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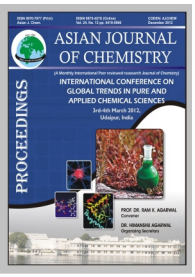

Synthesis and Study of Chemical Delivery System for Targeting Nitrogen Mustard to the Brain†

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The aim of the present study is to develop the redox delivery of *bis*-(2-chloroethyl) amine using nicotinic acid as carrier for the site specific and site enhanced sustained delivery of alkylating anticancer moiety to the brain. Structures of all the synthesized compounds were characterized by UV, IR and ^{1}H NMR techniques. An *in vitro* chemical oxidation study with silver nitrate of target compound (**4**) indicates that it can be readily converted into its corresponding salt (**3**). *In vitro* kinetic studies of final compound (**4**) in biological media showed its rate of oxidation followed pseudo-first-order kinetics with very short half-lives in rat plasma, human blood and tissue homogenate. The study of some other physicochemical parameter calculated by online software such as lipophilicity, polar surface area, rule of five, number of NH or OH hydrogen bond donors and nON value indicates that it can be permeable to blood-brain barrier.

Key Words: Anticancer agent, Blood-brain barrier, NBP assay, Physico-chemical parameters.

INTRODUCTION

The present approach in treatment of brain tumour is difficult by the presence of blood-brain barrier. Many potential useful drugs including those active against peripheral tumours such as chlorambucil and melphalan cannot enter the brain are therefore ineffective in treating cerebral neoplasms[1]. Various attempts have been made to overcome the limited access of anticancer agent into the brain by synthesizing the lipophilic analogues of alkylating anticancer agent[2] or by linking the active anticancer moiety to lipophilic carrier[3,4]. Chemical drug delivery systems represent novel and systematic ways of targeting active biological molecules to specific target sites or organs based on predictable enzymatic activation. One of the most promising approaches for chemical drug delivery is the concept of brain-specific drug delivery system based on a redox system analogous to the endogenous NADH↔NAD^+ coenzyme system developed by Bodor *et al.*[5]. The dihydropyridne↔quaternary salt redox system based chemical delivery system has been investigated earlier as a method to enhance the selective delivery of anticancer drugs to the brain[6-8]. In order to obtain anticancer agent with better blood-brain barrier penetration, we designed, synthesized and study reversible redox delivery system for nitrogen mustard agent.

EXPERIMENTAL

All the reagents and solvents used are purchased from the commercial suppliers. The melting points were determined on Veego-programmable melting point apparatus (microprocessor based) and are uncorrected. Infrared spectra were obtained on Perkin Elmer 882 using KBr disc. ^{1}H NMR spectra in DMSO-d_6 were recorded on Brucker AC-400 F, 400 MHz spectrometer using tetramethylsilane as internal standard. The ultraviolet spectra were recorded on Shimadzu, UV-1800 spectrophotometer. Reactions were monitored and the homogeneity of the products was checked by TLC which were prepared with silica gel G and activated at 110 ºC for 0.5 h.

Synthesis of 3-{N-{2-*bis*-(2-chloroethyl}aminoethyl} carbamoyl}pyridine (2): A mixture of nicotinamide (3.98 g, 10 mmol), *tris*-(2-chloroethyl)amine hydrochloride (**1**) (3.58 g, 20 mmol), which has previously been obtained by chlorination of triethanolamine, potassium carbonate (2.76 g, 20 mmol) and DMF was stirred overnight. The reaction mixture was poured into excess of water and extracted with ethyl acetate, washed and dried *in vacuo* to get compound as yellow oil (**2**) (1.42 g, 32 %).

Anal.: IR (KBr, ν_{max}, cm^{-1}): 3429 (N-H), 1638 (NH-C=O), 1050-1230 (C-N) and 740 (C-Cl). ^{1}H NMR ($CDCl_3$), δ 2.60-2.80 (m, 6H, J = 5.7 Hz, -[$CH_2NCH_2CH_2Cl)_2$], δ 3.3 (t, 4H, J = 7.16 Hz, -$N(CH_2CH_2Cl)_2$, δ 3.8 (t, 2H, J = 6.2 Hz, -$NHCH_2CH_2$-), δ 7.4 (t, 1H, J = 3.92 Hz, C_5 pyridine proton), δ 8.3 (d, 1H, C4 pyridine proton), δ 8.7 (d, 1H, C_6 pyridine proton) δ 9.2 (s, 1H, C_2 pyridine proton), δ 9.67 (brs, 1H, -$CONHCH_2$-).

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1-Methyl-3-{N-{2-*bis*{2-chloroethyl}aminoethyl}-carbamoyl}pyridinium iodide (3): To a solution of 2.9 g (0.01 mol) of *N,N-bis*-(2-chloroethyl)carbamoyl pyridine and 30 mL of acetone was added excess of methyl iodide (3 mL) of methyl iodide and the mixture was refluxed with stirring for 24 h. The yellow hygroscopic residue that separated was filtered, washed with acetone and dried. Yield: (65 %).

Anal.: IR (KBr, ν_{max}, cm^{-1}): 1660 (NH-C=O), 1050-1220 (C-N) and 745 (C-Cl). ^{1}H NMR (DMSO-d_6) δ 2.70-2.80 (m, 6 H, *J* = 5.7 Hz, -[$CH_2N(CH_2CH_2Cl^-)_2$], δ 3.3-3.4 (t, 4H, *J* = 7.16 Hz, -$NCH_2CH_2Cl)_2$, δ 3.7-3.8 (t, 2H, 6.2 Hz, -$NHCH_2CH_2$), δ 4.6 (s, 3H, -N+CH_3), δ 7.8 (s, 1H, C_4 pyridine proton), δ 8.3 (t, 1H, *J* = 6.8 Hz, C_5 pyridine proton), δ 9.3 (d, 1H, C_6 pyridine proton) and δ 9.5 (s, 1H, C_2 pyridine proton), δ 10.2 (brs, 1H, -$CONHCH_2$-).

1-Methyl-3-{N-{2-*bis*-{2-chloroethyl}aminoethyl}-carbamoyl}-1,4-dihydropyridines compound [dihydro derivative (4)]: To a solution of 1.47 g (3.4 mmol) of (**3**), 100 mL deaerated water and 100 mL of ethyl acetate were added 1.7 g (20.4 mmol) of sodium bicarbonate and 2.38 g (13.6 mmol) of sodium dithionite. The mixture was stirred under nitrogen for 1 h in ice bath. The ethyl acetate layer was separated and aqueous layer was re-extracted twice with 40 mL of ethyl acetate. The combined organic layer was washed with cold deaerated water, dried over anhydrous Na_2SO_4 and distilled on *vacuo*. The yellow coloured gummy residue preserved in desiccators protected from light and air. Yield: 60 %.

Anal.: IR (KBr, ν_{max}, cm^{-1}): 1645 (NH-C=O), 1050-1240 (C-N) and 748 (C-Cl). ^{1}H NMR ($CDCl_3$), δ 2.70-2.80 (m, 6 H, *J* = 5.7 Hz, -[$CH_2N(CH_2CH_2Cl^-)_2$], δ 3.0 (s, 3H, -NCH_3), δ 3.4 (t, 4H, *J* = 6 Hz, -$N(CH_2CH_2Cl)_2$, δ 3.8 (t, 2H, *J* = 6.2 Hz, -$NHCH_2CH_2$-), δ 4.8 (brs, 2H, C_4 pyridine proton), δ 5.6 (m, 1H, *J* = 4 Hz, C_5 pyridine proton), δ 5.64 (d, 1H, C_6 pyridine proton) and δ 7.0 (s, 1H, C_2 pyridine proton), δ 10.4 (brs, 1H, -$CONHCH_2$-).

In vitro chemical oxidation studies

By silver nitrate: One mL solution of 5 % dihydropyridine derivatives (**4**) was prepared in methanol. Then 5 mL of saturated methanolic solution of silver nitrate was added to above solution. The mixture was shaken and left for 2 min, centrifuged and the absorbance of the solution was determined after making 1.0 mL of solution diluted to 100 mL of methanol and concentration determined by standard curve. The same procedure was repeated for 4, 6, 8 and 10 min. (D.F. 100).

Kinetics of oxidation of the dihydro derivative (4): Calibration curves. A UV study of compound (**4**) revealed that they obey Beer's law with good correlation coefficient and at a wide range of dilution from 10 µg/mL to 60 µg/mL in both methanol and 2 % aqueous methanol. The study was done at 350 nm got the dihydro derivative.

Phosphate Buffer: In each of five tubes containing 0.2 mL of a 8.2×10^{-4} M methanolic solution of the freshly prepared dihydro derivative (**4**) was added 2 mL of freshly prepared phosphate buffer and the tubes were kept at 37 ºC in a water bath, at the end of the time period to be investigated, 8 mL of acetonitrile was added and the tubes were then shaken vigorously and centrifuged, the absorption of the supernatant solution at 350 nm was measured against reference.

100 % Whole human blood: Blood was withdrawn from a volunteer shortly before beginning each experiment. The blood was placed in heparinized tubes and stored on ice until needed, at which time it was incubated at 37 ºC. In each of 5 tubes containing 0.2 mL of a 8.2×10^{-4} M methanolic solution of the freshly prepared dihydro derivative (**4**) was added 2 mL of fresh heparinized whole human blood and the tubes were kept at 37 ºC in a water bath, at the end of the time period to be investigated, 8 mL of acetonitrile was added and the tubes were then shaken vigorously and centrifuged, the absorption of the supernatant solution at 350 nm was measured. A reference sample was made by addition of 0.1 mL of methyl alcohol instead of the sample solution following the same procedure.

In brain homogenate: Rat brain tissue (2.0 g) was homogenized in 10 mL of phosphate buffer, pH 7.4. The homogenate was centrifuged for 15 min at 3000 rpm, decanted, heated in a water bath at 50 ºC for 5 min and then centrifuged again. The supernatant solution was diluted to 100 mL with phosphate buffer (pH 7.4). To 10 mL of the freshly prepared homogenate was added 0.2 mL of a 6.25×10^{-4} M methanolic solution of the freshly prepared dihydro derivative (**4**). The mixture was scanned at 37 ºC from 400 nm to 220 nm every 5 min on a double beam UV spectrophotometer.

Reference sample: Methyl alcohol (0.2 mL) was diluted to 10 mL with the brain homogenate solution and the mixture was used to record the base line on a UV spectrophotometer and as a reference of the dihydro derivative (**4**) sample solution.

Liver homogenate: Rat liver tissue (5.0 g) was homogenized in 50 mL of phosphate buffer, pH 7.4. The homogenate was centrifuged for 15 min at 3000 rpm, decanted, heated in a water bath at 50 ºC for 5 min and then centrifuged again. The supernatant solution was diluted to 250 mL with phosphate buffer, pH 7.4. To 10 mL of the freshly prepated homogenate was added 0.2 mL of a 6.25×10^{-4} M methanolic solution of the freshly prepared dihydro derivative (**4**). The mixture was scanned at 37 ºC from 400 nm to 220 nm every 5 min on a double beam UV spectrophotometer.

Reference sample: Methyl alcohol (0.2 mL) was diluted to 10 mL with the liver homogenate solution and the mixture was used to record the base line on a UV spectrophotometer and as a reference of the dihydro derivative sample solution.

RESULTS AND DISCUSSION

Chemical characterization: The major steps for the synthesis of the dihydro derivative (**4**) agent are presented in Fig. 1. The nicotinamide was reacted with *tris*-(2-cholorethyl)-amine hydrocholoride (**1**) which was synthesized previously by chlorination of triethanolamine to obtain (**2**). The infrared spectra of (**2**) exhibited the disappearance of doublet of -$CONH_2$ peak at 3300 and 3350 cm^{-1} and appearance of single peak at 3300 of secondary amide and 750 cm^{-1} of C-Cl stretching. The ^{1}H NMR spectra of these compounds shows the six protons multiplets of -[$CH_2NCH_2CH_2Cl^-)_2$ at δ 2.60-2.80 ppm and four protons triplet at δ 3.3 ppm was assigned to the -CH_2- group adjacent to the -Cl group which is more deshielded due to the electronegativity effect of Chlorine. The compound (**2**) was then quaternized using methyl iodide in acetone to

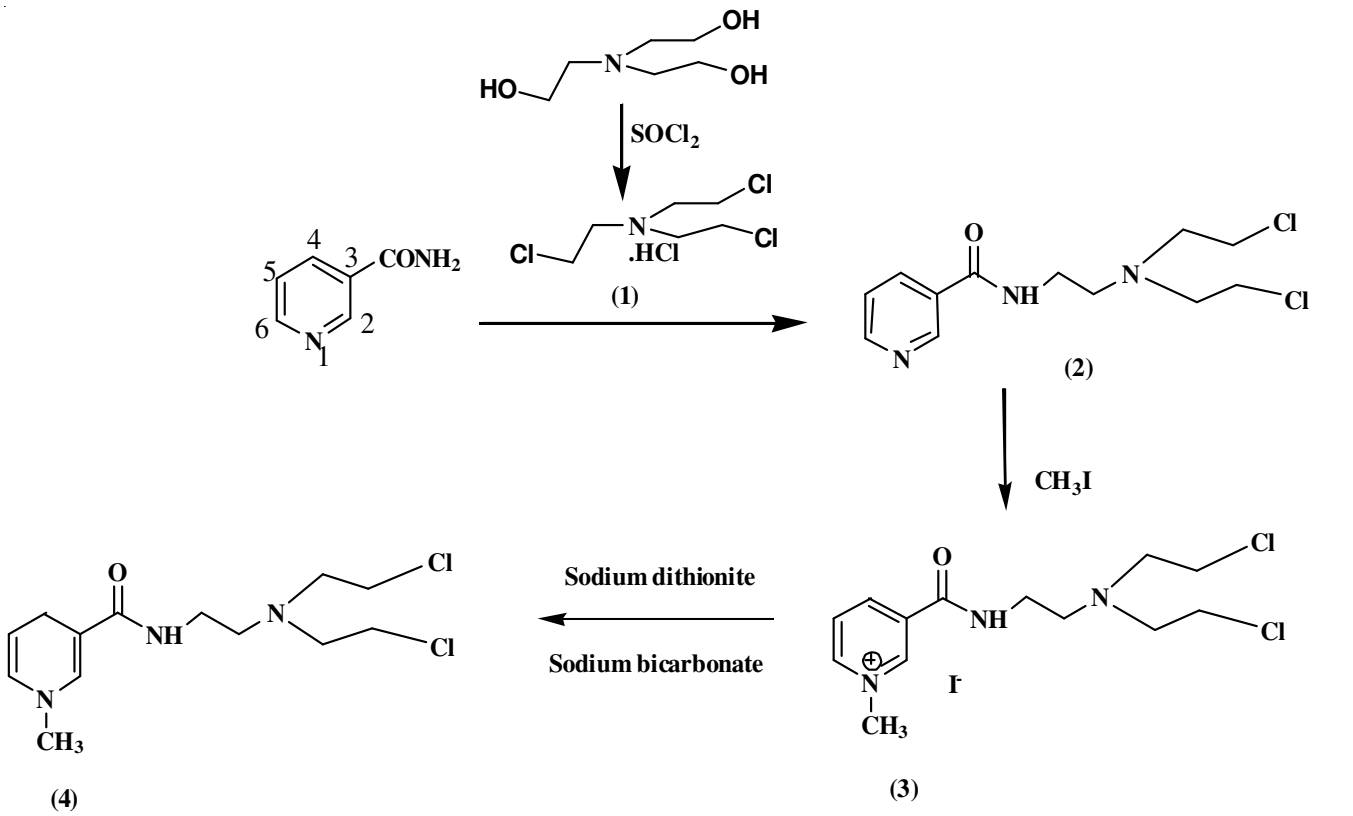

Fig. 1.

give the quaternary salts 1-methyl-3-[*bis*-(2-chloroethyl)amino]-carbamoyl-1,4-dihydro-pyridinium iodide (**3**). The structure was confirmed by appearance of 3*H* singlet of -NCH_3 group at δ 4.6. The obtained quaternary salt was then subjected to reduction process using sodium dithionite in alkaline medium, to give the corresponding dihydro derivative (**4**).

***In vitro* chemical and biological oxidation studies:** The prepared 1,4-dihydropyridines (**4**) was subjected to various chemical and biological investigations to evaluate the ability of these compounds to cross the BB and to be oxidized biologically into their corresponding quaternary compounds. In this study UV spectrophotometer was used to detect and monitor the oxidation of the tested 1,4-dihydropyridines into their corresponding quaternary salt either chemically or in biological fluids. All the kinetic studies were carried out in triplicate. The K values from the plot were calculated separately and average K and S.D. value was determined. Pseudo-first-order rate constants for the disappearance of compounds in biological media were determined by linear regression analysis from plot of log dihydro derivative (**4**) versus time. Quaternary salt which was thought to be converted by dihydro derivative (**4**), did not interfere with the absorption of CDS-mustard because its λ_{max} was found 340 nm, which was considerably different from that of dihydro derivative. The Table- 1 shows the calculated half-lives for dihydro derivative (**4**) in different media. The *in vitro* oxidation studies with $AgNO_3$ indicated the facile oxidative conversion of the *N*-methyl-1, 4-dihydropyridine analog (**4**) into the corresponding quaternary salt (**3**) with high oxidation rate (K = 4. 83 ± 0.06, $t_{1/2}$ = 14.2 min). The data indicate that the stability of the dihydro derivative (**4**) in aqueous media is much higher compared to that in biological media. The (**4**) was quite stable in Phosphate buffer pH 7.4 with K value of 1.12 ± 0.8 and $t_{1/2}$ of 61.8 min. Liver homogenate was the least stable medium for (**4**) with K value of 8.20 ± 3.6 and $t_{1/2}$ of 7.5 min. The stability of (**4**) in whole human blood was the highest among all tested biological materials (over 15 min). The (**4**) was readily oxidized ($t_{1/2}$ 11.0 min) in rat brain homogenate. The result of these *in vitro* stability tests were used to compare and evaluate the chemical delivery system to determine the final compound met the requirement of the ideal delivery system. This tissue homogenate study show, then, that brain targeting should be possible with these compounds.

TABLE-1
RATES OF OXIDATIVE CONVERSION IN VARIOUS MEDIA OF DIHYDRO DERIVATIVE (**4**) TO THE CORRESPONDING QUATERNARY PYRIDINIUM SALTS (**3**)

	Rate constant ($k \times 10^{-2}$ min^{-1})	$t_{1/2}$ min
Silver nitrate	4.83 ± 0.06	14.2
Phosphate buffer	1.12 ± 0.8	61.8
Whole human blood	3.8 ± 1.2	18.2
Brain homogenate	6.3 ± 2.7	11.0
Liver homogenate	8.20 ± 3.6	7.5

CNS active physicochemical parameters assessment: Since target compound is designed to be CNS active, hence the parameter were selected, which effect blood brain barrier. Physicochemical descriptors presented in Table-2 support the clinical potential of this dihydro derivative (**4**). Lipophilicity is one of the most important factors in controlling the interaction of drugs with biological system[9] has been calculated by various algorithm approaches. All methods show a significant increase

of lipophilic nature for the dihydro derivative (**4**). The polar surface area of a drug has been shown to be an effective means to predict drug transport[10,11]. The most active of CNS drug will have polar surface area of less than 70 A^2. Polar surface area of our dihydro derivative (**4**) compound is 35.57 A^2, which predicts that greater than 90 % of this agent will be absorbed by the intestine.

TABLE-2
COMPARISON OF PHYSICOCHEMICAL PARAMETERS OF DIHYDRO DERIVATIVE (**4**)

Physicochemical descriptors	Dihydro derivative (**4**)
C log P[1]	1.392
log P[1]	1.48
Mi log P[2]	1.115
Polar surface area[3]	35.57 A^2
Percent intestinal absorption[3] of drug	> 90 %
Molecular weight[2]	306
No. of violation[1] of rule of 5[2]	0
-NH and –OH[2]	1
nON values[2]	4

[1]C logP was calculated by Chem Draw Ultra 8.0
[2]Calculated by method of molinspiration
[3]Calculated by correlation of PSA to experimental intestinal absorption

A pharmacological parameter referred to as rule of 5 accurately predicts drug bioavailability and bioactivity[12]. Highest drug bioavailability and bioactivity is achieved when there are no violations of the following rules. According to the rule of five, compounds with number of violations not more than 1 shows good bioavailability and bioactivity. Analysis of molecular structure by molinspiration showed that **4** have zero violations of the rule of 5 (Table-2). The target compound **4** has only one NH or OH hydrogen bond donors, which show increase solubility in cellular membranes. The target compound has nON value 4 which is <10 and has molecular weight 306 which is < 500 preferable for compound to be CNS active. All this properties could permit a better penetration of the drug through the blood-brain barrier.

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