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ORIGINAL ARTICLE

Synthesis, physicochemical and kinetic studies of redox derivative of bis(2-chloroethylamine) as alkylating cytotoxic agent for brain delivery

Rajesh K. Singh [a,*], D.N. Prasad [b], T.R. Bhardwaj [c,1]

[a] *Pharmaceutical Chemistry Division, Shivalik College of Pharmacy, Nangal, Punjab 140126, India*
[b] *Shivalik College of Pharmacy, Nangal, Punjab 140126, India*
[c] *University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India*

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NBP assay;
Kinetic study

Abstract Nitrogen mustard (mustine) containing an established anticancer moiety (N,N-bis(2-chloroethyl)amino is the one of the most active and widely used alkylating anticancer agents but it is too polar to cross the blood–brain barrier. The present study evaluates the utility of the dihydropyridine↔pyridinium salt redox system for the specific delivery and sustained release of bis-(2-chloroethyl)amine as anticancer moiety to the brain as an initial effort in a search for agents that may prove effective as CNS antitumor agent. The cytotoxic moiety bis-(2-chloroethyl)amine was converted into the corresponding 1-methyl-3-[bis-(2′-chloroethyl)amino]carbamoyl-1,4 dihydropyridine (**CDS-mustard**) (**4**), in three steps. Structures of all the synthesized compounds were characterized by UV, IR and ^{1}H NMR and ^{13}C NMR spectroscopic studies. *In vitro* chemical oxidation studies with silver nitrate of CDS-mustard indicated that it can be readily converted into its corresponding salt (**3**). *In vitro* kinetic studies of CDS-mustard showed that its rate of oxidation followed pseudo-first-order kinetics with reasonable half-lives in biological media. The study of some other physicochemical parameter calculated by online software also indicates that it can be a potential CNS antitumor agent.

* Corresponding author. Tel.: +91 9417513730; fax: +91 1887221276.
E-mail addresses: rksingh244@gmail.com (R.K. Singh), shivalikcollegeofpharmacy01@rediffmail.com (D.N. Prasad), bhardwajrtilak@yahoo.com (T.R. Bhardwaj).
[1] Present address: ISF College of Pharmacy, Moga 142001, Punjab, India.

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1. Introduction

Primary and secondary metastatic tumors of the CNS represent a major health problem globally. They are the second leading cause of cancer death in male adults aged 20–29 and the fifth leading cause of cancer death in female adults aged 20–39. Male are affected more than females. More than 200,000 people in the United States are estimated to be diagnosed with a primary or metastatic brain tumor (Jemal et al., 2010). Still, where possible, surgery remains the pre-

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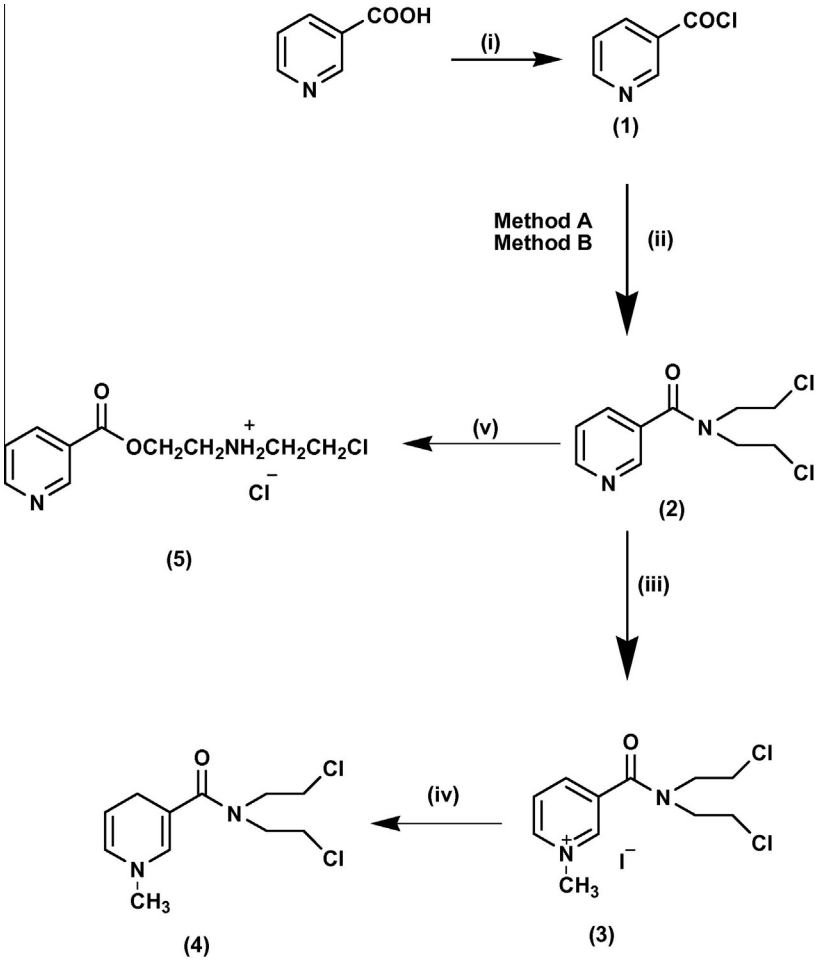
ferred method of treatment for most brain tumors and is often performed in conjunction with chemotherapy. But chemotherapy by antineoplastic agents is a challenge in the treatment of brain tumor. Many potential useful drugs including those active against peripheral tumors such as chlorambucil and melphalan due to their hydrophilicity cannot enter the brain and are therefore ineffective in treating cerebral neoplasms. The major obstruction to CNS drug delivery is the blood–brain barrier, which limits the access of drugs to the brain substance. The BBB comprises the endothelial lining of the microvessels in the brain, pericytes and astrocytes, and is the main barrier to drug transport into the CNS especially for labile hydrophilic compounds. In fact, it is estimated that only about 2% of potential CNS compounds can penetrate the BBB (Ambrose and Sheng-He, 2005).

Various attempts have been made to overcome the limited access of anticancer agent into the brain by synthesizing the lipophilic analogs of alkylating anticancer agent (Genka et al., 1993) or by linking the active anticancer moiety to lipophilic carrier (Peng et al., 1975; Bartzatt, 2004).

One of the most promising approaches for brain delivery is the concept of brain-specific drug delivery system based on a redox system analogous to the endogenous $NADH \leftrightarrow NAD^+$ coenzyme system developed by Bodor et al. (1981). The dihydropyridine↔quaternary salt redox system based chemical delivery system (CDS) has been investigated extensively as a method to enhance the selective delivery of drugs to the brain (Prokai et al., 2000). After entry into the brain, the CDS moiety is oxidized to a polar pyridinium species that cannot egress from the brain which then undergoes ester or amide cleavage to release the active drug and trigonelline.

This redox delivery system was successfully applied for brain-specific delivery of various alkylating anticancer agents. Chlorambucil (chlorambucil–CDH) (Bodor et al., 1989) and 1-(2-chloroethyl)3-cyclohexyl-1-nitrosourea (CCNU–CDH) (Raghavan et al., 1987) have been investigated to provide improved brain delivery relative to the parent compounds chlorambucil and CCNU, respectively. This system has also successfully been utilized to deliver nitrogen mustard group to the brain with ethyl as spacer group with promising results (EI-Sherbeny et al., 2003). Clearly there is a great need to develop therapeutic strategies that will provide efficient anticancer drug delivery to brain tumors. So it was therefore of interest to synthesize redox derivative of bis-(2-chloroethyl)amine (normustard) as alkylating anticancer moiety to the Bodor CDS directly, without any spacer group to evaluate its efficacy as a CNS anticancer agent by the given synthetic Scheme 1.



Scheme 1 Reagents and conditions: (i) Thionyl chloride; (ii) *Method A*: diethanolamine, thionyl chloride), *Method B*: bis(2-chloroethylamine; (iii) methyl iodide, acetone; (iv) sodium bicarbonate, sodium dithionite; (v) Trace amount of moisture, rearrangement.

2. Experimental section

All the reagents and solvents used are purchased from the commercial suppliers. The melting points were determined on Veego-melting point apparatus and are uncorrected. Proton (^{1}H) nuclear magnetic resonance (^{1}H NMR) and ^{13}C NMR spectra were obtained using Brucker AC-400 F, 400 MHZ spectrometer and are reported in parts per million (ppm), downfield from tetramethylsilane (TMS) as internal standard. Infrared (IR) spectra were obtained with Perkin Elmer 882 Spectrum and RXI, FT-IR model using a potassium bromide pellets (in cm^{-1}). 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 °C for 30 min. The plates were developed by exposure to iodine vapors. Anhydrous sodium sulfate was utilized as drying agents. All the solvents were dried and freshly distilled prior to use according to the standard procedure. A computational study of titled compounds was performed for prediction of ADME properties. Polar surface area (TPSA), milog *P*, number of rotatable bonds, molecular volume, number of hydrogen donor and acceptor atoms and violations of Lipinski's rule of five were calculated using Molinspiration online property calculation toolkit.

*2.1. Synthesis of nicotinoyl chloride (**1**)*

The nicotinic acid 3.5 gm (28.6 mmol) and freshly distilled thionyl chloride (10.0 ml) were refluxed for 6 h on a water bath. Excess thionyl chloride was distilled on *vacuo* and the product coevaporated with chloroform to get needle shape pale yellow crystal which was used without further purification for the next reaction.

*2.2. Synthesis of N,N-bis(2-chloroethyl)carbamoyl pyridine (**2**)*

This can be prepared by two methods:

Method A: The 3.5 gm (28.6 mmol) of nicotinoyl chloride was added in 50.0 ml of dry toluene. The solution was cooled in an ice bath and a solution of 5.12 gm (28.6 mmol) of diethanolamine and 2.0 ml of triethylamine was dropped in while stirring over 10 min and the mixture was refluxed for 6 h and then stirred overnight. The precipitate formed was filtered and the residue evaporated to obtain the intermediate diol which was treated with thionyl chloride and refluxed under a water bath for 3 h. The excess thionyl chloride was distilled under vacuum to obtain the final compound which was recrystallized from isopropanol to obtain yellow oil in 62% yield (**3**).

Method B: The solution of nicotinoyl chloride (3.5 g, 28.6 mmol) in dry toluene (50 ml) was slowly added (10 min) to a dry toluene (20 ml) solution (ice-bath cooling) of the free amine from 5.12 g (28.6 mmol) of bis(2-chloroethyl)amine hydrochloride in the form of free base which was prepared by adding 5% NaOH solution and extracting with diethyl ether. The mixture was heated at reflux for 4 h. Removal of solvent from the filtrate led to compound (**2**) as yellow oil in 54% yield.

2.2.1. Anal.

R_f = 0.46 (chloroform:methanol, 9:1).
IR (KBr): 3016 cm^{-1}(C–H), 1661 cm^{-1} (C═O), 1226, 1129, 1020 cm^{-1} (C–N) and 760 cm^{-1} (C–Cl).
^{1}H NMR (DMSO-d^6): δ 3.2 (t, 4H, J = 5.7 Hz, –N($CH_2CH_2Cl)_2$), δ 3.7 (t, 4H, J = 7.8 Hz, –N($CH_2CH_2Cl)_2$), δ 7.4 (t, 1H, J = 2.3 Hz, C_5 pyridine proton), δ 8.3 (d, 1H, J = 2.0 Hz, C_4 pyridine proton), δ 8.7 (d, 1H, J = 1.70 Hz, C_6 pyridine proton), δ 9.2 ppm (s, 1H, C_2 pyridine proton).
^{13}C NMR (DMSO-d^6): δ (ppm): 167.2 (C═O), 152 (C-2 pyr), 147.2 (C-6 pyr), 134.0 (C-4 pyr), 129.8 (C-3 pyr), 122.5 (C-5 pyr), 51.4 (C–N), 42.2 (C–Cl).

*2.3. Synthesis of 1-methyl-3-[bis(2-chloroethyl)amino]carbamoyl-pyridinium iodide (**3**)*

To a solution of 1.0 gm (4 mmol) of N,N-bis(2-chloroethyl)carbamoyl pyridine and 30.0 ml of acetone was added 1.136 g (8 mmol) 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%).

2.3.1. Anal.

R_f = 0.2 (chloroform:methanol, 8:2).
UV_{max} (H_2O): 275 nm.
IR (KBr): 3016 cm^{-1}(C–H), 1661 cm^{-1} (C═O), 1226, 1129, 1020 cm^{-1} (C–N) and 760 cm^{-1} (C–Cl).
^{1}H NMR (DMSO-d^6): δ 3.2 (t, 4H, J = 7.16 –N($CH_2CH_2Cl)_2$), δ 3.6 (t, 4H, J = 6.20, –N($CH_2CH_2Cl)_2$), δ 4.6 (s, 3H, –N^+CH_3) δ 8.3 (t, 1H, J = 6.8, C_5 pyridine proton), δ 8.6 (d, 1H, J = 9.6, C_4 pyridine proton), δ 9.3 (d, 1H, J = 5.84, C_6 pyridine proton), δ 10.2 ppm (s, 1H,C_2 pyridine proton).
^{13}C NMR (DMSO-d^6): δ (ppm): 167.8 (C═O), 148.4 (C-2 pyr), 148.1 (C-4 pyr), 145.2 (C-6 pyr), 138.0 (C-3 pyr), 128.4 (C-5 pyr), 50.7 (C–N), 46.8 (N^+–Me), 43.4 (C–Cl).

*2.4. Synthesis of 1-methyl-3-[bis(2-chloroethyl)amino]carbamoyl-1,4-dihydropyridine (**4**)*

To a solution of 0.9 gm (3.4 mmol) of 1-methyl-3-[bis(2-chloroethyl)amino]carbamoyl-1,4-dihydropyridinium iodide, 20.0 ml deaerated water, and 20.0 ml of ethyl acetate were added 1.7 gm (20.4 mmol) of sodium bicarbonate and 2.38 gm (13.6 mmol) of sodium dithionite. The mixture was stirred under nitrogen for 1 h in an ice bath. The ethyl acetate layer was separated, and aqueous layer was re-extracted twice with 20.0 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 colored gummy residue preserved in desiccators protected from light and air. **Yield**: 60%.

2.4.1. Anal.

R_f = 0.55 (chloroform:methanol, 9:1).
UV_{max} (CH_3OH): 320 nm.
IR (KBr): 2961 cm^{-1}(C–H), 1638 cm^{-1} (C═O), 1110, 1210 cm^{-1} (C–N) and 742 cm^{-1} (C–Cl).

^1H NMR (DMSO-d^6): δ 2.8 (s, 3H, –NCH_3), δ 3.0 (t, 4H, J = 4.0 Hz, –$N(CH_2CH_2Cl)_2$), δ 3.5 (t, 4H, J = 4.7 Hz, –$N(CH_2CH_2Cl)_2$), δ 4.1 (br s, 2H, C_4 pyridine proton), δ 4.7 (t, 1H, J = 4.0 Hz, C_5 pyridine proton), δ 5.6 (d, 1H, J = 6.3 Hz, C_6 pyridine proton) and δ 7.0 ppm (s, 1H, C_2 pyridine proton).
^{13}C NMR (DMSO-d^6): δ (ppm): 172.2 (C═O), 147.8(C-2 pyr), 134.0 (C-6 pyr), 121.2 (C-3 pyr), 108.0 (C-5 pyr), 31.4 (C-4 pyr), 52.8 (C–N), 43.6 (N-Me), 36.8 (C–Cl).

2.5. Synthesis of N-[2-(2-chloroethylamino)ethoxycarbonyl] pyridine hydrochloride (5)

N,N-bis(2-chloroethyl)carbamoyl pyridine (**2**) (5.0 mmol) was dissolved in 10 ml of water and stirred for 30 min at room temperature. The reaction mixture was basified by dilute sodium hydroxide solution to precipitate oil which was soluble in hydrochloric acid.

IR (KBr): 3429 cm^{-1} (N–H), 1720 cm^{-1} (–OC═O), 1050–1230 cm^{-1} (C–N) and 740 cm^{-1} (C–Cl).
^1H NMR (DMSO-d^6): δ 3.7 (m, 4H, –CH_2NHCH_2–), δ 4.2 (t, 2H, J = 7.2 Hz, –CH_2CH_2Cl–), δ 4.7 (t, 2H, J = 7.4 Hz, –OCH_2CH_2–), δ 7.4 (t, 1H, J = 2.8 Hz, C_5 pyridine proton), δ 8.3 (d, 1H, J = 2.2 Hz, C_4 pyridine proton), δ 8.7 (d, 1H, J = 2.4 Hz, C_6 pyridine proton), δ 9.2 ppm (s, 1H, C_2 pyridine proton).

2.6. NBP alkylation assay

The alkylation activity of prepared Q-CDS-mustard (**3**) was determined as per the given literature procedure (Pen et al., 2002). Thus a solution of Q-CDS-mustard or N-di(2-chloroethyl amine)[–$NH(CH_2CH_2Cl)_2$] in different concentrations as indicated in Table 1 in acetone (1 ml), distilled water (1 ml) and acetate buffer (1 ml, 0.25 M, pH 6.0) were incubated at 100 °C for 20 min with a solution of 4-(4-nitro-benzyl) pyridine (NBP) (5% w/v) in acetone (0.4 ml) and cooled to 25 °C. After the addition of acetone (2 ml), ethyl acetate (5 ml) and sodium hydroxide solution (0.25 M, 1.5 ml), the reaction mixture was vortexed and allowed to stand to separate the organic layers. The absorbance in the organic layers was determined (within 2 min of NaOH addition) at 545 nm. The experiments were carried out in triplicates. The results were expressed in absorbance value (mean ± S.E.M., n = 3 in all the cases) Table 1.

2.7. Determination of experimental partition coefficient

Partition coefficient was determined in between *n*-octanol and distilled water using the reported procedure (Fujita et al., 1964).

Table 1 Determination of chemical alkylating activity expressed in absorbance.

Descriptor	Concentration of compounds (μM/ml)	
	0.5 μM/ml	1.0 μM/ml
CDS-mustard	0.51 ± 0.05	0.74 ± 0.03
N-di(2-chloroethyl)amine	0.49 ± 0.07	0.71 ± 0.04
Blank	0.07 ± 0.02	0.08 ± 0.02

2.8. Calculation of $M\log P$

$M\log P$ was calculated by the method of Moriguchi et al. (1992) by the formula:

$$M\log P = 1.464(CX)^{0.6} - 1.221(NO)^{0.9} \times 0.653(PRX) - 0.300(UB)^{0.8} + 0.335(POL) + 0.726(ALK) - 0.269(RNG) - 1.358$$

CX = summation of carbon and halogen atoms
NO = total number of nitrogen and oxygen atoms
PRX = proximity effect
UB = total number of unsaturated bonds
POL = number of polar substituents
ALK = alkane, alkene, cycloalkene, cycloalkane dummy variable
RNG = ring structures

2.9. Calculation of Log BB

Log BB was calculated by the method of (Clark, 1999) by the formula:

$$\text{Log BB} = -0.0145\ \text{PSA} + 0.172\ M\log P + 0.131$$

(PSA = polar surface area)
($M\log P$ = Moriguchi partition coefficient)

2.10. In vitro chemical oxidation studies

By silver nitrate: One milliliter solution of 5% dihydropyridine derivatives was prepared in methanol. Then 5.0 ml of saturated methanolic solution of silver nitrate was added to the 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**).

2.11. Kinetics of oxidation of the dihydro derivative (4) (CDS-mustard) Table 2

Calibration curves: A UV study of compound (**4**) revealed that they obey Beer's Law with a good correlation coefficient and at a wide range of dilutions from 10 to 60 μg/ml in both methanol and 2% aqueous methanol. The study was done at 320 nm got the dihydro derivative (CDS-mustard).

Phosphate buffer: In each of five tubes containing 0.2 ml of a 10×10^{-4} M methanolic solution of the freshly prepared dihydro derivative (CDS-mustard) was added 2 ml of freshly prepared phosphate buffer and the tubes were kept at 37 °C 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 320 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 °C. In each of five tubes containing 0.2 ml of a 10×10^{-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 °C 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 320 nm was measured. A reference sample was made by addition of 0.2 ml of methyl alcohol instead of the sample solution following the same procedure.

100% Whole rat blood: Blood was withdrawn from rats 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 °C. In each of five tubes containing 0.2 ml of a 10×10^{-4} M methanolic solution of the freshly prepared dihydro derivative (**4**) was added 2 ml of fresh rat blood and the tubes were kept at 37 °C 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 the addition of 0.2 ml of methyl alcohol instead of the sample solution following the same procedure.

In 20% human plasma: A freshly prepared solution of the dihydro derivative (**4**) (0.2 mL, 6.25×10^{-4} M) in methanol was diluted to 10 ml, with 20% plasma (diluted with phosphate buffer, pH 7.4). The solution was kept at 30 °C and the UV spectrum was scanned from 400 to 220 nm every 5 min for 30 min against a reference sample made by dilution of 0.2 mL of methanol with 20% plasma to 10 mL.

In brain homogenate: Rat brain tissue (2.0 gm) 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 °C for 5 min, and then centrifuged again. The supernatant solution was diluted to 100 ml with a 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. The mixture was scanned at 37 °C from 400 to 220 nm every 5 min on a double beam UV spectrophotometer.

References 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 gm) 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 °C 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 prepared homogenate was added 0.2 ml of 6.25×10^{-4} M methanolic solution of the freshly prepared dihydro derivative. The mixture was scanned at 37 °C from 400 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 (**4**) sample solution.

2.12. Stability studies on storage

The CDS-mustard was tested for stability at different storage conditions by the published method (Al-Obaid et al., 2006). Samples from the prepared CDS-mustard was dried and stored in dark brown bottles and stored under nitrogen and dry conditions at room temperature (25 °C) and in the refrigerator. At one month interval, each sample was analyzed for its content of the 1,4-dihydropyridine derivative using UV spectral analyses.

3. Result and discussion

3.1. Chemical characterization

The major steps for the synthesis of the CDS-mustard agent are presented in Scheme 1. Initially, the carboxyl group of nicotinic acid is activated utilizing thionyl chloride ($SOCl_2$), which forms the more reactive acyl halide (**1**). This acyl halide was reacted with diethanolamine to obtain intermediate, $N^?$-bis (2-hydroxyethyl)amino)nicotinamide which without further purification was chlorinated by thionyl chloride to obtained amide (**2**). The IR spectra of this final compound exhibited the disappearance of –COOH peak at 2400–3300 cm^{-1} and appearance of sharp absorption at 1661 and 1220–1020 cm^{-1} confirming the presence of the amide group and –C–N functionality. The ^1H NMR spectra of these compounds, using $CDCl_3$ as a solvent show the ethylene bridge yielding two triplets integrating for four protons each. One triplet appeared at δ 3.20 ppm which was assigned to $–CH_2$ group adjacent to nitrogen. The other triplet at δ 3.75 ppm was assigned to the $–CH_2–$ group adjacent to the –Cl group which is more deshielded due to the electronegativity effect of Chlorine. In addition, other signals were observed at δ 7.4 (t, 1H, J = 2.3 Hz, C_5 pyridine proton), δ 8.3 (d, 1H, J = 2.0 Hz, C_4 pyridine proton), δ 8.7 (d, 1H, J = 1.70 Hz, C_6 pyridine proton), and δ 9.2 (s, 1H, C_2 pyridine proton).

In another route, nicotinoyl chloride was directly reacted with neutral base of bis(2-chloroethylamine) to obtain the title compound in 25% yield. The m.p., IR and NMR spectra were same for both the compounds. The amide formed was very hygroscopic. It was sensitive to trace amounts of water and readily rearranged to 2-(2-chloroethylaminoethyl)esters (**5**). These observations are in better accord with an amide-ester rearrangement reaction (Pettit et al., 1963).

The compound (**2**) was then quaternized using methyl iodide in acetone to 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 3H singlet of $–NCH_3$ group at position 1. The obtained quaternary salt was then subjected to reduction process using sodium dithionite in alkaline medium, to give the corresponding final target compound (**4**).

3.2. In vitro chemical, biological oxidation and storage studies

The prepared 1,4-dihydropyridines CDS-mustard (**4**) was subjected to various chemical and biological investigations to evaluate the ability of these compounds to cross the BBB 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. Figs. 1–4 show the results of such an investigation. Pseudo-first-order rate constants for the disappearance of compounds in biological media were determined by linear regression analysis from a plot of log CDS-mustard versus time. Quaternary salt which was thought to be converted by CDS-mustard, did not interfere with the absorption of CDS-mustard because its λ_{max} was found 275 nm, which was considerably different from that of CDS-mustard (320 nm).

Table 2 shows the calculated half-lives for CDS-mustard 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 = .0023 ± 0.05, $t_{1/2}$ = 3.0 min). The CDS-mustard was quite stable in phosphate buffer pH 7.4 with K value of .0142 ± 0.8 and $t_{1/2}$ of 48.8 min. Liver homogenate was the least stable medium for CDS-mustard with K value of 0.0797 ± 3.6 and $t_{1/2}$ of 8.7 min. The stability of CDS-mustard in human plasma was the highest among all tested biological materials (over 25 min). The CDS mustard was readily oxidized ($t_{1/2}$ 8.7 min) in rat liver homogenate.

A parallel study of the storage stability of CDS-mustard at room temperature and refrigerator (4 °C) over a period of 30 days was conducted (Table 3). The acetonitrile solutions of these compounds were UV checked after the specified time period. The CDS-mustard was not much stable at both room temperature (over 50% decomposed) and in refrigerator (over 10% decomposed) (See Table 3).

The results of these *in vitro* stability tests were used to compare and evaluate the chemical delivery system to determine whether the final compound met the requirement of the ideal delivery system. These studies show, then, that brain targeting is possible but fair stability is required for this compound.

3.3. Alkylating activity assessment

The Q-CDS-mustard was evaluated by its alkylating activity using 4-(4-nitro-benzyl) pyridine (NBP) as an analytical reagent by the method of Pen et al., using spectrophotometric quantitation. The 4-(4-nitro-benzyl) pyridine reacts with alkylating agents and gives a purple color upon basification (Fig. 5). The intensity of the produced color is directly proportional to the degree of alkylation. It is hypothesized that there is a

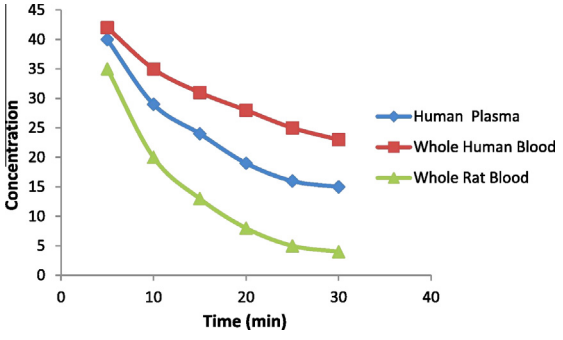


Figure 1 Concentration (μg/ml) against time of 1-methyl-3-[bis(2-chloroethyl)amino]carbamoyl-1,4-dihydropyridine (4) CDS-mustard in Human Plasma, Whole Human Blood and Whole Rat Blood.

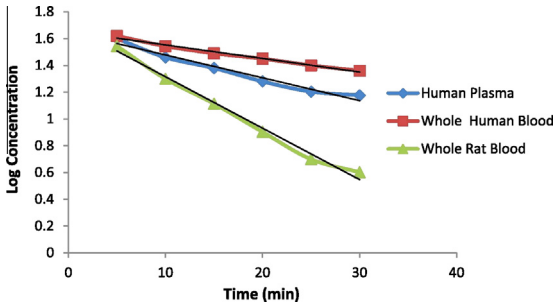


Figure 2 Log concentration against time of 1-methyl-3-[bis(2-chloroethyl)amino]carbamoyl-1,4-dihydropyridine (**4**) CDS-mustard in human plasma, whole human blood and whole rat blood.

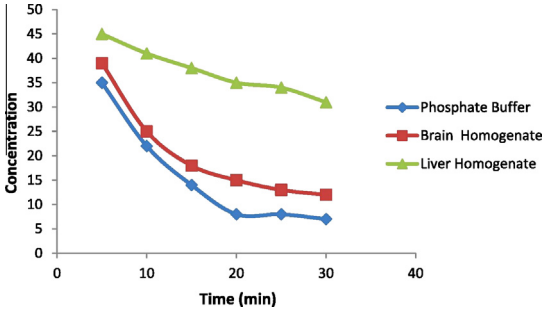


Figure 3 Concentration (μg/ml) against time of 1-methyl-3-[bis(2-chloroethyl)amino]carbamoyl-1,4-dihydropyridine CDS-mustard (4) in Phosphate Buffer, Brain homogenate and Liver Homogenate.

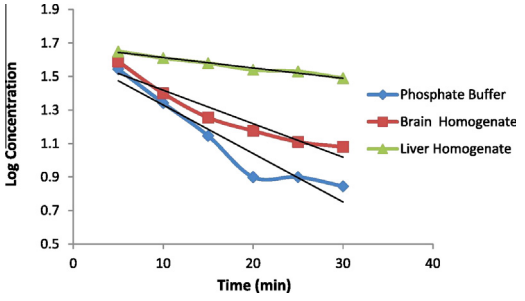


Figure 4 Log concentration against time of 1-methyl-3-[bis(2-chloroethyl)amino]carbamoyl-1,4-dihydropyridine (**4**) CDS-mustard in phosphate buffer, brain homogenate and liver homogenate.

Table 2 Rates of oxidative conversion in biological fluids of CDS-mustard (**4**) to the corresponding quaternary pyridinium salts (**3**).

Medium	Rate constant $K \times 10^{-2}$ min^{-1} ± S.E.	$t_{1/2}$ min ± S.E.
Silver nitrate	0.231 ± 0.05	3.0
Phosphate buffer	1.42 ± 0.8	48.8
Human plasma	2.46 ± 1.2	28.1
Whole human blood	4.47 ± 1.6	15.5
Whole rat blood	9.6 ± 2.4	7.22
Brain homogenate	5.46 ± 2.7	12.7
Liver homogenate	7.97 ± 3.6	8.7

S.E. is ±10% of the presented values.

Table 3 Stability of CDS-mustard (**4**) upon storage.

Conditions	CDS-mustard		
	Zero days	30 Days	% Decomposed
Room temperature (25 °C)	0.948	0.426	55.0
Refrigerator (4 °C)	0.948	0.816	13.9

correlation between the chemical alkylating activity and antitumor activity. The Q-CDS-mustard proved to be an active alkylating activity comparable to that of N-di(2-chloroethyl)amine as standard alkylating compound.

3.4. CNS active physicochemical parameters assessment

Since the target compound is designed to be CNS active, the parameters which affect the blood–brain barrier were selected. Physicochemical descriptors presented in Table 4 support the clinical potential of this CDS-mustard agent. Lipophilicity is one of the most important factors in controlling the interaction of drugs with biological system (Liu et al., in press).The log *P* value of CDS-mustard is determined experimentally and compared with other partition coefficient *M*log *P*. Both methods show a significant increased lipophilicity for the CDS-mustard agent. The polar surface area (PSA) of a drug has been shown to be an effective means to predict drug transport (Ertl et al., 2000; Palm et al., 1996, 1997) and its numerical value is inversely correlated with BBB penetration (Kelder et al., 1999). The most active CNS drug will have PSA of less than 70 A^2. PSA of our CDS-mustard compound is 23.54 A^2, which predicts that greater than 90% of this agent will be absorbed by the intestine.

Partitioning of drugs between the blood and the CNS can be expressed in concentration terms C_{brain}/C_{blood} (or BB), which can be utilized as Log BB similar to Log *P*. The parameter BB or C_{brain}/C_{blood} is profoundly increased for the CDS-mustard construct 1.16.

A pharmacological parameter referred to as ''Rule of 5'' accurately predicts drug bioavailability and bioactivity (Lipinski et al., 1997). Highest drug bioavailability and bioactivity are achieved when there are no violations of the rules.

According to the rule of five, compounds with number of violations not more than 1 show good bioavailability and bioactivity. Analysis of molecular structure by Molinspiration showed that CDS-mustard has zero violations of the rule of five (Table 4). The target compound CDS-mustard has no NH or OH hydrogen bond donors which show increased solubility in cellular membranes. The target compound has nON value of three which is < 10 and has molecular weight of 263 which is < 500 preferable for a compound to be CNS active. All this properties could permit a better penetration of the drug through the blood–brain barrier.

Table 4 Physicochemical parameters of CDS-mustard agent.

Descriptor	CDS-mustard agent
Polar surface area[a]	23.54 A^2
Percent intestinal absorption[b] of drug	> 90%
Exp. Log *P*	1.98
*M*log *P*[c]	1.60
Log BB[d]	0.064
$BB^3 = C_{brain}/C_{blood}$	1.16
Molecular weight[a]	263.17
No of violation[a] of rule of 5	0
–NH and –OH[a]	0
nON values[a]	3

[a] Calculated by method of Molinspiration.
[b] Calculated by correlation of PSA to experimental intestinal absorption.
[c] *M*log *P* was calculated by the method of Moriguchi et al. (1992).
[d] Log BB, where $BB = C_{brain}/C_{blood}$, $\text{Log BB} = \text{Log BB} = -0.0145\ \text{PSA} + 0.172\ M\log P + 0.131$.

4. Conclusion

Redox derivative of bis (2-chloroethyl)amine as alkylating anticancer moiety was designed in the hope to obtain CNS active antitumor agent. Structures of all the synthesized compounds were confirmed by UV, IR, and ^{1}H NMR techniques. The *in vitro* chemical alkylation activity studies (NBP) of Q-CDS-mustard was comparable to that of N,N-bis(2-chloroethyl)amine moiety as standard alkylating agent. The *in vitro* chemical and biological oxidation studies showed that CDS-mustard could be oxidized into their corresponding quaternary compounds (**3**). The obtained values indicate a reasonable stability of the CDS-mustard. The study of some other physicochemical parameters calculated by online software such as lipophilicity, Log BB, polar surface area, rule of five,

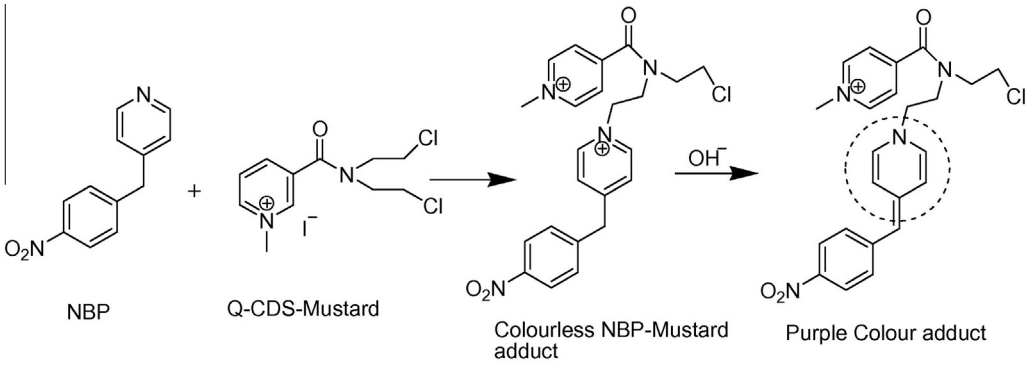


Figure 5 NBP reacts specifically with quaternary salt of CDS-mustard to produce chromophore upon basification with sodium hydroxide (see broken oval) giving purple color and has a strong absorbance peak at 541 nm.

number of NH or OH hydrogen bond donors, and nON value also indicates that CDS-mustard can be a potential CNS antitumor agent. But the drawback of this *in vitro* study is of course, that CDS-mustard is not so stable in biological media and it lacks stability when stored at room temperature. Still, we expect that the results in this work provide a necessary knowledgebase for future design of novel CDS-mustard by incorporating various electronegative substituents on the ring nitrogen of the 1,4-dihydropyridine moiety to enhance the stability of the prodrug.

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