Trakia Journal of Sciences, Vol. 8, Suppl. 2, pp 144-148, 2010

Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

GENERATION OF LIPID RADICALS IN MICE LIVER AND KIDNEY TISSUES AFTER TREATMENT BY SPIN LABELLED NITROSOUREAS – AN EX VIVO EPR SPECTROSCOPY STUDY

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ABSTRACT

Formerly by *in vitro* EPR spin-trapping technique we have demonstrated that nitroxyl free radical containing (spin-labeled) nitrosoureas 1-ethyl-1-nitroso-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-urea (SLENU) and N-[N'-(2-chloroethyl)-N'-nitrosocarbamoyl]-glycine amid of 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (SLCNUgly) possessed excellent superoxide anion scavenging activity (SSA). The aim of the present study was to investigate generation of lipid radicals in liver and kidney tissues of mice treated by SLENU or SLCNUgly, by EPR spin-trapping technique. N-tert-butyl-alpha-penylnitrone (PBN) was used as a spin-trapping agent.

The higher levels of lipid radicals in both kind of tissues were registered in mice treated by SLCNUgly comparing to those of the controls and mice treated by SLENU.

Based on these preliminary EPR spectroscopy results it might be concluded that the lower levels of lipid radicals generated in livers and kidneys of mice treated by SLENU might be explained by its slight alkylating activity when is compared to that of the other spin labeled nitrosourea SLCNUgly.

Key words: EPR spectroscopy, spin labeled, antitumor drug, lipid radicals, spin-trapping agent

INTRODUCTION

2-chloroethylnitrosourea drugs such as N'-cyclohexyl-N-(2-chloroethyl)-N-nitrosourea (CCNU), N,N'-bis (2-chloroethyl)-N-nitrosourea (BCNU) and N'-(trans-4-methyl cyclohexyl)-N-(2-chloroethyl)-N-nitrosourea (MeCCNU), exhibit comparatively good therapeutic properties against human cancer mainly lymphomas, gliomas, a few solid tumors and melanomas (1, 2). Clinical application of these antitumor drugs have been limited because their delayed and cumulative hematological toxicity (3). It was found that introducing of stable nitroxyl radical moieties such as 2,2,6,6-tetramethyl-4-aminopiperidin-1-oxyl (4-amino-TEMPO) in the structure of CCNU led to decrease of its *in vivo* general toxicity and improved its antitumor activity against some experimental tumor models in mice (4, 5). Moreover, a number of studies on biological activity of stable nitroxide radicals, demonstrated that these class of radicals possess radiosensible properties. Having in mind biological activity of the nitroxide radicals we have synthesized two different classes of spin labeled nitrosourea derivatives: first class as spin labeled antioxidants with radiosensibility properties and the second as potential antitumor agents.

For further biological and oncopharmakological studies have been selected both nitrosoureas:1-ethyl-1-nitroso-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-urea (SLENU) an representative of the spin labeled antioxidants and N-[N'-(2-chloroethyl)-N'-nitrosocarbamoyl]-glycine amid of 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (SLCNUgly) an representative of the potential antitumor agents **(Figure 1)**.

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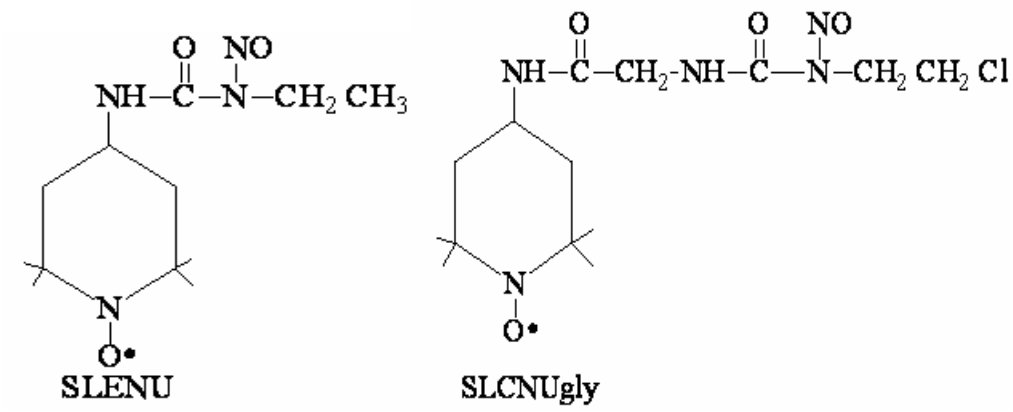


Figure 1. Chemical structures of spin labelled nitrosoureas

It was established that SLENU could successfully prevented *in vivo* oxidative damages induced by clinically used nitrosourea drug CCNU (6), while for the other nitrosourea was found that possessed *in vivo* high antitumor activity and comparatively lower general toxicity comparing to its clinically used analogue CCNU (7). Moreover, the nitrosoureas SLENU and SLCNUgly exhibited *in vitro* good superoxide anion scavenging activity (SSA) (8, 9).

The aim of the present study was, using proper EPR spectroscopy method to evaluate how the *in vitro* determined good SSA activity of SLENU and SLCNUgly would affect their behaviors at *in vivo* conditions.

MATERIALS AND METHODS

Chemicals

Nitrosoureas SLCNUgly and SLENU **(Figure 1)** were synthesized as previously described (7, 8). The spin-trapping agent, PBN and dimethylsulfoxide (DMSO) were purchased by Sigma Chemical Co, St. Louis, USA. All other chemicals used in this study were analytical grade.

Animals and treatment

White laboratory mice with weight 20-40 g were used. The mice were housed in polycarbonate cages in controlled conditions (12 h light/dark cycles), temperature of 18–23^{o}C and humidity of 40–70%, with free access to tap water and standard laboratory chow. Experiments were carried out in accordance with European directive 86/609/EEC of 24.11.1986 for protection of animals used in scientific and experimental purposes. Mice were divided in two groups (5 mice in each group) and inoculated i.p. with SLENU or SLCNUgly in Tween 20. Before treatment every mouse was weighed out and inoculated i.p. with a volume of the nitrosourea that corresponded to a dose of 60 mg/kg for SLENU and 66.6 mg/kg for SLCNUgly. Control group was inoculated with solvent only. After 3 hours of the treatment all animals in the tested and control group were exsanguinated under light ether anesthezia and the livers and kidneys were collected and washed in cool saline.

Electron paramagnetic resonance (EPR) study

All EPR measurements were performed at room temperature on a X-band EMX^{micro}, spectrometer Bruker, Germany, equipped with standard Resonator. All EPR experiments were carried out in triplicate and repeated thrice. Spectral processing was performed using Bruker WIN-EPR and Simphonia software.

EPR ex vivo ROS production in mice liver and kidney tissues.

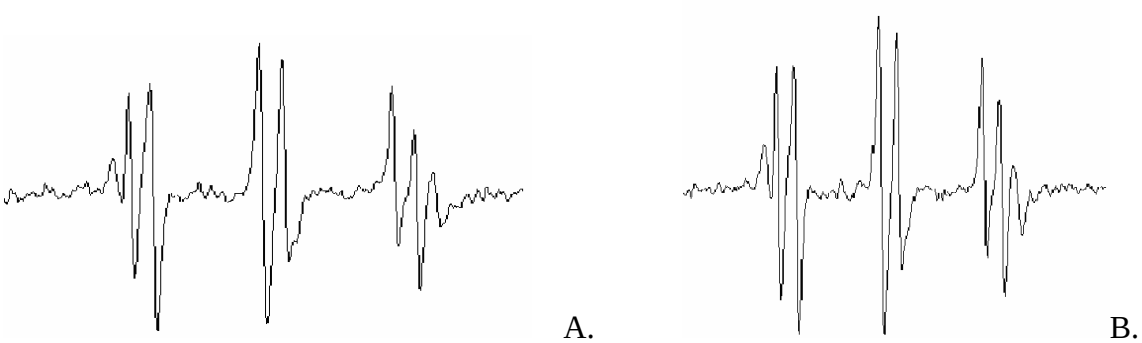
Preparation of homogenates and EPR study of ROS production and was performed according to Shi et al., 2005 with some modifications (10). Briefly, about 0.1 g of liver or kidney tissue was homogenized for 2 min after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. After centrifugation, 0.4 ml supernatant of homogenized tissue was taken in quartz tube and stored in liquid nitrogen for EPR measurement. EPR spectra were recorded at room temperature. EPR settings were as follows: 3503.74 G center field, 20.49 mW microwave power, 0.50 G modulation amplitude, 100 G sweep width, a receiver gain 1x10^{6}, 327.68 ms time constant, 81.92 s sweep time.

Statistical analysis

Statistical analysis was performed with Statistica 6.1, StaSoft, Inc. and results were expressed as means ± standard error (SE). Statistical significance was determined by the Student's t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Results from EPR *ex vivo* study on the levels of ROS production in liver and kidney tissues of tested and control mice are presented on **Figure 2, Figure 3, Figure 4.** Three hours after nitrosoureas treatment, ROS production marked by EPR spectra signals of the studied mice liver and kidney homogenates could be detected. EPR spectra of mice liver and kidney free radicals trapped by PBN exhibited six-lines (**Figure 2A, 2B**).



A. B.

Figure 2. EPR spectra of PBN –adducts registered in livers (**A**) and kidneys (**B**) of mice

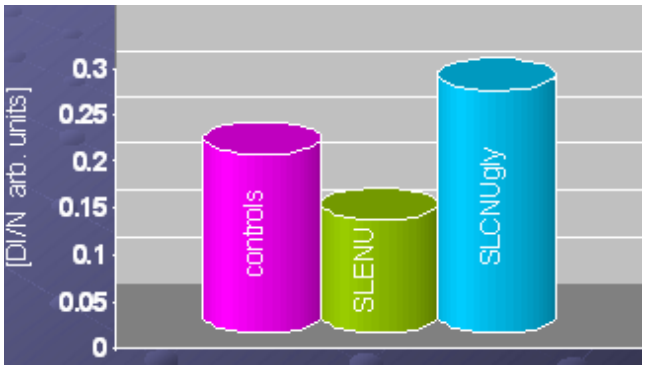


Figure 3. ROS production expressed in arbitrary units in livers of treated and control mice

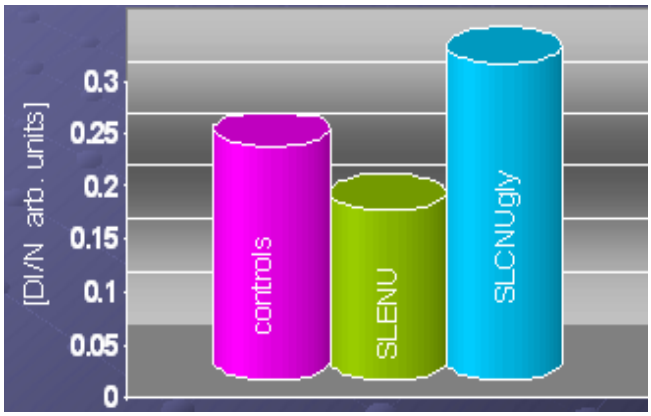


Figure 4. ROS production expressed in arbitrary units in kidneys of treated and control mice

The calculated hyperfine splitting constants of the spin adducts registered were: a^N = 13.88 G and a^H = 2.35 G for liver tissues and a^N = 13.90 G and a^H = 2.35 G for kidney tissues,

respectively. Based on the values of their splitting constants the spin adducts were identified as PBN/$^{\cdot}OCH_3$ radicals (11). To confirm that the radicals trapped by PBN originated, only from the livers and kidneys of mice, additional control samples containing nitrosoureas plus DMSO solution of PBN or only DMSO solution of PBN, were also studied but no PBN spin adducts were observed (data not shown). As is seen on **Figure 3** almost 2 times higher levels of ROS production (calculated as double integrated plots of EPR spectra of the PBN adducts) was found in liver homogenates of mice treated by SLCNUgly comparing to that of the mice treated by SLENU, while lower ROS production in the livers of mice treated by SLENU was found when was compared to that in livers of control mice. The levels of ROS production in kidney homogenates of the tested and control mice are presented on **Figure 4.** As is seen, about 2.5 times higher levels of ROS production was registered in the kidney homogenates of mice treated by SLCNUgly comparing to that of the mice treated by SLENU and a lower levels of ROS production were observed after treatment by SLENU in comparison with those of the control mice.

DISCUSSION

Formerly reported SSA activities of SLENU and SLCNUgly were correspondingly 5 and 6 times higher than that of the well known antioxidant Trolox (8). Results presented by this EPR *ex vivo* study demonstrated that SLENU does not provoke lipid peroxidation (LPO) process in mice tissues for the studied period, while SLCNUgly instead of its good SSA activity expressed higher levels of LPO products comparing to those of the controls and mice treated by SLENU. Increased levels of LPO products registered by the present EPR *ex vivo* study in liver and kidney tissues of mice treated by SLCNUgly might be explained by alkylating activity of the nitrosoureas (12)]. As it is accepted the alkylating activity of this class antitumor agents is responsible for their *in vivo* antitumor activity but is also assumed alkylating activity to be involved in the toxicity of the nitrosoureas, as well (12, 13). Bearing in mind that SLENU does not posses alkylating activity, while SLCNUgly exhibits *in vitro* high alkylating activity we suppose during alkylation SLCNUgly to cause generation of some toxic reactive free radical species like $^{\cdot}OH$. This our assumption is supported by the type of the spin adducts registered by the present *ex vivo* EPR spin trapping technique. It was demonstrated that the reaction of DMSO with $^{\cdot}OH$ produced $^{\cdot}CH_3$ radicals, and that oxidation of $^{\cdot}CH_3$ in aerobic conditions produced $^{\cdot}OCH_3$ radicals (11, 14). Since, for the present *ex vivo* EPR study the mice tissues homogenates were prepared in DMSO solution of PBN at aerobic conditions we accepted that *in vivo* SLCNUgly can cause generation of $^{\cdot}OH$ radicals which were trapped as finals PBN/$^{\cdot}OCH_3$ radical adducts. In conclusion, this preliminary EPR study demonstrates, ones again that SLENU is a nontoxic compound, with excellent *ex vivo* antioxidant properties and might be selected as proper synthetic antioxidant for further *in vivo* EPR studies. At the same time SLCNUgly exhibits higher toxicity comparing to that of SLENU. In spite of this finding since, SLCNUgly is a 2-chloroethylnitrosourea with *in vivo* high antitumor activity and is less toxic than its clinically used analogue CCNU, is proper to be extended the studies on its biological and oncopharmacological properties as a promising antitumor agent.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Education, Youth and Science of Bulgaria, 2006 and 2008.

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