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The Pharmacokinetics of Prednimustine and Chlorambucil in the Rat

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Summary. *In the rat prednimustine, the prednisolone ester of chlorambucil, is much less toxic than equimolar doses of chlorambucil, when administered subcutaneously (SC). This is due to differences in alkylating agent pharmacokinetics. Prednimustine injected SC produced low plasma concentrations (< 5 μM) of the alkylating metabolites chlorambucil and phenyl acetic mustard, which were maintained for 48 h. No unhydrolysed prednimustine could be detected. Chlorambucil, in contrast, was rapidly absorbed, peak levels (40 μM) occurring within 2 h, after which chlorambucil and phenyl acetic mustard plasma levels decreased with half-lives of 2.4 h and 2.9 h respectively.*

The toxicity of chlorambucil could be similarly reduced by administering either the methyl ester of chlorambucil or by giving chlorambucil in a multiple-treatment low-dose schedule. Neither of these treatments inhibited the Yoshida alkylating agent-resistant tumour, however, whereas prednimustine or a combination of chlorambucil and prednisolone produced significant tumour growth inhibition. Prednisolone did not alter chlorambucil pharmacokinetics. Thus the reduced toxicity of prednimustine is due to chlorambucil esterification and the subsequent alteration in pharmacokinetics, whilst inhibition of alkylating agent-resistant tumours results from the combination of chlorambucil and prednisolone.

Introduction

Chlorambucil [4(4-bis(2-chloroethyl)aminophenyl) butyric acid] is a bifunctional alkylating agent used widely in the treatment of human cancers [10, 17]. Prednimustine [pregna-1,4-diene-11β,17α,21-triol-3,20-dione,21 (4(4-bis(2-chloroethyl)aminophenyl)-butyric acid)], the prednisolone ester of chlorambucil, was synthesised by A. B. Leo (Helsingborg, Sweden) [9], in an attempt to improve the antitumour selectivity of chlorambucil by facilitating uptake into tumours possessing high concentrations of glucocorticoid receptors. In practice, prednimustine has proved effective in the management of a number of leukaemias and lymphomas [1].

We have previously shown [5] that prednimustine is much less toxic than chlorambucil when given SC to rats. Although the concomitant administration of prednisolone does significantly reduce chlorambucil-induced mortality, the combination of chlorambucil and prednisolone is consistently more toxic than the ester prednimustine [5]. This latter observation has been confirmed in an independent study [3]. In an attempt to explain the differential toxicities of chlorambucil and prednimustine further, their pharmacokinetics and tissue distributions have been investigated following SC administration to the rat.

Metabolism and distribution of chlorambucil in the rat have been investigated extensively. Chlorambucil is metabolised by β-oxidation of the butyric side chain [4, 11, 13], the products of β-oxidation, phenyl acetic mustard [2(4-bis(2-chloroethyl)aminophenyl)acetic acid] and an intermediate, 3,4-dehydrochlorambucil, constituting the major metabolites in rat blood 1 h after administration [11]. Radiochemical studies have shown that chlorambucil is excreted mainly by the kidneys, with 40%–60% of the administered dose being present in the urine at 24 h [7, 12, 13]. These studies also demonstrate that active uptake of chlorambucil into specific tissues does not occur, so that plasma concentrations generally exceed tissue levels [7, 13].

The metabolism of prednimustine in the rat has not previously been reported. However, in vitro

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studies from this laboratory have shown that the ester linkage is rapidly hydrolysed by esterases present in a number of tissues, including rat plasma [19]. The hydrolysis of prednimustine releases equimolar quantities of chlorambucil and prednisolone, whereupon the chlorambucil moiety is available for β-oxidation.

In the present study, the comparative pharmacokinetics and tissue distributions of chlorambucil and prednimustine have been investigated. This work has been facilitated by a recently developed high-performance liquid chromatography (HPLC) technique, which allows the simultaneous estimation of prednimustine, chlorambucil and phenyl acetic mustard in biological fluids [15]. These pharmacokinetic studies have allowed more accurate definition of the factors responsible for the toxicological and antitumour properties of prednimustine.

Materials and Methods

Chemicals. Prednimustine, both radioactive and non-radioactive, was a gift from A. B. Leo (Helsingborg, Sweden). $^{14}C/^{3}H$-prednimustine was universally ^{14}C-labelled in the chloroethyl side chains and ^{3}H-labelled in the 6 and 7 positions of the prednisolone molecule. Chlorambucil was a gift from the Wellcome Foundation (Beckenham, Kent, England). Phenyl acetic mustard was synthesised by Prof. W. C. J. Ross [2], and the methyl ester of chlorambucil by Dr M. Jarman, both at the Institute of Cancer Research (London, England). ^{3}H-Chlorambucil, ^{3}H-labelled in the ring ortho to the butyric side chain, was also prepared by Dr M. Jarman [8]. Prednisolone (11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) was obtained from the Sigma Chemical Company Ltd (Poole, Dorset, England). All other chemicals were supplied by BDH Chemicals Ltd (Poole, Dorset, England), Fisons Scientific Ltd (Loughborough, England); or Hopkin and Williams Ltd (Romford, Essex, England) and were of analytical grade.

Animal Studies. Female Wistar rats (150–200 g) were used throughout the investigation. Toxicity studies were performed on non-tumour-bearing rats as previously described [5]. Metabolic studies were performed on rats bearing the sensitive strain of the Walker 256 carcinosarcoma grown as an ascites. Antitumour studies were carried out in rats bearing the alkylating agent-resistant strain of the Yoshida sarcoma, as previously described [5].

For metabolic studies, tumour-bearing rats received either 10 mg ^{3}H-chlorambucil/kg (25.4 mCi/mmole) or 20 mg $^{14}C/^{3}H$ prednimustine/kg (^{14}C: 3.01 mCi/mmole; ^{3}H: 9.12 mCi/mmole). In a separate experiment to determine the effect of subsequent prednisolone administration on chlorambucil pharmacokinetics, animals received ^{3}H-chlorambucil 40 mg/kg (4.11 mCi/mmole) followed by prednisolone (40 mg/kg) 4 h later. All drugs were dissolved in dimethyl sulphoxide (DMSO) and administered SC. Animals were anaesthetised with diethyl ether at various times after drug administration and exsanguinated by direct cardiac puncture. Blood was placed in heparinised tubes (10 iu/ml) and plasma prepared by centrifuging at 600 *g* for 10 min at 4° C. Plasma was removed and stored at −20° C until analysed. In tissue distribution studies, organs were removed immediately after exsanguination, weighed, placed in liquid nitrogen, and stored at −20° C prior to analysis. Bone marrow (aspirated from femurs) and tumour cells were counted on a Model ZF Coulter Counter (Coulter Electronics Ltd., Harpenden, Herts, England), and washed once in reticulocyte standard buffer (0.01 *M* Tris-HCl pH 7.4, 0.01 *M* NaCl, 0.0015 *M* $MgCl_2$) prior to storage at −20° C.

Sample Analysis. Plasma levels of chlorambucil, phenyl acetic mustard and prednimustine were analysed by HPLC as previously described [15]. Samples (1 ml) of tissue homogenates (50% w/v) were extracted and analysed in a similar manner following homogenisation in ice-cold reticulocyte standard buffer in a Teflon/glass homogeniser. Compounds present in the effluent of the HPLC were quantitated by scintillation counting.

Scintillation Counting. Samples (0.1 ml) of plasma or tissue homogenates were counted following solubilisation in NCS solubiliser [14]. Fractions (1 ml) of HPLC effluent were collected directly into scintillation vials, 10 ml PCS scintillant (Hopkin and Williams, Romford, Essex, England) was added, and radioactivity was counted on a Model SL 30 Intertechnique Liquid Scintillation Spectrometer (Kontron Intertechnique, St. Albans, Herts., England). Counting efficiencies were determined by using ^{3}H- and ^{14}C-hexadecane (The Radiochemical Centre, Amersham, Bucks, England). In the calculation of molar quantities of prednimustine-derived compounds the ^{14}C radioactivity was employed.

Pharmacokinetic Analysis. Where possible, plasma levels of chlorambucil and phenyl acetic mustard were fitted to a mathematical function, a non-linear least-squares analysis being used [16]. Following chlorambucil administration, levels of parent drug and phenyl acetic mustard were fitted to the exponential function:

$$C = Ae^{-\beta t}$$

where C is the drug plasma concentration, t is the time after dosing, A is a concentration constant and β the first-order disposition rate constant. The plasma terminal phase half-life, $t_{1/2}\beta$, was calculated in the form:

$$t_{1/2}\beta = \frac{0.693}{\beta}.$$

After chlorambucil administration data were analysed following the initial absorption/distribution phase, i.e., from 2 h onwards.

Following prednimustine administration chlorambucil plasma levels were fitted to the expression:

$$C = A\left(\frac{\alpha}{\alpha-\beta}\right)(e^{-\beta t} - e^{-\alpha t})$$

as described by Wagner [18], where in addition to the previously defined terms, α is the first-order absorption rate constant. Phenyl acetic mustard levels following prednimustine administration were plotted manually, the mean value being used for each time point.

The areas under the plasma concentration vs time curves were determined by the trapezoidal rule [18]. The extrapolated volume of distribution (V_D) was calculated as:

$$V_D = \frac{\text{Dose (}\mu\text{moles/kg)}}{A}$$

where, as previously, defined, A is the time zero concentration constant.

Table 1. Total drug-derived materials in rat tissues following SC administration of ^{3}H-chlorambucil (10 mg/kg)

Tissue	Time (h)					
	0.5	1.0	2.0	4.0	8.0	16.0
Plasma[a]	71.4	99.4	69.8	53.4	37.6	24.1
Liver[b]	27.9	27.1	20.9	15.7	12.5	5.7
Fat[b]	1.5	3.0	2.6	2.9	1.7	1.8
Small intestine[b]	26.8	26.2	114.5	90.8	21.1	3.7
Lung[b]	10.0	11.7	7.7	8.7	5.0	3.6
Bone marrow[c]	ND	8.1	4.5	4.9	6.6	ND
Tumour[c]	8.4	14.2	15.5	13.0	10.5	11.9

Each point represents the mean of duplicate estimations in two animals
ND, not detectable
[a] nmoles/ml
[b] nmoles/g wet weight
[c] nmoles/10^{9} cells

Table 2. Total drug-derived materials in rat tissues following SC administration of ^{14}C/^{3}H-prednimustine (20 mg/kg)

Tissue	Time (h)						
	0.5	1.0	2.0	4.0	8.0	16.0	24.0
Plasma[a]	0.77	1.28	3.51	6.40	11.55	12.65	12.89
Liver[b]	0.46	1.19	1.67	3.72	4.85	5.11	4.04
Fat[b]	ND	ND	ND	0.34	0.52	0.97	0.87
Small intestine[b]	ND	5.16	1.58	22.64	12.44	3.57	3.39
Lung[b]	ND	ND	0.75	1.44	2.46	2.92	2.23

Each point represents the mean of duplicate estimations in two animals
ND, not detectable
[a] nmoles/ml
[b] nmoles/g wet weight

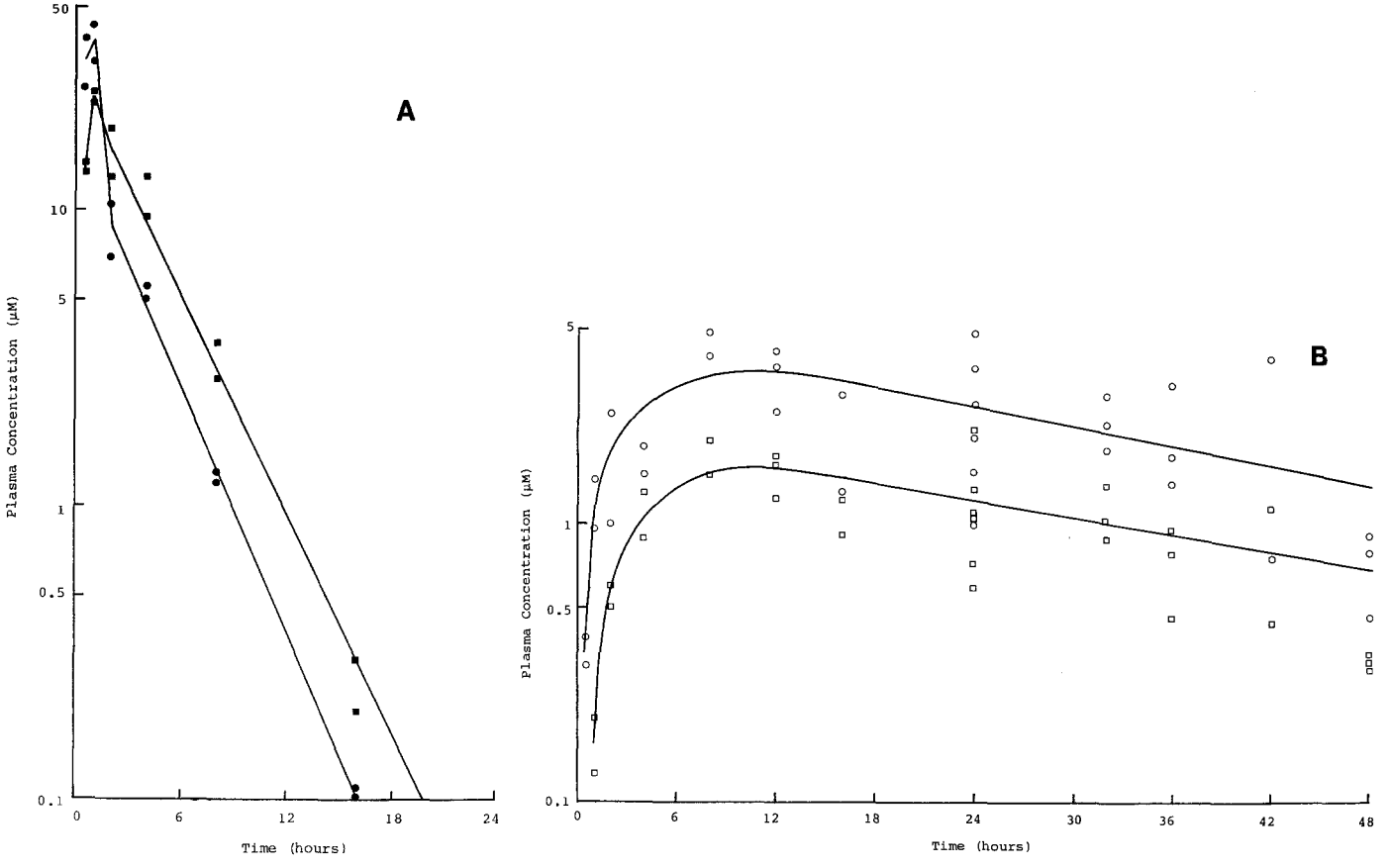

Fig. 1A and B. The pharmacokinetics of chlorambucil (———) and phenyl acetic mustard (———) in the rat following the SC administration of 10 mg ^{3}H-chlorambucil/kg (*closed symbols*) (**A**) and 20 mg ^{3}H/^{14}C-prednimustine/kg (*open symbols*) (**B**). *Lines* were fitted to *points* as described in the *Methods* section under *Pharmacokinetic Analyses*. Each *point* represents a determination made on a single animal

Results

Pharmacokinetic and Tissue Distribution Studies

Following chlorambucil administration (10 mg/kg) the levels of total drug-derived materials (total radioactivity) were consistently lower in all the tissues studied, with the exception of the small intestine, than in the plasma (Table 1): in the intestine, levels exceeded those in the plasma at 2 and 4 h. HPLC analysis of the free chlorambucil and phenyl acetic mustard levels in all the tissues examined also revealed lower concentrations than in the plasma.

Data from a similar study with prednimustine (20 mg/kg) are presented in Table 2. Again, the levels of total drug-derived materials were higher in the plasma than in any of the tissues examined at all time points, with the exception of the intestine. At no time was there sufficient radioactivity present in the bone marrow or tumour to produce a significant number of counts. HPLC analysis of plasma and tissue samples from prednimustine-treated animals also failed to reveal the presence of unhydrolysed prednimustine.

The plasma levels of chlorambucil and phenyl acetic mustard detected following SC injections of chlorambucil (10 mg/kg) or prednimustine (20 mg/kg) are presented in Fig. 1. Following chlorambucil administration, the peak circulating chlorambucil concentration was 40 nmoles/ml plasma and that of phenyl acetic mustard, 24 nmoles/ml plasma; chlorambucil and phenyl acetic mustard had plasma half-lives of 2.4 and 2.9 h, respectively, with phenyl

Table 3. AUC[a] values for the chlorambucil and phenyl acetic mustard levels detected following SC administration of chlorambucil (10 mg/kg) or prednimustine (20 mg/kg)

Drug administered	AUC[a] values (nmoles · ml^{-1} × h^1)	
	Chlorambucil	Phenyl acetic mustard
Chlorambucil	73	91
Prednimustine	117	54

[a] AUC, area under the plasma concentration-vs-time curve. Data from Fig. 1

Table 4. Pharmacokinetic parameters (plasma half-life, $t_{1/2}\beta$, and extrapolated volume of distribution, V_D) following the administration of chlorambucil (40 mg/kg) or chlorambucil (40 mg/kg) + prednisolone (40 mg/kg) 4 h later[a]

Drug treatment	Total chlorambucil-derived material		Chlorambucil		Phenyl acetic mustard	
	$t_{1/2}\beta$ (h)	V_D (ml/kg)	$t_{1/2}\beta$ (h)	V_D (ml/kg)	$t_{1/2}\beta$ (h)	V_D (ml/kg)
Chlorambucil	7.5 ± 1.6	298 ± 12	1.5 ± 0.3	355 ± 56	1.8 ± 0.2	445 ± 30
Chlorambucil + prednisolone	8.7 ± 1.3	282 ± 7	1.4 ± 0.2	249 ± 36	1.9 ± 0.3	459 ± 24

[a] Values ± standard error

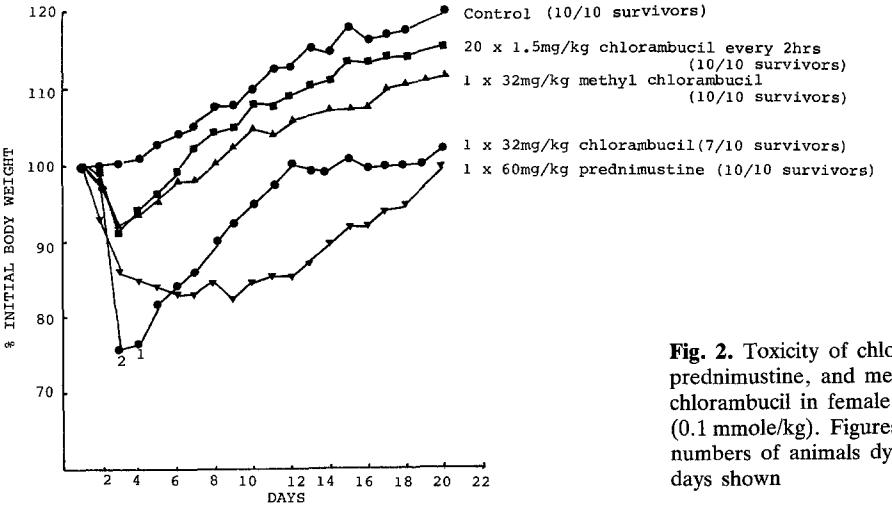

Fig. 2. Toxicity of chlorambucil, prednimustine, and methyl chlorambucil in female Wistar rats (0.1 mmole/kg). Figures indicate the numbers of animals dying on the days shown

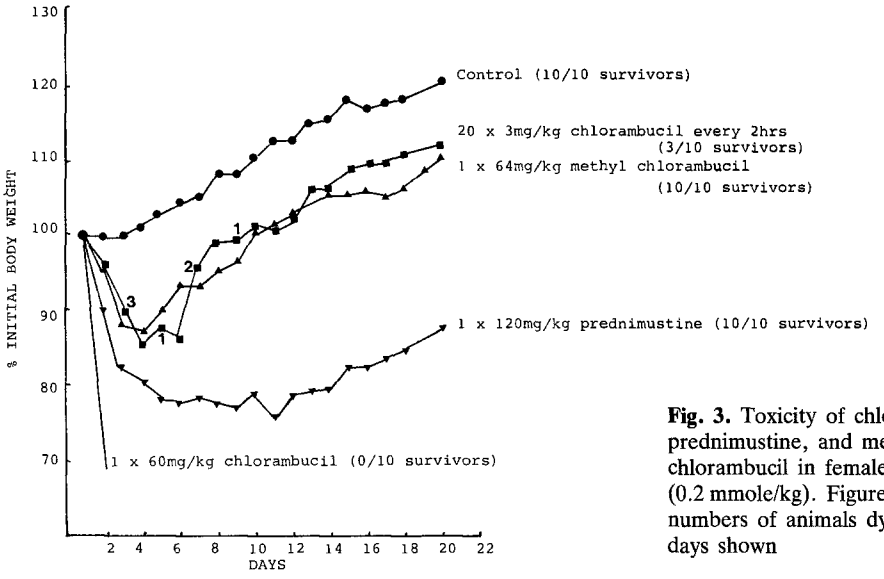

Fig. 3. Toxicity of chlorambucil, prednimustine, and methyl chlorambucil in female Wistar rats (0.2 mmole/kg). Figures indicate the numbers of animals dying on the days shown

Table 5. Antitumour effects of prednimustine, chlorambucil, chlorambucil + prednisolone, and methyl chlorambucil against the alkylating agent-resistant Yoshida sarcoma in vivo

Treatment	% Surviving cells 72 h after treatment[a]
Control	100
Chlorambucil 20 mg/kg × 1	100
Chlorambucil 1 mg/kg × 20 (every 2 h)	106
Prednimustine 40 mg/kg × 1	57
Chlorambucil + Prednisolone 1 mg/kg × 20 (every 2 h)	57
Methyl ester of chlorambucil 20 mg/kg × 1	100

[a] Each figure is the mean of two separate experiments in five rats each

acetic mustard the predominant compound after 2 h. Following prednimustine administration, plasma levels of chlorambucil and phenyl acetic mustard rose gradually over the first 12-h period and were then maintained at a relatively constant level for 48 h. Chlorambucil was the predominant circulating product of prednimustine metabolism. Comparison of the area under the plasma concentration vs time curves in Fig. 1 indicates that both prednimustine and chlorambucil gave rise to similar total quantities of chlorambucil plus phenyl acetic mustard (Table 3).

The pharmacokinetics of chlorambucil were also investigated in the rat at a toxic dose (40 mg/kg), and the influence of prednisolone (40 mg/kg) administered 4 h later was studied. As shown in Table 4, subsequent prednisolone treatment did not substantially alter the pharmacokinetics of total chlorambucil-derived material, chlorambucil, or phenyl acetic mustard.

Toxicity Studies

In an attempt to explain the reduced toxicity of prednimustine compared with chlorambucil, we first tested the toxicity of the methyl ester of chlorambucil. As seen in Figs. 2 and 3 the methyl ester was much less toxic than chlorambucil and was also less toxic than an equimolar dose of prednimustine at the two dose levels investigated.

The pharmacokinetics of prednimustine indicated that it provided a sustained low circulating level of chlorambucil. This might account for the low toxicity of prednimustine. Therefore we also compared the toxicity of chlorambucil given as a single high dose with that of multiple low-dose administration. As seen in Figs. 2 and 3, at two dose levels the multiple low doses of chlorambucil was much less toxic than the single high dose.

Antitumour Studies

We have shown previously that prednimustine or a combination of chlorambucil and prednisolone killed a significant percentage of alkylating agent-resistant Yoshida sarcoma cells [5]. Table 5 shows that multiple low doses of chlorambucil did not kill

chlorambucil-resistant tumour cells. Neither did the methyl ester of chlorambucil. However, multiple doses of chlorambucil plus prednisolone were as effective as prednimustine, killing 43% of the resistant tumour cells.

Discussion

Following the administration of prednimustine no unhydrolysed ester could be detected either in the plasma or in any of the tissues examined. These data indicate that prednimustine is rapidly hydrolysed in vivo, which is consistent with our observations of hydrolysis by rat tissues in vitro [19]. The differences in the pharmacokinetic profiles observed following the administration of chlorambucil and prednimustine (Fig. 1) indicate that prednimustine is released gradually, either from the site of injection or from another tissue depot, following which it is rapidly hydrolysed. This hydrolysis, catalysed by tissue and/or plasma esterases [19], results in the production of chlorambucil, which is metabolised subsequently to phenyl acetic mustard. Failure to detect either circulating prednimustine or unhydrolysed prednimustine in any of the tissues examined suggests that the depot of drug is in fact the site of injection. Chlorambucil, in contrast, is rapidly absorbed following SC administration.

The observed differences in the pharmacokinetic profiles offer an explanation for the reduced toxicity of prednimustine compared with chlorambucil, since we have shown that repeated low doses of chlorambucil are less toxic than the same total dose given as a single injection.

The similarity in the toxicological properties of prednimustine and the methyl ester of chlorambucil indicates, however, that the major factor in inducing a reduction in chlorambucil toxicity is esterification in general, rather than esterification with prednisolone in particular. The reduction in aqueous solubility induced by the esterification of chlorambucil presumably promotes retention of the toxic alkylating moiety at the site of injection, thus producing an alteration in pharmacokinetics. The reduced, but sustained, circulating levels thereby produced are inherently less toxic.

In the case of prednimustine, the prednisolone moiety could also reduce the toxicity, since Harrap et al. [5] have previously reported that the toxicity of chlorambucil can be significantly reduced by the administration of prednisolone 4 h after the alkylating agent. Under these conditions the reduction in chlorambucil toxicity could result from an alteration in the pharmacokinetic profile, i.e., similar to that produced following the administration of prednimustine. As demonstrated in the present study, however, subsequent prednisolone administration did not alter the pharmacokinetics of chlorambucil.

Other studies from this laboratory have described the mechanism of chlorambucil/prednisolone interaction in tumour cells [20]. It is possible that the enhanced therapeutic index of the combination derives from a selective elevation of chlorambucil binding to tumour nucleoproteins, in association with a concomitant decrease in binding to comparable nuclear protein fractions isolated from intestinal epithelial cells [6].

As previously reported [5], one of the most exciting properties of prednimustine is the antitumour activity that this compound displays against alkylating agent-resistant tumours. This effect can also be produced by using a binary combination of chlorambucil with prednisolone administered 4 h later. The lack of antitumour activity elicited by the methyl ester of chlorambucil in the present study indicates that it is in fact the prednisolone moiety of prednimustine that is important in re-establishing cytotoxicity against the resistant tumour rather than the alteration in pharmacokinetics induced by esterification.

From these results we conclude that it is esterification of the chlorambucil molecule that is important for inducing a reduction in whole-animal toxicity following SC injection, whereas the potentiation of activity against alkylating agent-resistant tumour cells is dependent upon the presence of prednisolone. These two characteristics are conveniently incorporated in the drug prednimustine. Nonetheless, the rapid hydrolysis of prednimustine in vivo must preclude any selective uptake of the chlorambucil moiety into tissues or tumours containing glucocorticoid receptors. These results have stimulated further studies into binary combinations of alkylating agents and steroids with regard to both the toxicological properties of these drug treatments and the molecular mechanisms operating [6, 20].

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