ACS Chemical Neuroscience

Research Article

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PP2A Ligand ITH12246 Protects against Memory Impairment and Focal Cerebral Ischemia in Mice

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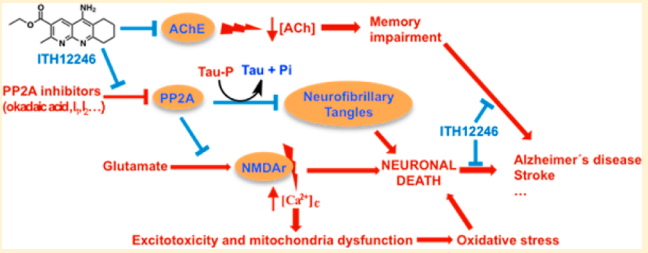
Supporting Information

ABSTRACT: ITH12246 (ethyl 5-amino-2-methyl-6,7,8,9-tetrahydrobenzo[*b*][1,8]naphthyridine-3-carboxylate) is a 1,8-naphthyridine described to feature an interesting neuroprotective profile in in vitro models of Alzheimer's disease. These effects were proposed to be due in part to a regulatory action on protein phosphatase 2A inhibition, as it prevented binding of its inhibitor okadaic acid. We decided to investigate the pharmacological properties of ITH12246, evaluating its ability to counteract the memory impairment evoked by scopolamine, a muscarinic antagonist described to promote memory loss, as well as to reduce the infarct volume in mice suffering phototrombosis. Prior to conducting these experiments, we confirmed its in vitro neuroprotective activity against both oxidative stress and Ca^{2+} overload-derived excitotoxicity, using SH-SY5Y neuroblastoma cells and rat hippocampal slices. Using a predictive model of blood-brain barrier crossing, it seems that the passage of ITH12246 is not hindered. Its potential hepatotoxicity was observed only at very high concentrations, from 0.1 mM. ITH12246, at the concentration of 10 mg/kg i.p., was able to improve the memory index of mice treated with scopolamine, from 0.22 to 0.35, in a similar fashion to the well-known Alzheimer's disease drug galantamine 2.5 mg/kg. On the other hand, ITH12246, at the concentration of 2.5 mg/kg, reduced the phototrombosis-triggered infarct volume by 67%. In the same experimental conditions, 15 mg/kg melatonin, used as control standard, reduced the infarct volume by 30%. All of these findings allow us to consider ITH12246 as a new potential drug for the treatment of neurodegenerative diseases, which would act as a multifactorial neuroprotectant.



KEYWORDS: *Alzheimer's disease, ITH12246, memory, neuroprotection, PP2A, Ser/Thr phosphatases, stroke*

Phosphoprotein phosphatase (PPP)-2A (PP2A) is an ubiquitous enzyme that catalyzes the dephosphorylation of serine (Ser) and threonine (Thr) residues on eukaryotic cell proteins.[1,2] It is the principal member of the Ser/Thr phosphatase family, which is involved in a huge amount of physiological pathways where phosphorylation/dephosphorylation processes are key regulatory events.[3,4] Activity of PP2A is highly regulated by endogenous inhibitors (I_1 and I_2)[5] and activators (PTPA: PP2A/Tyr phosphatase activator),[6] changes in the substrate specificity, and post-translational modifications,[4] with this last characteristic being the object of therapeutic interest. Thus, pharmacological intervention on either PP2A activity or PP2A-carboxymethylation regulatory enzymes (e.g., LCMT1[7] or PME-1[8,9]) has shown interesting therapeutic implications, mainly centered on the down-regulation of pro-oncogenic signaling pathways.[10] In spite of its essential role, drug research and development has scarcely paid attention to PPP enzymes, compared to what has been done on protein kinases, in part due to phosphatase enzymes being considered much less specific than kinases. Currently, this

Received: February 19, 2013
Accepted: May 28, 2013
Published: May 28, 2013

dx.doi.org/10.1021/cn400050p | *ACS Chem. Neurosci.* 2013, 4, 1267−1277

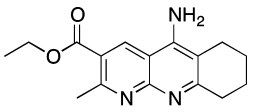
point of view is being abandoned, as it is now acknowledged the coordinated role of the regulation of both kinases and phosphatases.[11]

In this context, there is an emerging view that PP2A may become an attractive target in the field of neuroprotection in Alzheimer's disease (AD),[12] as this enzyme is the main one responsible for dephosphorylation of the protein tau (τ).[13] One of the hallmarks of AD pathogenesis is τ hyperphosphorylation. Briefly, τ hyperphosphorylation leads to microtubules disassembly and self-aggregation, forming the so-called neurofibrillary tangles (NFT),[14] due to an imbalance between protein kinases and protein phosphatases, as a result of either overactivity of the former or underactivity of the latter. Consistent with this view is the lower expression and down-regulation of PP2A found in AD brains,[5] which has been associated to the loss of activity of peptidyl prolyl *cis*−*trans* isomerase (Pin1) deriving to inefficient up-regulation of PP2A.[15] PP2A has also been described to play a role in the regulation of kinase activity of GSK3β and Cdk5, among other enzymes.[16]

On the other hand, PP2A is also being implicated in the pathogenesis of stroke, where the apoptotic cascade regulated by phosphorylation/dephosphorylation events plays a key role. Down-regulation of PP2A B-subunit takes place during focal cerebral ischemia induced by occlusion of the middle cerebral artery (MCAO) in rats.[17] Zhu et al. have recently studied the positive outcomes of the administration of simvastatin to rats subjected to ischemic injury by MCAO, as simvastatin pretreatment decreased both neurological deficit and infarct areas. In this model, PP2A expression and activity was diminished in MCAO-subjected rats, and such reduction was effectively counteracted by the presence of simvastatin.[18] The authors ascribe the protective effects of PP2A enzymatic activity to a fine regulation of NMDA-sensitive glutamate receptors (NMDARs) by two ways, direct dephosphorylation in two serines of the NMDAR subunit NR1, what induces a lesser Ca^{2+} influx through the NMDA channel, and a still unclear positive effect on the expression of NR3A, another NMDA-forming subunit that confers more rapid desensitization kinetics to NMDARs, as well as lower open probability and conductance.[19] The fact that PP2A-derived phosphatase activity is suppressing NMDAR activity gives us the clue to the potential therapeutic interest of PP2A ligands in stroke, where an overstimulation of glutamate receptors by an excessive extracellular glutamate is described.[20] Hence, a massive Ca^{2+} entry through NMDARs is triggered, being this event the principal factor for the neuronal excitotoxicity injury during ischemic stroke.[18] Unfortunately, there are no efficient pharmacological strategies to reduce the serious consequences of the cerebrovascular accidents currently. In the last 30 years, dozens of neuroprotectant compounds studied for brain ischemia have provided disappointing negative outcomes in clinical trials. Causes for this failure were treated by international committees, which elaborated a guideline to follow, in order to optimize the preclinical studies on in vitro models, but particularly on in vivo models of cerebral ischemia, to ensure better translation from the preclinical to clinical trials.[21] However, after nearly a decade since the STAIR criteria were established, clinical trials continue to provide negative outcomes.[22] Thus, novel strategies should be approached in the search for compounds with neuroprotective actions in stroke.

In summary, increasing observations support the validation of the maintenance of PP2A enzymatic activity as a new biological target, as already established for cancer treatment, for the search of new families of drugs against neurodegenerative diseases or stroke, due to its ability to protect against τ hyperphosphorylation and the subsequent NFT generation, or down-regulate NMDARs activity. In the case of τ hyperphosphorylation, many kinases can phosphorylate τ, but PP2A is the major phosphatase enzyme dephosphorylating τ by far,[12] so the search of optimal drugs acting on the kinase activity to mitigate τ hyperphosphorylation seems to be more complicated.

With all of these precedents, in the last 4 years, we have been interested in the study of pharmacological strategies to promote the PP2A enzymatic activity, as we consider it an exciting therapeutic target for many diseases such as Alzheimer's, cancer or stroke. We hypothesize that compounds able to promote PP2A activity or prevent inhibitory actions on PP2A should exhibit a neuroprotective profile in various biological models of neurodegeneration. Thus, we recently found interesting pharmacological properties related to the PP2A activity when describing a series of 1,8-naphthyridine derivatives able to inhibit cholinesterase enzymes.[23] They exhibited neuroprotective properties against oxidative stress in cultures of the human neuroblastoma cell line SH-SY5Y and in rat hippocampal slices. One of these derivatives (ethyl 5-amino-2-methyl-6,7,8,9-tetrahydrobenzo[*b*][1,8]naphthyridine-3-carboxylate), which we have named ITH12246 (Figure 1), showed additional



ITH12246

Figure 1. Chemical structure of the PP2A ligand ITH12246.

neuroprotective properties against amyloidogenesis, that is, exposure to the amyloid beta peptide$_{1-42}$ (Aβ_{1-42}) 30 μM for 24 h, and τ hyperphosphorylation, that is, exposure to the PP2A inhibitor okadaic acid (OA)[24] 30 nM for 24 h. Measuring the inorganic phosphorus released, by the method of malachite green, we ascribed the neuroprotective actions of ITH12246 against OA-induced toxicity to its capacity for interacting with PP2A and preventing the inhibitory action of OA on PP2A.[23]

Stock and co-workers have classified PP2A activators into three main families:[12] inhibitors of a inhibitory interaction on PP2A, allosteric activators, and ligands for post-translation modifying enzymes. According to our computational studies, compound ITH12246 could belong to the first family, that is, as an inhibitor of an inhibitory interaction on PP2A. We located by molecular field-based similarity essential features for recognition of both ITH12246 and OA binding to the same receptor, as well as electronic congruence.[23] These data led us to carry out the present investigation to explore whether compound ITH12246 could exhibit neuroprotective properties on in vivo models of pharmacological memory impairment and brain ischemic damage.

RESULTS AND DISCUSSION

Because PP2A seems to be involved in the pathogenesis of AD and stroke, in the present study, we have explored whether the PP2A regulator ITH12246 could mitigate memory loss in the scopolamine mouse model and if it could afford neuroprotection in the photothrombotic model of cortical infarct in

the mouse. We also performed in vitro experiments to further understand its neuroprotective properties. We have evaluated its ability to cross the blood-brain barrier on an in vitro model and explored its potential hepatotoxicity in cell cultures. Overall, the experiments here performed demonstrate two novel in vivo properties of ITH12246, namely, mitigation of memory impairment and reduction of cortical infarct. Data suggest that compound ITH12246 may have potential therapeutic application in AD and/or stroke.

Effects of ITH12246 on the Neurotoxicity Elicited by Rotenone/Oligomycin A (O/R). There is a rising interest in the oxidative stress-induced mechanisms leading to AD,[25] as a huge amount of contributions highlight such a relationship. For instance, elevated biomarkers of oxidative stress have already been found in the light-moderate stage of the disease.[26] The exposure of SH-SY5Y neuroblastoma cells to rotenone and oligomycin A (O/R) constitutes a good model of oxidative stress, having its origin in mitochondria. As far as stroke, mitochondrial complex I blockade by rotenone has been considered a very reproducible in vitro model of hypoxia occurring in physiopathological events related to cerebral ischemia.[27] O/R block complexes I and V, respectively, of the mitochondrial electron transport chain, thereby causing free radical generation and blockade of ATP synthesis.[28] Thus, SH-SY5Y cells were incubated for 24 h with ITH12246 before the addition of O/R, and coincubated with compounds plus O/R for an additional 24 h period. Melatonin was used as reference compound.[23] We previously described that 1 μM of ITH12246 protected by 48% SH-SY5Y cells against cell death induced by O/R, measured as lactate dehydrogenase (LDH) release.[23] However, this method only provides information about cell necrosis. In this study, we have evaluated the effect of ITH12246 in terms of cell viability, by the method of MTT reduction.[29] Mitochondrial enzymes of viable cells chemically modify MTT, yielding a measurable colored dye. This process cannot occur in either apoptotic or necrotic cells. Hence, we were able to assess whether our compound can protect against signals of both apoptotic and necrotic death. Following this protocol, we observed that 30 nM to 3 μM ITH12246 protected SH-SY5Y cells against loss of cell viability exerted by O/R with statistically significant values ($p < 0.05$) (Figure 2).

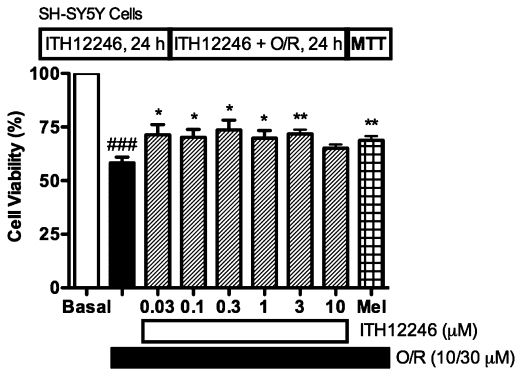


Figure 2. Protection by ITH12246 against the cytotoxic effects of O/R in SH-SY5Y neuroblastoma cells. Cell viability was measured via MTT reduction (ordinate), and data were normalized as % basal (white column; cells incubated only with cell culture medium). Data are mean ± SEM of triplicates of five different cell batches: $^{###}p < 0.001$, comparing to basal and O/R-lesioned cells; $^{**}p < 0.01$ and $^{*}p < 0.05$, comparing to O/R-lesioned cells in the absence of drugs.

Maximal protection was found at 0.3 μM, affording 37% protection, a figure slightly better than that of melatonin at 30 nM (25%). Thus, we confirmed that ITH1246 behaves as a neuroprotectant against an in vitro model of oxidative stress-related cytotoxic damage, across a wide range of concentrations.

These data extend the in vitro neuroprotective profile of ITH12246, which also protected SH-SY5Y cells against both Aβ exposure and τ hyperphosphorylation.[23] Nevertheless, although encouraged by the PP2A up-regulatory activity of ITH12246, the additional antiamyloidogenic and antioxidant activities are not sufficient to lead this potential drug to in vivo models of neurodegeneration, due to, despite the relationship between reactive oxygen species (ROS) generation leading to oxidative stress, and the AD-derived neurodegeneration being well documented, that none of the drugs clinically studied, acting as antioxidants, have been approved for the treatment of AD, nor those against Aβ-derived degeneration. For this reason, prior to the design of in vivo studies with ITH12246, we were interested in evaluating its ability to protect against other physiological biomarkers of neurodegeneration, that is, Ca^{2+} overload, induced by glutamate in rat hippocampal slices.

Effect of ITH12246 on Glutamate-Lesioned Rat Hippocampal Slices. In order to select the closest physiological model of Ca^{2+} dysregulation-based neuronal death, glutamate receptor-mediated Ca^{2+} overload appeared to be the most relevant from a pathogenic point of view, due to the fact that it reproduces the Ca^{2+} signaling pathways implicated in neurodegenerative disorders.[30] In fact, the only noncholinergic drug of those five approved by drug regulatory agencies for the treatment of AD is memantine (MEM), a NMDA-sensitive glutamate receptor blocker.[31] Moreover, recent observations have confirmed the influence of mitochondria-mediated cell Ca^{2+} regulation on glutamate-induced excitoxicity.[32] As far as stroke, right after ischemia occurs, the activation of glutamate receptors initiates the ischemic cascade, contributing to the early stage injury in the cerebral ischemia.[33]

Thus, to fully characterize the neuroprotective profile of ITH12246, previously confirmed against neurotoxicity induced by Aβ, τ, and oxidative stress, we evaluated the neuroprotective activity of ITH12246 on rat hippocampal slices subjected to glutamate (Glu) excitotoxicity. Following the experimental protocol indicated for the preparation of the rat hippocampal slices, after a stabilization period of 45 min at 34 °C, slices were coincubated with glutamate at 1 mM and ITH12246 at concentrations of 1, 3, 10, or 30 μM for 4 h at 37 °C. Glutamate caused statistically significant reduction of cell viability (28%) in nontreated tissues, mirroring data observed in previous reports from our group and others.[34,35] Melatonin was used as reference compound, as it has been recently described to attenuate glutamate-induced PP2A subunit B decrease in neuronal cells.[36] In our experiments, melatonin mitigated the tissue damage evoked by glutamate at 1 mM in a concentration-dependent manner, with a maximal protection at 30 μM, which counteracted the glutamate lesion by 74% (Figure 3a; MTT reduction of 91% compared to basal). Along the wide range of concentrations assayed, compound ITH12246 prevented such neuronal lesion, with a maximal protection at 3 μM, though higher concentrations showed a very similar profile, lacking statistically significant differences between 1 and 30 μM. At 3 μM, MTT was reduced by 84% compared to basal, having 42% more viability than that found in glutamate-treated control slices. This protection was similar to that shown by melatonin at 30 μM, used as reference

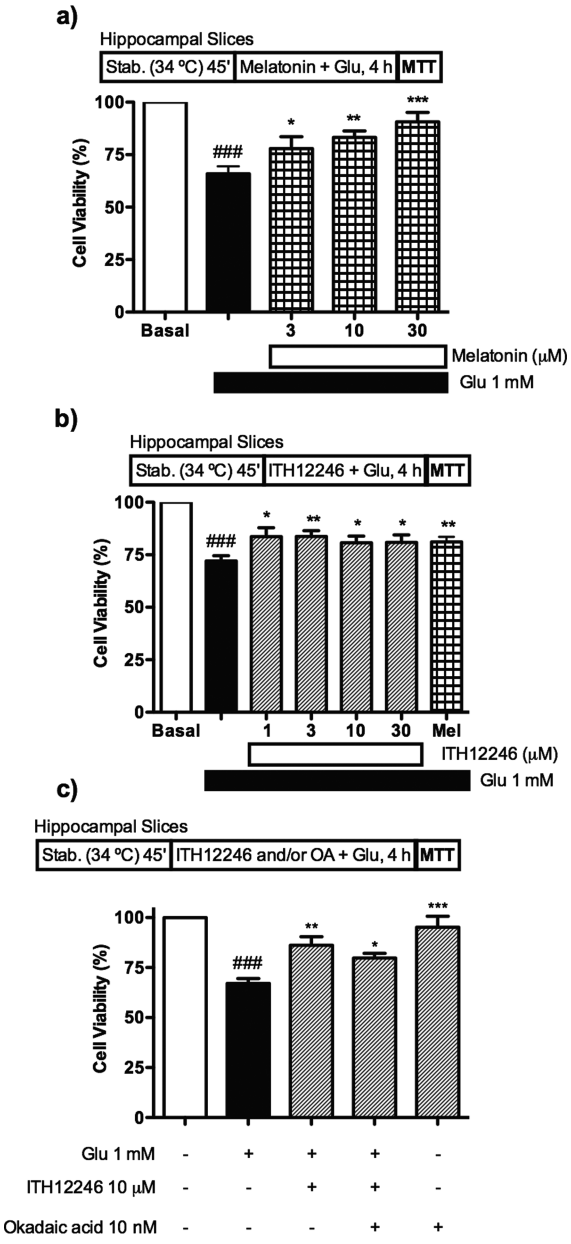


Figure 3. Melatonin (a) and ITH12246 (b) protected hippocampal slices against the neurotoxic effects of glutamate (Glu). Neuroprotective effects of ITH12246 were diminished by OA at 10 nM (c). Cell viability was measured via MTT reduction (ordinate) and data were normalized as % basal (white column; slices incubated only with cell culture medium). Data are mean ± SEM of quadruplicates of five independent experiments: $^{###}p < 0.001$ comparing basal and glutamate-lesioned slices; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{ns}p > 0.05$ comparing to glutamate group in the absence of drugs.

compound (Figure 3b). The coincubation with OA 10 nM reduced the neuroprotective effect of ITH12246 (10 μM) (Figure 3c), though this reduction is not statistically significant. It is important to note that this concentration of OA was not able to reduce per se the cell viability in these hippocampal slices, what would be suggesting that the PP2A inhibitor OA is an antagonist of the neuroprotective action of ITH12246 against glutamate exposure. Hence, ITH12246 would protect neurons from the glutamate overload by up-regulating the PP2A-derived phosphatase activity, what leads to the NMDAR deactivation, according to the observations of Zhu et al.[18] Under these experimental conditions, other metabolic pathways recruiting kinase enzymes do not seem to be involved in the reduction of the glutamate-induced toxicity promoted by ITH12246, as coincubation with LY294002, PD98059, or chelerythrine, which inhibit PI3K, ERK1/2, and PKC, respectively, did not counteract such neuroprotective action (Supporting Information Figure 1).

The neuroprotective activity of ITH12246 on the observed glutamate-evoked damage in the tissue model of hippocampal slices deserved our attention, taking into account that an enhanced glutamate release is observed in ischemic stroke, after oxygen and glucose deprivation (OGD)-induced energy depletion, leading to Ca^{2+} overload.[20] This finding prompted us to extend our study not only to the eligibility of ITH12246 as a new candidate for AD, but also for the treatment of cerebral ischemia, as the search for an efficient medicine for acute stroke has been disappointing.[37] However, prior studying this compound in in vivo models, we considered worthwhile evaluating its ability to reach cerebral targets in an in vitro predictive model of crossing the blood-brain barrier, as well as anticipating possible adverse effects in an in vitro model of hepatotoxicity.

In Vitro Blood-Brain Barrier (BBB) Permeation Assay. To evaluate the passive brain penetration of ITH12246, we used the PAMPA-BBB method described by Di et al.[38] and subsequently optimized by Rodríguez-Franco and co-workers for molecules with limited water-solubility.[39−45] The in vitro permeability (P_e) values of ITH12246 through a lipid extract of porcine brain were determined by using a mixture of PBS and ethanol in the ratio of 70:30. In the same assay, 10 commercial drugs of known CNS penetration were also tested and their experimental values were compared to reported values (Table 1), giving a good lineal correlation, P_e (exp) = 0.5659P_e (bibl) + 2.3581 (R^2 = 0.9185). From this equation and taking account the limits established by Di et al. for BBB permeation,[38] we found that compounds with permeability values above 4.6 × 10^{-6} cm s^{-1} could penetrate into the CNS, whereas molecules with permeability values below 3.5 × 10^{-6} cm s^{-1} were predicted to exhibit a low BBB permeation. Between these two limits, compounds showed an uncertain CNS penetration. Compound ITH12246 showed a permeability value in the uncertain range (4.39 ± 0.08) × 10^{-6} cm s^{-1}, very close to a guaranteed passive penetration. P_e values below 3.5 × 10^{-6} cm

Table 1. Permeability (P_e × 10^{-6} cm s^{-1}) in the PAMPA-BBB Assay of 10 Commercial Drugs, Used in the Experiment Validation, and Compound ITH12246

compd	bibl[a]	exp[b]	prediction
testosterone	17.0	13.71 ± 0.33	cns+
verapamil	16.0	10.20 ± 0.50	cns+
imipramine	13.0	7.92 ± 0.28	cns+
desipramine	12.0	10.47 ± 0.44	cns+
corticosterone	5.1	4.62 ± 0.05	cns+
ITH12246		4.39 ± 0.08	cns+/−
piroxicam	2.5	4.09 ± 0.14	cns+/−
hydrocortisone	1.9	3.23 ± 0.02	cns−
caffeine	1.3	3.50 ± 0.04	cns−
aldosterone	1.2	3.59 ± 0.01	cns−
ofloxacin	0.8	2.32 ± 0.02	cns−

[a]Taken from ref 39. [b]Data are the mean ± SD of three independent experiments.

s^{-1} would have led us to discontinue the in vivo study of ITH12246. Taking into account the predictive nature of this assay, we decided that this threshold value was not enough to prevent up further experiments. Hence, we planned to evaluate the possible side effects related to liver damage, as found in many CNS drugs.

Evaluation of Potential Hepatotoxicity of ITH12246 in HepG2 Cells. The liver is the principal target organ for most of toxic chemicals, as well as the vast majority of CNS drugs that, due to their high hydrophobicity, are easily oxidized to increase polarity and subsequently aid their elimination. Sometimes, metabolites are more reactive than the drug and become hepatotoxic. For instance, the first-prescribed AD drug tacrine, which shares some structural analogy with ITH12246, is double oxidized to a highly toxic quinonemethide-like metabolite, as glutathione (GSH) is wasted either due to the generation of GSH-linked conjugates with such metabolite or because GSH is oxidized to induce the reduction of the quinone structure back to tacrine.[46] For these reasons, we were interested in measuring the potential hepatotoxicity of ITH12246, comparing it to that elicited by tacrine. It is worth mentioning that we had designed this compound, and other analogues before, in such a manner to avoid this metabolic pathway, by incorporating a methyl group at C2 of the naphthyridine ring, that behaves as a metabolic blocker of the quinonemethide synthetic pathway. To carry out these experiments, we used the HepG2 cells, a cell line from a human hepatocellular carcinoma, which is considered an eligible model of the "liver" for toxicity assays.[47,48]

Figure 4 shows that at the concentration of 30 μM and above tacrine, used as standard for comparative purposes, presents a significant hepatotoxicity, with a reduction of cell viability of 20%, while ITH12246 was not toxic at this concentration. Hence, ITH12246 presented less hepatotoxicity than tacrine, since loss of cell viability was only significant at concentrations of 0.1 mM and above.

Effect of ITH12246 on Scopolamine-Treated Mice Measured by the Object Placement Test. After confirming that the prediction of pharmacokinetic properties of ITH12246 regarding BBB crossing and hepatotoxicity were not refraining its further in vivo studies, we proceeded to the in vivo determination of the memory enhancing properties of ITH12246, using the object placement test (OPT), inducing memory impairment with the administration of scopolamine in mice.[49] The object placement test is a spatial memory task first described in rats.[50] Briefly, in a familiar environment, novel or relocated objects attract rodents. In a first moment (T1) they are allowed to freely explore two similar objects during a learning trial (both objects were similarly explored in T1, as shown in Figure 2 of Supporting Information), after which animals are subjected to a test trial, where the position of one of the sample objects is changed. Global exploration time between T1 and T2 trials did not change in a statistical significant manner (Figure 3, Supporting Information). Should rodents show more interaction with the changed object, it is assumed they remember that unchanged sample object. Thus, the changed object preference is used as an indication of memory.

Administration of scopolamine, a muscarinic receptor antagonist,[51] induces memory impairment due to the loss of cholinergic neurotransmission elicited. A single administration of scopolamine induces transient memory impairment,[52] which can be easily shown in the OPT. Several groups have correlated the effect of scopolamine with those of AD in terms of memory,

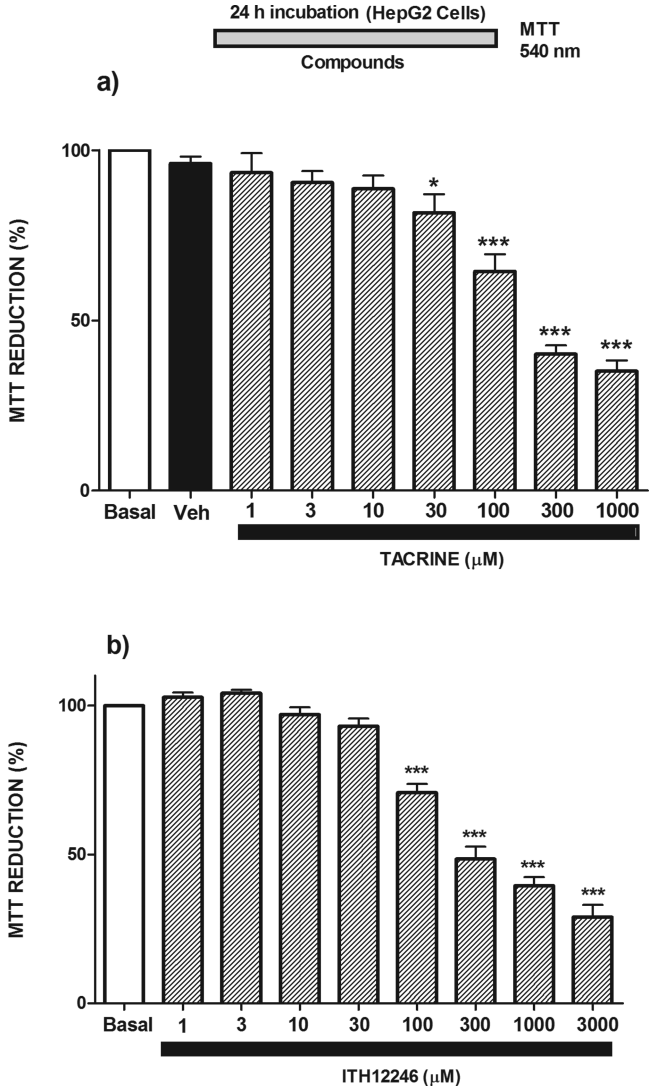


Figure 4. Panels (A) and (B) illustrate a concentration–response curve of tacrine and ITH12246, respectively. Cell viability was measured via MTT reduction (ordinate), and data were normalized as % basal (white column). Cells treated with DMSO (0.1%) were the negative control (Veh, black column). Data are the mean ± SEM of triplicates of four different cell batches: $*p < 0.05$ and $***p < 0.001$, comparing to cells in the absence of drugs.

recall, recognition, and learning capacity.[49] In this study, we also evaluated galantamine, as a positive control in the scopolamine test.[53] Thus, following the protocol described in the Methods section, scopolamine group showed a memory index, measured as the relative discrimination index (DI) (see Methods), significantly reduced (0.22 ± 0.04) compared to nontreated basal group (0.50 ± 0.04). This decrease in the memory index was partially reversed by ITH12246, as animals treated with this compound, at the concentration of 10 mg/kg i.p., showed a DI of 0.35 ± 0.03 (Figure 5). Galantamine at 2.5 mg/kg i.p.[53] also reduced the scopolamine-evoked loss of memory, with a memory index of 0.34 ± 0.02. These data demonstrate that ITH12246 improves mnemonic performance in mice subjected to the memory impairment elicited by scopolamine, in a similar fashion as galantamine that is currently used in AD patients. Insterestingly, this action on memory retrieval is observed at much lower concentrations of ITH12246 than those potentially hepatotoxic (Figure 5).

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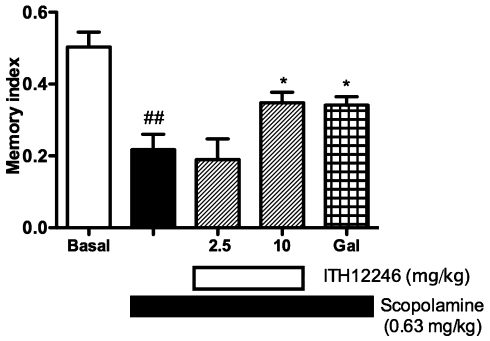

Figure 5. Memory index, measured as the relative discrimination index (DI) between a changed and a familiar object, in mice treated with scopolamine with or without drugs. After a 2-day familiarization phase, animals were treated with vehicle (basal group), scopolamine (control group), or drugs plus scopolamine. Galantamine was used as a reference and ITH12246 was assayed at two different concentrations (2.5 and 10 mg/kg). Data correspond to the mean ± SEM of at least nine animals. $^{##}p < 0.01$, compared to basal; $^{*}p < 0.05$ compared to scopolamine group in the absence of drugs.

Effect of ITH12246 on Cortical Infarct Volume in a Photothrombotic Model of Stroke in Mice. The positive outcomes on neuroprotection achieved with ITH12246 in in vitro slice models that mimic pathological events occurring during cerebral ischemia, that is, glutamate excitotoxicity (Figure 3) and OGD plus reoxygenation,[23] prompted us to design in vivo experiments to figure whether this effect was robust in a more relevant model of stroke, that is, the focal cortical infarct, elicited by permanent occlusion of small vessels by photothrombosis in the mouse.[54] We also hypothesize that PP2A up-regulators, such as ITH12246, could present a beneficial effect on focal cerebral ischemic injury, as PP2A activity is decreased in these situations.[17]

In these experiments, upon administration of Rose Bengal, oxygen singlet species are generated after illumination of brain cortex, causing peroxydation of endothelial cell membranes, occlusive platelet aggregation, microthrombi formation and focal cerebral ischemia.[55] Following the protocol described in the Methods section, we injected i.p. five times saline, ITH12246, or melatonin, both dissolved in saline; with the first injection being 20 min before ischemia (day 0), following two doses per day (day 1 and 2 postischemia). On the third day, after observing that there were no variations in monitored physiological or behavioral parameters or weight loss, we sacrificed the animals. Brain slices of control (saline) animals showed a mean volume of cortical infarct of 6.1 ± 0.3%. Melatonin (15 mg/kg), used as a reference,[54] reduced by 28% the infarct volume. At 10 mg/kg, ITH12246 slightly reduced, by 16%, infarct volume compared to saline group, with no statistical significance. By contrast, at the lowest dose used (2.5 mg/kg), ITH12246 reduced by as much as 59% the infarct volume. The lack of protection found at 10 mg/kg is surprising, and could be a consequence of loss of stability of the blood-brain barrier induced by the photothrombosis protocol, what would allow ITH12246 to massively penetrate into the brain. Thus, the effective concentration of the compound in the infarct area could reach to those predicted to be toxic in the experimental model of the HepG2 cells (Figure 4).

Taking into account that lower doses of ITH12246 than those of the well-known antioxidant melatonin were able to reduce the infarct volume 2.2-fold in extension, we can

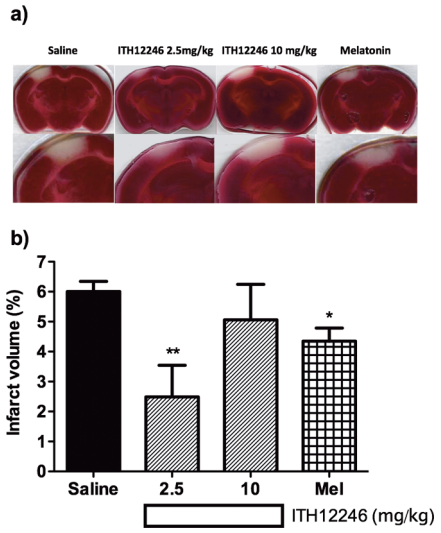

Figure 6. ITH2246 given before and after photothrombotic focal ischemia induction reduced infarct volume. (A) Representative images of brain slices stained with TTC of the four experimental groups; the images below show an amplification of the infarct area. Panel (B) represents the averaged data of cerebral infarct volume after photothrombosis for each treatment group; control group was treated with saline, melatonin was used as a positive control, and ITH2246 was assayed at two different doses (2.5 and 10 mg/kg). Data correspond to the mean and SEM of at least four animals per group. Statistically significant differences were determined with ANOVA test followed by Bonferroni post hoc. $^{*}p < 0.05$; $^{**}p < 0.01$ compared to saline.

hypothesize that ITH12246 properties to mitigate cortical infarct do not have to be only associated to its antioxidant profile, demonstrated in O/R-stimulated neuroblastoma cells (Figure 3) and OGD-subjected hippocampal slices[23] experiments, but also to additional activities, likely PP2A up-regulation, as our molecular modeling studies and phosphatase activity experiments[23] proposed.

■ CONCLUSIONS

On the basis of the findings presented, we propose 1,8-naphthyridine ITH12246 as a promising drug with potential therapeutic interest as neuroprotectant. This is based on its positive effects in the scopolamine model of memory impairment and the phototrombosis-induced focal cerebral ischemia. The best memory index was found at 10 mg/kg of ITH12246, but infarct volume reduction was only found at 2.5 mg/kg. We believe that the pharmacological properties of ITH12246 regarded to the mitigation of the O/R damage in SH-SY5Y cells, in addition to the reduction of OGD plus reox-induced lesion, are stabilizing the cell redox levels to physiological parameters. Moreover, through the reduction of glutamate-elicited damage observed in hippocampal slices, ITH12246 would be preventing the Ca^{2+} overload-derived cell death, and the reduction of inhibitory actions toward PP2A would help to maintain a physiological ratio of kinase/phosphatase metabolic pathways. Indeed, increasing evidence has related the progression of neurodegeneration with kinase overactivity and phosphatase underactivity.[56]

Therefore, we propose the compound ITH12246 as a new promising lead candidate for the treatment of neurodegenerative diseases like AD, as well as for stroke. Its demonstrated activities on cholinesterase enzymes and PP2A, position this

compound as a multitarget compound for AD, due to, as mentioned in the introduction part, maintenance of PP2A activity not only avoids τ hyperphosphorylation, but also would reduce the Ca^{2+} overload through NMDARs, and the anticholinesterase activity, what clearly has favored the memory index increase in the scoplomine-treated mice, would allow maintaining the cholinergic neurotransmission. These proposals are also valid for stroke, taking into account its close relationship with neurodegenerative diseases. In the complex scenario of the neuronal death, physiopathological events related to oxidative stress,[25] Ca^{2+} overload,[57−60] and misbalance in the phosphatase/kinase activity are found in most neurodegenerative diseases described.[11] Indeed, stroke is formally considered as an "acute" neurodegenerative disease, taking into account some observations, like the fact that neurons surrounding the central ischemia core show well-characterized cell damage reminding neurodegeneration.[61] Interestingly, it has been shown that cerebral ischemia upregulates the expression of the amyloid precursor protein (APP) in healthy rats,[62] and its amyloidogenic cleavage[63] to form Aβ, which would promote the release of inflammatory mediators amplifying such inflammation after ischemia.[64] In transgenic mice overexpressing APP, the susceptibility of the brain to ischemic injury was increased.[65] Clinical studies have demonstrated that ischemic lesions enhanced the cognitive deficits in AD patients.[66] For all of these reasons, Iadecola and Gorelick hypothesize that the pathogenic factors implicated in both AD and stroke are synergistic rather than additive.[67] As a consequence, it seems reasonable that a potential drug like ITH12246, by targeting PP2A, which has been implicated in both diseases, shows positive in vivo outcomes and becomes of interest for further preclinical studies focusing these two pathologies.

Recently, PP2A enzyme has been considered an interesting target to develop novel compounds for neuroprotection.[12] In this respect, compound ITH12246 could be considered as a wide spectrum neuroprotectant, a profile that is interesting taking into account the multifactorial nature of neurodegenerative diseases that, however, currently do not possess a huge battery of therapeutic strategies to slow down or counteract the disease. This is more dramatic in the case of AD, as there are no optimal medicines to treat AD patients, although the scientific community is tirelessly looking for new promising drugs and compromised targets, focusing mainly on the best-known physiopathological markers, that is Aβ[68] or aggregated τ protein,[69] as well as the so-called cholinergic hypothesis;[70] unfortunately, all of these approaches have failed.[71] As mentioned in the introduction section, stroke does not possess efficient medicines, too. Unlike AD, the physiopathological targets that trigger the ischemic damage are validated. The most logical strategy seems to modulate the activity of the glutamate receptors. However, glutamate receptors antagonists have shown severe side effects, for example, psychotomimesis, respiratory depresion, or cardiovascular dysregulation.[20] Thus, our hypothesis states that, by stabilizing the PP2A activity, ITH12246 could indirectly modulate the glutamate overexposure without affecting some other physiological functions of the glutamate receptors.

For this reason, the search of new drugs for neurodegenerative diseases acting by newly proposed mechanisms becomes mandatory, for example, the pharmacological intervention over PP2A enzyme. We believe that the results obtained in the present study with ITH12246 give confidence to further investigate its effects for stroke and AD.

METHODS

Reagents. Dimethyl sulfoxide (DMSO), glutamic acid, melatonin, rotenone, oligomycin A, scopolamine, and tacrine were purchased from Sigma Aldrich (Madrid, Spain). Galantamine was purchased from Tocris House (Bristol, U.K.).

Synthesis of Ethyl 5-Amino-2-methyl-6,7,8,9-tetrahydrobenzo[*b*][1,8]-naphthyridine-3-carboxylate (ITH12246). The synthesis of the 1,8-naphthyridine derivative ITH12246 was carried out as described.[23] Hydrochloride salt of ITH12246 was obtained according to the procedure described,[72] showing good analytical and spectral data according to its structure.

Culture of SH-SY5Y Neuroblastoma Cells. SH-SY5Y cells were seeded, subcultured, and treated similarly to what was previously described.[34]

Neuronal Viability Experiments in Rat Hippocampal Slices Stressed with Glutamate. Experiments were performed in hippocampal slices prepared from brains of 2-month-old Sprague−Dawley rats (275−325 g weight), following the European Union Council Directive issued for these purposes and were approved by the Ethics Committee of the Facultad de Medicina, Universidad Autónoma de Madrid, Spain. All efforts were made to minimize the number of animals and their suffering. We followed the protocol described by Egea and co-workers[73] with slight modifications.[23] Rats were quickly decapitated under sodium pentobarbital anesthesia (60 mg kg^{-1}, i.p.). Forebrains were rapidly removed from the skull and placed into ice-cold Krebs-bicarbonate dissection buffer (pH 7.4) containing the following: NaCl 120 mM, KCl 2 mM, $CaCl_2$ 0.5 mM, $NaHCO_3$ 26 mM, $MgSO_4$ 10 mM, KH_2PO_4 1.18 mM, glucose 11 mM, and sucrose 200 mM. Hippocampi were quickly dissected, and slices (300 μm thick) were rapidly prepared using a McIlwain tissue chopper, separated in Krebs buffer at 4 °C, and allowed to recover for 45 min in Krebs-bicarbonate buffer at 34 °C. Experiments were performed at 37 °C. A control and a neurotoxicity group was included in all experiments. To perform the experiments, we followed the protocols shown on top of the figures and briefly described in their legends. Hippocampal slices were collected immediately after the neurotoxic compound exposure period and were incubated with MTT (0.5 mg mL^{-1}) in Krebs-bicarbonate solution for 30 min at 37 °C. Hippocampal slice viability was determined by the ability of cells to reduce MTT.[29] Formazan production was measured as described below.

Cell Incubation with Compound Solutions. To prepare stock solutions of the various reagents, they were dissolved in DMSO, except for ITH12246 and galantamine, which were dissolved in water, at the concentration of 10^{-2} M. All solutions were stored in aliquots at −20 °C. Once defrosted for a given experiment, the aliquot was discarded. The final concentrations of DMSO used (always <0.1%) did not cause cell toxicity.

MTT Assay. Cell viability, virtually the mitochondrial activity of living cells, was measured by quantitative colorimetric assay with the dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), as described previously.[29] SH-SY5Y cells were seeded into 48-well culture. MTT was added to the wells (5 mg mL^{-1}) and allowed to incubate in the dark at 37 °C for 2 h, followed by cell lysis. The tetrazolium ring of MTT can be cleaved by active reductases in order to produce a precipitated formazan derivative. The generated formazan was dissolved by adding 200 μL of DMSO, resulting in a colored solution whose optical density was measured in a colorimetric plate reader at 540 nm (FLUOstar Optima, BMG, Germany). All MTT assays were performed in triplicate and quadruplicate for cultured cells and hippocampal slices, respectively. Data were expressed as percentage of MTT reduction, taking the maximum control tissue capability in each individual experiment as 100%. In some part of the text, data are discussed as percentage of protection afforded by a given treatment; for instance, a 50% decrease of MTT reduction means 50% cell death; hence, a decrease of 25% cell death by a given treatment means 50% neuroprotection.

In Vitro Blood-Brain Barrier Permeation Assay by PAMPA Method. Prediction of the brain penetration was evaluated using a parallel artificial membrane permeation assay (PAMPA), in a similar manner as described previously.[39−45] Pipetting was performed with a semiautomatic robot (CyBi-SELMA) and UV reading with a microplate spectrophotometer (Multiskan Spectrum, Thermo Electron Co.). Commercial drugs, phosphate buffered saline solution at pH 7.4 (PBS), and dodecane were purchased from Sigma, Aldrich, Acros, and Fluka. Millex filter units (PVDF membrane, diameter 25 mm, pore size 0.45 μm) were acquired from Millipore. The porcine brain lipid (PBL) was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μm), and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 200 μL of PBS/ethanol (70:30), and the filter surface of the donor microplate was impregnated with 4 μL of PBL in dodecane (20 mg mL^{-1}). Compounds were dissolved in PBS/ethanol (70:30) at 100 μg mL^{-1}, filtered through a Millex filter, and then added to the donor wells (200 μL). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which was left undisturbed for 240 min at 25 °C. After incubation, the donor plate was carefully removed and the concentration of compounds in the acceptor wells was determined by UV−vis spectroscopy. Every sample is analyzed at five wavelengths, in four wells, and at least in three independent runs, and the results are given as the mean ± standard deviation. In each experiment, 11 quality control standards of known BBB permeability were included to validate the analysis set.

Evaluation of Potential Hepatotoxicity of ITH12246. To evaluate potential hepatotoxicity of ITH12246, cultures of HepG2 cell line were used, and cell viability in presence of different concentrations of ITH12246 was assessed by the method of the MTT reduction, as described above. The human hepatoma cell line HepG2 was cultured in Eagle's minimum essential medium (EMEM) supplemented with 15 non essential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin into 75 mL flasks. For assays, cells were subcultured in 48-well plates at a density of 1×10^5 cells per well. Once seeded, they were allowed 24 h for a good wall adhesion, after which drugs were incubated in presence of 1% FBS. Cell viability by MTT reduction was measured 24 h after. Experiments were carried out by triplicate from four different batches.

Evalutation of Spatial Memory by the Object Placement Test Using Scopolamine as Memory Impairment Inducer. Adult male Swiss mice (12−14 weeks old, weighing 35−40 g; Charles River, Wilmington, MA) were used. The experimental procedures were performed following the *Guide for the Care and Use of Laboratory Animals* and were previously approved by our Hospital's ethics committee for the care and use of animals in research, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the Spanish *Real Decreto* of October 10th 2005 (RD 1201/2005). All efforts were made to minimize animal suffering and to reduce the number of animals used. Sample size was estimated according to the parameters observed in a previous object placement test,[74] using the formula described by Snedecor and Cochran,[75] with an online calculator, obtaining the number 7 as the minimum number of animals needed. Mice were housed under controlled temperature and lighting conditions with food and water provided ad libitum. Mice were placed for 5 min on a field ($40 \times 40 \times 40$ cm^3 made up of gray polyvinyl chloride) for 2 days prior to the day of the test to reduce stress and neophobic responses. On the test day, mice ($n \geq 9$) were randomly divided into five groups and injected with the drugs. The experimental groups, according to the substances injected, were as follows: basal (saline), scopolamine, scopolamine +2.5 mg/kg galantamine, scopolamine +2.5 mg/kg ITH12246, and scopolamine +10 mg/kg ITH12246. Mice were placed on the field with two identical objects (cylindrical glass bottles, heavy enough to prevent mice from moving them; height, 22 cm; diameter, 9 cm) and allowed to explore them for 30 s (T1, sample trial). Exploration of the objects was timed with stopwatches when mice sniffed at, whisked at, or looked at the objects from no more than 2 cm away. After 15 min, mice were placed in the field again, but one of the two objects had been moved to a new location. The time spent exploring the objects in new (novel) and old (familiar) locations (T2, recognition trial) was observed and timed with stopwatches for 3 min by an observer who was unaware of the treatments. All locations for the objects were counterbalanced among groups, and objects and field were washed with 0.1% acetic acid between trials to remove any olfactory cues. The time measured as an exploration behavior was used to calculate a memory discrimination index (DI) as previously reported:[76] DI = $(N - F)/(N + F)$, where N is the time spent exploring the new located object and F is the time spent exploring the familiar located object. Higher DI is considered to reflect greater memory ability.

Induction of Focal Ischemia in Mice. To induce ischemia, animals were anesthetized with 1.5% isoflurane in oxygen under spontaneous respiration. Mice were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and body temperature was maintained at 37.0 ± 0.5 °C using a servo-controlled rectal probe heating pad (Cibertec, Madrid, Spain). A midline scalp incision was made, the skull was exposed with removal of the periosteum, and both bregma and lambda points were identified. A cold-light (Zeiss KL 1500 LCD, Jena, Germany) was centered using a micromanipulator at 2.0 mm posterior and 3.0 mm lateral to bregma on the right side using a fiber optic bundle of 1.9 mm in diameter. One milligram (0.1 mL) of the photosensitive dye Rose Bengal (Sigma-Aldrich, St. Louis, MO) dissolved in sterile saline was injected i.p., and 20 min later the brains were illuminated through the intact skull for 30 min. After completion of the surgical procedures, the incision was sutured and the mice were allowed to recover. Animals showed no visible neurological or behavioral damage.

For the drug administration, mice were randomly divided into four groups ($n \geq 4$), subjected to ischemia and treated as follows: 0.9% NaCl sterile saline solution (saline group); 2.5 mg/kg ITH12246 (2.5 group); 10 mg/kg ITH12246 (10 group) and 15 mg/kg melatonin (melatonin group). ITH12246 was dissolved in saline and given i.p. 1 h before the onset of ischemia and twice a day thereafter. Doses were chosen according to previous in vitro data (Figure 3). Melatonin (Sigma-Aldrich, St. Louis, MO) was used as a positive control, and it was dissolved in saline with 5% DMSO and administered by i.p. injection 30 min before the onset of ischemia as well as 24 and 48 h after ischemia, as previously described.[54]

Three days after ischemia, mice were decapitated under deep isoflurane anesthesia. Brains were extracted and freshly cut using acrylic brain matrices (Stoelting Co., Wood Dale, IL) into eight coronal 1 mm thick slices. The slices were then stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Panreac, Castellar del Vallés, Spain) in 0.1 M phosphate buffer, pH 7.4, for 30 min at 37 °C and then fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer. Both rostral and caudal sides of the slices were digitized and cortical infarct volume was assessed by an observer blinded to the experimental groups using Image J (National Institutes of Health, Bethesda, MD). Briefly, after image calibration, ipsilateral cortex, contralateral cortex, and cortical infarct borders were delineated to obtain the areas; volumes were then calculated and the edema's effect was corrected. After addition of the corrected infarct volumes of the eight slices, and doing the same with the edema, results were expressed as percentage of the ratio relative to the contralateral cortex in order to correct for normal size differences between animals. Finally, the mean of rostral and caudal sides was calculated for each animal.

Data Analysis. Data are presented as mean ± standard error of the mean (SEM). Statistical differences were determined with one-way ANOVA followed by Dunnet post hoc test. Differences were considered to be statistically significant when $p \leq 0.05$. All statistical procedures were carried out using GraphPad Prism software version 5.0 for Mac OS X compatible computer.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Protection experiments by ITH12246 in hippocampal slices lesioned with glutamate in presence of kinase inhibitors, and

graphical treatment of the learning trial T1, and time spent in both trials in the object placement test. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

S. Lorrio conducted and analyzed in vivo experiments; A. Romero conducted hepatotoxicity assays and the experiment of melatonin protection against glutamate-induced hippocampal slice damage; L. Gonzalez-Lafuente and R. Lajarín-Cuesta conducted experiments on SH-SY5Y cells; F. J. Martinez-Sanz contributed to ITH12246 synthesis and purification; M. Estrada and M. I. Rodríguez-Franco performed PAMPA experiments, A. Samadi synthesized the hydrochloride salt of ITH12246; J. Marco-Contelles conceived the ITH12246 structure; M. G. López and M. Villarroya oversaw both in vivo and in vitro pharmacological experiments; C. de los Ríos synthesized ITH12246 and designed and oversaw in vitro experiments.

Funding

C. de los Rios and L. Gonzalez-Lafuente thank IS Carlos III for research contract under Miguel Servet Program. R. Lajarín-Cuesta thanks Universidad Autonoma de Madrid for predoctoral fellowship. M. Estrada thanks COLCIENCIAS (Colombia) for a Ph.D. fellowship. This work was supported by the following grants from Spanish Institutions to: C. de los Ríos, Programa Miguel Servet (CP10/00531, IS Carlos III); M. G. López, Spanish Ministry of Science and Innovation (SAF2009-12150); M. I. Rodríguez-Franco, Spanish Ministry of Science and Innovation (SAF2012-31035); and J. Marco-Contelles (SAF2009-07271).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Prof. Antonio G. Garcia (Instituto Teofilo Hernando, Universidad Autonoma de Madrid) for his invaluable scientific help. We thank the continued support of Fundacion de Investigación Biomedica, Hospital Universitario de la Princesa, Madrid, Spain, Fundación Teofilo Hernando, Madrid, Spain, and CSIC, Madrid, Spain.

ABBREVIATIONS

Aβ, amyloid beta peptide; AD, Alzheimer's disease; BBB, blood-brain barrier; CNS, central nervous system; LDH, lactate dehydrogenase; GSH, gluthatione; MCAO, middle cerebral arterial occlusion; MEM, memantine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFT, neurofibrillary tangles; NMDA, *N*-methyl-D-aspartate; OA, okadaic acid; OGD, oxygen and glucose deprivation; O/R, rotenone plus oligomycin A; OPT, object placement test; Pin-1, peptidyl prolyl *cis*–*trans* isomerase; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; SEM, standard error of the mean; τ, tau protein

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