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Synthesis of fluorescent C_{24}-ceramide: Evidence for acyl chain length dependent differences in penetration of exogenous NBD-ceramides into human skin

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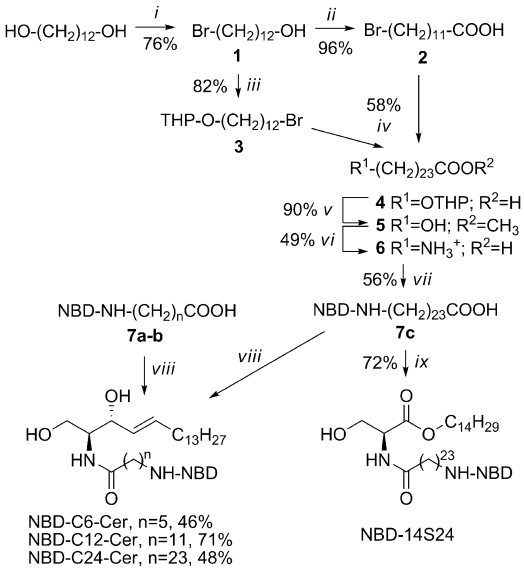
ABSTRACT

Topical skin lipid supplementation may provide opportunities for controlling ceramide (Cer) deficiency in skin diseases such as atopic dermatitis or psoriasis. Here we describe the synthesis of a long-chain 7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl (NBD)-labeled Cer and its different penetration through human skin compared to widely used short-chain fluorescent Cer tools.

Depletion in total ceramide (Cer) content with alteration in the Cer pattern is a common sign for skin disorders with diminished skin barrier function, including atopic dermatitis[1] and psoriasis.[2] The formulations containing physiological lipids and, in particular, Cer supplementation can improve permeability barrier homeostasis.[3–5] In addition, several pseudoCer and Cer analogues were able to recover perturbed skin barrier,[6–8] for example, a simple L-serine derivative 14S24 showed excellent activity.[9,10]

The mechanisms by which exogenous Cer correct the barrier abnormalities have been under debate for some time. In contrast to petrolatum, which remained restricted to the stratum corneum (SC),[5] short-chain fluorescent 7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl (NBD)-C_6-Cer penetrated into the nucleated epidermal cells within two hours,[5] was transported to the distal Golgi apparatus and then reprocessed.[11] Such fast epidermal uptake and metabolism has been generally accepted as the mechanism by which all topical Cer repair the perturbed skin barrier (for recent reviews, see Refs. 12–14).

However, no such evidence exists for natural long-chain Cer, which have an average acyl chain length of 24 carbons, as no such long-chain fluorescent tools have been commercially available. Although short-chain Cer have been widely used as soluble and easier-to-handle Cer mimics, many substantial differences



Scheme 1. Synthesis of NBD-labeled Cer and pseudoCer 14S24. Reagents: (i) 48% HBr, toluene; (ii) CrO_3, AcOH, H_2O, acetone; (iii) DHP, PPTS, $CHCl_3$; (iv) **3**, CH_2Br_2, Mg, CH_3MgCl, Li_2CuCl_4, THF; (v) $SOCl_2$, MeOH; (vi) HN_3 in benzene, PPh_3, DIAD, THF; (vii) NBD-Cl, $NaHCO_3$, MeOH; (viii) (1) *N*-hydroxysuccinimide, DCC, 4-DMAP, $CHCl_3$; (2) sphingosine, $CHCl_3$; (ix) L-Ser-O-$C_{14}H_{29}$, WSC, DMAP, $CHCl_3$.

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between naturally occurring long-chain Cer and their short-chain analogues have been described recently regarding their role in both cell signaling[15,16] and skin permeability.[17]

Thus, we hypothesized that the Cer penetration may be chain length dependent and that the previously observed fast (within 2 h) uptake by nucleated epidermal layers[5] may be specific only for the short-chain Cer. In order to test this hypothesis, we aimed to synthesize a series of fluorescent Cer analogues with different acyl chain length, namely 6, 12 and 24 carbons, and to evaluate their penetration into both disrupted and intact human skin. Furthermore, we aimed at comparing the penetration of NBD-C_{24}-Cer with 14S24, that is, a non-physiological pseudoCer, which has different polar head structure but the same chain length.

For the preparation of the fluorescent long-chain NBD-C_{24}-Cer, 24-aminotetracosanoic acid was synthesized first (Scheme 1). Mono-bromation of dodecan-1,2-diol was achieved under microwave activation in a much shorter time compared to previous method.[18] The bromo alcohol **1** was oxidized to give acid **2**.[19] Hydroxyl group of **1** was protected as THP ether **3**, and converted to a Grignard reagent. Coupling of the chloromagnesium salt of **2** with the Grignard reagent in the presence of freshly prepared lithium tetrachlorocuprate[20] afforded acid **4** in 58% yield. Its hydroxyl was deprotected and the carboxylic group was esterified to yield methyl ester **5** in one step. The formal one-pot conversion of hydroxyl to primary amino group was achieved by combination of Mitsunobu reaction with Staudinger reduction of the azido group.[21] The resulting hydrochloride of 24-aminotetracosanoic acid **6** was alkalized and labeled with NBD-Cl to yield **7c**. The commercially available ω-amino hexanoic and dodecanoic acids were labeled in the same manner to yield **7a–b**. Sphingosine was synthesized by alkynylation of protected L-serinal as described previously.[17] Then, it was N-acylated by succinimidyl esters of the NBD acids. NBD-labeled pseudoCer 14S24 was prepared by N-acylation of tetradecyl L-serine[10] with **7c** using carbodiimide coupling.

The fluorescent lipids were applied on viable human skin, intact or acetone-treated, maintained in Dulbecco's Modified Eagle's Medium at air/liquid interface. The first penetration experiment was realized under the same conditions as described previously for the short-chain Cer,[5] that is, 10 μM solution of the lipids in propylene glycol/ethanol 7:3 (v/v) were applied on the skin for 2 h. The skin sections, counterstained with Hoechst dye, were then examined under a fluorescent microscope (for experimental details, see

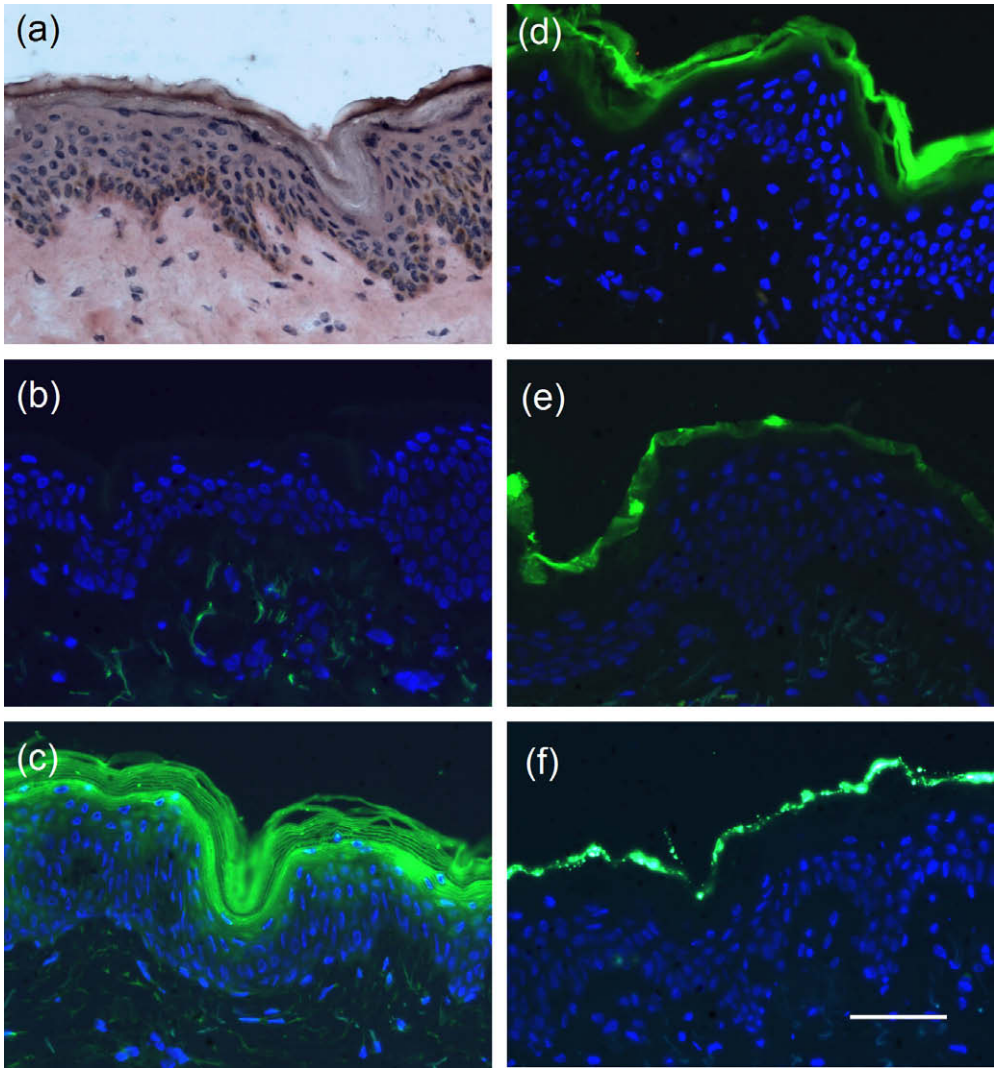

Figure 1. Penetration of exogenous fluorescent Cer with different chain length and pseudoCer 14S24 into human skin. Cer (green fluorescence) were applied on the ex vivo viable human skin in an equimolar mixture with cholesterol and lignoceric acid in propylene glycol/ethanol 7:3 vehicle at a total lipid concentration 1% for 12 h. Histological sections are shown using brightfield microscopy with H&E staining (panel a), fluorescence microscopy with nuclei of cells (blue) stained with the Hoechst 33258 dye (b–f). (a) and (b) controls, (c) NBD-C_6-Cer, (d) NBD-C_{12}-Cer, (e) NBD-C_{24}-Cer, (f) NBD-14S24. Only acetone-treated skin is shown, as no significant differences in Cer penetration were found between intact and acetone-treated skin despite significantly higher transepidermal water loss in the latter. Scale bar 50 μm.

Supplementary data). However, neither long nor short-chain NBD-Cer penetrated into nucleated epidermal layers (Supplementary data, Fig. S1). The reason for this discrepancy in NBD-C_6-Cer penetration between Man et al.[5] and our study may be the different barrier properties of mice and human skin. It has been repeatedly shown that some compounds penetrate in an almost similar manner, others differ in at least one logarithmic order, the human skin being the less permeable.[22] Human skin has, for example, thicker stratum corneum[23] and responds differently to various substances and treatments.[24] Moreover, other studies using NBD-C_6-Cer also gave contradictory results suggesting that this effect may be vehicle- and concentration-dependent. For example, no uptake of NBD-C_6-Cer into hairless mice epidermis from petrolatum was found.[25] In contrast, when using a complex lipid vehicle for 2 h[25] and 1.7% solution in dimethyl sulfoxide, which is actually a potent permeation enhancer, for 4 h,[26] respectively, the fluorescence was visible in the viable epidermis.

In order to reproduce the above mentioned in vivo results[5] and to be able to distinguish between Cer with various acyl chain length, the tissue was exposed to approximately three orders-of-magnitude higher concentration of the lipids for 12 h (Fig. 1, see Supplementary data for details).

Under these conditions, fluorescence of the short-chain NBD-C_6-Cer was observed within the viable epidermis in both intact and acetone-treated skin, which is fully in accordance with Man et al.[5] On the contrary, both lipids with 24 carbons acyl chain length, that is, NBD-C_{24}-Cer and NBD-14S24, stayed in several upper SC layers. One experiment was also performed on a non-viable skin to exclude the possibility of Cer metabolism and it yielded essentially the same results. This suggests that the long-chain lipids (a) display much delayed kinetics compared to NBD-C_6-Cer or (b) they may not be able to reach nucleated epidermis at all.

The reason for the observed chain length dependence in Cer penetration may be the difference in partitioning and mobility of the short- and long-chain Cer. The better penetration of shorter Cer through the lipid membranes may be explained by their smaller molecule and faster exchange between the lipid bilayers. For example, it has been found that the exchange of natural Cer between lipid membranes requires days,[27] while it takes less than a minute for short chain, fluorescent Cer to exchange between lipid vesicles.[28] An easier transbilayer movement of C_6-Cer compared to C_{16}-Cer was observed in phospholipid membranes.[29] Short-chain Cer were also found to increase skin permeability.[17]

Moreover, the long-chain Cer may be too hydrophobic to partition from SC into a hydrophilic viable epidermis. Indeed, free natural long-chain Cer cannot exist in solution in biological fluids or in cytosol. Long-chain Cer stay relatively tightly bound to the membrane where they are generated,[30,31] while short Cer can leave the membrane and translocate to other membranes. Another example is an elegant study comparing the effects of short- and long-chain Cer that showed that although exogenous NBD-C_6-Cer taken up via the plasma membrane was converted into glucosylCer in the Golgi (the same was suggested to occur with exogenous Cer in the skin[11]), long-chain Cer generated in the plasma membrane did not reach the Golgi and thus were not glycosylated.[32]

However, it should be kept in mind that these initial results were obtained using NBD-labeled lipids and should be confirmed using more physiological fluorescent Cer, for example, with polyene fluorophores, or radiolabeled lipids (for an excellent comparison and discussion on these Cer tools, see Refs. 33,34). Nevertheless, these results clearly showed chain length dependent differences in penetration of exogenous NBD-Cer across SC. Although short-chain Cer are useful experimental tools in many sphingolipid studies, they cannot be used as general Cer mimics, particularly when considering Cer effects in lipid membranes, including SC lipid lamellae. Thus, the mechanism of action of Cer in skin barrier repair, which was based on a behavior of a short-chain Cer, should be reconsidered.

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Supplementary data

Supplementary data (experimental procedures and Fig. S1) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.047.

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