

Modulation of amyloid precursor protein processing by synthetic ceramide analogues

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ABSTRACT

Previous studies suggest that membrane lipids may regulate proteolytic processing of the amyloid precursor protein (APP) to generate amyloid-beta peptide (Aβ). In the present study, we have assessed the capacity for a series of structurally related synthetic ceramide analogues to modulate APP processing in vitro. The compounds tested are established glucosylceramide synthase (GS) inhibitors based on the *D*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) structure. PDMP and related compounds PPMP and EtDO-P4 inhibited Aβ secretion from Chinese hamster ovary cells expressing human APP (CHO-APP) with approximate IC₅₀ values of 15, 5, and 1 μM, respectively. A trend for reduced secretion of the APP alpha-secretase product, sAPPα, was also observed in PDMP-treated cells but not in PPMP- or EtDO-P4-treated cells, whereas levels of the cellular beta-secretase product APP C-terminal fragment, CTFβ, were increased by both PDMP and PPMP but unaltered with EtDO-P4 treatment. Our data also revealed that EtDO-P4 inhibits endogenous Aβ production by human neurons. In conclusion, this study provides novel information regarding the regulation of APP processing by synthetic ceramide analogues and reveals that the most potent of these compounds is EtDO-P4.

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1. Introduction

1.1. Role of amyloid-β peptide (Aβ) in Alzheimer's disease (AD)

A prominent feature of AD is the presence of amyloid plaques in brain regions associated with memory and learning. Amyloid plaques contain Aβ peptides as a major constituent, and it is established that Aβ is derived from the amyloid precursor protein (APP) which undergoes two major pathways of enzymatic cleavage in the neuron [1]. The α-secretase pathway, which represents the major pathway for APP processing, does not generate Aβ as the metalloproteases (such as ADAM-10) responsible cleave in the middle of the Aβ sequence. In the second pathway, however, sequential cleavage of APP by β-secretase (BACE-1) and γ-secretase (a complex containing presenilins PS-1 or PS-2 as the catalytic subunit) generates Aβ peptide predominantly of 40 or 42 amino acids [2,3]. Once formed, Aβ peptides may assemble as soluble oligomeric species that lead to protofibrils (Fig. 1), which are neurotoxic at submicromolar concentrations [4,5]. It is clear that different macromolecular forms of Aβ regulate inflammation, oxidative stress, and lipid metabolism; all

processes that are implicated in AD neurodegeneration [6–8]. In addition to factors that regulate the net production of Aβ in the brain, the ratio of Aβ1–40 to Aβ1–42 species generated, their propensity to form macromolecular complexes, and their clearance from the central nervous system (CNS) are all potential therapeutic targets for AD [2,9]. Despite the recognised role for Aβ in AD neurodegeneration, the factors that modify Aβ production and deposition are not completely understood.

1.2. Glycosphingolipids (GSLs), Aβ, and AD

The brain is a rich source of GSLs that represent a large family of complex lipids derived from the sphingolipid biosynthetic pathway (Fig. 2). The initial rate-limiting enzyme for GSL synthesis is glucosylceramide synthase (GS), an enzyme that catalyses the conversion of ceramide to glucosylceramide (GlcCer). Through the action of glycosyl transferases, GlcCer may be further acted upon to form more complex GSLs that may contain sialic acid residues in which case the GSLs become negatively charged and are referred to as gangliosides (e.g., monosialylated gangliosides GM1, GM2, and GM3). Nonsialylated “neutral” GSLs such as lactosyl ceramide (LacCer) and ceramide trihexoside (CTH) are also present in the brain [10].

More than 30 years ago, it was reported that reductions in the levels of specific gangliosides were associated with AD; however, it

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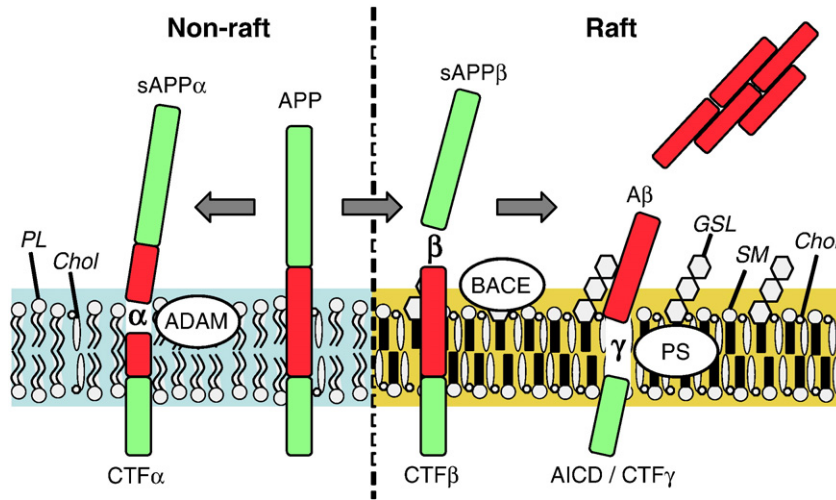


Fig. 1. Schematic representation of amyloid precursor protein (APP) processing. Amyloidogenic processing by β -secretase and γ -secretase generates A β peptides in the cholesterol- and GSL-enriched lipid raft microdomains within cell membranes. Nonamyloidogenic processing by α -secretase occurs predominantly in nonraft microdomains. Abbreviations are explained in the text.

was concluded that this was a “phenomenon accompanying extensive degradation of brain tissue rather than a factor in the aetiology of dementia” [11]. Similar studies performed almost a decade later also reported ganglioside reductions in AD brains and suggested this was due to “reduced density of nerve endings in the demented brains” [12]. These data suggest that the reduction of gangliosides observed in the AD brain may be a consequence of the disease rather than a cause.

Subsequent *in vitro* and *in vivo* studies indicated that ganglioside (particularly GM1) administration could potentiate the trophic effects of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [13–15]. Perhaps prematurely, these observations were set against the background data indicating “reduced” GSL levels in AD brains, and this led to the proposal that intracerebroventricular administration of GM1 could be used to treat AD [16,17]. Overall, this approach appeared to be unsuccessful as a treatment for AD and concerns were raised regarding immunological responses to GM1 administration [18–21].

Separate studies suggested that GM1 and other GSLs may in fact promote A β production and its assembly into neurotoxic complexes. It is known that GSLs are colocalised with A β in amyloid plaques and it has been proposed that GM1 may interact with A β to form a seed for amyloid plaque formation [22,23]. In addition, when GD3 synthase gene knockout mice (phenotype characterised by reductions in the levels of several brain gangliosides) were crossed with APP^{swe}+PSEN1DE9 amyloidogenic mice, both soluble A β and plaque load were reduced (85–95%), and this was associated with improved performance in cognitive tests [24]. Other recent studies have shown that GM1 also resolubilises mature A β fibrils to regenerate neurotoxic A β protofibrils from amyloid plaque [25]. Finally, *in vitro* studies indicate

that GSLs may stimulate both BACE and γ -secretase activity to promote A β generation [26,27].

Together, these findings suggest that therapeutic intervention to reduce GSL synthesis may be worth investigating as a novel strategy to reduce A β -associated neurodegeneration *in vivo*. Related to this, the synthetic ceramide analogue *D*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is an established GSL synthesis inhibitor that has been shown to inhibit A β secretion from SH-SY5Y neuroblastoma cells [28]. Although PDMP is not suitable for long-term animal studies due to its high hepatic metabolism (plasma $t_{1/2}$ ~ 1 h), PDMP derivatives (Fig. 3) including *D*-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP) and *D*-threo-ethylendioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (EtDO-P4) may provide viable alternatives [29,30]. EtDO-P4, in particular, has been successfully used in long-term studies in mice [31,32].

The aim of the present study was to investigate the impact that synthetic ceramide analogues PDMP, PPMP, and EtDO-P4 have on APP processing and A β production using CHO cells that stably express human APP695.

2. Materials and methods

2.1. Materials

Synthetic ceramide analogues *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), *D*-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (*D*-PPMP), and *L*-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (*L*-PPMP) were purchased from Matreya (Pleasant Gap, PA, USA). *D*/*L*-Threo-

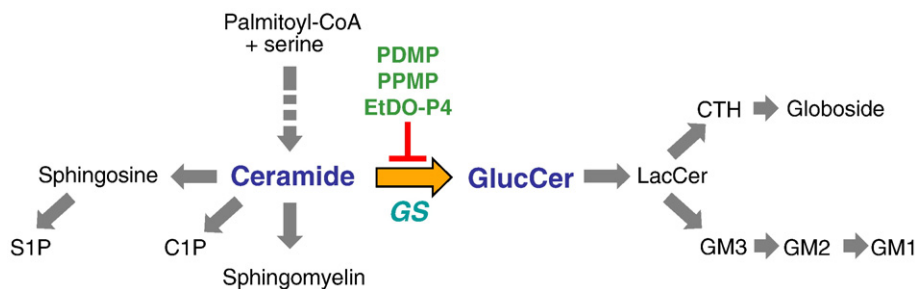


Fig. 2. Simplified scheme of sphingolipid biosynthesis. PDMP, PPMP and EtDO-P4 inhibit glucosylceramide synthase (GS) which catalyses the first step in glycosphingolipid synthesis. S1P, sphingosine-1-phosphate; C1P, ceramide-1-phosphate; Other abbreviations are explained in the text.

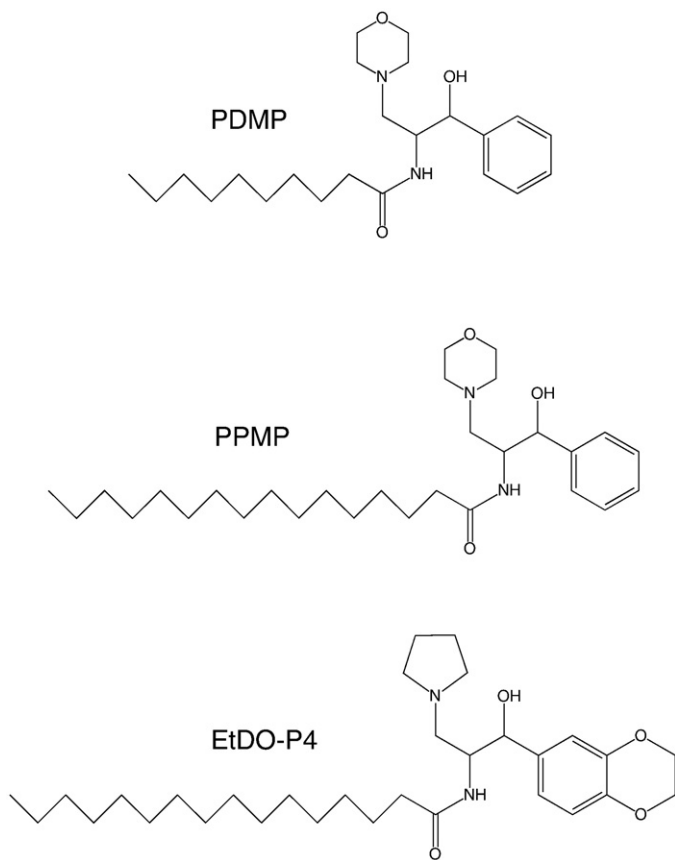


Fig. 3. Chemical structures of synthetic ceramide analogues used in this study.

ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (*D/L*-EtDO-P4) was synthesised as described previously [30]. The *D*-EtDO-P4 enantiomer was purified by preparative normal phase HPLC using a Lux 5 μm Cellulose-2 AXIA packed chiral 250×21.2 mm column (Phenomenex, Lane Cove NSW, Australia). An Agilent 1100 HPLC system was used with a mobile phase hexane:isopropanol: diethylamine (85:15:0.1, vol./vol./vol.), a flow rate of 10 ml/min and UV detection at 220 nm. *D*-EtDO-P4 eluted at 25 min and was collected from the column and dried under vacuum before use in experiments. Unless stated otherwise, all synthetic ceramide analogues used were in the *D*-threo configuration. The γ -secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) was purchased from Sigma (Castle Hill, NSW, Australia). Cell culture media and additives were obtained from Invitrogen (Melbourne, VIC, Australia) unless stated otherwise. Organic solvents were of analytical grade and were purchased from Ajax Finechem (Sydney, NSW, Australia). All other reagents were purchased through standard commercial suppliers.

2.2. Cell culture

The CHO cell line stably expressing the human 695-amino acid APP (CHO-APP) was generated and maintained as described previously [33]. This CHO cell model is an established method for analysis of APP metabolism [34–36]. CHO-APP cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The recombinant plasmid was maintained using puromycin (7.5 $\mu\text{g}/\text{ml}$). Cells were treated with synthetic ceramide analogues or DAPT as indicated for up to 48 h. These compounds were added to cells in complete growth medium containing 10% FCS. Human fetal brain tissues were obtained

from 14- to 18-week-old fetuses collected after therapeutic termination following informed consent (ethical approval from the University of New South Wales Human Research Ethics Committee, HREC03187). Neurons were isolated from the brain tissues and cultured as previously described [37].

2.3. Western blotting

CHO-APP cells were routinely cultured in 12-well plates, rinsed with cold phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors). Bicinchoninic acid protein assays were performed on lysates, and equal amounts of protein were separated on 12% SDS-PAGE gels and transferred onto 0.2- μm nitrocellulose membranes at 100 V for 30 min. Membranes were blocked at 22 °C for 2 h in PBS containing 5% nonfat dry milk and probed with the relevant antibodies at 4 °C for 16 h to analyse APP (WO2 monoclonal 1:400), APP plus APP β C-terminal fragment (CTF β , rabbit polyclonal 1:10,000; Sigma, Cat No. A8717) and β -actin (rabbit polyclonal 1:2000; Sigma, Cat No. A5060). The membranes were washed three times in PBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase-conjugated goat anti-rabbit (Dako; 1:2500) or rabbit anti-mouse (Dako; 1:2500) secondary antibody for 2 h. Signals were detected using enhanced chemiluminescence (ECL, Amersham Biosciences) and X-ray films. Membranes were stripped and reprobbed with β -actin antibody (rabbit polyclonal 1:5000; Sigma, Cat No. A5060) as a loading control. The signal intensity was quantified using NIH ImageJ software.

Western blotting of secreted A β peptides and the secreted products of APP cleavage by α -secretase (sAPP α) was carried out as previously described [35]. Briefly, A β in the culture medium was separated on 12% SDS-PAGE gels and transferred onto 0.2- μm nitrocellulose membranes at 65 V for 17 min. Membranes were boiled in PBS for 5 min, probed with anti-APP WO2 monoclonal antibody followed by rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody and ECL detection applied as described above. Where indicated, the optical density of bands detected in Western blots was measured using NIH ImageJ software and the data regarding expression of APP and its proteolytic products presented as a ratio relative to nontreated cells as previously described [35].

2.4. Cellular GSL analysis

GSL profiles of CHO-APP cells were analysed as described previously [38]. Briefly, cells grown to confluence in 6-well plates were treated with synthetic ceramide analogues as described above, rinsed three times with PBS and extracted in 2:1 (vol./vol.) chloroform/methanol. The GSL fractions were isolated by silicic acid chromatography, and the glycan moiety was released by ceramide glycanase addition [39]. The GSL glycans were then fluorescently labeled and analysed by normal phase HPLC as described previously [38]. Total peak area units for the glycan peaks were pooled to calculate the reduction of cellular GSL levels after treatment with ceramide analogues. Values were expressed as a percentage of total GSL levels present in vehicle-treated CHO-APP cells.

2.5. Cell viability assay

CHO-APP cells were seeded in 96-well plates at 80% confluence. Cells were treated with the indicated doses of ceramide analogues for 48 h. Culture media was removed, and 100 μl of medium (DMEM, 10% FCS) containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cat. No. M2128, Sigma) was added, and the cells were incubated for 1 h at 37 °C. The medium was discarded, and the cells were dissolved in DMSO (100 $\mu\text{l}/\text{well}$) and the absorbance of the cell lysates was measured at 550 nm. Higher absorbance values indicate increased cell viability. Cell number and morphology were also assessed

by direct counting of cells in five randomly selected fields in each of three triplicate wells. The cells remaining attached were captured in digital images recorded under phase contrast (20 \times), and the printed images were used for cell counting.

2.6. Statistical analysis

Unless stated otherwise, experiments were performed in triplicate and repeated at least three times. Data are presented as means with SE shown by error bars. Differences were considered significant where $P < 0.05$ as determined by the 2-tailed Student's t -test for unpaired data.

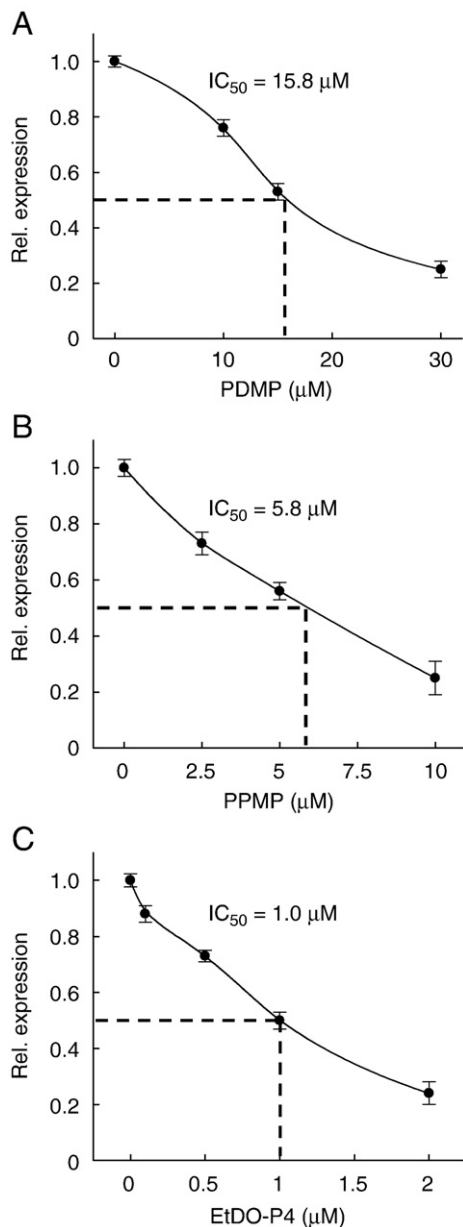


Fig. 4. Determination of IC_{50} values for inhibition of A β production by synthetic ceramide analogues. CHO-APP cells were treated with the concentrations of PDMP (A), PPMP (B), or EtDO-P4 (C) indicated for 48 h, and A β in the culture medium was measured by Western blotting. Optical density measurements of the Western blots were used to quantify A β . Data are derived from 4, 9, and 9 experiments for PDMP, PPMP, and EtDO-P4, respectively. Data are mean values with error bars indicating SE.

3. Results

3.1. Regulation of A β production by ceramide analogues

A previous study reported that PDMP could inhibit the secretion of A β from SH-SY5Y neuroblastoma cells [28]. Our initial experiments aimed to examine the impact that two derivatives of PDMP (PPMP and EtDO-P4), which have more potent GS inhibitory activity than PDMP [30], might have on cellular A β secretion. Our data indicate that both PPMP and EtDO-P4 inhibit A β secretion from CHO-APP cells. Using a 48-h treatment period, we found that the IC_{50} values for the inhibition of A β secretion by PDMP, PPMP, and EtDO-P4 were 15.8 μM , 5.8 μM , and 1.0 μM , respectively (Fig. 4). The reduction in extracellular A β levels induced by these compounds (when used at values that are close to their IC_{50} values) is illustrated by the Western blots shown in Fig. 5. Similar to the previous studies with SH-SY5Y cells [28], we noted a trend for PDMP to also inhibit the secretion of sAPP α from CHO-APP cells (Fig. 5), although overall, this did not reach statistical significance ($P = 0.051$). There were no obvious changes in sAPP α secretion when cells were treated with either PPMP or EtDO-P4 at concentrations that reduced A β secretion by $\sim 50\%$ (Fig. 5). Full-length cellular APP levels were significantly increased by $\sim 20\%$ with PPMP treatment, whereas a nonsignificant trend for increased cellular APP was observed with EtDO-P4 treatment (Fig. 5). These data suggest that there may be subtle differences in the mechanisms by which these structurally related ceramide analogues modulate APP processing and A β secretion. Intracellular A β was not detectable in CHO-APP cells either with or without ceramide analogue treatment (data not shown). The fact that A β is very efficiently secreted by the CHO-APP cells is consistent with previous studies [33,35,36].

3.2. Toxicity profiles of ceramide analogues

EtDO-P4 is a potent GS inhibitor that has already been used in long-term mouse studies [31,32]. However, because these ceramide analogues may also exhibit cytotoxicity, especially towards cancer cell lines [40,41], we also compared their affect on cellular MTT reduction as a marker of cytotoxic potential. Significant decreases in cellular MTT reduction were detected for all three ceramide analogues when tested at concentrations that inhibit A β secretion by $\sim 50\%$ (Fig. 6). The MTT reduction assay is a reliable method for the detection of cell death; however, it may also detect changes in mitochondrial electron transport, cellular metabolic activity, and cellular stress well in advance of cell death, particularly under conditions where cells are exposed to A β [42,43]. Therefore, cell survival was also checked by analysing phase contrast images of CHO-APP cells as described in the Materials and methods section. Gross morphological changes and overt cytotoxicity were only observed when the compounds were used at concentrations ~ 2 -fold greater than their IC_{50} values for A β secretion. As an example, phase-contrast images of CHO-APP cells treated with up to 5 μM EtDO-P4 for 48 h reveal significant loss of the cell monolayer and a clearly “rounded-up” cellular morphology (Fig. 6). Overall, however, these data indicate that the cytotoxicity of these ceramide analogues is reasonably low when used at concentrations that inhibit A β secretion by $\sim 50\%$.

3.3. Impact of ceramide analogues on APP CTF β generation

To better understand how PDMP, PPMP, and EtDO-P4 may be inhibiting cellular A β secretion, we also assessed the formation of the APP CTF β fragment using a specific antibody that recognises the APP C-terminal amino acids 676–695. Increased cellular CTF β levels in association with reduced A β secretion are associated with inhibition of γ -secretase activity. Our data indicate that PDMP and PPMP, when used at their $\sim \text{IC}_{50}$ concentrations, both caused a marked 6- to 12-fold increase in cellular CTF β (Fig. 7). In contrast, EtDO-P4 induced only a

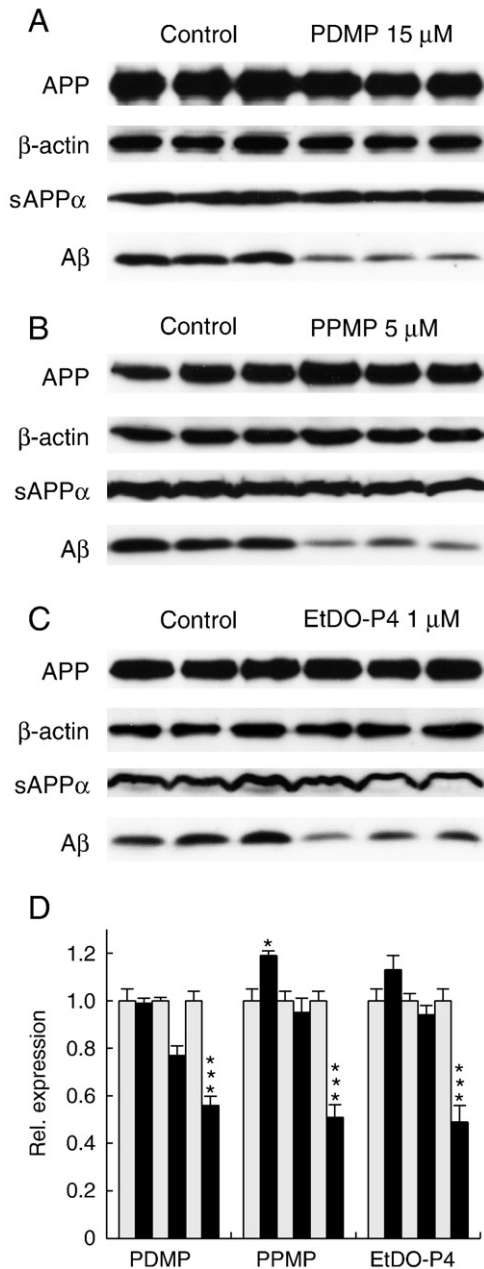


Fig. 5. Impact of synthetic ceramide analogues on sAPP α and A β production. CHO-APP cells were treated with 15 μ M PDMP (A), 5 μ M PPMP (B), or 1 μ M EtDO-P4 (C) for 48 h, and cellular APP and secreted sAPP α and A β were measured by Western blotting. β -Actin was used as a loading control. (D) Optical density measurements of the Western blots: control (grey bars) and treated (black bars). The bars in the histograms represent (from L to R) levels of APP, sAPP α and A β for each analogue tested. Data are mean values with error bars indicating SE, * P <0.05, *** P <0.001.

moderate 20% increase in CTF β levels (Fig. 7). The γ -secretase inhibitor DAPT was used as a positive control in these experiments and was found to increase CTF β levels by 50-fold and reduce A β secretion to undetectable levels (Fig. 7). We also assessed the activity of the L enantiomer of PPMP, which does not inhibit GS activity. L-PPMP had no significant impact on either cellular CTF β levels or A β secretion (Fig. 7). These data indicate that, although the inhibition of A β secretion that is induced by PDMP and PPMP is associated with increased CTF β accumulation (and thus presumably γ -secretase inhibition), EtDO-P4 may have a different anti-amyloidogenic mechanism of action. Furthermore, since L-PPMP did not have an impact on either CTF β or A β levels, this suggested that direct

inhibition of GS activity might be required for the anti-amyloidogenic activity of these ceramide analogues. One possible caveat with the data related to L-PPMP is that a previous study has indicated that, when used at low micromolar concentrations, it may stimulate sphingolipid synthesis (assessed after 48 h of treatment of Colo-205 cells) and thereby introduce a noncontrolled confounding variable in the present study [44].

3.4. Impact of EtDO-P4 on secretion of endogenous A β from primary human neurons

The data above indicate that EtDO-P4 is a potent inhibitor of cellular A β secretion and that the mechanism for this ceramide analogue is associated with only a moderate CTF β accumulation. To assess whether this action of EtDO-P4 may also be relevant to endogenously synthesised A β , we also treated primary human neurons with EtDO-P4. Similar to the results derived from the CHO-APP cell experiments, EtDO-P4 also inhibited A β production by human neurons (Fig. 8). In this case, the IC₅₀ for A β secretion was found to be \sim 3 μ M, and under these conditions, total cellular GSL levels were reduced by \sim 50% (Fig. 8).

4. Discussion

Increasing evidence suggests that there may be a link between dysregulation of cerebral lipid homeostasis and AD. For example, there are data indicating that synaptic terminals and isolated lipid raft fractions from AD brains are enriched in cholesterol and specific GSLs, respectively [45,46]. While causation has not been proved, it has been speculated that such changes in membrane lipid composition could contribute to the acceleration of A β deposition in AD [45–47]. If increased neuronal GSL and cholesterol levels do increase A β formation *in vivo*, then it would be predicted that, in genetic diseases resulting in the accumulation of these lipids in the brain, an increase in APP processing to produce A β would be observed. While this idea has not been extensively examined, studies in human Niemann-Pick Type C (NPC) brains and NPC mice (in which cholesterol and GSLs accumulate due to defects in endocytic trafficking) do indicate that although amyloid plaques are not a feature of NPC, A β production is significantly increased and it is thought that this may contribute to NPC neurodegeneration [48–50].

There are potentially several mechanisms to explain how GSLs may affect APP processing, amyloid deposition, and A β neurotoxicity. It is clear that the amyloidogenic processing of APP by β - and γ -secretases occurs within cell membranes in lipid raft microdomains that are enriched with GSLs and cholesterol (Fig. 1) [47,51–55]. Importantly, even subtle increases in the amount of raft lipids (including GSLs and cholesterol) in the cell membrane enhance APP processing to generate A β , and there may be multiple mechanistic explanations for this. These include an increase in the proportion of APP and β -secretase (BACE) localised to lipid rafts and direct modulation of BACE proteolytic activity [26,56]. There are also data derived from purified γ -secretase complex reconstituted in phosphatidylcholine (PC) liposomes that indicate strong induction of γ -secretase activity upon inclusion of porcine brain gangliosides to account for from 5% to 25% of the total liposome lipid (whereas increasing the ganglioside content to 50% or above inhibited γ -secretase activity) [27].

In addition, there may be direct interaction of cholesterol and GSLs in the membrane that together have an impact on APP processing. Related to the present experiments, our previous research showed that enrichment of cells with GSL promoted membrane cholesterol accumulation and that the PDMP potently stimulated cellular cholesterol efflux via an ABCA1 transporter-dependent pathway [38]. We also showed that PDMP treatment (10 μ M for 72 h) not only reduced cellular GSL expression but also led to a depletion of cholesterol from Triton X-100-insoluble membrane microdomains [38]. A subsequent study

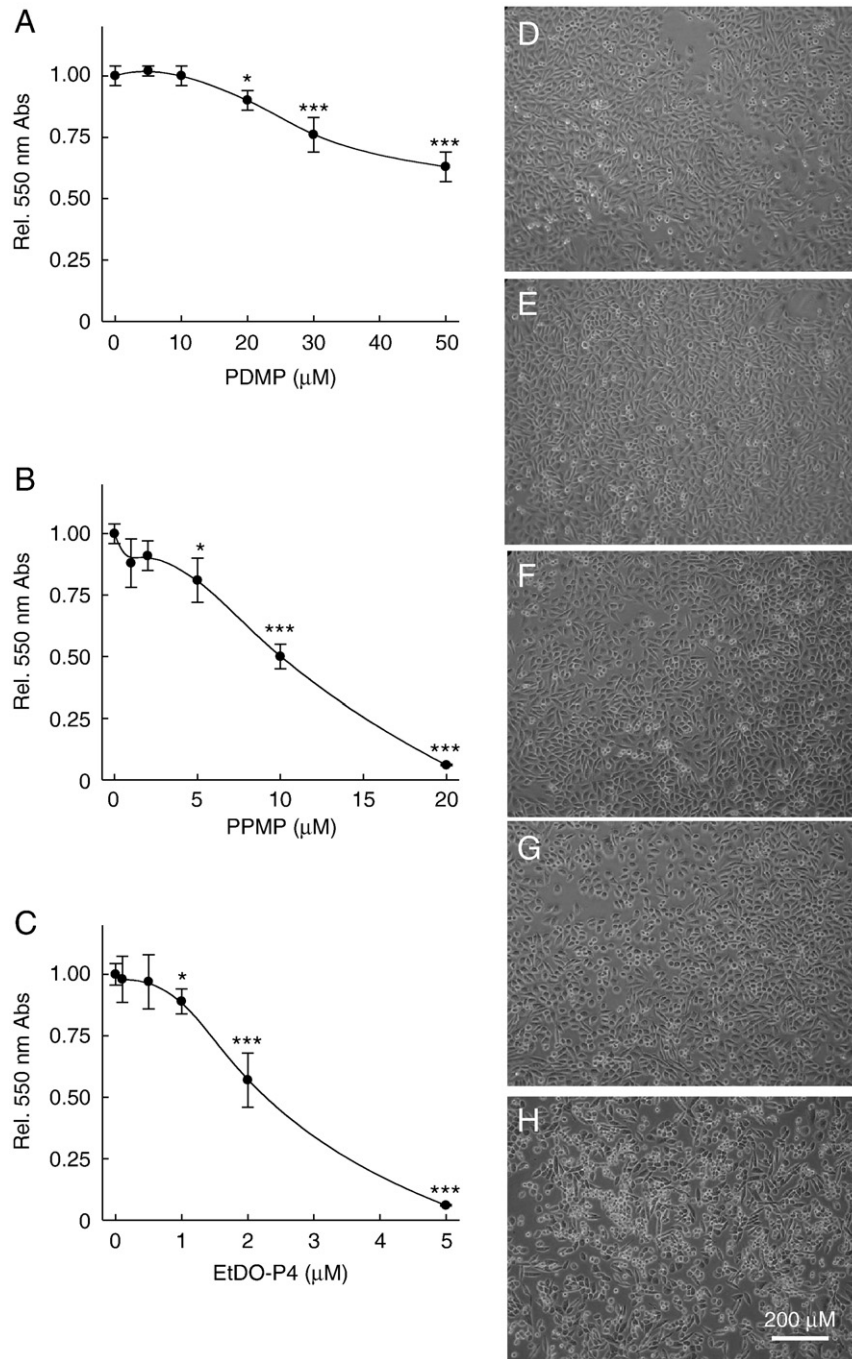


Fig. 6. Cytotoxicity assay of synthetic ceramide analogues. CHO-APP cells were treated with PDMP (A), PPMP (B), and EtDO-P4 (C) for 48 h, and cytotoxicity was measured using the MTT assay as described in Materials and methods. Data are mean values ($n=3$) with error bars indicating SE, * $P<0.05$, *** $P<0.001$. Phase-contrast microscopy of CHO-APP cells treated with EtDO-P4 at 0 μM (D), 0.1 μM (E), 0.5 μM (F), 1 μM (G), or 5 μM (H) for 48 h.

showed that 25 μM PDMP inhibited A β secretion from SH-SY5Y neuroblastoma cells by ~50% over 48 h [28]. These studies led us to examine PDMP-related ceramide analogues that may be used to test for possible anti-amyloidogenic activity in future studies of amyloidogenic transgenic mice.

The aim of the present study was therefore to examine the potential for synthetic ceramide analogues to inhibit A β secretion *in vitro*. We showed that PDMP and related compounds PPMP and EtDO-P4 inhibited A β secretion from CHO-APP cells with IC₅₀ values of 15.8 μM , 5.8 μM , and 1.0 μM , respectively. For reasons that remain to be defined, there were no universal effects of the different compounds on the levels of full-length APP or APP proteolytic products sAPP α and cellular CTF β . Based on the IC₅₀ values and the inactivity of L-PPMP, we

speculate that the mechanism by which these ceramide analogues inhibited A β may be related to their inhibition of GS activity; however, further experiments are clearly required to identify the precise pathways involved. It has been suggested that changes in cellular ceramide and diglyceride levels may correlate better than GS inhibitory activity with the cytostatic effects of ceramide analogues [40]. Whether differential changes in ceramide and diglyceride homeostasis also contribute to the different effects we have observed using synthetic ceramide analogues and their capacity to modulate different aspects of APP processing remains to be determined.

Regardless of the remaining details regarding the anti-amyloidogenic mechanism of action, the fact that EtDO-P4 inhibits A β secretion from both CHO-APP cells and human neurons at low micromolar

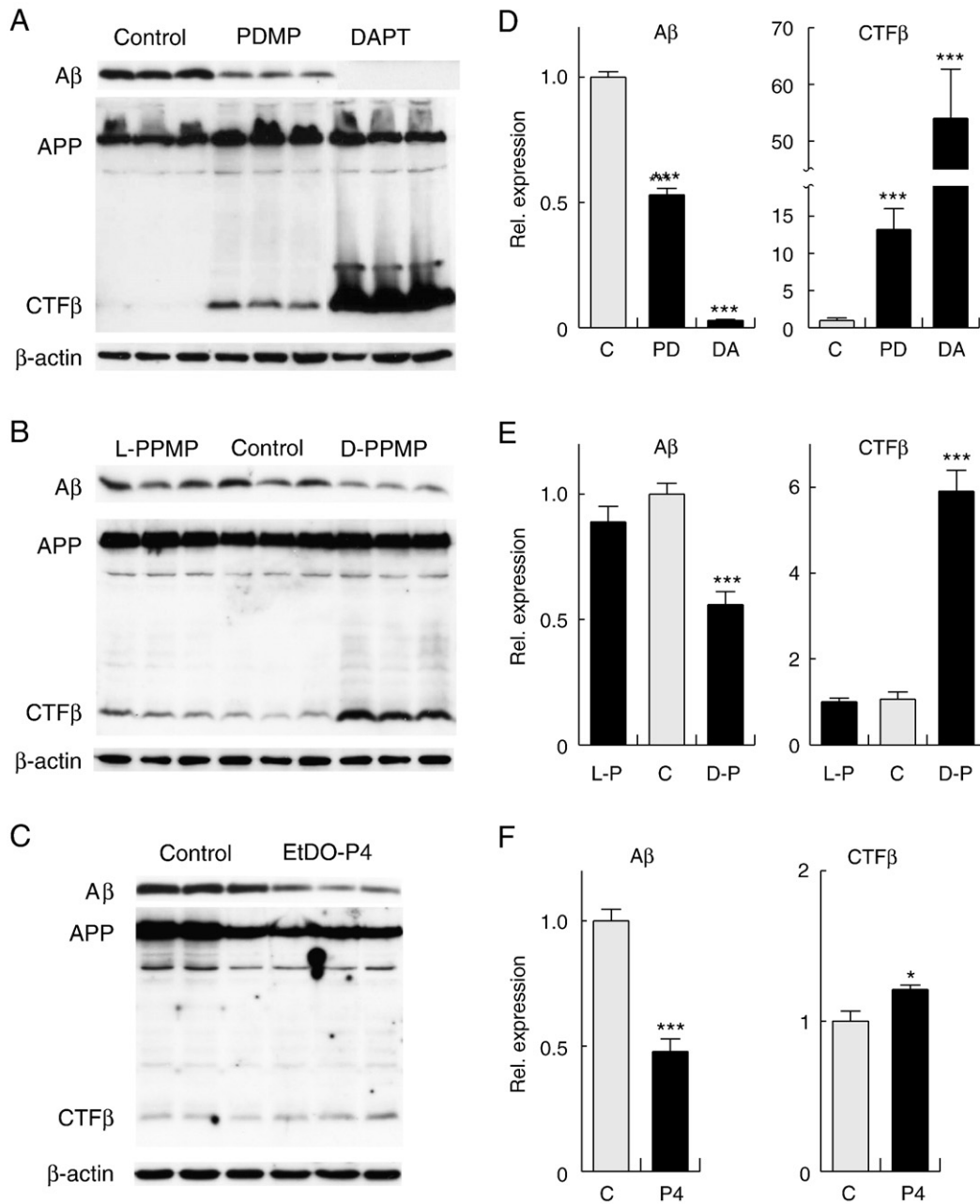


Fig. 7. Effect of synthetic ceramide analogues on the intracellular processing of APP. CHO-APP cells were treated with 15 μ M PDMP or DAPT (positive control, 1 μ M) (A), 5 μ M L-PPMP or 5 μ M D-PPMP (B) or 1 μ M EtDO-P4 (C) for 48 h, and cellular APP and CTF β and secreted A β were measured by Western blotting. β -Actin was used as a loading control. (D–F) Optical density measurements of the Western blots, respectively: control (grey bars) and treated (black bars). Data are mean values with error bars indicating SE, * P <0.05, *** P <0.001. C, control; PD, PDMP; DA, DAPT; L-P, L-PPMP; D-P, D-PPMP; P4, EtDO-P4.

concentrations and is applicable to *in vivo* mouse studies indicates that further investigation of this compound in an experimental animal model of AD is worth pursuing. Because of the rapid hepatic metabolism of PDMP (plasma $t_{1/2}$ ~ 1 h), this compound is not suitable for long-term animal studies. However, EtDO-P4 is ideal for *in vivo* studies with a $t_{1/2}$ in mice of ~7 h and our own data indicating i.p. injection of apoE $^{-/-}$ mice with EtDO-P4 (10 mg/kg) 3 times per week for 4 months is well tolerated and results in 49% inhibition in plasma GSL levels [32].

EtDO-P4 has a reported IC $_{50}$ for GS (Fig. 2) of 100 nM and, at levels 100-fold higher than its IC $_{50}$ for GS, has no impact on ceramide, sphingomyelin, phospholipid, or cholesterol synthesis [30,31]. EtDO-P4 is a hydrophobic molecule that appears to cross the blood–brain barrier (BBB) and has been shown to reduce GlcCer levels in the brains of Fabry disease (a GSL storage disease) mice by 16% when administered at 10 mg/kg as a complex with phospholipid vesicles (PLV) [31]. The

transport of EtDO-P4 across the blood–brain barrier is consistent with several earlier short term-(few days to 2 weeks) studies that have used the less potent PDMP analogues to modulate cerebral GSL metabolism in rats, gerbils, and mice [29,57–59]. In these “acute” studies, doses of 20 to 40 mg/kg administered i.p. twice per day (using detergent Tween 80 as vehicle) were used. One of these latter studies [29] revealed that a peak brain concentration of 50 μ M for D-PPMP was achieved 30 min after a single i.p. injection of 80 mg/kg, after which the brain concentration dropped to 6 μ M at 3 h and plateaued resulting in 4 μ M PDMP detected in the brain at the 8 h (final) time point. Considering the development of human therapeutics, it is potentially relevant that a GS inhibitor (Genz-112638) is currently in phase II clinical trials (<http://clinicaltrials.gov/show/NCT00358150>) to treat type 1 Gaucher disease, and it may be the case that this compound can also modulate neuronal A β secretion, although we are not aware of any study in which this has been assessed.

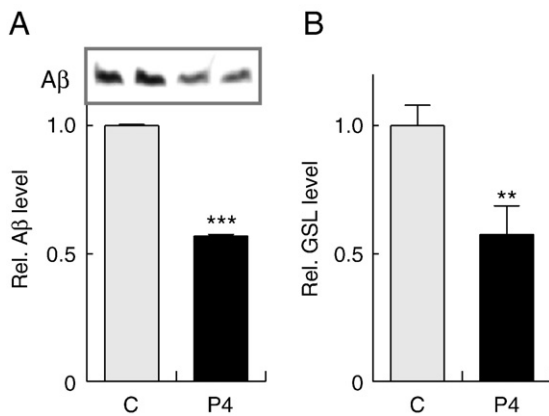


Fig. 8. Effect of EtDO-P4 on A β production and glycosphingolipid levels in primary human neurons. Human neurons were treated with 3 μ M EtDO-P4 for 48 h; (A) secreted A β was measured by Western blotting; (B) cellular glycosphingolipid (GSL) was measured by HPLC: control (grey bars) and EtDO-P4 (black bars). Data are mean values ($n=3$) with error bars indicating SE, ** $p<0.01$, *** $p<0.001$.

5. Conclusion

In conclusion, our current study provides novel information regarding the regulation of APP processing by synthetic ceramide analogues and reveals that the most potent of these compounds, EtDO-P4, may regulate APP processing through inhibition of GS. Future studies of the potential impact that EtDO-P4 or related compounds have on A β homeostasis in amyloidogenic mouse models appear to be worth pursuing.

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