

Small-molecule aggregation inhibitors reduce excess amyloid in a trisomy 16 mouse cortical cell line

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ABSTRACT

We have previously characterized a number of small molecule organic compounds that prevent the aggregation of the β -amyloid peptide and its neurotoxicity in hippocampal neuronal cultures. We have now evaluated the effects of such compounds on amyloid precursor protein (APP) accumulation in the CTb immortalized cell line derived from the cerebral cortex of a trisomy 16 mouse, an animal model of Down's syndrome. Compared to a non-trisomic cortical cell line (CNh), CTb cells overexpress APP and exhibit slightly elevated resting intracellular Ca^{2+} levels ($[\text{Ca}^{2+}]_i$). Here, we show that the compounds 2,4-dinitrophenol, 3-nitrophenol and 4-anisidine decreased intracellular accumulation of APP in CTb cells. Those compounds were non-toxic to the cells, and slightly increased the basal $[\text{Ca}^{2+}]_i$. Results indicate that the compounds tested can be leads for the development of drugs to decrease intracellular vesicular accumulation of APP in trisomic cells.

Key terms: Alzheimer's disease, Down syndrome, intracellular amyloid, murine trisomy 16, small molecule inhibitors.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia in the elderly population. The disease is neuropathologically characterized by the presence of neurofibrillary tangles and senile or amyloid plaques in vulnerable brain regions, such as the cerebral cortex and hippocampus. Despite considerable efforts aimed to understand the molecular and

cellular basis of AD, there are currently no effective treatments to cure it or to arrest its progression (De Felice and Ferreira, 2002).

It is widely accepted that the β -amyloid peptide ($\text{A}\beta$), derived from proteolytic processing of the membrane-bound amyloid precursor protein (APP), plays a central role in the neuropathology of AD (Hardy and Selkoe, 2002). Amyloid plaques are thought to arise from gradual extracellular $\text{A}\beta$ deposition in fibrillar aggregates that have been extensively implicated in

ABBREVIATIONS: $\text{A}\beta$, β -amyloid peptide; AD, Alzheimer's disease; CNh, neuronal cell line derived from the cerebral cortex of a normal mouse; CTb, neuronal cell line derived from the cerebral cortex of a trisomy 16 mouse; FBS, fetal bovine serum; PBS, phosphate buffered saline; 2,4-DNP, 2,4-dinitrophenol; 3-NP, 3-nitrophenol; 4-AA, 4-anisidine; NGF, nerve growth factor; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} levels; PMSF, phenylmethylsulfonylfluoride.

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neurotoxicity (for early examples, see Lorenzo and Yankner, 1994, Pike et al., 1995). More recent studies have indicated, however, that early synaptic dysfunction in AD is caused by accumulation and synaptic targeting by soluble A β oligomers (Lacor, 2007, Ferreira et al., 2007, Haass and Selkoe, 2007). Moreover, recent reports have also shown that early intracellular A β accumulation can be detected in the brains of affected individuals (Gouras et al., 2000) and in transgenic mice that develop A β plaques (Wirhth et al., 2001) and that accumulation occurs especially in intracellular vesicular bodies (Langui et al., 2004, Takahashi et al., 2002). Intracellular A β has been proposed to play an important role in neurotoxicity (Glabe, 2001, Kienlen-Campard et al., 2002) and to be involved in early AD pathogenesis (Gouras, 2005).

Down syndrome (DS), the major cause of mental retardation in the world, is characterized by an extra copy of chromosome 21 (trisomy 21). Indicating a relationship between the pathophysiologies of AD and DS, all DS individuals eventually develop AD neuropathology at an early age, with characteristic accumulation of cerebral amyloid plaques by the fourth decade of life (Geller and Potter, 1999). This is consistent with the fact that the APP gene is located on chromosome 21 and, therefore, is overexpressed in DS individuals (Galdzicki and Siarey, 2003). Furthermore, early intracellular accumulation of A β has also been observed in DS (Gouras et al., 2000).

Due to the partial genetic homology between mouse chromosome 16 and human chromosome 21, murine trisomy 16 is considered a model for both DS and AD (Galdzicki and Siarey, 2003). Specifically, the APP gene is present in murine autosome 16 (Galdzicki and Siarey, 2003). However, the murine trisomy 16 condition is not viable and animals die *in utero*. This drawback has limited the use of those animals to studies of early developmental stages and to the use of primary cell cultures (Galdzicki and Siarey, 2003). In this regard, an interesting development has been the establishment of an immortalized

cell line (CTb) derived from the cerebral cortex of a trisomy 16 mouse fetus (Allen et al., 2000, Cárdenas et al., 1999). This cellular model has been well accepted for the study of DS and AD pathologies (for a review, see Saud et al., 2006). Compared to an age-matched immortalized cortical cell line (CNh) derived from the cerebral cortex of a normal animal, CTb cells present slightly increased basal intracellular calcium levels and glutamatergic and cholinergic dysfunction (Allen et al., 2000, Cárdenas et al., 1999). Interestingly, CTb cells exhibit abnormally elevated intracellular levels of APP, which accumulates in vesicular bodies that positively stain with Congo Red (Arriagada et al., 2007, Opazo et al., 2006). Further, using immunoblotting and immunohistochemical techniques, such deposits have been shown to react with various antibodies that recognize different sequences in the APP molecule (Arriagada et al., 2007). Additionally, Opazo et al. (2006) recently showed that knockdown of APP in CTb cells normalizes cholinergic function, and proposed that specific gene overdose in Ts16 could explain membrane-related dysfunction associated with receptors and ion channels, particularly in cholinergic cells.

We have previously characterized a number of small molecule inhibitors of the oligomerization and fibrillization of A β , which potently protect hippocampal neurons against A β -induced neurotoxicity (De Felice et al., 2001, 2004). Considering their moderately hydrophobic nature, we hypothesized that some of those compounds would be able to cross cell membranes and could also be effective in decreasing amyloid accumulation in intracellular compartments. Here, we show that treatment with low, non-toxic concentrations of 2,4-dinitrophenol, 3-nitrophenol and 4-anisidine decreases the intracellular accumulation of amyloid in vesicular bodies in the CTb cell line. These results suggest that these compounds could be used as leads for the development of inhibitors that target both extracellular and intracellular amyloid accumulation.

METHODS

The CNh and CTb immortalized cell lines were established and characterized as previously reported (Allen et al., 2000, Cárdenas et al., 1999). For standard growth conditions, cell lines were maintained in DMEM/F12 medium (Sigma, St. Louis, MO) containing 6g/L glucose, 1g/L sodium bicarbonate, 10% (v/v) fetal bovine serum (FBS) (Cultilab, Campinas, Brazil), 10,000 U/mL penicillin/streptomycin (Gibco, Grand Island, NY). Passages were carried out by detaching the cells with 0.25% trypsin-1mM tetrasodium EDTA (Gibco, Grand Island, NY). For differentiation, cells were plated on coverslips pre-coated with poly-L-lysine (Sigma, St. Louis, MO) and incubated in DMEM/F12 medium containing 6g/L glucose, 1g/L sodium bicarbonate, 3% (v/v) FBS, 10,000 U/mL penicillin/streptomycin and 100 ng/mL nerve growth factor (NGF) (Calbiochem, La Jolla, CA). The cultures were kept in a 5% CO₂ atmosphere at 37 °C and 100% humidity.

After 5 days of differentiation as described above, CNh and CTb cell lines were incubated with 20 μM 2,4-dinitrophenol (2,4-DNP; Sigma, St. Louis, MO), 3-nitrophenol (3-NP; Aldrich Chem., St. Louis, MO) or 4-anisidine (4-AA; kindly provided by Dr. J. D. Figueroa-Villar, Military Institute of Engineering, Rio de Janeiro, Brazil) for 48 hours. Neuronal survival was determined using the Live/Dead kit (Molecular Probes, Inc., Eugene, OR) following manufacturer's instructions. Cells were examined and counted on a Nikon Eclipse TE300 microscope. Live cells were identified by green calcein fluorescence, while dead cells were identified by red etidium fluorescence. Two to five random fields were chosen per coverslip, and about 500 cells were counted on each field. Three independent assays were performed (2-4 cover slips per assay). Viabilities are expressed as percentage of cell survival (means ± SEM). Statistical significances were evaluated using unpaired Student's t-test.

For Congo Red (CR) staining, CNh and CTb cells were differentiated for five days

and were then treated with 20 μM 2,4-DNP, 3-NP or 4-AA for 48 hours, as described above. CR staining of intracellular amyloid-containing vesicular bodies was performed following a slight modification of the protocol described in (Puchtler and Sweat, 1965). Briefly, cells were fixed with 4% formaldehyde for 20 min at room temperature and washed 3 times with PBS. The coverslips were sequentially incubated in 80% ethanol saturated with sodium chloride plus 1% KOH for 30 min and in alkaline Congo Red (Sigma, St. Louis, MO) solution (0.2% in 80% ethanol saturated with sodium chloride plus 1% KOH) for 30 min. Dehydration was performed by washing the cover slips sequentially in solutions of increasing ethanol concentrations ranging from 50% to 96%. The coverslips were cleared in xylene and mounted in Entellan mounting medium (Sigma, St. Louis, MO). Congo Red staining was scored by examination of 3-5 random fields per cover slip under bright field microscopy and was expressed as percentage of cells containing intracellular Congo Red-positive vesicular bodies.

For intracellular calcium level measurements, CNh and CTb cells were plated on cover slips. After 5 days of differentiation, cells were incubated with 20 μM 2,4-DNP, 3-NP or 4-AA for 48 hours. [Ca²⁺]_i was assessed by microfluorometry using Indo-1 AM (Molecular Probes, Eugene, OR). Cells were incubated at 37 °C for 40 min with 5 μM Indo-1 AM (Molecular Probes) supplemented with 0.005% Pluronic (0.1% dimethyl sulfoxide). The composition of the extracellular solution was (in mM): 140 NaCl, 5 KCl, 2 MgCl₂, 1.5 or 2.5 CaCl₂, 10 4-(2-hidroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)-NaOH and 10 Dextrose (pH = 7.4). The coverslips were mounted in a perfusion chamber placed on the stage of an inverted fluorescence microscope (Diaphot-200, Nikon Corp.) equipped with two dichroic mirrors. One dichroic mirror was used for excitation (355 nm) and the fluorescence emitted by Indo-1 (> 400 nm) was split by the second dichroic mirror into beams of light centered at 410 and 485 nm, respectively. The intensities at

both wavelengths were continuously measured using two photomultipliers, and the analog signal was digitized using an A/D converter board (Labmaster, Scientific Solutions Inc, Mentor, OH) installed in a dedicated PC compatible computer. Data were stored in the hard disk for later analysis. A computer program calculated the F410/F485 ratio and yielded the corresponding $[Ca^{2+}]_i$ from a calibration curve obtained with a calcium calibration buffer kit (Molecular Probes, Inc.). Recordings were independently obtained from 10-15 individual cells per cover slip (three cover slips per experiment). Statistical significances were assessed using unpaired Student's t-test, at values of $p < 0.05$.

RESULTS

Figure 1 shows results from the determination of the percentages of Congo Red (CR)-positive cells in CNh and CTb cultures treated for 48 hours with 20 μ M 2,4-DNP, 3-NP or 4-AA. Control, untreated CNh (Figure 1A) and CTb (Figure 1B) cultures, exhibited cells containing intracellular vesicular bodies that were positively stained with CR (52% CR-positive cells in control CTb cultures *versus* 30% CR-positive cells in control CNh cultures). Treatment with 4-AN induced a large reduction in the percentage of CR-positive CTb cells (down to 19 % CR-positive cells; Fig. 1C). Figure 1C also shows that treatment with 2,4-DNP and 3-NP caused significant decreases in the percentages of CR-positive CTb cells. In the case of CNh cells, there were no statistically significant changes in the percentages of cells containing intracellular amyloid in the presence of any of the compounds investigated.

Figure 2 shows cell viabilities of CNh and CTb cultures treated with 20 μ M 2,4-DNP, 3-NP or 4-AN. For both cell lines, viabilities ranged from 91-93% in both control and drug-treated cultures, indicating that 2,4-DNP, 3-NP and 4-AN exhibited no toxicity to CNh and CTb cells at the concentrations used in this study.

Figure 3 shows the effects of 2,4-DNP, 3-NP and 4-AN on intracellular calcium levels of CNh and CTb cells. In agreement with a previous report (Cárdenas et al., 1999), trisomic cells exhibited a slightly higher basal $[Ca^{2+}]_i$ than normal cells. Treatment with 2,4-DNP and 4-AN caused slight increases in intracellular calcium levels of CNh cells. On the other hand, an increase in intracellular calcium level of CTb cells was only noted in the presence of 3-NP.

DISCUSSION

The presence and intracellular processing of APP are normal features in normal neurons, as this protein plays major roles in the regulation of several important cellular functions, especially in the nervous system, where it is involved in synaptogenesis and synaptic plasticity (Gralle and Ferreira, 2007). In agreement with the latter and previous results (Opazo et al, 2006, Arriagada et al. 2007), both CNh and CTb cells presents APP immunoreactivity in intracellular compartments. The results presented here show that 2,4-DNP, 3-NP and 4-AN decrease intracellular accumulation of amyloid in CTb cells without affecting cell viability. It is also interesting to note that the decrease in intracellular amyloid content induced by these compounds was quite significant in trisomic cells, but the percentages of CR-positive cells in normal CNh cultures were not affected by the inhibitors. Moreover, 4-anisidine, the most effective compound in terms of the decrease in the percentage of CR-positive cells (Fig. 1C), caused no alterations in intracellular calcium concentration in CTb cells (Fig. 3).

It remains to be elucidated whether the compounds investigated here disaggregate amyloid aggregates in the cytoplasmic vesicular bodies, interfere with intracellular formation and accumulation of APP, or both. Considering the incubation time of the cells with the compounds (48 h), it is possible that they interfere with one or more steps of amyloid processing pathways. However, the compounds tested here have been shown to

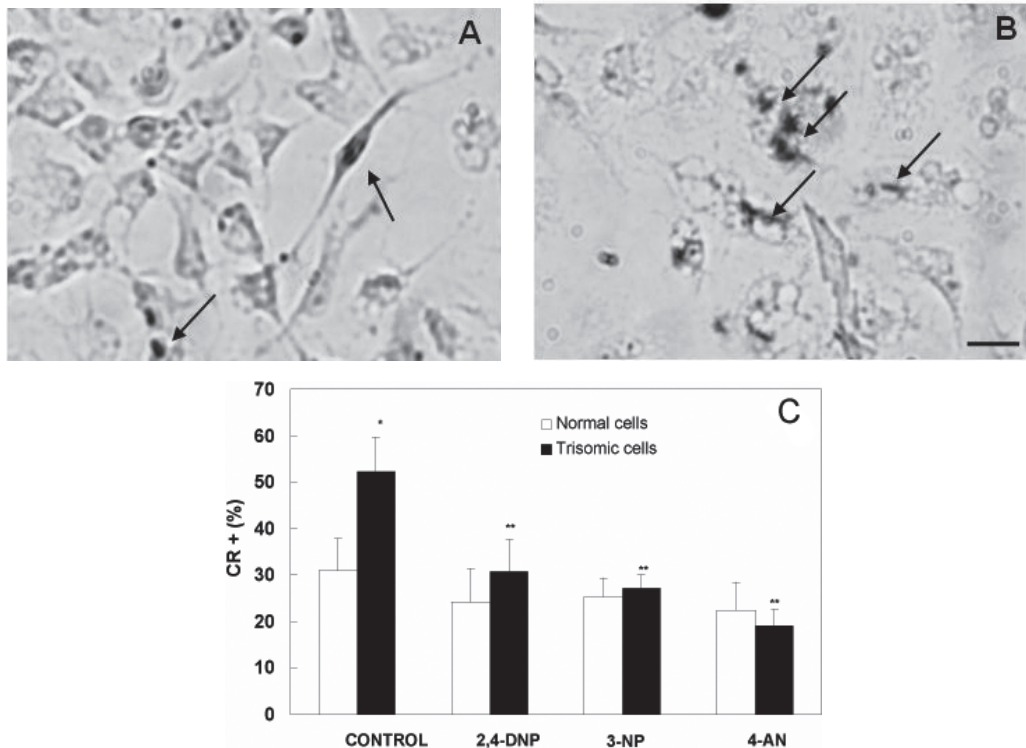


Figure 1: Amyloid aggregation inhibitors reduce intracellular amyloid accumulation in CTb cells. CNh and CTb cells were differentiated for 5 days (see “Methods”) and were maintained for two additional days in culture in the absence or in the presence of different compounds (20 μ M) before staining with Congo Red (CR). Panels A and B: CNh (A) and CTb (B) cells stained with CR and observed under bright field microscopy. Arrows point to some examples of cells containing CR-positive vacuoles in both cell lines. Scale bar: 25 μ m. Panel C: Percentage of CR-positive cells in cultures treated with different compounds. Statistical significances were evaluated using unpaired Student’s t-test. * $p < 0.02$ compared to untreated CNh cells; ** $p < 0.02$ compared to untreated CTb cells.

prevent the formation and to disaggregate previously formed amyloid fibrils and soluble oligomers *in vitro* (De Felice et al., 2001, 2004), and to prevent aggregation of amyloidogenic variants of human lysozyme, which are involved in hereditary systemic amyloidosis (Vieira et al., 2006).

The observed effects on Ca^{2+} homeostasis are also interesting. Increased APP level in DS neurons has been linked to lipoperoxidation (Begnia et al., 2003), which could affect Ca^{2+} channel function. Moreover, Pollard et al. (1995) described that the $A\beta$ peptide forms Ca^{2+} channels in artificial bilayers, with conductances of the order of 400 to 4000 pS. A channel of such conductance would dissipate the Ca^{2+} gradient quickly, altering the intracellular

homeostasis of the cation, which could deeply impair cell function. As well, altered APP metabolism in DS impairs mitochondrial function, which could render cells more susceptible to oxidative stress and alter Ca^{2+} homeostasis (Busciglio et al., 2002). The compounds tested here not only did not reduce $[Ca^{2+}]_i$ but induced further small increases in its concentration. One possible explanation for this effect could reside in the well-known effect of phenol-derived molecules in the uncoupling of oxidative phosphorylation, which could contribute to an energy crisis that further alters Ca^{2+} regulation. However, we note that measurements of oxygen consumption and mitochondrial potential in neuroblastoma cells treated with 20 μ m of

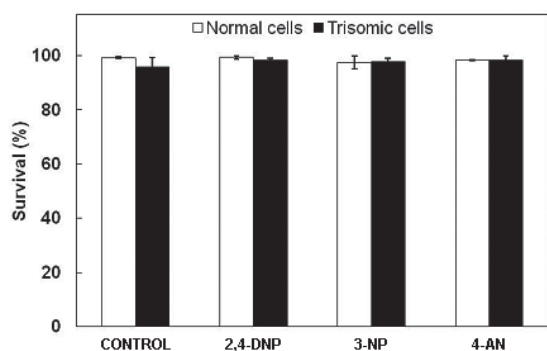


Figure 2: Amyloid aggregation inhibitors are non-toxic to CNh and CTb cells. Viabilities, measured using the Live/Dead assay (as described in “Methods”), of CNh and CTb cultures treated with 2,4-DNP, 3-NP and 4-AN for 48 hours. Three independent assays were performed (2-4 cover slips per assay). Viabilities are expressed as percentage of live cells. The data correspond to means \pm SEM of three independent assays. From three to seven thousand cells were counted in each experimental condition. No statistically significant differences in viabilities in comparison to control were found after the treatment with the small molecule inhibitors.

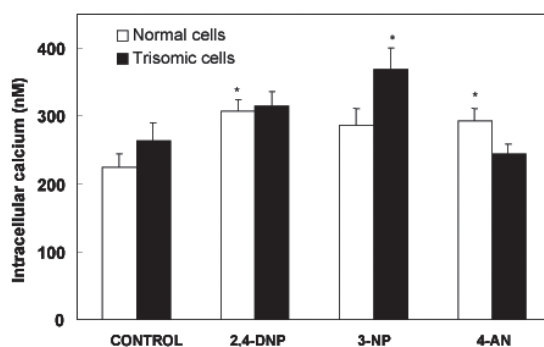


Figure 3: Intracellular calcium concentrations in drug-treated cells. After 48 hours of incubation in the absence or in the presence of 20 μ M 2,4-DNP, 3-NP or 4-AN, CTb or CNh cells were loaded with the fluorescent calcium indicator Indo-1. Recordings were obtained from 10-15 individual cells per cover slip (three cover slips per experiment) and were converted to intracellular calcium concentrations using a calibration curve, as described in “Methods”. Data are means \pm SEM of three independent experiments. Statistical significances were evaluated using unpaired Student’s t-test. * p < 0.02 compared to the respective untreated control.

the compounds investigated here showed that, at this low concentration, the compounds do not interfere with mitochondrial function (Wasilewska-Sampaio, 2005). Furthermore, 4-anisidine, a non-phenol, had no effect on $[Ca^{2+}]_i$ of trisomic cells but induced a marked reduction in the number of CR(+) cells. It is, thus, tempting to speculate that the effects of the compounds on APP metabolism and intracellular amyloid accumulation may encompass other mechanisms not involving regulation of intracellular Ca^{2+} , but we acknowledge that this hypothesis requires further study.

Recent results indicate that intracellular amyloid accumulation in the CTb cell line may be due to impairment in the lysosomal/endosomal pathways (Arriagada et al., 2007). In agreement with this hypothesis, it has been shown that trisomy 21 leads to endosomal abnormalities that are associated with increased soluble $A\beta$ levels and with the presence of intraneuronal $A\beta$

immunoreactivity, which co-localizes to enlarged endosomes (Cataldo et al, 2000). It has also been shown that a segmental trisomy 16 mouse model of Down syndrome exhibits endosomal abnormalities (Cataldo et al, 2003) and it was proposed that progressive alterations in lysosomal function in AD and DS may contribute to the neurodegenerative processes. This mechanism, associated with the gene overdose effect in the trisomic condition, could greatly impair APP metabolism and lead to its intracellular accumulation. Recently, Almeida et al. (2006) shed light on the mechanism by which such abnormalities of late-endosomal sorting occur, by showing that this impairment is related to an inhibition of ubiquitin-proteasome system in response to intracellular amyloid accumulation.

In the present work, we have not determined which fragments of APP accumulated in CR-positive vesicular bodies. Western blot analysis of cell lysates

revealed an APP fragment of about 98 kDa, which is immunoreactive to a commercial antibody against A β 1-40/42 (data not shown), indicating that it encompasses the A β sequence. This suggests that the intracellular vesicular bodies probably contain aggregates of an amyloidogenic nature that are positively stained with CR. Regardless of the precise nature of the amyloid species present, the results presented here show that the small-molecule compounds tested were capable of reducing the percentage of CTb cells containing CR-positive vesicular bodies.

CONCLUSION

Taken together with previous results (De Felice et al., 2001, 2004), the data presented here suggest that 2,4-DNP, 3-NP and 4-AN may be useful lead compounds for the development of drugs that target both extracellular deposition and intracellular accumulation of amyloid fragments.

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