Orally Bioavailable Metal Chelators and Radical Scavengers: Multifunctional Antioxidants for the Coadjutant Treatment of Neurodegenerative Diseases

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Supporting Information

ABSTRACT: Neurodegenerative diseases are associated with oxidative stress that is induced by the presence of reactive oxygen species and the abnormal cellular accumulation of transition metals. Here, a new series of orally bioavailable multifunctional antioxidants (MFAO-2s) possessing a 2-diacectylamino-5-hydroxypyrimidine moiety is described. These MFAO-2s demonstrate both free radical and metal attenuating properties that are similar to the original published MFAO-1s that are based on 1-N,N′-dimethylsulfamoyl-1-4-(2-pyrimidyl)piperazine. Oral bioavailability studies in C57BL/6 mice demonstrate that the MFAO-2s accumulate in the brain at significantly higher levels than the MFAO-1s while achieving similar neural retina levels. The MFAO-2s protect human neuroblastoma and retinal pigmented epithelial cells against hydroxyl radicals in a dose-dependent manner by maintaining cell viability and intracellular glutathione levels. The MFAO-2s outperform clioquinol, a metal attenuator that has been investigated for the treatment of Alzheimer’s disease.

■ INTRODUCTION

Oxidative stress results from an imbalance between oxidants, such as reactive oxygen species (ROS), and antioxidants, such as superoxide dismutase (SOD). An imbalance favoring oxidants can lead to protein, lipid-oxidation, and DNA tissue damage that is associated with age-related cataract, age-related macular degeneration (AMD), and Alzheimer’s disease (AD).1-4 In addition to oxidative stress, an age-related accumulation of redox-active metals, such as iron and copper, also occurs in the lens, retina, and brain of patients with age related cataracts, AMD, and AD.5-8 When combined with ROS, the increased presence of redox-active metal ions can promote further tissue damage by generating hydroxyl radicals through the Fenton reaction.1 In AD, ROS is generated by mitochondrial dysfunction, the presence of β-amyloid peptide (Aβ) and redox metal ions such as Fe2+ and Cu2+, and activated glial cells.15 Increased Mn levels can also contribute to mitochondrial dysfunction as well as impaired energy metabolism, excitotoxic neuronal death, apoptotic death, and oxidation of cellular components.10-13 Aβ also binds Zn to form neurotoxic plaque that results in localized oxidative stress and neuronal death.14 Aβ plaque is also present in the retinas of patients with AMD5,16 and in the lenses of AD patients with cataracts.17 The increased presence of Aβ plaque in these age-related diseases is accompanied by the dramatic loss of αβ crystallin,18 a zinc binding protein chaperone.

Currently, there is no treatment for preventing the onset, slowing the progression, or reversing these age-related diseases. For AD, approved treatments such as acetylcholinesterase inhibitors (AChEIs) and memantine, an N-methyl-D-aspartate (NMDA) receptor antagonist, only target the symptoms of AD. While these can temporarily improve cognitive and daily function, they do not significantly modify the progression of this disease. The majority of emerging disease-modifying AD treatments target Aβ based on the amyloid hypothesis19 including reducing Aβ production, increasing Aβ removal from the brain immune-therapeutically, or modulating Aβ toxicity.20 In addition, an alternative strategy has evolved that links metal dyshomeostasis, i.e., the modulation of metal–protein interactions linked to Aβ and tau neuropathology, to the treatment of AD.21 In the brain, metal dyshomeostasis of the transition metals copper, zinc, and iron has been linked to not only AD but several other neurodegenerative disorders.22 For example, the availability of zinc at the glutamatergic synapse is crucially involved in learning and memory and its availability has been shown to decline with age and AD.23 Copper levels have also been linked to learning and memory,24 and the onset of Huntington’s disease (HD) is associated with increased copper–protein interactions in the brain.14,25 Iron deposition in the brain (mainly in the basal ganglia) has also been linked to oxidative stress and the onset of Parkinson’s disease (PD).26

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Metal dyshomeostasis has been evaluated with the metal attenuators clioquinol (PBT1, 5-chloro-7-iodoquinolin-8-ol), a first generation 8-hydroxyquinoline (8-OHQ) analog, and PBT2 (5,7-dichloro-2-((dimethylamino)methyl)quinolin-8-ol), a second generation 8-OHQ analog, and shown to be effective in animal models of AD, HD, and PD.27,28 In a phase IIa clinical trial in AD patients, a 250 mg daily dose of PBT2 has also been shown to be safe and effective in reducing cerebrospinal fluid (CSF) Aβ levels and improving cognitive performance.29,30 A similar dose of PBT2 has been shown to be generally safe and well-tolerated in patients with HD, where an improvement in a measure of executive function (cognition) was observed.31 However, the clinical efficacy of PBT2 in both AD and HD studies is controversial.32 This may be related to the fact that in addition to the metal dyshomeostasis of the transition metals iron, copper, or zinc, neurodegenerative diseases are also linked to oxidative stress and mitochondrial dysfunction.33

Because multiple mechanisms are involved in the pathogenesis of neurodegenerations, we have designed compounds that are aimed at simultaneously combating multiple targets in these complex diseases.34 We have previously reported the development of an initial series of multifunctional antioxidants (MFAO-1s) where 4-(5-hydroxypyrimidin-2-yl)-N,N-dimethyl-3,5-dioxopiperazine-1-sulfonamide (JHX-4) and 4-(5-hydroxy-4,6-dimethoxyxypyrimidin-2-yl)-N,N-dimethyl-3,5-dioxopiperazine-1-sulfonamide (JHX-8) possess a 2-amino-5-hydroxypyrimidine free radical scavenging group (FRS) and a chelating group (CHL) consisting of two carboxyls directly adjacent to an amino group that function independently.35 This initial series of MFAO-1s demonstrated protective effects in human lens epithelial cells (hLECs), retinal pigmented epithelial cells (RPEs), and hippocampal astrocytes.36 In rats, oral administration of these MFAO-1s significantly delayed the progression of cataracts induced by ROS in diabetes and whole head γ-irradiation.36 Furthermore, in a rat model of dry atrophic AMD, they demonstrated retinal neuroprotection against light-induced injury where ROS is generated and iron is released from degenerating photoreceptors.37 While tissue analyses confirmed their presence in the lens and neural retina, their accumulation in the brain was minimal.36,37

Here, we report the synthesis and in vitro biochemical evaluation of a new series of MFAO-2 that accumulate in the brain following oral administration (Figure 2). This has been achieved by modifying the upper ring portion of MFAO-1s while retaining the 2-diacylamino-5-hydroxypyrimidine moieties required for independent antioxidant and metal attenuation activity. These MFAO-2s have subsequently been shown to reduce both Aβ40 and Aβ42 isoform levels in transgenic JAX Alzheimer’s mice by oral administration as well as remove zinc from the Aβ:Zn complex so that Aβ plaques can be subsequently degraded by matrix metalloproteinase 2.38

**CHEMISTRY**

The upper ring system, 1-N,N'-dimethylsulfonylperazine, in the first series of MFAO-1s was replaced with either a succinimide ring or glutarimide ring to give the second series of MFAO-2s as outlined in Scheme 1. According to the established procedure,35 the coupling reaction of the 2-

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**Figure 1.** Structures of the first generation of multifunctional antioxidants (MFAO-1s).

**Figure 2.** Structures of synthesized compounds in the MFAO-2 series. Shown are multifunctional compounds 2, 4, 6, and 8; monofunctional chelators (CHL) 1, 3, 5, and 7; monofunctional free radical scavengers (FRS) 10, 12, 14, and 16; and the nonfunctional parent compounds 9, 11, 13, and 15.

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**Scheme 1**

Reagents and conditions: (a) (1) acetone/toluene, 85 °C; (2) Ac₂O, 85 °C; (b) Pd–C/H₂/acetone, rt. 

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aminopyrimidines 19–22 with commercially available succinic anhydride 17 or glutaric anhydride 18 gave the 1-(2-pyrimidyl)piperidine-2,5-dione analogs 1, 3, 23, and 25 or the 1-(2-pyrimidyl)piperidine-2,6-dione analogs 5, 7, 24, and 26. Removal of the benzoxyl protecting groups by hydrogenation gave the MFAO-2s 2, 4, 6, and 8. The aminopyrimidines 19 and 20 were obtained as previously described while 21 was commercially available. The aminopyrimidine 22 was obtained from commercially available 2-chloro-5-nitropyrimidine and benzylamine followed by hydrogenation as outlined in Scheme 2.

**Scheme 2**

Reagents and conditions: (a) BnNH₂/K₂CO₃/dioxane, reflux; (b) 20% Pd(OH)₂/H₂/MeOH, rt.

Compounds 9–16 were synthesized from the 2-chloropyrimidines 31, 32, 33, and 34 by coupling with commercially available pyrrolidine 29 or piperidine 30 followed by hydrogenation as outlined in Scheme 3. The 2-chloropyrimidines 33 and 34 were commercially available, while 32 was obtained as previously described. The 2-chloropyrimidine 31 was obtained from commercially available 2-chloro-5-nitropyrimidine 39 by reduction of the nitro group to amine 5-amino-2-chloropyrimidine 40, acidic hydroxylation, and subsequent protection of the hydroxyl group as outlined in Scheme 4.

**Scheme 4**

Reagents and conditions: (a) Fe/AcOH/Er₂O₃/H₂O, at 70 °C; (b) 2 M H₂SO₄, reflux; (c) BnBr, K₂CO₃/MeOH, rt.

**RESULTS AND DISCUSSION**

In vitro analyses were conducted on the multifunctionals containing an upper ring succinimide ring (2, 4) and glutarimide ring (6, 8), their monofunctional FRS analogs (10, 12, and 14, 16, respectively) and their monofunctional CHL analogs (1, 3 and 5, 7, respectively), as well as their nonfunctional parent analogs (9, 11 and 13, 15, respectively).

**Metal Binding Studies.** The stoichiometry of the chelator–metal complexes was obtained using the method of continuous variation (Job plots) as previously described. Several solutions of each compound and metal ion of interest were examined at a constant total concentration (0.1–0.5 mM) of the MFAOs 2, 4, 6, and 8 and their monofunctional chelating analogs 1, 3, 5, 7 and the ion Fe²⁺, Fe³⁺, Cu²⁺, Cu³⁺, Zn²⁺, Mn²⁺, Ca²⁺, and Mg²⁺ by altering the mole fraction of one of the components. As summarized in Table 1, compounds 1–8 bound the transition metals Fe²⁺, Fe³⁺, Cu²⁺, Cu³⁺, Zn²⁺, and Mn²⁺ in a 1:2 ratio; however, these compounds failed to bind Ca²⁺ and Mg²⁺.

In Vitro Evaluation of Cytotoxicity and Antioxidant Activity. The cytotoxicity of compounds 1–16 was evaluated using a MTS cell viability assay in SH-SYSY human neuroblastoma cells (hNBs) and ARPE-19 human retinal pigmented epithelial cells (RPEs). All cells were exposed for 2 h to 1 mM of the MFAOs 2, 4, and 6, 8, their monofunctional FRS analogs 10, 12, and 14, 16, respectively, and their monofunctional CHL analogs (1, 3 and 5, 7, respectively) as well as their nonfunctional parent analogs 9, 11 and 13, 15, respectively, as well as the previous MFAO-1 JHX-4 (Figure 1), the metal attenuator clioquinol (PBT-1), and Trolox, a watersolubilized vitamin E derivative. As shown in Figure 3, no apparent cytotoxicity was observed with either compounds 1–16 or Trolox; however, clioquinol demonstrated a significant 30% reduction of the cell viability in human retinal pigmented epithelial cells (RPEs) and a 20% reduction in human neural neuroblastoma cells (hNBs).

To confirm the protective effect of MFAOs against the reactive hydroxyl radicals, both cells were treated with 1 mM of Fenton reagent (1 mM H₂O₂ and 1 mM Fe²⁺) with or without 1 mM compound 1–16, JHX-4, clioquinol, or Trolox. As shown in Figure 4, the multifunctional antioxidants 2, 4, 6, and 8 protected the hNB and RPE cells against hydroxyl radical generated ROS similar to that observed for MFAO-1 JHX-4. In both cells, this protection was significantly higher than that of the metal attenuator clioquinol. The activity of Trolox was only less in RPE cells. In order of protection, the MFAOs 2, 4, 6, 8, and JHX-4 provided significantly more protection than the monofunctional free radical scavengers 10, 12, 14, and 16 or Trolox. Even less protection was provided by the monofunctional chelators 1, 3, 5, and 7 or clioquinol, and no protection was provided by the multifunctional parent compounds 9, 11, 13, and 15. The cellular levels of reduced GSH, which is a sensitive biomarker of oxidative stress, were also measured under the same conditions as the cell viability experiments. As summarized in Figure 5, cells exposed to MFAOs were able to maintain GSH levels similar to JHX-4 and these levels were significantly higher than in cells exposed to Trolox or clioquinol. The relative activities of compounds with multifunctional versus monofunctional groups showed results similar to those observed with the viability studies.

The ability of MFAOs to protect cells against superoxide radicals was also assessed in both hNBs and RPEs.
Mitochondrial superoxide was generated using xanthine oxidase, and the levels of superoxide radicals generated were detected by selective MitoSOX Red fluorescence. For these studies, all cells were exposed to xanthine oxidase (XO, 25 μU/mL) for 1 h to generate approximately 100 μM superoxide with/without the presence of either the enzyme superoxide dismutase (SOD) or 1, 10, 100, and 1000 μM MFAOs 2, 4, and JHX-4, the nonfunctional parent compounds 9 and 11, clioquinol, or Trolox. As shown in Figure 6, superoxide levels generated were reduced by MFAOs, clioquinol, and Trolox in a similar dose-dependent manner in both cell lines. A similar dose-dependent cell protection against hydroxyl radicals was also observed using LIVE/DEAD viability/cytotoxicity staining (Figure 7). Exposure to hydroxyl radicals generated by 100 μM Fenton reagent for 2 h resulted in only 40% of RPE and hNBs cells remaining viable. Both cells were protected against hydroxyl radicals by the dose-dependent presence of MFAOs

2, 4, and JHX-4 or clioquinol; however, the nonfunctional parent compounds 9 and 11 provided no protection.

**Bioavailability Study.** Both the first generation MFAOs JHX-4 and JHX-8 and the second generation MFAOs 2, 4, 6, and 8 were administered to C57BL/6 mice by feeding chow containing 0.05% of the each compound for 14 days. Consumption studies indicated that this corresponded to an

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*The stoichiometry of the compound to metal complexes was obtained from the intersection of lines in Job’s plots. The total concentration of the compounds 1–8 and metal ion was 0.1–0.5 mM. Shown are the mean ± SD; n = 3. *Nonintersecting lines on the Job plot.*
average daily dose of 80 mg/kg. After whole body perfusion at the time of sacrifice, the drug levels in the lens, neural retina, and brain were measured by HPLC−MS. All MFAOs examined showed good oral bioavailability to at least one target tissue. This was anticipated because all compounds with the exception of JHX-8, which possesses 12 hydrogen bond acceptors, comply with Lipinski’s rule of 5.39 As illustrated in Figure 8A, all MFAO-2s accumulated in the brain (60−254 ng/mg protein) at 20- to 85-fold higher levels than MFAO-1s (JHX-4, 3 ng/mg protein, and JHX-8, 4 ng/mg protein). In contrast, both MFAO-2s and MFAO-1s achieved similar levels in the neural retina (Figure 8B); however, lower levels of MFAO-2s accumulated in the lens compared with those of MFAO-1 (Figure 8C). While it has generally been assumed that more lipophilic compounds should easily get into both the brain and the avascular lens, the present results suggest that differences exist in the passage of lipophilic drugs through the blood-aqueous barrier that is required to reach the lens and the blood−brain barrier that is required to reach the brain. Ongoing studies are investigating the molecular physicochemical parameters of these compounds that are associated with their uptake into the retina, brain, and lens.

CONCLUSION

Replacing the N,N-dimethylpiperazine-1-sulfonamide group in the first generation of multifunctional antioxidants with either a pyrrolidine or piperidine ring resulted in a new class of multifunctional antioxidants that have increased bioavailability to the brain. These second generation multifunctional antioxidants retain both the FRS activity against ROS and metal attenuation activity and protected RPEs and hNBs against both hydroxyl and superoxide radicals. While both generations of multifunctionals demonstrated similar retinal bioavailability, distinct differences in uptake to lens and brain were observed. The MFAO-2s demonstrated 20- to 85-fold improved drug penetration to the brain compared to MFAO-1s. These results suggest that MFAO-2 may have coadjutant therapeutic potential for AMD as well as AD, since ROS and redox-active metals are all present in these diseases.

EXPERIMENTAL SECTION

General. All solvents and reagents were obtained from commercial sources in the highest purity available. Clioquinol was purchased from TCI (>98%, Tokyo, Japan) and further purified by recrystallization. NMR spectra were obtained with a Varian 500 MHz spectrometer. Uncorrected melting points were obtained with the melting point apparatus MPA-120 EZ-Melt (Stanford Research Systems, Sunnyvale, CA). Column chromatography (CC) utilized SELECTRO SCIENTIFIC silica gel (200−400 mesh). UV−visible spectra were measured on a Molecular Devices SpectraMax Plus384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Final compound purities were assessed as >99%, and intermediate compound purities were assessed as ≥96% by reverse phase HPLC using a 250 mm × 4.6 mm
C18 Luna 5 μm column (Phenomenex Inc., Torrance, CA) with a mobile phase of 75:25 methanol/water at a flow rate of 0.9 mL/min and detection at 220, 254, and 280 nm. Elemental analyses were done by M-H-W Laboratories, Phoenix, AZ.

N-Benzyl-4,6-dimethoxypyrimidin-2-amine (28). A mixture of 2-chloro-4,6-dimethoxypyrimidine (27) (50.0 g, 0.29 mol), BnNH₂ (93.3 mL, 0.85 mol), and K₂CO₃ (62.5 g, 0.45 mol) in dioxane (1.0 L) was refluxed for 4 days. The reaction was filtered and the filtrate concentrated in vacuo to give a yellow oil. Silica gel column chromatography with 20:1 to 10:1 hexanes/EtOAc gave 61.7 g (87%) of white solid 28. 1H NMR (CDCl₃) δ 7.36 − 7.27 (m, 5H), 5.42 (s, 1H), 5.25 (s, 1H), 4.61 (d, J = 5.86 Hz, 2H), 3.83 (s, 6H).

2-Amino-4,6-dimethoxypyrimidine (22). Compound 28 (27.0 g, 0.11 mol) in 400 mL of MeOH was hydrogenated for 2 days at room temperature in the presence of 5.4 g of 20% Pd(OH)₂ catalyst under 1 atm of hydrogen gas (balloon). After filtration and solvent evaporation, a white solid was obtained which after silica gel column chromatography using 50:1 CHCl₃/MeOH yielded 16.8 g (98%) of 22. 1H NMR (CDCl₃) δ 5.47 (s, 1H), 4.90 (s, 2H), 3.84 (s, 6H).

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General Procedure for the Preparation of 1-(2-Pyrimidyl)pyrrolidine-2,5-dione (1), 1-(4,6-Dimethoxy-2-pyrimidyl)pyrrolidine-2,5-dione (3), 1-(2-Pyrimidyl)piperidine-2,6-dione (5), 1-(4,6-Dimethoxy-2-pyrimidyl)piperidine-2,6-dione (7), 1-(5-Benzoxyl-2-pyrimidyl)pyrrolidine-2,5-dione (23), 1-(5-Benzoxyl-2-pyrimidyl)piperidine-2,6-dione (24), 1-(4,6-Dimethoxy-2-benzoxyl-5-pyrimidyl)pyrrolidine-2,5-dione (25), 1-(4,6-Dimethoxy-5-benzoxyl-2-pyrimidyl)piperidine-2,6-dione (26). 42.0 g (0.42 mol) of succinic anhydride (17) dissolved in 300 mL of toluene and 20 g (0.21 mol) of 2-aminopyrimidine (21) was added, and the mixture was heated to 85 °C for 3 days. After cooling to room temperature, the precipitated product was filtered and washed with toluene. The filtrate was dried in vacuo, and the dried product was then dissolved in 300 mL of anhydrous Ac₂O and again heated to 85 °C for 3 h. After removal of the remaining Ac₂O in vacuo, the product was purified by silica gel column chromatography with CHCl₃ and recrystallized from EtOAc to give compound 1 as a white solid, mp 148.5−149.5 °C in 32% yield. 1H NMR (CDCl₃) δ 8.92 (d, J = 4.88 Hz, 2H), 7.42 (t, J = 4.88 Hz, 1H), 2.96 (s, 6H). Anal. Calc'd for C₈H₇N₃O₂: C, 54.24; H, 3.98; N, 23.72. Found: C, 54.29; H, 3.51; N, 23.90.

Figure 7. Dose-dependent protection of cells against hydroxyl radicals measured with the LIVE/DEAD viability assay. Part A illustrates the results of hNB cells that were preincubated for 1 h with/without the presence of 1, 10, 100, 1000 μM compounds 2, 4, 9, 11, JHX-4, chloquinol, or Trolox for 1 h and exposed to 100 μM Fenton reagent for 2 h. Part B illustrates the results of similar exposure of RPEs as above. The results represent the mean ± SEM, n = 6.

Figure 8. Oral bioavailability of MFAOs in mice. Tissue levels of MFAOs measured in the brain (A), neural retina (B), and lens (C) of C57BL/6 mice fed chow containing 0.05% of each MFAO (80 mg kg⁻¹ day⁻¹ average dose) for 14 days. The results represent the mean ± SEM (n = 6−7). The present MFAO-2s achieved higher brain levels compared to the previously synthesized MFAO-1s (A) but failed to reach adequate levels in the lens (C). Similar neural retinal levels were observed with both MFAO-1 and MFAO-2 (B).
Compound 3 was obtained as a white solid, mp 166.9–168.7 °C, in 60% yield. 1H NMR (CDCl3) δ 6.09 (s, 1H), 3.94 (s, 6H), 2.92 (s, 4H). Anal. Calc'd for C10H17N3O2: C, 59.17; H, 7.67; N, 18.82. Found: C, 58.94; H, 7.49; N, 18.56.

Compound 4 was obtained as a white solid, mp 170.5–172.0 °C, in 84% yield after recrystallization from Et2O. 1H NMR (CDCl3) δ 7.47–7.35 (s, 5H), 5.03 (s, 2H), 3.96 (s, 6H), 2.80 (t, J = 6.59 Hz, 4H), 2.15–2.13 (m, 2H).

General Procedure for the Preparation of 1-(5-Hydroxy-2-pyrimidyl)pyrrolidine-2,5-dione (2), 1-(4,6-Dimethoxy-5-hydroxy-2-pyrimidyl)pyrrolidine-2,5-dione (4), 1-(5-Hydroxy-2-pyrimidyl)piperidine-2,6-dione (8).

To 9.53 mL (116 mmol) of pyrrolidine (16) was added with 4.4 g of 10% Pd/C catalyst under a 1 atm of hydrogen gas with brine, dried over magnesium sulfate, and filtered. After removal of solvent in vacuo, the residue was purified by silica gel column chromatography using 20:1 hexanes/EtOAc as eluent. The product was then rechromatographed on silica gel using 20:1 hexanes/EtOAc as eluent. The product was then recrystallized from EtO to give a 12.0 g (76%) of compound 9 as a yellow solid, mp 39.0–39.6 °C. 1H NMR (CDCl3) δ 8.31 (d, J = 4.88 Hz, 2H), 6.45 (t, J = 4.88 Hz, 1H), 3.57 (t, J = 4.4 Hz, 4H), 2.10–1.98 (m, 4H). Anal. Calc'd for C7H19N3O4: C, 64.40; H, 7.43; N, 28.16. Found: C, 64.42; H, 7.53; N, 27.97.

Compound 11 was obtained as a white solid, mp 63.3–63.7 °C, 71% yield after recrystallization from EtO. 1H NMR (CDCl3) δ 5.34 (s, 1H), 3.87 (s, 6H), 3.56 (t, J = 6.59 Hz, 4H), 1.93–1.96 (m, 4H). Anal. Calc'd for C7H19N3O4: C, 64.40; H, 7.43; N, 28.16. Found: C, 57.20; H, 7.03; N, 19.91.

Compound 13 was obtained as a colorless oil in 90% yield after vacuum distillation at 90 °C under 0.25 mmHg. 1H NMR (CDCl3) δ 8.22 (d, J = 4.64 Hz, 2H), 6.35 (t, J = 4.64 Hz, 1H), 3.71 (t, J = 5.37 Hz, 4H), 1.64–1.58 (m, 2H), 1.56–1.51 (m, 4H). Anal. Calc'd for C7H19N3O4: C, 66.23; H, 8.03; N, 25.74. Found: C, 66.49; H, 7.95; N, 25.81.

Compound 15 was obtained as a white solid, mp 59.8–60.4 °C, in 74% yield. 1H NMR (CDCl3) δ 5.25 (s, 1H), 3.77 (6H), 3.68 (t, J = 5.49 Hz, 4H), 1.60–1.55 (m, 2H), 1.52–1.48 (m, 4H). Anal. Calc'd for C11H17N3O4: C, 59.17; H, 7.67; N, 18.82. Found: C, 58.94; H, 7.49; N, 18.56.

Compound 35 was obtained in 86% yield. 1H NMR (CDCl3) δ 8.06 (s, 2H), 7.34–7.25 (m, 5H), 4.93 (s, 2H), 3.45 (t, J = 6.59 Hz, 4H), 1.93–1.90 (m, 4H).

Compound 36 was obtained in 75% yield. 1H NMR (CDCl3) δ 8.04 (s, 2H), 7.33–7.25 (m, 5H), 4.94 (s, 2H), 3.63 (t, J = 4.88 Hz, 4H), 1.58–1.52 (m, 6H).

Compound 37 was obtained in 80% yield. 1H NMR (CDCl3) δ 7.47–7.32 (m, 5H), 4.84 (s, 2H), 3.90 (s, 6H), 3.51 (t, J = 6.59 Hz, 4H), 1.95–1.92 (m, 4H).

Compound 38 was obtained in 75% yield. 1H NMR (CDCl3) δ 7.46–7.27 (m, 5H), 4.85 (s, 2H), 3.89 (s, 6H), 3.69 (t, J = 5.49 Hz, 4H), 1.66–1.57 (m, 6H).

General Procedure for Preparation of 5-Hydroxy-2-(piperidin-1-yl)pyrimidine (10), 5-Hydroxy-4,6-dimethoxy-2-(piperidin-1-yl)pyrimidine (12), 5-Hydroxy-2-(piperidine-1-yl)-pyrimidine (14), 5-Hydroxy-4,6-dimethoxy-2-(piperidine-1-yl)-pyrimidine (16). Hydrogenation at room temperature for 12 h of compound 38 (17.6 g, 53.4 mmol) dissolved in 350 mL of acetone with 4.4 g of 10% Pd/C catalyst under a 1 atm of hydrogen gas (balloon) gave compound 16 as a pale red solid after filtration and solvent evaporation. Recrystallization from EtO gave 12.7 g (80%) of 16, mp 120.1–120.5 °C. 1H NMR (DMSO-d6) δ 7.63 (bs, 1H), 3.81 (s, 6H), 3.59 (t, J = 5.25 Hz, 4H), 1.60–1.56 (m, 2H), 1.52–1.47 (m, 4H). Anal. Calc'd for C11H17N3O2: C, 55.22; H, 7.16; N, 17.56. Found: C, 55.15; H, 7.21; N, 17.48.

Compound 10 was obtained as a pale yellow solid, mp 151.5–151.8 °C in 84% yield after recrystallization from EtO. 1H NMR (CDCl3) δ 8.05 (s, 2H), 3.55–3.75 (m, 4H), 1.96–1.92 (m, 4H). Anal. Calc'd for C7H19N3O2: C, 58.17; H, 6.71; N, 25.44. Found: C, 57.92; H, 6.67; N, 25.18.

Compound 12 was obtained as a yellow solid, mp 108.7–109.2 °C, in 78% yield after recrystallization from EtO. 1H NMR (DMSO-d6) δ 7.49 (brs, 1H), 3.82 (s, 6H), 3.41 (t, J = 6.47 Hz, 4H), 1.89–1.86 (m, 4H). Anal. Calc'd for C7H19N3O2: C, 53.32; H, 6.71; N, 18.66. Found: C, 53.51; H, 6.80; N, 18.80.

Compound 14 was obtained as a pale red solid, mp 94.3–94.7 °C, in 79% yield after recrystallization from EtO. 1H NMR (CDCl3) δ 8.06 (s, 2H), 3.47 (t, J = 6.59 Hz, 4H), 1.70–1.40 (m, 4H). Anal. Calc'd for C7H19N3O2: C, 60.32; H, 7.31; N, 23.45. Found: C, 60.15; H, 7.23; N, 23.26.

5-Amino-2-chloropyrimidine (40). 140 g (0.88 mol) of 2-chloro-5-nitropyrimidine (39) dissolved in 700 mL of EtOH. 197 g of iron powder (70 mesh, <212 μm), 700 mL of water, and 1400 mL of acetic acid were added together. The mixture was heated at 70 °C overnight, then cooled to room temperature and filtered. EtOH was removed from the filtrate in vacuo, and the pH was adjusted to 8.
with 12 N NaOH, and the product was extracted overnight with continuous liquid—liquid extraction with EtOAc. The resulting filter cake was washed with EtOAc, and the combined EtOAc layers were washed with water, then brine, dried over magnesium sulfate, and filtered. After removal of the solvent in vacuo and recrystallization with EtOH, 10 g (25%) of yellow solid 41 was obtained. 1H NMR (CDCl3) δ 10.93 (brs, 1H), 6.45 (t, J = 4.88 Hz, 1H), 3.57 (t, J = 4.4 Hz, 4H), 2.01—1.98 (m, 4H).

2-Chloropyrimidine-5-ol (41). Compound 40 (40 g, 0.31 mol) in 2 N sulfuric acid was refluxed for 2 h. After cooling to room temperature, the reaction mixture was extracted with EtOAc using continuous overnight liquid—liquid extraction. The combined EtOAc layers were washed with brine, dried over magnesium sulfate, and filtered. After solvent removal in vacuo and recrystallization with EtOH, 97.2 g (85%) of pale brown solid product 41 was obtained.

Metal Binding Studies. The stoichiometry of complexes formed between compounds 1—8 and the ions Cu2+, Zn2+, Fe3+, Mn2+, Ca2+, and Mg2+ at a constant total concentration (0.1—0.5 mM) was determined as previously described52 using the method of continuous variation (Job plots).53 Because of their low solubility, compounds 1—8 were first dissolved in acetone/tritritile (HPLC grade, UV cutoff 200 nm). These concentrated solutions were then diluted to 0.1 mM stock solutions with 1 mM acetate, pH 6.5. In a typical experiment (at 23 °C), several solutions of the compound and metal ion of interest were prepared at a constant total concentration of compound and ion but with a different mole fraction of one component. After an equilibration period of 45 min, the change in UV absorbance was monitored from 210 to 310 nm (PerkinElmer Lambda 10 spectrophotometer). The maximum absorbance for each compound was then recorded as a function of the mole fraction of the metal ion. Two linear dependences were obtained, at low and high molar fractions, which intersect at a mole fraction value that corresponds to the stoichiometry of the complex.

Cell Culture Studies. SH-SY5Y human neuroblastoma cells (hNBs, American Type Culture Collection (ATCC, Manassas, VA) and ARPE-19 human retinal pigmented epithelial cells (RPEs, ATCC, Manassas, VA) were cultured at 37 °C and 5% CO2 atmosphere in cell media (1:1 Eagle minimum essential medium with Earle’s balanced salt solution and Ham’s F12 (EMEM-F12) media containing 10% fetal bovine serum (FBS) for hNBs and Dulbecco’s modified Eagle medium (DMEM) containing 4% FBS for RPEs. Both media contained 1% penicillin/streptavidin solution. The cells were passaged when 80—90% confluent by treatment with trypsin—EDTA and then plated at a density of 1 × 105 cells onto 96-well plates or 1 × 105 cells onto 150 mm dishes.

In vitro cell viability studies were conducted as previously described58 using the Cell Titer 96 Aqueous One solution cell proliferation assay (MTS, Promega, Madison, WI) with 2 h of exposure to 1 mM Fenton reagent (1 mM H2O2, and 1 mM Fe2+) with/without 1 mM compounds 1—16, JHX-4, Trolox, or clioquinol dissolved in 0.16% DMSO. The results were normalized to blank control (100%) of cells not treated with Fenton reagent. On cells cultured in either 60 or 150 mm dishes also, glutathione (GSH) measurements were conducted as previously described58 with the same exposure as above.

Superoxide assays were conducted in 96-well plates using the MitoSOX Red reagent (Life Technologies Corporation, Carlsbad, CA). After removing cell media and washing with phosphate buffered saline (PBS), cells were preincubated in fresh media (150 μL) without FBS containing 1, 10, 100, or 1000 μM of each compound 2, 4, 9, 11, JHX-4, clioquinol, Trolox, or 25 μg/mL of superoxide dismutase (approximately 100 μM) at 37 °C for 1 h. Xanthine oxidase (100 μM/ mL, 50 μL) which generates approximately 100 μM of superoxide was added and cultured for an additional hour. After removing the media and washing with PBS, the cells were stained with 100 μM of the MitoSOX Red reagent (5 μM) and incubated for 2 h. The fluorescence of the each well was measured using fluorescent microplate reader at Ex/Em = 510/580 nm.

LIVE/DEAD viability/cytotoxicity assays were conducted in 96-well plates using the LIVE/DEAD viability/cytotoxicity assay kit (Life Technologies Corporation). After removing cell media and washing with PBS, cells were preincubated in fresh media (210 μL) without FBS containing 1, 10, 100, or 1000 μM of each compound 2, 4, 9, 11, JHX-4, clioquinol, or Trolox, at 37 °C for 1 h. Fenton reagent (Fe2+ and hydrogen peroxide dissolved in appropriate media without FBS to give a final concentration of 100 μM, 40 μL) was added to each well and incubation continued for 2 h. After 2 h of incubation with Fenton reagent, the medium was removed and the cells were washed with PBS. The cells were stained with 100 μL of the LIVE/DEAD reagent containing calcein AM (AM, 8 μM) and ethidium homodimer 1 (EthD-1, 16 μM) for 15 min at 37 °C. The fluorescence of each well was measured with a fluorescent microplate reader at Ex/Em = 494/517 nm (Fsam for LIVE Cells) and Ex/Em = 528/617 nm (Fsam for DEAD Cells). The fluorescence of control samples was also measured as follows: Fmax for LIVE cells (the fluorescence at Ex/Em = 494/517 nm in live cells samples labeled with AM only); Fmax for LIVE cells (the fluorescence at Ex/Em = 494/517 nm in live cells samples labeled with EthD-1 only); Fmax for DEAD cells (the fluorescence at Ex/Em = 528/617 nm in dead cells samples labeled with EthD-1 only); Fmax for DEAD cells (the fluorescence at Ex/Em = 528/617 nm without dye and cells); Blank494/517 (the fluorescence at Ex/Em = 494/517 nm without dye and cells); Blank528/617 (the fluorescence at Ex/Em = 528/617 nm without dye and cells). For the dead cells control, the cells were incubated in 70% of ethanol for 30 min at 37 °C. The percentage of live cells was calculated from the fluorescence readings using the equation:

\[
\% \text{LIVE cells} = \frac{F_{\text{max for LIVE cells}} - F_{\text{blank 494/517}} - F_{\text{blank 528/617}}}{F_{\text{max for LIVE cells}} - F_{\text{blank 494/517}}} \times 100
\]

The percentage of dead cells was calculated from the fluorescence readings using the equation:

\[
\% \text{DEAD cells} = \frac{F_{\text{max for DEAD cells}} - F_{\text{blank 528/617}} - F_{\text{blank 528/617}}}{F_{\text{max for DEAD cells}} - F_{\text{blank 528/617}}} \times 100
\]

Animal Studies. All procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of UNMC (Permit Number 11-086-1-1-FC).

Bioavailability Studies. Bioavailability studies were conducted in C57BL/6 mice (equal sexes, body weight 14—17 g, n = 6) fed chow containing 0.05% of compounds 2, 4, 6, 8, JHX-4, and JHX-8 for 14 days. Food consumption and body weights were monitored and indicated that each mouse received an average dose of 80 mg kg⁻¹ day⁻¹ of drug. Following whole body perfusion with PBS (5 mL) at the time of sacrifice, the lens, neural retina, and brain were removed and homogenized in ice-cold 10 mM acetate buffer, pH 4 (1 mL for three lenses and three retinas, 3 mL for two brains), containing N,N'-dimethyl-4-(pyridin-2-yl)pyrrole-1-sulfonamide (0.5 mM) as the internal standard. The protein concentration in an aliquot of the supernatant was determined according to Bradford.41 The remaining supernatant was then deproteinized with equal volumes of 20%
trichloroacetic acid (TCA). Following an additional centrifugation at 15 000g for 30 min at 4 °C, the supernatant was transferred to a clean test tube and dried in vacuo overnight. The residue was dissolved in chloroform, centrifuged at 15 000g for 30 min at 4 °C, and dried in vacuo. The remaining organic residue containing the internal standard and extracted compounds 2, 4, 6, 8, JHX-4, and JHX-8 was dissolved in 200 μL of HPLC grade acetonitrile and separated by reverse phase HPLC (Luna 5 μm, C18, 350 mm × 4 mm) column (Phenomenex Inc., Torrance, CA). The samples were eluted with 75% aqueous methanol. The eluent was monitored by UV at 261/273 nm and ESI-MS in negative mode on a Thermo Finnigan LCQ (Thermo Fisher Scientific, Waltham, MA). Samples were quantified against standard curves of compounds 2, 4, 6, 8, JHX-4, and JHX-8. All analyses were conducted in triplicate.

**Statistical Analysis.** Statistical comparisons between groups were performed with ANOVA test (Origin 8.1, OriginLab Corporation, Northampton, MA). Differences with $p < 0.05$ were considered significant.

## ASSOCIATED CONTENT

Supporting Information
An xlsx file containing molecular formula strings. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00272.

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The manuscript was written by H.K. as part of his Ph.D. requirements and revised by P.F.K. All authors have given approval to the final version of the manuscript.

**Notes**
The authors declare the following competing financial interest(s): P. F. Kador has financial interest as the sole inventor on U.S. Patent Patent 8,877,766.

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## ABBREVIATIONS USED

Aβ, amyloid β; AcO, acetic anhydride; AD, Alzheimer’s disease; AMD, age-related macular degeneration; CC, column chromatography; CHL, chelating group; CSF, cerebrospinal fluid; DMSO, dimethyl sulfoxide; EtOAc, ethyl acetate; EtOH, ethanol; EtO, ether; FBS, fetal bovine serum; FRS, free radical scavenging group; GSH, reduced glutathione; hLEC, human lens epithelial cell; hNB, human neuroblastoma cell; HPLC–MS, high performance liquid chromatography–mass spectrometry; HD, Huntington disease; i-ProOH, isopropanol; JHX-4, 4-((5-hydroxypyrimidin-2-yl)-N,N-dimethyl-3,5-dioxopiperazine-1-sulfonamide; JHX-8, 4-(5-(4-hydroxy-4,6-dimethoxyoxypiridin-2-yl)-N,N-dimethyl-3,5-dioxopiperazine-1-sulfonamide; MeOH, methanol; MFAO, multifunctional antioxidant; MFAO-1, first series of multifunctional antioxidants; MFAO-2, second series of multifunctional antioxidants; NMDA, N-methyl-D-aspartate; 8-OHQ, 8-hydroxyquinoline; PBS, phosphate buffered saline; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPE, retinal pigmented epithelial cell; SOD, superoxide dismutase; THF, tetrahydrofuran; XO, xanthine oxidase

## REFERENCES


