ACS Chemical Neuroscience

Research Article

pubs.acs.org/chemneuro

Mitigation of ROS Insults by *Streptomyces* Secondary Metabolites in Primary Cortical Neurons

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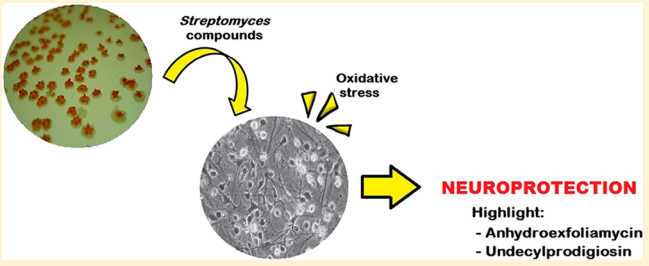
ABSTRACT: Oxidative stress is a common point in neurodegenerative diseases, widely connected with mitochondrial dysfunction. In this study, we screened seven natural products from *Streptomyces* sources against hydrogen peroxide insult in primary cortical neurons, an oxidative stress in vitro model. We showed the ability of these compounds to inhibit neuronal cytotoxicity and to reduce ROS release after 12 h treatment. Among the tested compounds, the quinone anhydroexfoliamycin and the red pyrrole-type pigment undecylprodigiosin stand out. These two compounds displayed the most complete protection against oxidative stress with mitochondrial function improvement, ROS production inhibition, and increase of antioxidant enzyme levels, glutathione and catalase. Further investigations confirmed that anhydroexfoliamycin acts over the Nrf2-ARE pathway, as a Nrf2 nuclear translocation inductor, and is able to strongly inhibit the effect of the mitochondrial uncoupler FCCP over cytosolic Ca^{2+}, pointing to mitochondria as a cellular target for this molecule. In addition, both compounds were able to reduce caspase-3 activity induced by the apoptotic enhancer staurosporine, but undecylprodigiosin failed to inhibit FCCP effects and it did not act over the Nrf2 pathway as was the case for anhydroexfoliamycin. These results show that *Streptomyces* metabolites could be useful for the development of new drugs for prevention of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases and cerebral ischemia.



KEYWORDS: *Marine natural products, Streptomyces, oxidative stress, Nrf2, neuroprotection, neurodegenerative diseases*

The incidence of neurodegenerative diseases, like Alzheimer's and Parkinson's diseases, has increased due to higher life expectancy, reaching epidemic proportions in all industrialized countries and becoming an important socioeconomic problem.[1] Neurodegenerative disorders are characterized, among other cellular pathologies, by mitochondrial dysfunction and reactive production of oxygen species (ROS), and therefore, they are related to oxidative stress.[1−3] The central nervous system is especially sensitive to free radical oxidative damage due to a high oxygen consumption ratio, a rich content of phospholipids, easily oxidizable, and high levels of iron, which can catalyze oxidative reactions and contribute to an increase in production of free radicals.[4] This is coupled with a low content of antioxidant defenses in the brain that are even more altered in Alzheimer's disease. In fact, it has been described that oxidative damage is an early event in this pathology and that there is even an increased brain oxidative damage before the appearance of dementia symptoms.[5,6] This oxidative damage occurs due to an imbalance between ROS production and antioxidant cell defenses.[2,7,8] The elevation of oxidative stress actions lead to mitochondrial dysfunction, causing an increase in free radical production and an exacerbation of the oxidative stress cycle.[2] Normal mitochondrial respiration produces molecular oxygen, but in the presence of defects on the electron transport chain the molecular oxygen is increased and promotes deficits in several enzymes in charge of reducing this molecular oxygen,[8] converting the mitochondria into the main producers of ROS.

The main oxygen species involved in neuronal oxidative injury are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$).[1] Their increase causes protein, DNA and RNA oxidation, and lipid peroxidation.[1,9] Neurons have enzymatic and nonenzymatic defenses to protect themselves from oxidative stress and its consequences. The enzymatic defense is based on enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) that convert free radicals into nontoxic molecules.

It has been described that an increase of antioxidant defenses activity protects cell models from neurodegeneration and that antioxidants can lower the risk of neurodegenerative diseases.[3] These antioxidant systems are part of the antioxidant response elements (AREs), and they are regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 works as a transcription factor when it is present in the nucleus and

Received: October 18, 2013
Published: November 12, 2013

regulates the activation of protective genes, including the above-mentioned enzymes, antioxidant and antiapoptotic proteins and proteasomes.[10] In nonoxidative stress conditions, Nrf2 is located in the cytosol, attached to Kealch-like ECH associated protein 1 (Keap1), forming the Nrf2-Keap1 complex. Nevertheless, in oxidative stress states, the Nrf2-Keap1 complex is destabilized, leading to Nrf2 release and its translocation to the nucleus. Therefore, Nrf2 and Keap 1 are able to work as free radical damage sensors. Once in the nucleus, Nrf2 attaches to Maf proteins, thereby intensifying the ARE response and consequently the cellular antioxidant machinery.[11] Small molecules have been described as Nrf2 inducers with protective effects in vitro and in vivo. Neurons lacking Nrf2 are more susceptible to H_2O_2 induced oxidative stress, but overexpression of Nrf2 elicited neuronal protection. It has also been described that an increase of Nrf2 expression in vivo is an effective strategy against ROS production and oxidative stress in the brain.[12] There are different pieces of evidence about Nrf2 dysfunction in neurodegenerative diseases such as Alzheimer's disease and Lewy body dementia. In Alzheimer's disease, a decrease in nuclear Nrf2 levels compared to age-matched controls is observed;[13] thus, nuclear Nrf2 overexpression can be a therapeutical option to increase the antioxidant defenses of neurons in neurodegenerative disorders.

The genus *Streptomyces* is the largest genus of *Actinobacteria*, a group of Gram-positive filamentous bacteria which includes over 500 species, primarily located in soil and marine ecosystems.[14] Their differentiation is a complex process whereby they produce branching mycelia, aerial hyphae, and spores.[15,16] *Streptomyces* species are well-established producers of secondary metabolites and over the last decades have generated a vast diversity of compounds. Some of these metabolites have pharmaceutically relevant properties such as anti-inflammatory, antiviral, antimicrobial, anticancer activities,[17] while others have been reported to elicit protection against neurodegenerative disorders.[18] Various *Streptomyces* secondary metabolites have been described as potential neuroprotective agents against oxidative stress by scavenging free radical species.[19] Moreover, several compounds isolated from these sources have the ability of promoting neuritogenesis,[20] and they have demonstrated neuroprotective capacity in various neurodegeneration models.[21] Considering these nervous system protection mechanisms, the natural products from marine- and desert-derived *Streptomyces* spp. are promising compounds for neuroprotection studies against oxidative stress and potential candidates for preventive drugs against neurodegenerative disorders.

The objective of this work was to conduct an in vitro screen of seven natural products isolated from *Streptomyces* spp. from the hyper-arid Atacama Desert and from the marine habitat against the oxidative stress cellular damage elicited by H_2O_2 in primary neurons cultures. The present study provides the first description of neuroprotective effects against H_2O_2 damage of *Streptomyces* metabolites with a highlighted effect of the compounds anhydroexfoliamycin and undecylprodigiosin.

RESULTS AND DISCUSSION

Effect of *Streptomyces* Compounds on Primary Cortical Neuron Viability. Cell viability was studied by MTT assay. It has been shown that in neuronal cells there is a good correlation between drug-induced decrease in mitochondrial activity and its cytotoxicity.[22] Cortical neurons were exposed for 48 h to compounds A–E, and no signs of cytotoxicity were observed, even at the highest concentration tested of 1 μM (data not shown). In contrast, compounds F and G, previously described as cytotoxic in P388, HL60, A-549, BEL-7402, and SPCA4 cell lines,[23] caused a dose-dependent cytotoxic effect in primary cortical neurons (Figure 2). Compound F produced a complete cell death at 1 μM (99.5 $\pm$ 0.4%), and the same effect was observed for compound G (98.4 $\pm$ 1.1%). However at lower concentrations compound F exhibited a higher cytotoxic effect than compound G as can be seen in Figure 2. For these compounds, only one concentration tested was nontoxic, 0.01 μM, and it was chosen for all the experiments.

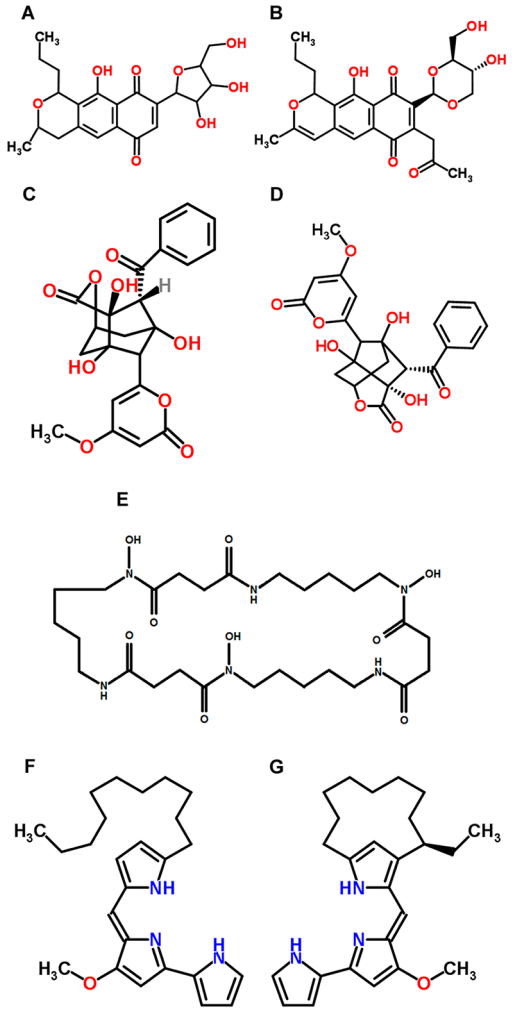


Figure 1. Compound structures.

Neuroprotective Effect of *Streptomyces* Compounds against H_2O_2 Insult. Since marine natural compounds can be potential neuroprotectors against oxidative stress, we used H_2O_2 as an oxidative stress inducer. H_2O_2 has a short half-life, and its high solubility promotes its dissociation into hydroxyl and superoxide ions, which leads to breaking bonds, altering the membrane permeability by lipid peroxidation, hence causing loss of membrane integrity and finally cellular damage.[24] This oxidative stressor has been widely used for oxidative stress studies. Since neurons are particularly sensitive to oxidative stress conditions and we have previous experience with them as an oxidative stress model (work under review), we use primary cortical neuron cultures obtained from mouse fetuses as a

dx.doi.org/10.1021/cn4001878 | ACS Chem. Neurosci. 2014, 5, 71–80

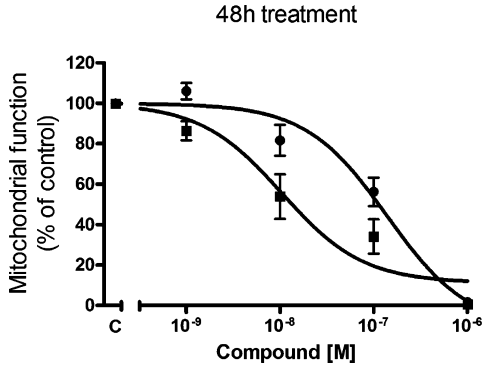


Figure 2. Cytotoxicity effects of compounds F and G after 48 h incubation. Dose response effect of F (■) and G (●) compounds in concentrations ranging from 10 to 1000 nM.

model. Therefore, primary cortical neurons of 4 days in vitro (div) were incubated for 12 h with 200 μM H_2O_2 that decreased cellular viability by 20−30% with respect to control cells. We evaluated the potential of these compounds to rescue primary cortical neurons against H_2O_2 insult. The neuroprotective action of the compounds was evaluated through two different tests, that is, MTT and LDH. Both assays provide viability measurements, but MTT determines the mitochondrial function activity while the LDH release assay is focused on the cell membrane integrity.[25] Neurons were coincubated for 12 h with 200 μM H_2O_2 and two different concentrations of the test compounds (1 and 0.1 μM). Only compounds F and G were used in lower concentrations (0.01 μM) due to the previous cytotoxicity results. As can be seen in Figure 3A, H_2O_2 produced a decrease in viability of 28.6 ± 3.4% ($p < 0.001$). It was observed that only compound E produced a significant increase in mitochondrial function at both concentrations, 0.1 and 1 μM, whereas compounds B and C enhanced mitochondrial function only at 0.1 μM. However, it was remarkable that when neurons were treated with 0.1 μM, the increase of cellular viability observed with A, B, and C was more pronounced than with 1 μM (Figure 3A). Compound F was tested at 0.01 μM, and as can be observed in Figure 3A it also improved mitochondrial function significantly.

LDH release was studied as a parameter of cellular membrane integrity. Treatments with 200 μM H_2O_2 produced a decrease of 27.3 ± 2.6% ($p < 0.001$) with respect to control cells, measured by this test. However, although compounds A, F, and G seem to diminish H_2O_2 induced cytotoxicity, none of the compounds tested produced a significant decrease of LDH release. LDH assays are summarized in Table 2.

To further understand the effects of *Streptomyces* compounds against H_2O_2 insult in the mitochondrial respiratory chain, the ΔΨm was studied. A decrease in ΔΨm is observed in mitochondrial dysfunction and, consequently, the same treatments previously used in the viability assays; that is, 200 μM H_2O_2 and the compounds in two different concentrations (1 and 0.1 μM, except for compounds G and F which were tested at 0.01 μM) were used to perform ΔΨm evaluation by TRMR assay. TRMR is a lipophilic indicator selectively accumulated in the mitochondria and active with negative membrane potential. Cortical neurons treated with 200 μM H_2O_2 presented a decrease of 29.8 ± 4.2% ($p = 0.001$) in ΔΨm in comparison to nontreated cells. As can be seen in Figure 3B, compounds A, B, D, and F maintained the ΔΨm more elevated than in H_2O_2

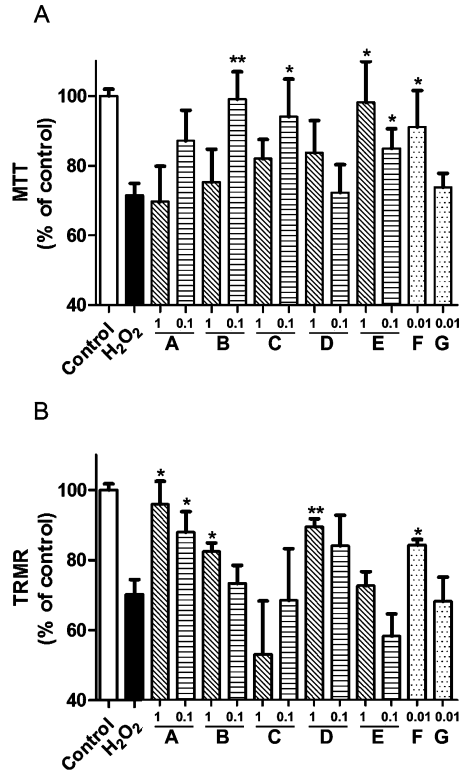


Figure 3. Evaluation of the neuroprotective effects at mitochondrial level. (A) Mitochondrial function was studied by MTT. Cellular viability was increased in neurons treated with B, C, E, and F compounds. (B) TRMR assay was performed to elucidate the ΔΨm. Co-incubation of cortical neurons with 200 μM H_2O_2 and compounds showed an increase of Ψm in A, B, D, and F presence. All values are shown in percentage versus nontreated control and compared to cells treated with 200 μM H_2O_2 alone. *$p < 0.05$ and **$p < 0.01$. Data are mean ± SEM of three or more independent experiments performed by triplicate.

insulted cells. Only compound A produced a complete recovery of ΔΨm with 95.9 ± 6.6% ($p = 0.015$) versus control cells.

Since some of the natural products tested showed beneficial effects against H_2O_2-induced cellular damage, we next studied the effect of the compounds over intracellular ROS generation, which occurs as a result of a secondary imbalance between the oxidant attack and antioxidant defenses. DCFH-DA was used to measure ROS levels in primary cortical neurons coincubated with H_2O_2 and the studied compounds at the same concentrations used in the previous assays. In Figure 4, it can be observed that ROS production in neurons treated with H_2O_2 was significantly increased with respect to basal levels. Neurons in the presence of H_2O_2 revealed a level of 119.0 ± 2.7% ($p < 0.001$) versus control cells whereas the coincubation with compounds E and B at 1 μM revealed a significant decrease of ROS levels relative to cells treated with H_2O_2 alone. Once again compounds A, B, and C showed the most pronounced effect at 0.1 μM as described above for the MTT assay. Compound F (0.01 μM) was also effective against H_2O_2-mediated induction of ROS (Figure 4).

Restoration of Antioxidant Defenses by *Streptomyces* Compounds. In view of the effects observed in mitochondrial function and ROS levels achieved by these natural compounds, we studied their ability to reestablish the antioxidant protection in an oxidative stress environment by analyzing two important antioxidant cell defenses, glutathione (GSH) and catalase

dx.doi.org/10.1021/cn4001878 | ACS Chem. Neurosci. 2014, 5, 71−80

Table 1. Compound Information

compd	chemical name	formula	MW	source organism
A	anhydroexfoliamycin	$C_{22}H_{24}O_8$	416.421	*Streptomyces* sp. Lt 005
B	naphthopyranomycin	$C_{25}H_{28}O_9$	472.484	*Streptomyces* sp. Lt 005
C	3-epi-5-deoxyenterocin	$C_{22}H_{20}O_9$	428.389	Atacama *Streptomyces* C1
D	5-deoxyenterocin	$C_{22}H_{20}O_9$	428.389	Atacama *Streptomyces* C1
E	nocardamine/deferroxamine E	$C_{27}H_{48}N_6O_9$	600.705	Atacama *Streptomyces* C1
F	undecylprodigiosin	$C_{25}H_{35}N_3O$	393.565	*Streptomyces* sp. CBS 198.65
G	metacycloprodigiosin/streptorubin A	$C_{25}H_{33}N_3O$	391.549	*Streptomyces* sp. CBS 198.65

Table 2. Cell Viability[a]

compd (μM)		cell viability (% of control)
control		100.0 ± 2.4
200 μM H_2O_2		72.7 ± 2.6
A	1	80.1 ± 4.9
	0.1	78.2 ± 4.5
B	1	68.6 ± 8.6
	0.1	73.8 ± 8.0
C	1	69.7 ± 8.8
	0.1	57.0 ± 10.0
D	1	72.0 ± 2.8
	0.1	78.9 ± 4.2
E	1	71.2 ± 9.3
	0.1	64.5 ± 9.2
F	0.01	75.5 ± 6.2
G	0.01	75.7 ± 9.5

[a]Neurons were coincubated with 200 μM H_2O_2 and the compounds in two different concentrations. Cell viability was determined by studying LDH release, after 12 h incubation. Results are mean ± SEM of three or more independent experiments performed in triplicate. Data are presented in percentage versus nontreated control.

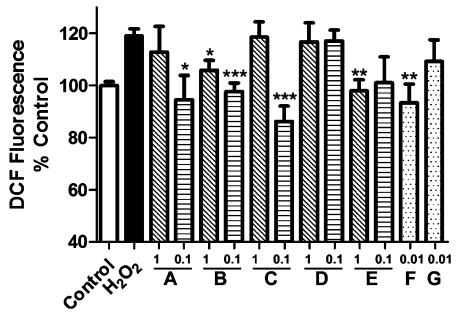


Figure 4. Inhibition of ROS production after treatment with compounds A, B, C, E, and F. Compounds were coincubated with 200 μM H_2O_2 at 1 and 0.1 μM. Just compounds F and G were assayed at 0.01 μM. Data are expressed in percentage respect nontreated control and compared to cells treated with 200 μM H_2O_2 by Student's *t* test. *p < 0.05, **p < 0.01, and ***p < 0.001. Values are mean ± SEM of three or more independent experiments performed by triplicate.

(CAT). GSH has an important role in detoxifying neurons from H_2O_2.[26] It acts as an electron donor by reducing H_2O_2 to water through GPx. In this reaction, GSH is oxidized to GSH disulfide (GSSG) and then reduced back to GSH by the GSH reductase enzyme.[27] Levels were evaluated in cortical neurons cotreated with H_2O_2 and the tested compounds. Since GSH is the most important intracellular source of thiol groups,[28] the Thiol-Tracker Violet dye reaction was used to determine its levels after cotreatment with H_2O_2 and compounds, as previously described. GSH levels are represented in percentage with respect to control cells in Figure 5. H_2O_2 treatment produced a decrease of 25.8 ± 3.1% (p < 0.001) versus GSH

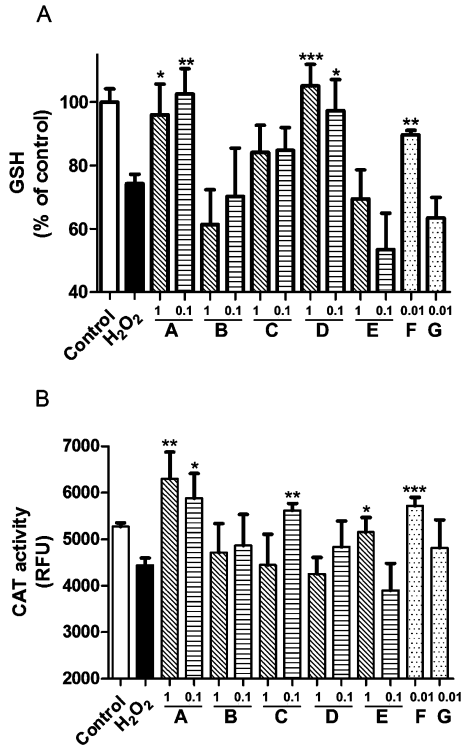


Figure 5. Restoration of GSH levels and CAT activity in an oxidative stress in vitro model. Cortical neurons were incubated for 12 h with 200 μM H_2O_2 and two different concentrations of the compounds (0.1 and 1 μM). Just compounds F and G were studied at 0.01 μM. (A) GSH results are presented in percentage versus the nontreated cells. Cells treated with 200 μM H_2O_2 and compounds A, D, and F showed GSH levels similar to control neurons. (B) CAT activity is represented in relative fluorescence units (RFU). Cells treated with H_2O_2 showed a decreased CAT activity which is later restored in the presence of compounds A, C, E. and F. *p < 0.05, **p < 0.01, and ***p < 0.001. Data are mean ± SEM of three or more independent experiments performed by triplicate.

control levels; moreover, coincubations with compounds A and D (1 and 0.1 μM) and F (0.01 μM) recovered GSH levels (Figure 5A). Compounds A and D were more effective than compound F (89.7 ± 1.4%, p = 0.002), with a total restoration of GSH levels after 12 h of treatment.

CAT detoxifies neurons through decomposition of H_2O_2 into H_2O and O_2.[29] Figure 5B shows the results as graphic bars, expressed as relative fluorescence units (RFU) of H_2O_2 cotreatment with compounds, obtaining positive results with compounds A, C, E, and F. Neurons treated with H_2O_2 presented a lower CAT activity (4440.3 ± 158.7 RFU) with

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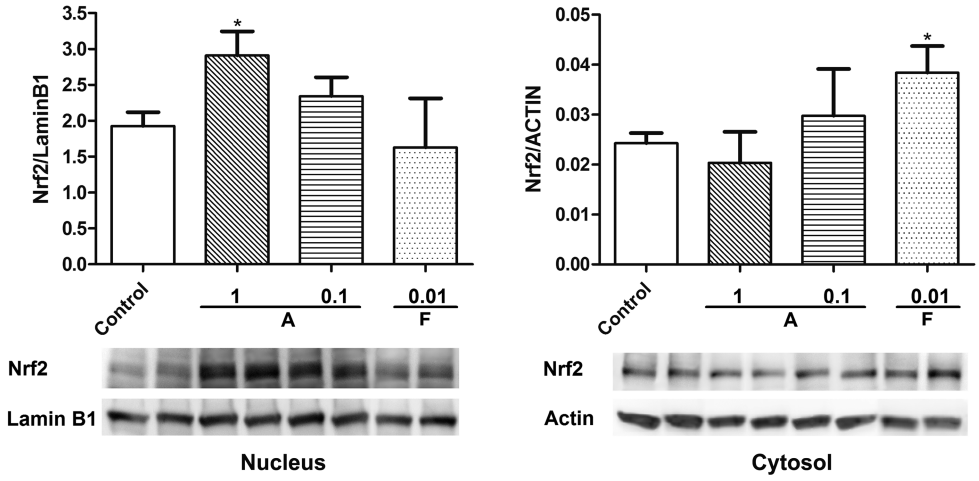

Figure 6. Induction of Nrf2 translocation by compound A. Nrf2 levels were studied after 6 h incubation with compound A and F. Results are presented in ratio of Nrf2/Lamin B1 for nuclear samples and Nrf2/Actin for cytosolic lysates. Compound A increases Nrf2 expression in nuclear fractions. *$p < 0.05$. Data are mean ± SEM of three or more independent experiments.

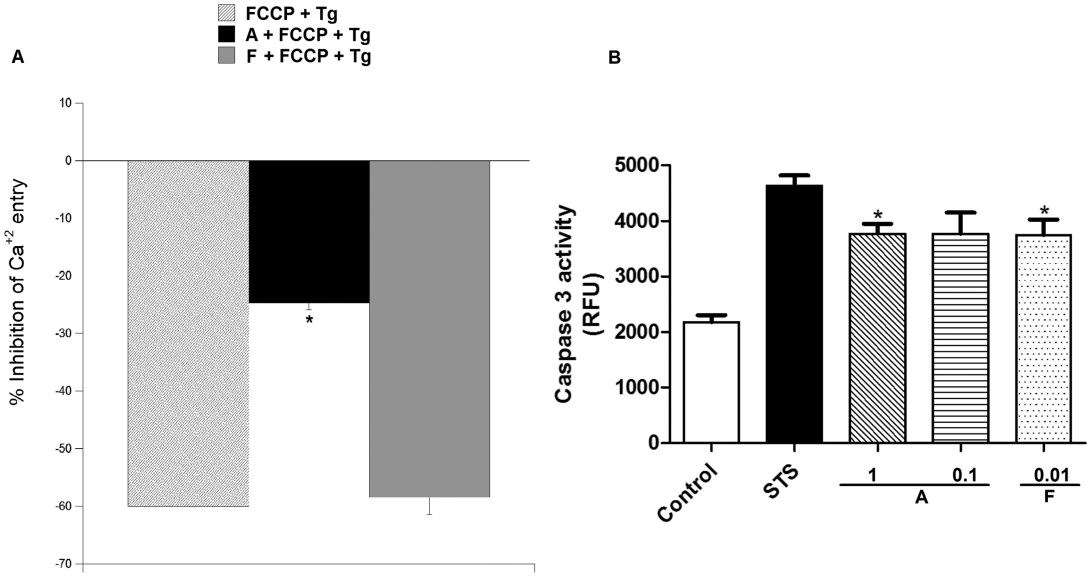

Figure 7. (A) Blockage of the FCCP inhibition of Ca^{2+} entry after Tg-induced endoplasmic reticulum empty. Compound A inhibited the FCCP effect, whereas compound F did not affect the Ca^{2+} entry decrease produced by FCCP. (B) Caspase-3 activity was measured as an apoptosis signal. Cotreatments of 0.5 μM STS and compounds A (1 and 0.1 μM) and F (0.01 μM) showed a decrease of the caspase-3 activity respect to cells only treated with STS. All values are shown in RFU and compared to cells treated with 0.5 μM STS. *$p < 0.05$. Data are mean ± SEM of three or more independent experiments.

respect to control cells (5270.0 ± 78.8 RFU, $p = 0.002$). Compound A highlights with the most pronounced increase in CAT activity in primary neurons at 1 μM (6301.3 ± 571.7 RFU, $p = 0{,}008$) and at 0.1 μM (5881.2 ± 532.2 RFU, $p = 0.020$). Additionally compounds C at 0.1 μM (5621.7 ± 152.0 RFU, $p = 0.002$), E at 1 μM (5153.5 ± 311.7 RFU, $p = 0.044$), and F at 0.01 μM (5721.0 ± 181.8 RFU, $p = 0.001$) restored CAT activity to control levels (5270.0 ± 78.8 RFU, $p = 0.002$) (Figure 5B).

Nrf2 Induction by *Streptomyces* Compounds. Nrf2/ARE pathway activation plays a protective role in neurons. Nrf2 is a transcription factor of several genes involved in antioxidant and neuroprotective activities, which makes it an interesting target for oxidative stress and neurodegenerative conditions.[11,30] Compounds A and F were chosen due to the protective effects observed in the oxidative stress assays to further explore their effect in cortical neurons on Nrf2 translocation to the nucleus. To determine whether their neuroprotective effects would involve Nrf2/ARE pathway activation, cortical neurons were incubated with 1 and 0.1 μM compound A and 0.01 μM compound F for 6 h, and cytosolic and nuclear fractions were separated for Western blot assays. Compound A produced an increase in Nrf2 expression in the nucleus compared to nontreated control cells but no significant variations in Nrf2 cytosolic levels. However, compound F showed no variations in Nrf2 nuclear levels and an augmentation in the cytosol (Figure 6), pointing out that compound A increases Nrf2 nuclear translocation while compound F did not affect Nrf2 pathway.

***Streptomyces* Compounds Effects over Cytoplasmatic Calcium Levels and Caspase-3 Activity.** Mitochondria are able to accumulate Ca^{2+} rapidly and release it slowly to

dx.doi.org/10.1021/cn4001878 | ACS Chem. Neurosci. 2014, 5, 71−80

maintain a certain level of cytosolic Ca^{2+} concentration when it increases, acting as a buffer in physiological conditions.[31] The breakdown of the Ca^{2+} homeostasis leads to mitochondrial dysfunction and finally to the activation of apoptosis pathways through cytochrome *c* release and activation of caspases.[32] The effect of compounds A and F on cytoplasmic Ca^{2+} was studied in the neuroblastoma cell line SH-SY5Y. Cells were loaded with FURA-2 AM to analyze fluorescent changes in this ion. Thapsigargin, a SERCA pump blocker, was used in a Ca^{2+} free medium to empty the intracellular Ca^{2+} stores and open the calcium release activated channels (CRAC) in the plasma membrane. Thereafter, a fast increase in cytosolic Ca^{2+} is observed when the ion is added to the extracellular medium.[33] Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) is an oxidative phosphorylation uncoupler that avoids the depletion of the mitochondrial calcium pools and that reduces this Ca^{2+} readmission due to mitochondrial depolarization. SH-SY5Y cells were preincubated with compound A at 1 μM or F at 0.01 μM for 5 min and later FCCP was added to block the cytosolic Ca^{2+} entry. As can be seen in Figure 7A, only compound A produced a strong inhibition of the FCCP effect on Ca^{2+} entry, whereas compound F did not block FCCP effects.

Following the calcium experiments, a potential effect on activity of caspases was studied. Caspase activity elevation is a clear apoptosis signal in cells. Apoptotic neurons show significant morphological changes as granulation and weakening of neurites, followed by cytoplasmic vacuolation and cellular swelling. Due to the high intrinsic activity of caspases in neurons, the treatments were carried out on 3 div with STS 0.5 μM, which is reported to be an effective apoptotic inductor.[34] Figure 7B shows that cortical neurons treated for 6 h with STS duplicate the caspase-3 activity of control cells without treatment. In addition, neurons were coincubated with compound A at 1 and 0.1 μM and compound F at 0.01 μM. In all treatments, caspase-3 activity was reduced with respect to neurons treated with STS alone (Figure 7B), but the decrease was only significant for compound A at 1 μM and F at 0.01 μM.

Microbial secondary metabolites obtained from a variety of representatives of the genus *Streptomyces*, one of the most abundant bacteria present in soil and marine ecosystems, have led to most of the currently used antibiotics. Therefore, *Streptomyces* strains recovered from marine and unusual environments are of great interest in the search for new drugs. One of these extreme environments is the Atacama Desert (Chile), a hyper-arid large desert with harsh life conditions. Despite these conditions, novel *Actinomycetes* were isolated for studies of their secondary metabolites,[14,35] and among these are the compounds investigated in the present work.

This study characterizes the effect of seven *Streptomyces*-derived compounds with diverse chemical scaffolds in an in vitro oxidative stress model. In this primary neuronal model, we use H_2O_2 to recreate oxidative stress conditions [36] and, thus, to induce an imbalance between ROS generation and antioxidant defenses. As a result of this lack of balance, a mitochondrial dysfunction and an elevation of free radical levels take place with the consequent cellular damage.[1,9]

We report here that several of the compounds tested were able to protect primary cortical neurons against H_2O_2 insult, assessed by measurements of mitochondrial activity (function and membrane potential) or intracellular ROS levels, and improvement of the depressed antioxidant molecules CAT and GSH. Most of them, that is, compounds A, B, C, E, and F diminished intracellular ROS production when primary cortical neurons were incubated with H_2O_2 and all of them, except compound A, also inhibited H_2O_2 effect over mitochondrial function. It is remarkable that in general the activity was higher at a lower concentration (0.1 μM) than at a higher one (1 μM) which may imply that the observed effects could be mediated receptor interactions or through a cellular signaling transduction pathway. For example, coumaric acid and resveratrol promoted antioxidants effects at low doses (0.5 μM), while at higher doses produced a dose-dependent pro-oxidant effect, with ROS elevation, cellular damage, and phospho-Akt down regulation.[37] In this regard, it has also been reported that products can exert high affinity for receptors at low concentrations while higher doses produced a direct enzyme inhibition or a receptor desensitization, which could explain the observed effect with better results at lower concentrations.[38,39]

There is little published evidence in the literature with regard to the cellular activity of the screened compounds, most of them have been described as antitumor agents,[40−42] and only compounds E and F have been described previously as antioxidants but not in neuronal systems. Compound E was compared to the well-known antioxidant butylatedhydroxyanisole, while compound F has shown antioxidant and UV protective properties in pigmented bacteria.[43] We found that the most effective compounds were compound A (anhydroexfoliamycin) and compound F (undecylprodigiosin) with a great antioxidant protection but achieved through different mechanisms. In this sense, anhydroexfoliamycin was not able to recover the mitochondrial function, but this compound showed a remarkable neuroprotection effect by eliminating ROS, maintaining the mitochondrial membrane potential and recovering CAT and GSH levels. Anhydroexfoliamycin was only described as an antibiotic with no other reported bioactivity.[44] The obtained promising results encouraged further investigation of the possible pathway for achieving this neuroprotection. We report that anhydroexfoliamycin increases Nrf2 translocation to the nucleus in cortical neurons that activate the ARE genes transcription for oxidative stress protection, including among others SOD, CAT, or Gpx, that can explain the results obtained in this work. Additionally, this compound is able to inhibit the calcium effects of the mitochondrial uncoupler FCCP by more than 50% and showed an inhibition of caspase-3 activity, offering a complete neuroprotection against oxidative stress.

The other highlighted compound is the red pigment undecylprodigiosin, which in this work we refer to as compound F. At 0.01 μM it was the only one that elicited neuroprotection in all the antioxidant assays (mitochondrial function, membrane potential, ROS production, CAT activity and GSH levels), indicating that this compound could have a great potential as an antioxidant molecule and that further experiments on its effect in vivo will be needed. Undecylprodigiosin effects are not a consequence of an increased Nrf2 translocation to the nucleus, as happens with anhydroexfoliamycin, but its ability to inhibit caspase-3 and, thus, to protect cells from apoptosis supports the idea that undecylprodigiosin directly suppresses against oxidative stress. This is supported by previous reports of its effects in bacteria. This compound delayed H_2O_2 lipid peroxidation and conferred the pigmented bacteria pronounced antioxidative and UV-protective properties,[43] that provide a survival advantage to the producing species. It is remarkable that compounds F and G

dx.doi.org/10.1021/cn4001878 | ACS Chem. Neurosci. 2014, 5, 71−80

(metacycloprodigiosin) are analogues included in the prodigiosin family. Both compounds have been shown to elicit immunosuppressive properties through the inhibition of T-lymphocyte proliferation and antitumor characteristics.[42] However, despite being in the same family, the first was one of the most active chemicals in the present work while the metacycloprodigiosin had no effect in any of the tested assays, which could be attributed to the additional cyclization of this compound. Compounds from the prodigiosin family were reported to inhibit MAPK, JNK, and p38 kinases and the activation of NF-κB in macrophages, which results in a decrease in NO production and activation of anti-inflammatory genes.[45] p38MAPK inhibitors are therapeutic agents to treat inflammatory and neurodegenerative disorders.[46] These studies support the findings of the present work and open other investigations about therapeutic opportunities for these natural products.

Marine *Streptomyces* compounds have displayed beneficial effects on numerous disease processes.[17] They have even showed that they can be potential candidates for neuroprotection treatments and neurocognitive improvement.[18,47] Further studies of the specific mechanism of action of these *Streptomyces*-derived natural products and the kinases involved are needed to elucidate if the effects described in the present work are related to intracellular signaling cascade modulation, mitochondrial interplay or gene expression.

In conclusion, this study demonstrated the ability of some *Streptomyces*-derived compounds to protect primary neuronal cultures against oxidative stress. Their capacity to protect cells from oxidative damage, with reduction of ROS and increase in the level of antioxidant enzymes, points them out as potential candidates in neurodegenerative studies for Parkinson's and Alzheimer's diseases or cerebral ischemia with a highlighted activity of the anhydroexfoliamycin and the pyrrole-based pigment undecylprodigiosin.

METHODS

Compound Information. The library of compounds was provided by the Marine Biodiscovery Centre (Department of Chemistry, University of Aberdeen). In this work, we focus our studies on seven secondary metabolites of *Streptomyces* origin (Table 1). These compounds were isolated upon large scale fermentation in ISP2 medium (5 L each) and subjecting the crude extract to multiple steps of liquid/liquid fractionation, SiO_2, Sephadex LH-20, and RP-C-18 chromatography. The structure elucidation of these compounds was based on their HRESIMS analysis as well as direct comparison with the previously reported NMR spectral data as described for anhydroexfoliamycin (A),[44] naphthopyranomycin (B),[48] 3-epi-5-deoxyenterocin (C),[49] 5-deoxyenterocin (D),[49] nocardamine (E),[50] undecylprodigiosin (F),[23] and metacycloprodigiosin (G).[23] The chemical information and the structures of these compounds are presented in Table 1 and shown in Figure 1.

Cell Culture. Swiss mice were used to obtain primary cultures of cortical neurons. All protocols described in this work were revised and authorized by the University of Santiago de Compostela Institutional animal care and use committee and fulfill with European legislation on use and management of experimental animals.

Primary cortical neurons were obtained from embryonic day 15−18 mice fetuses as described.[51] Briefly, 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 Dulbecco's modified Eagle's medium (DMEM) supplemented with *p*-amino benzoate, insulin, penicillin, and 10% fetal calf serum. The cell suspension was seeded in 96- or 12-multiwell 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 before 48 h of culture to prevent growing of non-neuronal cells. Cortical neurons were seeded in 96-well plates for neuroprotection, ROS, GSH, and CAT assays. Treatments were performed as coincubations of 200 μM H_2O_2 and the compounds at two different concentrations (1 and 0.1 μM) for 12 h on 4−5 days in vitro (div). Compounds F and G were tested in only one concentration (0.01 μM). For caspase-3 experiments and Nrf2 determination, incubations were carried out as described below for 6 h.

Neuroblastoma cell line SH-SY5Y was purchased from American Type Culture Collection (ATCC), number CRL-2266. The cells were plated in 25 cm^2 flasks at a cultivation ratio of 1:10. The cells were maintained in Eagle's minimum essential medium (EMEM) from ATCC and F12 Medium (Invitrogen) in a 1:1 proportion supplemented with 10% fetal bovine serum, 100 UI/mL penicillin, and 100 μg/mL streptomycin. The neuroblastoma cells were dissociated weekly using 0.05% trypsin/EDTA (1×) (Invitrogen). Neuroblastoma cells were used in calcium experiments.

Chemicals and Solutions. Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). Fetal calf serum was obtained from Gibco (Glasgow, U.K.), and DMEM was from Biochrom (Berlin, Germany). Thapsigargin (Tg) was from Alexis Corporation (Läufelfingen, Switzerland), FURA-2AM was obtained from Molecular Probes (Leiden, The Netherlands), and carbonyl cyanide *p*-(trifluoromethoxy) (FCCP) and all other chemicals were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain).

Cytotoxicity Assay. Cell viability was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) test, as previously described.[52,53] The assay was performed in cultures grown in 96-well plates and exposed to different compound concentrations (0.01, 0.05, 0.1, and 1 μM) added to the culture medium. Cultures were maintained in the presence of pure compounds at 37 °C in humidified 5% CO_2/95% air atmosphere for 48 h. Saponin was used as a cellular death control and its absorbance was subtracted from the other data. After treatment time, cells were rinsed and incubated for 1 h with a solution of MTT (500 μg/mL) dissolved in saline buffer. After washing off excess MTT, cells were disaggregated with 5% sodium dodecyl sulfate and the absorbance of the colored formazan salt was measured at 595 nm in a spectrophotometer plate reader.

Mitochondrial Function and Mitochondrial Membrane Potential (ΔΨm) Assays. Mitochondrial function was measured by MTT test following the method described above and changes in ΔΨm were studied with the tetramethylrhodamine methyl ester (TMRM) assay.[54] For TMRM assays, cells were washed twice with saline solution and incubated with 1 μM TMRM for 30 min. Then, neurons were solubilized with 50% DMSO/water. Fluorescence values were obtained using a spectrophotometer plate reader (535 nm excitation, 590 nm emission).

Cell Survival Measurement. LDH release was used as an indicator of cell survival. The In Vitro Toxicology Assay kit (TOX7, Sigma) was used for measuring its activity, following the commercial protocol.

dx.doi.org/10.1021/cn4001878 | ACS Chem. Neurosci. 2014, 5, 71−80

Determination of ROS Production. ROS determination was carried out by a fluorescence assay using 7′,2′-dichlorofluorescein diacetate (DCFH-DA), as described previously.[55] Briefly DCFH-DA enters the cell and it is de-esterified to the ionized free acid (DCFH). ROS reacts with the DCFH, forming the fluorescent 7′,2′-dichlorofluorescein (DCF). Upon experimentation, cells were first washed in saline solution and then were loaded with 20 μM DCF-DA for 30 min at 37 °C. Cells were washed and kept at room temperature for 30 min to allow a complete de-esterification. DCF accumulation was measured using a fluorescence plate reader where excitation was monitored at 475 nm and emission at 525 nm.

Glutathione Assay. Reduced glutathione represents the majority of intracellular free thiols in cells, so we used ThiolTracker Violet dye to estimate their levels in treated cells. Neurons were washed with phosphate buffer solution and loaded with 10 μM ThiolTracker Violet dye for 1 h at 37 °C. After incubation, neurons were washed once and fluorescence was read at 404 nm excitation and emission at 526 nm.

Catalase Activity Measurement. Catalase activity was measured with Amplex Red Catalase Assay kit after exposure of samples to H_2O_2. Cell lysates were processed following the commercial protocol and fluorescence was read at 530 nm excitation and 590 nm emission. Enzymatic activity was calculated by subtracting sample values to the no-catalase control.

Western Blot Assays. Western blot was used to determine Nrf2 levels in the nucleus and in the cytosol. After 6 h of treatment, cortical neurons were washed twice with ice-cold PBS. Samples were homogenized in 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 inhibitors cocktail from Roche) for 15 min, then scrapped, 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 an 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 and vortexing in 10 min intervals. Samples were then centrifuged at 14 000g at 4 °C for 30 min. The supernatants were collected as protein 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 (BIORAD) and transferred onto PVDF membranes (Millipore). Membrane blocking and antibody incubation was performed by Snap i.d protein detection system. The immunoreactive bands were detected using the Supersignal West Pico Chemiluminiscent Substrate or Supersignal West Femto Maximum Sensitivity Substrate (Pierce) and the Diversity 4 gel documentation and analysis system (Syngene, Cambridge, U.K.). Chemiluminiscence was measured with the Diversity GeneSnap software (Syngene). Nrf2 was detected with anti-NF-E2-related factor 2 antibody (1:1000, Millipore). Nrf2 signal was normalized by using β-actin (1:20 000, Millipore) for cytosolic samples and lamin B1 (1:1000, ABCAM) for nuclear samples.

Measurements of Cytosolic Calcium. For cytosolic Ca^{2+} measurements, cells were seeded onto 18 mm glass coverslips and used 48−72 h after plating at a density of 120 000 cells/glass coverslip. They were washed twice with saline solution supplemented with 0.1% bovine serum albumin (BSA). Physiological saline solution (Umbreit) was composed of the following (in mM): NaCl 119, $Mg(SO_4)$ 1.2, PO_4H_2Na 1.2, CO_3HNa 22.85, KCl 5.94, $CaCl_2$ 1. Glucose 1g/L was added to the medium with an osmotic pressure of 290 mOsm/kg of H_2O. In all the assays, the solutions were equilibrated with CO_2 before use, adjusting the final pH between 7.2 and 7.4.

Neuroblastoma cells were loaded with the calcium-sensitive fluorescent dye FURA 2 AM (0.5 μM). Neuroblastoma cells were plated in coverslips and shaken during 10 min at 37 °C and 300 rpm in a saline solution plus 0.1% BSA (described above). Loaded cells were washed twice, and coverslips were placed in a thermostatted chamber (Life Sciences Resources, U.K.). Cells were view using a Nikon Diphot 200 microscope equipped with epifluorescence optics (Nikon 40× immersion UV-Fluor objective). Addition of test compounds was made by aspiration and addition of fresh bathing solution to the chamber. Cytosolic Ca^{2+} ratio was obtained from the images collected by fluorescent equipment (Lambda-DG4). The light source was a xenon arc bulb, and the different wavelengths used were chosen with filters. For FURA-2AM, cells were excited at 340 and 380 nm light alternately and emission was collected at 510 nm. The experiments were carried out at least three times.

Caspase-3 Activity. The EnzChek Caspase-3 Assay kit was used for measuring caspase-3 activity through a nonfluorescent bisamide substrate (Z-DEVD-R110) that caspase-3 transforms first to a monoamide and then to the green-fluorescent rhodamine 110. Treatments for this assay were carried out on 3 div, and the caspase-3 activity was induced with 0.5 μM staurosporine (STS) during 6 h. Samples were obtained and processed following the commercial protocol and fluorescence was read at 496 nm excitation, 520 nm emission.

Statistical Analysis. All the results are expressed as means ± SEM of three or more experiments. Experiments were performed by triplicate. Statistical comparison was performed by Student's t test. P values < 0.05 were considered statistically significant.

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Funding

Funding is gratefully acknowledged from the following. From Ministerio de Ciencia y Tecnología, Spain: [SAF2009-12581 (subprograma NEF)], [AGL2009-13581-CO2-01], [TRA2009-0189, AGL2010-17875]. From Xunta de Galicia, Spain: [GRC 2010/10] [PGDIT 07MMA006261PR], [PGIDIT (INCITE) 09MMA003261PR], [PGDIT (INCITE) 09261080PR], [2009/XA044], and [10PXIB261254 PR]. Funding by the EU FP7 program: [211326 - CP (CONffIDENCE)], [265896 BAMMBO], [265409 μAQUA], [262649 BEADS], and [312184 PharmaSea]. Through the Atlantic Area Programme (Interreg IVB Trans-national): [2009-1/117 Pharmatlantic]. M.E.R. thanks the Government of the Arab Republic of Egypt for a Ph.D. Scholarship. M.J. thanks the Scottish University Life Science Alliance which provided funding to set up the compound library.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Melo, A., Monteiro, L., Lima, R. M., Oliveira, D. M., Cerqueira, M. D., and El-Bacha, R. S. (2011) Oxidative stress in neurodegenerative diseases: mechanisms and therapeutic perspectives. *Oxid. Med. Cell. Longevity 2011*, 467180.

(2) Gandhi, S., and Abramov, A. Y. (2012) Mechanism of oxidative stress in neurodegeneration. *Oxid. Med. Cell. Longevity 2012*, 428010.

(3) Bonda, D. J., Wang, X., Perry, G., Nunomura, A., Tabaton, M., Zhu, X., and Smith, M. A. (2010) Oxidative stress in Alzheimer disease: a possibility for prevention. *Neuropharmacology 59*, 290−294.

(4) Floyd, R. A. (1999) Antioxidants, oxidative stress, and degenerative neurological disorders. *Proc. Soc. Exp. Biol. Med. 222*, 236−245.

(5) Pratico, D., Clark, C. M., Liun, F., Rokach, J., Lee, V. Y., and Trojanowski, J. Q. (2002) Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. *Arch. Neurol. 59*, 972−976.

(6) Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E. K., Jones, P. K., Ghanbari, H., Wataya, T., Shimohama, S., Chiba, S., Atwood, C. S., Petersen, R. B., and Smith, M. A. (2001) Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol. 60*, 759−767.

(7) Wang, X., and Michaelis, E. K. (2010) Selective neuronal vulnerability to oxidative stress in the brain. *Front. Aging Neurosci. 2*, 12.

(8) Feng, Y., and Wang, X. (2012) Antioxidant therapies for Alzheimer's disease. *Oxid. Med. Cell. Longevity 2012*, 472932.

(9) Basli, A., Soulet, S., Chaher, N., Merillon, J. M., Chibane, M., Monti, J. P., and Richard, T. (2012) Wine polyphenols: potential agents in neuroprotection. *Oxid. Med. Cell. Longevity 2012*, 805762.

(10) Kaspar, J. W., Niture, S. K., and Jaiswal, A. K. (2009) Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radicals Biol. Med. 47*, 1304−1309.

(11) Joshi, G., and Johnson, J. A. (2012) The Nrf2-ARE pathway: a valuable therapeutic target for the treatment of neurodegenerative diseases. *Recent Pat. CNS Drug Discovery 7*, 218−229.

(12) Shih, A. Y., Imbeault, S., Barakauskas, V., Erb, H., Jiang, L., Li, P., and Murphy, T. H. (2005) Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo. *J. Biol. Chem. 280*, 22925−22936.

(13) Ramsey, C. P., Glass, C. A., Montgomery, M. B., Lindl, K. A., Ritson, G. P., Chia, L. A., Hamilton, R. L., Chu, C. T., and Jordan-Sciutto, K. L. (2007) Expression of Nrf2 in neurodegenerative diseases. *J. Neuropathol. Exp. Neurol. 66*, 75−85.

(14) Rateb, M. E., Houssen, W. E., Harrison, W. T., Deng, H., Okoro, C. K., Asenjo, J. A., Andrews, B. A., Bull, A. T., Goodfellow, M., Ebel, R., and Jaspars, M. (2011) Diverse metabolic profiles of a Streptomyces strain isolated from a hyper-arid environment. *J. Nat. Prod. 74*, 1965−1971.

(15) Allenby, N. E., Laing, E., Bucca, G., Kierzek, A. M., and Smith, C. P. (2012) Diverse control of metabolism and other cellular processes in Streptomyces coelicolor by the PhoP transcription factor: genome-wide identification of in vivo targets. *Nucleic Acids Res. 40*, 9543−9556.

(16) Yamanaka, K., Oikawa, H., Ogawa, H. O., Hosono, K., Shinmachi, F., Takano, H., Sakuda, S., Beppu, T., and Ueda, K. (2005) Desferrioxamine E produced by Streptomyces griseus stimulates growth and development of Streptomyces tanashiensis. *Microbiology 151*, 2899−2905.

(17) Zhang, H., Wang, Y., and Pfeifer, B. A. (2008) Bacterial hosts for natural product production. *Mol. Pharmaceutics 5*, 212−225.

(18) Hong, K., Gao, A. H., Xie, Q. Y., Gao, H., Zhuang, L., Lin, H. P., Yu, H. P., Li, J., Yao, X. S., Goodfellow, M., and Ruan, J. S. (2009) Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Mar. Drugs 7*, 24−44.

(19) Kim, W. G., Ryoo, I. J., Park, J. S., and Yoo, I. D. (2001) Benzastatins H and I, new benzastatin derivatives with neuronal cell protecting activity from Streptomyces nitrosporeus. *J. Antibiot. 54*, 513−516.

(20) Sunazuka, T., Hirose, T., and Omura, S. (2008) Efficient total synthesis of novel bioactive microbial metabolites. *Acc. Chem. Res. 41*, 302−314.

(21) Tadtong, S., Meksuriyen, D., Tanasupawat, S., Isobe, M., and Suwanborirux, K. (2007) Geldanamycin derivatives and neuroprotective effect on cultured P19-derived neurons. *Bioorg. Med. Chem. Lett. 17*, 2939−2943.

(22) Varming, T., Drejer, J., Frandsen, A., and Schousboe, A. (1996) Characterization of a chemical anoxia model in cerebellar granule neurons using sodium azide: protection by nifedipine and MK-801. *J. Neurosci. Res. 44*, 40−46.

(23) Liu, R., Cui, C. B., Duan, L., Gu, Q. Q., and Zhu, W. M. (2005) Potent in vitro anticancer activity of metacycloprodigiosin and undecylprodigiosin from a sponge-derived actinomycete Saccharopolyspora sp. nov. *Arch. Pharm. Res. 28*, 1341−1344.

(24) Triana-Vidal, L. E., and Carvajal-Varona, S. M. (2013) Protective Effect of Galantamine Against Oxidative Damage Using Human Lymphocytes: A Novel In Vitro Model. *Arch. Med. Res. 44*, 85−92.

(25) Lobner, D. (2000) Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? *J. Neurosci. Methods 96*, 147−152.

(26) Mytilineou, C., Kramer, B. C., and Yabut, J. A. (2002) Glutathione depletion and oxidative stress. *Parkinsonism Relat. Disord. 8*, 385−387.

(27) Aoyama, K., Watabe, M., and Nakaki, T. (2008) Regulation of neuronal glutathione synthesis. *J. Pharmacol. Sci. 108*, 227−238.

(28) Zampagni, M., Wright, D., Cascella, R., D'Adamio, G., Casamenti, F., Evangelisti, E., Cardona, F., Goti, A., Nacmias, B., Sorbi, S., Liguri, G., and Cecchi, C. (2012) Novel S-acyl glutathione derivatives prevent amyloid oxidative stress and cholinergic dysfunction in Alzheimer disease models. *Free Radicals Biol. Med. 52*, 1362−1371.

(29) Armogida, M., Nistico, R., and Mercuri, N. B. (2012) Therapeutic potential of targeting hydrogen peroxide metabolism in the treatment of brain ischaemia. *Br. J. Pharmacol. 166*, 1211−1224.

(30) Calkins, M. J., Johnson, D. A., Townsend, J. A., Vargas, M. R., Dowell, J. A., Williamson, T. P., Kraft, A. D., Lee, J. M., Li, J., and Johnson, J. A. (2009) The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. *Antioxid. Redox Signaling 11*, 497−508.

(31) Friel, D. D. (2000) Mitochondria as regulators of stimulus-evoked calcium signals in neurons. *Cell Calcium 28*, 307−316.

(32) Potterat, J. J., Woodhouse, D. E., Rothenberg, R. B., Muth, S. Q., Darrow, W. W., Muth, J. B., and Reynolds, J. U. (1993) AIDS in Colorado Springs: is there an epidemic? *AIDS 7*, 1517−1521.

(33) Bakowski, D., and Parekh, A. B. (2002) Permeation through store-operated CRAC channels in divalent-free solution: potential problems and implications for putative CRAC channel genes. *Cell Calcium 32*, 379−391.

(34) Kajta, M., Trotter, A., Lason, W., and Beyer, C. (2006) Impact of 17beta-estradiol on cytokine-mediated apoptotic effects in primary hippocampal and neocortical cell cultures. *Brain Res. 1116*, 64−74.

(35) Rateb, M. E., Houssen, W. E., Arnold, M., Abdelrahman, M. H., Deng, H., Harrison, W. T., Okoro, C. K., Asenjo, J. A., Andrews, B. A., Ferguson, G., Bull, A. T., Goodfellow, M., Ebel, R., and Jaspars, M. (2011) Chaxamycins A-D, bioactive ansamycins from a hyper-arid desert Streptomyces sp. *J. Nat. Prod. 74*, 1491−1499.

(36) Zhao, G., Yao-Yue, C., Qin, G. W., and Guo, L. H. (2012) Luteolin from Purple Perilla mitigates ROS insult particularly in primary neurons. *Neurobiol. Aging 33*, 176−186.

(37) Pasciu, V., Posadino, A. M., Cossu, A., Sanna, B., Tadolini, B., Gaspa, L., Marchisio, A., Dessole, S., Capobianco, G., and Pintus, G. (2010) Akt downregulation by flavin oxidase-induced ROS generation mediates dose-dependent endothelial cell damage elicited by natural antioxidants. *Toxicol. Sci. 114*, 101−112.

(38) Williams, R. J., and Spencer, J. P. (2012) Flavonoids, cognition, and dementia: actions, mechanisms, and potential therapeutic utility for Alzheimer disease. *Free Radicals Biol. Med. 52*, 35−45.

(39) Jiang, F., Li, W., Huang, Y., Chen, Y., Jin, B., Chen, N., Ding, Z., and Ding, X. (2013) Antioxidant, antityrosinase and antitumor activity comparison: the potential utilization of fibrous root part of Bletilla striata (Thunb.) Reichb.f. *PLoS One 8*, e58004.
(40) Kalinovskaya, N. I., Romanenko, L. A., Irisawa, T., Ermakova, S. P., and Kalinovsky, A. I. (2011) Marine isolate Citricoccus sp. KMM 3890 as a source of a cyclic siderophore nocardamine with antitumor activity. *Microbiol. Res. 166*, 654−661.
(41) Moore, B. S., Kalaitzis, J. A., and Xiang, L. (2005) Exploiting marine actinomycete biosynthetic pathways for drug discovery. *Antonie Van Leeuwenhoek 87*, 49−57.
(42) Montaner, B., and Perez-Tomas, R. (2003) The prodigiosins: a new family of anticancer drugs. *Curr. Cancer Drug Targets 3*, 57−65.
(43) Stankovic, N., Radulovic, V., Petkovic, M., Vuckovic, I., Jadranin, M., Vasiljevic, B., and Nikodinovic-Runic, J. (2012) Streptomyces sp. JS520 produces exceptionally high quantities of undecylprodigiosin with antibacterial, antioxidative, and UV-protective properties. *Appl. Microbiol. Biotechnol. 96*, 1217−1231.
(44) Potterat, O., Zahner, H., Volkmann, C., and Zeeck, A. (1993) Metabolic products of microorganisms. 264. Exfoliamycin and related metabolites, new naphthoquinone antibiotics from Streptomyces exfoliatus. *J. Antibiot. 46*, 346−349.
(45) Huh, J. E., Yim, J. H., Lee, H. K., Moon, E. Y., Rhee, D. K., and Pyo, S. (2007) Prodigiosin isolated from Hahella chejuensis suppresses lipopolysaccharide-induced NO production by inhibiting p38 MAPK, JNK and NF-kappaB activation in murine peritoneal macrophages. *Int. Immunopharmacol. 7*, 1825−1833.
(46) Munoz, L., and Ammit, A. J. (2010) Targeting p38 MAPK pathway for the treatment of Alzheimer's disease. *Neuropharmacology 58*, 561−568.
(47) Eftekharzadeh, B., Hamedi, J., Mohammadipanah, F., Khodagholi, F., Maghsoudi, N., and Klenk, H. P. (2010) Inhibition of oxidative stress-induced amyloid beta formation in NT2 neurons by culture filtrate of a strain of Streptomyces antibioticus. *Appl. Microbiol. Biotechnol. 86*, 1805−1811.
(48) Shindo, K., and Kawai, H. (1992) A novel antibiotic, naphthopyranomycin. *J. Antibiot. 45*, 584−586.
(49) Sitachitta, N., Gadepalli, M., and Davidson, B. S. (1996) New α-pyrone-containing metabolites from a marine-derived actinomycete. *Tetrahedron 52*, 8073−8080.
(50) Lee, H. S., Shin, H. J., Jang, K. H., Kim, T. S., Oh, K. B., and Shin, J. (2005) Cyclic peptides of the nocardamine class from a marine-derived bacterium of the genus Streptomyces. *J. Nat. Prod. 68*, 623−625.
(51) Vale, C., Alonso, E., Rubiolo, J. A., Vieytes, M. R., LaFerla, F. M., Gimenez-Llort, L., and Botana, L. M. (2010) Profile for amyloid-beta and tau expression in primary cortical cultures from 3xTg-AD mice. *Cell. Mol. Neurobiol 30*, 577−590.
(52) Alonso, E., Vale, C., Vieytes, M. R., Laferla, F. M., Gimenez-Llort, L., and Botana, L. M. (2011) 13-Desmethyl spirolide-C is neuroprotective and reduces intracellular Abeta and hyperphosphorylated tau in vitro. *Neurochem. Int. 59*, 1056−1065.
(53) Alonso, E., Vale, C., Vieytes, M. R., Laferla, F. M., Gimenez-Llort, L., and Botana, L. M. (2011) The cholinergic antagonist gymnodimine improves Abeta and tau neuropathology in an in vitro model of Alzheimer disease. *Cell. Physiol. Biochem. 27*, 783−794.
(54) White, M. G., Wang, Y., Akay, C., Lindl, K. A., Kolson, D. L., and Jordan-Sciutto, K. L. (2011) Parallel high throughput neuronal toxicity assays demonstrate uncoupling between loss of mitochondrial membrane potential and neuronal damage in a model of HIV-induced neurodegeneration. *Neurosci. Res. 70*, 220−229.
(55) Kim, E. J., Kwon, K. J., Park, J. Y., Lee, S. H., Moon, C. H., and Baik, E. J. (2002) Neuroprotective effects of prostaglandin E2 or cAMP against microglial and neuronal free radical mediated toxicity associated with inflammation. *J. Neurosci. Res. 70*, 97−107.