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SEPARATION OF ISOMERS OF 4-[BIS-(2-BROMOPROPYL)AMINO]-2′-CARBOXY-2-METHYLAZOBENZENE (CB 10-252) AND INVESTIGATION OF ITS ACTIVITY TOWARDS SOME HUMAN TUMOUR XENOGRAFTS

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Abstract—A number of azomustard derivatives have been shown by t.l.c. to consist of two components. In the case of CB 10-252, 4-[bis-(2-bromopropyl)amino]-2′-carboxy-2-methylazobenzene, these have been separated by preparative h.p.l.c. They are apparently diastereoisomers, chloroform solutions of which exhibit identical *trans*→*cis* photoisomerism and a thermal *cis*→*trans* reaction in the dark. They are also reduced identically by the soluble fraction of liver homogenate. The ability of a number of human tumour xenografts to reduce CB 10-252 was measured but the tumour with the most potent reductase activity was not inhibited by the drug *in vivo*.

The azomustard derivative 4-[bis-(2-bromopropyl)amino]-2′-carboxy-2-methylazobenzene (CB 10-252, NSC 240419; I, R = COOH, $R^1 = R^2 = CH_3$, X = Br) was the product of an attempt to design a drug to be specific for primary hepatocellular carcinoma [1]. This particularly rapidly growing tumour is common in parts of Africa and the Far East [2] and in the majority of cases treatment is impossible or unhelpful [3]. This contrasts with the primary liver tumours seen in Europe and the U.S.A. which grow much more slowly.

In 1955, Ross and Warwick [4, 5] showed that, because of their highly conjugated structure, nitrogen mustards of this type, I, tend to be chemically unreactive and therefore presumably non-toxic. Following enzymic reduction of the azo group, two metabolites would be expected, an aniline derivative II and a *p*-phenylenediamine derivative III [1, 5]. Ross and Warwick [5] found a striking correlation between reducibility of the azo group and antitumour activity against the Walker 256 rat carcinoma. This investigation also showed that electron donating substituents *ortho* to the azo group are necessary for activity. The most active compound of this early series was 4-[bis-(2-chloroethyl)amino]-2′-carboxy-2-methylazobenzene (CB 1414, NSC 16498; I, R = COOH, $R^1 = CH_3$, R^2 = H, X = Cl), which underwent clinical trial [6] but was devoid of significant benefit; however, it should be noted that liver tumours were not investigated.

Some fifteen years later Connors and coworkers [7] confirmed that the enzyme system necessary for the reductive cleavage of the azo mustards is actually present in liver, and that the expected metabolites are in fact formed. Of the two metabolites resulting from the reduction of CB 1414, one is anthranilic acid (II, R = COOH), which has no antitumour activity and only a moderate host toxicity. The second metabolite (III, $R^1 = CH_3$, R^2 = H, X = Cl) is a *p*-phenylenediamine mustard. Nitrogen mustards of this type are extremely potent alkylating agents with known antitumour activity [8].

The ability to reduce azo compounds was later shown to be a property of human primary hepatocellular carcinoma [9]. CB 1414 therefore fulfilled two of the criteria for a drug to be truly selective, i.e. it is inactive in itself but capable of metabolism to a potent cytotoxic agent by a rapidly proliferating tumour. The third criterion for selectivity, that the toxic metabolite should remain within the confines of the tumour, is more difficult to achieve. However, investigation of a further series of azomustards (see Scheme, I, R = COOH, $R^1 = CH_3$) containing var-

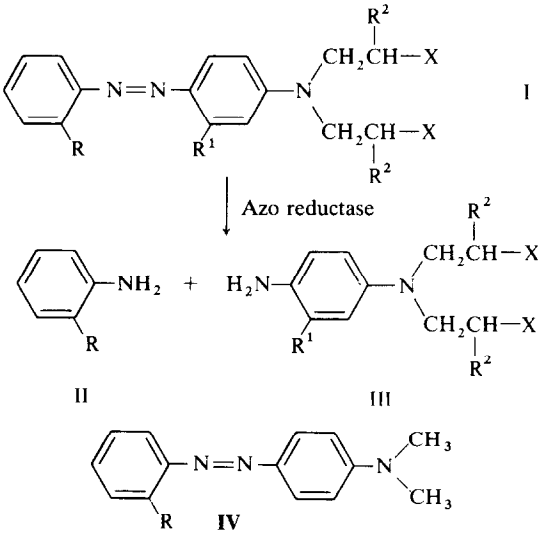
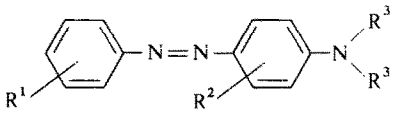


Table 1. Structural and t.l.c. properties of a range of azomustards



No.	R^1	R^2	R^3	R_f values Main spots
1	H	H	CH_2CH_2Br	0.75
2	H	2-CH_3	CH_2CH_2Br	0.77
3	2′-COOH	H	CH_2CH_2Br	0.37
4	2′-COOH	2-CH_3	CH_2CH_2Br	0.39
5	2′-COOH	2-CH_3	CH_2CH_2Cl	0.35
6	2′-COOH	3-OCH_3	CH_2CH_2Cl	0.30
7	H	2-CH_3	CH_2CH_2I	0.81
8	2′-COOH	H	$CH_2CH(CH_3)Br$	0.34, 0.41
9	2′-COOH	2-CH_3	$CH_2CH(CH_3)Br$	0.39, 0.46
10	2′-COOH	H	$CH_2CH(CH_3)Cl$	0.33, 0.40
11	2′-COOH	2-CH_3	$CH_2CH(CH_3)Cl$	0.37, 0.44
12	H	H	$CH_2CH(CH_3)Cl$	0.61, 0.76
13	2′-COOH	2-OCH_3	$CH_2CH(CH_3)Br$	0.05, 0.07
14	4′-NO_2	2-COOH	$CH_2CH(CH_3)Br$	0.10, 0.16

ious substituents R^2 and X, together with the corresponding phenylenediamine derivatives (II), by Bukhari and coworkers [1] produced an alkylating metabolite, 4-bis-(2-bromopropyl)amino-2-methylaniline (III, $R^1 = R^2 = CH_3$, X = Br) with a chemical half life of 41 sec at pH 7.5 and 37°. Thus the parent azo compound, CB 10-252, is a relatively nontoxic drug, which is reduced to a very toxic metabolite in tissues containing the appropriate enzyme system. The rapid hydrolysis of the cytotoxic metabolite should prevent its toxic effect from spreading to other proliferating tissues.

Confirmation of this selectivity was produced by Connors *et al.*, [10]. Implantation of the Walker 256 tumour in the liver of rats rendered it more susceptible to the action of CB 10-252 than to cyclophosphamide, whereas when the tumour was implanted in the flank the reverse was true.

The original supposition was that the enzyme responsible for the reduction of the azobond would be azoreductase. Although this is true in the case of azo compounds such as 4-dimethylaminoazobenzene (DAB; IV, R = H), it is not true for CB 10-252 or methyl red (IV, R = COOH). When various fractions of liver homogenate were examined for their ability to reduce this selection of azo compounds, it was observed that DAB was reduced in the presence of the microsomal fraction with a pH optimum of 6.9. CB 10-252 and methyl red, on the other hand, are reduced by the soluble fraction at an optimum of pH 6.2 [11]. There is no cross reactivity between the two systems and it is therefore necessary to specify the appropriate azo reductase enzyme system being considered. The significant feature distinguishing the two types of azo compound is the presence or absence of a carboxylic acid group *ortho* to the azo group. It has been shown that in such compounds the carboxyl group is hydrogen bonded to the adjacent nitrogen of the azo group to form a six membered ring (V) such that no hydroxyl stretching frequency is visible in the infrared spectrum [12]. The resulting major disruption in the electron distribution of the azo bond compared with the unsubstituted compounds, such as DAB, may explain the differences observed in the enzymic reduction.

Renewed clinical interest in CB 10-252 prompted a more detailed study of the chromatographic properties of the drug and the reductive ability of a selection of human tumours grown in immune deprived mice.

MATERIALS AND METHODS

CB 10-252 was from a batch prepared by Ward Blenkinsop & Co. Ltd., for clinical formulation. The other azo mustards were synthesized within this Institute [1, 5]. Thin layer chromatography (t.l.c.) was undertaken on silica precoated on aluminium (Merck 5554) and developed with toluene–ethyl acetate (4:1). Preparative high performance liquid chromatography (Prep h.p.l.c.) was carried out using a Jobin–Yvon Chromatospec Prep 100. The column was formed from 140 g t.l.c. silica gel 60H (Merck 11695), with 1 g of CB 10-252, absorbed on additional silica, applied to the top of the column. Elution was with toluene-ethyl acetate (97:3) at an initial flow rate of 30 ml/min. The chromatographically pure fractions were evaporated and the residues crystallized from benzene. Ultraviolet and visible spectra were recorded in chloroform solution in a Pye SP8-150 spectrophotometer.

The CB 10-252 azo reductase activity of ten human tumour xenografts and three rodent tumours, relative to the liver of a similar non-tumour-bearing animal, was determined using the method of Autrup and Warwick [11]. The xenografts were maintained in T lymphocyte-deficient male CBA/LAC mice [13] and the rodent tumours passaged in the appropriate species [14–16].

RESULTS AND DISCUSSION

Thin layer chromatographic studies of CB 10-252 showed the material to consist of two components in roughly equal proportions which showed no tendency to re-equilibrate on standing in either daylight or darkness. If the t.l.c. plate was run a second time perpendicular to the initial run there was no indication of the components separating a second time. The slow running component CB 10-252S had R_f 0.39 and the faster component CB 10-252F R_f 0.46. A number of other azo mustards were available at the time and their t.l.c. properties were similarly investigated. These compounds could be readily separated into two groups based on their appearance on t.l.c. in this system. One group produce a single spot and the others separate into two spots similar to those seen from CB 10-252. The structural features and the t.l.c. pattern of these compounds are correlated in Table 1. From this it can be seen that irrespective of the nature of the ring substituents the number of spots is governed by the nature of the haloalkyl groups. In particular, haloethyl groups (compounds 1–7) produce one spot, whereas 2-halopropyl groups give rise to two spots. The explanation for this is that each 2-halopropyl group contains an asymmetrically substituted carbon atom at C-2; the presence of two such centres in the molecule resulting in diastereomers.

A possible alternative explanation could be that the components being separated are the *cis* and *trans* isomers about the azo group. It would not be expected, however, that the inclusion of additional methyl groups at a position so remote from the azo bond could have such a marked effect on the stability of these isomers. In order to investigate this possibility in more detail and also to determine whether the soluble fraction of liver supernatant possessed the same activity with respect to the two components, an attempt was made to separate them in bulk. One gram of CB 10-252 was subjected to Prep/h.p.l.c. and produced following crystallization of the components from benzene, 160 mg CB 10-252F, m.p. 172–5° (Anal. $C_{20}H_{23}Br_2N_3O_2$. C_6H_6 requires C 54.3, H 5.1, Br 27.8, N 7.3; Found C 54.2, H 5.4, Br 27.1, N 7.8) and 30 mg CB 10-252S, m.p. 182–4° (Anal. $C_{20}H_{23}Br_2N_3O_2$ requires C 48.3, N 4.7, Br 32.1, N 8.5; Found C 48.5, N 5.0, Br 31.2, N 8.9). Elemental analysis showed that CB 10-252F crystallized with one molecule of benzene of crystallization. Each isomer had identical mass, n.m.r. and ultraviolet/visible spectra to the mixture.

The visible spectrum of CB 10-252 in chloroform solution has maximum absorbance at 478 nm. However, when the solution has been previously exposed to daylight there is a progressive increase in absorbance when the solution is placed in the dark. Measurements were made of the rate of this increase, assuming it to be a first order reaction. This assumption is valid as the observed spectral change is due to a thermal *cis*→*trans* rearrangement which occurs in the dark (Fig. 1). For the kinetic measurements, therefore, the absorbance was measured at 1-min intervals; the sample was in the light beam for only 5 sec and the remainder of the time it was in darkness. In daylight, photoisomerism occurs, converting the wholly *trans* isomer solution, formed in the dark, to a mixture of *cis* and *trans* isomers. The actual position of the equilibrium is dependent on the total illumination. Such isomeric changes are well established for azo compounds [17], but are not always demonstrable. The changes occur very rapidly in aqueous solution [18] and it proved impossible to detect them in this instance. The kinetic results obtained for the thermal *cis*→*trans* rearrangement are shown in Table 2. The fast and slow isomers exhibit identical kinetics for this reaction, within the limits of the determination. In order to demonstrate that an azo compound that does not separate chromatographically into two components undergoes the same rearrangements, the rate for CB 1414 (I, R = COOH, R^1 = CH_3, R^2 = H, X = Cl) was determined under similar circumstances (Table 2).

The reductase activity of the thirteen tumour systems are shown in Table 3. The number of passages since the initial tumour implant and the age of the particular tumour are indicated in the case of the xenografts. The reductase activity is calculated as nmoles of CB 10-252 reduced per 100 mg of protein per minute as a percentage of the activity of that of the liver of a similar non-tumour-bearing animal. The results show that the age and passage number of a tumour has little or no effect on the azo reductase activity.

Table 2. Rate constant and half-life of dark *cis*→*trans* rearrangement of CB 10-252 components in chloroform at 25°

	Rate constant (min^{-1})	Half-life (min)
CB 10-252 F	0.23 ± 0.02	3.1 ± 0.2
CB 10-252 S	0.24 ± 0.03	2.9 ± 0.3
CB 1414	0.19 ± 0.01	3.7 ± 0.2

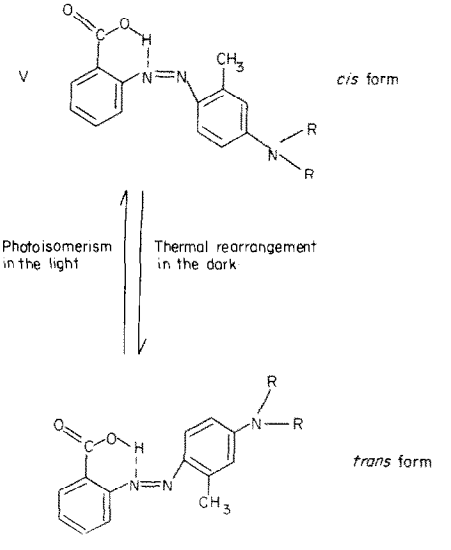


Table 3. Ability of a range of human tumour xenografts to reduce CB 10-252

Tumour	Passage number	Time since implant (days)	Relative activity (Liver = 100)	Mean ± S.D.
Lung–Bush	15	36	55.4, 45.2	47.9 ± 8.9
	17	65	54.7, 36.5	
Lung–Mount	43	42	44.1, 42.9	55.6 ± 20.3
	44	49	55.9, 57.1	
	7	57	38.5, 34.3	
	8	47	87.4, 84.5	
Lung–Oat cell	24	80	19.5, 21.3	19.2 ± 1.6
	24	101	17.5, 18.6	
Lung–P246	9	79	28.0, 25.9	23.4 ± 4.2
	9	82	20.9, 18.9	
Kidney	35	36	34.9, 40.5	30.6 ± 8.5
	35	47	23.8, 23.1	
Melanoma-HX47	7	101	29.1, 31.1	23.0 ± 8.3
	9	87	17.2, 14.5	
Melanoma-HX41	7	150	17.0, 24.9	20.9 ± 5.6
Ovary	25	56	42.9, 28.4	36.6 ± 6.1
	25	86	38.8, 36.4	
Squamous cell carcinoma	17	101	19.8, 19.8	21.2 ± 2.2
	18	80	24.5, 20.8	
Teratoma	10	150	11.0, 5.5	8.6 ± 2.4
	11	91	9.7, 8.3	
TLX 5			39.6, 28.8	34.2 ± 7.6
Adj PC6/A			65.3, 66.5	65.9 ± 0.8
Walker 256			68.5, 63.1	65.8 ± 3.8

Two of the tumours have been tested *in vivo* for their response to CB 10-252 treatment. These were the Lung–Mount xenograft and the Walker 256 carcinosarcoma. Although these two tumours showed the highest levels of enzyme activity amongst the group investigated, no significant anti-tumour activity was detected. In the case of the Walker tumour there is less response than when the tumour is transplanted in the liver [10]. The inactivity towards the Lung–Mount xenograft could be due to a number of causes; the level of azo reductase may be insufficient to produce the necessary levels of the active metabolite within the tumour or the tumour could be inherently unresponsive to alkylating agents as cyclophosphamide is also inactive against this tumour.

These results suggest that a tumour enzyme level approaching that of normal liver may be necessary for effective use of this agent, or that the tumour must be situated within the liver itself. CB 10-252 was tested clinically in Kenya [11] but no results were published. A trial on patients from the United Kingdom and the Middle East showed no significant clinical effect on tumour growth [19] but this could be due to the tumour having a slower growth rate to those seen in East Africa. Currently, CB 10-252 is undergoing Phase II/III clinical trial in Zambia.

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