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Immunomodulation and the quorum sensing molecule 3-oxo-C₁₂-homoserine lactone: The importance of chemical scaffolding for probe development†

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Abstract

As a guide for chemical probe design, focused analogue synthetic studies were undertaken upon the lactone ring of 3-oxo-C₁₂-homoserine lactone. We have concluded that hydrolytic instability of the heterocyclic ring is pivotal for its ability to modulate immune signaling and probe preparation was aligned with these findings.

The immune system relies on innate and adaptive components that are essential for protection against microbial assault. Upon infection, the innate immune system triggers an inflammatory response through microbial product-mediated activation of Toll-like receptor (TLR) signaling effectors, such as the transcription factor NF-κB and protein kinase p38, and the production of immune modulators, including tumor necrosis factor (TNF) among other cytokines.^{1,2} The goal of this inflammatory response is not only pathogen elimination but also activation of the adaptive immune system via cytokine- and T- and B-cell receptor-driven induction of similar effector mechanisms, thereby triggering lymphocyte proliferation essential for the generation of efficient immunity.^{1,2} Certain microbes, however, including the human opportunistic pathogen *Pseudomonas aeruginosa*,³ are armed with specific factors that can impair signaling within both components of the immune systems, often leading to pathological complications accompanying acute or persistent infections.⁴

P. aeruginosa is a nosocomial Gram-negative bacteria responsible for a number of infections, particularly in immunocompromised individuals such as those suffering from cystic fibrosis.^{5–7} In order to promote such persistent infections, *P. aeruginosa* secretes a variety of virulence factors⁸ and signaling molecules,⁹ including the quorum sensing signaling molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (acylhomoserine lactone (AHL), **C12**, Figure 1).^{9,10} In contrast to other pathogenicity-promoting factors produced by *P. aeruginosa*,¹¹ **C12** has been demonstrated to exert a broad spectrum of biological activities in mammalian cells.^{11–19} With respect to innate and adaptive immunities, **C12**

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suppresses both TLR-mediated cytokine expression in macrophages^{12,19} and proliferation of activated lymphocytes,¹⁶ two key responses of immune cells to invading pathogens.^{1,2,4} Our laboratory has reported evidence linking the immunosuppressive nature of **C12** to its ability to selectively disrupt the regulation of NF- κ B functions in macrophages and other cell types activated by TLR agonists, TNF or whole bacteria.¹⁹ Interestingly, despite inhibition of NF- κ B-dependent immune system effectors, exposure of mammalian cells to **C12** resulted in activation of the endoplasmic reticulum (ER) stress response, protein kinase p38 pathway and apoptosis.^{15,19} Notably, however, unlike TLR agonists and other pathogen-associated molecular patterns (PAMPs), **C12**-mediated activation of mammalian cells occurred through mechanism(s) distinct from currently known PAMP recognition receptor pathways.^{18,19}

In order to better understand the structural significance of **C12** on mammalian cell activation, we²⁰ and others^{21–24} have been actively interested in the chemical synthesis of **C12** analogues. To date, only a few of these reports have examined the effect of conservative structural changes around the **C12** scaffold on leukocyte proliferation^{21,22} or the activation of signaling pathways in mammalian cells,^{18,20} and a systematic analysis of the role that hydrolytic stability of the lactone plays has yet to be interrogated. In this report, we describe focused analogue synthesis studies and cell-based biochemical analyses to elucidate the functional importance of lactone stability with respect to **C12**-mediated activation of the ER stress response, p38 pathway and apoptosis. Counterintuitive to most medicinal chemistry efforts of ligand-receptor interactions, our findings reveal that hydrolytic *instability* of the heterocyclic ring is critical for mammalian cell activation. Based on these studies, a new chemical probe was prepared, which will be useful for mammalian cell receptor identification.

In Gram-negative bacteria, AHLs are produced as quorum sensing signaling molecules to promote bacterial multicellularity and virulence.^{9,25} With respect to mammalian cell immunoregulation, previous experiments have demonstrated that AHLs containing a L-homoserine lactone, 3-oxo substituent and an alkyl chain of 10–14 carbons were necessary for activity.^{18,20–22} Moreover, our studies have indicated the importance of lactone ring opening, as the corresponding lactam derivative was completely inactive in mammalian cell activation experiments¹⁸ and a thiolactone analogue was >35 times less active than **C12** in a mouse splenocyte proliferation assay.²¹

To better understand the effect of lactone stability on mammalian cell activation by AHLs, **C12-N** and **C12-S** were prepared as previously reported by our laboratory^{18,26} and others,^{21,27,28} respectively. Bone marrow-derived macrophages (BMDMs) were then treated with **C12-N** or **C12-S** (50 μ M) over a period of 2 h, and macrophage responsiveness was analyzed by Western blot for the cleavage of poly(ADP)-ribose polymerase (PARP) and the phosphorylation of eukaryotic translation initiation factor 2 α (p-eIF2 α) and protein kinase p38 (p-p38), biochemical markers indicative of **C12**-induced apoptosis, the ER stress response and the p38 pathway, respectively.¹⁸ As Figure 2 shows, neither analogue induced **C12**-mediated biological effects, thus, corroborating with previous reports.^{18,21}

Because previous studies by our laboratory showed that the lactone of **C12** is readily hydrolyzed upon addition to cells with the cellular fraction containing ~10 times more hydrolyzed compound than the supernatant,¹⁸ we were interested to examine if lactone hydrolytic stability was a determinant in the ability of **C12** to activate mammalian cells. Half-lives of **C12**, **C12-N** and **C12-S** have been previously reported, and were measured as ~18 h, >48 h and 82.3 h, respectively.^{26,28} Coupling this data with our biochemical analyses, we hypothesized that hydrolytic *instability* of the lactone ring may be an important factor for AHL-mediated activation of mammalian cells. To examine this possibility, we designed and synthesized **β C12**, which contains a β -lactone in place of the homoserine lactone and should

exhibit greater hydrolytic lability.²⁹ The synthesis of **βC12** began with the activation of acid **1** with DCC followed by nucleophilic displacement of the resultant activated ester with Meldrum's acid and coupling with methanol to yield **2**. The 3-oxo substituent was then protected with ethylene glycol and the methyl ester was hydrolyzed to the corresponding acid **3**. **βC12** was then obtained following coupling with amino-β-lactone **4** and deprotection of the 3-position. As anticipated, the measured half-life of **βC12** in buffered aqueous solution was ~2 h, which is drastically reduced compared to the natural **C12** and analogues **C12-N** and **C12-S**.

With the **βC12** in hand, similar to the above-described experiments for the other AHLs, BMDMs were treated with **βC12** (50 μM) over a period of 2 h, and cellular extracts were examined by Western blot analysis for **C12**-mediated biomarkers. As Figure 3a shows, a 15-min treatment with both **C12** and **βC12** resulted in comparable activation of PARP cleavage as well as phosphorylation of eIF2α and p38, thus demonstrating that hydrolytic instability is a critical feature for AHL activity in mammalian immune cells. We also noted that in contrast to **C12**, **βC12**-mediated biological activity rapidly diminished over time. As this AHL ring analogue contains a β-lactone motif, which could readily modify proteins,²⁹ this may explain the observed results. Alternatively, it may not be able to reclose due to ring strain and remains in its hydrolyzed (open) form. Interestingly, this structural modification was not tolerated by *P. aeruginosa*, as **βC12** was not active as an autoinducer in a *P. aeruginosa* quorum sensing assay (see Supporting Information). These results mirror our previously reported findings, which indicated that the structural requirements for autoinducer activity and mammalian cell activation are distinct.²⁰

Because many in the field, including our laboratory, are actively interested in uncovering the mammalian cell receptor(s) of **C12** to aid in delineating the mechanisms by which AHLs regulate immune cell processes,^{20,30,31} we were eager to design an analogue of **βC12** for future target identification studies in immune cells. In particular, as the possibility of covalent protein modification exists with this AHL derivative, we were encouraged to use this scaffold for probe development. **βC12-N₃** (Figure 3b, see the Supporting Information for synthetic details) was designed to utilize the β-lactone functionality as a chemical warhead coupled with the installation of an azide moiety as an affinity tag following click chemistry with a biotinylated or fluorophore-labeled alkyne. The ability of **βC12-N₃** to activate mammalian cells was then examined, as probe activity is critical for its use in target identification studies. As Figure 3a shows, **βC12-N₃** showed similar activity to the parent **βC12** and recapitulated **C12**-mediated biochemical phenotypes with modified kinetics, again demonstrating that incorporation of an azido functionality into the AHL tail is a conservative structural change.²⁰ Target identification studies in immune cells utilizing this AHL probe will be reported in due course.

In conclusion, by completing focused analogue synthesis studies around the lactone of **C12** produced by *P. aeruginosa*, we have identified new structural analogues that can be used to explore the diverse biochemical activities of this bacterial signaling molecule, which is both an activator of mammalian cells and immunomodulator. Moreover, we have also uncovered that hydrolytic instability of the lactone ring is significant for the ability of **C12** to activate mammalian cells. As our over-arching goal is to identify the protein target(s) responsible for the paradoxical biological activities of **C12**, our future studies will focus on the employment of **βC12-N₃**-based compounds as probe molecules to begin to understand the link between AHL-induced ER stress and immunosuppression. We hope that these future studies will help us begin to understand not only how AHLs impact mammalian cell biology but also how bacterial signaling molecules are able to impair the human immune system to promote virulence and pathogenicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Structures of *N*-(3-oxododecanoyl)-L-homoserine lactone (**C12**) and its lactam and thiolactone analogues.

Fig. 2. Biological activities of **C12-N** and **C12-S** in BMDMs. BMDMs were treated with 50 μ M AHL for the indicated period of time, and cellular extracts were analyzed by Western blot for the cleavage of PARP, phosphorylated forms of eIF2 α (p-eIF2 α) or p38 (p-p38) and actin as a loading control. **C12** was used as a positive control.

Fig. 3. β C12 AHLs. (a) Biological activities of C12, β C12 and β C12-N₃ in BMDMs. BMDMs were treated as previously described and analyzed by Western blot for the cleavage of PARP, phosphorylated forms of eIF2 α (p-eIF2 α) or p38 (p-p38) and actin as a loading control. (b) Structure of β C12-N₃.

Scheme 1.
Synthesis of β C12