SMAMP **3** shows a marginal decrease in the LPS induced TNF production (SI, Figure S7).

The anti-inflammatory cytokine IL-10 itself is known to inhibit LPS-induced TNF production in RAW cells.^{28, 29} Thus, in order to evaluate the possible correlation, if any, between the pro- and anti-inflammatory cytokine release activities, the ability of SMAMP **4** to enhance LPS-induced TNF production in RAW cells pre-incubated with externally added mouse recombinant IL-10 (rIL-10) was investigated. Figure 2 shows that the addition of rIL-10 did not significantly affect the SMAMP **4**-mediated self-agonistic effect in RAW 264.7 cells as only a slight decrease in the overall TNF level was observed. However, upon LPS stimulation, the presence of rIL-10 resulted in the abrogation of SMAMP **4**'s capacity to enhance TNF production. This observation suggests that in LPS-stimulated RAW 264.7 cells, the SMAMP **4**-mediated decrease in IL-10 production was at least partially responsible for increased TNF production. Therefore, SMAMP **4** seems to orchestrate the balance of the pro- and anti-inflammatory cytokine responses in macrophages. Many fundamental questions still remain, but these unique immunomodulatory properties of

SMAMP **4** can be used to trigger immune responses in a very specific way. For example, MLA, a TLR4 (toll-like receptor 4) agonist that induces enhanced TNF production similar to SMAMP **4**, is already an effective adjuvant for hepatitis B and influenza.¹⁷

The specific self-agonistic effect of SMAMP **4** along with its elevated agonistic effect with LPS stimulation in RAW 264.7 cells prompted us to evaluate SMAMP **4** activity in primary mouse cells, murine bone marrow derived macrophages (BMDMs). In the absence of LPS, SMAMP **4** induced a small amount (30 pg/ml) of TNF in BMDMs (Figure 3A); however, upon LPS stimulation, SMAMP **4** significantly increased the LPS-induced TNF production and inhibited the LPS-induced IL-10 production. This observation is very similar to the immunomodulatory effect of SMAMP **4** in RAW 264.7 cells.

Beside its ability to modulate pro- and anti-inflammatory cytokine production in RAW 264.7 and BMDM cells, SMAMP **4** also induced significantly higher levels of murine KC (chemokine CXCL1, a neutrophil chemo-attractant) compared to the DMSO control in BMDMs (Figure 3C). Increased KC expression has been found to be associated with neutrophil influx in a range of inflammatory conditions.³⁰ It was previously reported that the protective activity of a synthetic cationic peptide against bacterial infection was associated with the induction of chemokines such as CXCL1 from macrophages and/or monocytes.³¹ Thus, the ability of this non-toxic, non-peptidic, antimicrobial SMAMP to modulate both cytokine and chemokine production is encouraging for the design of synthetic molecules with multiple biological functions.

The new series of aryl-based SMAMPs described here, designed via systematic tuning of hydrophobicity and cationic charge, exhibited potent antibacterial activity compared to **MSI-78**, while being non-toxic to host cells. Additionally, SMAMP **4** exhibited unique immunomodulatory properties. The dual-functional role of SMAMPs with direct antimicrobial activity and immunomodulatory response is very encouraging since immunomodulatory compounds have gained importance in recent years in anti-infective therapy, cancer therapy and vaccine development. These SMAMPs were originally designed to execute antimicrobial activity, but their ability to boost the innate immune response represents a promising approach to prevent or treat infectious diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

RAW 264.7 cells were pre-incubated with SMAMPs (5.0 $\mu\text{g/ml}$) or DMSO (0.05% DMSO) for 1 h and stimulated with or without LPS (100 ng/ml) for 18 h. The supernatants were analyzed for (A, B) TNF, (C) IL-6, and (D) IL-10 production by ELISA. The data are presented as the average $\pm$ s.e.m. of triplicate samples and are representative of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, non-significant; the means were compared by Student's t-test).

Figure 2.

RAW 264.7 cells were pre-incubated with or without recombinant mouse IL-10 (50 ng/ml), then with SMAMP 4 (5.0 μ g/ml) or DMSO (0.05% DMSO) for 1 h, and stimulated with or without LPS (100 ng/ml) for 18 h. The supernatants were analyzed for TNF. The data are presented as the average $\pm$ s.e.m. of triplicate samples. (***, $P < 0.001$; ns, non-significant; the means were compared by Student's t-test).

Figure 3.

Mouse BMDM cells were pre-incubated with SMAMP **4** (5.0 μ g/ml) or 0.01% DMSO for 1 h and stimulated with or without LPS (100 ng/ml) for 15 h. The supernatants were analyzed for (A) TNF, (B) IL-10, and (C) murine KC. The data are presented as the average $\pm$ s.e.m. of triplicate samples and are representative of two to three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; the means were compared by Student's t-test).

Table 1

Antibacterial activity and selectivity of SMAMPs

SMAMP	R ₁	R ₂	R ₁ ^a (min)	MIC (μg/ml)		HC ₅₀ (μg/ml)		Selectivity (HC ₅₀ /MIC)	
				<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>
1		H	22.9	12.5	50	>1000	>20	>80	>20
2		H	26.4	12.5	25	195	7.8	15.6	7.8
3		H	28.8	12.5	3.13	537	171.5	43	171.5
4			23.5	3.13	3.13	656	209.6	209.6	209.6

SMAMP	R ₁	R ₂	R _t ^a (min)	MIC (μg/ml)		HC ₅₀ (μg/ml)	Selectivity (HC ₅₀ /MIC)	
				<i>S. aureus</i>	<i>E. coli</i>		<i>S. aureus</i>	<i>E. coli</i>
5			25.7	6.25	6.25	>1000	>160	>160
				8-16	16-32	120	8-15	4-8
MSI-78 ^b	-	-	-	-	-	-	-	-

^a Measured by HPLC using C8 column with a gradient of 1% acetonitrile/min starting with 100% water

^b Literature values (reference 18)