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Original Contribution

DETERMINATION LD_{50} OF POTENTIAL ANTITUMOR AGENT SLCNUgly AND ITS EFFECT ON ANTIOXIDANT ENZYME DEFENSE *IN VIVO*

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ABSTRACT

The aim of the present study is to determine LD_{50} of the antitumor agent SLCNUgly and to evaluate its *in vivo* effect on antioxidant enzyme defense in erythrocytes of mice treated by SLCNUgly in a dose 66.6 mg/kg i.p. and compared to that of antitumor drug CCNU - 40 mg.kg i.p. The dose of 113.8 mg/kg was determined as LD_{50} for the spin-labeled nitrosourea SLCNUgly in mice. Based on the results, obtained from groups, treated with maximal effective doses of drugs we might conclude that at our experimental conditions spin-labeled nitrorsourea SLCNUgly exhibits lower general toxicity when was compared to clinically used antitumor drug CCNU.
Key words: spin-labeled nitrosourea, glycine, catalase activity, superoxide dismutase activity

INTRODUCTION

2-chloroethylnitrosourea drugs such as CCNU, N, N'-bis (2-chloroethyl)-N-nitrosourea (carmustine, BCNU), N'-(trans-4-methyl cyclohexyl)-N-(2-chloroethyl)-N-nitrosourea (MeCCNU), exhibit comparatively good therapeautic properties against human cancer mainly lymphomas, gliomas, a few solid tumors and melanomas [1,2]. Their clinical application is limited because they show delayed and cumulative hematological toxicity [6].To achieve a more selective cytotoxic effect, various carrier molecules have been selected for synthesis of new nitrosourea derivatives. Since, L-amino acids participate in transport through mammalian cell membranes series of amino acid [15]; dipeptide [13] and oligopeptide [19] nitrosourea derivatives have been synthesized and their antitumor activity in vivo evaluated. It has been found that introducing of 2,2,6,6-tetramethyI-4-aminopiperidin-l-oxyl (4-amino-Tempo) in the structure of CCNU led to decrease of its *in vivo* general toxicity and improve its antitumor activity against some experimental tumor models in mice [10,11]. At present it is accepted that the aminoxyl radicals possess low toxicity and are not mutagenic by themselves [12]. Bearing in mind, the advantages of amino acids and stable aminoxyl free radicals we have synthesized a number of spin labeled (aminoxyl radical containing) amino acid nitrososureas [23,25]. Derivatives containing alanine (SLCNUala) and glycine (SLCNUgly) moieties exhibited *in vivo* higher anhitumor activity against lymphoid leukemia LI210 and higher antimelanomic effect against B16 melanoma in comparison with antitumor drug CCNU [8,25]. Moreover, using Jerne hemolytic plaque assay we have reported a lower immunosuppression in healthy mice treated with SLCNUgly in comparison with those treated with CCNU [26]. By EPR spin-trapping technique Gadjeva et al., have found that spin labeled nitrosoureas including SLCNUgly exhibit high superoxide anion scavenging activity (SSA) which was comparatively with that of some referent antioxidants as Vitamin C and Vitamin E. The lower general toxicity of spin labeled hitrosoureas and lower immunosuppression of SLCNUgly we have explained with their high SSA activity [3,22]. In view of its *in*

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vivo expressed properties, SLCNUgly was selected for further oncopharmakological studies. By the present studies we have reported LD50 of SLCNUgly and the preliminary results concerning its effect on antioxidant enzymes SOD and CAT in erythrocytes isolated from mice treated by SLCNUgly and compared to that in mice treated with antitumor drug CCNU. Glucose level in plasma of mice treated by SLCNUgly and compared to that in mice treated with antitumor drug CCNU, was also studied.

MATERIALS AND METHODS

Glycine, dicyclohexylcarbodiimide, 2-chloroethylisocyanate and all other reagent and chemicals necessary for synthesis of SLCNUgly were purchased by Sigma Chemical Co, St. Louis, USA. Catalase, xanthine oxidase and all other reagent for study of SOD and CAT activity were purchased by Sigma Chemical Co, St. Louis, USA. Kit for determination of blood glucose was purchased by Human, Germany.

Spectrophotometric measurements were carried out on Ultrospec LKB Spectrophotometer. White laboratory mice with weight 20-40 g were used. The laboratory mice were housed in polycarbonate cages in controlled conditions (12 h light/dark cycles), temperature of 18-23 C and humidity of 40-70%, with free access to tap water and standard laboratory chow. Experiments were carried out in accordance with recommendations of National normative documents and European directive. 86/609/EEC of 24.11.1986 for protection of animals used in scientific and experimental purposes.

Mice were divided in groups of 6 and inoculated i.p. with suspension of SLCNUgly or CCNU prepared in Tween 20: saline (1:9). Before treatment every mouse was weighed and was inoculated with SLCNUgly in a volume according to the dose and its weight. Doses of 66.6 mg/kg for SLCNUgly and 40.0 mg/kg for CCNU, applied intraperitonealy were chosen to study enzyme activities of SOD and CAT and level of glucose in blood of tested mice. These doses were the maximal effective doses ("curing doses") against the tumor models of L1210 and P388 in mice. All studied parameters were mesured on 3rd, 6th, 24th and 48th h after treatment.

Determination of LD50 of SLCNUgly. The dose of 150.0 mg/kg was chosen as a maximal starting dose because at that dose, 100 % mortality was found when antitumor activity and antimelanomic effect of SLCNUgly were *in vivo* studied. Six doses were used - 150, 130, 120, 115, 110 and 100 mg/kg, and mice were divided in groups of 6 and inoculated i.p. with suspension of SLCNUgly prepared in Tween 20: saline (1:9).

Glucose was measured spectrophotometrically and expressed in mmol/1. Determination of SOD activity was based on the generation of O_2‘ in a system containing hypoxanthine/xanthine oxidase. SOD activity was determined in erythrocyte lysate according to Sun et al. [14], with some modifications [24]. SOD activity was expressed as enzyme units per g Hemoglobin (U/gHb). CAT activity was determined in erythrocyte lysate isolated from blood of tested mice according to Johanson and Borg [9], with some modifications [24]. CAT activity was expressed as enzyme units per g Hemoglobin (U/gHb).

Statistical analysis. Statistical analysis was performed with Statistica 6.1, StaSoft, Inc. and results were expressed as means ± SD. $p<0.05$ was considered statistically significant.

RESULTS

Results from determination of LD50 of spin labeled amino acid nitrosourea SLCNUgly are presented on Fig 1. KORELIA-DINAMICS programme was used for evaluation of LD50 [17,18]. Data are interpolated with a cubic spline and LD50 was calculated from described engraving. As is seen 113.8 mg/kg was the dose determined as LD50.

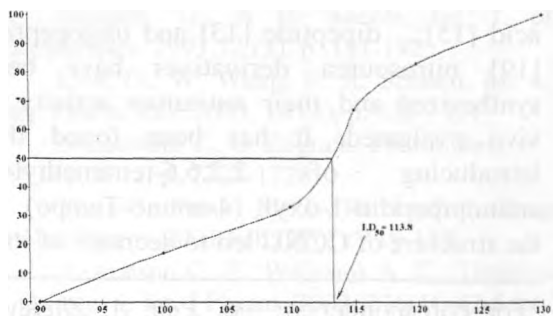


Fig. 1. Determination of LD_{S0} of SLCNUgly

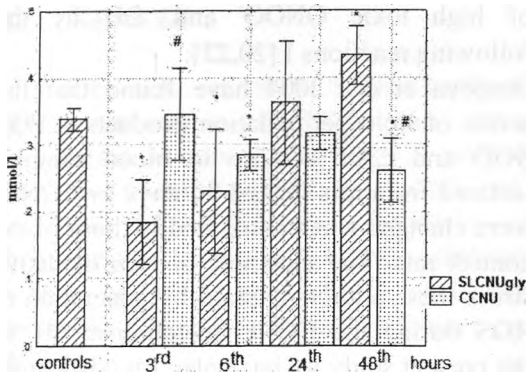


*Fig 2. Levels of glucose in plasma isolated from blood of mice treated by SLCNUgly or CCNU. * mice treated by SLCNUgly at 3rd, 6th u 48th h and treated by CCNU at 48th h vs, control group, p< 0.05; # mice treated by CCNU at 3rd and 48th h* vs, *treated by SLCNUgly at the same hours, p< 0.05.*

Results concerning the glucose levels in plasma of the tested mice and control group are shown on Fig 2. On 3rd h after treatment of mice with SLCNUgly a statistical significant decrease in glucose of tested mice was found compared either to that of the control group or to the group treated with CCNU (mean 1.84 ± 0.63 mmol/1 for SLCNUgly .vs. 3.38 ± 0.16 mmol/1 for controls and 3.45 ± 0.69 mmol/1 for CCNU). For the followed period the glucose level gradually increases and at 48th h it was statistical significant higher either comparing to that at 3rd h after the treatment with SLCNUgly or to the control group (mean 4.32 ± 0.44 mmol/l at 48th h vs. 1.84 ± 0.63 mmol/1 at 3rd h and 3.38 ± 0.16 mmol/1 for the controls).

Results obtained for effect of SLCNUgly on SOD activity are presented on Fig 3. At 3rd h after treatment the mice by SLCNUgly, the level of SOD activity was statistical significant decreased comparing to that of control group and to the group treated by CCNU (mean 150.36 ± 64.69 U/gHb for SLCNUgly vs. 1428.51 ± 232.55 U/gHb for control group and 2009.73 ± 331.96 U/gHb for CCNU). Moreover, at 6th h after treatment with SLCNUgly, SOD activity was statistical significant increased in comparison with that at 3rd h after its treatment and comparing to that at 6th h after treatment with CCNU (mean 1762.4 ±279.86 U/gHb at 6th h vs. 150.36 ± 64.69 U/gHb at 3rd h and 1111.74 ± 347.07 U/gHb at 6lh h for CCNU). As is seen at 48th h after SLCNUgly treatment SOD activity was commensurable with that of the control group (mean 1482.20 ± 65.60 U/gHb for SLCNUgly and 1428.51 ± 232.55 U/gHb for controls).

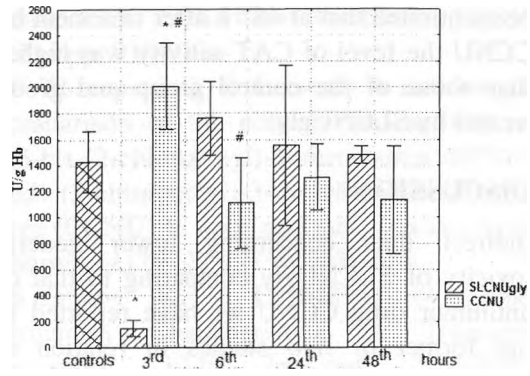


Fig 3. In vivo SOD activity in erythrocyte lysates isolated from blood of mice treated by SLCNUgly or CCNU.
** mice treated by SLCNUgly at 3rd h and by CCNU at 3rd h* vs, *control group, p<0.05.*
 # mice treated by CCNU at 3rd and 6th h vs, those treated by SLCNUgly at the same hours ,p<0.05.

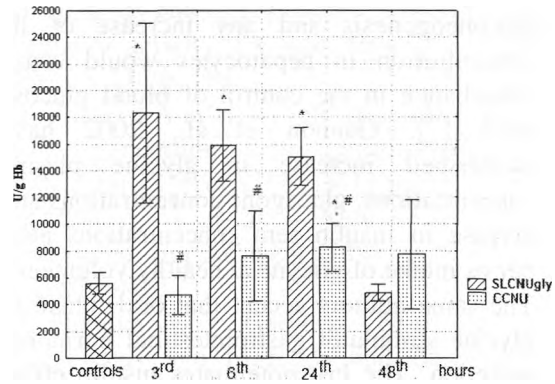


Fig 4. In vivo CAT activity in erythrocyte lysates isolated from blood of mice treated by SLCNUgly or CCNU.
** mice treated by SLCNUgly at 3rd, 6th u 24th h and treated by CCNU at 24th h vs, control group, p<0.05.*
 # mice treated by CCNU at 3rd, 6th, 24th h vs, those treated by SLCNUgly at the same hours, p<0.05.

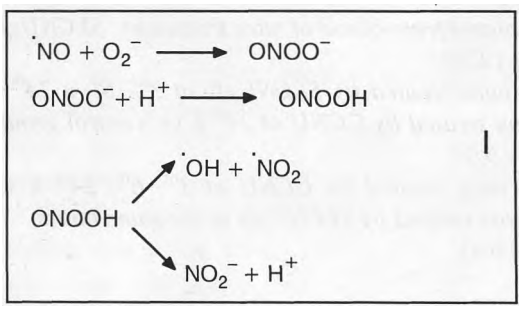
Effect of SLCNUgly on CAT activity is shown on Fig 4. After treatment by SLCNUgly it was found statistical significant increase in CAT activity at 3rd h when compared either to that of the control group or to the group treated by CCNU at 3rd h (mean 18306.49 ± U/gHb for SLCNUgly vs. 5551.73 ±812.16 U/gHb for controls and 4673.03 ± 1445.95 U/gHb for CCNU). For the followed period the level of CAT activity decreased and at 48th h was statistical commensurable with CAT activity of the

control group (mean 4864.06 ± 610.80 U/gHb for SLCNUgly and 5551.72 ± 812.16 U/gHb for controls). Furthermore, it should be mentioned that at 48th h after treatment by CCNU the level of CAT activity was higher than those of the control group and group treated by SLCNUgly.

DISCUSSION

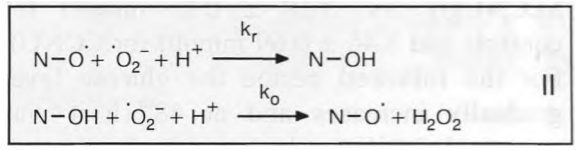
Indirect data concerning lower general toxicity of SLCNUgly comparing to that of antitumor drug CCNU we have reported in our former *in vivo* studies in relation to antitumor activity of this spin labeled amino acid nitrososurea [23,21]. By the present study the dose of 113.8 mg/kg was etermined as LD50 for SLCNUgly. This dose is twofold higher than that reported for CCNU (56 mg/kg) [16]. It is obvious, SLCNUgly exhibits twofold lower general toxicity in comparison with that of the clinic used drug CCNU.

It is well known that glycine participates in gluconeogenesis and any increase of its concentration in hepatocytes would cause disturbance in the control of blood glucose level [7]. Gannon et al., 2002 have established increase in glycine plasma concentrations, glucagons concentrations and ncrease in insulin sera concentrations after per os intake of glicyne at healthy volunteers. The same authors accept that oral intake of glycine stimulates gastrointestinal hormones secretion. The last potentiates insulin effect and as a result that lead to decrease in blood glucose level [5].



Bearing in mind above reported facts, we suppose the low levels of glucose in plasma of mice treated by SLCNUgly on 3rd and 6th h probably are due to the presence of glycine moiety in molecule of spin labeled nitrosourea. By our former UV and Visible spectrophotometrical experiments we have established a light dependent nitric oxide ('NO) generation from CCNU [20] and hypothesized that if CCNU could generate 'NO *in vivo,* it might contribute to formation of high toxic ONOO' and ˙OH by the following reactions **I** [20,22]:

Gadjeva et ah, 2004 have found that the levels of lipid peroxidation products (LPO), SOD and CAT activity in blood samples isolated from rats treated 30 days by CCNU, were changed when compared to those of the control rats. The inducible *in vivo* oxidative stress these authors explain by generation of ROS during the CCNU metabolism [4]. In the present study we have also found changes in the levels of SOD and CAT activity after a single treatment of mice by SLCNUgly or CCNU comparing to those of the control group. These data support the assumption that during their *in vivo* metabolism both nitrosoureas probably generate radical metabolites such as '0_2\ H_20_2, NO'. The low SOD activity at 3rd h in mice treated by SLCNUgly in comparison with that of the control group might be explained by the high SSA activity of the spin labeled nitrosourea dependent on presence of nitroxyl group (N-O') in its structure. It seems very likely, *in vivo* N-θ' group to compete with cell SOD for scavenging and neutralizing of ,0_2' according the scheme Π [3]:



Since, CAT is an enzyme that comparatively fast respond to the pathological increase of H_20_2 concentrations (probably to above scheme) this might explain almost three-fold increased CAT activity at 3rd h after treatment mice by SLCNUgly comparing to that of control group.

It should also be mentioned that the levels of SOD and CAT activities at 48th h after treatment the mice by SLCNUgly were commensurable with those of the control group, while those of the mice treated by CCNU were different from controls. It is evident, treatment the mice by SLCNugly or CCNU initiates oxidative stress which attenuates at 48th h after SLCNUgly treatment, while after CCNU treatment this process persists in the followed period.

Based on the LD50 determination for SLCNUgly and preliminary results obtained about its effect on SOD and CAT activities of tested mice we might conclude that spin labeled nitrosourea is a compound with lower general toxicity in comparison with antitumor drug CCNU used in the clinic.

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