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Design and Biological Evaluation of Novel Antioxidants Containing *N*-*t*-Butyl-*N*-hydroxylaminophenyl Moieties

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Abstract—In order to develop therapeutic agents against neurodegenerative diseases, we designed novel antioxidants containing *N*-*t*-butyl-*N*-hydroxylaminophenyl moieties and evaluated in vitro and in vivo neuroprotective properties as well as anti-ischemic effects.

One of main causative agents of neurologic disorders is reactive oxygen species (ROS). ROS-mediated oxidative stress leads to cell damage directly by modifications of polyunsaturated membrane lipids, proteins and nucleic acids or indirectly by increased levels of intracellular free calcium ions.[1] Central nervous system is especially sensitive to cell injury and/or death by ROS due to increased oxygen consumption rate and low levels of natural antioxidants, as well as high concentrations of iron ions and polyunsaturated fatty acids.[2]

Human and animals have adopted various defense mechanisms to regulate intracellular concentration of ROS.[3] Superoxide dismutase, catalase and glutathione peroxidase are used in enzymatic defense systems, and α-tocopherol and ascorbic acid are involved in non-enzymatic defense mechanisms. However, with aging these intricate defense mechanisms become less efficient, particularly owing to decreased activity of a large number of enzymes involved in ROS defense. This explains why oxidative stress induced by ROS is one of the main etiological factors of cerebral aging, as well as acute neurodegenerative diseases such as stroke, Parkinson's and Alzheimer's diseases.[1a]

To date, many efforts have been devoted to the development of efficient antioxidants, in particular, phenol-type antioxidants to protect neuron cells from neurodegenerative disorders.[3] Recently, novel therapeutic agents to control multiple events were also developed.[4] For example, it was reported that a dual inhibitor of neuronal nitric oxide synthase and lipid peroxidation was a useful therapeutic agent to treat neurodegerative disorders.[4a] Herein we describe design and biological evaluation of novel antioxidants containing *N*-*t*-butyl-*N*-hydroxylaminophenyl moieties.

The *N*-*t*-butyl-*N*-hydroxylaminophenyl derivatives (*t*-Bu-NR-OH) have been examined for the development of molecule-based magnets since their radicals (*t*-Bu-NR-O$^{\cdot}$) are stable enough to be employed as organic magnetic materials.[5] Therefore, we reasoned that *N*-*t*-butyl-*N*-hydroxylaminophenyl derivatives could have therapeutic potentials as novel antioxidants because of high stability of radical species generated from abstraction of hydrogen on N-OH by ROS.

Target compounds **1**–**3** were prepared by oxidation of aldehyde **4** synthesized by a known procedure and

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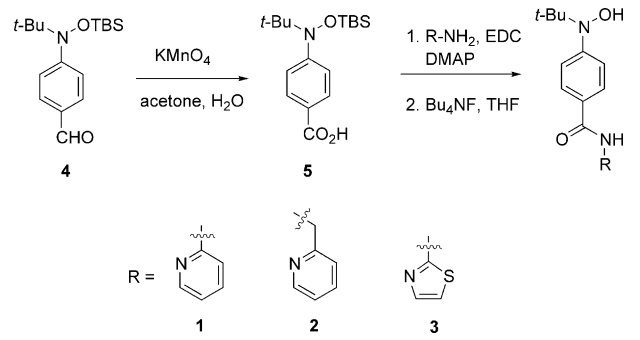

Scheme 1. Synthesis of compounds **1**–**3**.

subsequent coupling of the resulting acid to appropriate amines followed by deprotection of TBS group (Scheme 1).[6] Having these compounds at hand, we initially examined lipid peroxidation (LPO) inhibition to evaluate their antioxidant activities in vitro by measuring the extent of the repression of the radical chain reaction of multilayer liposomes.[7] As a positive control, ebselen, which is a synthetic small molecule mimicking the action of glutathione peroxidase and one of the most promising antioxidants, was used.[8] Compounds **1**–**3** had superior LPO inhibition activities in comparison to ebselen as shown in Table 1. Interestingly, LPO inhibition activity depends on the amino group attached to *N*-*t*-butyl-*N*-hydroxylaminophenyl moieties. Thiazole-containing compound **3** showed the best LPO inhibition effect among three compounds and 2-pyridylamine group (**1**) exhibits better inhibition than 2-pyridylmethylamine group (**2**).

We also investigated radical-scavenging ability of *N*-*t*-butyl-*N*-hydroxylaminophenyl group by incubating **1** with galvinoxyl which is known to be stable at room temperature.[9] EPR spectrum of incubation mixture showed a triplet structure by nitrogen atom of N–O• which was further splitted by hydrogens on **1** (Fig. 1).[6] This demonstrates that a hydrogen atom of N-OH is readily transferred to phenoxide radical of galvinoxyl to form a more stable N–O• species. This also suggests that *N*-*t*-butyl-*N*-hydroxylaminophenyl derivatives can remove ROS more efficiently than di-*t*-butylphenol-type compounds.

We then examined neuroprotective effects of the compounds on neuronal cell death induced by Fe^{2+} ions that generate ROS by their auto-oxidation to Fe^{3+} ions.[10] For these studies, we first determined the effective optimal concentration of compounds by measuring the activity of lactate dehydrogenase (LDH) released by damaged or destroyed cells into the extracellular fluid.[10] It was found that **1**–**3** showed little neurotoxicity up to 100 μM (data not shown). Based on these observations, we examined neuroprotective properties of **1**–**3** at concentrations where their neurotoxicity is low (below 100 μM). Neurotoxicity induced by ROS was remarkably decreased in the presence of **1**–**3** at 10 μM concentrations, indicating that compounds **1**–**3** have excellent neuroprotective activities (Fig. 2).

Table 1. IC_{50} values for compounds **1** ~**3** and ebselen in inhibition of lipid peroxidation

Compd	**1**	**2**	**3**	Ebselen
IC_{50} (μM)	4.3	11.1	1.44	68.9

Finally, we evaluated the anti-ischemic properties of thiazole-possessing compound **3** which showed the best LPO inhibition and in vivo neuroprotective activities using gerbils as animal models. Neuronal cell survival rate was determined by counting the CA1 neuron cells in the gerbil hippocampus.[11] Neuron cells of gerbils suffering ischemic injury were almost killed (less than 15% neuronal cell survival relative to sham) as shown in Figure 3a. However, **3** exhibited 57% and 81% of neuronal cell survival when gerbils were treated with **3** before and after ischemic injury, respectively.[12] Ebselen-treated gerbils as a positive control showed 58% of neuronal cell survival in both pre- and post-treated groups. This demostrates that **3** efficiently protects neuronal cell death from ischemic injury, particularly in post-treated groups. All the results of LPO inhibition activity, in vivo neuroprotective activity and animal studies indicate that **3** can be used as a novel therapeutic agent to treat neurodegenerative disorders that involve ROS.

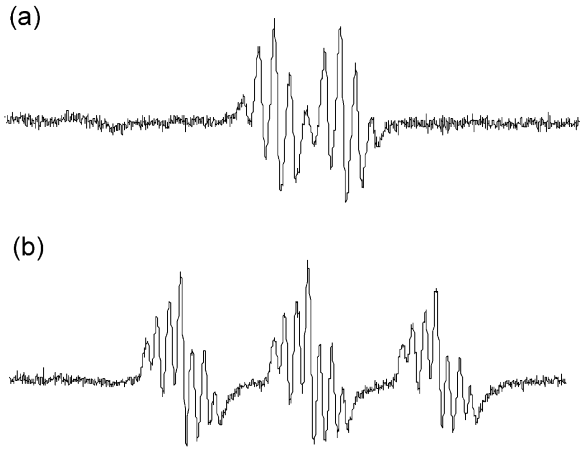

Figure 1. EPR spectra of (a) galvinoxyl and (b) radical species generated from incubation of galvinoxyl with compound **1** in DMSO at 25 °C. The EPR spectra were recorded on an X-band Bruker spectrometer: microwave frequency, 9.77 GHz; microwave power, 1.00 mW; modulation frequency, 100.0 kHz; modulation amplitude, 0.52 G.

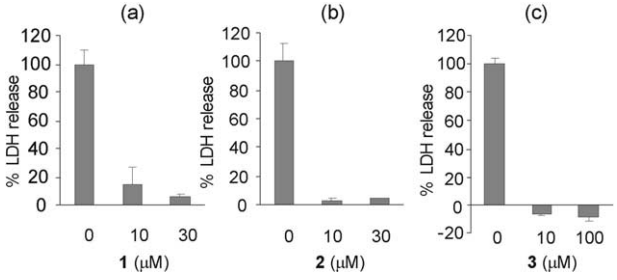

Figure 2. Neuroprotective effect of compounds **1**–**3** on neuronal cell death induced by Fe^{2+} ions.

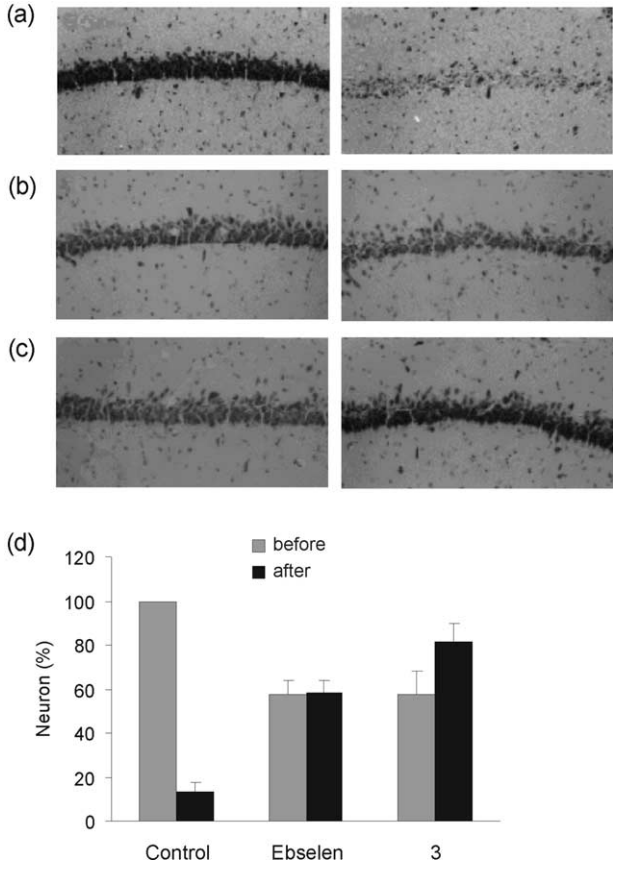

Figure 3. Staining images of the hippocampus of (a) sham (left) and global ischemic control (right), and gerbils suffering ischemic injury before (left) or after (right) treatment with (b) ebselen and (c) compound **3**. (d) Quantitative data of neuronal cell survival.

In summary, we developed novel antioxidants containing *N*-*t*-butyl-*N*-hydroxylaminophenyl moieties that afforded excellent neuroprotective activities against ROS and ischemic injury although in vivo neuroprotective mechanisms remain open to future studies.

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12. Twenty gerbils were treated with a single dose (60 mg/kg gerbil) of compound **3** prior and posterior to ischemic injury.