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International Journal of Pharmaceutics 247 (2002) 39–45

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Two spin labeled triazenes: relationship between biochemical and biological activities

Vesselina G. Gadjeva *

Department of Chemistry and Biochemistry, Medical Faculty, 11 Armeiska Street, 6000 Stara Zagora, Bulgaria

Received 5 September 2001; received in revised form 11 June 2002; accepted 26 June 2002

Abstract

Biochemical and biological activities of two recently synthesized spin labeled triazenes, containing the nitroxyl free radical moiety at different places of the triazene structure have been studied and compared with those of the antitumor drug Dacarbazine (DTIC). Tissue distribution of the triazenes was investigated in vitro in organ homogenates, tumor (B16 melanoma) and blood of C57BL mice using the electron paramagnetic resonance (EPR) method. The spin labeled triazenes were mainly localized in the tumor and in the brain. Normal leucocites, YAC-1 mNK target Moloney lymphoma cells and B16 melanoma cells were treated with spin labeled triazenes in vitro and the effects on cell viability were compared. Spin labeled 3,3-dimethyl triazene with nitroxyl radical as a substituent in the benzen ring was more cytotoxic to B16 melanoma cells than to YAC-1 Moloney lymphoma cells and normal leucocites in comparison to the spin labeled monomethyl triazene. The spin labeled derivatives were assessed with low toxicity for BDF1 mice hybrids in vivo. These results could be interpreted in terms of a possible correlation between tissue distribution and the selective antimelanoma activity of the spin labeled triazenes.

Keywords: Triazenes; Spin labeled; B16 melanoma; Tissue distribution; EPR-method

1. Introduction

Metastatic malignant melanoma remains a frustrating and almost invariable fatal disease. Alkylating agents are the most frequently used drugs in chemotherapeutic regiment for the treatment of malignant melanoma. The most common drug in use is 5-(3,3-dimethyltriazene-1-yl)-imidazole-4-carboxamide (Dacarbazine, DTIC) (Comis, 1976; Lucas et al., 1982; Jungelius et al., 1998). 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). The hydrosoluble triazene, *p*-(3,3-dimethyl-1-triazeno) benzoic acid potassium salt (DM-COOK, CAS 70055-49-1) has been described as a highly active antimetastatic and antidisseminative agent and in consideration of these properties, DM-COOK has been proposed as an advantageous substitute for its clini-

* Tel.: +359-42-2819-326; fax: +359-42-600-705
E-mail address: vgadjeva@mf.uni-sz.bg (V.G. Gadjeva).

0378-5173/02/$ - see front matter
PII: S0378-5173(02)00360-5

cally employed analog DTIC (Varnavas et al., 1991).

It is believed that the stable nitroxyl radicals (spin labels) can act as a transport vehicle through cell membranes (Sosnovsky, 1990) and in general aminoxyl radicals possess low toxicity (Sosnovsky, 1992). It is also known that nitroxyl radicals accumulate predominantly into pigmented melanoma tumors (Blagoeva et al., 1979). In order to achieve a more selective cytotoxic effect, we synthesized a number of spin labeled nitrosoureas and triazenes as potential selective antimelanomic agents (Raikov et al., 1993; Zheleva and Gadjeva, 2001). In our preliminary studies, a higher cytotoxicity to B16 melanoma cells than to YAC-1 and to normal lymphocytes was demonstrated for some of the spin labeled triazenes in comparison with DTIC (Zheleva and Gadjeva, 2001). It was deduced that spin labeled triazenes are selective cytotoxic agents towards B16 cells.

By this paper we present our comparative study on two spin labeled triazenes, analogues of 3,3-dimethyl-(4-carboxyphenyl)-triazene (DM-COOH), containing the nitroxyl free radical moiety at different places of the triazene structure with respect to their selective accumulation in B16 melanoma tumors and selectivity towards B16 melanoma cells.

2. Materials and methods

2.1. Compounds

The spin labeled triazene derivatives 2,2,6,6-tetramethyl-4-[(3-(4-carboxyphenyl)-1-methyl-2-triazenyl)]piperidyl-1-oxyl (SLTA6) and 2,2,6,6-tetramethyl-4-[(4-(3,3-dimethyl-1-triazenyl)-benzenyl)-benzoylamino]piperidyl-1-oxyl (SLTA8) used for this study were synthesized by the procedure formerly reported (Raikov et al., 1993; see Fig. 1). The antitumor drug DTIC was purchased from Bristol-Myers Squibb Co. All other reagents used were of the best quality commercially available.

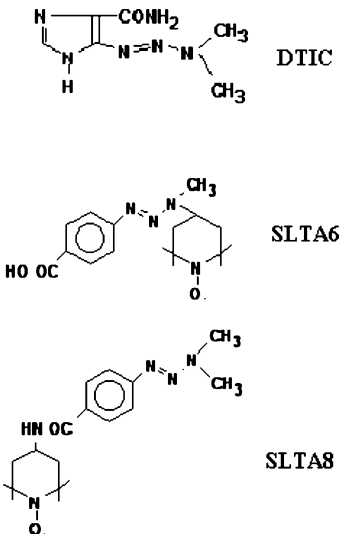

Fig. 1. Chemical structures of spin labeled triazenes and DTIC used for this study.

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 chemical half-life

The half-lives ($\tau_{1/2}$) of the triazenes were determined by following the change in absorbance in solution during incubation at 37 °C using the method of Cameron et al. (1985). The solutions were prepared by dissolving the compounds (0.26 mM) in DMSO/0.01 M phosphate buffer (pH 7.2) and distilled water. The decrease in absorbance was monitored at a wavelength near 300 nm corresponding to the absorption maxima for the respective compounds. Measurements were carried out on a LKB spectrophotometer (Sweden).

2.4. Drug distribution studies

Distribution of the spin labeled triazenes SLTA6 and SLTA7 in organ homogenates (liver, spleen, brain, kidneys) and blood was evaluated in four groups of C57BL mice with 20–25 g body weight and subcutaneously implanted with tumor

B16 melanoma. Tumor bearing animals were tested at day 14th after the implantation. Spin labeled triazenes were administrated i.p. at a dose of 50 mg/kg. Animals were decapitated after appropriate time intervals (10, 30, 60 and 90 min following injection) and dissected. Blood samples were taken from the free streaming blood and were collected into heparinised tubes containing PBS (pH 7.4). Tissue from visceral organs (liver, spleen, brain, kidneys) and from the tumor were taken, weighed and homogenized in PBS (10% w/v) and centrifuged at $2000 \times g$ for 15 min. Supernatants were extracted with chloroform for evaluation of triazene concentration by EPR spectroscopy. Before determination of concentration of the spin label, the samples were reoxidized 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 of animals, (Raikov et al., 1985). The concentration was estimated from the first derivative of the low-field line of the triplet spectrum. The EPR spectra were measured at room temperature at 9.765 GHz with 0.8 Gpp field modulation intensity and 15 dB microwave power.

2.5. Cytotoxicity in vitro

Cytotoxicity of triazenes on normal leukocytes (NL) 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–Paqwe gradient. Mononuclear cells were collected in interphace, washed and cultured in RPMI 1640 medium for 3 days. The drug dose was varied from 12.5 to 100 μM/ml. Drugs were administered in Me_2SO solution so that the final 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 370 in 48 well plates. The viability of the cells was assessed at the 3rd day by trypan blue exclusion. The drug efficiency was evaluated by calculating the IC50, (dose required to achieve 50% decrease in cell growth) according to the method already described (Emond and Page, 1982).

2.6. Antitumor activity and toxicity in vivo

The antitumor activity and toxicity of spin labeled triazenes were evaluated on lymphoid leukemia L1210 in mice. The BDF1 male mice with 20–25 g body weight, in groups of six, were inoculated i.p. with 10^5 tumor cells. On day 1 after transplantation the mice received various doses of the studied compounds (25–800 mg/kg). The animals were then observed according to the protocol of Geran et al. (1972). The oncostatic index, $T/C \times 100$ (T, the mean survival time of the treated group of mice and C, mean survival time of the tumor bearing control group) was calculated. This index expressed prolongation of survival. When $T/C \times 100 \geq 125$, the agent was considered active at the given dose; $T/C \leq 85\%$, the given dose was considered nontoxic.

3. Results and discussion

The spin labeled triazenes shown in Fig. 1 were synthesized as potential antimelanomic agents in 1993 and later some biological activities were studied and compared with that of the clinically used drug DTIC. Studies on modulating effects on DOPA oxidase activity of mushroom tyrosinase of the spin labeled triazenes as a preliminary prognosis for their antimelanomic activities have been made (Gadjeva et al., 1999). All studied spin labeled compounds activated the enzyme reaction, whereas the clinically used nonlabeled triazene DTIC, showed an inhibitory effects. So, the activating effect of the spin labeled triazenes on DOPA-oxidase activity was explained only by the presence of nitroxide moiety in their structures.

Resently, we established that introducing of a nitroxyl radical in the triazene structure increased their stability and leaded to a selectivity towards B16 melanoma cells (Zheleva and Gadjeva, 2001). Further, it was of interest to study whether the position of the nitroxyl moiety would affect the stability and the selectivity of spin labeled triazenes.

Our recent study demonstrated that replacement of the imidazole ring with an aryl- or other heteroaryl ring stabilized the triazenes (Zheleva and Gadjeva, 2001). In the present study, results for the half-lives of SLTA6 and SLTA8 showed that the spin labeled triazenes were stable in phosphate saline ($\tau_{0.5} > 200$ min; Table 1).

A very promising approach for achieving selective sensitization of tumors involves the use of compounds which are not only nontoxic to normal cells but are also selectively accumulating into tumor cells. Simeonova et al. (1994) have found that a spin-labeled nitrosourea was localized in the lungs of Lewis lung carcinoma-bearing mice (Simeonova et al., 1994). Our recent study showed that the nitrosourea 1-methyl-3-[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)]-1-nitrosourea was also mainly concentrated in the lungs, in the spleen and in the brain (Gadjeva and Koldamova, 2002). On the other hand Blagoeva and co-workers found that a selective accumulation of the nitroxide 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TMPO) occurred in hamster and mice melanotic melanomas (Blagoeva et al., 1979). Based on this last finding the tissue distribution of the spin labeled triazenes was investigated in organ homogenates and in tumor (B16 melanoma) of C57BL mice. The pharmacokinetic profiles for the spin labeled triazenes were different than these previously reported for spin labeled nitrosoureas and for the nitroxide radical TMPO.

Table 1
Half-lives of spin labeled triazene derivatives in 0.01 M phosphate buffer, at 37 °C

Compound	λ_{max} (nm)	$\tau_{0.5}$ (min)	Degradation (%)[a]
DTIC	325	30	74
SLTA6	320	> 200	20
SLTA8	320	> 200	20

[a] After illumination with UV light for 120 min at 55 °C.

Our results from tissue distribution of SLTA6 and SLTA8 in organ homogenates from liver, spleen, brain, kidneys in blood and in tumor (B16 melanoma) of C57BL mice, studied by EPR-method are presented in Fig. 2. As is seen the maximum concentration of these compound was reached 30 min after i. p. injection in spleen, and in the melanoma B16 tumor. The spin labeled triazenes were also found in the brain and it could be due to the fact that the spin labeled triazenes cross the blood brain barrier and could be the 'drug of choice' in chemotherapy of brain tumors. A low accumulation was found 30 min after i.p. injection in the liver which characterizes the compounds possibly with a low acute toxicity.

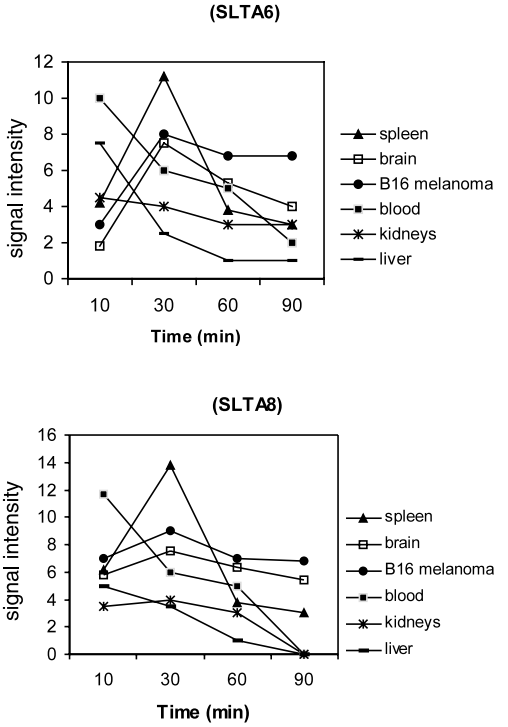

Fig. 2. Tissue distribution of SLTA6 and SLTA8 in blood and organ homogenates from liver, spleen, brain, kidneys and tumor (B16 melanoma) of C57BL mice after i.p. administration at a dose of 50 mg/kg. Measurements were performed on four groups of mice with three animals in each group. The S.E.s associated with the data of this figure are within 6% of the presented values.

The spin labeled triazenes were mainly localized in the melanoma B16. Moreover, a high concentration of either SLTA6 or SLTA8 in melanoma B16 remained constant in the tumor for all of the time period studied. This result could be explained by the selective accumulation of the carrier part the nitroxyl moiety of the spin labeled triazene, in melanoma B16 tumor, and could be prerequisite for its selective cytotoxicity. We did not find any significant difference between the concentration of SLTA6 and SLTA8 in B16 melanoma.

Table 2 shows the results from in vitro assay of SLTA6, SLTA8 and DTIC against B16 melanoma cells, Moloney lymphoma YAC-1 cells and human lymphocytes (NL). Spin labeled triazenes were less toxic against NL than DTIC. Thus, for SLTA6 and SLTA8 the IC50 were 120.0 and 100.8 μM/ml, respectively, whereas for DTIC, IC50 was 62.2 μM/ml. Moreover, spin labeled triazenes appears to be more toxic to B16 cells than to YAC-1 cells and to NL in comparison with DTIC. The magnitude of the difference between the cell types B16 and YAC-1 depended on the drug; the difference was greater for spin labeled triazenes than for DTIC (Fig. 3). However, spin labeled 3,3-dimethyl triazene SLTA8 inhibited the proliferation of B16 melanoma cells more than the spin labeled monomethyl triazene SLTA6 inhibited (IC50 were 17.2 and 37.8 μM/ml, respectively). SLTA8 completely inhibited B16 melanoma cell growth at dose 100 μM/ml (Fig. 3).

Results from in vivo tests against lymphoid leukemia L1210 in BDF1 male mice of the spin labeled triazenes are presented in the Table 3. As expected, neither of the spin labeled triazenes possessed antitumor properties under these experimental conditions ($T/C \leq 125\%$). However, it should be noted that all of the triazene derivatives exhibited low toxicity at doses 25–800 mg/kg for

Table 2
Inhibition of cell growth by spin labeled triazenes

Compound	Tumor cells	IC50 (μmol/ml)[a]
SLTA6	B16	37.8
	YAC-1	75.0
	NL	120.0
SLTA8	B16	17.2
	YAC-1	37.0
	NL	100.8
DTIC	B16	38.9
	YAC-1	50.5
	NL	62.2

[a] IC50 (dose required to achieve 50% decrease in cell growth).

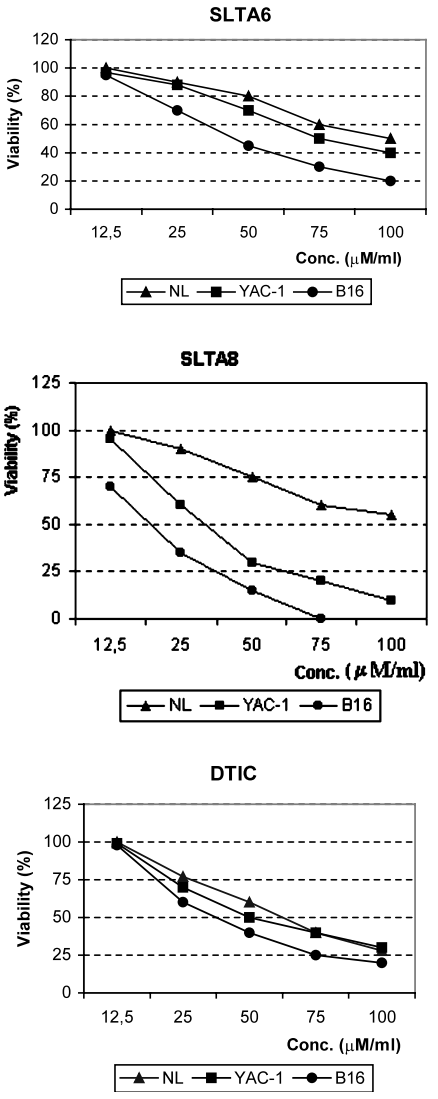

Fig. 3. Concentration-dependence of the effect of spin labeled triazenes on cell proliferation of Normal leucocides, YAC-1 mNK target Moloney lymphoma cells and B16 melanoma cells. Cells were exposed to drug for 2 h; cell viability was determined on day 3. Values represent the mean of three experiments. The S.E.s associated with the data of this figure are within 10% of the presented values.

Table 3
Antitumor activity in vivo of spin labeled triazenes on lymphoid leukemia L1210

Compound	Dose (mg/kg)	T/C (%)[a]	Survivors (survivors/total) (5 days)
SLTA6	25.0	112.0	0/6
	125.0	108.0	0/6
	250.0	103.9	0/6
	500.0	100.0	0/6
	540.0	94.7	0/6
	640.0	91.6	0/6
	800.0	47.6	6/6
SLTA8	25.0	120.0	0/6
	125.0	113.0	0/6
	250.0	111.7	0/6
	500.0	108.0	0/6
	540.0	96.7	0/6
	640.0	81.8	0/6
	800.0	50.6	6/6

1×10^5 Tumor cells in 0.1 ml, i.p. transplanted.
[a] T/C (%), (MST treated/MST control) $\times$ 100; $T/C \geq 125\%$ minimal criteria for antitumor activity; $T/C \leq 85\%$-'toxicity' values (Geran et al., 1972).

BDF1 mice hybrids in vivo (see number of animals, survivors/total in Table 3)

Using the EPR method we showed that spin-labeled triazenes and their precursor 4-amino-TMPO, scavenge $^{\bullet}O_2^-$ and so possessed high superoxide scavenging activity (SSA) while the clinically used triazene DTIC exhibited no SSA (Gadzheva et al., 1994). It was proved that the mechanism of this SSA activity was the result of a redox cycling between nitroxide and its corresponding hydroxylamine (Gadzheva et al., 1994) according to the following equations:

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

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

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 anti-melanomic activity and low toxicity of spin labeled triazenes could be attributed to the antioxidant effect of the incorporated nitroxide which is derived efficiently from the redoxcycling equations.

We can conclude that introducing of nitroxide moiety in the structure of triazene derivatives leads to selective accumulation in the B16 melanoma tumor and in the brain and to a selective anti-melanoma activity in vitro. We also consider that spin labeled triazenes can be studied in the treatment of melanomas with cerebral metastases.

Acknowledgements

The measurements cytotoxicity of the triazenes were carried out during a special leave of Dr V. Gadjeva in School of Medicine, Tokay University, Isehara, Japan. The author wish to thank Professor Nakazawa, Department of Physiology, Professor Habu and Dr Matsuyama, Department of Cellular Biology, for the useful technical help.

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