Biochemical Pharmacology, 1962, Vol. 11, pp. 1079–1087. Pergamon Press Ltd., Printed in Great Britain.

HAEMOTOLOGICAL AND TUMOUR-INHIBITORY EFFECTS OF PEPTIDE DERIVATIVES OF MELPHALAN

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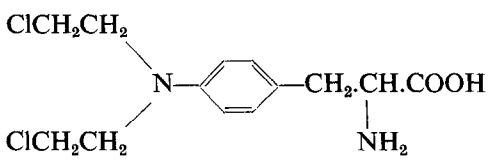
(*Received* 18 *May* 1962; *accepted* 20 *June* 1962)

Abstract—The haematological and tumour-inhibitory effects of a number of peptide derivatives of *p*-di(2-chloroethyl)amino-L-phenylalanine (Melphalan) have been investigated. Melphalan is the C-terminal amino acid in all but one of the peptides. Blocking of the free COOH groups of Melphalan or its peptides by esterification does not reduce the physiological activity. In contrast, blocking of the free NH_2 group by formylation or acetylation causes considerable reduction in activity, though there is no corresponding decrease in the chemical reactivity of the N-mustard group. High tumour-inhibitory and haematological activity can be maintained in di-, tri- and tetra-peptide esters of Melphalan provided that the terminal NH_2 group of the peptide chain remains free.

IN THE search for chemotherapeutic agents for treatment of malignant diseases many compounds have been developed by attaching various chemical structures to the cytotoxic di(2-chloroethyl)amine (nitrogen mustard) grouping, $(ClCH_2CH_2)_2N$-.

With the idea of determining whether any desirable specificity of action could be achieved by the use of a naturally occurring amino acid as carrier of the nitrogen mustard group Bergel and Stock,[1] and Bergel *et al.*[2] prepared the DL, L and D isomers of *p*-di(2-chloroethyl)aminophenylalanine (I). These compounds were named respectively Merphalan, Melphalan and Medphalan.



I

The racemic (DL) form, Merphalan, was prepared independently by Larionov *et al.*[3] who gave it the name of Sarcolysin.

The interesting therapeutic properties of these α-amino acid compounds led to the development of Melphalan and Merphalan derivatives, and particularly to the elaboration of a series of peptides of Melphalan.[4, 5] In most of these peptides the *p*-di(2-chloroethyl)amino-L-phenylalanine (Melphalan) is the C-terminal amino acid. This series thus contrasts with the series described by Russian workers[6–8] in which the DL-isomer (Sarcolysin) is N-terminal, i.e. joined to the other amino acids through its carboxyl group.

The haematological and tumour-inhibitory effects of some of these Melphalan peptides are now described. The haematological effects of an homologous series of compounds related to Merphalan have been described by Elson.[9]

EXPERIMENTAL

Rats of a Wistar albino colony were used for all the experiments. Compounds were administered by intraperitoneal injection dissolved in either arachis oil or water, or in the form of a fine suspension in arachis oil.

Blood counts and toxicity determinations were carried out as described by Elson.[9] Tumour-inhibitory action against the implanted Walker rat carcinoma 256 was assessed as described by Connors *et al.*[10]

An assessment of the chemical reactivity of some of the compounds was made by finding the extent of reaction under standard conditions. The method was based on that of Ross,[11] but the reaction was carried out in the presence of calcium carbonate to avoid the possibility of hydrolysis of the N-formyl group (where present) by liberated hydrogen ion. In general, the compound (0·500 m-mole) was heated 30 min under reflux in acetone (25 ml) and water (25 ml), together with precipitated calcium carbonate (0·20 g; 2 m-mole). The solution was rapidly cooled, acidified with concentrated nitric acid (previously boiled until colourless), and 0·1 N silver nitrate solution (10·0 ml) was added. The mixture was shaken to assist coagulation of the silver chloride, filtered, and the excess silver nitrate in the combined filtrate and washings titrated against standard potassium thiocyanate using ferric alum as indicator. In one experiment (with Melphalan) one equivalent of chloride ion was introduced (see Table 3, CB 3025 Cl). The procedure was exactly as above except that an aqueous solution of calcium chloride (0·500 mequiv.; prepared by diluting 5·0 ml 0·1 N $CaCl_2$ solution to 25 ml with water) was used in place of distilled water. Duplicate hydrolyses and blanks were run in all cases. The percentage total available mustard chlorine liberated as chloride (percentage reaction) is given for several representative compounds in Table 3.

RESULTS AND DISCUSSION

Fig. 1 shows the leucocyte response patterns to Merphalan, Medphalan and Melphalan. The graphs represent the percentage of the normal number of lymphocytes, and neutrophils circulating in the blood on successive days after treatment with a single dose of the compound. Each curve represents a mean value for four rats as described by Elson.[9] The ratios L/d and N/d give an assessment of the absolute activity of the compounds in depressing the number of lymphocytes and neutrophils, respectively. They are obtained by dividing the maximum percentage fall in numbers of circulating blood cells by the dose (d) in mg/kg.[9]

Melphalan is the most active of the three optical forms, a dose of 5 mg/kg producing approximately the same haematological effect as 20 mg/kg of the D-isomer, Medphalan. Merphalan (Sarcolysin) is of intermediate activity. The same order of effectiveness with Melphalan, again showing the highest activity, is also seen in the inhibitory response of the Walker rat carcinoma 256 (Table 1). A single dose of 1 mg/kg of Melphalan can almost completely suppress the growth of the tumour, whereas the same dose of Medphalan has very little effect.

Since therefore it is established that the highest activity is obtained with the L-isomer, the peptides investigated were derivatives of Melphalan rather than of Medphalan.

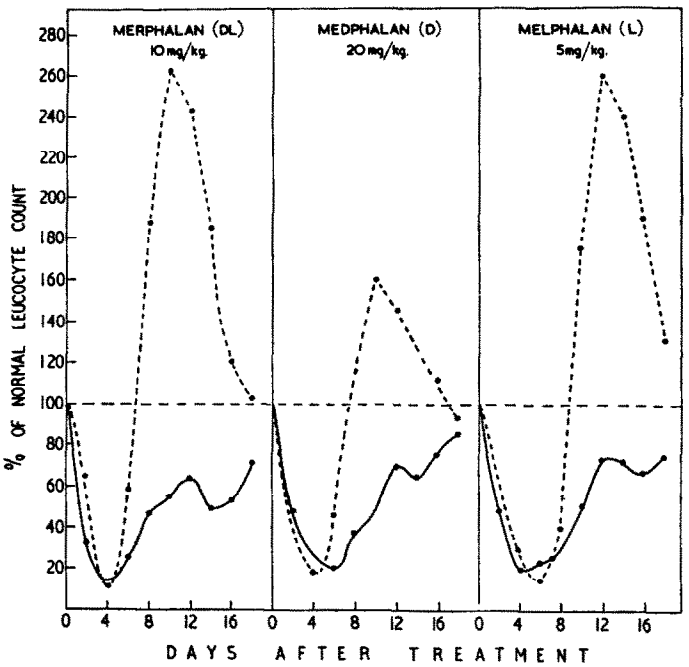


FIG. 1. Effect of single doses of Merphalan, Medphalan and Melphalan on the numbers of leucocytes circulating in the blood of the normal rat. —•——•— lymphocytes. – – · – – · – – neutrophils.

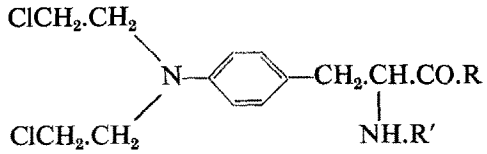
TABLE 1. INHIBITORY ACTION OF MERPHALAN (DL), MEDPHALAN (D) and MELPHALAN (L) ON THE GROWTH OF WALKER RAT CARCINOMA 256

Control (C)	Treated (T)		
	DL	D	L
115	15	44	0·5
90	5	34	0
81	4	30	0
39	3	28	0
37	1	23	0
31	0	20	0
30	0	19	0
28	0	13	0
22	0	10	0
	0	6	0
Mean 52·6	2·8	22·7	0·05
C/T	18·8	2·3	>1000

Weight (g) of individual tumours at 13 days after implantation and 12 days after administration of the DL, D and L, forms of *p*-di(2-chloroethyl)aminophenylalanine, at a dose of 1 mg/kg intraperitoneally in oil.

Merphalan was also avoided because the use of racemic acids in peptide synthesis is likely to lead to products of uncertain stereochemical composition.[4] The toxicity and haematological effects of the Melphalan peptides on the normal albino rat and their inhibitory action on the growth of the Walker carcinoma implanted in rats of the same strain are summarized in Table 2. The notation used in these tables is that

Table 2. Melphalan derivatives



Compound							Haematological Action					Toxicity	Tumour inhibiting action		
No.	Code No. C.B.	R	R¹	Notation*	Configuration†	Dose *d* mg/kg	% Fall from normal: Lymphocytes L.	% Fall from normal: Neutrophils N.	*L/d* (*LF/d*‡)§	*N/d* (*NF/d*)	*L/N*	Approx. lethal dose *D* (*D/F*)	Dose (mg/kg)	Medium	Inhibition of Walker tumour *C/T*
1	3025	—OH	H	Mel.OH	L	5	80	90	16 (14·6)	18 (16·4)	0·90	9 (9·9)	1·0 0·5	Oil Water	∞ 4
2	3177	—OC_2H_5	H.HCl	Mel.OEt	L	5	75	90	15 (15)	18 (18)	0·83	10 (10)	1·0 0·5 1·0	Water Water Oil	∞ 5 6
3	3206	—OC_2H_5	—CO.CH.NH_2 (HCl.H_2O); $CH(CH_3)_2$	Val.Mel.OEt	LL	5	75	90	15 (19·6)	18 (23·6)	0·83	12·5 (9·6)	1·0 1·5	Oil Oil	∞ 45
4	3262	—OC_2H_5	—CO.CH.NH_2 (HCl.H_2O); $CH_2CH(CH_3)_2$	Leu.Mel.OEt	LL	6·5	75	98	11·5 (15·6)	15 (20·3)	0·77	11·5 (8·5)	2·0 1·0	Water Water	∞ 96
5	3207	—OC_2H_5	—CO.CH.NH_2 (HCl); CH_2 (C6H5)	Phe.Mel.OEt	LL	6	78	90	13 (18·2)	15 (21·0)	0·87	12 (8·6)	1·5 1·0	Oil Oil	∞ 1·5
6	3232	—OC_2H_5	—CO.CH_2.NH.CO.CH.NH_2; $CH(CH_3)_2$; 2HCl	Val.Gly.Mel.OEt	LL	6	81	87	13·5 (20·5)	14·5 (22·1)	0·93	20 (13·1)	10 2	Oil Oil	∞ 1
7	3248	—OC_2H_5	—CO.CH.NH.CO.CH.NH_2; CH_2; CH_3; $CH(CH_3)_2$.2 HCl	Ala.Leu.Mel.OEt	LLL	6·5	72	87	11 (17·5)	13·5 (21·5)	0·81	25 (15·7)	10 1·2 0·8	Water Water Water	∞ 17 6

TABLE 2.—*continued.*

Compound						Haematological Action					Toxicity	Tumour inhibiting action		
No.	Code No. C.B.	R	R^1	Notation*	Configuration†	Dose *d* mg/kg	% Fall from normal: Lymphocytes L.	% Fall from normal: Neutrophils N.	*L/d* (*LF/d*‡)§	*N/d* (*NF/d*)	*L/N*	Approx. lethal dose *D* (*D/F*)	Dose (mg/kg)	Medium	Inhibition of Walker tumour *C/T*
8	3263	$-NHCH_3$ $H_5C_2O_2C$	$-CO.CH.NH_2$ 2 HCl $CH(CH_3)_2$ 2 H_2O	Val.Mel.Gly.OEt	LL	6	71	81	12 (19)	13 (20·5)	0·92	20 (12·5)	5·0 2·0	Water Water	∞ 22
9	3252	$-OC_2H_5$	$-CO.CH_2.NH.CO.CH_2.NH.CO.CH.NH_2$ 2 $HCl.2H_2O$ $CH(CH_3)_2$	Val.Gly.Gly.Mel.OEt	LL	10	63	77	6·3 (10·8)	7·7 (13·2)	0·82	30 (17·4)	10 0·84	Water Water	∞ 5
10	3208	—OH	—CHO	Fo.Mel.OH	L	50	90	40	1·8 (1·6)	0·8 (0·7)	2·2	150 (167)	20 10	Oil Oil	∞ 30
11	3239	$-OC_2H_5$	—CHO	Fo.Mel.OEt	L	50	80	55	1·6 (1·56	1·1 (1·07)	1·4	200 (205)	20 10	Oil Oil	∞ 5
12	3224	$-OC_2H_5$	$-CO.CH.NH.CO.CH_3$ CH_2 	Ac.Phe.Mel.OEt	LL	45	80	95	1·8 (2·5)	2·1 (3·0)	0·86	100 (71)	12·5 10	Oil Oil	41 44
13	3258	$-OC_2H_5$	$-CO.CH.NHCOCH_3$ $CH_2CH(CH_3)_2$	Ac.Leu.Mel.OEt	LL	20 12·5	80 65	90 77	mean 4·6 (6·1)	mean 5·3 (7·1)	mean 0·87	51 (40)	6 2	Oil Oil	210 4

* The abbreviated nomenclature follows that proposed for peptides and proteins by Brand and Edsall[18] and now widely adopted; Mel = melphalan moiety.

† Configuration of amino acid residue, reading from left to right where there is more than one optically active centre.

‡ $F = \frac{\text{Mol. wt. of compound}}{\text{Mol. wt. of Mel.OEt}}$.

§ Figures in parentheses in the haematology and toxicity columns are the values corrected for molecular weight differences; Melphalan ethyl ester hydrochloride (CB 3177) is the reference compound.

commonly employed in amino acid and peptide chemistry.[12] For example, Mel.OH represents the free amino acid Melphalan, and Mel.OEt its ethyl ester. Similarly, Val.Mel.OEt signifies L-Valylmelphalan ethyl ester.

The compounds may be considered under two headings: (1) Melphalan esters and peptides with an unsubstituted amino group (free —NH_2); (2) compounds with acylated amino groups (blocked —NH_2). Both classes contain compounds with free or esterified carboxylic acid groups (—COOH or —$COOC_2H_5$). Blocking of the —COOH group by formation of the ethyl ester appears to have very little effect on the biological activity of the compound. It may have some practical advantages since the ester hydrochlorides are usually water soluble.

An assessment of haematological activity and of lethality adjusted for differences in molecular weight has also been made (given in parentheses in Table 2), the activity of Melphalan ester, CB 3177, being taken as a standard. By the use of the factor *F*, allowance can be made for increases in molecular weight with increasing complexity of the peptide, so that a direct comparison of absolute activity can be made. The effect of the carrier moiety on the intrinsic cytotoxic action of the nitrogen mustard group can thus be estimated.

Table 2 demonstrates that a high degree of haematological and tumour-inhibitory activity can be retained in di-, tri- and even tetra-peptides of Melphalan provided that a free —NH_2 group is present in the molecule. It is clearly not necessary for this free amino group to be attached to the Melphalan residue; it can, in fact, be far removed from the ester group as in the tetrapeptide CB 3252.

In Fig. 2 are shown the blood response patterns to Melphalan ester (CB 3177); Val.Mel.OEt, a dipeptide ester (CB 3206); Val.Gly.Mel.OEt, a tripeptide ester (CB 3232); and Val. Gly.Gly.Mel.OEt, a tetrapeptide ester (CB 3252). All show the typical nitrogen mustard pattern of response to a single dose,[13] the most characteristic features of which are the rapid fall in all blood elements followed by the very marked transient neutrophilia observable from about 8 days after administration of the drug. The most rapid fall in lymphocytes and platelets is shown by Melphalan ester CB 3177, the minimum values being reached in from 2 to 3 days. With the peptides there appears to be some increase in the duration of the depressive action, roughly in proportion to the increasing length of the peptide chain. With the tetrapeptide CB 3252 in particular there is more effect on the neutrophils than on lymphocytes and the minimum value for the circulating neutrophils is not reached until 6 or 7 days after administration of the compound. This tetrapeptide does appear to be somewhat less active than Melphalan ester and other peptides when the assessment is based on the absolute fall of leucocytes (*LF/d*; *NF/d*; Table 2). This figure, however, makes no allowance for the duration of action, so that when the prolonged response to CB 3252 is also taken into account, the tetrapeptide must undoubtedly be considered a highly active compound. In its toxic and growth-inhibitory effects it compares very favourably with that of some of the other derivatives, the lethal dose being approximately three times that of Melphalan.

Compounds 1 to 9 of Table 2, all containing a free NH_2 group, thus show high biological activity, comparable with that of Melphalan. If, however, as in compounds 10 to 13 (Table 2), one of the H atoms of the NH_2 group is substituted by a formyl or acetyl group, there is a very marked diminution in haematological activity. The compounds are also much less toxic and less inhibitory to the growth of the Walker

tumour. This depression of the biological activity of the dichloroethylamine (N mustard) moiety by acylation of the α-amino group of the phenylalanine carrier has been noted previously.[4, 14, 15]

The rates of reaction under standard conditions of several representative compounds indicated that the reduction in biological activity caused by formylation or

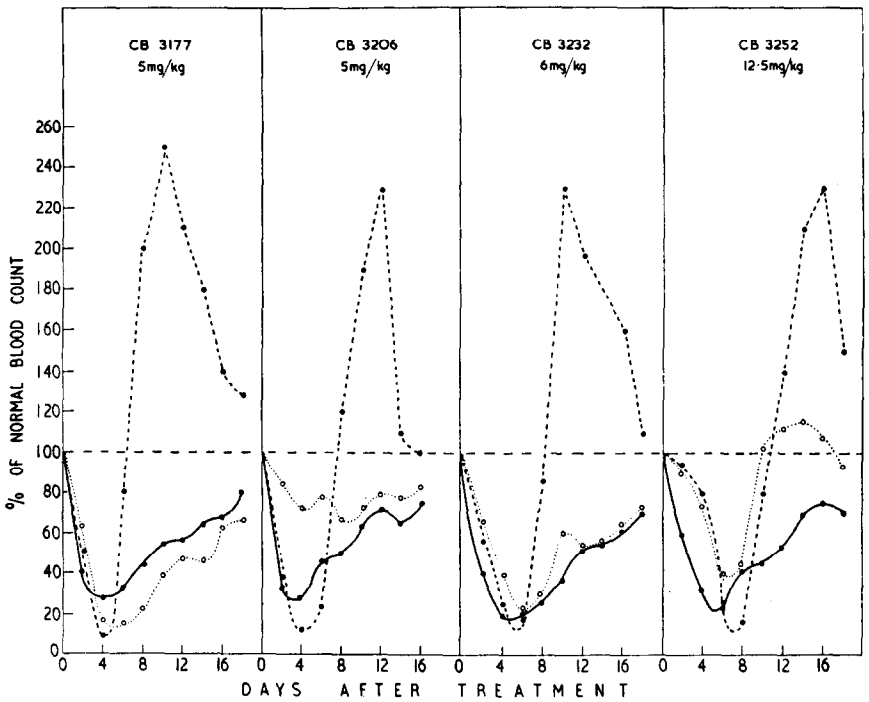


FIG. 2. Blood response patterns of the normal rat to Melphalan ester (Mel.OEt; CB 3177), and to a dipeptide (Val.Mel.OEt; CB 3206), a tripeptide (Val.Gly.Mel.OEt; CB 3232), and a tetrapeptide (Val.Gly.Gly.Mel.OEt; CB 3252) ester of Melphalan. —•—•— lymphocytes. – – · – – · – – neutrophils. . . . o . . . o . platelets.

acetylation is not necessarily related to a decrease in the chemical reactivity of the mustard group. Relevant data are given in Table 3, where the derivatives are listed in order of increasing reactivity. The compounds were hydrolysed in the presence of excess calcium carbonate to avoid a fall in pH with the consequent possibility of hydrolysis of the N-formyl group, where present. The extent of reaction was given by the proportion of total mustard chlorine released as chloride. The results show that compounds with similar chemical reactivities may have quite different intensities of biological activity, though the latter is always of the "mustard" type. For example, Melphalan (CB 3025) reacted at the same rate as formylmelphalan ester (CB 3239), yet its minimum tumour-inhibiting dose is only about one-twentieth that of the formyl compound, and the haematological activity is also much greater. The hydrolysis of Melphalan ester hydrochloride (CB 3177) would be expected to be suppressed to some extent by the chloride ions initially present, since the first step in the reaction is a reversible ionization[16] (equilibrium (1) in reaction scheme below). Consequently the

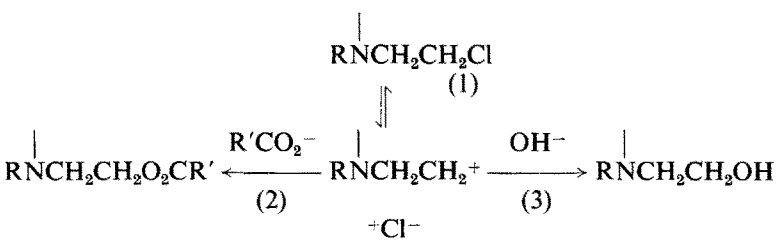
experimental figure of 20 per cent is not strictly comparable with the rates for the other compounds in Table 3. To assess the influence of chloride ion under the experimental conditions used, Melphalan was hydrolysed in the presence of one equivalent of chloride (added as calcium chloride). It can be seen from Table 3 (CB 3025 Cl and CB 3025) that the extent of reaction was thereby reduced from 27 to 22 per cent,

TABLE 3. REACTION RATES
(30 min in boiling 1:1 acetone–water with excess $CaOC_3$)

Code no. CB	Compound	% Reaction	Minimal dose (mg/kg i.p.) giving complete tumour inhibition	Haematological *LF/d*
3224	N-Acetyl-L-phenylalanyl-* Melphalan ethyl ester	19	12·5 (oil)	2·5
3177	Melphalan ethyl ester hydrochloride	20	1 (water)	15
3025Cl	Melphalan + ½ $CaCl_2$	22	—	—
3025	Melphalan	27	1 (oil)	14·6
3239	N-Formylmalphalan ethyl ester	27	20 (oil)	1·6
3208	N-Formylmelphalan	41	20 (oil)	1·6

* Contaminated with some D-L dipeptide (racemization during synthesis[4, 5]).

indicating that the "true" value for Melphalan ester (CB 3177) is around 25 per cent (20 + 5 per cent). The chemical reactivity of this ester is thus not substantially different from that of the *biologically* much less active formyl derivative, CB 3239. The relatively high reactivity figure (41 per cent) of formylmelphalan is no doubt a result of the compound being in the form of carboxylate ion during hydrolysis. As indicated in the scheme below, the esterification reaction (2) with this anion supplements hydrolysis (3) in removing carbonium ions, and competes with the chloride ion recombination process (equilibrium (1)). The



concentration of free chloride consequently tends to be higher than in reactions not involving additional anion competition. The conditions used in obtaining a measure of comparative reactivities do not, of course, simulate a physiological environment, and it would be of interest to determine reaction rates in conditions of this kind, as suggested recently by Ross for some related compounds in an appendix to a paper by one of us (L.A.E.).[9] However, it already seems clear that, given sufficient chemical

reactivity, the degree of biological activity of the Melphalan derivatives discussed here is greatly influenced by the nature of the carrier of the mustard group.

Acknowledgements—We are indebted to Dr. W. C. J. Ross for his helpful comments on some of the chemical aspects.

This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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