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RADIO STABILITY AND BIOLOGICAL EVALUATION OF NEW CLASS NITROXYL-LABELLED AGAENTS AS RADIOTRACERS

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ABSTRACT
Recently, a new class of *in vitro* and *in vivo* radiotracers, the nitroxyl -labelled agents SLENU and SLCNUgly, analogs of CCNU have been discovered. Previous investigations demonstrated that SLENU and SLCNUgly have low molecular-weight stable free radicals which have freely membrane permeable. The nitroxyls easily crossed 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- SLENU (97 %) and ^{99m}Tc- SLCNU gly (98.7 %) as a good radiotrasers, to evaluate their labeling efficiency, potential use as organ/tumor early-diagnosis agents and compared to clinically used ^{99m}Tc-CCNU. *In vitro* EPR spectra of the drugs were characterized by symmetric triplet constant strong signals with a_N = 16-17G, typical for nitroxyl-labelled six-membered cyclic (nitroxyl) radicals. Blood kinetic and lungs location studies of nitroxyl- radio- conjugates showed a biexponential pattern, as well as quick reduced duration from the blood circulation and homogenates. This study characterizes nitroxyl-labelled nitrosoureas as a general class of new spin-labeled diagnostic markers that have high radio- stability, can reduce the negative lateral effects of anticancer therapy and might be applied in *ex/in vivo* organ-visualization.

Key words: ^{99m}Tc-SLENU, ^{99m}Tc-SLCNU gly, ^{99m}Tc- CCNU, *Ex Vivo* EPR, Stability, Radiotracers.

NTRODUCTION

In radiation oncology, research and development of anticancer agents to be applied individually or conjugated with radioisotops, is the basis of radiotherapeutic treatments (1, 2). For this reason several new synthetic anticancer agents were traced for *in vitro* stability, toxicity and protective effects for the treatment of human cancers and therapy (3). Isotope radiolabeling strategy has been introduced for attaching the radio-isotope to the antitumor drugs to increase the effectiveness of interaction at cell levels. Utilization of direct isotopic radio-conjugation (^{99m}Tc) of active agents is the subject of many investigations (4, 5). To reduce the negative effect of radiotherapy and drugs damages, radiotracers are usually used for labeling of diagnostic markers, and for localization to the individual organs and tissues. For this purpose, needs of stable, inert compounds with lower toxicity and good stability which affected tumor cells only with high activity were felt (6).

The clinically used drug (N'-cyclohexyl-N-(2-chloroethyl)-N-nitrosourea) CCNU indicated higher clinical sensitivity and activity against human malignancies, neoplasm, Hodgkin's disease, but exhibits high toxicity against the normal cells, responsible for distortions to the integrity of the subsequent DNA and induced chromosomal aberration (7, 8). These facts were a prerequisite for the synthesis and research of new drugs SLENU (1-ethyl-1-nitroso-3-[4-(2,2,6,6-tetramethylpiperidine-1-

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oxyl)]-urea) and SLCNUgly (1-ethyl-1-nitroso-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-urea), the spin-labeled analogues of CCNU, with a strategy based on reduction of cytotoxicity and passive delivery to tumors (8, 9, 10, 11). Nitroxyl radicals have aplication for labeling of conventional therapeutics easily crossed the blood-brain barrier, as tracers for EPR-MRI investigations and in radiation oncology (12, 13, 14) and as paramagnetic agents are attractive for electron paramagnetic resonance (EPR) structure diagnostics (15).

In the present study, we introduce the pharmacokinetic studies and biological evaluation of paramagnetic nitrosoureas after conjugation with radionuclide Technetium-99m. Electron paramagnetic resonance (EPR) structure investigations, stability and further examination on short/long time blood circulation and organ allocation, were investigated and compared to the clinical used non-spin-labeled nitrosourea CCNU.

MATERIALS AND METHODS

Chemicals

Nitrosoureas, nitroxyl-labelled analogues of CCNU, were synthesized and over the last 22 years their properties and activities have been investigated (7, 8, 9, 10). Stannous Chloride dehydrated ($SnCl_2$.$2H_2O$) 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 grade.

99mTc-labeling study

Drugs were dissolved in distillated water and was labeled with freshly eluted 2mci (74MBq) ^{99m}Tc-pertecnetate by direct labeling method (16). After incubation (22^{o}C) of the radio-conjugates, the radiochemical purity and *in vitro* studies up to 1- 24 hr were carried out by ITLS.

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. EPR measurements were performed on a X-band EMX^{micro} spectrometer (Bruker, Germany), equipped with standard Resonator. The direct EPR spectra were measured at 12 G with 1.03 G field modulation intensity and 6.29 mW microwave power.

Stability study of the ^{99m}Tc-conugates

The percentage labeling efficiency and stability of ^{99m}Tc-nitroxyl conjugates and ^{99m}Tc-CCNU at a particular time point was performed as per the method described earlier (17). It was estimated by ascending ITLC and (PAW) (3:5:1,5 v/v) as mobile phases, and SG strips as the stationary phase. The free ^{99m}Tc-pertechnetate (Rf = 0.9- 1.0) and reduced/hydrolyzed (R/H) technetium were determined.

Blood kinetics

Blood clearance of ^{99m}Tc-SLENU, ^{99m}Tc-SLCNUgly, and ^{99m}Tc-CCNU were determined in animals by administering intravenously 18.5 MBq of the radiolabeled drugs into the dorsal ear vein and collecting blood samples (60 min - 24 h) were placed 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 Indian Institutional Animals ethics Committee.

Statistical analysis

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 $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

SLENU and SLCNUgly formally, consist of two parts: N-nitroxyl spin-labeled part (characterized with low toxic effects, sensitiveness to the reduction of biological samples) and non-cycle structure (responsible for the anticancer activity and toxicological characteristics) (8, 12, 13, **Figure 1**).

Preclinical studies demonstrated that nitroxyls possessed excellent cell and blood-brain barrier permeability, selectiveness uptake in B16 melanomas, C57BL tumor, testicular carcinomas, Hodgkin's disease (7-11). The compounds exhibited *in vivo* low general toxicity, short half-life time and possessed high super scavenging activity (SSA) with the dose-limited toxicity and good therapeutic index (8, 9, 10) (**Table 1**).

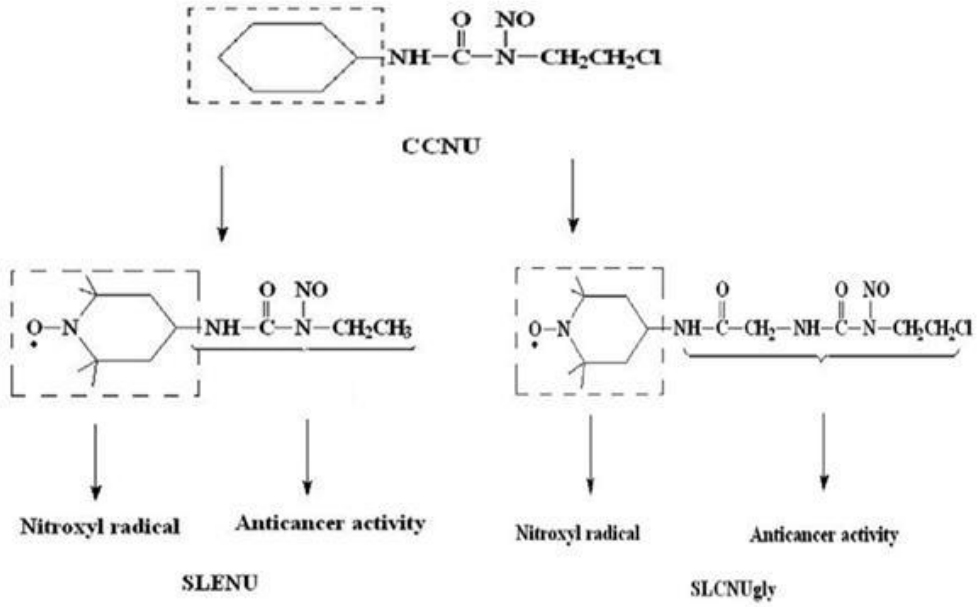

Figure 1. The structures of spin-labeled nitrosoureas, analogues of the clinically used drug CCNU.

Table 1. *Physicochemical properties of the spin-labeled nitrosoureas and CCNU(8, 9).*

Compound	***$\tau_{0.5}$ (min)***	***Alkilating activity (A_{560}/Mm/h)***	***Carbomoylating activity (%)***	***SSA IC_{50} (μM)***
CCNU	54	0.34	63.17	~
SLENU	75	0.008	54.46	67
SLCNUgly	35	0.50	37.78	10.22

In solution form spin-labelled nitrosoureas exhibit EPR spectra consisting of strong symmetric triplet signals with aN = 16-17 G, typical for the nitroxyl radical moiety, presenting in their structures (**Figure 2**).

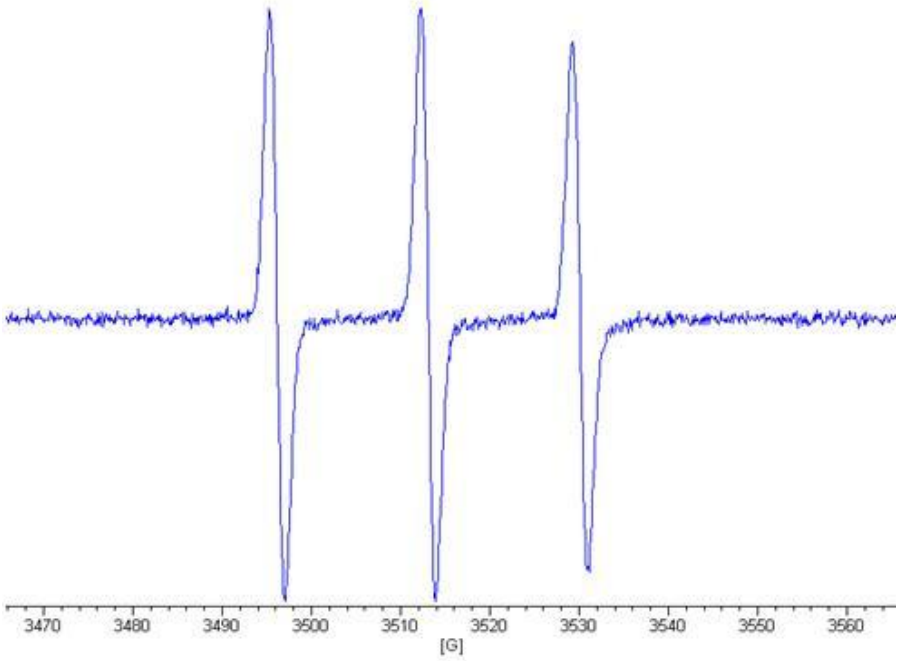

Figure 2. Typical EPR spectrum of a nitroxyl-labelled nitrosourea.

Radiolabeled 99mTc– nitroxyl conjugates and 99mTc– CCNU were challenged to test the stability and the labeling efficiency and optimum conditions are presented in **Table 2**.

Table 2. *The labelling efficiency, reduced/hydrolyzed and percent of free technetium of the conjugates.*

Compound	***%, 99m-Tc-labeled***	***%, R/ H***	***% free TcO_4^{2-}***
^{99m}Tc–CCNU	98	0.7	1.3
^{99m}Tc–SLENU	96.9	1.1	2.0
^{99m}Tc–SLCNUgly	97	1.3	1.0

The nitroxyl-labelled agents were found chemically pure and in vivo stable **(Table 2)** with more than 97% labeling efficiency, compared to CCNU - 97.8 %. Only 1- 2 % degradation of the complex were observed at 1 to 24 hr. Stability of the conjugates with time was studied in saline at standard conditions (37°C, pH=7). The reduced/hydrolyzed (R/H) technetium for nitrosyl-labelled compounds was 1.0-1.3%, respectively, compared to CCNU - 0.7%. The spin-labeled agents demonstrated high metal stability for long time, uncompromised reactivity and isotop-labelling activity will determine their rapid organ allocation.

Some authors have found that spin-labelled nitrosourea loaded with nanoparticles were localized in lungs of Lewis lung-carcinoma-bearing mice (18), possessed low toxicity and quick reduced duration in blood/ lungs homogenates of C57BL mice, and antioxidant effect to the incorporated nitroxide (8, 9). Based on these findings, the blood kinetics data and lungs localization of nitroxyl-labeled nitrosourea loaded with 99mTc were investigated and compared to the clinical used compound CCNU. It was established that the half-lifes of the 99mTc–conjugates were greater than those of the drugs in a free state.

The maximum concentration of 99mTc-nitroxyl-conjugates in blood **(Figure 3)** reached at 1h post injection, declined gradually and completely observed to 4 h after administration, in comparison with 99mTc-CCNU.

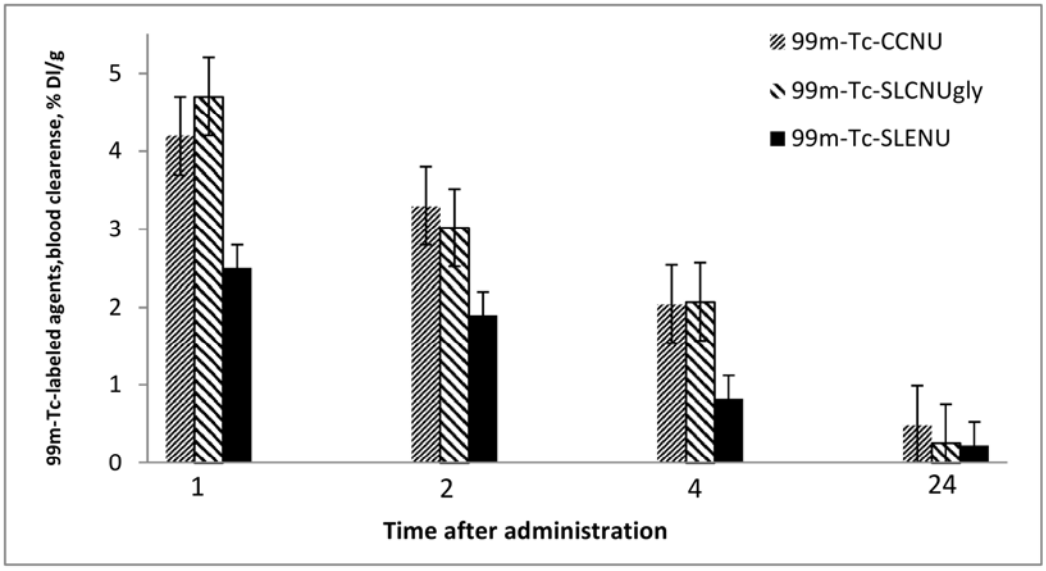

Figure 3. Blood clearance of mice treated by 99mTc- spin-labelled conjugates and 99mTc-CCNU (% DI/g).

To evaluate the potential significance of nytroxil- labeled drugs uptake/ elimination by lungs tissues with regard to cytotoxicity, the biodistribution of the drugs labeled with 99mTc as shown in **Figure 4** was carried out.

Results from lungs accumulation of 99mTc-nitroxyl-conjugates **(Figure 4)** in reached at 1h p. i. and in compliance with the allocation in blood circulation, confirm a quickly reduced exhaustion of spin-labeled products in comparison to the Lomustine.

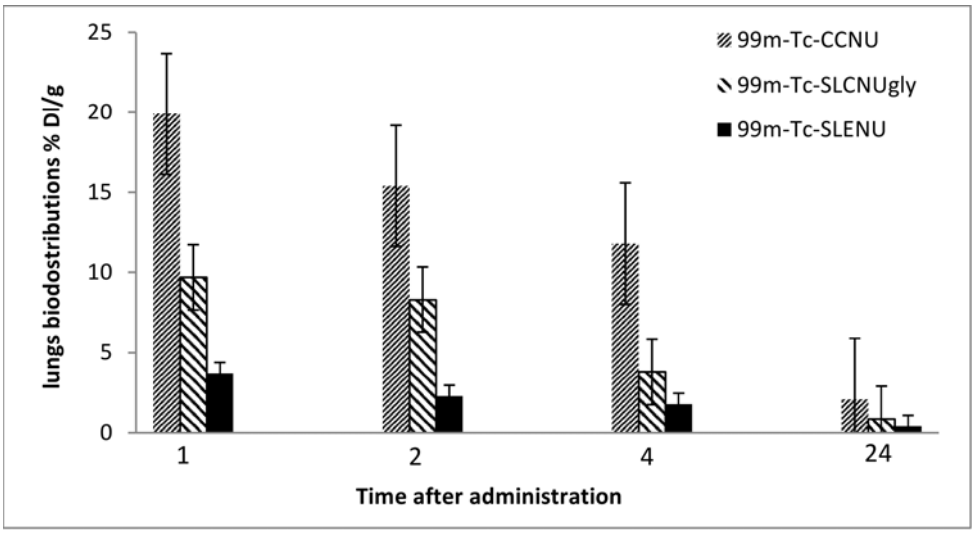

Figure 4. Lungs distibution of mice treated by 99mTc-spin-labelled conjugates and 99mTc-CCNU (% DI/g).

CONCLUSION

Spin-labelled nitrosoureas represent a new class of agents that might have simultaneously application as general antioxidants and antitumor drugs, as well. Because selectiveness of the nitroxyl moiety and radio stability of the 99m Tc- conjugated spin- labeled nitrosoureas they might to be further developed as radiotracers for in vivo organ-visualization and tumour targeting.

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