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Effect of the spin-labelled 1-ethyl-1-nitrosourea on CCNU-induced oxidative liver injury

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This study was carried out to determine the effects of 1-ethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea (SLENU), recently synthesised in our laboratory, and vitamin E as positive control on 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) – free radical induced oxidative injuries in the liver of mice. Specifically, alterations in malonyl dialdehyde (MDA) level and activities of some antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), were measured in liver homogenates from tumour-bearing C57 black mice after treatment with solutions of CCNU (30 mg/kg) and SLENU (100 mg/kg), both administered intraperitoneally. CCNU-induced increase in MDA level, SOD and CAT activities were suppressed by SLENU. The present results and those from a previous report demonstrated superoxide scavenging activities (SSA) of the nitrosourea SLENU and enabled us explain the protective effect of the spin-labelled nitrosourea on CCNU-induced oxidative stress in the liver of mice. This protective effect is through the scavenging of $^{\bullet}O_2^-$ and by an increased production of $^{\bullet}NO$. Thus, a potential for developing new combination chemotherapy in cancer is seen.

1. Introduction

Reactive oxygen species (ROS), such as $^{\bullet}O_2^-$, H_2O_2, and $OH^{\bullet}$ are produced in all mammalian cells as a result of mitochondrial oxidative respiration or cell exposure to toxicants (Freeman et al. 1982; Marks et al. 1996). Low levels of ROS are indispensable as mediators in many cell processes, including differentiation, cell cycle progression or growth arrest, apoptosis and immunity (Mates and Sanchez-Jimenez 2000; Shackelford et al. 2000). Reaction of nitric oxide $NO^{\bullet}$ with oxygen or other free radicals generates reactive nitrogen species (RNS), which cause multiple biological effects (Moncada et al. 1991; Patel et al. 1999). $NO^{\bullet}$ is either cytostatic or cytotoxic, interacting with a number of molecular targets within cells. Cellular defence mechanisms have evolved to protect cells from ROS, and these include repair systems, generations of detoxifying enzymes such as superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase (GPX) and the production of small molecule scavengers such as glutathione (GSH) (Freeman et al. 1982; Patel et al. 1999; Davies 2000). An imbalance between the mechanisms that generate and protect against ROS results in oxidative stress, which may cause severe metabolic malfunctions and damage of biological macromolecules (Marks et al. 1996; Beckman and Ames 1997).

The final common pathway in the mechanism of action of ionising radiation and many chemotherapeutic agents is through reactive oxygen species. Some anticancer drugs can result into increased production of oxidising radical species (Hug et al. 1997; Davies 2000) and could be the reason for some toxic side effects seen as hepatotoxicity, cardiotoxicity, pulmonary fibrosis, etc (Minow et al. 1976; Hug et al. 1997; Mates and Sanchez-Jimenez 2000; Kalender et al. 2001).

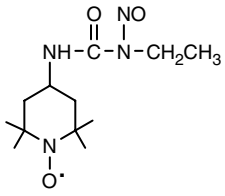
2-Chloroethylnitrosoureas (NUs), like CCNU, Me-CCNU, BCNU, chlorozotocin, etc. find wide application in the management of various human cancers, notably lymphomas, gliomas, a few solid tumours and melanomas (Hahn et al. 1973; Carter et al. 1988; Grochow 2001). Unfortunately, the clinical efficacy of these drugs is limited by their delayed and cumulative haematological toxicity (Gnewuch and Sosnovsky 1997). All clinically used nitrosoureas, alone or in combination, may also cause delayed, cumulative dose-related chronic hepatotoxicity that is irreversible and thus may alter liver function. NUs-induced liver abnormalities such as ascites, hyperbilirubinaemia, and thrombocytopenia leading to fatalities have been reported (De Vita et al. 1990; Wolff et al. 1986; Ducastelle et al. 1988).

Efforts are going on globally to develop new nitrosoureas that may exhibit better therapeutic efficacy but having lower toxicity (Monneret et al. 2000). Reduced toxicity and increased antineoplastic properties were achieved when nitroxyl (aminoxyl) groups, such as 2,2,6,6-tetramethylpiperidine-1-oxyl (TMPO), were introduced in chemical structure of certain antitumour drugs (Raikov et al. 1985; Gnewuch and Sosnovsky 1997). Following this finding, we synthesised a number of spin-labelled analogues of the anticancer drug CCNU. Some of these compounds showed

advantages over CCNU, by having lower toxicity and higher anticancer activity against some experimental tumour models (Zheleva et al. 1995; Gadjeva and Raikov 1999; Gadjeva and Koldamova 2001; Gadjeva et al. 2003). Using the EPR method we have shown that spin-labelled nitrosoureas and their precursor 4-amino TMPO could scavenge $^{\bullet}O_2^-$ and thus exhibit high superoxide scavenging activity (SSA) (Gadzheva et al. 1994).

Although a number of studies have examined the protective effects of antioxidants such as vitamins C and E, carotenoids and selenium, these studies have not provided consistent evidence in favour of hepatoprotection (Perez et al. 1986; Lenzhofer et al. 1983; Kalender et al. 2001). Recently, we have demonstrated that CCNU-induced increased plasma MDA level and decrease in erythrocyte SOD and CAT activities in rat blood were prevented by SLENU, but not by vitamin E (Gadjeva et al. 2005). This state of affairs has informed our decision to pursue the situation much further.

Therefore, the aim of the present study was to determine whether the nitroxide antioxidant 1-ethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea (SLENU), an analogue of the antitumour drug CCNU, would prevent CCNU-induced oxidative liver damage and thus provide some level of hepatoprotection. In studying this, we investigated the level of lipid peroxidation products and the activities of antioxidant defence enzymes superoxide dismutase and catalase in liver homogenates isolated from mice treated with CCNU in combination with SLENU.



SLENU

2. Investigations and results

Figure 1 illustrates the levels of lipid peroxidation in liver homogenates isolated from mice treated with CCNU alone and in combination with either Vitamin E or SLENU. It was found that 1 h after administration of CCNU, the levels of MDA were significantly increased in liver homogenates isolated from both tumour-bearing mice and healthy mice, compared to corresponding control groups (mean 1.478 nM vs 0.596 nM, $p < 0.0001$ and 0.787 nM vs 0.508 nM, $p < 0.01$). Tumour-bearing mice treated with CCNU had an about 50% higher level of MDA compared to healthy mice treated with CCNU at the same dose. Vit. E and SLENU did not increase the levels of MDA in healthy mice. However, tumour-bearing mice treated with Vit. E alone increased significantly the level of MDA compared to controls (mean 1.140 nM, $p < 0.001$). The combination of CCNU and Vit. E showed a slight decrease in the levels of MDA in both tumour-bearing and healthy mice compared to cases of CCNU administered alone (mean 1.275 nM, $p > 0.05$ and 0.740 nM, $p > 0.05$). MDA levels in liver homogenates from mice treated with SLENU alone were increased but not significantly compared to control (mean 0.449 nM and 0.816 nM, $p > 0.05$). However, combined application of CCNU and SLENU led to a strong decrease in the levels of MDA, compared to the levels when CCNU was administered alone (mean 0.761 nM, $p < 0.0001$ and 0.634 nM, $p < 0.05$); the levels

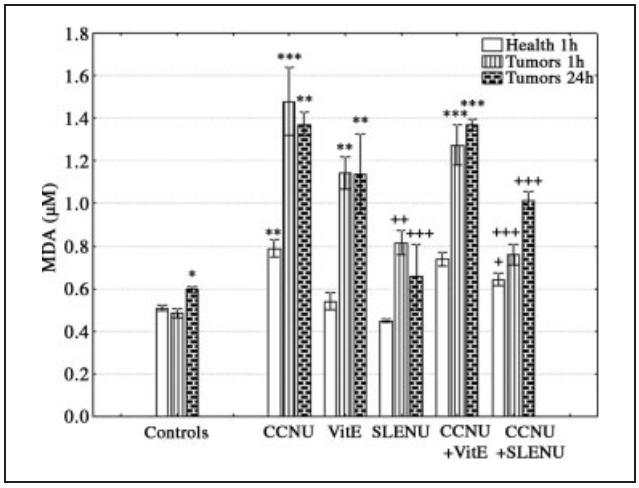


Fig. 1: Lipid peroxidation in liver homogenates isolated from mice treated with CCNU, Vit. E, SLENU, and their combinations. Values are expressed as mean ± S.E. * $p < 0.05$ vs. healthy controls; ** $p < 0.001$ and *** $p < 0.0001$ vs. corresponding controls; + $p < 0.05$, ++ $p < 0.001$ and +++ $p < 0.0001$ vs. corresponding groups with CCNU administered alone

of the former were close to those obtained from SLENU when administered alone. Lipid peroxidation at the 24th h in tumour-bearing mice treated with CCNU remained as high as the value obtained during the 1st h post administration (mean 1.371 nM, $p > 0.05$) (Fig. 1). MDA remained significantly high also at 24 h in tumour-bearing mice treated with Vit. E alone. However, it should be noted that there was significant difference after treatment with SLENU at the 24th h compared with CCNU alone (mean 0.656 nM, $p < 0.001$) and there was no significant difference compared to control. Whereas a combination of CCNU and Vit. E at the 24th h did not show any difference in the level of MDA compared to CCNU administered alone (mean 1.352 nM, $p > 0.05$), the combination of CCNU and SLENU showed significantly lower level of MDA (mean 1.150 nM, $p < 0.0001$).

As can be seen from the data in presented Fig. 2, the activities of SOD in liver homogenates isolated from tumour-bearing control mice at 1 h and 24 h were significantly decreased compared to SOD activity of liver homogenates isolated from healthy controls (mean 5.294 U/gPr, and

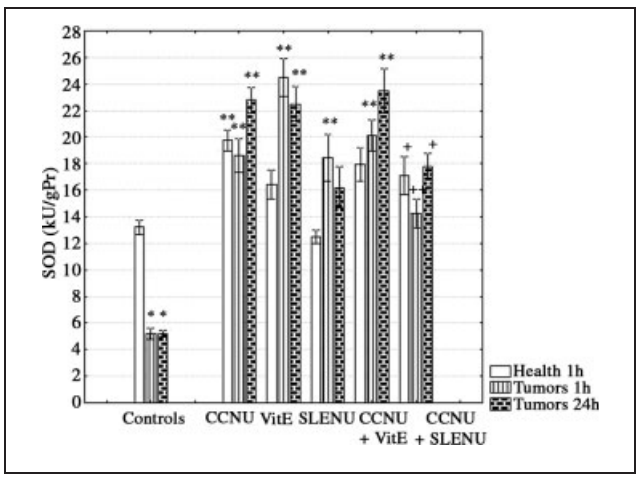


Fig. 2: SOD activity of liver homogenates isolated from mice treated with CCNU, Vit. E, SLENU and their combinations. Values are expressed as mean ± S.E. * $p < 0.001$; ** $p < 0.0001$ vs. healthy controls; + $p < 0.05$ and ++ $p < 0.001$ vs. CCNU alone

5.23 U/gPr vs. 13.216 U/gPr, $p < 0.001$). In contrast, 1 h after treatment with CCNU, SOD activities of both healthy and tumour-bearing liver homogenates were found to be much higher than those of the corresponding controls (mean 19.756 U/gPr and 18.621 U/gPr, $p < 0.0001$). We found that 24 h after treatment with CCNU, SOD activity continued to increase (mean 22.777 U/gPr, $p < 0.0001$). Tumour-bearing mice treated with Vit. E alone had significantly higher SOD activities compared to healthy controls (mean 24.453 U/gPr and 22.492 U/gPr, $p < 0.0001$). No significant differences, compared to the healthy controls, were observed among the groups of healthy mice at 1 h and tumour-bearing mice at 24th h treated with SLENU (mean 12.492 U/gPr, $p > 0.05$ and 14.733 U/gPr, $p > 0.05$). A combined application of CCNU and Vit. E did not lead to a significant decrease in the level of SOD compared to that of CCNU administered alone in tumour-bearing mice (mean 20.149 U/gPr and 23.478 U/gPr, $p > 0.05$). When both healthy and tumour-bearing mice were treated with the combination of CCNU and SLENU the levels of the antioxidant enzyme SOD were significantly decreased compared to those of mice treated with CCNU alone (mean 17.102 U/gPr, $p < 0.05$, 14.256 U/gPr, $p < 0.001$ and 17.797 U/gPr, $p < 0.05$). Moreover, in tumour-bearing mice, 1 h after administration of the combinations CCNU + SLENU, SOD activity was close to that of the healthy controls ($p > 0.05$).

Figure 3 represents the activity of the antioxidant enzyme CAT in liver homogenates isolated from healthy and tumour-bearing mice. The activity of CAT in tumour-bearing control mice was significantly increased at the 24th h compared to the healthy controls (mean 54.053 U/gPr, vs. 26.856 U/gPr, $p < 0.001$). One hour after treatment with CCNU the activities of CAT in both healthy and tumour-bearing mice were also significantly increased compared to the corresponding controls (mean 48.058 U/gPr and 59.486 U/gPr and, $p < 0.0001$). The activities of CAT were very high in the liver homogenates 1 h after treatment of tumour-bearing mice with either Vit. E or SLENU (mean 66.596 U/gPr and 63.558 U/gPr respectively). However, when a CCNU and Vit. E combination was administered to both healthy and tumour-bearing mice the levels of the antioxidant enzyme CAT were significantly decreased compared to CCNU administered alone (mean 36.952 U/gPr 31.07825 U/gPr, $p < 0.0001$). Moreover, after treatment of

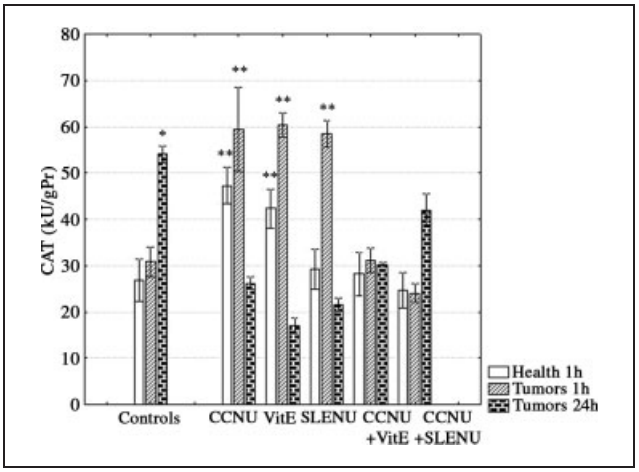


Fig. 3: CAT activity of liver homogenates isolated from mice treated with CCNU, Vit. E, SLENU and their combinations. Values are expressed as mean ± SE. * $p < 0.001$ vs. healthy controls; ** $p < 0.0001$ vs. corresponding controls; + $p < 0.0001$ vs CCNU alone

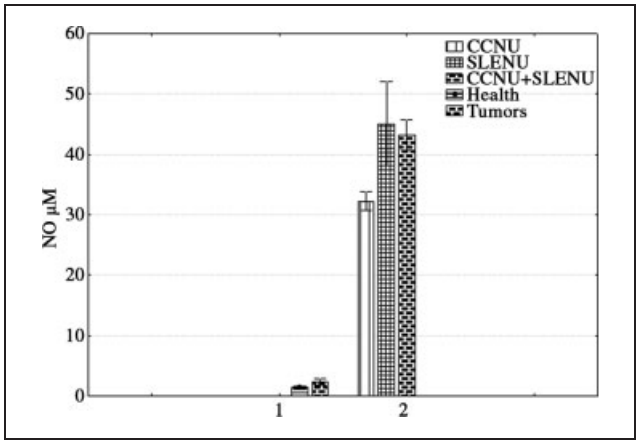


Fig. 4: NO$^{\bullet}$ expressed as total end products of NO_2^- and NO_3^-. * $p < 0.0001$; ** $p < 0.001$ vs. controls

both healthy and tumour-bearing mice with combination of CCNU and SLENU, CAT activities were found to be close to those of the controls (mean 24.700 U/gPr and 24.015 U/gPr vs. 26.855 U/gPr, $p > 0.05$). However, no significant differences, compared to the healthy controls, were observed in the antioxidant enzyme CAT levels 24 h after drug administration among the groups of mice treated with CCNU, Vit. E, SLENU or their combinations.

Figure 4 shows the levels of NO$^{\bullet}$ expressed as total end products of NO_2^- and NO_3^-. The levels were found to be significantly increased in tumour-bearing mice compared to healthy controls (mean 2.321 µM vs. 1.373556 µM, $p < 0.05$). Mice treated with CCNU had 23 times increased levels of NO as compared to the control mice (mean 32.252 µM vs. 1.373 µM, $p < 0.0001$). It is interesting that after treatment with SLENU the level of NO was significantly higher than that in mice treated with CCNU alone (mean 45.088 µM, $p < 0.001$). When the combination of CCNU and SLENU was administered the level of NO remained as high as that of mice treated with SLENU alone (mean 43.137 µM).

3. Discussion

Even with molecular advances that have identified genetic lesions that cause cancer, we still rely upon chemotherapy and radiation treatment in cancer. Such treatments are given to seriously ill patients and result in serious, and sometimes fatal, side effects. There is a growing interest in devising strategies for prevention of CCNU-induced liver abnormalities (Kristal et al. 2004).

1-Alkyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosoureas were synthesized in our research laboratory and during the last years some properties and activities have been investigated and compared with those of their nonlabeled analogue CCNU (Gadzheva et al. 1994, 1997; Gadjeva and Koldamova 2001). Our formerly reported results for alkylating, carbamoylating activities and half-life times of the 1-ethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea (SLENU) showed that the alkylating activity was lower than that of CCNU, but the carbamoylating activity of SLENU was high and comparable with that of CCNU. The half-lives were also comparable (75 min for SLENU and 54 min for CCNU), (Gadjeva and Koldamova 2001).

The present study was conducted to investigate the role of 1-ethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitro-

sourea (SLENU), the spin-labelled analogue of the antitumour drug CCNU, as potential for alleviating the oxidative stress produced after CCNU-induced oxidative injury, using the mice liver homogenates as test model. We used vitamin E as a positive control since Vitamin E influences the efficacy of many drugs currently used in cancer treatment (Kalender et al. 2001). In furtherance of our earlier reported findings of (1) excellent superoxide scavenging activity (SSA) of the spin-labelled nitrosoureas SLENU (Gadjeva et al. 1994), and (2) beneficial effects of SLENU on CCNU-induced oxidative stress in rat blood (Gadjeva et al. 2005), we have, accordingly, tried to explain here the protective effect of the spin-labelled nitrosourea on CCNU-induced oxidative stress in the liver of mice as possible antioxidant mechanisms. The level of MDA and the activities of the antioxidant enzymes, SOD and CAT, in liver homogenates were used as indicators of oxidative stress.

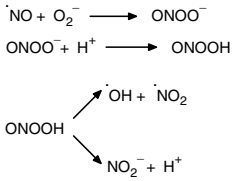
The first measurement was carried out 1 h after administration of drugs. We chose this time point in view of half-lives of SLENU and CCNU, and also because of our last electron spin resonance (ESR) study of tissue distribution of the spin-labelled nitrosourea SLENU in organ homogenates of C57BL where it was demonstrated that the maximum concentration of SLENU in lungs, brain, liver and spleen was reached 30 min after intraperitoneal administration of the drug, and a complete disappearance of the nitrosourea within 90 min in all tissues studied (Gadjeva and Koldamova 2001). In the present study we found that within 1 h following treatment with CCNU, liver homogenates of both healthy and tumour-bearing mice had higher productions of lipid peroxidation materials compared to controls ($p < 0.001$) and this was accompanied by increased activities of the antioxidant defence enzymes SOD and CAT ($p < 0.001$). This disturbance might be a sequel of the augmented generation of toxic reactive oxygen species (ROS) in the liver, and possible products of the metabolism of CCNU. It is well known that increased production of lipid peroxides might be due to increased levels of ROS and could be connected with the presence of oxidative stress.

Augmented generation of toxic reactive oxygen species (ROS), which are products of CCNU metabolism, finds support in previous studies. For example, Acikgoz et al. (1995) demonstrated enhanced membrane lipid peroxidation caused by CCNU. We have reported earlier the light dependent $NO^{\bullet}$ generation from CCNU; we had proposed an *in vivo* formation of high toxic $ONOO^-$ and $^{\bullet}OH$ with the potential of these two to contribute to the severe toxicity of this drug (Gadjeva et al. 2001). We have also reported the presence of oxidative stress in blood of rats after 30 days oral treatment with CCNU (Gadjeva et al. 2005).

Based on this last finding we have hypothesized that if CCNU could generate $^{\bullet}NO$ *in vivo*, within might contribute to tissue $ONOO^-$ and $^{\bullet}OH$ production by the mechanism proposed in Scheme 1.

Mice were treated with both typical antioxidants studied – SLENU and vitamin E (both possessing high SSA). Our experiment showed that Vit. E and SLENU did not increase the levels of MDA in healthy mice indicating that the compounds did not induce oxidative stress. Although we observed that tumour mice treated with SLENU alone had increased levels of MDA 1 h after treatment compared to controls we consider that SLENU did not induce oxidative stress because of the significantly lower MDA levels for SLENU compared to Vit. E and an about 50% lower level of MDA compared to mice treated with CCNU at the same time. Furthermore, complete suppression of the oxidative stress was observed after adding the combinations

Scheme 1



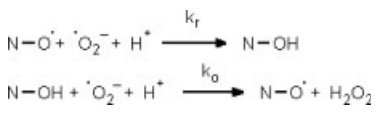
CCNU and SLENU. MDA levels were decreased, and SOD and CAT activities were restored to levels close to the control. Our results showing decreased MDA levels of mice treated with the combinations CCNU and SLENU suppose a reduced ROS production that might be attributed to the effect of SLENU on CCNU-induced oxidative injury. Such a chemopreventive effect of the nitroxide Tempol had been reported by several authors (Mitchell et al. 2001; Samuni et al. 2002; Thiemermann 2003). Mitchell et al. demonstrated that nitroxides at non-toxic concentrations were effective as *in vitro* and *in vivo* antioxidants when oxidation was induced by the superoxide, hydrogen peroxide, organic hydroperoxides, ionising radiation, or specific DNA-damaging anticancer agents (Mitchell et al. 2001).

Using ESR studies we have established that clinically used nitrosourea drugs, like CCNU, could not scavenge $^{\bullet}O_2^-$ while the spin-labelled nitrosourea derivatives, such as SLENU, could successfully scavenge $^{\bullet}O_2^-$ by exhibiting high SSA (Gadjeva et al. 1994). We also showed that the mechanism of SSA activity was through redox cycling between nitroxide and its corresponding hydroxylamine moiety, according to the mechanism proposed in Scheme 2.

The non-toxic effect of the spin-labelled nitrosourea, SLENU, and its ability to reverse the CCNU-induced oxidative stress (increase in MDA level and alteration in SOD and CAT activities) in our study have led us to propose the following hypothesis. The nitroso group in the spin-labelled nitrosourea SLENU may lead to the generation of $NO^{\bullet}$ when SLENU is used alone or jointly with CCNU. However, the nitroxyl free radical moiety incorporated only in the spin-labelled compounds might successfully compete with the self-generated $NO^{\bullet}$ and that produced by CCNU in the scavenging of $^{\bullet}O_2^-$. This effect could prevent formation of highly toxic species such as $ONOO^-$ and $^{\bullet}OH$ and at the same time could increase the level of $NO^{\bullet}$. In this regard, our present results are consistent with the notion that the protective effects of SLENU are due to both SSA and its increased release of $NO^{\bullet}$.

In our study plasma levels of nitrite (NO_2^-) and nitrate (NO_3^-) were used to estimate the level of $NO^{\bullet}$ formation, since $NO^{\bullet}$ is highly unstable and has a very short half-life. We observed significantly higher $NO^{\bullet}$ end products in the plasma of mice treated with CCNU, SLENU and the combination of them. This could be produced by CCNU and SLENU or reflect increased $NO^{\bullet}$ degradation promoted by oxidative stress.

Scheme 2



k_r and k_o are second-order rate constants for the reduction of nitroxide and oxidation of hydroxylamine by superoxide, respectively

Several *in vitro* studies have demonstrated the protective effect of NO in oxidative injury, both in the generalised case and in hepatocytes. Rubbo et al. (1994) suggested that NO$^\bullet$ might act as a primary antioxidant in biological systems by limiting lipid peroxidative chain propagation. Using a model system, authors demonstrated that NO$^\bullet$ was a potent terminator of radical chain propagation and that NO$^\bullet$ inhibits peroxynitrite-dependent lipid peroxidation reactions.

In view of these facts we can conclude that pretreatment with SLENU can markedly suppress the hepatotoxic manifestations, observed in CCNU-treated mice by scavenging of $^\bullet O_2^-$ and causing increased $^\bullet$NO production. Further studies are, however, needed to clarify the effect of this combination in antitumour chemotherapy.

4. Experimental

4.1. Compounds tested

CCNU was obtained from Bristol-Myers Squibb Co. (Connecticut, USA). Buttermilk xanthine oxidase, trolox, SULF (sulfanilamide), NEDD (N-(1-naphtyl) ethylenediamine dihydrochloride) and VCl_3 were obtained from Fluka (Germany). TMPO was purchased from Aldrich (Milwaukee, U.S.A.). SLENU was synthesized according to Gadjeva and Koldamova (2001). The test compounds were dissolved *ex tempore*: first step in Tween and the second step in saline.

4.2. Animals

All procedures performed on animals were done in accordance with guidelines of the Bulgarian government regulations and were approved by the authorities of Trakia University, Bulgaria. The animals were housed in plastic cages, fed a normal laboratory diet and water ad libitum.

The study was carried out on 108 mice C57 black (bred in the Laboratory of Oncopharmacology, National Cancer Institute, Sofia), average weight 18–22 g, divided into groups of 6 animals per group (equal number of either sex).

4.3. Drug treatment

On day 0, mice were inoculated i.p. with 0.5 ml 10^5 tumour cell suspension of lymphoid leukaemia L1210 in saline. On day 3, an LD50 dose (30 mg/kg) of CCNU and spin-labelled nitrosourea SLENU (100 mg/kg) and combinations of them were administered intraperitoneally in a single injection in volume 0.01 ml per body weight. The injected materials were prepared in 10% Tween solutions in accordance with routine methods (Geran et al. 1972). Mice were sacrificed by cervical decapitation 1 and 24 h after administration of the drugs. Livers were removed and kept on ice until homogenisation on the same day. The samples were first washed with deionised water to separate the blood and then homogenised. The tissue homogenates were centrifuged for 10 min at 15000 rpm at 4 °C and the final supernatants were collected. They were used for determination of lipid peroxidation and superoxide dismutase and catalase activities. The protein concentration of the supernatants was determined using the method of Lowry et al. (1951).

4.4. Analyses of oxidative stress-related parameters

4.4.1. Analysis of lipid peroxidation

The levels of lipid peroxidation were determined using the thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive product (Draper and Hadley 1990).

In the TBA assay 1 ml of the supernatant, 1 ml of normal saline and 1 ml of 25% trichloro-acetic acid (TCA) were mixed and centrifuged for 20 min at 2000 rpm. One ml of protein free supernatant was taken, mixed with 0.25 ml of 1% TBA and boiled 1 h at 95 °C. After cooling the absorbance of the pink colour of the obtained fraction product was read at 532 nm.

4.4.2. Measurement of antioxidant enzymes activities

Total SOD activity was determined by the xanthine/xanthine-oxidase/nitro-blue tetrazolium (NBT) method according to Sun et al. (1988). Briefly, superoxide anion radical ($^\bullet O_2^-$) produced by the xanthine/xanthine-oxidase system reduces NBT to formazan, which can be assessed spectrophotometrically at 560 nm. SOD competes with NBT for the dismutation of $^\bullet O_2^-$ and inhibits its reduction. The level of this reduction is used as a measure of SOD activity. The total SOD activity is expressed in units/mg of protein, where one unit was equal to SOD activity that causes 50% inhibition of the reaction rate without SOD.

The assay of CAT activity was done according to Beers and Sizer (1952). Briefly, hydrogen peroxide (30 mM) was used as a substrate and the decrease in H_2O_2 concentration at 22 °C in a phosphate buffer (50 mM, pH 7.0) was followed spectroscopically at 240 nm for 1 min. The activity of the enzyme was expressed in units per mg of protein and 1 unit was equal to the amount of an enzyme that degrades 1 μM H_2O_2 per minute.

4.4.3. Measurement of nitric oxide

Serum nitric oxide was measured in terms of its products, nitrite and nitrate, by the method of Griess modified by Miranda et al. (2001). This method is based on a two-step process. The first step is the conversion of nitrate to nitrite using vanadium(III) and the second is the addition of sulphanilamide and N(-naphthyl) ethylenediamine (Griess reagent). This converts nitrite into a deep purple azo-compound, which is measured colorimetrically at 540 nm. Nitric oxide products were expressed in μM.

4.5. Statistical analysis

Data are expressed as mean ± SE. Student's t-test was used to determine the statistical differences between groups. $P < 0.05$ was considered statistically significant.

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