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Natural products inspired synthesis of neuroprotective agents against H_2O_2-induced cell death

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ABSTRACT

Stroke is a debilitating disease and the third leading cause of death in the USA, where over 2000 new stroke cases are diagnosed every day. Treatment options for stroke-related brain damage are very limited and there is an urgent need for effective neuroprotective agents to treat these conditions. Comparison of the structures of several classes of neuroprotective natural products such as limonoids and cardiac glycosides revealed the presence of a common structural motif which may account for their observed neuroprotective activity. Several natural product mimics that incorporate this shared structural motif were synthesized and were found to possess significant neuroprotective activity. These compounds enhanced cell viability against H_2O_2 induced oxidative stress or cell death in PC12 neuronal cells. The compounds were also found to enhance and modulate Na^+/K^+-ATPase activity of PC12 cells, which may suggest that the observed neuroprotective activity is mediated, at least partly, through interaction with Na^+/K^+-ATPase.

Stroke is a major health concern in the industrialized countries. It is the third leading cause of death in the USA, ranking after heart disease and cancer.[1] It is also the leading cause of long term disability, resulting in enormous financial burden on affected families and on the health care system. Stroke-related healthcare costs are rising, with nearly 1 million hospitalizations each year in the U.S. and an estimated direct medical cost of 25.2 billion for the year 2007.[2] Currently available treatment options for stroke-related brain damage are limited. The only FDA approved drug for acute ischemic stroke, IV recombinant tissue plasminogen activator (rt-PA), unfortunately can be received by only 5–10% of acute ischemic stroke (AIS) patients because of the less than 3 h restrictive therapeutic time window within which it must be administered.[3] Therefore, there is an urgent need for identification of new neuroprotective drug candidates and drug targets for treatment of ischemic stroke.

Natural products are a prolific source of bioactive agents of diverse structure and varying biological activity. They are the single most productive source of lead molecules for development as clinically useful drugs for human disorders. In the search for neuroprotective agents from natural sources, a number of plant extracts and several natural products isolated from them have been reported to provide neuroprotection against ischemic stroke.[4–6] In addition, a wide range of natural product derivatives and natural product mimics of synthetic origin such as estrogen-like compounds,[7] kavalactone derivatives,[8] glucose-containing flavones,[9,10] arylidenepregnenolone derivatives,[11] lignophenol derivatives,[12] triazine derivatives,[13] pyrazolyl-2,4-thiazolidinedione derivatives,[14] phenolic thiazoles,[15] and indole derivatives[16] have been shown to protect neurons against oxidative damage induced by H_2O_2 or by other oxidative stress conditions. Among neuroprotective natural products, a group of naturally occurring limonoids dictamnusine **1**, dictamdiol **2**, fraxinellone **3**, calodendrolide **4**, obacunone **5** and limonin **6** (Fig. 1) isolated from *Dictamnus dasycarpus* were reported to have significant neuroprotective activity against glutamate-induced neurotoxicity in rat cortical cells.[5] Obacunone **5** was also reported to be protective against glutamate-induced oxidative damage in mouse hippocampal HT22 cells.[17] Further, it induced cell resistance to oxidative injury from glutamate-induced cytotoxicity in HT22 cells, presumably mediated through p38 MAPK pathway-dependent heme oxygenase-1 expression.[17]

The cardiac glycoside neriifolin **7** isolated from the yellow oleander *Thevetia neriifolia* and its 16-β-acetoxy derivative oleandrin,

Abbreviations: IV, intravenous; FDA, food and drug administration; AIS, acute ischemic stroke; rt-PA, recombinant tissue plasminogen activator; NMR, nuclear magnetic resonance; NOE, nuclear overhauser effect; *m*-CPBA, *meta*-chloroperoxybenzoic acid; ORTEP, oak ridge thermal ellipsoid plot; ROS, reactive oxygen species; GSH, glutathione; Pi, inorganic phosphate; TLC, thin layer chromatography; HRMS, high resolution mass spectrometry.

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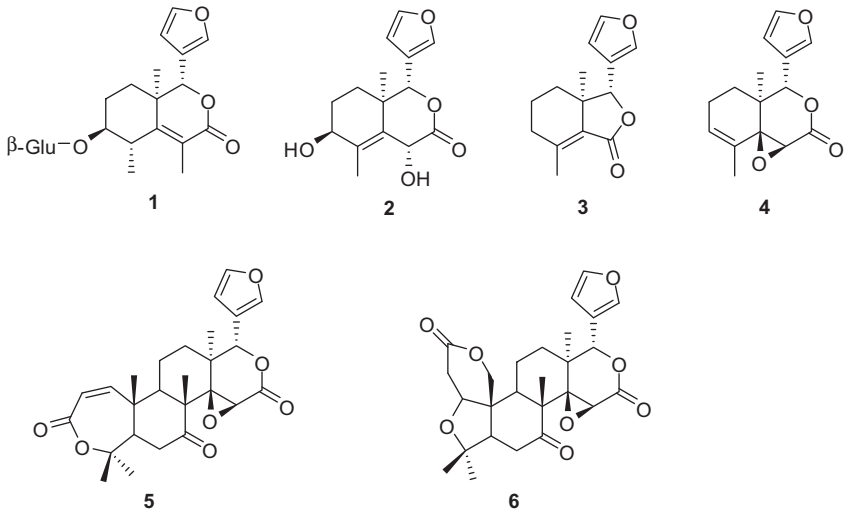


Figure 1. Neuroprotective limonoids.

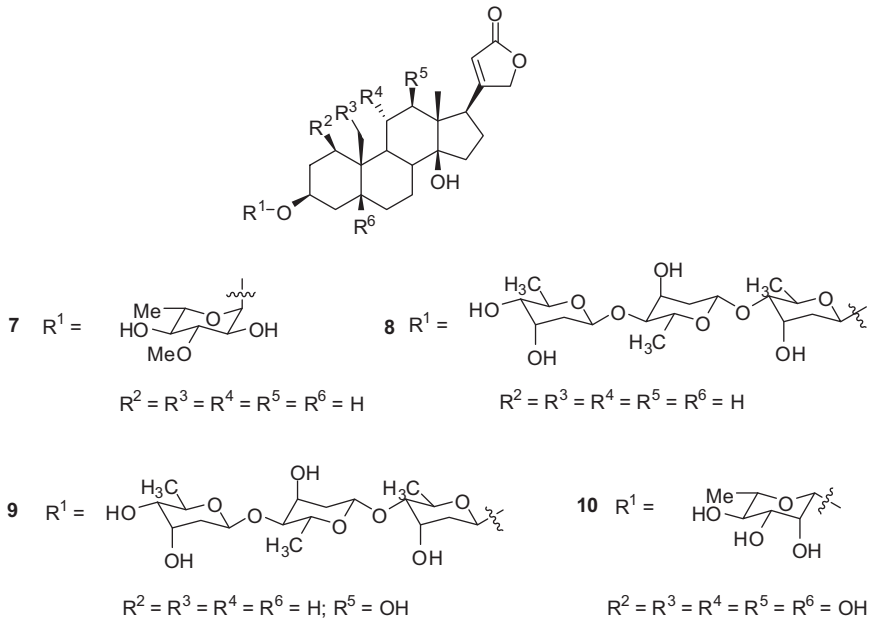


Figure 2. Neuroprotective cardiac glycosides.

and to a lesser extent the glycosides digitoxin **8**, digoxin **9** and ouabain **10** (Fig. 2), provided protection against neuronal cell death induced by oxygen and glucose deprivation in a brain slice assay, an effect believed to be mediated through their putative target the Na^+/K^+-ATPase.[6,18] Sublethal concentrations of ouabain has been shown to have neuroprotective activity against excitotoxicity mediated neuronal injury and this effect has been attributed to intracellular cascades linked to ouabain interaction with Na^+/K^+-ATPase leading to modulation of subcellular Bcl-2 levels.[19] However, cardiac glycosides are substrates for P-glycoprotein, which precludes their usefulness as therapeutics for ischemic stroke, as effective penetration of the blood brain barrier to build up therapeutic levels of the drugs within the CNS is inhibited.[6] Nevertheless, they may serve as lead molecules for designing more effective drug candidates.

A comparison of the structures of these naturally occurring neuroprotective molecules revealed that they share a common bicyclic ring system with a five-membered heterocycle (furanyl or γ-lactone) side chain and with or without an oxygen function at the ring junction (Fig. 3). Intrigued by the presence of this common structural motif in neuroprotective natural products, we designed and synthesized several structural congeners of this unit to explore their neuroprotective properties. The molecules designed for synthesis are shown in Figure 4.

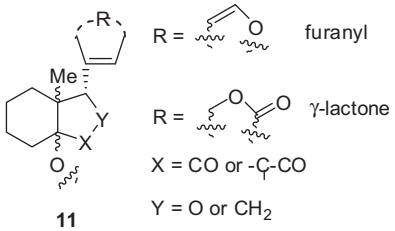


Figure 3. Structural motif shared by neuroprotective natural products.

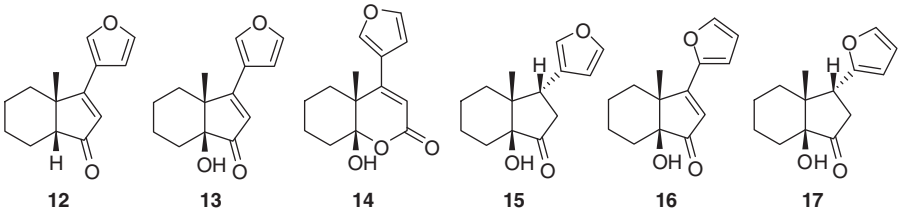
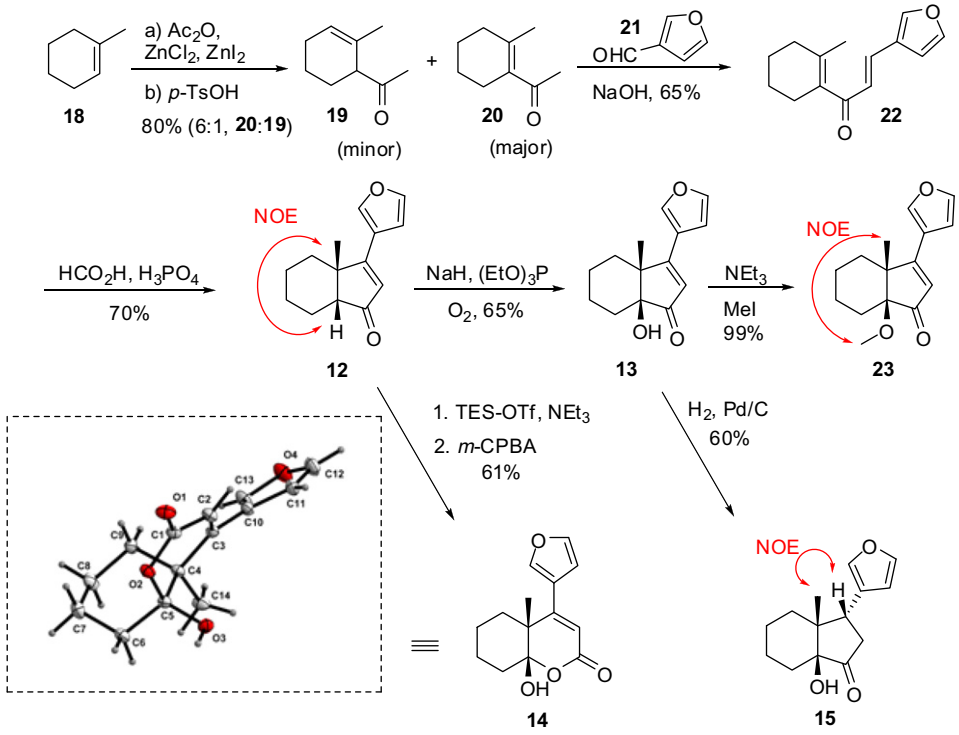


Figure 4. Target molecules designed for synthesis.

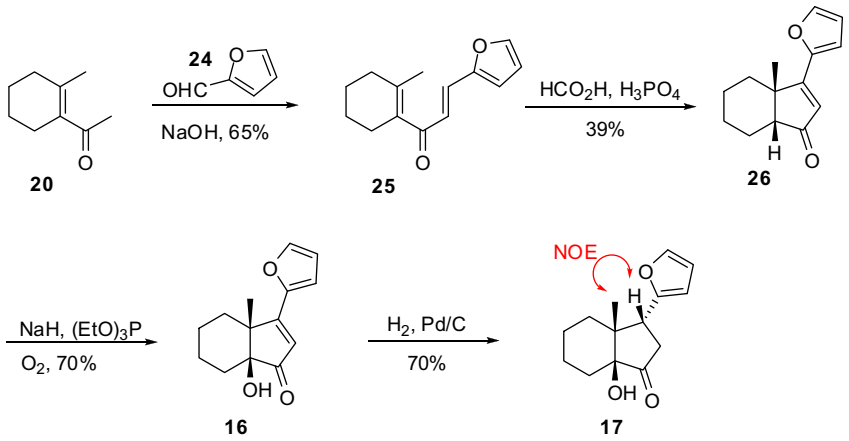


Scheme 1. Synthesis of target compounds **12**–**15**. Inset: ORTEP drawing derived from the single-crystal X-ray analysis of racemic **14**. The crystal structure of compound **14** has been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 871250.

The synthesis of target molecules **12**, **13**, **14** and **15** is shown in Scheme 1. Lewis acid-catalyzed acetylation of 1-methylcyclohexene **18** gave a nearly 1:1 mixture of the two ketones **19** and **20**. Acid-catalyzed isomerization using *p*-toluenesulfonic acid in benzene followed by bulb-to-bulb distillation of the product gave a mixture of the two ketones in which the α,β-unsaturated ketone **20** was the major isomer (**19**:**20**: ~1:6 by ^{1}H NMR).[20] No attempt was made to separate the mixture and it was subjected to aldol condensation with 3-furfuraldehyde **21**. Chromatography separation gave the divinylketone **22**. It was converted to **12**, consisting of the bicyclic motif of our interest, by acid-catalyzed Nazarov cyclization[21] using a mixture of formic acid and phosphoric acid. Rubottom oxidation[22] of **12** with triethylsilyl triflate and triethylamine followed by *m*-CPBA to obtain α-hydroxylation product **13** resulted in mainly the Baeyer–Villiger oxidation product lactone **14** with low yields of **13**. However, α-peroxidation via the enolate, followed by reduction with triethyl phosphate gave quantitative yields of the *cis*-fused alcohol **13**.[23] The *cis* configuration of **13** was confirmed by conversion to the methyl ether **23** in which an NOE was observed between the angular methyl group and the methyl ether group. The *cis*-configuration of **14** was confirmed by single crystal X-ray crystallography (Scheme 1). Catalytic hydrogenation of the α,β-unsaturated ketone **13** gave the ketone **15**, the configuration of the newly generated stereo center being confirmed by an NOE observed between its H atom and the angular methyl group.

A similar synthetic approach using 2-furfuraldehyde **24** instead of 3-furfuraldehyde **21** gave the target compounds **16** and **17** (Scheme 2). The configuration at the newly generated stereo center in **17** was confirmed by NOE experiment as before. The ^{1}H NMR, ^{13}C NMR and HRMS data of all the compounds synthesized were in full agreement with the proposed structures.[36]

Oxidative damage mediated by reactive oxygen species (ROS) has been implicated as a major cause of cellular injuries in a vast variety of clinical abnormalities including cancer, diabetes, aging, cardiovascular disease, and neurodegenerative disorders. Reactive oxygen intermediates are produced in all mammalian cells, partly as a result of normal cellular metabolism and partly due to activation of a variety of oxidant-producing enzymes in response to exogenous stimuli. Excessive accumulation of reactive oxidants and the intracellular level of reactive oxidants are toxic.[24] It is well established that treatment of PC12 cells with H_2O_2 results in nuclear damage, decrease in mitochondrial transmembrane potential, cytosolic accumulation of cytochrome-c, activation of caspase-3, increase in the formation of reactive oxygen species. (ROS) and depletion of glutathione (GSH).[25] Protection of PC12 cells against



Scheme 2. Synthesis of target compounds **16** and **17**.

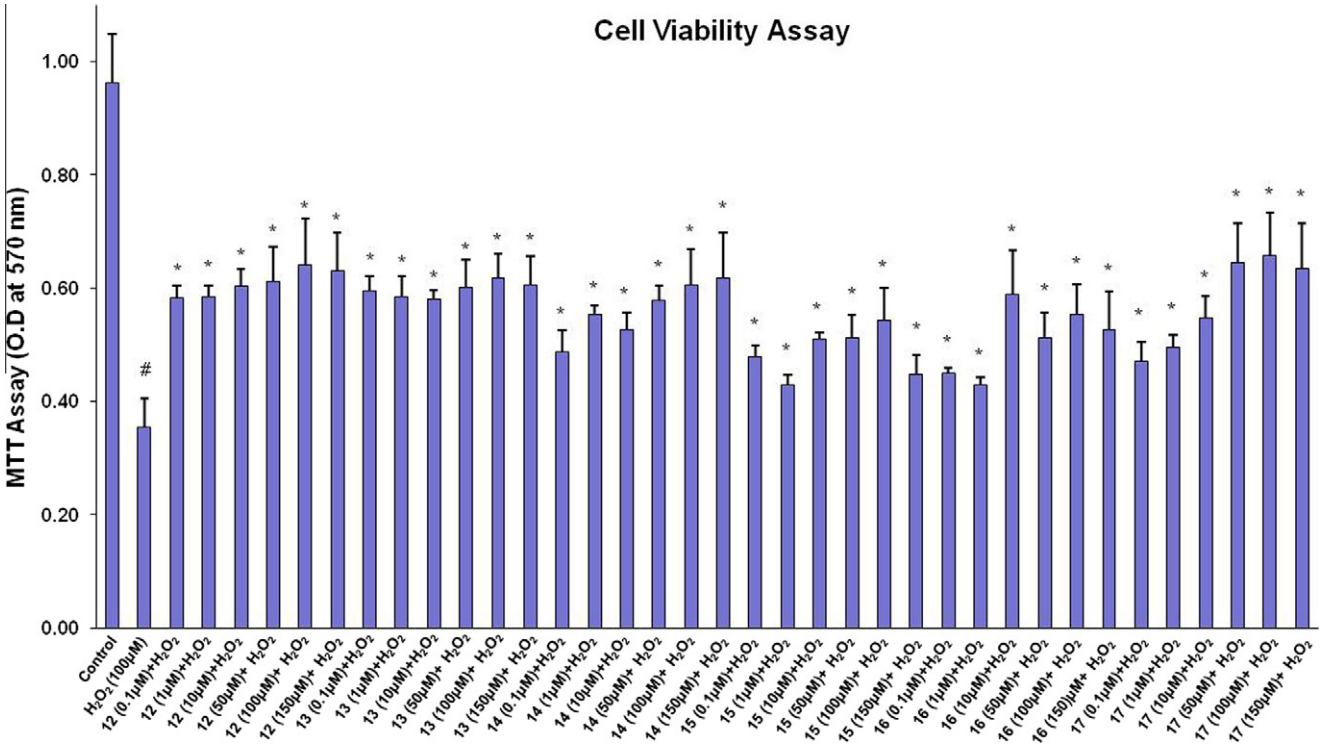


Figure 5. Neuroprotective activity of the test compounds. Experiments were repeated in triplicates with three separate batches of cultures. *Control*—naïve or no treatment; H_2O_2—cells treated with 100 μM H_2O_2 only; **12**–**17**—cells pretreated with different concentrations of test compounds (**12**, **13**, **14**, **15**, **16** and **17**) for 6 h followed by 18 h exposure to 100 μM H_2O_2. At the end of the treatment, cells were harvested for cell viability assay. Data are presented as means ± SEM. Student *t*-test was performed for statistical comparison between control and other treatments. A value of p <0.05 was considered to be statistically significant. #versus control and *versus H_2O_2.

H_2O_2 induced cell death can therefore be used to measure the neuroprotective activity of test agents. In order to test the effects of these compounds on the viability of PC12 cells against H_2O_2 induced cell death, cultured cells were pretreated with different concentrations (0.1, 1, 10, 50, 100 and 150 μM) of test compounds 6 h before adding 100 μM of H_2O_2 for additional 18 h of incubation. Treating cells with 100 μM of H_2O_2 alone induced profound cell death compared to control (Fig. 5). Control cells did not receive any treatment, therefore, 100% cell survival was observed. Cells pretreated with concentrations 0.1–150 μM of all compounds significantly (p <0.0001) enhanced cell viability and reversed the H_2O_2 induced oxidative stress. Compounds **15**, and **16** did not show a pattern of increased protection with increasing concentrations (0.1–150 μM) in terms of cell viability. Compounds **12** and **13** were observed to have the highest protection at the lowest concentrations of 0.1 and 10 μM and also showed a pattern of increasing protective potential with increasing concentration in terms of cell viability, which gradually increased and started plateauing at 150 μM (Fig. 5). Lowest concentrations of compounds **14** and **17** were not as potent as compound **12** and **13**, nevertheless showed a pattern of increasing protection with increasing concentration with **17** plateauing at 150 μM concentration. These results suggest that all these compounds, especially compounds **12** and **13** have neuroprotective properties and may have potential to be developed as neuroprotective agents. It is interesting to note that both compounds **12** and **13** possess furan-3-yl moieties attached to the bicyclic system, as are commonly found in naturally occurring limonoids. Corresponding analogue **16** with a furan-2-yl moiety is less active at lower concentrations. Although compounds **14** and **15** contain a furan-3-yl moiety, they lack an unsaturated cyclopentenone ring as a part of the bicyclic system. These results indicate that hexahydro-1*H*-ineden-1-one structural motifs with a

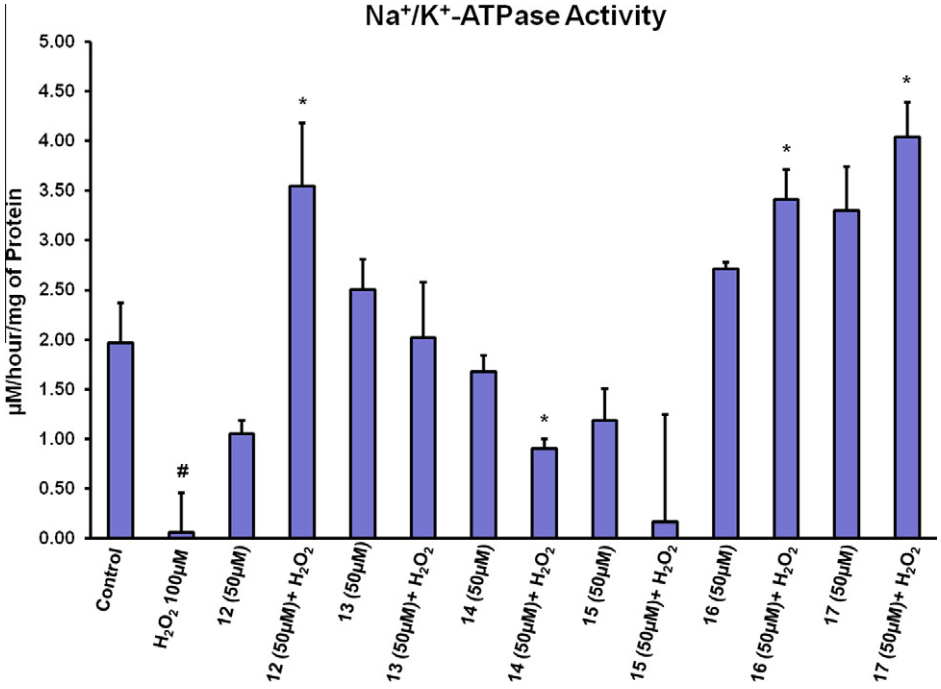


Figure 6. Effect of compounds on Na^+/K^+-ATPase activity: Experiments were repeated in triplicate. *Control*—naïve or no treatment; *H_2O_2*—cells treated with 100 μM H_2O_2 only; **12**–**17**, cells co-treated with 50 μM of test compounds (**12**, **13**, **14**, **15**, **16** and **17**) and 100 μM of H_2O_2 for 1 h. At the end of the treatment, cells were harvested for Na^+/K^+-ATPase assay. Data are presented as means ± SEM. Student *t*-test was performed for statistical comparison between control and other treatments. A value of p <0.05 was considered to be statistically significant. #versus control and *versus H_2O_2.

furan-3-yl moiety attached to C3 position and with or without a hydroxyl function at the ring juncture possess significant neuroprotective properties and are potential lead molecules to be developed as neuroprotective agents.

The neuroprotective properties of cardiac glycosides are believed to be mediated through interaction with the Na^+/K^+-ATPase pump.[6,18,19,26] Na^+/K^+-ATPase is an ATP-powered ion pump that establishes concentration gradients for Na^+ and K^+ ions across the plasma membrane in all animal cells by pumping Na^+ from the cytoplasm into the outside of cell and K^+ from the extracellular medium into the cell.[27,28] Blocking the Na^+/K^+-ATPase pump may lead to apoptosis. Neurodegeneration upon administration of ouabain has been attributed to inhibition of Na^+/K^+-ATPase pump.[19,29,30] However, ouabain has been shown to be able to initiate cell proliferation or cell death in a dose-dependent manner,[31] with non-toxic concentrations of it being neuroprotective.[19] Sublethal concentrations of ouabain have been shown to provide neuroprotection against excitotoxicity mediated neuronal injury through interaction with Na^+/K^+-ATPase leading to modulation of subcellular Bcl-2 levels.[19] Other studies have shown that restoring Na^+/K^+-ATPase activity protect cells against oxidative cell damage.[32,33] We therefore, tested the compounds for their effect on the activity on Na^+/K^+-ATPase pump by direct method using adherent PC12 cells as described in previous studies,[34,35] with some modifications. The assay was run in two parallel steps; one for the evaluation of total ATPase activity and the other for the evaluation of ouabain insensitive ATPase activity. ATPase activity was calculated by colorimetric quantification of inorganic phosphate (Pi) formed in 30 min, and Na^+/K^+-ATPase activity was determined by the difference between the results in the absence and presence of ouabain. In order to test the effects of these compounds on Na^+/K^+-ATPase pump, PC12 cells were treated with each compound at 50 μM in the presence and absence of 100 μM H_2O_2 for one hour prior to the Na^+/K^+-ATPase assay. Control cells did not receive any treatment and the Na^+/K^+-ATPase activity observed in these cells was considered to be endogenous or basal activity. The results are shown in Figure 6. A significant reduction in Na^+/K^+-ATPase activity was observed in the presence of 100 μM H_2O_2 in comparison to control (p <0.02). Treatment with compounds only (without H_2O_2) did not show any significant effects on the Na^+/K^+-ATPase activity as compared to control. However, when the cells were challenged with 100 μM H_2O_2 in the presence of test compounds, Na^+/K^+-ATPase activity was observed to be significantly restored with compound **12**, (p <0.014), compound **14** (p <0.006), compound **16** (p <0.003) and compound **17** (p <0.002). No direct correlation could be drawn between structural variations and the variations in Na^+/K^+-ATPase activity. However, the results may suggest that the Na^+/K^+-ATPase enhancing and modulating effects of these compounds may be responsible, at least in part, for their observed neuroprotective activity. Since, Na^+/K^+-ATPase has a significant role in cerebral ischemia pathophysiology, developing such compounds with Na^+/K^+-ATPase enhancing activity would be an ideal proposition for drug discovery in the treatment of neurologic disorders such as stroke and cerebral ischemia. Further studies are continuing to elucidate the mechanism(s) of action and the putative target of these molecules.

In summary, we report the design and synthesis of several natural product mimics that incorporate a common structural unit shared by several classes of natural products with neuroprotective activity. Neuronal viability was restored with different concentrations of these compounds against H_2O_2 induced oxidative stress. Compounds with hexahydro-1*H*-ineden-1-one structural motifs with a furan-3-yl moiety attached to C3 position and with or without a hydroxyl function at the ring juncture in particular possessed significant neuroprotective properties and may be useful as lead molecules for developing neuroprotective agents.

Oxidative stress is an important factor in the initiation and progression of many neurodegenerative diseases. The results suggest that enhancing and modulating effects on Na^+/K^+-ATPase activity may be responsible, at least in part, for the neuroprotective

properties of these compounds. Na^+/K^+-ATPase has a significant role in cerebral ischemia pathophysiology. Therefore, compounds with Na^+/K^+-ATPase enhancing activity will be useful as agents in the treatment of neurologic disorders such as stroke and cerebral ischemia. Further studies are in progress to validate their efficacy as potential lead molecules for developing therapeutic agents for neurodegenerative diseases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.013.

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36. *Compound characterization data:*
Compound ***12***: Yellow oil, mp 70–73 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 1.19–1.25 (m, 1H), 1.4–1.57 (m, 2H), 1.65–1.68 (m, 1H), 1.43 (s, 3H), 1.91–1.94 (m, 1H), 2.09–2.14 (m, 1H), 2.21–2.24 (m, 1H), 6.18 (s, 1H), 6.60 (m, 1H), 7.48 (m, 1H), 7.80 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): 20.17, 21.38, 21.63, 24.97, 36.76, 45.73, 55.18, 110.03, 120.17, 125.82, 142.67, 143.89, 173.96, 208.96 ppm. HRMS: (ESI) calcd for $C_{14}H_{16}O_2$ $[M+Na]^+$ 239.1048; found 239.1056.
Compound ***13***: White crystals, mp 74–80 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 1.25–1.31(m, 1H),1.32 (s, 1H), 1.44–1.49 (m, 1H), 1.65–1.77 (m, 5H), 1.96–2.02 (m, 1H), 2.59 (s, 1H), 6.19 (s, 1H), 6.62 (d, J = 1.2 Hz, 1H), 7.51 (m, 1H), 7.85 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.56, 20.83, 26.18, 32.83, 33.93, 50.20, 80.98, 109.95, 120.30, 123.25, 142.85, 144.10, 172.75, 210.19 ppm. HRMS: (ESI) calcd for $C_{14}H_{16}O_2$ $[M+Na]^+$ 255.0998; found 255.0992.
Compound ***14***: White crystals, mp 110–112 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 1.35 (s, 3H), 1.48–1.49 (m, 2H), 1.71–1.72 (m, 3H), 1.88–2.02 (m, 3H), 3.40 (br, 1H), 6.05 (s, 1H), 6.51–6.52 (m, 1H), 7.46 (s, 1H), 7.61 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): 20.02, 20.59, 22.72, 34.98, 36.89, 42.91, 104.80, 110.44, 115.93, 122.33, 141.46, 143.68, 157.49, 164.28 ppm. HRMS: (ESI) calcd for $C_{14}H_{16}O_2$ $[M+Na]^+$ 271.0946; found 271.0936.
Compound ***15***: White solid, mp 70–73 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 0.96–1.04 (m, 1H), 1.13 (s, 3H), 1.22–1.25 (m, 2H), 1.42–1.47 (m, 1H), 1.59–1.67 (m, 2H), 1.98–2.04 (m, 1H), 2.38–2.46 (m, 1H), 2.74–2.81 (m, 1H), 3.46–3.51 (t, J = 9.6 Hz, 1H), 6.29 (s, 1H), 7.26 (s, 1H), 7.39 (m, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): 16.51, 20.81, 23.74, 28.73, 31.39, 38.55, 38.88, 45.01, 80.37, 111.12, 122.63, 140.24, 143.03, 215.70 ppm. HRMS: (ESI) calcd for $C_{14}H_{16}O_2$ $[M+Na]^+$ 257.1154; found 257.1154.
Compound ***16***: White crystals, mp 76–81 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 1.24–1.35 (m,1H),1.36 (s, 3H) 1.42–1.49 (m, 1H), 1.59–1.83 (m, 5H), 2.07–1.13 (m,1H), 2.55 (s,1H), 6.36 (s,1H), 6.56 (m, 1H), 6.90 (d, J = 3.66 Hz, 1H), 7.59 (d, J = 1.83 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.28, 20.51, 25.73, 32.80, 33.73, 49.22, 80.73, 112.81, 115.51, 121.06, 145.57, 149.33, 167.45, 209.85 ppm. HRMS: (ESI) calcd for $C_{14}H_{16}O_2$ $[M+Na]^+$ 255.0998; found 255.0997.
Compound ***17***: White solid, mp 68–72 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 1.01–1.09 (m, 2H), 1.23 (s, 3H), 1.44–1.45 (m, 2H), 1.57–1.67 (m, 3H), 1.99–2.03 (m, 1H), 2.66–2.83 (m, 2H), 3.61–3.66 (t, J = 8.0 MHz, 1H), 6.11 (d, J = 1.2 Hz, 1H), 6.33–6.34 (m, 1H), 7.37 (s,1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.68, 20.79, 23.67, 28.61, 31.50, 37.05, 41.59, 45.83, 80.35, 107.24, 110.30, 141.80, 153.96, 215.14 ppm. HRMS: (ESI) calcd for $C_{14}H_{16}O_2$ $[M+Na]^+$ 257.1154; found 257.1147.