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New Carrier for Specific Delivery of Drugs to the Brain

Mahmoud Sheha,[a,*] Ali Al-Tayeb,[b] Hosny El-Sherief[b] and Hassan Farag[a]

[a]*Medicinal Chemistry Department, Faculty of Pharmacy, Assiut University, Assuit 71526, Egypt*
[b]*Medicinal Chemistry Department, Faculty of Pharmacy, Al-Azhar University, Assiut 71526, Egypt*

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Abstract—1,4-Dihydropyridines were thoroughly investigated as carriers for specific drug delivery to the brain and were found very efficient. The main problem which arises in their application in pharmaceutical preparations, is the short shelf-life time due to hydration and/or oxidation. To overcome these problems, a new carrier system is suggested. Many of 1,4-dihydropyridine-3,5-dicarboxylate derivatives are used as calcium channel blockers which are known to have long shelf-life time, and at the same time, they are safe, with no reported neurotoxicity. Since efficiency of brain specific delivery depends on the rate of oxidation of the dihydropyridine carrier, (the faster the rate, the higher the efficiency), these 3,5-dicarbonyl compounds have almost of no brain-specific delivery properties. *N*-alkoxycarbonylmethyl derivatives of 1,4-dihydropyridine-3,5-dicarboxylate, a new carrier system is suggested and expected to be stable enough for formulation and storage. The drug moiety will be connected to the 3,5-dicarbonyl groups in the form of esters or amides. The suggested drug-carrier once delivered and distributed in the body, will be subjected to several sequential enzymatic hydrolytic and oxidative processes. The D-carrier is designed so that, the rate of hydrolysis of the ester group at the nitrogen atom should be faster than that of the hydrolysis and release of the drug moiety. The stability and efficiency of brain specific delivery of model drugs were investigated. The in vitro and in vivo studies proved the efficiency of brain specific delivery of the carrier and at the same time its shelf life stability. The results suggest that the designed carrier is promising to be used in application of pharmaceutical formulation.

Introduction

One of the most important goals of pharmaceutical research and development is the targeted drug delivery, which is the most efficient way to improve the drug therapeutic index (the ratio of safety to efficacy). This goal could be achieved by physical, biological, or molecular systems that result in producing high concentrations of pharmacologically active agents at the pathologically relevant site.[1]

The chemical approaches of drug delivery are the most advanced chemical-enzymatic-based drug targeting systems or, better stated, 'retro-metabolic drug design approaches'.[2] Chemical drug delivery systems (CDS) are defined as compounds that are produced by synthetic chemical reactions forming covalent bonds between the drug and the so-called 'carrier' and other moieties. At least one chemical bond needs to be broken for release of the active component. This concept was successfully used for targeting drugs to specific organs like the brain, lung and within the eye.[3]

The delivery of drugs to the brain is often seriously limited by the blood–brain barrier (BBB).[4] It is generally accepted that the ability of the molecule to cross the BBB is a function of its partition coefficient. The ionized compounds fail to achieve cerebrospinal fluid over plasma distribution ratio, unless they are actively transported. The approach of derivatizing the compound and forming a prodrug that exhibits improved physicochemical properties for the transport through the BBB should be treated with caution. This approach may improve the delivery of the drug to the brain and simultaneously improves transport to other tissues, thus increasing the incidence of systemic side effects.

A general method for brain specific delivery with sustained release of the drug was developed based on dihydroazine ↔ azinium salt redox chemical delivery systems.[5,6] This delivery system reduces the systemic toxicity by accelerating the elimination of the hydrophilic drug-quaternary carrier from the general circulation. On the other hand, it provides a low-level

*Corresponding author. Tel.: +20-12-3971-632; fax: +20-88-332-776; e-mail: sheha@acc.aun.eun.eg

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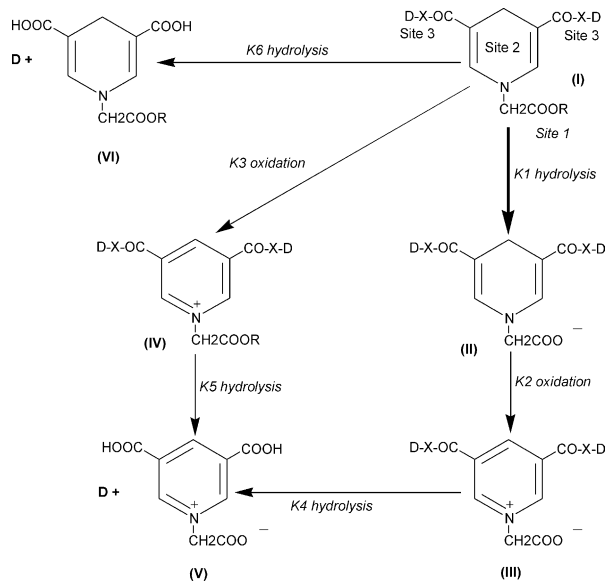


Scheme 1. Representative outline for the chemical delivery system design and work rationale; D = drug; X = NH, or O; R = alkyl.

sustained release of the active species in the brain that even reduces the central toxicity. The main factor is the choice of the quaternary carrier, which must be of low toxicity alone and when conjugated with the drug.[7]

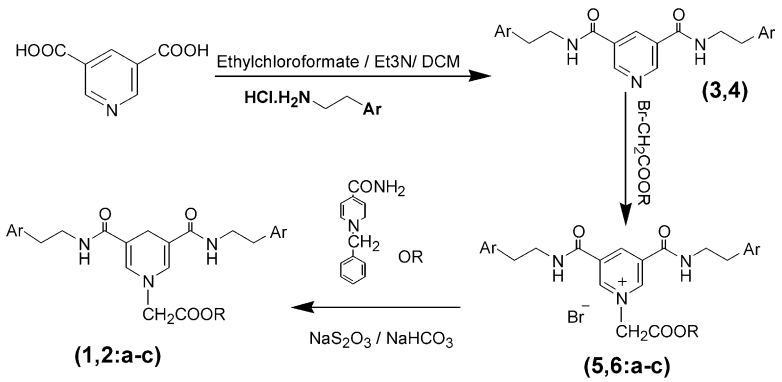
A class of organ-targeting systems that use dihydropyridine conjugates to enhance brain delivery of drugs with low toxicity has been described.[8] These chemical delivery systems rely on the conversion of dihydropyridine to pyridinium salts, biomimicking the NADH→NAD^+ oxidation, to achieve selective CNS sequestration. The lipophilicity of the designed pyridine chemical delivery systems (PCDS) is essential for significant brain uptake. In the brain the PCDS should be relatively rapidly oxidized to the polar quaternary salt to prevent efflux. The release of the active drug occurs subsequently in a sustained manner at a rate that allows reaching active concentrations at the site of action. Two main problems arise in the application of the above approach in pharmaceutical preparations. (1) Short shelf-life stability due to acid-catalyzed hydration at C_5=C_6 double bond that gives 6-hydroxy-1,4,5,6-tetrahydropyridine derivatives.[9,10] This hydrated product will not be oxidized enzymatically in vivo to the corresponding quaternary derivative. Accordingly, the chemical delivery system loses the most important step in its specific delivery pathway, and hence loses its brain lock-in property. (2) Isomerization into 1,2-dihydro-derivatives during preparation.[11]

Many of *N*-substituted-1,4-dihydropyridine-3,5-dicarboxylate derivatives are used as calcium channel blockers which are known to have long shelf-life time, and at the same time, they are safe, with no reported neurotoxicity. Previous studies on these compounds as brain-specific chemical delivery systems showed that they are very stable toward oxidation and require a very long time to get oxidized to the corresponding quaternary salts.[6] Since efficiency of brain specific delivery depends on the rate of oxidation of the dihydropyridine carrier, (the faster the rate, the higher the efficiency). These 3,5-dicarbonyl compounds were almost of no brain-specific delivery properties.

The rationale of the present work based on insertion of an alkoxycarbonylmethyl group on the nitrogen of 1,4-dihydropyridine-3,5-dicarboxylic acid to give a carrier system with expected brain locked in properties and enough stability during formulation and storage. The suggested drug-carriers **I** once delivered and distributed in the body, will be subjected to several enzymatic hydrolytic and oxidative processes (Scheme 1). The D-carrier **I** is designed so that, the rate of hydrolysis of the alkyl ester (site 1) is faster than that of the hydrolysis and release of the drug moiety (site 3): $K_1 > K_6$. Selecting the suitable linkages of the drug and type of R group can control this difference.[12] Once the ester is hydrolyzed, compound **I** will be transformed to the corresponding anion **II**. The negative charge on the acid anion is expected to decrease the energy of activation of oxidation (ΔE) of the 1,4-dihydropyridine to the corresponding quaternary, since the quaternary product **III**, with the close location of opposite charges, resembles the zwitterions of α-amino acids. The quaternary ion formed inside the brain will be locked-in, while that formed in blood and other tissues will be cleared easily. Two simple model drugs, phenethylamine and tryptamine, were chosen, each to be linked through amide bonds on both C_3 and C_5 of pyridine-3,5-dicarboxylic acid. Representative compounds will be used in the in vitro and in vivo studies to investigate their stabilities and efficiency of brain-specific delivery.

Results and Discussion

The 3,5-di(*N*-β-phenethyl)carbamoylpyridine (**3a–c**) and 3,5-di(*N*-2-[3-indolyl]ethyl)carbamoylpyridine (**4a–c**) reported in this work were prepared by coupling pyridine-3,5-dicarboxylic acid with the appropriate amine as shown in Scheme 2. The reaction proceeded at very mild conditions, was less time consuming and afforded the corresponding pure amide (**3** and **4**). The structures of the prepared amides were ascertained by spectral analysis (IR and ^{1}H NMR) and elemental analysis. The quaternary derivatives (**5**,**6**:**a–c**) were obtained by treating the derivatives (**3** and **4**) with the appropriate alkyl halide (ethyl bromoacetate, isopropyl bromoacetate and bromoacetic acid) under mild condition. The quaternization process was observed to be easier in the case of tryptamine derivatives than that of phenethylamine derivatives, where reaction times were shorter and with better yields. The spectral analysis (IR and ^{1}H NMR) as well as elemental analysis confirmed the structures of the synthesized compounds (**5**,**6**:**a–c**). It was reported that reduction of 3-substituted or 3,5-disubstituted pyridinium salts with sodium dithionite in mildly basic solution affords only the corresponding 1,4-dihydropyridines.[6] Reduction of the quaternaries (**5**,**6**:**a**,**b**) was accomplished by using sodium dithionite in aqueous solution of $NaHCO_3$. The use of dithionite for reduction of quaternaries is limited by water solubility of the quaternary salt and/or stability of the dihydropyridine



Scheme 2. Preparation of the carrier system with tryptamine (Ar = 3-indolyl) or β-phenethylamine (Ar = phenyl) as model drugs.

in the aqueous alkali solution. A novel method for the reduction of pyridinium salts to the corresponding dihydropyridine using 1-benzyl-1,2-dihydroisonicotinamide was described by Nuvole et al.[13] A disproportionation reaction occurs in organic solvent resulting in reduction of the pyridinium salt to the corresponding dihydropyridine derivative, while 1,2-dihydroisonicotinamide is oxidized to the corresponding quaternary isonicotinamide that is easily removed by filtration.[14] This method was successfully used in the reduction processes of drug-carrier quaternaries that are particularly sensitive to aqueous basic media. Attempts to prepare the 1,4-dihydropyridine derivatives (**1c** and **2c**) were successful but the product could not be obtained in analytical pure form. Trials for purification of the product from 1-benzylisonicotinamide were unsuccessful, the purity was found to be about 90–95% as checked by HPLC. The structure of dihydropyridine derivatives (**1**,**2**:**a**–**c**) were confirmed based on their ^{1}H NMR and UV-spectra.

The stabilities of the 1,4-dihydropyridine-3,5-dicarboxylic acid derivatives (**1** and **2**) were determined in the presence of the oxidizing agents (alcoholic AgNO3 and potassium ferricyanide), buffer solution (stability after reconstitution), 80% plasma, and 20% brain homogenate. The dihydropyridine derivatives (**1a** and **2a**) were selected for the in vivo study. To carry out these investigations, a reverse-phase HPLC method of analysis of these compounds and their metabolites in solutions, plasma and brain homogenate was developed, and validated before use.

Table 1. The rates of oxidation of prepared phenethylamine-CDS (**1a**–**c**) and tryptamine-CDS (**2a** and **2c**) ($n = 3$)

Oxidizing agent	Compd	k_{disapp}[a] (min^{-1})	$T_{1/2}$ (h)	r
Silver nitrate	**1a**	8.03×10^{-4}	14.4	0.94
	1b	6.83×10^{-4}	17	0.99
	1c	Too fast to be monitored		
	2a	8.4×10^{-4}	13.8	0.97
	2c	2.23×10^{-2}	0.5	0.99
Ferricyanide reagent	**1a**	1.15×10^{-2}	1.0	0.98
	1b	1.17×10^{-3}	9.9	0.98
	1c	2.36×10^{-3}	4.9	0.98
	2a	8.67×10^{-3}	1.33	0.96
	2c	1.17×10^{-3}	9.9	0.95

[a]k_{disapp} is pseudo first order rate constant for the dihydropyridine derivatives (**1a**–**c**, **2a**, and **2c**) calculated from three replicate determination at each time.

The 1,4-dihydropyridine derivatives (**1**,**2**:**a**–**b**) with *N*-acetic acid esters (ethyl and isopropyl) were found to be relatively stable towards oxidation with silver nitrate (Table 1). While their corresponding acids (**1c** and **2c**) were found to be oxidized much faster, to the extent that oxidation of compound **1c** could not be monitored and compound **2c** was found to have $t_{1/2}$ of oxidation of about 0.5 h. The ferricyanide oxidation of dihydropyridine is commonly used to study their sequential electron–proton–electron transfer mechanism.[15] During oxidation study with ferricyanide, it was found that the ethyl esters (**1a** and **2a**) are partially hydrolyzed to the corresponding acid (minor quantity) in addition to the oxidation product. The isopropyl ester **1b** showed a slower rate of disappearance, by both hydrolysis and oxidation, throughout the time of the experiment. These results prove the idea that the acid anion catalyzed the oxidation process of the dihydro compounds to the corresponding quaternaries.

The stability in both slightly alkaline or acidic buffers of the prepared dihydropyridine CDS was investigated to help in selecting the buffer system to be used as the solvent, if needed in solution during its formulation. The stabilities of tryptamine CDS ester **2a**, and its corresponding acid **2c** were investigated in phosphate buffers (0.2 M, pHs 7.4 and 5.8) at room temperature (Table 2). The concentration of the parent CDS and its oxidation or degradation products were monitored by HPLC for 4 days. Pseudo first order rate of disappearance of each CDS was determined and its $t_{1/2}$ was calculated (Table 2). It was observed that the tryptamine CDS ester **2a** disappears more than twice as fast at pH 7.4 than at pH 5.8 ($t_{1/2} = 97$ and 246 h, respectively). Hydrolysis was observed in both buffers, where the hydrolysis product was detected after 24 h at pH 7.4 and after 48 h at pH 5.8. The acid **2c** was found only as quaternary acid **6c** at both pHs. The results showed that these CDS should be stored in solid forms to be reconstituted in slightly acidic buffer prior to administration.

To know the possible metabolic pathways of the synthetized CDS in human plasma, the stability of the ester

Table 2. Stability of tryptamine CDSs in buffers at various pHs ($n=3$)

pH	Compd	($k_{disapp}\pm$SD)[a]$\times10^{-3}$ h^{-1}	$t_{1/2}$ (h)	r
7.4	**2a**	7.14 ±0.304	97	0.99
	2c	11.66±0.42	59.5	0.97
5.8	**2a**	2.87±0.468	246	0.96
	2c	15.56±2.99	45.6	0.98

[a]k_{disapp} is pseudo first order rate constant for the dihydropyridine derivatives (**2a** and **2c**) calculated from three replicate determination at each time.

1a and **1b** in 80% human plasma was determined. The pseudo first order rate constant and $t_{1/2}$ of disappearance of the CDS was calculated (Table 3). It was observed that only the quaternary acid was found in plasma as a metabolite in addition to the dihydropyridine **1a** or **1b**. The isopropyl ester is more stable than the ethyl ester that may be due to the difference in the rate of hydrolysis of the different esters.

The stabilities of the synthesized dihydropyridines (**1a**–**c** and **2c**) were investigated in 20% rabbit brain homogenate. The homogenate was equilibrated at 37 °C and freshly prepared solution of 1,4 dihydro compound was added. The concentration of the spiked compound and its oxidation or degradation products were monitored by HPLC. As shown in Table 4, the isopropyl ester **1b** is more stable toward enzymatic oxidation in brain homogenate than the ethyl ester **1a** and the corresponding acid derivative **1c**, with half-life times of 24.0, 9.5 and 5.6 h, respectively. The results were concurrent with the expected results calculated by the molecular orbital calculation.[12]

The results prove the idea that these types of esters are more stable even against enzymatic oxidation, once the ester is hydrolyzed to the corresponding acid, which will exist as the anion, the rate of oxidation will be accelerated to give the corresponding quaternary compound. The tryptamine-acid derivative **2c** is oxidized at a slower rate than the phenethyl-acid derivative **1c**; $t_{1/2}=7.7$ and 5.6 h, respectively. These results may indicate the importance of the steric factor on the rates of enzymatic oxidation. According to the results of the in vitro study, the ethyl ester has enough stability in plasma to reach the brain and at the same time it is hydrolysed at a reasonable rate to give the acid anion, in the brain, which is needed to enhance the rate of oxidation of the CDS to the corresponding quaternary. The dihydropyridine derivatives (**1a** and **2a**) were selected for the in vivo study. A solution in DMSO was injected through the jugular vein to a group of male Sprague–Dawley rats which were then sacrificed at various time intervals; their blood and brains were analyzed for the quaternary precursors of phenethylamine (**5a** and **5c**) and tryptamine (**6a** and **6c**). The results of these experiments are presented in Figures 1 and 2. With respect to phenthylamine-CDS **1a**, analysis of blood samples taken after 10 min proved the presence of **1a** in high concentration (17.14 μg/g) in addition to its products of hydrolysis. At 20 min only the dihydro and quaternary acids (**1c** and **5c**) could be detected. Neither quaternary acid nor other oxidation or hydrolysis products could be detected after 2 h. The picture looked different in the brain samples. The only product detected after 20 min was the quaternary acid and this increased with time. With respect to tryptamine-CDS **2a**, almost the same trend of delivery was observed, while delivery to the brain was slower than that of Ph-CDS **1a**. The amount of **2a** delivered to the brain

Table 3. The rate of hydrolysis phenethylamine CDSs **1a** and **1c** in 80% human plasma

Compd	($k_{disapp}\pm$SD)[a] $\times$ 10^{-3} min^{-1}	$t_{1/2}$ (h)	r
1a	1.35±0.0145	8.56	0.95
1b	0.5±0.00428	23.1	0.92

[a]k_{disapp} is pseudo first order rate constant for the dihydropyridine derivatives (**1a** and **1b**) calculated from three replicate determination at each time.

Table 4. The rate of disappearance of CDSs (**1a**–**c** and **2c**) in 20% rabbit brain homogenate

Compd	($k_{disapp}\pm$SD)$\times10^{-3}$ min^{-1}	$t_{1/2}$ (h)	r
1a	1.213±0.0956	9.5	0.98
1b	0.480±0.0866	24.0	0.81
1c	2.058±0.0971	5.6	0.96
2c	1.499±0.1066	7.7	0.96

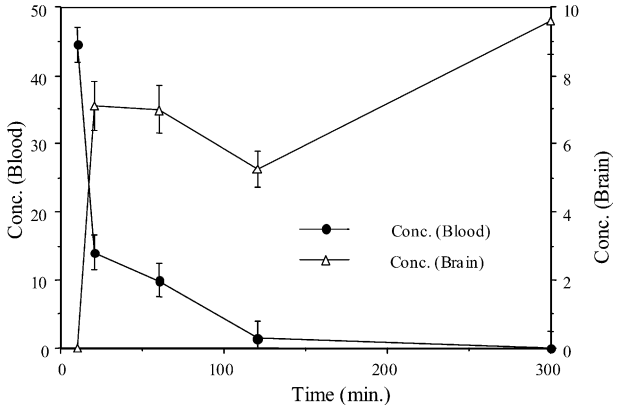


Figure 1. In vivo distribution of phenethylamine-CDS (**1a**) in blood and in rat brain homogenate (concd in μg/g against time in minutes).

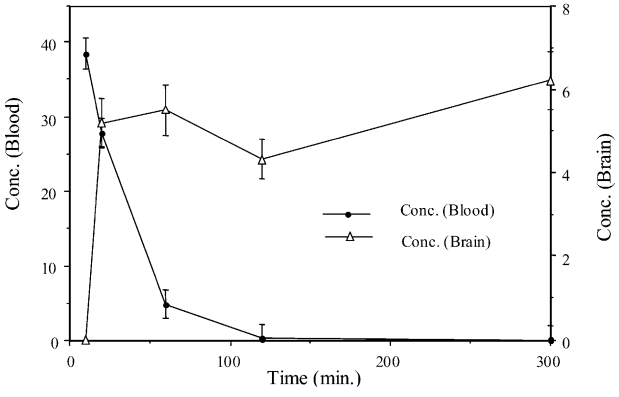


Figure 2. In vivo distribution of tryptaine-CDS (**2a**) in blood and in rat brain homogenate (concd in μg/g against time in minutes).

after 5 h was about two-thirds that of **1a**. This may be due to the rapid clearance of **2a** from blood so its concentration necessary for BBB penteration was less than **1a**.

In general, the results show in both CDSs that, the corresponding quaternary acid was detected at higher concentrations in blood than in brain within the first hour. The concentration of this quaternary was below detection limit in blood after 2 h from injection while it increases in the brain until 5 h, that is, increase in the brain and decrease in the blood with time until 5 h. The presence of the quaternary acid as the only product in brain, proves the main idea of the new CDS. The lipoidal CDS **1a** or **2a** penetrates the BBB and rapidly hydrolyzed by brain esterases to the corresponding acid, present as anion in pH 7.4. Oxidation of the resultant anion, by brain dehydrogenases, to the corresponding quaternary is accelerated due to the negative charge on the carboxylate anion. Accordingly, the results prove the following sequential processess after brain penetration, the D-CDS is sequentially; (i) hydrolyzed to give the dihydro acid anion (ii) oxidized to the quaternary acid anion 'the locked-in moiety', which (iii) hydrolyzed in a timely manner to release the parent drug.

Conclusions

1,4-Dihydropyridine-3,5-dicarboxylic acid with alkoxycarbonylmethyl substituent on the nitrogen atom was investigated as a novel shelf-stable carrier system to deliver biologically active compounds to the brain. The results proved that these types of esters-CDS, specially *N*-ethoxycarbonylmethyl substituent, are more stable even against enzymatic oxidation. Once the ester is hydrolyzed to the corresponding acid, the rate of oxidation will be accelerated to give the corresponding quaternary acid that has fast clearance from the blood and lock-in property in the brain to release the drug.

Experimental

General methods

Melting points were determined on electrothermal melting point apparatus and are uncorrected. Elemental microanalysis for carbon, hydrogen, and nitrogen were performed on Elementar Analysensysteme GmbH VarioEL (Germany). IR spectra were recorded as KBr disks on a Shimadzu IR 200-91527 spectrophotometer.

^{1}H NMR spectra were run on Varian Em-360L NMR spectrophotometer (60 MHz) (Varian, USA) and on Jeol, Lambda, Oxford NMR YH (400 MHz, Japan) using TMS as internal standard, chemical shifts are expressed in δ (ppm).

The high performance liquid chromatographic system consisted of Pump (TSP Thermo Separation Products, ConstaMetric® 3500, CT, USA). The column used was an Eurosepher-100 C18 (Knauer) (25 cm×4.6 mm internal diameter, 5 μm particle diameter). The sample loop was 20 μL. The chromatographic peaks were UV-scanned with the use of a photodiode array detector (TSP Thermo Separation Products, Spectromonitor® 5000, CT, USA). The system controlled with LC talk program (LC talk Version 2.03 Software, Copyright 1993 by Thermo Separation Products, CT, USA). An isocratic solvent system was prepared by mixing 0.1% aqueous solution of trifluoroacetic acid with acetonitrile in a ratio of 45:55, v/v. The mobile phase was degassed and filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA) and pumped at a flow rate of 1.0 mL/min. Acetonitrile (ACN), methanol (MeOH) and trifluoroacetic acid (TFA) used were of HPLC grade and obtained from E-Merck, Darmstadt, Germany. All other chemicals and solvents were of analytical reagent grade and were used without further purification.

Preparation of 3,5-di-(*N*-[aryl]ethyl)carbamoylpyridine (3 and 4). To an ice-cold solution of 3,5-pyridinedicarboxylic acid (1.67 g; 0.01 mol) in 30 mL dichloromethane, triethylamine (10 mL; 0.072 mol) was added, while stirring. To the mixture, ethyl chloroformate (4.8 mL; 0.05 mol) was added drop wise over a period of 10 min, and stirring was continued for 1 h. The amine (0.03 mol) was then gradually added within 10 min and stirring was continued for an additional 6 h. The solvent was then distilled under reduced pressure, and the residue was extracted with ethyl acetate. The extract was washed with 0.01 N HCl, dried, distilled, and the crude solid product was crystallized from ethanol to yield the diamide derivatives of pyridine-3,5-dicarboxylic acid. The following compounds were prepared according to this procedure.

3,5-Di(*N*-β-phenethyl)carbamoylpyridine (3). β-Phenethylamine (3.8 mL; 0.03 mol) was used as the amine to yield 3 g (80%) of white crystals; mp 189–190 °C. ^{1}H NMR ($CDCl_3$) δ 2.8 (t, 4H, $NHCH_2\mathit{CH_2}$), 3.5 (q, 4H, $NH\mathit{CH_2}CH_2$), 7.2 (s, 10H, Ar–H_5), 8.3 (t, 2H, NH), 8.6 (s, 1H, C_4–H), 9.1 (s, 2H, C_2–H, C_6–H).

3,5-Di(*N*-2-[3-indolyl]ethyl)carbamoylpyridine (4). Tryptamine hydrochloride (5.89 g; 0.03 mol) was used as amine to yield 4 g (73%) of white crystals; mp 198–200 °C. ^{1}H NMR (DMSO-d_6) δ 3.0 (t, 4H, $NHCH_2\mathit{CH_2}$), 3.6 (t, 4H, $NH\mathit{CH_2}CH_2$), 6.9–7.5 (m, 10H, indole protons), 8.5 (s, 2H, NH of indole), 8.6 (s, 1H, C_4–H of pyridine), 9.1(s, 2H, C_2-H, C_6-H of pyridine), 10.3 (s, 2H, CONH). Elemental analysis, $C_{27}H_{25}N_5O_2$ (451.52); calcd C, 71.82; H, 5.58; N, 15.51. Found C, 71.45; H, 5.46; N, 15.22.

Preparation of 1-(alkyl)-pyridinium bromide derivatives (5,6:a–c)

To a solution of compound **3** or **4** (0.003 mol) in 30 mL of methanol, (0.004 mol) of bromoacetic acid (ester) was added, the reaction mixture was refluxed overnight, and then cooled to room temperature. The formed crystals were filtered, washed with methanol, dried to give the corresponding pyridinium bromide derivative. The following compounds were prepared by this procedure.

1-(Ethoxycarbonylmethyl)-3,5-bis(*N*-β-phenethylcarbamoyl)-pyridinium bromide (5a). Compound **3** and ethyl bromoacetate (0.22 mL) were used to yield 1.2 g (76%) of white crystals; mp 212–215 °C. ^{1}H NMR (DMSO-d_6/$CDCl_3$) δ 1.2 (t, 3H, CH_2CH_3), 2.8 (t, 4H, $NHCH_2CH_2$), 3.5 (t, 4H, $NHCH_2CH_2$), 4.1 (m, 2H, CH_2CH_3), 5.7 (s, 2H, NCH_2COO), 7.2 (s, 10H, Ar–H_5), 9.2 (s, 1H, C_4), 9.8 (s, 2H, C_2, C_6). Elemental analysis, $C_{27}H_{30}BrN_3O_4$ (540.45); calcd C, 60.00; H, 5.60; N, 7.78. Found C, 60.17; H, 5.68; N, 7.59.

1-(Isopropyloxycarbonylmethyl)-3,5-bis(*N*-β-phenethylcarbamoyl)-pyridinium bromide (5b). Compound **3** and isopropyl 2-bromoacetate (1 mL) were used to yield 1.1 g (81%) of white crystalline solid; mp 217–219 °C. ^{1}H NMR (DMSO-d_6/$CDCl_3$) δ 1.3 (d, 6H, CH_3), 2.8 (t, 4H, $NHCH_2CH_2$), 3.5 (t, 4H, $NHCH_2CH_2$), 5.0 (m, 1H, $CHCH_3$), 5.7 (s, 2H, NCH_2COO), 7.3 (s, 10H, Ar–H_5), 9.3 (s, 1H, C_4-H), 9.8 (s, 2H, C_2–H, C_6H). Elemental analysis, $C_{28}H_{32}BrN_3O_4$ (554.48); calcd C, 60.65; H, 5.82; N, 7.58. Found C, 60.77; H, 5.81; N, 7.48.

1-(Carboxymethyl)-3,5-bis(*N*-β-phenethylcarbamoyl)-pyridinium bromide (5c). Compound **3** (0.75 g; 0.002 mol) and bromoacetic acid (0.8 g, 0.006 mol) were used to yield 0.7 g (68%) of yellow crystals; mp 140–142 °C. ^{1}H NMR (DMSO-d_6) δ 2.8 (t, 4H, $NHCH_2CH_2$), 3.5 (t, 4H, $NHCH_2CH_2$), 5.2 (s, 2H, NCH_2COO), 7.2 (s, 10H, Ar-H_5), 9.2 (s, 1H, C_4–H), 9.4 (s, 2H, C_2-H, C_6-H). Elemental analysis, $C_{25}H_{26}BrN_3O_4$ (512.40); calcd C, 58.60; H, 5.11; N, 8.20. Found C, 57.88; H, 5.26; N, 8.16.

1-(Ethoxycarbonylmethyl)-3,5-bis{*N*-[2-(3-indolyl)ethyl]-carbamoyl}pyridinium bromide (6a). Compound **4** (0.451 g, 0.001 mol) and ethyl bromoacetate (0.55 mL; 0.005 mol) were used to yield 0.5 g (81%) of yellow crystals, mp 194–196 °C. ^{1}H NMR (DMSO-d_6) δ 1.2 (t, 3H, CH_2CH_3), 3.0 (t, 4H, $NHCH_2CH_2$), 3.6 (t, 4H, $NHCH_2CH_2$), 4.2 (m, 2H, CH_2CH_3), 5.7 (s, 2H, N–CH_2COO), 6.9–7.5 (m, 10H, indole protons), 9.4 (s, 2H, NH of indole), 9.5 (s, 1H, C_4-H of pyridine), 9.6 (s, 2H, C_2-H, C_6-H of pyridine), 10.8 (s, 2H, CONH). Elemental analysis, $C_{31}H_{32}BrN_5O_4$ (618.52); calcd C, 60.20; H, 5.21; N, 11.32. Found C, 59.97; H, 5.08; N, 11.20.

1-(Isopropyloxycarbonylmethyl)-3,5-bis{*N*-[2-(3-indolyl)-ethyl]-carbamoyl}pyridinium bromide (6b). Compound **4** (0.451 g; 0.001 mol) and 1 mL of isopropyl-2-bromoacetate were used to yield 0.55 g (87%) of yellow crystal; mp 218–220 °C. ^{1}H NMR (DMSO-d_6) δ 1.2 (d, 6H, $CHCH_3$), 3.0 (t, 4H, $NHCH_2CH_2$), 3.6 (t, 4H, $NHCH_2CH_2$), 5.0 (m, 1H, $CHCH_3$), 5.7 (s, 2H, N–CH_2COO), 6.9–7.5 (m, 10H, indole protons), 9.4 (s, 2H, NH of indole), 9.5 (s, 1H, C_4–H of pyridine), 9.6 (s, 2H, C_2–H, C_6–H of pyridine), 10.8 (s, 2H, CONH). Elemental analysis, $C_{32}H_{34}BrN_5O_4$ (632.55); calcd C, 60.76; H, 5.42; N, 11.07. Found C, 60.49; H, 5.40; N, 10.94.

1-(Carboxymethyl)-3,5-bis{*N*-[2-(3-indolyl)ethyl]-carbamoyl}pyridinium bromide (6c). Compound **4** (0.451g; 0.001 mol) and bromoacetic acid (1.1 g; 0.008 mol) were used to give 0.45 g (76%) of yellow crystal mp 213–216 °C. ^{1}H NMR (DMSO-d_6) δ 3.0 (t, 4H, $NHCH_2CH_2$), 3.6 (t, 4H, $NHCH_2CH_2$), 5.6 (s, 2H, N–CH_2COO), 6.9–7.5 (m, 10H, indole protons), 9.4 (s, 2H, NH of indole), 9.5 (s, 1H, C_4-H of pyridine), 9.6 (s, 2H, C_2-H C_6-H of pyridine), 10.8 (s, 2H, CONH). Elemental analysis, $C_{29}H_{28}BrN_5O_4$ (590.47); calcd C, 58.99; H, 4.78; N, 11.86. Found C, 58.60; H, 4.67; N, 11.61.

Preparation of 1-(alkyl)-1,4-dihydropyridine derivatives (1,2:a–b)

To a solution of compound **5**,**6**:**a**–**b** (0.4 mmol) in 200 mL of deaerated water, sodium bicarbonate (1 g; 0.012 mol) and 200 mL of ether were added. The mixture was stirred in an ice bath, and sodium dithionite (0.7 g; 4 mmol) was added portion-wise over a period of 5 min and stirring was continued for 3 h under nitrogen stream. The ether layer was then separated, washed with water, dried over anhydrous Na_2SO_4 and evaporated under vacuum, to give the corresponding dihydropyridine derivative. The following compounds were prepared by the abovementioned procedure.

1-(Ethoxycarbonylmethyl)-3,5-bis(*N*-β-phenethylcarbamoyl)-1,4-dihydropyridine (1a). Compound **5a** (0.216 g) was used to yield 0.1 g (54%) of yellow solid, mp 160–162 °C. ^{1}H NMR (DMSO-d_6) δ 1.2 (t, 3H, CH_2CH_3), 2.7 (t, 4H, $NHCH_2CH_2$), 3.0 (s, 2H, C_4–H), 3.3 (t, 4H, $NHCH_2CH_2$), 4.1 (s, 2H, NCH_2COO), 4.2 (m, 2H, CH_2CH_3), 6.8 (s, 2H, C_2-H, C_6-H), 7.1–7.3 (m, 10H, Ar-H_5).

1-(Isopropyloxycarbonylmethyl)-3,5-bis(*N*-β-phenethylcarbamoyl)-1,4-dihydropyridine (1b). Compound **5b** (0.22 g) was used to give 0.17 g (66%) of yellow semisolid, which could not be crystallized. ^{1}H NMR (DMSO-d_6) δ 1.2 (d, 6H, $CHCH_3$), 2.7 (t, 4H, $NHCH_2CH_2$), 3.0 (s, 2H, C_4–H), 3.3 (t, 4H, $NHCH_2CH_2$), 4.1 (s, 2H, NCH_2COO), 4.9 (m, 1H, $CHCH_3$), 6.8 (s, 2H, C_2–H, C_6–H), 7.1–7.3 (m, 10H, Ar–H_5).

1-(Ethoxycarbonylmethyl)-3,5-bis{*N*-[2-(3-indolyl)ethyl]-carbamoyl}-1,4-dihydropyridine (2a). Compound **6a** (0.246 g) was used to yield 0.119 g (55%) of pale-yellow solid of mp 178–80 °C. ^{1}H NMR (DMSO-d_6) δ 1.2 (t, 3H, CH_2CH_3), 2.8 (t, 4H, $NHCH_2CH_2$), 3.1 (s, 2H, C_4-H), 3.3 (t, 4H, $NHCH_2CH_2$), 4.1 (s, 2H, NCH_2COO), 4.2 (m, 2H, CH_2CH_3), 6.8 (s, 2H, C_2-H, C_6-H), 6.9–7.5 (m, 10H, indole protons).

1-(Carboxymethyl)-3,5-bis(N-β-phenethylcarbamoyl)-1,4-dihydropyridine (1c). To a solution of **5c** (0.2 g; 0.4 mmol) and the 1-benzyl-1,2-dihydroisonicotinamide (0.1 g; 0.5 mmol) in anhydrous methanol (20 mL) was stirred at 0 °C for 4 h under nitrogen. The solid that separated was filtered and washed with methanol and ether. The solid was identified as 1-benzyl-4-carbamoyl-pyridinium bromide. The filtrate was evaporated by stream of nitrogen to give semisolid yellow residue, which was identified as 1,4-dihydro acid (**1c**).

1-(Carboxymethyl)-3,5-bis{*N*-[2-(3-indolyl)ethyl]carbamoyl}-1,4-dihydropyridine (2c). The same procedure used for the preparation of compound **1c** was followed to give semisolid yellow residue, which was identified to be the 1,4-dihydro acid (**2c**) with a little impurities of 1-benzyl-4-carbamoylpyridinium bromide (about 5% by HPLC) and could not be separated.

Chemical oxidation and in vitro studies

Chemical oxidation of the prepared dihydropyridine CDSs (1,2:a–c). Oxidation with silver nitrate. In a series of tubes, 1 mL of 2×10^{-4} M methanolic solution of the PCDSs (**1**,**2**:**a**–**c**) was added to 5 mL of 10% methanolic silver nitrate solution and the mixture was mixed for 3 min. At the specific time intervals (5, 15, 30, 60, and 120 min), one tube is vortexed, centrifuged and the supernatant was filtered by using 0.22 μm nylon-membrane filtration disc and analyzed by HPLC. The rate of disappearance of the PCDSs and the appearance of the corresponding quaternaries was determined. The apparent pseudo first order rate constant of disappearance of PCDSs (k_{disapp}, min^{-1}) was determined by linear regression of the log of the mean peak area of three experiments against time in minutes.

Oxidation with ferricyanide reagent. To a series of tubes, each containing 3 mL of freshly prepared ferricyanide reagent,[16] 1 mL of the freshly prepared PCDSs (**1**,**2**:**a**–**c**) in concentration of (2×10^{-4} M) were added at room temperature. The mixture was shaken and set aside. At a suitable time interval (5, 15, 30, 60, and 120 min) one tube is vortexed, filtered, and analyzed by HPLC. The rate of disappearance of the PCDSs and the appearance of the corresponding quaternaries was determined. The apparent pseudo first order rate constant of disappearance of PCDSs (k_{disapp}, min^{-1}) was determined by linear regression of the log of the mean peak area three experiments against time in min.

Stability of the prepared 1,4-dihydropyridine CDSs (2a and 2c) in buffer systems (*shelf stability*). Two series; each of three tubes, each tube contain 5 mL of phosphate buffers (pH 5.8 or 7.4), 0.5 mL of freshly prepared dihydropyridine derivatives solution (2×10^{-4} M) was added. The mixtures were kept stirring in a dark at room temperature all the time of experiment (4 days). At appropriate time intervals (2, 24, 48, 72, and 96 h), 20 μL of the mixture was taken and analyzed by HPLC for the dihydropyridine derivative (**2a** or **2c**) and corresponding metabolites. The apparent pseudo first order rate constant of disappearance of PCDSs (k_{disapp}, min^{-1}) was determined by linear regression of the log peak area of the corresponding dihydropyridine against time in minutes.

In vitro stability in biological fluids

In human plasma (1a and 1b). A freshly collected heparinized human blood was centrifuged at 4000 rpm for 20 min. The supernatant (plasma) was collected by a Pasteur pipette. To 5 mL of 80% freshly collected plasma (diluted with phosphate buffer (0.11 M, pH 7.4), prewarmed in a water bath at 37 ± 1 °C for 5 min, 300 μL of 2×10^{-4} M methanolic solution of freshly prepared PCDSs (**1a** or **1b**) was added and mixed thoroughly. The mixture was kept at 37 ± 1 °C the whole time of the experiment. At time intervals (5, 15, 30, 60, 90, 120, 180, and 240 min) 200 μL withdrawn from the tested mixture, added immediately to 2 mL of ice-cold methanol, vortexed, and placed in deep freezer (−20 °C). When all samples were collected, they were centrifuged, and supernatants were filtered through nylon membrane 0.22 μm pore size and analyzed by HPLC for their contents of the CDSs (**1a** or **1b**) and corresponding quaternary derivatives.

In 20% rabbit brain homogenate (1a–c and 2c). About 4 g of rabbit brain were taken, washed with ice-cold saline solution and homogenized in a tissue homogenizer with about 20 mL of aqueous ice-cold isotonic phosphate buffer (0.2 M, pH 7.4), while keeping the homogenizer tube in ice bath. Methanolic solution (300 μL of 2×10^{-4} M) of freshly prepared PCDSs (**1a**–**c** and **2c**) were mixed with 10 mL of homogenate, previously equilibrated at 37 ± 1 °C in a water bath. Samples of 0.5 mL were withdrawn from the tested mixture at different time intervals (15, 60, 120, and 300 min) and immediately added to 2 mL ice-cold methanol, vortexed, and placed in the deep freezer (−20 °C). When all the samples have been collected, centrifuged, and the supernatants were filtered through nitrocellulose membrane filter (0.22 μm) and analyzed by HPLC for their content of the PCDSs (**1a**, **1c**, and **2c**) and corresponding quaternary derivatives.

In vivo distribution. Five groups, each of three Sprague–Dawley female rats of average weight of 120–140 g were anesthetized with urethane. A freshly prepared solution of dihydropyridine (**1a** or **2a**) in DMSO (25 mg/mL) was injected through the jugular vein in a dose level of 20 mg/kg of animal body weight. After the appropriate time intervals, 1 mL of blood was withdrawn from the eye and added immediately to a centrifuge tube containing 4 mL of acetonitrile, which was afterwards weighed to determine the amount of blood added. The sample kept freezing till analysis. The animal was then decapitated, and the brain was weighed and kept in the freezer overnight then homogenized in 1 mL of water. Four milliliter of 5% DMSO in acetonitrile was added and the mixture homogenized again then centrifuged at 4000 rpm for 10 min. The amount of the quaternary derivatives was determined from the HPLC spectrum in relation to a recovery experiment made by adding a specific amount of the quaternary to a blank brain and hybrid in the same manner of homogenization and extraction.

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