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The Melatonin–*N*,*N*-Dibenzyl(*N*-methyl)amine Hybrid ITH91/IQM157 Affords Neuroprotection in an in Vitro Alzheimer's Model via Hemoxygenase-1 Induction

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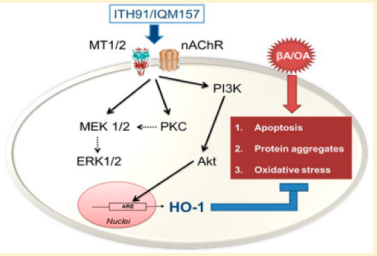
Supporting Information

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 Alzheimer's disease (AD)-like pathology that combines amyloid beta (Aβ) and tau hyperphosphorylation induced by okadaic acid (OA), in the human neuroblastoma cell line SH-SY5Y. Combination of subtoxic concentrations of Aβ and OA caused a significant toxicity of 40% cell death, which mainly was apoptotic; this effect was accompanied by retraction of the cells' 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 Aβ 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 Aβ 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, acetylcholinestaerase inhibitor, ITH91/IQM157, neuroprotection*

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 (Aβ) deposition, both related to 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

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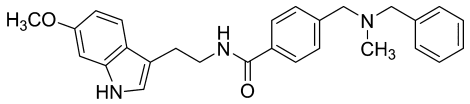
neuroprotective effect in several AD models,[8,11−14] and it has also shown beneficial effects in a double blind study on the sleep−wake rhythm and cognitive and noncognitive functions in Alzheimer's type dementia.[15] For all these reasons, melatonin could be beneficial in AD.[16] (iii) Previous results 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 (Figure 1) that shares chemical features of melatonin and the



ITH91 / IQM-157

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

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 μM), displaces propidium from the peripheral anionic site of AChE (25% at 1.0 μM), presents antioxidant properties (ORAC = 1.5 trolox equiv), and protects neural cells against mitochondrial free radicals (26% at 1.0 μM).[18]

As mentioned earlier, there are two characteristic histopathological features in post-mortem brains of patients suffering from AD: senile plaques caused by accumulation of βA 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 βA pathology with tau pathology, such as the double transgenic mice APPswe/TauVLW[21] or the triple transgenic PS1M146 V, 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 $A\beta_{25-35}$ 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 DISCUSION

In order to set up the cytotoxicity model, we first performed concentration−response curves with Aβ and okadaic acid in the human neuroblastoma cell line SH-SY5Y. Okadaic acid (OA), a phosphatase inhibitor that causes hyperphospholylation of tau protein,[23] was more effective to induce cell death than Aβ; in fact, maximum cell death achieved with Aβ was near 40% (10 μM βA, Figure 2A), while with OA maximum cell death reach over 80% (30 nM OA, Figure 2B). Interestingly, when subeffective concentrations of both stimuli (1 μM of Aβ and 3 nM of OA) were combined, we observed a significantly higher cytotoxic effect compared to each toxin alone (Figure 2C). This result was corroborated in a primary neuronal culture, in which a similar toxicity was observed (Supporting Information SI-1). Therefore, this result validates the use of a

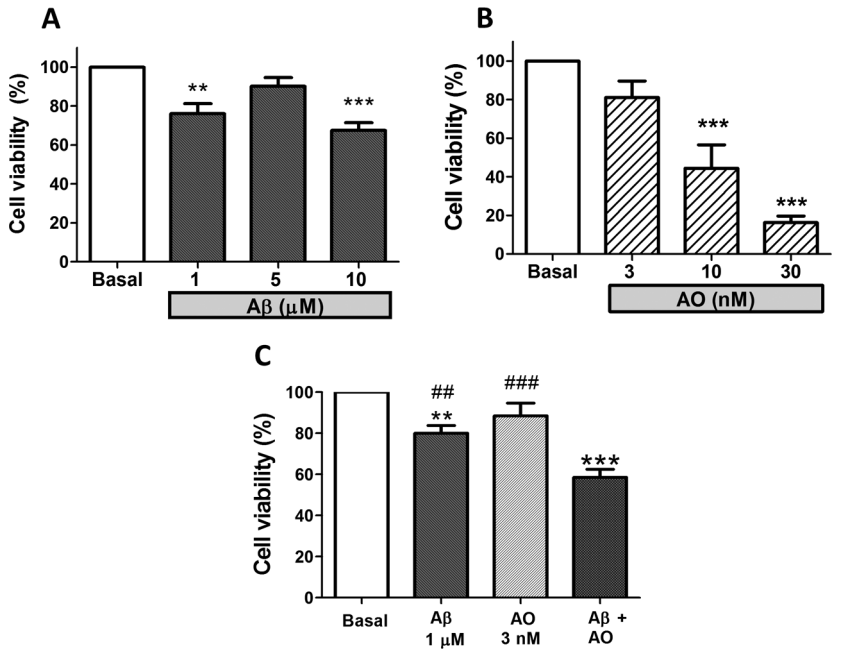


Figure 2. $A\beta_{25-35}$ (βA) and okadaic acid (OA) reduce cell viability of SH-SY5Y cells: combination of subeffective concentrations of Aβ and OA cause significant cell death. Cells were incubated with the toxic stimuli for 20 h, and cell viability was assessed by the MTT technique. (A) Concentration−response curve with 1, 5, and 10 μM Aβ. For reasons that we do not completely understand, at 5 μM Aβ, we did not achieve a significant reduction in cell viability. (B) Concentration−response curve with 3, 10, and 30 nM OA. (C) Effect of 1 μM Aβ, 3 nM OA, and their association on SH-SY5Y cell viability. Values are expressed as means ± SEM of five different cultures, $^{***}P < 0.001$, $^{**}P < 0.01$ compared to basal; $^{###}P < 0.001$, $^{##}P < 0.01$ with respect to combination of both toxic stimuli.

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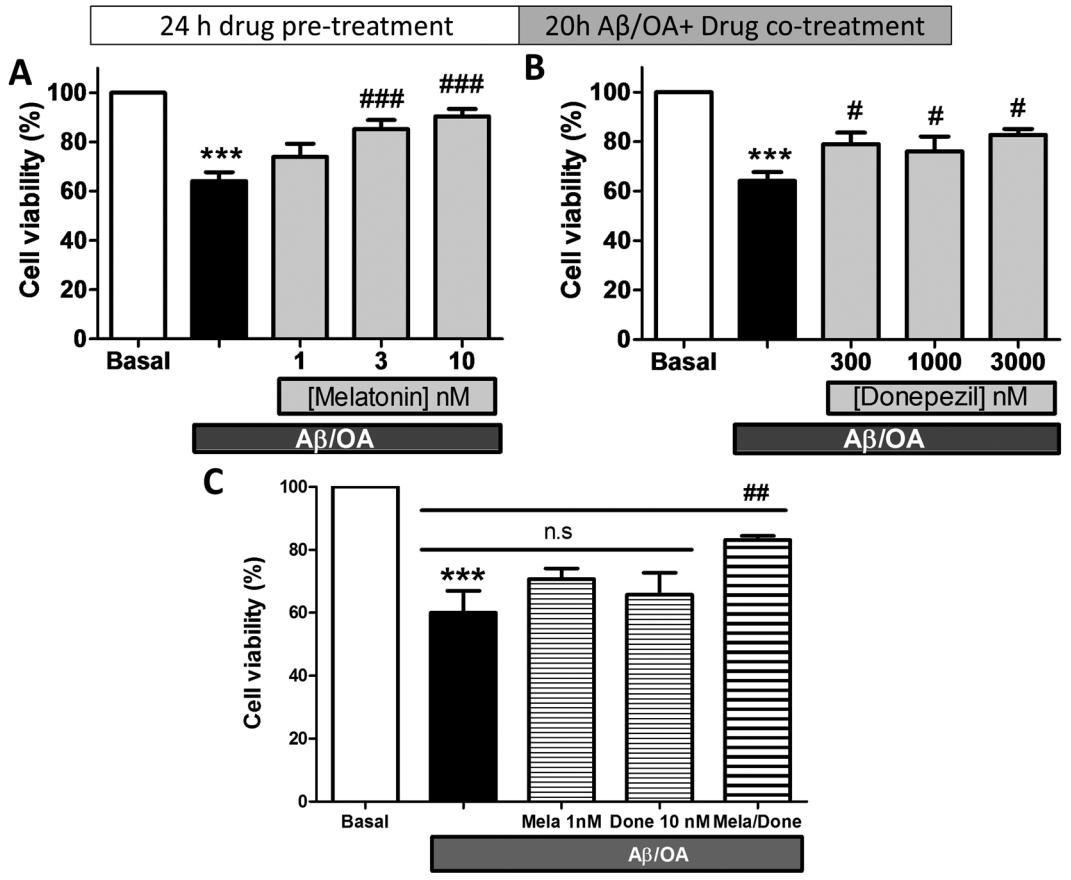

Figure 3. Combination of subeffective concentrations of melatonin and donepezil provide significant protection against Aβ/OA-induced toxicity. On the top part of the figure, a schematic illustration of the protocol used is represented. Cells were exposed for 20 h to 1 μM Aβ plus 3 nM OA (Aβ/OA). When the neuroprotective compounds melatonin or donepezil were used, they were preincubated 24 h before adding the toxics and during the exposure to the toxics. Effect of increasing concentrations of melatonin (A) and donepezil (B) on the cell viability of SHSY5Y cells exposed to the combination of Aβ/OA, measured as MTT reduction. (C) Neuroprotective effect afforded by the association of subeffective concentrations of melatonin (1 nM) plus donepezil (10 nM). Data represent the mean ± SEM from seven different cultures, $^{***}P < 0.001$ compared to basal; $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$ compared with βA/OA group.

neuronal cell line instead of primary neuronal cultures which "replaces" the use of animals.

When we analyzed the apoptotic and necrotic populations in SH-SY5Y cells exposed to Aβ (1 μM) in combination with OA (3 nM), from now on Aβ/OA, we found that cell death was mainly apoptotic (Figure 4B). These results are consistent with those described in animal models of AD where mutations associated with overexpression of Aβ 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 monotransgenics. Besides the effects on cell death, subtoxic concentrations of Aβ/OA caused neurite retraction (Figure 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 Aβ/OA. We also found aggregates of thioflavin S staining as an indication of Aβ aggregation (Figure 5E). Taken together, by combining subtoxic concentrations of Aβ 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 Aβ/AO, we evaluated the potential cytoprotective effect of melatonin, the acetylcholinesterase inhibitor donepezil, and the association of subeffective concentrations of both. The experimental protocol consisted of preincubating SH-SY5Y cells for 24 h with increasing concentrations of the neuroprotective compounds prior to the addition of the toxic stimuli (Aβ /OA), and maintaining the protective compounds for an additional 20 h period together with the toxins (see protocol on top of Figure 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) (Figure 3A). We also evaluated the potential neuroprotective effect of donepezil; the range of concentrations was selected based on previous data from our group.[25] As represented in Figure 3B, donepezil was protective at concentrations ranging from 0.3 to 3 μM; however, a concentration-dependent effect was not observed.

To test the hypothesis that a significant neuroprotective effect could be achieved with the combination of subeffective concentrations of melatonin and an AChEI, we used 1 nM of melatonin plus 10 nM donepezil in the Aβ/OA toxicity model;

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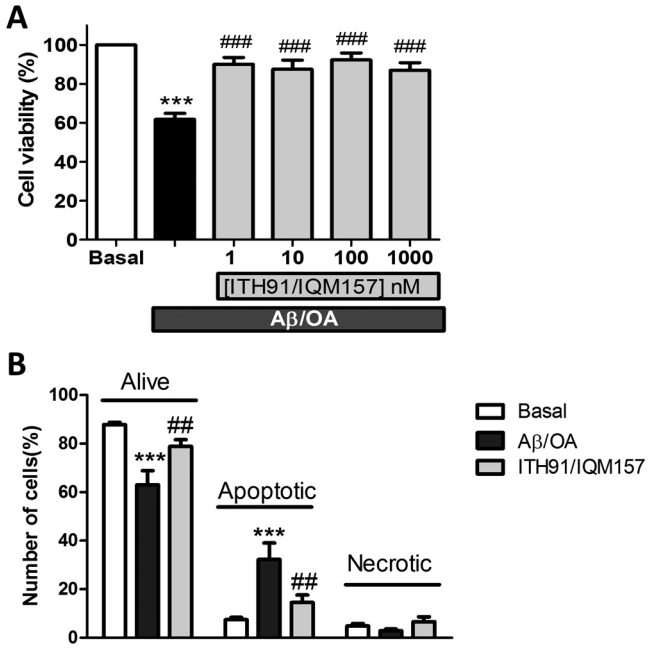

Figure 4. ITH91/IQM157 is neuroprotective against Aβ/OA toxicity by an antiapoptotic mechanism. (A) Effect of increasing concentrations of ITH91/IQM157 on the cell viability of cells exposed to Aβ/OA. (B) Percentage of alive, apoptotic, and necrotic cells, measured by flow cytometry in control cells or cells exposed to Aβ/OA alone or in the presence of ITH91/IQM157 at 100 nM. Data correspond to the mean ± 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 βA/OA-induced apoptotic cell death.

indeed, the drug combination afforded significant protection (57% protection) compared to the drugs alone (Figure 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 acetylcholinestarase inhibitor donepezil, the neuroprotective actions found with ITH91/IQM157 were achieved at lower concentrations; at 1 nM, ITH91/IQM157 already offered maximum protection (Figure 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 versus 57% with the combination strategy (Figure 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 versus 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 "backup" systems yielding the same effect such as gene expression, protein synthesis, receptors response, and protein degradation. Proteins and intermediates involved in these backup systems can be completely different and therefore, drugs targeting primary pathways will have no effect over this backup pathway, an effect known as redundancy. Multitarget 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

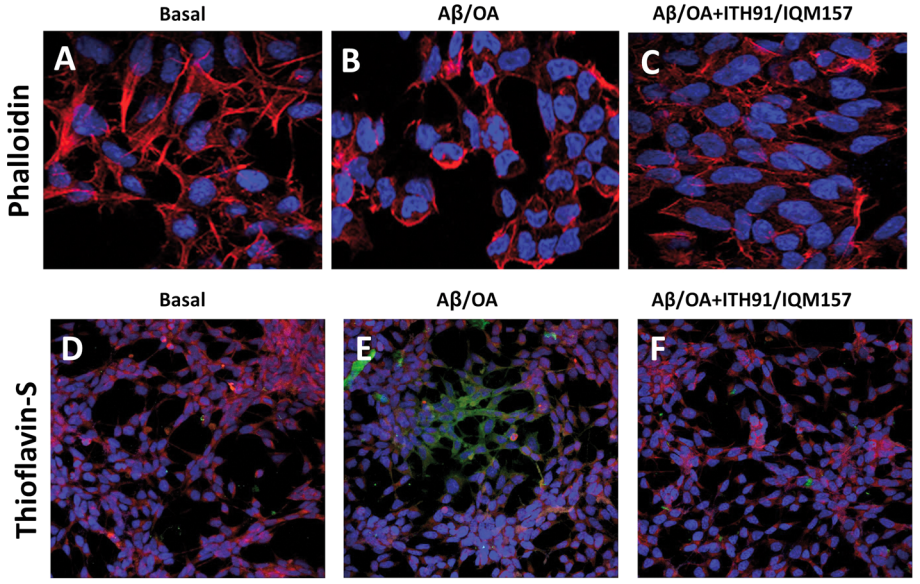

Figure 5. ITH91/IQM157 recovered cytoskeletal alterations and thioflavin-S aggregates induced by exposure of SH-SY5Y cells to Aβ/OA. Top part shows images of SH-SY5Y cells double stained with Hoechst 33342 (nuclei in blue) and phalloidin (cytoskeleton in red) under basal conditions (A), treated with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

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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.

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 (Figure 6A). The

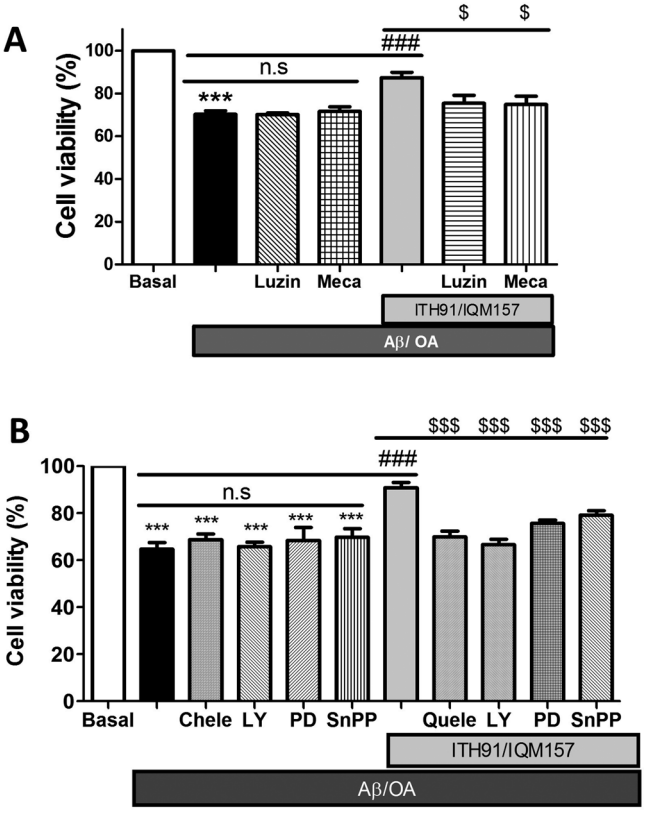

Figure 6. Neuroprotection elicited by ITH91/IQM157 involves melatonin receptors, nicotinic acetylcholine receptors, PI3K/Akt, ERK1/2, PKC, and induction of HO-1. (A) The melatonin receptor antagonist luzindole (3 μM) and the nAChR antagonist mecamylamine (10 μM) partially block the protective action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

involvement of nAChRs has also been implicated in the protective effects of other AChE inhibitors like galantamine and donepezil.[26−28] The neuroprotective effect of ITH91/IQM157 was accompanied by the recovery of the cytoarchitecture and a reduction of thioflavin-S aggregates (Figure 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 Aβ and prevent its aggregation[29,30] and it can also interfere with APP processing.[31−33] Furthermore, we previously reported that compound ITH91/IQM157 displaces propidium iodide from the peripheral acetylcholinesterase site, which is known to participate in βA aggregation.[34] Interaction with MT_2 receptors can stimulate phospholipase C and activate protein kinase C (PKC) via diacylglycerol, which in turn phosphorylates and inactivates GSK-3β, whose participation in APP synthesis[35,36] and tau hyperphosphorylation is 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 (Figure 6B).

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[17,37].[38] Indeed, ITH91/IQM157 increased phosphorylation of ERK1/2 and Akt (Figure 7A and B) and its protective actions were prevented in the presence of inhibitors of these kinases (Figure 6B). Akt can phosphorylate GSK-3β at position Ser-9, inactivating it[39−41] and can improve neuronal survival by (i) contributing to reduction of βA and tau pathology as mentioned above and/or (ii) promoting the nuclear translocation of Nrf2 (nuclear factor E2-related factor 2) to increase the cells defense mechanisms.[42]

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 (Figure 7C), and, most interesting, its protective actions were prevented when an inhibitor of this antioxidant enzyme (SnPP) was added to the cells (Figure 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.[37,43,44]

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 Aβ 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.[37,45] Also induction of HO-1 by the ACEI galantamine has been related to protection of microvascular endothelial cells.[46] 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 Aβ 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, and 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.

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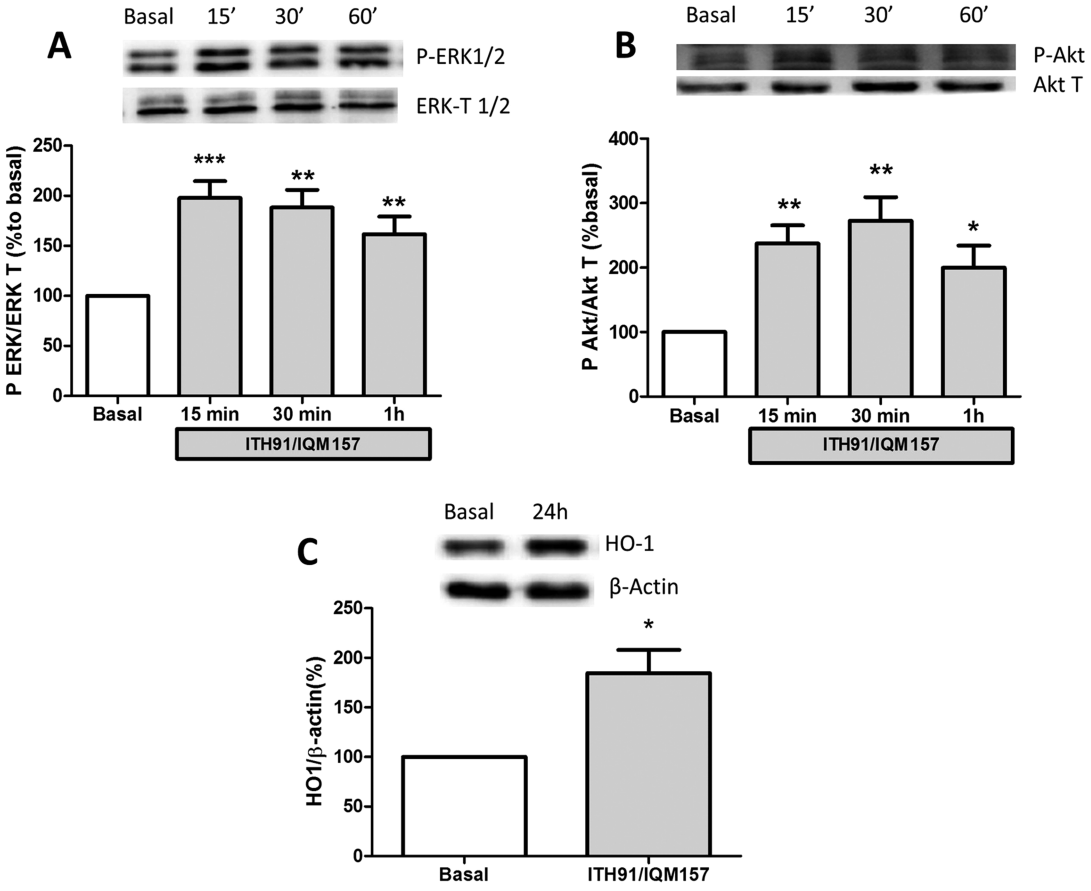

Figure 7. ITH91/IQM157 increases ERK1/2 and Akt phosphorylation and induces the antioxidant enzyme HO-1. ERK1/2 phosphorylation with respect to total-ERK1/2 (A) and Akt phosphorylation with respect to total-Akt (B) was analyzed, by Western blot, in SH-SY5Y cells treated for 60, 30, or 15 min with 100 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 a 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 ± SEM of five experiments. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ significantly different from untreated cells.

METHODS

Materials. Amyloid beta ($A\beta_{25-35}$), okadaic acid (AO), chelerythrine, PD98059 (2-(2-amino-3-methoxyphenyl)-(4*H*-1-benzopyran-4-one)), and LY294002 (morpholino-4-yl-8-phenylchromen-4-one), mecamylamine, were from Tocris scientific/Biogen, Madrid, Spain. Tin protoporphyrin (IV) from was Frontier Scientific Europe, Lancashire, U.K. Donepezil and melatonin were 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 amino acids, 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 μg/mL streptomycin (Invitrogen, Madrid, Spain) in Milli-Q H_2O. Cells were grown initially in a flask and subcultured 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 and 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 and Lang.[47] Upon completion of the experiments, 50 μL 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% CO_2 and 95% air. Finally, 200 μL 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-Aminoactinomycin 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 μL of 1× binding buffer, 5 μL of annexin V−PE, and 5 μL of 7-AAD. Cells were incubated at room temperature for 15 min in darkness, and then 100 μL of 1× 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-rhodamine 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 three times with PBS (NaCl 9 g/L, 10 mM NaH_2PO_4, 10 mM K_2HPO_4) 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 min. Later, the

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cells were washed three times with PBS every 5 min; staining of the nuclei with Hoechst (5 μg/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 three times with PBS every 5 min. Later, they were permeabilized with 0.1% Triton for 1 min and washed three times with PBS before staining them with Thioflavin-S 0.5% for 10 min. Then, three consecutive washes with ethanol 80%, Milli-Q H_2O, and PBS were performed. Later, cells were stained with phalloidin-rhodamine in PBS 1:1000 (Sigma-Aldrich, Madrid, Spain) for 20 min, followed by three washes with PBS every 5 min; staining of the nuclei with Hoechst (5 μg/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 Na_3VO_4. 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 h at constant amperage. Proteins were transferred to PVDF membranes (Millipore Ibérica SA, Madrid, Spain) for 2 h at 70 mA. Later on, membranes were blocked for 2 h with TTBS + 4% albumin (Sigma-Aldrich, Madrid, Spain), incubated with anti-P-Akt, anti-total Akt (Santa Cruz Biotechnology, Santa Cruz, CA,), anti-P-ERK, anti-total ERK, anti-HO-1 (1:1000) (Chemicon, Temecula, CA), and anti-β actin (1:10 000) (Sigma-Aldrich, Madrid, Spain) for 2 h. After washing several times with TTBS, the corresponding secondary antibodies (1:100 000) were added (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min. Finally, the membranes were revealed using ECL Advance Western Blotting Detection Kit (GE Healthcare, Barcelona, Spain) and quantified by using 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 $p < 0.05$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures showing (SI-1) effect of 1 μM Aβ, 3 nM OA, and their association on primary neuronal cell culture viability, and (SI-2) concentration−response curves of compounds ITH90/IQM156 and ITH91/IQM157 on the viability of SH-SY5Y cells exposed to Aβ/OA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥I.B. and J.E. have contributed equally to this work. I.B. has contributed to the concept/design, acquisition of data, data analysis/interpretation, and critical revision of the manuscript. J.E. has contributed to the concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript, critical revision of the manuscript, and approval of the manuscript. 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 the concept/design, drafting of the manuscript, critical revision of the manuscript, and approval of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

AD, Alzheimer's disease; Aβ, amyloid beta; AO, okadaic acid; AChEI, acetylcholine esterase inhibitors; α7 nAChRs, α7 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)-(4*H*-1-benzopyran-4-one); SnPP, tin(IV) protoporphyrin IX dichloride

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