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Bromoalkaloids Protect Primary Cortical Neurons from Induced Oxidative Stress

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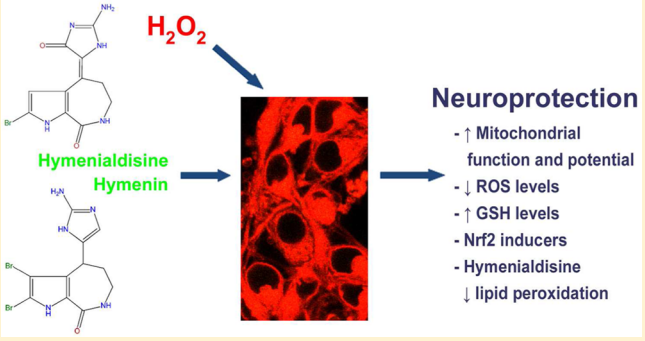
ABSTRACT: Bromoalkaloids are secondary metabolites with a demonstrated high activity in several therapeutic areas. In this research, we probe the neuroprotective and antioxidant activities of hymenialdisine and hymenin. Both structures were tested in an oxidative stress cellular model, consisting of cortical neurons that are incubated with the oxidative stress inducer hydrogen peroxide and the tested compound. Several oxidation biomarkers were analyzed, and the results of the oxidative stress induced neurons in the presence and absence of bromoalkaloids were compared. Both compounds demonstrated significant neuroprotective ability under stress conditions at low nanomolar concentrations, with hymenialdisine highlighted for demonstrating a more complete protection. Also, the activity of hymenialdisine and hymenin was studied in the nuclear factor erythroid 2-related factor 2 (Nrf2)−antioxidant response element (ARE) pathway, and, for the first time, these halogenated metabolites are described as Nrf2 inducers, reinforcing the antioxidant capacity observed and therefore opening a new path of investigation. These results, added to the previously described effect of this compound family in negatively modulating several kinases and proinflammatory cytokines, position hymenialdisine and hymenin as good candidates for the development of new drugs for neurodegenerative diseases.



KEYWORDS: *Bromoalkaloids, cortical neurons, neuroprotection, Nrf2, oxidative stress, sponges*

Oceans are lively ecosystems that cover 70% of Earth. Their vast geographical distribution entails a huge variety of environments and living organisms that are able to adapt to extreme temperatures and high salinity and pressure conditions. To survive in these hostile environments, they produce a high number of diverse compounds.[1] These secondary metabolites, produced for different biological purposes, represent a unique compound library with excellent efficacy and specificity for many different therapeutic targets. In addition, owing to their liberation into water, these substances are usually very active, and their effect occurs at very low concentrations.[2] For all of these reasons, marine compounds have been placed as promising candidates for drug development investigations. Among all sea organisms, the large variety of compounds produced by sponges stands out, comprising rare nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides, and amino acid derivatives that are often halogenated.[3] Alkaloids are nitrogen-containing secondary metabolites for which several pharmacological properties have been ascribed.[4] Bromopyrrolealkaloids are a well-known class of sponge metabolites, often found in genera such as *Axinella*, *Agelas*, *Hymeniacidon*, *Stylotella*, and *Pseudoceratina*,[5] and can even comprise 7−12% of the sponge's dry weight.[6] Bromopyrrole alkaloids have been described as being anticancer, antibacterial, antifungal, antihistaminic, and anti-inflammatory compounds among other numerous effects.[4,7−12] Hymenialdisine (HD) and hymenin (H) are included in this brominated metabolites group, and both compounds are characterized by a $C_{11}N_5$ structure containing guanidine, the above-mentioned bromo, and a fused bicyclic pyrrolo[2,3-*c*]azepin-8-one ring system that bears either a 2-aminoimidazole or a glycocyamidine appendage, but only HD contains a monobromopyrrole moiety in which the bromine atom is situated in the α position (Figure 1).[13] H has shown an adrenoceptor blocking effect and good antibacterial activity against *Bacillus subtilis* and *Escherichia coli*.[2] HD has exhibited inhibitory activity against NF-κB and consequently produces a decrease in IL-8, IL-2, and TNF-α generation, marking it as a

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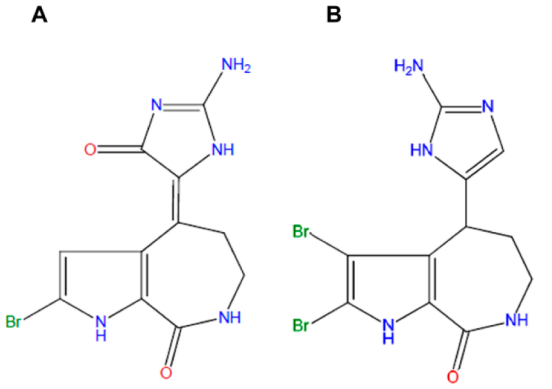

Compound	Abbreviation	Formula	Mol. Weight	Source organism
Hymenialdisine	HD	$C_{11}H_{10}BrN_5O_2$	324.133	*Porifera* (Fijian sample)
Hymenin	H	$C_{11}H_{11}Br_2N_5O$	389.046	*Porifera* (Fijian sample)

Figure 1. Structures of the bromoalkaloids hymenialdisine (A) and hymenin (B).

potential anti-inflammatory agent.[14] In addition, HD and related compounds inhibit several kinases such as glycogen synthase kinase-3beta (GSK3β), cyclin-dependent kinases (CDKs), and casein kinase 1. Specifically, GSK3β and CDK5 are involved in Alzheimer's disease (AD), playing an important role in tau hyperphosphorylation; therefore, their inhibition by HD also diminishes the levels of phosphorylated tau in an *in vitro* model.[15] Thus, HD is a promising compound for neurodegenerative disorder research.

Neurodegenerative diseases comprise a large group of pathologies characterized by a progressive and selective functional loss or sensory dysfunction in nerve cells from the brain and spinal cord and include illness such as AD, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease.[16] Cells of the nervous system are particularly vulnerable to oxidative stress-mediated damage because they have elevated metabolic activity and a limited threshold of cell regeneration. Even though it is not clear if oxidation processes are the initial trigger in neurodegenerative disorders, these stress conditions are intimately implicated in the loss and injury of neurons.[17] An oxidative stress condition occurs when radical oxygen species (ROS) generation increases or antioxidant defenses fail. As a consequence, the overproduction of ROS causes oxidative damage to proteins and DNA as well as lipid peroxidation, which leads to pathology.[18] In addition, ROS are key molecules in the regulation of cellular functions such as activation of the mitogen-activated protein kinase (MAPK) cascade, ion transport, calcium mobilization, and activation of the apoptosis program.[19] However, the principal sensor and modulator of the oxidative stress pathway is the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant response element (ARE), which is responsible for regulating the expression of antioxidant and detoxifying genes. Under normal conditions, Nrf2 is attached to kelch-like ECH associating protein 1 (Keap1), which prevents its translocation to the nucleus and allows it to be ubiquitinated by an E3 ligase, resulting in its subsequent degradation. Nevertheless, under conditions of oxidative stress-mediated damage, the Nrf2−Keap1 junction is broken, and Nrf2 translocates to the nucleus where it binds Maf proteins and, finally, ARE, triggering the transcription of genes like superoxide dismutase 1, catalase, sulforedoxin, thioredoxin, peroxiredoxin, and others whose protein products are in charge of the synthesis and metabolism of glutathione (GSH). Hence, previous research in neurodegenerative animal models has demonstrated that activation of the Nrf2−ARE pathway ameliorates the symptoms of these

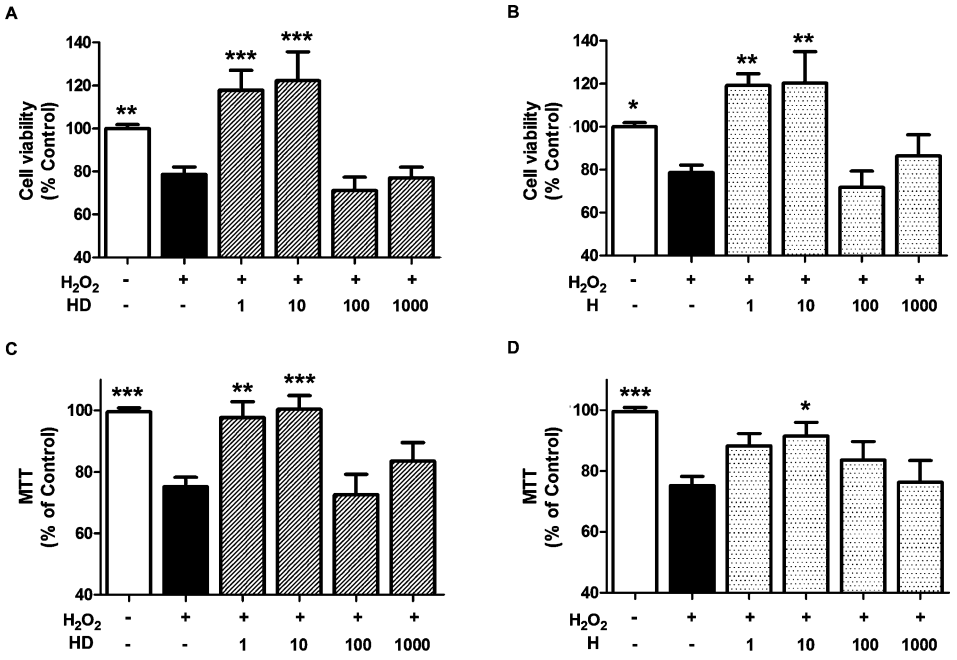

Figure 2. Both bromoalkaloids protect neurons at the cell membrane and mitochondrial levels. Cell viability was increased when neurons were treated with 10 and 1 nM HD (A) and H (B) compared to that of cells incubated with H_2O_2 only, whereas mitochondrial function was preserved with 10 and 1 nM HD (C) but only with 10 nM H (D). Results are presented as a percentage, with the nontreated control being 100%, and are compared to cells treated with 200 μM H_2O_2 alone. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Data are the mean ± SEM of five independent experiments performed in triplicate.

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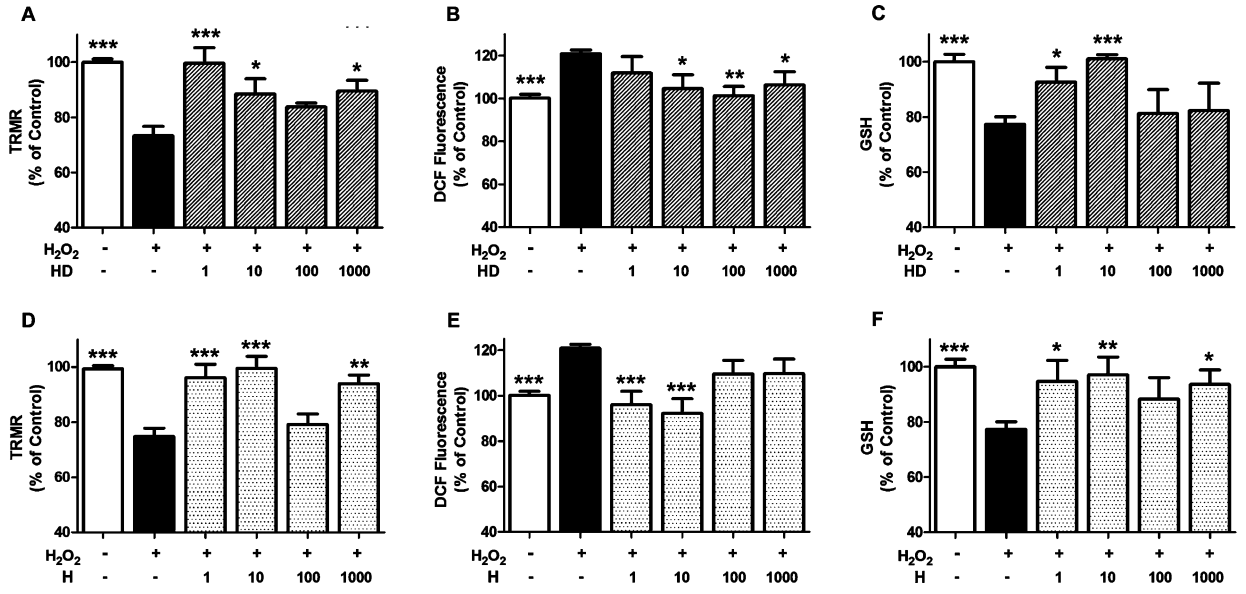

Figure 3. HD and H defend neurons against induced oxidative stress. Variations in Ψ_m (A, D), ROS production (B, E), and GSH levels (C, F) were studied in cells co-incubated with 200 μM H_2O_2 and compounds. HD demonstrated antioxidant ability in all assays at 10 nM, whereas H did so at 10 and 1 nM. All values are shown as a percentage, with the nontreated control being 100%, and are compared to that of cells treated with 200 μM H_2O_2 alone. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Data are the mean ± SEM of six independent experiments performed in triplicate.

pathologies.[20] Many neurodegenerative disease investigators are focusing their efforts on the modulation of enzymatic regulatory components responsible for antioxidant protection to imitate therapeutic effects and thus to retard disease advancement.[17]

The aim of this research is to evaluate the neuroprotection ability of HD and H under oxidative stress conditions. Toward this purpose, the activity of these compounds was analyzed at various subcellular levels: membrane, mitochondria, cytosol, and, finally, nucleus, by studying the antioxidant ARE−Nrf2 pathway. As stated above, oxidative stress is a common feature of neurodegenerative diseases and therefore this information will contribute to completing an understanding of the actions of HD and H for their potential use in pharmacology development.

■ RESULTS AND DISCUSSION

In this study, we analyzed the neuroprotective capacity of hymenialdisine (HD) and hymenin (H) in primary cortical neurons exposed to oxidative stress agents. First, we evaluated their activity regarding cell viability, as their effects on primary cortical neurons have not been previously tested. Cells were incubated with compounds for 48 h in the nanomolar range. Then, an MTT assay was performed; none of the compounds produced a decrease in viability at these concentrations (data not shown).

In recent work, we demonstrated the antioxidant activity and neuroprotective ability of several compounds from *Porifera* sources in primary neurons, so we tested HD and H in this previously established cellular oxidative stress model.[21] To study different aspects of the compounds' antioxidant capacity, we co-incubated cortical neurons with H_2O_2 (200 μM) and the compounds at several concentrations (1000, 100, 10, and 1 nM) for 12 h to evaluate cell survival, mitochondrial membrane potential (Ψ_m), reactive oxygen species (ROS) generation, and glutathione (GSH) levels.

Cell viability was analyzed by LDH and MTT assays. Both tests are commonly used to determine neuronal survival, but in the LDH assay, we measured LDH release to the media to examine cellular membrane damage, and in the MTT assay, we verified mitochondrial function, which, in neurons, is well-related with cell survival.[22] Cortical neurons were treated with H_2O_2 and compounds as previously described, and after 12 h of incubation, their viability was determined. A survival reduction of 21.4 ± 3.6% was detected in cells treated with H_2O_2 compared to that of control neurons by the LDH test. Moreover, this diminution of viability was corrected when cells were co-incubated with the compounds at their lowest concentrations. As can be seen in Figure 2A,B, HD and H at 10 and 1 nM achieved a higher percentage of survival even than that of the nontreated control, around 120% viability, so they differed statistically from the H_2O_2-treated cells. In addition, in the MTT assay we observed a viability decrease of 24.8 ± 3.1% in neurons treated with H_2O_2 with respect to that of nontreated cells. In Figure 2C, it can be seen that HD improved mitochondrial function when neurons were co-incubated with H_2O_2 and hence increased neuronal survival at 10 nM (100.3 ± 6.0%, $p < 0.001$) and 1 nM (97.8 ± 5.1%, $p < 0.01$), maintaining the survival percentage of control cells and confirming the LDH assay results. Furthermore, H demonstrated a significant defensive activity against oxidation only at 10 nM (86.0 ± 3.4%, $p < 0.05$) (Figure 2D). To complete the examination of mitochondrial function, Ψ_m was studied. The same treatments as those for the viability assays were carried out, consisting of 12 h co-incubations with H_2O_2 and the compounds, and the TRMR test was then performed. A Ψ_m reduction of 28.1 ± 3.6% in neurons incubated with H_2O_2 was observed compared to that in control cells without treatment (Figure 3A,D). HD and H seem to have similar effects, maintaining the Ψ_m at nontreated cell levels when the compounds were present at 1000, 10, and 1 nM, but no neuroprotective effects were observed at 100 nM for either of the compounds. It is noteworthy that HD at 1 nM completely preserved Ψ_m (99.6 ± 5.7%, $p < 0.001$), maintaining a basal

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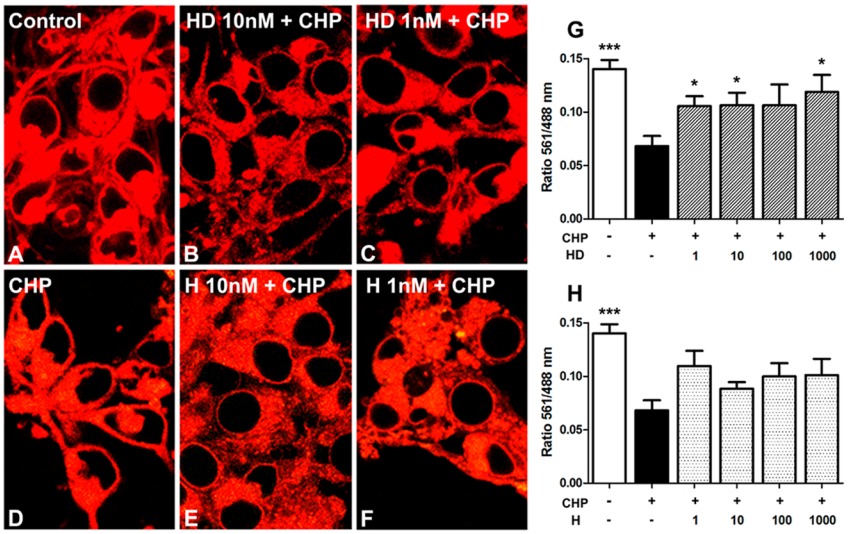

Figure 4. HD prevents lipid peroxidation in cortical neurons. Lipid peroxidation was studied by confocal microscopy. Representative images from the experiments are presented that correspond to untreated control cells (A), neurons treated with 100 μM CHP (D), and coincubations of CHP with compounds HD (B, C) and H (E, F). Neurons were cotreated with HD (G) or H (H) and 100 μM CHP, and HD prevented peroxidation at 10 and 1 nM. All values are presented as a ratio of 561/488 nm and are compared to that of neurons treated with 100 μM CHP alone. *, $p < 0.05$; ***, $p < 0.001$. Data are the mean ± SEM of two coverslips from six different cell preparations.

potential. Following the examination of neuroprotection against oxidative stress, we analyzed the amount of ROS as a useful indicator of cellular stress. Consequently, we measured ROS using a nonfluorescent compound, 7′,2′-dichlorofluorescein diacetate (DCFH-DA), that penetrates cells and reacts with ROS, resulting in fluorescence emission. Once again, neurons were co-incubated with H_2O_2 and compounds at the concentrations specified above, and ROS levels were determined by a DCFH-DA assay. Results are expressed as a percentage of the level of ROS in untreated control cells. H_2O_2 treatment produced an increase of ROS of 20.0 ± 1.7%. In this experiment, HD was able to reduce ROS levels at 1000 nM (106.3 ± 6.2%, $p < 0.05$), 100 nM (101.3 ± 4.3%, $p < 0.01$), and 10 nM (104.5 ± 6.6%, $p < 0.05$) with respect to that observed in neurons treated with H_2O_2 alone (Figure 3B), and H reduced ROS levels at 10 nM (92.2 ± 6.4%, $p < 0.001$) and 1 nM (95.9 ± 6.0%, $p < 0.001$), achieving control levels (Figure 3E).

In order to study the antioxidant capacity of these compounds in depth, we studied their effects on cellular GSH levels, which is a thiol that protects neurons against oxidative stress and whose decrease indicates a dramatic alteration in the cellular antioxidant defense.[23] Once more, neurons were treated with H_2O_2 and the bromoalkaloids at different concentrations, and after 12 h, GSH was detected with a thiol tracker dye provided by a commercial kit. H_2O_2 produced a GSH levels reduction of 21.3 ± 2.7% with regard to that of the no-treatment control (Figure 3C,F). However, HD was able to increase GSH, reaching control levels, at 10 nM (101.1 ± 1.5%, $p < 0.001$) and 1 nM (92.6 ± 5.4%, $p < 0.05$), and H repeated the behavior observed for Ψ_m, enhancing GSH levels at 1000, 10, and 1 nM but without significant effect at 100 nM.

ROS-mediated lipid peroxidation is a fundamental element in neurodegenerative diseases due to the high lipid content of the brain. Thus, primary cortical neurons exposed to an oxidative stress inducer undergo membrane lipid degradation. To study this, cumenehydroperoxide (CHP) at 100 μM was used as an oxidant. Cells were treated for 2 h with or without CHP or were co-incubated with the halogenated compounds and CHP; during the last 15 min of the incubation, cells were labeled with BODIPY 581/591 C11 reagent. Untreated control neurons labeled with BODIPY 581/591 C11 emit red fluorescence (Figure 4A). However, cells treated with CHP have transformed the phenylbutadiene segment of the BODIPY 581/591 C11 reagent, due to oxidation, and the fluorescence changes from red to green. These neurons emit red and green fluorescence, resulting in a yellow image (Figure 4D). Thus, the fluorescence ratio is halved with regard to that of the control, as can be seen in Figure 4G,H, which implies an increase in lipid peroxidation. When cortical neurons were cotreated with HD at 1000, 100, 10, and 1 nM and CHP, lipid peroxidation was reduced at all concentrations, which was statistically significant at 1000, 10, and 1 nM with respect to that of cells treated with CHP (Figure 4G). Moreover, in the cotreatments with compound H (1000, 100, 10, and 1 nM) and CHP, even though lipid peroxidation seemed to decrease, the difference compared to that with CHP treatment alone was not significant (Figure 4H).

Considering all of the results obtained in the neuroprotection assays, we studied the effects of the halogenated compounds on the translocation of Nrf2 to the nucleus. To study the possible effects on this target that precede the observed effects on ROS levels and antioxidant defense, neurons were treated with HD or H at selected concentrations (1000, 100, 10, and 1 nM) for 6 h. Subsequently, cells were lysed, and cytosolic and nuclear fractions were recovered. The lysates were processed by western blotting, and Nrf2 signal was corrected by that of lamin B1 in the nuclear fraction and actin in the cytosolic fraction, both of which are structural proteins. Results are expressed as a ratio of the corrected nuclear Nrf2 versus the corrected cytosolic Nrf2 to verify translocation. HD produced an augmentation of Nrf2 in the nucleus at all concentrations tested, but in the cytosolic fraction, it caused an increase (1000 and 100 nM) or a reduction (10 and 1 nM) in Nrf2 depending on its concentration. As a consequence, the ratio did not

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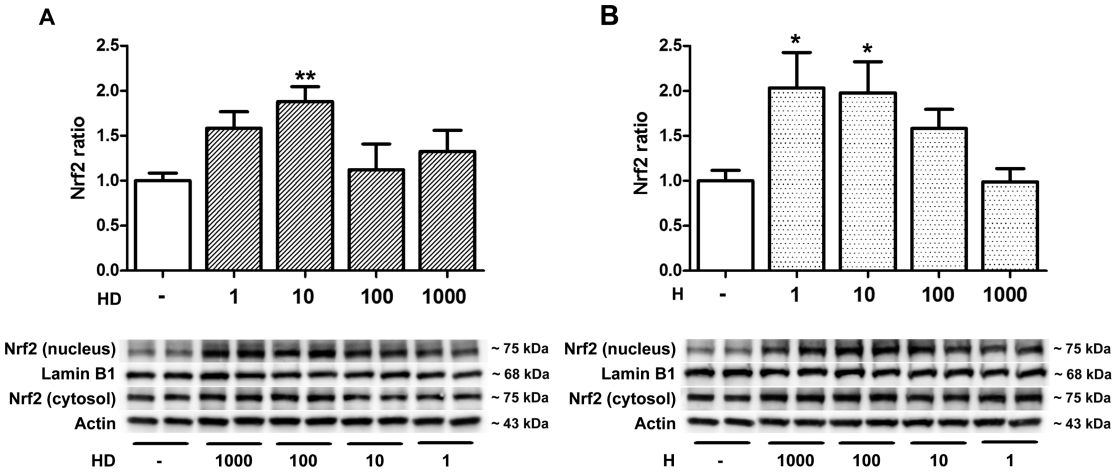

Figure 5. Nrf2 translocation induced by bromoalkaloids. Nrf2 levels were studied in nuclear and cytosolic fractions after a 6 h incubation with compounds to evaluate its translocation. Results are presented as a ratio of nuclear-Nrf2/cytosolic-Nrf2, both of which are corrected the abundance of fraction-selective structural proteins (lamin B1 for nuclear samples and actin for cytosolic lysates). Nrf2 translocation was induced by HD at 10 nM (A), whereas H promoted its translocation at 10 and 1 nM (B). *, $p < 0.05$; **, < 0.01. Data are the mean ± SEM of four independent experiments.

statistically differ from that of control cells at 1000 and 100 nM; however, it rose above control levels at 10 nM, confirming the translocation of Nrf2 at lower concentrations. The augmented nuclear levels of Nrf2 at 1 nM were not enough to achieve a significant increase in the ratio, even though the cytosolic signal was lower than that of the control (Figure 5A). We found similar results with compound H, which resulted in an elevation of nuclear Nrf2 at all concentrations tested. Again, the high concentrations of Nrf2 in the cytosol counteracted the nuclear Nrf2 levels and thus the ratio between both did not vary with respect to that of the control. However, when neurons were treated with H at 10 and 1 nM, both concentrations produced a significant increase in the Nrf2 ratio, demonstrating Nrf2 translocation (Figure 5B).

Natural marine compounds, such as sponge-derived secondary metabolites, are very attractive molecules for the development of pharmaceuticals. These products are structurally diverse and include elements such as bromine, which is not usually found in terrestrial secondary metabolism.[24] The bromopyrrolealkaloids hymenialdisine and hymenin have demonstrated high activity in several fields. In this research, we have explored the antioxidant ability of hymenialdisine and hymenin at nanomolar concentrations. In fact, both compounds are clearly more effective at low nanomolar concentrations, between 1 and 10 nM, resulting in a surprising full recovery of cellular viability in the presence of H_2O_2. This lack of a concentration-dependent effect may indicate that these compounds are interacting with a receptor or a cellular signal transduction pathway that could have a maximal threshold effect or, more likely, a receptor that could suffer desensitization. These kinds of effects have been reported for other natural compounds, such as as coumaric acid and resveratrol, which exhibited antioxidant effects at 500 nM or lower concentrations, whereas higher doses induced oxidation processes, increasing ROS levels and cell injury and downregulating Akt.[25] Moreover, hymenialdisine and hymenin elicited a general neuronal protection against oxidative stress, preserving cell membrane integrity and mitochondrial activity, reducing ROS levels, and increasing the antioxidant defense. Nevertheless, we cannot explain the intermittent, but surprising, results obtained for the mitochondrial membrane potential, GSH, or lipid peroxidation assays, where compounds were effective at 1, 10, and 1000 nM but not at 100 nM.

In addition, HD prevents lipid peroxidation at these concentrations, confirming a higher antioxidant activity of this compound. Lipid peroxidation is one of the principal ROS-mediated mechanisms of damage to neuronal membranes, producing an increase of rigidity, a diminution of activity of membrane-associated enzymes, damage to receptors, permeability alterations, and the generation of numerous secondary products that consequently spread the cellular injury. ROS accumulation attacks the membrane bilayer lipids, resulting in highly reactive electrophilic aldehydes such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and acrolein. These aldehydes post-translationally modify proteins, usually leading to a dysregulation of cellular homeostasis. These lipid peroxidation indicators have been detected in brain and body fluids in diverse neurodegenerative disorders. Thus, preventing lipid peroxidation can be a good strategy to prevent or retard the progression of the pathology.[26]

It is remarkable that, as was demonstrated in the present work, HD and H activate the Nrf2−ARE pathway, producing an augmentation of Nrf2 expression and its effective translocation to the nucleus at low concentrations. This observation agrees with the complete recovery of normal GSH levels shown at these concentrations and may be responsible for the antioxidant protective effect of these compounds. In several neurodegenerative diseases, a reduction of Nrf2 expression has been observed, and inducers of this pathway can produce a beneficial effect in *in vivo* and *in vitro* models of these disorders.[27] Additionally, high activity of HD and H family compounds at nanomolar concentrations was previously observed in kinase inhibition studies, where hymenialdisine also elicited a more potent effect than hymenin. In these investigations, hymenialdisine was able to inhibit some kinases such as MEK-1,[28] casein kinase 1 (CK1), CDK1, CDK2, CDK5, and GSK-3β, which was highlighted by its inhibition activity toward CDK5 with an IC_{50} of 28 nM and toward GSK-3β with an IC_{50} of 10 nM. These kinases are well-related with Alzheimer's disease; consequently, it was observed that both compounds produced a reduction of tau hyperphosphorylation, which was observed in *in silico* and *in vitro* experiments.[15]

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Furthermore, HD also inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which is a transcription factor that regulates the expression of several pro-inflammatory genes, such as those encoding cytokines.[29] Nevertheless, the inhibition of these signaling proteins takes place at much higher concentrations than that used in the kinases studies, with an IC_{50} of 600 nM for interleukin-1 (IL-1),[30] 2400 nM for IL-2, and 1400 nM for tumor necrosis factor α (TNF-α).[31] A significant increase of TNFα, IFNγ, and interleukins in plasma, brain, and cerebrospinal fluid has been observed in numerous neurodegenerative disorders such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, and AD, where neuroinflammation is a key factor in the development of pathology and, moreover, where a relationship between inflammation and AD risk has been suggested.[32,33] In fact, the blockage of these molecules in AD diminishes cognitive impairment and reduces pathological changes in mouse models.[34]

Microglia is the main cell type responsible for inflammation in neurodegenerative disorders, where an overactivated state of these cells results in an increased release of the inflammatory mediators mentioned above and ROS, resulting in oxidative stress-mediated inflammation. The high levels of nitric oxide synthase (NOS) and NADPH oxidase (NOX) enzymes in activated microglia are responsible for the elevated production of ROS and reactive nitrogen species (RNS) that lead to a pathological cycle in which inflammation increases ROS production by cytokine release and ROS lead to oxidative stress, mitochondrial dysfunction, and the consequent inflammation that completes the cycle.[35] Moreover, it is well-established that ROS can activate MAPK pathways, even though the mechanisms of this are not well described. Consequently, the reduction of ROS generation by increasing the antioxidant defense can also inhibit MAPK signaling, preventing the pathological hallmarks of oxidative stress-related diseases.[36] Therefore, regardless of whether ROS are the cause or result of the pathology, the bromoalkaloids studied in this work elicited neuroprotection either by their antioxidant capacity, presented in this work, or by their ability to inhibit the kinases described earlier, as shown previously.

Finally, hymenialdisine and hymenin have been demonstrated to modulate the Nrf2−ARE pathway as Nrf2 inducers, with a resulting downregulation in the response to oxidative insults and an enhancement of GSH, which promotes the cellular antioxidant defense. Therefore, because Nrf2 is a therapeutic target in neurodegenerative disorders, this work opens a new research path, which is also supported by the kinase and IL inhibition data previously described, and suggests that these two bromoalkaloids may be interesting candidates for drug development targeting neuroinflammatory processes.

METHODS

Compounds Information. Hymenialdisine (HD) and hymenin (H) (Figure 1) were provided by the Marine Biodiscovery Centre (Department of Chemistry, University of Aberdeen). These compounds were isolated from an Axinellid sponge collected from Snake Island in Fiji at a depth of 9 m (S16.46.442 E179.59.997) by subjecting its methanolic extract to multiple steps of liquid/liquid fractionation and Sephadex LH-20 and RP-C-18 chromatography. The structural elucidation of these compounds was based on their HRESIMS analysis as well as direct comparison with the previously reported NMR spectral data described for HD and H.[13]

Cell Culture. Primary cultures of cortical neurons were performed from Swiss mice, and all protocols presented in this study were revised and authorized by the University of Santiago de Compostela Institutional animal care and use committee and complied with European legislation on the use and management of experimental animals.

Primary cortical neurons were obtained from embryonic day 15−18 mouse fetuses as described.[37] Briefly, the cerebral cortex was removed, and neuronal cells were dissociated by trypsinization at 37 °C followed by mechanical titration in DNase solution (0.005% w/v) with a soybean-trypsin inhibitor (0.05% w/v). Cells were suspended in DMEM supplemented with *p*-amino benzoate, insulin, penicillin, and 10% fetal calf serum. The cell suspension was seeded in 96- or 12-well plates precoated with poly-D-lysine and incubated in a humidified 5% CO_2/95% air atmosphere at 37 °C. Cytosine arabinoside (20 μM) was added prior to 48 h of culture to prevent non-neuronal cells from growing. Cells were seeded in 96- and 12-well plates for experiments. LDH, MTT, TMRM, ROS, and GSH assays were carried out in 96-well plates, and the treatments consisted of co-incubations of 200 μM H_2O_2 and the bromoalkaloids at 1000, 100, 10, and 1 nM for 12 h on 4−5 days *in vitro* (div) neurons. Lipid peroxidation and Nrf2 assays were performed in 12-well plates as described below.

Chemicals and Solutions. Plastic tissue-culture dishes were purchased from Jet Biofilm (VERTEX Technics, Madrid, Spain). Fetal calf serum was obtained from Gibco (Glasgow, UK), and Dulbecco's modified Eagle's medium (DMEM) was from Biochrom (Berlin, Germany).

Cell Viability Assay. Cytotoxicity of the compounds was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test, as previously described.[38,39] Primary cortical neurons were grown in 96-well plates and treated with several concentrations of the bromoalkaloids (range 1−1000 nM) and kept at 37 °C in humidified 5% CO_2/95% air atmosphere for 48 h. After the incubation period, neurons were washed and exposed for 1 h to a MTT solution (500 μg/mL) dissolved in saline buffer. Then, excess MTT was rinsed from the cells, cells were disaggregated with 5% sodium dodecyl sulfate, and the absorbance of the colored formazan salt was measured at 595 nm using a spectrophotometer plate reader. A lethal dose of saponin was used as a positive control for cell death, and its absorbance was subtracted from that of the other results.

Treatments for the Oxidative Stress Cell Model. For the determination of cell survival, mitochondrial function and membrane potential, and the evaluation of ROS and GSH levels, 4−5 div neurons were co-incubated with 200 μM H_2O_2 and the halogenated compounds at 1, 10, 100, or 1000 nM for 12 h. In all assays, a cellular control (with solvent) and an oxidant positive control (cells treated with 200 μM H_2O_2) were included.

LDH Release Measurement. Lactate dehydrogenase (LDH) release is accepted as a sign of cell survival. After 12 h of treatment with H_2O_2 and the two alkaloids, the In Vitro Toxicology Assay Kit (TOX7, Sigma) was used to determine the compounds' neuroprotective ability by following the manufacturer's protocol.

Mitochondrial Function Assay. Neurons were incubated following the oxidative stress model previously described for the mitochondrial function assay, and an MTT test was performed according to the protocol detailed.

Mitochondrial Membrane Potential (Ψ_m) Assay. Variations in Ψ_m were evaluated with the tetramethylrhodamine methyl ester (TMRM) assay[40] using the following steps. Upon experimentation, cells were rinsed twice with saline solution and incubated with 1 μM TMRM for 30 min. Finally, neurons were solubilized with 50% DMSO/water. Fluorescence values were measured in a spectrophotometer plate reader (535 nm excitation, 590 nm emission).

Determination of ROS Generation. ROS evaluation was carried out by a fluorescence assay using 7′,2′-dichlorofluorescein diacetate (DCFH-DA), as previously described.[41] Cells were treated with H_2O_2 and the compounds, and then they were washed with saline solution and loaded with 20 μM DCF-DA for 30 min at 37 °C. Neurons were rinsed and maintained at room temperature for 30 min to allow a complete de-esterification of DCF-DA to fluorescent 7′,2′-dichlorofluorescein (DCF) by reacting with ROS. DCF accumulation was

measured using a fluorescence plate reader, with excitation at 475 nm and emission at 525 nm.

Glutathione Assay. GSH is the reduced form of glutathione and is the main intracellular free thiol in cells. Thus, to measure its levels, ThiolTracker Violet dye was used. Once the treatment was finished, cells were rinsed with phosphate buffer and then incubated with 10 μM ThiolTracker Violet dye for 1 h at 37 °C. Finally, neurons were washed, and fluorescence was read with 404 nm excitation and 526 nm emission.

Confocal Microscopy Imagining for Determination of Lipid Peroxidation. Lipid peroxidation was analyzed with the Image-iT Lipid Peroxidation Kit for live cell analysis (Molecular Probes). The kit provides an oxidant inducer, cumenehydroperoxide (CHP), which was utilized at 100 μM. Neurons were treated with CHP and the bromoalkaloids at 1, 10, 100, or 1000 nM for 2 h and labeled with C11-BODIPY (581/591) reagent (2.5 μM) for the last 15 min of the incubation time. Then, cells were washed twice with cold saline buffer, and confocal images were taken within 2 h following the staining. These images were obtained using a 40× oil immersion objective with a Nikon Eclipse TE2000-E inverted microscope attached to the C1 laser confocal system and EZ-C1 V.2.20 software (Nikon Instruments Europe B.V., Amstelveen, Netherlands). C11-BODIPY (581/591) was excited using a 488 nm argon laser, and fluorescence was measured using a band-pass filter from 590/50 and a 650 nm long-pass filter. Under basal conditions, the fluorophore emits a red signal, but in the presence of an oxidant, fluorescence changes from red to green, and its ratio indicates the degree of lipid peroxidation. All data presented were obtained from two coverslips and six different cell preparations.

Western Blot Assays. The expression of Nrf2 in the nucleus and in the cytosol was determined by western blot. The treatments with the bromoalkaloids were carried for a 6 h incubation period, and afterward, cortical neurons were washed twice with cold saline buffer. Lysates were obtained by adding an ice-cold cytosolic hypotonic buffer solution (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$, containing a complete phosphatase/protease inhibitor cocktail from Roche) to the cells for 15 min, which were then scraped, and, finally, centrifuged at 3000 rpm, 4 °C, for 10 min to obtain the cytosolic fraction. The supernatant was collected, and the pellet was resuspended in ice-cold nuclear extraction buffer (100 mM Tris, pH 7.4, 2 mM Na_3VO_4, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 0.5% deoxycholate, and 20 mM $Na_4P_2O_7$, containing 1 mM PMSF and a protease inhibitor cocktail) for 30 min with vortexing in 10 min intervals. Samples were then centrifuged at 14 000*g* at 4 °C for 30 min. The supernatants were collected as nuclear fractions. Protein concentration was determined by Bradford assay, and samples of cell lysates containing 10 μg (nuclear fraction) and 20 μg (cytosolic fraction) of total protein were used for electrophoresis. Electrophoresis was resolved in a 10% polyacrylamide gel (Bio-Rad) and transferred onto PVDF membranes (Millipore). Membrane blocking and antibody incubation was performed using the SNAP i.d. protein detection system. The immunoreactive bands were detected using the Supersignal West pico chemiluminescent substrate or Supersignal West femto maximum sensitivity substrate (Pierce) and the Diversity 4 gel documentation and analysis system (Syngene, Cambridge, U.K.). Chemiluminescence was measured with Diversity GeneSnap software (Syngene). Nrf2 was detected with anti-NF-E2-related factor 2 antibody (1:1000, Millipore). Nrf2 signal was normalized by that of β-actin (1:20 000, Millipore) for cytosolic samples and by that of lamin B1 (1:1000, Abcam) for nuclear samples.

Statistical Analysis. All neuroprotection results are expressed as the mean ± SEM of three or more experiments, and experiments were performed by triplicate. For all data analysis, an ANOVA statistical test with Dunnett's posthoc analysis was used, and p values < 0.05 were considered to be statistically significant.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AD, Alzheimer's disease; ARE, antioxidant response element; CDKs, cyclin-dependent kinases; GSH, glutathione; GSK-3β, glycogen synthase kinase-3beta; HD, hymenialdisine; H, hymenin; Keap1, kelch-like ECH associating protein 1; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; Ψ_m, mitochondrial membrane potential; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, radical oxygen species

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