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Neuroprotective Effects of *N*-Alkyl-1,2,4-oxadiazolidine-3,5-diones and Their Corresponding Synthetic Intermediates *N*-Alkylhydroxylamines and *N*-1-Alkyl-3-carbonyl-1-hydroxyureas against in vitro Cerebral Ischemia

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Herein we report the synthesis and neuroprotective effects of new *N*-alkyl-1,2,4-oxadiazolidine-3,5-diones and their corresponding synthetic intermediates, *N*-alkylhydroxylamines and *N*-1-alkyl-3-carbonyl-1-hydroxyureas, in an in vitro model of ischemia. We found five analogues that protect HT22 cells from death in the concentration range of 1–5 μM. Because members of the MAP kinase family are known to be key players in nerve cell survival and death, we characterized the role of these kinases in the neuroprotective mechanisms of the newly synthesized analogues. The results indicate that these compounds provide neuroprotection through distinct mechanisms of action.

Introduction

Epidemiological, clinical, and biochemical studies have shown that various types of dietary fatty acids can modify the risk of stroke.[1,2] This has led to an increased focus on the potential neuroprotective activities of free fatty acids in pharmacological research. Besides being key biomolecules in metabolic processes, free fatty acids serve as substrates for cell membrane biogenesis (glyco- and phospholipids) and as precursors of intracellular signaling molecules such as prostaglandins, leukotrienes, thromboxanes, and platelet-activating factor. Polyunsaturated fatty acids have been implicated in the prevention of various human diseases including obesity, diabetes, coronary artery disease, stroke, and inflammatory and neurological disorders.[3] Saturated fats are usually regarded as unhealthy, but nutritionists believe that the type of saturated fat is also important. Stearic acid is biochemically classified as a saturated fatty acid, both for the purposes of food labeling and dietary recommendations. Stearic acid, one of the most common fatty acids in brain phospholipids, originates in the circulation and is sequestered from the blood by the brain along with precursor fatty acids.[4] The neuroprotective effects of stearic acid against the toxicity of oxygen or glucose deprivation, or of glutamate toward rat cortical or hippocampal slices,[5] have been reported. The behavior of stearic acid is especially unique with respect to its effects on serum cholesterol levels. A beneficial effect of stearic acid toward clotting factors can result in a diminished thrombogenic state. In the present study, we first assayed the neuroprotective properties of various major fatty acids in our in vitro ischemia assay using the mouse hippocampal cell line, HT22. Several fatty acids were assayed including linoleic, abietic, docosahexaenoic, and stearic acids. Using the bioisosterism approach, we then synthesized new fatty acid bioisostere derivatives bearing different *N*-alkyl chains and evaluated the neuroprotective properties of this new series of stearic acid analogues and their intermediates in the in vitro ischemia assay, as well as a different assay of oxidative-stress-induced nerve cell death using the same cell line. We also looked at potential neuroprotective signaling pathways.

The bioisosterism approach

A lead compound with a desired pharmacological activity may have associated with it undesirable side effects, characteristics that limit its bioavailability, or structural features that adversely influence its metabolism and excretion from the body. Bioisosterism represents an approach used by medicinal chemists for the rational modification of lead compounds into safer and more clinically effective agents.

Carboxylate bioisosteres have been extensively studied.[6] Of these, those most frequently used as biomimetics are phosphonic, sulfonic, and tetrazole groups. For example, at physiological pH, the tetrazole group has a similar degree of ionization (pK_a 4.9) as carboxylic acid but is ~10-fold more lipophilic (Figure 1). Another carboxylate bioisostere that is less studied is the 1,2,4-oxadiazolidine-3,5-dione bioisostere group (Figure 1). This carboxylic bioisostere group (X = O) is found in quisqualic acid, a natural compound isolated from the seeds of various varieties of *Quisqualis chinesis*,[7] *Q. indica*,[8] and *Q. fructus*.[9]

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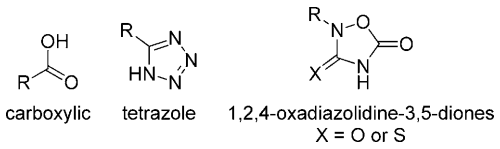


Figure 1. Three non-classical carboxylic acid bioisosteres.

It turns out that the biological properties of quisqualamine are very similar to those of the neurotransmitter γ-aminobutyric acid (GABA; Figure 2).[10] Quisqualic acid is an agonist for

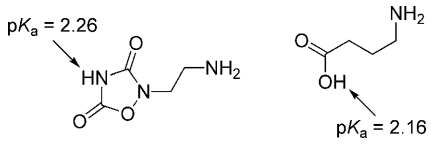
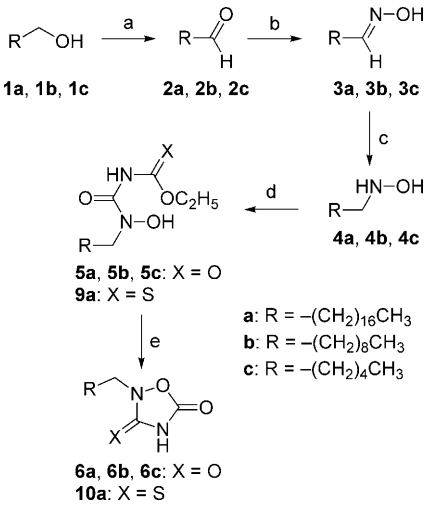


Figure 2. Quisqualamine (left) and GABA (right) bioisosteres.



Scheme 1. a) PCC, CH_2Cl_2, RT, 2 h; b) NH_2OH, HCl, H_2O, NaOH, RT; c) $NaBH_3CN$, MeOH, RT; d) $OCNCO_2C_2H_5$ or $SCNCO_2C_2H_5$, CH_2Cl_2, RT; e) NaOH, dioxane, RT.

both metabotropic glutamate receptors coupled to phosphoinosotide hydrolysis[11] and the non-NMDA ionotropic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor.[12] Compounds containing 1,2,4-oxadiazolidine-3,5-dione moieties were also found to be active as anti-hyperglycemic agents.[13]

Starting from the hypothesis that the 1,2,4-oxadiazolidine-3,5-dione group, through its acidic properties, is very similar to that of carboxylic acid and therefore could have similar neuroprotective effects to those of stearic acid, the synthesis and biological activity of new *N*-alkyl-1,2,4-oxadiazolidine-3,5-dione derivatives and their synthetic intermediates were investigated. Three alkyl chain lengths were selected: hexyl, decyl, and stearyl.

Results

Chemistry

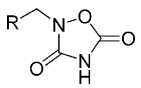
The new 1,2,4-oxadiazolidine-3,5-dione carboxylic bioisosteres **6a**, **6b**, and **6c** were synthesized as depicted in Scheme 1. Starting from commercially available aldehydes **2b** and **2c** or prepared from stearyl alcohol **1a** in the case of stearyl aldehyde **2a**, the corresponding aldoximes **3a**, **3b**, and **3c** were obtained in good yields. The reductive step leading to the corresponding hydroxylamines **4a**, **4b**, and **4c** was achieved using sodium cyanoborohydride in methanol as a reductive reagent. The compounds were purified by column chromatography and immediately used for the next step, as these hydroxylamine intermediates are rather unstable. The hydroxylamines reacted quite easily with ethyl isocyanatoformate to give the corresponding 3-ethoxycarbonyl-1-hydroxy-1-alkylureas **5a**, **5b**, and **5c** in acceptable yields. The stearylhydroxylamine intermediate **4a** was also treated with ethyl isothiocyanatoformate to give the corresponding 3-ethoxycarbonyl-1-hydroxy-1-stearylthiourea **9a**. The *N*-alkyl-1,2,4-oxadiazolidine-3,5-diones **6a**, **6b**, **6c**, and the corresponding thiooxo derivative **10a** were obtained directly by stirring the urea or thiourea intermediates in 3.5 N NaOH for a few hours at room temperature.

Comparison of physicochemical properties of the *N*-alkyl-1,2,4-oxadiazolidine-3,5-dione moiety and carboxylic acid

We considered pK_a (ionization state) and Clog*P* (hydrophobicity) as the two most important parameters, as it is well known that these molecular properties can influence passive absorption of which solubility and permeability are the key factors. Table 1 summarizes the pK_a and Clog*P* values for the stearyl-, decyl-, and hexyl-3,5-dioxo-1,2,4-oxadiazolidine bioisosteres and their corresponding tetrazole and carboxylic acid analogues.

As can be seen, replacement of the carboxylic acid group by a 3,5-dioxo-1,2,4-oxadiazolidine or tetrazole moiety does not significantly change the acidic properties of the resulting bioisosteres, and only slight differences in hydrophobicity are ob-

Table 1. Comparison of the physicochemical properties of 3,5-dioxo-1,2,4-oxadiazolidines and those of the corresponding carboxylic acids.



Compound	R	Clog*P*[a]	pK_a[b]	Ref.
6a	$-(CH_2)_{16}CH_3$	7.98	9.87	
6b	$-(CH_2)_8CH_3$	4.27	ND	
6c	$-(CH_2)_4CH_3$	2.69	2.26	
stearic acid		8.27	10.5	[15]
decanoic acid		4.03	4.90	[16]
hexanoic acid		2.45	2.16	[14]

[a] Clog*P* values were calculated using ChemDraw software (CambridgeSoft, Cambridge, MA, USA). [b] Values were determined by UV/Vis spectroscopy (Sirius Analytical Instruments Ltd., "Applications and Theory Guide to pH-Metric pK_a and log*P* Determination", 1993.

served. For example, in the case of the stearyl-3,5-dioxo-1,2,4-oxadiazolidine, the Clog*P* and pK_a values for stearic acid and its bioisostere **6a** are quite similar: 8.27 and 7.98 for the Clog*P* values, respectively, and 10.5 and 9.87 for the respective pK_a values.

Biological results

Neuroprotective effects of fatty acids

Before testing the newly synthesized derivatives, we first assayed the neuroprotective properties of stearic acid versus other known unsaturated and cyclic fatty acids (linoleic, abietic, and docosahexaenoic) as reference fatty acids, using a cell-based assay that reproduces a number of the pathophysiological changes associated with ischemia. Indeed, the changes observed in nerve cells following treatment with iodoacetic acid (IAA) are very similar to the changes that have been observed in the brain in animal models of ischemia. This primary assay was recently described by Maher et al.[17] and uses the mouse HT22 hippocampal nerve cell line in combination with IAA to induce ischemia in vitro. IAA is a known irreversible glyceraldehyde-3-phosphate dehydrogenase inhibitor[18] that induces chemical ischemia.[17] Treatment of HT22 cells with IAA induced a dose-dependent increase in cell death 20 h post-treatment with $<5\%$ survival at 20 μM. Figure 3 shows the dose–response curves for four fatty acids: linoleic, abietic, docosahexaenoic, and stearic acids. As can be seen, the maximal neuroprotective effects of the acids never exceeded 50% cell survival and were observed at concentrations between 1 and 2 μM. In contrast to the other fatty acids, which all gave bell-shaped curves, abietic acid gave a more typical neuroprotection curve with a distinct plateau.

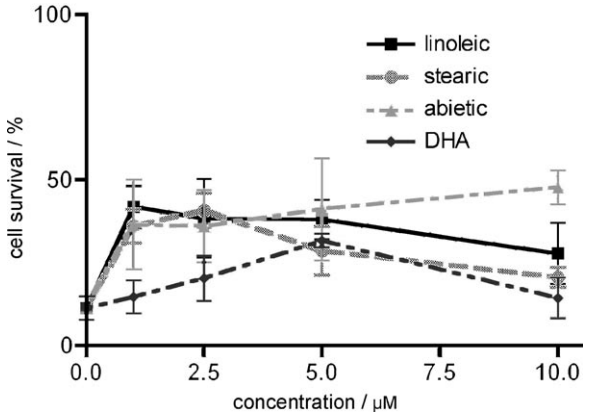


Figure 3. Dose–response curves for neuroprotection from IAA toxicity: representative HT22 cell protection curves for fatty acids after IAA injury. HT22 cells were treated with 20 μM IAA for 2 h alone or in the presence of increasing doses of linoleic, stearic, abietic, or docosahexaenoic acid. The same concentrations of fatty acids were also included in the fresh medium added after the 2 h treatment with IAA. Percent survival was measured after 24 h by MTT assay. Similar results were obtained in 3–5 independent experiments.

Neuroprotective effects of the new fatty acid bioisosteres and their synthetic intermediates

The new fatty acid bioisostere analogues and their synthetic intermediates were tested for their neuroprotective effects in two different models of nerve cell death. The IAA model was already described. The second model, used to test the neuroprotective effects of the new analogues, was oxidative glutamate toxicity. In this widely used model of oxidative-stress-induced death, treatment of HT22 cells with 5 mM glutamate induces cell death within 24 h via a well-characterized pathway involving glutathione depletion and the production of reactive oxygen species.[19] The flavonoid fisetin,[20] which was previously reported to be neuroprotective in this assay,[17] was used as reference compound for comparison with the new analogues. The results obtained with the HT22/IAA assay as well as the glutamate toxicity assay are listed in Table 2.

Having identified new compounds, primarily **4a** and **4b**, with neuroprotective effects, the question of their mechanism of action became an important issue. We first investigated the p38 MAPK and JNK pathways.[21] Using specific antibodies to the phosphorylated forms of JNK and p38 MAPK as well as their specific substrates, the effects of some of the most active compounds (**4a**, **4c**, **4b**, **5b**, and **6a**) on these pathways were examined. We also looked at the effects of these compounds on ERK activation, because this pathway is implicated in neuroprotection. The results are listed in Table 3.

Discussion

From these results it is clear that the majority of the compounds—bioisosteres 3,5-dioxo-1,2,4-oxadiazolidines **6a**, **6b**, and **6c**, the thiodiazoline **10a**, as well as the synthetic intermediates **5a**, **5b**, **5c**, **4a**, **4b**, **4c**, **3a**, **3b**, and **3c**—show neuroprotective effects at concentrations ranging from 1.0 to 20 μM. The most active in our screening model are **4b** and **4a**, with respective EC_{50} values of 1.0 and 2 μM. Both **4a** and **4b** contain a hydroxylamine function linked to a linear stearyl or decyl alkyl chain. Surprisingly, replacement of the carboxylic acid function by a hydroxylamine group appears to greatly enhance the observed neuroprotective effect, as these analogues have EC_{50} values better than that of stearic acid ($EC_{50}=6.3$ μM). We emphasize this point because, to our knowledge, the neuroprotective effects of *N*-alkylhydroxylamine compounds have never been reported. Because *N*-hydroxylamine compounds are known to be relatively unstable and sensitive to oxidation, their potential use as chemotherapeutic agents could be limited. However, it is possible to stabilize such compounds through the formation of their corresponding acid salts.[22]

The data also indicate that the octadecyl (stearyl) alkyl chain is not required for the neuroprotective effect. Interestingly, compounds **4a** and **4b** have a potency similar to that of the flavonoid fisetin, which was previously reported to be neuroprotective in the IAA toxicity assay;[17] however, whereas fisetin provided nearly 100% protection, the other analogues (with the exception of compound **4b**, which provided 83% protection) only protected the cells between 50 and 76%.

Table 2. Protection of HT22 cells from IAA and glutamate toxicity by fatty acid bioisosteres.

Compound	EC_{50} [μM] HT22/IAA[a]	Max. Protection [%][b]	Toxicity [%][c]	HT22/Glutamate [%][d]	JNK/p38 MAPK Activation[e]	ERK Activation[f]
6a	6.0	60	0	9.9	No effect	No effect
10a (thio)	18.6	37	6	7.7	ND	ND
6b	6.0	40	4	8.7	ND	ND
6c	8.1	50	7	9.5	ND	ND
5a	8.9	50	11	12.8	No effect	No effect
5b	6.0	62	6	9.6	No effect	Increases
5c	ND	ND	ND	ND	ND	ND
4a	2	62	50	13.5	No effect	Increases
4b	1.0	83	3	62.0	No effect	Increases
4c	2.7	76	6	9.2	No effect	Increases
3a	8.9	49	61	ND	ND	ND
3b	5.8	62	12	9.4	No effect	No effect
3c	ND	ND	ND	ND	ND	ND
Stearic acid	6.3	41	16	ND	ND	ND
Fisetin	2.8	95	15	95	Decreases	Increases

[a] Half-maximal effective concentrations for protection from IAA toxicity were determined by exposing HT22 cells to various concentrations of each analogue in the presence of 20 μM IAA for 2 h; cell viability was determined after 24 h by the MTT assay. [b] The maximal percent survival at the most effective concentration (which varied from 1 to 10 μM depending on the analogue). [c] HT22 cells were exposed to each analogue at 10 μM, and cell viability was determined after 24 h by MTT assay. [d] HT22 cells were exposed to various concentrations of each analogue in the presence of 5 mM glutamate for 24 h; cell viability was determined after 24 h by MTT assay, and the maximal percent survival at the most effective dose (which varied from 1 to 10 μM depending on the analogue) was determined; the average percent survival for cells treated with glutamate alone was 15 ± 5%. [e] The effect of the analogues on JNK and p38 MAPK activation by IAA treatment was determined by SDS-PAGE and western blot with phospho-specific antibodies as described.[23] [f] The effect of the analogues on ERK activation in the presence of IAA was determined by SDS-PAGE and western blot with phospho-specific antibodies as described.[23]

Table 3. Effect of analogues **4a**, **4c**, **4b**, **5b**, and **6a** on JNK, p38 MAP kinase, and ERK activation.

Compound	JNK/p38 MAPK Activation[a]	ERK Activation[b]
4a	No effect	Increases
4b	No effect	Increases
4c	No effect	Increases
5b	No effect	Increases
6a	No effect	No effect
Fisetin	Decreases	Increases

[a] Effects of new analogues on JNK and p38 MAP kinase activation: HT22 cells were untreated or treated with 10 μM **4a**, **4b**, **4c**, **5b**, or **6a** in the absence or presence of 20 μM IAA for 2 h. Following recovery for 2 h, cell lysates were prepared, and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting with antibodies to phospho- and total JNK and p38 MAP kinase. [b] Effects of new analogues on ERK phosphorylation: HT22 cells were untreated or treated with 10 μM **4a**, **4b**, **4c**, **5b**, or **6a** in the absence or presence of 20 μM IAA for 2 h for comparative ERK activation estimation. Following recovery for 2 h, cell lysates were prepared, and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting with antibodies to phospho- and total ERK.

Notably, the acidic properties of the compounds (pK_a values reported in Table 1) weakly influence the observed neuroprotective effect. The neuroprotective potency of compound **6a** (pK_a 9.87), a true bioisostere of stearic acid (pK_a 10.5), is slightly higher than that of stearic acid. Replacement of the oxygen atom in bioisostere **6a** by a sulfur atom in **10a** lowered the observed neuroprotective effect by a factor of three, indicating that the electronic parameters of the hydrophilic moiety (sulfur being more nucleophilic and bulkier than oxygen) greatly influence the neuroprotective effect. The results of the reaction mechanism studies reported in Table 3 suggest that analogues **4a**, **4b**, **4c**, **5b**, and **6a** exert their neuroprotective effects through distinct mechanisms, as **4a**, **4b**, **4c**, and **5b** induce ERK activation, while **6a** has no effect. Moreover, none of the tested analogues affect JNK or p38 MAP kinase activation; this indicates that all these analogues induce neuroprotective effects through pathways that are at least partially distinct from the reference compound fisetin and more similar to that of the GM1-sialoganglioside analogues we recently described.[23]

Together these data strongly suggest that the new analogues **4a**, **4c**, **4b**, **5b**, and **6a** do not act as antioxidants, but rather as inducers of antioxidant and/or neuroprotective protein synthesis. This conclusion is supported by their complete lack of activity in the Trolox equivalent antioxidant capacity (TEAC) assay, an in vitro assay for antioxidant activity[24] (data not shown). Although their mechanism of action has yet to be fully elucidated, our preliminary experiments suggest that the whole group of newly designed compounds does not provide neuroprotection through the same mechanism as the reference compound fisetin, because they have no effect on JNK or p38 MAP kinase activity. However, we did observe that, similar to fisetin analogues, **4a**, **4b**, **4c**, and **5b**, but not analogue **6a** activate ERK kinase. In summary, we have successfully synthesized a series of new *N*-alkyl-1,2,4- oxadiazolidine-3,5-diones bearing alkyl chains of varying lengths (hexyl, decyl, and stearyl), and, along with their synthetic intermediates, we evaluated their neuroprotective effects. Some of the new analogues exhibit potent neuroprotective effects toward nerve cells exposed to ischemia-like insults. Analogues **4a**, **4c**, **4b**, and **5b** are of particular interest, as they are very efficacious, with EC_{50} values ranging between 1 and 2 μM. Moreover, although the mechanism by which these new analogues exert their neuroprotective effect remains unclear, it appears to be distinct from that of the reference compound fisetin. Given these potent effects in cell-based assays, further exploration of the neuroprotective effects of analogues in animal models of ischemia is clearly warranted.

Experimental Section

Chemistry

General methods: Starting materials and reagents were obtained from commercial suppliers and were used without purification. THF was distilled over sodium benzophenone ketyl immediately prior to use; CH_2Cl_2 was distilled over P_2O_5 just prior to use. ^{1}H NMR spectra were recorded at 250 MHz on a Bruker AC-250 spectrometer. Elemental analyses were within 0.4% of theoretical values for all compounds. Chemical shifts (δ) are expressed ppm downfield from $(CH_3)_4Si$. ESIMS data were obtained on a Waters Micromass ZMD spectrometer by direct injection of the sample solubilized in CH_3CN. Analytical and preparative TLC was performed with silica gel plates 0.2 and 1 mm thick, respectively (60 F_{254}, Merck). Preparative flash column chromatography was carried out on silica gel (230–240 mesh, G60, Merck). Compound purity was determined by elemental analysis.

Adehydes 2b and **2c** are commercially available compounds, whereas stearaldehyde **2a** was prepared as follows: Pyridinium chlorochromate (PCC; 1 g, 5.4 mmol) in anhydrous CH_2C1_2 (10 mL) was suspended in a 500-mL round-bottomed flask fitted with a reflux condenser. 1-Stearol (1 g, 3.6 mmol) in CH_2Cl_2 (20 mL) was added in one portion to the magnetically stirred solution. After 2 h, dry Et_2O (15 mL) was added, and the supernatant was decanted. The insoluble residue was washed thoroughly with anhydrous Et_2O (3×10 mL), whereupon it became a black granular solid. The combined organic solution was dried, and the solvent was evaporated. A solid white compound was obtained (808 mg, 83%): R_f=0.8 (*c*Hex/EtOAc 9:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ=9.73 (s, 1H, C(O)*H*), 2.40 (m, 2H, -CH_2C(O)), 1.57 (s, 2H, -CH_2CH_2C(O)), 1.22 (m, 28H, -$(CH_2)_{14}$-), 0.96 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ=203.6, 44.0, 32.0, 29.8 (2), 29.5, 22.8, 22.2, 21.4, 14.3 ppm; ESIMS: *m/z* 269 $[M+H]^+$.

Oximes: Oximes **3b** and **3c** are known compounds prepared from the corresponding commercially available aldehydes according to published procedures.[25] Stearaldehyde oxime **3a** was prepared as follows: A solution of hydroxylamine hydrochloride (1 g, 14.5 mmol) in H_2O (10 mL) was added to a solution of stearaldehyde (0.8 g, 2.9 mmol) in EtOH (10 mL), followed by the addition of 10% NaOH until a precipitate was formed. The reaction mixture was heated (T=70 °C) for 2 h, cooled to room temperature, and the resulting white precipitate was filtered, washed with cold H_2O and dried. A white solid was obtained in quantitative yield: R_f= 0.87 (*c*Hex/EtOAc 3:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.43 (m, 1H, -*CH*=N(OH), *trans*), 6.93 (m, 1H, -*CH*=NOH, *cis*), 2.44 (m, 2H, -CH_2CH=N(OH)), 1.50 (m, 2H, CH_2CH_2CH=N(OH)), 1.29 (m, 28H, -$(CH_2)_{14}$), 0.87 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ= 152.5, 31.8, 30.2, 29.8 (12C), 25.2, 22.2, 21.4, 14.5 ppm; ESIMS: *m/z* 284 $[M+H]^+$, 100%; Anal. calcd for $C_{18}H_{37}NO$: C 76.32, H 13.14, N 4.94, O 5.64.

Hydroxylamines 4a, 4b, and 4c

General procedure A

***N*-octadecylhydroxylamine (4a)**: A solution of $NaBH_3CN$ (133 mg, 2.2 mmol) in MeOH (2 mL) was added with concurrent dropwise addition of aqueous 6 N HCl/MeOH 1:1 to a stirred solution of the oxime (500 mg, 1.7 mmol) in MeOH (10 mL) at −60 °C. The mixture was then allowed to warm to −20 °C over 2 h while maintaining pH 3. After evaporation the entire workup was carried out at 0 °C: addition of saturated aqueous NaCl and basification with 6 N KOH. Extraction with Et_2O, drying over $MgSO_4$, and evaporation of the organic phase gave the corresponding hydroxylamine, which was used either with or without purification: oily compound (62%); R_f=0.3 (*c*Hex/EtOAc 2:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ=2.87 (m, 2H, -CH_2CHN (OH)), 1.50 (m, 2H, -CH_2CH_2CHN (OH)), 1.24 (m, 28H, -$(CH_2)_{14}$-), 0.83 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ= 44.5, 31.8, 29.7, 29.5 (10C), 29.3, 27.2, 22.7, 14.1 ppm; ESIMS: *m/z* 286 $[M+H]^+$, 100%; Anal. calcd for $C_{18}H_{39}NO$: C 75.72, H 13.70, N 4.91, O 5.60.

***N*-decylhydroxylamine (4b)**: According to general procedure A, the reaction of (*E*)-decanaloxime (200 mg, 1.1 mmol) with $NaBH_3CN$ (293 mg, 4.6 mmol) afforded the *N*-decylhydroxylamine as a white solid (180 mg, 94%): R_f=0.16 (*c*Hex/EtOAc 3:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ=2.92 (m, 2H, -CH_2CHNOH), 1.52 (m, 2H, -CH_2CH_2CHNOH), 1.48 (m, 12H, -$(CH_2)_6$-), 0.87 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ=44.6, 31.8, 29.3 (2C), 29.2 (2C), 27.0, 26.2, 22.6, 14.1 ppm; ESIMS: *m/z* 174 $[M+H]^+$, 100%; Anal. calcd for $C_{10}H_{23}NO$: C 69.34, H 13.38, N 8.08, O 9.23.

***N*-hexylhydroxylamine (4c)**: According to general procedure A, the reaction of (*E*)-hexanaloxime (1.43 g, 12.1 mmol) with $NaBH_3CN$ (0.936 g, 14.8 mmol) afforded the *N*-hexylhydroxylamine as a white solid (828 mg, 55%): R_f=0.37 (*c*Hex/EtOAc 2:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ=2.87 (m, 2H, -CH_2CHN(OH)), 1.50 (m, 2H, -CH_2CH_2CHN(OH)), 1.24 (m, 6H, -$(CH_2)_3$-), 0.83 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ=44.5, 31.5, 26.5, 26.7, 22.7, 14.0 ppm; ESIMS: *m/z* 118 $[M+H]^+$, 100%; Anal. calcd for $C_{14}H_{28}N_2O_4$: C 61.49, H 12.90, N 11.95, O 13.65.

General procedure B

***N*-octadecylhydroxylamine ethyl formylcarbamate (5a)**: Hydroxylamine-*N*-octadecylhydroxylamine (0.24 g, 0.8 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL). Ethyl isocyanotoformate (0.098 g, 0.8 mmol) was added, and the reaction mixture was maintained at room temperature for 6 h. The solution was evaporated under reduced pressure, EtOAc was added (10 mL), and the residue was washed with H_2O (3×10 mL). The organic phase was dried over $MgSO_4$, and after filtration and evaporation under reduced pressure, silica gel chromatography (*c*Hex/EtOAc 2:1) of the residue gave a transparent oil (62 mg, 18%): R_f=0.5 (*c*Hex/EtOAc 2:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ=9.36 (s, 1H, N*H*), 8.09 (s, 1H, NO*H*), 4.25 (q, J=7.12 Hz, 2H, CH_3CH_2OC(O)), 3.68 (t, J=7.5 Hz, 2H, -CH_2N(OH)), 1.69 (m, 2H, -CH_2CH_2N(OH)), 1.24 (m, 31H, -$(CH_2)_{14}$- and CH_3CH_2OC(O)), 0.83 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ=153.5, 151.5, 58.8, 52.5, 31.5, 30.2, 29.8 (13C), 26.6, 23.7, 22.4, 14.5 ppm; ESIMS: *m/z* 401 $[M+H]^+$, 100%; Anal. calcd for $C_{22}H_{44}N_2O_4$: C 65.96, H 11.14, N 6.99, O 15.98.

Ethyl 2-(3-hydroxy-3-decylureido)acetate (5b): According to general procedure B, the reaction of *N*-decylhydroxylamine (100 mg, 0.5 mmol) with ethyl isocyanatoformate (66 mg, 0.5 mmol) afforded **5b** as a white solid in quantitative yield: R_f=0.8 (EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ=8.71 (s, 1H, N*H*), 8.52 (s, 1H, NO*H*), 4.18 (q, J=6.8 Hz, 2H, CH_3CH_2OC(O)), 3.49 (t, J=7.1 Hz, 2H, -CH_2NOH), 1.61 (m, 2H, -CH_2CH_2NOH), 1.25 (m, 17H, -$(CH_2)_7$- and CH_3CH_2OC(O)), 0.86 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ=153.5, 151.8, 61.6, 51.5, 48.5, 31.6, 29.3 (2), 29.1, 26.4, 22.4, 21.4, 13.9 ppm; ESIMS: *m/z* 289 $[M+H]^+$, 100%; Anal. calcd for $C_{14}H_{28}N_2O_4$: C 58.31, H 9.79, N 9.71, O 22.19.

Ethyl 2-(3-hexylureido)acetate (5c): According to general procedure B, the reaction of *N*-hexylhydroxylamine (0.8 g, 0.5 mmol) with ethyl isocyanatoacetate (0.87 g, 0.5 mmol) afforded **5c** as a white solid in quantitative yield: R_f=0.8 (EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.94 (s, 1H, N*H*), 6.47 (s, 1H, NO*H*), 4.14 (m,

2H, $CH_3CH_2OC(O)$), 3.90 (m, 2H, -OC(O)CH_2NH), 3.44 (m, 2H, -CH_2NOH), 1.56 (m, 2H, -CH_2CH_2NOH), 1.25 (m, 9H, -$(CH_2)_3$- and $CH_3CH_2OC(O)$), 0.83 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ = 169.5, 156.5, 61.0, 52.8, 44.5, 31.2, 26.3, 23.7, 22.7, 14.1 ppm; ESIMS: *m/z* 247 $[M+H]^+$, 100%; Anal. calcd for $C_{11}H_{22}N_2O_4$: C 53.31, H 9.09, N 11.31, O 25.99.

General procedure C

2-octadecyl-1,2,4-oxadiazolidine-3,5-dione (6a): NaOH (3.5 N, 4 mL) was added slowly under stirring to a solution of **5a** (0.062 g, 0.15 mmol) in dioxane (4 mL) at 0 °C. The mixture was stirred at 0 °C for 15 min, and then at room temperature for 7 h. The solvent was evaporated, the residue was taken up in H_2O, and the aqueous solution was acidified to pH 5 with 1 N HCl. The solution was extracted with EtOAc, the organic layers were dried over $MgSO_4$, and the solvent was evaporated to yield a white solid (34 mg, 62%): R_f = 0.5 (EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 3.68 (t, *J* = 7.5 Hz, 2H, -CH_2N(OH)), 1.70 (m, 2H, -CH_2CH_2N(OH)), 1.24 (m, 30H, -$(CH_2)_{15}$-), 0.83 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ = 157.3, 153.2, 48.7, 31.5, 29.5 (12C), 26.3, 23.5, 22.7, 14.2 ppm; ESIMS: *m/z* 355 $[M+H]^+$, 100%; Anal. calcd for $C_{20}H_{38}N_2O_3$: C 67.72, H 10.80, N 7.86, O 13.54.

2-decyl-1,2,4-oxadiazolidine-3,5-dione (6b): According to general procedure C, the reaction of **5b** (100 mg, 0.34 mmol) with NaOH (10 mL) afforded the 2-decyl-1,2,4-oxadiazolidine-3,5-dione as a white solid (48 mg, 58%): R_f = 0.6 (EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 9.58 (s, 1H, N*H*), 3.66 (t, *J* = 7.1 Hz, 2H, -CH_2NO), 1.70 (m, 2H, -CH_2CH_2NO), 1.25 (m, 14H, -$(CH_2)_7$- and $CH_3CH_2OC(O)$), 0.87 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ = 153.5, 151.8, 48.5, 31.6, 29.3 (2), 29.1, 26.4, 22.4, 21.4, 13.9 ppm; ESIMS: *m/z* 243 $[M+H]^+$, 100%; Anal. calcd for $C_{12}H_{22}N_2O_3$: C 59.48, H 9.15, N 11.56, O 19.81.

2-hexyl-1,2,4-oxadiazolidine-3,5-dione (6c): According to general procedure C, the reaction of **5c** (1.8 g, 0.3 mmol) with 3.5 M NaOH (10 mL) afforded the 2-hexyl-1,2,4-oxadiazolidine-3,5-dione as a transparent oil in quantitative yield: R_f = 0.6 (EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 9.77 (s, 1H, N*H*), 3.66 (t, *J* = 6.9 Hz 2H, -CH_2N), 1.69 (m, 2H, -CH_2CH_2N), 1.31 (m, 6H, -$(CH_2)_3$-), 0.88 (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ = 157.5, 153.9, 48.9, 31.7, 26.2, 23.6, 22.7, 14.2 ppm; ESIMS: *m/z* 187 $[M+H]^+$, 100%; Anal. calcd for $C_8H_{14}N_2O_3$: C 51.60, H 7.58, N 15.01, O 25.78.

Thio derivatives

***N*-octadecylhydroxylamine ethyl thioformylcarbamate (9a)**: According to general procedure B, the reaction of *N*-octadecylhydroxylamine (44 mg, 0.15 mmol) with ethoxycarbonyl isothiocyanate (30 mg, 0.23 mmol) afforded compound **9a** as a yellow oil in quantitative yield: R_f = 0.8 (EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 9.69 (s, 1H, N*H*), 7.98 (s, 1H, NO*H*), 4.20 (q, *J* = 7.12 Hz, 2H, $CH_3CH_2OC(O)$), 3.61 (t, *J* = 7.5 Hz, 2H, -CH_2N(OH)), 1.69 (m, 2H, -CH_2CH_2N(OH)), 1.24 (m, 31H, -$(CH_2)_{14}$- and $CH_3CH_2OC(O)$), 0.83 ppm (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ = 183.2, 153.4, 59.1, 57.5, 31.8, 30.2, 29.7 (12C), 27.6, 24.0, 22.7, 14.5, 13.8 ppm; ESIMS: *m/z* 417 $[M+H]^+$, 100%; Anal. calcd for $C_{22}H_{44}N_2O_3S$: C 63.42, H 10.14, N 6.72, O 11.52, S 7.70.

2-octadecyl-3-thioxo-1,2,4-oxadiazolidin-5-one (10a): According to general procedure C, the reaction of **9a** (0.67 g, 0.16 mmol) with NaOH (10 mL) afforded the 2-octadecyl-3-thioxo-1,2,4-oxadiazolidin-5-one as a white solid (28 mg, 47%): R_f = 0.6 (EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$): δ = 3.70 (t, *J* = 7.5 Hz, 2H, -CH_2N(OH)), 1.75 (m, 2H, -CH_2CH_2N(OH)), 1.29 (m, 30H, -$(CH_2)_{15}$-), 0.88 (m, 3H, -CH_3); ^{13}C NMR (250 MHz, $CDCl_3$): δ = 183.3, 159.2, 48.7, 31.5, 29.5 (12C), 26.3, 23.5, 22.7, 14.2 ppm; ESIMS: *m/z* 371 $[M+H]^+$, 100%; Anal. calcd for $C_{20}H_{38}N_2O_2S$: C 64.82, H 10.30, N 7.56, O 8.54, S 8.65.

Biology

Cell culture: Fetal calf serum (FCS) and dialyzed FCS (DFCS) were obtained from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA). HT22 cells were grown in DMEM supplemented with 10% FCS and antibiotics.

Cytotoxicity assays: Cell viability was determined with a modified version of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the standard procedure. Cells were seeded onto 96-well microtiter plates at a density of 5×10^3 cells per well. For the in vitro ischemia assay, the next day, the medium was replaced with DMEM supplemented with 7.5% DFCS, and the cells were treated with iodoacetic acid (IAA; 20 μM) alone or in the presence of test compounds. After 2 h, the medium in each well was aspirated and replaced with fresh medium without IAA, but containing the compounds to be screened. After 20 h, the medium in each well was aspirated and replaced with fresh medium containing 2.5 μg mL^{-1} MTT. After incubation for 4 h at 37 °C, the cells were solubilized with a solution containing 50% *N*,*N*-dimethylformamide (DMF) and 20% SDS (100 μL, pH 4.7). For the oxidative glutamate toxicity assay, the next day, the medium was replaced with DMEM supplemented with 7.5% DFCS, and the cells were treated with the test compounds alone or in the presence of 5 mM glutamate. After 24 h, the medium in each well was aspirated and replaced with fresh medium containing 2.5 μg mL^{-1} MTT. After incubation for 4 h at 37 C°, the cells were solubilized with a solution containing 50% DMF and 20% SDS (100 μL, pH 4.7). For both assays, the absorbance at λ 570 nm was measured on the following day with a microplate reader (Molecular Devices). Results were confirmed by visual inspection of the wells. Controls included compound alone to test for toxicity and compound with no cells to test for interference with the assay chemistry.

SDS-PAGE and immunoblotting: HT22 cells from the same density cultures as used for the cell death assays were untreated or treated with the analogues alone or in the presence of 20 μM IAA for 2 h followed by 2 h of recovery in the presence of the analogues. The cells were washed twice in phosphate-buffered saline (PBS) and solubilized in SDS sample buffer containing 0.1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride (PMSF), boiled for 5 min, and either analyzed immediately or stored frozen at −70 °C. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Uniform loading and transfer of the samples were confirmed by staining the nitrocellulose with Ponceau-S. Transfers were blocked for 1 h at room temperature with 5% nonfat milk in TBS/0.1% Tween-20 and then incubated overnight at 4 °C in the primary antibody diluted in 5% bovine serum albumin (BSA) in TBS/0.05% Tween-20. The primary antibodies used were phospho-p44/42 MAP kinase antibody (no. 9101, 1:1000), phospho-p38 MAP kinase (no. 9211, 1:1000), phospho-SAPK/JNK (no. 9255, 1:1000), phospho *c*-Jun (no. 9261, 1:1000), phospho-MAPKAPK-2 antibody (no. 3041, 1:1000), and total MAPKAPK-2 (no. 3042, 1:1000) from Cell Signaling (Beverly, MA, USA); total p38 antibody (no. sc-728, 1:500) and *c*-Jun/AP-1 antibody (no. sc-44-G) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); JNK1 antibody (no. 15701A, 1:500) from Pharmingen; and pan-ERK antibody (no. E17120, 1:10000) from Transduction Laboratories (San Diego, CA, USA). The

transfers were rinsed with TBS/0.05% Tween-20 and incubated for 1 h at room temperature in horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse (Biorad, Hercules, CA, USA) diluted 1:5000 in 5% nonfat milk in TBS/0.1% Tween-20. The immunoblots were developed with the Super Signal reagent (Pierce, Rockford, IL, USA).

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