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Improved delivery through biological membranes. XLI. Brain-enhanced delivery of chlorambucil

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Summary

Brain-enhanced delivery of chlorambucil was achieved using a dihydropyridine pyridinium salt chemical delivery system (CDS). Application of the CDS approach to the carboxylic acid-containing anticancer agent required the development of novel, alcoholic redox carriers. Several *N'*-(ω-hydroxyalkyl), -(ω-hydroxycycloalkyl) and -(ω-hydroxy branched alkyl)nicotinamide derivatives were therefore synthesized. After in vitro characterization of the dihydropyridine delivery forms of chlorambucil, these compounds were tested in vivo in the rat. The CDS derivative in which an ethyl group separated the 1-methyl-1,4-dihydronicotinamide and chlorambucil fragments generated sustained levels of chlorambucil in the brains of test animals after i.v. administration ($t_{1/2}$ in brain = 2.4 days) while blood levels rapidly fell ($t_{1/2}$ = 2 h) producing a favorable brain/blood ratio. This compound (**1d**) was well tolerated at doses of 60 mg/kg while equimolar chlorambucil (39 mg/kg) caused greater than 80% mortality in test animals within 2 h. Subsequently, a cyclohexyl-containing CDS derivative was tested. This sterically more hindered system produced a lower level of chlorambucil in the periphery but also reduced central nervous system (CNS) concentration of the drug.

Introduction

Primary and secondary metastatic tumors of the central nervous system (CNS) represent a major health problem. It has been estimated that the incidence of these cancers exceeds 12.5 per 100,000 U.S. residents, meaning that more than 27,000 new cases of these usually fatal diseases occur annually (NINCDS, 1977). In addition, 24% of all people dying from peripheral cancers have intracranial metastases (Posner, 1977). While neurosurgery can be useful, pharmacological intervention has, by far, the broadest application (Neuwelt and Frenkel, 1980). Unfortunately, many potentially useful drugs including those active against peripheral tumors cannot enter the brain and are therefore ineffective in treating cerebral neoplasms. The basis of this impermeability is the blood–brain barrier (BBB).

The BBB results from the tight joining of the cerebral capillaries eliminating intracellular transport of most substances (Rapoport, 1976; Suckling et al., 1986; Pardridge et al., 1986). This ultrastructural architecture forces compounds to pass through the phospholipoidal cell membranes if they are to access the brain parenchyma and this excludes all but the most lipophilic molecules. It has been shown that for compounds with octanol : water partition coefficients less than 0.1,

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penetration into the CNS is limited by BBB permeability (Greig, 1987; Fenstermacher et al., 1981). Compounds in this category include many useful antineoplastic agents such as melphalan and chlorambucil (Greig et al., 1988).

One method for improving BBB transit for these and similar drugs is by prodrug formation (Bundgaard, 1985; Stella et al., 1985; Bodor and Kaminski, 1987). By esterifying the carboxylic acid of chlorambucil, for example, the lipophilicity of the drug can be augmented and high brain concentrations can be obtained. Unfortunately, as has been pointed out previously, there is little organ selectivity with simple prodrugs and while BBB transit may be enhanced, the extraction of the cytotoxic agent into other body compartments is likewise increased (Stella and Himmelstein, 1980). This increased tissue burden often translates into higher toxicity for the drug with no net increase in the therapeutic index of the compound. A more involved chemical latentiation for increasing brain delivery and selectivity is the chemical delivery system (CDS) approach (Bodor et al., Bodor and Brewster, 1983; Bodor, 1984, 1987).

In this method, a molecular carrier is covalently linked to the drug to be transported. Derivatives of 1-substituted-1,4-dihydronicotinic acids have been most used in this capacity. After systemic administration, the increased lipophilicity imparted by the above-mentioned chemical modifications allows for an increased tissue distribution so that the drug conjugate can enter compartments, such as the CNS, inaccessible to the parent compound. With time, the enzymatically labile carrier is oxidized to give the corresponding trigonellinate (1-methylnicotinate) salt. This conversion results in lipophilicity changes (log P) of between three and five orders of magnitude and acts to accelerate the peripheral elimination of the drug conjugate from the systemic circulation. In the brain, the hydrophilic drug-oxidized carrier conjugate is trapped behind the BBB. The "locked-in" salt can subsequently hydrolyze releasing the active drug and the oxidized carrier molecule which is readily lost from the cerebrospinal fluid (CSF) via active mechanisms (Bodor et al., 1986). The released drug can then act at the tumor site. This scheme allows for the enhanced delivery of cytotoxic agents to the CNS thereby sparing the periphery and increasing the therapeutic index of the drug. The carrier molecules in and of themselves are innocuous in the CNS (Brewster et al., 1988b; Brewster et al., 1988c). The present report details the application of the CDS to a widely used antineoplastic agent, chlorambucil.

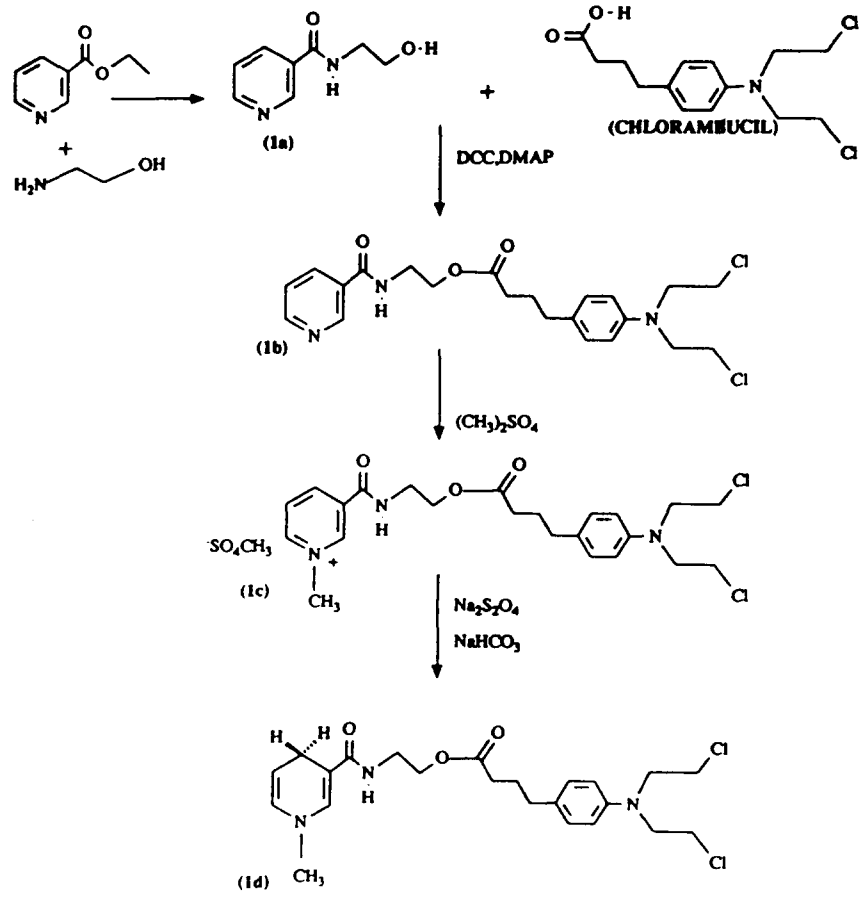
Materials and Methods

Elemental analysis of compounds synthesized were performed by Atlantic Microlab, Atlanta, GA. Melting points were determined using Hoover–Thomas capillary melting point apparatus and were uncorrected. Nuclear magnetic resonance spectra were recorded on a Varian EM360 Spectrometer. Samples were dissolved in an appropriate deuterated solvent and chemical shifts (δ) reported relative to an internal standard (tetramethyl silane, TMS). Ultraviolet (UV) spectroscopy was performed on a Hewlett Packard 8451A Diode array spectrophotometer. All chemicals used were of reagent grade. Chlorambucil was obtained from Sigma Chemical Co., St. Louis, MO.

*Preparation of **1a–1d** (Scheme 1)*

*N-4(hydroxyethyl)pyridine-3-carboxamide **(1a)**.* A mixture containing ethyl nicotinate (70.0 g, 0.46 mol) and ethanolamine (28.29 g, 0.46 mol) was heated at reflux overnight. A light yellow solid was formed. The reaction mixture was then cooled. The solid was filtered, washed with dry ether and crystallized from isopropanol. The white hygroscopic crystals were filtered under argon, washed with ether and dried over P_2O_5. Yield 90%, m.p. 90–91°C. NMR (d_6DMSO) δ 9.2–9.0 (br s, 1H, C-2, pyridine H); 8.8–8.4 (m, 2H, C-6, pyridine H + –NH); 8.3–8.1 (m, 1H, C-4, pyridine H); 7.6–7.3 (m, 1H, C-5 pyridine H); 5.1–4.7 (t, 1H, OH, exchanges with D_2O); 3.9–3.3 (m, 4H, CH_2–CH_2, alkyl side chain H).

*N{1[-4([4-[bis(2-chloroethyl)]amino)phenyl)butanoyloxy]ethyl}-pyridine-3-carboxamide **(1b)**.* Chlorambucil (2.5 g, 0.08 mol) was dissolved in 80 ml of dry acetonitrile. To this was added 1.5 g



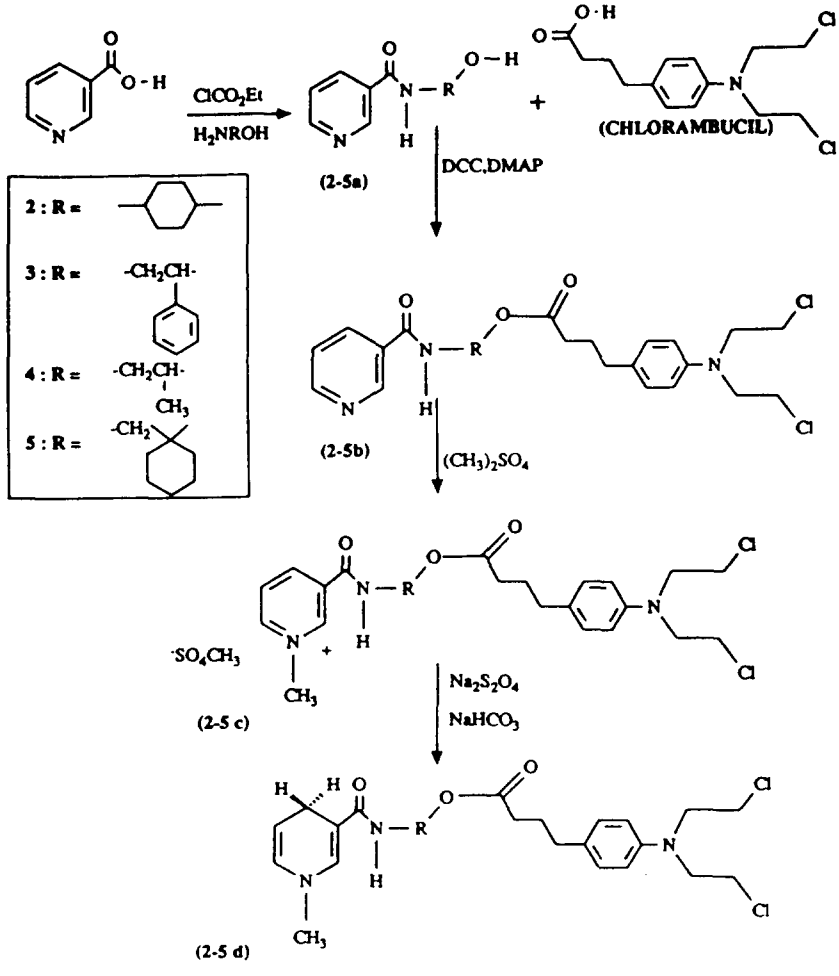
Scheme 1

(0.009 mol) of **1a**. To the stirred solution kept over argon was added 1.86 g (0.009 mol) DCC and DMAP (0.1 g, 0.008 mol). The reaction mixture was stirred overnight at room temperature and under argon. At the end of the reaction the solid DCU that was formed was filtered and washed with 25 ml cold acetonitrile. The filtrate was evaporated in vacuo in low heat. The yellow residue obtained was applied to a column packed with 100 g of silica gel (Davisil, grade 634, mesh 100–200). Elution with $CHCl_3$–THF (8 : 2) yielded the product. A yellow solid is obtained upon evaporation of the eluent in vacuo. Yield 82.7%, m.p. 73–75 °C. NMR ($CDCl_3$) 9.1–8.9 (d, 1H, C-2, pyridine H); 8.7–8.5 (dd, 1H, C-6, pyridine H); 8.2–8.1 (dt, 1H, C-4, pyridine H); 7.5–7.3 (m, 1H, C-5, pyridine H); 7.2–6.8 (AB, quartet 4H, chlorambucil phenyl H); 4.4–4.1 (br t, 2H, $-CH_2-O-C$); 3.9–3.5 (br s, 10H, 2H CH_2–NH, 8H, N–(CH_2–$CH_2Cl)_2$); 2.6–1.6 (m, 6H, chlorambucil butyl H). Anal. C_{22} $H_{27}Cl_2N_3O_3$. Theory: C 58.41; H 6.01; N 9.28, Cl 15.67. Found: C 58.31; H 5.90; N 9.17; Cl 15.54.

1-Methyl-3-({N-[4-{[4-(bis(2-chloroethyl)]amino}phenyl)butanoyloxy]ethyl}carbamoyl)pyridinium methane sulfate (1c). To 2 g, (0.04 mol) **1b** in 200 ml of dry acetonitrile was added dimethyl sulfate (0.613 g, 0.04 mol) and the mixture was heated at reflux overnight under dry conditions. The solvent was removed in vacuo and the residue obtained was washed with dry ether. A red viscous liquid was obtained. Yield, 97%. NMR ($CDCl_3$) 9.4–9.3 (s, 1H, C-2, pyridinium H); 9.2–8.7 (m, 2H, C-4, C-6, pyridinium H); 8.3–7.9 (m, 1H, C-5,

pyridinium H); 7.2–6.6 (AB quartet, 4H, chlorambucil phenyl H); 4.6–4.4 (br s, 3H, CH_3SO_4); 4.3–4.1 (br t, 2H, $-CH_2-O-C$); 3.8–3.4 (br s, 13H, 3H, CH_3, 2H, CH_2-NH, 8H, $N-(CH_2-CH_2Cl)_2$); 2.6–1.6 (m, 6H, chlorambucil butyl H).

*1-Methyl-3-{ {N-[4-{ {4-[bis(2-chlorethyl)]amino}phenyl-butanoyloxy] ethyl} carbamoyl) 1,4-dihydropyridine (**1d**).* The methylsulfate quaternary salt (**1c**) (2.49 g, 0.004 mol) was dissolved in 350 ml ice-cold water flushed with nitrogen. To this solution was added 150 ml ethyl acetate, 2.17 g (0.026 mol) of $NaHCO_3$ and 2.97 g (0.017 mol) of $Na_2S_2O_4$. The mixture was stirred at 5 °C for 1 h. At the end of the reaction period the two layers were separated. The aqueous layer was extracted 3 times with 50 ml portions of ethyl acetate. The combined organic extracts were dried over $MgSO_4$ and evaporated to dryness. The residue was applied to a short (20 g) neutral alumina column (100–140 mesh) and eluted with chloroform. The product was obtained within the first 50 ml of eluent. The solvent was evaporated to dryness and the residue washed with anhydrous ether and dried to give a light yellow solid. Yield 70%, m.p. 90–92 °C. UV (MeOH) 218, 358 nm. NMR ($CDCl_3$) 7.2–6.5 (AB quartet, 5H, C-2, pyridine H +4 chlorambucil phenyl H); 5.7–5.4 (m, 1H, C-6, pyridine H); 4.9–4.4 (m, 1H, C-5, pyridine H); 4.3–4.1 (t, 2H, $-CH_2-O-C$); 3.8–3.5 (br s, 10H: 2H, CH_2-NH, 8H, $(NCH_2CH_2Cl)_2$); 3.4–3.1 (m,



Scheme 2

2H, C-4, pyridine H); 2.9–2.7 (s, 3H, N–CH_3); 2.7–1.7 (m, 6H, chlorambucil butyl H). Anal. $C_{23}H_{31}Cl_2N_2O_3$. Theory: C, 58.97; H, 6.67; Cl 15.13; N, 8.97. Found: C, 58.90; H, 6.72; N, 8.94; Cl 15.05.

General procedure for the synthesis of N-(4-hydroxyalkyl)pyridine-3-carboxamides ***2a–5a*** *(Scheme 2)*

Nicotinic acid was suspended in dry THF. One equivalent of freshly distilled triethylamine was added. The clear solution obtained was cooled to −4°C. One equivalent of ethyl chloroformate in THF was added (with the exclusion of moisture) such that the temperature of the reaction did not exceed 0°C. One equivalent of the appropriate amino alcohol was added directly to the above reaction mixture. The reaction mixture was then allowed to warm to room temperature and stirred for 2 h. The precipitated triethylamine hydrochloride was removed by filtration. The filtrate was evaporated under reduced pressure and the product recrystallized from isopropanol to give:

N-(4-hydroxycyclohexyl)pyridine-3-carboxamide ***(2a)***. Yield 85%, m.p. 208–210°C. NMR d_6-DMSO 9.2–9.1 (d, 1H, C-2, pyridine H); 8.9–8.7 (dd, 1H, C-6, pyridine H); 8.5–8.4 (br s, 1H, C–NH); 8.3–8.1 (dt, 1H, C-4, pyridine H); 7.6–7.4 (m, 1H, C-5, pyridine H); 4.7–4.5 (br d, 1H, OH (D_2O exchangeable); 3.8–3.3 (m, 2H, trans 1.6-cyclohexyl H); 2.1–1.1 (m, 8H, cyclohexyl H). Anal. $C_{12}H_{16}N_2O_2$. Theory: C 65.43; H 7.32; N 12.71. Found: C 65.37; H 7.35; N 12.65.

N-(2-hydroxy-2-phenyl)ethyl-3-pyridine carboxamide ***(3a)***. Yield 91%, m.p. 122–124°C. NMR d_6-DMSO 9.2–9.1 (d, H, C-2, pyridine H); 8.7–8.4 (br dd, 1H, C-6, pyridine H + C-NH); 8.3–8.0 (dt, 1H, C-4, pyridine H); 7.5–7.1 (m, 6H, C-5, pyridine H + phenyl H); 5.4–5.1 (br d, OH, (D_2O exchangeable)); 5.0–4.7 (q, 1H, CH); 3.7–3 (m, 2H, CH_2). Anal. $C_{14}H_{14}N_2O_2$. Theory: C 69.40; H 5.82; N 11.56. Found: C 69.34; H 5.87; N 11.52.

N-(2-hydroxy)propyl-3-pyridine carboxamide ***(4a)***. Yield 85%, m.p. 40–41°C. NMR d_6-DMSO 9.2–9.1 (d, 1H, C-2, pyridine H; 8.7–8.5 (dd, 1H, C-6, pyridine H); 8.3–7.9 (dt, 2H, NH + C-4, pyridine H); 7.4–7.2 (m, 1H, C-5, pyridine H); 5.1–4.9 (br s, 1H, OH, (D_2O); 4.3–3.9 (m, 1H, CH); 3.3–2.9 (m, 2H, CH_2); 1.6–1.4 (d, 3H, CH_3). Anal. $C_9H_{12}N_2O_2$. Theory: C 59.98; H 6.71; N 15.54. Found: C 59.82; H 6.62; N 15.39.

N-(1-hydroxy)cyclohexyl-1-methyl-3-pyridinecarboxamide ***(5a)***. m.p. 110–112°C. NMR d_6-DMSO 9.2–9.1 (d, 1H, C-2, pyridine H); 8.7–8.5 (dd, 1H, C-6, pyridine H); 8.2–8.0 (dt, 1H, C-4, pyridine H); 7.5–7.2 (m, 2H, NH + C-5, pyridine H); 3.8–36.6 (br s, 1H, OH, D_2O exchangeable); 3.5-3.3 (br m, 2H, CH_2); 1.5–1.3 (br s, 10H, cyclohexyl H). Anal. $C_{13}H_{18}N_2O_2$. Theory: C 66.43; H 7.74; N 11.95. Found: C 66.59; H 7.79; N 11.86.

General procedure for the condensation of chlorambucil with various amino alcohols ***(2b–5b)***.

Chlorambucil was mixed with 1.1 equivalents of an appropriate amino alcohol, one equivalent of DCC and 0.1 equivalent of DMAP in dry acetonitrile. The reaction mixture was stirred at room temperature in the presence of argon for two days. Precipitated DCU was removed by filtration. The filtrate was evaporated to dryness and the residue applied to a silica gel column as described earlier and eluted with $CHCl_3$: THF 8 : 2. The appropriate eluting fractions were combined and evaporated to dryness in vacuo.

N{1-[4([4-[bis(2-chloroethyl)]amino}phenyl)butanoyloxy]cyclohexyl}-pyridine-3-carboxamide ***(2b)***. Yield 81%, m.p. 120–122°C. NMR ($CDCl_3$) 9.1–9.0 (d, 1H, C-2, pyridine H); 8.8–8.6 (dd, 1H, C-6, pyridine H); 8.3–8.1 (dt, 1H, C-4, pyridine H); 7.7 (br s, 1H, NH); 7.6–7.3 (m, 1H, C-5, pyridine H); 7.2–6.5 (AB quartet 4H, chlorambucil phenyl H); 4.9–4.4 (br m, 2H, cyclohexyl trans H); 3.7–3.4 (br s, 8H, $N(CH_2CH_2Cl)_2$); 2.6–1.3 (m, 14H, cyclohexyl + chlorambucil butyl H). Anal. $C_{26}H_{33}Cl_2N_3O_2$ 1/4 H_2O. Theory: C 63.00; H 5.95; N 7.88; Cl 13.33. Found: C 62.87; H 5.99; N 7.78; Cl 13.11.

N-({2-phenyl-2-[4-[4-(bis-2-chloroethyl)]amino phenyl)butanoyloxy]ethyl}-3-pyridinecarboxamide ***(3b)***. Yield 70%, m.p. 99–101°C. NMR $CDCl_3$ 9.1–8.9 (d, 1H, C-2, pyridine H); 8.7–8.5 (dd, 1H, C-6, pyridine H); 8.2–8.0 (dt, 1H, C-4, pyridine H); 7.8 (s, 1H, NH); 7.5–7.2 (m, 6H, C-5, pyridine H + phenyl H) 7.1–6.5 (AB quartet, 4H, chlorambucil phenyl H); 6.1–5.9 (t, 1H, CH); 4.1–3.7

(m, 2H, CH_2); 3.6–3.5 (br s, 8H, N, $(CH_2CH_2Cl)_2$); 2.6–1.6 (m, 6H, butyl H). Anal. $C_{28}H_{31}Cl_2N_3O_3$. Theory: C 63.64; H 5.91; Cl 13.42; N 7.95. Found: C 63.50; H 5.99; Cl 13.11; N. 7.78.

N-{2-[4-(4-[bis-(2-chloroethyl)amino}phenyl)butanoyloxy]propyl}-3-pyridinecarboxamide ***(4b)***. Yield 84%. NMR ($CDCl_3$) 9.1–8.9 (d, 1H, C-2, pyridine H); 8.7–8.5 (dd, 1H, C-6, pyridine H); 8.2–7.9 (dt, 1H, C-4, pyridine H); 7.7–7.5 (m, 2H, C-5, pyridine H + NH); 7.2–6.4 (AB quartet, 4H, chlorambucil phenyl H); 5.3–4.9 (m, 1H, CH); 3.6–3.4 (br s, 10H, CH_2 + N, $(CH_2CH_2Cl)_2$); 2.6–1.6 (m, 6H, butyl H) 1.6–1.4 (d, 3H, CH_3). Anal. $C_{23}H_{29}Cl_2N_3O_3$. Theory: C 59.23; H 29.22; N 9.00; Cl 15.20. Found: C 59.08; H 29.34; N 8.86; Cl 14.98.

N-({1-cyclohexyl-2-[4-[4-{bis-2-chloroethyl]amino}phenyl)butanoyloxy]methyl}-2-pyridine carboxamide ***(5b)***. Yield 40%, m.p. 92–94°C. NMR ($CDCl_3$). 9.2–9.0 (d, 1H, C-2, pyridine H); 8.8–8.6 (dd, 1H, C-6, pyridine H); 8.5–8.3 (dt, 1H, C-4, pyridine H); 7.5–7.3 (m, 2H, C-5, pyridine H + NH); 7.2–6.5 (AB quartet, 4H, chlorambucil phenyl H); 3.8–3.5 (m, 10H, CH_2 + N $(CH_2CH_2Cl)_2$); 2.6–1.7 (m, 6H, butyl H); 1.6–1.3 (br s, 10H, cyclohexyl H). Anal. $C_{27}H_{35}N_3Cl_2O_3$. Theory: C 62.30; H 6.78; N 8.07; Cl 13.62. Found: C 62.34; H 6.79; N 8.06; Cl 13.57.

General procedure for the preparation of pyridinium salts ***(2c–5c)***.

The substituted nicotinamide was dissolved in dry acetonitrile and one equivalent of dimethyl sulfate was added to it. The reaction mixture was heated at reflux overnight. The solvent was evaporated in vacuo and the residue was washed with ether and dried.

1-Methyl-4-[(N-{1-cyclohexyl-4-[4-{4-[bis-2-chloroethyl)]amino}phenyl-butanoyloxy]}carbamoyl]pyridinium methanesulfate ***(2c)***. Yield 80%. UV (MeOH) 220, 265 nm. NMR ($CDCl_3$) 9.5–9.3 (d, 1H, C-2, pyridinium H), 9.2–8.7 (m, 2H, C-4 + C-6, pyridinium H); 8.3–7.9 (m, 1H, C-5, pyridinium H); 7.2–6.6 (AB quartet, 4H, chlorambucil phenyl H); 4.6–4.4 (br s, 3H, CH_3SO_4); 3.7–3.4 (br s, 13H, N-CH_3 + trans cyclohexyl H + $N(CH_2CH_2Cl)_2$); 2.6–1.2 (m, 14H, cyclohexyl H + chlorambucil butyl H).

1-Methyl-3[(N-{2-phenyl-2[4-[{4-[bis-(2-chloroethyl)] amino}phenyl)-butanoyloxy]}ethyl)carbamoyl]pyridinium methanesulfate ***(3c)***. Yield 91%. UV (MeOH) 219, 272 nm. NMR ($CDCl_3$) 9.5–9.3 (d, 1H, C-2 pyridinium H); 9.2–8.7 (m, 2H, C-4 + C-6, pyridinium H); 8.3–7.4 (m, 1H, C-5, pyridinium H); 7.6–7.3 (br s, 5H, phenyl H); 7.2–6.5 (AB quartet, 4H, chlorambucil phenyl H); 5.5–5.2 (m, 1H, CH); 4.6–4.4 (br s, 3H, CH_3SO_4); 3.7–3.4 (br s, 13H, N-CH_3 + CH_2 + $N(CH_2CH_2Cl)_2$); 2.6–1.6 (m, 6H, chlorambucil butyl H).

1-Methyl-3(N-{1-[4-({4[bis(2-chloroethyl)]amino}-phenyl) butanoyloxy] propyl} carbamoyl} pyridinium methanesulfate ***(4c)***. Yield 92%. UV (MeOH) 218, 269 nm. NMR ($CDCl_3$) 9.5–9.3 (d, 1H, C-2, pyridinium H); 9.2–8.7 (m, 2H, C-4 + C-6 pyridinium H); 8.3–7.9 (m, 1H, C-5, pyridinium phenyl H); 7.2–6.6 (AB quartet, 4H, chlorambucil phenyl H); 5.2–4.9 (m, 1H, CH); 4.6–4.4 (br s, 3H, CH_3SO_4); 3.7–3.4 (br s, 13H, N-CH_3 + CH_2 + N $(CH_2CH_2Cl)_2$); 2.6–1.6 (m, 6H, chlorambucil butyl H); 1.7–1.5 (d, 3H, CH_3).

1-Methyl-3(N{1-[4-({4-[bis(2-chloroethyl)]amino}-phenyl)butanoyloxy]methylcyclohexyl}carbamoyl]pyridinium methanesulfate ***(5c)***. Yield 90%. UV (MeOH) 220, 278 nm. NMR ($CDCl_3$) 9.5–9.3 (d, 1H, C-2, pyridinium H); 9.2–8.6 (m, 2H, C-4 + C-6, pyridinium H); 8.3–7.9 (m, 1H, C-5, pyridinium H); 7.2–6.5 (AB quartet, 4H, chlorambucil phenyl H); 4.6–4.4 (br s, 3H, CH_3SO_4); 4.0–3.5 (m, 13H: CH_2 + N-CH_3 + $N(CH_2CH_2Cl)_2$); 2.6–1.6 (m, 6H, chlorambucil butyl H); 1.6–1.3 (br s, 10H, cyclohexyl H).

General procedure for the synthesis of the dihydropyridines ***(2d–5d)***

This synthesis is as described for (**1d**).

1-Methyl-3({N-[4-({4-[bis(2-chloroethyl)]-amino}phenyl)-butanoyloxy]cyclohexyl}carbamoyl)-1,4-dihydropyridine ***(2d)***. Yield 65%. UV (MeOH) 216, 360 nm. NMR ($CDCl_3$) 7.2–6.9 (br, AB quartet, 5H, C-2, pyridine H, chlorambucil 4-phenyl H); 5.7–5.4 (dt, C-6, pyridine H); 5.2–4.9 (m, 1H, C-5, pyridine H); 4.7–4.4 (m, 2H, 1.6 trans cyclohexyl H); 3.6–3.4 (s, 8H, chlorambucil $N(CH_2CH_2Cl)_2$); 3.2–3.0 (bs, 2H, C-4, pyridine H); 2.9–2.8 (s, 3H, N–CH_3); 2.6–1.3 (m, 14H, 8H

cyclohexyl H + 6H, chlorambucil butyl H). Anal. $C_{27}H_{37}Cl_2N_3O_3$. Theory: C62.06; H7.13; N 8.04; Cl 13.57. Found: C 61.88; H 7.19; N 8.00; Cl 13.50.

1-Methyl-3{(N-{-2-phenyl-2-[4-({4-[bis(2-chloroethyl)]amino}phenyl)butanoyloxy]}ethyl)carbamoyl]1,4-dihydropyridine (3d). Yield 45%. UV (MeOH) 216, 362. NMR ($CDCl_3$) 7.7–7.5 (br s, 5H, phenyl H); 7.2–6.4 (br, AB quartet 5H, C-2, pyridine H, chlorambucil 4-phenyl H); 5.5–5.2 (m, 1H, CH) 4.7–4.5 (m, 1H, C-5, pyridine H); 3.8–3.6 (br s, 10H, CH_2 + chlorambucil $N(CH_2CH_2Cl)_2$); 3.1–2.9 (br s, 2H, $N-CH_3$); 2.6–1.6 (m, 6H, chlorambucil butyl H). Anal. $C_{29}H_{35}Cl_2N_3O_3$. $1H_2O$. Theory: C 61.91; H 6.62; Cl 12.60; N 7.47. Found: C 61.79, H 6.71; Cl 12.49; N 7.23.

1-Methyl-3-[(N-{2-[4-({4-[bis(2-chloroethyl)]-amino}phenyl)butanoyloxy]propyl})carbamoyl]-1,4-dihydropyridine (4d). Yield 60%. UV (MeOH) 220, 364 nm. NMR ($CDCl_3$) 7.2–6.5 (br, AB quartet, 5H, C-2, pyridine H + chlorambucil phenyl H); 5.6–5.4 (dt, C-6, pyridine H); 5.1–4.4 (m, 2H, C-5, pyridine H + CH); 3.5–3.3 (br s, 10H, chlorambucil $N(CH_2CH_2Cl)_2$, $+CH_2$); 1.2–1.1 (d, 3H, CH_3). Anal. $C_{24}H_{33}Cl_2N_3O_3.0.5$ H_2O. Theory: C 58.69; H 6.96; Cl 14.42; N 8.55. Found: C 58.52; H 6.84; Cl 14.26; N 8.31.

1-Methyl-3({N-[1-({4-[bis(2-chloroethyl)amino}phenyl)butanoyloxy]methyl cyclohexyl}carbamoyl]-1,4-dihydropyridine (5d). Yield 55%. UV (MeOH) 216, 368 nm. NMR ($CDCl_3$); 7.2–6.5 (br AB quartet, 5H, C-2, pyridine H + chlorambucil phenyl H); 5.8–5.6 (dt, C-6, pyridine H); 4.8–4.6 (m, 1H, C-5, pyridine H); 3.7–3.5 (br s, 10H, CH_2 + chlorambucil $N(CH_2CH_2Cl)_2$); 3.2–3.0 (bs, 2H, C-4, pyridine H); 2.9–2.8 (s, 3H, $N-CH_3$); 2.6–1.7 (m, 6H, chlorambucil butyl H); 1.6–1.4 (br s, 10H, cyclohexyl H). Anal. C_{28} $H_{39}Cl_2N_3O_3.1/2$ H_2O. Theory: C 61.64; H 7.39; Cl 12.99; N 7.70. Found: C 61.59; H 7.28; Cl 12.79; N 7.58.

Analytical methodology

All detection and quantitation of dihydropyridine derivatives, pyridinium salt derivatives and chlorambucil was performed using high-performance liquid chromatography (HPLC). The system configuration included a Waters Model 510 pump, a Kratos Spectroflow 757 variable wavelength detector, a Kontron MSI660 autosampler and a Hewlett-Packard 3390 A integrator. All separations were achieved on a Hamilton PRP-1 polystyrene-divinylbenzene analytical column (15 cm × 4.6 mm i.d.). For the pyridinium salts (**1c–5c**) as well as for chlorambucil a single mobile phase was used which consisted of 55 : 40 : 5 acetonitrile : 0.05 M KH_2PO_4 : H_2O and 0.05 M tetramethylammonium phosphate and 0.005 M tetrabutylammonium phosphate. These derivatives were detected at 260 rm and the flow rate was 1 ml/min. All determinations were made at ambient temperatures. In the above indicated conditions the retention time for chlorambucil was 8.18 min. The quaternary salts (**1c–5c**) eluted at 5.24, 5.46 min, 5.74 min, 3.54 min and 5.76 min, respectively. For the dihydropyridines, the mobile phase was made by combining 85 parts of acetonitrile and 15 parts H_2O. These compounds were detected at 360 nm and, with the exception of compound (**4c**) which was eluted using a flow rate of 0.8 ml/min, the flow rate was 1 ml/min. The retention times for the CDS's (**1d–5d**) were 5.47, 4.25 min, 3.91 min, 3.9 min, and 3.65 min, respectively.

Lipophilicy (log P) determinations

Solutions of chlorambucil, as well as compounds (**1d–5d**) were prepared in octanol. Two milliliters of each solution were then partitioned against 2 ml of pH 7.4 phosphate buffer which had been saturated with octanol. The partitioning was carried out at ambient temperature for 2 h. The concentration of each compound in both phases was then determined by HPLC as previously described. The log of the ratio of the peak heights of a compound in octanol and in water was determined and reported as log *P*. Each determination was run in triplicate and data are reported as the average of the 3 values.

In vitro studies

Ferricyanide oxidation studies. The rate of ferricyanide-mediated oxidation of (**1d–5d**) was determined using a modification of published methods. In the procedure, the rate of decrease of the 358 nm absorbance band of a particular dihydro-

pyridine derivative was determined in buffered 20% aqueous acetonitrile solutions [0.1 mM $K_4Fe(CN)_6$, 60 mM KCl and 1.0 mM K_2CO_3] containing various concentrations of $K_3Fe(CN)_6$ (1–50 mM). Each of the CDS compounds (**1d–5d**) was added, using a Hamilton syringe, to the buffered oxidant which was contained in an anaerobic teflon-lined screw-top cuvette (Spectrocell, Inc., Oreland, PA). The cuvette and its contents were maintained at 37°C in a thermostated cell holder. For a given $Fe(CN)_6^{-3}$ concentration, the pseudo-first-order rate constant was determined and then plotted as a function of the ferricyanide ion concentration. The slope of this line gave the second order rate constant (k_0, s^{-1} M^{-1}). Measurements were made using a Hewlett-Packard 8451A diode array spectrophotometer with an HP85 microprocessor.

Buffer and biological matrices. The stability of the CDS derivatives (**1d–5d**) as well as for the corresponding quaternary salt metabolites (**1c–5c**) was studied in isotonic phosphate buffer (pH 7.4), 20% rat brain homogenate and whole rat blood. Rat brain was freshly obtained and homogenized in isotonic phosphate buffer. The homogenate was centrifuged and the supernatant diluted to make a 20% w/v matrix. Whole rat blood was obtained via cardiac puncture. In these studies, a small volume (50 μl) of the test compound dissolved in dimethyl sulfoxide (DMSO) was added to buffer, rat brain homogenate or whole blood, each maintained at 37°C. At various times, after this addition, an aliquot (100 μl) of the appropriate matrix was removed, mixed with ice-cold acetonitrile (400 μl) and vortexed. The sample was then centrifuged at 13,000 *g* (Beckman Microfuge 12) for 8 min and the supernatant removed, filtered through 0.45 μm polyvinylidene difluoride membrane (Millipore) and analyzed by HPLC.

Acute toxicity studies

Groups of 5 Sprague–Dawley rats were restrained and injected via the tail vein with vehicle (DMSO, 0.5 ml/kg) or **2d, 3d, 4d 5d** at a dose equimolar to 20 mg/kg **1d**. Immediately after drug administration, the general robustness of animals was observed and mortality, if it occurred before 2 h was noted. At 2 h posttreatment animals were sacrificed and a gross necropsy was performed. Obvious changes in organ appearance were noted.

In vivo tissue distribution studies

Conscious restrained Sprague–Dawley rats (200 g b.wt.) were given either **1d**, chlorambucil or vehicle in doses equimolar to 60 mg/kg **1d** and 0.5 ml/kg, respectively. In a separate study rats were injected with 20 mg/kg (**2d**). At various times posttreatment groups of animals ($n = 5$) were sacrificed and brain and whole blood was collected, weighed and frozen. In preparing tissue and blood for analyses 1 ml of blood or 1 g of brain was homogenized with 1 ml of water. To this were then added 4 ml of ice-cold acetonitrile and 1 ml of saturated NaCl. The mixture was thoroughly mixed and cooled to −15°C for 1 h. The organic layer which separated under these circumstances was removed, filtered through 0.45 μm polyvinylidene difluoride membranes and analyzed by HPLC. Standard curves were constructed using low quantities of drug added to organ or blood homogenates which were subsequently extracted as described above. Phenylacetic mustard (PAM) was detected but not quantitated in these studies. The retention time of this material was 4.0 min using the HPLC system described for **1c** and **2c**.

In vitro alkylating potency

The ability of chlorambucil, the chlorambucil quaternary salt derivatives (**1c** and **2c**) and the chlorambucil CDS'S (**1d** and **2d**) to quaternize 4-(4-nitrobenzyl) pyridine was studied as an assay of their alkylating potency. Studies were performed as described in previous accounts (Freidman and Boger, 1961).

Results and Discussion

Chemistry

Chlorambucil differs from other drugs which have been applied to the CDS in that it is a carboxylic acid. Novel carriers were, therefore, designed to allow for connection of chlorambucil with nicotinic acid derivatives. The first type of carriers generated were *N'*-hydroxyalkylnico-

tinamides. Synthetically, ethyl nicotinate was reacted with 2-aminoethanol giving the *N'*-hydroxyethylnicotinamide (**1a**) (Casadio et al., 1977). Reaction of this compound with chlorambucil in the presence of a dehydrating agent (dicyclohexylcarbodiimide, DCC) and a nucleophilic catalyst (4-(dimethylamino) pyridine, DMAP) gave the amide-ester (**1b**). Quaternization of this nicotinamide with methyl sulfate gave the pyridinium methanesulfate salt (**1c**) which was reduced in aqueous sodium dithionite to give the CDS (**1d**). Four other CDS were subsequently prepared. These derivatives were designed to examine the effect of substitution on the position α to the alcoholic ester oxygen on the rate of hydrolytic cleavage. The compounds incorporated various alkyl bridges producing secondary and tertiary ester of chlorambucil. The synthetic routes for preparing the carriers involved a mixed anhydride method. Nicotinic acid, ethyl chloroformate and triethylamine were reacted with either 4-aminocyclohexanol, 2-amino-1-phenylethanol, 2-amino-1-methylethanol or 1-aminomethylcyclohexanol to give the corresponding alcohol-amides (**2a–5a**). Each of these carriers was then condensed with chlorambucil using DCC and DMAP giving (**2b–5b**). The DMAP catalysis is known to facilitate the esterification of hindered alcohols (Hassner and Alexanian, 1978). Each of the derivatives was then quaternized with methyl sulfate to give (**2c–5c**) and reduced with aqueous sodium dithionite to yield the corresponding dihydropyridines (**2d–5d**) or CDS's.

TABLE 1

Octanol: water partition coefficients (log P) for chlorambucil and various chlorambucil CDS's (1d–5d)

Compound	log *P*	Lipophilicity relative to chlorambucil
Chlorambucil	−0.40	1
1d	2.16	362
2d	2.85	1748
3d	2.29	485
4d	2.75	1400
5d	2.30	497

According to the CDS scheme, derivatization of the parent drug should result in an increase in the lipophilicity of the conjugate. To examine this point, the octanol: water partition coefficients were determined for chlorambucil as well as for the CDS's (**1d–5d**)[21]. The results of this study are collected in Table 1. All of the delivery systems are substantially more lipophilic than chlorambucil itself. The cyclohexyl derivative (**2d**) has the highest octanol: water partition coefficient while the simple ethyl compound (**1d**) has the lowest log P of the CDS's tested. All of the compounds (**1d–5d**) are lipophilic enough to readily penetrate the BBB.

In vitro studies

The CDS scheme requires that after brain penetration the CDS is oxidized to generate the "locked-in" pyridinium salt and that this salt hydrolyzes in a timely manner to release the parent drug. The stability of the synthesized dihydropyridines (**1d–5d**) was, therefore, assayed in pH 7.4 phosphate buffer, in rat brain homogenate and in whole rat blood. These data are presented in Table 2. In all cases, the rate of oxidation of the CDS in biological matrices was faster than in phosphate buffer suggesting an enzymatically mediated degradation. This has been observed in many cases with other chemical delivery systems (Bodor and Brewster, 1983). In these studies, three groups of compounds could be identified based on their reactivity. The CDS in which a simple ethyl group separated the nicotinamide and chlorambucil (**1d**) was oxidized in brain homogenate the fastest (relative rate = 1) followed by the cyclohexyl (**2d**) and 1-methyl ethyl (**4d**) containing derivatives (relative rates of oxidation = 0.5 and 0.61, respectively). The bulkiest groups, i.e. the 1-aminomethylcyclohexanol (**5d**) and the 1-phenylethyl (**3d**) derivatives were oxidized the slowest (relative rates of oxidation = 0.147 and 0.141, respectively). This effect of structure on the oxidation rates may indicate the involvement of enzymes of the NADH transhydrogenase type. A similar observation was previously made for the biological oxidation of a series of 1-methyl-1,4-dihydronicotinic acid esters (Bodor and Brewster, 1983). A second study was performed to examine

TABLE 2

Rate constants for oxidation for a series of chlorambucil-CDS derivatives (1d–5d)

Compound	Medium	Rate constant k (min^{-1})	Half-life $t_{1/2}$ (min)	Correlation coefficient r
1d	Buffer (pH 7.4)	0.0109	63.3	0.998
	Brain homogenate	0.102	6.8	0.994
	Whole blood	0.289	2.4	0.992
2d	Buffer (pH 7.4)	0.0045	153.7	0.999
	Brain homogenate	0.0509	13.6	0.997
	Whole blood	0.0308	22.5	0.998
3d	Buffer (pH 7.4)	0.0059	117.8	0.996
	Brain homogenate	0.0150	46.3	0.989
	Whole blood	0.860	8.06	0.996
4d	Buffer (pH 7.4)	0.0051	137.10	0.999
	Brain homogenate	0.0616	11.25	0.998
	Whole blood	0.0197	35.12	0.997
5d	Buffer (pH 7.4)	0.0043	161.5	0.997
	Brain homogenate	0.0143	48.40	0.996
	whole blood	0.0041	169.75	0.959

oxidative stability. Dihydropyridines react with potassium ferricyanide in solution to generate the corresponding pyridinium salt (Powell et al., 1984; Okamoto et al., 1980; Okamoto et al., 1977). Values obtained for the rates of this oxidation are useful in that they examine chemical stability of the drugs separate from enzymatic stability. The data shown in Table 3 indicate the similarity in rates for the compounds tested. This parameter is extremely useful in predicting pharmaceutical robustness in that ferricyanide oxidizes dihydropyridine via a mechanism similar to air oxidation, i.e., it is radical-mediated. Enzymatic oxidation of dihydronicotinamides is thought to be mediated by hydride transfer. All of these values are in the range of stable "formulatable" dihydropyridines such as the CDS based on estradiol which is currently clinically tested (Brewster et al. 1988a; Estes et al., 1987).

TABLE 3

Second-order rate constants for the ferricyanide-mediated oxidation of various chlorambucil CDS

Compound	Rate constant k_0 (s^{-1} M^{-1})	Correlation coefficient r
1d	24.97	0.9993
2d	52.01	0.9999
3d	30.18	0.997
4d	24.92	0.9997
5d	10.20	0.9995

The rate of hydrolytic cleavage of chlorambucil from its oxidized carrier in vitro is given in Table 4. These data indicate the rate of hydrolysis is generally more rapid in buffer than in organ homogenates and suggest a high degree of tissue binding of the quaternary salts. In these studies, (**1c**) is clearly the most labile in biological matrices. Compound (**4c**), i.e. the 1-methylethyl derivative is hydrolyzed next fastest followed by the phenylethyl (**3c**) and cyclohexyl (**2c**) derivatives. Compound (**5c**) is the most sluggishly hydrolyzed. Interestingly, the compounds which contain the ethyl spacer are the least stable to both oxidative and hydrolytic decomposition, i.e. (**1c** and **1d**) and the 1-aminomethyl-cyclohexanol derivatives (**5c** and **5d**) are the most stable with respect to these two metabolic pathways.

In vivo distribution

Compound (**1d**) was first tested in vivo in rats. Sprague–Dawley rats received a dose of 60 mg/kg (**1d**) or an equimolar dose of chlorambucil, both in a dimethylsulfoxide (DMSO) vehicle. This dose of chlorambucil proved to be toxic and even by

TABLE 4

Rate constants for hydrolysis for a series of chlorambucil quaternary salt derivatives (1c–5c)

Compound	Medium	Rate constants k (min^{-1})	Half-life $t_{1/2}$ (min)	Correlation coefficient r
1c	Buffer (pH 7.4)	0.0221	31.4	0.993
	Brain homogenate	0.133	5.20	0.994
	Whole blood	0.0162	42.79	0.989
2c	Buffer (pH 7.4)	0.0292	23.8	0.998
	Brain homogenate	0.00149	464.8	0.994
	Whole blood	0.00167	416.3	0.988
3c	Buffer (pH 7.4)	0.0239	28.9	0.998
	Brain homogenate	0.0016	446.4	0.999
	Whole blood	0.0037	188.0	0.986
4c	Buffer (pH 7.4)	0.0201	34.4	0.998
	Brain homogenate	0.0067	103.7	0.995
	Whole blood	0.0056	123.3	0.997
5c	Buffer (pH 7.4)	0.0213	32.5	0.998
	Brain homogenate	0.0009	708.9	0.996
	Whole blood	0.0006	1205.5	0.986

pooling samples only a 1 h time point could be obtained. At various times after the administration of (**1d**), animals were sacrificed and blood and brain were removed and assayed for chlorambucil and the quaternary salt (**1c**) and (**1d**) itself. The data obtained from this study are collected in Figs. 1–4. None of the dihydronicotinamide derivatives were detected in either brain or blood at any of the time points investigated. The rapid disappearance of this material is due to its large volume of distribution as well as its in vivo metabolic lability. As shown in Figs. 1 and 2, chlorambucil is produced in high initial levels in both brain and blood. Brain concentrations of chlorambucil were maintained, however (Fig. 1), as the levels of the quaternary salt slowly fell. The estimated half life for chlorambucil in the CNS is approximately 2.4 days while that for the quaternary salt is only 2.0 hours. In the blood (Fig. 2) the chlorambucil quaternary derivative (**1c**) is also converted to the parent alkylating agent as well as phenylacetic mustard (PAM) (Mitoma et al., 1977; McLean et al., 1980). No PAM was detected in brain. In this study, the

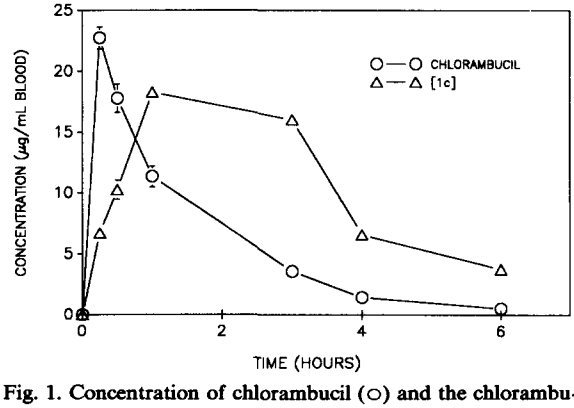


Fig. 1. Concentration of chlorambucil (○) and the chlorambucil quaternary salt **1c** (△) in brain after an i.v. dose of **1d** (60 mg/kg). Each time point represents the average ±S.E.M. for 5 animals.

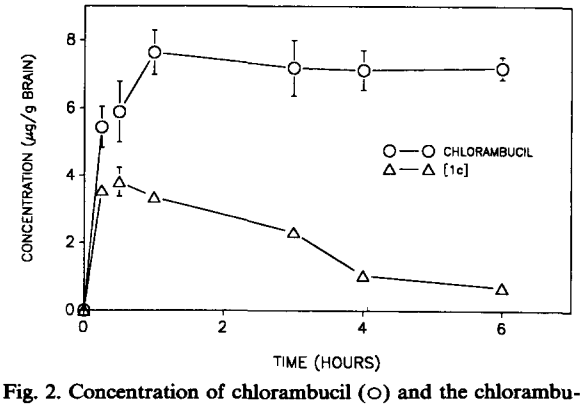


Fig. 2. Concentration of chlorambucil (○) and the chlorambucil-quaternary salt **1c** (△) in blood of rats given an i.v. dose of 60 mg/kg **1d**. Each time point represents the average ± SEM for five animals.

half-life of chlorambucil in blood was 1.1 h while that of the quaternary salt was approximately 2 h. The levels of chlorambucil in brain are stable while those in blood rapidly fell generating a favorable brain/blood ratio (Fig. 3). Importantly, no symptomatology indicative of neurotoxicity was observed. There have been sporadic reports in the literature that chlorambucil overdoses have produced neurotoxic reactions (Ichida et al., 1985; Byrne, et al., 1981). These effects are not seen in this study even though the dose of (**1d**) administered is equimolar to many times the LD_{50} dose of chlorambucil. In previous studies the ratio of the areas under the chlorambucil concentration curve for brain and blood after administration of chlorambucil was calculated to be 0.021. In this study the brain/blood ratio is approximately 1.119, which represents an improvement of more than 50-fold. While these results were encouraging, it was decided to examine other CDS derivatives of chlorambucil to see if peripheral levels of chlorambucil could be reduced over those produced after (**1d**) dosing.

In selecting a second compound to test in vivo, a pilot toxicity study was performed. Acute i.v. effects of each of the CDS compounds **2d–5d** was examined in rats and the derivative with the highest tolerated dose would be subsequently used in distribution studies. In this study, animals received (i.v.) a dose of **2d–5d** equimolar to 20 mg/kg **1d**. Compounds **4d** and **5d** were acutely toxic at this dose with 4 out of 4 animals dying within 2 h. In

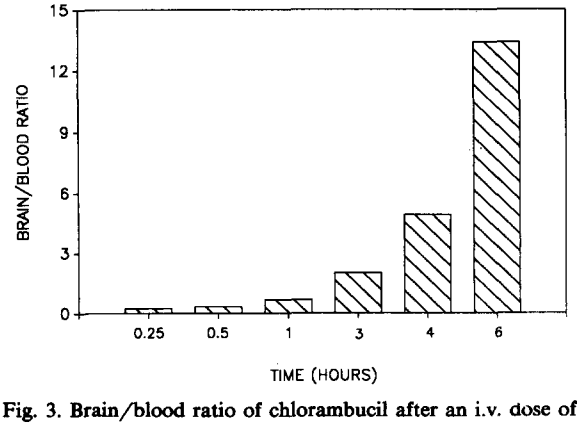


Fig. 3. Brain/blood ratio of chlorambucil after an i.v. dose of **1d** (60 mg/kg).

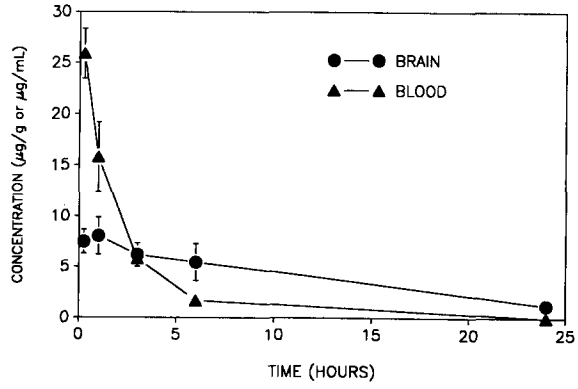


Fig. 4. Concentration of **2c** in the brain (●) and blood (▲) of test animals given an i.v. dose of 20 mg/kg **2d**. Each time point represents the average ± S.E.M. for 5 animals.

the case of **3d**, one out of 5 animals died but others were anoxic and displayed respiratory distress. Compounds **2d** caused no mortality and animals generally tolerated the dose well. A distribution study was therefore performed, using **2d**. The amount of free chlorambucil was dramatically reduced in blood after **2d** dosing compared with **1d** administration and made up less than 1% of the total circulating concentration of drug (alkylating agent i.e. the quaternary salt, CDS and the parent drug), while in the case of (**1d**), this figure ranged from 20 to 80%. The CDS also provides a sustained presence of the quaternary salt in the brain but the amount of chlorambucil released was below the limits of detection of the assay. In the CNS, the half-life for the quaternary salt was approximately 9 h while in the blood this species disappeared with an apparent $t_{1/2}$ of 1.5 h (Fig. 4).

One last point addressed is whether the precursor chlorambucil quaternary salts have alkylating activity. Since the mustard is intact in these species and the chemical modifications take place at some distance away from the active portion of the molecule, it might be expected that the quaternary salt may also have anticancer activity. Classical chemical alkylating methods have been employed to address this point (Friedman and Boyer, 1961). It appears that the in vitro alkylating potency of those species is approximately 80% of that of chlorambucil. This being the case

the distributional advantages afforded by the CDS become of even greater importance in reducing toxicity. In general, modification of a drug to generate a CDS usually diminishes or abolishes pharmacological activity. In this case, the potency of the agent is not necessarily reduced, thus the advantage achieved by using the CDS is the rapid elimination of the drug from the periphery relative to the CNS. The acute toxicity of the CDS is certainly lower than that of the parent drug. As stated, animals well tolerated a 60 mg/kg dose of **1d** while an equimolar dose of chlorambucil (39 mg/kg) caused mortality in $> 80\%$ of animals within 2 h.

In summary, 5 chemical delivery systems were prepared for the classical alkylating agent, chlorambucil. These derivatives were studied in vitro and were found to possess characteristics associated with successful brain delivery. In vivo testing of the ethyl-containing delivery system (**1d**) resulted in sustained brain delivery of chlorambucil while blood levels rapidly dissipated. This generated a brain/blood ratio of 13 at 6 h posttreatment. A second derivative was subsequently studied. This CDS contained a cyclohexyl bridge (**2d**). While peripheral levels of chlorambucil were reduced using this derivative, brain delivery of chlorambucil was also lower.

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