

NATURALLY OCCURRING 22R-HYDROXYCHOLESTEROL DERIVATIVES PROTECT NEURONAL CELLS AGAINST β -AMYLOID (1-42) TOXICITY

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Abstract

22R-hydroxycholesterol is an intermediate in the steroid biosynthesis pathway shown to be present in decreased levels in Alzheimer's disease brain specimens compared to those from healthy age-matched patients (Yao and Papadopoulos, *J Neurochem*, 2002). This steroid was then shown to exhibit a neuroprotective property against β -amyloid(1-42) (A β) toxicity in rat PC12 and human NT2 neuronal cells by binding and inactivating the peptide (Yao and Papadopoulos, *J Neurochem*, 2002). In search of potent 22R-hydroxycholesterol derivatives, we assessed the ability of a series of naturally occurring entities containing the 22R-hydroxycholesterol structure to protect PC12 cells against A β -induced neurotoxicity. Sixteen compounds sharing a common spirost-5-en-3-ol or a furost-5-en-3-ol structure were tested. Although some of these compounds exhibited neuroprotective activity against 0.1 μ M A β , only three protected against the 1-10 A β -induced toxicity. These three entities shared a common structural feature, a long chain ester in position 3, which could be the determinant factor for sustained neuroprotection. The neuroprotective property of these 22R-hydroxycholesterol derivatives was coupled to their ability to displace radiolabeled 22R-hydroxycholesterol from A β , examined using a cholesterol-protein binding blot assay, suggesting that the A β -22R-hydroxycholesterol physicochemical interaction contributes to the beneficial effect of these compounds. Computer docking simulations of 22R-hydroxycholesterol and its derivatives on A β was accomplished using Monte Carlo simulated annealing implemented in modified versions of Autogrid/Autodock. This method identified two 22R-hydroxycholesterol binding sites on A β . Chemical entities which, as 22R-hydroxycholesterol, do not have the long ester chain in position 3, seem to bind preferentially only to one site. In contrast, the presence of this ester chain seems to confer the ability to bind to both sites on A β . Taken together, the *in vitro* and computational simulations studies suggest that occupancy of both binding sites on A β is required for neuroprotection against high concentrations of A β . The relevance of the second binding site as a potential therapeutic target remains to be determined. In conclusion, these results suggest that spirost-5-en-3-ol- and furost-5-en-3-ol naturally occurring derivatives of 22R-hydroxycholesterol might offer a new approach for Alzheimer's disease therapy.

Background

- 22R-hydroxycholesterol levels are lower in hippocampus and cortex from AD brain specimens compared to age-matched controls
- 22R-hydroxycholesterol protects rat PC12 and human NT2N cells against A β_{1-42} toxicity
- 22R-hydroxycholesterol exerts its neuroprotective effect by binding and inactivating A β_{1-42} (Yao et al., 2002; *J. Neurochem*, 85:1110-1119)

Materials and methods

Cells culture Rat pheochromocytoma PC12 cells were cultured (8x10⁴ cells/well) in 96-well plates in RPMI 1640 without glutamine medium supplemented with 5% horse serum and 10% FBS. After an overnight period to let them attach, PC12 were incubated with A β_{1-42} with or without SP compounds for 3 days before being assayed for various viability assays.

22R-hydroxycholesterol derivatives-protein binding blot assay (CPBBA) Purified A β_{1-42} protein (50 μ M) and 3H-22R-hydroxycholesterol were incubated either alone or in the presence of increasing concentrations of 100 μ M unlabeled 22R-hydroxycholesterol (SP222) or its derivatives (SP223-238) in 20 μ l volume for 8 or 24 h at 37°C. At the end of the incubation time, samples were separated by 1.5% agarose (Type I-B) gel electrophoresis under native conditions and transferred to nitrocellulose membrane in 10XSSC buffer. The membrane was exposed to tritium-sensitive screen and analyzed by phosphorimaging using the Cyclone Storage phosphor system (Packard BioScience). Image-densitometric analysis was performed using the OptiQuant software (Packard). This method allows for the separation, visualization and identification of AB complexes, which have incorporated radiolabeled cholesterol (Yao and Papadopoulos, 2002; FASEB J 16:1677-1679) and 22R-hydroxycholesterol or SP222 derivatives under native conditions. Low molecular weight unincorporated 22R-hydroxycholesterol and derivatives are separated and eliminated during electrophoresis.

Peptide modeling and SP222 derivatives docking Computer docking of SP222 and 16 of its derivatives with A β_{1-42} was accomplished using an A β structure initialized by the solution structure of A β_{1-40} Met(O) (MMDB Id: 7993 PDB Id:1BA) resulting from data generated by CD and NMR spectroscopy (Watson et al., 1998). The Met(O) SME 35 residue was replaced by Met retaining the adjacent backbone dihedral angles and the I41 and A42 residues appended. The energy of the structure was then minimized using the Alchemy 2000 program (Tripos Inc.). The SP222 derivative structures were also developed using Alchemy 2000. The docking was accomplished using Monte Carlo simulated annealing as previously described (Li et al., 2001) implemented in modified versions of Autogrid/Autodock (Morris et al., 1998). For each of the derivative A β_{1-42} pairs approximately 10⁸ conformations were evaluated to obtain the selected one of minimum energy. Three sessions consisting of 100 runs, each starting at a random initial relative location and orientation of the ligand with respect to the target were executed. Each run was comprised of 100 annealing cycles using about 2 x 10⁴ improvement steps.

Statistical analysis Statistical analysis was performed by one-way analysis of variance (ANOVA) and unpaired Student's t test using the INSTAT 3.00 (GraphPad, San Diego, CA).

References

- Yao and Papadopoulos, 2002; FASEB J 16:1677-1679
 Yao et al., 2002; *J. Neurochem*, 85:1110-1119
 Watson et al., 1998; *Biochem*, 37:1270-1276
 Li et al., 2001, *PNAS* 98: 1267-1272
 Morris et al., 1998, *J. Comput. Chem*, 19: 1639-1662

Conclusion

Many drugs are actually used in an attempt to treat Alzheimer's disease including acetylcholine-esterase inhibitors, COX-1 and COX-2 inhibitors, statins, antioxidants and brain vessels vasodilators. Unfortunately, up to now, slowing down the progression of neurodegeneration and of the associated symptoms is the best that can be done. We report herein the protective properties of a natural steroid present in *Gynura japonica* (asteraceae) against A β_{1-42} , the neurotoxic peptide accumulating in Alzheimer's disease brain. Our results indicate that naturally occurring spirostanol compounds may be of interest to protect neuronal cells against A β_{1-42} . The neuroprotective activity of SP233 may be due, at least in part, to its 22R-hydroxycholesterol-like ability to bind and inactivate the A β_{1-42} . Moreover, SP233 is a more potent A β_{1-42} inactivator compared SP222. This potency seems to be related to the existence of a second binding site on A β_{1-42} for this compound but this preliminary result remains to be validated. SP233 may also have others properties which might contribute to the neuroprotection displayed by this compound. Further *in vitro* and *in vivo* studies are required to precise its exact pharmacology. However, the data presented suggest that SP233 might offer a new therapeutic means in the treatment of Alzheimer's disease.

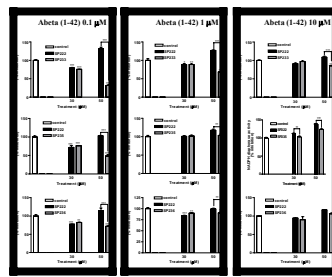


Fig 1: Cytoprotective effect of SP222 derivatives against A β (1-42) on PC12 cells assessed by MTT test. * p<0.05 ** p<0.01 *** p<0.001

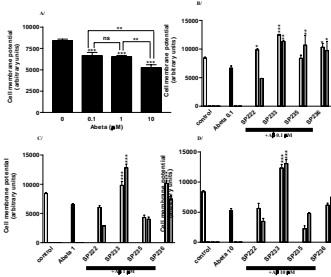


Fig 2: Cytoprotective effect of SP222 derivatives against A β (1-42) on PC12 cells assessed by Cytalix™ kit assay. * p<0.05 ** p<0.01 *** p<0.001

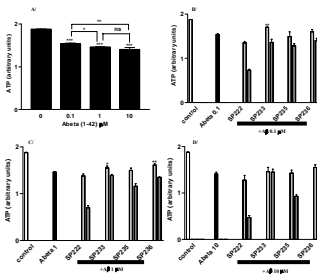


Fig 3: Effect of SP222 derivatives on A β (1-42)-induced intracellular ATP decrease on PC12 cells. * p<0.05 ** p<0.01 *** p<0.001

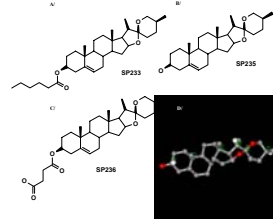


Fig 4: This figure shows the common stereochemistry displayed by the 3 protective SP compounds. The stereochemistry of the different asymmetric carbons is 3R, 10R, 13S, 20S, 22S, 25S.

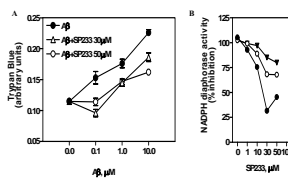


Fig 5: Protective effect of SP233 on A β (1-42)-induced toxicity on rat PC12 neuronal cell. A: PC12 cells were treated for 72 h with increasing concentrations of A β (1-42) in the presence or absence of either 30 or 50 μ M SP233. Levels of viability were measured using the trypan blue assay as described under Experimental procedures. B: PC12 cells were treated for 72 h with increasing concentrations of A β (1-42) in the presence or absence of the indicated concentrations of SP233. Levels of viability were measured using the MTT assay as described under Materials and Methods. A β (1-42): 0.1 nM (black circle), 1 nM (open circle), 10 nM (black triangle). Results shown are mean \pm SD (n=6-12). These results confirmed the data described above with MTT assay and Cytalix™ assay.

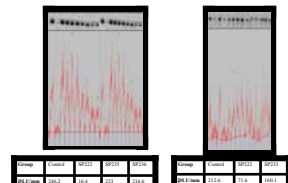


Fig 6: 22R-hydroxycholesterol derivatives-protein binding blot assay. As shown by these electrophoresis of the different SP-A β complexes, SP233-236 and 233 displaced SP222 from its bound with A β (1-42) showing their respective ability to bind A β (1-42). The intensity of this displacement seems to correlate with the intensity of the neuroprotective properties displayed by the different SP compounds.



Fig 7: A β peptide modeling and SP222 derivative docking simulations. A second binding site different from the SP222 binding site seems to exist on A β (1-42). This site looks more specific for the 3-position long chain substituted compounds like SP233 and SP236. Although further investigations are needed to confirm the increased protective effect of SP233 and SP236 seems to be related to their capacity to bind this second site on A β (1-42).

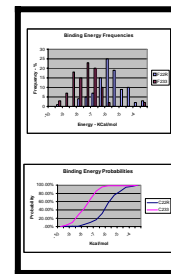


Fig 8: Comparison of Binding Energies of SP222 and SP233 with A β_{1-42} . This is an analysis of 100 docking runs with each of the compounds. The data shows that about 23% of the time SP233 docks with energy of -7.0 to -7.5 kcal/mol while SP222 docks about 25% of the time with only -5.5 to -6.8 kcal/mol. The probability of SP233 having a stronger (more negative) docking energy is significantly greater than that for SP222. Almost 100% of the time SP233 binds with less than -6 kcal/mol while the equivalent number for SP222 is only about -4 kcal/mol. Both distributions may indicate that they are bimodal perhaps indicating two sites. For SP233 we may have peaks both at -7 to -7.5 and -8 to -8.5 while with SP222 we may have peaks at -5.5 to 6.8 and -6.8 to -4.5. Whether these peaks are associated with A β_{1-42} sites has yet to be determined.