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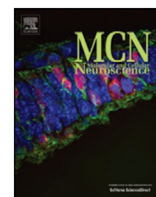

Anti-amyloidogenic effects of glycosphingolipid synthesis inhibitors occur independently of ganglioside alterations

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ABSTRACT

Evidence has suggested that ganglioside abnormalities may be linked to the proteolytic processing of amyloid precursor protein (APP) in Alzheimer's disease (AD) and that pharmacological inhibition of ganglioside synthesis may reduce amyloid β-peptide (Aβ) production. In this study, we assessed the usefulness of two well-established glycosphingolipid (GSL) synthesis inhibitors, the synthetic ceramide analog D-PDMP (1-phenyl 2-decanoylamino-3-morpholino-1-propanol) and the iminosugar N-butyldeoxynojirimycin (NB-DNJ or miglustat), as anti-amyloidogenic drugs in a human cellular model of AD. We found that both GSL inhibitors were able to markedly inhibit Aβ production, although affecting differently the APP cleavage. Surprisingly, the L-enantiomer of PDMP, which promotes ganglioside accumulation, acted similarly to D-PDMP to inhibit Aβ production. Concurrently, both D- and L-PDMP strongly and equally reduced the levels of long-chain ceramides. Altogether, our data suggested that the anti-amyloidogenic effects of PDMP agents are independent of the altered cellular ganglioside composition, but may result, at least in part, from their ability to reduce ceramide levels.
Moreover, our current study established for the first time that NB-DNJ, a drug already used as a therapeutic for Gaucher disease (a lysosomal storage disorder), was also able to reduce Aβ production in our cellular model. Therefore, our study provides novel information regarding the possibilities to target amyloidogenic processing of APP through modulation of sphingolipid metabolism and emphasizes the potential of the iminosugar NB-DNJ as a disease modifying therapy for AD.

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1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder leading to a progressive deterioration of cognitive functions with loss of memory. AD is characterized neuropathologically by intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein, senile plaques of aggregated amyloid-β peptide (Aβ) and neuronal death. The pathogenic mechanisms contributing to neuronal loss and dysfunction in AD are not clear. It appears likely that a pivotal role is played by the amyloid precursor protein (APP), and in particular by its proteolytic fragment - β-amyloid (Aβ) - which accumulates in brain and causes lesions believed to be involved in the pathogenesis of the disease. Aβ is generated from APP by the sequential action of two proteolytic enzymes, i.e., β-secretase producing a C-terminal fragment (βCTF) and γ-secretase to release Aβ (Lichtenthaler et al., 2011). However, under physiological conditions, APP is predominantly cleaved by α-secretase, resulting in the formation of soluble APP (sAPPα) and C-terminal fragment (αCTF), respectively (Lammich et al., 1999).

There is mounting evidence linking Aβ generation with lipid homeostasis in AD, suggesting that lipid metabolism is involved in the control of APP processing and vice versa (Grimm et al., 2012; Haughey et al., 2010; Piccinini et al., 2010). Among the various membrane lipid species, gangliosides, a family of complex glycosphingolipids (GSLs), have been receiving increasing attention because they were shown to be involved in the pathogenesis of AD (Ariga et al., 2008). Gangliosides are glycosylated sphingolipids containing sialic acids derived from an initial binding of glucose to ceramide generating glucosylceramide (GlcCer) by the glucosylceramide synthase (GCS). By addition of galactose, lactosylceramide (LacCer) is formed, and further addition of sialic acids and N-acetylgalactosamine leads to gangliosides (Yu and Saito, 1989). Gangliosides are highly enriched in neuronal and glial membranes, and involved in the regulation of early development, differentiation and proliferation of these cells (Lopez and Schnaar, 2009; Yu et al., 2009).

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Several studies reported significant alterations of ganglioside composition in the brains of AD patients and in transgenic mouse models of AD (Barrier et al., 2007; Gottfries et al., 1996; Kracun et al., 1992; Molander-Melin et al., 2005; Svennerholm, 1994). This is characterized as an increase in simple gangliosides (GM2 and GM3) whereas complex gangliosides (GT1b, GD1b, GD1a and GM1) tend to decrease. Additionally, specific gangliosides may promote Aβ production and/or its assembly into neurotoxic complexes (Ariga et al., 2001; Matsuzaki, 2011; Yanagisawa, 2011). Conversely, Aβ aggregation as well as Aβ induced-cell death are reduced in APPswe/PSEN1ΔE9 mice lacking GD3 synthase, a phenotype characterized by a reduction in the levels of several brain gangliosides (Ariga et al., 2013; Bernardo et al., 2009). Altogether, these data strongly suggest that altered ganglioside composition, leading to anomalous lipid raft/membrane organization, affects APP amyloidogenic versus non-amyloidogenic processing. Accordingly, targeting sphingolipid metabolism by molecules able to down-regulate ganglioside biosynthesis may be relevant for novel therapeutic approaches in AD.

However, until now, very few studies were interested in determining the usefulness of GSL synthesis inhibitors as anti-amyloidogenic agents. In one study, the well-known inhibitor D-1-phenyl 2-decanoylamino-3-morpholino-1-propanol (D-PDMP), a synthetic ceramide analog, was shown to strongly reduce Aβ secretion from SH-SY5Y neuroblastoma cells (Tamboli et al., 2005). More recently, Li et al. (2010, 2012) confirmed these results, demonstrating that other related ceramide analog GSL inhibitors, based on the PDMP structure, also reduced Aβ secretion from CHO cells stably expressing human APP695 (CHO-APP) and from primary human neurons. In contrast, a very recently published study reported that D-PDMP up-regulated Aβ production from HEK293 cells stably expressing the Swedish mutant of APP and Neuro2a cells, respectively, while a non-ceramide type GSL inhibitor, the iminosugar N-butyldeoxynojirimycin (NB-DNJ), did not (Takasugi et al., 2015).

In view of the discrepancy in the few data available, the present study was undertaken to examine whether the well-established GSL inhibitor D-PDMP, but also the iminosugar NB-DNJ, may impact on the APP processing and Aβ secretion from another cellular AD model, namely H4 neuroglioma cells expressing the double Swedish mutation (K595N/M596L) of human APP. This cell line is characterized by a 15-fold increased Aβ production, reproducing a "degenerative state" that mimics the AD pathological condition (Colombo et al., 2009; Shin et al., 2010).

2. Material and methods

2.1. Reagents and antibodies

Opti-MEM, penicillin, streptomycin, Blasticidin S, Fetal Bovine Serum (FBS), were obtained from Invitrogen (Gibco-Invitrogen, Cergy Pontoise, France). Hygromycin B was from InvivoGen (Cayla-Invivogen, Toulouse, France). All reagent-grade chemicals for buffers were purchased from Sigma (St. Quentin Fallavier, France) and all organic solvents (analytical grade) were from VWR International (Strasbourg, France). Antibodies used for western blot analysis were obtained from the following sources: Anti β-actin and anti C-terminal residues (amino acids 676–695) of APP were from Sigma (St Quentin Fallavier, France). Aβ was immunodetected using WO-2 clone (Millipore, St Quentin Yvelines, France). Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse) were from Cell Signaling and Amersham Biosciences (Orsay, France), respectively. D- and L-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP and L-PDMP) and highly purified ganglioside GM3 were purchased from Matreya (Biovalley, Marne La Vallée, France). N-butyldeoxynojirimycin (NB-DNJ) was from EnzoLife Sciences (distributed by Covalabs, Villeurbanne, France).

2.2. Cell culture and treatments

We used the human neuroglioma H4 cell line stably transfected with the pAG3 vector containing human APP695 with the double Swedish mutation K595N/M596L that was kindly provided by Dr A. Colombo and Dr. T. Borsello (Institute for Pharmacological Research "Mario Negri", Milan, Italy). This H4APPsw cell model provides a valid system for testing compounds that interfere with APP processing (Crestini et al., 2011; Imbimbo et al., 2007; Lanz et al., 2006) since these cells have the advantages that they accumulate very high levels of Aβ (Colombo et al., 2009; Czvitkovich et al., 2011), are of human origin, and are CNS derived thus containing higher concentrations of glycosphingolipids than any other tissue (Ledeen and Yu, 1982). Moreover, the expression of APPsw in these cells was shown to modulate global gene expression towards AD pathogenesis (Shin et al., 2010). H4APPsw cells were grown in Opti-MEM culture medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2, as previously described (Colombo et al., 2009; Noel et al., 2015). Hygromycin B (200 μg/mL) and Blasticidin S (2.5 μg/mL) were added to the culture medium to select cells expressing the transgene. For experiments, cells were seeded on 6-well plates (~2.5×10^4 cells/well), and treated either with 20 μM D-PDMP, 20 μM L-PDMP or 50 μM NB-DNJ for times specified in results. D- and L-PDMP were dissolved in ethanol at a concentration of 10 mM and NB-DNJ was dissolved at a concentration of 20 mM in sterile water. Control cells were incubated with the carrier alone.

In some experiments, H4APPsw cells were treated for 12 h with 1, 10 or 50 μM exogenous GM3 added to the culture medium in the form of complexes with BSA at a ratio of 1:1 (mol/mol) in Opti-MEM. For this, ganglioside GM3 was solved in methanol, dried under nitrogen flow and dissolved at the moment of use in serum-free medium containing BSA fatty acid free (Sigma, France). At the end of the treatment, the culture medium was collected, and the cells were washed three times with ice-cold PBS to remove GM3 loosely bound to the cell surface.

2.3. Cell lysis

Total protein extracts were obtained by scraping cells in ice-cold lysis buffer (Tris–HCl 50 mM, NaCl 50 mM, pH 6.8, NaF 500 mM, Triton X-100 1%, PMSF 100 mM, protease and phosphatase inhibitors 10 μg/mL). The lysates were sonicated and then centrifuged at 15,000 ×g for 15 min at 4 °C. The resulting supernatants were collected for BioRad protein assay and western blot analysis.

2.4. Media protein precipitation

After treatment, media were mixed with 100 mM PMSF and protease inhibitor cocktail at 10 μg/mL final concentration. Four hundred μL of medium were incubated overnight at 4 °C with 100 μL of 50% trichloroacetic acid (TCA). After centrifugation at 21,900 ×g for 30 min at 4 °C, the pellets were washed twice with 500 μL acetone and reconstituted in 50 μL urea 4 M.

2.5. Cell viability assay

Cell viability was determined exactly as previously described (Noel et al., 2011, 2015) by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using a commercial kit (MTS CellTiter 96 Aqueous kit, Promega, Madison, WI). The results were expressed as a percentage of the untreated controls. All MTS assays were performed in triplicate.

2.6. Western blot analysis

Cell lysate proteins (30 μg) or media proteins (47 μL) were separated on a 4–12% NuPAGE Bis-Tris gel and blotted onto a nitrocellulose

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