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Synthetic Mimics of Antimicrobial Peptides with Immunomodulatory Responses

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Supporting Information

ABSTRACT: A new series of aryl-based synthetic mimics of antimicrobial peptides (SMAMPs) with antimicrobial activity and selectivity have been developed via systematic tuning of the 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 *Staphylococcus aureus* and *Escherichia coli*, compared with 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 in the absence of lipopolysaccharide, a TLR agonist. SMAMP **4** also induced the production of a neutrophil chemoattractant, murine KC, in mouse primary cells. This is the first nonpeptidic SMAMP demonstrating both good antimicrobial and immunomodulatory activities.

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), which are found in almost every multicellular organism, have attracted considerable attention as models for the design of new therapeutic agents because of 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 chemoattraction 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 because of their toxicity, high manufacturing cost, and poor *in vivo* efficacy.[3] This has driven research toward the development of non-natural AMPs or synthetic mimics of AMPs (SMAMPs), including non-natural peptide mimics[4−7] as well as synthetic polymers[8−17] and oligomers,[17−19] which aim to reproduce critical AMP biophysical characteristics such as cationic charge and amphiphilic structure.

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.[20] However, greater potential may lie in harnessing the innate immune system to combat bacterial infections.[21] Toward this end, 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.[22] The combination of direct antimicrobial activity and controlled immunomodulation in an antimicrobial system would present a novel strategy for treating 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 activities, some of which have significantly enhanced selectivity and reduced toxicity compared with the well-known magainin analogue **MSI-78**, which is in phase-III clinical trials as a topical antibiotic.[20] One of these new SMAMPs also showed unique immunomodulatory responses.

We previously reported a new series of SMAMPs based on simple aryl scaffolds synthesized via Suzuki coupling.[23] 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 to achieve antimicrobial activity and selectivity comparable to those of the previously described oligomeric SMAMPs.

In this work, a new series of aryl SMAMPs was designed in order to evaluate the effect of charge and aromatic group hydrophobicity on the biological activity while still maintaining an amphiphilic topology. Four and six cationic charges were investigated, and three different central rings were used to tune the overall hydrophobicity: benzene, naphthalene, and phenylbenzene [see the Supporting Information (SI) for the synthesis and characterization of the SMAMPs]. The hydrophobicities of the aryl SMAMPs were quantified using reversed-phase HPLC

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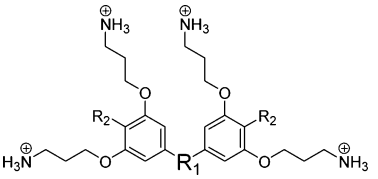
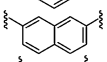
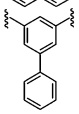
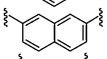
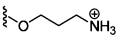
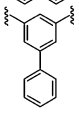
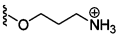
Table 1. Antibacterial Activities and Selectivities of SMAMPs



SMAMP	R_1	R_2	R_t[a] (min)	MIC (μg/ml)		HC_{50} (μg/ml)	Selectivity (HC_{50}/MIC)	
				S. aureus	*E. coli*		*S. aureus*	*E. coli*
1		H	22.9	12.5	50	>1000	>80	>20
2		H	26.4	12.5	25	195	15.6	7.8
3		H	28.8	12.5	3.13	537	43	171.5
4			23.5	3.13	3.13	656	209.6	209.6
5			25.7	6.25	6.25	>1000	>160	>160
MSI-78[b]	-	-	-	8-16	16-32	120	8-15	4-8

[a]Measured by HPLC using a C8 column with a gradient of 1% acetonitrile/min starting with 100% water. [b]Data from ref 23.

retention times (R_t). The antimicrobial activities [expressed as minimum inhibitory concentration (MIC)] of these SMAMPs were tested against four pathogens, including both Gram-negative and Gram-positive bacteria, and their hemolysis [evaluated in terms of HC_{50}, the lowest concentration that causes 50% hemolysis of red blood cells (RBCs)] was tested against human RBCs (Tables S1 and S2 in the SI).

Table 1 summarizes the biological activities of the SMAMPs in comparison with **MSI-78**. Relative to the previously studied triaryl benzene oligomers,[23] SMAMP **1** containing a benzene central ring showed a significant decrease in hemolytic activity (HC_{50} > 1000 μg/mL vs 36 μg/mL), although the antimicrobial activity was still low. The reduced toxicity was attributed to the increase in hydrophilicity due to the greater number of cationic charges (4 vs 2).[23] 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 than SMAMP **1**. SMAMP **3** with a pendant phenyl group was the most hydrophobic among the three compounds in the series containing four charges (R_t = 28.8 min). Relative to SMAMP **2**, SMAMP **3** showed an 8-fold increase in antimicrobial activity against *Escherichia coli* (MIC = 3.13 μg/mL) as well as a higher HC_{50} and thus an improved selectivity of 172. The increased 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.[24]

To elucidate the role of charge, SMAMPs **4** and **5**, each with six charges, were designed and synthesized for comparison to their analogues with four charges (SMAMPs **2** and **3**, respectively). SMAMPs **4** and **5** containing naphthalene and phenylbenzene central rings, respectively, had higher HC_{50} values (i.e., were less toxic against RBCs) than SMAMPs **2** and **3**. SMAMP **4** showed improved antimicrobial activity against both *Staphylococcus aureus* and *E. coli* relative to SMAMP **2**, resulting in a very high selectivity of 200. This was almost 20 times higher than the selectivity of **MSI-78**, and the potency was also increased (MIC ≈ 3 μg/mL vs 16 μg/mL for **MSI-78**). SMAMP **5** did not show a significant improvement in the antimicrobial activity relative to 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 nonhemolytic and active polymers.[25] These data confirm that increasing the charge improves the selectivity.

In addition to the direct antimicrobial activity demonstrated by 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 such as human neutrophil α-defensins are known to stimulate TNF production in monocytes.[26] Lipopolysaccharide (LPS),[27] a bacterial cell wall component, and bacterial DNA (CpG-ODN)[28] are also known for their direct stimulation of TNF in macrophages, and some synthetic analogues such as polysaccharides (branched chitins)[29] and monophosphoryl lipid A (MLA) have been reported to induce TNF production.[30] 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 pretreatment of RAW 264.7 cells with polymyxin B, known for its high LPS binding affinity,[27] did not affect the ability of SMAMP **4** to increase TNF production (Figure S6 in the SI). In addition, the TNF production varied with the SMAMP concentration, indicating controlled stimulation by SMAMP **4**. To evaluate the immunomodulatory effect of these SMAMPs in the presence of LPS, RAW 264.7 cells were preincubated with the SMAMPs and then stimulated with LPS (Figure S7). SMAMP **4** increased the LPS-induced production of the pro-inflammatory cytokines TNF and IL-6 (Figure 1B,C) but inhibited the production of the anti-inflammatory cytokine IL-

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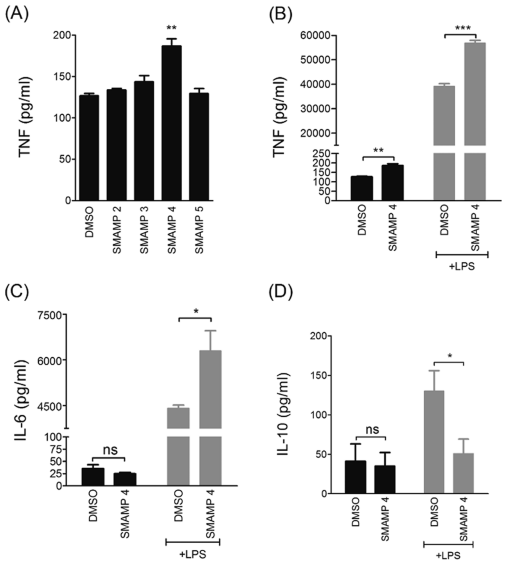

Figure 1. RAW 264.7 cells were preincubated with SMAMP (5.0 μg/mL) or 0.05% DMSO for 1 h and stimulated with or without LPS (100 ng/mL) for 18 h. The supernatants were analyzed by ELISA for (A, B) TNF, (C) IL-6, and (D) IL-10 production. The data are presented as mean ± standard error of the mean (sem) for triplicate samples and are representative of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant; the means were compared using Student's t test).

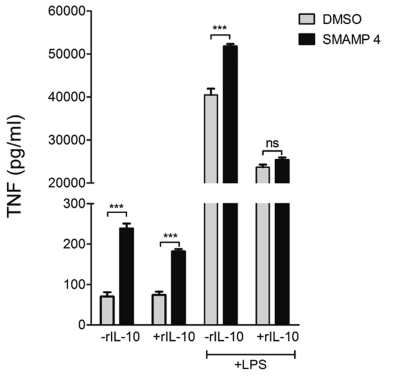

Figure 2. RAW 264.7 cells were preincubated with or without mouse recombinant IL-10 (50 ng/mL) and then with SMAMP **4** (5.0 μg/mL) or 0.05% DMSO for 1 h, followed by stimulation with or without LPS (100 ng/mL) for 18 h. The supernatants were analyzed for TNF. The data are presented as mean ± sem of triplicate samples (***, $P < 0.001$; ns, nonsignificant; the means were compared using Student's t test).

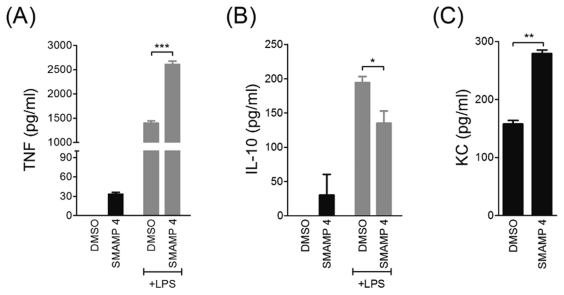

Figure 3. Mouse BMDM cells were preincubated with SMAMP **4** (5.0 μg/mL) or 0.05% DMSO for 1 h and stimulated with or without LPS (100 ng/mL) for 15 h. The supernatants were analyzed for (A) TNF, (B) IL-10, and (C) murine KC. The data are presented as the mean ± sem of triplicate samples and are representative of two to three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; the means were compared using Student's t test).

10 to background levels (Figure 1D). This unique ability of SMAMP **4** distinguishes it from peptides such as LL-37 and IDR peptides, which do not directly stimulate TNF production but do suppress LPS-induced TNF production by upregulation of IL-10 and, in the case of LL-37, also by binding to LPS.[31,−33] On the basis of 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** showed a marginal decrease in LPS-induced TNF production (Figure S7).

The anti-inflammatory cytokine IL-10 itself is known to inhibit LPS-induced TNF production in RAW cells.[34,35] Thus, 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 preincubated 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 a 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 toll-like receptor 4 (TLR4) agonist that induces enhanced TNF production similar to SMAMP **4**, is already an effective adjuvant for hepatitis B and influenza.[22]

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 the SMAMP **4** activity in primary murine bone marrow-derived macrophages (BMDMs). In the absence of LPS, SMAMP **4** induced a small amount of TNF production (30 pg/mL) 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 (Figure 3A,B). This observation is very similar to the immunomodulatory effect of SMAMP **4** in RAW 264.7 cells.

Besides 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 chemoattractant) relative 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.[36] 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.[37] Thus, the ability of this nontoxic, nonpeptidic, antimicrobial SMAMP to modulate both cytokine and chemokine production is encouraging for the design of synthetic molecules with multiple biological functions.

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The new series of aryl-based SMAMPs described here, which were designed via systematic tuning of hydrophobicity and cationic charge, exhibited potent antibacterial activities relative to **MSI-78** while being nontoxic 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.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, HPLC data, broad-spectrum antimicrobial activity, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

‖H.D.T. and A.S. contributed equally.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Taubes, G. *Science* **2008**, *321*, 356.
(2) Zasloff, M. *Nature* **2002**, *415*, 389.
(3) Hancock, R. E. W.; Sahl, H.-G. *Nat. Biotechnol.* **2006**, *24*, 1551.
(4) Park, Y.; Lee, D. G.; Jang, S. H.; Woo, E. R.; Jeong, H. G.; Choi, C. H.; Hahm, K. S. *Biochim. Biophys. Acta* **2003**, *1645*, 172.
(5) Porter, E. A.; Wang, X.; Lee, H. S.; Weisblum, B.; Gellman, S. H. *Nature* **2000**, *404*, 565.
(6) Liu, D.; DeGrado, W. F. *J. Am. Chem. Soc.* **2001**, *123*, 7553.
(7) Chongsiriwatana, N. P.; Patch, J. A.; Czyzewski, A. M.; Dohm, M. T.; Ivankin, A.; Gidalevitz, D.; Zuckermann, R. N.; Barron, A. E. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 2794.
(8) Mowery, B. P.; Lee, S. E.; Kissounko, D. A.; Epand, R. F.; Epand, R. M.; Weisblum, B.; Stahl, S. S.; Gellman, S. H. *J. Am. Chem. Soc.* **2007**, *129*, 15474.
(9) Tew, G. N.; Liu, D.; Chen, B.; Doerksen, R. J.; Kaplan, J.; Carroll, P. J.; Klein, M. L.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5110.
(10) Lienkamp, K.; Madkour, A. E.; Musante, A.; Nelson, C. F.; Nusslein, K.; Tew, G. N. *J. Am. Chem. Soc.* **2008**, *130*, 9836.
(11) Kuroda, K.; DeGrado, W. F. *J. Am. Chem. Soc.* **2005**, *127*, 4128.
(12) Arnt, L.; Nusslein, K.; Tew, G. N. *Polym. Sci., Part A: Polym. Chem.* **2004**, *42*, 3860.
(13) Ilker, M. F.; Nusslein, K.; Tew, G. N.; Coughlin, E. B. *J. Am. Chem. Soc.* **2004**, *126*, 15870.
(14) Gabriel, G. J.; Madkour, A. E.; Dabkowski, J. M.; Nelson, C. F.; Nusslein, K.; Tew, G. N. *Biomacromolecules* **2008**, *9*, 2980.
(15) Arnt, .; I, L.; Tew, G. N. *J. Am. Chem. Soc.* **2002**, *124*, 7664a.
(16) Sambhy, V.; Peterson, B. R.; Sen, A. *Angew. Chem. Int. Ed.* **2008**, *47*, 1250.
(17) Choi, S.; Isaacs, A.; Clements, D.; Liu, D.; Kim, H.; Scott, R. W.; Winkler, J. D.; DeGrado, W. F. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 6968.
(18) Tang, H.; Doerksen, R.; Jones, T.; Klein, M.; Tew, G. *Chem. Biol.* **2006**, *13*, 427.
(19) Tew, G. N.; Clements, D.; Tang, H.; Arnt, L.; Scott, R. W. *Biochim. Biophys. Acta* **2006**, *1758*, 1387.
(20) Fjell, C. D.; Hiss, J. A.; Hancock, R. E. W.; Schneider, G. *Nat. Rev. Drug Discovery* **2012**, *11*, 37.
(21) Finlay, B. B.; Hancock, R. E. *Nat. Rev. Microbiol.* **2004**, *2*, 497.
(22) Nicholls, E. F.; Madera, L.; Hancock, R. E. *Ann. N.Y. Acad. Sci.* **2010**, *1213*, 46.
(23) Thaker, H. D.; Sgolastra, F.; Clements, D.; Scott, R. W.; Tew, G. N. *J. Med. Chem.* **2011**, *54*, 2241.
(24) White, S. H.; Wimley, W. C. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 319.
(25) Al-Badri, Z. M.; Som, A.; Lyon, S.; Nelson, C. F.; Nusslein, K.; Tew, G. N. *Biomacromolecules* **2008**, *9*, 2805.
(26) Chaly, Y. V.; Paleolog, E. M.; Kolesnikova, T. S.; Tikhonov, I. I.; Petratchenko, E. V.; Voitenok, N. N. *Eur. Cytokine Network* **2000**, *11*, 257.
(27) Gough, M.; Hancock, R. E.; Kelly, N. M. *Infect. Immun.* **1996**, *64*, 4922.
(28) Lipford, G. B.; Sparwasser, T.; Bauer, M.; Zimmermann, S.; Koch, E. S.; Heeg, K.; Wagner, H. *Eur. J. Immunol.* **1997**, *27*, 3420.
(29) Shimojoh, M.; Kojima, T.; Nakajima, K.; Hatta, K.; Katoh, A.; Kurita, K. *Biomacromolecules* **2010**, *11*, 1212.
(30) Henricson, B. E.; Manthey, C. L.; Perera, P. Y.; Hamilton, T. A.; Vogel, S. N. *Infect. Immun.* **1993**, *61*, 2325.
(31) Scott, M. G.; Dullaghan, E.; Mookherjee, N.; Glavas, N.; Waldbrook, M.; Thompson, A.; Wang, A.; Lee, K.; Doria, S.; Hamill, P.; Yu, J. J.; Li, Y.; Donini, O.; Guarna, M. M.; Finlay, B. B.; North, J. R.; Hancock, R. E. *Nat. Biotechnol.* **2007**, *25*, 465.
(32) Mookherjee, N.; Hamill, P.; Gardy, J.; Blimkie, D.; Falsafi, R.; Chikatamarla, A.; Arenillas, D. J.; Doria, S.; Kollmann, T. R.; Hancock, R. E. *Mol. BioSyst.* **2009**, *5*, 483.
(33) Rosenfeld, Y.; Papo, N.; Shai, Y. *J. Biol. Chem.* **2006**, *281*, 1636.
(34) Dagvadorj, J.; Naiki, Y.; Tumurkhuu, G.; Hassan, F.; Islam, S.; Koide, N.; Mori, I.; Yoshida, T.; Yokochi, T. *Innate Immun.* **2008**, *14*, 109.
(35) Clarke, C. J. P.; Hales, A.; Hunt, A.; Foxwell, B. M. J. *Eur. J. Immunol.* **1998**, *28*, 1719.
(36) Lira, S. A.; Zalamea, P.; Heinrich, J. N.; Fuentes, M. E.; Carrasco, D.; Lewin, A. C.; Barton, D. S.; Durham, S.; Bravo, R. *J. Exp. Med.* **1994**, *180*, 2039.
(37) Nijnik, A.; Madera, L.; Ma, S. H.; Waldbrook, M.; Elliott, M. R.; Easton, D. M.; Mayer, M. L.; Mullaly, S. C.; Kindrachuk, J.; Jenssen, H.; Hancock, R. E. W. *J. Immunol.* **2010**, *184*, 2539.