Article:
Kim, E.-S., Shin, Y., Kim, E.-H. et al. (4 more authors) (2022) Neuroprotective efficacy of N-t-butylhydroxylamine (NtBHA) in transient focal ischemia in rats. Toxicological Research, 38 (4). pp. 479-486. ISSN 1976-8257

https://doi.org/10.1007/s43188-022-00131-7

Neuroprotective efficacy of N-t-butylhydroxylamine (NtBHA) in transient focal ischemia in rats

Eun-Sun Kim[1], Yusun Shin[1], Eun-Hye Kim[1], Donghyun Kim[1], Milena De Felice[2], Arshad Majid[2,*], and Ok-Nam Bae[1,*]

1 College of Pharmacy, Institute of Pharmaceutical Science and Technology, Hanyang University, Ansan 15588, Korea

2 Sheffield Institute for Translational Neuroscience, University of Sheffield, Sheffield S10 2TN, UK

* Correspondence: arshad.majid@sheffield.ac.uk (A.M.); onbae@hanyang.ac.kr (O.-N.B)

Abstract

The pharmacological or toxicological activities of the degradation products of drug candidates have been unaddressed during the drug development process. Ischemic stroke accounts for 80% of all strokes and is responsible for considerable mortality and disability worldwide. Despite decades of research on neuroprotective agents, tissue plasminogen activators (t-PA), a thrombolytic agent, remains the only approved acute stroke pharmacological therapy. NXY-059, a free radical scavenger, exhibited striking neuroprotective properties in preclinical models and met all the criteria established by the Stroke Academic Industry Roundtable (STAIR) for a neuroprotective agent. In phase 3 clinical trials, NXY-059 exhibited significant neuroprotective effects in one trial (SAINT-I), but not in the second (SAINT-II). Some have hypothesized that N-t-butyl hydroxylamine (NtBHA), a breakdown product of NXY-059 was the actual neuroprotective agent in SAINT-I and that changes to the formulation of NXY-059 to prevent its breakdown to NtBHA in SAINT -II was the reason for the lack of efficacy. We evaluated the neuroprotective effect of NtBHA in N-methyl-D-aspartate (NMDA)-treated primary neurons and in rat focal cerebral ischemia. NtBHA significantly attenuated infarct volume in rat transient focal ischemia, and attenuated NMDA-induced cytotoxicity in primary cortical neurons. NtBHA also reduced free radical generation and exhibited mitochondrial protection.

Key word: Ischemic stroke**,** Neuroprotection, N-t-butylhydroxylamine (NtBHA)

Introduction

Despite considerable preclinical promise, neuroprotective drugs have failed to exhibit efficacy in clinical trials. The only clinically approved treatment, tissue plasminogen activator (t-PA), has a short therapeutic time window and carries the risk of hemorrhage [1-3]. There is an urgent need for safe and effective neuroprotective stroke therapies [4, 5]. One of the major processes involved in neuronal cell death due to ischemic injury is free radical mediated neurotoxicity, and tissue oxidation caused by free radicals [6, 7]. Compounds that can trap free radicals could have significant potential for treating ischemic stroke [6].

NXY-059 is a nitrone-based free radical trapping agent that was developed to treat acute stroke patients and works by preventing the progression of free radical-induced damage in cerebral ischemia [8-10]. In studies using various experimental ischemic stroke models, NXY-059 exhibited robust efficacy by improving neurological impairment and reducing cerebral infarct volume [9-12]. As an adjunct to t-PA, it reduced bleeding and increased the therapeutic time window [8]. Taken together, the preclinical studies met the criteria established by the Stroke Academic Industry Roundtable (STAIR) for the development of neuroprotective agents [13].

These promising preclinical findings were also observed in a large phase 3 clinical trial, (SAINT-I). NXY-059 exhibited improved functional outcomes when administered to stroke patients within 6 h of stroke onset compared to placebo [14]. However, a subsequent trial, SAINT-II exhibited no difference between NXY-059 and placebo. Consequently, further development of NXY-059 was stopped [15]. Many reasons for the differences in the observed outcomes between SAINT-I and SAINT-II have been advanced. One proposed explanation is that the actual neuroprotective agent in SAINT-I was N-t-butylhydroxylamine (NtBHA) and not NXY-059. It was suggested that the use of an old and unstable formulation of NXY-059 in SAINT-I (but not SAINT-II), allowed the degradation of NXY-059 to a powerful antioxidant,

(NtBHA) during storage [16].

In this study, we investigated the protective effect of NtBHA on *in vitro* and *in vivo* models of stroke and explored potential mechanisms of action.

Materials and Methods

Animals

The animal experiments carried out in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Hanyang University. For the ischemic stroke model, 250-270 g male Sprague-Dawley rats (Koatech, Pyeongtaek, Korea) were used. For primary cortical neuronal cultures, C57/BL6 female and male mice (Koatech) were mated to obtain newborn pups.

Blinding of randomization

Experimental animals were randomly assigned to treatment and outcomes were evaluated by blinded assessors.

Drug Administration schedule

NtBHA was obtained from Sigma-Aldrich (St. Louis, MO, USA), and dissolved in distilled water. NtBHA was administered intravenously in the tail vein 3 h after onset of ischemia at 10 mg/kg IV bolus. Carnosine, obtained from Sigma-Aldrich, was used as a positive control [17, 18].

Induction of MCAo (Middle Cerebral Artery Occlusion)

Rats were anesthetized with 5% isoflurane and maintained under 2.5% isoflurane anesthesia during surgery [17]. Occlusion of middle cerebral artery was induced by an intraluminal insertion of silicon-coated monofilament suture [19]. Rectal temperature was maintained at 37°C. Cerebral blood flow (CBF) was monitored by laser Doppler (Perimed, North Royalton, OH, USA) and measured pre, during and after reperfusion. A monofilament (Doccol Corp,

Redlands, CA, USA). was inserted into the common carotid artery (CCA), and advanced 18 mm into the internal carotid artery (ICA). The monofilament was removed 3 h after ischemia induction and blood flow was monitored.

Quantification of infarct volume by TTC staining

Twenty-four hours after induction of ischemia, animals were anesthetized with 5% isoflurane and euthanized by decapitation. The rapidly removed brain is sliced into thin tissue using a matrix slicer to obtain a total of five slices. Brain tissue was stained in 2,3,5-triphenyl tetrazolium chloride (TTC) solution at 37°C for 5 min to identify the region of cerebral infarction, and then fixed in 4% paraformaldehyde [19]. The fixed brain slices were analyzed for cerebral infarct volume using the Image J. The hemisphere area for each section was calculated by averaging the measured areas on each side of the section.

Primary cortical neuronal cultures

Primary cortical neurons were isolated from newborn mouse pups and maintained to investigate N-methyl-D-aspartate (NMDA)-induced neuronal damage [20]. Cerebral cortices isolated from newborn mouse pups were dissociated at 37°C for 30 min in dissection medium (Na_2SO_4 81.8 mM, K_2SO_4 30 mM, $MgCl_2$ 5.8 mM, $CaCl_2$ 0.252 mM, HEPES 1.5 mM, glucose 20 mM, and phenol red 0.001%, pH 7.6) supplemented with L-cysteine (4 mM), papain (10 U/ml) and DNase (1000 U/ml). The dissociated cells were washed by Neurobasal A and then pulverized by pipette, and the cells were seeded onto well plates coated with poly-D-lysine. On days 3 and 6 after seeding, 50% of the medium was replaced with Neurobasal A containing B27. Neuronal cultures were maintained in a 5% CO_2/95% air incubator at 37°C and experiments were conducted between 8 and 10 days in vitro. NtBHA (5 μM) or NXY-059 (5 μM) was treated to cells before and after NMDA exposure.

Assessment of cell viability

Cell viability was analyzed by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [21]. Neuronal cells were seeded in 24 well plates at a density of 1 x 10^5 cells / well and incubated at 37°C for 9 days. Cells were exposed to NMDA for 20 min, then replaced with the previously collected medium and maintained in a CO_2 incubator for 24 h. MTT at a final concentration of 5 mg/mL was added to each well and incubated at 37°C for 2 h. Insoluble formazan was dissolved by adding 100 µL of DMSO and absorbance as measured at 570 nm using an EnSpire multimode spectrophotometer (PerkinElmer, Santa Clara, CA, USA).

Cell death analysis

To assess NMDA-induced cytotoxicity, cell death was measured using propidium iodide (PI) staining [22]. After exposure to NMDA cytotoxicity, PI (final concentration 5 µg/mL) was added to each well and maintained for 30 min at 37°C. Fluorescence images of PI-stained cells were determined by fluorescence microscopy (Nikon Eclipse Ti, Nikon, Japan). Nine visual fields were randomly selected in each well. Selected fluorescence images were analyzed for fluorescence image signal scores through a region of interest (ROI) statistical program.

Measurement of reactive oxygen species (ROS)

ROS production during NMDA-induced cytotoxicity was investigated by CM-H2-DCFDA (DCF) staining assay [23]. After cytotoxicity induction, DCF (final concentration 10 µM) was added to each well and the cells were incubated in the dark for 20 min at 37°C. ROS stained with DCF showed green fluorescence and the image was confirmed by fluorescence microscope (Nikon Eclipse Ti). The fluorescence signal score of the randomly obtained images was analyzed through a region of interest (ROI) statistics program for fluorescence microscopy.

Evaluation of Mitochondrial membrane potential

JC-1 staining was used to assess mitochondrial membrane potential [24]. JC-1 (final concentration 5 μM) was added to each well and incubated in the dark for 30 min at 37°C. The red filter stained with JC-1 was a dimer formed from the normal membrane potential of mitochondria, and the monomer form due to mitochondrial destruction was observed as a green filter. Fluorescence images obtained through fluorescence microscopy (Nikon Eclipse Ti) were analyzed by the region of interest (ROI) program of interest and the ratio of red/green fluorescence intensity was calculated to confirm mitochondrial membrane potential.

Data analysis

Mean and standard error of the mean (SEM) were calculated for all experimental groups. A t-test was performed to determine the statistical significance between the treatment groups, and in all cases, a p-value of < 0.05 was considered significant.

Results

Efficacy in transient focal ischemia

The neuroprotective effect of NtBHA was determined using rat transient focal ischemia. Cerebral blood flow (CBF) before, during ischemia and during reperfusion were monitored by laser doppler (Fig 1A). No differences were observed between groups. At 3 h after induction of ischemia, NtBHA and carnosine (positive control) were administered via tail veins and the monofilaments were removed to attain reperfusion. NtBHA significantly reduced cerebral infarction by 21.5 % compared to control (Fig 1B).

Effect of NtBHA on N-methyl-D-aspartate (NMDA)-induced neurotoxicity in primary cortical neurons

Primary mouse cortical neurons were used to investigate the protective potential of NtBHA or NXY-059 against NMDA toxicity. PI staining and MTT assays were performed to measure the extent of NMDA-induced cellular injury and death in the presence and absence NtBHA or NXY-059. Treatment with NMDA for 20 min induced significant cell death and decreased cell viability (Fig 2A, B). Treatment with NtBHA (5 μM) significantly reduced NMDA-induced neurotoxicity both in PI-staining and MTT assay (Fig 2A, B), while NXY-059 (5 μM) failed to show statistically significant improvement of neuronal cell damage.

Effect of NtBHA on NMDA-induced oxidative stress and mitochondrial damage in primary cortical neurons

To explore the mechanism of action of NtBHA, we investigated the antioxidative effect of NtBHA or NXY-059, by measuring ROS generation in neurons after NMDA exposure using DCF fluorescence staining. NtBHA, but not NXY-059, significantly attenuated the NMDA-induced increase in ROS generation in neurons (Fig 3).

Since mitochondrial damage resulting from ROS generation is an important mediator of cellular injury and death, we investigated whether NtBHA reduces damage to mitochondrial membrane potential. We used JC-1 fluorescence staining to assess mitochondrial health. Red JC-1 fluorescence indicates normal mitochondrial membrane potential, and green fluorescence indicates the presence of monomer forms (impaired low membrane potential). Green fluorescence was increased after NMDA exposure confirming impairment in mitochondrial membrane potential. Treatment with NtBHA and NMDA simultaneously increased red fluorescence (Fig 4), suggesting that NtBHA protects against NMDA-induced impairment of mitochondrial membrane potential. Neuronal cells treated with NXY-059 showed a trend in the restoration of disrupted mitochondrial membrane potential, however, the effect did not reach statistical significance.

Taken together, these results indicate that NtBHA attenuates NMDA-induced cell death and injury by decreasing ROS generation and protection mitochondrial membrane potential.

Discussion

As seen in recent drug product recalls due to the genotoxic potential of the nitrosamine impurities [25], the degradation products and/or impurities of drugs or drug candidates may play a critical role in the pharmacological or toxicological activities of concern. The primary goal of this study was to determine the neuroprotective potential of NtBHA which may provide clues to why NXY-059 exhibited efficacy in SAINT-I but not SAINT-II.

NXY-059, a free radical trapping agent, is a nitrone-based drug that was selected for clinical trials after extensive preclinical testing in accordance with STAIR guidelines [13]. However, NXY-059 is unstable and is broken down to NtBHA which has substantial radical scavenging properties [16]. In this study, we investigated the protective effects of NtBHA using both in vitro and in vivo models of experimental stroke. Our data show that NtBHA attenuated infarct volume in transient ischemia. It may be possible that previous studies used NXY-059 had degraded to NtBHA and that the active drug may be NtBHA.

NtBHA itself is a potent radical scavenger, and NtBHA oxidizes to 2-methyl-2-nitrosopropane (MNP). MNP can be reduced back to NtBHA by mitochondria, or it can synthesize nitric oxidie (NO) that dilates the blood vessels [8]. MNP also neutralizes reactive free radicals by forming spin adducts with them [16]. Thus, it is possible that the observed efficacy in SAINT-I may have been due to NtBHA, generated by hydrolysis of NXY-059. The patent application of Astra Zeneca states that α-(2,4-disulfophenyl)-N-tertbutylnitrone and its salts are easily degraded and that the shelf-life of such formulations is very short. The formulation of NXY-059 was changed for SAINT-II to reduce hydrolysis and this may explain the lack of efficacy in SAINT II. Interestingly, consistent with this view, NtBHA has been suggested as an effective decomposition product of α-phenyl-N-t-butyl nitrone (PBN) in delaying senescence in human lung fibroblasts [26].

The restoration of cell viability, the reduction of cellular ROS generation, and the reverse of impaired mitochondrial membrane potential were found in NtBHA-treated cortical neurons after NMDA stimulation. NXY-059 treatment did not demonstrate a statistically significant difference compared to the NMDA-treated group. At least in our experimental system, NtBHA was found to be more potent in neuroprotection than NXY-059. Still, our data do not mean that the preclinical *in vivo* efficacy of NYX-059 in the previous reports were absolutely mediated by the effect of NtBHA. Direct comparison of *in vivo* efficacy of NXY-059 and NtBHA would be worthy to be investigated in future studies with optimization of dose/ durations of treatment of each drug.

Oxidative stress is a major mediator of cerebral ischemia/reperfusion injury [27]. Transient cerebral ischemia induces the production of excessive amounts of ROS into ischemic tissue during reperfusion [28-30]. During ischemia/reperfusion extracellular glutamate accumulates and induces over stimulation of several receptors but particularly of N-methyl-D-aspartate (NMDA) receptors. Excessive activation of NMDA receptors is a major step leading to neuronal damage after stroke [31]. In this study, we used NMDA-induced cell damage in mouse cerebral cortical neurons to explore the protective effects of NtBHA. Our results showed that NtBHA significantly reduced NMDA-induced cytotoxicity. The influx of calcium through the NMDA receptor results in a steady increase in the calcium of the mitochondria, which enhances ROS production and disruption of the mitochondrial membrane potential [31]. Increases in ROS formation in brain tissue after ischemia have been widely documented [32]. The brain is particularly vulnerable to oxidative stress because of its high consumption of oxygen and low endogenous antioxidant levels [33, 34]. Oxidative stress created by excessive ROS oxidizes major cellular components such as lipids, proteins and DNA, leading to cellular damage due to extensive damage [35]. Thus, reducing ROS production following reperfusion could be an effective treatment for ischemic stroke [36]. Our results show that NtBHA inhibits the

production of ROS. Intracellular ROS production induced by NMDA leads to a decrease in mitochondrial membrane potential. Mitochondrial dysfunction due to reduced mitochondrial membrane potential results in biochemical sequalae such as release of inducers of apoptosis and induction of cell suicide [37]. We showed that NtBHA attenuates NMDA-induced mitochondrial membrane potential reduction.

There are several papers regarding the bioactivities of NtBHA, and we could not exclude the possibilities that these effects may potentially contribute to the neuroprotective action of NtBHA against ischemic stroke. NtBHA has been suggested to generate nitric oxide (NO) [26, 38], a potent vasodilator, which could affect the blood flow in penumbra or ischemic region. NtBHA attenuated apoptotic pathways including the release of cytochrome C or activation of caspase-3, up-regulation of Bax, and down-regulation of Bcl-2 in isolated islet cells or U937 cells [39-41], and these anti-apoptotic effects may help to reduce brain infarct. NtBHA delayed senescence-associated changes in mitochondria and cellular senescence [42]. Further studies warrant the detailed mechanism for *in vivo* neuroprotection of NtBHA.

Although this study only demonstrated the neuroprotective effects of NXY-059, a further toxicological study of NXY-059 needs to be considered. Along with recent advances in the biological role and therapeutic potential of NO donors as well as nitrone-based drugs, the precise evaluation of potential toxicity would be required to enhance the clinical importance of these drugs.

In conclusion, we demonstrated that NtBHA reduced brain injury in transient focal ischemia. This protective effect may be mediated by reduction in ROS generation and reduced mitochondrial injury. These results provide the first evidence that NtBHA produced during the hydrolysis of NXY-059 could account for the neuroprotection observed in SAINT-I but not SAINT-II.

Declarations

The authors declare no conflict of interests or competing interests.

Acknowledgements

This work was supported by a grant from the Ministry of Health and Welfare of Korea (HI14C2180) and grants from the National Research Foundation of Korea, supported by the Ministry of Science, ICT & Future Planning (NRF-2017R1C1B3002626). A.M. was partially supported/funded by the NIHR Sheffield Biomedical Research Centre (BRC). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health and Social Care (DHSC).

Figures and Figure legends

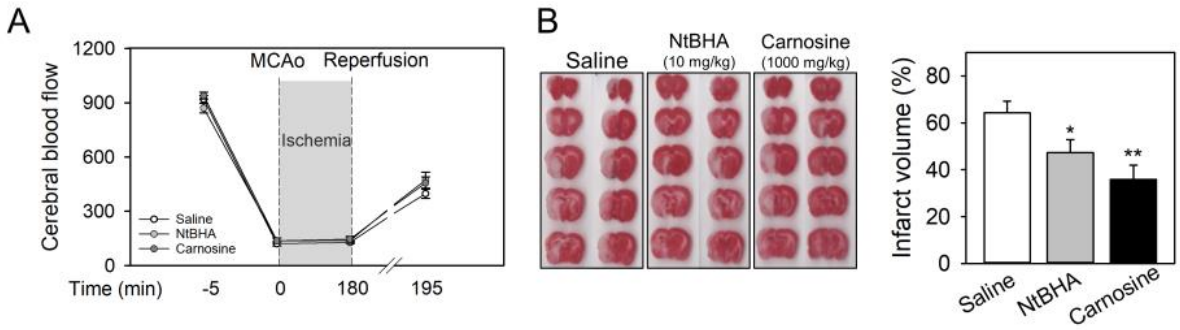

Figure 1. The protective effect of NtBHA on infract volume after transient focal ischemia

NtBHA (10 mg/kg) and carnosine (positive control; 1,000 mg/kg) were intravenous administered at 3 h after MCAO induction, and the brain was isolated after 3 h ischemia/ 21 h reperfusion. (A) Changes in blood flow by ischemic induction and reperfusion were monitored in real time. (B) Cerebral infarction volume was calculated using the Image J program after TTC staining. All experimental values were analyzed by ± SEM and t-test. *$p<0.05$, **$p<0.01$ vs. control. N=15 rats/group.

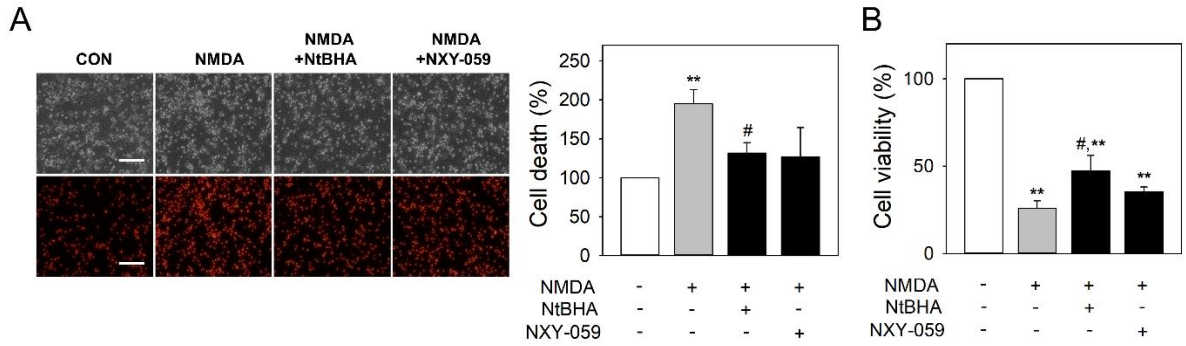

Figure 2. Protective effect of NtBHA on NMDA-induced cytotoxicity in primary neuronal cells.

Primary neuronal cells isolated from mice were treated with NtBHA (5 μM) or NXY-059 (5 μM) for 24 h before and after NMDA exposure. (A) Cell death was observed by PI staining. Fluorescence image signal score was analyzed through the area of interest program. Scale bar; 100 μm. (B) Cell viability in primary neuronal cells was measured by MTT assay. All experimental values were analyzed by ± SEM and t-test. *$p<0.05$, **$p<0.01$ vs. control. #$p<0.05$ vs. NMDA-treated cells. N=3.

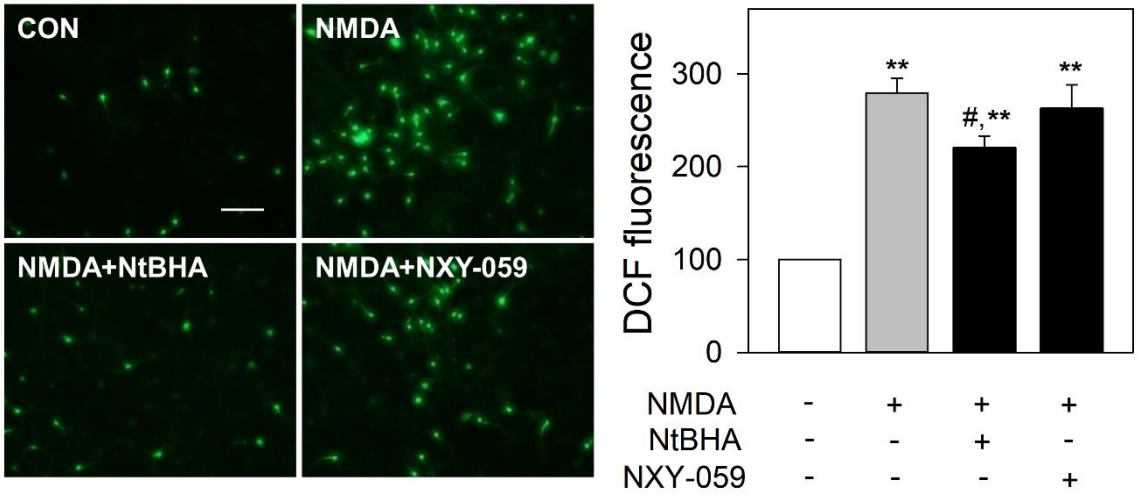

Figure 3. Protective effect of NtBHA on NMDA-induced generation of reactive oxygen species in primary neuronal cells.

Generations of cellular reactive oxygen species (ROS) after stimulation of NMDA was monitored by DCF fluorescence staining. Fluorescence images were analyzed through the area of interest program. Scale bar; 100 μm. All experimental values were analyzed by ± SEM and t-test. **p<0.01 vs. control. #p<0.05 vs. NMDA-treated cells. N=3.

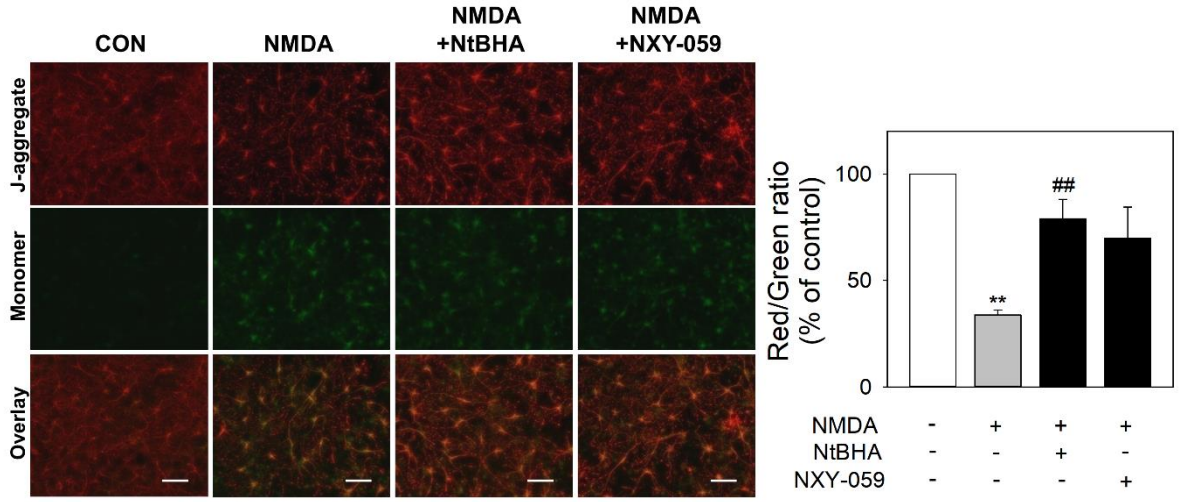

Figure 4. Protective effect of NtBHA on NMDA-induced perturbation of mitochondrial membrane potential in primary neuronal cells.

The mitochondrial membrane potentials analyzed by JC-1 show red fluorescence in the aggregate form of JC-1 at the normal membrane potential and green fluorescence in the monomer form at the damaged low membrane potential. Fluorescence images were analyzed through the area of interest program. Scale bar; 100 μm. All experimental values were analyzed by ± SEM and t-test. **$p<0.01$ vs. control. ##$p<0.01$ vs. NMDA-treated cells. N=3.

References

1. Bae ON and Majid A (2013) Role of histidine/histamine in carnosine-induced neuroprotection during ischemic brain damage. Brain Res 1527:246-54. 10.1016/j.brainres.2013.07.004
2. Hacke W, Donnan G, Fieschi C, Kaste M, von Kummer R, Broderick JP, Brott T, Frankel M, Grotta JC, Haley EC, Jr., Kwiatkowski T, Levine SR, Lewandowski C, Lu M, Lyden P, Marler JR, Patel S, Tilley BC, Albers G, Bluhmki E, Wilhelm M, Hamilton S, Investigators AT, Investigators ET and Investigators Nr-PSG (2004) Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. Lancet 363:768-74. 10.1016/S0140-6736(04)15692-4
3. Wang M, Zhang Y, Feng L, Zheng J, Fan S, Liu J, Yang N, Liu Y and Zuo P (2017) Compound porcine cerebroside and ganglioside injection attenuates cerebral ischemia-reperfusion injury in rats by targeting multiple cellular processes. Neuropsychiatr Dis Treat 13:927-935. 10.2147/NDT.S129522
4. Lees KR, Davalos A, Davis SM, Diener HC, Grotta J, Lyden P, Shuaib A, Ashwood T, Hardemark HG, Wasiewski W, Emeribe U, Zivin JA and Investigators SI (2006) Additional outcomes and subgroup analyses of NXY-059 for acute ischemic stroke in the SAINT I trial. Stroke 37:2970-8. 10.1161/01.STR.0000249410.91473.44
5. Muir KW, Lees KR, Ford I, Davis S and Intravenous Magnesium Efficacy in Stroke Study I (2004) Magnesium for acute stroke (Intravenous Magnesium Efficacy in Stroke trial): randomised controlled trial. Lancet 363:439-45. 10.1016/S0140-6736(04)15490-1
6. Sydserff SG, Borelli AR, Green AR and Cross AJ (2002) Effect of NXY-059 on infarct volume after transient or permanent middle cerebral artery occlusion in the rat; studies on dose, plasma concentration and therapeutic time window. Br J Pharmacol 135:103-12. 10.1038/sj.bjp.0704449
7. Lees KR, Zivin JA, Ashwood T, Davalos A, Davis SM, Diener HC, Grotta J, Lyden P, Shuaib A, Hardemark HG, Wasiewski WW and Stroke-Acute Ischemic NXYTTI (2006) NXY-059 for acute ischemic stroke. N Engl J Med 354:588-600. 10.1056/NEJMoa052980
8. Slemmer JE, Shacka JJ, Sweeney MI and Weber JT (2008) Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging. Curr Med Chem 15:404-14. 10.2174/092986708783497337
9. Lees KR, Sharma AK, Barer D, Ford GA, Kostulas V, Cheng YF and Odergren T (2001)

Tolerability and pharmacokinetics of the nitrone NXY-059 in patients with acute stroke. Stroke 32:675-80. 10.1161/01.str.32.3.675

10. Lees KR, Barer D, Ford GA, Hacke W, Kostulas V, Sharma AK, Odergren T and Investigators S-N- (2003) Tolerability of NXY-059 at higher target concentrations in patients with acute stroke. Stroke 34:482-7. 10.1161/01.str.0000053032.14223.81
11. Bath PM, Gray LJ, Bath AJ, Buchan A, Miyata T, Green AR and Investigators NXYEM-aiIAwS (2009) Effects of NXY-059 in experimental stroke: an individual animal meta-analysis. Br J Pharmacol 157:1157-71. 10.1111/j.1476-5381.2009.00196.x
12. Green AR, Ashwood T, Odergren T and Jackson DM (2003) Nitrones as neuroprotective agents in cerebral ischemia, with particular reference to NXY-059. Pharmacol Ther 100:195-214. 10.1016/j.pharmthera.2003.07.003
13. Macleod MR, van der Worp HB, Sena ES, Howells DW, Dirnagl U and Donnan GA (2008) Evidence for the efficacy of NXY-059 in experimental focal cerebral ischaemia is confounded by study quality. Stroke 39:2824-9. 10.1161/STROKEAHA.108.515957
14. Diener HC, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, Shuaib A, Ashwood T, Wasiewski W, Alderfer V, Hardemark HG, Rodichok L, Saint I and Investigators II (2008) NXY-059 for the treatment of acute stroke: pooled analysis of the SAINT I and II Trials. Stroke 39:1751-8. 10.1161/STROKEAHA.107.503334
15. Hill MD (2007) Stroke: the dashed hopes of neuroprotection. The Lancet Neurology 6:2-3. 10.1016/s1474-4422(06)70658-5
16. Proctor PH and Tamborello LP (2007) SAINT-I worked, but the neuroprotectant is not NXY-059. Stroke 38:e109; author reply e110. 10.1161/STROKEAHA.107.489161
17. Kim ES, Kim D, Nyberg S, Poma A, Cecchin D, Jain SA, Kim KA, Shin YJ, Kim EH, Kim M, Baek SH, Kim JK, Doeppner TR, Ali A, Redgrave J, Battaglia G, Majid A and Bae ON (2020) LRP-1 functionalized polymersomes enhance the efficacy of carnosine in experimental stroke. Sci Rep 10:699. 10.1038/s41598-020-57685-5
18. Baek SH, Noh AR, Kim KA, Akram M, Shin YJ, Kim ES, Yu SW, Majid A and Bae ON (2014) Modulation of mitochondrial function and autophagy mediates carnosine neuroprotection against ischemic brain damage. Stroke 45:2438-2443. 10.1161/STROKEAHA.114.005183
19. Kim EH, Kim ES, Shin D, Kim D, Choi S, Shin YJ, Kim KA, Noh D, Caglayan AB, Rajanikant GK, Majid A and Bae ON (2021) Carnosine Protects against Cerebral

Ischemic Injury by Inhibiting Matrix-Metalloproteinases. Int J Mol Sci 22:10.3390/ijms22147495

20. Jain S, Kim ES, Kim D, Burrows D, De Felice M, Kim M, Baek SH, Ali A, Redgrave J, Doeppner TR, Gardner I, Bae ON and Majid A (2020) Comparative Cerebroprotective Potential of d- and l-Carnosine Following Ischemic Stroke in Mice. Int J Mol Sci 21:10.3390/ijms21093053
21. Lee H, Park J and Park K (2022) Assessment of agonistic and antagonistic properties of humidifier disinfectants to the estrogenic and androgenic receptors by transactivation assay. Toxicol Res 38:99-109. 10.1007/s43188-021-00111-3
22. Choi SM, Cho YS, Park G, Lee SK and Chun KS (2021) Celecoxib induces apoptosis through Akt inhibition in 5-fluorouracil-resistant gastric cancer cells. Toxicol Res 37:25-33. 10.1007/s43188-020-00044-3
23. Kim D, Kim EH, Choi S, Lim KM, Tie L, Majid A and Bae ON (2021) A Commonly Used Biocide 2-N-octyl-4-isothiazolin-3-oneInduces Blood-Brain Barrier Dysfunction via Cellular Thiol Modification and Mitochondrial Damage. Int J Mol Sci 22:10.3390/ijms22052563
24. Kim D, Kim EH and Bae ON (2021) Comparative study of two isothiazolinone biocides, 1,2-benzisothiazolin-3-one (BIT) and 4,5-dichloro-2-n-octyl-isothiazolin-3-one (DCOIT), on barrier function and mitochondrial bioenergetics using murine brain endothelial cell line (bEND.3). J Toxicol Environ Health A 84:932-943. 10.1080/15287394.2021.1955786
25. Bharate SS (2021) Critical Analysis of Drug Product Recalls due to Nitrosamine Impurities. J Med Chem 64:2923-2936. 10.1021/acs.jmedchem.0c02120
26. Atamna H, Paler-Martinez A and Ames BN (2000) N-t-butyl hydroxylamine, a hydrolysis product of alpha-phenyl-N-t-butyl nitrone, is more potent in delaying senescence in human lung fibroblasts. J Biol Chem 275:6741-8. 10.1074/jbc.275.10.6741
27. Ma S, Yin H, Chen L, Liu H, Zhao M and Zhang X (2012) Neuroprotective effect of ginkgolide K against acute ischemic stroke on middle cerebral ischemia occlusion in rats. J Nat Med 66:25-31. 10.1007/s11418-011-0545-7
28. Yamato M, Egashira T and Utsumi H (2003) Application of in vivo ESR spectroscopy to measurement of cerebrovascular ROS generation in stroke. Free Radic Biol Med 35:1619-31. 10.1016/j.freeradbiomed.2003.09.013

29. Abe K, Tonomura M, Ito M, Takai N, Imamoto N, Rokugawa T, Momosaki S, Fukumoto K, Morimoto K and Inoue O (2015) Imaging of reactive oxygen species in focal ischemic mouse brain using a radical trapping tracer [(3)H]hydromethidine. EJNMMI Res 5:115. 10.1186/s13550-015-0115-1
30. Terasaki Y, Liu Y, Hayakawa K, Pham LD, Lo EH, Ji X and Arai K (2014) Mechanisms of neurovascular dysfunction in acute ischemic brain. Curr Med Chem 21:2035-42. 10.2174/09298673216661312282234 00
31. Shen H, Yuan Y, Ding F, Hu N, Liu J and Gu X (2010) Achyranthes bidentata polypeptides confer neuroprotection through inhibition of reactive oxygen species production, Bax expression, and mitochondrial dysfunction induced by overstimulation of N-methyl-D-aspartate receptors. J Neurosci Res 88:669-76. 10.1002/jnr.22221
32. Chao X, Zhou J, Chen T, Liu W, Dong W, Qu Y, Jiang X, Ji X, Zhen H and Fei Z (2010) Neuroprotective effect of osthole against acute ischemic stroke on middle cerebral ischemia occlusion in rats. Brain Res 1363:206-11. 10.1016/j.brainres.2010.09.052
33. Margaill I, Plotkine M and Lerouet D (2005) Antioxidant strategies in the treatment of stroke. Free Radic Biol Med 39:429-43. 10.1016/j.freeradbiomed.2005.05.003
34. Schreibelt G, van Horssen J, van Rossum S, Dijkstra CD, Drukarch B and de Vries HE (2007) Therapeutic potential and biological role of endogenous antioxidant enzymes in multiple sclerosis pathology. Brain Res Rev 56:322-30. 10.1016/j.brainresrev.2007.07.005
35. Sugawara T and Chan PH (2003) Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. Antioxid Redox Signal 5:597-607. 10.1089/152308603770310266
36. Yang S and Li W (2016) Targeting oxidative stress for the treatment of ischemic stroke: Upstream and downstream therapeutic strategies. Brain Circulation 2:153. 10.4103/2394-8108.195279
37. Saeed SA, Shad KF, Saleem T, Javed F and Khan MU (2007) Some new prospects in the understanding of the molecular basis of the pathogenesis of stroke. Exp Brain Res 182:1-10. 10.1007/s00221-007-1050-9
38. Chamulitrat W, Jordan SJ, Mason RP, Saito K and Cutler RG (1993) Nitric oxide formation during light-induced decomposition of phenyl N-tert-butylnitrone. J Biol Chem 268:11520-7.
39. Zhao Z, Zhang X, Zhao C, Choi J, Shi J, Song K, Turk J and Ma ZA (2010) Protection

of pancreatic beta-cells by group VIA phospholipase A(2)-mediated repair of mitochondrial membrane peroxidation. Endocrinology 151:3038-48. 10.1210/en.2010-0016

40. Lee JH, Tak JK, Park KM and Park JW (2007) N-t-Butyl hydroxylamine regulates ionizing radiation-induced apoptosis in U937 cells. Biochimie 89:1509-16. 10.1016/j.biochi.2007.07.010

41. Kim HJ, Shin SW, Oh CJ, Lee MH, Yang CH and Park JW (2005) N-t-Butyl hydroxylamine regulates heat shock-induced apoptosis in U937 cells. Redox Rep 10:287-93. 10.1179/135100005X83662

42. Atamna H, Robinson C, Ingersoll R, Elliott H and Ames BN (2001) N-t-Butyl hydroxylamine is an antioxidant that reverses age-related changes in mitochondria in vivo and in vitro. FASEB J 15:2196-204. 10.1096/fj.01-0134com