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Identification, design, synthesis, and pharmacological activity of (4-ethyl-piperazin-1-yl)-phenylmethanone derivatives with neuroprotective properties against β-amyloid-induced toxicity

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Abstract

In search of novel therapeutic approaches for Alzheimer's disease (AD), we report herein the identification, design, synthesis, and pharmacological activity of (4-ethyl-piperaz-1-yl)-phenylmethanone derivatives with neuroprotective properties against β-amyloid-induced toxicity. (4-ethyl-piperaz-1-yl)-phenylmethanone is a common substructure shared by molecules isolated from plants of the Asteraceae genus, traditionally used as restorative of lost or declining mental functions. (4-Ethyl-piperaz-1-yl)-phenylmethanone displayed strong neuroprotective properties against $A\beta_{1-42}$ and reversed $A\beta_{1-42}$-induced ATP depletion on neuronal cells, suggesting a mitochondrial site of action. $A\beta_{1-42}$ has been described to induce a hyperactivity of the glutamate network in neuronal cells. (4-Ethyl-piperaz-1-yl)-phenylmethanone also inhibited the neurotoxic effect that glutamate displayed on PC12 cells, suggesting that the reduction of glutamate-induced neurotoxicity may be one of the mechanisms by which this compound exerts its neuroprotective properties against the deleterious effects of the $A\beta_{1-42}$. These data suggest that the identified (4-ethyl-piperaz-1-yl)-phenylmethanone chemical entity exerts neuroprotective properties and may serve as a lead compound for the development of novel therapies for AD.

Keywords: Alzheimer's disease; Glutamate; Piperazinyl; Amyloid; ATP

1. Introduction

Alzheimer's disease (AD) is the most common dementia occurring in the elderly, affecting about 10% of people above 65 years and 40% above 80 years. Familial AD is the early-onset form of the disease, involving different mutations of the amyloid protein precursor (APP) gene, and accounting for no more than 5% of the total AD cases (Rocchi et al., 2003). The late-onset form of the disease accounts for more than 95% of the AD cases and its origin remains elusive. Several risk factors have been identified but a causality relationship of the onset or progression of the disease has not been yet established. AD is clinically characterized by a progressive and, by now, irreversible impairment of cognitive processes commonly associated with a non-cognitive symptomatology including depression (Robert et al., 1996). Histologically, AD is defined by the

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presence in postmortem human brain specimens of amyloid neuritic plaques, the formation of neurofibrillary tangles and degeneration of the cholinergic neurons (Whitehouse et al., 1981; Selkoe et al., 1986).

During the past decades, improving the cholinergic network dysfunction was the main focus of the scientific community. This led to the creation of the therapeutic class of acetylcholinesterase inhibitors (AchEI) with tacrine as the class leader. Despite promising clinical data, the beneficial effects of tacrine were modest and the new generation of AchEI, represented by galantamine and donezepil, although safer, did not dramatically improve the delay of symptom onset. This short 1–2 years delay (Tariot and Winblad, 2001; Waldemar et al., 2001), although priceless for the patients and their relatives is a limitation of the use of AchEI. Since then, despite major breakthroughs in understanding the mechanisms underlying the progression of the disease, no major advances have been made in AD drug development, even though memantine, an antagonist of the glutamatergic NMDA-subtype receptor has just been approved to be released in the US market (Marder, 2004).

Local anesthetics were shown to exhibit neuroprotective properties in vivo, during cerebral ischemia in gerbils (Fujitani et al., 1994; Chen et al., 1998; Adachi et al., 1999), and in vitro, during a hypoxic episode in hippocampal neurons (Lucas et al., 1989; Liu et al., 1997; Raley-Susman et al., 2001). Concomitantly, procaine and lidocaine have been showed to inhibit NMDA receptor activity (Nishizawa et al., 2002), suppress the anoxia-induced increase of the intracellular calcium concentration in gerbil hippocampus (Liu et al., 1997) and prevent the ischemia-triggered increase of extracellular glutamate concentration in gerbil brain (Fujitani et al., 1994). Procaine containing formulations have been tested for decades as a possible treatment for cognitive disorders in geriatric subjects (Kent, 1976; Goodnick and Gershon, 1984). Several possible mechanisms mediating the effects of procaine have been studied, such as cerebral blood vessel dilation, monoamine oxidase inhibition or an anti-depressant effect but none were really convincing. We recently demonstrated that procaine chlorhydrate itself down-regulated the stressor-induced increase of adrenal corticosteroid synthesis in vitro and in vivo in rats (Xu et al., 2003). Such a stressor-induced imbalance of the hypothalamo-pituitary-adrenal axis has been described in many diseases including AD (Swaab et al., 1994; O'Brien et al., 1996; Giubilei et al., 2001; Rasmuson et al., 2002). High concentrations of cortisol have been reported to contribute to the neurodegeneration that occurs in AD either by a direct effect on the neuronal cells or by sensitizing them to $A\beta_{1-42}$ and glutamate neurotoxicity (Abraham et al., 2000; Polleri et al., 2002). In addition, excessive serum concentrations of cortisol have been associated with impaired memory processes and suggested to trigger non-cognitive conditions like depression and mood variability (Kiraly et al., 1997; Tafet et al., 2001). However, the high metabolism rate of procaine to *p*-aminobenzoic acid by various esterases present in the blood provides a challenge for the use of this molecule in the therapy of chronic diseases (Brodie et al., 1948; Traut, 1952).

This consideration led us to screen a database of natural compounds, using procaine as a substructure. The goal was to select stable biologically active compounds and to identify the common chemical core structure bearing the activity. This chemical core will be then used as a starting point to develop a series of analogs in further studies. In this prospective, we report herein the isolation of three biologically active compounds (Fig. 1) which led to the characterization, design, synthesis, and pharmacological activity of (4-ethyl-piperaz-1-yl)-phenylmethanone derivatives with neuroprotective properties against $A\beta_{1-42}$-induced neurotoxicity in rat pheochromocytoma (PC12) cells.

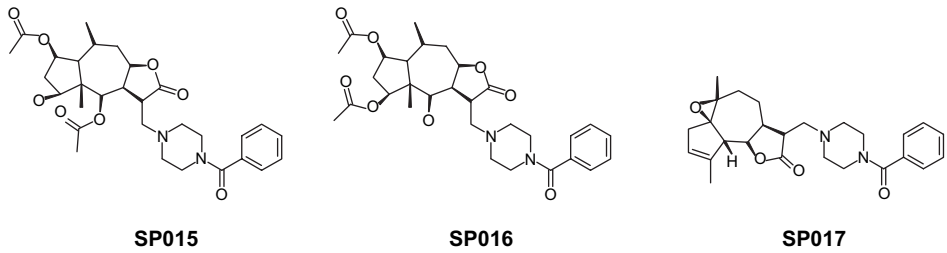
2. Materials and methods

2.1. Materials

$A\beta_{1-42}$ peptide was purchased from American Peptide Co. (Sunnyvale, CA). The anti-oxidant *tert*-butyl-phenylnitrone (PBN) and the *N*-methyl-D-aspartate (NMDA) receptor antagonist (+)-MK801 were purchased from Sigma (St Louis, MO). Structures of SP015, SP016 and SP017 are shown in Fig. 1. The chemical names of SP015, SP016 and SP017 are described in Table 1. SP008 was synthesized by Taros, Inc. (Marburg, Germany) as described below. Cell culture supplies were purchased from GIBCO (Grand Island, NY) and cell culture plasticware was from Corning (Corning, NY) and Packard BioSciences Co. (Meriden, CT).

2.2. In silico screening for procaine derivatives

The Interbioscreen Database of naturally occurring entities was screened for compounds containing the procaine structure using the ISIS software (Information Systems Inc., San Leandro, CA). Acetic acid 7-acetoxy-3-(4-benzoyl-piperazin-1-yl-methyl)-5-hydroxy-4a,8-dimethyl-2-oxo-dodecahydro-azuleno[6,5-*b*]furan-4-yl ester (SP015), acetic acid 5-acetoxy-3-(4-benzoyl-piperazin-1-yl-methyl)-4-hydroxy-4a,8-dimethyl-2-oxo-dodecahydro-azuleno[6,5-*b*]furan-7-yl ester (SP016) and 3-(4-benzoyl-piperazin-1-yl-methyl)-6,6a-epoxy-6,9-dimethyl-3a,4,5,6,6a,7,9a,9b-octahydro-3*H*-azuleno[4,5-*b*]furan-2-one (SP017) compounds identified were purchased from Interbioscreen (Moscow, Russia) (Table 1).



	Chemical denomination	Origin
SP015	Acetic acid 7-acetoxy-3-(4-benzoyl-piperazin-1-yl-methyl)-5-hydroxy-4a,8-dimethyl-2-oxo-dodecahydro-azuleno[6,5-b]furan-4-yl ester	*Inula Britanica (Asteraceae)*
SP016	Acetic acid 5-acetoxy-3-(4-benzoyl-piperazin-1-yl-methyl)-4-hydroxy-4a,8-dimethyl-2-oxo-dodecahydro-azuleno[6,5-b]furan-7-yl ester	*Inula Britanica (Asteraceae)*
SP017	3-(4-benzoyl-piperazin-1-yl-methyl)-6,6a-epoxy-6,9-dimethyl-3a,4,5,6,6a,7,9a,9b-octahydro-3H-azuleno[4,5-b]furan-2-one	*Artemisia glabella (Asteraceae)*

Fig. 1. Chemical formula and chemical denomination of *Artemisia genus* isolated compounds.

2.3. Cell culture and treatments

PC12 cells (rat pheochromocytoma) (ATCC, Manassas, VA) were cultured in RPMI 1640 without glutamine medium containing 10% of fetal bovine serum and 5% of horse serum at 37 °C and 5% CO_2. PC12 cells were incubated for 24 h in 96-well plates (5×10^4 cells per well) with increasing concentrations of SP015, SP016, SP017 (1, 10 and 100 μM) or SP008 (1 and 10 μM). $A\beta_{1-42}$ was incubated overnight at 4 °C and then added to the cells at 0.1, 1 or 10 μM final concentrations for a 24 h time period.

To study the role played by the NMDA receptor in the $A\beta_{1-42}$-induced neurotoxicity, increasing concentrations (0, 1, 5, 10, 25 and 100 μM) of the NMDA receptor antagonist (+)-MK801 were added to the cell media immediately before $A\beta_{1-42}$. Cell viability was assessed 4 h later using the MTT assay. To assess the effect of SP008 on the glutamate-induced excitotoxicity, PC12 cells were pre-treated with SP008 at 0.3, 1 and 3 μM for 24 h and then submitted to glutamate exposure for another 24 h time period. Cell viability was subsequently assessed using the MTT assay. The involvement of the oxidative stress in the toxicity of $A\beta_{1-42}$ was assessed by incubating the PC12 in presence of 10, 100 or 500 μM phenyl-*tert*-butyl-nitrone PBN for 24 h. $A\beta_{1-42}$ was then added to the incubation media. Cell viability was assessed by MTT 24 h later.

2.4. Cell viability determination

The cellular toxicity of Aβ was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Trevigen, Gaithersburg, MD) as previously described (Lecanu et al., 2004). Briefly, 10 μl of the MTT solution were added to the cells cultured in 100 μl of medium. After an incubation period of 4 h in the same conditions as above, 100 μl of detergent were added and cells incubated overnight at 37 °C. The blue coloration was quantified at 600 nm and 690 nm using the Victor spectrophotometer (EGG-Wallac, Gaithersburg, MD). The effect of $A\beta_{1-42}$ was expressed as ($DO_{600}-DO_{690}$). To compare the protective effect of the compounds tested, the decrease of MTT signal observed with $A\beta_{1-42}$ was considered to be the 100% inhibition of the NADPH diaphorase activity and the effect of the compounds tested is shown as an increase or decrease of this percentage.

2.5. ATP measurement

ATP concentrations were measured using the ATPLite-M™ assay (Packard BioSciences Co.), as previously described (Lecanu et al., 2004). In brief, cells were cultured on black 96-well ViewPlate™ and the ATP concentrations measured on a TopCount NXT™ counter (Packard BioSciences Co.) according to the manufacturer's recommendations. The effect of $A\beta_{1-42}$ was expressed in arbitrary units. To compare the potential protective effect of the compounds tested on ATP recovery, the decrease of ATP concentration induced by $A\beta_{1-42}$ was considered to be the 100% reduction and the effects of the compounds tested are shown as changes of this percentage.

Table 1
Assessment of the neuroprotective effect of the SP compounds against $A\beta_{1-42}$ cytotoxicity on PC12 cells

		SP015			SP016			SP017		
Aβ 1–42	Control	1	10	100	1	10	100	1	10	100
0.1	100.0±8.8	86.6±19.6	95.9+9.0	115±14.7	88.1±25.5	89.6±31.6	124±26.8	94.1±8.5	70.1±21.9*	208±10.0
1	100.0±6.6	78.8±24.7	82.8±21.9	116±22.3	87.2±32.5	86.1±25.9	126±29.6	65.7±10.7***	71.9±14.6**	231±11.1
10	100.0±5.3	78.1±15.0*	78.5±16.6*	120±25.1	88.2±23.6	78.1±43.9	118±34.3	54.6±20.6**	69.4±21.7*	226±11.1

Data presented as means±SD ($n=6$). *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control. Statistical analysis performed by ANOVA followed by a Dunnett's test.

2.6. Free radical production

Oxidative stress was assessed by measuring the free radical production using the fluorescent probe dihydroxy di-chlorofluorescein diacetate (2,7-DCF) (Molecular Probes, Eugene, OR), as previously described (Lecanu et al., 2004). For these experiments, cells were cultured in polylysine coated microplates. Cells were washed once with RPMI 1640 and medium was then replaced by 100 μl of RPMI 1640. Cells were incubated for 45 min at room temperature in the dark with 100 μl of 2,7-DCF 50 μM and the fluorescence (excitation $\lambda=485$ nm, emission $\lambda=535$ nm) was measured using the Victor multilabel counter (EGG-Wallac, Gaithersburg, MD).

2.7. SP008 synthesis

Solvents were purified by standard methods. MS: recorded on a VG Tribid, Varian CH7 (EI). Thin-layer chromatography (TLC) analyses were performed on silica gel 60 F_{254} with a 0.2 mm layer thickness. NMR spectroscopy: Bruker AMX300. All resonances are given in ppm and referenced to residual solvent signals ($CDCl_3$: 7.25 ppm).

2.8. 2,3,4-Trimethoxybenzoyl chloride (*1*)

2,3,4-Trimethoxybenzoic acid (5.00 g, 23.6 mmol) was dissolved in dry toluene (2 ml). A catalytic amount of *N*,*N*-dimethylformamide (two drops) was added. To this mixture was added dropwise a solution of oxalyl chloride (4.27 g, 33.6 mmol) in toluene (11 ml). Stirring was continued at room temperature for 3.5 h. Excess reagent and solvents were removed in a vacuum (yield: 5.13 g **1**, 94%).

^{1}H NMR ($CDCl_3$) δ 7.82 (d, 1H, 9 Hz), 6.68 (d, 1H, 9 Hz), 3.89 (s, 3H), 3.86 (s, 3H), 3.80 (s, 1H). MS (EI) m/z 230 (M^+), 212, 195, 179, 152.

2.9. 4-Ethyl-1-(2,3,4-trimethoxybenzoyl)-piperazine (*2*)

To a solution of crude 2,3,4-trimethoxybenzoyl chloride (0.93 g, 4.0 mmol) in dry dichloromethane (40 ml) was added drop wise *N*-ethylpiperazine (0.92 g, 8.1 mmol) at 0 °C. Stirring was continued for 30 min. The mixture was washed with saturated aqueous NH_4Cl. The aqueous layer was extracted twice with dichloromethane. The combined organic layers were washed with brine, dried ($MgSO_4$) and concentrated. The crude product was recrystallized from ether/petroleum ether to give **2** as a solid (0.63 g, 51%).

^{1}H NMR ($CDCl_3$) δ 6.88 (d, 1H, 8.5 Hz), 6.62 (d, 1H, 8.5 Hz), 3.83 (s, 3H), 3.81 (s, 3H), 3.80 (s, 3H), 3.76 (m, 2H), 3.25 (m, 2H), 2.43 (m, 4H), 2.35 (q, 2H, 7 Hz), 1.02 (t, 3H, 7 Hz). MS (EI) m/z 308 (M^+), 237, 195, 97.

2.10. Statistical analysis

Data are expressed as mean ± SD. Data obtained were assessed between experimental groups by a one-way ANOVA and Dunnett's test was used for comparison. A difference was considered significant when $p<0.05$.

3. Results

3.1. $A\beta_{1-42}$ neurotoxicity assessed by MTT assay, ATP measurement and free radical production in PC12 cells (Fig. 2)

$A\beta_{1-42}$ induces a dose-dependent decrease of PC12 cell viability ($p<0.001$) (Fig. 2A) and of the intracellular ATP concentrations ($p<0.001$) (Fig. 2B). A dose-dependent relationship is also observed on the free radical production as $A\beta_{1-42}$ at 1 and 10 μM concentrations induced a significant increase of the oxidative stress ($p<0.01$ and $p<0.001$, respectively) (Fig. 2C).

3.2. Effect of SP015, SP016 and SP017 on the cell viability assessed by MTT, on $A\beta_{1-42}$-induced ATP decrease and on the $A\beta_{1-42}$-induced free radical production

The identified naturally occurring SP015, SP016 and SP017 derivatives displayed neuroprotective properties against $A\beta_{1-42}$ neurotoxicity in PC12 cells (Table 1). SP015 protected only at 1 and 10 μM concentrations against the highest concentration of $A\beta_{1-42}$, whereas SP016 had no protective activity. SP017 at 1 μM reduced the diaphorase inhibition induced by $A\beta_{1-42}$ but the best effect was observed with SP017 (10 μM) which was able to protect against the three concentrations of $A\beta_{1-42}$ tested (70.1 ± 21.9% versus 100.0 ± 8.8% for the control, $p<0.05$, $n=6$; 71.9 ± 14.6% versus 100.0 ± 6.6% for the control, $p<0.01$, $n=6$, and 69.4 ± 21.7% versus 100.0 ± 5.3% for the control, $p<0.05$, $n=6$). SP017 at 100 μM potentiated the toxic effect of $A\beta_{1-42}$ suggesting a probable toxicity. A toxic effect was observed when these piperazines have been given at 100 μM in absence of $A\beta_{1-42}$ although this effect was significant only for SP017 (100 μM). Among the natural derivatives of procaine, SP015 at 1 μM and SP017 at 1 and 10 μM concentrations were able to reverse the effect of $A\beta_{1-42}$ on ATP (Table 2). As shown in Fig. 2C, $A\beta_{1-42}$ induced in a dose-dependent manner the production of free radicals in PC12 cells. SP015 (Fig. 3A), SP016 (Fig. 3B) and SP017 (Fig. 3C) compounds did not affect the $A\beta_{1-42}$-induced oxidative stress. On the contrary, these compounds amplified the $A\beta_{1-42}$-induced free radical production.

3.3. Effect of PBN on $A\beta_{1-42}$-induced free radical production and cell viability

$A\beta_{1-42}$ has been described to induce oxidative stress in brain tissue (Varadarajan et al., 1999; McLellan et al., 2003) and an excessive oxidative stress is known to be neurotoxic. Therefore, we assessed (1) if an oxidative stress accounts for $A\beta_{1-42}$-induced neurotoxicity in our experiments, and (2) if an anti-oxidative effect takes place in the neuroprotective effect of procaine and derivatives. $A\beta_{1-42}$ induced a dose-dependent increase of free radical production in PC12 cells which is significantly reduced by PBN 10, 100 and 500 μM ($p<0.05$ against 0.1 μM $A\beta_{1-42}$, and $p<0.05$, $p<0.01$ and $p<0.01$ against 10 μM $A\beta_{1-42}$, respectively; Fig. 4A). PBN had no effect on the $A\beta_{1-42}$-induced decrease in cell viability (Fig. 4B).

3.4. SP008 synthesis

Based on the data presented above on the effects of naturally occurring SP015, SP016 and SP017, the

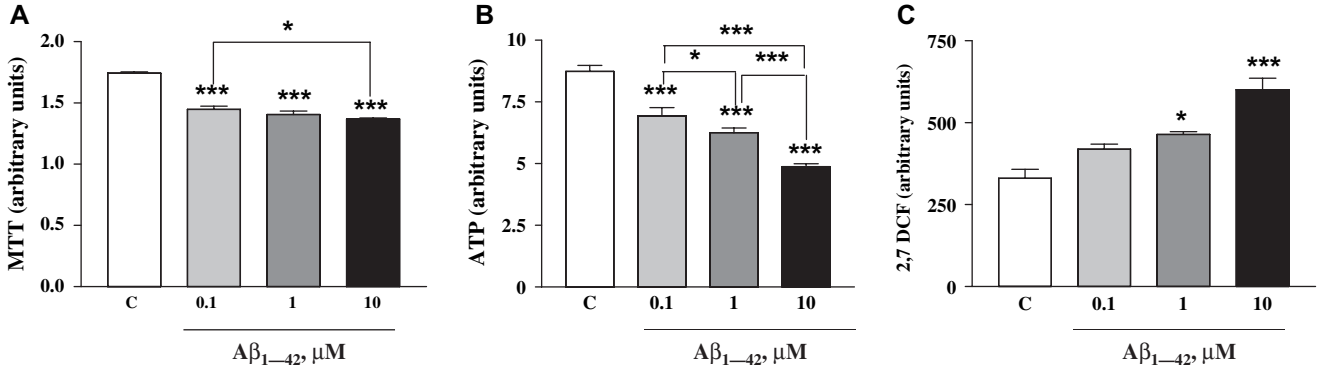


Fig. 2. Effect of $A\beta_{1-42}$ on rat pheochromocytoma PC12 cell viability assessed using the MTT assay (A) and by measuring the intracellular ATP concentrations (B). The effect of $A\beta_{1-42}$ on the free radical production was assayed using the fluorescent probe 2,7-DCF (C). PC12 cells were exposed to increasing concentrations of $A\beta_{1-42}$ (C, control) and the different parameters were assayed after 24 h exposure. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Data shown as means ± SD, $n=6$. *$p<0.05$, ***$p<0.001$ compared to control unless otherwise stated.

Table 2
SP compounds reverse the ATP stock depletion induced by $A\beta_{1-42}$ on PC12 cells

		SP015			SP016			SP017		
Aβ 1–42	Control	1	10	100	1	10	100	1	10	100
0.1	100.0±13.4	46.3±10.1**	99.2±13.1	91.5±1.5	49.5±10.2**	101±7.8	86.3±15.7	28.1±7.2**	54.9±15.6**	115±21.4
1	100.0±18.9	55.6±5.3**	124±24.7	85.6±12.3	81.5±4.2	105±10.1	100±17.8	42.6±5.8**	44.9±8.1**	108±20.8
10	100.0±7.3	32.8±9.4**	104±22.5	73.8±6.8*	96.8±30.6	110±10.1	121±29.0	73.0±12.1*	68.9±7.3**	122±6.7

Data presented as means±SD ($n=6$). *$p<0.05$, **$p<0.01$ compared to control. Statistical analysis performed by ANOVA followed by a Dunnett's test.

common 4-ethyl-1-benzoyl-piperazine structure was identified and used as the basis to chemically synthesize the SP008 compound (Figs. 5 and 6). As polyphenols have been described for years now to exert anti-oxidant properties (Rice-Evans, 1995) that have been claimed to be responsible for curative properties in different diseases (Damianaki et al., 2000; Soleas et al., 2002; Wang et al., 2002; Cuevas et al., 2000; Bastianetto et al., 2000), we designed SP008 to display a polyphenolic group. This polyphenolic group is expected to exert a strong anti-oxidant activity that might contribute to the neuroprotective effect of our compounds. The structure–activity relationship of flavonoids regarding their anti-oxidant properties was published years ago and it has been demonstrated that the compounds displaying a trihydroxybenzoic group like epicatechin- or epigallocatechin-gallate had the strongest anti-oxidant activity (Rice-Evans et al., 1996).

3.5. Effect of SP008 on cell viability and ATP level of PC12 cells exposed to increasing concentrations of $A\beta_{1-42}$

SP008 at 10 μM exerted a protective effect against 0.1 μM $A\beta_{1-42}$-induced cytotoxicity ($p<0.01$, $n=6$) (Fig. 7A) although this concentration did not preserve the $A\beta_{1-42}$-depleted ATP stock. Paradoxically, 1 μM SP008 did not reduce the 0.1 μM $A\beta_{1-42}$-induced NADPH diaphorase inhibition (Fig. 7A) but it prevented the ATP decrease ($p<0.05$) (Fig. 7D). SP008 demonstrated neuroprotective effects against 1 μM $A\beta_{1-42}$, assessed using the MTT assay, when used at 1 ($p<0.05$) and 10 ($p<0.01$) μM (Fig. 7B). This effect was accompanied by a dose-dependent ATP preservation (Fig. 7E).

SP008 administered at 10 μM concentrations displayed neuroprotective properties against 10 μM $A\beta_{1-42}$-induced toxicity in PC12 cells; this effect was statistically significant ($p<0.05$, $n=6$) (Fig. 7C). This effect of SP008 was accompanied by a dose-dependent restoration of ATP levels, although this effect was not statistically significant (Fig. 7F).

3.6. Assessment of an anti-glutamatergic effect of SP008 in the protective activity displayed against the $A\beta_{1-42}$-induced neurotoxicity

The NMDA receptor has been demonstrated to contribute to $A\beta_{1-42}$ neurotoxicity (Parks et al., 2001; Harkany et al., 1999). Therefore, in order to assess if a neuroprotective effect of SP008 could be due to the blockade of the NMDA neurotransmission, we first study if an NMDA hyperactivity occurs in our experimental conditions. This was studied by using (+)-MK801, a non-competitive inhibitor of NMDA receptor (Wong et al., 1986), on $A\beta_{1-42}$ neurotoxicity.

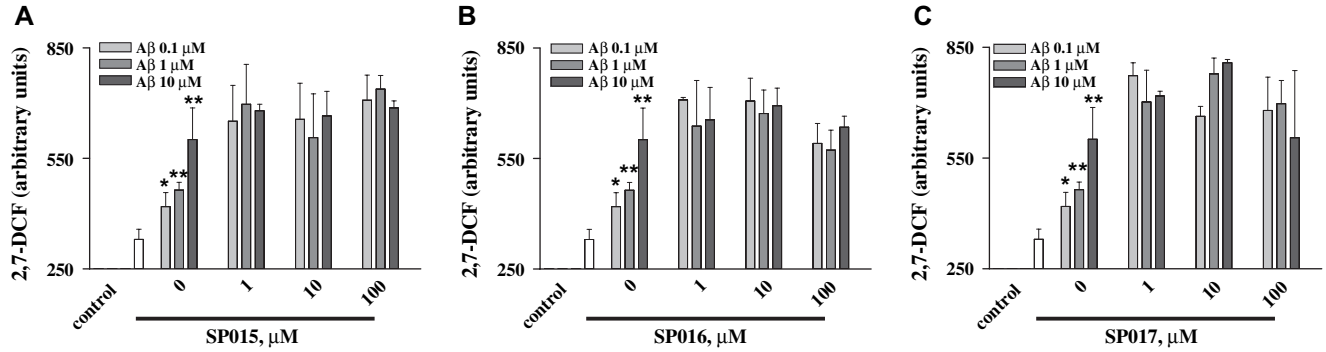


Fig. 3. Effect of SP015, SP016 and SP017 on the $A\beta_{1-42}$-induced free radical production on PC12 cells. PC12 cells were pre-incubated for 24 h with increasing concentrations of SP015 (A), SP016 (B) and SP017 (C) before being exposed to increasing concentrations of $A\beta_{1-42}$. Free radical production was measured using the fluorescent probe 2,7-DCF after 24 h of $A\beta_{1-42}$ exposure. Control cells were exposed to vehicle only. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Results shown are means ± SD, $n=6$, compared to SP compounds 0 μM.

(+)-MK801 improves PC12 cell viability decrease induced by $A\beta_{1-42}$ in a dose-dependent manner (Fig. 8A). (+)-MK801 used at 25 μM concentrations protected PC12 cells against 0.1 and 1 μM $A\beta_{1-42}$-induced toxicity ($p<0.05$). (+)-MK801 used at 100 μM concentrations provided the most significant neuroprotective effect against all concentrations of $A\beta_{1-42}$ tested ($p<0.001$). Glutamate 100 μM dramatically reduced PC12 cell viability ($p<0.001$, $n=6$; Fig. 8A). The effect of SP008 was then studied against glutamate toxicity. The effect of SP008 was dose-dependent with a significant neuroprotective effect observed at a concentration as low as 0.3 μM ($p<0.001$ compared to control, $n=6$) (Fig. 8B).

4. Discussion

In our endeavor to discover a potential cure for AD, we developed an interest in plants traditionally used to treat mental illnesses. Among them, plants from *Artemisia* genus have been used traditionally as restoratives of lost or declining mental functions (Wake et al., 2000). This led us to screen natural compound databases and to isolate the active compounds SP015, SP016 and SP017 from plants of the Asteraceae family, *Inula britanica* and *Artemisia glabella*.

Among the natural compounds screened, SP017 showed the highest protective effect on the mitochondrial function, as evidenced by the changes seen in mitochondrial diaphorase activity, with an efficacy range of 30–70% inhibition of $A\beta_{1-42}$ toxicity. Interestingly, despite the important chemical similarity between SP015 and SP016, SP016 displayed a significant effect only against low $A\beta_{1-42}$ concentrations (0.1 μM) when administered at 1 μM whereas 1 μM SP015 offered an important protection even against the highest $A\beta_{1-42}$ concentration examined. Surprisingly, the effect of these different compounds on PC12 viability submitted to $A\beta_{1-42}$ exposure did not completely match the effect observed on the restoration of ATP content. In particular, SP015 displayed a neuroprotective effect at 1 and 10 μM only against 10 μM $A\beta_{1-42}$, whereas the restoration of the ATP content was observed against

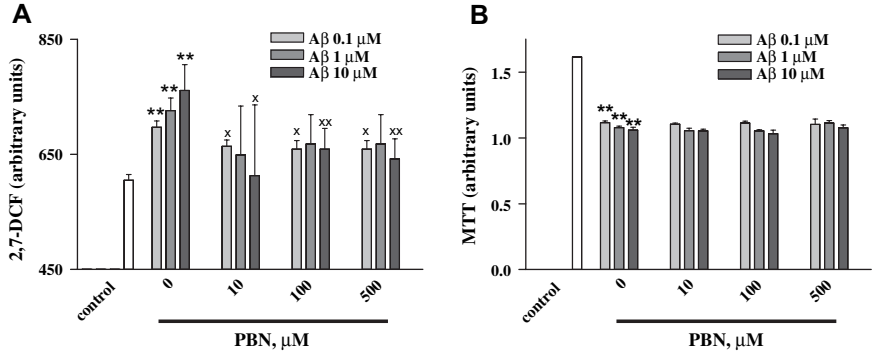


Fig. 4. Effect of the anti-oxidant phenyl-*tert*-butylnitrone (PBN) on the $A\beta_{1-42}$-induced free radical production and cell viability on PC12 cells. PC12 cells were pre-incubated for 24 h with increasing concentrations of PBN before being exposed to increasing concentrations of $A\beta_{1-42}$. Free radical production was measured using the fluorescent probe 2,7-DCF (A) and cell viability assessed using the MTT assay (B) after 24 h of $A\beta_{1-42}$ exposure. Control cells were exposed to vehicle only. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Data shown are means ± SD, $n=6$, compared to the respective 0 μM group. $^{**}p<0.01$ compared to the control. $^{x}p<0.05$, $^{xx}p<0.01$ compared to 0 μM.

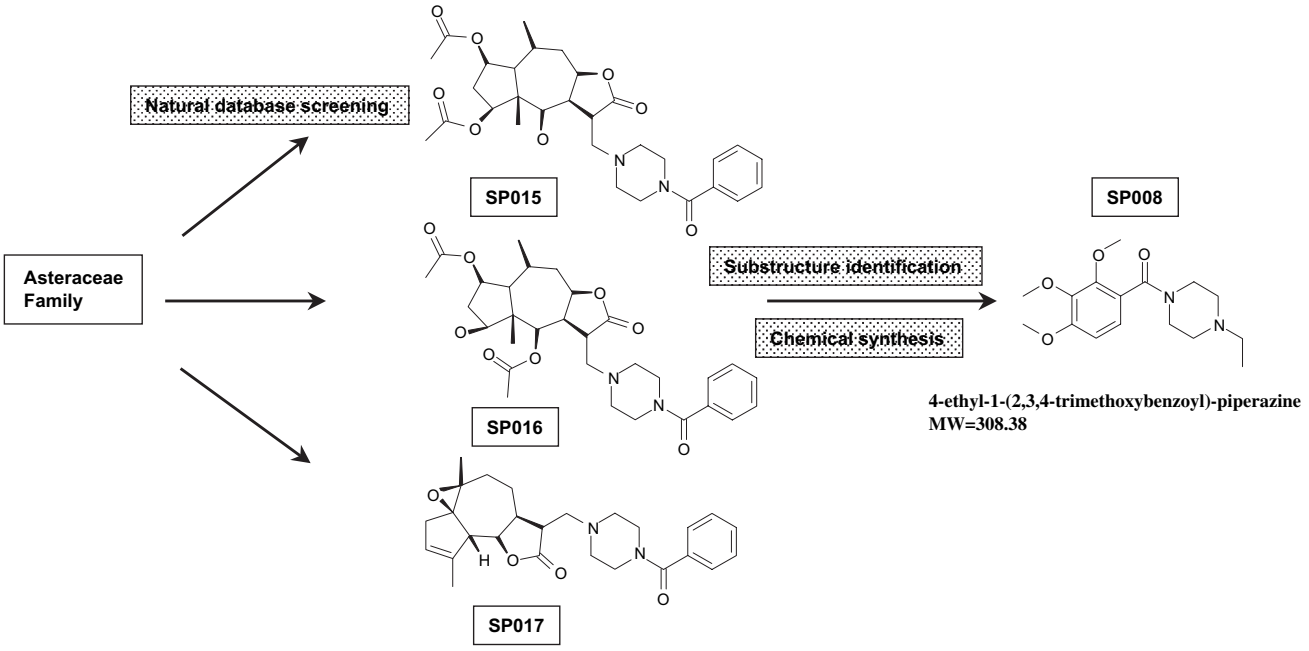


Fig. 5. Screening process and substructure isolation leading to SP008 identification.

the same concentration of $A\beta_{1-42}$ with SP015 given at 1 and 100 μM but not at 10 μM. In addition, SP016 did not exert any effect on the $A\beta_{1-42}$-induced ATP decrease. This apparent discrepancy suggests that the preservation of the intracellular ATP stock might not be the only mechanism by which these natural compounds exert their neuroprotective properties.

The involvement of an oxidative stress has been proposed as one of the fundamental pathogenic mechanisms occurring in AD (Volicer and Crino, 1990; Pappolla et al., 1996; Gotz et al., 1994; Tateyama et al., 2003), although its real importance remains unclear. Thus, we still do not know if the oxidative stress reported in AD is due to an increase of free radical production or to an increased sensitivity to an aging-related oxidative process. However, because in both cases it might be of interest to reduce the formation of the free radicals, we studied the implication of a possible anti-oxidant property in the neuroprotective activity of the compounds tested herein. Surprisingly, SP015 and SP017 displayed a free radical generating effect, which was unexpected considering the neuroprotective effect exhibited by these compounds. This observation was further confirmed by the total absence of a rescuing effect by the potent anti-oxidant PBN on PC12 cells despite a dramatic decrease of the $A\beta_{1-42}$-induced free radical production and by previous data reporting that free radicals and lipid peroxidation do not mediate the acutely induced neuronal cell death by $A\beta_{1-42}$ (Yao et al., 1999). Therefore, these data suggest that the neuroprotective effect of the naturally occurring derivatives is not mediated by any anti-oxidant effect.

Despite potentially interesting neuroprotective properties, these natural compounds had the major disadvantage of displaying a toxic effect with increasing concentrations. We therefore tried to solve and bypass this main issue. The chemical structures of SP015, SP016 and SP017 share a common 4-ethyl-1-benzoyl-piperazine substructure. The neuroprotection obtained with SP015 and SP017 and the preservation of the ATP cellular stocks induced by SP015, SP016 and SP017 against $A\beta_{1-42}$ led us to hypothesize that this common substructure might be responsible, at least in part, for the "anti-amyloid" effects we report herein for these natural compounds. We modified this substructure to obtain a two-step chemical synthesis of the 4-ethyl-1-(2,3,4-trimethoxybenzoyl)-piperazine compound, which we named SP008. SP008 exhibited significant

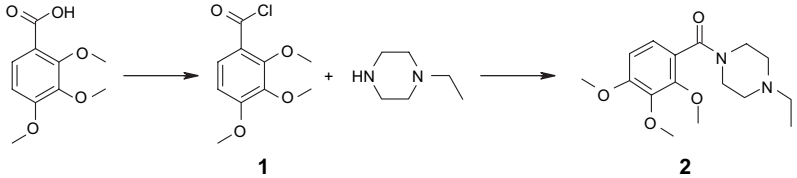


Fig. 6. SP008 chemical synthesis.

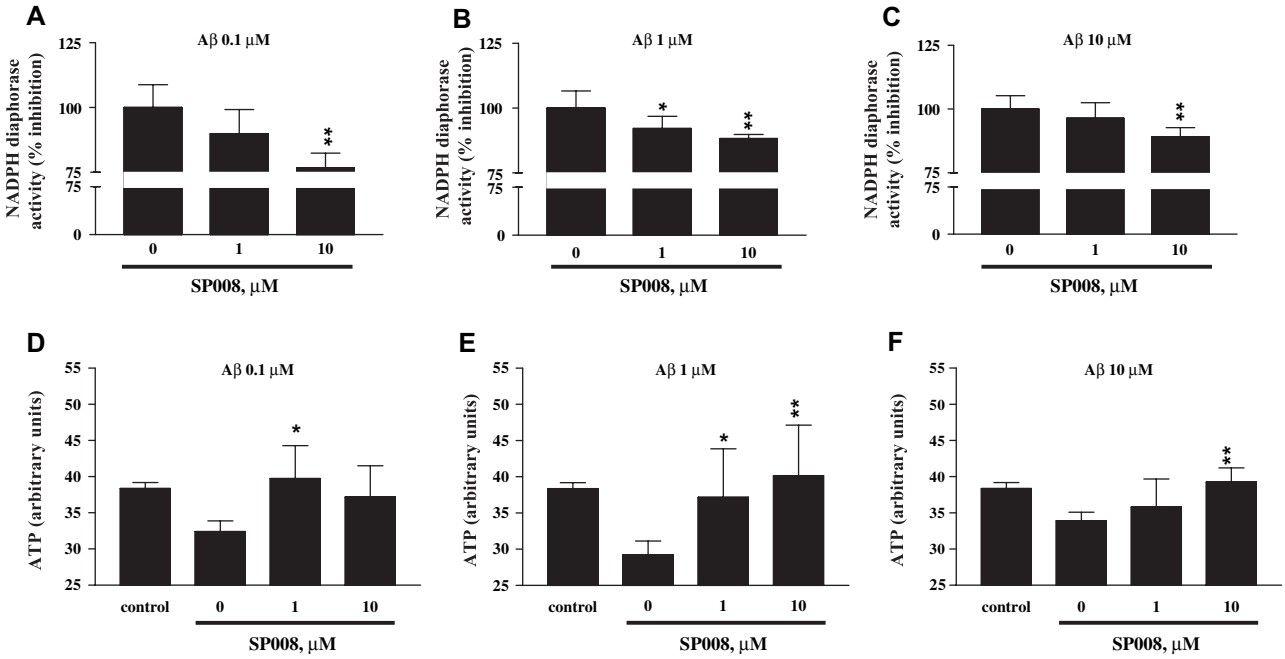


Fig. 7. Effect of SP008 on cell viability and $A\beta_{1-42}$-induced ATP depletion on PC12 cells. PC12 cells were pre-incubated with increasing concentrations of SP008 for 24 h before being exposed to increasing concentrations of $A\beta_{1-42}$ for 24 h. Cell viability was assessed using the MTT assay (A,B,C) and the free radical production was measured using the fluorescent probe 2,7-DCF (D,E,F). Cell viability results are presented as inhibition of the NADPH-diaphorase activity, considering that 100% inhibition corresponds to the effects obtained with $A\beta_{1-42}$. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Results shown are means ± SD, $n=6$. *$p<0.05$, **$p<0.01$ compared to 0 μM group.

neuroprotective properties against $A\beta_{1-42}$. SP008 displayed an interesting dose–effect relationship against 10 μM $A\beta_{1-42}$ predicting a lack of toxicity at high concentration compared to SP017, the most potent natural compound of the series. The beneficial effect of SP008 on PC12 viability was further confirmed by its ability to prevent the $A\beta_{1-42}$-induced intracellular ATP stock depletion even against 10 μM $A\beta_{1-42}$.

The disruption of calcium homeostasis is one of the hypotheses that have emerged in an attempt to explain the pathophysiology of AD (Kachaturian, 1987). Previous reports have clearly indicated that $A\beta_{1-42}$ increases intra-neuronal calcium concentrations leading to an excitotoxicity-type neuronal death (Olney et al., 1997) and the intracellular calcium increase seems to have multiple extra- and intracellular origins. In particular, the glutamatergic network is targeted by the β-amyloid peptides since $A\beta_{1-40}$ (Wu et al., 1995) and $A\beta_{25-35}$ (Mogensen et al., 1998) have been described to selectively augment NMDA-receptor-mediated synaptic transmission in rat hippocampus. Interestingly, the NMDA receptor antagonist MK-801

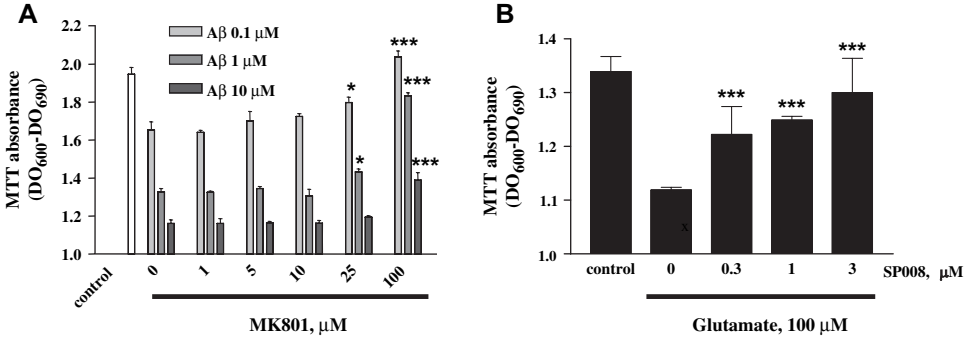


Fig. 8. Assessment of an anti-glutamatergic effect of SP008 in the protective activity displayed against the $A\beta_{1-42}$-induced neurotoxicity. (A) PC12 cells were pre-incubated for 24 h with increasing concentrations of (+)-MK801 before being exposed for 24 h to increasing concentrations of $A\beta_{1-42}$. Cell viability was assessed using the MTT assay. Control cells treated with vehicle only. (B) PC12 cells were pre-incubated with increasing concentrations of SP008 for 24 h before being exposed to 100 μM glutamate for 24 h. Cell viability was assessed using the MTT assay. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Results shown are means ± SD, $n=6$. *$p<0.05$, ***$p<0.001$ compared to 0 μM.

protected cholinergic nucleus basalis neurons and striatal neurons from amyloid peptides neurotoxicity in vivo (Parks et al., 2001; Harkany et al., 1999) and in vitro on neuroblastoma cells whereas AP-5, which binds specifically the glutamate site, did not (Le et al., 1995). These results led these authors to conclude that amyloid peptides might act more by stabilizing the opening state of the NMDA-associated calcium channel after inserting the plasma membrane rather than by directly binding the glutamate site. In our study, MK-801 reduced the neurotoxicity induced by $A\beta_{1-42}$ in a dose-dependent manner, suggesting therefore the involvement of an over-stimulation of the NMDA receptors in the neurotoxicity we report herein. SP008 was able to dramatically reduce the glutamate-induced neurotoxicity on PC12 cells in a dose-dependent manner even when given at concentrations as low as 0.3 μM, which probably accounts for its neuroprotective effect against $A\beta_{1-42}$. Although the possible blockade of the NMDA receptor needs to be clarified, these data suggest that the inhibition of the NMDA-induced calcium inward current might account for the protective effect provided by SP008 in our experiments, sharing therefore some pharmacological mechanisms with memantine, the NMDA-antagonist in use as an AD treatment.

AD is an evolving neurodegeneration involving many pathological pathways and for which there is not yet an available cure. The screening of a natural compounds database, using procaine as a starting point, allowed us to isolate natural compounds extracted from plants, which were historically known to be used against cognitive performance decline, in particular from the *Artemisia* genus. The identification of a common chemical substructure shared by these protective, but cytotoxic at high concentration, naturally occurring compounds led to the synthesis of SP008. SP008 exhibited a strong anti-amyloid activity and did not display the toxicity at high concentration that has been exhibited by SP017 and from which SP008 was derived. Moreover, SP008 was able to prevent $A\beta_{1-42}$-induced ATP depletion and inhibited the glutamate-induced excitotoxicity on PC12 cells. Therefore, our results suggest that there might be some interest in developing such an attractive compound and to use it as a starting point to synthesize a series of analogs. However, a wise caution should prevail as we are aware that further studies are required to establish the complete pharmacological profile of SP008 and to definitively determine if this compound is an interesting drug candidate/lead compound to be developed as an AD treatment.

Acknowledgements

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