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Original article

Nitroxyl-Labeled Glycine Containing 2-Chlorethylnitrosourea: A Study Of ^{99m}Tc-Radiolabeling, EPR Spectroscopy And Biological Evaluation Of New Potential Anticancer Agent For Tumor Imaging And Radiotherapy

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ABSTRACT:
Recently, a new class of *in vitro* and *ex vivo* radiotracers/radioprotectors, the nitroxyl–labeled agent N-[N'-(2-chloroethyl)-N'-nitrosocarbamoyl-glycine amide of 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (SLCNUgly), has been discovered. Our previous investigations demonstrated that SLCNUgly is a low-molecular-weight stable free radical which is freely membrane permeable, easily crosses the blood brain barrier and exhibited *in/ex vivo* the lowest general toxicity and higher anticancer activity against some experimental tumor models. Further investigation was aimed to develop a ^{99m}Tc-labeled SLCNUgly (96.5%) as a chelator and evaluate its labeling efficiency and potential use as a tumor seeking agent and for early diagnosis. Tissue biodistribution of ^{99m}Tc- SLCNUgly was determined in normal mice at 1, 2, and 24 h (n=4/ time interval, route of administration i.v.). The distribution data was compared to that using male albino non-inbred mice

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and EPR investigation. The imaging characteristics of ^{99m}Tc– SLCNUgly conjugate examined in Balb/c mice grafted with Ehrlich Ascitis tumor in the thigh of hind leg demonstrated major accumulation of the radiotracer in organs and tumor. Planar images and auto-radiograms confirmed that the tumors could be visualized clearly with ^{99m}Tc- SLCNUgly. Blood kinetic study of radio-conjugate showed a biexponential pattern, as well as quick reduced duration from the blood circulation. This study establishes Glycine Containing nitroxyl (SLCNUgly) as a new spin-labeled diagnostic marker which reduce the negative lateral effects of radiotherapy and for tumor- localization.

KEYWORDS: *SLCNUgly, Ex Vivo* EPR, ^{99m}Tc-conjugate, Biodistribution, EAT Tumor Imaging

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INTRODUCTION

Strategies to attenuate drugs and radiation toxicity in modern chemotherapy along with radiation therapy were dosage optimization, synthesis and the use of analogues having lower toxicity or a combined therapy. Clinical and experimental trials have been directed toward development of new drugs as anticancer agents to be applied individually or conjugated with toxins, drugs, natural extracts and antitumor radioisotops for chemotherapeutic and radiotherapeutic treatments[1-3]. Isotope radiolabeling (^{99m}Tc-labeled) of active anticancer agents has been also introduced for attaching the radio-isotope to the antitumor drugs to increase the effectiveness of interaction at cell levels[4,5]. Currently, the radiopharmaceuticals combined with direct radiography are the only options for investigation of location of tumor malformations and their visualization by gamma scintigraphic (*real time*) imaging of the body[6-8].

The clinically used antitumor drug Lomustine 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) indicates: 1) higher clinical activity against human malignancies, variety of human neoplasms, lymphomas, melanomas, Hodgkin's disease and brain tumors; 2) high toxicity against the normal cells, responsible for distortions to the integrity of the subsequent DNA and induced chromosomal aberration[9,10]. Different chemical structures have been used as selective carriers for the 2-chloroethyl- N-nitrosocarbamoyl cytotoxic group to optimize the antitumor action and/or decrease the toxicity of 2-chloroethylnitrosourea drugs[11-14.] It was found that presence of nitroxyl free radical moiety caused beneficial modifying effects on the toxicity and activity of the antitumor TEPA and Thio TEPA drugs[15,16]. Reduced toxicity and increased antineoplastic properties were achieved when cyclohexyl amino moiety in the structure of antitumor drug CCNU was replaced by the nirtoxyl free radical 4-Amino TEMPO[17,18]. Further a number of spin-labeled analogues of the anticancer drug CCNU was synthesized and their biological activity was studied. Some of these spin-labeled nitrosoureas showed advantages over CCNU, having lower toxicity and higher anticancer activity against experimental tumor models[9,19-22]. By EPR spectroscopy Gadjeva et al., 1994 shown that spin-labeled nitrosoureas and their precursor 4-amino TEMPO could scavenge .O^{2-} and so exhibited high superoxide scavenging activity (SSA)[23].

N-[N'-(2-chloroethyl)-N'-nitrosocarbamoyl-glycine amide of 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (SLCNUgly) (Fig. 1), has been synthesized as a spin-labeled analog of CCNU(Zheleva et al., 1995)[19]. Formerly reported results about its in vitro determined physico-chemical properties showed higher alkylating activity, and almost twice lower carbamoylating activity compairing to those of CCNU[24,25]. Half-life of SLCNUgly was also shorter comparing to that of CCNU (29 min for SLCNUgly and 54 min for CCNU). *In vivo* SLCNUgly exhibited higher antileukaemic activity, higher antimelanomic effect and better immunomodulatory properties when was compared to its nonlabeled analogue antitumor drug CCNU[19,25].

As the nitroxyl radicals possess high T1 contrast properties they could be used in MRI and EPR imaging investigations[26]. Because the chemical and biological properties of SLCNUgly this nitroxyl containing drug is attractive for further studies that include *in vivo* and/or *ex vivo* techniques like as MRI and EPR organ diagnostics studies. The conventional method for investigation of paramagnetic (spin-labeled) agents, their organ distribution, including the *ex vivo* tissue homogenates and *in vivo* live animals is the electron paramagnetic resonance (EPR) spectroscopy[27]. This unique technique allows measuring and representing processes of the metabolism of free radicals, the reactive oxygen species (ROS), organ/tissue oxygenation and nitrous oxide production (RNS) in the normal physiology and cancer processes. Unfortunately with EPR/EPRI spectroscopy cannot be determined the precise organ-specific location of the tumor in the body[28,29]. A thorough examination and understanding of the targeting, the visualization and biodistribution in different organs of the spin-labeled compounds could be achieved by radiolabeling with Technetium-99m (99m-Tc)[1-3,30]. 99m-Tc is the radionuclide of choice in the development of diagnostic imaging agents by virtue of its wide availability, convenient half-life, and ideal energy for imaging[6-8,29,30].

Therefore, the aim of the present study was to determine and compare the pharmacokinetic/biodistribution of the spin-labeled glycine containing 2-chloroethylnitrosourea SLCNUgly before and after its labeling with 99m-Tc. The solubility, the pharmacodynamics of elimination through blood circulation for a short/long time of SLCNUgly were also investigated by EPR distribution in organs of healthy mice. In the present study we also study the accumulation and specific tumor uptake of the SLCNUgly radioactive conjugate in solid *Ehrlich Ascites Tumor,* toxicity and permeability for BBB by gamma imaging assay.

MATERIAL AND METHODS

CHEMICALS

Spin-labeled drug SLCNUgly was synthesized according to Zheleva et al., 1995[19]. Stannous Chloride dehydrated ($SnCl_2.2H_2O$), the spin-trapping agent, PBS and $K_3[Fe(CN_6)]$were purchased from Sigma-Aldrich Chemical Co, St. Louis, USA. ^{99m}-Tc was procured from Regional Center for Radiopharmaceuticals, Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India. All other chemicals and solvents were of analytical reagent grade.

^{99m}Tc-LABELING STUDY

SLCNUgly (4 mg) was dissolved in 1 ml distillated water and was labeled with ^{99m}Tc by direct labeling method[31]. Briefly 0.1 mL stannous chloride solution (1mg/mL) was added to it and pH was adjusted to 7.0 using 1M sodium bicarbonate sol. Freshly eluted 2mci(74MBq) 99mTc-pertecnetate in 0.5ml saline was added, mixed thoroughly and the reaction mixture was incubated for 10-20 min at room temperature (22°C). After incubation of the radio-conjugate, the radiochemical purity and *in vitro* studies up to 24 hr were carried out by paper chromatography using ITLC-SG (instant thin layer chromatography-silica gel) paper as the stationary phase and acetone and saline as the mobile phases.

INSTRUMENTATION

HPLC analyses were performed on a Waters chromatograph efficient with 600 coupled to a Waters 2487 photodiode array UV detector. Instant thin layer chromatography (ITLS-SG) (Gelman Sciences Inc, Ann Arbor, MI) was used for labeling efficiency determination. Gamma imaging and biodistribution studies were done using a planner gamma camera (Hawkeye, Germany) and gamma-scintillation counter (Capintec,USA) respectively. All EPR measurements were performed on X-band EPR^{micro}, spectrometer (Bruker, Germany), equipped with standard Resonator, Bulgaria. Experiments were carried out in triplicate. The EPR spectra were measured at room temperature (300K) at modulation amplitude 10.00 G and microwave power 1.28 mW.

STABILITY STUDY OF THE ^{99m}Tc-SLCNUgly

The percentage labeling efficiency and stability of ^{99m}Tc-SLCNUglyat a particular time point was performed as per the method described earlier[4].It was estimated by ascending instant thin layer chromatography (ITLC) (Gelman Sciences Inc, Ann Arbor, MI) using acetone and pyridine, acetic acid and distillated water (PAW) (3:5:1,5 v/v) as mobile phases, and silica gel (SG)-coated fiber glass strips as the stationary phase. Approximately 2 to 3 μL of the radio-labeled complex was applied at a point 1 cm from one end of an ITLC-SG strip. The strip was developed in acetone or 0.9% saline and the solvent front was allowed to reach 8 cm from the point of application. The strip was cut horizontally into 2 halves, and the radioactivity in each segment was determined in a well-type gamma ray counter calibrated for ^{99m}Tc energy. The free ^{99m}-Tc-pertechnetate that moved with the solvent (R_f = 0.9-1.0) was determined. The reduced/hydrolyzed (R/H) technetium remained at the point of application whereas free pertechnetate and labeled complex moved with the solvent front in PAW.

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CODEN JPBSCT
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BLOOD KINETICS

For EPR experiments of SLCNUgly (administrated i.p. at a dose of 40 mg/kg) blood samples were taken from (male albino non-inbred mice) the free streaming blood and were collected into heparinized tubes containing PBS (pH=7-7.4). Blood clearance of ^{99m}Tc-SLCNUgly was determined in rabbit by administering intravenously 18.5MBq of the radiolabeled complex into the dorsal ear vein and thereafter collecting blood samples from the other ear veins of the rabbit starting from15min to 24 hour post injection and then counting the samples in the gamma counter. Decay-corrected radioactivity in the blood was expressed as percent injected dose in blood, using total blood volume as 7% of the body weight. Animal protocols have been approved by Bulgarian/Indian Institutional Animals ethics Committee.

TUMOR LINE

Experimentally, monolayer cultures for cell experiments of murine cell line EAT (*Ehrlich Ascitis tumour*) were used. EAT was maintained in the peritoneum of the mice in the ascites form by serial weekly passage. The exponentially growing cells were washed, and suspended in phosphate buffered saline (PBS) pH=7.4. Approximately 10-15 million cells were subcutaneously injected into the thigh of the right hind leg of the mice (8–12 week old, weighing about 25-30 gm). Tumors were allowed to grow for 7-10 days to reach a diameter of approximately 0.9–1cm and thereafter used for further studies. An injected dose of 120 µCi (100 µL) of ^{99m}Tc-SLCNUgly was used.

EPR ORGAN AND BLOOD DISTRIBUTION

Biodistribution of SLCNUgly in organ homogenates (liver, lungs, spleen, pancreas, brain, kidneys) and blood of male albino non-inbred mice (35-40 g body weight, normal diet) was evaluated by EPR spectroscopy as previously described by Gadzheva and Koldamova., 2001[10]. Spin-labeled nitrosourea was administrated i.p. at a dose of 40 mg/kg. Animals were decapitated at appropriate time points following injection (10, 30, 60, 90 min and on the 4h, 24 h) and dissected. Tissues from lungs, liver, spleen, brain, kidneys, pancreas and blood were collected and processed immediately. For nitrosourea extraction, samples were weighed and homogenized in PBS (10% w/v) and centrifuged at 2000 g for 15 min. Supernatants were collected and the concentration of nitrosourea was evaluated by EPR spectroscopy. Before measuring, the spin-labeled concentration, the samples were deoxidized by $K_3[Fe(CN_6)]$ (a spectroscopic broadening reagent), because of the fast reduction of the nitroxide function (10-20 min) in the tissues.

^{99m}Tc-BIODISTRIBUTION

The study was performed to assess the distribution and localization of ^{99m}Tc-labeled SLCNUgly. An intravenous injection of ^{99m}Tc-SLCNUgly in a volume of 100 µl was injected through the tail vein of each mouse (Balb/c mice, 22-30g. body weight, and normal diet). At 1, 4, and 24h after injection, mice were sacrfii ced and dissected. Tissues from different organs (liver, kidneys, lungs, muscle, spleen, brain, heart) were removed, made free from adhering tissues, weighed, and then their radioactivity was measured in a shielded well-type gamma scintillation counter calibrated for ^{99m}Tc-energy. Uptake of the radiotracer in each tissue was calculated and expressed as percent injected dose (activity) per gram of the tissue (% ID/g).

GAMMA SCINTIGRAPHIC IMAGING IN EAT bearing mice

Image viewing was performed using planar gamma camera (Hawkeye)[32]. To enable qualitative radiolabeled-agent localization over time imaging was performed in EAT (10-15 million) cells implanted tumor bearing mice by injecting 100µci ^{99m}Tc-SLCNUgly in the tail vein. Images were obtained at different time intervals starting from 1h, 2h, 4h, and 24 h post injection. Data presented are results from experiments performed in quadruplicate or (3 mice in each four groups). Ellipsoid regions of interest (*ROIs*) based on the gamma images were drawn on the heart, lungs, stomach, muscle, liver, spleen, and brain, around the kidneys, and around the total body. The ROI was also drawn on the contralateral muscle of the mice in the left hind limb. For delineation of the tumor, at threshold of at least 25% of the maximum pixel value was chosen. For the calculation of tumor uptake at 2,5 h after injection, this threshold value was individually adjusted to obtain the same ROI volume as the tumor ROI at 1 h for the same animal. Uptake was calculated as the counts in the tissue divided by the injected activity and normalized for the ROI size (%ID/g). Tracer elimination at 1h and 24 h was calculated by subtracting the total body counts at the time of imaging from the injected activity and expressed as percentage by multiplying with 100 and dividing by the injected activity.

STATISTICAL ANALYSIS

J PBMS
Journal of Pharmaceutical and Biomedical Sciences
ISSN NO- 2230 – 7885
CODEN JPBSCT
NLM Title: J Pharm Biomed Sci.

Statistical analysis was performed with Statistica 6.1, Sta-Soft, Inc. and results were expressed as mean ± standard error (SE) or standard deviation (SD). Statistical significance was determined by the Student's t-test. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

After synthesis the structure of SLCNUgly (Fig.1), was confirmed by IR spectroscopy and elemental analysis. The result for τ0,5 (29 min) lower carbamoylating activity (37.78±0.17/ EPR research) and higher alkylating activity (0.831 A560/mM/h) exhibited the best combination for a good therapeutic index, high cell membrane transport, high antitumor activity and low cytotoxicity. The EPR spectrum registered was symmetric triplet constant strong signals with aN = 16-17 G, characteristic for the nitroxyl-labeled six-membered cycle (Fig. 1).

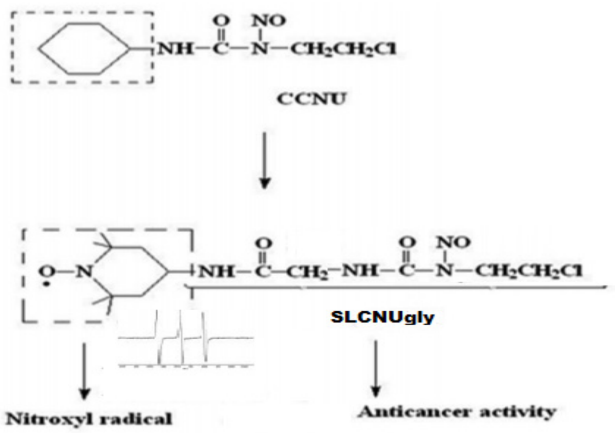

Figure 1. Chemical structures of non-labeled drug CCNU, nitroxyl-labeled analog SLCNUgly and EPR spectrum of the spin-labeled agent.

QUALITY CONTROL TEST

Radiolabeled ^{99m}Tc–SLCNUgly spin-labeled conjugate was challenged to test the stability and the labeling efficiency and results are presented in Table 1.

Table 1. *In vitro* stability of ^{99m}Tc-labeled SLCNUgly conjugate at different time intervals.

Time, h	% Labeled complex	% Free TcO_4	% Reduced hydrolyzed
0	96.5%	1,3%	1-2,2%
1	96.5%	1.3%	1-2.2%
4	96.5%	1,3%	1-2.2%
24	96.5%	1,3%	1-2.2%

Optimum conditions required for maximum labeling efficiency were established. The drug was found chemically pure (98.9 %) and *in vivo* stable. It was labeled with ^{99m}-Tc with more than 96.5% labeling efficiency and only 2-2.5% degradation was observed at 24 hr. Stability of the labeled complex with time was studied in saline at standard conditions (37°C, pH=7), as shown in Table 1. The high stability of labeled radioactive product for a long time ensures continuing suitability for *in/ex vivo* use. This 2-chloroethylnitrosourea has high metal stability, rapid organ allocation and uncompromised reactivity.

BLOOD KINETICS

Simeonova et al., 1994[33] have found that polybutylcyanoacrylate nanoparticles loaded with spin-labelled nitrosourea were localized in the lungs and blood of Lewis lung carcinoma-bearing mice[33]. Based on this finding the blood kinetics data of the spin labeled 2-chloroethylnitrosourea, registered by EPR (Fig. 2A) and ^{99m}-Tc-SLCNUgly in blood of healthy mice after labeling with radioisotope (Fig.2B) were investigated, respectively.

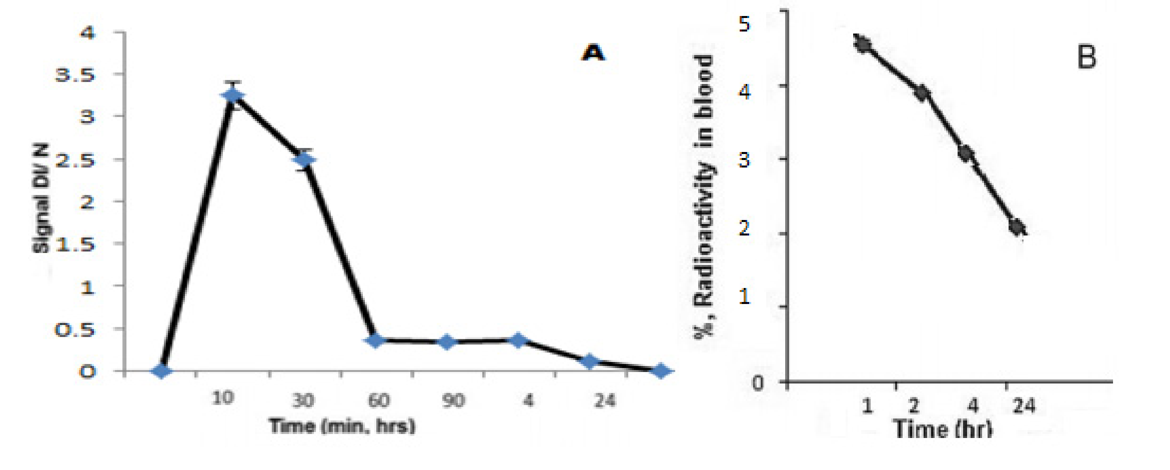

Figure 2. **(A)** Blood kinetic curve of SLCNUgly of male albino non-inbred mice after i.p. administration. EPR-Measurements were performed on four groups of three animals. The SE associated with 6% of the presented values. **(B)** Blood kinetic curve of ^{99m}Tc- SLCNUgly of Balb/c mice after i.v. administration. Data from the groups of five mice are expressed as a mean % ID/g ± SD.

The blood samples were collected using a micro-capillary at 10, 30, 60, 90 min and 4, and 24h p.i. of SLCNUgly and 1h, 2h, 4h and 24h p.i. with ^{99m}Tc-SLCNUgly. The blood clearance studies of free drug conducted showed that the half-life of the drug was greater than the drug in its free state. The maximum concentration of SLCNUgly, registered by EPR (arbt. units), in blood reached at 10 min after i.p. injection and almost completely observed to 24 h. The maximum concentration of ^{99m}Tc–conjugate reached at 60 min p. i. and then declined gradually.

EPR *Ex vivo* ORGAN/TISSUE DISTRIBUTION

EPR biodistribution of SLCNUgly was investigated in organ homogenates (lungs, liver, spleen, pancreas, brain, kidneys, and blood) of male albino non-inbred mice (Fig.3).

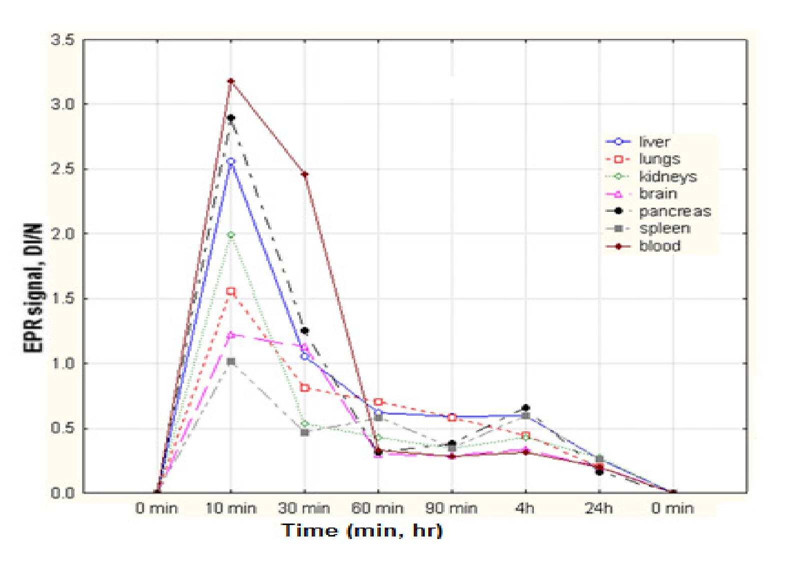

Figure 3. Tissue distribution of SLCNUgly in organ homogenates (liver, spleen, lung, kidneys, pancreas and brain) and blood of albino non-inbred mice after i.p. administration. EPR-Measurements were performed on four groups of mice/ three animals. The SE associated with 6% of the presented values.

The data showed almost complete absence of the spin labeled 2-chloroethylnitrosourea within 4h in all tissues studied. In liver, lungs, kidneys, brain, pancreas and spleen the maximum concentration of nitrosourea was seen 10 min after administration of the drug. A relatively low accumulation found in the lungs, brain and spleen was a prerequisite for a low toxicity in these

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NLM Title: J Pharm Biomed Sci.

organs. The spin labeled nitrosourea was mainly localized in the blood, pancreas and in the liver on the 10 min.

RADIOCONJUGATE BIODISTRIBUTION

To evaluate the potential significance of SLCNUgly uptake/elimination by various tissues with regard to cytotoxicity, the biodistribution of the drug labeled with ^{99m}Tc as shown in Table 2 was carried out. The data demonstrated that the major accumulation of the radio-conjugate activity in terms of percent injected dose per gm organ/tissue was in lung, liver, spleen, kidney, stomach and intestines.

Table 2. *In vitro* biodistribution study of ^{99m}Tc- SLCNUgly in whole body organ homogenates and blood of Balb/c health mice after i.v. administration. Data from the groups of five mice are expressed as a mean % ID/g ± SD at different time intervals.

Organs	% ID/g 1h	% ID/g 4h	% ID/g 24h
Blood	4,7±0.98	3,4±0,76	2,07±0.17
Heart	0,98±0.22	0,92±0.2	0,43±0.08
Lungs	9,7±1.25	8,3±1.12	3,8±0.21
Liver	38,6±3.84	34,5±3.62	21,8±1.06
Spleen	21,7±1.77	15,3±1.09	13,2±0.23
Kidney	7,7±1.00	6,62±0.97	4,01±0.36
Stomach	0,48±0.18	0,47±0.16	0,36±0.03
Intestine	2,35±0.52	1,22±0.5	0,32±0.07
Muscle	0,34±0.12	0,29±0.11	0,22±0.02
Brain	0.11±0.001	0.09±0.001	0.00±0.00

SCINTIGRAPHY IMAGING IN EAT BEARING MICE

Localization of ^{99m}Tc-SLCNUgly in Balb/c mice with EAT tumor at 2.5h post injection as seen by gamma camera imaging, is presented in Fig. 5.

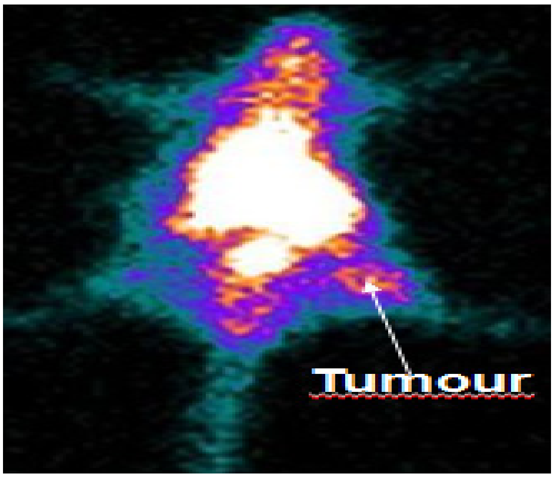

Figure 5. Whole body scintigraphic images of radiolabeled 99mTc- SLCNUgly nitrosourea in female BALB/c mice bearing s.c. EAT (*in the right thigh*) tumor implant.

Imaging was carried out at different time intervals. The mice depicted the beginning of accumulation of activity in tumor at 1h, which reached to maximum at 2,5 h.

DISCUSSION

Spin-labeled amino acid nitrosourea (SLCNUgly), a nitroxyl free radical analog of CCNU, was synthesized and over the last 27 years its modifying effects on the toxicity and antitumor activity of the 2-chloroethyl-N-nitrosocarbamoyl cytotoxic group have been *in vivo* investigated[19,22,25]. All beneficial effects of SLCNUgly have been attributed to its high superoxide scavenging activity which was explained by the nitroxide presence in the nitrosourea structure[22,34,35].
By this research we extend our investigation on the pharmaco-kinetic profile of spin-labeled amino acid nitrosourea estimating its EPR biodistribution

ISSN NO- 2230 – 7885
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in organs. SLCNUgly reached the maximum localization in the blood, liver, pancreas, lungs, kidneys and brain even at 10 min after administration. Moreover, the high concentration in bloodstream remained almost constant in the period from 10th to 30th min of the study. This result can be explained by the presence of amino acid residue in the structure of SLCNUgly, which is easily recognized by cell receptors, and is a prerequisite for high and selective accumulation. Low drug concentrations established after 30 min of the SLCNUgly injection were in accordance with its formerly reported comparatively short half-life[24,22]. This fact also explains why 30 min after injection concentrations of SLCNUgly gradually decrease in all measurements.

As is seen (Fig. 3) to the 30th min was measured higher SLCNUgly concentration in the pancreas in comparison with those found in the other organs. It is well known that pancreas maintains the body's blood glucose (sugar) balance by the primary hormones insulin and glucagon that regulate blood glucose levels. Gannon et al., 2002 have reported that oral intake of glycine causes increase in glucagon and insulin secretion in sera of healthy volunteers which led to decrease of glucose levels measured in blood of the same volunteers[36]. Bearing in mind the results of Gannon et al., we consider that the high pancreas concentration of the glycine containing SLCNUgly demonstrarted by the present study corresponds to our formerly reported low glucose levels measured in blood of healthy mice treated by the same nitrosourea[35]. It seems likely formerly measured low blood glucose levels to due to the higher accumulation of the glycine containing SLCNUgly in the pancreas comparing to the other organs.

Fast accumulation of SLCNUgly in brain (10 min) indicates that the compound successfully can cross the BBB and may be used in further *in vivo* experiments to find application for treatment of brain tumors.

At present the low organ/ tissue toxicity of the nitroxyl labeled 2-chloroethylnitrosoureas was attributed to their high SSA explained by the presence of the stable nitroxyl radical structure[23,22]. Other authors based on their studies propose nitroxyl radicals to be used for labeling of conventional therapeutics and noninvasive magnetic resonance imaging[37,38]. A higher EPR signal intensity of tissue homogenates than the same signal from the TEMPOL-treated animals was also cinfirmed[38]. Although nitroxyls have a lower relativity than conventional contrast agents such as gadolinium and technetium complexes, the volume distribution of the SLCNUgly can be explained by its high cell permeability. It is well known that amino acids participate in transport through mammalian cell membranes. The presence of glycine amino acid moiety in SLCNUgly could be the reason for its good cell permeability and to act as a high transport cell vehicle.

Based on the above-mentioned facts, we have made the following assumptions to explain the *ex vivo* maximum labeling efficiency and low toxicity effect of the ^{99m}Tc-SLCNUgly conjugate; and to verify the possible use as a contrast marker for early detection/screening of body-tumor formation and to confirm the tumor-localization in the brain tissue.

The experimental data revealed that at a period of 24 hours incubation, more than 96.5% binding with ^{99m}Tc signifies not only the high stability of the ^{99m}Tc-labeled product but also its suitability for *in vivo* use. The gradual decline of the labeled compound from the circulation suggests its high binding with the plasma proteins. Distribution data demonstrated that the maximum accumulation of radio-conjugate was in liver (38.6±3.84%) followed by spleen (21.7± 1.77%), lungs (9.7± 1.25%) and kidneys (7.7± 1.00 %) at 1st hour post injection (Fig. 4A).

The accumulation of the radio-conjugate in the liver hepatocytes (38.6 %/g) at 1st h post injection shows, that the major portion of the conjugate is excreted through hepatobiliary route. The decreased liver radioactivity established at 24th h (7.6 %/g) also confirms the clearance of the conjugate through hepatobiliary route. This may be therapeutically acceptable, since the liver is the usual metabolizing organ for most of the drugs. Accumulation in kidney (7.7 %/g) at 1st h that is reduced to 1.04 %/g at 24th h post injection indicates that a part of the radiolabeled drug is eliminated from the body via renal route. Accumulation and retention in intestines (Table 2) have been observed to be stable at 1-4 hrs time points studied, associated probably with the good absorption function of the radio-conjugate. In previous in vitro studies we have shown SLCNUgly almost completely counteracted CCNU bactericidial effect towards fresh overnight cultures of *Escherichia coli* strain[34]. Based on this finding[34] we consider that in spite comparatively good absorption and retention of the radio-conjugate found in the intestine, SLCNUgly probably would not exhibit *in vivo* high toxicity in intestine. The insignificant uptake of activity in the stomach (0.48

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%/g) is highly suggestive of its *in vivo* stability. In the Fig. 4A are presented the compartmental distribution of tissues between 1 hour and 24 hours after injection. Radio-conjugate was rapidly and randomly distributed into the brain tissue (0.11 %/g for 1 hr) but has disappeared quickly and reaches a minimum value of 24 hours. Recently, some authors also reported that the MRI signal of a BBB quickly disappeared after transportation from the blood vessels to the brain (Fig. 4B)[39,40].

Localization of ^{99m}Tc-SLCNUgly in Balb/c mice with EAT tumour at 2.5 h post-injection as seen by gamma camera imaging is presented in Fig. 5. Imaging was carried out at different time intervals. The mice depicted the beginning of accumulation of activity in tumour at 1 h, which reached the maximum at 2.5 h.

A number of studies on possible clinical applications of the nitroxides as well as to provide insight into the mechanisms of radiation cytotoxicity have been carried out[41]. The nitroxides allow to explore the mechanisms of action of different chemotherapeutic agents for tumor imaging. Understanding these processes is important for the process of ameliorating the toxicity of therapies and to the rationale design of future agents[42,43,44]. Scintigram of EAT bearing mice at 2.5 h corroborated with biodistribution data. In C57BL/Lymphoma L_{1210} bearing mice the optimal dose for anticancer activity of SLCNUgly was 66.6 mg/kg[19]. Maximum target-to-non-target ratio in Balb/c mice was obtained at 2.5 h which substantiates the potential of the nitroxides, represent as a new class for tumor scintigraph[37,39], radioprotectors which may have application as general antioxidants and for *in vivo* radiotherapy[40].

CONCLUSION

Nitroxides represent a new class of spin-labeled radioprotectors which could find application as antioxidants. The excellent EPR results, stability and affinity of SLCNUgly towards the solid Ehrlich ascites tumor and other experimental tumor models indicates that this compound has substantial promise for use in the *in vivo* visualization of tumors. Present findings have led to the development of potential and selectiveness of SLCNUgly for further toxicological studies and radio-therapy with promising applications for active brain-tumor targeting.

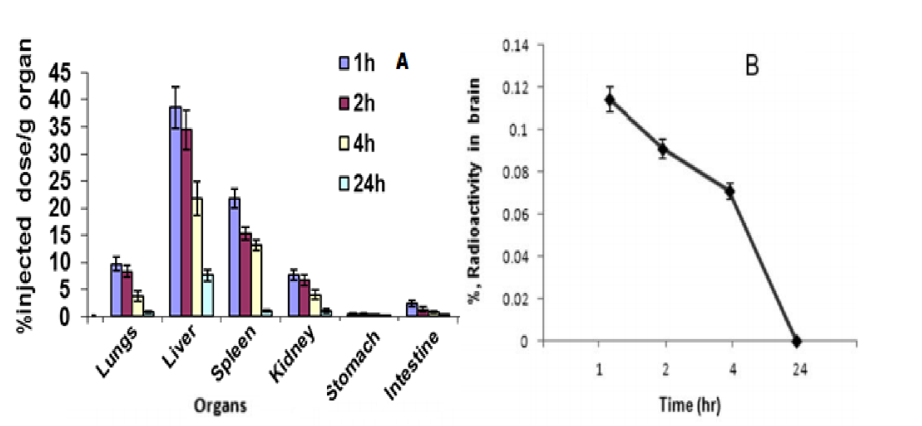

Figure 4. **(A):** Tissue biodistribution of 99mTc-SLCNUgly in organ homogenates (liver, spleen, lung, kidneys); and **(B):** in brain homogenates of Balb/c mice after i.v. administration. Data from the groups of five mice are expressed as a mean % ID/g± SD.

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