

Undifferentiated and Differentiated PC12 Cells Protected by Huprines Against Injury Induced by Hydrogen Peroxide

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Abstract

Oxidative stress is implicated in the pathogenesis of neurodegenerative disorders and hydrogen peroxide (H₂O₂) plays a central role in the stress. Huprines, a group of potent acetylcholinesterase inhibitors (AChEIs), have shown a broad cholinergic pharmacological profile. Recently, it has been observed that huprine X (HX) improves cognition in non transgenic middle aged mice and shows a neuroprotective activity (increased synaptophysin expression) in 3xTg-AD mice. Consequently, in the present experiments the potential neuroprotective effect of huprines (HX, HY, HZ) has been analyzed in two different *in vitro* conditions: undifferentiated and NGF-differentiated PC12 cells. Cells were subjected to oxidative insult (H₂O₂, 200 μM) and the protective effects of HX, HY and HZ (0.01 μM–1 μM) were analyzed after a pre-incubation period of 24 and 48 hours. All huprines showed protective effects in both undifferentiated and NGF-differentiated cells, however only in differentiated cells the effect was dependent on cholinergic receptors as atropine (muscarinic antagonist, 0.1 μM) and mecamylamine (nicotinic antagonist, 100 μM) reverted the neuroprotection action of huprines. The decrease in SOD activity observed after oxidative insult was overcome in the presence of huprines and this effect was not mediated by muscarinic or nicotinic receptors. In conclusion, huprines displayed neuroprotective properties as previously observed in *in vivo* studies. In addition, these effects were mediated by cholinergic receptors only in differentiated cells. However, a non-cholinergic mechanism, probably through an increase in SOD activity, seems to be also involved in the neuroprotective effects of huprines.

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder and the most prevalent cause of dementia with ageing. The presence of β-amyloid (Aβ)-containing senile plaques and neurofibrillary tangles in the AD brain are the main hallmarks of the disease and are widely believed to be responsible for neuronal degeneration and cell death in this disorder [1]. Although the AD aetiology and pathogenesis are still unknown, several reports point out that excitotoxicity, reduced energy metabolism, mitochondrial dysfunctions, and oxidative stress are very important mechanisms involved in cell death in AD [2]. This hypothesis is supported by the finding that Aβ peptides are associated with free-radical oxidative stress and are the main cause of cellular dysfunctions [3],[4],[5],[6],[7]. As a consequence of that, necrotic and apoptotic processes occur and are the main pathways of cell death in AD [8],[9],[10],[11].

The key symptoms of AD are primarily caused by cholinergic dysfunction, and a significant correlation has been found between a cortical decrease in cholinergic activity and cognitive deterioration (the cholinergic hypothesis). The present

approved therapeutic approach is mainly based on increasing cholinergic transmission using cholinesterase inhibitors (ChEI) [12],[13],[14],[15],[16],[17]. However, the therapeutic benefit of such agents is not entirely explained by increasing activity of the cholinergic system, and a large body of evidence shows that ChEI have multiple effects on the central nervous system, some of which could be regarded as broadly neuroprotective [13],[18],[19]. Thus, donepezil, the most widely prescribed AD therapy, markedly decreases lactate dehydrogenase (LDH) release in cortical cells previously exposed to oxygen-glucose deprivation (OGD), and in addition it has a protective effect in gerbils [19]. Similar results have been observed in cell cultures in which neurotoxicity was induced using Aβ protein [20]. It has been suggested that the efficacy of galantamine, a modest AChEI, with allosteric modulator activity on the nicotinic receptor, can be ascribed to its neuroprotective activity mediated by α7 nicotinic receptors [18]. Rivastigmine, another AChEI recently approved, also has a neuroprotective effect although it is completely independent of nicotinic receptors [21]. Consequently, AChEI

might be able to act as disease-modifying anti-Alzheimer drugs rather than as mere palliative drugs [22].

It has been demonstrated that huprines, a group of anticholinesterase drugs obtained by the molecular hybridization of tacrine and (\pm)-huperzine A, show high selectivity and a potent inhibitory action on AChE in both *in vitro* and *ex vivo* studies [23],[24], an agonistic action on muscarinic and nicotinic receptors [25],[26], and can affect the binding of ligands to the peripheral site of AChE, thereby inhibiting the amyloidogenic process induced by the enzyme [27],[28]. Despite controversial data about the ability of tacrine to exhibit a neuroprotective effect [21],[29], it has been widely demonstrated that huperzine A, the other component of the huprine molecule, shows a neuroprotective activity against different stimuli in several experimental conditions [29],[30]. Interestingly, a recent *in vivo* study has revealed the neuroprotective effect of huprine X, since in 3xTg mice treated with the drug, it significantly increased the synaptophysin content to levels close to those of non-transgenic mice [31] and improved cognition by regulating some neurochemical processes, such as alpha secretases and glycogen synthase kinase 3-beta, in the same transgenic mice [32].

Clonal cell-lines, such as rat pheochromocytoma PC12 cells, provide a useful model system for the investigation of neuronal injury. In contrast with central nervous system primary cultures, which contain many types of nerve cells as well as glial populations, clonal nerve cell-lines yield homogeneous populations, allow an easy manipulation and control over the extracellular milieu, and provide definite advantages over animal experiments. Due to the multitarget pharmacological profile of huprines and because of the importance of oxidative stress in most neurodegenerative diseases, especially in AD, in the present study the potential neuroprotective effects of huprine X (12-amino-3-chloro-9-ethyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride), huprine Y (12-amino-3-chloro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinoline hydrochloride) and huprine Z (12-amino-3-fluoro-6,7,10,11-tetrahydro-9-methyl-7,11-methanocycloocta[*b*]quinoline hydrochloride) were assessed. For this purpose, undifferentiated and differentiated PC12 cells were subjected to a necrotic insult (hydrogen peroxide), this being the most common process in all degenerative disorders [11],[33].

Results

Effect of huprines on cell death induced by H₂O₂ in PC12 cells

1. Undifferentiated PC12 cells. Firstly, the cells were incubated in the presence of HX, HY, and HZ (1 μ M, 0.1 μ M, 0.01 μ M) during 1 h and 2 h prior to H₂O₂ addition. In these experimental conditions no protective effects were found (data not shown). In the light of these data, we proceeded to increase the incubation time of drugs to 24 h and 48 h prior to H₂O₂ addition. The effect of H₂O₂ treatment and of the pre-treatment with huprines prior to H₂O₂ addition in cell survival is shown in Fig. 1. In the 24 h pre-treatment of huprines prior to H₂O₂ addition, drugs induced a percentage of protection between 47% and 65% at 1 μ M and 0.1 μ M but no significant changes were observed at 0.01 μ M (Table 1). When the pre-treatment period was increased to 48 h, a significant increase in the protection percentage was observed at all concentrations used, even at 0.01 μ M (between 12% and 70%).

In no case modifications in cell survival were observed when huprines were incubated alone (without H₂O₂) at concentrations of 0.01 μ M, 0.1 μ M, and 1 μ M, nevertheless, at concentrations as

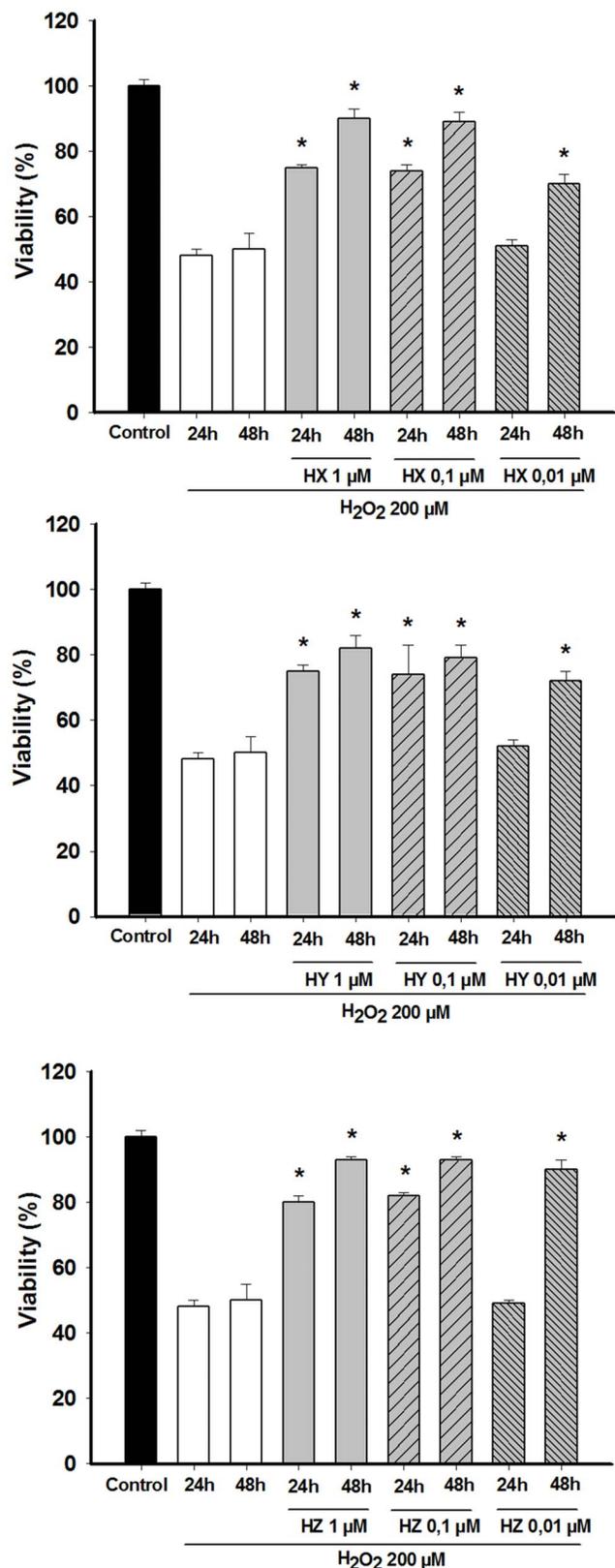


Figure 1. Attenuation of H₂O₂-induced cell damage by different concentrations (0.01 μ M, 0.1 μ M, 1 μ M) of (A) HX, (B) HY and (C) HZ in undifferentiated PC12 cells. Cells were incubated with 200 μ M H₂O₂ for 2 h. Huprines were added to the culture 24–48 h prior to H₂O₂ addition. Cell viability was assessed by

measuring the MTT reduction. At least three independent experiments were carried out in triplicate. The data are means \pm s.e.m. expressed as percentage of control value. * $P < 0.05$ compared with H₂O₂ group (Dunnett's test).
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high as 10 μ M and 100 μ M, huprines reduced cellular viability (data not shown).

2. NGF-differentiated PC12 cells. Similar to undifferentiated PC12 cells, nerve growth factor (NGF)-treated PC12 cells suffered around a 50% reduction of cellular viability when they were exposed to the H₂O₂ insult (200 μ M) for 2 h. Taking the results obtained with undifferentiated PC12 cells as starting point, in this set of experiments drugs were pre-incubated for 48 h and HX, HY, and HZ were added at concentrations of 0.1 μ M and 1 μ M (Fig. 2). In this experimental approach a percentage of protection ranging from 44% to 56% was obtained with all of the huprines at both concentrations used (Table 2).

Role of muscarinic and nicotinic receptors in the neuroprotective effects of huprines

1. Undifferentiated PC12 cells. Because previous studies in our laboratory have shown that huprines interact with muscarinic and nicotinic receptors [26],[34],[35], we studied the possible role of cholinergic receptors in the neuroprotective effect exerted by huprines. For this purpose, MEC (100 μ M), a nicotinic receptor (nAChR) antagonist, or ATR (0.1 μ M), a muscarinic receptor antagonist, were used. Each antagonist was added to the medium concomitantly with huprines. Neither MEC nor ATR antagonised the increase of the survival induced by 48 h of pre-treatment with huprines in undifferentiated PC12 cells (Table 1). These results suggest that the neuroprotective effect of huprines in this cell line is not mediated by an interaction with muscarinic or nicotinic receptors.

Worthy of note, addition of MEC and ATR alone did not modify cell viability.

2. NGF-differentiated PC12 cells. The role of muscarinic and nicotinic receptors in NGF-differentiated PC12 cells was also assessed. In this case, MEC (100 μ M) significantly prevented the protective effect of huprines, reducing significantly the neuroprotection percentage induced by drugs (Table 2). ATR (0.1 μ M) also significantly reduced the neuroprotection effects of huprines, however this reduction was lower than that observed in the presence of MEC.

Effect of huprines on SOD activity

1. Undifferentiated PC12 cells. PC12 cultures exposure to H₂O₂ (200 μ M) for 2 h produced a marked decrease of superoxide dismutase (SOD) activity, as compared with control cells. The 48 h pre-treatment of the undifferentiated PC12 cells with HX, HY, and HZ (0.01 μ M–1 μ M) prior to H₂O₂ addition led to a significant increase (24%–86%) of SOD activity, when compared with the H₂O₂-treated group (Fig. 3). The levels of SOD activity after the huprines (0.1 μ M–1 μ M) treatment could also surpass the SOD activity levels of the control cells. This increase in SOD activity might be related to the neuroprotective effect of the huprines against H₂O₂.

Incubation of huprines in the absence of H₂O₂ did not modify the enzyme activity.

2. NGF-differentiated PC12 cells. The 2 h exposure to H₂O₂ (200 μ M) led to a remarkable reduction in SOD activity, while pre-treatment with huprines prior to H₂O₂ addition significantly increased the enzymatic activity. This modification was found in all of the huprines at the concentration of 1 μ M (Fig. 4). Given that cell viability is greatly reduced in the presence of MEC in NGF-treated PC12 cells, and the fact that nAChRs have been involved in SOD activation [36], the role of such receptors in the SOD activity was assessed. MEC slightly reversed the enzyme activity, but this reversion was not statistically

Table 1. Effect of huprines pretreatment on undifferentiated PC12 cell survival after exposure of cells to H₂O₂ (200 μ M).

TREATMENT	% PROTECTION 24 H	% PROTECTION 48 H
Huprine X (1 μ M)	47 \pm 0.6*	70 \pm 4.8*
Huprine X (0.1 μ M)	49 \pm 2.9*	74 \pm 4.6*
Huprine X (0.01 μ M)	3 \pm 0.9	12 \pm 5.4*
Huprine X (1 μ M) +MEC (100 μ M)		68 \pm 0.5 ^a
Huprine X (1 μ M) +ATR (0.1 μ M)		66 \pm 10.2 ^a
Huprine Y (1 μ M)	52 \pm 2.3*	60 \pm 3.6*
Huprine Y (0.1 μ M)	47 \pm 8.7*	51 \pm 3.3*
Huprine Y (0.01 μ M)	4 \pm 5.5	22 \pm 5.5*
Huprine Y (1 μ M) +MEC (100 μ M)		61 \pm 9.3 ^b
Huprine Y (1 μ M) +ATR (0.1 μ M)		58 \pm 1.2 ^b
Huprine Z (1 μ M)	62 \pm 2.2*	65 \pm 0.5*
Huprine Z (0.1 μ M)	65 \pm 1.3*	65 \pm 0.5*
Huprine Z (0.01 μ M)	0.5 \pm 2.4	27 \pm 3.7*
Huprine Z (1 μ M) +MEC (100 μ M)		59 \pm 4.7 ^c
Huprine Z (1 μ M) +ATR (0.1 μ M)		55 \pm 7.6 ^c

Effect of antagonists mecamylamine (MEC) and atropine (ATR) on huprines protective effect on PC12 cells survival after exposure of the cells to H₂O₂. See Methods for details of cell treatment and conditions. Values are expressed as percentage of protection (mean \pm s.e.m.) obtained from at least three independent experiments run in triplicate. * $P < 0.05$ as compared with H₂O₂-treated group (Dunnett's test).^{a,b,c} No significant changes compared with huprine X, Y and Z (1 μ M), respectively.

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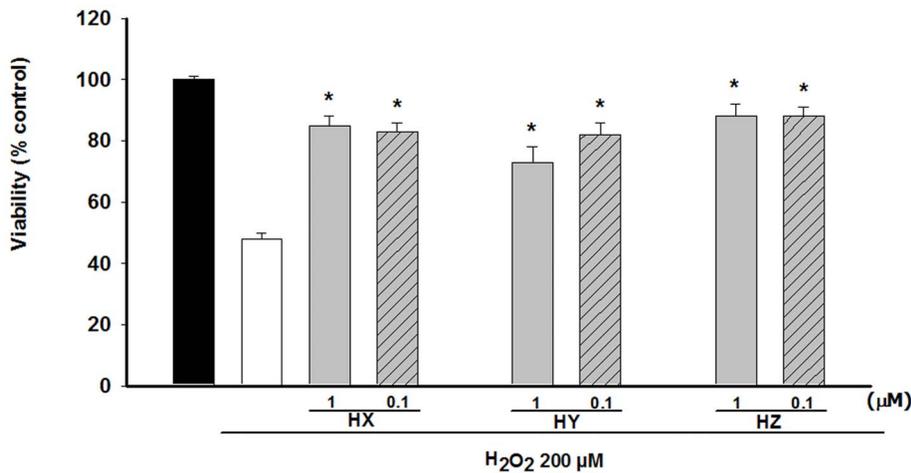


Figure 2. Attenuation of H₂O₂-induced cell damage by 0.1 μM and 1 μM concentrations of huprines: HX, HY and HZ in NGF differentiated PC12 cells. Cells were incubated with 200 μM H₂O₂ for 2 h. Compounds were added to the culture 48 h prior to H₂O₂ addition. Cell viability was assessed by measuring the MTT reduction. At least three independent experiments were carried out in triplicate. The data are means ± s.e.m. expressed as percentage of control value. *P<0.05 compared with H₂O₂ group (Dunnett's test). doi:10.1371/journal.pone.0074344.g002

significant (Fig. 4). ATR did not modify the increase of SOD induced by drugs (data not shown).

Discussion

Besides the pathological hallmarks of AD, which include the accumulation of protein deposits in the brain as Aβ plaques and neurofibrillary tangles (NFT), AD brains exhibit constant evidence of reactive oxygen species (ROS) - and reactive nitrogen species (RNS)-mediated injury [37]. In some circumstances the produc-

tion of oxidant species can exceed the endogenous antioxidant ability to destroy them, and an oxidative imbalance occurs. Consequently, the protection of neurons from oxidative damage and death is an important challenge in the development of new treatments against neurodegenerative diseases. PC12 is a cell line characterised by showing sympathetic neuronal cell properties, morphologically, physiologically, and biochemically [38]. In addition, when they are incubated with NGF, PC12 cells increase the expression of cholinergic receptors, choline acetyltransferase expression and acetylcholinesterase activity [39]. Therefore, PC12 cells represent an appropriate experimental approach to study the role of cholinergic receptors in the neuroprotective effect of acetylcholinesterase inhibitors under different conditions.

First of all, we subjected the undifferentiated PC12 cells to an oxidative insult, such as hydrogen peroxide, not only because it is a hydrogen free-radical generator, but also because the toxicity of Aβ, the major pathological hallmark for AD, is partially mediated by hydrogen peroxide [40]. Using this experimental approach, the present results have revealed that HX, HY, and HZ induced a significant protective effect against hydrogen peroxide. All huprines showed a similar effect, suggesting that the slight structural differences among the three compounds are not relevant for their protective activity.

Neuroprotection is a very common feature among different AChEIs in response to different harmful insults. Thus, the acetylcholinesterase inhibitor galantamine showed a protective effect after Aβ or thapsigargin stimulus in the human neuroblastoma cell line SH-SY5Y, as well as in bovine chromaffin cells [41]. Additionally, donepezil, rivastigmine and galantamine showed a neuroprotective effect against okadaic acid in undifferentiated SH-SY5Y cells [21]. Huperzine A, one of the parent compounds from which huprines were designed, showed a protective effect after inducing oxygen-glucose deprivation in pheochromocytoma cells, most likely by alleviating disturbances of oxidative and energy metabolism [42] or in the presence of hydrogen peroxide, which improved expression of apoptosis-related genes [43]. Nevertheless, it is necessary to state that the protective effect of huprines was only apparent when cells were preincubated with the drugs for periods of 24 h or longer (48 h), suggesting that a prolonged treatment before hydrogen peroxide exposure is necessary to elicit

Table 2. Effect of huprines pretreatment on NGF differentiated PC12 cells survival after exposure of cells to H₂O₂ (200 μM).

TREATMENT	% PROTECTION 48 H
Huprine X (1 μM)	53±5.0*
Huprine X (0.1 μM)	44±6.0*
Huprine X (1 μM) +MEC (100 μM)	7±3.2 ^b
Huprine X (1 μM) +ATR (0.1 μM)	32±2.7 ^b
Huprine Y (1 μM)	56±5.0*
Huprine Y (0.1 μM)	49±6.1*
Huprine Y (1 μM) +MEC (100 μM)	2.3±0.9 ^b
Huprine Y (1 μM) +ATR (0.1 μM)	16±1.6 ^b
Huprine Z (1 μM)	56±7.0*
Huprine Z (0.1 μM)	47±7.5*
Huprine Z (1 μM) +MEC (100 μM)	17±3.4 ^b
Huprine Z (1 μM) +ATR (0.1 μM)	27±7.1 ^b

Effect of antagonists mecamylamine (MEC) and atropine (ATR) on huprines protective effect on PC12 cells survival after exposure of the cells to H₂O₂. See Methods for details of cell treatment and conditions. Values are expressed as percentage of protection (mean ± s.e.m.) obtained from at least three independent experiments run in triplicate. *P<0.05 as compared with H₂O₂-treated group; ^bP<0.05 as compared with 1 μM concentration of corresponding drug (Dunnett's test).

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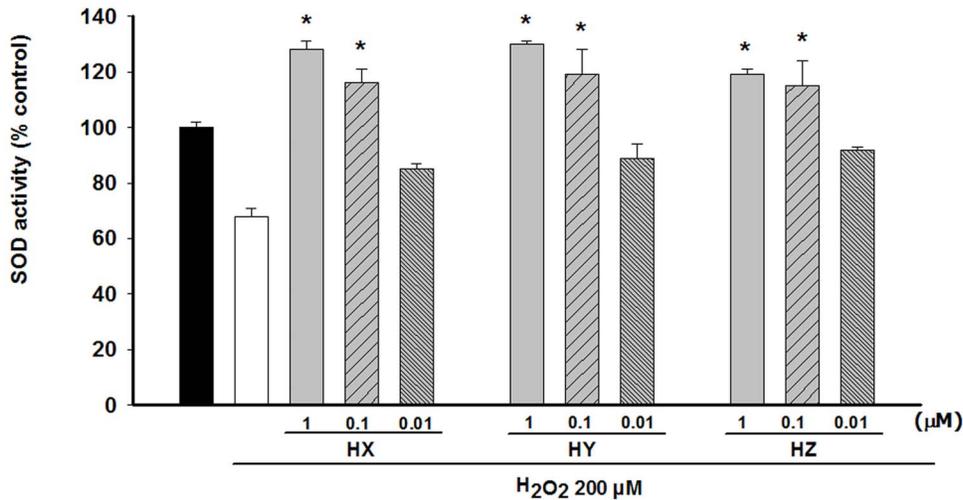


Figure 3. Modulation of the SOD activity in undifferentiated PC12 cells after preincubation with HX, HY and HZ. Cells were incubated with 200 μM H₂O₂ for 2 h. Huprines were added to the culture 48 h prior to H₂O₂ addition. SOD activity was measured with a SOD activity assay from Fluka. At least three independent experiments were carried out in triplicate. The data are means ± s.e.m. expressed as percentage of control value. *P<0.05 compared with H₂O₂ group (Dunnett's test). doi:10.1371/journal.pone.0074344.g003

their neuroprotective activity. Indeed, when drugs were added concomitantly with hydrogen peroxide or even a few hours before inducing the insult, no protective effects of huprines were observed (data not shown). The relevance of the incubation time prior to the addition of some toxic agents is well established in the literature. Studies performed with donepezil, galantamine, and tacrine in cortical neurons using glutamate as a toxic agent have shown that the protective effects of these drugs were more pronounced at longer incubation periods [44],[45]. It has been suggested that a delay in the neuroprotective effects of some AChEIs could be ascribed to the cascade of events that take place after nicotinic receptor activation [44]. Herein, the neuroprotective effects of huprines were higher when the preincubation time was extended up to 48 h, and in these conditions the effects of the drugs were

also observed at a concentration as low as 10 nM. These data could also suggest that the effects of huprines are less apparent when the cell damage has been already established. In fact, HX improved cognitive deficits in 3xTg-AD mice 7 months old [41] but such effect was not observed in 12 month old 3xTg-AD mice (non published data).

In addition to their high affinity inhibition of AChE (picomolar range) these compounds are able to stimulate muscarinic and nicotinic receptors [25],[34],[46]. These data led us to speculate that the agonistic effect of the huprines on cholinergic receptors could trigger the neuroprotective effect together with the potentiation of the cholinergic system associated with the inhibitory effect on AChE. In fact, the neuroprotective effect observed in some AChEIs has been ascribed to their activity on

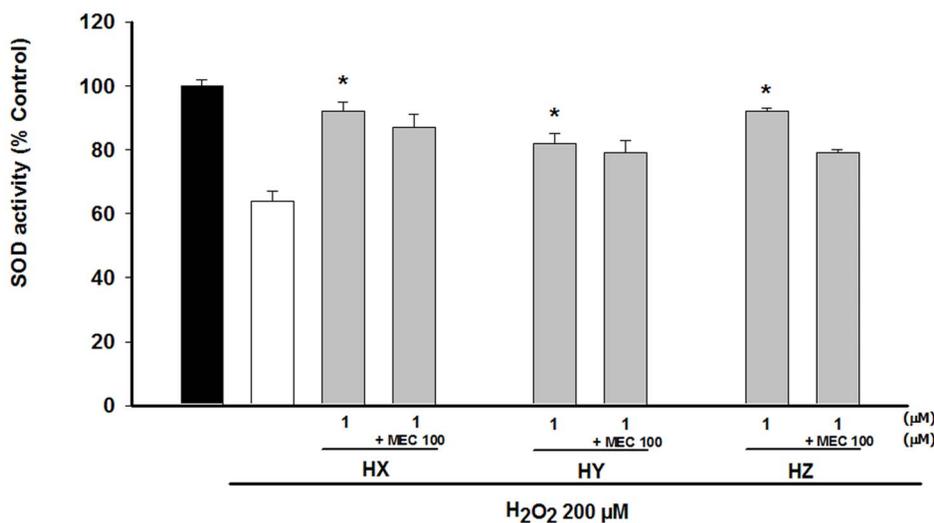


Figure 4. Modulation of the SOD activity in NGF-PC12 differentiated cells by HX, HY and HZ. Huprines were added alone or concomitantly with MEC (100 μM) to the culture 48 h prior to H₂O₂ addition. Then, cells were incubated with 200 μM H₂O₂ for 2 h. SOD activity was measured using a SOD activity assay from Fluka. At least three independent experiments were carried out in triplicate. The data are means ± s.e.m. expressed as percentage of control value. *p<0.05 compared with H₂O₂ group (Dunnett's test). doi:10.1371/journal.pone.0074344.g004

nicotinic receptors [18]. In order to analyse the possible involvement of cholinergic receptors in the protection induced by huprines, MEC and ATR were used to block nicotinic and muscarinic receptors, respectively. Both antagonists failed to reverse the effect of all compounds in undifferentiated cells, suggesting that the protection induced by huprines was not related to their activity on cholinergic receptors. The latency period between the addition of drugs to the incubation medium and detection of the protective effect seems to indicate that the mechanism of these drugs might include a chain of events inducing the activation/inactivation of different factors.

Hydrogen peroxide is a free-radical generator that induces cell and tissue damage. SOD is an antioxidant enzyme that plays a pivotal role in maintaining a very low, steady state of intracellular O₂⁻ [47]. The activity of this enzyme was significantly increased by huprines in undifferentiated cells, suggesting that changes in SOD expression and/or activity might be involved in the protective effects of huprines.

It is known that exposure of PC12 cells to NGF leads to changes in their properties, increasing the cholinergic receptors expression. In a second set of experiments we subjected PC12 cells to the presence of NGF and once they were differentiated, cells were treated for 48 h with huprines. As it was observed in undifferentiated cells, HX, HY, and HZ elicited a significant protective effect after adding hydrogen peroxide to the culture medium; however the percentage of protection was lower than in the case of undifferentiated cells. Differentiated cells seem to be more sensitive to oxidant stimulus than undifferentiated cells [48], however, in the present study all cells showed a similar sensitivity to hydrogen peroxide. Huprines were not able to restore cell viability with the same efficacy than in undifferentiated cells.

As mentioned above, NGF-differentiated PC12 cells increase the presence of cholinergic factors, especially nicotinic and muscarinic receptors, therefore cells were incubated with huprines and concomitantly with MEC or ATR to block nicotinic and muscarinic receptors, respectively. In contrast to that observed in undifferentiated cells, in NGF-differentiated PC12 cells a significant reduction of the neuroprotective effects of huprines was observed when the antagonists were added, achieving survival values similar to those obtained in non-treated cells, especially in the presence of MEC. ATR only induced a partial, but significant reversion, of the protective effects of huprines. The role of nicotinic receptors in neuroprotection has been widely described. The higher transcription of nAChR, and especially the $\alpha 5$, $\alpha 7$, and $\beta 4$ subunits of the nAChR in NGF-differentiated cells [49], might explain the differences obtained in undifferentiated and NGF-differentiated PC12 cells. It has also been shown that the neuroprotective effect of donepezil and galantamine against glutamate could be reverted by $\alpha 7$ and $\beta 4$ nAChR antagonists and by some inhibitors of the IP₃-kinases pathway [50]. This complex pathway implies changes in a large number of proteins involved in cellular proliferation and/or apoptosis, modifying the balance between these two processes. Our data suggest that the protection induced by huprines could be mainly related to the nAChRs in NGF-differentiated cells. The higher reduction of the neuroprotective effects of the huprines in the presence of the nicotinic antagonist, as compared with the muscarinic antagonist, is in consonance with the results obtained in other studies, which highlight the role of nAChR in AD development [44],[50]. However, muscarinic receptors have also been related to neuroprotection [51],[52]. Thus, activation of muscarinic receptors could increase the antiapoptotic protein Bcl2 via MAPK, thus preserving the viability of mouse cerebellar granule cells [53].

Herein, the effects of huprines on cell viability were also partially mediated by muscarinic receptors in NGF-differentiated cells.

Finally, the activity of SOD in NGF-differentiated cells was also analysed. As in the case of undifferentiated PC12 cells, a decrease of SOD activity was induced by hydrogen peroxide. Again, the presence of huprines promoted the recovery of enzyme activity. *In vivo* administration of galantamine, an AChEI with nicotinic activity, provides neuroprotection to gerbils in an ischaemia model increasing, among others, SOD activity, and this effect was inhibited by MEC [36]. Rivastigmine and also huperzine A and huperzine B increase SOD activity [54],[55],[56], however, none of these drugs has affinity to nAChRs. It has been demonstrated that nAChRs modulate SOD activity by the activation of PI3K/Akt, nevertheless, the same authors have highlighted the complex regulation of this enzyme, suggesting that multiple positive and negative regulatory elements can be involved [57]. In order to rule out the role of nAChRs in the increase of SOD activity induced by huprines, MEC was also tested in NGF-differentiated cells. Antagonist MEC was unable to modify the effect of huprines on SOD, suggesting that the increase of the enzyme activity induced by huprines is not directly mediated by cholinergic receptors.

In summary, HX, HY, and HZ have shown neuroprotective effects, thereby supporting previous data observed in *in vivo* studies [31]. In addition, the subtle structural differences among huprines seem to be not relevant for the neuroprotective effect, as all of them provided similar neuroprotective activity. In addition, the protection against hydrogen peroxide was independent of cholinergic receptors in undifferentiated cells, whereas in NGF-differentiated cells nAChRs seem to be involved in this effect, as MEC was able to reverse the effects of huprines. Finally, huprines increased SOD activity, which suggests the ability of the drugs to decrease ROS accumulation in response to oxidative stress. The fact that huprines induced their protective effect by different mechanisms in differentiated cells, as compared to undifferentiated ones, and that their action on SOD activity was totally independent of nicotinic receptors, indicates that multiple pathways can contribute to the protective activity of drugs, and cellular mechanisms involved in such processes are also dependent on the cellular system and environmental conditions in which they are studied. These *in vitro* results could support *in vivo* data observed in NTg and 3xT-AD mice treated by HX in which an improvement in cognition and a positive regulation of synaptophysin have been described [31],[32]. Consequently, more experiments must be carried out to shed more light on the molecular mechanisms involved in the neuroprotective effect of huprines.

Material and Methods

Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), EDTA, foetal bovine serum, glutamine, HEPES, horse serum, hydrogen peroxide, nerve growth factor β (NGF- β), penicillin, poli-L-lysine hydrobromide, potassium phosphate monobasic, protease inhibitor cocktail, RPMI-1640, sodium chloride, sodium phosphate dibasic dihydrate, sodium pyruvate, and streptomycin were purchased from Sigma-Aldrich (St. Louis, USA). Glucose was purchased from Merck (Darmstadt, Germany) and the enzyme trypsin-0.2 g EDTA from Sigma-Aldrich (Saint Louis, USA). Huprines X, Y, and Z (HX, HY and HZ) were synthesized at the Laboratori de Química Farmacèutica, Facultat de Farmàcia, Universitat de Barcelona. The antagonists atropine (ATR) and mecamylamine (MEC) hydrochloride were obtained from Sigma-

Aldrich (St. Louis, USA). Superoxide dismutase activity was determined using the SOD assay kit from Fluka, Sigma-Aldrich, and the Bradford protein assay from Bio-rad (Hercules, California, USA) was used to quantify proteins.

Cell culture and treatment

The rat pheochromocytoma cell line PC12 is a classic *in vitro* neuroendocrine cell model. Unlike primary neurons, undifferentiated PC12 cells do not require NGF for survival, but they respond to it by producing lengthy neurite extensions and by undergoing other neural-specific changes such as an increase of cholinergic receptors expression [58],[59]. NGF-treated PC12 cells exhibit many of the hallmarks of differentiated neurons.

PC12 cells (ATCC, CRL-1721) were generously provided by Dr. N. Gomez (Biochemistry and Molecular Biology Department, UAB, Barcelona, Spain) and grown on polystyrene tissue-culture dishes in RPMI-1640 containing 10% horse serum and 5% foetal bovine serum, supplemented with 2 mM glutamine, 1 mM pyruvate, 100 unit/mL penicillin and 100 mg/mL streptomycin at 37°C with 95% air–5% CO₂. All experiments were carried out 24 h after cells were seeded in 24-well or 6-well plates previously treated with poly-L-lysine hydrobromide. The cellular density in 6-well plates was 200,000 cells/mL and in 24-well plates about 30,000 cells/mL. For experiments in undifferentiated cells 24-well plates were used and the drug treatments were directly started 24 h after seeding the cells. For cell differentiation, the 6-well plates were used and cells were treated with NGF-β 100 ng/mL for 7 days prior to huprines addition. To induce necrosis, cells were incubated with the indicated concentration of H₂O₂ for 2 h. To study the effects of huprines on PC12 cells, cells were preincubated with different concentrations of drugs for 24 h or 48 h before addition H₂O₂. When antagonists mecamlamine (MEC, nicotinic receptor antagonist) and atropine (ATR, muscarinic receptor antagonist) were used they were added concomitantly with agonists.

Cell viability assay

Cell viability was measured by the method of MTT described by Mosmann [60]. Briefly, cells in 24-well plates were rinsed with phosphate-buffered saline (PBS), MTT (0.5 mg/mL) was added to each well and incubated for 30 min at 37°C. After removal of the medium with MTT, cells and dye crystals were solubilised with

200 μL DMSO, and optical density was measured at 570 nm on a microplate reader. The percentage of protection was calculated according to the following equation:

$$\% \text{Protection} = \frac{[\text{survival}(\text{drug} + \text{H}_2\text{O}_2) - \text{survival}(\text{H}_2\text{O}_2)] \times 100}{\text{survival}(\text{control}) - \text{survival}(\text{H}_2\text{O}_2)}$$

Enzymatic assay

For the assay of SOD activity, the cultures were washed with ice-cold PBS and then pooled in 0.1 M PBS, 0.05 mM EDTA buffered solution and homogenized. The homogenate was centrifuged for 1 h at 10,000xg. The resulting supernatants were used in this assay. Superoxide dismutase activity was determined using the SOD assay Kit-WST, obtained from Fluka and was measured in accordance with the instructions supplied by the manufacturer. Briefly, samples were incubated at 37°C for 20 min and the SOD activity, as an inhibition activity, was quantified by measuring the decrease in the color development (amount of superoxide anion) at 440 nm. The effect of huprines on SOD activity was evaluated as the percentage of control group values. The protein level in cells was measured by Bradford method, using bovine serum albumin as standard [61].

Statistical analysis

Data are expressed as means ± SEM and evaluated for statistical significance (P<0.05) with one-way ANOVA followed by Duncan's multiple-range test.

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Author Contributions

Conceived and designed the experiments: MVC AB. Performed the experiments: MP MVC BP. Analyzed the data: MP MVC AB BP. Contributed reagents/materials/analysis tools: DMT PC. Wrote the paper: MVC MP. Design and synthesis of huprines: DMT PC.

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