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Incorporation and visualization of azido-functionalized N-oleoyl serinol in Jurkat cells, mouse brain astrocytes, 3T3 fibroblasts and human brain microvascular endothelial cells†

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The synthesis and biological evaluation of azido-N-oleoyl serinol is reported. It mimics biofunctional lipid ceramides and has shown to be capable of click reactions for cell membrane imaging in Jurkat and human brain microvascular endothelial cells.

Ceramide 1 regulates physiological functions including apoptosis, cell-growth, differentiation, migration and adhesion and has been implicated in cancer, neurodegeneration, diabetes, microbial pathogenesis, obesity, and inflammation. Bieberich et al. described N-oleoyl serinol 2 (S18) as a ceramide mimicking molecule (see Fig. 1). The validity of this suggestion is reflected in the fact that it induces apoptosis in rapidly dividing neuroblastoma cells, but not in resting or differentiated cells. Also, S18 activates atypical protein kinase C (aPKC) and induces formation of aPKC associated complexes with polarity proteins in stem cell experiments. The similarity of S18 to ceramide is also represented by the fact that a highly specific antibody against ceramide, also developed by Bieberich et al., binds to S18. aPKC has been also evaluated successfully as a target for small molecule drug candidates against pancreatic cancer cells. Here we present preliminary data on the synthesis and biological studies of 3a and 3b azido analogues of S18. Ceramide analogs with fluorescent tags proved to be useful for the study of dynamics and visualizing membrane architecture. However, the suitability of such lipids is limited to its ability to behave similar to its unmodified counterpart. Bioorthogonal chemical reporter strategies have addressed this issue and successfully applied, i.e. the alkyne–azide “click” chemistry. In this context, we aimed to apply click chemistry to N-oleoyl serinol ceramide by introducing an azide group and examining if substitution of one serinol-hydroxyl group by an azide will preserve its biological capabilities. For the synthesis of 3 we used a modified protocol of Hawkins et al. with an enantiomer excess (ee) of 92 for 3a and 95 for 3b. For detailed Information of the synthesis see Scheme 1 and ESI.

Recently the Golgi apparatus has been imaged using a clicked ceramide analogue by 3D confocal and STED microscopy in HeLa cells. Building on those studies, we first examined if the azido-modified analogs 3a, 3b used in this work.

Scheme 1. Synthesis of azide modified R-N-oleoyl serinol 3a from L-serine methyl ester hydrochloride 4a or analogue with D-serine methyl ester hydrochloride 4b. Reagents and conditions: (a) N,N-Diisopropylethylamine, CH₂Cl₂, rt, 24 h, 84% (6a), 83% (6b); (b) DIBAL-H, thf, 0 °C – > rt, 24 h, 74% (7a), 94% (7b); (c) MsCl, N,N-Diisopropylethylamine, DCM, 0 °C, 1 h, (d) HCl, 60 °C, 16 h 80% over two steps (8a), 86% over two steps (8b); (e) 4 N HCl, 107 °C, 16 h. (f) Oleic acid, DCC, NHS, DIPEA, dmf, rt, 24 h 47% over two steps (3a), 73% over two steps (3b).

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...astrocytes because they were previously shown to enrich ceramide in Golgi and other vesicular compartments.\(^{13}\) Fig. 4 shows that there are three anti-ceramide antibody labeled compartments that were also positive for 3b. Consistent with previous results obtained with anti-ceramide antibody and NBD-C6 ceramide labeling of the Golgi, these compartments partially colabeled with an antibody against GM130, a marker for cis-Golgi (see Fig. S7, ESI\(^+\)). In a third approach we used human brain microvascular endothelial cells (HBMEC) as serinol acceptors. HBMEC are unique EC representatives as they have continuous intercellular tight junctions (TJs), which control movement of molecules through the EC layer. The EC–pericyte interactions lead to induction of TJ formation. Under inflammatory conditions, pericytes stimulate immune response by production of cytokines.\(^{14}\) Also it has been shown that Neisseria meningitidis, which targets brain ECs, induces the formation of ceramide-enriched platforms on HBMEC to favor bacterial uptake.\(^{15,16}\)

One of the fundamental biological functions of ceramides is to induce apoptotic cell death.\(^{17,18}\) HBMEC were incubated with unmodified N-oleoyl serinol 2 and the enantiomeric enriched compounds 3a and 3b and cell death were determined. Annexin V binding and propidium iodide (PI) staining enabled to discriminate between early apoptotic, late apoptotic and necrotic cell death. Treatment of HBMEC with 25 µM for 1 h did not result in any toxic effects (Fig. S2A, ESI\(^+\)), however, incubation overnight (16 h) with 10 µM or 50 µM resulted in an increase of late apoptotic and in particular necrotic cells (Fig. S2B, ESI\(^+\)). 3a and 3b were comparable with unmodified N-oleoyl serinol 2. They are not toxic below concentrations of 50 µM (see Fig. S2, ESI\(^+\)). Bieberich et al. observed that N-oleoyl serinol 2 induces apoptosis only in rapidly dividing neuroblastoma cells. Here 3 also induced apoptosis at similar concentrations. 3b incorporation in HBMEC was efficiently (see Fig. 3). While 3b is found in the membrane after 5 min

**Fig. 2** Jurkat T cells were incubated with 25 µM 3b (left panels) or 2 (right panels) in HBSS for 30 min at RT. After washing, the cells were exposed to 20 µM DIBO 488 dye and the click reaction was performed for 5 min at RT (upper panels) or for 60 min at 37 °C (lower panels). Cells were washed three times with HBSS and kept at RT until microscopic analysis.

**Fig. 3** HBMEC were treated with 25 µM 3b (left panels) in RPMI medium for 5 min at RT or were left untreated (right panels). After washing, the cells were exposed to 25 µM DIBO 488 alkyne and click reaction was performed for 5 min at RT (upper panels) or for 60 min at 37 °C (lower panels). Cells were washed three times with PBS and kept at RT until microscopic analysis.
interaction is necessary for the establishment and maintenance of the cell polarity complex in non-transformed cells.\textsuperscript{19} Moreover it has been shown that infection of brain ECs with \textit{N. meningitidis} result in recruitment of the polarity complex Par3/Par6/Pkc\textsubscript{\textsuperscript{z}} underneath the attached bacterial microcolonies, which is followed by the delocalization of junctional proteins from the intercellular junctions.\textsuperscript{20}

In summary we have demonstrated the incorporation and visualization of azido-functionalized N-oleoyl serinol by bioorthogonal click chemistry in Jurkat cells and human brain microvascular endothelial cells. We have also shown that apoptosis of the HBMEC can be titrated. It should be noted that more than half of the mitotic glia cells and neurons die during fetal brain development by apoptosis. The stained ceramide analogue can be localized. Thus it might be a helpful tool to investigate sphingosine-1-phosphate and ceramide, and their metabolites roles in endothelial barrier function, which may be a further advancement in this field.

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Notes and references