*Bull. Pharm. Sci., Assiut University, Vol. 33, Part 1, June 2010, pp. 69-78.*

# SPECIFIC BRAIN DELIVERY OF PYRIDYL CHLOROETHYLUREA DERIVATIVES AS POTENTIAL ANTITUMOR AGENTS: SYNTHESIS, EVALUATION AND MOLECULAR MODELING STUDY

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يتناول البحث تشييد وتقييم وإرساء جزيئي لمشتقات ثنائى الهيدروبيريدين كلوروايثيل يوريا **IIIa-e** كمضادات لأورام المخ. واحتواء هذه المركبات على ثنائى الهيدروبيريدين تمكنها من التوصيل التفاضلى للمخ. وتم تشييد المركبات المستهدفة خلال عملية الإختزال لمركبات أملاح البيريدينيم **IIa-e**. وتم اختبار فاعلية هذه المركبات بطرق مختلفة منها الأكسدة الكيميائية والأكسدة فى سوائل بيولوجية والاتاحة الحيوية فى المخ وتعيين الفاعلية الألكيلية باستخدام ٤-(٤-نيتروبنزيل)بيريدين بالاضافة الى الإرساء الجزيئى للمركبات باستخدام برنامج MOE وقد اظهرت النتائج أن المركب **IIe** له فاعلية مؤلكلة مقارنة بالعقار المرجعى كلورامبيوسيل.

*The present investigation describes the synthesis; evaluation and molecular modeling studies of a series of 1-substituted-1,4-dihydropyridine-3-chloroethylurea derivatives **IIIa-e** as potential agents for treatment of brain tumors. The incorporation of the 1,4-dihydropyridine moiety in the structure attains an efficient site specific chemical delivery system (CDS) of the chloroethylurea (CEU) as a known antitumor pharmacophore to the brain. The target compounds **IIIa-e** were synthesized through reduction of the corresponding quaternary compounds **IIa-e**. The in-vitro oxidation studies showed that, compounds **IIIa-e** could be oxidized into their corresponding quaternary compounds **IIa-e**, respectively which attains their "locked in" characteristics as brain antitumor agents. The in-vivo studies showed that compound **IIIa** was able to cross the BBB at detectable concentration. In addition the in-vitro alkylating activity studies using 4-(4-nitrobenzyl)pyridine (NBP) revealed that compound **IIe** is an efficient alkylating agent with activity comparable to reference drug chlorambucil. The target compounds were tested for their binding to the colchicine-binding site (CBS) of β-tubulin using Molecular Operating Environment (MOE) software.*

## INTRODUCTION

Brain tumors are one of the most lethal forms of cancer, that are extremely difficult to treat. Clinical failure of several potentially effective therapeutics for the treatment of brain tumors is usually not due to a lack of drug potency, but rather can be attributed to shortcomings in the delivery of a drug to the brain. Extensive efforts have been made to develop novel strategies to overcome such obstacles.

The use of dihydropyridine / pyridinium redox carrier as brain-specific chemical delivery system (CDS)[1-3] provides an important flexible method and sustained delivery of drugs to the brain. *In-vivo* the lipohillic characteristics of the dihydropyridine affects the transport of the drug into the brain. Meanwhile, enzymatic oxidation of the carrier moiety to the quaternary pyridinium inside the brain hinders rapid elimination affording a means for sustained effect "locked in" and accelerate its clearance from the peripheral circulation and subsequently reduce the possible side effects of the parent drug[4-7].

Substituted phenyl chloroethylureas were designed whereby affording a combination of the known antitumor agent chlorambucil and the unnitrosated pharmacophore of carmustine to give more active compounds than chlorambucil and carmustine[8-11]. Moreover, the lipophillic 1,4-dihydropyridine moiety attains a good mean for site specific delivery to the

*Received in 13/5/2010 & Accepted in 23/6/2010*

brain. Incorporation of alkyl or substituted benzyl to N-1 of the 1,4-dihydropyridine moiety provides targeting in a manner similar to that of CDSs[8].

Interestingly, Chloroethylureas (CEUs) mediate their cytotoxicity by covalent binding to cell proteins, and the main alkylated protein for antimitotic CEUs is *β*-tubulin[12]. The electrophilic 2-chloroethylamino moiety of the molecule was initially proposed to interfere with the cysteinyl-239 residue of human *β*-tubulin[12], whereas the migrational modification of *β*-tubulin following treatment with antimitotic CEUs was shown to be related to the esterification of glutamic acid-198 of mouse *β*-tubulin[13], located near the colchicine-binding site[14]. This modification blocks the entry of tubulin in the mitotic spindle or destabilizes it, leading to a $G_2$/M cell-cycle arrest.

These observations prompted us to synthesize a new series of pyridylchloro-ethylurea derivatives, studing their alkylating activity chemically using NBP and investigating the molecular modeling characteristics to the colchicine-binding site of β-tubulin using MOE.

## EXPERIMENTAL

Melting points were determined in open glass capillaries and are uncorrected. UV spectra were recorded on Pye Unicam SP 1750 UV/Vis Spectrophotometer. $^1$H-NMR spectra were recorded on a 60 MHz EM-390 spectrometer in DMSO-$d_6$ and TMS as internal standard. Chemical shifts are expressed in δ ppm, and coupling constant (J) are given in Hz. Elemental analyses for C,H, and N were performed at the unit of microanalysis, Cairo University. Thin-layer chromatography was performed on precoated (0.25 mm, 60 F254) silica gel plates, compounds were detected with a 254-nm UV lamp. The HPLC system consisted of a Knauer model 64 solvent delivery module (Knauer, Germany), a Knauer variable wave length UV detector at 20 μL sample loop, and Shimadzu CR-6A chromatopac integrator (Shimadzu, Tokyo, Japan). The column used was a Knauer C18 (250 mm x 4.6 mm ID, 5μm). The effluent monitored at 262 nm at flow rate 1.5 mL/min. The mobile phase used consisted of 10% acetonitrile in 0.2% acetic acid.

### Synthesis of 1-(2-chloroethyl)-3-pyridin-3-yl-urea I[15]

A stirred solution of 3-aminopyridine (39 mmol) in THF (50 mL) was treated dropwise at 0-5°C with 2-chloroethylisocyanate (39 mmol) in THF (10 mL). The reaction mixture was stirring at room temperature for 1.5 hrs. The solvent was removed under reduced pressure and the product was crystallized from ethyl acetate. Yield (80%), m.p. 137-8°C.

### General procedure for preparation of 1-alkyl(4-substituted benzyl)-3-[3-(2-chloro-ethyl)ureido]pyridinium halides IIa-e

To a solution of compound **I** (10 mmol) in ethanol (30 mL), appropriate alkyl or substituted benzyl halide (15 mmol) in ethanol (10 mL) was added with stirring, the mixture was refluxed 8-10 hrs. Solvent was removed under reduced pressure and the obtained residue was triturated with ether till solidification. The obtained solid was crystallized from ethanol to give **IIa-e**.

### 3-[3-(2-Chloroethyl)ureido]-1-methyl-pyridinium iodide IIa

Yield (65%), m.p. 166-167°C. IR (KBr) ν: 3345, 3225 (NH), 1688 (C=O) $Cm^{-1}$. $^1$H-NMR (DMSO-$d_6$) δ (ppm): 3.47 (t, J= 6.1 Hz, 2H, $\underline{CH_2}CH_2Cl$), 3.69 (t, J= 6.0 Hz, 2H, $CH_2\underline{CH_2}Cl$), 4.33 (s, 3H, $N^+CH_3$), 6.93 (s, 1H, $\underline{NH}CH_2CH_2$), 7.80-8.40 (m, 2H, C4- and C5-pyr.), 8.56 (d, J= 7 Hz, 1H, C6-pyr.), 9.10 (s, 1H, C2-pyr.), 9.73 (s, 1H, $\underline{NH}CO$).

Anal. ($C_9H_{13}ClIN_3O$) (Cald/ Found) C, 31.62/31.20; H, 3.80/3.60; N 12.30/12.00).

### 3-[3-(2-Chloroethyl)ureido]-1-ethylpyridin-ium iodide IIb

Yield (60%), m.p. 147-8°C. IR (KBr) ν $cm^{-1}$: 3345, 3225 (NH), 1670 (C=O) $^1$H-NMR (DMSO-$d_6$) δ (ppm): 1.60 (t, J= 7.0 Hz, 3H, $CH_2\underline{CH_3}$), 3.45 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$), 3.65 (t, J= 6.0 Hz, 2H, $CH_2\underline{CH_2}Cl$), 4.70 (q, J= 7.0 Hz, 2H, $N^+\underline{CH_2}CH_3$); 6.95 (s, 1H, $\underline{NH}CH_2CH_2$), 7.80-8.50 (m, 2H, C4- and C5-pyr.), 8.73 (d, J= 7.0 Hz, 1H, C6-pyr.), 9.30 (s, 1H, C2-pyr.), 9.80 (s, 1H, $\underline{NH}CO$).

Anal. ($C_{10}H_{15}ClIN_3O$) (Cald/ Found) C, 33.75/33.50; H, 4.21/4.00; N, 11.61/11.50).

**1-(4-Chlorobenzyl)-3-[3-(2-chloroethyl)-ureido]pyridinium chloride IIc**

Yield (90%), m.p. 186-7°C. IR (KBr) ν $cm^{-1}$: 3340, 3225 (NH), 1680 (C=O) $^1$H-NMR (DMSO-$d_6$) δ (ppm): 3.48 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$). 3.70 (t, J= 6.0 Hz, 2H, $CH_2\underline{CH_2}Cl$), 6.10 (s, 2H, $N^+\underline{CH_2}$), 7.50 (s, 1H, $\underline{NH}CH_2CH_2$), 7.60-8.30 (m, 5H, C5-pyr., 4 ArH), 8.56 (d, J= 7.0 Hz, 1H, C4-pyr.), 9.06 (d, J= 7.0 Hz, 1H, C6-pyr.), 9.63 (s, 1H, C2-pyr.), 10.10 (s, 1H, $\underline{NH}$CO).

Anal. ($C_{15}H_{16}Cl_3N_3O$) (Cald / Found) C, 49.93/49.70; H, 4.44/4.20; N, 11.65/11.40.

**1-(4-Bromobenzyl)-3-[3-(2-chloroethyl)-ureido]pyridinium bromide IId**

Yield (75%), m.p. 190-191°C. IR (KBr) ν $cm^{-1}$: 3340, 3225 (NH), 1688 (C=O). $^1$H-NMR (DMSO-$d_6$) δ (ppm): 3.45 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$), 3.68 (t, J= 6.1 Hz, 2H, $CH_2\underline{CH_2}Cl$), 5.93 (s, 2H, $N^+\underline{CH_2}$), 7.10 (s, 1H, $\underline{NH}$ $CH_2CH_2$), 7.33-7.83 (m, 4H, ArH), 7.70-8.53 (m, 2H, C4- and C5-pyr.), 8.86 (d, J= 7.0 Hz, 1H, C6-pyr.), 9.43 (s, 1H, C2-pyr.), 10.20 (s, 1H, $\underline{NH}$CO).

Anal. ($C_{15}H_{16}$ $Br_2ClN_3O$) (Cald/Found) C, 40.04/39.80; H, 3.55/3.50; N, 9.34/9.10).

**3-[3-(2-Chloroethyl)ureido]-1-(4-nitro-benzyl)pyridinium bromide IIe**

Yield (88%), m.p. 210-12°C. IR (KBr) ν $cm^{-1}$: 3387, 3265 (NH), 1693 (C=O), 1564, 1354 (N=O). $^1$H-NMR (DMSO-$d_6$) δ (ppm): 3.44 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$). 3.67 (t, J= 6.0 Hz, 2H, $CH_2\underline{CH_2}Cl$), 6.13 (s, 2H, $N^+\underline{CH_2}$), 7.10 (s, 1H, $\underline{NH}CH_2CH_2$), 7.80 (d, J= 7.0 Hz, 2H, ArH), 8.10-8.20 (m, 2H, C4-,C5-pyr.), 8.30 (d, J= 7.0 Hz, 2H, ArH), 8.90 (d, J= 7.5 Hz, 1H, C6-pyr.), 9.50 (s, 1H, C2-pyr.), 10.26 (s, 1H, $\underline{NH}$CO).

Anal. ($C_{15}H_{16}BrClN_4O_3$) (Cald/ Found) C, 43.32/43.10; H, 3.85/3.60; N, 13.47/13.40).

**General procedure for preparation of 1-(2-chloroethyl)-3-[1-alkyl(4-substituted benzyl)-1,4-dihydropyridin-3-yl]ureas IIIa-e**

To a suspension of 1-alkyl (4-substituted benzyl)-3-[3-(2-chloroethyl) ureido]pyridinium halides **IIa-e** (10 mmol) in degassed water (100 mL) and methylene chloride (25 mL) previously cooled to 0°C and stirred under nitrogen stream, sodium bicarbonate (60 mmol) was added portionwise over a period of 15 min. Sodium dithionite (40 mmol) was then added portionwise over 10 minutes. Stirring was continued under nitrogen stream at 0°C for 2 hrs. The reaction mixture was extracted with three portions of methylene chloride. The extract was washed with cold deaereated water, dried with anhydrous sodium sulfate and evaporated in *vacuo* to give the crude dihydropyridyl compounds **IIIa-e**.

**1-(2-Chloroethyl)-3-[1-methyl-1,4-dihydro-pyridin-3-yl]urea IIIa**

Yield (55%, oily). $^1$H-NMR ($CDCl_3$) δ (ppm): 2.9 (s, 3H, N-$CH_3$). 3.0-3.2 (m, 2H, C4-H), 3.42 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$), 3.63 (t, J= 6.0 Hz, 2H, $CH_2\underline{CH_2}Cl$), 4.78 (m, 1H, C5-H), 5.53 (s, 1H, C6-H), 6.76-6.90 (s, 1H, $\underline{NH}CH_2CH_2$), 7.1 (s, 1H, C2-H), 9.64 (s,1H, NHCO).

**1-(2-Chloroethyl)-3-[1-ethyl-1,4-dihydro-pyridin-3-yl]urea IIIb**

Yield (52%, oily). $^1$H-NMR ($CDCl_3$) δ (ppm): 1.3 (t, J= 7.0 Hz, 3H, N-$CH_2\underline{CH_3}$), 3.0-3.2 (m, 2H, C4-H), 3.4 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$), 3.65 (t, J= 6.0 Hz, 2H, $CH_2\underline{CH_2}Cl$), 4.6 (q, J= 7.0 Hz, 2H, N-$\underline{CH_2}CH_3$), 4.70 (m, 1H, C5-H), 5.57 (s, 1H, C6-H), 6.76-6.90 (s, 1H, $\underline{NH}CH_2CH_2$), 7.2 (s, 1H, C2-H), 9.64 (s,1H,NHCO).

**1-[1-(4-Chlorobenzyl)-1,4-dihydropyridin-3-yl]-3-(2-chloroethyl)urea IIIc**

Yield (54%, oily); $^1$H-NMR ($CDCl_3$) δ (ppm): 3.0-3.2 (m, 2H, C4-H). 3.45 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$), 3.65 (t, J= 6.0 Hz, 2H, $CH_2\underline{CH_2}Cl$), 4.70 (m, 1H, C5-H), 5.57 (m, 3H, C6-H, $\underline{CH_2}$-Ph), 6.76-6.90 (s, 1H, $\underline{NH}CH_2CH_2$), 7.2 (s, 1H, C2-H), 7.52-7.30 (dd, J= 7.0 Hz, 4H, ArH), 10.9 (s, 1H, $\underline{NH}$CO).

**1-[1-(4-Bromobenzyl)-1,4-dihydropyridin-3-yl]-3-(2-chloroethyl)urea IIId**

Yield (51%, oily); $^1$H-NMR ($CDCl_3$) δ (ppm): 2.80-3.0 (m, 2H, C4-H). 3.44 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$), 3.66 (t, J= 6.0 Hz, 2H, $CH_2\underline{CH_2}Cl$), 4.60 (m, 1H, C5-H), 5.57 (m, 3H, C6-H, $\underline{CH_2}$-Ph), 6.76-6.80 (s, 1H, $\underline{NH}CH_2CH_2$), 7.1 (s, 1H, C2-H), 7.33-7. 83 (dd, J= 8.5 Hz, 4H, ArH), 10.20 (s, 1H, $\underline{NH}$CO).

**1-(2-Chloroethyl)-3-[1-(4-nitrobenzyl)-1,4-dihydropyridin-3-yl]urea IIIe**

Yield (50%, oily); $^1$H-NMR ($CDCl_3$) δ (ppm): 2.80-3.0 (m, 2H, C4-H), 3.46 (t, J= 6.0 Hz, 2H, $\underline{CH_2}CH_2Cl$), 3.67 (t, J= 6.1 Hz, 2H, $CH_2\underline{CH_2}Cl$), 4.60 (m, 1H, C5-H), 5.80 (m, 3H, C6-H, $\underline{CH_2}$-Ph), 6.80-6.90 (s, 1H, $\underline{NH}CH_2CH_2$), 7.3 (s, 1H, C2-H), 7.87-8.1 (dd, J= 7.0 Hz, 4H, ArH), 10.26 (s, 1H, $\underline{NH}CO$).

**Chemical oxidation of the 1,4-dihydropyridine derivatives by hydrogen peroxide**

The respective 1,4-dihydropyridine derivative (0.1 mmol) was added to 30% hydrogen peroxide (2 mL). The mixture was stirred and samples were monitored by HPLC to follow the formation of the quaternary products. Acetonitrile (10% in 0.2% acetic acid solution) was used as mobile phase, flow rate 1.5 mL/ min and UV detector was used at $\lambda_{max}$ 262 nm. The apparent pseudo first-order rate constants of appearance of quaternary ($k_{app.}$, $min^{-1}$) were determined from linear regression analysis of ln AUC against time in minutes.

**Kinetics of oxidation of the 1,4-dihydropyridine derivatives in 20% brain homogenate**

About four grams of rat brain was 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 an ice bath. To 5 mL of the freshly prepared brain homogenate, previously equilibrated at 37±1°C in a water bath for 5 minutes, 300 µL of 0.2 mmol methanolic solution of freshly prepared compounds **IIIa-e** was added. The mixture was kept at 37±1°C during the experiment. Samples of 0.5 mL were withdrawn from the tested mixture at different time intervals (10, 30, 60, 90, 120, and 160 minutes) 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, they were centrifuged at 4000 rpm for 10 minutes, and the supernatants were analyzed by HPLC for their content of the dihydro-compounds and their corresponding quaternary derivatives. The apparent pseudo first-order rate constants of appearance of quaternary ($k_{app.}$, $min^{-1}$) were determined from linear regression analysis of ln AUC against time in minutes.

**Bioavailability study of 1,4-dihydropyridine derivatives**

Six groups, each of three Sprague Dawley female rats of average weight of 120-140 g were anesthetized with urethane. The freshly prepared solution of compound **IIIa** at a concentration of 25 mg/mL in dimethylsulfoxide (DMSO) was injected through the external jugular vein at a dose level of 30 mg/kg of body weight. At appropriate time intervals (10, 30, 60, 90, 120, and 160 minutes), 1 mL blood was withdrawn from the eye and added immediately to a centrifuge tube containing 4 mL of acetonitrile. The animal was then decapitated, and the brain was collected, weighed, and kept frozen together with the blood samples. The whole brain was homogenized in 1 mL of water and mixed with 4 mL of 5% DMSO in acetonitrile. Both brain and blood samples were homogenized again and centrifuged at 4000 rpm for 10 minutes. The supernatants from both brain and blood samples were analyzed by HPLC. Control tests were performed on six (one for every test time) Sprague Dawley female rats injected with DMSO at a dose of 1 mL/kg animal weight and treated similarly as experiment.

**Colorimetric evaluation of alkylating activitiy of the prepared quaternary compounds IIa-e**

Methyl ethyl ketone (M & B) and acetone AR (Merck) were used as solvents. 4-(4-Nitrobenzyl) pyridine (NBP) was freshly recrystallized from cyclohexane and used as 5% solution in methyl ethyl ketone. Triethylamine was used as 17% w/v solution in acetone. Chlorambucil was obtained by extraction with acetone from leukeran tablets (Wellcome) and was used as the reference drug. The intensity of the purple colors produced in the test was measured on a Shimadzu UV 120-02 spectrophotometer. The apparent pseudo first-order rate constants of appearance of quaternary ($k_{app.}$, $min^{-1}$) were determined from linear regression analysis of ln absorbance against time in minutes.

**Method**

To a solution of the quaternary alkylating agents **IIa-e** (10 mg) in methyl ethyl ketone (5 mL), 4-(4-nitrobenzyl) pyridine reagent (5 mL of 5% in methyl ethyl ketone) and water (1 mL) were added. The mixture was then heated to 79°C in water bath. 1.5 mL were withdrawn from the reaction mixture at different time interval (5, 10, 15, 20, 25, 30 min) cooled for one minute in an ice bath. Triethylamine reagent (1.5 mL) was added and mixed. The intensity of the purple color formed was measured within 2 minutes at the $\lambda_{max}$ 560 nm against the reagent as blank. Chlorambucil (10 mg) in methyl ethyl ketone (5 mL) was used as reference drug to compare its activity under the same conditions with the activity of the tested compounds.

**Molecular docking**

All the molecular modeling studies were carried out on Intel Pentium 1.6 GHZ processor, 512 MB memory with Windows XP operating system using Molecular Operating Environment (MOE 2008-10 Chemical Computing Group, Canada)[16] as the computational software. All minimizations were performed with MOE until a RMSD gradient of 0.05 kcal and $A^{-1}$ with MMFF94X force-field and the partial charges were automatically calculated.

Crystal structure of the complex of tubulin-colchicine: stathmin-like domain was recovered from the Brookhaven Protein Data Bank (http:/www.rcsb.org/pdb/with the entry code 1SA0).

Colchicine was extracted from model and CEU were merged into the colchicine binding site in such a way that (2-chloroethyl) urea moiety was adjacent to the CBS.

The enzyme was prepared for docking studies where (i) Ligand molecule was removed from the enzyme active site. (ii) Hydrogen atoms were added to the structure with their standard geometry. (iii) MOE Alpha Site Finder was used for the active sites search in enzyme structure and dummy atoms were created from the obtained alpha spheres. (iv)The obtained model was then used in predicting the ligand-enzyme interactions at the active site.

A 3-D model was built to correlate the activity with the structure to design new and more active CEU derivatives.

The interactions obtained between CEU and the CBS, and the mean distances between the carbon atom bearing the chlorine atom of CEU (electrophile) and the carboxyl of Glu-β198 (nucleophile) were measured to evaluate the minimal distance required to initiate the nucleophilic reaction. As illustrated in Table (5), the distances between the heteroatoms of CEU and the amino acids of β-tubulin were calculated and were taken as a mode of stabilization, and the lowest energy scores of the docking of the CEU complex were used to describe the molecular stabilization of the CEU–CBS complexes. It is postulated that the distance and the ligand position are important parameters involved in the initiation or not of the acylation of Glu-β198.

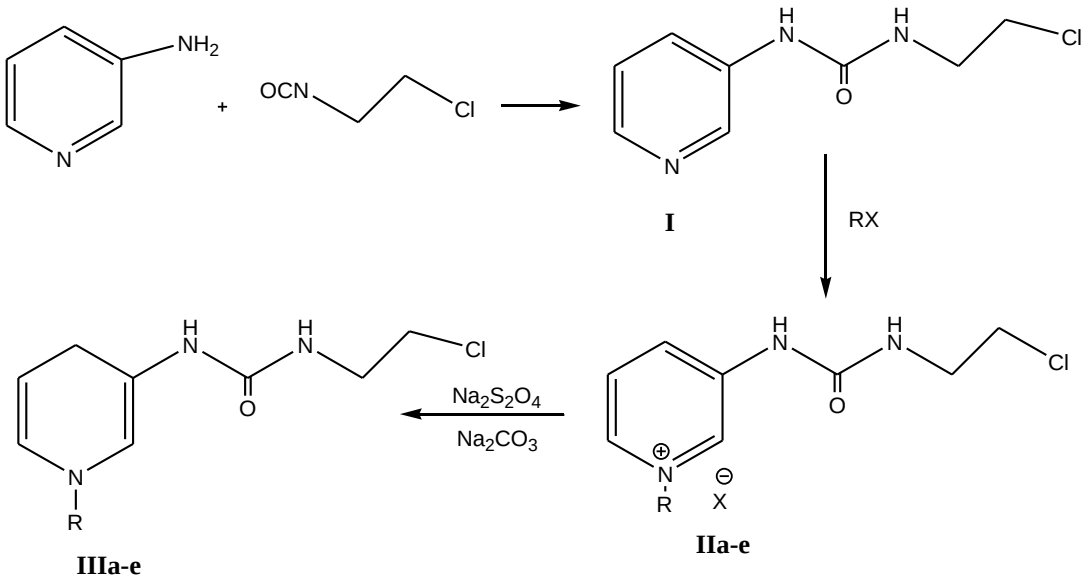
Concomitantly, the urea moiety anchors the drug to Cys-β239 through a hydrogen bond locking the CEU in a spatial conformation favoring the nucleophilic addition. The latter hydrogen bond favors also the electronic induction of the 2-chloroethylamino group by decreasing the energy requirements for the acylation. Then, the acylation occurs between the carboxyl group of Glu-β198 and the carbon atom (electrophilic) bearing the chlorine atom of CEU. Finally the acylation of Glu-β198 leads to important conformational changes of the α,β-tubulin heterodimer that is possibly in the same order of magnitude as the ones described with the Glu-β198-Gly mutation[17-21].

## RESULTS AND DISCUSSION

**A- Chemistry**

A synthetic strategy was adopted as described in Scheme 1 to obtain the final target compounds, 1-(2-chloroethyl)-3-[1-alkyl(4-substituted benzyl)-1,4-dihydropyridin-3-yl]-urea (**IIIa-e**). Compound **I** was synthesized through reaction of 3-aminopyridine with 2-chloroethylisocyanate in THF. Direct quaternization of 1-(2-chloroethyl)-3-pyridin-3-yl urea (**I**) with appropriate alkyl (4-substituted benzyl) halides gave compounds (**IIa-e**). The obtained quaternary compounds were reduced using sodium dithionite in alkaline medium to give the corresponding dihydropyridines (**IIIa-e**) in analogy to the reported procedures[22&23].

The structures of the compounds **IIa-e** were confirmed by their elemental microanalyses, UV, IR and $^1$H-NMR data.




R, X; **a**: $CH_3$, I; **b**: $C_2H_5$, I; **c**: -$CH_2$-$C_6H_4$(4-Cl), Cl; **d**:-$CH_2$-$C_6H_4$(4-Br), Br; **e**:-$CH_2$-$C_6H_4$(4-$NO_2$), Br

**Scheme 1:** Synthesis of the target compounds **IIa-e** and **IIIa-e**.

Structures of the 1,4-dihydropyridines **IIIa-e** were established by their $^1$H-NMR and UV spectra which show absorption maxima at 348-366 nm characteristic for the 1,4-dihydropyridine derivatives[22&23].

**B- Chemical and biological investigation**

The prepared 1,4-dihydropyridine derivatives were subjected to various chemical and biological investigations to evaluate their ability to cross the BBB, and to be oxidized biologically into their corresponding quaternary compounds. HPLC analyses has been applied to monitor the oxidation of the tested 1,4-dihydropyridines into their corresponding quaternary compounds. The HPLC analyses showed that the 1,4-dihydropyridines were detected in blood and brain homogenate at retention time of 5.1-5.6 min while the quaternaries were detected at retention time of 6.2-6.5 min. The mean calibration curve was plotted and best-fit linear regression equation was derived, and used to estimate the concentrations of the quaternary salts.

Hydrogen peroxide oxidizes the dihydropyridines by free radical mechanism. The increase in concentration of the quaternary compounds were monitored by HPLC using UV detector at $\lambda_{max}$ 262 nm. The results in Table (1) indicate the ease of oxidation of 1-methyl-1,4-dihydropyridine **IIIa** to the corresponding quaternary **IIa** with high oxidation rate ($K_{app}$ 17.54x$10^{-2}$). The substituted 1-benzyl-1,4-dihydropyridines **(IIIc-e)** converted to the corresponding quaternaries **IIc-e** with oxidation rates (three to four fold) less than **IIIa** ($K_{app}$ 6.52, 6.90, 4.42 x $10^{-2}$ respectively). The 1-(4-nitrobenzyl)-1,4-dihydropyridinyl-3-chloroethylurea **IIIe** exhibits the lowest rate of oxidation. The electron withdrawing effect of the nitro group decreases the electron density on the ring nitrogen of 1,4-dihydropyridine more than the corresponding bromo and chloro derivatives.

**Table 1:** Kinetics of oxidation of the 1,4-dihydropyridine derivatives **IIIa-e** with hydrogen peroxide.

| Compound No. | $K_{app}$ $min^{-1}$ x $10^{-2}$ | $t_{½}$ (min) | r |
|---|---|---|---|
| **IIIa** | 17.54 | 3.9 | 0.968 |
| **IIIb** | 14.31 | 4.8 | 0.979 |
| **IIIc** | 6.52 | 10.6 | 0.990 |
| **IIId** | 6.90 | 10.0 | 0.990 |
| **IIIe** | 4.42 | 15.7 | 0.980 |

$K_{app}$ is the apparent pseudo first order rate constant of appearance of quaternary derivatives, $t_{1/2}$ is the half-time of the reaction, r= correlation coefficient.

The enzymatically oxidation of the prepared 1,4-dihydropyridine derivatives in the brain could be predicted by spiking the test compounds in brain homogenate. The data summarized in Table (2) indicates facile oxidation of 1-methyl-1,4-dihydropyridine derivative **IIIa** into corresponding quaternary **IIa** with high oxidation rate ($K_{app}$= 42.63x$10^{-2}$, $t_{½}$ 1.6 min). The 4-substituted benzyl-1,4-dihydropyridines **IIIc-e** converted to the corresponding quaternaries **IIc-e** with oxidation rates (seven to twelfth fold) less than **IIIa** ($K_{app}$ 6.56, 4.74, 3.55 x $10^{-2}$ respectivelly). Compounds **IIIa-e** exhibited similar behavior as in case of the chemical oxidation.

**Table 2:** kinetics of oxidation of the 1,4-dihydropyridine derivatives **IIIa-e** in brain homogenate.

| Compd. No. | Brain homogenate $K_{app}$ $min^{-1}$ x $10^{-2}$ | $t_{½}$ (min) | r |
|---|---|---|---|
| **IIIa** | 42.63 | 1.6 | 0.994 |
| **IIIb** | 34.82 | 2.0 | 0.989 |
| **IIIc** | 6.56 | 10.6 | 0.986 |
| **IIId** | 4.74 | 14.6 | 0.980 |
| **IIIe** | 3.55 | 19.5 | 0.990 |

$K_{app}$ is the apparent pseudo first order rate constant of appearance of quaternary derivatives, $t_{1/2}$ is the half-time of the reaction, r= correlation coefficient.

Compound **IIIa** was selected for *in-vivo* study due to its ease of chemical and enzymatic oxidation. A dose of 30 mg/kg, was injected to rats and at selected time intervals, blood and the brain samples were collected. The concentrations of the quaternary derivative **IIa**, was measured in both blood sample and brain homogenate using HPLC assay. The results are given in Table (3).

**Table 3:** *In-vivo* distribution of **IIIa** in blood (mg/mL) and (brain homogenate (mg/g) in rats.

| Time (min) | Mean conc. | |
|---|---|---|
| | Blood | Brain |
| 10 | 75.7 ± 6.2 | 18.5 ± 1.8 |
| 30 | 56.3 ± 5.4 | 20.3 ± 2.1 |
| 60 | 34.9 ± 3.3 | 27.2 ± 2.6 |
| 90 | 20.1 ±1.2 | 56.4 ± 5.4 |
| 120 | - | 33 ± 4.2 |
| 160 | - | 7.62 ± 0.9 |

The analyses of blood samples after 10 minutes proved the presence of compound **IIa** in high concentration. The concentration declined rapidly in blood and undetected after 90 min. The picture looked different in the brain samples. The concentration of **IIa** increased steadily in the brain, and reach its maximum at 90 min followed by decline indicating the sustained release of the quaternary compound.

**Determination of the alkylating potential**

The alkylating potential of the prepared compounds **IIa-e** were evaluated colorimetrically using NBP as analytical reagent[24-26]. NBP reacts with alkylating agents and gives a purple color on alkalinization (Fig. 1). The intensity of the produced color is directly proportional to the degree of alkylation. Chlorambucil was used as a reference. All of the tested compounds proved to be active alkylating agents as shown in Table (4). The *p*-nitrobenzyl substituted derivative **IIe** was the most active member among this series, with alkylating activity ($K_{app}$= 0.219 $min^{-1}$, $t_{½}$= 3.2 min) comparable to that of chlorambucil ($K_{app}$= 0.192 $min^{-1}$, $t_{½}$= 4.9 min).

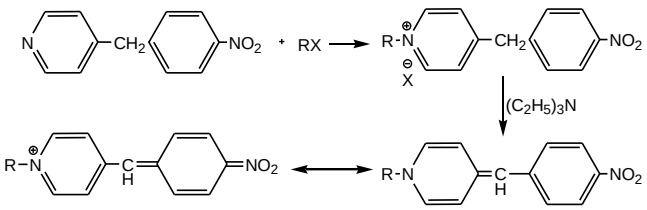



**Fig. 1:** Reaction of alkylating agents with NBP reagent.

**Table 4:** Alkylating activity of compounds **I** and **IIa-e**.

| Compd. No. | R | x | Regression data | | |
|---|---|---|---|---|---|
| | | | $K_{app}$ | $t_{½}$ | r |
| **I** | H | - | 0.032 | 21.7 | 0.999 |
| **IIa** | $CH_3$ | I | 0.041 | 16.5 | 0.998 |
| **IIb** | $C_2H_5$ | I | 0.053 | 13.0 | 0.999 |
| **IIc** | p-Cl-$C_6H_4$-$CH_2$- | Cl | 0.125 | 5.5 | 0.999 |
| **IId** | p-Br-$C_6H_4$-$CH_2$- | Br | 0.178 | 4.0 | 0.999 |
| **IIe** | P-$NO_2$-$C_6H_4$-$CH_2$- | Br | 0.219 | 3.2 | 0.987 |
| **Chlorambucil** | | | 0.192 | 4.9 | 0.994 |

Each reading was triplicate, reaction temp. 79°C, $\lambda_{max}$= 560 nm.

**Molecular modeling study**

Based on the hypothesis brought about by the X-ray structure by Ravelli[20], showing that the tubulin-colchicine complex is compatible with acylation (alkylation through ester formation) of Glu-β198 by CEU[17]. Molecular modeling study was carried out using MOE on CEU structure. These studies were intended to develop novel CEU that would be more active and more selective toward the Glu-β198 residue imbedded in the colchicine-binding site. The synthesized series of CEU derivatives **IIa-e** and **IIIa-e** were selected for molecular modeling study using crystal structure of tubulin-colchicine complex. The biological activity is expressed in terms of the alkylating potentials using NBP.

Docking studies were performed by MOE using crystal structure of the complex of tubulin-colchicine: stathmin-like domain (recovered from the Brookhaven Protein Data Bank; http:/www.rcsb.org/pdb/with the entry code 1SA0). Fifty docking interactions for each ligand and the top-scoring configuration of each of the ligand-enzyme complexes was selected on energetic grounds.

In this study, the analyses were based on the position of the ligand and the nature of the interactions occurring into the pocket with CEU, which are more important for the acylation of Glu-β198. All models are showing that the 2-chloroethylurea moiety of CEU is protruding inside the adjacent pocket toward Glu-β198 in such a way that the acylation is favored. Cys-β239 is probably one of the most important amino acid residue involved in the stabilization of the CEU–tubulin complex prior to the acylation of Glu-β198.

It is also important to note that all models of the CEU–tubulin complexes show hydrogen bonds interactions between the SH group of Cys-β239 and the C=O and NH groups of urea moiety of CEU. In addition to vander-Walls interactions between substituent on pyridine ring and CBS. These interactions probably lock the 2-chloroethylurea moiety into the pocket containing Glu-β198. The distance between the urea moiety of CEU and the SH group of the binding site is an important parameter favoring Glu-β198 acylation. As shown from Table (5), the above mentioned distances ranging from (3.04-3.61A°) for quaternary compounds **IIa-e** and (2.53-6.09 A°) for dihydropyridine compounds **IIIa-e**. The ideal distance range from (2.4-3.5 A°)[17] which is in agreement with this result. The shorter the distance between the electrophilic group of CEU and the –COOH of Glu-β198 is, the higher the rate of acylation. This distances range from (5.77-7.82 A°) for quaternary compounds **IIa-e**, and (5.93 – 9.64 A°) for dihydropyridine compounds **IIIa-e**. As shown from Table (5) and Figure (2), compound **IIe** exhibit the shortest distance of all tested compounds which is in agreement with its alkylating potential using NPB.

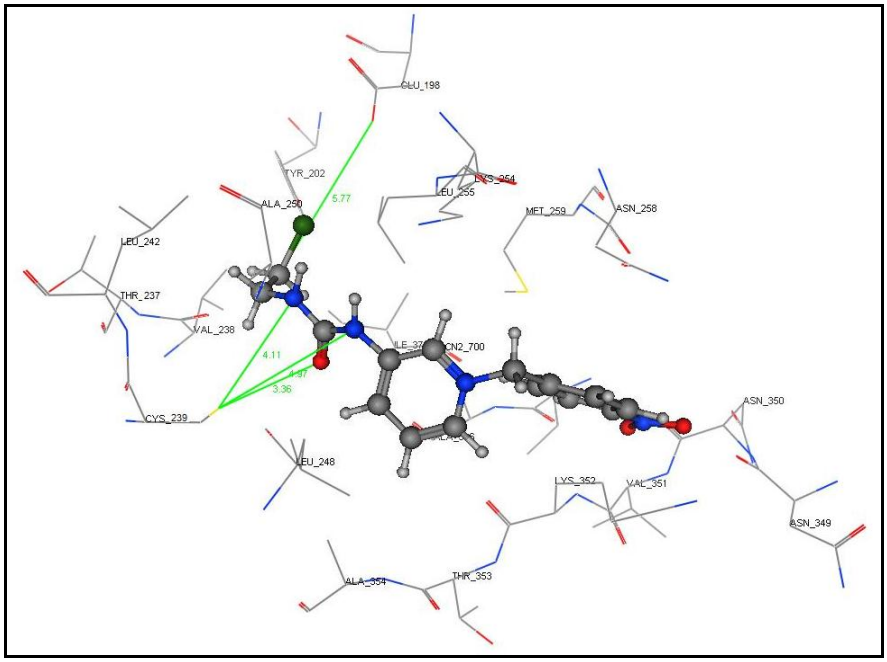



**Fig. 2:** 3D docked structure of **IIe** (ball and stick) at Tubulin-Colchicine active site.

**Table 5:** Docking scores and distances (A°) between heteroatoms of the key amino acids and the CEU in ten different CEU models.

| Compd. No. | Lower energy score | CEU interacting group | Amino acid involved | Amino acid interacting group | Interatomic distance (A°) |
|---|---|---|---|---|---|
| **IIa** | -8.0242 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-β198 | SH<br>SH<br>O-C=O | 3.20<br>4.17<br>7.11 |
| **IIb** | -7.7477 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-β198 | SH<br>SH<br>O-C=O | 3.49<br>3.65<br>7.82 |
| **IIc** | -8.5050 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-β198 | SH<br>SH<br>O-C=O | 3.61<br>3.52<br>5.89 |
| **IId** | -9.2048 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-β198 | SH<br>SH<br>O-C=O | 3.04<br>4.42<br>5.89 |
| **IIe** | -8.9546 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-198 | SH<br>SH<br>O-C=O | 3.36<br>4.54<br>5.77 |
| **IIIa** | -7.3995 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-β198 | SH<br>SH<br>O-C=O | 4.55<br>5.91<br>7.74 |
| **IIIb** | -7.6120 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-β198 | SH<br>SH<br>O-C=O | 6.09<br>5.63<br>9.64 |
| **IIIc** | -7.8666 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-198 | SH<br>SH<br>O-C=O | 2.53<br>1.73<br>7.06 |
| **IIId** | -9.5689 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-β198 | SH<br>SH<br>O-C=O | 2.76<br>3.89<br>7.23 |
| **IIIe** | -9.2957 | C=O (urea)<br>NH (urea)<br>C-Cl | Cys-β239<br>Cys-β239<br>Glu-β198 | SH<br>SH<br>O-C=O | 2.74<br>3.00<br>5.93 |

**Conclusion**

We can concluded that *in-vitro* oxidation studies showed the ability of the tested dihydropyridine derivatives **(IIIa-e**) to be oxidized into the corresponding quaternary compounds (**IIa-e**). The rate of oxidation was affected by substitution on nitrogen of pyridine nucleus. The 4-nitrobenzyl group optimized the rate of oxidation to ensure the delivery of the drug into the brain and sustained release of the quaternary compound. *In-vitro* studies showed good alkylating potential of compound **IIe** comparable to reference standard, chlorambucil. The *in-vivo* studies revealed that 1,4-dihydropyridines, could cross the BBB at detectable concentrations.

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