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Original article

Structure-based design of nitrosoureas containing tyrosine derivatives as potential antimelanoma agents

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Abstract

Two new nitrosoureas (TNUs), containing tyrosine derivatives as carriers of nitrosourea cytotoxic group have been synthesised. The physicochemical properties such as half-life time ($\tau_{0.5}$), alkylating and carbamoylating activities were determined. The nitrosoureas showed a higher inhibiting effect on the DOPA-oxidase activity of mushroom tyrosinase than that of the antitumour drug *N*′-cyclohexyl-*N*-(2-chloroethyl)-*N*-nitrosourea (lomustine, CCNU). In vitro cytotoxic effects of newly synthesised tyrosine containing nitrosoureas have been studied and compared to those of CCNU. A higher cytotoxicity to B16 melanoma cells than to YAC-1 and to lymphocytes was demonstrated for the tyrosine containing nitrosoureas in comparison with CCNU. Based on the results presented, we accept that a new trend for synthesis of more selective and less toxic nitrosourea derivatives as potential antimelanomic drugs might be developed.

Keywords: Nitrosourea; B16 melanoma cells; DOPA-oxidase activity; Tyrosinase

1. Introduction

Human malignant melanoma represents a difficult therapeutic challenge to the scientists and physicians. Complete responses to chemotherapy are rare, immunotherapy is virtually ineffective, and the overwhelming majority of melanoma is not radioresponsive [1]. In reviewing the major advances in the chemotherapy of cancer, it is immediately apparent that the most remarkable successes have occurred when the biological uniqueness of a given tumour was exploited. The biological uniqueness of the melanoma cell resides in their tendency to retain the biochemical apparatus for the conversion of tyrosine to melanin in the highly specialised organelle, the melanosome [2]. Tyrosine serves as the starting material for the biosynthesis of melanin. The degree and type of melanin pigmentation of melanoma cells can be influenced by changing the concentration of tyrosine in growth medium [3]. Through an increased melanotic pigmentation by raising the concentration of tyrosine, one can induce significant toxicity to melanin-producing cells as compared with their non-pigmented counterparts [4]. In 1993, Jimbow et al. provided a rationale for utilizing melanin precursors such as tyrosine, DOPA, dopaquinone for selective chemotherapeutic agents for melanoma [5]. These *N*-acetyl and α-methyl derivatives, which are substrates of tyrosinase, showed significant cytotoxicity that is selective to melanocytes and melanoma cells.

Chloroethylnitrosoureas, such as *N*′-cyclohexyl-*N*-(2-chloroethyl)-*N*-nitrosourea (lomustine, CCNU) and *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (carmustine, BCNU) are chemotherapeutic agents of most interest for treatment of malignant melanoma [6,7]. However, the clinical efficacy of nitrosourea drugs is limited because they show delayed and cumulative haematological toxicity [8,9].

Since L-amino acids participate in the transport through mammalian cell membranes series of amino acid nitrosourea derivatives have been synthesised in order to achieve a more selective cytotoxic effect [10–12]. Bearing in mind the above-mentioned facts that L-tyrosine occupies a central position in the synthesis of melanin while it is concentrated mainly in pigment melanomas, we set the task of designing new nitrosoureas as potential antimelanomic agents in which structure tyrosine derivatives are used.

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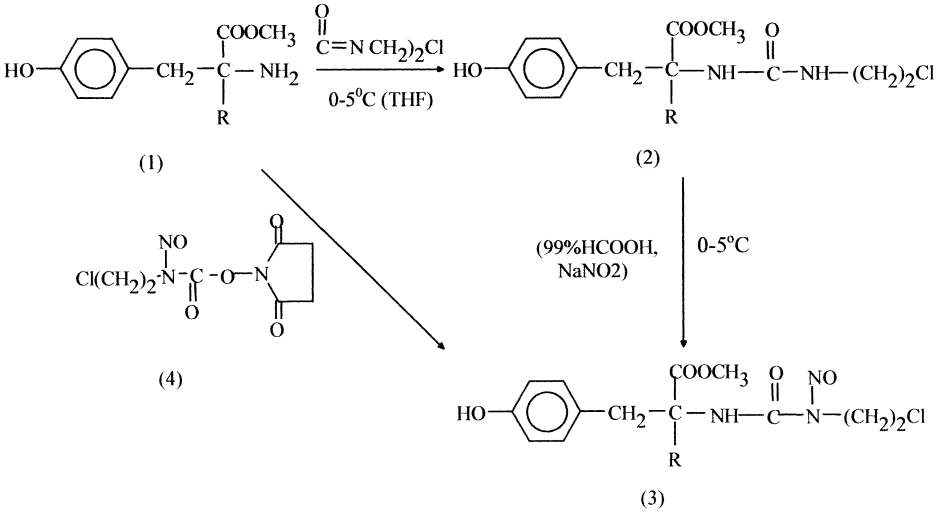
In the present study, we report the synthesis and biological evaluations of two new nitrosoureas (TNUs) utilizing tyrosine derivatives as carriers of the nitrosourea cytotoxic group. A relationship between their physicochemical properties, modulating effects on DOPA-oxidase activity of tyrosinase and their in vitro antimelanomic effects has been discussed (Fig. 1).

2. Chemistry

TNUs were prepared by two different synthetic pathways according to Fig. 1. The conventional path through condensation of the tyrosine esters **1a**–**b** with 2-chloroethyl isocyanate and nitrosation of intermediate ureas **2a**–**b** afforded the TNUs **3a**–**b** (see Section 5). In order to ascertain the position of the nitroso group in the nitrosourea derivatives, a regio-selective method was used to transfer the chloroethyl moiety containing the nitroso group to the corresponding tyrosine ester. Nitrosoureas **3a**–**b** were also synthesised using *N*′-hydroxysuccinimide-*N*-(2-chloroethyl)-*N*-nitrosocarbamate (**4**) as a regio-selective transfer reagent (see Section 5). The synthesised nitrosoureas were characterised by elemental analysis, IR, NMR and MS.

3. Pharmacology, results and discussion

Results for half-life time ($\tau_{0.5}$), alkylating activity and carbamoylating activity of TNUs and antitumour drug CCNU are presented in Table 1. TNUs showed shorter $\tau_{0.5}$ and lower carbamoylating activity when compared with those of CCNU. Moreover, their alkylating activities were higher than that of CCNU. Previously we reported that introducing amino acid moiety in the nitrosourea structure leads to a faster decomposition, higher alkylating, lower carbamoylating activity, better antimelanomic activity and lower general toxicity when compared with those of CCNU [12]. It is obvious from the present results that the replacement of the cyclohexyl moiety with tyrosine residues in the structure of CCNU leads to a fast decomposition of the nitrosoureas, a decrease in their carbamoylating activities



R	TE	TU-s	TNU-s
H	TE (1a)	TU (2a)	TNU (3a)
CH_3	MTE (1b)	MTU (2b)	MTNU(3b)

Fig. 1. Chemical structures of TNUs.

Table 1
Physicochemical properties of TNUs and CCNU

Compound (code)	Half-life time ($\tau_{0.5}$) (min)	Alkylating activity [a] ($A_{560} \times mM^{-1} \times h^{-1}$)	Carbamoylating activity[b] (%)
TNU	36	0.56	34.65
MTNU	30	0.60	32.06
CCNU	54	0.34	62.68

[a] Note comments on its evaluation made in Section 5.2.2.
[b] Note comments on its evaluation made in Section 5.2.3.

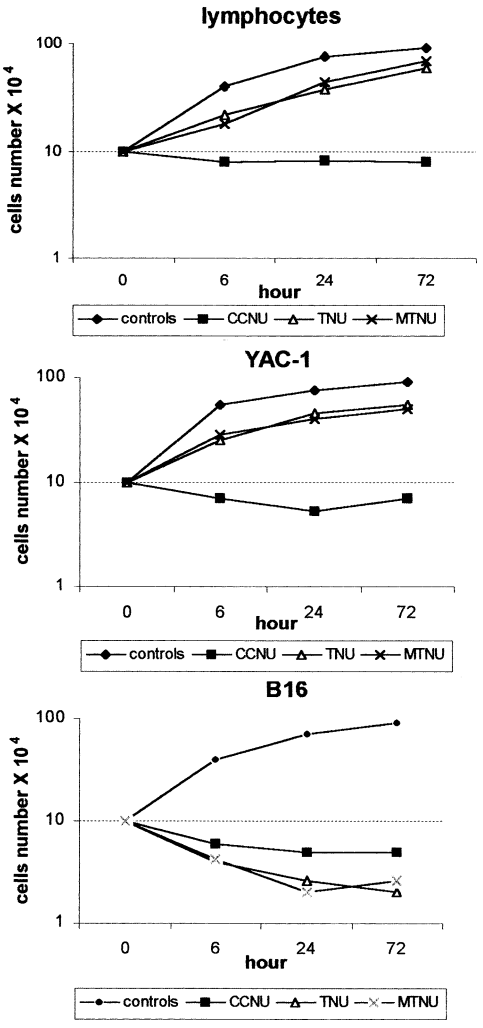


Fig. 2. Effect of TNUs and CCNU on the proliferation of B16, YAC-I and human lymphocytes. Cells were treated with 12.5 μM of the indicated drug for 2 h and cell number was determined vs. time. Values represent the mean of three measurements. The S.E. values associated with the data of this figure are within 10% of the presented values.

and on the other hand, an increase in their alkylating activities. It is known that nitrosourea derivatives decompose under physiological conditions to yield alkylating (carbonium ion) and carbamoylating (isocyanate) moieties responsible, respectively, for alkylation of nucleic acids and proteins and for carbamoylation of proteins and enzymes [13]. At present, it is believed that antitumour activity of nitrosoureas is due to the alkylating property whereas severe toxic effects such as myellosuppression are due to the carbamoylating property [14,15]. Our results for $\tau_{0.5}$, alkylating activity and carbamoylating activity of **3b** exhibited the best combination for a good therapeutic index, i.e. short half-life, high alkylating activity and low carbamoylating activity.

B16 melanoma cells, moloney lymphoma YAC-1 cells and human lymphocytes (NL) were treated in vitro with TNUs and CCNU. TNUs were less toxic against NL than CCNU. Moreover, TNUs appear to be much more toxic to B16 cells than to YAC-1 cells in comparison with CCNU (see Fig. 2). When examined as a function of drug concentration, the effects on cell viability were compared for concentrations between 5 and 50 μM. The magnitude of the difference between the cell types B16 and YAC-1 depended on the drug; the difference was greater for TNUs than for CCNU (see Fig. 3). It was deduced that TNUs are selective cytotoxic agents towards B16 cells.

Tyrosinase is a 529 amino acid membrane-associated glycoprotein with enzymatic activity involved in melanin biosynthesis. Tyrosinase is present in a significant quantity in the malignant melanoma tumours. In

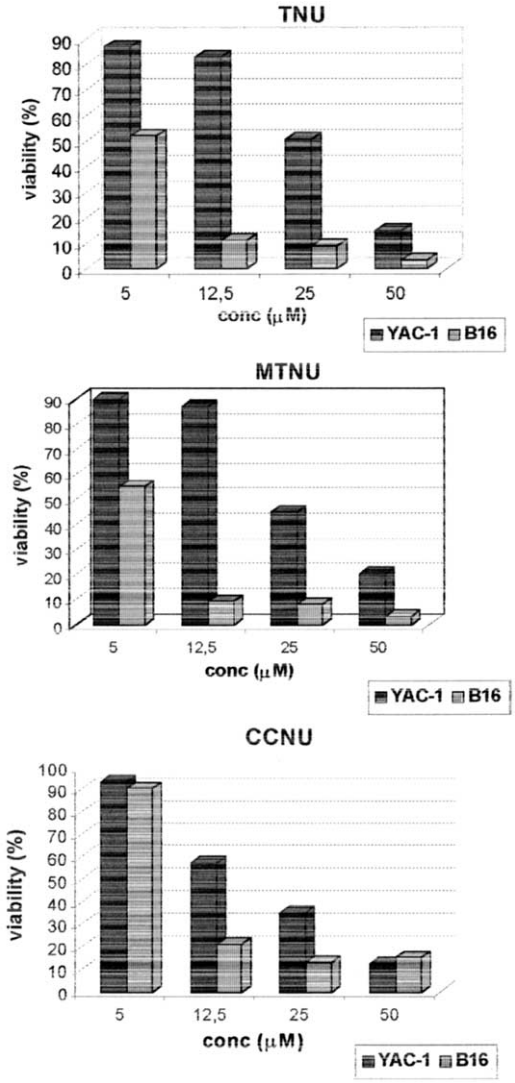


Fig. 3. Concentration dependence of the effect of TNUs and CCNU on cell proliferation. Cells were exposed to drug for 2 h and cell viability was determined on the day 3. Values represent the mean of three measurements. The S.E. values associated with the data of this figure are within 10% of the presented values.

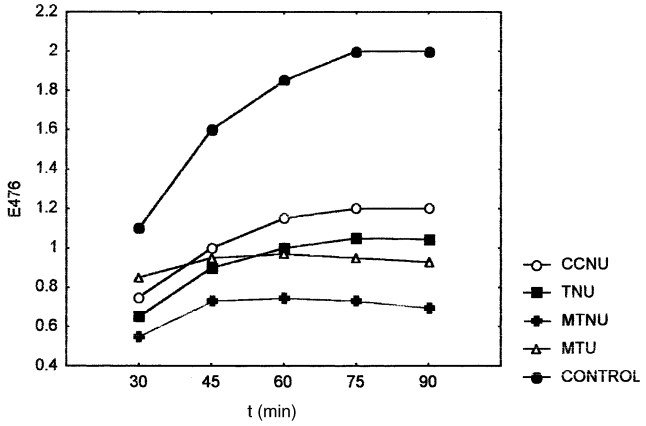


Fig. 4. Time course for the modulating effects of TNUs, the urea precursor MTU and antitumour drug CCNU on DOPA-oxidase activity of mushroom tyrosinase. Control, DOPA-oxidase activity of mushroom tyrosinase. Values represent the mean of three measurements. The S.E. values associated with the data of this figure are within 3% of the presented values.

this connection, we have assumed that a relationship might exist between the antimelanomic activity of newly synthesised nitrosoureas and their influence on the tyrosinase activity.

In our previous studies, a strong dependence between modulating effect on DOPA-oxidase activity of mushroom tyrosinase of formerly synthesised spin labeled nitrosoureas and their chemical half-life time was established [16]. In the present study, modulating effects of TNUs on DOPA-oxidase activity of mushroom tyrosinase have been studied in comparison with the effect of CCNU and the precursor of MTNU, tyrosine urea MTU (see Fig. 4). It was demonstrated that TNUs had an higher inhibiting effect on DOPA-oxidase activity of tyrosinase in comparison with that of CCNU. The mechanism of this inhibiting effect could be explained by the possible role of both the carbamoylating isocyanate and the tyrosine residue as competitive inhibitor for the tyrosinase according to the substrate DOPA. In view of the fact that TNUs nitrosoureas had short half-life times, we accepted that one because of their fast decomposition, the inhibiting effect of the isocyanate manifested. During the enzyme reaction, which completed at 90 min, full decomposition of the TNUs occurred and the active isocyanate metabolites were formed. The lower inhibiting effect of the urea MTU in comparison with that of the corresponding nitrosourea MTNU could be explained by the stability of the urea; it does not decompose to isocyanate and influences only as competitive inhibitor. Bearing in mind the above demonstrated in vitro higher cytotoxicity towards B16 melanoma cells for TNUs than CCNU, we suppose that it is closely related to their inhibiting effects on DOPA-oxidase activity of tyrosinase as well.

4. Conclusions

In conclusion, we accept that the selectivity towards B16 melanoma cells of TNUs demonstrated in vitro is due to the presence of tyrosine moiety in their structures and is closely related to their inhibiting effect on DOPA-oxidase activity of tyrosinase. It is also obvious that in advance in vitro evaluation of DOPA-oxidase activity of newly synthesised nitrosoureas as potential antimelanomic agents would be quite useful as a preliminary prognosis for their antimelanomic activity. We also consider that introducing of tyrosine esters in the structure of nitrosourea derivatives might turn out to be a new trend for preparing more selective and less toxic potential antimelanomic drugs.

5. Experimental protocol

5.1. Chemistry

L-Tyrosine methyl ester, α-methyl-*p*-tyrosine methyl ester, and chloroethylisocyanate were purchased from Aldrich Chemical Co. (Milwaukee, USA). CCNU was kindly gifted by Bristol-Myers Squibb Co. (Wallingford, CT, USA). The mushroom tyrosinase and DL-dihydroxyphenylalanine (DL-DOPA) were purchased from Sigma.

N′-Hydroxysuccinimide-*N*-(2-chloroethyl-*N*-nitrosocarbamate (**4**) was prepared as described in Ref. [17]. All melting points were determined in a Kofler apparatus and are reported uncorrected. IR spectra were determined in UR-20 Carl Zeiss Jena spectrophotometer in KBr pellets. Mass spectra were obtained in a Finningan-MAT 711 spectrometer (100 eV). ^{1}H-NMR spectra were measured in $CDCl_3$ using a Bruker AM 500 spectrometer operated at 70 eV ionizing energy. Elemental analyses were performed in Perkin–Elmer Model 240-elemental analyzer. Thin layer chromatography (TLC) experiments were carried out in Merck silica gel GR_{254} plates.

5.1.1. Synthesis of N-[N′-(2-chloroethyl)-N′-nitrosocarbamoyl]-tyrosine methyl ester (TNU) (**3a**)

L-Tyrosine methyl ester (2.56 mM) was dissolved in 10 mL anhydrous THF cooled to 0–5 °C. After 10 min of stirring, a solution of chloroethyl isocyanate (2.56 mM) in 10 mL anhydrous THF was added dropwise. The reaction mixture was stirred for a further 1 h at −5 to 0 °C. The nitrosation was performed in the reaction mixture with anhydrous gas of ethylnitrite, obtained by adding H_2SO_4 dropwise (5 mL, 98%) to a mixture of $NaNO_2$ (3 mM), EtOH (10 mL) and H_2O (44 mL). The reaction mixture was stirred for 3 h at −5 to 0 °C and the solution was concentrated in

vacuo. The residue was kept overnight at −5 to 0 °C under ether to give an orange crystalline material which was recrystallised twice from (EtOH–ether 1:1 v/v), yield: 65%.

M.p. 82–84 °C; $R_f = 0.76$ ($CHCl_3$–CH_3OH 9:1 v/v); ^{1}H-NMR ($CDCl_3$): δ 3.4–3.6 (4H, m, $-CH_2CH_2Cl$); δ 3.69 (3H, s, $-OCH_3$); δ 7.5–7.8 (m, 4H arom); MS; m/z: 329 [M^+], 309 [$M^+ - NO$]; IR (KBr, cm^{-1}) ν :3370, 1721, 1495, 830; Anal. $C_{13}H_{16}ClN_3O_5$: Calc.: C, 47.41; H, 4.90; N, 12.77. Found: C, 47.30; H, 4.36; N, 12.70%.

5.1.2. Synthesis of N-[N′-(2-chloroethyl)-carbamoyl]-α-methyl-tyrosine methyl ester (MTU) (**2b**)

α-Methyl-*p*-tyrosine methyl ester (2.5 mM) was dissolved in 10 mL anhydrous THF cooled to 0–5 °C. After 10 min of stirring, a solution of chloroethylisocyanate (2.5 mM) in 10 mL ether was added dropwise. The reaction mixture was stirred for a further 1 h at −5 to 0 °C and concentrated under reduced pressure. The precipitated urea was filtered off and recrystallised from ether as white crystals, yield 87%.

M.p. 176–178 °C; R_f: 0.49 ($CHCl_3$–CH_3OH 9:1 v/v); ^{1}H-NMR ($CDCl_3$): δ 1.12 (3H, s, $-CH_3$); δ 3.66–4.20 (4H, m, $-CH_2CH_2Cl$); δ 6.66 (1H, s, NH–CH_2CH_2Cl); δ 5.67 (1H, s, –NH–CO); δ 3.84 (3H, s, $-OCH_3$); δ 7.32–7.70 (m, 4H arom); MS; m/z: 314 [M^+]; IR (KBr, cm^{-1}) ν : 3250, 2500, 1715, 830; Anal. $C_{14}H_{19}ClN_2O_4$: Calc.: C, 53.42; H, 6.08; N, 8.90. Found: C, 53.80; H, 5.60; N, 9.00%.

5.1.3. Synthesis of N-[N′-(2-chloroethyl)-N′-nitroso-carbamoyl]-α-methyl-tyrosine methyl ester (MTNU) (**3b**)

N-[*N*′-(2-Chloroethyl)-carbamoyl]-α-methyl-tyrosine methyl ester (MTU) (**2b**) (2.4 mM) was dissolved in 98% HCOOH (10 mL) and anhydrous $NaNO_2$ (24 mM) was added in small portions to the solution at −5 to 0 °C. The reaction mixture was stirred for 3 h at −5 to 0 °C and the solution was poured into ice/H_2O (70 ml). Stirring was continued for another 30 min and then the yellow crude product was extracted with methylenechloride (3 × 10 mL). The combined extracts were successively washed with 10% $NaHCO_3$ (2 × 10 mL) and H_2O. The organic layers were dried over anhydrous $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was crystallised twice from $(C_2H_5)_2O$–*n*-hexane 1:1 v/v as pale yellow crystals, yield 63%.

M.p. 154–156 °C; $R_f = 0.87$ ($CHCl_3$–CH_3OH 9:1 v/v); ^{1}H-NMR ($CDCl_3$): δ 1.20 (3H, s, $-CH_3$); δ 3.83 (3H, s, $-OCH_3$); δ 3.66–4.00 (4H, m, $-CH_2CH_2Cl$); δ 5.68 (1H, s, –NH–C=O); δ 7.32–8.07 (m, 4H arom); MS; m/z: 343 [M^+], 323 [$M^+ - NO$]; IR (KBr, cm^{-1}) ν: 3390, 1110, 1740, 1490, 800; Anal. $C_{14}H_{18}ClN_3O_5$: Calc.: C, 48.97; H, 5.29; N, 12. 24. Found: C, 49.00; H, 5.10; N, 12.13%.

5.1.4. Synthesis of TNUs by the use of N′-hydroxy-succinimide-N-(2-chloroethyl)-N-nitrosocarbamate

α-Methyl-*p*-tyrosine methyl ester or L-tyrosine methyl ester (2.5 mM) was dissolved in DMF (5 mL at 0–5° C). The mixture was stirred vigorously for 15 min and *N*′-hydroxysuccinimide-*N*-(2-chloroethyl)-*N*-nitrosocarbamate (2.5 mM) was added. After 3 h of stirring the mixture was poured into ice–water (70 mL). The yellow crude product obtained was extracted with ether (3 × 10 mL). The combined extracts were successively washed with 10% $NaHCO_3$ (2 × 10 mL), 10% citric acid (2 × 10 mL), and a saturated solution of NaCl (2 × 10 mL). The organic layers were dried over anhydrous $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure, crystallised twice from $(C_2H_5)_2O$–*n*-hexane as yellow crystals and dried in a dessicator, yield 72%.

5.2. Pharmacology

5.2.1. Determination of half-life times ($\tau_{0.5}$) of the nitrosoureas

The half-life times of the nitrosoureas were determined according to Gadjeva et al. [18]. 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.

5.2.2. Determination of alkylating activity of the nitrosoureas

Alkylating activity of the compounds was determined according to Gadjeva et al. [18]. Briefly, various concentrations of the nitrosoureas were incubated for 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 $A_{560} \times mM^{-1} \times h^{-1}$.

5.2.3. Determination of carbamoylating activity of the nitrosoureas

Carbamoylating activities of the nitrosoureas were determined following the method of Gadjeva et al. [19]. Briefly, 4-amino-TMPO (50 μmol) was dissolved in ethanol and PBS for a stock solution. To 450 μL of this

solution was added 5 μmol of the corresponding nitrosourea dissolved in ethanol. The reaction mixtures were incubated at 37 °C in a water bath and aliquots were withdrawn for analysis each hour for 6 h. Ten microliters of each mixture was spotted on TLC plates and chromatograms were developed with chloroform–methanol (9:1, v/v), dried and visualised 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 JEOL JES-FE2XG EPR spectrometer (Tokyo, Japan). Carbamoylating activity was determined by the following equation and expressed as a percent carbamoylation of 4-amino-TMPO: $\{([\text{4-amino-TMPO}]_0 - [\text{4-amino-TMPO}]_T)/[Y]_0\} \times 100$, where $[\text{4-amino-TMPO}]_0$, $[\text{4-amino-TMPO}]_T$ 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.

5.2.4. Determination of the tyrosinase activity

Among in vitro methods used for the determination of the tyrosinase activity Burnett's spectrophotometric method is more utilizable [20]. Briefly: compound's solutions (1×10^{-2} mM) in ethanol–0.1 M phosphate buffer, pH 6.8 (1/10, v/v) were incubated at 37 °C with 150 mg% DL-DOPA and 200 U mL^{-1} mushroom tyrosinase. DOPA-chrome product formed during the enzyme reaction was detected at 30, 45, 60, 75 and 90 min during the incubation at $\lambda_{max} = 475$ nm on Pharmacia LKB, Ultrospec III spectrophotometer (Sweden). Both DOPA-oxidase activity of pure mushroom tyrosinase and autooxidation of the substrate DL-DOPA were determined, as well.

5.2.5. Cytotoxicity of the spin labeled triazenes. In vitro test

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

Cytotoxicity of nitrosoureas on normal leukocytes was investigated by the method of Weisenthal et al. [21]. Cells were separated by the modified method of Boyum [22]. Heparinized vascular blood from healthy donors was layered on Ficoll-Paqwe gradient. Mononuclear cells were collected in interphase, washed and cultured in RPMI 1640 medium for 3 days. The drug dose was changed from 5 to 50 μM mL^{-1}. 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 2 h in RPMI 1640 medium supplemented with following components: FBS, L-glutamine, penicillin C and streptomycin. The dose was changed from 5 to 50 μM mL^{-1}. 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.

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