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Pharmacokinetic basis for the comparative antitumour activity and toxicity of chlorambucil, phenylacetic acid mustard and β,β-difluorochlorambucil (CB 7103) in mice

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Summary. This report describes the relationship between the pharmacokinetics, antitumour activity and toxicity of chlorambucil (CHL), phenylacetic acid mustard (PAAM) and β,β-difluorochlorambucil (β-F_2CHL) in mice. Pharmacokinetics were studied by HPLC, antitumour activity by a regrowth delay assay using the KHT murine sarcoma and toxicity by acute LD_{50}. For both antitumour activity and acute toxicity the order of potency was: PAAM > CHL > β-F_2CHL. CHL and PAAM exhibited identical therapeutic indices, whereas that for β-F_2CHL was somewhat improved. CHL is metabolized by mitochondrial β-oxidation to the 3,4-dehydro derivative (DeHCHL) and PAAM, and the latter is further metabolized to its monodechloroethylated derivative DeC-PAAM, presumably by hepatic microsomal enzymes. Administered PAAM gave only one metabolite, DeC-PAAM. Unexpectedly, despite β,β-disubstitution, β-F_2CHL was also β-oxidized to give DeHCHL and PAAM, but at reduced rates. Further, metabolic switching was demonstrated with the appearance in large amount of 2 new, unidentified metabolites, which may be dechlorethylation products. The pharmacokinetics of administered CHL, PAAM and β-F_2CHL differ in that the plasma clearance was fastest for CHL, slowest for PAAM and intermediate for β-F_2CHL. For the metabolites, CHL produced peak plasma concentrations of DeHCHL and PAAM, respectively, 7-fold and 2-fold greater than those produced by β-F_2CHL. However, despite these differences, exposures to total bifunctional nitrogen mustards were similar following administration of the 3 drugs and therefore cannot account for their differential activity. In contrast, there was a good correlation between potency and PAAM exposure, which is highest after treatment with PAAM, intermediate after CHL and lowest after β-F_2CHL. In plasma, 3.2% of PAAM is present as nonprotein-bound free drug, compared to 1.3% for DeHCHL, 0.9% for CHL and 0.45% for β-F_2CHL. We propose the amount of free bifunctional nitrogen mustard, itself partly dependent on the extent of metabolism, to be of major importance for the in vivo potency of CHL analogues.

Introduction

Chlorambucil (4-[4-bis(2-chloroethyl)aminophenyl]butyric acid; CHL) has been used in a number of malignant diseases such as chronic lymphocytic leukemia [24], Hodgkin's [13] and non-Hodgkin's lymphoma [14], ovarian cancer [10] and breast cancer [6]. It has also been used for inflammatory disorders of immune origin, e.g. rheumatoid arthritis [1].

CHL is metabolised extensively and rapidly via β-oxidation of its butyric acid side-chain both in rats [5, 8, 15, 16, 19] and in man [17, 21]. In rats, 3,4 dehydrochlorambucil (DeHCHL) and phenylacetic mustard (PAAM), respectively the intermediate and final products of β-oxidation, have been identified as major metabolic products, together with monodechloroethylated PAAM (DeC-PAAM)

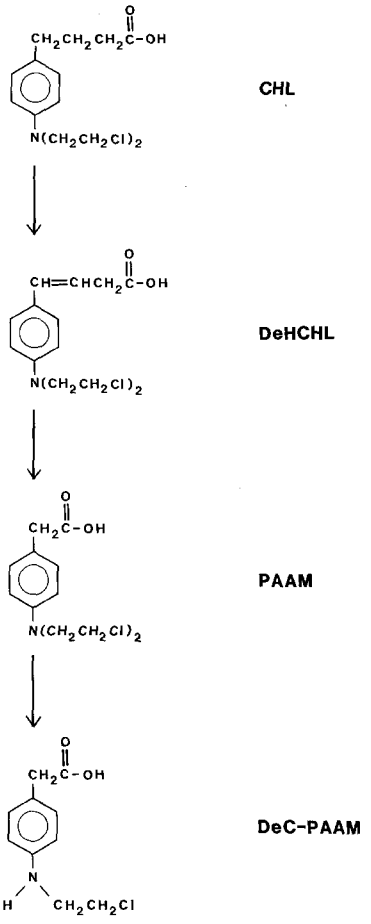

Fig. 1. Structures and metabolism of CHL and its metabolites

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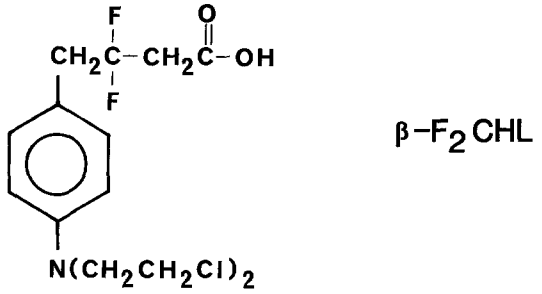

Fig. 2. Structure of the CHL analogue β-F_2CHL

(Fig. 1). In man, only PAAM has so far been identified. Despite its established usage over a long period and relatively well understood metabolic fate, the pharmacokinetics of CHL and its active metabolites are not well documented. Early studies with radiolabelled CHL provided data that are difficult to interpret since the parent CHL was not separated from its active and inactive metabolites or breakdown products. However, recent improvements of HPLC technology have made quantitation of individual species readily possible. Even so, detailed reports of the pharmacokinetics of CHL are still sparse, particularly in experimental animals. For example, there have only been two reported studies in rats [5, 22] and none in mice, the species in which antitumour testing is usually conducted. Further, no information is available on the pharmacokinetics of DeHCHL in any species, although it is known to be one of the major metabolites in the rat [16]. In this paper we report the first description of the pharmacokinetics of CHL and its metabolites, including DeHCHL, in the mouse.

A most interesting feature of the pharmacology of CHL is the possibility of improving its therapeutic index by controlling the supposedly adverse metabolism to PAAM, an approach known as 'metabolic switching' [5]. The idea was first conceived following the observation that PAAM has antitumour potency similar to the parental mustard, but has a therapeutic index two-fold lower because of greater normal tissue toxicity [16]. It was suggested that greater selectivity might be obtained if β-oxidation of CHL is retarded or blocked. However, Farmer et al. [5] found that deuterium substitution at the β-carbon position of the butyric acid side-chain of CHL resulted in only a small reduction of the rate of β-oxidation, which had no effect on the therapeutic index. For this reason, β,β-difluorochlorambucil (β-F_2CHL, Fig. 2) was synthesized [3] in the hope that the very much stronger chemical bond between the fluorine and the carbon atoms would block β-oxidation more effectively. We now describe the comparative pharmacokinetics and metabolism of injected CHL, PAAM and β-F_2CHL in mice, together with data on their hydrolysis rates and plasma protein binding, all obtained using HPLC analysis. We also report on the antitumour activity and toxicity of these compounds, and provide some explanation of these based on differences in pharmacokinetic behaviour. Finally, this paper also forms the basis for the following article on the effects of the chemosensitizer misonidazole and other chemical modifiers on the pharmacokinetics and activity of these agents [11].

Materials and methods

Mice and tumours. Inbred male C3H/He mice were bred from our own colony or obtained from Olac (Bicester, UK) and were used at 25–35 g throughout these studies. The KHT fibrosarcoma was grown in the gastrocnemius muscle of the hind leg as described by Twentyman et al. [27]. Mice were treated when tumours were between 200 and 400 mg. The time taken by individual tumours to reach 4 times their initial treatment volume was determined and growth delay was calculated as the geometric mean of individual values in a group. Each group contained 6–8 mice.

Acute toxicity tests. Groups of three mice were treated with various doses of the different mustards, and the dose of each required to cause 50% lethality at 7 days ($LD_{50/7}$) was determined by computerized probit analysis using the Generalized Linear Interactive Modelling Programme (GLIM) of the Royal Statistical Society of London.

Drugs and drug administration. CHL was kindly provided by Dr D. E. V. Wilman of the Institute of Cancer Research, Sutton, UK and by the Wellcome Foundation, and was also purchased from Sigma. The following were also provided as generous gifts: PAAM (2-[4-bis(2-chloroethyl)aminophenyl]acetic acid) from Dr D. E. V. Wilman and DeC-PAAM (2-[4-(2-chloroethyl)aminophenyl]acetic acid) from Dr A. McLean of the Department of Pharmacy, University of Texas, Austin, USA. β-F_2CHL (4-[4-bis(2-chloroethyl)aminophenyl]-3,3-difluorobutyric acid) was synthesized in the Department of Chemistry, University of Birmingham, UK; details will be given elsewhere [3]. CHL, PAAM and β-F_2CHL were dissolved in acidified ethanol (4.8 ml conc HCl and 95% v/v ethyl alcohol in a volume of 100 ml) and diluted 1:9 with propylene glycol/K_2HPO_4 buffer (20 g K_2HPO_4 plus 450 ml propylene glycol in a final volume of 1 l), final pH 7.4. This was injected immediately by the i. p. route in a volume of 0.01 ml/g body weight. Control animals received the appropriate vehicle.

Sample preparation. Blood was collected under diethyl ether anaesthesia by heart puncture into heparinized syringes and was immediately cooled on ice. This was then centrifuged for 10 min in a refrigerated Du Pont Sorvall RC-5B Superspeed Centrifuge at 4000 *g* at a temperature of 4 °C. Aliquots (200 µl) of plasma from individual mice were deproteinized with 2 volumes of methanol (HPLC grade, Rathburns, Walkerburn, UK) and cooled on dry ice. The protein precipitate was then removed by centrifugation for 10 min at 4000 *g* at a temperature of −20 °C. The clear supernatant was then removed and stored at −20 °C prior to analysis within a week.

High-performance liquid chromatography. This was carried out using modular equipment from Waters Assoc. (Milford, Mass, USA). CHL and its analogues were separated by paired-ion HPLC on Waters Radial-PAK reversed-phase bonded octadecylsilane (C18, 8 mm I. D.) cartridge columns containing spherical particles of 5 µm or 10 µm diameter, with and without C18 guard columns. Two elution methods were used. In the standard isocratic elution method the mobile phase consisted of 65% methanol in phosphate buffer, pH 4, containing 5 m*M* *tetra*-butylammonium hydroxide [TBAH (Fisons, Loughborough, UK)]. The gradient elution method, used to identify the more hydrophilic metabolites and hydrolysis products of CHL, was based on that described by Newell et al. [21]. The line-

ar gradient was from 35%–80% methanol in buffer containing 5 m*M* TBAH, pH 7.4, over a period of 9 min commencing at the time of injection. In some experiments, absorption of the effluent was monitored at 254 nm; in others both 254 nm and 280 nm UV-detection were used. Quantitation of all compounds, except DeHCHL ((E)-4-[4-bis(2-chloroethyl)aminophenyl]3-butenoic acid), was by peak height with reference to linear standard curves prepared from authentic standards. DeHCHL concentration was calculated by reference to the relative extinction coefficients of CHL and the methyl ester of DeHCHL ((E)-methyl-4[4-bis(chloroethyl)aminophenyl] 3-butenoate), data on the latter being supplied by Dr D. Newell, Institute of Cancer Research, Sutton.

Plasma protein binding. Using the procedures described earlier, plasma was obtained from mice at various times after treatment with CHL, PAAM or β-F_2CHL, all at a dose of 15 mg/kg. Plasma from groups of four mice was pooled. Quadruple 200 µl aliquots of the pooled plasma were then subjected to ultrafiltration at 2000 *g* across YMB or YMT membranes in an MSE Chilspin Centrifuge using the Amicon Micro-partition System at a temperature of 5 °C. Aliquots (35 µl) of the plasma water were analysed by injecting directly onto the column for comparison with total plasma drug concentrations (bound plus free) estimated as described above. Unless stated otherwise, plasma concentrations represent the total amount present (i.e. bound plus free).

Spontaneous hydrolysis. CHL, PAAM and β-F_2CHL were incubated at 37 °C at a concentration of 10 µg/ml in 0.1 *M* sodium phosphate buffer, pH 7.4. Triplicate 150 µl aliquots were removed at various times and mixed with an equal volume of ice-cold methanol. Aliquots of the mixture were then stored at −20 °C and analysed within 2 days.

Pharmacokinetic parameters. In regions where exponential decays were judged to operate, best-fit lines were obtained by least-squares linear regression analysis, yielding half-lives with 95% confidence limits. Area under the concentration-time curve (AUC) from time 0 to the final time *t* was estimated by Simpson's rule, the remaining AUC from *t* to infinity (t-∞) being given Ct/k, where *k* is the elimination rate constant and Ct is the concentration at *t*. Values of $AUC_{0\text{-}\infty}$ are the sums of $AUC_{0\text{-}t}$ and $AUC_{t\text{-}\infty}$.

Statistical analysis. Degrees of significance were analysed by Student's *t*-distribution.

Results

Tumour response and normal tissue toxicity

Figure 3 shows the response of the KHT sarcoma to CHL, PAAM and β-F_2CHL and Figure 4 shows the results of the acute toxicity tests, all deaths occurring within 24 h. The results are summarized quantitatively in Table 1 in terms of the ED_{15d} (dose required to produced 15 days tumour growth delay), the LD_{50} (dose required to cause 50% lethality) and the therapeutic index (TI) calculated from their ratio. Comparing a range of isoeffect doses, PAAM showed consistently 1.8–1.9 times greater antitumour potency than

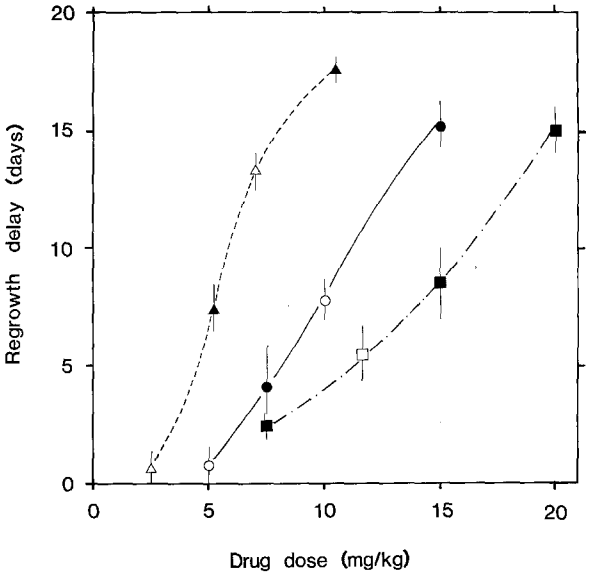

Fig. 3. Dose-response relationship of the KHT tumour to CHL (●, ○), PAAM (▲, △) and β-F_2CHL (■, □). Each *datum point* represents the geometric mean of 6–8 mice. *Error bars* show ±SE. Different symbols represent independent experiments

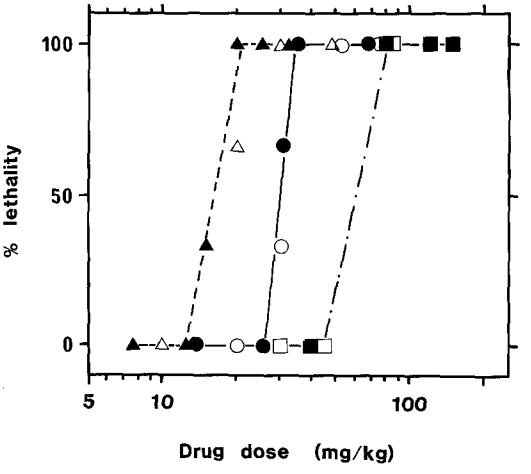

Fig. 4. The acute lethality ($LD_{50/7}$) of CHL (●, ○), PAAM (▲, △) and β-F_2CHL (■, □). Each *datum point* represents a group of three mice. *Closed* and *open symbols* represent independent experiments

Table 1. Antitumour activity, toxicity and therapeutic indices of PAAM, CHL and β-F_2CHL in mice

	LD_{50}[a] (mg/kg)	$ED_{15\ days}$[a] (mg/kg)	$LD_{50}/ED_{15\ days}$[a] (TI)
PAAM	15.9	8.0	2.0
CHL	30.0	14.6	2.1
β-F_2CHL	60.2	20.0	3.0

[a] LD_{50} and $ED_{15\ days}$ are the doses required to give 50% lethality and 15 days regrowth delay respectively, while TI is the therapeutic index given by their ratio. Values were calculated using the combined data from two independent experiments (Figs. 3, 4)

CHL, but the LD_{50} was likewise 1.9 times lower and the TI was therefore identical to that for the parent compound. β-F_2CHL exhibited 1.3–1.5 times less antitumour potency compared to CHL but was only half as toxic, and the TI was therefore increased by 50%.

HPLC of CHL, β,β-difluorochlorambucil and their metabolites

It was previously shown in the rat and in man that the butyric acid side-chain of CHL is metabolized rapidly by β-oxidation to form the major metabolites DeHCHL and PAAM, which could be detected in the plasma [5, 15, 16, 19]. In addition, DeC-PAAM, probably (but not necessarily solely) formed by microsomal dechloroethylation of PAAM, has also been identified as the major metabolite in rat urine but has not been found in the plasma [16].

Typical chromatograms obtained in the present study are shown in Figure 5 (gradient method) and Fig. 6 (isocratic method). It is clear from the chromatograms of the plasma extract of mice treated with CHL that at least three metabolites were also present in the plasma (Figs. 5B, 6A). Peaks I and II are, respectively, DeC-PAAM and PAAM, identified by co-chromatography with synthetic standards. Peak III has the same UV-absorption characteristics ($A_{254\,nm}/A_{280\,nm}$) as DeHCHL (compare Fig. 6A, upper and lower panels). Furthermore, the chromatographic behaviour of peak III is closely similar to that observed by Farmer et al. [5], which also had identical UV-absorption characteristics and which was unambiguously confirmed as DeHCHL by GC-MS. We have therefore assigned peak III as DeHCHL. Peak IV is the parent CHL.

Plasma extracts of mice treated with β-F_2CHL were also analysed using HPLC. At least five extra peaks were seen besides that of the parent drug (peak V; Figs. 5C, 6B). Peaks I, II and III have identical retention times to DeC-PAAM, PAAM and DeHCHL respectively. Note that these three metabolites were found at much lower concentrations when compared with those for CHL treated mice. In fact, the presence of DeHCHL can only be detected at 280 nm (Fig. 6B, upper panel). Additional evidence in support of the finding that β-F_2CHL is metabolized to these three species was afforded by mixing experiments of the type illustrated in Figure 6D. Plasma extracts from mice given β-F_2CHL were mixed with those from mice receiving CHL. Peaks for metabolites I–III remained as sharp as in unmixed samples, with no suggestion of peak splitting or broadening. In contrast, CHL and β-F_2CHL could be resolved from each other. It can also be seen from these chromatograms that β-F_2CHL was not metabolized to CHL. The identities of peaks VI and VII are unknown except that they are not the products of spontaneous hydrolysis of β-F_2CHL. Finally, as expected, the chromatogram of the plasma extract of mice treated with

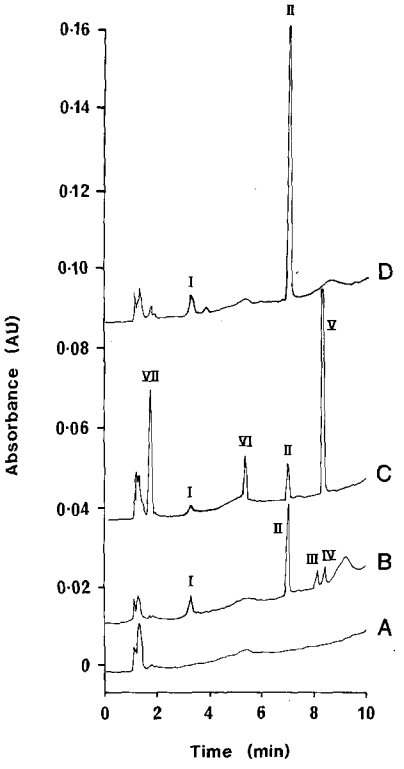

Fig. 5. Gradient HPLC chromatograms of methanol extracts of plasma obtained from mice 40 min after receiving *(A)* vehicle, *(B)* CHL, *(C)* β-F_2CHL, *(D)* PAAM, all at 15 mg/kg. *Peak I:* DeC-PAAM; *peak II:* PAAM; *peak III:* DeHCHL; *peak IV:* CHL; *peak V:* βF_2CHL; *peaks VI and VII:* unidentified metabolites

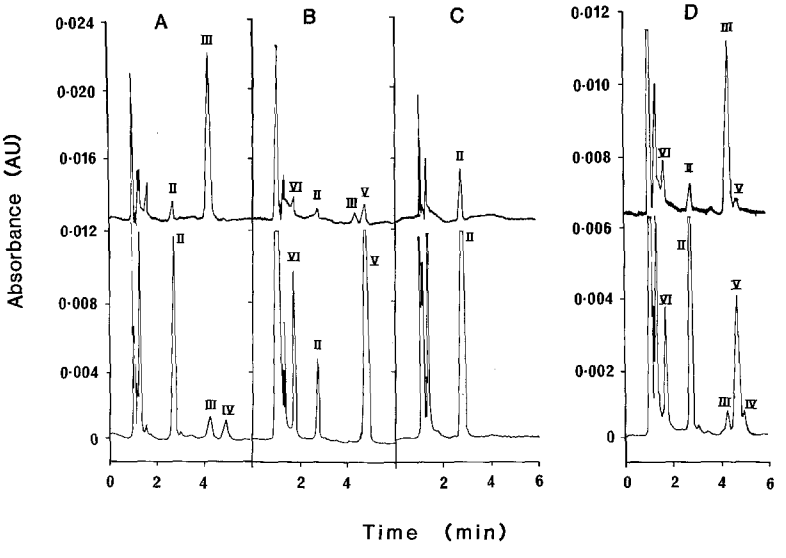

Fig. 6A–D. Isocratic HPLC chromatograms of methanol extracts of plasma obtained from mice 40 min after receiving **(A)** CHL, **(B)** β-F_2CHL, **(C)** PAAM, all at 15 mg/kg. Chromatogram **D** is for a 1 : 1 mixture of extracts **A** and **C**. *Upper section:* detection at 280 nm UV. *Lower section:* detection at 254 nm UV. *Peak II:* PAAM; *peak III:* DeHCHL; *peak IV:* CHL; *peak V:* β-F_2CHL; *peak VI:* an unidentified metabolites of β-F_2CHL

Table 2. Pharmacokinetic parameters of PAAM, CHL and β-F_2CHL in mice (15 mg/kg i.p.). Values are calculated from data of a typical experiment (Fig. 7)

Injected drug	PAAM		DeHCHL		CHL		β-F_2CHL		Total mustards	
	$t_{1/2}$ (min)	$AUC_{0-\infty}$ (m*M* min)	$t_{1/2}$ (min)	$AUC_{0-\infty}$ (m*M* min)	$t_{1/2}$ (min)	$AUC_{0-\infty}$ (m*M* min)	$t_{1/2}$ (min)	$AUC_{0-\infty}$ (m*M* min)	$t_{1/2}$ (min)	$AUC_{0-\infty}$ (m*M* min)
PAAM	65.8	20.4	–	–	–	–	–	–	65.8	20.4
CHL	74.7	8.66	62.2	5.25	13.8	4.2	–	–	63.1	18.0
β-F_2CHL	59.8	4.2	109	0.35	–	–	37.2	16.0	54.3	20.6

PAAM shows the presence of only one metabolite, DeC-PAAM (peak I; Figs. 5D, 6C).

Pharmacokinetics of CHL, β-F_2-CHL and PAAM

The ability of the HPLC technique to quantitate the various parent mustards individually as well as their metabolites allows detailed quantitative studies of the pharmacokinetics and metabolism of these agents. Figure 7 shows the comparative mouse pharmacokinetics of CHL, β-F_2CHL and PAAM, all given at a dose of 15 mg/kg (or 49, 44 and 54 μmol/kg, respectively), and Table 2 summarizes their pharmacokinetic parameters, viz. plasma clearance half-life ($t_{½}$) and area under the concentration-time curve (AUC). All three drugs were absorbed rapidly after IP administration. It is worth noting that the plasma peak concentrations of the injected parent compounds and that of total, known, bifunctional mustards are at least 3–4 times higher than would be expected if distributed evenly in the body volume. This is reflected in the low apparent volume of distribution ($V_{d\ ext}$) found for these compounds, the values being 0.253, 0.234 and 0.147 l/kg for PAAM, CHL and β-F_2CHL respectively. This observation is consistent with the results of a number of reports [7, 19, 22], which showed that the levels of CHL and PAAM were lower in a range of rat tissues than in plasma. These findings suggest that CHL, β-F_2CHL and PAAM are subject to retention in the plasma.

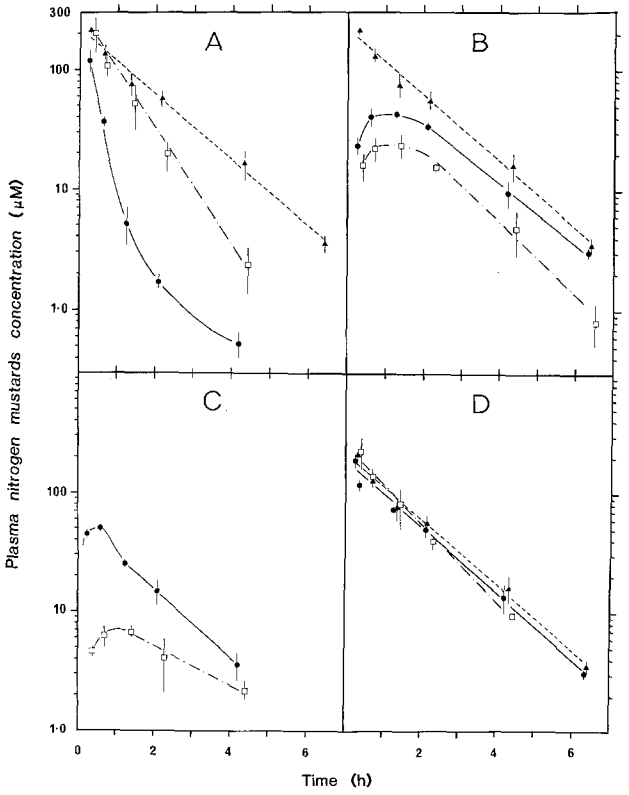

Fig. 7 A–D. Plasma pharmacokinetics after injection of 7.5 mg/kg CHL (● – ●), β-F_2CHL (□ – □) and PAAM (▲ – ▲). **(A)** Injected parent compounds; **(B)** PAAM, injected or metabolite; **(C)** the metabolite DeHCHL and **(D)** total nitrogen mustards. Data are from a typical experiment. Each *datum point* represents the average of three mice. *Error bars* show ±SD

In terms of the behaviour of the injected parent drugs (Fig. 7A), the most striking differences between CHL, PAAM and DeHCHL are in their elimination kinetics. It can be seen from Fig. 7 that drug elimination was usually exponential. However, with CHL there was a tendency, more marked in some experiments (e.g. Fig. 7, [29]) than others, for the clearance profiles to be biexponential. The relative clearance half-lives, calculated assuming monoexponential decay, were in the order: CHL (13.8 min) > β-F_2-CHL (37.2 min) > PAAM (65.8 min). The AUC values, more useful where the precise nature of the CHL elimination kinetics are in doubt, were 4.2 m*M* min for CHL, 16 m*M* min for β-F_2CHL and 20 m*M* min for PAAM. This suggests quantitative differences in the handling of these drugs by the body. That this is indeed the case can be seen when the pharmacokinetics of their metabolites are considered. Figure 7B shows the pharmacokinetics of PAAM, the end product of β-oxidation. As expected, the highest concentration was found when PAAM was injected directly, the peak concentration being approximately 200 μ*M*. With CHL treatment, the peak concentration of PAAM was 4–5 times lower (approx. 42 μ*M*). A further twofold decrease in PAAM peak concentration was seen with β-F_2CHL, the value being approximately 23 μ*M*. The clearance half-lives of PAAM for the three cases were, however, very similar (Table 2). Figure 7C illustrates the pharmacokinetics of DeHCHL following treatment with CHL and β-F_2CHL. The peak DeHCHL concentration was 7 times greater after CHL than β-F_2-CHL. Note that no DeHCHL was formed from PAAM. Finally, Figure 7D shows the pharmacokinetics of total nitrogen mustards (parent drug plus the above metabolites) for all three compounds. It can be seen that despite the disparities in the metabolism of CHL, PAAM and β-F_2CHL, giving rise to different concentrations of various metabolites, the total bifunctional nitrogen mustard concentrations were very similar for all three compounds. Quantitative studies of

Table 3. Plasma protein binding of CHL, DeHCHL, PAAM and β-F_2CHL. Plasma samples were obtained from mice treated with 15 mg/kg. Data are from a typical experiment

	Total drug plasma conc. (μ*M*)	Plasma free drug conc. (μ*M*)	% free drug
β-F_2CHL	51.8 (1.11)[a]	0.2377 (0.0058)	0.46 (0.02)
CHL	52.83[c] (1.38)	0.5006 (0.0078)	0.95 (0.04)
	23.19[c] (1.82)	0.2210 (0.0112)	0.95 (0.12)
DeHCHL	29.65[b] (0.85)	0.3605 (0.0079)	1.22 (0.06)
	27.16[b] (0.95)	0.3451 (0.0290)	1.27 (0.15)
PAAM	50.43[c] (0.71)	1.593 (0.0430)	3.16 (0.13)
	14.77[b] (0.71)	0.4510 (0.030)	3.05 (0.28)
	9.15[b] (0.33)	0.2851 (0.0045)	3.12 (0.16)

[a] 1 SD in parenthesis
[b] Values are for metabolites
[c] Values are for injected drugs

plasma DeC-PAAM were carried out with CHL. The detailed results are given in the following paper [11]. Plasma concentration rose slowly to a peak of about 20 μ*M* 1–2 h after injection.

Plasma protein binding

Since CHL and similar compounds are acidic drugs, ionized at plasma pH, it is likely that they will bind to plasma albumin, possibly curtailing the amount of active free drug available to target tissue. We have therefore evaluated the relative plasma protein binding ability of the various mustards. The results of these investigations are shown in Table 3. All the nitrogen mustards studied are highly bound to plasma protein. This binding was reversible since all the drug was recovered from plasma by alcohol precipation. The extent of protein binding is in the order: β-F_2CHL > CHL > DeHCHL > PAAM. The differences in the percent plasma-free drug concentration between the four mustards are all significant ($P<0.01$). Thus, at equal total plasma drug concentrations, the amount of free PAAM is 2.5 times that of free DeHCHL, 3 times that of free CHL and 6.0 times that of free β-F_2CHL.

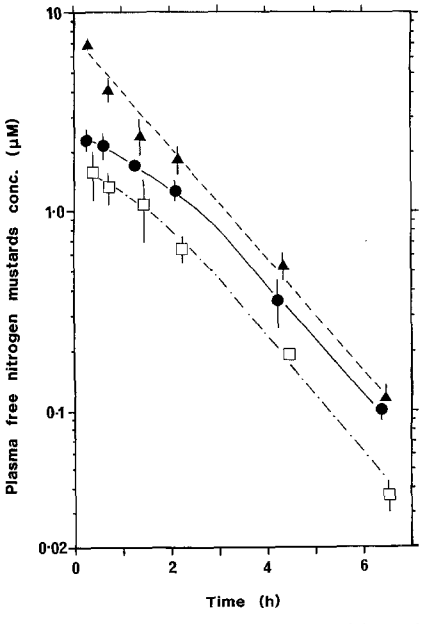

Fig. 8. Plasma pharmacokinetics of free nitrogen mustards in mice following treatment with 7.5 mg/kg CHL (●–●), β-F_2CHL (□–□) and PAAM (▲–▲). Data are from a typical experiment. Each *datum point* is for three mice. *Error bars* show ±SD

Pharmacokinetics of free mustards in plasma

Figure 8 shows the plasma pharmacokinetics of total free bifunctional nitrogen mustards following the administration of CHL, PAAM and β-F_2CHL. It can be seen that the relative exposures to active free mustards are in the order PAAM > CHL > β-F_2CHL, the AUCs being respectively 658, 353 and 223 μ*M* min.

Spontaneous hydrolysis

CHL, PAAM and β-F_2CHL undergo spontaneous hydrolysis in phosphate buffer to form at least four breakdown products of unknown identity. Decay kinetics were monoexponential. The hydrolysis rates were similar for PAAM ($t_{½}$ 16.9 min) and CHL ($t_{½}$ 14.1 min) whereas β-F_2CHL hydrolysis was slower ($t_{½}$ 36.5 min). None of the breakdown products were seen in the plasma.

Discussion

We have shown that in mice CHL is converted by β-oxidation, presumably in the mitochondria, to form PAAM via the intermediate DeHCHL. The same metabolic pathway has been found in the rat [15, 16, 19] and PAAM has also been shown to be the major metabolite in man [17, 21]. Previous work in the rat has shown the dehydro metabolite to be predominantly in the 3,4-dehydro form, although evidence was found for another unidentified isomer, presumed to be an initially formed 2,3-dehydro derivative [16]. As noted in the study by Farmer et al. [5], the unavailability of synthetic material for this and other isomers means that their presence cannot be excluded. Our identification of a substantial quantity of DeC-PAAM in the plasma is consistent with the initial observation of McLean and co-workers [17] that DeC-PAAM is the major 'post-β-oxidation' metabolite. This conclusion is further supported by the finding that DeC-PAAM was the only metabolite detected following PAAM treatment. It is, of course, possible that some DeC-PAAM could also be formed by monodechlorethylation of CHL and/or DeHCHL, subsequently followed by β-oxidation, and further evidence for this is given in the following paper [11]. It should also be noted that Mitoma et al. [19] found evidence of ten urinary metabolites and degradation products in the rat, most of which had undergone oxidation of the butyric acid side-chain to phenylacetic and benzoic acid derivatives, and many had undergone metabolism on the chloroethyl moieties.

The pharmacokinetics of CHL have been reported in the rat [22] and in man [21], but not in mice, despite the fact that the latter species is normally used for drug testing and analogue development. Further, there are no data on the pharmacokinetics of DeHCHL in any species, although it has been shown to be a major metabolite in rat [15, 16]. We showed here that after the administration of CHL, DeHCHL is found in similar concentrations to PAAM and the parent compound in mouse plasma. Also,

as far as we are aware, the pharmacokinetic behaviour of administered PAAM has not been investigated previously in any species. An interesting observation, predicted from its behaviour as a metabolite, is that the clearance of injected PAAM was much slower than that of CHL, suggesting that the liver enzymatic dechlorethylation of PAAM is a much slower process than the β-oxidation of CHL. Rather unexpectedly, we found both DeHCHL and PAAM to be present in the plasma of mice administered β-F_2CHL, indicating the ability of this analogue to undergo β-oxidation. The mechanism of this is unknown, but initial metabolism to CHL was ruled out. It is clear therefore that β,β-disubstitution does not block β-oxidation completely. Nevertheless β-oxidation is slowed down with peak plasma concentrations of DeHCHL and PAAM reduced sevenfold and twofold, respectively, compared to those after CHL. The greater reduction in DeHCHL concentration suggests that the initial α,β-dehydrogenase reaction is the most strongly affected. In addition, the appearance of new, unidentified metabolites in apparently large amounts does demonstrate the successful achievement of 'metabolic switching' [5], whereby alternative pathways become prominent when a primary route is inhibited. Evidence will be presented in the following paper that the new metabolites may be dechlorethylation products [11].

Our demonstration that the acute toxicity of PAAM is about twice that of CHL in mice is consistent with previous findings in both mice and rats [16, 7]. Regarding antitumour activity, however, the agreement is less universal. The present results with the solid KHT sarcoma in mice and those of Godeneche et al. [7] with the ascites Moloney sarcoma, also in mice, both show that PAAM is again twofold more active than CHL. In contrast, McLean et al [16] observed that PAAM has similar potency to CHL against the Walker 256 transplanted rat tumour. In addition, Goodman et al. [9] have found similar cytotoxic activity for CHL, PAAM and DeHCHL against human tumour cells in vitro. These discrepancies may be due to the differences in the tumour systems and/or host species used. Whatever the reasons, it is clear that the therapeutic index of PAAM may be similar to or twofold lower than that for CHL.

It is important to consider the pharmacokinetic factors which might be particularly important for CHL toxicity and antitumour activity, and which might also contribute to the differences observed between the various analogues. It is probable that the major component responsible for the effects of chlorambucil is PAAM. Because of its long half-life, this represents about 50% of the total bifunctional nitrogen mustard AUC in the plasma, and it exhibits in vitro antitumour activity very similar to that of CHL [7]. Likewise, DeHCHL has the same in vitro activity to the parent drug [9] and also exhibits identical toxicity and antitumour activity in the rat [17]. Hence DeHCHL will also contribute to the pharmacological properties of administered CHL, since it represents about 30% of the plasma AUC. Owing to its rapid metabolism, the parent drug is responsible for only the remaining 20% of the plasma AUC. Finally, although present in quite large amounts in the plasma, it is probable that DeC-PAAM has comparatively little effect. It shows diminished activity compared to the bifunctional nitrogen mustards in vitro [9] and exhibits considerably reduced toxicity and no antitumour activity in the rat [16].

Turning now to a comparison of administered PAAM, β-F_2CHL and CHL, total plasma bifunctional nitrogen mustard concentrations were virtually identical for the three drugs, and this could not account for the differences in potency described earlier. On the other hand, there does appear to be a correlation between potency and exposure to PAAM. Thus PAAM levels were highest following administration of PAAM itself and lowest after β-F_2CHL, with intermediate levels after CHL. It is not clear, however, why PAAM should be more potent than the other circulating bifunctional nitrogen mustards. Hydrolysis rate studies showed CHL and PAAM to be similarly reactive (although both were more reactive than β-F_2CHL) and, as mentioned earlier, both compounds exhibit similar cytotoxicity in vitro [9].

A possible explanation was revealed by the reversible protein binding results. These showed that total free plasma mustard concentrations were highest after PAAM, lowest after β-F_2CHL and intermediate after CHL. Compared to CHL, exposure was 1.9 times greater for administered PAAM and 1.6 times less for β-F_2-CHL. These differences were due to the fact that the extent of plasma protein binding is in the order: PAAM < DeHCHL < CHL < β-F_2CHL. Extensive reversible binding to plasma protein has been noted previously for CHL [12]. Binding of these acidic drugs most probably involves association with cationic groups on albumin, although binding to immunoglobulin G has also been described [2]. The physiocochemical properties most likely to affect this type of binding are the pK_a of the carboxyl group and the overall lipophilicity of the drug. The pK_a value for CHL is 4.46 [4] and those for the other derivatives will be fairly similar: thus all will be fully ionized at physiological pH. Previous studies have shown that the binding of short-chain fatty acid anions to albumin increases with increasing chain length from acetate to caprylate [26]. Thus, the shorter alkyl chain length probably accounts for the reduced binding of PAAM compared to the other longer-chain-length mustards. Godenche and co-workers [7] have previously suggested that for a small series of nitrogen mustards, including CHL and PAAM, the toxicity was greatest for the most hydrophilic. It is quite possible that lipophilicity was, in fact, providing an indirect measure of protein binding capacity.

A possible hypothesis then is as follows. Other factors being equal, the toxicity of CHL analogues increases as the amount of free drug in the plasma rises and a greater amount of drug becomes available for uptake into tissues. Of course, extensive protein binding per se will not necessarily impair drug distribution: this will only occur if the binding affinity is high [18]. Two types of evidence can be cited in support of this possibility. Firstly, the acidic drugs *p*-chlorophenoxy-acetic acid and *p*-chlorophenoxyisobutyric acid increase the cytotoxicity of CHL in vitro by displacing it from its serum protein binding sites [25]. This clearly shows that protein binding of CHL does restrict cell uptake. It is also interesting to note that the acidic drug indomethacin can in some circumstances increase the antitumour activity of CHL in vivo [23]. The second line of evidence comes from tissue uptake work. Tissue levels were not determined here, but the low apparent volumes of distribution are consistent with restricted uptake due to protein binding. In support of this, Newell and colleagues [22] have shown that, following the administration of ^{3}H-CHL in the rat, tumour and normal tissue levels were con-

siderably lower than those in plasma throughout the time course. Similar results were obtained using ^{14}C-CHL [7, 19].

The pharmacological activity of CHL and its analogues and metabolites is likely to be dependent upon a number of variables. Taken overall, however, we feel that the amount of free bifunctional nitrogen mustards available in the plasma, itself partly dependent on the extent of metabolism to PAAM, is likely to be a very important parameter. It certainly ranks the derivatives correctly in terms of potency. The question of how to explain differences in therapeutic index is more difficult. Thus we appear to be able to explain why administered PAAM is twice as potent as CHL in terms of both toxicity and antitumour activity, and also why βF_2CHL is less potent than CHL, but not why the therapeutic index is greater for β-F_2CHL. Unfortunately, normal tissue damage is usually assessed by acute LD_{50}. Since the animals die within 24 h, lethality may not be due to classical alkylation, but to some other effect such as neurotoxicity, for example. This effect, rather than the myelosuppression common to related mustards, is the one which limits high dose CHL therapy in man (Calvert, personal communication [20, 28]. An additional complication is that we do not know the identity or pharmacological properties of the new metabolites formed after metabolic switching with β-F_2CHL. In the above argument we have made the assumption that they are comparatively inactive, and this may not be correct. We would predict, however, on both drug potency and metabolic grounds, that metabolism would be switched to the dechlorethylation pathway. We plan to characterize these metabolites by mass spectrometry in due course.

To assess the possible clinical significance of the 50% improvement in therapeutic index for β-F_2CHL over that of CHL, we can compare the therapeutic indices we have obtained with other commonly used alkylating agents in the KHT sarcoma (unpublished data). It is noteworthy that the value of 3.0 for β-F_2CHL is also consistently better than those for CCNU (2.1) cyclophosphamide (2.5) and melphalan (< 1.0).

The present results clearly show that the therapeutic index can be increased when the metabolic production of PAAM is sufficiently reduced, which may warrant further effort to block this process completely. In addition to metabolic switching by structural modification, one might also consider the use of metabolic inhibitors, an approach considered in the following paper [11]. The demonstration that β,β-difluro substitution does not completely block β-oxidation may provide useful information as to the precise mechanism of this metabolic process. Finally, the possible influence of protein binding on toxicity and therapeutic index of CHL introduces another important consideration in the design of anticancer agents of this type.

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