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Physicochemical and pharmacokinetic parameters of seven lipophilic chlorambucil esters designed for brain penetration

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Summary. This report describes the physicochemical and pharmacokinetic parameters of seven chlorambucil esters, which were compared with those of chlorambucil. These esters were designed as chlorambucil prodrugs to increase the brain penetration and concentration vs time profile of chlorambucil within the CNS for potential treatment of brain tumors. They include four aliphatic esters from one to eight carbon chains in length (chlorambucil-methyl, -propyl, -hexyl, and -octyl esters) and three aromatic esters, including the phenylmethyl, phenylethyl and prednisolone ester of chlorambucil, prednimustine. The esters were lipophilic and possessed log octanol : water partition coefficients (log P values) that ranged from 4.05 to >8.0. All retained alkylating activity, which was reduced compared with that of chlorambucil. In addition, all were metabolized in vivo in the rat to yield chlorambucil alone. Measurement of the in vitro rate of ester hydrolysis of the compounds to yield chlorambucil in rat plasma demonstrated that short-chain aliphatic and aromatic chlorambucil esters were rapidly broken down to their parent compound. The plasma half-lives of the compounds increased with the increasing length and complexity of their ester chain. This may have been related to an increase in the binding of the long-chain esters to plasma proteins, protecting the ester from nonspecific plasma esterases, and to a reduced affinity of plasma esterases to these esters. Pharmacokinetic analysis of chlorambucil-hexyl, -octyl, and -prednisolone esters by HPLC demonstrated that following their intravenous administration in the rat (in doses equivalent to equimolar chlorambucil, 10 mg/kg), they yielded only low concentrations of active compounds in plasma and brain. The brain : plasma ratio of these was low and similar to that of chlorambucil, and no ester demonstrated anticancer activity superior to that obtained after the administration of equimolar chlorambucil (5 mg/kg i.v., days 1–5) against brain-sequestered Walker 256 carcinosarcoma in the rat.

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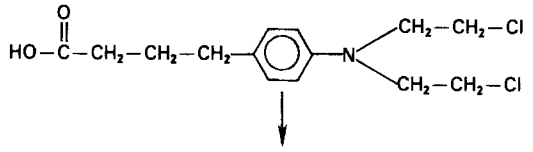
Introduction

Primary malignant brain tumors occur at an annual rate of 4.5 cases/100,000 population and metastatic brain tumors, at 8.5/100,000. Although several types of systemic malignancy (choriocarcinoma, testicular carcinoma, oat-cell carcinoma of the lung, and breast cancer in women) can be successfully managed by chemotherapy even when disseminated to multiple sites, when the same responsive tumors are identified as metastatic foci in the brain, the effect of chemotherapy at the CNS sites has been minimal [15, 16, 35]. Malignant brain tumors are almost always fatal [15, 48, 49]. The median survival of patients following surgery and radiation therapy for primary brain tumors is approximately 7.5–9.5 months [15, 49]. The prognosis for patients with metastatic brain tumors is more pessimistic; their median survival is approximately 4–6 months after surgery and/or radiation therapy [15, 35].

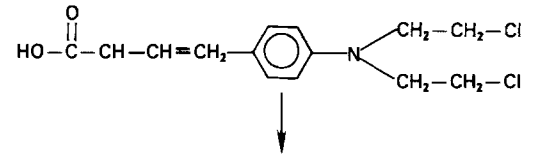
The lipophilic nitrosoureas have been the mainstay of brain tumor chemotherapy, but their effects on patient survival have been disappointing [15, 49]. The failure of chemotherapy to cure a cancer patient is ultimately due to the overgrowth of both insensitive and resistant cells; this, in essence, is the same for tumors located within the brain. It is therefore not surprising that single-drug regimens used in the treatment of tumors comprised from a heterogeneous cell population at best only minimally increase patient survival. Both the cell-kill and Goldie-Coldman hypotheses agree that the best responses are achieved by combination therapies [14, 42, 46]. For tumors in the brain this situation is complicated by the difficulty of delivering chemotherapeutic drugs into the CNS [15, 16, 35]. Thus, ironically, whereas a wide variety of anticancer agents are presently available to the oncologist, few possess the required physicochemical properties that render them suitable for the treatment of CNS-sequestered tumors [15–17]. Consequently, the list of agents that are suitable to combine with the nitrosoureas is meager.

Several investigations have demonstrated that alkylating agents other than the nitrosoureas, particularly classic nitrogen mustards, are among the most active compounds against human glioma and medulloblastoma xenografts in immune-deprived animals [12, 45]. In addition, studies by Schabel et al. [43] and Hill [22] indicate that for many tumors there is a lack of cross-resistance between different types of anticancer alkylating agents, as cellular mechanisms for their activity as well as those involved in the

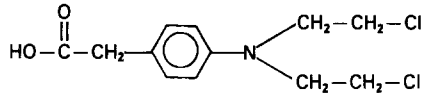
CHLORAMBUCIL



3,4 DEHYDROCHLORAMBUCIL



PHENYLACETIC MUSTARD



METABOLISM OF CHLORAMBUCIL BY β-OXIDATION

PHENYLPROPRIONIC MUSTARD

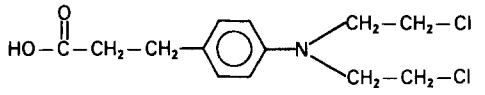
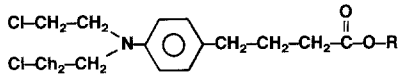


Fig. 1. Chemical structures of chlorambucil, its products of β-oxidation (3,4-dehydrochlorambucil and phenylacetic mustard), and phenylproprionic mustard

4-[p-[Bis(2-chloroethyl)amino]phenyl]butyrates



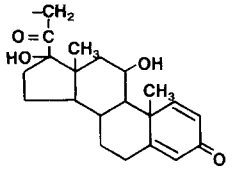
Compound	-R	Formula
Chlorambucil	-H	$C_{14}H_{19}Cl_2NO_2$
Chlorambucil-methyl ester	$-CH_3$	$C_{15}H_{21}Cl_2NO_2$
Chlorambucil-propyl ester	$-(CH_2)_2CH_3$	$C_{17}H_{25}Cl_2NO_2$
Chlorambucil-hexyl ester	$-(CH_2)_5CH_3$	$C_{20}H_{31}Cl_2NO_2$
Chlorambucil-octyl ester	$-(CH_2)_7CH_3$	$C_{22}H_{33}Cl_2NO_2$
Chlorambucil-phenylmethyl ester	$-CH_2-C_6H_5$	$C_{21}H_{25}Cl_2NO_2$
Chlorambucil-phenylethyl ester	$-(CH_2)_2-C_6H_5$	$C_{22}H_{27}Cl_2NO_2$
Chlorambucil-prednisolone ester		$C_{35}H_{45}Cl_2NO_6$

Fig. 2. Chemical structures of chlorambucil and chlorambucil esters

development of resistance are different [6]. As a consequence, combinations of alkylating agents have been used in clinical therapy, particularly in conjunction with autologous bone marrow transplantation [7].

Chlorambucil is a bifunctional, nitrogen-mustard alkylating agent that has demonstrated wide clinical activity [5, 8]. Due to its ionization at physiological pH, however, the compound only minimally enters the brain. Furthermore, the compound binds to plasma constituents, which further reduces its brain uptake. Greig and Rapoport [18] have reported that the brain : plasma integral concentration ratio for chlorambucil and its active metabolites is only 0.017; its activity against alkylating-agent-sensitive, brain-sequestered tumors is minimal [18]. This paper describes the physicochemical and pharmacokinetic parameters of chlorambucil and seven lipophilic ester derivatives of the drug. Masking of chlorambucil's ionizable carboxylic acid moiety by a lipophilic ester may increase the compound's delivery to the brain and yet maintain its alkylating activity.

Materials and methods

Chemicals. Chlorambucil, 4-[*p*-[bis(2-chloroethyl)amino] phenyl]butyric acid, was purchased from Sigma Chemical Co. (St Louis, Mo). Phenylacetic mustard, 2-[*p*-[bis(2-chloroethyl)-amino]phenyl]acetic acid, and phenylproprionic mustard, 4-[*p*-[bis(2-chloroethyl)-amino]phenyl]proprionic acid, were obtained from the Pharmaceutical Resources Branch of the National Cancer Institute (Bethesda, Md). The latter is not a product of the in vivo metabolism of chlorambucil and was used as an internal standard during chlorambucil analysis (Fig. 1).

Chlorambucil-methyl ester, 2-methyl-4-[*p*-[bis(2-chloroethyl)amino]phenyl]-butyrate, and chlorambucil-propyl ester, 2-propyl-4-[*p*-[2-chloroethyl)amino]-phenyl]butyrate (Fig. 2), were prepared from chorambucil and the appropriate alcohol (methanol and propanol, respectively) by reaction with *p*-toluenesulfonic acid (Sigma) in benzene under azeotropic conditions to remove water formed during the reaction. Formation of the appropriate esters was confirmed by analysis of nuclear magnetic resonance (NMR) data and infrared (IR) spectral bands. Chlorambucil-hexyl ester, -octyl ester, -phenylmethyl ester, and -phenylethyl ester (2-hexyl-, 2-octyl-, 2-phenylmethyl-, and 2-phenylethyl-4-[*p*-[2-chloroethyl)amino]phenyl]butyrate, respectively) were obtained from the Chemical Resources Branch of the National Cancer Institutes. These compounds have previously been synthesised by Roehrig et al. [41]. Chlorambucil-prednisolone ester, 11B,17,21-trihydroxypregna-1,4-diene-3,20-dione 21-[4-[*p*[2-chloroethyl)amino] phenyl]butyrate (prednimustine) was obtained from Hoffmann-La Roche (Nutley, NJ). All chlorambucil esters were >95% pure by HPLC analysis and were prepared as salts. Methanol, ethanol, propanol, acetone, and acetonitrile were of HPLC grade and were supplied by Burdick & Jackson Laboratories (Muskegon, Mich).

Alkylating activity. The alkylating activity of each agent was determined at concentrations between 0.1 and 100 m*M*. Drug was dissolved in 50% (v/v) acetone/50% ethanol, and 0.2 ml was added to 1 ml 0.2 *M* acetate buffer

(pH 5.6). Then, 0.5 ml 5% *p*-nitrobenzyl pyridine (Sigma) in acetone was added and the mixture was incubated for 2 h at 37° C. Finally, 3 ml 25% 3-amino-1-propanol (Sigma) in tertiary butyl alcohol (Sigma) was added and coloration of the reaction product was measured by spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) at 560 nm. The alkylating activity of each agent was then compared with that of equimolar chlorambucil.

Pharmacokinetic study. Adult male Wistar rats (Charles River Laboratories Inc., Wilmington, Mass) weighing approximately 120 g each were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The left saphenous vein was exposed and either 10 mg/kg chlorambucil (Sigma) or chlorambucil ester (equimolar to 10 mg/kg chlorambucil) in dimethyl sulfoxide was injected i.v. (250 µl/kg). At 15 and 30 min following drug administration, blood was collected by cardiac puncture and the brain was removed and placed on 0.9% NaCl, ice-chilled filter paper. A minimum of three animals were killed per time point. The blood was centrifuged (7,000 *g*, 45 s) and the plasma was removed and stored immediately at −70° C. Plasma and brain samples from animals given chlorambucil were analyzed by HPLC for chlorambucil and for its active metabolites, 3,4-dehydrochlorambucil and phenylacetic mustard. Samples from animals given a chlorambucil ester were additionally analyzed for that compound.

Instrumentation and conditions. HPLC analysis was carried out using a Waters Associates system (Milford, Mass) consisting of a Model 6000A solvent pump, a Wisp 710B automatic injector, and a model 480 variable wavelength UV detector, which was set at 254 nm wavelength. Separation was done on a 10-µm Partosil 10 ODS 3 column (Ace Scientific, East Brunswick, NJ), with a guard column packed with pellicular C_{18} material (Waters). Chromatograms were recorded on a Waters Model 720 data module. The mobile phase for pump A was a mixture of water, acetic acid, and acetone (97.1 : 2 : 0.9, by vol.), and that for pump B was 97.5% acetonitrile and 2.5% water (containing 2% acetic acid). The flow rate was set at 1.8 ml/min, produced at 30% from pump A and 70% from pump B, which resulted in a column pressure of 2,000 psi. The system was run under these isocratic conditions for 10 min and then linearly for a further 10 min to achieve 10% from pump A and 90% from pump B. The retention times were: phenylacetic mustard, 4.5 min; phenylproprionic mustard, 5.5 min; 3,4-dehydrochlorambucil, 6.3 min; chlorambucil, 7.0 min; chlorambucil-methyl ester, 12.1 min; chlorambucil-propyl ester, 13.3 min; chlorambucil-hexyl ester, 16.0 min; chlorambucil-octyl ester, 18.0 min; chlorambucil-phenylmethyl ester, 16.2 min; chlorambucil-phenylethyl ester, 17.3 min; and chlorambucil-prednisolone ester, 19.1 min.

Concentrations of chlorambucil and its metabolites were calculated from the ratio of their peak height measurements with an internal standard. These were then quantified from calibration curves of six points, two samples per point, which were run daily and intermixed with the unknown samples. Different internal standards were used for the quantitation of each derivative. Phenylproprionic acid was used as an internal standard for the quantitation of chlorambucil, 3,4-dehydrochlorambucil, and phenylacetic mustard. For each chlorambucil ester, the ester with the closest retention time was used. To ensure that no ester hydrolysis occurred during sample preparation and extraction procedures samples were maintained at 4° C.

Sample preparation and extraction. Plasma and brain samples were thawed and placed in an ice bath at 0° C. In all, 2 ml chilled water and 6 ml ethyl acetate were added to each plasma sample, which then was shaken and centrifuged (2,000 *g* for 10 min at 4° C). The ethyl acetate phase was removed and evaporated to dryness, and 100 µl methanol containing 2% acetic acid was added. The samples were then injected onto the HPLC. For brain, 4 ml chilled water was added and the sample was sonicated (Model 225, Heat Systems-Ultrasonics Inc., Farmingdale, NY) for 30 s; it was then extracted as described for the plasma samples. For esters whose log octanol : water partition coefficients (log P values) were >6.0, double extractions were required to obtain a high extraction coefficient and reproducible results.

Protein binding studies. Plasma protein binding of chlorambucil and chlorambucil esters was measured at concentrations from 1 to 100 nmol/ml by centrifugal ultrafiltration. Amicon centrifree micropartition systems (Amicon Corp., Danvers, Mass) were used for the rapid preparation of protein-free ultrafiltrates of the agents in either pooled rat plasma or a suspension of human serum albumin and λ-globulin (Sigma) (5 g/dl and 3 g/dl, respectively, in physiological buffer), as previously described [20]. Samples of both protein-free ultrafiltrate and original drug solution were stored at −70° C prior to HPLC analysis. Drug-membrane binding was measured with drug solutions prepared in isotonic physiological buffer. The recovery of all was approximately 100%.

Log P values. Log P values were calculated according to Leo et al. [27] and Hansch [21].

In vitro plasma half-life studies. The rate of chlorambucil ester hydrolysis to chlorambucil was measured in rat and human plasma. Plasma was maintained at 37° C, and chlorambucil or chlorambucil ester was added to a final concentration of 10 µg/ml. The plasma was then rigorously shaken, and 1-ml samples were removed at intervals of 15 s to 24 h. Samples were immdiately frozen to −70° C and maintained at this temperature prior to HPLC analysis.

Anticancer activity studies. Female Wistar rats (Charles River) weighing approximately 120 g each were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Walker 256 carcinosarcoma tumor cells (1×10^4) in 10 µl Dulbecco's modified Eagle's tissue-culture medium (Gibco Laboratories, Chagrin Falls, Ohio) were then injected intracerebrally into the parietal cortex through a 30-gauge needle attached to a Hamilton syringe. With the exception of control animals, which were injected with vehicle alone, all animals were given either 5 mg/kg i.v. chlorambucil daily for 5 consecutive days starting 36 h following tumor-cell implantation of an equimolar dose of chlorambucil ester. This dose was previously determined to be the maximum tolerated dose for chlorambucil [18]. Drug activity

Table 1. Log octanol/water partition coefficient, in vitro alkylating activity, in vitro half-life in rat plasma, and percentage of binding to plasma proteins of chlorambucil and chlorambucil esters

Compound	Log octanol/water Partition coefficient[a]	Alkylating activity (% of chlorambucil)[b]	In vitro plasma half-life (rat)	Plasma protein binding[c]
Chlorambucil	−0.66 (unionized, 3.70)	100	stable for hours	99.0%
Chlorambucil-methyl ester	4.05	57	< 10 s	99.4%
Chlorambucil-propyl ester	5.05	39	10 s	99.6%
Chlorambucil-hexyl ester	6.55	30	2 – 5 min	99.8%
Chlorambucil-octyl ester	7.55	23	15 – 20 min	99.9%
Chlorambucil-phenylmethyl ester	6.18	19	1 – 2 min	99.4%
Chlorambucil-phenylethyl ester	6.63	17	2 min	99.7%
Chlorambucil/prednisolone ester	> 8.00	37	2 – 5 min	99.9%

[a] Calculated from Hansch [21] and Leo et al. [27]
[b] Measured by reaction with *p*-nitrobenzyl pyridine
[c] Measured in vitro at 37° C at concentrations between 1 and 100 nmol/ml by centrifugal ultrafiltration

against the intracerebrally implanted tumor was assessed by comparing the mean survival of the drug-treated animals to that of the vehicle-treated controls. Controls were run concurrently with treated animals in all studies; a minimum of seven animals were used in each control and treatment group.

Calculations. Brain concentrations of chlorambucil, its active metabolites, and chlorambucil esters were calculated from the net regional brain concentration, as measured with HPLC, by subtracting the intravascular concentration at the time of death (T). The intravascular concentration equalled the plasma concentration of the compound (nmol/ml) at time T, multiplied by the regional blood volume (ml/g brain).

Regional blood volume was measured by injecting three anesthetized rats i.v. with [^{14}C]-methyl bovine serum albumin (17 μCi/mg; New England Nuclear Research Products, Boston, Mass), which was determined to be 99.5% pure by polyacrylamide slab electrophoresis. Blood and tissue samples were collected at 2 min as described for chlorambucil. Tissue samples were digested overnight at 50° C in 1 ml Protosol (New England Nuclear) and prepared for scintillation counting by adding 10 ml Ready Solv MP liquid scintillation cocktail (Beckman Instruments, Palo Alto, Calif). Counting was done on a Beckman LS9000 liquid scintillation spectrometer. [^{14}C]-Methyl bovine serum albumin (mol. wt., 69,000 daltons) remains within the cerebral vasculature during the 2-min experiment [39]. Regional blood volume was calculated by dividing the [^{14}C] activity in a brain sample by that in blood ($dpm \cdot g^{-1}/dpm \cdot ml^{-1}$); it equalled 2.4% and 1.7% in cerebral cortex and cerebellum, respectively.

Chlorambucil ester concentration-vs-time data obtained from in vitro plasma studies were fitted by nonlinear regression analysis [30] to a single exponential equation:

$$C = Ae^{-\alpha\tau}, \quad (1)$$

where C equals the concentration of chlorambucil ester (μg/ml) at time t (min), A is the theoretical zero-time concentration in a central compartment, and α is the apparent first-order elimination rate constant (min^{-1}). The plasma half-life ($t_{1/2}$), in minutes was calculated from the parameters by the general formula

$$t_{1/2\alpha} = \frac{0.693}{\alpha} . \quad (2)$$

Areas under the concentration-time profiles (between 15 and 30 min) were calculated by the trapezoidal rule.

Statistical analysis. A two-tailed Student's *t*-test was carried out to compare the two means. When more than two means were compared, one-way analysis of variance and the Bonferroni multiple-test were used [33]. Statistical significance for all tests was taken as $P < 0.05$. Unless otherwise stated, means ± SEM are given routinely.

Results

Table 1 lists the log P value, alkylating activity, rate of ester hydrolysis in rat plasma, and percentage of binding to plasma proteins of chlorambucil and its seven lipophilic ester derivatives. The log P of unionized chlorambucil is 3.70; at physiological pH, however, chlorambucil exists preferentially in an ionized form (p*Ka*, 4.46 [9] – 5.6 [13]) and possesses a log P value of −0.66. All seven ester derivatives are lipophilic, with log P values ranging from 4.05 for the methyl ester to > 8.0 for the prednisolone ester. The alkylating activity of the esters, determined by in vitro chemical reaction with *p*-nitrobenzyl pyridine, was significantly reduced compared with that of equimolar chlorambucil. With the exception of the prednisolone ester, increasing the size and complexity of the ester by the addition of carbons caused a decline in the alkylating activity of the compound.

All compounds bound significantly to plasma proteins, primarily to serum albumin. The percentage of binding of chlorambucil to pooled rat plasma and resuspended human serum albumin and λ-globulin was similar (99.0%). Binding of the ester derivatives to resuspended serum albumin and λ-globulin was greater than that of chlorambucil and increased with the increasing lipophilicity of the compound. This binding was reversible for each agent, as all could be recovered by alcohol precipitation. Concentration-dependent binding was not determined at drug levels between 1 and 100 nmol/ml for any agent. The binding of the derivatives to rat plasma could not be measured due to the occurrence of ester hydrolysis.

Table 2. Brain and plasma concentrations of chlorambucil esters, chlorambucil, and active metabolites nmol/ml plasma, nmol/g brain following i. v. administration of chlorambucil (10 mg/kg) or equimolar chlorambucil ester to rats

	15 min:					30 min:					AUC (15–30 min):
	CHL-E	CHL	D.CHL	PAM	TOTAL	CHL-E	CHL	D.CHL	PAM	TOTAL	TOTAL
Chlorambucil:											
Plasma		141.78 ±5.92	2.28 ±0.40	13.22 ±0.47	157.28 ±6.26		87.04 ±11.08	3.04 ±0.16	19.30 ±1.14	109.38 ±12.00	1999.95 ±136.95
Cerebellum		1.45 ±0.21	ND	0.80 ±0.04	2.25 ±0.20		0.48 ±0.19	ND	0.67 ±0.23	1.15 ±0.09	25.50 ±2.18
Cerebral cortex		0.92 ±0.43	ND	0.62 ±0.04	1.54 0.51		0.48 ±0.12	ND	0.56 ±0.01	1.04 ±0.12	19.35 ±4.57
Chlorambucil-hexyl ester:											
Plasma	0.34	14.05 4.11	ND	0.90 ±0.43	15.37* ±4.88	ND	16.02 4.37	ND	3.06 ±0.10	19.08* ±4.47	258.34* ±70.16
Cerebellum	0.15 ±0.15	0.16 ±0.01	ND	ND	0.31* 0.15	ND	0.01 ±0.00	ND	ND	0.01** 0.00	2.42*, ** ±1.10
CCerebral cortex	0.12 ±0.12	0.22 ±0.01	ND	ND	0.34 ±0.12	ND	0.02 ±0.00	ND	ND	0.02 ±0.00	2.75* ±0.85
Chlorambucil-octyl ester:											
Plasma	ND	14.05 ±1.81	ND	0.98 ±0.36	16.03* ±2.06	ND	25.08 ±6.18	ND	2.58 ±0.89	27.76* ±6.50	328.42*
Cerebellum	0.30 ±0.02	0.17 ±0.09	ND	0.12 ±0.10	0.59 0.20	0.30 ±0.02	0.29 ±0.29	ND	0.12 ±0.12	0.71 ±0.31	9.75*
Cerebral cortex	0.16 ±0.01	0.14 ±0.04	ND	0.03 ±0.03	0.33 ±0.07	0.15 ±0.02	0.24 ±0.09	ND	0.01 ±0.01	0.39 ±0.10	5.40* ±3.38
Chlorambucil-prednisolone ester:											
Plasma	ND	7.04 ±2.50	ND	1.20 ±0.04	8.23* ±2.51	ND	12.17 ±4.76	ND	1.99 ±0.20	14.16* ±4.90	167.98* ±55.52
Cerebellum	ND	ND	ND	ND	ND*, **	0.09 ±0.06	ND	ND	ND	0.09 ±0.06	0.67*, ** ±0.44
Cerebral cortex	0.09 ±0.09	ND	ND	ND	0.09* ±0.09	ND	ND	ND	ND	ND	0.67* ±0.67

All values represent the means (±SEM) from 3 animals

AUC, Area under the concentration vs time curve of total compounds possessing alkylating activity (nmol·min/ml or nmol·min/g; CHL-E, concentration of chlorambucil ester (nmol/ml or nmol/g); CHL, concentration of chlorambucil (nmol/ml or nmol/g); D.CHL, concentration of 3,4-dehydrochlorambucil (nmol/ml or nmol/g); PAM, concentration of phenylacetic mustard (nmol/ml or nmol/g); TOTAL, total concentration of compounds possessing alkylating activity (nmol/ml or nmol/g); ND, No detectable concentration

* Significantly different from value achieved after chlorambucil administration, $P < 0.05$

** Significantly different from value achieved after chlorambucil-octyl ester administration, $P < 0.05$

Rates of enzyme-induced cleavage of the chlorambucil ester derivatives by nonspecific plasma esterases were measured in vitro in rat plasma at 37° C. Chlorambucil proved to be relatively stable over a period of hours; thereafter, minor hydrolysis of the chloroethyl moieties occurred. All chlorambucil ester derivatives broke down to release chlorambucil alone. The relative rates of breakdown varied with the complexity and log P value of the ester: simple, short aliphatic esters were cleaved rapidly to release chlorambucil within seconds, whereas longer aliphatic esters were cleaved less rapidly and possessed half-lives on the order of minutes. Enzymatic cleavage of aromatic esters was also rapid and occurred within minutes; the rate of breakdown likewise decreased with the increasing complexity and lipophilicity of the derivative.

Brain and plasma concentrations of chlorambucil, 3,4-dehydrochlorambucil, phenylacetic mustard, and of the chlorambucil esters were quantitated after the administration of chlorambucil and chlorambucil esters, respectively, at 15 and 30 min (Table 2). Significant concentrations of chlorambucil were detected in plasma at both times after chlorambucil administration, and low amounts of 3,4-dehydrochlorambucil and phenylacetic mustard, the active products of β-oxidation [11, 31, 32], were also found. Chlorambucil concentrations in cerebellum and cerebral cortex were low, and small amounts of phenylacetic mustard were present. Concentrations of compounds that possessed alkylating activity were summed in each individual tissue sample to give the total concentration of all agents possessing alkylating activity (i. e., agents possessing anticancer activity). The concentration integrals (measured between 15 and 30 min) of these active agents in plasma, cerebellum, and cerebral cortex were 1,999.95 nmol/ml, 25.50 nmol/g, and 19.35 nmol/g, respectively. Calculated from these, the mean brain : plasma ratio of active agents following chlorambucil administration was 0.011.

With the exception of those described below, the pharmacokinetic profiles of the chlorambucil esters were similar to those obtained after chlorambucil administration. Following equimolar administration of chlorambucil-hexyl ester, minimal concentrations of the agent were detected in plasma and brain. However, the product of

ester hydrolysis, chlorambucil, together with the product of its subsequent β-oxidation, phenylacetic mustard, were detected in plasma and brain at low concentrations. Total amounts of compounds possessing anticancer activity in plasma, cerebellum, and cerebral cortex were significantly lower than those detected following chlorambucil administration, and the concentration integrals (measured between 15 and 30 min) were 258.34 nmol·min/ml, 2.42 nmol·min/g, and 2.75 nmol·min/g, respectively, representing 13%, 9.5%, and 14%, respectively of those achieved after chlorambucil administration. Calculated from the concentration integrals, the mean brain:plasma ratio of total active agents after chlorambucil-hexyl ester administration was 0.010.

Following the i.v. administration of chlorambucil-octyl ester, minimal concentrations were detected in brain and none was found in plasma. Total concentrations of agents possessing anticancer activity occurred predominantly in the form of chlorambucil and phenylacetic mustard and were significantly lower than those detected after chlorambucil administration. The concentration integrals (measured between 15 and 30 min) of total active agents following chlorambucil-octyl ester administration were 328.42 nmol·min/l, 9.75 nmol·min/g, and 5.40 nmol·min/g, respectively, representing 16%, 38%, and 28%, respectively, of those achieved after chlorambucil administration. The mean brain : plasma ratio calculated from the total concentration integrals for chlorambucil-octyl ester was 0.023.

The prednisolone ester of chlorambucil could not be detected in plasma following its i.v. administration, and only minimal amounts were detected in brain. Whereas low concentrations of chlorambucil and phenylacetic mustard were present in plasma following chlorambucil-prednisolone ester administration, neither was detected in brain. Total concentrations of agents possessing alkylating activity were low at both 15 and 30 min in all tissues. These and their concentration integrals, 167.98 nmol·min/ml, 0.67 nmol· min/g, and 0.67 nmol·min/g in plasma, cerebellum, and cerebral cortex, respectively, were significantly lower than those achieved following chlorambucil administration, representing 8.4%, 2.6%, and 3.5%, respectively, of the latter. Calculated from the concentration integrals, the mean brain : plasma ratio of total active agents following chlorambucil-prednisolone ester was 0.004.

Following the implantation of Walker 256 carcinosarcoma tumor in the rat brain, untreated control animals died, with a mean survival of 9 days. Animals given chlorambucil (5 mg/kg i.v., days 1–5) survived for 11 days, 22% longer than controls. The survival of all rats given chlorambucil esters was not significantly different from that of either untreated controls or animals given chlorambucil.

Discussion

Three major factors determine the delivery of a drug to the brain [16, 17]. The first is the permeability of the blood-brain barrier to the compound, which is related to its lipid : water partition coefficient in the absence of a facilitated transport system, as occurs for melphalan [19]. Because only the unionized and unbound fraction of a compound is considered to be free to penetrate a cell, this partition parameter is dependent on the fractional concentration of the unionized and unbound fraction in the aqueous phase, times the lipid solubility of the unionized agent, from its octanol:water partition coefficient [39]. In cases in which drug is rapidly released from plasma proteins to form a new equilibrium [28], its steady-state binding to plasma protein may not be informative [40]. The second factor is the time-dependent free-plasma concentration profile of the drug, which is related to its route of administration, its tissue distribution, and the mechanisms for its metabolism and elimination. The final factor is local cerebral blood flow. The cumulative effect of these factors determines the actual concentration of drug that eventually reaches the brain and, dependent on its distribution and binding within the brain, its maintenance therein with time. Greig [16, 17] has reported that the maintenance of high, unbound concentrations of unionized drug in plasma is optimal for its delivery and retention in brain.

One of the most important properties of a drug, with regard to drug delivery, is a balanced lipo/hydrophilicity [17]. Transport within the body primarily occurs via the hydrophilic phases of plasma and extracellular fluid to enable an agent to reach its target cell from its site of administration, whereas transport into the target cell (to elicit a pharmacological response) and transport across biological membrane barriers (such as those at the blood-brain interface) occur via lipid phases. Rapoport [39] has demonstrated that there is a linear relation between the cerebrovascular permeability of a compound (i.e., its brain penetration) and its lipophilicity as determined by its octanol : water partition coefficient (log P valaue). Thus, in the case of a lipophilic drug with a log P value of >0 (i.e., equal partition in octanol and water), transfer across the blood-brain barrier is rapid and distribution from the blood to the brain is limited by the rate of local blood flow. Conversely, a lipid-insoluble, polar compound that is not transported into the brain, such as chlorambucil (log P value, -0.6), is rate-limited from entering the brain by its low permeability at the blood-brain barrier.

The addition of a lipophilic group to a compound alteres the partition coefficient of the agent and increases its log P value. Extensive studies by Hansch [21] and Leo et al. [27] have enabled the calculation and assignment of numerical constants, π, for substituents in terms of how much they alter the lipid solubility of a derivative compared with a parent compound. One of the simplest ways of increasing the lipophilicity of an agent is by the sequential addition of a CH_2 group into it, which adds approximately 0.5 to the log P value [21, 27]. The structures of chlorambucil and phenylacetic mustard (Fig. 1) differ by two CH_2 substituents. From this, the log P value of the latter can be predicted to be 1 log less than that of chlorambucil and the agent can be expected to be 10 times less lipophilic. Studies by McLean et al. [31], Godeneche et al. [13], and Lee et al. [26] indicate that the activities of both agents against extracerebral tumors are similar, although the therapeutic efficacy of chlorambucil may be slightly higher than that of phenylacetic mustard [13, 26, 31]. Furthermore, Greig and Rapoport [18] have demonstrated that the brain]:[plasma ratios of the concentration integrals of both compounds are small and similar in rats (0.021 and 0.013, respectively) and that both are restricted from entering the brain by their carboxylic acid residue. It is possible

to mask this carboxylic acid moiety by esterification, which forms neutral nitrogen mustards of differing lipophilicities.

Reaction of chlorambucil with the appropriate alcohol under azeotopic conditions can yield multiple esters. In the present study, we analyzed the physicochemical properties and pharmacokinetic characteristics of a series of seven chlorambucil esters. In each case, replacement of the carboxylic acid moiety of chlorambucil by a lipophilic ester reduced the alkylating activity of the compound compared with that of the parent agent. Larger esters caused greater reductions, except that the alkylating activity of the prednisolone derivative was greater than that of the phenylethyl ester. However, it should be remembered that the in vivo biological activity of these chlorambucil esters derives from the resultant actions of the derivative, in addition to several transitory alkylating entities including chlorambucil, 3,4-dehydrochlorambucil, and phenylacetic mustard. Therefore, the relative rates of enzymatic breakdown to each metabolite of different activity and physicochemical character is important. Furthermore, the in vivo efficacy of most anticancer agents is dependent on the tumor system studied and its site of implantation [18, 26, 41]. All of the ester derivatives possessed significant intrinsic alkylating activity that was lower than that of chlorambucil but comparable with that of melphalan, which is of significant clinical value despite the fact that its alkylating activity is only 38.4% of that of chlorambucil [5, 8]. In addition, each ester was metabolized, albeit at different rates, to products of higher alkylating activity. The alkylating activity of phenylacetic mustard, measured under identical conditions, is 100.8% of that of chlorambucil, a value that compares favorably with that reported by Godeneche et al. [13].

Our finding that the plasma protein binding of chlorambucil was 99% is in agreement with other studies [10, 18, 26], which have additionally reported that phenylacetic mustard is 96.6% plasma protein-bound. Binding of these acidic agents most probably involves association with cationic groups on the proteins, although binding to immunoglobulin G has also been reported [3]. Binding is most likely determined by the p*K*a of the carboxylic acid moiety and the lipophilicity of the agent. The p*K*a of chlorambucil lies between 4.46 [9] and 5.6 [13], and its metabolites are expected to have similar values, suggesting that all are predominantly ionized at physiological pH. Therefore, any differences in binding to plasma proteins are probably related to differences in lipophilicity. The binding of the chlorambucil esters was greater than that of chlorambucil and increased in proportion to log P. However, unlike the binding of chlorambucil and its metabolites, binding between the esters and plasma constituents was probably due to hydrophobic interactions [34, 44]. These results are in accord with those of Scholtan [44], who demonstrated a positive linear relation between log P and binding constants to human albumin for a variety of agents including derivatives of sulfapyrimidine, tetracycline, sulfonamide, penicillin, and steroids. In each case, the addition of a CH_2 or phenyl substituent increased the binding capacity by a constant that was specific to the series of agents studied [44]. Furthermore, our demonstration of extensive reversible binding of chlorambucil-prednisolone ester to plasma proteins is in agreement with studies by Wilkinson et al. [50].

Ehrsson et al. [10] have reported that chlorambucil is 100-fold more chemically stable in vitro when bound to albumin than when unbound in aqueous solution. Similar or greater stability is probably afforded the lipophilic esters, as binding protects the chloroethyl moieties from rapid decomposition and inactivation by hydrolysis [26] or formation of a cyclic aziridinium ion [38] prior to delivery to its target. Disposition studies in the rat have demonstrated that tumor and normal tissue concentrations are lower than those of plasma throughout the time course following the administration of [^{3}H]-chlorambucil [36]. Plasma protein binding may therefore protect the compound from β-oxidation and, additionally, protect chlorambucil and the esters, 3,4-dehydrochloroambucil and phenylacetic mustard from hepatic cytochrome P-450-catalyzed monodechlorination [11, 32] but restrict the delivery of drug to target tissues [25, 28]. Our studies demonstrate a decreased rate of enzymatic breakdown of chlorambucil esters with increasing aliphatic ester chain length and lipophilicity of the compound. Extensive binding to plasma proteins may protect the longer-chain chlorambucil esters from hydrolysis by plasma esterases, thereby extending their plasma half-life.

Nonspecific esterases, predominantly carboxylesterases and arylesterases, are primarily responsible for the hydrolysis of ester links in uncharged substrates in plasma [4, 24, 47]. A major factor that affects the plasma half-life of a chlorambucil ester is the length and shape of the hydrophobic ester, which influences the reactivity and affinity of plasma esterases for the hydrophobic groups on either side of the ester link. Hofstee [23] and Malhotra and Philip [29] have reported that the occurrence of ester hydrolysis requires an optimal number of carbon atoms in an ester. The reactivity and affinity of plasma esterases for a substrate are also dependent on the acyl and alkyl parts of the specific compound [4, 47]. Taking into account the small number of chlorambucil esters analyzed in the present study, it appears that aromatic esters are more rapidly hydrolyzed than aliphatic esters of equal carbon number and that large esters are more stable in plasma than shorter ones.

As predicted by their rapid in vitro hydrolysis in plasma, all of the agents studied quickly broke down in vivo to release chlorambucil following their i.v. administration in rats. Indeed, the rates of ester hydrolysis were so rapid that, with the exception of the chlorambucil-hexyl ester, none could be detected in plasma. The pharmacokinetics of each, with the exception of the chlorambucil-hexyl, -octyl, and -prednisolone esters, were identical to those obtained after the administration of equimolar chlorambucil.

The brain and plasma pharmacokinetics of chlorambucil in rats have recently been reported by Greig and Rapoport [18]. After i.v. administration, chlorambucil disappeared from plasma with a half-life of 26 min; primarily undergoing β-oxidation to phenylacetic mustard. Phenylacetic mustard was present in substantial amounts in plasma after 15 min and was the primary alkylating agent from 60 min onward. Neither these agents nor the intermediate metabolite, 3,4-dehydrochlorambucil, readily entered the brain. The brain : plasma concentration integral ratio, calculated between 5 and 240 min, of agents possessing alkylating activity following chlorambucil administration was 0.017 [18]. Our results indicate that the

brain : plasma concentration integral of compounds possessing alkylating activity was 0.011, calculated between 15 and 30 min after chlorambucil administration.

Administration of chlorambucil-hexyl, -octyl, and -prednisolone esters produced total concentrations of compounds possessing alkylating activity in plasma and brain that were significantly lower than those achieved after equimolar chlorambucil administration. The brain : plasma integral ratio of each concentration however, was similar and low. The presence of chlorambucil as the predominant alkylating agent in plasma following the administration of the chlorambucil esters, together with negligible amounts of the chlorambucil esters themselves, indicates that rapid hydrolysis of the esters occurs in vivo and in vitro. Indeed, our inability to detect chlorambucil-prednisolone ester in plasma is in accord with studies of Wilkinson et al. [50], who reported rapid metabolism of chlorambucil-prednisolone ester in human and rat plasma, and with studies of Newell et al. [36, 37], who failed to detect either unhydrolyzed drug in plasma following the subcutaneous administration of chlorambucil-prednisolone ester in rats or active drug in plasma following oral administration in man.

The achievement of only low plasma concentrations of total compounds possessing alkylating activity after the administration of chlorambucil esters, compared with equimolar chlorambucil administration, is probably due to an initially larger volume of distribution of the lipophilic ester derivatives. The combination of the presence of active drug in a predominantly ionic form due to rapid in vivo chlorambucil ester hydrolysis and the maintenance of only low concentrations of unbound active drug in plasma accounts for the low active drug concentrations in brain and the insignificant activity of chlorambucil and its esters against intracerebral implants of the alkylating agent-sensitive tumor Walker 256 carcinosarcoma. These factors may additionally account for the poor therapeutic response of chlorambucil-prednisolone ester in the treatment of patients with glioblastoma multiforme [1].

This study indicates that none of the lipophilic chlorambucil esters examined helped to deliver and maintain high concentrations of chlorambucil in the brain of rats. Despite differences in the abundance, reactivities, and affinities of plasma and tissue esterases between rats and humans [2, 4], we do not consider that any agent shows sufficient promise for administration in humans for brain tumor treatment. Nevertheless, analysis of these agents may be of value in the development of compounds with a sufficient plasma half-life and free, unbound concentration to achieve and maintain therapeutic brain concentrations in our ongoing evaluation of agents for the potential treatment of brain-sequestered tumors.

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