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Synthesis and evaluation of a tag-free photoactive phospho-ceramide analogue-1 (PCERA-1) probe to study immunomodulation in macrophages†

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Phospho-ceramide analogue-1 (PCERA-1), a synthetic analogue of ceramide-1-phosphate (C1P), has been previously shown to act as a potent modulator of macrophage activity and inflammation. We have developed an efficient synthesis of PCERA-1 from readily available starting materials, and designed and prepared derivatives of this analogue, including a photoaffinity probe to tag and identify putative proteins that bind PCERA-1.

Sphingolipids are major constituents of biological membranes of eukaryotic cells. Ceramide-1-phosphate (C1P), a sphingoid analogue of phosphatidic acid, is one of the metabolites in the 'sphingomyelin cycle'. C1P has been shown to regulate numerous cell functions including cell proliferation, apoptosis, cell migration, arachidonic acid production, mast cell degranulation, calcium mobilization, and glucose uptake.[1] While some activities, such as arachidonic acid production and proliferation, are carried out by intra-cellular C1P, migration and glucose uptake are stimulated in RAW264.7 macrophages by exogenous C1P, presumably acting *via* an unknown Giα protein-coupled receptor.[2] Interestingly, a synthetic C1P analogue, named phospho-ceramide analogue-1 (PCERA-1), is a potent modulator of macrophage activity and inflammation *in vivo*[3,4] and *in vitro*.[5,6] The enzyme that catalyzes arachidonic acid production, cPLA2α, is a shared target of C1P and PCERA-1, stimulated by both analogues and inhibited by their non-phosphorylated derivatives.[7] However, we have previously shown that PCERA-1 (but not C1P) down-regulates pro-inflammatory cytokine production (TNFα, IL-12 and IL-23 p40) and simultaneously up-regulates production of at least one anti-inflammatory cytokine (IL-10).[5,8c] The mechanism of cytokine modulation by PCERA-1 involves elevation of intra-cellular cyclic adenosine monophosphate (cAMP) levels, presumably *via* an unknown Gsα protein-coupled receptor,[8b] followed by transcriptional activation of cAMP response element binding protein (CREB).[8c] We have recently reported that despite their structural similarity, C1P and PCERA-1 have distinct receptor-mediated effects on RAW264.7 macrophages.[8c] However, the receptor targets for both PCERA-1 and C1P are yet to be determined. Improving our understanding of the proteins involved in this process could open the door to alternate therapeutic routes for the treatment of inflammatory conditions, including those that are currently treated by anti-TNFα biologics (*e.g.* rheumatoid arthritis, inflammatory bowel disease and psoriasis),[11,12] as well as additional inflammation-associated diseases, such as atherosclerosis, diabetes type II *etc.* As a part of our ongoing investigation of the anti-inflammatory functions of PCERA-1,[3–8] we now report the synthesis of a tag-free PCERA-1 probe **9** (shown in Fig. 1), designed to label and identify putative proteins that bind PCERA-1.

In the past decade, activity-based protein profiling (ABPP) has emerged as a powerful strategy in the field of chemical proteomics to characterize enzyme function directly in native biological systems.[9] Our group has developed a toolbox of probes in order to investigate the binding of molecules of interest to unknown proteins in various biological systems,[10] and have recently shown that we can use our chemical proteomics platform to target specific

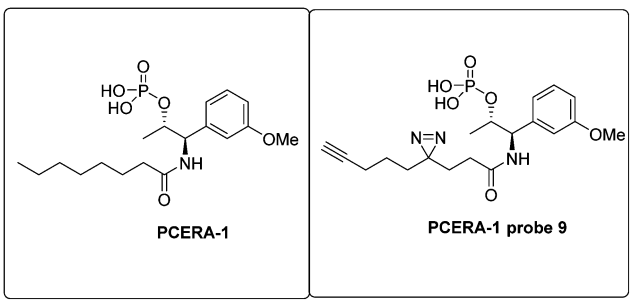

Fig. 1 Structures of PCERA-1 and the biomimetic tag-free photoactivatable PCERA-1 probe **9**.

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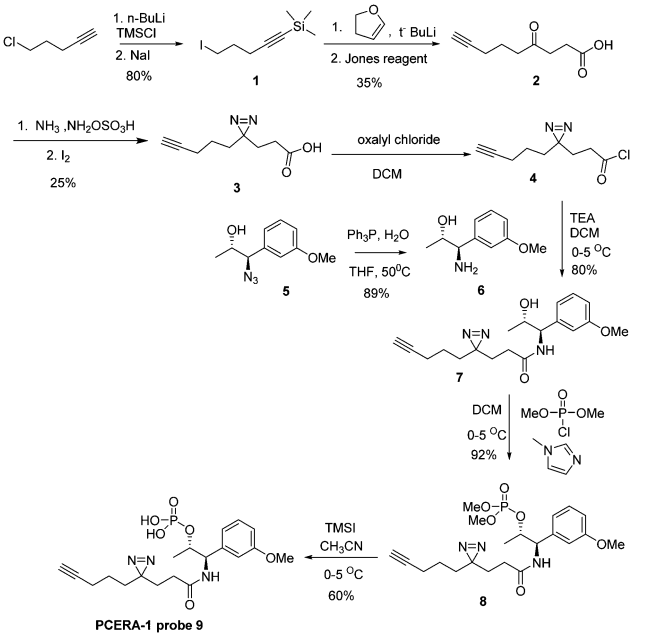
bacterial receptors, and that slight changes in probe design can result in significant functional differences when the probes are assessed in bacteria and in mammalian cells.[10b]

The design of PCERA-1 probe **9** required firstly the development of a synthetic strategy that introduces modifications at the alkyl side chain without affecting aromaticity and chirality. Secondly, modifications of the parent molecule should be as small as possible, in order to minimize any loss of affinity to target proteins. We therefore designed and synthesized a probe scaffold that contains a diazirine photo-cross-linker and a small alkyne handle (Fig. 1). The diazirine is used to establish a covalent bond between the probe scaffold and the target receptor upon irradiation with UV light, which is required for purification, enrichment, and analysis of the labeled proteins. The choice of diazirine (the smallest photoreactive moiety) as a reactive group was motivated by its small size in comparison to other photoreactive groups such as benzophenone, thereby enabling maximal receptor recognition, and the relatively mild irradiation conditions it requires. The alkyne serves as a benign handle through which an azide-containing tag is linked after cell labeling *via* the copper(I)-catalyzed alkyne–azide cycloaddition reaction (CuAAC). This bioorthogonal reaction enables highly specific and efficient coupling of labeled proteins to a fluorophore for in-gel analysis, or to biotin for streptavidin-based affinity purification and identification using mass spectrometry.

The PCERA-1 probe was synthesized based on a previously published protocol,[13] with some significant improvements (see ESI† for details). The synthesis of PCERA-1 probe started from a commercially available chloropentyne that was converted to (5-iodo-1-pentynyl)-trimethylsilyl-silane **1**, as in the reported procedure (Scheme 1). The 4-ketoacid **2** was accessed *via* lithiation of 2,3-dihydrofuran and alkylation with (5-iodo-1-pentynyl)-trimethylsilyl-silane **1**, followed by direct oxidation of the crude alkylated dihydrofuran using Jones' reagent. The alkylated product **2** was then treated with liquid ammonia and hydroxylamine-*O*-sulfonic acid to afford the diaziridine intermediate, which was oxidized with iodine to afford the desired diazirine acid moiety **3**. Diazirine carboxylic acid **3** was converted to acid chloride **4** and then coupled with amine **6** to afford the coupled product **7**. Then, the alcohol group in **7** was converted to dimethyl phosphate **8** by using dimethyl phosphorochloridate (DCP) and *N*-methyl imidazole (NMI). Finally, removal of methoxy groups was carried out using trimethyl silyl iodide (TMSI) to afford the PCERA-1 probe. For improved solubility and usage in cell culture, we converted PCERA-1 probe **9** to its corresponding sodium salt **10** with sodium hydroxide in ethanol. We also synthesized the sodium salt form of PCERA-1 **11** (see ESI†) to compare its biological activity with that of the PCERA-1 probe.

Next, we checked the biological activity of the PCERA-1 probe 9 as compared to PCERA-1 in the modulation of cytokine expression in RAW264.7 macrophages. The cells were co-stimulated by LPS and either PCERA-1 or probe at increasing concentrations, in order to obtain synergistic induction of the anti-inflammatory cytokine IL-10. The probe's activity proved to be almost identical to PCERA-1, in terms of both potency and efficacy of IL-10 induction (Fig. 2). Additionally, the two compounds similarly suppressed LPS-stimulated TNFα expression and release (Fig. 3). The native molecule C1P was included as a negative control, as it has only a minor effect on TNFα release when added exogenously.[8c]



Scheme 1 Synthesis of PCERA-1 probe **9**.

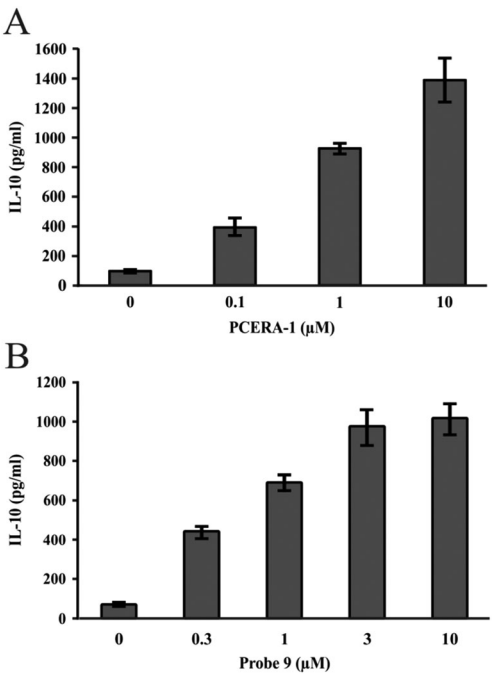


Fig. 2 The PCERA-1 probe stimulates LPS-induced IL-10 release with similar potency and efficacy to the parent molecule. RAW264.7 macrophages were incubated for 2 hr at 37 °C with LPS (100 ng ml^{-1}) and increasing concentrations of either PCERA-1 (A) or probe **9** (B). IL-10 secretion to the medium was measured by ELISA. Data expressed as mean ± SD (n = 6). $p < 0.006$ relative to cells stimulated with LPS alone.

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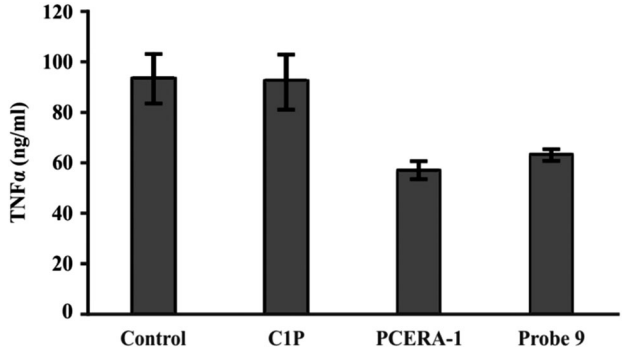

Fig. 3 The PCERA-1 probe inhibits LPS-induced TNFα release similarly to the parent molecule. RAW264.7 macrophages were incubated for 5 hr at 37 °C with LPS (100 ng ml^{-1}) and either PCERA-1 (3 μM), PCERA-1 probe **9** (3 μM) or C16:0 C1P (50 μM). TNFα secretion to the medium was measured by ELISA. Data expressed as mean ± SD (n = 6). $p < 0.004$ relative to cells stimulated with LPS alone.

Finally, we performed an initial proteome labeling experiment. RAW264.7 macrophages lysate was incubated with the PCERA-1 probe, briefly irradiated, then coupled to a fluorescent tag, rhodamine azide, using CuAAC chemistry. In parallel, a competition experiment was set up in which increasing concentrations of the parent molecule PCERA-1 were added alongside the probe, in order to determine whether the probe and parent molecule compete for the same binding sites. The samples were analyzed by fluorescence in-gel visualization and multiple proteins were found to be labeled, with two bands in the 30 kDa region being the most intense (Fig. 4). The intensity of these bands was reduced by ~20–30% when the ratio of probe to PCERA-1 was 1 : 1, and by more than 50% for a 1 : 5 ratio, indicating that the probe is indeed interacting with the same proteins and binding sites as the parent molecule.

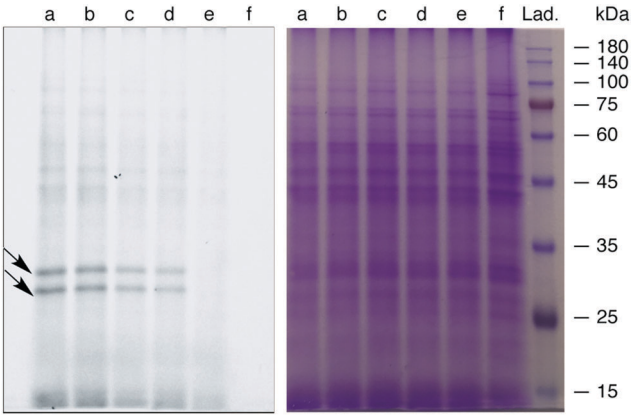

Fig. 4 The PCERA-1 probe **9** specifically labels proteins in macrophage. RAW264.7 macrophages lysates were incubated with 100 μM of probe **9** for 30 min, then irradiated with UV light (365 nm) for 10 min, coupled to rhodamine azide, and analyzed *via* in-gel fluorescence imaging (lane a; see ESI† for details). In parallel, a competition experiment was set up in which increasing concentrations of PCERA-1 (100, 250, 500 μM) were added together with the probe (lane b, c and d respectively), causing the intensity of the labeled bands to decrease (arrows). Control conditions with no probe added yielded only background fluorescence, indicating that the labeling is specific to the probe (lane e). No fluorescence was observed for control with no rhodamine added (lane f). Left, fluorescence; right, Coomassie staining.

In conclusion, we have developed an efficient synthesis that allows expedient preparation of a PCERA-1 based tag-free photoaffinity probe from readily available starting materials. Our results demonstrate that this probe mimics PCERA-1 by activating the receptor expressed in macrophages with similar potency and efficacy to that of the parent molecule, indicating that binding has not been impaired by the minimal diazirine and alkyne modifications. The probe also proved effective in labelling proteins in macrophages, and was found to compete effectively with the parent molecule. In future proteomics studies, this probe will be used to investigate PCERA-1-receptor interactions in macrophages, and as such may help to elucidate the mechanism of action for this molecule and potentially identify novel therapeutic targets for the treatment of chronic inflammatory and inflammation-linked diseases.

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