Protection Against ROS-Induced Damage by Streptomyces Secondary Metabolites in Primary Cortical Neurons

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ABSTRACT:

Oxidative stress plays a significant role in neurodegenerative diseases and is closely linked to mitochondrial dysfunction. In this investigation, we evaluated seven natural products derived from Streptomyces against hydrogen peroxide-induced oxidative stress in primary cortical neurons, serving as an in vitro model. Our findings demonstrated that these compounds effectively reduced neuronal cytotoxicity and decreased reactive oxygen species (ROS) release after 12 hours of treatment. Notably, the compounds quinoneanhydroexfoliamycin and the red pyrrole-type pigment undecylprodigiosin exhibited remarkable protective properties against oxidative stress. They enhanced mitochondrial function, inhibited ROS production, and increased levels of antioxidant enzymes, including glutathione and catalase. Further studies revealed that anhydroexfoliamycin activates the Nrf2-ARE signaling pathway, inducing Nrf2 nuclear translocation, and significantly counteracts the impacts of the mitochondrial uncoupler FCCP on cytosolic Ca2+ levels, suggesting that mitochondria are a critical target for this compound. Additionally, while both compounds reduced staurosporine-induced caspase-3 activity, undecylprodigiosin did not mitigate the effects of FCCP and did not influence the Nrf2 pathway, unlike anhydroexfoliamycin. These findings indicate that metabolites from Streptomyces may hold promise for developing new therapies aimed at preventing neurodegenerative conditions such as Parkinson's and Alzheimer's diseases, as well as cerebral ischemia.

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

INTRODUCTION:

The prevalence of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, has surged due to increased life expectancy, reaching epidemic levels across industrialized nations and presenting a substantial socioeconomic challenge. Neurodegenerative disorders are characterized by various cellular pathologies, notably mitochondrial dysfunction and increased reactive oxygen species (ROS) production, which are closely associated with oxidative stress. The central nervous system is particularly vulnerable to oxidative damage from free radicals, attributed to its high oxygen consumption, abundant phospholipids that are prone to oxidation, and elevated iron levels, which can catalyze oxidative reactions and foster free radical generation. Moreover, the brain possesses limited antioxidant defenses, which are further diminished in Alzheimer's disease. Research indicates that oxidative damage is an early event in this condition, occurring even before cognitive decline manifests. This oxidative damage results from the imbalance between ROS production and the brain's antioxidant defenses. As oxidative stress escalates, it leads to mitochondrial dysfunction, amplifying free radical production and perpetuating the cycle of oxidative stress.

Under normal conditions, mitochondrial respiration produces molecular oxygen; however, defects in the electron transport chain can lead to excess molecular oxygen and impair several antioxidant enzymes, causing mitochondria to become major sources of ROS. The primary species of oxygen involved in neuronal oxidative injury include superoxide anion (O2•−), hydrogen peroxide (H2O2), and hydroxyl radicals (OH•). Their accumulation results in the oxidation of proteins, DNA, RNA, and lipid peroxidation. Neurons utilize both enzymatic and non-enzymatic defenses to mitigate oxidative stress. Enzymatic defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), which convert free radicals into non-toxic substances. It has been reported that enhancing antioxidant activity protects cellular models from neurodegeneration, and antioxidants may lower the risk of developing neurodegenerative diseases. These antioxidant systems are regulated by antioxidant response elements (AREs) and depend on the nuclear factor erythroid 2-related factor 2 (Nrf2). As a transcription factor, Nrf2 controls the activation of protective genes, including the aforementioned enzymes, as well as antioxidant and anti-apoptotic proteins and proteasomes.

Under non-oxidative stress conditions, Nrf2 resides in the cytoplasm in a complex with Keap1. However, during oxidative stress, this Nrf2-Keap1 complex is destabilized, allowing Nrf2 to translocate to the nucleus. Both Nrf2 and Keap1 serve as sensors for free radical damage. Once in the nucleus, Nrf2 binds to Maf proteins to enhance the ARE response, thus boosting the cellular antioxidant capacity. Several small molecules have been identified as Nrf2 inducers, demonstrating protective effects in vitro and in vivo. Neurons deficient in Nrf2 exhibit heightened susceptibility to oxidative stress from H2O2, while Nrf2 overexpression provides protective benefits. An increase in Nrf2 levels in vivo has proven effective in combating ROS production and oxidative stress within the brain. Evidence indicates that Nrf2 dysfunction is prevalent in neurodegenerative conditions like Alzheimer's disease and Lewy body dementia; for instance, reduced nuclear Nrf2 levels have been observed in Alzheimer's patients compared to age-matched controls. Consequently, enhancing nuclear Nrf2 expression may offer a therapeutic strategy to bolster antioxidant defenses in neurons suffering from neurodegenerative diseases.

The genus Streptomyces, comprising over 500 species of Gram-positive filamentous bacteria primarily found in soil and marine ecosystems, is recognized as the largest genus of Actinobacteria. Their complex differentiation process includes the production of branching mycelia, aerial hyphae, and spores. Streptomyces species are renowned for their ability to synthesize a wide array of secondary metabolites, many of which exhibit pharmaceuticals properties, including anti-inflammatory, antiviral, antimicrobial, and anticancer activities. Notably, some of these metabolites have demonstrated protective effects against neurodegenerative disorders. Various secondary metabolites extracted from Streptomyces have been identified as potential neuroprotective agents, efficiently scavenging free radicals and promoting neuritogenesis, as well as exhibiting neuroprotective capabilities in numerous neurodegeneration models. Given these protective mechanisms, natural products derived from marine and desert Streptomyces species are promising candidates for neuroprotection studies aimed at countering oxidative stress and developing preventive treatments for neurodegenerative diseases.

The aim of this study is to perform an in vitro screening of seven natural products isolated from Streptomyces spp. sourced from the hyper-arid Atacama Desert and marine environments against oxidative stress-induced cellular damage from H2O2 in primary neuronal cultures. This research provides the first account of the neuroprotective effects of Streptomyces metabolites against H2O2 damage, highlighting the significant roles of 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 ± 0.4%), and the same effect was observed for compound G (98.4 ± 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.

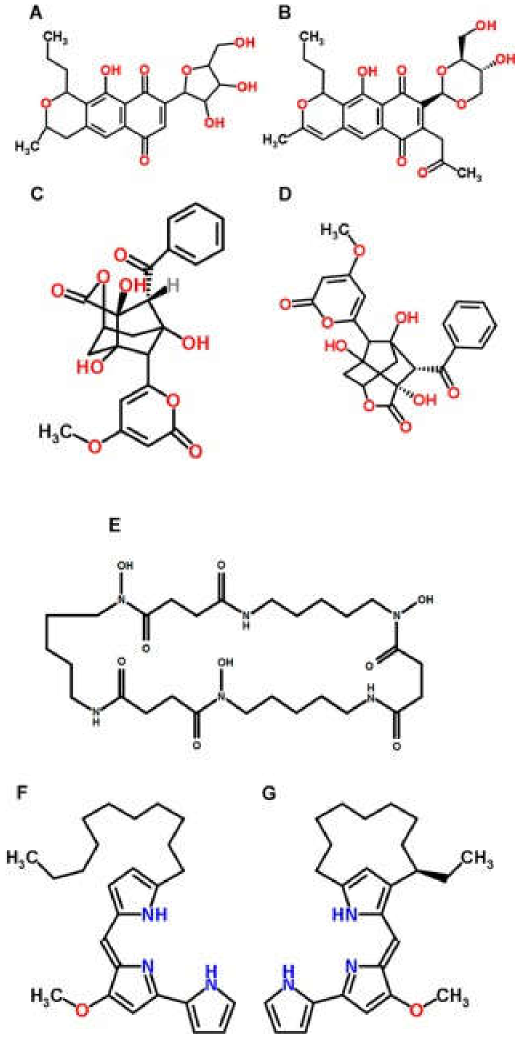

Figure 1. Compound structures.

Neuroprotective Effect of Streptomyces Compounds against H2O2 Insult. Since marine natural compounds can be potential neuroprotectors against oxidative stress, we used H2O2 as an oxidative stress inducer. H2O2 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

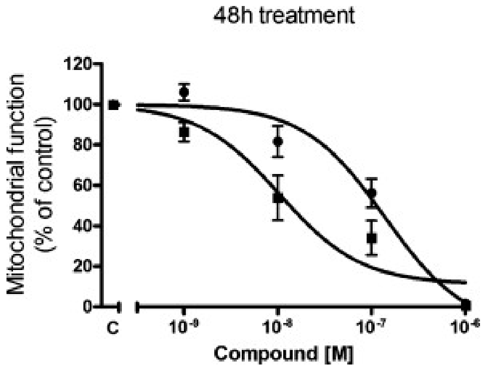

Figure 2. Cytotoxicity effects of compounds F and G after 48 h incubation. Dose response effect of F (■) and G (●) compounds in concentrations ranging from 10 to 1000 nM.

model. Therefore, primary cortical neurons of 4 days in vitro (div) were incubated for 12 h with 200 μM H2O2 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 H2O2 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 H2O2 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, H2O2 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.

mproved mitochondrial function significantly. LDH release was studied as a parameter of cellular membrane integrity. Treatments with 200 μM H2O2 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 H2O2 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 H2O2 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 H2O2 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 H2O2 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 H2O2

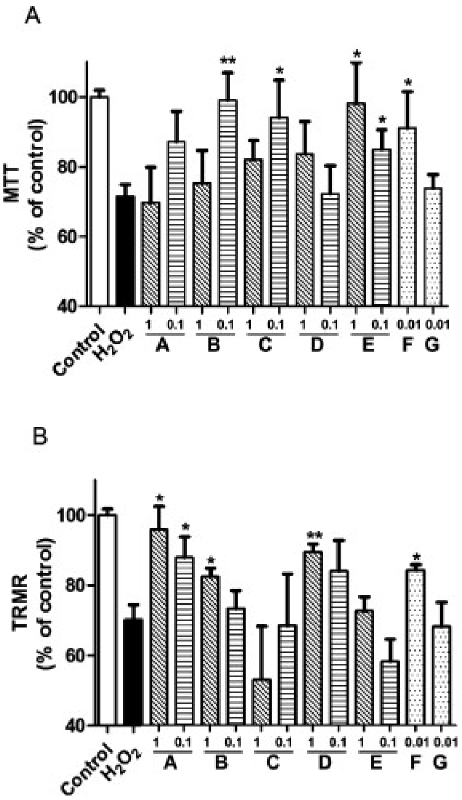

Figure 3. Evaluation of the neuroprotective effects at mitochondrial level. (A) Mitochondrial function was studied by MTT. Cellular viability was increased in neurons treated with B, C, E, and F compounds. (B) TRMR assay was performed to elucidate the ΔΨm. Co-incubation of cortical neurons with 200 μM H2O2 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 H2O2 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 H2O2-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 H2O2 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 H2O2 was significantly increased with respect to basal levels. Neurons in the presence of H2O2 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 H2O2 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 H2O2-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

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 H2O2 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.

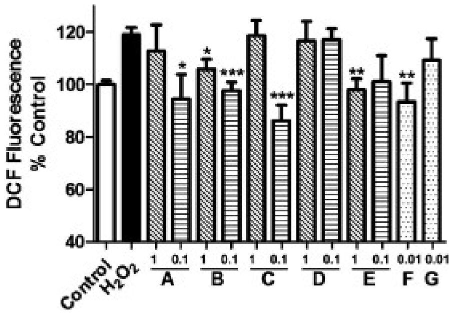

Figure 4. Inhibition of ROS production after treatment with compounds A, B, C, E, and F. Compounds were coincubated with 200 μM H2O2 at 1 and 0.1 μM. Just compounds F and G were assayed at 0.01 μM. Data are expressed in percentage respect nontreated control and compared to cells treated with 200 μM H2O2 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 H2O2. 26 It acts as an electron donor by reducing H2O2 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 H2O2 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 H2O2 and compounds, as previously described. GSH levels are represented in percentage with respect to control cells in Figure 5. H2O2 treatment produced a decrease of 25.8 ± 3.1% (p < 0.001) versus GSH

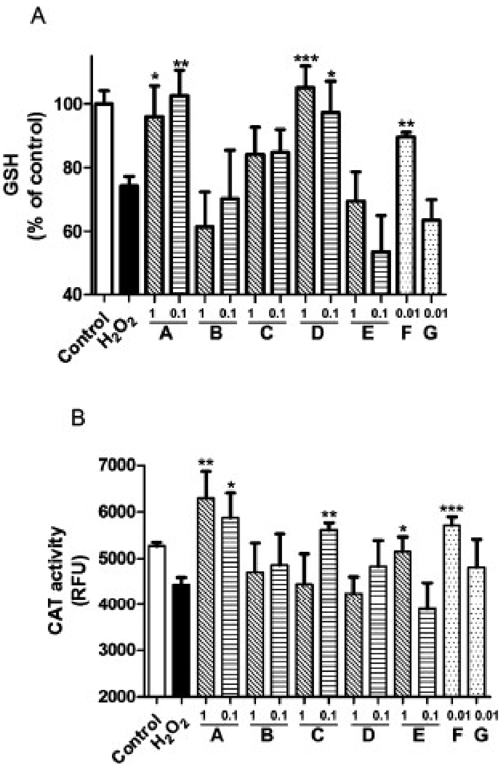

Figure 5. Restoration of GSH levels and CAT activity in an oxidative stress in vitro model. Cortical neurons were incubated for 12 h with 200 μM H2O2 and two different concentrations of the compounds (0.1 and 1 μM). Just compounds F and G were studied at 0.01 μM. (A) GSH results are presented in percentage versus the nontreated cells. Cells treated with 200 μM H2O2 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 H2O2 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 H2O2 into H2O and O2. 29 Figure 5B shows the results as graphic bars, expressed as relative fluorescence units (RFU) of H2O2 cotreatment with compounds, obtaining positive results with compounds A, C, E, and F. Neurons treated with H2O2 presented a lower CAT activity (4440.3 ± 158.7 RFU) with

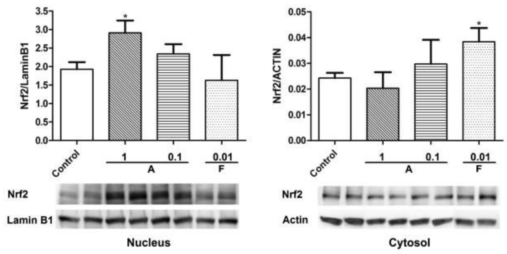

Figure 6. Induction of Nrf2 translocation by compound A. Nrf2 levels were studied after 6 h incubation with compound A and F. Results are presented in ratio of Nrf2/Lamin B1 for nuclear samples and Nrf2/Actin for cytosolic lysates. Compound A increases Nrf2 expression in nuclear fractions. *p < 0.05.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

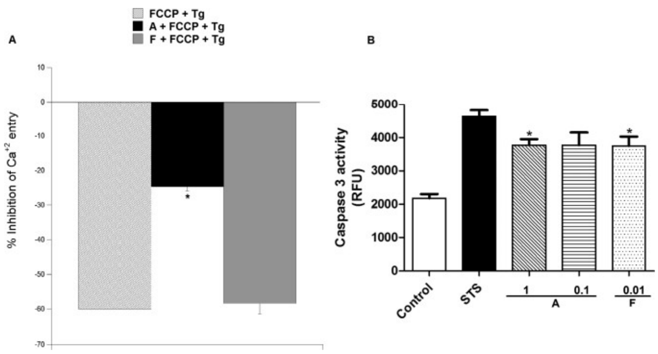

Figure 7. (A) Blockage of the FCCP inhibition of Ca2+ entry after Tg-induced endoplasmic reticulum empty. Compound A inhibited the FCCP effect, whereas compound F did not affect the Ca2+ entry decrease produced by FCCP. (B) Caspase-3 activity was measured as an apoptosis signal. Cotreatments of 0.5 μM 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 Ca2+ rapidly and release it slowly to maintain a certain level of cytosolic Ca2+ concentration when it increases, acting as a buffer in physiological conditions.31 The breakdown of the Ca2+ 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 Ca2+ 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 Ca2+ free medium to empty the intracellular Ca2+ stores and open the calcium release activated channels (CRAC) in the plasma membrane. Thereafter, a fast increase in cytosolic Ca2+ 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 Ca2+ 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 Ca2+ entry. As can be seen in Figure 7A, only compound A produced a strong inhibition of the FCCP effect on Ca2+ 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 Streptomycesderived 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.

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 (metacycloprodigiosin) are analogues included in the prodigiosin family. Both compounds have been shown to elicit immunosuppressive properties through the inhibition of Tlymphocyte 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, SiO2, 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-epi5-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-Dlysine and incubated in a humidified 5% CO2/95% air atmosphere at 37 °C. Cytosine arabinoside (20 μM) was added before 48 h of culture to prevent growing of nonneuronal cells. Cortical neurons were seeded in 96-well plates for neuroprotection, ROS, GSH, and CAT assays. Treatments were performed as coincubations of 200 μM H2O2 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 CRL2266. The cells were plated in 25 cm2 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 (Laufel¨ fingen, Switzerland), FURA2AM 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 coloredformazan 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.

Determination of ROS Production. ROS determination was carried out by a fluorescence assay using 7′,2′-dichlorofluoresceindiacetate (DCFH-DA), as described previously.55 Briefly DCFH-DA enters the cell and it is deesterified 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 μMThiolTracker 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 nonfluorescentbisamide 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 μMstaurosporine (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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