Biochemical Pharmacology, Vol. 30, pp. 289–291.

0006-2952/81/040289–03 $02.00/0

IN VIVO INVESTIGATION OF SOME METABOLIC CHANGES OF 3-[BIS-(2-CHLORETHYL)-CARBOHYDRAZIDE]-2,2,5,5-TETRAMETHYLPYROLINE-1-OXYL BY THE NBP-REACTION AND ESR METHOD

ELENA RAÏKOVA, DIANA GRUEVA and EVGENY GOLOVINSKY*

Institute of Oncology, Academy of Medicine, 1156 Sofia, and *Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

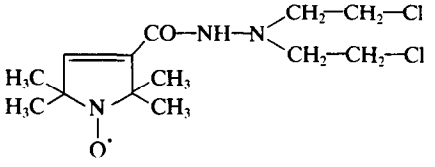
(*Received* 29 *April* 1980; *accepted* 25 *August* 1980)

Abstract—The reaction with 4-(4-nitrobenzyl)-pyridine (NBP) and the method of electron spin resonance (ESR) were used for the quantitative investigation of some time-dependent metabolic changes of 3-[bis(2-chlorethyl)-carbohydrazide]-2,2,5,5-tetramethylpyroline-1-oxyl (HMSL) occurring in hamster blood, liver and transplantable pigment melanoma. The alkylating property of HMSL was found to decrease slowly in liver tissue. This property was retained longest in melanoma tissue, but at a relatively lower degree. In the tissues HMSL was probably reduced to the respective hydroxylamine. After the addition of $K_3Fe(CN)_6$ to the material studied, diamagnetic hydroxylamine was oxidized back again to the corresponding radical, and by the ESR method considerably higher HMSL concentrations were established in the tissues and for a period longer than that determined by the NBP-reaction. The data obtained with the latter indicate that despite the presence of HMSL in the tissues (ESR), the compound was hydrolyzed and turned into an inactive hydroxy derivative. These results correlate with the established weak antitumor effect of HMSL on transplantable pigment melanoma.

The spin-labelled derivatives of nitrogen mustard permit the study of some aspects of the mechanisms of carcinogenesis and mutagenesis and the antitumor action of alkylating agents by means of the electron spin resonance method (ESR).

The spin-labelled derivatives of nitrogen mustard synthesized by us proved to be suitable spin labels for nucleic acids [1]. It was also found that some of the studied spin-labelled nitrogen mustards possessed an antitumor action [2].

The object of the present work is the quantitative investigation of some metabolic changes of 3-[bis-(2-chlorethyl)-carbohydrazide]-2,2,5,5-tetramethyl-pyroline-1-oxyl (HMSL) in hamster blood, liver homogenates and transplantable pigment melanoma after the i.p. administration of the compound:



The chlorethylamine and iminoxyl residues in the molecule of HMSL made possible the quantitative determination of the compound respectively by the reaction with 4-(4-nitrobenzyl)-pyridine (NBP) and ESR method.

MATERIALS AND METHODS

HMSL was obtained by a method described by us previously [1]. 4-(4-nitrobenzyl)-pyridine was a commercial preparation (Riedel de Haen). The ESR investigations were performed with a 3BS-X spectrometer. The spectrophotometric investigations were carried out with a SF-16 spectrophotometer. The NBP-reaction was realized according to a method described in the literature [3, 4].

The experiments were performed with 2-month-old hamsters of both sexes having two-sided transplantations of pigment melanoma [5]. On the 30th day of transplantation and tumor size of about 1 cm the animals were injected i.p. HMSL suspended in Twin-80 in a dose of 100 mg/kg. Other groups of hamsters with transplanted pigment melanoma were i.p. injected a water solution of 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TMPO) in a dose of 100 mg/kg [6].

The animals distributed into groups of 6 were killed by decapitation at the 5th, 10th, 20th, 30th, 60th, 90th, 120th min and 3rd hour from the HMSL administration. Blood samples were taken for tests, and from the liver and melanoma 10% homogenates were prepared which were studied by the NBP-reaction and ESR.

The determination of the amount of paramagnetic centres was achieved by the ESR method before and after treatment of the samples with 10% water solution of potassium ferricyanide (0.1 ml per ml of the sample).

The ESR spectra of HMSL and TMPO are steady symmetrical triplet signals. The quantity of paramagnetic centres has been calculated in μg/ml with respect of a control having a known concentration [7].

RESULTS AND DISCUSSION

The results obtained from the NBP experiments have shown that the spin-labelled derivatives of

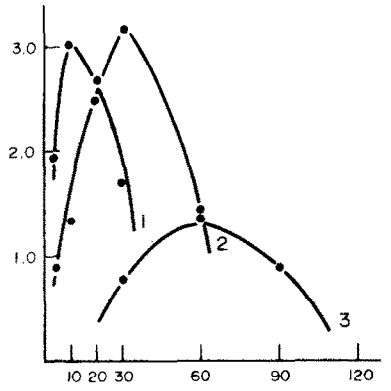


Fig. 1. Changes of HMSL concentration with time determined by the NBF-reaction. Abscissa—HMSL concentration (μg/ml) and ordinate—time (min). (1) blood; (2) liver; (3) melanoma.

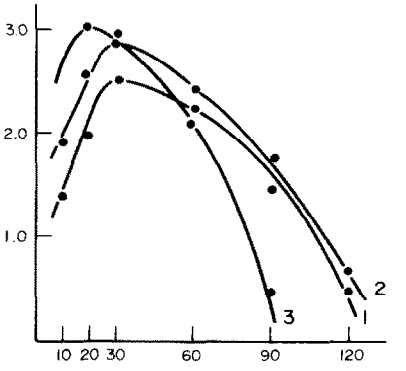


Fig. 3. Changes of TMPO concentration with time determined by the ESR method. Abscissa—TMPO concentration (μg/ml) and ordinate—time (min). (1) melanoma without $K_3Fe(CN)_6$; (2) melanoma with $K_3Fe(CN)_6$; (3) liver with $K_3Fe(CN)_6$. TMPO is metabolized in the first 5 min.

nitrogen mustard [1], like other alkylating agents such as chlorethylamines (no spin-labelled), ethylenimines, etc. [3], produce a coloured reaction with NBP.

The highest alkylating property of HMSL in blood was recorded in the first 10 min after which they are not observed because of the tissue distribution of HMSL by blood (Fig. 1).

The investigations performed with NBP-reagent in liver and melanoma homogenates showed that the HMSL concentration in liver was much higher than that in melanoma up to the first hour after the administration (Fig. 1). In melanoma the alkylating function of the compound was highest at the end of the first hour after which it did not decrease abruptly and had relatively significant values to the end of the second hour (Fig. 1).

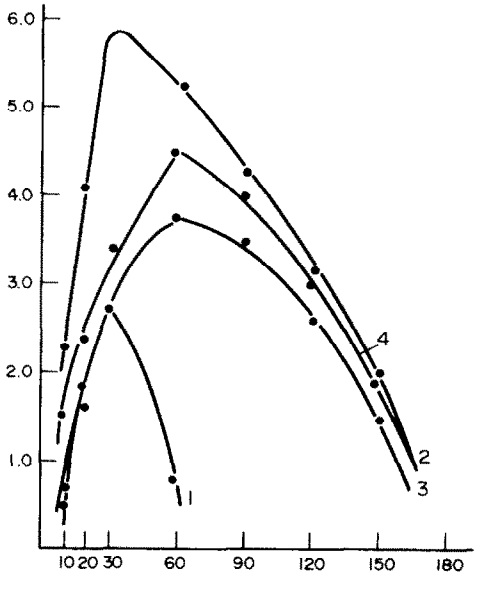
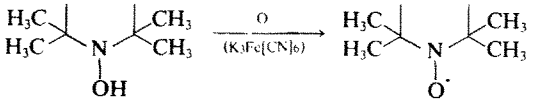


Fig. 2. Changes of HMSL concentration with time determined by the ESR method. Abscissa—HMSL concentration (μg/ml) and ordinate—time (min). (1) liver without $K_3Fe(CN)_6$; (2) liver with $K_3Fe(CN)_6$; (3) melanoma without $K_3Fe(CN)_6$; (4) melanoma with $K_3Fe(CN)_6$.

The ESR investigations indicated that in liver HMSL changed relatively quickly, while in melanoma tissue the changes of this compound were slowed down and it could be detected there up to the 3rd hour (Fig. 2). Unlike HMSL, the TMPO concentration in both liver and melanoma tissues decreased considerably more quickly; thus, at the 2nd hour the TMPO concentration in melanoma was less than 0.5 μg/ml, while that of HMSL was about 2.5 μg/ml. TMPO was not detected in liver tissue after the 10th min (without $K_3Fe(CN)_6$) from the administration [6].

Our investigations have shown that in these tissues the iminoxyl radicals are reduced to the respective hydroxylamine which has also been found by other authors [8]. Due to this reduction the ESR method proved to be inapplicable for the determination of the quantity of the radical converted into hydroxylamine. The reversing oxidation of diamagnetic hydroxylamine to the corresponding radical with the help of potassium ferricyanide has permitted, by means of the ESR method, the detection of the reduced and unchanged amount of free iminoxyl radical:



After the addition of potassium ferricyanide to the samples tested, it was found that the HMSL concentration in liver (Fig. 2) and in melanoma were considerably higher in comparison with the concentrations determined by the NBP-reaction.

As a result of the investigations performed with the NBP-reaction and the ESR method, the alkylating property of HMSL proved to decrease relatively quickly (Figs. 1 and 2). At the same time by means of the ESR method it was established that HMSL was present in liver and melanoma tissues probably mainly in a nonimmobilized state (sym-

metric triplet ESR signal of the compound), because HMSL remains covalently bound to low molecular fragments removed from biological macromolecules by the action of the repairing enzyme systems.

The data of the NBP-reaction show that despite the presence of HMSL in the tissues investigated, the compound was hydrolyzed to the corresponding inactive hydroxy derivative. We demonstrated that the chlorethylamine group and nitroxide radical in HMSL have one and the same metabolic pathway; that means that *in vivo* HMSL is not hydrolysed through the —CO–NH– bond. For the purpose as references we applied inactive hydroxy derivative and hydroxylamine derivative which we had previously synthesized and characterized in our laboratory as pure substances [7] (in liver homogenate prepared 1 hour post i.p. administration of HMSL; TLC on silicagel H; developing solvent $CH{\cdot}Cl_3/CH_3OH$ 9:1). This explains why HMSL exhibits a relatively weak antitumor effect with transplantable melanoma.

The results also show that unlike TMPO the elimination of HMSL in the organism is slower which is probably due to the high reactivity of the chlorethylamine residues through which the compound is related to the biological substrates.

The considerably quicker changes of HMSL in liver tissue as compared with those in melanoma are most probably due to the activity of the redox enzymes in liver.

Transplantable pigment melanoma, in contrast to other transplantable tumours has lower capability to reduce the nitroxides radicals because of low content of reduced glutatione and other compounds containing SH groups [9, 10].

REFERENCES

1. E. T. Raïkova, *C.r. Acad. bulgare Sci.* **30**, 1779 (1977).
2. D. V. Popov and E. T. Raïkova, *Problems of Oncology* **VII**, 49 (1979).
3. J. Epstein, R. W. Rosenthal and R. J. Ess, *Analyt. Chem.* **27**, 1435 (1955).
4. S. M. Lozeva and E. V. Golovinsky, *C.r. Acad. bulgare Sci.* **28**, 947 (1975).
5. I. Chernozemsky and R. Raïchev, *Neoplasma* **13**, 577 (1966).
6. P. Blagoeva, Z. Raïkov and N. Yordanov, *C.r. Acad. bulgare Sci.* **29**, 881 (1976).
7. V. Spassov, Z. Raïkov, D. Getova and E. Raïkova, Bulg. Pat. No. 41229, 27.10.1978
8. A. T. Quintanilha and L. Packeer, *Proc. natn. Acad. Sci. U.S.A.* **74**, 570 (1977).
9. M. Slomianko-Winnicka, *Ann. Acad. Med. Stetinensis* **4**, 165 (1971).
10. W. Shimmack and K. H. Summer, *Int. J. Radiat. Biol.* **34**, 293 (1978).