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Spin labelled nitrosoureas and triazenes and their non-labelled clinically used analogues — a comparative study on their physicochemical properties and antimelanomic effects

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Abstract

Physicochemical properties, such as half life time ($\tau_{0.5}$), alkylating and carbamoylating activity and in vivo antimelanomic effects against B16 melanoma of spin labeled (containing nitroxyl free radical moiety) amino acid nitrosoureas, synthesized in our laboratory, have been studied and compared to those of the antitumor drug N′-cyclohexyl-*N*-(2-chloroethyl)-*N*-nitrosourea (lomustine, CCNU). We have shown that the introduction of amino acid moieties and the replacement of cyclohexylamine with nitroxyl moiety leads to a faster decomposition, higher alkylating, lower carbamoylating activity, better antimelanomic activity and lower general toxicity, when compared to those of CCNU. It was also established that spin labeled triazenes, previously synthesized by us, were more stable in phosphate saline than their nonlabeled analogue, 5-(3,3-dimethyltriazene-1-yl)-imidazole-4-carboxamide (dacarbazine, DTIC). A higher cytotoxicity to B16 melanoma cells than to YAC-1 and lymphocytes was demonstrated for all spin labeled triazenes, in comparison with DTIC. An assumption has been made to explain the lower general toxicity of the spin labeled nitrosoureas compared to that of CCNU. Based on the results presented, we accept that a new trend for synthesis of more selective and less toxic nitrosourea and triazene derivatives as potential antimelanomic drugs might be developed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Spin labeled; Nitroxyl free radical; Malignant melanoma; Triazenes; Nitrosoureas; DOPA-oxidase activity

1. Introduction

2-Chloroethylnitrosoureas and triazenes belong to the alkylating chemotherapeutic agents. Some, such as CCNU, *N*,N′-bis (2-chloroethyl)-*N*-nitrosourea (carmustine, BCNU), N′-(*trans*-4-methyl cyclohexyl)-*N*-(2-chloroethyl)-*N*-nitrosourea (MeCCNU), DTIC and 3,3-dimethyl-(4-carboxyphenyl)-triazene (DM-COOH) have been applied for the treatment of human cancer, mainly lymphomas, gliomas, a few solid tumors and melanomas (Comis and Carter 1974; Comis, 1976

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Carter et al., 1988). Single agents of most interest in the treatment of malignant melanomas include DTIC and nitrosoureas, such as CCNU (Comis and Carter, 1974; Vorobiof and Falkson, 1982), but complete responses to chemotherapy with these drugs are rare. The addition of interferons or interleukin-2 to selected chemotherapeutic agents has additive or even synergistic effects in metastatic melanoma (Legha et al., 1989 Atkins, 1997 Margolin et al., 1998). Pyrhonen et al. has investigated the addition of α-interferon to the combination of DTIC, vincristine, bleomycin and CCNU for metastatic melanoma. The same authors reported an overall response rate of 62% in 45 assessable patients (Pyrhonen et al., 1992). Instead of high in vivo activity, clinical efficacy of nitrosourea drugs is limited because they show delayed and cumulative hematological toxicity (Green et al., 1981). A serious disadvantage of DTIC as a chemotherapeutic agent derives not only from its high toxic side-effects, but also from its photosensitivity that leads to a rapid decomposition (Baker, 1980). Replacement of the imidazole ring with an aryl- or other heteroaryl ring stabilizes the triazenes and in most of cases, does not adversely affect their activity (Cameron et al., 1985). In order to achieve a more selective cytotoxic effect, various carrier molecules have been selected for synthesis of new nitrosourea and triazene derivatives. Since, L-amino acids participate in the transport through mammalian cell membranes series of amino acid (Tang and Eisenbrand, 1981) dipeptide (Sosnovsky et al., 1993) and oligopeptide (Zeller, 1986), nitrosourea derivatives have been synthesized and their antitumor activity in vivo evaluated. Bearing in mind that the presence of the nitroxyl free radical moiety could modify the toxicity and activity of TEPA and Thio TEPA derivatives (Gutierrez et al., 1981), a spin labeled analogue of the anticancer drug CCNU was prepared (Raikov et al., 1985; Sosnovsky and Li, 1985). This compound showed advantages over CCNU — it had lower toxicity and higher anticancer activity against some experimental tumor models (Sosnovsky and Li, 1985; Ilarionova et al., 1985). At present, it is believed that the aminoxyl moiety acts as a transport vehicle through cell membranes and a conclusion has been made that, in general, aminoxyl radicals possess low toxicity and are not mutagenic by themselves (Sosnovsky, 1992). On the other hand, Blagoeva et al. found that a selective accumulation of 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TMPO) occurred in hamster and mice melanotic melanomas (Blagoeva et al., 1979). Both the advantage of amino acids and formerly reported selective accumulation of the stable nitroxyl radicals (spin labels) in melanoma tissues, led us to synthesize several spin labeled amino acid 2-chloroethylnitrosoureas as potential antimelanomic drugs. All these nitrosoureas showed in vivo high antileukemic activity against lymphoid leukemia L1210 (Zheleva et al., 1991, 1995). Moreover, a good correlation between antileukemic activity of the spin labeled amino acid nitrosoureas and some of their biochemical properties was found (Ilarionova et al., 1993). Bearing in mind the above mentioned selective accumulation of the nitroxyl radicals in melanoma tissues, we synthesized a number of spin labeled 1-aryl and 1-heteroaryl triazenes, as potential antimelanomic drugs (Raikov et al., 1993), as well. Recently, we have investigated modulating effects on DOPA oxidase activity of mushroom tyrosinase of the spin labeled nitrosoureas and triazenes, as a preliminary prognosis for their antimelanomic activities (Gadjeva et al., 1999). In the present study, we report our investigations on spin labeled amino acid nitrosourea and triazene derivatives, formerly synthesized in our laboratory with respect to their antimelanomic effects and some physicochemical properties in comparison with clinically used nitrosourea CCNU and triazene DTIC. Furthermore, bearing in mind the formerly reported facts: (1) an excellent expressed superoxide scavenging activity (SSA) of the spin labeled nitrosoureas and triazenes (Gadjeva et al., 1994); and (2) light dependent nitric oxide generation from antitumor drug CCNU (Zheleva et al., 1997), we have tried to explain the lower general toxicity of the spin labeled nitrosoureas in comparison with their nonlabeled clinically used analogue, CCNU.

2. Materials and methods

2.1. Compounds

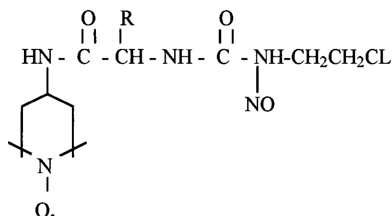
The spin labeled nitrosourea and triazene derivatives used for this study were synthesized by the procedures previously described (Zheleva et al., 1991, 1995 Raikov et al., 1993) (Figs. 1 and 2). The antitumor drugs DTIC and CCNU were purchased from Bristol-Myers Squibb Co. The free stable nitroxyl radical 4-amino-TMPO was purchased from Aldrich Chemical Company. All other reagents used were of the best quality commercially available.

2.2. Cells

YAC-1 mNK target Moloney lymphoma cells and B16 melanoma cells were kind gifts of the Department of Cellular Biology, Tokay University, Isehara, Japan.

2.3. Determination of $\tau_{0.5}$ of the spin labeled nitrosoureas

The half-life times of the nitrosoureas were determined according to Wheeler et al., 1974 with slight modifications. The compounds were dissolved in absolute ethyl alcohol in concentrations 5×10^{-3} M. The same volume of 0.1 M phosphate buffer pH 7.4 was added to each solution. Solutions were incubated at 37°C. Absorptions of the solutions were periodically measured at 230 nm on an Ultrospec LKB Spectrophotometer, Sweden. The results obtained for $\tau_{0.5}$ are expressed in minutes.



Compound	R
SLCNUgly	H
SLCNUala	CH_3
SLCNUleu	CH_2-$CH(CH_3)_2$
SLCNUmet	CH_2CH_2-S-CH_3
SLCNUphe	CH_2-C_6H_5

Fig. 1. Chemical structures of the spin labeled aminoacid nitrosoureas.

2.4. Determination of $\tau_{0.5}$ of the spin labeled triazenes

Chemical stability of triazenes was determined in 0.01 M phosphate buffer/Me_2SO, by following the absorption change in UV spectrum at the wavelength ≈ 300 nm, corresponding to the absorption maximum for the compound under investigation (Lucas and Huang, 1982) A sample of a solution of the drug in Me_2SO was added to 0.01 M phosphate buffer (pH 7.2) and was incubated at 37°C for 200 min. Measurements were carried out on an Ultrospec LKB spectrophotometer, Sweden. Spectra were recorded and the half-life determined from a logarithmic plot of the extent of decomposition against the time.

2.5. Determination of alkylating activity of the spin labeled nitrosoureas

Alkylating activity of the compounds was determined according to Gadjeva et al., 1989. Briefly, various concentrations of the nitrosoureas were incubated 2 h at 37°C reactive medium containing acetone, 0.025 M acetate buffer of pH 7 and 0.8 ml 4-(n-nitrobenzyl)-piridine (NBP) solution in acetone. Then the reaction mixture was cooled on ice and 0.25 M NaOH and ethylacetate were added to it. The colour of the ethylacetic layer was recorded on an Ultrospec LKB spectrophotometer, Sweden. Alkylating activities of the compounds are expressed through $A_{560} \times mM^{-1} \times h^{-1}$.

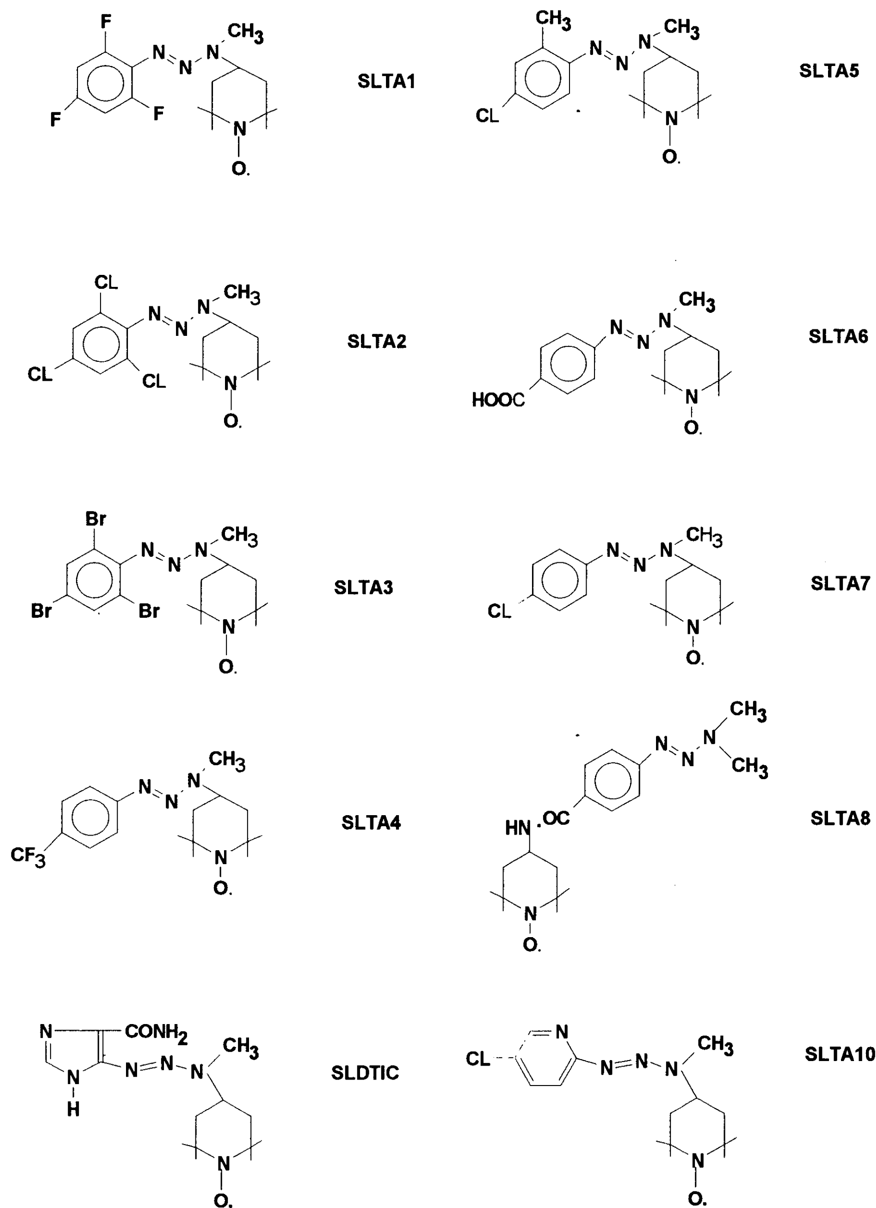


Fig. 2. Chemical structures of the spin labeled triazenes.

2.6. Determination of carbamoylating activity of the spin labeled nitrosoureas

Carbamoylating activities of the nitrosoureas were determined following the method of Gadjeva et al., 1997. Briefly, 4-amino-TMPO (50 μmol) was dissolved in ethanol and PBS for a stock solution. To 450 μl of this solution, 5 μmol of the corresponding nitrosourea dissolved in ethanol was added. The reaction mixtures were incubated at 37°C in a water bath and aliquots were withdrawn for analysis each hour for 6 h. Each mix-

Table 1
Physicochemical properties of the spin labeled aminoacid nitrosoureas and CCNU

Compound (code)	$\tau_{0.5}$ (min)	Alkylating activity[a] ($A_{560} \times mM^{-1} \times h^{-1}$)	Carbamoylating activity[b] (%)
SLCNUmet	25	0.85	32.44
SLCNUala	28	0.62	34.47
SLCNUgly	29	0.83	37.78
SLCNUleu	33	0.67	39.98
SLCNUphe	40	0.12	46.31
CCNU	54	0.34	62.68

[a] Note comments on its evaluation made in Section 2.5.
[b] Note comments on its evaluation made in Section 2.6.

ture (10 μl) was spotted on TLC plates and chromatograms were developed with chloroform/methanol (9:1,v/v), dried and visualized on UV light at 254 nm. The smears corresponding to a nitroxide radical were scraped off and extracted. Nitroxides present at different position from 4-amino-TMPO or corresponding nitrosourea were attributed to the products of carbamoylation. Then, each extract was measured on a JEOLJES-FE2XG EPR spectrometer (Tokyo, Japan). Carbamoylating activity was determined by the following equation and expressed as a percent of carbamoylation of 4-amino-TMPO: $\{([4\text{-amino-TMPO}]_0\text{-}[4\text{-amino-TMPO}]_T)/[Y]_0\} \times 100$, where $[4\text{-amino-TMPO}]_0$, $[4\text{-amino-TMPO}]_T$ and $[Y]_0$ are the concentrations of 4-amino-TMPO at time 0, at time T and the concentration of the nitrosourea Y at time 0, respectively.

2.7. Antimelanomic activity of the spin labeled nitrosoureas — in vivo test

All experimental procedures and the type of mice used, hybrid BDF1 (DBA/2xC57Bl/6) were in accordance with the routine methods described in the literature (Geran et al., 1972), with slight modifications. On day 0, BDF1 mice (average weight: 18–22 g) were inoculated subcutaneously with 10% tumor cell suspension in saline in volume of 0.5 ml. On day 3, various doses of spin labeled amino acid nitrosoureas (Table 2) were administrated i.p. in a single injection in volume 0.01 ml per body weight, as 10% ethanol solutions in saline. The control group (22 mice) received only the same volume of 10% ethanol in saline. Six mice for each treated group were used. The antimelanomic effect against B16 melanoma was evaluated by comparing the weights of the tumors of the control animals to those of the treated mice. The TGI parameter was calculated by the formula $[Tc - Tt/Tc] \times 100$, where Tc represents the weights of the tumours of the control mice and Tt represents the weights of the tumors of the treated mice (Geran et al., 1972).

2.8. Cytotoxicity of the spin labeled triazenes — in vitro test

Cytotoxicity of triazenes on normal leukocytes was investigated by the method of Weisenthal et al., 1984. Cells were separated by the modified method of Boyum, 1968. Heparinizised vascular blood from healthy donors was layered on Ficoll–Hypaque gradient. Mononuclear cells were collected in interphase, washed and cultured in RPMI 1640 medium for 3 days. The drug dose was varied from 12.5 to 150 μM/ml. Drugs were administered in Me_2SO solution, so that the final

Table 2
Half-life time of the spin labeled triazene derivatives in 0.01 M phosphate buffer, at 37°C

Compound[a]	λ_{max} (nm)	$\tau_{0.5}$ (min)	Degradation[b] (%)
DTIC	325	30	74
SLTA5	300	>200	18
SLTA7	320	>200	16

[a] The half-life times of the other spin labeled triazenes demonstrated similarity and are not shown here.
[b] After illumination with UV light for 120 min at 55°C.

Me_2SO concentration was usually 0.5%. Controls were treated with the same concentration of Me_2SO in PBS buffer (pH 7.4).

Approximately 10^4, either B16 cells or YAC-1 cells were grown with drug for 3 h in RPMI 1640 medium supplemented with following components: FBS, L-glutamine, penicillin C and streptomycin. The dose was varied from 12.5 to 150 μM/ml. Control cultures received medium without drug. Cells were washed three times with 0.154 M NaCl, resuspended in RPMI 1640 medium at a final concentration of 1×10^4 and then seeded with 1 ml of cell suspension per well and incubated for 3 days at 37°C in 48 well plates. The viability of the cells was assessed on the third day by Trypan blue exclusion. The drug efficiency was evaluated by calculating the IC_{50}, (dose required to achieve 50% decrease in cell growth) according to the method already described (Emond and Page, 1982).

3. Results and discussion

Results for alkylating activity, carbamoylating activity and $\tau_{0.5}$ of the spin labeled amino acid nitrosoureas and antitumor drug CCNU are presented in Table 1. All spin labeled nitrosoureas showed shorter $\tau_{0.5}$ and lower carbamoylating activity, when compared to those of CCNU. Moreover, their alkylating activities were higher than that of CCNU. Only the spin labeled phenylalanine nitrosourea showed lower alkylating activity in comparison with CCNU. It is obvious that, as a whole, both the introducing of amino acid moieties and the replacement of the cyclohexyl moiety with nitroxyl free radical ones in the nitrosourea structure lead to a fast decomposition of the compounds and also decrease their carbamoylating activities and, on the other hand, increase their alkylating activities.

Cameron et al. have established that some acetoxymethylaryltriazenes are stable in phosphate saline for > 2 h and have shown antitumor activity against mouse tumor models comparable with other arylalkyltriazenes (Cameron et al., 1985). The results from the half-life times of the spin labeled triazene derivatives are shown in Table 2. As can be seen, the introducing of nitroxyl free radical in the triazene structure increases the stability of the compounds. All spin labeled triazenes were stable in phosphate saline ($\tau_{0.5} > 200$ min). After UV exposure spin labeled triazenes decomposed into the corresponding aryldiazonium ions that were identified on thin-layer chromatograms.

Results from in vivo test against B16 melanoma of the spin labeled amino acid nitrosoureas and CCNU are presented in Table 3. The highest antimelanomic effect was found for the spin labeled alanine nitrosourea. This compound completely inhibited melanoma B16 growth (TGI = 100%) at a dose of 34.1 mg/kg. All other spin labeled nitrosoureas (with the exception of SLCNUmet) also showed better activity than antitumor drug CCNU. Moreover, the spin labeled nitrosoureas showed a lower general toxicity than CCNU (Table 3, number of animals, deaths/total).

Table 4 shows the results from in vitro assay of spin labeled triazenes and DTIC against B16 melanoma cells, moloney lymphoma YAC-1 cells and human lymphocytes (NL). Spin labeled triazenes were twice less toxic against NL than DTIC. Thus, for SLDTIC, the IC_{50} was 120 μM, whereas for DTIC, IC_{50} was 62.2 μM. Moreover, spin labeled triazenes appears to be more toxic to B16 cells than to YAC-1 cells and to NL in comparison with DTIC (Table 4). The magnitude of the difference between IC_{50} for the cell types depended on the drug; the difference was greater for SLDTIC and SLTA5 than for DTIC. It was deduced that spin labeled triazenes were selective cytotoxic agents towards B16 cells.

Studies on the modulating effects of the spin-labeled nitrosourea and triazenes derivatives on DOPA-oxidase activity of mushroom tyrosinase have recently been made (Gadjeva et al., 1999). Modulating effects of the spin-labeled triazenes on DOPA-oxidase activity of mushroom tyrosinase have been studied in comparison with the effect of the free stable nitroxyl radical, 4-amino-TMPO. All studied spin labeled triazenes activated the enzyme reaction, whereas clinically used nonlabeled triazene DTIC showed inhibiting effect. The nitroxyl radical showed the highest activating effect. So, the activating effect of the spin

Table 3
Antimelanomic effects of the spin labeled aminoacid nitrosoureas and CCNU against B16 melanoma in mice

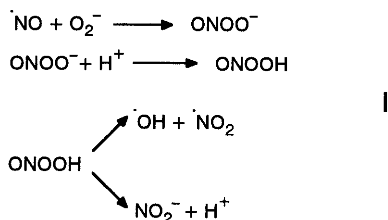
Compound	Dose (mg/kg)	TGI[a] (%)	Number of animals (deaths/total)
SLCNUala	5.7	33.3	0/6
	11.4	62.0	0/6
	22.8	85.2	0/6
	34.1	100.0	1/6
	51.2	100.0	2/6
	64.0	–	6/6
SLCNUgly	61.4	4.8	0/6
	76.8	52.0	0/6
	96.0	85.7	0/6
	120.0	85.7	0/6
	150.0	–	6/6
SLCNUleu	49.2	66.7	0/6
	61.4	76.2	0/6
	76.8	85.7	1/6
	96.0	90.7	1/6
	120.0	–	6/6
SLCNUmet	37.0	61.9	0/6
	55.6	61.9	0/6
	83.3	–	6/6
SLCNUphe	32.0	48.1	0/6
	64.0	77.8	0/6
	96.0	88.9	1/6
	120.0	–	4/6
CCNU	22.2	63.0	0/6
	33.3	85.2	2/6
	50.0	85.2	2/6

[a] TGI (%) parameter was calculated by the formula $[Tc-Tt/Tc]\times 100$ where Tc represents the weights of the tumours of the control mice and Tt represents the weights of the tumours of the treated mice (Geran et al., 1972).

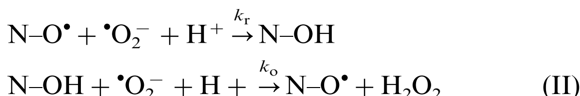
labeled triazene on DOPA-oxidase activity could be explained only by the presence of nitroxyl moiety in their structures (Gadjeva et al., 1999). We also demonstrated that the spin labeled amino acid nitrosourea derivatives had a dual modulating effect on DOPA-oxidase activity — activating at the beginning of the enzyme reaction and inhibiting later on — in comparison with that of CCNU, which expressed an inhibiting effect only. In view of the fact that all spin labeled amino acid nitrosoureas had short half life times, we accepted that at the beginning of the enzyme reaction activating effect, the nitroxyl moiety dominated, while later on because of their decomposition, the inhibiting effect of the isocyanate manifested (Gadjeva et al., 1999). Bearing in mind the above demonstrated in vitro selectivity towards B16 melanoma cells for the spin labeled triazenes and high in vivo antimelanomic activity for the spin labeled aminoacid nitrosoureas, we suppose that they are due to the presence of nitroxyl free radical moiety and are closely related to their modulating effects on DOPA-oxidase activity of mushroom tyrosinase, as well.

Our formerly electron spin resonance (ESR) study demonstrated that after UV irradiation in benzene, CCNU could be activated to a free radical intermediate (Raikov et al., 1990). By other recently performed UV and visible spectrophotometrical experiments, we have established a light dependent nitric oxide ($^{\bullet}NO$) generation from CCNU (Zheleva et al., 1997).

Based on this last finding, we have hypothesized that if CCNU could generate $^{\bullet}NO$ in vivo, it might contribute to tissue $ONOO^-$ and $^{\bullet}OH$ production (Freeman, 1994) by the following reactions I:



By ESR method, we have shown that spin-labeled nitrosoureas, spin-labeled triazenes and their precursor 4-amino-TMPO, scavenged $^{\bullet}O_2^-$ and so possessed high SSA, while clinically used nitrosourea CCNU and triazene DTIC exhibited no SSA (Gadjeva et al., 1994). It was proven that the mechanism of SSA activity was as a result of a redox cycling between nitroxide and its corresponding hydroxylamine (Gadjeva et al., 1994) according to the following equations II:



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

$$\text{N–OH} + {}^{\bullet}O_2^- + H + \xrightarrow{k_o} \text{N–O}^{\bullet} + H_2O_2 \qquad \text{(II)}$$

where k_r and k_o were second-order rate constants for the reduction of nitroxide and oxidation of hydroxylamine by superoxide, respectively. Thus, the beneficial effects, such as high antimelanomic activity and low toxicity of spin labeled nitrosoureas and triazenes could be attributed to the antioxidant effect of the incorporated nitroxide, which is derived efficiently from the redoxcycling equations (II) (Gadjeva et al., 1994).

Based on both above-mentioned facts, we have made the following assumption to explain in vivo lower general toxicity of the spin labeled amino acid nitrosoureas compared to that of CCNU. Since both nonlabeled CCNU and spin labeled nitrosoureas possess nitroso groups in the structures, during their in vivo methabolism they probably might generate nitric oxide which by means of reactions I, might lead to the generation of the high toxic $ONOO^-$ and $^{\bullet}OH$. However, only spin labeled nitrosoureas that contain nitroxyl free radical moiety might successfully compete with $^{\bullet}NO$ generated from the same nitrosourea molecule for scavenging of $^{\bullet}O_2^-$ and thus, a redox cycling between nitroxide and its corresponding hydroxylamine according to the equations II would be realized and that might prevent formation of the above mentioned high toxic species.

In conclusion, we also consider that the introduction of nitroxyl moieties in the structure of nitrosourea and triazene derivatives might start a new trend for the preparation of more selective and less toxic potential antimelanomic drugs.

Table 4
Inhibition of cell growth by spin labeled triazenes

Compound	Tumour cells	IC_{50}[a] (μmole/ml)
SLDTIC	B16	30.0
	YAC-1	75.0
	NL	120.0
SLTA5	B16	32.5
	YAC-1	73.3
	NL	100.5
SLTA7	B16	33.6
	YAC-1	74.8
	NL	100.5
DTIC	B16	38.9
	YAC-1	50.5
	NL	62.2

[a] IC_{50} (dose required to achieve 50% decrease in cell growth).

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