Improved Delivery Through Biological Membranes. XLV. Synthesis, Physical-Chemical Evaluation, and Brain Uptake Studies of 2-Chloroethyl Nitrosourea Delivery Systems

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The dihydropyridine ⟷ pyridinium redox chemical delivery system (CDS) was supplied to two 2-chloroethylnitrosoureas, i.e., HECNU and CCNUOH, and the physicochemical properties of the delivery systems were studied to assess their potential as improved delivery forms to the CNS. Detailed physicochemical evaluation and brain uptake studies were performed on one of the delivery systems (CCNUOH-CDS) derived from *trans*-4-hydroxy-CCNU, an active metabolite of CCNU. Two aqueous-based formulations derived from hydroxypropyl-β-cyclodextrin (HPβCD) and Tween 80:ethanol:water system were developed for CCNUOH-CDS to overcome the poor aqueous solubility conferred upon it by its high lipophilicity. The formulations enabled a 200- to 400-fold improvement in the water solubility of CCNUOH-CDS. Dose- and vehicle-dependent comparative tissue distribution studies in rats indicated improved brain-to-organ ratios of the delivery system at lower doses.

KEY WORDS: chloroethylnitrosoureas; dihydropyridine chemical delivery system; physicochemical evaluation; hydroxypropyl-β-cyclodextrin; blood–brain barrier; sustained delivery of drugs.

INTRODUCTION

The (2-chloroethyl)nitrosoureas are among the most effective antitumor agents, with demonstrated clinical activity against a variety of malignant diseases (1–3), particularly brain tumors (4,5) and lymphomas (6). Most of these agents are nonionized and lipid soluble and therefore capable of crossing the blood-brain barrier and penetrating deeply into brain tumor tissue.

Unfortunately, a variety of physicochemical and metabolic factors complicates the use of nitrosoureas in the treatment of central cancers including their peripheral toxicity and their poor CNS retention.

A method for achieving a "lock-in" effect in delivering the nitrosoureas to the brain may offer a significant advantage in the treatment of intracranial malignancies. The method chosen to address this problem is based on a dihydropyridine⟷pyridinium salt redox system. The principles and specific applications of the chemical delivery system [CDS] have been discussed in detail elsewhere (7).

In the present investigation this chemical approach has been applied to 1-(2-chloroethyl)-1-nitroso-3-(2-hydroxyethyl)urea [HECNU], a water-soluble nitrosourea-type anticancer drug which is in clinical phase I/II studies (8,9). This nitrosourea derivative, compared with other nitrosoureas, has shown superior anticancer efficacy and less toxicity, especially after repeated applications (9,10). HECNU induced remission in 100% of animals after intracerebral inoculation of L1210 and L5222 leukemias and was clearly superior to BCNU (11,12). In this report, we describe the synthesis of (a) HECNU-CDS, (b) an isostere of HECNU-CDS in which the oxygen in the ester is replaced by an NH to obtain an amide-linked carrier nitrosourea, and (c) a nicotinamide analogue with the nitrosourea derivatized at the pyridine nitrogen. A preliminary examination of the stability in buffers and biological matrices of the carrier-linked derivatives is also described.

In another application the CDS was applied to 1-(2-chloroethyl)-1-nitroso-3-(*trans*-4-hydroxycyclohexyl)urea [CCNU-OH], an active metabolite of CCNU, the rationale and synthesis of which have been described elsewhere (13). As previously reported, CCNU-OH-CDS was demonstrated to have the appropriate characteristics to serve as an effective delivery system for sustained delivery of CCNU-OH to the brain. Additional studies to evaluate the CDS are presented here. These include stability characterization studies in buffer solutions. In addition, tissue distribution studies in rats of CCNU-OH-CDS in the formulations were conducted to compare the organ uptake of the drug with respect to the dose and the vehicle of administration. CCNU-OH-CDS exists as a mixture of 1,4-isomer **15a** and 1,6-isomer **15b** in approximately 80:20 proportions, respectively. No effort was made to separate these isomers and all studies discussed in this report pertaining to CCNU-OH-CDS were done on the mixture.

Cyclodextrins have been widely used as excipients to increase the aqueous solubility and often the stability of pharmaceutical preparations (14,15). Cyclodextrins are cyclic oligosaccharides consisting of six (α-cyclodextrin), seven (β-cyclodextrin), or eight (γ-cyclodextrin) α-1,4-glucose units. The size of the lipophilic cone-like cavity is a decisive criterion for the formation of stable inclusion complexes, as the guest molecule must fit at least partly into this opening. For many drugs, β-cyclodextrin forms the most stable complexes. However, β-cyclodextrin has low aqueous solubility (about 1.8%, w/v, at 25°C) and its complexes are frequently only slightly water soluble. This low solubility is due to rather stable hydrogen bonding in the crystal lattice.

The solubility of β-cyclodextrin can be increased by partial alkylation of the hydroxy groups (15). Several amorphous, water-soluble derivatives of α-, β-, and γ-cyclodextrins have been prepared and their physicochemical and toxicological properties evaluated (16,17). One of the derivatives tested was 2-hydroxypropyl-β-cyclodextrin (HPβCD), prepared from the reaction of β-cyclodextrin with propylene oxide. This derivative was shown to be an effective solubilizer for a wide variety of drugs and to exert no untoward

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effects in several animal models or humans even at very high doses (15,18). The very physicochemical parameters which allow for successful CDS functioning complicate the development of acceptable pharmaceutical formulations. The high lipophilicity of CCNU-OH-CDS (log P = 2.53) (13) allows the species to partition into deep brain compartments but also confers poor aqueous solubility (0.01 mg/ml at 25°C). In addition, the oxidative lability, which provides for CNS retention, and the hydrolytic instability, which generates sustained CCNU-OH release, combine to limit the shelf-life of CCNU-OH-CDS. In our efforts to improve the aqueous solubility of CCNU-OH-CDS, two aqueous-based formulations were developed. The solubility, stability profiles, and tissue distribution in rats of CCNU-OH-CDS in these formulations were studied (Fig. 1).

MATERIALS AND METHODS

Synthesis

Melting points (m.p.) were determined on an Electrothermal apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian EM 360 A spectrometer. Elemental analyses were performed by Atlantic Microlabs, Inc. (Atlanta, GA). UV spectra were recorded on a Hewlett Packard 8451A diode array spectrophotometer. TLC was performed on EM Reagents silica gel 60 F_{254} plates. Column chromatography was performed on either silica gel (Davisil, 100–200 mesh, 60 A) or aluminum oxide (activated, neutral, Brockman I, 150 mesh, 58 A, Aldrich). The following compounds were prepared according to the literature methodology: 2,4,5-trichlorophenyl-*N*-(2-chloroethyl)carbamate (14), 2,4,5-trichlorophenyl-*N*-(2-chloroethyl)-*N*-nitrosocarbamate (19), 1-benzyl-1,2-dihydroisonicotinamide (20), and 2-hydroxypropyl-β-cyclodextrin (HPβCD) (21).

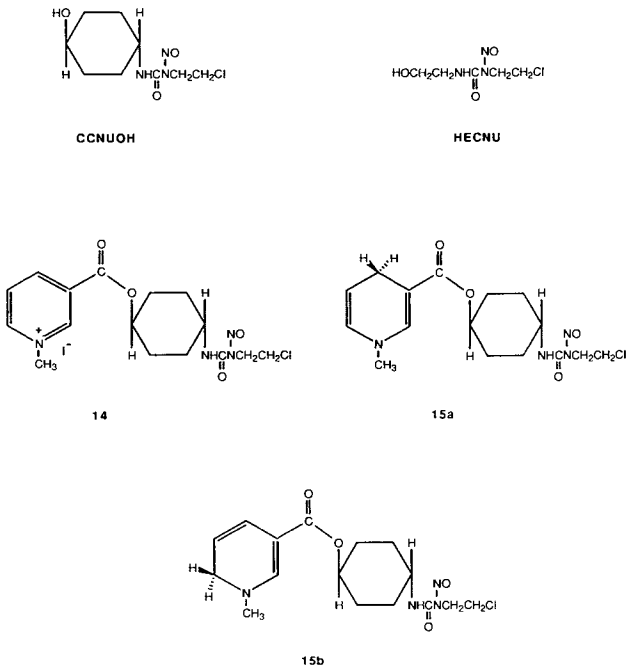

Fig. 1. Structure of selected compounds.

N-(2-Chloroethyl)-N'-[2-(3-pyridinylcarbonyloxy)ethyl]-N-nitrosourea (4) and N-(2-chloroethyl)-N'-[2-(3-pyridinylcarbonylamino)ethyl-N-nitrosourea (5)

A solution of the appropriate amine, **1** or **2**, (5 m*M*) and 2,4,5-trichlorophenyl-*N*-(2-chloroethyl)-*N*-nitrosocarbamate (6 m*M*, 2 g) in pyridine (30 ml) was stirred under argon at room temperature until completion of the reaction (2–24 hr).

Workup for 4. The mixture was evaporated to dryness *in vacuo* and the oily residue was chromatographed on a column of silica gel. Elution, initially with benzene, to remove the reaction by-products, and, finally, with chloroform gave **4**. Yield, 91%; m.p., 63–64°C. TLC, chloroform:ethylacetate (1:1), R_f 0.26. ^{1}H NMR ($CDCl_3$) δ 3.1–3.65 (m, 2, $COOCH_2$), 3.65–4.4 (m, 4, CH_2Cl, CH_2N), 4.7 (t, 2, $CH_2N(NO)$), 7.25–7.5 (m, 1, pyridine-H_5), 7.6–8.0 (br s, 1, NH; exchanged with D_2O), 8.15–8.45 (m, 1, pyridine-H_4), 8.7–8.9 (m, 1, pyridine-H_6), 9.2 (m, 1, pyridine-H_2). *Anal.* ($C_{11}H_{13}ClN_4O_4$)C,H,N.

Workup for 5. The mixture was evaporated to dryness *in vacuo* and the residue was triturated with chloroform to obtain **5**. Yield, 86%; m.p., 140–141°C. TLC, chloroform:ethylacetate (9:1), R_f 0.25. ^{1}H NMR (DMSO-d_6) δ 3.35–3.85 (m, 6, NCH_2CH_2N, $CH_2N(NO)$), 4.1 (t, 2, CH_2Cl), 7.4–7.7 (m, 1, pyridine-H_5), 8.1–8.35 (m, 1, pyridine-H_4), 8.65–9.15 (m, 4, pyridine-H_6, pyridine-H_2, NH; exchanged with D_2O). *Anal.* ($C_{11}H_{14}ClN_5O_3$)C,H,N.

1-Methyl-3-{[[[(2-chloroethyl)nitrosoamino]carbonyl]amino]-ethoxycarbonyl}pyridinium Iodide (6) and 1-Methyl-3-{[[[(2-chloroethyl)nitrosoamino]carbonyl]amino]-ethylaminocarbonyl}pyridinium Iodide (7)

A solution of **4** (5 m*M*) in THF (40 ml) or **5** (5 m*M*) in acetonitrile (50 ml) was treated with an excess of iodomethane (40 mM, 5.68 g) and the solution was heated at gentle reflux until completion of the reaction (2–4 hr).

Workup for 6. The mixture was cooled to 5°C, and the solid was collected by filtration. Yield, 82%; m.p., 120–121°C. ^{1}H NMR (DMSO-d_6) δ 3.25–3.55 (m, 2, $COOCH_2$), 3.6–3.9 (m, 2, CH_2N), 4.1–4.3 (m, 2, $CH_2N(NO)$), 4.35–4.8 (m, 5, CH_2Cl, NCH_3), 7.5 (br s, 1, NH; exchanged with D_2O), 8.2–8.75 (m, 1, pyridine-H_5), 8.9–9.45 (m, 2, pyridine-H_4 and -H_6), 9.65 (br s, 1, pyridine-H_2). *Anal.* ($C_{12}H_{16}ICl N_4O_4$)C,H,N.

Workup for 7. The mixture was cooled to room temperature and evaporated to dryness *in vacuo*. The residue was crystallized from a mixture of acetonitrile and ether. Yield, 85%; m.p., 122–124°C. ^{1}H NMR (DMSO-d_6) δ 3.35–3.7 (m, 6, $-NCH_2CH_2N-$,$N(NO)CH_2$), 4.1 (t, 2, CH_2Cl), 4.35 (s, 3, NCH_3), 8.1–8.35 (m, 1, pyridine-H_5), 8.6–9.3 (m, 5, pyridine-H_4, -H_6, H_2, and NH). *Anal.* ($C_{12}H_{17}ICl N_5O_3$)C,H,N.

1-[[[(2-Chloroethyl)nitrosoamino]carbonyl]amino]ethyl-3-carbonylaminopyridinium Bromide (12)

A suspension of **11** (1 g, 3 m*M*) in a 1:1 mixture of pyridine:dimethyl sulfoxide (30 ml) was treated with 2,4,5-trichlorophenyl-*N*(2-chloroethyl)-*N*-nitrosocarbamate (1.1 g, 3.3 m*M*) and stirred under argon at room temperature for 3 days. The mixture was evaporated to dryness *in vacuo* with ethanol as a cosolvent. The residue was dissolved in acetonitrile (10 ml) and diluted with acetone (100 ml). The solid was isolated by filtration and dried under reduced pres-

sure. Yield, 0.58 g (50%); m.p., 130–132°C. ^{1}H NMR (DMSO-d_6) δ 3.25–3.65 (m, 4, CH_2CH_2N), 3.7–4.2 (m, 4, (NO)NCH_2CH_2Cl), 4.75–5.05 (br s, 2, $CONH_2$ exchanged with D_2O), 8.1–8.4 (m, 1, pyridine-H_5), 8.45–8.75 (br s, 1, NH; exchanged with D_2O), 8.9–9.3 (m, 2, pyridine-H_6, -H_4), 9.6 (br s, 1, pyridine-H_2). *Anal.* ($C_{11}H_{15}BrClN_5O_3$)C,H,N.

N-(2-chloroethyl)-N'-[2-(1,4-dihydro-1-methyl-3-pyridinylcarbonyloxy)ethyl]-N-nitrosourea (9), N-(2-chloroethyl)-N'-[2-(1,4-dihydro-1-methyl-3-pyridinylcarbonylamino)ethyl]-N-nitrosourea (10), and N-(2-chloroethyl)-N'-[2-(3-carbonylamino-1,4-dihydropyridinyl)ethyl]-N-nitrosourea (13)

A solution of the appropriate pyridinium salt (1 m*M*) and *N*-benzyl-1,2-dihydroisonicotinamide (1 m*M*) in anhydrous acetonitrile (25 ml) was stirred under argon at 0°C for 2 hr. The solution was filtered and the filtrate evaporated to dryness *in vacuo* at ~30°C. The residue was suspended in methylene chloride and filtered. The filtrate was evaporated to a small volume and flash chromatographed over a column of aluminum oxide by eluting with acetonitrile:1,2-dichloroethane (1:9) mixtures. The eluate was evaporated *in vacuo* at 30°C to obtain a syrup which was dried under reduced pressure on an oil pump for 1 hr to obtain a glass. Yield of **9**, 63%. ^{1}H NMR($CDCl_3$) δ 2.9 (s, 3, CH_3), 3.1–3.2 (m, 2, pyridine-H_4), 3.3–4.0 (m, 6, CH_2Cl, -NCH_2CH_2OCO), 4.05–4.3 (m, 2, N(NO)CH_2), 4.6–4.9 (m, 1, pyridine-H_5), 5.6–5.8 (m, 1, pyridine-H_6), 7.0 (d, 1, pyridine-H_2), 7.6 (br s, 1, NH; exchanged with D_2O). *Anal.* ($C_{12}H_{17}ClN_4O_4$)C,H,N. Yield of **10**, 62%. ^{1}H NMR ($CDCl_3$) δ 2.9 (s, 3, CH_3), 3.1 (m, 2, pyridine-H_4), 3.2–3.9 (m, 6, NCH_2CH_2N, CH_2Cl), 4.15 (t, 2, N(NO)CH_2), 4.5–4.9 (m, 1, pyridine-H_5), 5.5–5.8 (m, 1, pyridine-H_6), 5.85–9.15 (m, 1, NH; exchanged with D_2O), 7.0 (d, 1, pyridine-H_2), 7.9–8.2 (m, 1, NH; exchanged with D_2O). Yield of **13**, 50%. ^{1}H NMR ($CDCl_3$) δ 2.8–3.9 (m, 8, pyridine-H_4, N-CH_2CH_2N, CH_2Cl), 4.15 (t, 2, N(NO)CH_2), 4.5–4.95 (m, 1, pyridine-H_5), 5.5–6.3 (m, 3, $CONH_2$, pyridine-H_6), 7.0 (d, 1, pyridine-H_2), 7.8 (s, 1, NH).

Analytical Methods

High-performance liquid chromatographic (HPLC) methods were developed for quantitative analysis of all key components of the chemical delivery systems. The chromatographic system consisted of an LDC-Milton Roy constametric III G metering pump, Kontron MSI 660 autosampler, Kratos spectroflow 757 UV absorbance detector, and Perkin-Elmer LCI-100 laboratory computing integrator. For the analysis of HECNU and **6**, **7**, and **12**, a 4.6-mm-i.d. × 250-mm analytical column (5 μm, C8, Alltech) operated at ambient room temperature was used. The mobile phase consisted of acetonitrile:10 m*M* KH_2PO_4:tetramethylammonium perchlorate in the following proportions: **6** and **12** (45:55:20 m*M*), **7** (45:55:10 m*M*). At a flow rate of 1 ml/min **6**, **7**, and **12** were detected at 266 nm with retention times of 6.30, 6.36, and 5.60 min, respectively. For the analysis of **9**, **10**, and **13** the mobile phase consisted of acetonitrile and water at proportions of 60:40, 55:45, and 50:50, respectively. The dihydropyridines were detected at 358 nm with retention times of 6.54 min at 1 ml/min for **9**, 6.3 min at 0.8 ml/min for **10**, and 5.58 min at 0.8 ml/min for **13**.

For the analysis of **15a** and **15b**, a Fisher Resolvex C18 (4.6-mm-i.d. × 250-mm) analytical column was used. The mobile phase consisted of acetonitrile, tetrahydrofuran, and water (55:5:40) and the retention time was 6.5 min for **15b** and 8.8 min for **15a** at a flow rate of 1 ml/min. The peaks were detected at 350 nm. For the analysis of the precursor quaternary derivative **14** and CCNU-OH, a Spherisorb C-8 column and a mobile phase containing 0.05 *M* KH_2PO_4 with 0.02 *M* tetramethyl ammonium perchlorate:acetonitrile (65:35) were used. At a flow rate of 2 ml/min **14** eluted at 7 min, while CCNU-OH eluted at 4 min. The peaks were detected at 254 nm.

In Vitro Stabilities of 6, 7, 9, 10, 12–14 and 15a

The stability of the quaternary (**6**, **7**, **12**, and **14**) and the dihydropyridine derivatives (**9**, **10**, **13**, and **15a**) in biological matrices was investigated. An aliquot of a DMSO stock solution (5×10^{-3} *M*) of the quaternary salt or the dihydropyridine derivative was incubated in 5 ml of rat whole blood or rat brain homogenate (20%, w/v, in 0.01 *M* isotonic phosphate buffer, μ = 0.154, pH 7.4) preequilibrated at 37°C to obtain an initial concentration of 5×10^{-5} *M*. At appropriate time intervals 100-μl samples were withdrawn and mixed with 1 ml of cold acetonitrile to precipitate the proteins. The solutions were centrifuged at 10,000 rpm for 3 min and the supernatants were analyzed by HPLC.

The stability of the derivatives was also studied in 0.04 *M* phosphate buffer (μ = 0.154, pH 7.4) at 37°C. The initial concentration of the compounds was 5×10^{-5} *M*. At appropriate times, samples were taken and chromatographed.

Solubility Studies on 15a and 15b

The effect of HPβCD on the aqueous solubility of **15a** and **15b** was determined. An excess of **15a** and **15b** was added to aqueous solutions of HPβCD ranging in concentration from 0 to 40% (w/v). The suspensions were sonicated in an ultrasonic bath for 1 hr and then placed in a 30 ± 0.2°C shaking water bath. After equilibration for 3 hr, an aliquot was filtered through a 0.45-μm membrane filter unit and analyzed by HPLC.

Kinetic Studies on 15a and 15b

To determine the chemical stabilities of **15a** and **15b**, pH vs rate of decomposition profiles were constructed using chloroacetate (pH <3), acetate (pH 4–5), phosphate (pH 5–8.1), and Tris (pH >8.1) buffers and at a constant ionic strength (μ) of 0.1 *M* maintained for each buffer by adding calculated amount of sodium chloride or sodium nitrate. The buffer solutions were saturated with nitrogen to minimize degradation due to dissolved oxygen.

The influence of HPβCD on the degradation of **15a** and **15b** was investigated. In the kinetic runs, the reactions were initiated by adding a stock solution of **15a** and **15b** in acetonitrile to 3 ml of buffer solution preequilibrated at the desired temperature, the final concentration of CCNU-OH-CDS (both isomers) being 0.03 mg/ml and that of acetonitrile being 0.5%. The solutions were kept in constant-temperature baths, and at appropriate times, samples were taken and analyzed by HPLC.

Animal Studies on 15a and 15b

The *in vivo* tissue distribution behavior of **15a** and **15b** in DMSO, aqueous HPβCD, and Tween 80:ethanol:water (1:1:2) vehicles was studied in rats at two doses, 15 and 5 mg/kg. In the first experiment, one group of six conscious restrained male Sprague-Dawley rats weighing ~200 g was injected i.v. via the lateral tail vein with 15 mg/kg of **15a** and **15b** in DMSO (vehicle dose, 0.5 ml/kg), and another group of six rats was treated with 15 mg/kg of **15a** and **15b** in 46% (w/v) aqueous HPβCD (vehicle dose, 2.5 mg/kg). At 4 hr posttreatment, animals were sacrificed by cervical fracture and organs were collected, weighed, and frozen immediately on dry ice. In preparing the organs for analysis, the organ was homogenized in 1 ml of water. To this were added 4 ml of cold acetonitrile and 1 ml of saturated sodium chloride. The mixture was then rehomogenized, centrifuged, and cooled at −15°C, and the upper acetonitrile layer was removed and analyzed by HPLC.

In the second experiment, three groups of rats, six rats per time point in each group, received i.v. 5 mg/kg of **15a** and **15b** in (a) DMSO (vehicle dose, 0.5 mg/kg), (b) 46% (w/v) aqueous HPβCD (vehicle dose, 2.5 mg/kg), and (c) Tween 80:ethanol:water (1:1:2; vehicle dose, 2 mg/kg). At 15, 30, 60, and 120 min posttreatment, animals were sacrificed and organs were collected, weighed, and processed for analysis as described before.

Standard calibration curves were constructed for **14** in appropriate biological media. The limit of detection for the method of analysis was determined by subjecting a number of standard solutions of **14** in appropriate biological media to the chromatographic procedure. The concentration that gave a peak height of about 5 mm, under the conditions of analysis, was taken as the limit of detection.

The extent of recovery of **14** from appropriate biological media was determined by comparing the standard calibration curves in the appropriate biological media with a calibration graph obtained by treating identically a number of standard solutions of similar concentrations of **14** in acetonitrile. The extent of recovery in general was found to be equivalent in all cases, with an average of 98 ± 1.6% for **14**.

RESULTS AND DISCUSSION

Chemistry

The synthesis of dihydropyridine derivatives **9**, **10**, and **13** was accomplished as shown in Fig. 2. BOC-protected ethanolamine was reacted with nicotinic acid, which, after removal of the BOC group with hydrogen chloride in ethylacetate, gave rise to **1**. The synthesis of **2** was accomplished by heating at reflux ethyl nicotinate with a large excess of ethylenediamine and purifying the product by open column chromatography. The reaction of **1** and **2** with 2,4,5-trichlorophenyl(2-chloroethyl)nitrosocarbamate, **3**, in pyridine readily afforded the required nitrosoureas **4** and **5** in good yields. The poor solubility of **11** in pyridine necessitated the use of DMSO as a solvent and longer reaction times, resulting in a moderate yield of **12**. Quaternization of **4** and **5** with methyl iodide provided the pyridinium derivatives **6** and **7** in good yield. In view of the known lability of 2-chloroethylnitrosoureas under alkaline conditions (22) usually employed in the preparation of 1,4-dihydropyridines using sodium dithionite (23), reduction of **6**, **7**, and **12** to their corresponding dihydropyridines **9**, **10**, and **13**, respectively, was accomplished by a direct hydride transfer reaction with 1-benzyl-1,2-dihydroisonicotinamide, **8**, under anhydrous conditions (20). The structures of the dihydropyridine derivatives were established by ^{1}H NMR and UV spectra. HECNU was prepared by a method analogous to the synthesis of CCNU-OH described earlier (13) by reacting aminoethanol with the activated chloroethylnitrosocarbamate **3**.

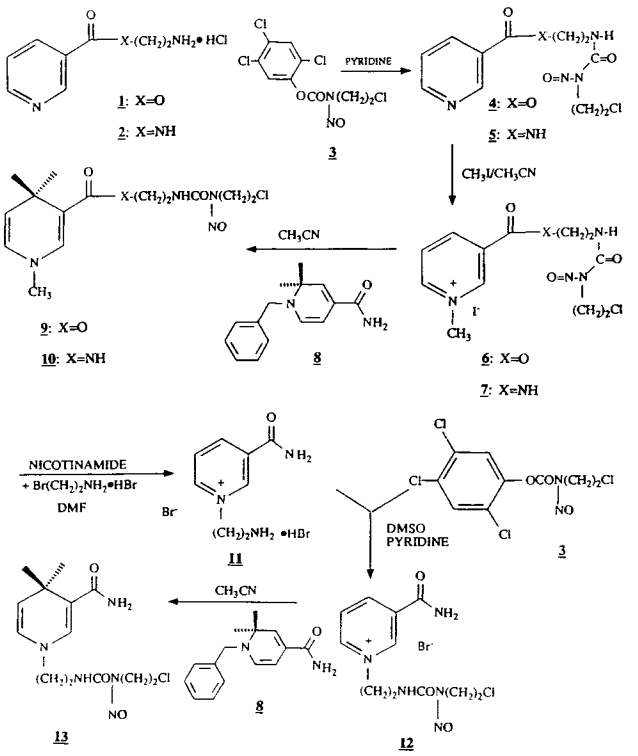

Fig. 2. Synthesis of some of the chemical delivery systems studied.

Chemical and Enzymatic Stability Studies

The pseudo first-order rate constants and half-lives for the degradation of the dihydropyridine derivatives **9**, **10**, **13**, and **15a**, and their quaternary precursors **6**, **7**, **12**, and **14**, respectively, in different biological media and pH 7.4 isotonic phosphate buffer are shown in Table I. The delivery scheme proposed by Bodor and Brewster (7) requires that the dihydropyridines be more stable in blood than in the brain, where they should be oxidized rapidly to the quaternary hydrophilic species, and that the drug be released slowly from the latter to achieve sustained levels in the brain.

The reactivity of the pyridinium derivatives in phosphate buffer followed a predictable pattern, with **12** > **6** > **14** > **7**. In both rat blood and rat brain homogenate, the pyridinium derivatives varied in their reactivity. The most reactive in rat blood was the ester-linked pyridinium salt, **6**, which was enzymatically hydrolyzed to HECNU, and the most stable was the amide-linked pyridinium derivative, **7**. The latter was also the most stable in rat brain homogenate.

The relative stability of the lipophilic dihydropyridine

Table I. Pseudo First-Order Rate Constants (k_{obs}) and Half-Lives ($t_{1/2}$) for the Disappearance of **6**, **7**, **9**, **10**, **11**, **13**, **14**, and **15a** from Various Media at 37 ± 0.1°C

Compound	Medium	$(k_{obs}) \times 10^2$ min^{-1}	$T_{1/2}$ (min)
6	Buffer (A)	1.9457	35.61
	Rat blood (B)	4.9947	13.88
	20% rat brain homogenate (C)	1.411	49.11
7	(A)	1.5881	43.6
	(B)	1.174	59.00
	(C)	0.9404	73.68
11	(A)	2.406	28.8
	(B)	2.3624	29.33
	(C)	1.676	41.34
14	(A)	1.744	39.73
	(B)	1.4459	47.90
	(C)	1.152	60.15
9	(A)	1.8115	38.2
	(B)	7.9482	8.72
	(C)	2.2878	30.29
10	(A)	5.856	11.83
	(B)	4.593	15.09
	(C)	5.977	11.59
13	(A)	1.0486	66.08
	(B)	1.481	46.76
	(C)	1.8469	37.52
15a	(A)	2.556	27.1
	(B)	3.7903	18.3
	(C)	1.587	43.65

derivative compared with its polar pyridinium salt is an important factor in evaluating the redox CDS (24). The stability of the dihydropyridines **9**, **10**, **13**, and **15a** in rat brain homogenate was compared with the stability of the corresponding pyridinium compounds. The pyridinium derivatives **6**, **7**, **12**, and **14** were more stable than their dihydropyridine analogues, **9**, **10**, **13**, and **15a**, respectively. This behavior predicts accumulation of the charged pyridinium derivatives in the brain following administration of the CDS.

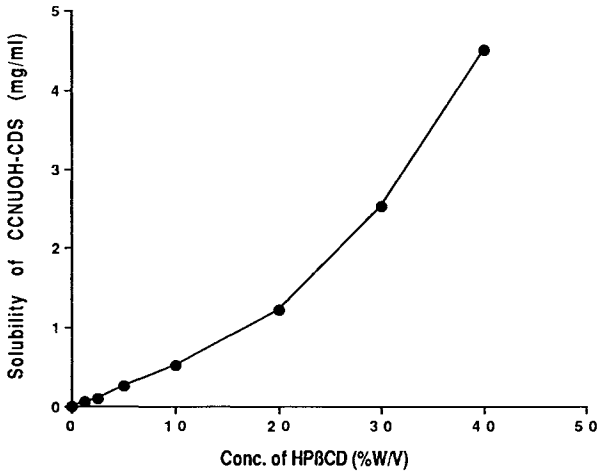

Fig. 3. Phase-solubility diagram of CCNUOH-CDS in aqueous HPβCD at 30 ± 0.2°C.

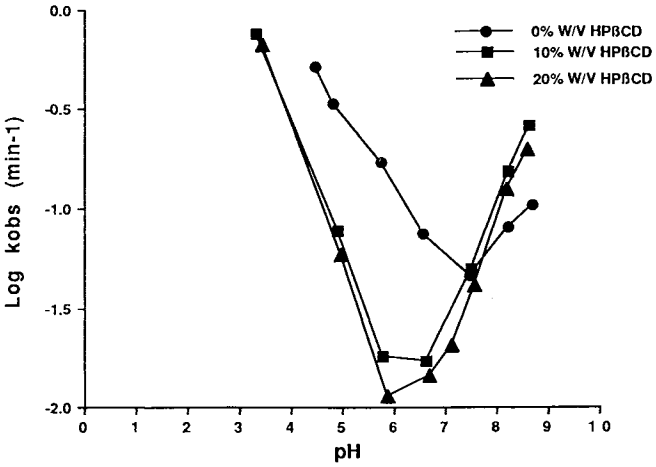

Fig. 4. The log k_{obs}–pH profile for the degradation of **15a** in aqueous buffers at 40 ± 0.2°C.

The dihydropyridines were very reactive in rat blood undergoing rapid oxidation to the corresponding pyridinium derivatives. The most reactive dihydropyridine in rat blood was **9**, whereas **10** was very sensitive to brain enzymes. Interestingly, the sensitivity of **9** to both chemical and enzymatic catalytic processes contrasts the stability of its pyridinium precursor.

Solubility Studies

The effect of HPβCD on the aqueous solubility of CCNU-OH-CDS is shown in Fig. 3. A 40% (w/v) HPβCD solution (about isotonic solution) resulted in about a 400-fold increase in the solubility, from 0.01 to 4.5 mg/ml. The phase-solubility diagram is of the Ap type (25). The solubility of CCNU-OH-CDS in Tween 80:ethanol:water (1:1:2) vehicle was 2.5 mg/ml, which is about a 250-fold increase in the solubility.

Stability in Aqueous Solutions

At constant pH the disappearance of **15a** and **15b** displayed first-order kinetics for several half-lives. The influ-

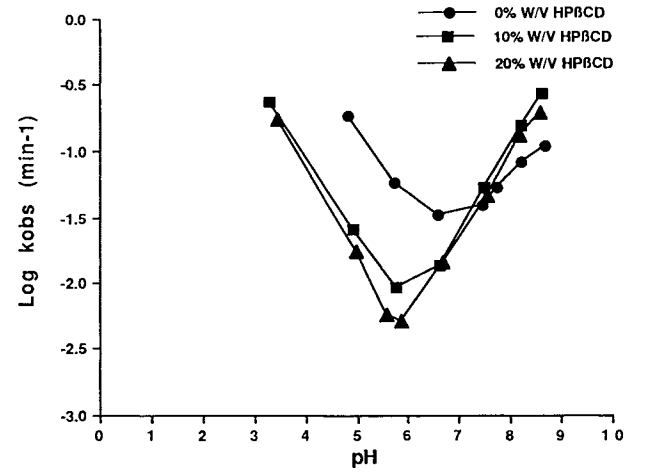

Fig. 5. The log k_{obs}–pH profile for the degradation of **15b** in aqueous buffers at 40 ± 0.2°C.

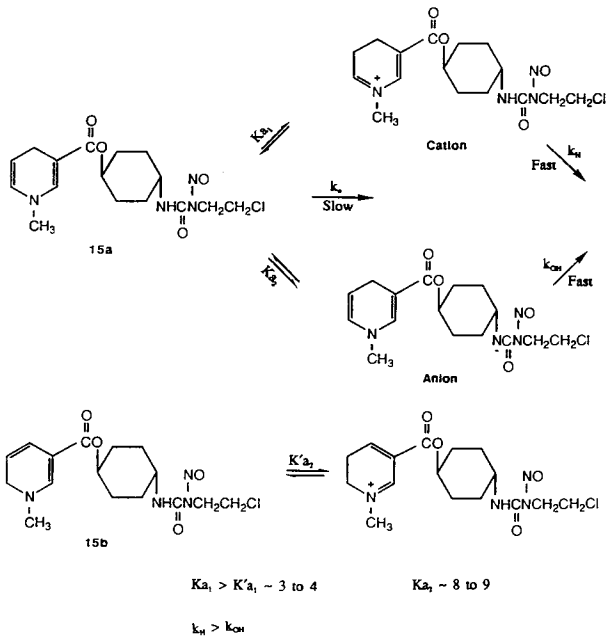

Fig. 6. Ionization and the degradation pathway of **15a** and **15b**.

ence of pH on the pseudo first-order rate constants for the degradation of **15a** and **15b** is shown in Figs. 4 and 5. CCNU-OH-CDS exists as a cation at pH <3 (pK_a, 3–4), is nonionized at pH 4–8, and exists as an anion at pH >8 (pK_a, 8–9). Both the cationic and the anionic forms of CCNU-OH-CDS are very unstable, the nonionized form being the most stable (Fig. 6). Compound **15a** has maximum stability at pH about 7.2, t_{90} = 2.5 min at 40°C. Compound **15b** has a maximum stability at pH 6.7, t_{90} = 3.5 min at 40°C. Compound **15b** is more stable than the 1,4-isomer **15a** over the entire pH range studied, but the largest difference is observed at pH <7.

The influence of HPβCD on the stability of **15a** and **15b** in aqueous buffers are shown in Figs. 4 and 5. While HPβCD has very little effect at neutral pH, it increases the stability at lower pH; e.g., at pH 6, 20% (w/v) HPβCD results in about a 60-fold increase in the stability of the 1,6-isomer. The minimum of the profiles shifts toward lower pH with increasing HPβCD concentration and the slopes become steeper. This is due to differences in the degree of complexation of the different ionized species degree of complexation: nonionized form > cation > anion. In 20% (w/v) HPβCD aqueous buffer solution **15b** has maximum stability at pH 5.7, t_{90} = 23 min at 40°C.

Tissue Distribution Studies

In our earlier report (13) we have demonstrated that CCNU-OH-CDS is capable of delivering sustained levels of CCNU-OH to rat brain by slow hydrolysis of the oxidized form **14**. However, the advantages of the CDS as applied to nitrosoureas and a wide array of drugs are not limited to simply gaining entry to the brain but encompass the broader prospect of minimizing the dose-dependent and dose-limiting toxicities associated with many drugs. The latter goals may be achieved by administering substantially lower doses of drug-CDS to result in therapeutic concentrations of the drug in the brain but reducing levels in the peripheral compartments achieving a higher brain to organ ratio of the drug. The present study was designed to study the effect of dose and vehicle on the distribution of CCNU-OH-CDS to rat brain with respect to peripheral organs. Neither CCNU-OH-CDS nor CCNU-OH could be detected in the tissues studied. The level of the pyridinium precursor **14** in an organ was measured as an indication of the effect of dose and the vehicle of administration of CCNU-OH-CDS. In our earlier study (13) CCNU-OH-CDS, when administered at 60 mg/kg in DMSO, produced higher levels of **14** in the lungs, with a brain-to-lung ratio of 0.3 at 15 min. The ratio was close to unity at 4 hr postadministration.

In the present study, CCNU-OH-CDS, when administered i.v. to rats at a dose of 15 mg/kg in DMSO, produced **14** levels in the peripheral tissues that were disproportionately lower, giving an improved brain/organ ratio (Table II). At 4 hr posttreatment the brain/lung ratio was 2.65. In comparing these levels of **14** to those obtained after administration of an equivalent dose of CCNU-OH-CDS in aqueous HPβCD i.v. to rats, the brain/lung ratio of 3.71 suggests a 139% improvement in favor of the aqueous-based HPβCD vehicle.

These encouraging results led to the second study, the results of which are shown in Table II. In this study the dose of CCNU-OH-CDS was reduced further, to 5 mg/kg, and administered to three groups of rats in three vehicles, viz., DMSO, Tween 80:ethanol:water (1:1:2), and aqueous HPβCD. The results suggest that at lower doses peripheral levels of the drug are reduced considerably, resulting in brain/organ ratios of >1. Even though the brain/lung ratio for **14** remains close to unity at early time points (15 and 30 min posttreatment), the ratio of 1.39, 1.27, and 1.09 for **14** levels 2 hr posttreatment in DMSO-, Tween 80-, and HPβCD-treated rats, respectively, suggests a rapid elimination of the polar quaternary species from the periphery. In comparing

Table II. Tissue Levels of **14** After i.v. Administration of CCNU-OH-CDS (**15a** and **15b**) to Rats

Dose: 15 mg/kg			Dose: 5 mg/kg						
	Conc. (μg/g ± SE)		Time	Brain conc. (μg/g ± SE)			Lung conc. (μg/g ± SEM)		
Tissue	HPβCD	DMSO	(min)	DMSO	Tween 80	HPβCD	DMSO	Tween 80	HPβCD
Brain	8.16 ± 0.48	11.16 ± 0.44	15	11.36 ± 1.02	8.08 ± 0.61	8.72 ± 0.55	11.32 ± 1.18	8.32 ± 0.73	8.16 ± 0.34
Lung	2.20 ± 0.24	4.20 ± 0.6	30	10.92 ± 0.98	9.0 ± 0.27	8.52 ± 0.46	10.2 ± 1.56	7.76 ± 1.09	8.64 ± 1.05
			60	8.28 ± 0.95	6.54 ± 0.27	6.68 ± 0.24	5.8 ± 0.53	5.52 ± 0.23	5.0 ± 0.36
			120	5.68 ± 0.30	3.56 ± 0.50	4.68 ± 0.25	4.08 ± 0.37	2.8 ± 0.34	2.76 ± 0.36

the three vehicles, the levels of **14** in Tween 80- and HPβCD-treated rats were lower in both brain and lungs in comparison with those obtained in DMSO-treated rats. However, the progressive increment in brain/lung ratio for HPβCD over the period of analysis was an improvement of 121 and 133% over DMSO and Tween 80, respectively.

This paper has evaluated the usefulness of two aqueous-based vehicles to solubilize and stabilize CCNU-OH-CDS in aqueous solutions. Both have the advantage of being nontoxic. The results obtained in this study with regard to their ability to deliver CCNU-OH-CDS to the brain at different doses are very encouraging. A detailed toxicological evaluation of CCNU-OH-CDS in these vehicles is worthwhile.

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