Bcl-2 overexpression prevents apoptosis induced by ceramidase inhibitors in malignant melanoma and HaCaT keratinocytes

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Dedicated to the 65th birthday of Professor Werner Reutter

Abstract We examined the biological effects of the ceramide analogues (1*S*,2*R*)-2-*N*-myristoylamino-1-phenyl-1-propanol (D-*e*-MAPP) and (1*R*,2*R*)-2-*N*-myristoylamino-1-(4-nitrophenyl)-1,3-propandiol (D-NMAPPD) on human HaCaT keratinocytes and human melanoma cells. We could demonstrate that D-*e*-MAPP and D-NMAPPD are able to suppress acid ceramidase activity. The elevation of the endogenous level of ceramide is followed by induction of apoptosis and suppression of proliferation in HaCaT keratinocytes. Moreover, we recently identified a group of human melanoma cell populations which are heterogeneously susceptible to C_2-ceramide-mediated apoptosis. Studies with these melanoma cells revealed correlation between ceramide-mediated apoptosis and D-NMAPPD-induced apoptosis, confirming the effect of this inhibitor on ceramide signaling in human melanoma cells. These findings suggest ceramidase inhibitors as a potential new therapeutical class of antiproliferative and cytostatic drugs.

Key words: Ceramide; Ceramidase; Melanoma; HaCaT keratinocyte; Bcl-2

1. Introduction

Ceramide is a signaling molecule involved in cellular responses to a variety of apoptotic stimuli [1,2]. It is now evident that ceramide is involved in the induction of apoptosis, cell cycle arrest and cell differentiation in various cell systems [3–5]. Three main metabolic pathways are known to participate directly in reducing intracellular ceramide levels: (i) biosynthesis of sphingomyelin through sphingomyelin synthase, (ii) biosynthesis of cerebrosides through glycosyltransferases and (iii) production of sphingosine via ceramidase [6]. Metabolic manipulation of ceramide levels, such as the addition of bacterial sphingomyelinase or 1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an inhibitor of glycosylceramide synthases [7], causes accumulation of intracellular ceramide followed by ceramide-specific biological responses. Recently, elevation of endogenous ceramide levels and growth suppression in HL-60 cells by inhibition of alkaline ceramidase has been shown [8].

Ceramidases (*N*-acylsphingosine deacylases) hydrolyze ceramide producing sphingosine and a free fatty acid. Three isoforms of ceramidases, acid, alkaline and neutral, assigned by their catalytic pH optimum have been described [9,10]. In order to evaluate the cellular and biochemical activities of ceramidase inhibitors we examined the biological effects of the previously described agents D-*erythro*-2-*N*-myristoylamino-1-phenyl-1-propanol (D-*e*-MAPP), its inactive enantiomer L-*erythro*-2-*N*-myristoylamino-1-phenyl-1-propanol (L-*e*-MAPP) and a new member of this group, (1*R*,2*R*)-2-*N*-myristoylamino-1-(4-nitrophenyl)-1,3-propandiol (D-NMAPPD). D-*e*-MAPP has been recently shown to elevate endogenous ceramide levels and suppress growth of HL-60 leukemia cells by inhibition of ceramidase, whereas D-NMAPPD is a novel member of the ceramidase inhibitor group [8]. Very recently D-NMAPPD, previously termed B13, has been shown to induce apoptosis in human colon cancer cells in vitro and in vivo conditions [11]. Our study on HaCaT keratinocytes and melanoma cells provides evidence that the ceramidase inhibitors D-*e*-MAPP and D-NMAPPD are able to suppress acid ceramidase activity. The subsequent elevation of the endogenous level of ceramide is followed by induction of apoptosis and suppression of proliferation in HaCaT keratinocytes. However, L-*e*-MAPP, the enantiomer of D-*e*-MAPP, was without effect. Previously we described a group of human melanoma cell populations which are heterogeneously susceptible to (2*S*,3*R*)-*N*-acetylsphingosine (C_2-ceramide)-mediated apoptosis [12]. Treatment of melanoma cells with D-NMAPPD, but not D-*e*-MAPP, revealed suppression of acid ceramidase activity and elevation of endogenous ceramide levels in all melanoma cell populations. However, induction of apoptosis and suppression of proliferation succeeded only in ceramide-sensitive cells, while the ceramide-resistant cells stayed insensitive to D-NMAPPD. On the other hand, Bcl-2 overexpression abolished apoptosis triggered by D-NMAPPD in HaCaT keratinocytes and A375 melanoma cells.

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Abbreviations: D-*e*-MAPP, (1*S*,2*R*)-2-*N*-myristoylamino-1-phenyl-1-propanol; L-*e*-MAPP, (1*R*,2*S*)-2-*N*-myristoylamino-1-phenyl-1-propanol; D-NMAPPD, (1*R*,2*R*)-2-*N*-myristoylamino-1-(4-nitrophenyl)-1,3-propandiol; C_2-ceramide, (2*S*,3*R*)-*N*-acetylsphingosine; LDH, lactate dehydrogenase

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2. Materials and methods

2.1. Materials

D-*e*-MAPP, L-*e*-MAPP and D-NMAPPD were synthesized as described [13]. *N*-Palmitoyl-[1-^{14}C]D-sphingosine (55 mCi/mmol) was purchased from Biotrend (Cologne, Germany) and L-[^{3}H]serine (33 Ci/mmol) was purchased from Amersham (Brunswick, Germany). Remaining lipids and detergents were obtained from Sigma (Munich, Germany). The construction of pIRES/mbcl-2 has been reported previously and has been shown to be functional in HaCaT keratinocytes [14] and A375 cells [15].

2.2. Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT was grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 0.35 g/l glutamine, 100 IU/ml penicillin and 0.1 g/l streptomycin. Media and culture reagents were obtained from Biochrom Seromed (Berlin, Germany). Prior to treatment, HaCaT keratinocytes were cultivated for 24 h in serum-free KGM (Clonetics, St. Katharinen, Germany). For L-[^{3}H]serine labeling of cells, serine-free modified Eagle's medium (MEM; Biochrom Seromed KG, Berlin, Germany) was used. The applied agents were added to the cells in KGM. M186 and M221 were obtained from patients with histologically confirmed metastatic melanoma by surgical intervention. A375 is an established cell line originated from primary tumors [16] and Mel2A from metastasis [17].

The construction of pIRES/mbcl-2 has been reported previously and has been shown to be functional in HaCaT keratinocytes [14] and A375 cells [15].

Briefly, transfection of melanoma cells and HaCaT keratinocytes with pIRES1neo/mbcl-2 or pIRES1neo was carried out according to the supplier's protocol using the lipofection reagent pFx-2 from Invitrogen (Groningen, The Netherlands) and FuGene 6 from Boehringer Mannheim (Mannheim, Germany), respectively. After a selection period with geneticin of approximately 10 weeks, individual cell clones were isolated. Bcl-2 overexpression in these cell lines was confirmed by Western blotting (data not shown).

All melanoma cell populations were grown and treated in Dulbecco's MEM (Gibco BRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum and 100 IU/ml penicillin and 0.1 g/l streptomycin.

2.3. Determination of cytotoxicity

Cytotoxicity was determined with the cytotoxicity detection kit (lactate dehydrogenase, LDH) from Roche Diagnostics (Mannheim, Germany) exactly as described elsewhere [5].

2.4. Cell death detection

Subconfluent cells in 24-well plates were treated with the indicated agents or respective control vehicle. After incubation, cell death was measured in a photometric enzyme immunoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) with the kit 'Cell death detection ELISA^{PLUS}' from Roche Diagnostics as described elsewhere [18].

2.5. Cell proliferation assay

HaCaT keratinocytes and melanoma cells were seeded in 24-multiwell plastic culture dishes and treated with the respective agents or control vehicle at 40% confluence. After incubation, cell proliferation was determined by crystal violet staining as described in detail [19].

2.6. Measurement of ceramidase activity in HaCaT keratinocytes and melanoma cells

Ceramidase activity was measured by a modification of the method of Gatt and Yavin [20] and Sugita [21]. Cells were disrupted by homogenization in a Dounce homogenizer and subsequent sonication in 0.25 M sucrose, 1 mM EDTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM $CaCl_2$ and 20 μg/ml of each leupeptin, pepstatin and aprotinin. Protein was measured in samples by the BCA assay [22]. Substrate solution was prepared by combining 1.5 μl 10 μCi/ml *N*-palmitoyl-[1-^{14}C]D-sphingosine, 2.5 μl 1 mM *N*-stearoyl-D-sphingosine, 25 μl 0.1% Triton X-100 (v/v) in chloroform/methanol, 2:1 (v/v) and 25 μl 0.2% sodium cholate (w/v) in chloroform/methanol, 2:1 (v/v). Solvents were evaporated, resolved in 7.5 μl H_2O, sonicated for 3 min, heated for 5 s at 80°C and cooled rapidly on ice. Then, to 25 μl of the cell extract 5 μl of 50 mM $MgCl_2$, 7.5 μl of substrate solution and 12.5 μl of one of the respective buffers were added. For measurement of alkaline ceramidase, 0.2 M CHES, pH 9.0 and for acid ceramidase 0.2 M sodium acetate, pH 4.5 were used. Ethanol, the solvent of the inhibitors, had no effect on the enzyme reaction. The reaction mixture was incubated for 1 h at 37°C. The [^{14}C]palmitic acid product of ceramidase was isolated by the addition of 0.5 ml of isopropyl alcohol/heptane/1 M NaOH (40:10:1, v/v/v), 0.3 ml of heptane and 0.25 ml of H_2O. Samples were mixed and centrifuged for 5 min at 13 000×*g* at 4°C. The upper phase was discarded and the lower phase washed twice with heptane. Finally, 0.25 ml of 0.5 M H_2SO_4 and 0.5 ml of heptane were added and after mixing and centrifugation, the upper phase was used for counting by liquid scintillation.

2.7. Measurement of ceramide levels

Cells were seeded in six-well plates and, after reaching a subconfluent state, metabolically labeled with 2 μCi/ml L-[^{3}H]serine in serine-free MEM medium for 24 h to reach steady-state conditions [23]. Subsequently, chasing for 3 h with 5 mM non-radioactive serine in KGM was performed, followed by treatment with agents or respective vehicle as indicated. Lipids were extracted as described [24]. The extracted lipids were diluted in 50 μl chloroform/methanol, 9:1 (v/v) and 20 μl of this solution was applied to precoated silica gel 60 high performance thin layer chromatography plates (Merck, Darmstadt, Germany) and developed in chloroform/methanol, 9:1 (v/v). The quantification of ceramide contents was achieved by scanning of autoradiographs using an imaging densitometer (Bio-Rad, Munich, Germany) and analyzed using MultiAnalyst Software (Bio-Rad).

3. Results

3.1. D-*e*-MAPP and D-NMAPPD inhibit the activity of acid ceramidase

Since earlier studies of D-*e*-MAPP and L-*e*-MAPP in HL-60 cells showed an inhibitory effect of D-*e*-MAPP but not L-*e*-MAPP on alkaline ceramidase, we examined the effect of these compounds and their analogue D-NMAPPD on acid as well as alkaline ceramidase isoforms. Ceramidase activity was examined for pH dependence using ^{14}C-labeled C_{16}-ceramide as substrate in cytosolic and membrane fractions of HaCaT keratinocytes and melanoma cells. The pH optima of ceramidase activity was found to exist at pH 4.5 and pH 9.0 as described for HL-60 cells [8]. D-*e*-MAPP served as a relatively poor inhibitor of acid ceramidase in a concentration-dependent manner in HaCaT keratinocytes, with an approximately half-inhibitory concentration of 500 μmol/l (IC_{50} = 500 μM). In contrast D-NMAPPD was found to be a potent inhibitor of acid ceramidase with an IC_{50} of about 10 μmol/l. These results demonstrate that D-NMAPPD is a much more potent inhibitor of acid ceramidase activity than D-*e*-MAPP in HaCaT keratinocytes (Fig. 1A). D-NMAPPD at a concentration of 500 μmol/l reduced the alkaline ceramidase activity only to 90% and in contrast the acid ceramidase activity to 18% compared to control in all investigated cells. D-NMAPPD served as a strong inhibitor of acid ceramidase, while alkaline ceramidase activity was inhibited marginally by D-NMAPPD (Fig. 1B) and D-*e*-MAPP (data not shown). L-*e*-MAPP inhibited neither acid nor alkaline ceramidase up to concentrations of 500 μmol/l (data not shown).

3.2. Only high concentrations of D-NMAPPD have significant cytotoxic effects on HaCaT and melanoma cells

Cells were treated with indicated concentrations of D-NMAPPD, D-*e*-MAPP or L-*e*-MAPP. After 12 h of incubation, cytotoxicity was detected by a measurement of the re-

lease of LDH activity into the cell culture supernatants. No significant LDH-release was observed when cells were incubated with 25 or 50 μM of D-*e*-MAPP (Fig. 2), indicating that membrane integrity was not impaired in this case. Also 25 μM D-*e*-NMAPPD was without any cytotoxic effect, whereas 50 μM D-NMAPPD increased the rate of LDH-release up to 210% of control in all investigated cells (Fig. 2, inset). L-*e*-MAPP as the stereoisomer of D-*e*-MAPP was without any effect at both concentrations.

3.3. Elevation of endogenous ceramide levels upon treatment with D-NMAPPD

After radiolabeling with L-[^{3}H]serine of HaCaT keratinocytes and melanoma cells for 24 h, cells were treated with 25 μM D-*e*-MAPP, D-NMAPPD or ethanol vehicle for various periods of time. An eight-fold ceramide elevation was observed in HaCaT keratinocytes already after 3 h of treatment and a maximum of 11-fold after 6 h of treatment with

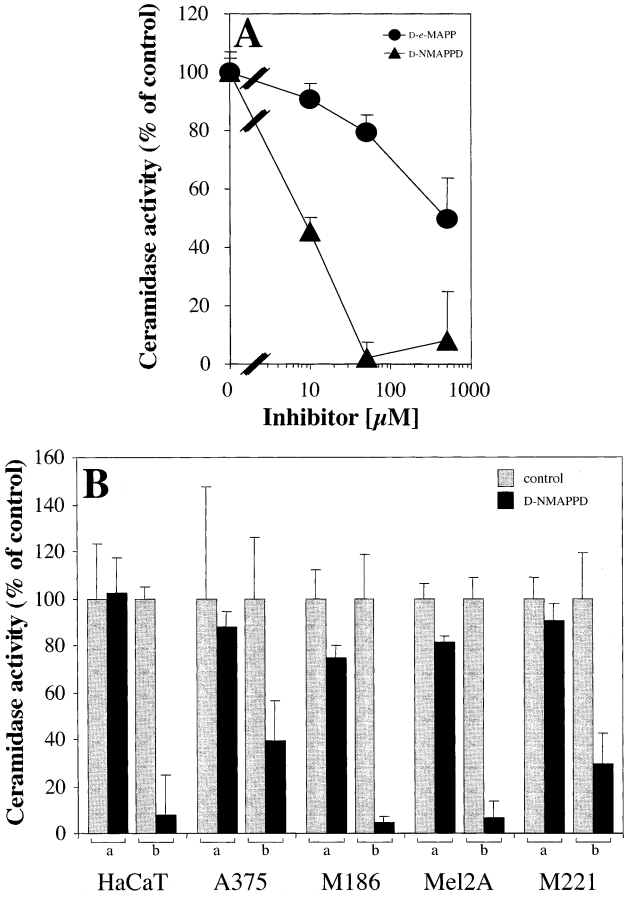

Fig. 1. Concentration-dependent effect of D-*e*-MAPP and D-NMAPPD on acid ceramidase activity in HaCaT keratinocytes (A) and the effect of D-NMAPPD on activity of acid and alkaline ceramidases in HaCaT keratinocytes and melanoma cells (B). In vitro activity of these enzymes was detected as described in Section 2. Using *N*-palmitoyl-[1-^{14}C]-D-sphingosine as substrate, solubilizates of cells were incubated for 60 min at the respective pH (acid ceramidase activity, pH 4.5; alkaline ceramidase activity, pH 9.0). (A) Represents acid ceramidase activity in the presence of D-*e*-MAPP and D-NMAPPD in HaCaT keratinocytes. (B) Represents activity of alkaline ceramidase (a) and acid ceramidase (b) in the presence of 500 μM D-NMAPPD in HaCaT keratinocytes and melanoma cell populations A375, M186, Mel2A and M221. Values are given as percent of control ± S.D. ($n = 4$).

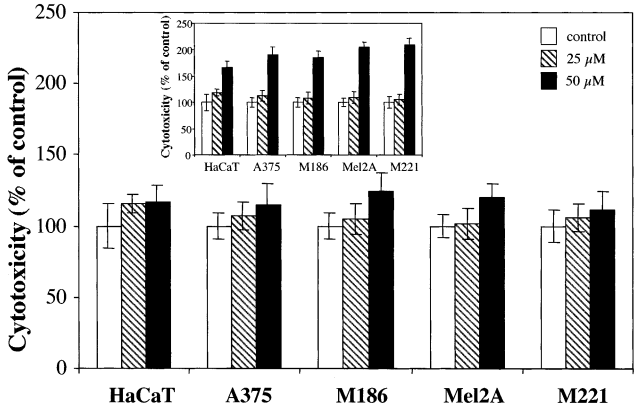

Fig. 2. Concentration-dependent cytotoxic effects of ceramidase inhibitors. Subconfluent HaCaT keratinocytes and A375, M186, Mel2A and M221 cell populations were treated with different concentrations of D-*e*-MAPP and D-NMAPPD (see inset). After 12 h of incubation, cytotoxicity was determined as described in Section 2. Values are given as percent of control ± S.D. ($n = 4$).

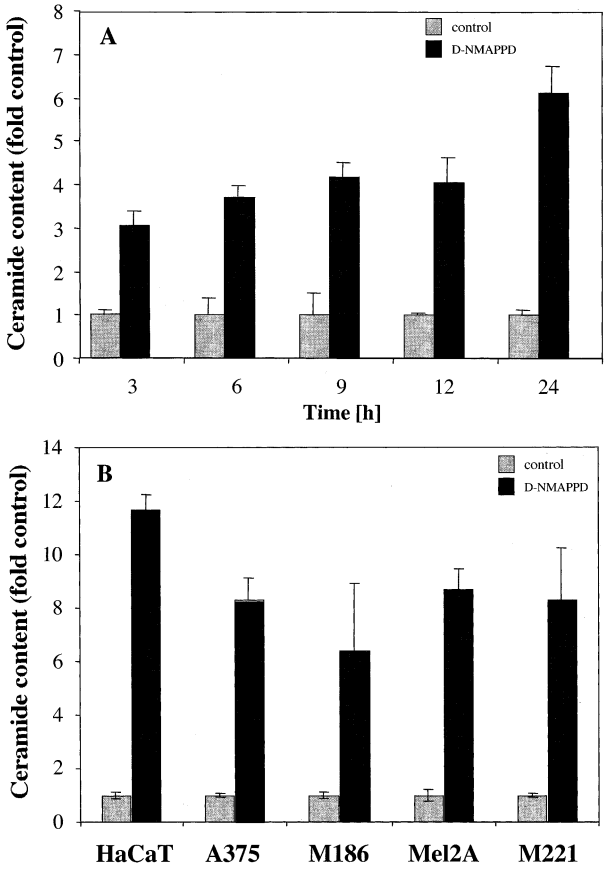

Fig. 3. Influence of D-NMAPPD on endogenous ceramide levels in HaCaT keratinocytes and melanoma cells. Subconfluent HaCaT keratinocytes and the melanoma cell populations A375, M186, Mel2A and M221 were radiolabeled with L-[^{3}H]serine for 24 h. Then, cells were treated with vehicle or with 25 μM D-*e*-MAPP and D-NMAPPD. Lipids were extracted for ceramide measurements at the indicated time points as described in Section 2. A: Time-dependent increase of endogenous ceramide in HaCaT keratinocytes. B: Ceramide increase in HaCaT keratinocytes and melanoma cell lines after treatment with D-NMAPPD or vehicle control for 24 h. Values are given as percent of control ± S.D. ($n = 3$).

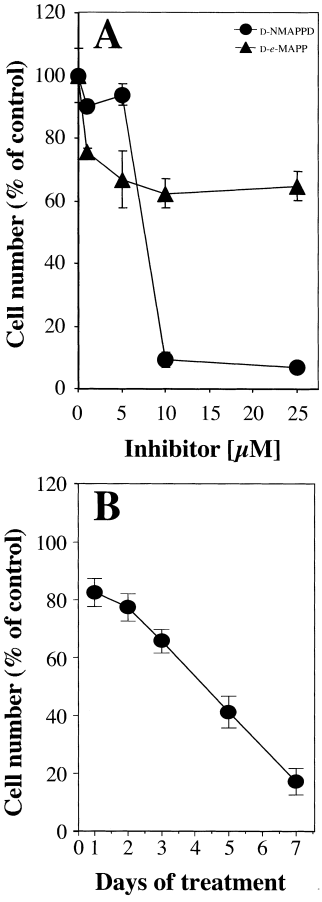

Fig. 4. Concentration-dependent antiproliferative effects of ceramidase inhibitors on HaCaT keratinocytes and melanoma cells. Cell growth was measured photometrically using DNA staining with crystal violet as described in Section 2. A: Subconfluent HaCaT keratinocytes were treated with non-cytotoxic concentrations of D-*e*-MAPP and D-NMAPPD for 48 h. B: Long time course proliferation experiment with A375 melanoma cells upon treatment with 25 μM D-NMAPPD. Values are given as percent of control ± S.D. ($n = 4$).

D-NMAPPD. A continuous ceramide increase was seen in HaCaT keratinocytes after treatment with D-*e*-MAPP, reaching a maximum of six-fold after 24 h (Fig. 3A). Moreover, treatment of four melanoma cell lines with 25 μM D-NMAPPD for 24 h resulted in a strong accumulation of ceramide (Fig. 3B) reaching more than nine-fold as compared to vehicle-treated cells.

3.4. Ceramidase inhibitors inhibit cell proliferation

After 48 h of incubation with the respective substances, the cell proliferation was measured. In a concentration-dependent manner in HaCaT keratinocytes, the most potent inhibitor of cell proliferation was D-NMAPPD with an IC_{50} of about 7 μM, whereas D-*e*-MAPP showed reduced antiproliferative activity. Cell growth was inhibited by only 40% at a concentration of > 10 μM D-*e*-MAPP (Fig. 4A). Two ceramide-sensitive melanoma cell populations (A375, M186) and two ceramide-resistant melanoma cells (Mel2A, M221) were treated for 48 h with 25 μM D-NMAPPD. D-NMAPPD reduced cell numbers in a concentration-dependent manner in ceramide-sensitive melanoma cell populations. In contrast, D-NMAPPD showed no antiproliferative effect on ceramide-resistant melanoma cells (data not shown). D-*e*-MAPP exhibited no antiproliferative effect on melanoma cell lines and 25 μM L-*e*-MAPP was also without antiproliferative effect on HaCaT keratinocytes and melanoma cells. In a long time course proliferation experiment, the number of A375 melanoma cells decreased continuously and after 7 days of treatment with 25 μM D-NMAPPD was reduced to 18% (Fig. 4B).

3.5. Ceramidase inhibitors induce apoptosis in HaCaT and melanoma cells

We tested the ability of D-*e*-MAPP, L-*e*-MAPP and D-NMAPPD to induce apoptosis in keratinocytes and melanoma cells. HaCaT keratinocytes, two ceramide-sensitive (A375, M186) and two ceramide-resistant (Mel2A, M221) melanoma cell populations were treated for 24 h with 25 μM D-*e*-MAPP, D-NMAPPD or L-*e*-MAPP. Apoptosis was determined morphologically by observation under light microscopy and measured photometrically using an enzyme immunoassay. D-NMAPPD was a very efficient proapoptotic stimulus and the rate of D-NMAPPD-treated HaCaT keratinocytes driven into apoptosis was 7.5-fold compared to control cells incubated with ethanol as a control vehicle. D-NMAPPD also induced apoptosis in the two ceramide-sensitive melanoma cell populations, A375 and M186. The rate of apoptotic A375 melanoma cells after treatment was 3.5-fold and that of apoptotic M186 melanoma cells was three-fold compared to vehicle-treated cells. In contrast, D-NMAPPD showed no apoptotic effect on ceramide-resistant melanoma cells. D-*e*-MAPP had no proapoptotic effects on all melanoma cell lines, whereas cell death rates of HaCaT keratinocytes after treatment with 25 μM D-*e*-MAPP was two-fold compared to control (data not shown). L-*e*-MAPP, the inactive enantiomer, was without apoptotic effect in all tested cell lines at a concentration of 25 μM.

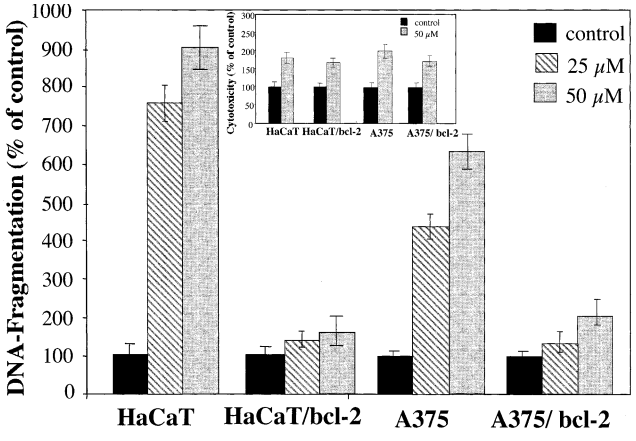

Fig. 5. The involvement of Bcl-2 in D-NMAPPD-induced apoptosis. Bcl-2 transfected A375 melanoma cells and HaCaT keratinocytes and respective vector transfected cells were treated with 25 and 50 μM D-NMAPPD for 24 h. The inset shows cytotoxicity measured in the same cell lines as above under the influence of 50 μM D-NMAPPD after 12 h. DNA fragmentation and cytotoxicity were measured as described in Section 2. DNA fragmentation or cytotoxicity of control cells was set as 100% and DNA fragmentation or cytotoxicity of treated cells was calculated as % of control. Values represent the mean of three experiments ± S.D.

3.6. Overexpression of Bcl-2 prevents D-NMAPPD-triggered apoptosis

Ceramide-induced cytochrome *c* release has been shown to be prevented by Bcl-2 overexpression. In order to further elucidate the role of ceramidase inhibitors in our experimental model, we used Bcl-2 overexpressing HaCaT keratinocytes and A375 melanoma cells published recently [14,15]. Bcl-2 overexpression completely abolished apoptosis induced by 25 μM D-NMAPPD, suggesting that the apoptosis induced by elevation of endogenous ceramide through inhibition of acid ceramidase activity is a mitochondrial activation-dependent process (Fig. 5). Bcl-2 overexpression also abolished apoptosis induced by 50 μM D-NMAPPD but not the cytotoxic effects of this concentration (Fig. 5, inset). The distinction between apoptotic and cytotoxic effects demonstrates that the apoptosis induced by D-NMAPPD is independent of its cytotoxicity in melanoma cells.

4. Discussion

We examined the cellular response to the ceramidase inhibitors, D-*e*-MAPP and its new analogue D-NMAPPD, in HaCaT keratinocytes and human melanoma cells.

In the first step, we investigated the influence of these compounds on the ceramidase activity and endogenous ceramide levels. Our experiments with D-*e*-MAPP and D-NMAPPD revealed inhibition of acid ceramidase in all investigated cell lines (D-NMAPPD served as a stronger inhibitor than D-*e*-MAPP), while the alkaline isoform was affected only slightly. These results are in contrast to previously published findings in HL-60 cells, where D-*e*-MAPP inhibited the acid ceramidase marginally ($IC_{50} > 500$ μM) but activity of alkaline ceramidase was completely abolished by an IC_{50} of 1–5 μM. By quantitative evaluation of ceramide content, we demonstrated an increase of the ceramide pool which was in accordance with the decrease of acid ceramidase activity. Furthermore, D-*e*-MAPP displayed a less antiproliferative and apopotic effect than D-NMAPPD in HaCaT keratinocytes. Moreover, it did not show any effect in melanoma cells, while D-NMAPPD significantly influenced both HaCaT and melanoma cells. Therefore, we suggest that D-NMAPPD is a more potent ceramidase inhibitor than D-*e*-MAPP strongly inhibiting the acid isoenzyme. A major difference between D-*e*-MAPP and D-NMAPPD is the presence of a hydroxyl group in a position corresponding to the 1-hydroxyl group of ceramide. This group may be responsible for substrate recognition of ceramidases and therefore it can lead to an increased binding of the inhibitor. Recently, we identified a group of human melanoma cell populations which are heterogenously susceptible to ceramide-mediated apoptosis paralleled by a low relative Bax to Bcl-2 protein ratio [15]. D-NMAPPD induced apoptosis in ceramide-sensitive melanoma cells, while the ceramide-resistant melanoma cells were insensitive to D-NMAPPD, thus confirming the effect of this inhibitor on the ceramide signaling pathway. Furthermore, Bcl-2 overexpression abolished apoptosis triggered by D-NMAPPD in HaCaT keratinocytes and A375 melanoma cells, pointing out the involvement of mitochondrial activation and cytochrome *c* release in D-NMAPPD-induced apoptosis.

The acid isoenzyme of ceramidase is predominantly localized in lysosomes and has been previously described mainly in connection with biodegradation of sphingolipid metabolites, most likely in the process of recycling membrane lipids through the lysosomal pathway. On the other hand, alkaline and neutral ceramidases have been described to be involved in sphingosine-mediated signal transduction pathways in eukaryotic cells [8]. Here, we demonstrate the capability of regulating endogenous ceramide levels through modulation of acid ceramidase activity. This elevation of intracellular ceramide has significant biological consequences in vitro and mimicks the effects obtained upon treatment with exogenous ceramides [5]. Therefore, we suggest that the action of exogenous cell-permeable ceramides elevates the cellular ceramide pool and can serve as an appropriate tool for studying ceramide biology.

The significance of lysosomal pool of ceramide in signaling processes has been a matter of controversial debate [25]. With regards to the intracellular accumulation of ceramide, Ségui et al. [26] have shown that elevated ceramide levels in acidic compartments neither affect cell growth nor the susceptibility of cells to apoptotic stimuli. This explains why fibroblasts having a genetic defect in acid ceramidase activity (Farber disease) and therefore accumulating ceramide in lysosomes do not display any abnormality in cell viability. On the other hand, Strelow et al. [27] clearly demonstrate the involvement of acidic ceramidase in apoptosis where mostly lysosomal ceramide is depleted. Overexpression of this enzyme protects from tumor necrosis factor α (TNFα)-induced cell death. Furthermore, other studies involving acidic sphingomyelinase (aSMase) have shown that the acidic compartment does indeed play an important role in signal transduction. TNFα signaling was shown to activate aSMase and therefore to involve lysosomes which could further be confirmed by using the lysosomotropic agent NH_4Cl and the vesicularization inhibitor brefeldin A [28,29]. Additionally, several recent papers show that aSMase can be secreted and can also be targeted to specific plasma membrane microdomains rich in sphingolipids, such as caveolae and rafts [30–32]. The targeting of aSMase requires different N-terminal proteolytic cleavages and glycosylation events [33]. It is conceivable that a similar mechanism might also exist for the acidic ceramidase.

Our findings provide an important insight into the physiologic significance of regulating ceramide metabolism and suggest ceramidase inhibitors as a new class of compounds for the treatment of hyperproliferative skin diseases.

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References

[1] Hannun, Y.A. (1994) J. Biol. Chem. 269, 3125–3128.
[2] Kolesnick, R. and Hannun, Y.A. (1999) Trends Biochem. Sci. 24, 224–225.
[3] Okazaki, T., Bielawska, A., Bell, R.M. and Hannun, Y.A. (1990) J. Biol. Chem. 265, 15823–15831.
[4] Geilen, C.C., Wieder, T. and Orfanos, C.E. (1997) Arch. Dermatol. Res. 289, 559–566.
[5] Bektas, M., Dullin, Y., Wieder, T., Kolter, T., Sandhoff, K., Brossmer, R., Ihrig, P., Orfanos, C.E. and Geilen, C.C. (1998) Exp. Dermatol. 7, 342–349.

[6] Hannun, Y.A. and Luberto, C. (2000) Trends Cell Biol. 10, 73–80.
[7] Abe, A., Inokuchi, J., Jimbo, M., Shimeno, H., Nagamatsu, A., Shayman, J.A., Shukla, G.S. and Radin, N.S. (1992) J. Biochem. (Tokyo) 111, 191–196.
[8] Bielawska, A., Greenberg, M.S., Perry, D., Jayadev, S., Shayman, J.A., McKay, C. and Hannun, Y.A. (1996) J. Biol. Chem. 271, 12646–12654.
[9] Hassler, D.F. and Bell, R.M. (1993) Adv. Lipid Res. 26, 49–57.
[10] Nikolova-Karakashian, M. and Merrill Jr., A.H. (2000) Methods Enzymol. 311, 194–201.
[11] Selzner, M., Bielawska, A., Morse, M.A., Rudiger, H.A., Sindram, D., Hannun, Y.A. and Clavien, P.A. (2001) Cancer Res. 61, 1233–1240.
[12] Raisova, M., Bektas, M., Wieder, T., Daniel, P., Eberle, J., Orfanos, C.E. and Geilen, C.C. (2000) FEBS Lett. 473, 27–32.
[13] Bielawska, A., Linardic, C.M. and Hannun, Y.A. (1992) J. Biol. Chem. 267, 18493–18497.
[14] Müller-Wieprecht, V., Riebeling, C., Stooss, A., Orfanos, C.E. and Geilen, C.C. (2000) Arch. Dermatol. Res. 292, 455–462.
[15] Raisova, M., Hossini, A.M., Eberle, J., Riebeling, C., Wieder, T., Sturm, I., Daniel, P.T., Orfanos, C.E. and Geilen, C.C. (2001) J. Invest. Dermatol. 117, 333–340.
[16] Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H. and Parks, W.P. (1973) J. Natl. Cancer Inst. 51, 1417–1423.
[17] Bruggen, J., Macher, E. and Sorg, C. (1981) Cancer Immunol. Immunother. 10, 121–127.
[18] Wieder, T., Orfanos, C.E. and Geilen, C.C. (1998) J. Biol. Chem. 273, 11025–11031.
[19] Geilen, C.C., Haase, A., Wieder, T., Arndt, D., Zeisig, R. and Reutter, W. (1994) J. Lipid Res. 35, 625–632.
[20] Gatt, S. and Yavin, E. (1969) Methods Enzymol. 14, 139–144.
[21] Sugita, M., Willians, M., Dulaney, J.T. and Moser, H.W. (1975) Biochim. Biophys. Acta 398, 125–131.
[22] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[23] Jones, M.J. and Murray, A.W. (1995) J. Biol. Chem. 270, 5007–5013.
[24] Geilen, C.C., Bektas, M., Wieder, T. and Orfanos, C.E. (1996) FEBS Lett. 378, 88–92.
[25] Kolesnick, R. and Hannun, Y.A. (1999) Trends Biochem. Sci. 24, 224–225.
[26] Ségui, B., Bezombes, C., Uro-Coste, E., Medin, J.A., Andrieu-Abadie, N., Augé, N., Brouchet, A., Laurent, G., Salvayre, R., Jaffrézou, J.-P. and Levade, T. (2000) FASEB J. 14, 36–47.
[27] Strelow, A., Bernardo, K., Adam-Klages, S., Linke, T., Sandhoff, K., Krönke, M. and Adam, D. (2000) J. Exp. Med. 192, 601–611.
[28] Wiegmann, K., Schutze, S., Machleidt, T., Witte, D. and Krönke, M. (1994) Cell 78, 1005–1015.
[29] Monney, L., Olivier, R., Otter, I., Jansen, B., Poirier, G.G. and Borner, C. (1998) Eur. J. Biochem. 251, 295–303.
[30] Marathe, S., Schissel, S.L., Yellin, M.J., Beatini, N., Mintzer, R., Williams, K.J. and Tabas, I. (1998) J. Biol. Chem. 273, 4081–4088.
[31] Kolesnick, N.R., Goni, F.M. and Alonso, A. (2000) J. Cell. Physiol. 184, 285–300.
[32] Dobrowsky, R.T. (2000) Cell. Signal. 12, 81–90.
[33] Schissel, S.L., Keesler, G.A., Schuchman, E.H., Williams, K.J. and Tabas, I. (1998) J. Biol. Chem. 273, 18250–18259.