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Pharmacokinetics of chlorambucil-tertiary butyl ester, a lipophilic chlorambucil derivative that achieves and maintains high concentrations in brain

Nigel H. Greig, Eileen M. Daly, Daniel J. Sweeney, and Stanley I. Rapoport

Laboratory of Neurosciences, National Institute on Aging, National Institutes of Health, Bethesda, MD 20892, USA

Summary. Equimolar doses of chlorambucil (10 mg/kg) and the lipophilic chlorambucil derivative, chlorambucil-tertiary butyl ester (13 mg/kg), were given i.v. to rats. Plasma and brain concentrations of chlorambucil and its active metabolites, 3,4-dehydrochlorambucil and phenylacetic mustard, as well as of chlorambucil-tertiary butyl ester were then determined by HPLC between 2 and 240 min after drug administration. Chlorambucil demonstrated a monophasic disappearance from plasma following its administration, with a half-life of 28 min. Significant amounts of phenylacetic mustard were detected after 15 min, and this agent maintained high levels of active compounds in plasma throughout the study. Only low concentrations of chlorambucil and phenylacetic mustard were detected in brain between 2 and 120 min. Following equimolar chlorambucil-tertiary butyl ester administration, it rapidly disappeared from plasma, with a half-life of approximately 2 min, and maintained low plateau concentrations between 15 and 120 min after treatment. It was not detected thereafter, although significant amounts of chlorambucil and phenylacetic mustard were detected throughout the study. Significant amounts of chlorambucil-tertiary butyl ester entered and remained within the brain, achieving a peak concentration at 15 min and disappearing thereafter with a half-life of 37 min. Low levels of chlorambucil and phenylacetic mustard were also detected. Calculated from the areas under the concentration vs time curves of total active compounds derived from chlorambucil and chlorambucil-tertiary butyl ester in brain and plasma, the brain:plasma concentration integral ratios were 0.018 and 0.68, respectively. Following equimolar doses of chlorambucil and chlorambucil-tertiary butyl ester, a 7-fold greater concentration integral was achieved by chlorambucil-tertiary butyl ester in brain at a 5-fold lower plasma concentration integral. Chlorambucil-tertiary butyl ester may be of value in the treatment of brain-sequestered tumors.

Offprint requests to: Nigel H. Greig, Laboratory of Neurosciences, Bldg. 10, Rm 6C 103, National Institutes of Health, Bethesda, MD 20892, USA

Introduction

Malignant brain tumors remain virtually incurable despite numerous attempts to modify their dismal outcome. Recent epidemiological studies indicate that annual brain cancer mortality is rising in the elderly [10], which is the fraction of the population that has the highest incidence of all cancers [9], including those of the brain [3, 41, 44]. Primary brain tumors account for approximately 10% of all cancers, excluding those of the skin; the median survival of patients following surgery and radiation therapy is only 9 months [42, 43]. Furthermore, approximately 24% of all patients dying from cancer have metastatic brain tumors, including those of the dura [33]. Cerebral metastases are a manifestation of widespread dissemination of an extracerebral primary tumor throughout the body; consequently, the prognosis for such patients is bleak. Their median survival after surgery and/or radiation therapy is approximately 6 months [14, 24, 30, 33].

A number of studies have demonstrated that adjuvant chemotherapy, particularly with bis-chloronitrosourea (BCNU) can induce brain tumor regression [24, 43]. However, regression is temporary; consequently, the effect of such agents on patient survival has proved to be disappointing [14]. There are a number of reasons why treatment regimens that have been successful in the management of a variety of extracerebral tumors have proved to be of little therapeutic value for brain tumors. Total surgical resections are not possible in the brain, and nervous tissue is sensitive to the detrimental effects of radiation therapy. Furthermore, systemic chemotherapeutic agents must contend with problems related to delivery to their site of action [15], specifically, a low tumor blood flow and the presence of a variably intact blood-brain tumor barrier, which restricts the uptake of water-soluble drugs into tumors, particularly at their infiltrative edge within the normal brain [16]. These factors reduce the effectiveness of many potentially effective, water-soluble anticancer agents and biological response modifiers [17, 20, 21]. Such agents cannot reach and maintain therapeutic concentrations throughout brain tumors and are therefore of little value when combined with nitrosoureas in treating intracerebral tumors, which invariably comprise heterogeneic cell types.

In previous reports we described the brain and plasma pharmacokinetics and anticancer activities of chlorambucil [18], melphalan [18, 19] and several lipophilic chloram-

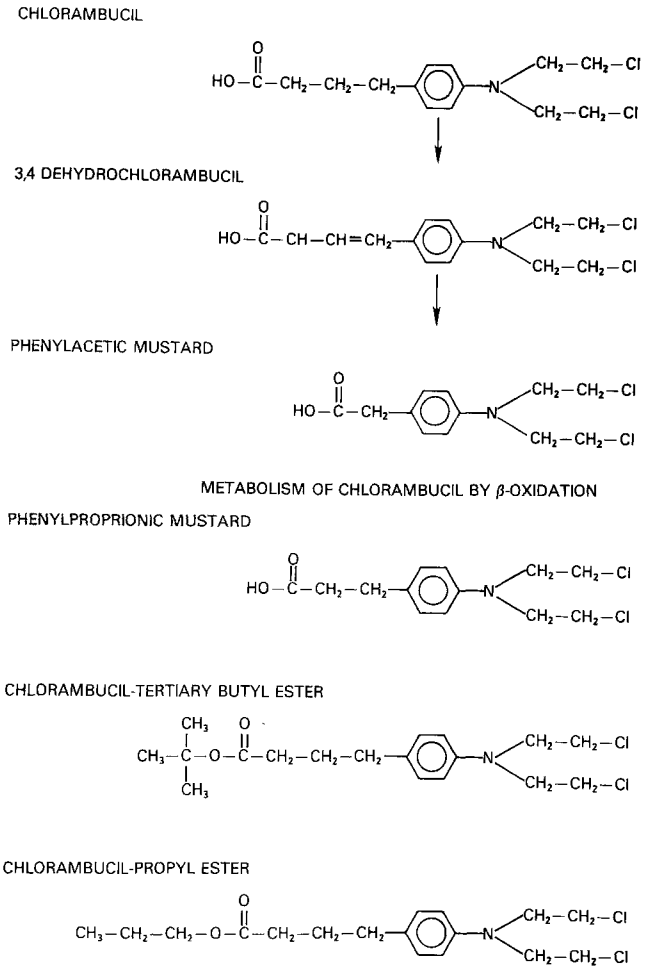


Fig. 1. Chemical structures of chlorambucil, its products of β-oxidation (3,4-dehydrochlorambucil and phenylacetic mustard), chlorambucil-tertiary butyl ester, and the internal standards phenylproprionic mustard and chlorambucil-propyl ester

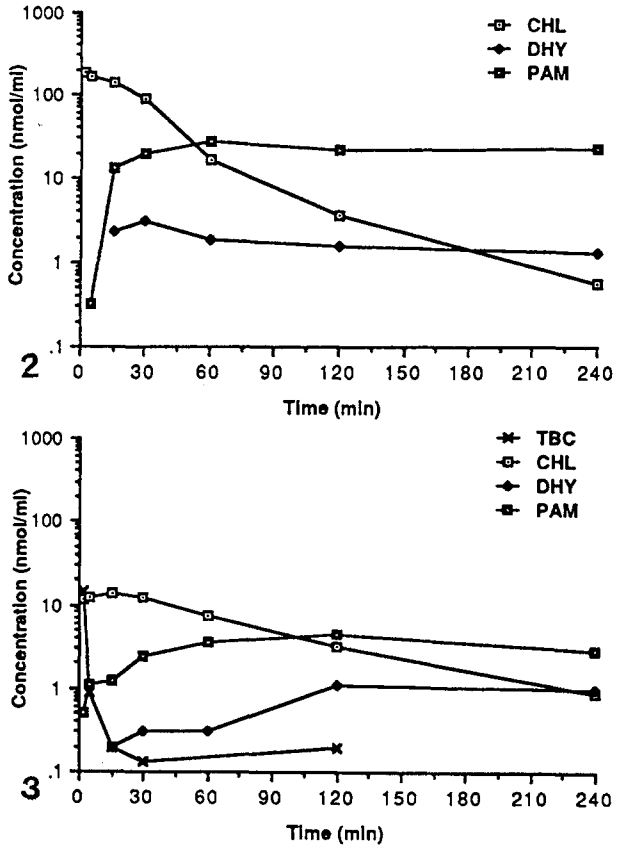
bucil-ester derivatives [22], as alkylating agents other than the nitrosoureas have proved to be active against extracerebral xenografts of primary brain tumors in immune-deprived animals [12, 39]. These agents are effective in the clinical treatment of malignant melanoma and cancer of the breast [8, 11], which frequently metastasize to the brain [16, 33]. However, none of these agents possesses the physicochemical properties required to achieve and maintain high brain concentrations. Both chlorambucil and melphalan are predominantly ionized in plasma at physiological pH [13, 18], and the chlorambucil esters are rapidly metabolized to chlorambucil in plasma prior to reaching the brain [22]. In the present paper we describe the physicochemical and pharmacokinetic characteristics of chlorambucil-tertiary butyl ester, a lipophilic chlorambucil derivative designed to remain sufficiently stable in plasma to enable significant accumulation in the brain.

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. Chlorambucil-tertiary butyl ester, 3-pentyl-4-[4-[bis(2-chloroethyl)amino]phenyl]butyrate, was synthesized from chlorambucil and tertiary butyl alcohol (Sigma) by reaction with *p*-toluenesulfonic acid (Sigma) in benzene under azeotropic conditions to remove water formed during the reaction. Formation of the chlorambucil-tertiary butyl ester was confirmed by analysis of nuclear magnetic resonance (NMR) data and infrared (IR) spectral bands. The compound was prepared as a hydrochloride salt and was >99% pure. Chlorambucil-propyl ester, 2-propyl-4-[2-chloroethyl)amino] phenyl]butyrate, was prepared by reaction of the appropriate alcohol with chlorambucil as previously described [22] and was used as an internal standard during HPLC analysis and quantitation of chlorambucil-tertiary butyl ester (Fig. 1). Methanol, propanol, acetone, and acetonitrile were of HPLC grade and were supplied by Burdick & Jackson Laboratories (Muskegon, Mich).

Pharmacokinetic study. Adult male Wistar rats (Charles River Laboratories, 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 or 13 mg/kg chlorambucil-tertiary butyl ester (equimolar to 10 mg/kg chlorambucil) in dimethyl sulfoxide was injected i.v. (250 μl/kg). From 2 to 240 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, together with a sample of brain, 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 chlorambucil-tertiary butyl ester were additionally analyzed for that compound.

Instrumentation, conditions, preparation, and extraction. HPLC analysis was carried out using a Waters Associates dual-pump system (Milford, Mass), described previously [18, 22]. The mobile phase for pump I was a mixture of water, acetic acid, and acetone (97.1:2:0.9, by vol.), and that for pump II was 97.5% acetonitrile and 2.5% water (containing 2% acetic acid). The flow rate, produced at 30% from pump I and 70% from pump II, was set at 1.8 ml/min, 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 I and 90% from pump II. Retention times were: phenylacetic mustard, 4.5 min; phenylproprionic mustard, 5.5 min; 3,4-dehydrochlorambucil, 6.3 min; chlorambucil, 7.0 min; chlorambucil-propyl ester, 13.3 min; and chlorambucil-tertiary butyl ester, 16 min (Fig. 1). Concentrations of chlorambucil-tertiary butyl ester and chlorambucil and its metabolites were calculated from the ratio of their peak height measurements with their appropriate internal standard. These were then quantified



Figs. 2, 3. Plasma concentrations of chlorambucil (*CHL*), 3,4-dehydrochlorambucil (*DHY*), phenylacetic mustard (*PAM*), and chlorambucil-tertiary butyl ester (*TBC*) following i. v. administration of **2** 10 mg/kg chlorambucil and **3** 13 mg/kg equimolar chlorambucil-tertiary butyl ester in the rat. Total concentrations of active agents in plasma and individual concentrations of chlorambucil, 3,4-dehydrochlorambucil, and phenylacetic mustard were significantly greater following chlorambucil administration than after chlorambucil-tertiary butyl ester, with the exception of chlorambucil concentrations at 120 and 240 min ($P < 0.05$)

from calibration curves of six points, two samples per point, which were run daily and interspersed among the unknown samples. Samples were maintained at 4° C to ensure that no ester hydrolysis occurred during sample preparation and extraction procedures, which have previously been described [22].

Alkylating activity. The alkylating activity of chlorambucil-tertiary butyl ester was determined at concentrations between 0.5 and 25 m*M* by reaction with *p*-nitrobenzyl pyridine (Sigma), as previously described [22], and compared with that of equimolar chlorambucil.

Plasma protein binding. The percentage of binding of chlorambucil-tertiary butyl ester to plasma proteins was measured at concentrations between 1 and 100 mmol/ml by centrifugal ultrafiltration, as previously described [22], using Amicon centrifree micropartition systems (Amicon Corp., Danvers, Mass).

Calculations. Brain concentrations of chlorambucil-tertiary butyl ester and chlorambucil and its active metabolites were calculated from the net regional brain concentrations, as measured with HPLC, by subtracting the intravascular volume 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 plasma 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) as previously described [18, 20–22]. Regional blood volume was calculated by dividing the [^{14}C]-activity in a brain sample by that in blood ($dpm \cdot g^{-1}/dpm \cdot ml^{-1}$) of animals killed at 2 min and equalled 1.7%. [^{14}C]-Methyl bovine serum albumin (mol. wt., 69,000 daltons) remains within the cerebral vasculature during a 2-min experiment [34].

Chlorambucil and chlorambucil-tertiary butyl ester concentration-vs-time data were fitted by nonlinear regression analysis [28] to a single exponential equation:

$$C = Ae^{-\alpha\tau},$$

where *c* equals the concentration of compound (nmol/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}) [28]. Plasma half-life ($t^1/_2$) was calculated from the parameters by the general formula

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

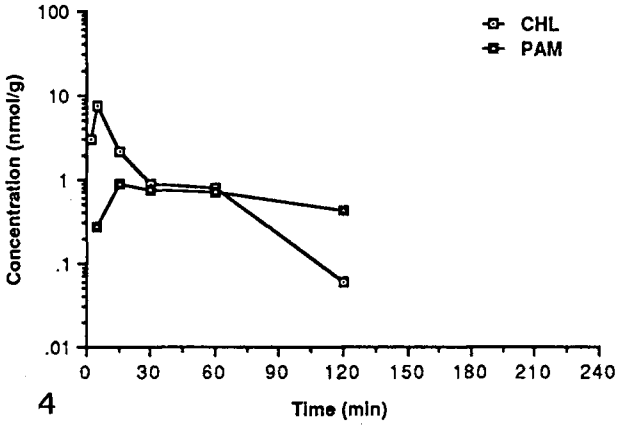
Areas under the concentration-time profiles (between 2 and 240 min) were calculated by the trapezoidal rule [40].

Statistical analysis. A two-tailed Student's *t*-test was carried out to compare two means. When more than two means were compared, one-way analysis of variance and the Bonferroni multiple-test were used [29]. Statistical significance for all tests was taken as $P < 0.05$. Unless otherwise stated, means ± SE are given routinely.

Results

Figures 2 and 3 illustrate the plasma vs time profiles of chlorambucil-tertiary butyl ester, chlorambucil, 3,4-dehydrochlorambucil, and phenylacetic mustard following the i. v. administration of chlorambucil (10 mg/kg) and equimolar chlorambucil-tertiary butyl ester, respectively. After i. v. administration of chlorambucil, it demonstrated a monophasic disappearance from plasma, with a half-life of 28 min. Low concentrations (approximately 2 nmol/ml) of 3,4-dehydrochlorambucil, the intermediate metabolite of β-oxidation, were detected after 15 min. Phenylacetic mustard, the active end metabolite of β-oxidation, was detected throughout the study; it reached a peak concentration of 27.8 nmol/ml at 60 min. The area under the concentration vs time curve of total compounds possessing anticancer activity, derived from chlorambucil, was 11,835 nmol·min/ml.

Following i. v. administration of chlorambucil-tertiary butyl ester in rats, it disappeared rapidly from plasma, with a half-life of 2 min. Low concentrations of the ester (approximately 0.2 nmol/ml) were detected between 15 and 120 min but not thereafter. Significant amounts of chlorambucil were present in plasma throughout the study. A peak concentration of 13.8 nmol/ml was achieved at



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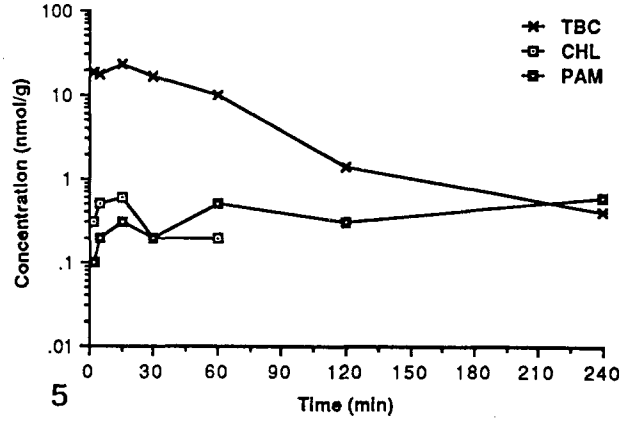

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Figs. 4, 5. Brain concentrations of chlorambucil (*CHL*), phenylacetic mustard (*PAM*), and chlorambucil-tertiary butyl ester (*TBC*) following i. v. administrationtion of **4** 10 mg/kg chlorambucil and **5** 13 mg/kg equimolar chlorambucil-tertiary butyl ester in the rat. Total concentrations of active agents derived from chlorambucil-tertiary butyl ester administration were significantly greater than those from chlorambucil ($P < 0.05$)

15 min; thereafter, it disappeared from plasma monophasically, with a half-life of 56 min. Low amounts of 3,4-dehydrochlorambucil and phenylacetic mustard were detected after 15 min and throughout the study, respectively. The area under the concentration vs time curve of total compounds possessing anticancer activity, derived from chlorambucil-tertiary butyl ester, was 2,225 nmol·min/ml.

Figures 4 and 5 illustrate the brain concentration vs time profiles of chlorambucil-tertiary butyl ester, chlorambucil, and phenylacetic mustard following i. v. administration of chlorambucil (10 mg/kg) and equimolar chlorambucil-tertiary butyl ester, respectively. A peak chlorambucil concentration of 7.6 nmol/g was achieved 5 min following its administration, after which brain levels declined, with a half-life of 19 min, and could not be detected after 120 min. Low amounts of phenylacetic mustard (approximately 0.5 nmol/g) were present up to 120 min, and no 3,4-dehydrochlorambucil was detected. The area under the brain concentration vs time curve of total compounds possessing anticancer activity, derived from chlorambucil, was 216 nmol·min/g.

Significant amounts of chlorambucil-tertiary butyl ester were detected and maintained in brain following the administration of this agent. A peak concentration of 22.7 nmol/g was achieved at 15 min; thereafter, it disappeared from the brain following a single exponential, with a half-life of 37 min. Low amounts of chlorambucil and phenylacetic mustard were detected between 2 and 120 min and after 15 min, respectively. 3,4-Dehydrochlorambucil was not detected in brain at any time. The area under the brain concentration vs time curve of total compounds possessing anticancer activity, derived from chlorambucil-tertiary butyl ester, was 1,509 nmol·min/g, a value >7 times that obtained after equimolar chlorambucil administration. Chlorambucil-tertiary butyl ester possessed 40% of the alkylating activity of chlorambucil and proved to be >99% plasma protein-bound at concentrations achievable in vivo.

Discussion

There have been several attempts to design lipophilic derivatives of chlorambucil [35] to increase its brain delivery [22, 23], as ionized and heavily plasma protein-bound drugs such as chlorambucil [18] minimally enter the brain, whereas lipophilic and unrestrictively bound agents generally reach and maintain significant intraparenchymal concentrations [17]. We have previously described the brain and plasma pharmacokinetics of chlorambucil [18], and our present studies confirm that the brain uptake of chlorambucil and its active metabolites is minimal after i. v. administration. Calculated from the concentration vs time profiles, chlorambucil has a brain/plasma concentration integral ratio of 0.023, and that of total active compounds derived from chlorambucil is 0.018. These values compare favorably with previous reports [18]. As a consequence, the activity of chlorambucil against intracerebral tumors that are sensitive to alkylating agents is minimal [18].

In our initial attempts to increase the brain uptake of chlorambucil, we analyzed and reported the physicochemical characteristics, brain and plasma levels, and intracerebral activities of seven lipophilic chlorambucil derivatives. These included a homologous series of chlorambucil alkyl esters (length between one and eight carbons), three aromatic esters, which included a phenylmethyl and phenylethyl ester, and the prednisolone ester, prednimustine. None reached or maintained significantly greater brain levels than did chlorambucil. Short-chain alkyl and aromatic esters were rapidly metabolized by plasma esterases to chlorambucil prior to significant delivery to the brain, and this may explain the ineffectiveness of prednimustine in the treatment of patients with glioblastoma multiforme [2]. Longer-chain alkyl esters, (length, six and eight carbons) proved to be more stable but bound extensively to plasma constituents, which restricted their brain penetration. The results of these studies are in accord with attempts to design lipophilic derivatives of methotrexate to enhance its brain uptake. Short dialkyl esters of methotrexate rapidly regenerate methotrexate, whereas longer-chain esters bind extensively and restrictively to plasma constituents, which minimizes the achievement of intraparenchymal concentrations of methotrexate and methotrexate ester [25, 36, 37]. Finally, the linkage of chlorambucil to a dihydropyridine $\rightleftharpoons$ pyridinium redox system, which has been suggested by Bodor and Brewster [6] to increase the delivery of water-sluble compounds to the brain, proved to be ineffective, as brain levels of drug were

not significantly different from those achieved after chlorambucil administration [23].

Greig [15, 17] has reported that optimal drug delivery and retention in the brain are achieved by the maintenance of high, unbound levels of unionized drug in plasma, increasing the integrated exposure (time × concentration). However, due to large amounts of unspecific aryl- and allyl-esterases in plasma and liver [1, 4, 5, 7], it is difficult to sustain significant and steady-state levels of ester derivatives of drug in plasma in vivo. To overcome this problem, we synthesized and tested a branched ester derivative, chlorambucil-tertiary butyl ester, whose steric hindrance reduces the rate of ester hydrolysis.

Following i.v. administration of the chlorambucil-tertiary butyl ester, it reached and maintained significant concentrations in the brain: peak levels were 3-fold greater than those achieved after equimolar chlorambucil administration, and its half-life was 2-fold longer than that of chlorambucil. As a consequence, the AUC of active compounds derived from chlorambucil-tertiary butyl ester in brain was 7-fold greater than that derived from equimolar administration of chlorambucil, and this was achieved with an AUC of total active compounds in plasma that was 5-fold less than that derived from chlorambucil.

The initial rapid disappearance of chlorambucil-tertiary butyl ester from plasma ($t_{1/2}$, 2 min) was probably due to (a) the large volume of distribution of lipophilic drugs, which accounts for the significantly lower plasma concentration of total active compounds derived from chlorambucil-tertiary butyl ester compared with equimolar chlorambucil, and (b) continuous plasma and tissue de-esterification of chlorambucil-tertiary butyl ester to chlorambucil. By comparing the plasma half-lives of chlorambucil derived from ester hydrolysis of chlorambucil-tertiary butyl ester and that derived from chlorambucil administration alone (56 and 28 min, respectively), it is evident that chlorambucil-tertiary butyl ester slowly and continuously back-diffuses from lipid stores into plasma and is enzymatically cleaved to chlorambucil. In accordance with this, total active compounds in plasma predominantly occurred in the form of chlorambucil after the administration of chlorambucil-tertiary butyl ester.

Conversely, in brain, total active compounds predominantly appeared in the form of chlorambucil-tertiary butyl ester itself, although small amounts of chlorambucil and phenylacetic mustard were also detected. The brain : plasma concentration vs time integral ratios of chlorambucil after chlorambucil-tertiary butyl ester administration and chlorambucil administration were identical (0.023). This suggests that the chlorambucil detected in brain after chlorambucil-tertiary butyl ester administration was derived chiefly from its penetration across the blood-brain barrier from plasma rather than from the ester hydrolysis of chlorambucil-tertiary butyl ester within the brain. Accordingly, the ester cleavage of chlorambucil-tertiary butyl ester in the brain appears to be slow. Its disappearance from the brain is therefore primarily due to back-diffusion into plasma and inactivation by hydrolysis of the chloroethyl moieties, rather than to ester cleavage.

Calculated from the AUCs of the concentration vs time profiles, chlorambucil-tertiary butyl ester has a brain: plasma concentration integral ratio of 33.4, which is 1,450-fold greater than that of chlorambucil. This ratio is similar to that of lipophilic and centrally acting drugs, such as the dopamine antagonist haloperidol, which has a brain: plasma concentration ratio of approximately 30 [26] and is used clinically as an antipsychotic agent. Although chlorambucil-tertiary butyl ester, like chlorambucil and other chlorambucil esters [22], binds extensively to plasma proteins, this binding appears to be non-restrictive [17] and enables its free entry into the brain. Reaction of chlorambucil-tertiary butyl ester with *p*-nitrobenzyl pyridine [13, 22] demonstrates that chlorambucil-tertiary butyl ester, like chlorambucil [38], possesses intrinsic alkylating capacity, as would be predicted from its structure. As with chlorambucil and prednimustine [18, 27, 31], one would expect the biological activity of chlorambucil-tertiary butyl ester to derive from the combined actions of the compound itself and its transitory alkylating metabolites, chlorambucil, 3,4-dehydrochlorambucil, and phenylacetic mustard. Whereas the alkylating activity of chlorambucil-tertiary butyl ester is lower than that of chlorambucil, its greater lipophilicity may enable a higher intracellular uptake and the achievement of similar or greater activity, compared with chlorambucil. The brain:plasma concentration integral ratio of total active compounds derived from chlorambucil-tertiary butyl ester was 0.68. This ratio is 35-fold greater than that derived from the administration of chlorambucil and compares favorably with that of BCNU (approximately 0.3) [32].

Analysis of the pharmacokinetics of the novel lipophilic chlorambucil derivative, chlorambucil-tertiary butyl ester, indicates that it reaches and maintains significant concentrations of compounds possessing alkylating activity in the brain and may therefore be of value in brain tumor chemotherapy. As levels of active agents in plasma are significantly lower than those derived from the administration of equimolar chlorambucil, it may be possible to deliver higher initial doses of chlorambucil-tertiary butyl ester before myelosuppression becomes dose-limiting, thereby further increasing brain concentrations. We are currently examining the systemic and intracerebral toxicity and anticancer activity of chlorambucil-tertiary butyl ester in the rat.

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