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On the effects of topical synthetic pseudoceramides: comparison of possible keratinocyte toxicities provoked by the pseudoceramides, PC104 and BIO391, and natural ceramides

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Summary

Background—Ceramides (Cer) in the stratum corneum are essential for epidermal permeability barrier function. Thus, topical Cer replacement therapy has been employed to improve barrier function in clinical situations associated with Cer deficiency, *e.g.*, atopic dermatitis. Because of the disadvantages of both natural- and skin identical-Cer (CNS origins and cost, respectively), synthetic chemical mimics, or pseudoceramides (pseudo-Cer), have been utilized as Cer substitutes. Whereas increased levels of intracellular Cer trigger cell growth inhibition and apoptosis, Cer levels are maintained by metabolic/catabolic pathways protecting cells from Cer-induced apoptosis. However, since the metabolic fates of each pseudo-Cer remain unknown, their wide-spread deployment in topical agents has raised concern about potential toxicities.

Objective—We compared the effects of two chemically-unrelated commercially-available pseudo-Cer to exogenous cell-permeant (C2)- or natural (C18)-Cer on cell growth and apoptosis thresholds in cultured human keratinocytes (CHK).

Methods—Cell growth and cell toxicity of CHK exposed to either C2-Cer or pseudo-Cer were assessed by MTT and lactate dehydrogenase release assays. Mitochondrial membrane potential, an indicator of apoptosis, was measured using membrane permeabilized semi-intact keratinocytes exposed C2-Cer, natural-Cer or pseudo-Cer.

Results—While the cell-permeant-Cer inhibits keratinocyte growth and increases cell toxicity, neither of the pseudo-Cer showed these effects. Decreased mitochondrial membrane potential occurred in CHK incubated with cell-permeant- and natural-Cer, but not pseudo-Cer.

Conclusions—Taken together with preclinical safety studies of these pseudo-Cer and their wide-spread use over the counter without evidence of toxicity, these studies provide further assurance about the safety of these pseudo-Cer for topical use.

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Conflict of Interests

Dr. Elias is a consultant to both Ceragenix and Symrise, who manufacture 'barrier repair' mixtures that incorporate PC-104 and BIO391, respectively.

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Keywords

apoptosis; atopic dermatitis; ceramide; permeability barrier; pseudoceramide

1. Introduction

A family of nine ceramides (Cer) is present in human stratum corneum (SC), which together accounts for about 50% of its lipid weight (5% of total SC weight) [1]. Cer, like the other two key SC lipid species, cholesterol and free fatty acids (FFA), segregate to SC extracellular domains [2], where they form a series of membrane bilayers that mediate permeability barrier homeostasis [3]. Hence, if only because of their sheer abundance, topical therapies that aim to reverse Cer deficiency, a characteristic of atopic dermatitis, typically employ Cer or Cer-like molecules, and often in large quantities [4]. Yet, three issues confront those who wish to use natural or skin-identical Cer in such 'barrier repair therapeutics': 1) nature-identical, synthetic Cer are extremely expensive (typically \$2,000–10,000/kg); 2) less-expensive, naturally-occurring Cer derive primarily from bovine central nervous system, raising concerns about transmission of bovine spongiform encephalopathy ('mad cow disease') [5]; and 3) excess intracellular Cer can be toxic to cells, both inhibiting growth and inducing apoptosis [6,7].

To circumvent these issues, relatively-inexpensive, synthetic mimics have been developed (pseudoceramides, pseudo-Cer), which are intended to substitute for Cer in topical products [1,8–10], including triple-lipid (Cer, cholesterol, FFA) barrier repair mixtures [4]. Earlier studies demonstrated that topical applications of such pseudo-Cer improved the water-holding properties of the stratum corneum in human skin [11]. Furthermore, while naturally-occurring Cer, when incorporated into such mixtures, facilitate permeability barrier recovery [12,13], recent studies suggest that certain pseudo-Cer can substitute efficiently for natural (bovine) Cer in triple lipid mixtures. For example, when present as the dominant species in a 3:1:1 molar ratio, they accelerate barrier recovery after acute abrogation in comparison to vehicle-treated sites [4,14]. Two pseudo-Cer, *i.e.*, *N*-(3-hexadecyloxy-2-hydroxypropyl)-*N*-2-hydroxyethylhexadecanamide (PC-104) and a similar structural compound, *N*-(2-hydroxyethyl)-2-pentadecanolyhexadecanamide (Bio391), can form lamellar/multilamellar structures *in vitro* with cholesterol and free fatty acids [15–17]. Accordingly, the cutaneous symptoms of atopic dermatitis appear to improve in patients treated with pseudo-Cer (PC-104 and others)-containing mixtures [4,18,19]. Moreover, human atopic dermatitis displays near normal-appearing lamellar membranes within the interstices of the SC after topical treatment with both of the pseudo-Cer-containing, triple-lipid mixtures [4,14]. Yet, while certain pseudo-Cer substitute effectively for natural Cer, not all pseudo-Cer are effective in such triple-lipid mixtures (Mao-Qiang M & Elias PM, unpublished observations). Hence, whether synthetic pseudo-Cer effectively mimic natural Cer needs to be determined on a molecule-by-molecule basis.

Because pseudo-Cer diverge substantially in molecular structure from endogenous Cer, legitimate concerns have been raised about the potential toxicity of these molecules [10]. When endogenous Cer levels increase, they can stimulate signal cascades in response to a variety of oxidative stressors that can lead to increased apoptosis and cell death [6]. Moreover, increased levels of endogenous Cer also inhibit growth of cultured human keratinocytes (CHK) [20] and induce apoptosis in CHK [21]. Yet, cellular Cer levels can be maintained by metabolic and catabolic pathways, *i.e.*, metabolic conversion of Cer to either glucosylceramide [20], sphingomyelin [22], Cer-1-phosphate [23], and/or Cer hydrolysis to sphingosine and fatty acids by ceramidase, followed by phosphorylation to sphingosine 1-phosphate*. However, excess Cer production can overwhelm these homeostatic systems, thereby increasing apoptosis. Indeed, exogenous cell-permeant Cer (C2- and C6-Cer) increase differentiation [24,25] and

induce apoptosis [26,27] in CHK, effects that are dependent upon the concentrations of these Cer species. Since natural Cer are considered nearly cell-impermeant, even if exogenous Cer penetrate into nucleated layers of epidermis, it is unlikely that intracellular Cer levels are substantially increased to cause deleterious effects in skin cells. In fact, natural Cer (C_≥16) have been utilized for Cer replacement therapy without apparent adverse effects. In contrast, the cellular fate of pseudo-Cer could differ, raising questions about the potential toxicity [10].

We compared here the effects of two chemically-unrelated pseudo-Cer in comparison to both cell-permeant Cer, N-acetylsphinganine (C2-Cer) and natural Cer, N-stearoylsphinganine (C18-Cer) (Fig. 1), in intact and semi-intact undifferentiated CHK. Cell-permeant Cer decreased cell growth and increased cell toxicity, while natural Cer also decreased mitochondrial membrane potential, an indicator of apoptosis. In contrast, the two pseudo-Cer, at comparable concentrations, did not exhibit these effects, suggesting that they have minimal effects on keratinocyte proliferation and/or apoptosis.

2. Materials and Methods

2.1. Samples

Cell-permeant N-acetylsphinganine (C2-Cer) and natural Cer, N-stearoylsphinganine (C18-Cer), were purchased from Matreya (Pleasant Gap, PA). BIO391 and PC-104 were kindly provided from Symrise GmbH & Co KG (Holzminden, Germany), and Ceragenix Pharmaceuticals Inc. (Denver, CO), respectively. These lipids were dissolved in ethanol and then prepared as a bovine serum albumin (BSA) complex (mole ratio of Cer to BSA=1:1).

2.2. Cell culture

Normal human keratinocytes were isolated from human neonatal foreskins by a modification of the method of Pittelkow and Scott [28] under an Institutional Review Board-approval protocol (University of California, San Francisco). Cells were grown in Keratinocyte Growth Medium, supplemented with bovine epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone and 0.07 mM calcium chloride (Cascade Biologics, Portland, OR). Immortalized, non-transformed, low passage number HaCaT cells, derived from human epidermis, were a gift from Dr. N. Fusenig (Heidelberg, Germany). The cultures were maintained at 37°C under 5% CO₂ in air.

2.3 Assays for Cell Growth

Cell growth was assayed by cellular dehydrogenase activities (MTT assay) using The Cell Titer 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). Values represent means±SD. Since C18-Cer is largely cell-impermeant, we did not investigate the effects of C18-Cer on cell growth and cellular toxicities (see below) in intact cells.

2.4 Cell Toxicity

Lactate dehydrogenase (LDH) release into the culture medium was utilized as a measure of cell viability, using the Cytotoxicity Assay Kit (Promega, Madison, WI). Values represent means±SD.

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2.5. Mitochondrial Membrane Potential

Membrane permeabilized-semi-intact HaCaT human keratinocytes were prepared with a hypotonic buffer as described previously [29]. Briefly, cells were rinsed with ice-cold phosphate-buffered saline followed by incubation with 10 mM HEPES buffer (pH 7.2 containing 15mM KCl and 0.1 mM MgCl₂), for 10 min on ice. Cells were rinsed with 25mM HEPES buffer (pH 7.2 containing 115 mM KCl). Greater than 95% of cells were permeabilized as assessed by the trypan blue dye-exclusion assay. Semi-intact cells were then incubated with either 10 μ M of pseudo-Cer, cell permeant C2-Cer, natural Cer (C18-Cer), or 250 μ M hydrogen peroxide. Mitochondrial membrane potential was assayed using the MitoPT™ assay system following the manufacture's protocol (B-Bridge International, Inc., Sunnyvale, CA). The results are reported as the ratio of aggregate form (red fluorescence; EX 490nm/EM 590nm), to monomeric form (green fluorescence; EX 490nm/EM 527nm) of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl- benzamidazolocarboxyanin iodide. Values represent mean $\pm$ SD.

3. RESULTS

3.1 Cell-permeant Cer, but not pseudo-Cer, inhibit keratinocyte proliferation

To address the issue of toxicity, we initially compared the effects of comparable concentrations of cell-permeant Cer (N-acetylsphinganine, C2-Cer) but not cell-impermeant natural Cer (N-stearoylsphinganine, C18-Cer) with two, chemically-unrelated pseudo-Cer (BIO391, N-palmitoyl-4-hydroxy-L-proline palmityl ester and PC-104, 1,3-bis(N-2-(hydroxyethyl) palmitoylamino)-2-hydroxypropane) (Fig. 1) on keratinocyte proliferation. While C2-Cer provoke a dose-dependent decline in proliferation, neither of the pseudo-Cer altered cell growth in cultured human keratinocytes (CHK), when deployed at comparable concentrations (Fig. 2).

3.2. Cell-permeant Cer, but not pseudo-Cer, increase cytotoxicity and cell death

We next studied the potential of cell-permeant Cer, C2-Cer, vs. pseudo-Cer to induce cytotoxicity, assessed by LDH release from cells, an indication not only of toxicity, but also of the late stages of apoptosis, in CHK. While exogenous, C2-Cer induced lactate dehydrogenase release in a dose-dependent fashion, neither of the pseudo-Cer exhibited toxic effects on intact CHK (Fig. 3).

Yet, because pseudo-Cer, like natural Cer, may not be able to penetrate intact CHK *in vitro*, we next assessed apoptosis by changes in mitochondrial membrane potential [30,31], in permeabilized, semi-intact HaCaT keratinocytes. Pertinent to the present studies, a number of prior studies have demonstrated that Cer-mediated decreases in mitochondrial membrane potential parallel apoptosis in several cell types, and Cer decreases mitochondrial membrane potential in isolated mitochondria [30,31]. Hydrogen peroxide (H₂O₂)-induced, oxidative stress, which served as a positive control in this assay, decreased mitochondrial membrane potential by over 50% in comparison to vehicle-treated control levels (Fig. 4). Likewise, both natural Cer (C18-Cer) and cell-permeant Cer (C2-Cer), impaired mitochondrial function to a somewhat lesser extent (20–35%) in comparison to H₂O₂. In contrast, PC-104 showed significantly lower levels of membrane depolarization than natural Cer, while Bio391 displayed no changes in membrane potential in comparison to vehicle-treated control (Fig. 4). Thus, pseudo-Cer, at the concentrations employed here, which were equivalent to the range of concentrations employed in topical reagents, are less toxic to membrane-permeabilized, semi-intact keratinocytes than are natural/cell-permeant Cer. These results suggest that these two pseudo-Cer, BIO391 and PC-104, are less toxic to intact and membrane-permeabilized keratinocytes than comparable concentrations of either nature-identical or cell-permeant Cer.

4. Discussion

While Cer are essential components of the epidermal permeability barrier, decreased Cer content and changes in Cer molecular distribution also have been demonstrated in several dermatoses, including atopic dermatitis [32–35] and psoriasis [36]. Not surprisingly, both atopic dermatitis and psoriasis display phenotype-dependent abnormalities in barrier function [37,38]. Accordingly, *in vivo* studies in both experimental models and preliminary human studies have demonstrated that topical Cer-containing lipid mixtures can improve barrier function [12,13,39]. More importantly, Cer-containing lipid mixtures currently are being assessed for their potential use as primary and/or ancillary therapy of atopic dermatitis. Because of the potential risks and high cost of natural- and skin-identical synthetic Cer, respectively, chemical synthetic mimics, or pseudo-Cer, have been developed as Cer-alternatives. Whereas natural Cer have potentially deleterious effects, including inhibition of cellular proliferation and induction of apoptosis, in a variety of cells [6,7], including keratinocytes [21,24–27], certain metabolic and catabolic pathways can suppress potential Cer accumulation, thereby protecting cells from Cer-induced apoptosis [20,22,23]. In addition, natural Cer are nearly cell-impermeant suggesting that the quantity of natural Cer absorbed into the nucleated cell layers may not be sufficient to induce apoptosis.

Despite the apparent efficacy of pseudo-Cer in the treatment of atopic dermatitis [4,18,19, 40], whether or not pseudo-Cer(s) exhibit(s) similar effects as seen with natural Cer on cellular growth and apoptosis has not been reported. Our present studies demonstrate that two pseudo-Cer, BIO391 and PC-104, have fewer adverse effects on cell growth and toxicity than exhibited by either comparable concentrations of nature-identical or cell-permeant Cer in intact CHK. Likewise, the two pseudo-Cer exhibited a lesser propensity to induce apoptosis-related, membrane depolarization in membrane-permeabilized, semi-intact cells. However, these studies do not exclude possible adverse effects of other, chemically-unrelated pseudo-Cer that were not tested here. Lack of toxicity to CHK at these concentrations, though relevant for topical therapy in human subjects [10], does not, however, exclude the possibility of toxicity by these pseudo-Cer to other cell types should substantial absorption occur. Since even more pseudo-Cer could penetrate into skin with barrier defects, we assessed unimpeded access of these agents to undifferentiated cultured keratinocytes, which represent the cells of nucleated cell layers in epidermis and do not have permeability barrier structures which could exclude pseudo-Cer from the nucleated layers of the epidermis. Our results demonstrate that pseudo-Cer do not show significant toxicity at concentrations that assume approximately 100% absorption of the agent applied topically. In fact, the doses (up to 25 μ M) tested in this study represent a similar concentration range of pseudo-Cer agent used for topical therapy (*i.e.*, 1–2%). Thus, even if 100% of topical applied pseudo-Cer penetrate into skin, it is not likely to show significant cell toxicities as demonstrated in this study. Moreover, PC-104 is not a topical irritant or sensitizer after applications at high concentrations, under occlusion, to intact and wounded rodent skin; or when instilled into rabbit eyes, and it is not mutagenic in the Ames (*S. typhimurium*) assay (FDA 510K Notification for EpiCeram Skin Barrier Emulsion, April 12, 2006). In addition, PC-104 has been widely used for over five years in the U.S., without reports of toxicity, in a triple-lipid formulation (TriCeram, Osmotics Corp.). Finally, it should be noted that TriCeram has been extensively employed as ancillary therapy for atopic dermatitis [4], a disease characterized by defective barrier function (*op cit*), without reports of cutaneous or extracutaneous side effects.

Even more extensive *in vivo* preclinical safety studies were performed on BIO391, which likewise revealed no evidence of toxicity, including oral administration (LD50 > 2000 mg/kg); lack of acute toxicity to *Daphnia magna*: neither skin nor eye irritation; and no evidence of skin sensitization; and no evidence of mutagenicity (tested according to OECD guidelines

#423, 402, 404, 405, 429, and 471, April, 2006). Thus, neither cutaneous nor extracutaneous toxicity appears likely to occur with either of these compounds.

We did not address whether these and other pseudo-Cer are metabolized into potentially-toxic metabolites. However, as both of these pseudo-Cer are amphiphilic, highly hydrophobic (log P o/w > 13) compounds with molecular weight > 500 [41], substantial penetration into deeper epidermal layers, a prerequisite for consequent metabolic conversion and/or systemic uptake, appears unlikely. Moreover, BIO391 reportedly is very stable over a pH range of 5–7 [14], excluding the possibility of a non-enzymatic, pH-driven degradation under physiologic or pathologic conditions.

Finally, prior studies demonstrated that Cer containing 2-OH fatty acid (2-OH Cer) are less potent than Cer containing non-hydroxy fatty acid for inducing apoptosis in U937 cells [42]. However, since both BIO391 and PC-104 are designed as Cer-containing, non-OH fatty acid mimetics, we did not compare the effects of 2-OH Cer in this study. Although it has not been determined whether 2-OH Cer either have less potent toxicities in CHK or improve epidermal permeability barrier function, 2-OH Cer mimetics pseudo-Cer may further assure safety for these therapeutic agents.

In summary, two chemically-unrelated, synthetic Cer mimics or pseudo-Cer, N-palmitoyl-4-hydroxy-L-proline palmitoyl ester (BIO391) and 1,3-bis(N-2-(hydroxyethyl) palmitoylamino)-2-hydroxypropane (PC-104), display lesser effects on CHK growth and viability than either exogenous natural or cell-permeant Cer, providing further evidence for the safety of these two agents when incorporated into topical therapeutics. Yet, further assessments of the safety pseudo-Cer still need to be performed *in vivo*.

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Figure 1.
Structures of pseudoceramides, natural ceramide (N-stearoylsphingenine or C18-Cer), and cell-permeant ceramide (N-acetylsphingenine or C2-Cer).

Figure 2.

Pseudo-Cer do not inhibit cell growth in human keratinocytes. Cultured human keratinocytes (CHK) isolated from human neonatal foreskins were incubated with pseudo-Cer or cell-permeant C2-Cer for twenty-four hours. Cell growth was assessed by dehydrogenase enzymes in metabolically active cells using aqueous One Solution Cell Proliferation Assay Kit (Promega). Values represent mean $\pm$ SD from three experiments.

Figure 3.

Pseudo-Cer do not induce toxicity in human keratinocytes. CHK were incubated with pseudo-Cer or cell-permeant C2-Cer for twenty-four hours. Cell toxicity was assessed by LDH release into the culture medium. Values represent mean $\pm$ SD from three experiments.

Figure 4.

Pseudo-Cer produce significantly less apoptosis than natural ceramides in cell-free mitochondrial Assay. Membrane permeabilized-semi-intact HaCaT human keratinocytes were prepared with a hypotonic buffer as described previously [29]. Semi-intact cells were incubated with 10 μ M of pseudo-Cer, cell-permeant C2-Cer, natural Cer (C18-Cer), or 250 μ M of hydrogen peroxide. Values represent means $\pm$ SD from three experiments.