The melatonin-*N,N*-dibenzyl(*N*-methyl)amine hybrid ITH91/IQM157 affords neuroprotection in an *in vitro* Alzheimer's model via Hemo-oxygenase-1 induction

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ABSTRACT: We have investigated the protective effects of ITH91/IQM157, a hybrid of melatonin and *N,N*-dibenzyl(*N*-methyl)amine, in an *in vitro* model of AD-like pathology that combines amyloid beta (A β) and tau hyperphosphorylation induced by okadaic acid (OA), in the human neuroblastoma cell line SH-SY5Y. Combination of sub-toxic concentrations of A β and OA caused a significant toxicity of 40% cell death, which mainly was apoptotic; this effect was accompanied by retraction of the cell's prolongations and accumulation of thioflavin-S stained protein aggregates. In this toxicity model, ITH91/IQM157 (1-1000 nM) reduced cell death measured as MTT reduction; at 100 nM, it prevented apoptosis, retraction of prolongations and β A aggregates. The protective actions of ITH91/IQM157 were blocked by mecamylamine, luzindol, chelerythrine, PD98059, LY294002 and SnPP. We show that the combination of melatonin with a fragment endowed with AChE inhibition in a unique chemical structure, ITH91/IQM157, can reduce neuronal cell death induced by A β and OA by a signaling pathway that implicates both nicotinic and melatonin receptors, PKC, Akt, ERK1/2 and induction of hemoxygenase-1.

Keywords: SH-SY5Y, Okadaic acid, beta-amyloid, Melatonin, Alzheimer's disease, Acetylcholinesterase inhibitor, ITH91/IQM157, neuroprotection

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. There are about 27 millions of patients in the world and this figure could increase to 107 million by the year 2050 if no treatment is found to delay the onset or the progression of the disease (1). Therefore, the development of an effective treatment is a social, economic and political global priority.

From a histopathological point of view, AD is characterized by two protein alterations, namely tau hyperphosphorylation and excessive amyloid beta ($A\beta$) deposition, both related to the neuronal degeneration (2-4.) This neurodegenerative process affects the cholinergic system, among others. Therefore, acetylcholinesterase inhibitors are the main drugs used today to treat these patients. For later stages of the disease, inhibition of NMDA receptors with memantine is also used. A meta-analysis for commercially available acetylcholinesterase inhibitors (AChEI) and memantine in combination for the treatment of patients with AD revealed only a modest trend favoring active treatment over placebo (5). Therefore, the search for new compounds to treat this disease is still mandatory.

The use of multitarget compounds is emerging as an interesting strategy to treat different pathologies. These compounds combine, in a single molecule, complementary activities over different pathways of the pathophysiological cascade of AD. More specifically, our group has become interested in compounds that combine fragments derived from an inhibitor of acetylcholinesterase (AChEI) and melatonin for the following reasons: (i) AChEI are the drugs mainly used in clinic to treat AD patients; their mechanism of action is based on the improvement of cholinergic neurotransmission, (ii) the levels of the neurohormone melatonin, endowed with antioxidant properties (6), are gradually reduced with age. In the cerebral spinal fluid

(CSF), melatonin levels can be reduced by 50% when compared to young subjects; this reduction is even greater in AD patients (below 20%)(7-9). It is also worth mentioning that hippocampal CA1 and CA3 pyramidal neuronal loss can be reproduced in rats by removing their pineal gland, while replacement of melatonin in the drinking water recovers such loss (10). Furthermore, melatonin has shown neuroprotective effect in several AD models (8, 11-14), and it has also shown beneficial effects in a double blind study on the sleep-wake rhythm, cognitive and non-cognitive functions in Alzheimer type dementia (15). For all these reasons, melatonin could be beneficial in AD (16) and, (iii) previous results from our group have shown that the combination of subeffective concentrations of galantamine and melatonin offer a significant neuroprotective effect in SH-SY5Y cells against mitochondrial intoxication with rotenone and oligomycin A(17). With these ideas in mind, we synthesized several melatonin – *N,N*-dibenzyl(*N*-methyl)amine hybrids (18); the idea of keeping the AChEI activity, even if modest, was based on the fact that this target remains clinically valid for the majority of drugs (donepezil, rivastigmine and galantamine) used today in AD patients. In this study we have focused on ITH91/IQM157 (**Fig 1**) that shares chemical features of melatonin and the AChEI AP2238, has low toxicity, is capable of crossing the blood brain barrier in a predictive model and has an interesting pharmacological profile with potential for the treatment of AD. It inhibits human AChE ($IC_{50} = 4.1 \mu M$), displaces propidium from the peripheral anionic site of AChE (25% at $1.0 \mu M$), presents antioxidant properties (ORAC = 1.5 trolox equiv.) and protects neural cells against mitochondrial free radicals (26% at $1.0 \mu M$) (18).

Figure 1. Chemical structure of compound ITH91/IQM157, a melatonin – *N,N*-dibenzyl(*N*-methyl)amine hybrid.

As mentioned earlier, there are two characteristic histopathological features in postmortem brains of patients suffering AD: senile plaques caused by accumulation of β A peptide and neurofibrillary tangles composed of hyperphosphorylated tau protein. It is also proposed that these alterations are not independent, but are interrelated (19, 20). Although there are several *in vivo* models that combine β A pathology with tau pathology, such as the double transgenic mice APPswe/TauVLW (21) or the triple transgenic PS1M146V, APPswe, and TauP301L (22), virtually no *in vitro* models combine these two alterations. Therefore we have implemented an *in vitro* model that combines beta and tau pathology by combining A β ₂₅₋₃₅ and okadaic acid in the human neuroblastoma cell line SH-SY5Y. We have used this model to evaluate the potential neuroprotective effects of the melatonin – *N,N*-dibenzyl(*N*-methyl)amine hybrid ITH91/IQM157.

RESULTS AND DISCUSSION

In order to set up the cytotoxicity model, we first performed concentration-response curves with A β and okadaic acid in the human neuroblastoma cell line SH-SY5Y. Okadaic acid (OA), a phosphatase inhibitor that causes hyperphosphorylation of tau protein (23), was more effective to induce cell death than A β ; in fact, maximum cell death achieved with A β was near 40 % (10 μ M β A, **Fig 2A**) while with OA, maximum

cell death reach over 80 % (30 nM OA, **Fig 2B**). Interestingly, when sub-effective concentrations of both stimuli (1 μ M of A β and 3 nM of OA) were combined, we observed a significantly higher cytotoxic effect compared to each toxin alone (**Fig. 2C**). This result was corroborated in a primary neuronal culture, in which a similar toxicity was observed (**Supplemental data1**). Therefore, this result validates the use of a neuronal cell line instead of primary neuronal cultures which “Replaces” the use of animals.

Figure 2. β A₂₅₋₃₅ (β A) and okadaic acid (OA) reduce cell viability of SH-SY5Y cells: combination of subeffective concentrations of β A and OA cause significant cell death. Cells were incubated with the toxic stimuli for 20 h and cell viability was assessed by the MTT technique. (A) Shows the concentration-response curve with 1 μ M, 5 μ M and 10 μ M of β A. (B) Concentration-response curve with 3 nM, 10 nM and 30 nM of OA. (C) Effect of 1 μ M β A, 3 nM OA or their association on SH-SY5Y cell viability. Values are expressed as means $\pm$ SEM of 5 different cultures, ***P < 0.001, **P < 0.01 compared to basal; ###P < 0.001, ##P < 0.01, #P < 0.05 with respect to combination of both toxic stimuli.

When we analyzed the apoptotic and necrotic populations in SH-SY5Y cells exposed to A β (1 μ M) in combination with OA (3 nM) - from now on A β /OA-, we found that cell death was mainly apoptotic (**Fig. 4B**). These results are consistent with those described in animal models of AD where mutations associated with over-expression of A β protein and mutations associated with hyperphosphorylation of tau, are combined (22, 24); these animals show greater pathology and functional alterations in a more precocious way compared to mono-transgenics. Besides the effects on cell death, subtoxic concentrations of A β /OA caused neurite retraction (**Fig. 5B**), an effect related to tau hyperphosphorylation, which causes microtubule destabilization, cytoarchitecture loss and, consequently, neurodegeneration (3, 23). This degeneration and cell death is also reflected in the emergence of more pyknotic nuclei in cells treated with A β /OA. We also found aggregates of thioflavin S staining as an indication of A β aggregation (**Fig. 5E**). Taken together, by combining subtoxic concentrations of A β with OA, we have established a cytotoxicity model that displays several pathological markers of AD, such as neurite retraction, accumulation of protein aggregates and apoptotic cell death. This model could, therefore, serve as a new cytotoxicity model to evaluate compounds with potential interest in the screening stage of AD-compounds, before moving into the in vivo studies that are more expensive and more time consuming.

Having set the experimental conditions of toxicity induced A β /AO, we evaluated the potential cytoprotective effect of melatonin, the acetylcholinesterase inhibitor donepezil and the association of sub-effective concentrations of both. The experimental protocol consisted of pre-incubating SH-SY5Y cells for 24 hours with increasing concentrations of the neuroprotective compounds prior to the addition of the toxic stimuli (A β /OA) and, maintaining the protective compounds for an additional 20 h

period together with the toxins (see protocol on top of **Fig. 3**). Melatonin showed a significant protective effect at the concentration of 3 nM (35.8 % protection), and this protection increased in a concentration-dependent manner, being maximum at 10 nM (73 % protection) (**Fig. 3A**). We also evaluated the potential neuroprotective effect of donepezil; the range of concentrations was selected based on previous data from our group (Arias y col., 2005). As represented in **Fig. 3B**, donepezil was protective at concentrations ranging from 0.3 to 3 μ M; however, a concentration-dependent effect was not observed.

Figure 3

Figure 3. Combination of sub-effective concentrations of melatonin and donepezil provide synergic against β A/OA-induced toxicity. On the top part of the figure, a schematic representation of the protocol used is represented. Cells were exposed for 20 h to 1 β M β A plus 3 nM OA (β A/OA). When the neuroprotective compounds melatonin or donepezil were used, they were pre-incubated 24 h before adding the toxics. Effect of increasing concentrations of melatonin (A) and donepezil (B) on the cell viability of SHSY5Y cells exposed to the combination of β A/OA, measured as MTT reduction. (C) Synergic neuroprotective effect afforded by the association of sub-effective concentrations of both compounds. Data represent the mean $\pm$ SEM from 7 different cultures, ***P < 0.001 compared to basal, ###P < 0.001, ##P < 0.01, #P < 0.05 compared with β A/OA group.

To test the hypothesis that a significant neuroprotective effect could be achieved with the combination of sub-effective concentrations of melatonin and an AChEI, we used 1 nM of melatonin plus 10 nM of donepezil in the A β /OA toxicity model; indeed the drug combination afforded significant protection (57% protection) compared to the drugs alone (**Fig. 3C**).

Next, we evaluated the potential neuroprotective effect of the melatonin – *N,N*-dibenzyl(*N*-methyl)amine hybrid ITH91/IQM157. Compared to melatonin or the acetylcholinesterase inhibitor donepezil, the neuroprotective actions found with ITH91/IQM157 were achieved at lower concentrations; at 1 nM, ITH91/IQM157 already offered maximum protection (**Fig. 4A**). This hybrid improved the neuroprotective activity in comparison to the combination strategy of subeffective concentrations of melatonin (1 nM) and donepezil (10 nM); protection was 75 % with 1 nM ITH91/IQM157 *vs* 57% with the combination strategy (**Fig. 3C**). This finding agrees with our previous observation that combination of subeffective concentrations of melatonin and the AChEI galantamine offers significant neuroprotection (17).

There are several potential advantages for a multifunctional molecule *vs* combination of different drugs covering the same mechanisms. First, association of several drugs may have different pharmacodynamics and pharmacokinetics; however, when a single molecule is developed, these properties can be optimized. Second, when two or more drugs are combined, frequently, there are complex pharmacological interactions that modify the effect of the other, giving increased secondary effects or reducing the effectiveness of one or more of the combined molecules. Finally, drugs directed to a single target might not always modify complex systems, even if they act in the way they are expected to precede. It is very common in the cell to have “back-up” systems yielding the same effect such as gene expression, protein synthesis, receptors

response and protein degradation. Proteins and intermediates involved in these back-up systems can be completely different and therefore, drugs targeting primary pathways will have no effect over this back-up pathway, an effect known as redundancy. Multi target therapeutics can be more efficacious making the biological system more sensitive to the action of a drug with two or more targets simultaneously, thereby, mitigating the redundancy effect. Therefore, the complexity of interactions in the drug-combination approach has led to the hypothesis that one single molecule, acting on several targets at the same time, might be more effective for the drug development in complex diseases like AD.

Figure 4. ITH91/IQM157 is neuroprotective against β A/OA toxicity by an anti-apoptotic mechanism. (A) Effect of increasing concentrations of ITH91/IQM157 on the cell viability of cells exposed to β A/OA. (B) Percentage of alive, apoptotic, and necrotic cells, measured by flow cytometry in control cells or cells exposed to β A/OA alone or in the presence of ITH91/IQM157 at 100 nM. Data correspond to the mean $\pm$ SEM of four different cell batches; *** $P < 0.001$ significantly different from basal apoptotic cell death. ### $P < 0.001$, ## $P < 0.01$ significantly different from β A/OA-induced apoptotic cell death.

Concerning the neuroprotective mechanism of action of ITH91/IQM157, both melatonin and nicotinic receptors seem to be implicated since both luzindole (a melatonin receptor antagonist) and mecamylamine (a nicotinic receptor antagonist) significantly reduced its protective effect (**Fig. 6A**). The involvement of nAChRs has also been implicated in the protective effects of other AChE inhibitors like galantamine and donepezil (25-27). The neuroprotective effect of ITH91/IQM157 was accompanied by the recovery of the cytoarchitecture and a reduction of thioflavin-S aggregates (**Fig. 5C and F**). The reduction of protein aggregates can be related to actions of the melatonin substructure, since it is reported that melatonin can directly interact with A β and prevent its aggregation (28, 29) and it can also interfere with APP processing (30-32). Furthermore, we previously reported that compound ITH91/IQM157 displaces propidium iodide from the peripheral acetylcholinesterase site, which is known to participate in β A aggregation (33). Interaction with MT₂ receptors can stimulate phospholipase C and activate protein kinase C (PKC) via diacylglycerol, which in turn phosphorylates and inactivates GSK-3 β , whose participation in APP synthesis (34, 35) and tau hyperphosphorylation are well documented; this could be an additional mechanism for compound ITH91/IQM157. In fact, the protective mechanism of ITH91/IQM157 was partially inhibited by the PKC inhibitor chelerythrine (**Fig. 6B**).

Figure 5. ITH91/IQM157 recovered cytoskeletal alterations and thioflavin-S aggregates induced by exposure of SH-SY5Y cells to β A/OA. Top part shows images of SH-SY5Y cells double stained with Hoechst 33342 (nuclei in blue) and phalloidin (cytoskeleton in red) under basal conditions (A) , treated with β A/OA in the absence (B) or presence of 100 nM of ITH91/IQM157 (C). Bottom figures show of SH-SY5Y cells double stained with Hoechst 33342 (nuclei in blue), phalloidin (cytoskeleton in red) and Thioflavin-S (β A aggregates in green) under basal conditions (D) or treated with β A/OA in the absence (E) or presence of 100 nM of ITH91/IQM157 (F). Images are representative of others obtained in 3 different cell batches.

Our group and others have shown that activation of melatonin and nicotinic receptors can promote survival pathways such as those related to PI3K/Akt and ERK1/2 (17, 36) 37). Indeed, ITH91/IQM157 increased phosphorylation of ERK1/2 and Akt (**Fig. 7A and 7B**) and its protective actions were prevented in the presence of inhibitors of these kinases (**Fig. 6B**). Akt can phosphorylate GSK-3 β at position Ser-9, inactivating it (38-40) and can improve neuronal survival by: (i) contributing to reduction of β A and tau pathology as mentioned above and/or (ii) promoting the nuclear

translocation of Nrf2 (nuclear factor E2-related factor 2) to increase the cell's defense mechanisms (41).

Figure 6. Neuroprotection elicited by ITH91/IQM157 involves melatonin receptors, nicotinic acetylcholine receptors, PI3K/Akt, ERK1/2, PKC and induction of HO-1. (A) The melatonin receptor antagonist luzindole (3 μ M) and the nAChR antagonist mecamylamine (10 μ M) partially block the protective action of ITH91/IQM157. Both antagonists per se had no effect on cell death caused by β A/OA. (B) The protective effect of ITH91/IQM157 is prevented by the PKC inhibitor chelerythrine (1 μ M), the PI3K/Akt antagonist LY294002 (10 μ M), the ERK1/2 antagonist PD98059 (10 μ M) and the HO-1 inhibitor Sn(IV) protoporphyrin IX dichloride (SnPP) (10 μ M). The antagonists per se had no effect on cell death caused by β A/OA. Values are means $\pm$ SEM of 7 experiments. ***P < 0.001 significantly different from untreated cells; ###P < 0.001, ##P < 0.01 in comparison to β A/OA; \$\$\$P < 0.001, \$\$P < 0.01, \$P < 0.05 with respect to ITH91/IQM157 treated cells.

Hemoxygenase-1 (HO-1) can be transcribed by Nrf2, it is an enzyme related to antioxidant, antineuroinflammatory and neuroprotective actions. Compound ITH91/IQM157 was capable of inducing *per se* HO-1 (**Fig 7C**), and, most interesting, its protective actions were prevented when an inhibitor of this antioxidant enzyme (SnPP) was added to the cells (**Fig. 6B**). These results indicate that part of its neuroprotective actions can be attributed to induction of HO-1 as already described for other neuroprotective drugs that interact with melatonin or nicotinic receptors (36, 42, 43).

Figure 7

Figure 7. ITH91/IQM157 increases ERK1/2 and Akt phosphorylation and induces the antioxidant enzyme HO-1. ERK1/2 phosphorylation with respect to total-ERK1/2 (A) and Akt phosphorylation with respect to total-Akt (B) was analyzed, by western blot, in SH-SY5Y cells treated for 60, 30 or 15 min with 100 nM ITH91/IQM157. The top part of the figures shows a representative immunoblot and the histogram below shows the mean densitometric quantification of both kinases. (C) HO-1 induction in cells treated for 24 h with ITH91/IQM157 at 100 nM. The top part of the figure illustrates a representative immunoblot and the bottom part an histogram with the densitometric quantification of HO-1 induction normalized with respect to β -Actin, under basal conditions or exposed to melatonin. Values correspond to the mean $\pm$ SEM of 5 experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ significantly different from untreated cells.

As a multifunctional drug, ITH91/IQM157 is endowed with different complementary mechanisms of action that could be useful to limit the complex physiopathological cascade of AD. One of those complementary actions, besides ACE inhibition and A β aggregation could be induction of HO-1 as part of its neuroprotective mechanism. In this study we have focused on HO-1 because this enzyme seems to participate in the protective action of drugs that have a similar mechanism to compound ITH91/IQM157, for example, melatonin or nicotinic agonists (Parada et al., 2013a; Parada et al., 2013b). Also induction of HO-1 by the ACEI galantamine has been related to protection of microvascular endothelial cells (Nakao et al., 2008). We see in this study that ITH91/IQM157 can induce HO-1 and that its protective actions are lost in the presence of the HO-1 inhibitor SnPP; this effect does not exclude the drug from having ACE inhibitory actions that could improve cognition or from reducing beta-amyloid aggregation that could contribute to reduce neuroinflammation and protecting neurons adjacent to the beta-amyloid plaques.

In conclusion, the melatonin-*N,N*-dibenzyl(*N*-methyl)amine hybrid ITH91/IQM157 reduces cell vulnerability as well as A β aggregates and disruption of the cytoskeleton in an *in vitro* AD-related model. The mechanism of action of ITH91/IQM157 involves melatonin and nicotinic receptors, activation of a signaling cascade that includes PKC, ERK1/2, PI3K/Akt and induction of the antioxidant and antineuroinflammatory enzyme HO-1; all of these actions can contribute to promote cell survival and, thereby prevent neurodegeneration.

MATERIALS AND METHODS

Materials

Amyloid beta ($A\beta_{25-35}$), okadaic acid (AO), chelerythrine, PD98059 (2-(2-amino-3-methoxyphenyl)-(4H-1-benzopyran-4-one)) and LY294002 (morpholino-4-yl-8-phenylchromen-4-one), mecamlamine, were from Tocris scientific/ Biogen, Madrid, Spain. Tin protoporphyrin (IV) from Frontier Scientific Europe, Lancashire, UK. Donepezil and melatonin was obtained from Sigma Aldrich, Madrid, Spain and ITH91/IQM157 was synthesized by the group of Dr. Rodríguez-Franco from the Instituto de Química Médica, Consejo Superior de Investigaciones Científicas (IQM-CSIC).

Culture of the human neuroblastoma cell line SH-SY5Y

SH-SY5Y cells were maintained in culture medium containing 10 % inactivated fetal bovine serum, 15 nonessential aminoacids, 1 mM sodium pyruvate (Invitrogen, Madrid, Spain), F12 nutrient medium (Ham12), MEM medium (Eagle's minimum essential medium) (Sigma Aldrich, Madrid, Spain), NaHCO_3 , 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin (Invitrogen, Madrid, Spain) in H_2O miliQ. Cells were grown initially in a flask and sub-cultured in 48-well plates at a density of 1×10^5 cells/well. Cells were maintained in an incubator in a humid atmosphere at 37°C with 5 % CO_2 ; they were used between 4-12 passages.

Measurement of cell viability using the MTT method

Cell viability was assessed by the detection of mitochondrial activity in living cells using the colorimetric analysis of Blue Tetrazolium Bromide Thiazolyl (MTT) (Sigma-Aldrich, Spain), previously described by Denizot.(44). Upon completion of the

experiments, 50 μ l of reagent MTT was added to each well to achieve a final concentration of 0.5 mg/ml; then, the cells were kept for 2 h in an incubator at 37 °C with 5% CO₂ and 95 % air. Finally, 200 μ l of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan salt and absorbance was measured in an ELISA reader at 540 nm. The absorbance obtained in basal conditions was taken as 100 % cell viability.

Measurement of apoptosis and necrosis with annexin V–phycoerythrin (PE) and 7-amino-actinomycin-D (7-AAD) by flow cytometry

Apoptosis was determined by flow cytometry using an annexin V–PE (phycoerythrin) and 7-AAD double staining kit (BD Bioscience, Madrid, Spain) according to the manufacturer's instructions. Briefly, at the end of the experiment, cells were collected after centrifugation and resuspended in a solution containing 100 μ l of 1 $\times$ binding buffer, 5 μ l annexin V–PE and 5 μ l 7-AAD. Cells were incubated at room temperature for 15 minutes in darkness and 100 μ l of 1 $\times$ binding buffer was added. Cells were then subjected to FACS analysis (Beckman Coulter, Madrid, Spain). Annexin V +/7-AAD– cells were considered as early apoptotic cells, annexin V +/7-AAD + as late apoptotic cells, and annexin V –/7-AAD– as viable cells.

Double staining of SH-SY5Y cells with phalloidin and Hoechst

We used phalloidin-rhodamin staining to detect the cellular cytoskeleton in our experimental conditions. Hoechst staining was concomitantly used to detect the nuclei. At the end of the experiment, SH-SY5Y cells were washed 3 times with PBS (NaCl 9 g/L, 10 mM NaH₂PO₄, 10 mM K₂HPO₄) and fixed with 2 % paraformaldehyde

dissolved in PBS for 15 min, permeabilized with 0,1% Triton in PBS for 1 min and stained with phalloidin-rhodamine in PBS 1:1000 (Sigma-Aldrich, Madrid, Spain) for 20 minutes. Later, the cells were washed 3 times with PBS every 5 min; staining of the nuclei with Hoechst (5µg/ml) was performed during the second wash (Invitrogen, Madrid, Spain). Finally, the slides were covered with coverslips adding glycerol-PBS (1:1 vol/vol) and imaged with a confocal microscope (TCS SPE, Leica, Wetzlar, Germany).

Triple staining of SH-SY5Y cells with Thioflavin-S, Hoechst and phalloidin

SH-SY5Y cells were fixed with 2 % paraformaldehyde dissolved in PBS for 15 min and washed 3 times with PBS every 5 min. Later, they were permeabilized with 0,1 % Triton for one minute and washed 3 times with PBS before staining them with Thioflavin-S 0.5 % for 10 minutes. Then, 3 consecutive washes with ethanol 80 %, mili-Q H₂O and PBS were performed. Later, cells were stained with phalloidin-rhodamine in PBS 1:1000 (Sigma-Aldrich, Madrid, Spain) for 20 minutes, followed by 3 washes with PBS every 5 min; staining of the nuclei with Hoechst (5µg/ml) was performed during the second wash (Invitrogen, Madrid, Spain). Finally, the slides were covered with coverslips adding glycerol-PBS (1:1 vol/vol) and imaged with a confocal microscope (TCS SPE, Leica, Wetzlar, Germany).

Measurement of protein expression by Western-Blot

SH-SY5Y cells were lysed with 100 µl of cold lysis buffer containing: 1 % Nonidet P-40, 10 % glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate and 1 mM Na₃VO₄. Once the amount of protein was quantified using

the BCA Protein Assay Kit Reagent (Fisher Scientific, Madrid, Spain), electrophoresis was performed running 30 µg of proteins in polyacrylamide gels (PAGE) for 2 hours at constant amperage. Proteins were transferred to PVDF membranes (Millipore Ibérica SA, Madrid, Spain) for 2 hours at 70 mA. Later on, membranes were blocked for two hours with TTBS + 4 % albumin (Sigma-Aldrich, Madrid, Spain), incubated with anti-P-Akt, anti-total Akt (Santa Cruz Biotechnology, Santa Cruz, CA , USA), anti-P-ERK, anti-total ERK, anti-HO-1 (1:1000) (Chemicon, Temecula, CA, USA) and anti-β actin (1:10,000) (Sigma-Aldrich, Madrid , Spain) for 2 hours. After washing several times with TTBS, the corresponding secondary antibodies (1:100,000) were added (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 minutes. Finally, the membranes were revealed using ECL Advance Western Blotting Detection Kit (GE Healthcare, Barcelona, Spain) and quantified by Scion-Image software.

Statistical analysis

Data are presented as means ± SEM. Differences between groups were determined by applying a one-way ANOVA followed by a Newman–Keuls post hoc analysis. The level of statistical significance was taken at $p < 0.05$.

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SUPPORTING INFORMATION

SI-1- Effect of 1 μ M A β , 3 nM OA or their association on primary neuronal cell culture viability. Values are expressed as means $\pm$ SEM of 4 different cultures, ***P < 0.001 compared to basal; ###P < 0.001, ##P < 0.01, with respect to both toxic stimuli combined. This information is available free of charge via de internet at <http://pubs.acs.org/>

SI-2- Concentration response curves of compounds ITH90/IQM156 and ITH91/IQM157 on the viability of SH-SY5Y cells exposed to A β /OA. Values are expressed as means $\pm$ SEM of 4 different cultures, ***P < 0.001 compared to basal; ###P < 0.001, ##P < 0.01, #P < 0.05 with respect to both toxic stimuli alone. As shown, compound ITH91/IQM157 (compound 4 in the former JMC paper by López-Iglesias et al. 2014) showed a higher potency than compound ITH90/IQM156 (compound 3 in the former JMC paper by López-Iglesias et al. 2014), so we selected compound ITH91/IQM157 instead of ITH90/IQM156 :This information is available free of charge via de internet at <http://pubs.acs.org/>

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Author contribution

I.B. has contributed to concept/design, acquisition of data and data analysis/interpretation and critical revision of the manuscript. J.E. has contributed to concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article. E.P. has contributed to acquisition of data and data analysis/interpretation. E.N. has contributed to acquisition of data and data analysis/interpretation. R.L. has contributed to acquisition of data, data analysis/interpretation and critical revision of the manuscript.

M.I.R.F. has contributed to chemical synthesis of compounds ITH90/IQM156 and ITH91/IQM175 and critical revision of the manuscript. M.G.L. has contributed to concept/design, drafting of the manuscript, critical revision of the manuscript and approval of the article.

Notes

All authors of this work declare that there are no conflicts of interest

Non-standard abbreviations: AD, Alzheimer disease; A β , amyloid beta; AO, Okadaic acid; AChEI, Acetylcholine esterase inhibitors; $\alpha 7$ nAChRs: $\alpha 7$ nicotinic Acetylcholine Receptors; HO-1: Heme oxygenase 1; MTT, Blue Tetrazolium Bromide Thiazolyl; DMSO, Dimethyl sulfoxide; PKC, Protein kinase C; PI3K, phosphatidylinositol 3-kinase; LY, LY294002 (morpholino-4-yl-8-phenylchromen-4-one); PD, PD98059 (2-(2-amino-3-methoxyphenyl)-(4H-1-benzopyran-4-one); SnPP, Tin (IV) protoporphyrin IX dichloride

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