REPORT ON THE
PRESENT
INVESTIGATION
3.1. L- Glutamic acid conjugate of Methotrexate (MTX-GLU)

Fig- 3. 2-(4-(((2,4-bis(2-amino-4-carboxybutanamido)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (MTX-GLU)
3.1.1. Experimental

3.1.1.1. Materials and Methods

3.1.1.1.1. Chemicals

Methotrexate was procured from IPCA lab, Buddi, India as gift sample. L-Glutamic acid, L-Lysine, L-Tyrosine, L-Phenylalanine and L-Leucine were purchased from Molychem, India. Di-tert-butyl dicarbonate (BOC)_2O was purchased from Sigma-Aldrich, St. Louis, Missouri, USA. 1,4-Dioxane, Dichloromethane, Dicyclohexylcarbodiimide (DCC), 4-Dimethyl amino pyridine (DMAP), Tetrahydrofuran (THF), Dimethylformamide (DMF) Trifluoroacetic acid (TFA) and sodium dihydrogen phosphate were purchased from SD Fine, India. Water, ethyl acetate, methanol and acetonitrile were of HPLC grade and purchased from Merck, Germany.

3.1.1.1.2. Synthesis

All the reactions were performed with reagents of commercial high purity without further purification. Reactions were monitored by thin-layer chromatography. The melting points were determined with open capillary method and were uncorrected.

3.1.1.1.3. Purification

Purifications of the compounds were performed by crystallization and column chromatography. Purities of the final compound were determined by an analytical RP-HPLC (Yunglin Liquid Instrument, Korea) on a promocil C18 column (4.6 mm x 250 mm, 5 µm).

3.1.1.1.4. HPLC

Chromatographic method were calibrated and verified by reported method with minor modification (Nagulu et al., 2009). Yunglin liquid chromatography system RP-HPLC equipped with a isocratic pump, dual wavelength UV/VIS absorbance detector (UV730D) and manual sample injector with switch (7725i) was used (YL Instrument, Korea). The chromatographic separations were achieved on a promocil C18 column (4.6 mm x 250 mm, 5 µm), thermo stated at 27˚C. The mobile phase was mixture of phosphate buffer (10 mmol sodium dihydrogen phosphates and 10 mmol sodium hydroxide, pH 7.4) premixed, filtered and degassed buffer in water: acetonitrile (90:10). The λ_max was set at 310 nm and flow rate was 1mL/min and retention time was found at 7.3 min. The chromatograms were shown in Fig- 7, Fig- 9 and Fig- 11.
3.1.1.1.5. UV spectroscopy

A double beam UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan) with spectral bandwidth of 1 mm and wavelength accuracy of ± 0.3 nm was used with a pair of 10 mm matched quartz cells.

3.1.1.1.6. FTIR

FTIR spectra were recorded in KBr discs for solid sample and liquid cell (fixed cell) of NaCl with nujol (mineral oil) were used for liquid sample on IR solution Version Affinity-1 (Shimadzu, Japan).

3.1.1.1.7. 1H NMR and 13C NMR

$^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance II 400 spectrometer (Bruker Biospin, Fällanden, Switzerland) operating at 400.136 MHz and 100.61 respectively, using tetramethylsilane (TMS) as an internal standard. Spectra were recorded at sophisticated analytical instrument facilities (SAIF) laboratory, Panjab University, Chandigarh.

3.1.1.1.8. Mass Spectroscopy

The final products were also characterized by mass spectroscopy with Q-Tof micromass spectrometer (Waters, Milford Massachusetts, USA) equipped with an electrospray ionization source. Spectra were recorded at sophisticated analytical instrument facilities (SAIF) laboratory, Panjab University, Chandigarh.

3.1.1.1.9. Elemental Analysis

Elemental Analysis was carried out on a 2400 series II CHNS/O analyser, Perkin Elmer, USA, value found were within ± 0.5% of theoretical ones. Analysis were performed at Indian association for cultivation of science (IACS), Jadavpur, Kolkata.

3.1.1.1.10. Animals

Healthy male albino rats of Sprague Dawley strain of uniform body weight (100±10 g), adult male/female wister rats of 140-150g body weight and Swiss albino mice (2-3 month old of either sex) weight 22-30g of BALB/c strain were procured from the animal house facilities of national centre of disease control, Sham Nath Marg, Delhi, India and Columbia institute of pharmacy, Raipur, C.G, India. The animals were reared on laboratory chow pallets, fed ad libitum and had free access to food and water at all the time. The room was maintained at 25±2°C with natural daytime light and no light after 12 h until morning. Experiments were carried out during the light phase. All procedures were reviewed and
approved by the animal ethics committee (Regd. No.-1283/c/09/CPCSEA) at Columbia Institute of Pharmacy, Raipur, C.G, India.

3.1.1.1.11. Radioisotopes and Scintillation

99mTc-pertechnetate was provided by Institute of nuclear medicine and allied science (INMAS), DRDO, Delhi, India. The radioactivity counter was a gamma ray counter (Type GRS23C, serial no. 458-425 Electronic Corporation of India Limited, India). Imaging of animal was performed using Single Photon Emission Computerized Tomography (SPECT, LC 75-005, Diacam, Siemens AG, Erlanger, Germany) gamma camera.
3.1.2. Synthesis of MTX-GLU

Scheme II. Glutamic acid conjugated of Methotrexate (MTX-GLU)
Reagents and conditions: i - L-Glutamic acid, NaOH, 1,4-Dioxane, (BOC)₂O, stir at r.t. for 16 h; ii - MTX, anhydrous CH₂Cl₂, DMAP, DCC, stir at r.t. for 24 h; iii - TFA, anhydrous CH₂Cl₂, stir at r.t. for 1.5 h.
3.1.2.1. General Procedure of Synthesis

Synthesis of 2-((tert-butoxycarbonyl)amino)pentanedioic acid (1)

Synthesis of 2-((tert-butoxycarbonyl) amino) pentanedioic acid was done by reported method with little modification (Laulloo, et al., 2007; Martin et al., 2002). Glutamic acid (20 mmol) was added to a stirred solution of NaOH (25 mmol) in 50 ml of water at ambient temperature and then diluted with 50 ml of 1,4-Dioxane and Di-tert-butyl dicarbonate (BOC)₂O (4.5g, 20 mmol, 1.00 equiv) was added drop wise. The reaction was brought to completion by further stirring for 16 h at room temperature. The solution was diluted with 50 ml of water. The solution was concentrated under reduced pressure. This mixture was cooled in an ice bath and covered with a layer of diethyl ether (10 ml). The reaction mixture was extracted with (20 ml x 2) of diethyl ether, and the organic phase extracted with (30 ml x 3) of saturated aqueous sodium bicarbonate solution. The combined aqueous layer was acidified to pH 2 by addition of potassium hydrogen sulfate solution. The mixture was extracted with (30ml x 2) of ethyl ether. The organic layer was dried over MgSO₄, filtered, and evaporated under rotary evaporator. Hexane was added to the thick oil to turbidity. The mixture is allowed to stand for 1 h. The white crystalline solid was collected by filtration, washed with (10ml x 3) portions of hexane, and dried under reduced pressure to get 2-((tert-butoxycarbonyl)amino)pentanedioic acid (1)

White crystalline powder, yield 85%, m.p.: 108±3°C, IR (KBr) cm⁻¹: 3440 (N-H), 3295 (O-H), 3120, 2976 (C-H str.), 1714 (C=O), 1427 (C-H bend) and 1330 (CH-(CH₃)₃) Appendix-I

Synthesis of 2-4-((4-bis((2-((2,4-bis((tert-butoxycarbonylamino)-4-carboxybutanamido)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid. (2)

Methotrexate (2 mmol) was taken in 50 mL of dry dichloromethane and 4-dimethylaminopyridine (1.6 mmol) was added to it. The mixture was stirred and cooled in an ice bath to 0°C. Compound 1 (6 mmol) was taken in dichloromethane (10 ml) and (2.2 mmol) of dicyclohexylcarbodiimide (DCC) in dichloromethane was added over a 5-min period. The pH (3) was monitored during this. This mixture was stirred at 0°C for 2 h and added to the ice cooled reaction mixture of MTX drop wise with stirring. The resulting reaction mixture was stirred for 24 h at room temperature. The precipitated was removed by filtration and the filtrate was concentrated under vacuum and taken up in ethyl acetate and washed with 5% of aqueous acetic acid solution (3 x 20 mL) and 1M sodium carbonate (3 x 20 ml). This washed organic solution was dried over anhydrous sodium sulfate and concentrated in vacuum. A viscous yellow crude product was obtained. This was recrystallized. Further purification of
crude product was done by column chromatography using dichloromethane: acetonitrile: methanol (1:1:2) as mobile phase to afford in of 2-(4-(((2,4-bis(2-tert-butoxycarbonylamino)-4-carboxybutanamido)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (2). Yellow solid, yield 53%, m.p.: 172±3ºC, IR (KBr) cm\(^{-1}\): 3327 (N-H), 3269 (O-H), 3095 (Ar-C-H), 2976 (C-H str.), 1702 (C=O), 1649 (C=O), 1525 (C=N), and 1458 (C-H bend).

(Appendix-II)

**Synthesis of 2-(4-(((2,4-bis(2-amino-4-carboxybutanamido)pteridin-6-yl)methyl)(methyl) amino)benzamido)pentanedioic acid.**

Compound 2 (1mmol) was taken and trifluoroacetic acid (TFA, 4.5 mL) was added drop wise in 5 ml dichloromethane at 0°C in an ice bath. The reaction was left to stir for 1.5 h then the reaction was saturated with diethyl ether until precipitate formed. The precipitate was decanted off, washed twice with diethyl ether then dried over anhydrous sodium sulfate and evaporated under vacuum to obtain the desire product as a dry light brownish solid. The crude product was triturated in ethyl acetate (10 ml) and purified in column chromatography using dichloromethane: methanol: water (1:2:1) as mobile phase. Following recrystallization the compound 3 recovered.

Yellow solid, Yield: 70%. m.p.: 205±3ºC. Analysis for C\(_{30}\)H\(_{36}\)N\(_{10}\)O\(_{11}\) (712.257); calculated: C, 50.56; H, 5.09; N, 19.65; found: C, 50.66; H, 5.07; N, 19.70. FTIR (KBr), \(\nu\) (cm\(^{-1}\)): 3564.45(O-H), 3206.03(N-H), 3198.03(N-H), 1705.07(C=O), 1683.86 (CONH), 1517.05(C=N). (Appendix-III) \(^1\)H NMR (DMSO) \(\delta\) (ppm): 11.28(s, 1H, OH), 11.18(s, 1H, OH), 8.55(s, 2H, NH\(_2\)), 8.31(s, 1H, NH), 8.31(s, 1H, NH), 8.30(s, 1H, NH), 8.16(s, 1H, NH), 8.15(s, 1H, Pyrazine), 6.59-7.80(m, Ar-H), 4.78(s,2H, CH\(_2\)), 4.37-4.41(m,1H, CH), 3.38(s,3H, CH\(_3\)), 4.78(s,2H, CH\(_2\)),3.22-3.40(t, 1H, CH), 2.52-2.54(m, 2H, CH\(_2\)), 2.32-2.36 (t, 2H, CH\(_2\)), 2.10-2.14(m, 2H, CH\(_2\)), 2.05-2.09(t, 2H, CH\(_2\)). (Appendix-IV) \(^{13}\)C NMR (DMSO) \(\delta\) (ppm): 174.15(COOH), 173.87 (COOH), 172.48(COOH), 171.25(CONH), 170.14(CONH), 166.25(CONH), 162.64(CN, Pyrimidine), 162.48(CN, Pyrimidine), 154.68(CN, Pyrazine), 150.84 (CN, Pyrazine), 148.83 (Pyrazine), 146.00, (Pyrazine),128.78(2C,1-benzene),121.49 (Benzene), 121.34 (Benzene), 11.91(2C,1-benzene), 55.48(CHNH\(_2\)), 53.87(CHNH\(_2\)), 52.94(CNH\(_2\)), 52.01(CN), 39.99(CH\(_3\)N), 33.68(CH\(_2\)), 33.05(CH\(_2\)), 31.57(CH\(_2\)), 30.59(CH\(_2\)), 30.15(CH\(_2\)), 27.59(CH\(_2\)). (Appendix-V) MS \(m/z\) (%):713.32.(M+1). (Appendix-VI)
3.1.2.2. HPLC Methods

The chromatographic separations were achieved on a promocil C18 (4.6 mm x 250 mm, 5 µm) analytical column. The mobile phase comprised of phosphate buffer (10 mmol dihydrogen phosphate and 10 mmol sodium hydroxide, pH 7.4) premixed, filtered and degassed buffer in water: methanol: acetonitrile (70:20:10) MTX-GLU. The retention time of MTX-GLU was 6.4 min at \( \lambda_{\text{max}} \) 295 nm. The chromatograms were shown in Fig- 13, Fig- 15 and Fig- 17

3.1.3. Analysis of MTX-GLU

The verification of analytical method was performed by spiked placebo technique. The method was verified for linearity and accuracy in accordance with ICH guidelines on analytical validation Q2 (R1) (22). The purity of the MTX-GLU was assessed by means of RP-HPLC. Test samples were dissolved in 5% methanol and filtered through a 0.45-µm polyamide filter before injection. Analysis of mixture of MTX-GLU (at 295 nm) always showed a good separation of the peaks under above conditions. The result was shown in Table 2

3.1.4. Stability studies in different buffer solutions

The MTX-GLU was investigated for their chemical stability in phosphate buffer solution of four different pH values: 2.0, 4.9, 7.4 and 8.0 at 37°C. Precisely, 1 mL methanol solution (0.140 µM/mL) of the conjugate was added into 4 mL of different buffer. After mixing, it was kept in a 37 ±1°C constant water bath and then 200 µL samples was withdrawn at different time points (0, 1, 2, 4, 8, 12 and 24 h). The disappearance of MTX-GLU was monitored by HPLC method. The rate constant (\( K_{\text{disapp}}, \text{ h}^{-1} \)) and half-lives (\( t_{1/2}, \text{ h} \)) of the compounds in aqueous solution were calculated by linear regression of peak area against time in hrs. The values were shown in Table 3.

3.1.5. Stability in plasma extracts and brain homogenate

Blood was drawn from mice though orbital sinus and was collected in a heparinized tube paved with heparin sodium. Samples were centrifuged at 15000 rpm for 15 min to separate plasma which was diluted with double volumes of water. The brain was removed and homogenized in cold phosphate buffer of pH 7.4 with proportion of 1:5 (w/v). Samples were then placed on ice and used immediately. 1 mL sol of MTX-GLU (0.140 µM/mL) in PBS 7.4 was added to plasma and brain homogenate respectively and gently vortexed. Samples were incubated at 37 °C and 200 µL aliquots were removed after 15, 30, 60, 90 and 120 min,
respectively. Following deproteinisation and centrifugation the supernatants were analyzed by HPLC. The result was shown in Table 4.

3.1.6. In vitro conversion of MTX-GLU to MTX in brain homogenate

The conjugate was treated in brain homogenate and the peak of MTX was analyzed by HPLC. Brain was removed and homogenized in cold phosphate buffer of pH 7.4 with proportion of 1:5 (w/v). Samples were then placed on ice and used immediately. 1 mL sol of MTX-GLU (0.140 µM/mL) in PBS 7.4 was added to brain homogenate and gently vortexted. The pH was adjusted to acidic with 10% phosphoric acid. Samples were incubated at 37°C and 200 µL aliquots were removed after 15, 30, 60, 120, 180, 240, and 480 min respectively. Samples were deproteinised, centrifuged and the supernatants were analyzed by HPLC for determination of MTX. The result and chromatogram was shown in Fig- 19 and Fig- 20

3.1.7. Distribution coefficients of the MTX-GLU in 1-octanol/PBS 7.4

The distribution coefficient (logD) was determined by shake flask method based on the OECD Test Guideline 107. The compound was dissolved in PBS 7.4 to a concentration of 1.40µM/mL. The solution was carefully diluted with PBS 7.4, to obtain a series of known concentrations in order to create a calibration curve (Fig- 21). The instrument was set to the maximum absorbance wavelength. Using aqueous (PBS) solutions of MTX-GLU (1.40 µM/mL) stock solutions were prepared with corresponding volumes of 1-octanol (the ratio varied with the compounds). The mixture was kept for 24 h on a mechanical shaker and then allowed to stand long enough to separate the phases and achieve a saturation state. Concentration in the aqueous phase was determined by using UV-visible spectrophotometer. Based on a simple mass balance, concentration in the octanol phase was determined by using formula

\[ V_{aq}C_{eq} + V_{oct}C_{oct} = V_{aq}C_{initial} \]  \hspace{1cm} (eq. 1)

Where, V is the volume of the respective phases; \(C_{eq}\) is the aqueous concentration at equilibrium; \(C_{initial}\) is the initial aqueous concentration and \(C_{oct}\) is the octanol phase concentration.

Therefore, the octanol phase concentration was determined from the following relationship:

\[ C_{oct} = V_{aq} (C_{initial} - C_{eq})/V_{oct} \]  \hspace{1cm} (eq. 2)

\[ Pow = C_{oct}/C_{eq} \]  \hspace{1cm} (eq. 3)

The logD was calculated as the ratio of the concentration in the octanol phase to the concentration in the aqueous phase result shown in Table 5.
3.1.8. Protein binding study

The plasma protein binding was estimated by equilibrium dialysis technique (Rajput, et al., 2012). Blood from goat was collected, heparinized and kept in deep freezer at -20°C. Plasma was separated with aid of cooling centrifuge at 6000 rpm. The conjugate was dissolved in plasma to obtain a series of known concentrations in order to create a calibration curve (Fig- 22). Various concentrations of MTX-GLU (10, 20, 50 and 100 μg/mL) were prepared in plasma. Each dialyzing bag filled with 5 mL of plasma, containing a known amount of the drug, was then immersed in flask containing 5 mL of phosphate buffer 7.4 and the flasks were incubated at 37°C for 24 h with orbital shaker at 50 rpm. At the end of the incubation period, the buffers as well as the contents of the dialyzing bags were analyzed separately by UV spectrophotometer and plasma protein binding was calculated by using formula and result was shown in Table 5

\[
\text{Percent of bound to plasma protein} = \frac{C_P - C_B}{C_P} \times 100
\]

Where, \( C_P \) is the concentration of MTX-GLU in the plasma after incubation, \( C_B \) is the concentration of MTX-GLU in the phosphate buffer after incubation and \( C_P \) is the concentration of MTX-GLU in the plasma before incubation.

3.1.9. Toxicological Studies

Toxicity studies were carried out as per the guidelines of Organization of Economic Co-operation and Development (OECD-423) for testing of chemicals. In-vitro and In vivo toxicological studies has been performed to determine the safety margin of the conjugate.

3.1.9.1 In vitro hemolytic toxicity study

The degree of hemolysis was determined by reported method (Singhai, et al., 1997). Human venous blood was collected using syringe prefilled with acid citrate dextrose buffer. The RBCs were separated from the whole blood by centrifugation (REMI, Mumbai, India) at 3,000 rpm for 5 min. The supernatants and buffy coats were removed and discarded. The packed cell volume (PCV) was washed with normal saline and centrifuged again at 2000 rpm for 2 min. The 5 mL of PCV was then diluted to 100 mL with normal saline to get 5% RBC suspension. 0.5 mL of suitably diluted (0.1, 0.2, 0.3 and 0.4 %) plain MTX and MTX-GLU were added to 4.5 mL of normal saline and incubated for 1 h with RBC suspension. After centrifugation, supernatants were taken and diluted with an equal volume of normal saline and absorbance was measured at 540 nm. RBC suspension was added to 5 mL of saline and 5 mL
distilled water; respectively to obtain 0% and 100% hemolysis. The degree of hemolysis was determined by the following equation: (Result was shown in Fig- 22)

\[
\text{Hemolysis (\%) = } \frac{\text{Abs}_{100} - \text{Abs}_0}{\text{Abs}_{100} - \text{Abs}_0} \times 100
\]

Where, Abs, Abs\(_{100}\) and Abs\(_0\) are the absorbance of sample, a solution of 100% hemolysis and a solution of 0% hemolysis, respectively.

### 3.1.9.2 In vivo hematological studies

Healthy male albino rats of Sprague Dawley strain of uniform body weight (100±10 g) were selected for the study of hematological parameters. Fifteen animals were selected and divided into three groups comprised of three rats in each group. 25 and 250 mg/kg body weight of MTX and MTX-GLU were separately administered, intravenously into second and third groups of animals, respectively daily up to 7 days. The first group was kept as control, which was maintained on same regular diet for 7 days. After 15 days blood samples were collected from the animals of all the groups and analyzed for RBC count, WBC count, hematocrit (HCT), hemoglobin content, differential monocyte count, lymphocytes and neutrophils by pathology laboratory. Result shown in Table 6

### 3.1.9.3. Neurotoxicity Studies

Methotrexate is known to cause local neurotoxicity at high dose by Intrathecal treatment (Bairy, K L. et al., 2005). So neurotoxicity study of the conjugate was needed to determine safety margin of the conjugates. This was assessed by studying motor coordination, loco motor activity and induction of catatonia in animals.

### 3.1.9.3.1. Motor Coordination test

This study was carried out in a Rota-rod apparatus following established procedure with modification (Parvathi, et al., 2013). Healthy adult male/female wister rats of 140-150g body weight were used. Animals were kept under standard laboratory conditions. Commercial pellet diet and water were provided ad libitum. Animals were randomly divided into four groups consisting of six rats each. Control group, received calculated dose of vehicle (1 mL/100g) oral route. Group one received Diazepam 2 mg/kg body wt./i.p., group two received MTX 3 mg/kg body wt./i.v., group three received MTX-GLU equivalent to 3 mg/kg body wt. of MTX/i.v for 7 days. The motor coordination and performance of each rat was evaluated 1 h after administration of vehicle, MTX and MTX-GLU and 30 min after
administration of diazepam for 7 days in a Rota-rod apparatus. This equipment consists of a horizontal metal rod of 3 cm in diameter and divided into six parts, and it is placed at a height of 50 cm, rotating at 20 rpm. Latency to fall from the rotating bar was registered and compared with control. Results shown in Table 7

3.1.9.3.2. Assessment of locomotor activity

The locomotor activity of animals following administration of drug and conjugate was studied by established procedure with modification (Parvathi, et al., 2013). Animals were randomly divided into four groups consisting of six rats each. Control group, received calculated dose of vehicle (1 ml/100g) oral route. Group one received Diazepam 2 mg/kg body wt./i.p., group two received MTX 3 mg/kg body wt./i.v., group three received MTX-GLU equivalent to 3 mg/kg body wt. of MTX/i.v for 14 days. The locomotor activity of each rat was recorded individually for 300 sec after 1 h administration of vehicle, MTX and MTX-GLU and after 30 min administration of diazepam for 14 days using an actophotometer. The movement of the animal cuts off a beam of light falling on the photocell, and the count is recorded digitally and compared with control. Results was shown in Table 8

3.1.9.3.3. Catalepsy

Induction of catatonia was studied by bar test method (Ahmad et al., 2012). Animals were randomly divided into four groups consisting of six rats each. Control group, received calculated dose of vehicle (1 mL/100g) oral route. Group one received haloperidol 2 mg/kg body wt./i.p., group two received MTX 3 mg/kg body wt./i.v., group three received MTX-GLU equivalent to 3 mg/kg body wt. of MTX/i.v for 14 days. Catalepsy was assessed by means of a standard bar test. Catalepsy induced with haloperidol (2.0 mg/kg i.p.) and locomotor activity on 1st, 4th, 8th and 14th day of the MTX and MTX-GLU treatment was compared with respect to control. The catalepsy was assessed in terms of the time in sec. The end point of the catalepsy was considered to occur when both the front paws were removed from the bar. Catalepsy of an individual rat was measured by a scoring method (Ahmad et al., 2012). Result was shown in Table 9

3.1.10. In vivo release of MTX from MTX-GLU

Mice were randomly divided into 2 groups, 24 in each group for different sampling time and housed in one cage. Each animal was injected with MTX or MTX-GLU in phosphate buffer of pH 7.4 through the tail vein at a single dose equivalent to 7.2 µM/kg body weight of MTX. At appropriate time interval (15, 30, 60, 90, 120, 150, 180 and 240 min), the animal was sacrificed and 1 mL blood samples withdrawn from cardiac puncture were
collected in heparinized tube. Plasma was immediately separated by centrifugation and diluted with PBS 7.4 to 1:3 which was stored at -20°C until assay. Meanwhile, the brain sample was removed, weighed. Each tissue sample was homogenized and diluted with PBS 7.4 to 1:3 (g/mL). The homogenates were also stored at -20°C until assay. Before analyzing conjugate, hydrolysis was performed. After deproteinization the mixture was centrifuged at 15000 rpm for 15 min. 200 µL aliquots were withdrawn, 200 µL of acetonitrile was added to each aliquot and vortexed. Samples were centrifuged for further 15 min to remove residual proteins and the supernatants were analyzed to determine the concentration of Methotrexate by HPLC method. HPLC chromatogram of MTX in plasma and brain are shown in Fig-25 and Fig-26 respectively. Result was shown in Table 10, Table 11 and Fig-24.

3.1.11. Radio labelling of MTX and MTX-GLU with $^{99m}$Tc and optimization

3.1.11.1. Preparation of radiolabeled conjugates with $^{99m}$Tc

The radiolabeling of MTX and MTX-GLU conjugates was done by dissolving the 2.0 mg equivalent of drug in 1 mL water for injection in a sterile glass vial. Then followed by addition of (30–200 µg) concentrations of a reducing agent, stannous chloride (1 mg/mL solution made in 1 N HCl) and pH was adjusted between 6.0 to 8.0 using 0.5M sodium bicarbonate solution. To the resulting mixture (filtered through 0.22 µm membrane filter), 1-2 mL of 74 MBq $^{99m}$Tc-pertechnete containing 2.0 to 3.0 mCi was added drop wise and the reaction mixture was incubated at 25 ± 5°C for 15-20 min and checked for radiolabeling efficiency.

3.1.11.2. Determination of the radiolabeling efficiency of conjugates, radiochemical impurity and in vitro stability in saline & plasma

The radiolabeling efficiency was ascertained by thin layer chromatography method (Reddy, et al., 2004) using the instant thin layer chromatography silica gel (ITLC–SG) strips as stationary phase and acetone as the mobile phase. The procedure involved spotting 2 µL samples of radiopharmaceuticals onto chromatographic strip 10 cm in length. After developing in the solvent, the strip was cut into two portions (top: bottom: 1:3) and activity in each portion was measured in the form of count using gamma scintillation counter. $^{99m}$Tc-labeled MTX and $^{99m}$Tc-labeled MTX-GLU remained at the origin and free technetium travelled with the solvent front ($R_f = 0.9-1.0$). The radiolabeling yield was expressed as a percentage of the total amount of radioactivity applied in the testing system. Percentage of
conjugate was determined using pyridine: acetic acid: water (3:2.5:1) as the mobile phase. The radiolabeling efficiency was calculated using established equation (Babar, et al., 2000)

\[
\text{Radioactivity (counts) retained in the lower half of the strip} \times 100
\]
\[
\text{Initial radioactivity associated (total count present with strip)}
\]

Radiochemical impurity that is likely to exist in the form of unconjugated technetium in $^{99m}$Tc-labeled MTX-GLU conjugates and $^{99m}$Tc-labeled MTX solution was determined by the instant thin layer chromatography-silica gel (ITLC–SG) strips as stationary phase. The effects of incubation time, pH, and stannous chloride concentration on labelling were studied to achieve optimum reaction conditions Table- 12. The in vitro stability of radiolabeled formulation was evaluated in 0.9% (w/v) sodium chloride and in mice plasma Table 13. After the optimization and evaluation, stable radiolabeled MTX and MTX-GLU were used for bio-distribution study in mice.

### 3.1.12. Bio-distribution study and Radioscintigraphy Imaging in mice

Swiss albino mice (2-3 month old of either sex) weighing 22-30g of BALB/c strain were used for bio-distribution studies. Three mice for each drug and conjugate per time point (15, 30, 60, and 120 min) were used in the study. 3.7 MBq of radiolabeled compound containing 2 mg of MTX and MTX-GLU (equivalent to 2.80 μM/kg body weight) was injected through the tail vein of each mouse. The mice were killed humanely at different time intervals and the blood was collected using cardiac puncture. Subsequently, brain, lungs, heart, liver, kidney, spleen, intestine and stomach were dissected, washed twice using normal saline, made free from adhering tissue/fluid, and weighed. Radioactivity present in each tissue/organ was counted using shielded well-type gamma scintillation counter and expressed as % of radioactivity in blood to tissue relative distribution shown in Table 14.

\[
\text{Counts in tissue (organ) sample} \times 100
\]
\[
\text{Counts in Blood sample after 15 min}
\]

Gamma scintigraphy imaging was performed on mice following i.v. administrations of radiolabeled drug and its conjugate to determine the localization of drug in brain. 18.5 MBq of radiolabeled compound containing 2 mg of MTX and MTX-GLU (equivalent to 2.80
µM/kg body weight) was injected through the tail vein of each mouse. The mice were
anaesthetized using 0.4 ml ketamine (50 mg/mL) intramuscular injection and placed on the
imaging board. Imaging was performed using gamma scintillation camera. Images were
shown in Fig- 27

3.1.13. Statistical analysis

All experiments were conducted at least in triplicate and results were expressed as
mean ±SD. The AUC_0-t, AUMC_0-t and MRT were calculated by nonlinear Trapezoidal rule.
Statistics (Graphpad prism 5.0 demo version) Statistical evaluation was performed by
unpaired t-test with 95% confidence interval. P< 0.001 was considered significant. The RE
and CE were calculated to evaluate the brain targeting property of conjugate. The value of RE
and CE were defined as following:

REs (relative uptake efficiencies) = (AUC_0-t)sample/(AUC_0-t)control
CEs (concentration efficiencies) = (C_max)Sample/(C_max) control

Where, sample represented (Conjugate) and control (Methotrexate)
3.2. L- Lysine conjugate of Methotrexate (MTX- LYS)

Fig-4. 2-(4-((2,4-bis(2,6-diaminohexanamido)pteridin-7-yl)methyl)(methyl)amino)benzamido) pentanedioic acid. (MTX- LYS)

3.2.1. Experimental
3.2.1.1. Materials and Methods
The materials and methods are as described in section 1.3.1.1.1
3.2.2. Synthesis of MTX-LYS

Reagents and conditions: i - L-lysine, NaOH, 1,4- Dioxane-Water, (BOC)$_2$O, stir at r.t. temp., for 20 h ii - MTX, Anhydrous DMF, DCC, Ethyl Acetate, stir at 0ºC for 2 h, Methotrexate (MTX) stir at r.t. for 24 h iii - TFA, Anhydrous DMF, stir at r.t. for 1.5 h.
3.2.2.1. General Procedure of Synthesis

Synthesis of 2,6-Bis((tert-butoxycarbonyl)amino)hexanoic acid (1)

2,6-Bis((tert-butoxycarbonyl)amino)hexanoic acid was prepared by using reported method with little modification (Keller et al., 1990; Laulloo, et al., 2007; Martin et al., 2002). L-lysine (10 mmol) was added to a mixture solution of NaOH (50 mmol) in 50 mL of water at ambient temperature and then diluted with 50 mL of 1,4-dioxane and Di-tert-butyl dicarbonate (BOC)\(_2\)O (6.54g, 30 mmol, 3.00 equiv) was added drop wise. The reaction was brought to completion by further stirring for 20 h at room temperature. The solvent was removed under reduced pressure. This mixture was cooled in an ice bath and covered with a layer of diethyl ether (10 mL). The combined mixture was acidified to pH 3 by addition of 1 M HCl aqueous solution. The mixture was extracted with (30mL x 2) of ethyl ether. The organic layer was dried over MgSO\(_4\), filtered, and evaporated under rotary evaporator. Clear oil was collected get 2,6-Bis((tert-butoxycarbonyl)amino)hexanoic acid (1)

Clear oil, IR (NaCl) cm\(^{-1}\): 3351(N-H), 3014(C-H str), 2934(C-H str.), 2848(C-H), 1739 (C=O), 1643(C=O), 1445 (C-H bend) and 1350 (CH-(CH\(_3\))\(_3\) of BOC). IR spectra in Appendix-VII

Synthesis of 2-(4-(((2,4-bis(2-tert-butoxycarboneylamino)-4-carboxybutanamido)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid. (2)

2,6-Bis((tert-butoxycarbonyl)amino)hexanoic acid (1g, 3mmol) was dissolved in dimethyl formamide (10 mL). To this solution dicyclohexylcarbodiimde (DCC) (0.7g, 3mmol, 1.1 eq.) dissolved in dimethyl formamide were added for 5 min with stirring. The stirring was continue at 0ºC for 2 h and then the solution of methotrexate (MTX) (1.36g, 3 mmol, 1 eq.) in anhydrous dimethyl formamide (10 mL) was added drop wise. The resulting reaction mixture was stirred for 24 h at room temperature. Ethyl acetate (50 mL) was added to the reaction mixture and then filtered to remove the precipitated N,N-dicyclohexylurea (DCU). The clear filtrate was extracted with (3 x 20 mL) of 0.1 N HCl, (2 x 20 mL) of water, (2 x 20 mL) of brine, (3 x 20 mL) of saturated aqueous solution of sodium bicarbonate and (2 x 20 mL) of brine. The solution was then dried over anhydrous sodium sulphate and concentrated in vacuum. The residue was purified by column chromatography on silica gel using ethyl acetate/hexanes (70:30) to provide 1.34 g of compound 2

Yellow solid, yield 53%, m.p.: 149±3ºC, IR (KBr) cm\(^{-1}\): 3366(N-H), 3255(O-H), 3023(Ar C-H), 2959(C-H str), 1680 (C=O), 1641(C=O), 1536(C=N), and 1485 (C-H bend). IR spectra in Appendix-VIII
Synthesis of 2-(4-((2,4-bis(2-amino-4-carboxybutanamido)pteridin-6-yl)methyl)(methyl) amino)benzamido)pentanedioic acid.(3)

Compound 2 (1.1g, 1mmol) was dissolved in dimethyl formamide, and TFA (trifluoro acetic acid) 6 mL was added at 0°C for 5 min with continuous stirring and kept at room temp for 1.5 h. Then drying under vacuum to remove completely TFA, recrystallization was carried out using ethyl acetate/ diethyl ether mixture (1:10). The precipitate was filtered off, washed with cold diethyl ether and solvent was removed under vacuum to obtain the desire product as a light yellow solid with 0.38g yield of compound 3 (MTX-LYS).

Light Yellow solid, yield: 55%, m.p.: 173±2ºC. Elemental analysis for C$_{32}$H$_{46}$N$_{12}$O$_7$ calculated C (54.07%), H (6.52%), N (23.65%) and found C (54.05%), H (6.56%), N (23.70%). FTIR (KBr),$\nu$(cm$^{-1}$): 3446.01(O-H broad), 3310.63(N-H asymm, str.), 3276.27(N-H symm. str), 2927.34 and 2867.28(C-H str),1732.08 (C=O (OH) , 1645.28(C=O(NH)str), 1529.05(C=N), 1475.51(CH$_2$, def), 1305.26(C-H def). (Appendix-IX) $^1$H NMR(DMSO) $\delta$ (ppm): 11.08(s, 1H,OH), 10.73(s, 1H,OH), 8.58(s, 4H, NH$_2$), 8.36 (m, 4H, NH$_2$), 8.23(s, 1H, NH), 8.21 (s, 1H, NH), 8.17(s, 1H,OH), 8.13 (s, 1H, CH(Pyrazine), 6.3-7.9(m, Ar-H), 4.78(s, 2H, CH$_2$), 4.41-4.28 (t,1H,CH), 3.73-3.75 (m,1H,CH), 3.13-3.03(m,2H, CH$_2$), 2.54-2.42(t,2H,CH$_2$), 2.22-2.06(m,2H,CH$_2$), 1.94-1.83(m,2H,CH$_2$), 1.72-1.67 (m,2H, CH$_2$),1.37-1.53(m,2H,CH$_2$).  (Appendix-X) $^{13}$CNMR DMSO $\delta$(ppm) : 174.04 (-C(=O)OC), 173.81(-C(=O)OC), 177.26(-C(=O)NC), 177.39(-C(=O)NC), 166.34(-C(=O)NC), 162.64(C(=N)CN Pyrimidine), 162.34 (C(=N)CN, Pyrimidine), 154.47(C(=N)C, Pyrazine), 150.82(C(=N)NNPyrimidine), 148.86, (C(=C)NC,Pryazine), 146.14(C(=C)NC, Benzene), 128.83(C(=C)C, Benzene), 121.26 (C(=C)C,Benzene), 121.28(C(=C)C, Benzene),110.91(C(=C)C, Benzene), 57.42 (C(N)CC), 57.39(C(N)CC), 54.99(C(N)CC), 51.81(C(N)C), 42.02(C(N)CH$_2$), 40.18(C(C)CH$_2$), 39.97(C(N)H$_3$), 39.76(C(N)CH$_2$), 39.55(C(N)CH$_2$), 32.64(C(C)CH$_2$), 30.46(C(C)CH$_2$), 29.08(C(C)CH$_2$), 26.11(C(C)CH$_2$),24.1(C(C)CCH2),24.08(C(C) CCH2). (Appendix-XI) MS (ESI+) m/z (%) (M+1): Calcd 711.36., Found 711.32. (Appendix-XII)

3.2.2.2. HPLC Methods

The chromatographic separations were achieved by methods and conditions are as described in section 1.3.1.1.1.4. The solvent system comprised of phosphate buffer (10 mmol dihydrogen phosphate and 10 mmol sodium hydroxide, pH 7.4) premixed, filtered and degassed buffer in water: acetonitrile (90:10) was used as mobile phase at a flow rate of 1
mL/min. The retention time of MTX-LYS was 5.3 min at wavelength of 289nm. HPLC chromatograms were shown in Fig- 28, Fig- 30 and Fig- 32

3.2.3. Analysis of MTX-LYS

MTX-LYS was analyzed following calibration of the method (1.3.1.3.) The calibration curve of MTX-LYS in PBS (Fig- 29), Plasma (Fig- 31) and brain homogenate (Fig- 33) were linear over the range of 10-50µg/mL. The purity of the MTX-LYS was assessed by method as described in section 1.3.1.3. (Table 15)

3.2.4. Stability studies in different buffer solutions

The MTX-LYS was investigated for their chemical stability by method as described in section 1.3.1.4. (Table 16)

3.2.5. Stability in plasma extracts and brain homogenate

The stability study in plasma and brain homogenate were accessed by method as described in section 1.3.1.5. (Table 17)

3.2.6. In vitro conversion of MTX-LYS to MTX in brain homogenate

The MTX-LYS was treated in brain homogenate to determine the conversion of conjugate to its parent drug. The method was followed as described in section 1.3.1.6. The results and HPLC chromatogram was shown in Fig- 34 and Fig- 35

3.2.7. Distribution coefficients of the conjugates in 1-octanol/water

The distribution coefficient (logD) was determined by method as described in section 1.3.1.7. The MTX-LYS was analyzed by UV spectroscopic methods following verification. (Fig- 36 and Table 18)

3.2.8. Protein binding study

The plasma protein binding study was carried out following earlier reported method (section 1.3.1.8) using verified of the UV- spectroscopic method for estimation of MTX-LYS (Fig- 37 and Table 18)

3.2.9. Toxicological Studies

In-vitro and In vivo toxicological studies was performed to determine the safety margin of the MTX-LYS following earlier reported method

3.2.9.1 In vitro hemolytic toxicity study

The degree of hemolysis was determined by method as described in section 1.3.1.9.1 and result was shown in Fig- 38.
3.2.9.2 *In vivo* hematological studies

The study of hematological parameters was done by method as described in section 1.3.1.9.2 and result was shown in Table 19.

3.2.9.3 Neurotoxicity Studies

Neurotoxicity study of the conjugate was done to determine effect of MTX-LYS on CNS.

3.2.9.3.1. Motor Coordination test

Motor coordination test was performed by method as described in section 1.3.1.9.3.1 and result was shown in Table 20.

3.2.9.3.2. Assessment of locomotors activity

The locomotor activity was performed by method as described in section 1.3.1.9.3.2 and result shown in Table 21.

3.2.9.3.3. Catalepsy

Induction of catatonia was studied by method as described in section 1.3.2.9.3.3 and result shown in Table 22.

3.2.10. *In vivo* release of MTX from MTX-LYS

Release of MTX from MTX-LYS was accessed by method as described in section 1.3.1.10. The HPLC chromatogram of MTX in plasma and brain was shown in Fig- 40 and Fig- 41 respectively and result was shown in Table 23, Table 24 and Fig- 39

3.2.11. Radio labelling of MTX and MTX-LYS with $^{99m}$Tc and optimization

The radiolabeling of MTX and MTX-LYS were done by method described in section 1.3.1.11. The optimized condition and radio-labelling efficiency was shown in Table 25. The in vitro stability study of radiolabeled conjugate was evaluated and shown in Table 26. After the optimization and evaluation, stable radiolabeled MTX and MTX-LYS were used for bio-distribution study in mice.

3.2.12. Bio-distribution study and Radioscintigraphy Imaging in mice

The bio-distribution study and radioscintigraphy imaging of MTX-LYS were done by method described in section 1.3.1.12. The result of bio-distribution study was shown in Table 27. To visualize the transportation of conjugate from blood to brain scintigraphy imaging was performed shown in Fig-42.

3.2.13. Statistical analysis

Statistical analysis was done as described in section 1.3.1.13.
3.3. L- Tyrosine conjugate of Methotrexate (MTX-TYR)

Fig- 5. 2-amino-3-(4-(5-(4-(2-amino-2-carboxyethyl)phenoxy)-2-(4-((2,4-diaminopteridin-6-yl) (methyl)amino)benzamido)-5-oxopentanoyloxy)phenyl)propanoic acid (MTX-TYR)

3.3.1. Experimental
3.3.1.1. Materials and Methods
The materials and methods were as described in section 1.3.1.1.1
3.3.2. Synthesis of MTX-TYR

Scheme IV. Tyrosine conjugated of Methotrexate (MTX-TYR)

Reagents and conditions: i - L-Tyrosine, NaOH, 1,4-Dioxane, (BOC)₂O and stir for 10 h at r.t.; ii - MTX, DMF, DCC, DMAP and stir for 18 h at r.t.; iii - Dioxane, 4N HCl, stir for 6 h at r.t.
3.3.2.1. General Procedure of Synthesis

Synthesis of 2-((tert-butoxycarbonyl) amino)-3-(4-hydroxyphenyl) propanoic acid (1)

Synthesis of 2-((tert-butoxycarbonyl) amino)-3-(4-hydroxyphenyl) propanoic acid (1) was done by modification of reported procedure (Banerjee et al., 2011; Keller et al., 2003). 2-amino-3-(4-hydroxyphenyl) propanoic acid (3.62g, 20.0mmol, 1.00 equiv) was taken in a mixture of 1,4 Dioxane (40 mL), water (20 mL) and 1M NaOH soln (20 mL) and stirred with cooling in ice bath. Di-tert-butyl dicarbonate (BOC)₂O (4.63g, 21.0mmol, 1.05equiv) was added and stirring was continued for 10 h at room temp. Resulting mixture was subjected to reduce the volume under vacuum and cooled at ice bath. Residue was covered with ethyl acetate (20 mL) and acidifies with a dilute aqueous solution of KHSO₄ to pH 2-3. The aqueous phase was extracted with ethyl acetate and this operation was done repeatedly. The organic layers was washed with water and dried over anhydrous MgSO₄ and evaporated under vacuum at a bath temperature not exceeding 30°C. The crude material was obtained as a waxy solid. The crude product was treated with hexane (50 mL) and allowed to stand overnight. The mixture was diluted with hexane (50 mL), stirred vigorously for 30 min and placed in refrigerator overnight, and filtered to collect a white solid, 4.6g (82%). This compound 1 was used in the next step without further purification.

White crystalline powder, yield 92%, m.p.: 135±3°C, IR (KBr) cm⁻¹: 3339 (N-H), 3296 (O-H), 3152 (C-H), 3099(C-H str.), 1698(C=O), 1658 (C=O), 1456(C-H bend) and 1365(CH-(CH₃)₃ of BOC). (Appendix-XIII)

Synthesis of 2-(tert-butoxycarboxylamino)-3-(4-(4-(2-carboxy-2-(tert-butoxycarbonyl amino)ethyl)phenoxy)-4-(4-((2,4-diaminopteridin-6-yl)(methyl)amino)benzamido)-5-oxopeptanoyloxy) phenyl) propanoic acid (2)

MTX (1.36g, 3 mmol, 1 eq.) was dissolved in dimethyl formamide (10 mL). To this solution dicyclohexylcarbodiimde (0.7g, 3mmol, 1.1 eq.) and 4-dimethylaminopyridine (0.3g, 2.4 mmol, 0.8 equiv) dissolved in dimethyl formamide were added for 5 min with stirring. The stirrer was continue at 0°C for 2 h and then the solution of compound 1 (2.5g, 9mmol, 3 eq.) in anhydrous dimethyl formamide (10 mL) was added drop wise. The resulting reaction mixture was stirred for 18 h at room temperature. Ethyl acetate (50 mL) was added to the reaction mixture and then filtered to remove the precipitated N,N-dicyclohexylurea (DCU). The clear filtrate was extracted with (3 x 20 mL) of 0.1 N HCl, (2 x 20 mL) of water, (2 x 20 mL) of brine, (3 x 20 mL) of saturated aqueous solution of sodium bicarbonate and (2 x 20 mL) of brine. The solution was then dried over anhydrous sodium sulphate and concentrated.
in vacuum. The residue was purified by column chromatography on silica gel using ethyl acetate/hexanes (70:30) to provide 1.48 g (51%) of compound 2.

Yellow solid, yield 53%, m.p.: 278±3°C, IR (KBr) cm⁻¹: 3310(N-H), 3060 (Ar C-H str), 2952 (C-H str), 1703 (C=O), 1643 (C=O), 1530(C=N) and 1445 (C-H bend). (Appendix-XIV)

**Synthesis of 2-amino-3-(4-(5-(4-(2-amino-2-carboxyethyl)phenoxy)-2-(4-((2,4-diaminopteridin-6-yl)(methyl)amino)benzamido)-5-oxopentanoyloxy) phenyl) propanoic acid (3)**

A solution of 2-(tert-butoxycarbonylamino)-3-(4-(5-(4-(2-carboxy-2-(tert-butoxy carbonylamino)ethyl)phenoxy)-4-(4-((2,4-diaminopteridin-6-yl)(methyl)amino)benzamido)-5-oxopentanoyloxy) phenyl)propanoic acid (1g, 1 mmol) in dioxane (20 mL) was treated with a solution of 4N HCl in dioxane (2.5 mL in 10 mL), stirred for 6 h and stored overnight at room temp. The precipitate was filtered off, washed with cold diethyl ether and evaporated under vacuum to obtain the desire product as a dry yellow solid in 0.50g (63%) yield.

Yellow solid, m.p.: 257±3°C. Elemental Analysis, Calcd, C (58.45%), H (5.16%), N (17.94%) and found C (57.92%), H (5.11%), N (17.86%). FTIR (KBr): cm⁻¹: 3580.66(O-H), 3497.18(N-H asymm. str.), 3210.29(N-H sym. str.), 1707.86(C=O str.), 1687.35(C=O str.), 1639.49(C=N str.), 1293.45(C-O str.), 1147.85(C-O str.). (Appendix-XV) ¹H NMR(DMSO): δ 11.792(s, 1H, (C=O)OH), 11.132(s, 1H, (C=O)OH), 8.576(s, 4H, 2NH₂), 8.165(s, 2H, NH₂), 7.952(s, 1H, NH), 7.563(s, 2H, NH₂), 7.083(s, 2H, NH₂), 7.744-7.828(m, 4H, Ar-H), 6.786-6.901(m, 4H, Ar-H), 5.138(s, 2H, CH₂), 4.799(s, 3H, CH₃), 4.447-4.393(m, 1H, CH), 2.240-2.079(q, 2H, CH₂), 1.879-1.742(t, 2H, CH₂), 1.651-1.716(t, 2H, CH₂). (Appendix-XVI)

¹³C NMR(DMSO): δ 174.28(COOH), 174.14(COOH), 171.67(C=O), 171.34(C=O), 166.37(Ar-C(=O)), 162.65(C, Pyrimidine), 161.91(C, Pyrimidine), 153.23(C, Pyrazine), 151.47(C, 1-Benzene), 151.29(C, 1-Benzene), 150.8(C, Pyrazine), 148.88(C, Pyrazine), 146.66(C, Pyrazine), 129.13(C, Benzene)128.89(C, Benzene), 128.63(C, Benzene), 121.94(C, 1-Benzene), 121.70(C, Benzene), 121.47(C, Benzene), 121.23(C, Benzene), 111.19(C, Benzene), 111.06(C, Benzene), 110.94(C, Benzene), 54.92(C=C=O), 51.98(C=NH₂), 51.74(C=OH), 51.49(C=O), 39.20(N-Methyl), 30.55(C=Ar-R), 27.34(C=Ar-R), 27.16(C=CH₂), 26.23(C=CH₃). (Appendix-XVII) MS (ESI+) for C₃₈H₄₀N₁₀O₉ (M+1): 781.30. (Appendix-XVIII)
3.3.2.2. HPLC Methods

The chromatographic separations were achieved by methods described in section 1.3.1.1.1.4. The solvent system was comprised of phosphate buffer (10 mmol dihydrogen phosphate and 10 mmol sodium hydroxide, pH 7.4) premixed, filtered and degassed buffer in water: methanol: acetonitrile (70:20:10) was used as mobile phase at a flow rate of 1mL/min. The retention time of MTX-TYR was found to be at 6.1 min at wavelength of 349 nm. HPLC chromatograms for PBS, plasma and brain homogenates were shown in Fig-43, Fig-45 and Fig-47 respectively.

3.3.3. Analysis of MTX-TYR

The calibration curve of MTX-TYR in PBS, Plasma and brain homogenate methods were linear over the range of 10-50µg/mL and shown in Fig-44, Fig-46 and Fig-48 respectively. The purity of the MTX-TYR was assessed by method as described in section 1.3.1.3 and result was shown in Table 28.

3.3.4. Chemical and enzymatic stability study of MTX-TYR

3.3.4.1. Stability studies in different buffer solutions

The MTX-TYR was investigated for their chemical stability followed by method described in section 1.3.1.4. The result was shown in Table 29.

3.3.4.2. Esterase activity of Plasma and brain homogenate

Carboxyl esterase activity was determined following reported method (Karanth et al., 2000; Clement et al., 1990) with p-nitro phenyl acetate as substrate. Blood collected from the trunk into heparinized tubes were centrifuged for 2minutes. Plasma was removed and stored at -70°C until assay. Brain homogenates were prepared in ice-cold Tris-HCL buffer (0.1M, pH 7.8 at 25°C with 1% Triton-X-100) in a tissue homogenizer. The homogenates were centrifuged for 20 minutes at 4°C and the supernatant was used for the assay. The tissue samples were added to buffer (0.1M Tris-HCL, pH 7.8 at 25°C containing 2 mm EDTA) and volume adjusted to 990 µl. Samples were pre incubated at 37°C for 10minutes and the reaction was started by adding 10µl of 50 mM stock p-nitro phenyl acetate solution in acetone (Conc. = 0.5 mM). Change in absorbance at 405 nm was recorded after 5 min against a reagent blank containing only substrate. Carboxyl esterase activity was calculated using p-nitro phenol standard curve and expressed as nmol/min/ml plasma or mg of homogenate. The result was shown in Table 30.
3.3.4.3. Stability in plasma extracts and brain homogenate

The stability study in plasma and brain homogenate was accessed by method described in section 1.3.1.5 and result was shown in Table 31.

3.3.5. In vitro conversion of MTX-TYR to MTX in brain homogenate

The MTX-TYR was treated with brain homogenate to determine the in vitro conversion of conjugate to MTX. The concentration of MTX was monitored by HPLC and method was followed as described in section 1.3.1.6. The HPLC chromatogram was shown in Fig- 50 and result was shown in Fig- 49

3.3.6. Distribution coefficients of the MTX-TYR in 1-octanol/PBS 7.4

The distribution coefficient (logD) was determined by method described in section 1.3.1.7. The calibration curve for MTX-TYR in PBS 7.4 by UV spectroscopy was shown in Fig- 51 and result of distribution coefficient was shown in Table 32.

3.3.7. Toxicological Studies

In-vitro and In vivo toxicological studies was performed to determine the safety margin of the MTX-TYR.

3.3.7.1 In vitro hemolytic toxicity study

The degree of hemolysis was determined by method as described in section 1.3.1.9.1 and result was shown in Fig- 52.

3.3.7.2 In vivo hematological studies

The study of hematological parameters was done by method described in section 1.3.1.9.2. The result was shown in Table 33.

3.3.7.3. Neurotoxicity Studies

Neurotoxicity study of the conjugate was done to determine safety margin of the conjugates.

3.3.7.3.1. Motor Coordination test

Motor coordination test was performed by method described in section 1.3.1.9.3.1 and result was shown in Table 34.

3.3.7.3.2. Assessment of locomotors activity

The locomotor activity was performed to determine the CNS effect of MTX-TYR by method described in section 1.3.1.9.3.2 and result was shown in Table 35.
3.3.7.3.3. Catalepsy

Induction of catatonia by MTX-TYR was studied by method described in section 1.3.1.9.3.3 and result was shown in Table 36.

3.3.8. In vivo release of MTX from MTX-TYR

Release of MTX after administration of MTX-TYR was accessed by method described in section 1.3.1.10. The concentration of MTX was observed by HPLC chromatogram shown in Fig- 54 and Fig- 55. The result was shown in Table 37, Table 38 and Fig- 53.

3.3.9. Radio labelling of MTX and MTX-TYR with $^{99m}$Tc and optimization

The radiolabeling of MTX and MTX- TYR were performed by method described in section 1.3.1.11. The optimized reaction condition and result of in-vitro stability study was shown in Table 39 and Table 40 respectively.

3.3.10. Bio-distribution study and Radioscintigraphy Imaging in mice

The bio-distribution study and radioscintigraphy imaging of MTX-TYR were done by method described in section 1.3.1.12. The result of relative tissues distribution of MTX-TYR was shown in Table 41 and images of scintigraphy was given in Fig- 56.

3.3.11. Statistical analysis

Statistical analysis was done as described in section 1.3.1.13.
3.4. L-Phenylalanine and L-Leucine conjugates of Methotrexate

Fig-6.  
3a. 2-(4-(((2,4-bis(2-amino-3-phenylpropanamido)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (MTX-PAL). R= C6H5

3b. 2-(4-(((2,4-bis(2-amino-4-methylpentanamido)pteridin-6-yl)methyl) (methyl)amino)benzamido)pentanedioic acid (MTX-LEU). R= -CH (CH₃)₂

3.4.1. Experimental

3.4.1.1. Materials and Methods
The materials and methods were as described in section 1.3.1.1.1
3.4.2. Synthesis of L-Phenylalanine and L-Leucine conjugates of Methotrexate

**Scheme V.** L-Phenylalanine and L-Leucine amide conjugated Methotrexate

Reagents and conditions for 3a (MTX-PAL) i: L-Phenylalanine, NaOH, Tert-butyl alcohol, (BOC)$_2$O, stir for 18 h at r.t. ii: MTX, DMF, DCC,MTX, stir for 24 h at r.t. iii: 4 N HCl, 1,4 Dioxane, stir for 6 h. at r.t.  
Reagents and conditions for 3b (MTX-LEU) i: L-leucine, NaOH, 1,4-dioxane, (BOC)$_2$O, stir for 18 h at r.t. ii: MTX, DMF, DMAP, DCC, stir for 24 h at r.t. iii: TFA, DMF, stir for 1.5h. at r.t.

3.4.2.1. General Procedure for Synthesis of compound 3a (MTX-PAL)
Synthesis of 2-(tert-butoxycarbonylamino)-3-phenylpropanoic acid (1a)

Synthesis of 2-(tert-butoxycarbonylamino)-3-phenylpropanoic acid (1a) was done by reported procedure (Keller et al., 2003). 2-amino-3-phenylpropanoic acid (2g, 12.7 mmol) was taken in a mixture of (0.4g, 10 mmol) NaOH soln (20 mL), water (20 mL) tert-butyl alcohol (20 mL), and stirred with cooling in ice bath. Di-tert-butyl dicarbonate (BOC)₂O (3.27g, 15 mmol, 1.18equiv) was added and stirring was continued for 18 h. at room temp. Resulting mixture was subjected to reduce the volume under vacuum and cooled at ice bath. Residue was covered with ethyl acetate (20 mL) and acidifies with a dilute aqueous solution of KHSO₄ to pH 1-1.5. The turbid reaction mixture was extracted with (30 mL x 3) of ethyl ether and this operation was done repeatedly. The organic layers was washed with water (20 mL x 2) and dried over anhydrous MgSO₄ and evaporated under vacuum at a bath temperature not exceeding 30°C. The yellowish oily residue was treated with hexane (50 mL) and allowed to stand overnight. The mixture was diluted with hexane (50 mL), stirred vigorously for 30 min and placed in refrigerator overnight, and filtered to collect a pure white solid, 5.1g (80%). This compound 1a was used in the next step without further purification.

White crystalline powder, yield 80%, m.p.: 88±2°C, IR (KBr) cm⁻¹: 3342 (N-H), 3212 (O-H), 3194 (C-H), 3068 (C-H str.), 2996 (C-H str.), 2931(C-H str.), 1715 (C=O), 1683 (C=O), 1436 (C-H bend) and 1309 (CH-(CH₃)₃ of BOC).(Appendix-XIX)

Synthesis of 2-(4-(((2,4-bis(2-(tert-butoxycarbonylamino)-3-phenylpropanamido)pteridin-6-yl) methyl) (methyl amino) benzamido) pentanedioic acid (2a)

2-(tert-butoxycarbonylamino)-3-phenylpropanoic acid (1g, 3.7 mmol) was dissolved in dimethyl formamide (10 mL). To this solution dicyclohexylcarbodiimde (0.82g, 4.0 mmol, 1.1 eq.) dissolved in dimethyl formamide were added for 5 min with stirring, The stirring was continue at 0°C for 2 h and then the solution of methotrexate (MTX) (1.4g, 3 mmol, 1 eq.) in anhydrous dimethyl formamide (10 mL) was added drop wise. The resulting reaction mixture was stirred for 24 h at room temperature. Ethyl acetate (50 mL) was added to the reaction mixture and then filtered to remove the precipitated N, N-dicyclohexyleurea (DCU). The clear filtrate was extracted with (3 x 20 mL) of 0.1 N HCl, (2 x 20 mL) of water, (2 x 20 mL) of brine, (3 x 20 mL) of saturated aqueous solution of sodium bicarbonate and (2 x 20 mL) of brine. The solution was then dried over anhydrous sodium sulphate and concentrated in vacuum. The residue was purified by column chromatography on silica gel using CH₃CN: CH₃OH (70:30) to provide 1.49 g (51.3%) of compound 2a.
Yellow solid, yield 53%, m.p.: 157±3ºC, IR (KBr) cm⁻¹: 3390 (N-H), 3292 (O-H), 3022 (Ar C-H), 2945 (C-H str.), 2854 (C-H str.), 1701(C=O), 1697 (C=O), 1547 (C=N) and 1446 (C=H bend). (Appendix-XX)

**Synthesis of 2-(4-(((2,4-bis(2-amino-3-phenylpropanamido)pteridin-6-yl)methyl)(methyl) amino)benzamido)pentanedioic acid (3a)**

The compound 2 (0.95g, 1 mmol) in dioxane (20 mL) was treated with 4N HCl in dioxane (2.5 mL in 10 mL), stirred for 6 h at RT and stored overnight at 4°C. The precipitate was filtered off, washed with cold diethyl ether and removed the solvent in vacuum to obtain the desire product as a light yellow solid. Triturate the crude product in ethyl acetate (10 ml) and purified in column chromatography using ethyl acetate: Methanol: water (1:2:1) as mobile phase to get 3a as yellow solid with 0.56 g yield (76%).

Light Yellow solid, Melting point 180±3ºC. Analysis for C₃₈H₄₀N₁₀O₇ (748.787); calculated: C 60.95 %; H 5.38%; N 18.71%; found: C 60.12 %; H 5.34%; N 18.17%. FTIR (KBr): cm⁻¹3363.20(N-H asymm. str.), 3250.56(N-H sym. Str.), 1751.36(C=O .str.), 1683.86(C=N str.). (Appendix-XXI)

**1H NMR (DMSO):** δ 8.56(s,2H,NH₂), 6.71-7.76(m,5H,Ar-H), 8.15(s, 1H, NH), 8.135(s, 1H, NH), 6.67-6.816(m,5H,Ar-H), 4.366-4.421 (m,1H, CH), 4.7895(d,2H, CH₂), 3.220(s, 3H, CH₃), 2.521-2.530(t, 2H, CH₂), 2.054-2.127(h, 2H, CH₂), 2.320-2.357(t, 1H, CH). (Appendix-XXII)

**13C NMR (DMSO):** δ 174.25 ( carbonyl -acid), 174.01 ( carbonyl -acid), 166.34 ( carbonyl -amide), 162.64 ( carbonyl -amide), 162.02 ( carbonyl -amide), 153.93 (imine-cyclic), 150.8 (imine-cyclic), 148.83 (imine-cyclic), 146.47, 110.93-128.80(Aromatic), 54.99(methanetriyl), 51.98(methanetriyl), 40.13(N-methyl), 39.93(methanediyli), 39.72 (methanediyli), 30.56(methanediyli), 26.28(methanediyli). C: 60.95, H: 5.38,N: 18.71. (Appendix-XXIII) MS (ESI+) for C₃₈H₄₀N₁₀O₇ (M+1): Calcd 749.79, Found 749.6. (Appendix-XXIV)

### 3.4.2.2. General Procedure for Synthesis of compound 3b (MTX-LEU)

**Synthesis of 2-(tert-butoxycarbonylamo)-4-methylpentanoic acid. (1b)**

Synthesis of 2-(tert-butoxycarbonylamino)-4-methylpentanoic acid was done by reported method with little modification (Laulloo, et al., 2007; Martin et al., 2002). 2-amino-4-methylpentanoic acid (2.6g, 20 mmol, 1.00 equiv) was added to a stirred solution of NaOH (1g, 25 mmol, 1.25 equiv) in 20 mL of water at ambient temperature and then diluted with 20 mL of 1,4-dioxane and Di-tert-butyl dicarbonate (BOC)₂O (4.5g, 20 mmol, 1.00 equiv) in dioxane was added drop wise. The reaction was brought to completion by further stirring for 18 h at room temperature. The solution was diluted with 50 mL of water and concentrated
under reduced pressure. This mixture was cooled in an ice bath and covered with a layer of ethyl acetate (10 mL). The reaction mixture was extracted with (20 mL x 2) of ethyl acetate, and the organic phase was washed with (30 mL x 3) of saturated aqueous sodium bicarbonate solution. The combined aqueous layers were acidified to pH 2 by addition of potassium hydrogen sulfate solution. The mixture was extracted with (30 mL x 2) of ethyl acetate. The organic layer was dried over MgSO₄, filtered, and evaporated under rotary evaporator. Hexane was added to the thick oil to turbidity. Crystallization occurs after cooling and stirring the mixture for a short time. The mixture is allowed to stand for 1 h. The white crystalline solid is collected by filtration, washed with (10 mL x 3) portions of hexane, and dried under reduced pressure to get Compound 1b with yield of 3.5g (75%).

White crystalline powder, yield 80%, m.p.: 88±2°C, IR (KBr) cm⁻¹: 3336 (N-H), 2961(C-H str.), 2870 (C-H str.), 1716 (C=O), 1674 (C=O), 1469 (C-H bend) and 1366 (CH-(CH₃)₃ of BOC). (Appendix-XXV)

**Synthesis of 2-(4-(((2,4-bis(2-(tert-butoxycarbonylamino)-4-methylpentanamido)pteridin-6-yl) methyl (methyl)amino)benzamido)pentanedioic acid (2b)**

Methotrexate (0.9g, 2 mmol, 1 equiv) was dissolved in 10 mL of dry dimethylformamide (DMF) and (0.2g, 1.6 mmol, 0.8 equiv) of 4-dimethylaminopyridine was added and stirred with cooling in an ice bath to 0°C. 2-(tert-butoxycarbonylamino)-4-methylpentanoic acid 1b (1.35g, 6 mmol, 3 equiv) was dissolved in 10 mL of DMF and (0.5g, 2.2 mmol, 1.1 equiv) of dicyclohexylcarbodiimide dissolved in DMF was added over a 5-min period. This mixture was stirred at 0°C for 2 h and ice cooled reaction mixture of MTX was added drop wise with stirring. The resulting reaction mixture was stirred for 24 h at room temperature. The precipitated was removed by filtration and the filtrate was concentrated under vacuum and taken up in ethyl acetate and washed with 0.1 N HCl, brine (3 x 20 mL) and 1M sodium carbonate (3 x 20 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuum. A viscous yellow crude product was obtained and recrystallized with mixture of ethyl acetate and hexane (10:1). Further purification of crude product was done by column chromatography using ethyl acetate: acetonitrile: methanol (1:1:2) as mobile phase to afford in 1.0g (53%) of compound 2b.

White crystalline powder, yield 80%, m.p.: 168±2°C, IR (KBr) cm⁻¹: 3367 (N-H), 3200 (O-H), 3127 (Ar C-H), 2965 (C-H), 2856 (C-H), 1697 (C=O), 1681 (C=O), 1541 (C=N) and 1456(C=H bend). (Appendix-XXVI)
**Synthesis of 2-(4-((2,4-bis(2-amino-4-methylpentanamido)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (3b)**

Compound 2 (1mmol) dissolved dimethyl formamide and trifluoroacetic acid (TFA, 4.5 mL) was added drop wise in 5 mL dimethyl formamide at 0°C in an ice bath. The reaction was left to stir for 1.5 h then the reaction was saturated with diethyl ether until precipitate formed. The precipitate was decanted off, wash twice with diethyl ether then dried over anhydrous sodium sulfate and evaporated under vacuum to obtain the desire product as a dry light brownish solid. It was triturated in ethyl acetate (10 mL) and purified in column chromatography using dichloromethane: methanol: water (1:2:1) as mobile phase. The compound was recrystallized to get yellow solid in 0.55g (80%) yield 3b.

Light yellow powder, melting point 132±3 ºC, Elemental Analysis, Calcd, C (56.46%), H (6.51%), N (20.58%) and found C (56.62%), H (6.54%), N (20.47%)

**FTIR (KBr): cm\(^{-1}\)** FTIR (KBr),\(\nu(\text{cm}^{-1})\): 3441.01(O-H broad), 3315.63(N-H asymm,str.), 3217.27(N-H symm. str), 1732.08(C=O(OH) ,1645.28 (C=O (NH)str), 1529.20(C=N) (Appendix-XXVII)

**\(^1\)H NMR(DMSO):\(\delta\)** 11.13-10.89 (br s 1H,Carboxylic acid), 7.72(br s 1H,NH\(_2\)), 7.70 (br s 1H,NH\(_2\)), 6.60 (br s 2H,NH\(_2\)), 8.56(s,1H,N=CH), 8.22-8.25(d,1H, NH of amide with leucine), 8.14-8.16(d,1H, NH of amide with leucine), 8.04(d,1H, NH of amide in MTX), 6.79(d,2H, Ar-H), 7.25(d,2H, Ar-H),4.78 (s,2H,CH\(_2\)), 4.2 (m,1H,CH) (Appendix-XXVIII)

**\(^13\)C NMR(DMSO):\(\delta\)** 162.65,162.74, 155.21,148.93,146.01, and 121.23(5C-Heterocyclicring), 54.96(s,1C,CH\(_3\)), 39(1C,CH\(_3\)), 151.85, 128.85, 121.46, 110.93(4C,Ar-C), 166.33(1C,CONHMTX), 51.76(1C,CH), 173.72,173.95 (2C,COOH), 171.63,171.47 (2C,CONH, amide with Leucine), 56.18,56.48(2C,CH-NH\(_2\)), 32.93-19.06(C, Alkyl) (Appendix-XXIX) MS (ESI+) for C\(_{32}\)H\(_{44}\)N\(_{10}\)O\(_{7}\) (M+1): Calcd 681.34., Found 681.62. (Appendix-XXX)

**3.4.2.3. HPLC Methods**

The chromatographic separations were achieved by method described in section 1.3.1.1.4 The solvent system was comprised of phosphate buffer (10 mmol dihydrogen phosphate and 10 mmol sodium hydroxide, pH 7.4) premixed, filtered and degassed buffer in water: methanol: acetonitrile (70:20:10) was used as mobile phase with flow rate of 1mL/min. The retention time of MTX-PAL and MTX-LEU was found to be at 6.7 min and 6.0 min at wavelength of 282 nm and 323 nm respectively. The HPLC chromatograms of MTX-PAL in PBS 7.4, plasma and brain homogenates were shown in Fig- 57, Fig- 59 and Fig- 61 and chromatograms of MTX-LEU were shown in Fig- 63, Fig- 65 and Fig- 67 respectively.
3.4.3. Analysis of MTX-PAL and MTX-LEU

The calibration curves of MTX-PAL and MTX-LEU in PBS (Fig- 58 and Fig- 64), Plasma (Fig- 60 and Fig- 66) and brain homogenate (Fig- 62 and Fig- 68) were linear over the range of 10-50 µg/mL. The purity of the conjugates was assessed by method as described in section 1.3.1.3 and results were shown in Table 42 and Table 43

3.4.4. Stability studies in different buffer solutions

The MTX-PAL and MTX-LEU were investigated for their chemical stability by method described in section 1.3.1.4 and results were shown in Table 44

3.4.5. Stability in plasma extracts and brain homogenate

The stability study of MTX-PAL and MTX-LEU in plasma and brain homogenate was accessed by method described in section 1.3.1.5 and results were shown in Table 45.

3.4.6. In vitro conversion of MTX-PAL and MTX-LEU to MTX in brain homogenate

The MTX-PAL and MTX-LEU were treated in brain homogenate to determine the conversion of conjugate to its parent drug and monitored by HPLC. The method was followed as described in section 1.3.1.6. The result and chromatograms was shown in Fig- 69, Fig- 70 and Fig- 71 respectively.

3.4.7. Distribution coefficients of MTX-PAL and MTX-LEU in 1-octanol/PBS 7.4

The distribution coefficient (logD) was determined by method as described in section 1.3.1.7. The calibration curve for MTX-PAL and MTX-LEU in PBS 7.4 by UV spectroscopy was verified and shown in Fig- 72 and Fig- 73. The result was shown in Table 46

3.4.8. Protein binding study

The plasma protein binding was estimated by method described in section 1.3.1.8. The calibration curve for MTX-PAL and MTX-LEU in goat plasma was shown in Fig- 74 and Fig- 75 respectively. The result was shown in Table 46

3.4.9. Toxicological Studies

In-vitro and In vivo toxicological studies was performed to access the safety margin of the MTX-PAL and MTX-LEU.

3.4.9.1 In vitro hemolytic toxicity study

To determine the degree of hemolysis by MTX-PAL and MTX-LEU method followed as described in section 1.3.1.9.1 and result was show in Fig- 76.
3.4.9.2 *In vivo* hematological studies

The effect of MTX-PAL and MTX-LEU on hematological parameters was study by method described in section 1.3.1.9.2 and result was shown in Table 47.

3.4.9.3. Neurotoxicity Studies

Neurotoxicity of the conjugates was studied to assess the safety margin.

3.4.9.3.1. Motor Coordination test

Motor coordination test were performed by rotarod test model as described in section 1.3.1.9.3.1 and results were shown in Table 48.

3.4.9.3.2. Assessment of locomotors activity

The locomotors activity was performed by method as described in section 1.3.1.9.3.2 and results were shown in Table 49.

3.4.9.3.3. Catalepsy

Catatonia Induce by MTX-PAL and MTX-LEU were studied by bar test method as described in section 1.3.1.9.3.3 and results were shown in Table 50.

3.4.10. *In vivo* release of MTX from MTX-PAL and MTX-LEU

Release of MTX from MTX-PAL and MTX-LEU was accessed by method as described in section 1.3.1.10. The concentration of MTX released by MTX-PAL and MTX-LEU were measured by HPLC chromatogram shown in Fig- 78, Fig- 79 and Fig- 80, Fig- 81 respectively. The results were shown in Table 51, Table 52 and Fig- 77.

3.4.11. Radio labelling of MTX and MTX-PAL and MTX-LEU with 99mTc and optimization

The radiolabelling optimization and in-vitro stability studies of MTX-PAL and MTX-LEU were performed by method described in section 1.3.1.11 and results were shown in Table 53 and Table 54 respectively.

3.4.12. Bio-distribution study and Radioscintigraphy Imaging in mice

The bio-distribution study and radioscintigraphy imaging of MTX-PAL and MTX-LEU were done by method mentioned in section 1.3.1.12. The results of bio-distribution study were shown in Table 55. Images of scintigraphy in mice for MTX-PAL and MTX-LEU were given in Fig- 82 and Fig- 83 respectively.

3.4.13. Statistical analysis

Statistical analysis was done as described in section 1.3.1.13.