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Beneficial effects of spin-labelled nitrosourea on CCNU-induced oxidative stress in rat blood compared with vitamin E

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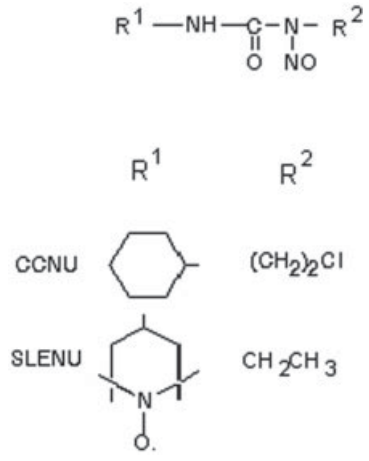
This study was carried out to determine the effects of a recently synthesized 1-ethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea (SLENU), compared with vitamin E as a positive control, on 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)-induced oxidative stress in rats. We determined plasma malonyl dialdehyde (MDA) levels and the activities of erythrocyte superoxide dismutase (SOD) and catalase (CAT). Forty two white albino healthy rats were treated once daily for 30 days with oral preparations of CCNU (12.5 mg/kg and 25 mg/kg), SLENU (25–200 mg/kg), and combinations of these. The CCNU-induced increase in plasma MDA level and the usual decrease in erythrocyte SOD and CAT activities were reversed by SLENU, but not by vitamin E. We have previously demonstrated that SLENU is a superoxide scavenger. A combination of our present findings with previous results thus leads us to proposing a new chemotherapeutic combination of CCNU and SLENU that is devoid of high toxicity.

1. Introduction

Chloroethylnitrosoureas have shown a significant clinical activity against human malignancies and some of them, such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) are used for the treatment of human cancers, mainly lymphomas, gliomas, a few solid tumours and melanomas (Carter et al. 1988; Pyrhonen et al. 1992; Perry et al. 1998). Unfortunately, the clinical efficacy of these drugs is limited because they show delayed and cumulative hematological toxicity (Gnewuch and Sosnovsky 1997). Reduced toxicity and increased antineoplastic properties were achieved when stable nitroxyl radicals, such as 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), were introduced in the chemical structure of certain antitumour drugs (Sosnovsky 1992; Gnewuch and Sosnovsky 1997). Therefore, we synthesized a number of spin-labelled analogues of the anticancer drug CCNU. These compounds showed advantages over CCNU by having lower toxicity and higher anticancer activity in some experimental tumour models (Gadjeva and Raikov 1999; Zheleva et al. 1995; Gadjeva and Koldamova 2001). By electron spin-trapping (ESR) we have shown that spin-labelled nitrosoureas and their precursor 4-amino TEMPO could scavenge $^{\bullet}O_2^-$, thus exhibiting high superoxide scavenging activity (Gadzheva et al. 1994). In addition, our studies demonstrated a light dependent nitric oxide ($NO^{\bullet}$) generation from the antitumour drug CCNU (Zheleva et al. 1997). These facts have led us to suggest a possible role of in vivo generated $ONOO^-$ and $^{\bullet}OH$ in severe CCNU toxicity, while lowered toxicities of its spin-labelled analogues could be due to their excellent superoxide scavenging activity (Zheleva and Gadjeva 2001).

The aim of the present study was to determine the protective effects of 1-ethyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea (SLENU) against CCNU-induced oxidative damage. SLENU is an analogue of the antitumour drug CCNU. Thus, we investigated the level of lipid peroxidation products and the activities of the antioxidant defence enzymes, superoxide dismutase and catalase, in blood of rats treated with CCNU in combinations with SLENU.



2. Investigations and results

2.1. Biochemical findings

The results of the levels of lipid peroxidation and the activities of antioxidant enzymes SOD and CAT are presented in the Table. The levels of MDA were significantly

increased in the plasma of rats after 30 days of treatment with CCNU p.o. at doses 12.5 mg/kg and 25 mg/kg, compared to the control group. There was no difference in MDA concentration, compared to the controls, after treatment with SLENU p.o. at doses between 25 and 200 mg/kg. The combination CCNU p.o. 12.5 mg/kg + Vit. E p.o. 200 mg/kg and CCNU p.o. 25 mg/kg + Vit. E p.o. 200 mg/kg, produced lower levels of MDA compared to CCNU administered alone. However, the combinations CCNU p.o. 12.5 mg/kg + SLENU p.o. 200 mg/kg and CCNU p.o. 25 mg/kg + SLENU p.o. 200 mg/kg, resulted in a significantly lower level of MDA, compared to CCNU administrated alone at the same dose, which were close to those of the controls.

The activity of SOD in the erythrocyte lysates significantly decreased after 30 days of treatment with CCNU at doses 12.5 mg/kg p.o. and 25 mg/kg p.o., compared to the controls. No significant differences, compared to the controls, were observed among the groups of rats treated with SLENU at doses between 25 and 200 mg/kg p.o. When rats were treated with combinations of CCNU and Vit. E, e.g. CCNU p.o. 12.5 mg/kg + Vit. E p.o. 200 mg/kg e.g., the level of SOD increased but not significantly, compared to CCNU administered alone. When rats were treated with the combination of CCNU p.o 25 mg/kg and SLENU p.o. 200 mg/kg the level of SOD remained as low as after treatment with CCNU alone. However, both the combinations CCNU p.o. 12.5 mg/kg + SLENU p.o. 200 mg/kg and CCNU p.o. 25 mg/kg + SLENU p.o. 200 mg/kg, showed activities of SOD close to those of the controls (see Table).

The activity of CAT in the erythrocyte lysates significantly decreased after 30 days of treatment with CCNU p.o. at doses 12.5 mg/kg and 25 mg/kg, compared to the controls. No significant differences, compared to the controls, were observed among the groups of rats treated with SLENU p.o. at doses between 25–200 mg/kg. Treatment with a combination of CCNU p.o. (12.5 mg/kg and 25 mg/kg) and Vit. E p.o. (200 mg/kg) caused a significant increase in the levels of the antioxidant enzyme CAT, compared to CCNU administrated alone. However, after treatment with a combination of CCNU p.o. (12.5 mg/kg and 25 mg/kg) and SLENU p.o. (200 mg/kg) CAT activity was found to be significantly lower and close to that of the controls.

3. Discussion

To the best of our knowledge, prevention of CCNU-induced oxidative stress by spin-labelled nitrosourea in blood of CCNU-administrated rats is reported here for the first time.

Chloroethyl nitrosourea derivatives exhibit *in vivo* activity by alkylation of nucleic acids and proteins (Wheeler et al. 1974; Babson and Reed 1978; Lucas et al. 1982). Involvement reactive oxygen species (ROS) in CCNU toxicity is supported by several findings, two of which are the following: 1) Acikgoz et al. (1995) demonstrated enhanced membrane lipid peroxidation caused by CCNU. 2) We have shown that the light dependent $NO^{\bullet}$ generation in CCNU could lead to *in vivo* formation of highly toxic $ONOO^{-}$ and $^{\bullet}OH$ that were thought to contribute to the severe toxicity of this drug (Zheleva and Gadjeva 2001).

In the present study we found that oral treatment with CCNU for 30 days led to increased levels of lipid peroxidation products and a decrease in the activities of SOD and CAT in the blood of rats. This points toward oxidative stress. This disturbance could be associated with the augmented generation of ROS, due to CCNU metabolism.

Another indirect evidence for involvement of ROS in CCNU toxicity was the complete reversal of oxidative stress, following administration of the antioxidants SLENU and vitamin E. Treatment with combinations of CCNU and SLENU decreased the elevated amounts of free radicals and increased the previously reduced antioxidant status to normal levels. Normalisation of the levels of antioxidant enzymes after applying combinations of CCNU and SLENU could be linked to the protection of cellular structures by SLENU from oxidative damage by CCNU.

By ESR studies we have established that clinically used nitrosourea drugs, like CCNU, could not scavenge $^{\bullet}O_2^-$ while spin-labelled nitrosourea derivatives, such as SLENU, could successfully scavenge $^{\bullet}O_2^-$ (Gadjeva et al. 1994). We also showed that this effect was through redox cycling between nitroxide and its corresponding hydroxylamine moiety, according to the following proposed equations:

$$\rangle N{-}O^{\bullet} + {}^{\bullet}O_2^- + H^+ \xrightarrow{k_r} \rangle N{-}OH$$

$$\rangle N{-}OH + {}^{\bullet}O_2^- + H^+ \xrightarrow{k_o} \rangle N{-}O^{\bullet} + H_2O_2$$

Table: MDA, SOD and CAT in blood of rats after 30 days of treatment with CCNU, SLENU and combinations of both

Groups	MDA (μM/l)	SOD (U/gHb)	CAT (U/gHb)
Control (non treated)	1.996 ± 0.142	6303.98 ± 1416.71	4004.46 ± 1657.61
CCNU p.o. 12.5 mg/kg	2.766 ± 0.416[a] p = 0.01	4992.48±638.15[a] p = 0.01	1605.90 ± 207.71[a] p = 0.01
CCNU p.o. 25 mg/kg	3.216 ± 0.988[a] p = 0.0003	4988.53 ± 609.00[a] p = 0.01	1238.10 ± 205.99[a] p = 0.01
SLENU p.o. 200 mg/kg	2.077 ± 0.218[c] p = 0.004	5903.98 ± 1072.65	4754.46 ± 1453.86
CCNU p.o. 12.5 mg/kg + Vit. E p.o. 200 mg/kg	2.458 ± 0.111	5923.69 ± 799.92	15241.64 ± 3101.01[b] p = 0.01
CCNU p.o. 25 mg/kg + Vit. E p.o. 200 mg/kg	2.594 ± 0.258[c] p = 0.01	5195.38 ± 345.98[a] p = 0.03	13856.94 ± 1445.35[c]
CCNU p.o.12.5 mg/kg+ SLENU p.o. 200 mg/kg	2.293 ± 0.146[b] p = 0.004	6163.69 ± 245.39[b] p = 0.02	6461.64 ± 2207.35
CCNU p.o. 25 mg/kg + SLENU p.o. 200 mg/kg	2.086 ± 0.156[c] p = 0.0008	5895.387 ± 466.83	5456.94 ± 2490.02

[a] – vs. control; [b] – vs. CCNU p.o. 12.5 mg/kg; [c] – vs. CCNU p.o. 25 mg/kg

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

The non-toxic effect of the spin-labelled nitrosourea SLENU, and its ability to reverse the CCNU-induced oxidative stress 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 *in vivo* generation of $NO^{\bullet}$ 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 $^{\bullet}OH$.

Furthermore, bearing in mind the formerly reported facts: (1) high *in vivo* antitumour effects of spin-labelled nitrosoureas against different tumour models (Zheleva et al. 1995; Gadjeva and Raikov 1999); (2) *in vitro* synergistic effect of the the spin-labeled nitrosourea SLENU on cytotoxicity of bleomycin and farmorubicin in human lymphoid leukemia tumour cells (Gadjeva and Koldamova 1991) and (3) an excellent expressed superoxide scavenging activity (SSA) of the spin labelled nitrosoureas (Gadzheva et al. 1994), we consider that new combination chemotherapeutic schemes containing lower dose of the highly toxic CCNU plus an adequate amount of the spin-labelled nitrosourea, such as SLENU, could decrease the toxic side effects of CCNU by increased $^{\bullet}O_2^-$ scavenging, while still providing the needed antitumour property of alkylation.

4. Experimental

4.1. Animal studies

42 white healthy rats (100–200 g body weight) were divided into groups of 6 animals (each group contains equal number of both genders). The animals were housed in plastic cages, fed a normal laboratory diet and water *ad libitum*.

4.2. Treatment

All rats were treated once daily for 30 days, with oral preparations of CCNU (12.5 mg/kg and 25 mg/kg), SLENU (25–200 mg/kg) and combinations of them in accordance with the routine methods described in the literature (Geran et al. 1972). The test compounds were dissolved ex tempore as follows: first step in DMSO (from 1 ml to 3 ml) and second step in double-distilled water (made to 10 ml) and kept at 37 °C.

4.3. Blood samples

Blood was collected in tubes containing EDTA, centrifuged 15 min at 3000 rpm, and plasma was carefully separated. The erythrocyte pellet was washed three times with saline and 0.5 ml of the cell suspension was diluted with 2 ml of cold water to lyse the erythrocytes. 1.8 ml of water and ethanol/chloroform (3 : 5/v : v) were added to 0.2 ml of lysate to precipitate hemoglobin. The tubes were shaken vigorously for 5 min and centrifuged for 20 min at 2500 rpm. The resulting supernatant was used for determination of enzyme activities.

4.4. Analyses of oxidative stress-related parameters

4.4.1. Products of lipid peroxidation (MDA)

The total amount of lipid peroxidation products in the plasma was determined using the thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products (Plaser et al. 1966). The intensity of the pink colour of the resulting fraction product was read at 532 nm. Results were expressed as μM/l.

4.4.2. Measurement of antioxidant enzymes activities

Cu-SOD activity was determined in the erythrocyte lysates as described by Sun and Oberley (1988) with minor modifications. Briefly, the xanthine/xanthine oxidase system was used to generate superoxide radical anion. This species reduces nitroblue tetrazolium (NBT) to formazan which is monitored at 560 nm. SOD in the sample removes the superoxide anion and inhibits the reduction. The level of this reduction is used as a measure of SOD activity. One unit of enzymatic activity is defined as the amount of enzyme causing 50% inhibition of the reduction of NBT to formazan. Results were expressed as units per g hemoglobin (U/gHb).

CAT activity was assessed in the erythrocyte lysates according to Beers and Sizer (1952). Briefly, hydrogen peroxide was used as a substrate and the decrease in H_2O_2 concentration at 22 °C in phosphate buffer (pH = 7.0) was followed spectroscopically at 240 nm. One unit of CAT activity is defined as the amount of enzyme that degrades 1 μM H_2O_2 per min. Results are presented as units per g haemoglobin (U/gHb).

The hemoglobin concentration of lysate was determined by the cyanmethemoglobin method (Mahoney et al. 1993).

4.5. Statistical analysis

The results were reported as means ± SD. Statistical analysis was performed with Student's t-test and Multiple regression analysis. $p < 0.05$ was considered statistically significant.

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