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Note

Evaluation of the alkylating activity of nitrosoureas by thin-layer densitometry

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The compounds 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride (ACNU; NSC-D 245382), 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; NSC-79037) and 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea (methyl-CCNU; NSC-95441), shown in Fig. 1, have been widely used as alkylating agents in the chemotherapy of human cancer. It has been suggested that the main carcinostatic mechanisms of nitrosourea might be based on the alkylation of tumour cell components by active alkyl fragments which were formed non-enzymatically[1,2].

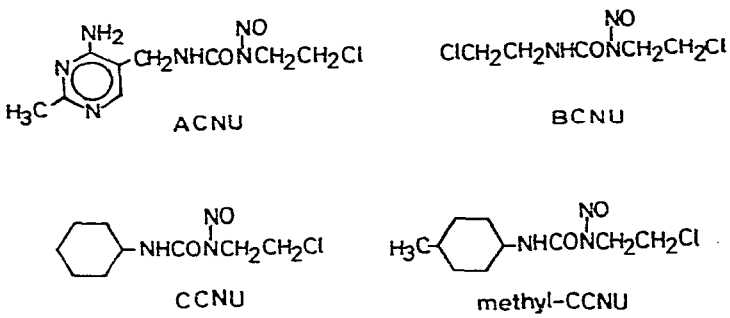

Fig. 1. Structures of ACNU, BCNU, CCNU and methyl-CCNU.

A colorimetric method for the determination of alkylating agents was reported in 1955 by Epstein *et al.*[3], based on the colour reaction of the agents with 4-(*p*-nitrobenzyl)pyridine (NBP). However, it is difficult to measure individual amounts of several active alkyl fragments generated simultaneously from one alkylating agent by this method.

This paper describes a method for the chemical evaluation of the alkylating activity of nitrosoureas by thin-layer densitometry. We have also identified the structure of each alkyl-NBP by mass spectrometry after isolating the corresponding spots by thin-layer chromatography.

EXPERIMENTAL

Apparatus

A Shimadzu CS-900 chromatoscanner equipped with a dual-pen recorder and an analogue integrator was used.

TLC plates and development

Pre-coated silica gel 60 F_{254} glass plates (20 × 20 cm) with a layer thickness of 0.25 mm were obtained from Merck (Darmstadt, G.F.R.). The plates were developed in *n*-hexane–acetone–28% ammonia solution (10:50:1) to a height of 10 cm at room temperature.

Reagents and solvents

All reagents and solvents were of guaranteed reagent grade and were used without further purification. NBP was obtained from Tokyo Chemical Industry (Tokyo, Japan). ACNU, BCNU, CCNU, and methyl-CCNU were synthesized according to methods described in the literature[4–6].

Standard solution. A freshly prepared solution containing 25 μmoles of each nitrosourea in 10 ml of acetone was used.

Procedure

To a 15-ml amber-coloured test-tube were successively added 2 ml of standard solution, 1 ml of phosphate buffer solution (0.025 *M*, pH 7.4), 1 ml of acetone and 0.4 ml of a 5% (w/v) solution of NBP in acetone. The mixture was placed in a boiling water-bath for 15 min. The solution was cooled and 5 ml of ethyl acetate were added. The test-tube was shaken vigorously, then centrifuged for 1 min at 400 *g*. The upper organic layer containing unreacted NBP was aspirated and discarded. 1,2-Dichloroethane (10 ml) and 0.25 *N* sodium hydroxide solution (1.5 ml) were also added successively to the aqueous layer. The tube was shaken vigorously and then centrifuged for 1 min at 400 *g*. The lower organic layer was evaporated to dryness and the residue was dissolved in 1 ml of acetone. A 5-μl aliquot of the solution was spotted on thin-layer plates using a Microcap capillary.

Measurement and relative quantitation

Immediately after development, the surface of the ammoniacal thin-layer plate was rapidly covered and shielded using a transparant glass plate (thickness 1 mm) of the same size and vinyl tape, in order to prevent discoloration of the spots. Measurement of each spot was carried out in the transmittance mode at a wavelength of 650 nm. The discoloration of each spot was neglected within 30 min after development. The densitometor was operated with a beam slit of 1.25 × 1.25 mm at a scanning speed of 10 mm/min with zig-zag scanning. The shielded plates were scanned in the direction of the solvent flow. The visible absorption profile of the thin-layer chromatogram and the integration curve of each spot were obtained simultaneously.

Calculation of the relative amounts of compounds in unknown products as alkyl-NBPs was achieved from the integrated total peak areas. The ratios were obtained from the equation

$$C = \frac{A}{B} \cdot 100$$

where A is the integrated intensity of each spot, B the integrated intensities of the total spots and C the relative amount (%).

RESULTS AND DISCUSSION

Typical thin-layer densitograms of alkyl-NBPs formed from the reaction of nitrosoureas and NBP are shown in Fig. 2. The relative amounts of alkyl fragments in the phosphate buffer solution (pH 7.4) of nitrosoureas after 15 min at 100° are listed in Table I.

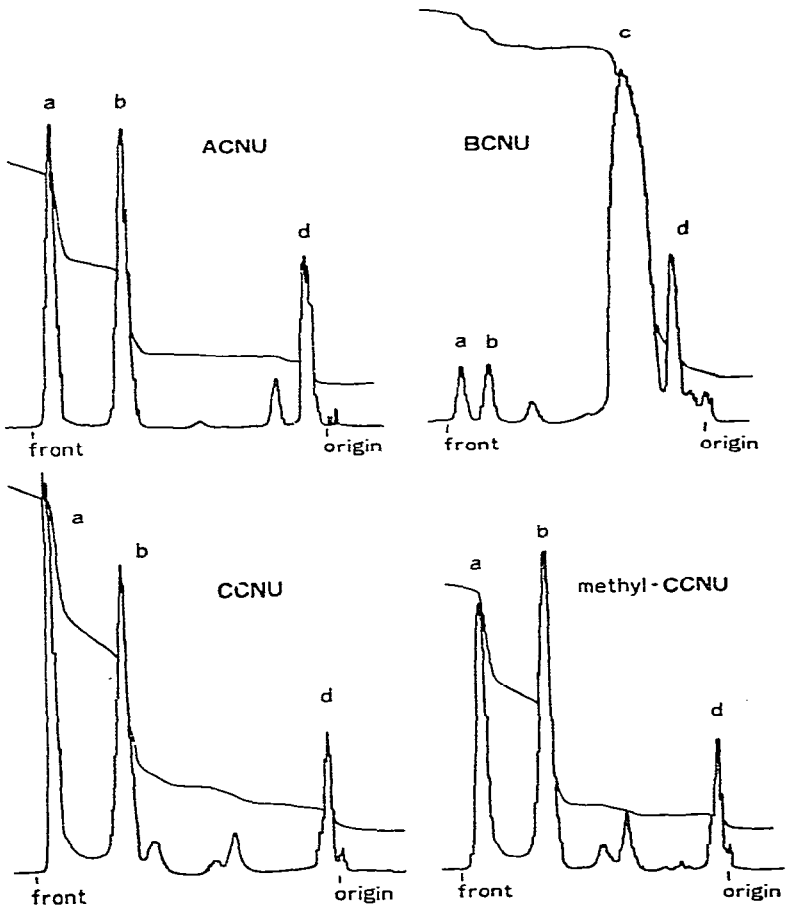

Fig. 2. Typical thin-layer densitograms of alkyl derivatives derived from the reaction of nitrosoureas and NBP. (a) NBP–$CH=CH_2$; (b) NBP–CH_2CH_2Cl; (c) NBP–$CH_2CH_2NH_2$; (d) NBP–CH_2CH_2OH.

The results indicate that ACNU, CCNU and methyl-CCNU gave similar densitograms to that of BCNU. For ACNU, CCNU and methyl-CCNU, 40–42% of the nitrosourea was converted into the vinyl fragment and 42–48% into the 2-chloroethyl fragment. However, with CCNU and methyl-CCNU, the formation of the 2-hydroxyethyl fragment was only 6%, which was about half of that with ACNU. With

TABLE I

FORMATION OF ALKYL FRAGMENTS FROM NITROSOUREAS AFTER 15 MIN IN PHOSPHATE BUFFER (pH 7.4) AT 100°

Nitrosourea	*Amount formed (%)*				
	$-CH=CH_2$	$-CH_2CH_2Cl$	$-CH_2CH_2NH_2$	$-CH_2CH_2OH$	*Unknown*
ACNU	40	42	0	12	6
BCNU	5	4	76	10	5
CCNU	41	42	0	6	11
Methyl-CCNU	42	48	0	6	6

BCNU, although the 2-aminoethyl fragment was the major product (76%), 5% of vinyl and 4% of 2-chloroethyl fragments were observed, but the amount of the 2-hydroxyethyl fragment formed (10%) was similar to that with ACNU (12%).

These alkyl fragments were identified as coloured alkyl-NBPs by mass spectrometry after extraction from the thin-layer plate with acetone. Table II shows the main fragment ions that served for the identification of alkyl-NBPs. In the mass fragmentation of the alkyl-NBPs, it was observed that the prominent ions, such as $(M-NO)^+$, $(M-NO_2)^+$ and $[M-(R+NO_2)]^+$, are common to all of them. By analysis of the mass spectrum, the alkyl fragments identified as NBP derivatives were found to be vinyl, 2-chloroethyl, 2-aminoethyl and 2-hydroxyethyl (see Table II). Of these, the 2-aminoethyl-NBP was obtained only from BCNU, as shown in Fig. 2. It has been suggested that 2-aminoethyl-NBP was derived by an S_N2 reaction of NBP with 2-chloroethylamine formed on decomposition of BCNU. However, it has been reported by Wheeler and Chumley[7] that 2-chloroethylamine shows no carcinostatic effect. Therefore, the anti-tumour effect of nitrosoureas *in vivo* are probably due to other alkyl fragments, such as vinyl, 2-chloroethyl and 2-hydroxyethyl cations. These alkyl fragments were also observed with each nitrosourea. The ultimate degradation products, acetaldehyde, vinylchloride and 2-chloroethanol, have been reported by Montgomery *et al.*[8] and Reed *et al.*[9] in studies of the chemical degradation of BCNU and CCNU. They hypothesized that these products are formed via a 2-chloroethyldiazene hydroxide intermediate. We have also determined acetaldehyde and 2-chloroethanol derived from these four nitrosoureas in phosphate buffer solution (pH 7.4) following incubation for 3 h at 37° [10]. With ACNU and BCNU, about 50% of the nitrosourea was converted into 2-chloroethanol. However, with CCNU and methyl-CCNU, the amounts of 2-chloroethanol formed were about two thirds of those from ACNU and BCNU.

TABLE II

CHARACTERISTIC FRAGMENTATION OF ALKYL-NBPs IN THE MASS SPECTRUM

NBP-R	*Main fragment ion (m/e)*			
	M^+	$(M-NO)^+$	$(M-NO_2)^+$	$[M-(R+NO_2)]^+$
NBP–$CH=CH_2$	240 (base peak)	210	194	167
NBP–CH_2CH_2Cl	276 (base peak)	246	230	167
NBP–$CH_2CH_2NH_2$	257 (base peak)	227	211	167
NBP–CH_2CH_2OH	258 (base peak)	228	212	167

We have also observed by thin-layer densitometry that the species of alkyl fragments did not change significantly in aqueous media ranging from pH 4.5 to 7.4 and heated at 37°, 60°, 80° and 100°. The maximum coloration was observed following incubation at 100° for 15 min. Further, it was found that these fragments were rapidly converted into acetaldehyde, 1,2-dichloroethane and 2-chloroethanol in the presence of OH^- and/or Cl^- ions in the medium. Although acetaldehyde did not react with NBP under the conditions used here, 2-chloroethanol reacted only about 100-fold less than the corresponding fragments from nitrosoureas, and 1,2-dichloroethane less readily.

CONCLUSIONS

The chemical differences between four nitrosoureas have been revealed by the determination of the relative amount of each alkyl fragment on a thin-layer densitogram. Active alkyl fragments such as vinyl, 2-chloroethyl and 2-hydroxyethyl should play an important role in the carcinostatic alkylation of tumour cells *in vivo* that are common to these nitrosoureas.

It should be possible to determine effectively the alkylating activities not only of nitrosoureas, but also of many other alkylating agents, including their active metabolites, by this method.

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