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Neuroprotective effects of mercaptoethylleonurine and mercaptoethylguanidine analogs on hydrogen peroxide-induced apoptosis in human neuronal SH-SY5Y cells

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ABSTRACT

A series of mercaptoethylleonurine and mercaptoethylguanidine derivatives were designed and synthesized. Their neuroprotective effects toward H_2O_2-induced apoptosis were investigated in human SH-SY5Y cells. The results from these studies identified several potent compounds, with compound **8k** emerging as the most effective. Further investigation demonstrated that **8k** reduced H_2O_2-induced activation of mitochondrial apoptosis by inhibiting the expression of Bax and elevating the expression of Bcl-2. Moreover, the molecular mechanism underlying the observed neuroprotective effects of **8k** was exerted via the Akt and JNK pathways. Compound **8k** can be a lead compound for further discovery of neuroprotective medicine.

Parkinson's disease (PD), the second most common neurodegenerative disorder after Alzheimer's disease, is mainly characterized by the loss of dopaminergic neurons in the substantial nigra pars compacta.[1,2] Although the underlying mechanisms in the neurodegenerative processes of PD remain unknown, many studies have suggested the involvement of oxidative stress and mitochondrial dysfunction in the activation of an apoptotic cascade that ultimately results in the loss of dopaminergic neurons.[3,4] Oxidative stress caused by excessive reactive oxygen species (ROS) production has been shown to lead to cellular dysfunction, culminating in cell death.[5] The most common ROS are oxygen radicals, such as superoxide and hydroxyl radicals, and non-free radicals, such as hydrogen peroxide. All of these reactive oxygen species are generated in various redox processes in the human body. The generation of ROS in normal cells is tightly regulated by biological antioxidants and antioxidant enzymes. These ROS cause oxidative damage to molecules such as carbohydrates, proteins, lipids, and DNA. Many antioxidant compounds can prevent these oxidative-stress-related disorders by scavenging ROS.[6] Hydrogen peroxide induces apoptosis in a variety of cells, including neuronal cells, and is a precursor of highly reactive free radicals.[7]

Many naturally occurring compounds have been utilized as leads in drug discovery, and efforts also have been made to search compounds with neuroprotective effects.[8–10] Leonurine (Fig. 1), a cardioprotective drug candidate, was reported to exhibit cardioprotective effects both in vivo and in vitro,[8,9] and its preclinical study is on-going. Moreover, Shi et al. recently reported that leonurine significantly reduced 6-hydroxydopamine (6-OHDA)-induced cell death in dopaminergic SH-SY5Y cells and attenuated apomorphine-elicited rotational behavior in 6-OHDA-lesioned rats.[11] Structurally, the guanidine moiety in leonurine may play a very important role in its activity. Similarly, mercaptoethylguanidine (MEG), another scavenger bearing a guanidine unit, can react with peroxynitrite and protect against peroxynitrite-induced oxidative damage.[12] MEG was also reported to have neuroprotective,[13] cardioprotective,[14–16] and radioprotective effects.[17] Although both leonurine and MEG have interesting neuroprotective effects, their structure–activity relationships (SAR) have not been extensively explored.

Herein, by considering the common structural features of leonurine and MEG, we designed and synthesized a series of new mercaptoethylleonurine (MEL) analogs (**1a–h**) by incorporating a mercaptoethyl group and changing the chain length (R^1 group) to find new chemical entities with better neuroprotective activity than leonurine and MEG. The in vivo biological activities could depend on many parameters, including bioavailability. Given that increasing aqueous solubility generally results in enhanced

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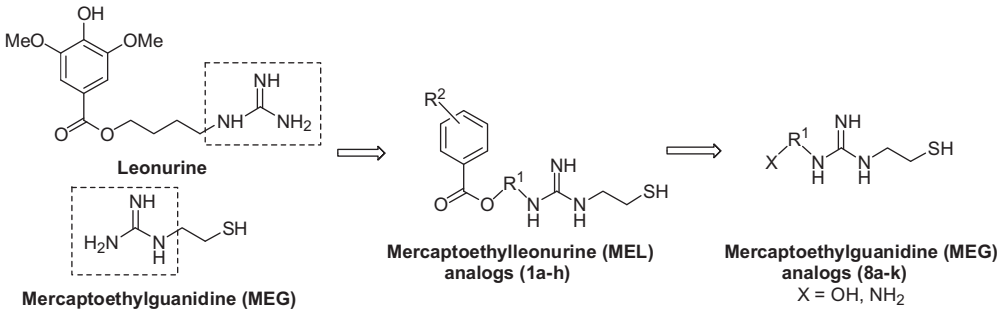
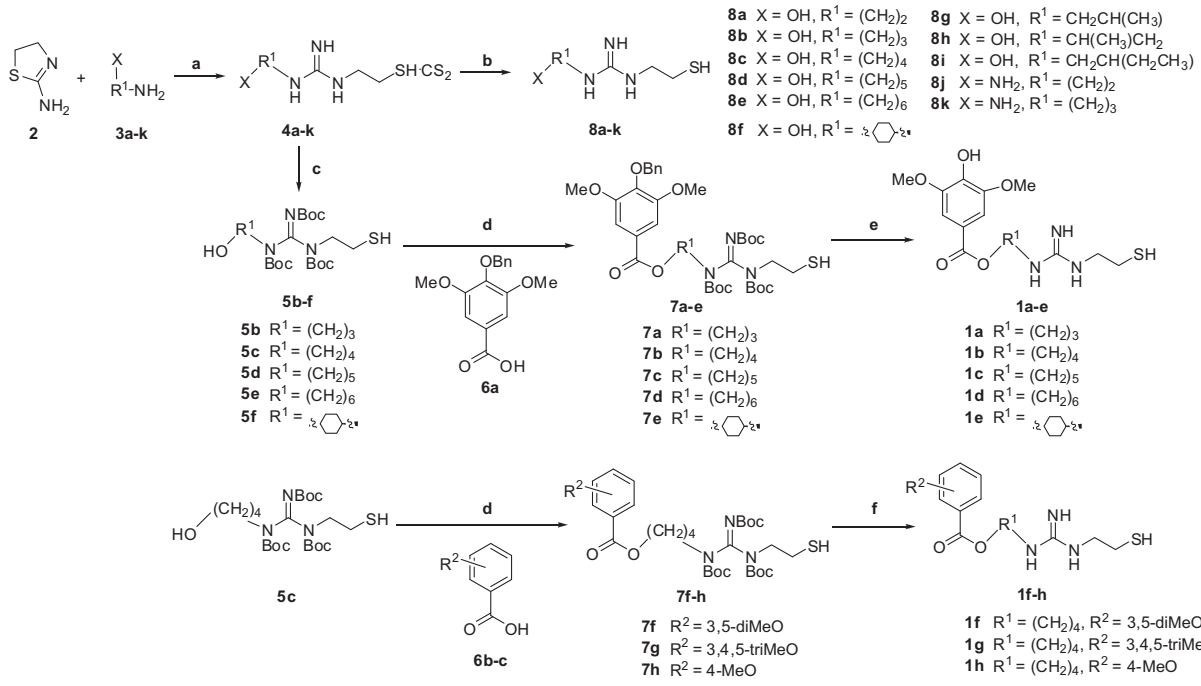


Figure 1. Design strategy.



Scheme 1. Reagents and conditions: (a) (i) HBr, MeCN, H_2O, 110 °C, 1–2 h; (ii) CS_2, H_2O, 0 °C, 12 h; (b) HCl, MeOH, 40–50 °C, 2–3 h; (c) Na_2CO_3, $(Boc)_2O$, dioxane:H_2O = 1.5:1, rt, 24–72 h; (d) EDC, DMAP, CH_2Cl_2, 0 °C, 3 h; then rt, 24–72 h; (e) (i) HCOOH, rt, 24–28 h; (ii) HCl, MeOH, rt, 2–3 h; (f) HCOOH, rt, 24–28 h.

bioavailabilities in vivo, we also designed and synthesized **8a–k** in an attempt to improve bioavailability by increasing the hydrophilic character of the molecules.

The synthesis of our target compounds started from 2-amino-2-thiazoline (**2**), a commercially available starting material, as illustrated in Scheme 1. The treatment of 2-amino-2-thiazoline (**2**) with amino alcohols (**3a–3i**) or diamines (**3j–3k**) in the presence of 40% hydrobromic acid followed by the addition of carbon disulfide afforded guanidine derivatives (**4a–k**).[18] Guanidines **4b–f** were converted to their corresponding tri-Boc substituted intermediates **5b–f** using excess Boc_2O and sodium carbonate as a base in 49–66% yield. The free hydroxyl groups in **5b–f** were coupled with 4-benzyloxy-3,5-dimethoxy-benzoic acid (**6a**) to afford esters **7a–e** in 36–48% yield in the presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP). After subsequently removing the Boc and benzyl protecting groups on **7a–e** with formic acid and hydrochloric acid, new MEL analogs (**1a–e**) were synthesized in desirable yields. It is worth mentioning that the debenzylation step was problematic. After numerous attempts of failure (using various palladium catalytic hydrogenations; ferric chloride; Li-naphthalene; etc.), it was found that the benzyl group could be only easily removed in 4 N HCl in MeOH. Analogs **1f–h** were obtained in good yields in a similar manner as **1a–e**. The MEG derivatives were prepared from **4a–k** using hydrochloric acid in methanol at 40 °C, followed by HPLC purification to provide compounds **8a–k** in 29–50% yield.[19]

Eight MEL analogs (**1a–h**) and 11 MEG analogs (**8a–k**) (Table 1) were evaluated for their neuroprotective activity toward H_2O_2-induced apoptosis in human SH-SY5Y cells.[20] The cytotoxic potential of **1a–h** and **8a–k** has been performed at 5–100 μM concentration by MTT assay and cytotoxic effect of them was not observed at the dosage used in this study. Among analogs **1a–h**, only **1a** exhibited greater neuroprotective activity than leonurine relative to the positive control *N*-acetyl-cysteine (NAC) at 5 mM. Increasing the side chain length (R^1 substitution) from three carbons (**1a**) to four, five, and six carbons (**1b–d**, respectively), did not improve their neuroprotective activity. Although compound **1d** had a similar activity to **1a** at low concentrations (5 μM), the activities were dramatically lower at higher concentrations (25 μM and 50 μM). Replacing the straight side chain with cyclohexyl linkage also decreased the activity of **1e** relative to **1a**. Compared with leonurine, compound **1b**, which contained a mercaptoethyl group on the guanidine of

Table 1
Neuroprotective activity of compounds **1a–1h**, and **8a–8k** on H_2O_2-induced SH-SY5Y cells

Compound	Control (%)	Model (%)	NAC, 5 mM (%)	Different concentrations of compounds		
				50 μM (%)	25 μM (%)	5 μM (%)
1a	100 ± 7.44	63.06 ± 4.92	76.42 ± 5.54	93.34 ± 8.79	83.30 ± 9.45	78.83 ± 8.96
1b	100 ± 7.91	75.66 ± 8.66	88.84 ± 9.48	60.38±9.86	66.4 ± 6.54	60.70 ± 5.48
1c	100 ± 7.44	63.06 ± 4.92	76.42 ± 5.54	65.92 ± 2.92	59.87 ± 1.75	53.85 ± 2.91
1d	100 ± 7.91	75.66 ± 8.66	88.84 ± 9.48	73.76 ± 8.03	80.58 ± 3.23	78.47 ± 3.83
1e	100 ± 7.91	75.66 ± 8.66	88.84 ± 9.48	65.87 ± 8.41	67.16 ± 6.69	76.38 ± 9.17
1f	100 ± 7.91	75.66 ± 8.66	88.84 ± 9.48	66.06 ± 14.78	63.51 ± 12.55	62.11 ± 11.4
1g	100 ± 7.02	66.32 ± 4.42	82.39 ± 6.29	49.12 ± 5.35	49.4 ± 7.81	32.48 ± 1.45
1h	100 ± 7.02	66.32 ± 4.42	82.39 ± 6.29	48.62 ± 3.12	37.55 ± 5.69	42.37 ± 2.42
8a	100 ± 7.44	63.06 ± 4.92	76.42 ± 5.54	65.09 ± 3.29	59.64 ± 1.86	56.36 ± 1.02
8b	100 ± 7.44	63.06 ± 4.92	76.42 ± 5.54	82.12 ± 6.22	80.99 ± 10.24	67.17 ± 2.88
8c	100 ± 7.02	66.32 ± 4.42	82.39 ± ± 6.29	70.16 ± 7.64	63.49 ± 1.76	68.04 ± 3.96
8d	100 ± 7.44	63.06 ± 4.92	76.42 ± 5.54	82.83 ± 6.50	80.60 ± 7.59	60.02 ± 6.13
8e	100 ± 7.02	66.32 ± 4.42	82.39 ± 6.29	52.88 ± 2.04	55.45 ± 2.96	72.41 ± 6.28
8f	100 ± 7.02	66.32 ± 4.42	82.39 ± 6.29	30.43 ± 0.52	38.64 ± 5.65	31.65 ± 1.67
8g	100 ± 7.91	75.66 ± 8.66	88.84 ± 9.48	65.49 ± 5.51	67.30 ± 3.65	77.06 ± 3.02
8h	100 ± 7.91	75.66 ± 8.66	88.84 ± 9.48	80.04 ± 3.41	67.25 ± 2.19	59.86 ± 7.84
8i	100 ± 7.91	75.66 ± 8.66	88.84 ± 9.48	71.1 ± 5.39	77.11 ± 0.45	75.82 ± 10.32
8j	100 ± 7.91	63.06 ± 4.92	76.42 ± 5.54	79.22 ± 3.80	75.36 ± 7.63	66.33 ± 9.43
8k	100 ± 7.02	66.32 ± 4.42	82.39 ± 6.29	96.54 ± 6.99	84.27 ± 9.67	77.29 ± 8.47
Leonurine	100 ± 7.02	66.32 ± 4.42	82.39 ± 6.29	45.95 ± 4.41	38.53 ± 6.28	76.65 ± 2.94

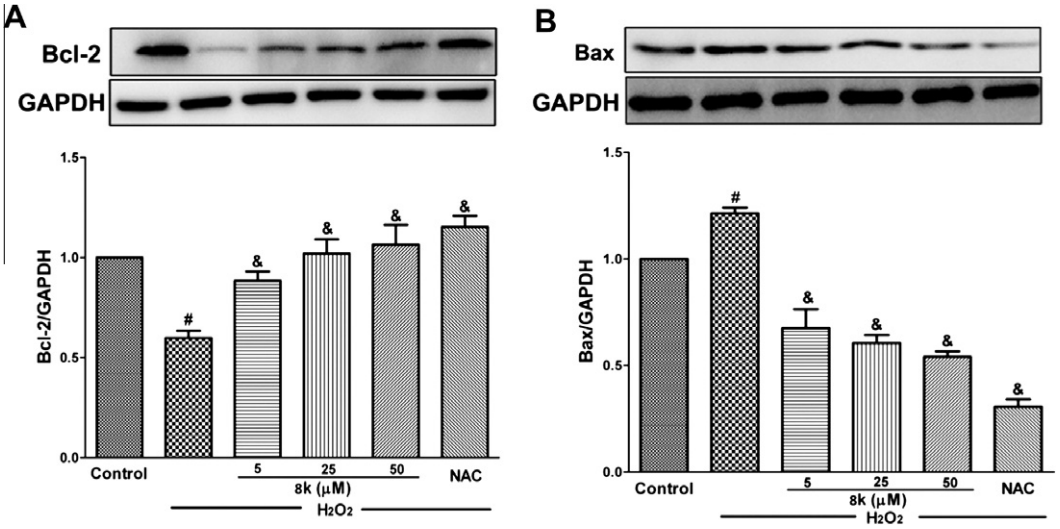


Figure 2. Compound **8k** modulated H_2O_2-induced Bax and Bcl-2 protein changes in SH-SY5Y cells. Cells were treated with **8k** (5, 25, 50 μM) or NAC (5 mM) for 4 h and then stimulated by H_2O_2 (100 μM) for another 12 h before western blotting. (A) Bar graph showing the quantitative analysis of Bcl-2 expression with GAPDH as the loading control. (B) Bar graph showing the quantitative analysis of Bax expression with GAPDH as the loading control. Data are mean ± SEM of results from at least three independent experiments, each performed in duplicate, $^{\#}P$ <0.05 versus control cells; $^{\&}P$ <0.05 versus H_2O_2-stimulated cells.

leonurine, also exhibited only moderate activity. Further modification of the substitution patterns on the phenyl ring (**1f–h**) did not produce any promising analogs. Interestingly, after removing the phenyl ring, some analogs in the free hydroxyl analog series (**8a–i**), such as **8b** and **8d**, retained good activity at all three doses. Further SAR development included the replacement of the hydroxyl group with an amino group to yield compound **8k**, a bioisostere of **8b**. Surprisingly, compound **8k** was the most potent analog both series. Based on its excellent neuroprotective activity, **8k** was chosen for further study.

The activation of phosphatidylinositol 3-kinase (PI3 K) is known to promote cell survival and prevent apoptosis.[21,22] The anti-apoptotic effects of PI3 K are mediated by its downstream target Akt, which may regulate the expression of several apoptosis-related genes, such as Bcl-2/Bax proteins, which mediate apoptosis.[23,24] Therefore, the elucidation of the molecular mechanism involved in this effect might provide novel insight into the process of neurodegeneration and suggest the potential utility of MEL and MEG analogs for protection against neurodegenerative diseases caused by oxidative stress and apoptosis.

In this study, H_2O_2 stimulation significantly inhibited the expression of Bcl-2 and increased Bax expression in SH-SY5Y cells (P <0.05). In the cells treated with compound **8k** and NAC, the effects of H_2O_2 on the expression of Bcl-2 and Bax were evidently reversed (Fig. 2). Additionally, the effects of **8k** were dose-dependent. As shown in Figure 3, compound **8k** can attenuate the H_2O_2-induced phosphorylation of JNK and Akt in SH-SY5Y cells but does not affect the phosphorylation of p38 and ERK relative to the H_2O_2-stimulated cells.

In summary, eight MEL analogs (**1a–h**) and eleven MEG analogs (**8a–k**) were designed and synthesized. Analogs **1a**, **8b**, **8d**, **8j**, and **8k** showed neuroprotective activity against H_2O_2-induced apoptosis in human SH-SY5Y cells, with **8k** exhibiting the highest neuroprotective activity. Further studies showed that compound **8k** can

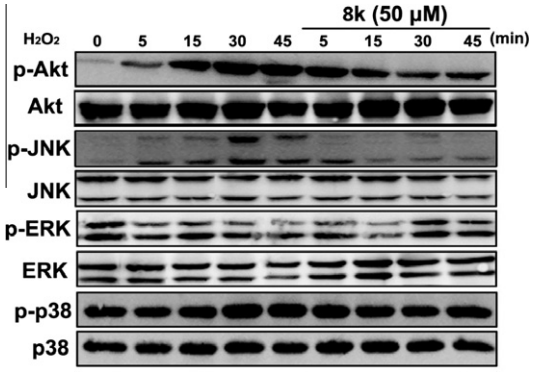


Figure 3. Compound **8k** attenuated H_2O_2-induced phosphorylation of Akt and JNK in SH-SY5Y cells. Cells were pretreated with 50 μM **8k** for 4 h. Next, the cells in model groups were stimulated with H_2O_2 (100 μM) for the indicated periods and then collected. The phosphorylation of Akt, JNK, ERK, and p38 was detected by Western blot using the sum of Akt, JNK, ERK, and p38 as the loading control. Data are results from at least three independent experiments, each performed in duplicate.

reduce the H_2O_2-induced activation of mitochondrial apoptosis by inhibiting the expression of Bax and elevating the expression of Bcl-2. Moreover, the molecular mechanism underlying the observed neuroprotective effects of **8k** was more probable via the Akt and JNK pathways. This finding may provide insight for our future design of leonurine analogs with optimal neuroprotective activities.

Acknowledgments

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19. *Representative experiment for the preparation of 1-(3-aminopropyl)-3-(2-mercaptoethyl)guanidine (**8k**)*: 2-Amino-2-thiazoline (**2**, 306 mg, 3.0 mmol) was dissolved in acetonitrile–water (5 mL, 3:2), and 40% hydrobromic acid (0.6 mL) was added. After the mixture was stirred for 5 min, compound **3k** (12.0 mmol) was added. The solution was heated in a sealed vessel at 110 °C for 1–2 h. After cooling to 0 °C, water (5 mL) and carbon disulfide (0.5 mL) were added with stirring, and the mixture was kept at 0 °C for 12 h. The yellow solid formed was filtered, washed with ethanol and ether, and dried to give compounds **4k** as a yellowish solid in 45% yield, which will be used directly in the next step. Compound **4k** was dissolved in MeOH (5 mL), and 4 N HCl in MeOH (5 mL) was added. The solution was heated to 40–50 °C and stirred until all of the yellow solid was dissolved (2–3 h). Next, the reaction mixture was concentrated and the residue was purified by HPLC to give compound **8k** as a yellowish oil (42%). ^{1}H NMR (CD_3OD, 400 MHz) δ ppm: 3.51 (2H, d, J = 6.26 Hz), 3.37 (4H, m), 2.91 (2H, d, J = 6.46 Hz), 1.96 (2H, m); ^{13}C NMR (CD_3OD, 100 MHz) δ ppm: 153.22, 43.73, 38.39, 23.00, 19.71; $C_6H_{16}N_4S$: ESIMS m/z 160.1 $[M-NH_3+H]^+$; HRMS m/z calcd 160.0864 $[M-NH_3+H]^+$, found 160.0888.
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