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Low-dose γ-secretase inhibition increases secretion of Aβ peptides and intracellular oligomeric Aβ

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ABSTRACT

γ-Secretase inhibitors have been considered promising drug candidates against Alzheimer's disease (AD) due to their ability to reduce amyloid-β (Aβ) production. However, clinical trials have been halted due to lack of clinical efficacy and/or side effects. Recent *in vitro* studies suggest that low doses of γ-secretase inhibitors may instead increase Aβ production. Using a stem cell-derived human model of cortical neurons and low doses of the γ-secretase inhibitor DAPT, the effects on a variety of Aβ peptides were studied using mass spectrometry. One major focus was to develop a novel method for specific detection of oligomeric Aβ (oAβ), and this was used to study the effects of low-dose γ-secretase inhibitor treatment on intracellular oAβ accumulation. Low-dose treatment (2 and 20 nM) with DAPT increased the secretion of several Aβ peptides, especially Aβx-42. Furthermore, using the novel method for oAβ detection, we found that 2 nM DAPT treatment of cortical neurons resulted in increased oAβ accumulation. Thus, low dose-treatment with DAPT causes both increased production of long, aggregation-prone Aβ peptides and accumulation of intracellular Aβ oligomers, both believed to contribute to AD pathology.

1. Introduction

Plaques, composed of the aggregation-prone peptide amyloid-β (Aβ), are key pathological hallmarks of Alzheimer's disease (AD). Ever since the amyloid cascade hypothesis was proposed in 1991 (Hardy and Allsop, 1991), much research on AD pathogenesis has focused on the mechanisms behind Aβ generation and degradation. In line with this, several drug candidates have been developed, aiming at reducing Aβ production by inhibiting or modulating the key enzymes involved in the production of Aβ. Aβ is generated by sequential cleavage of the amyloid precursor protein (APP) by β-secretase followed by γ-secretase (Blennow et al., 2006), and therefore both β- and γ-secretase inhibitors have been proposed as possible treatments for AD.

The first γ-secretase inhibitor (GSI) showing *in vivo* reduction of Aβ in the brain was *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester (DAPT) (Dovey et al., 2001), although failing to reach clinical testing. It has since then been widely used *in vitro* to study different aspects of APP cleavage and Aβ generation. Other GSIs, such as semagacestat (also called LY-450139) and avagacestat, have been developed thereafter, but the phase III trial with semagacestat and the phase II trial with avagacestat were struck by side effects (Coric et al., 2012; Imbimbo et al., 2011), and even lead to deteriorating cognition (Doody et al., 2013). γ-Secretase has many other substrates besides APP including NOTCH, a protein important during neuronal development and maintenance (Imayoshi et al., 2010). The inhibition of NOTCH cleavage may therefore be one cause for the severe side effects of GSIs (Henley et al., 2014), as could accumulation of toxic APP cleavage products (Mitani et al., 2012). In addition, upon treatment with GSIs, it has been reported that the initial decrease in Aβ levels in blood is followed by an increase in Aβ levels, as the drug concentration in plasma decreases (De Strooper, 2014). Interestingly, both *in vivo* and *in vitro* studies have shown that low doses of both semagacestat and DAPT increase Aβ levels, instead of decreasing them (Barnwell et al., 2014; Lanz et al., 2006).

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Because the Aβ peptide is hydrophobic, accumulation causes it to aggregate into soluble oligomers and further into insoluble fibrils, the constituent of Aβ plaques (Blennow et al., 2006). It has been reported that deposits of extracellular Aβ do not correlate with cognitive symptoms and that Aβ plaques may be present several years before symptom onset in AD (Perrin et al., 2009). Additionally, compact plaques consisting of Aβ fibrils are found in non-demented individuals (Guillozet et al., 2003), and it is now believed that the soluble oligomeric Aβ (oAβ) is the most toxic species (Benilova et al., 2012). Soluble forms of synthetic as well as naturally produced oAβ are toxic to cells *in vitro* and *in vivo* (Hoshi et al., 2003; Lambert et al., 1998; Takuma et al., 2004), and have the ability to disrupt cognitive function (Cleary et al., 2005).

Due to the importance of oAβ in AD pathogenesis, there is a need for reliable methods to detect oAβ. Previously, oligomer-specific antibodies such as the polyclonal anti-oligomer antibody A11, have been developed. However, this antibody also detects oligomers from other proteins such as insulin, prion protein and lysozyme (Kayed et al., 2003). Different dyes, such as congo red and thioflavin S/T stain Aβ fibrils and protofibrils, but do not recognize smaller oligomers of Aβ (Walsh et al., 1999). In addition, small probes (luminescent conjugated oligothiophenes; LCOs) have been shown to bind to aggregated Aβ in diffuse plaques (Nilsson et al., 2006), and seem to detect smaller Aβ aggregates than traditional dyes, but are not specific for Aβ aggregates. It is therefore of importance to develop new sensitive and specific methods for detection of oAβ. Recently, a method based on the proximity ligation assay (PLA) for detection of α-synuclein oligomers was described (Roberts et al., 2015). Here, we used the same approach to develop an assay for detection and quantification of intracellular oAβ. This oAβ method, along with immunochemical quantification of Aβx-38, Aβx-40, and Aβx-42 and immunoprecipitation-mass spectrometry (IP-MS) analysis of secreted Aβ peptides, was used to examine the effects of low-dose γ-secretase inhibition on production of a variety of Aβ peptides and the potential accumulation of intracellular oAβ in a previously established human neuronal model (Shi et al., 2012) that produces all relevant Aβ peptides (Bergström et al., 2016).

2. Results

2.1. Low-dose γ-secretase inhibition increases secretion of Aβ42 without affecting intracellular C-terminal fragment of APP

To test the hypothesis that low-dose γ-secretase inhibition increases production of long Aβ peptides, human induced pluripotent stem cell (iPSC)-derived cortical neurons were treated with 2, 20 or 200 nM DAPT for 48 h. Thereafter, cell-conditioned media were collected and analysed for secreted Aβx-38, Aβx-40, and Aβx-42. As the basal levels of Aβ secretion varied depending on the age of the neurons used in each repeat, the relative changes are presented here for statistical analysis. The actual concentrations of secreted Aβ from each experiment are available in Supplementary Fig. 1. Treatment with 2 or 20 nM DAPT tended to increase the secreted levels of Aβx-38 (Fig. 1A) and Aβx-40 (Fig. 1B) compared with control, although the difference did not reach statistical significance, while secretion of Aβx-42 (Fig. 1C) was significantly increased upon treatment with 2 and 20 nM DAPT. Treatment with 200 nM DAPT significantly decreased the secretion of all three peptides compared with control. This shows that low-dose treatment with the γ-secretase inhibitor DAPT significantly increased the secretion of Aβx-42, whereas a higher concentration (200 nM) decreased the secretion of all three Aβ peptides.

In addition, the effect of DAPT treatment on the Aβx-42/38 and Aβx-42/40 ratios was investigated. Treatment with 20 nM DAPT increased the Aβ42/38 ratio, which was further increased upon treatment with 200 nM DAPT (Fig. 1D). Treatment with 200 nM DAPT also significantly increased the Aβx-42/40 ratio (Fig. 1E). To investigate if the increased secretion of Aβ was accompanied by a change in the APP C-terminal fragment (the substrate for γ-secretase), western blot analysis was performed on cell lysates. While treatment with 2 or 20 nM DAPT did not change the levels of intracellular C-terminal fragments compared with control, they were significantly increased upon 200 nM DAPT treatment (Fig. 1F).

2.2. Low-dose γ-secretase inhibition reveals different patterns of Aβ peptide secretion

From earlier studies, we know that a number of Aβ peptides of varying length can be found in CSF and cell-conditioned media, and that the peptides are differently affected by secretase inhibition (Portelius et al., 2010; Portelius et al., 2011). However, this has not been investigated in human iPSC-derived neurons, why we performed a more detailed investigation of the effects of low-dose GSI treatment on Aβ peptide secretion by analysing cell-conditioned media from cortical neurons using IP-MS. The relative levels of all secreted peptides were calculated by dividing the peak area of each peptide by the total peak areas for all peptides detected. Relative secretion levels of the short Aβ1–16 (Fig. 2A) was not affected by 2 or 20 nM DAPT but was, as expected (Portelius et al., 2009), increased when cells were exposed to 200 nM DAPT. This supports that γ-secretase is not involved in the production of Aβ1–16, but rather that the decrease in γ-secretase activity allows for an increased activity of other secretases. In contrast, Aβ1–17 was significantly decreased upon treatment with 200 nM DAPT, with a trend towards decrease upon 20 nM DAPT treatment (Fig. 2B). A similar tendency was seen for Aβ1–19 (Fig. 2C), although not reaching statistical significance, indicating that production of these two peptides may be γ-secretase-dependent. Interestingly, Aβ1–20 had a similar secretion pattern as Aβ1–16, with a tendency to increase upon DAPT treatment (Fig. 2D). Secretion of Aβ1–33 was not significantly decreased by low doses of DAPT, but completely abolished upon treatment with 200 nM (Fig. 2E).

Aβ1–34 has earlier been shown to be the Aβ peptide most sensitive to γ-secretase inhibition (Portelius et al., 2010). We found a direct statistically significant dose-dependent decrease in secretion of Aβ1–34 (Fig. 2F) upon treatment with increasing doses of DAPT, showing that 2 nM DAPT is indeed inhibiting γ-secretase. To further verify this, we investigated the mRNA expression of HES1, which is stimulated upon γ-secretase dependent cleavage and activation of NOTCH (Kageyama et al., 2008), after treatment with 2, 20 and 200 nM DAPT. All three doses decreased HES1 expression to a similar level, compared to control (Supplementary Fig. 2), supporting that 2 nM DAPT is enough to reduce γ-secretase activity.

The longer peptides Aβ5–40 (Fig. 2G), Aβ1–37 (Fig. 2H), Aβ1–38 (Fig. 2I), Aβ1–39 (Fig. 2J), Aβ1–40 (Fig. 2K) and Aβ1–42 (Fig. 2L) all follow the same trend, however statistically non-significant, towards increased secretion upon treatment with 2 and 20 nM DAPT, but with strongly reduced secretion upon treatment with 200 nM DAPT.

2.3. A novel method to detect oligomeric Aβ

As low doses of the γ-secretase inhibitor DAPT increase the production/secretion of longer, more aggregation-prone Aβ peptides and decrease, or do not affect, the production of shorter, we were interested in investigating the effects of low-dose γ-secretase inhibition on intracellular oAβ accumulation. A novel method for oAβ detection was developed using proximity ligation assay (PLA; Duolink), by conjugating PLUS and MINUS Duolink probes to the N-terminal specific Aβ antibody 82E1 (Horikoshi et al., 2004). The method was optimized on oAβ1–42 (Supplementary Fig. 1A) fed to SH-SY5Y cells, as previously described (Domert et al., 2014). Using immunocytochemistry, it was evident that the 82E1 antibody was able to detect intracellular Aβ1–42 (Supplementary Fig. 1B, first panel) but as predicted, the control peptide Aβ42–1 was not detected (Supplementary Fig. 1B, second panel). SH-SY5Y cells were thereafter fed with increasing concentrations of