

Short Communication

The Anticonvulsant Zonisamide Inhibits Hydroxyl Radical Generated from Methylguanidine

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Methylguanidine (MG) is a known nephrotoxin and neurotoxin, and an intracisternal injection of MG can induce convulsions in experimental animals. In this *in vitro* study, we examined the inhibitory effects of the antiepileptic agent zonisamide (ZNS) on hydroxyl radicals ($\bullet\text{OH}$) generated from MG by using an electron spin resonance (ESR) technique. ZNS scavenged $\bullet\text{OH}$ generated from MG in a dose-dependent manner through direct scavenging during the auto-oxidation of MG. The rate constant of ZNS reacting with the $\bullet\text{OH}$ was at a near diffusion-controlled rate. These findings indicate that ZNS might detoxify MG and could thus protect against convulsive disorders.

Key words: methylguanidine, zonisamide, convulsions, hydroxyl radical, electron spin resonance

Methylguanidine (MG) is a known nephrotoxin and neurotoxin. Patients with chronic uremia show markedly high plasma levels of MG, and uremic toxicity due to excessive MG levels is a characteristic feature in chronic renal failure [1–3]. Mori *et al.* [4] were the first to report that γ -guanidinobutyric acid (GGBA) induced convulsions in rabbits. Other guanidino compounds including MG were subsequently shown to induce convulsions in experimental animals [5,6].

Guanidino compounds including GGBA, arginine, taurocyamine, glyocyamine, and N-acetylarginine are normally present at low levels in human brain tissues [7]. However, the accumulation of such compounds in uremic patients and in experimental animal models of uremia could possibly trigger characteristic convulsions [6,8]. Highly elevated levels of guanidino compounds in the brain may also cause metabolic abnormalities such as altered neurotransmitter metabolism [9], and the intracisternal injection of these com-

pounds induces convulsions in rats and rabbits [10,11]. In addition, the brains of animal models of epilepsy and in amygdala- or hippocampus-kindled rats showed sustained increases in MG [12,13].

In previous *in vitro* studies, we showed that extremely high levels of MG and other guanidino compounds such as α -guanidinoglutaric acid (α -GGA) generate reactive oxygen species (ROS), *i.e.*, hydroxyl radicals ($\bullet\text{OH}$), and superoxide anion radicals ($\text{O}_2^{\bullet-}$), all of which can damage cell membranes and lead to neuronal depolarization, which in turn may induce epileptogenicity [14,15].

The anticonvulsant zonisamide (ZNS) scavenges $\bullet\text{OH}$ [16], which is the most damaging ROS. In the present study, we examined the inhibitory effect of ZNS on $\bullet\text{OH}$ generation by MG by using electron spin resonance (ESR) with a spin trap, 5,5'-dimethyl-1-pyrroline N-oxide (DMPO).

Materials and Methods

Chemicals. ZNS (1,2-benzisoxazole-3-methanesulfonamide) ($C_8H_8N_2O_3S$; MW 212.23) was obtained from Dainippon Sumitomo Pharmaceutical Co. (Osaka, Japan). MG hydrochloride (MW 109.56) was obtained from Tokyo Kasei Kogyo Co. (Tokyo). L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-2H-1-benzopyran-6-yl) hydrogen phosphate) potassium salt (EPC- K_1) (MW 706.90), which we first found as a standard material for $\bullet OH$ scavenging, was a kind gift from Dr. Kazumi Ogata (Oga Research, Osaka) [17]. Diethylenetriaminepentaacetic acid (DETAPAC) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and the spin trap DMPO was from Labotec Co. (Tokyo). All other chemicals were sourced from Wako Pure Chemical Industries (Osaka).

Hydroxyl radical ($\bullet OH$) generation from MG. The ESR measurements were performed as follows: An exact amount of MG (10 mg; final concentration, 0.46 M) was taken to a test tube followed by the buffer or 0.1% methylcellulose-1500 (MC) in de-ionized ultra-pure water (pure water) (30 μl) (for the sample solution: EPC- K_1 in the buffer or ZNS in MC; control: without sample), 8.9 M DMPO (20 μl), 0.1 M Tris buffer (pH 6.0, 100 μl), and 0.2 mM Fe^{2+} -DETAPAC in pure water (50 μl) (final volume, 200 μl). The solution of MC and the sample solutions were prepared just before analysis.

The mixture was quickly mixed using a vortex mixer and immediately put into an ESR quartz flat cell (capacity, 200 μl). ESR recordings were started immediately after the addition of Fe^{2+} -DETAPAC. The generation of $\bullet OH$ was measured as the spin adducts of DMPO (DMPO-OH). The intensity of the peak height of the DMPO-OH adducts was normalized against the third peak of Mn^{2+} in an internal standard manganese oxide (MnO) fitted to the ESR spectrometer. The pH of the reaction mixture was double-checked after each ESR measurement, using pH test paper.

Inhibitory effect of EPC- K_1 or ZNS on $\bullet OH$ generated from MG. (i) EPC- K_1 was dissolved in the buffer solution just before analysis. The diluted solutions were examined for the scavenging activity of $\bullet OH$ generated from MG as described. (ii) An appropriate amount of ZNS was precisely weighed, and added with 0.1% MC solution just before analysis. The

ZNS solution was diluted immediately before measurement with 0.1% MC (ZNS final concentrations, 0–30 mM; control: without ZNS), and the scavenging activity of $\bullet OH$ generated from MG was determined as described.

ESR measurements. ESR spectrometers (JES-FA100, JEOL, Tokyo) equipped with MnO as an internal standard were used. Basically, the conditions for the $\bullet OH$ generation from MG were as follows: microwave frequency, 9.4 GHz; microwave power, 8 mW; magnetic field center, 335.6 mT; sweep width, 10 mT; modulation width, 0.1 mT; modulation frequency, 100 kHz; response time, 0.1 sec; gain, 1×500 ; sweep time, 0.5 min; accumulation, 16 times; temperature, 23°C. The ESR recordings were performed at 16-times accumulation to enhance the intensity of the DMPO-OH signal.

In previous experiments [18,19], the intensity of the DMPO-OH signal gradually increased and reached a plateau around 8–10 min after the addition of Fe^{2+} , and thus the ESR signals were accumulated for 8 min. The DMPO-OH signal intensity was almost constant at the maximum level in the pH range of 5.5–6.5, and we therefore used pH 6.0 as an optimal experimental condition in this study.

Results

Hydroxyl radical ($\bullet OH$) generation from MG. Signals typical of the DMPO-OH adducts (peak height ratio 1 : 2 : 2 : 1, quartet) (Hyperfine condition of the DMPO-OH adduct: $a_N = 1.49$ mT, $a_H^\beta = 1.49$ mT) were observed when DMPO was added to MG followed by Fe^{2+} -DETAPAC (Fig. 1). The linear

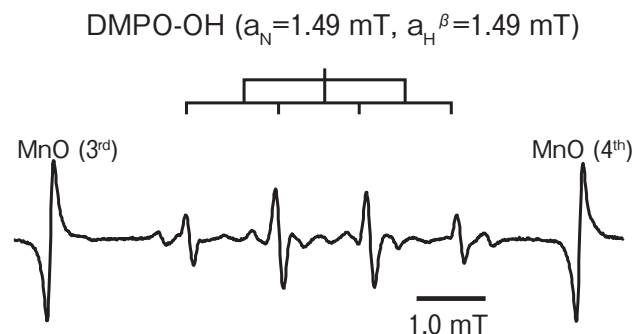


Fig. 1 Typical DMPO-OH ESR spectra generated from MG. The intensity of the DMPO-OH signal generated from MG (10 mg) (final concentration in assay, 0.46 M).

relationship was observed between the intensity of the DMPO-OH signals and the MG concentrations (0–0.92 M) as shown previously [18,19].

Inhibitory effect of EPC-K₁ on •OH generated from MG. EPC-K₁, known as a •OH scavenger,

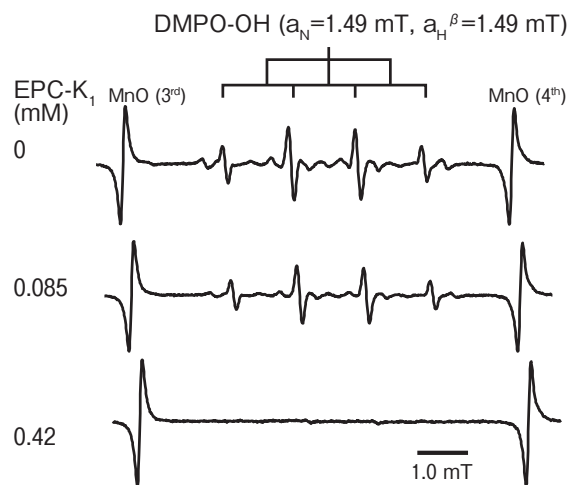


Fig. 2 Inhibitory effect of EPC-K₁ on •OH generated from MG. The intensity of DMPO-OH signals generated from MG (final concentration, 0.46 M) was recorded with or without EPC-K₁ (control: without EPC-K₁).

suppressed the generation of •OH from MG in a dose-dependent manner. The representative ESR spectra for various concentrations of EPC-K₁ (final concentrations, 0, 0.085 and 0.42 mM) are shown in Fig. 2. At these concentrations, EPC-K₁ suppressed 86% and 17% of •OH generation from MG, respectively.

Inhibitory effect of ZNS on •OH generated from MG.

We dissolved ZNS in 0.1% MC solution just before use, because it hardly dissolved at higher concentrations. ZNS inhibited •OH generated from MG in a dose-dependent manner (Fig. 3A, B), with an ID₅₀ of 18 mM. The estimated concentration of ZNS necessary to completely diminish •OH was 40 mM, based on the extension of the dose-response curve.

Discussion

EPC-K₁ (a phosphate diester of vitamins C and E) is a known •OH radical scavenger [17], and here it scavenged •OH generated from MG in a dose-dependent manner, with an estimated ID₅₀ of 200–250 μ M under the above-described experimental conditions. Unlike other •OH scavengers such as ascorbate, EPC-K₁ is not thought to act as a reductant, thus

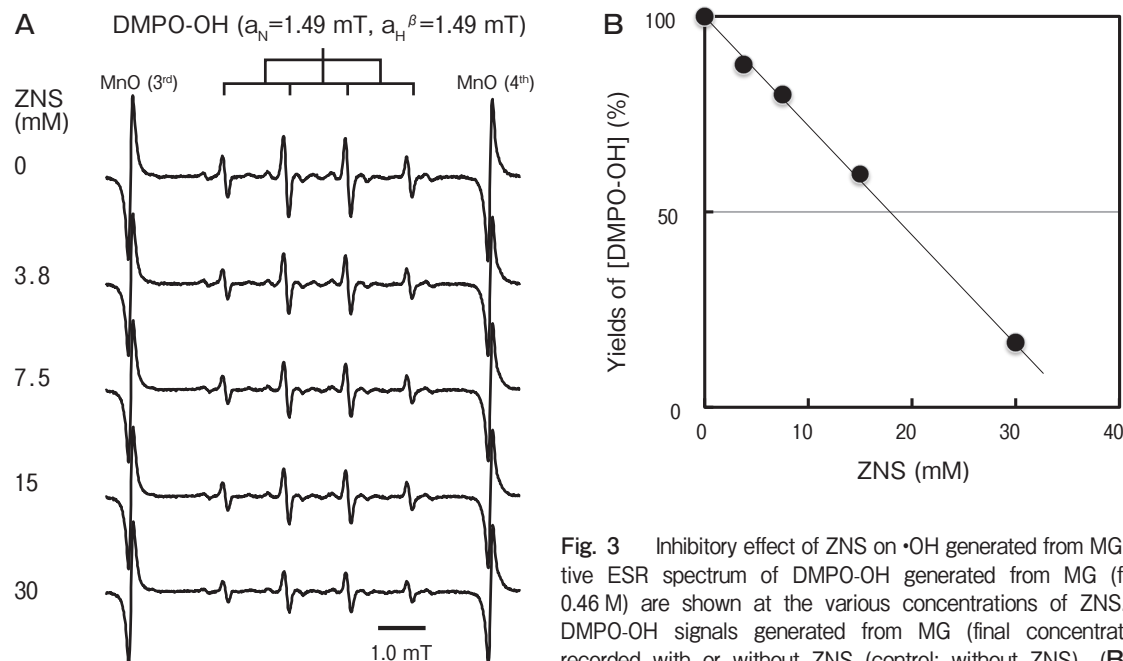


Fig. 3 Inhibitory effect of ZNS on •OH generated from MG. (A) A representative ESR spectrum of DMPO-OH generated from MG (final concentration, 0.46 M) are shown at the various concentrations of ZNS. The intensity of DMPO-OH signals generated from MG (final concentration, 0.46 M) was recorded with or without ZNS (control: without ZNS). (B) A representative dose-dependent curve for the inhibitory effect of ZNS on •OH generated from MG.

avoiding the possibility of reducing nitroxyl radicals or the DMPO-OH adducts, depending on the condition [20,21].

ZNS scavenged •OH generated by the Fenton reaction in a dose-dependent manner [16]. In our preliminary experiments on ZNS, we observed its direct •OH scavenging activity by changing the DMPO concentrations from 0.45 mM to 4.5 mM, and the value of ID₅₀ shifted by 10-fold when the concentration of DMPO was increased by 10-fold. Therefore, ZNS directly scavenges •OH when they are encountered, but does not interfere with the •OH generation system itself, such as via iron chelating [22]. The reaction rate constant k_2 in the present study was calculated as $2.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (the reaction rate constant of DMPO with •OH, $k_{\text{DMPO}} = 3.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [23]), and this was also confirmed by the estimation method used in our previous studies; *i.e.*, in the range of $10^9 - 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ [16], at near diffusion-controlled rates. The values of k_2 for some other known •OH scavengers are 2.7×10^{10} , 3.2×10^{10} , 4.7×10^9 , 3.7×10^9 , 2.7×10^9 , and $< 7.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for melatonin, salicylate, thiourea, sodium formate, mannitol, and urea, respectively [23,24].

In our previous studies [18,19], we found that the •OH generated from MG was diminished by the addition of either SOD (200 U/assay) or catalase (650 U/assay), effectively suppressed by ethanol (40% and 70% inhibition for 10% and 20% (v/v) ethanol, respectively) or dimethylsulfoxide (DMSO) (20% and 40% inhibition for 20% and 40% (v/v) DMSO, respectively), and slightly suppressed by mannitol (20% inhibition at a concentration of 280 mM), suggesting that MG oxidation may occur via the transient formation of $\text{O}_2^{\cdot-}$ from adjacent oxygen in the medium and then H_2O_2 generation, followed by the Fenton reaction to produce •OH with the existence of iron ions [14,15].

To examine the hypothesis [14,15] of MG oxidation via the formation of H_2O_2 followed by the generation of •OH under the existence of Fe^{2+} , we measured the H_2O_2 . By using highly sensitive fluorometry (detection limit: 0.1 nM), we detected H_2O_2 as the fluorescence quenching of chromotropic acid ($\lambda_{\text{ex}} = 440 \text{ nm}$; $\lambda_{\text{em}} = 235 \text{ nm}$) with a flow technology according to the method by Li *et al.* [25]. Here we observed that the test solution without MG (as a control) showed no H_2O_2 , whereas in the presence of MG, H_2O_2 was

detected in a dose-dependent manner.

Pyrogarol [26], homogentisic acid [27], and 6-hydroxyldopamine [28] are examples of agents known to non-enzymatically generate ROS via auto-oxidation, whereby $\text{O}_2^{\cdot-}$ is produced followed by •OH where the transition metal exists, although the underlying mechanisms might differ slightly among these agents and that of MG.

ZNS showed almost no scavenging activity against $\text{O}_2^{\cdot-}$, which reacts slowly with most substances and is thus less reactive, whereas ZNS directly scavenged •OH generated by the Fenton reaction [16].

MG levels increase in serum and urine with chronic renal failure in animal models and humans, and in epileptic foci of brain tissue in animal models [11,29]. Taking these findings together with the present results, we speculate that it is possible that ZNS contributes, at least in part, to suppressing excessive ROS generation in the presence of extreme levels of MG and particularly •OH, which is the most hazardous species for damaging biological membranes and thus for inducing neuronal depolarization and associated convulsive seizures.

In conclusion, in this *in vitro* study, ZNS inhibited •OH generated from MG—which is a known nephrotoxin and neurotoxin—in a dose-dependent manner, and ZNS directly scavenged •OH at near diffusion-control rates. These results suggest that ZNS may be beneficial, at least in part, for preventing MG-related convulsive disorders.

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References

1. Giovannetti S, Biagini M and Cioni L: Evidence that methylguanidine is retained in chronic renal failure. *Experientia* (1968) 24: 341–343.
2. Giovannetti S, Balestri PL and Barsotti G: Methylguanidine in uremia. *Arch Intern Med* (1973) 131: 709–713.
3. Yokozawa T, Mo ZL and Oura H: Comparison of toxic effects of methylguanidine, guanidinosuccinic acid and creatinine in rats with adenine-induced chronic renal failure. *Nephron* (1989) 51: 388–392.
4. Jinnai D, Sawai A and Mori A: γ -Guanidinobutyric acid as a convulsive substance. *Nature* (1966) 212: 617.
5. Mizuno A, Mukawa K, Kobayashi K and Mori A: Convulsive activity of taurocyamine in cats and rabbits. *IRCS Med Sci* (1975) 3: 385.
6. Mori A: Reactive oxygen species and mechanism of induction of seizure by guanidino compounds; in *Free Radicals in Brain*

- Physiology and Disorders, Yoshikawa T, Hiramatsu M and Packer L eds, Academic Press, San Diego (1996) pp 3–14.
7. Mori A, Hosotani M and Choong TL: Studies on brain guanidino compounds by automatic liquid chromatography. *Biochem Med* (1974) 10: 8–14.
 8. Matsumoto M, Kishikawa H and Mori A: Guanidino compounds in the sera of uremic patients and in the sera and brain of experimental uremic rabbits. *Biochem Med* (1976) 16: 1–8.
 9. Cubeddu L, Mosimann W and Weiner N: The effects of methylguanidine on norepinephrine metabolism in isolated, perfused spleen and heart. *J Pharmacol Exp Ther* (1974) 188: 93–106.
 10. Mori A, Watanabe Y and Akagi M: Guanidino compound anomalies in epilepsy; in *Advances in Epileptology; XIIIth Epilepsy International Symposium*, Akimoto H, Kazamatsuri H, Seino M and Ward M eds, Raven Press, New York (1982) pp347–351.
 11. Mori A: Biochemistry and neurotoxicology of guanidino compounds: History and recent advances. *Pavlov J Biol Sci* (1987) 22: 85–94.
 12. Hirayasu Y, Morimoto K and Otsuki S: Increase of methylguanidine and guanidinoacetic acid in the brain of amygdala-kindled rats. *Epilepsia* (1991) 32: 761–766.
 13. Shimizu Y, Morimoto K, Kuroda S and Mori A: Sustained increase of methylguanidine in the rats after amygdala and hippocampal kindling. *Epilepsy Res* (1995) 21: 11–17.
 14. Mori A, Kohno M, Masumizu T and Packer L: α -Guanidinoglutaric acid as a free radical generator. *Biochem Mol Biol Int* (1995) 37: 371–374.
 15. Mori A, Kohno M, Masumizu T, Noda Y and Packer L: Guanidino compounds generate reactive oxygen species. *Biochem Mol Biol Int* (1996) 40: 135–143.
 16. Mori A, Noda Y and Packer L: The anticonvulsant zonisamide scavenges free radicals. *Epilepsy Res* (1998) 30: 153–158.
 17. Mori A, Edamatsu R, Kohno M and Ohmori S: A new hydroxyl radical scavenger: EPC-K1. *Neurosciences* (1989) 15: 371–376.
 18. Noda Y, Murakami S, Mankura M and Mori A: Inhibitory effect of fermented papaya preparation on hydroxyl radical generation from methylguanidine. *J Clin Biochem Nutr* (2008) 43: 185–190.
 19. Noda Y and Mankura M: Inhibitory effect of antioxidants on hydroxyl radical generation from methylguanidine: an ESR study. *Neurochem Res* (2009) 34: 734–738.
 20. Buettner GR: The pecking order of free radicals and antioxidants: lipid peroxidation, α -tocopherol, and ascorbate. *Arch Biochem Biophys* (1993) 300: 535–543.
 21. Quintanilha AT and Packer L: Surface localization of sites of reduction of nitroxide spin-labeled molecules in mitochondria. *Proc Natl Acad Sci USA* (1977) 74: 570–574.
 22. Kimura K: Mechanism of active oxygen species reduction by non-steroidal anti-inflammatory drugs. *Int Biochem Cell Biol* (1997) 29: 437–446.
 23. Halliwell B and Gutteridge JMC: *Free Radicals in Biology and Medicine*, 3rd Ed. Oxford University Press, New York (1999) pp357–358.
 24. Matuszak Z, Reszka KJ and Chignell CF: Reaction of melatonin and related indoles with hydroxyl radicals: EPR and spin trapping investigations. *Free Radic Biol Med* (1997) 23: 367–372.
 25. Li ZH, Li DH, Oshita K and Motomizu S: Flow-injection determination of hydrogen peroxide based on fluorescence quenching of chromotropic acid catalyzed with Fe (II). *Talanta* (2010) 82: 1225–1229.
 26. Siegel SM and Siegel BZ: Autoxidation of pyrogallol: general characteristics and inhibition by catalase. *Nature* (1958) 181: 1153–1154.
 27. Martin JP Jr and Batkoff B: Homogentisic acid autoxidation and oxygen radical generation: Implications for the etiology of alkaptonuric arthritis. *Free Radic Biol Med* (1987) 3: 241–250.
 28. Adams RN, Murrill E, McCreery R, Blank L and Karolczak M: 6-Hydroxydopamine, a new oxidation mechanism. *Eur J Pharmacol* (1972) 17: 287–292.
 29. Matsumoto M, Kishikawa H and Mori A: Guanidino compounds in the sera of uremic patients and in the sera and brain of experimental uremic rabbits. *Biochem Med* (1976) 16: 1–8.