REVIEW OF LITERATURE
2.1 **Pathophysiology CNS metastases**

Incidences of secondary brain tumour or metastatic brain tumour are high compared to primary brain tumour. As brain receives more than 15 to 20% of blood supply, cancer cells in systemic circulation reach the brain tissue and proliferate to become a brain tumour (Scheme I) (Gavrilovic and Posner, 2005). However tumour metastasis varies with tumour type and the site of brain metastasis is influenced by the site of primary tumour (Delattre et al., 1988). Besides at each step tumour cells may fail leading to a probability of 0.01% brain metastasis (Luzzi et al., 1998) Distribution of brain metastasis is governed by the size of region and degree of vasculature. Accordingly 85% occur in cerebral hemispheres, 10 to 15% occur in cerebellum and only about 3% occur in brain stem (Lisa et al., 2008)

Malignant neoplasm arises in an organ distant from the central nervous system

↓

Develops its own vascular supply

↓

Clone(s) of malignant cells with metastatic potential enter blood or lymph channels and eventually reach the venous circulation

↓

The malignant cells enter the right heart with the venous circulation and either exit through the pulmonary artery to the lung or cross a patent foramen ovale to enter the systemic circulation

↓

From systemic circulation it enters cerebral circulation

↓

Tumour cells entering the cerebral circulation arrest in brain capillaries or venules, cross the vessel wall, and grow within the brain

**Scheme I.** Pathophysiology CNS metastases
2.2. BBB in primary and metastatic brain tumours

The integrity of BBB is suggested to be compromised in case of brain tumour (Wiranowska et al., 1992). A number of alterations in the brain endothelial cells have been described including change in tight junction structure, swelling of perivascular space, irregular basal lamina with increased fenestration and pinocytic activity (Liebner et al., 2000; Shibata S 1989). However these alterations may be a sign of tissue of tumour origin rather than normal brain endothelial cells. Besides altered BBB has been reported to only marginally increase the vascular permeability of drugs across it (Groothuis et al., 1982; Blasberg et al., 1990). This also depends on type of tumour and stage of tumour development (Bart et al., 2000; Fidler et al., 2002) In a recent study Ngoc et al., 2013 examined integrity of BBB in a mouse brain tumour model at various stages of tumour development and suggested that the BBB remains intact throughout the early and intermediate stages of tumour development with BBB permeability increasing only during the final stages of tumour progression. These studies indicate that BBB is still an obstacle and require novel approaches to improve delivery of chemotherapeutic in the brain.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Chemical structure</th>
<th>Mode of crossing BBB</th>
<th>Mode of action</th>
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<tr>
<td>Carmustine</td>
<td><img src="image" alt="Carmustine_structure" /></td>
<td>High lipid solubility</td>
<td>Cross-links in DNA and RNA</td>
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<tr>
<td>Lomustine</td>
<td><img src="image" alt="Lomustine_structure" /></td>
<td>High lipid solubility</td>
<td>Alkylation and cross-linking of DNA and RNA.</td>
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<tr>
<td>Procarbazine</td>
<td><img src="image" alt="Procarbazine_structure" /></td>
<td>-</td>
<td>Inhibit transfer of methyl groups of methionine into t-RNA.</td>
</tr>
<tr>
<td>Temozolamide</td>
<td><img src="image" alt="Temozolamide_structure" /></td>
<td>-</td>
<td>Alkylation agent used for treatment glioblastoma multiforme</td>
</tr>
</tbody>
</table>
Topotecan

Binds to the topoisomerase I-DNA complex

Cisplatin

Poor permeability

Crosslinking of DNA leading to apoptosis.

Cyclophosphamide

Poor permeability

Alkylation of DNA bases

Etoposide

Poor permeability

Inhibits DNA topoisomerase II

Daunorubicin

Poor permeability

Intercalation of DNA

Doxorubicin

Poor permeability

Complexation of DNA and inhibition of topoisomerase II.
2.3. Methotrexate

Methotrexate (MTX) is a folic acid analogue commonly used in cancer chemotherapy. It inhibits dihydrofolate reductase (DHFR), an enzyme which reduces dihydrofolate to tetrahydrofolate. Tetrahydrofolic acids are utilized for the synthesis of purine nucleotides and thymidylate. Therefore, MTX interferes with DNA synthesis and cellular replication. (Rajagopalan, 2002). Generally, neoplastic cells and epithelial cells are most sensitive to this effect of MTX because the rate of proliferation is much higher than normal cells.

Oral absorption of MTX is dose dependent and highly variable whereas following intramuscular administration absorption is achieved rapidly and completely. Peak serum levels are generally achieved within 1-2 hours or 0.5-2 h after oral or intramuscular administration respectively. At doses of 30 mg/m² or less, MTX is generally well absorbed with a mean bioavailability of about 60%. Absorption of doses greater than 80 mg/m² is significantly less probably due to saturation effect (Thomson PDR 2004). After intravenous administration, plasma proteins binding of MTX is approximately 50%. Highest concentrations are found in the kidneys, gall bladder, spleen, liver, and skin. MTX does not cross the blood-brain barrier and enter CSF in therapeutically insignificant amount when given orally or parenterally.

MTX undergoes hepatic and intracellular metabolism and converts into polyglutamated conjugates which may convert back to MTX by hydroxylase enzymes. It may convert into 7-hydroxy derivatives in small amount of the administered dose. MTX may be partly metabolised into pharmacologically inactive metabolite 2,4-diamino-N10-methylpteroic acid by intestinal flora (Thomson PDR 2004; McEvoy et al., 2005). Plasma clearance of MTX is triphasic: first phase involves distribution into organs, the second renal excretion and third phase is passage into the enterohepatic circulation. However renal excretion is primary route of elimination and it is depend on dose and route of administration.
After IV administration, the terminal half-life is in the range from 0.7 to 5.8 hours or 0.9 to 2.3 hours, 80% to 90% of the administered dose is excreted unchanged in the urine within 24 hours.

MTX is used in the treatment of gestational choriocarcinoma, acute lymphocytic leukemia, meningeal leukemia, lymphosarcoma. In high dose therapy, methotrexate is used alone or in combination with other anticancer agents in the treatment of breast cancer, epidermoid cancers, lung cancer and non-Hodgkin’s lymphomas. Because of its inability to cross BBB it is not used against any form of brain tumour.

Chemically, MTX is N-[4-[(2,4-diamino-6-pteridinyl)ethyl]methylamino] benzoyl]-L-glutamic acid (Fig-2). It has partition co-efficient (logp) value of -1.85 and 50% bound to protein, primarily with albumin. These physicochemical properties suggest its polar nature which prevents it from crossing BBB. Although it is believed to be transported by reduced folate carrier, limited capacity of this transporter is unable to ensure enough bioavailability for any therapeutic utility against brain tumour. So far less is detectable in the brain tissue than in the serum. Thus, MTX is administered in high doses, up to 8 g/m², in order to achieve therapeutic drug concentrations in brain. Very high (1–8 g/m²) doses may enhance brain availability, but being a folate antagonist at these dose levels, it becomes too toxic for any practical use as it suffers from toxicities including cause bone marrow depression, ulcerative colitis, and hematologic toxicity such as, hematopoiesis aplastic anemia, pancytopenia, leukopenia, neutropenia, thrombocytopenia and CNS toxicity (Batchelor et al.,2003; DeAngelis et al.,2002; O’Brien et al.,2000; Holmboe et al.,2012; Widemann and Adamson 2006). So, this is a major challenge to enhance brain availability of MTX to increase its therapeutic utility.

2.4. Strategy for enhancing application of methotrexate against brain tumour

2.4.1 Blood Brain Barrier disruption (BBBD)

2.4.1.1 Magnetic resonance imaging (MRI)-guided focused ultrasound (FUS) BBBD

Mei et al., (2009) adopted BBBD approach to deliver MTX to brain. In this rabbits were sonicated at the optimal exposure time after intravenous injection of MTX. The MTX concentration in the sonicated group was found to be higher the control group. It was found that magnetic resonance imaging–guided focused ultrasound can disrupt the BBB reversibly and deliver IV administered MTX to targeted brain locations to achieve greater than 10-fold increase in the drug level.
2.4.1.2 Transient reversible BBB disruption with intracarotid mannitol infusion

Neuwelt et al., (1979) described transient reversible BBB disruption with intracarotid hyperosmolar mannitol infusion. Studies were performed in canine model which showed that osmotic BBB disruption before chemotherapy (methotrexate) resulted in markedly elevated levels of drug in the ipsilateral cerebral hemisphere. After understanding the feasibility of BBB disruption, Neuwelt and co-worker (1982) further investigated the effect of BBB disruption to elevate the concentration of MTX in brain. However, this time study was performed to investigate the effect of adrenal cortical steroids and osmotic blood-brain barrier modification on methotrexate delivery to normal and glioma-bearing rats. The concentration of MTX was found to be more in tumour-bearing hemisphere following osmotic blood-brain barrier modification. However the level was less when steroids were used for barrier modification.

2.4.1.3 Intracarotid administration of alkylglycerols for BBBBD

The BBB disruption by intracarotid administration of alkylglycerols has been a novel strategy for increased delivery of chemotherapeutic drugs to the normal brain and brain tumours in rats (Erdlenbruch et al., 2003b). Effectiveness of pentyl- and hexyl glycerol derivatives have been elucidated in vivo by analyzing the transfer of methotrexate (MTX) across the blood–brain barrier (BBB) in normal rats. The effects were compared with BBB disruption using hypertonic mannitol or intracarotid infusion of bradykinin. Apart from 1-O-pentyldiglycerol, all alkylglycerols induced a concentration-dependent increase in MTX delivery to the brain varying from 1.1 to more than 300-fold compared to intra-arterial MTX alone. Although BBB disruption with different approaches, such as radiation, mannitol, bradykinin and intracarotid administration of alkyl glycerol allows higher concentration of MTX in brain, it also predisposes brain to other toxic agents circulating in body and hence may enhance toxicity. Besides this approach requires skillful administration in specialized hospital facility and hence may not be affordable.

2.4.2 Convection-enhanced delivery

Convection-enhanced delivery (CED) is a novel delivery method that uses pressure fluid convection established by pressure gradient during interstitial infusion. This convection allows drugs to diffuse to brain which otherwise could not have diffused over required distances by this drug concentration is increase at the site if administration significantly in Comparison to the systemic administration. Using this technique Salkmon et al., (2011) demonstrated delivery of biodegradable maghemite nanoparticles of MTX into brain. This
improved effectiveness of MTX against cancer cells in brain due to higher diffusion of MTX. In another attempt Wu et al., 2006 developed bioconjugate MTX for CED to brain. As tumour cells overexpress EGFR /EGFRvIII, MTX was conjugated to each fifth-generation PAMAM dendrimer molecule, and subsequently conjugated to cetuximab. This was supposed to enhance targeted delivery of MTX directly at the tumour cells. However the bioconjugate showed only small reduction in EC50 and it did not yield any therapeutic gain.

2.4.3 Nano carrier

Nano particles in the range of 80 nm are reported to have the ability to cross BBB (Shenoy et al., 2005). Accordingly efforts have been made to improve MTX brain delivery through formation of nano particle. In 2006 Jiang et al., demonstrated higher brain transport of methotrexate across blood brain barrier by formation of polybutylcyanoacrylate nanoparticles coated with polysorbate 80. In another effort, Young et al., (2009) synthesized monodisperse Fe$_3$O$_4$ nanoparticles (NPs) of Methotrexate (MTX). This was reported with higher uptake and targeting efficiency against tumour cell line (9L rat glioma).

2.4.4 Receptor mediated Transport

Friden et al., 1991 examined the ability of a monoclonal antibody (OX-26) for brain delivery of MTX. OX-26 preferentially, binds to capillary of endothelial cells in the brain after intravenous administration (Jefferies et al., 1984) by recognizing the rat transferrin receptor which allows it to act as a carrier for the delivery of drugs across the blood-brain barrier. MTX conjugate of this antibody was found to enhance accumulation of MTX in brain parenchyma.

2.4.5 Transnasal Delivery of Methotrexate

Nasal chemotherapy is an interesting approach for delivering drug in CNS while circumventing BBB. Accordingly this was examined for MTX in tumour-bearing rats (Shingaki et al., 2010). It showed a significant inhibitory effect on in vitro growth of 9L glioma cells. The pharmacokinetic studies suggested the direct transport of MTX from nasal cavity to both the CSF and to the brain. Nasal chemotherapy with MTX significantly reduced the tumour weight as compared to non-treatment control and IP group. However there are
several issues like inadequate availability, mucosal irritation and cytotoxicity which do not favour its application.

2.5 **Rationale of amino acid conjugate**

The methods adopted so far have not been able to address many issues successfully and there is scope for evaluation of other approaches to enhance its brain delivery. One approach that has recently received attention is reengineering of drugs based on the knowledge of the endogenous amino acid/oligopeptide transportation system within the BBB for brain transport (Gabathuler R., 2010; Pardridge WM., 2012). Amino acids are required in the brain for their role in the regulation of several pathways of brain amino acid metabolism, including neurotransmitter synthesis, S-adenosylmethionine production, and protein synthesis. Considering the polar nature of amino acids BBB is the major barrier for their passive transport across brain. Accordingly several transportation systems at BBB exist that facilitate amino acid transport for normal functioning of brain. Some of these transporters are flexible enough to accept larger molecules. So these transporters have been capitalized to transport drug molecules in the disguise of their amino acid substrates. However this requires optimization of some structural features that is necessary of substrate identification by these transporters. Based on this techniques amino acid conjugates have been demonstrated to cross BBB capitalizing on transporters - including ATB 0,+ , PEPT1, LAT1, LAT2 and several other transporters (Tsuji et al., 2005; Ganapathy ME and Ganapathy V. 2005; Kido et al., 2001; Toyobuku et al., 2003). Considering these, it is worthwhile to develop and analyse reversible amino acid conjugates of MTX for enhancement of brain availability of MTX.

2.5.1 **Rationale of selection of MTX-GLU**

Many studies has been demonstrated that uptake of L-glutamate and L-aspartate from plasma to brain is mediated by sodium independent x̂ transporter system. (Al-Sarraf et al., 1995, 1997a, b; Drewes et al., 1977; Hawkins et al., 1995; Benrabh and Lefauconnier 1996). Furthermore, Hutchinson et al., (985) and Lee et al., (1998) shown L- glutamate uptake at the abluminal membrane of the capillary endothelial cell was mediated by sodium independent saturable mechanism similar to x̂ transporter system. The particular protein which facilitates this uptake has not been identified.
Chemically, MTX is N-[4-[[2,4-diamino-6-pteridinyl] methyl] methylamino] benzoyl]-L-glutamic acid. However, glutamic acid moiety is a poor carrier as transport capacity for anionic amino acid system (system xG-) is quite low compared to neutral amino acids (Al-Sarraf et al., 1995; 1997a, b; Drewes et al., 1977; Hawkins et al., 1995). Unlike glutamic acid, its amide glutamine (GLU) is a good substrate for large neutral amino acid transporter L1 (LAT1). This transport system is the most important source by which essential amino acids gain access to the brain and it has capacity to transport relatively larger molecule provided they meet the basic structural feature including free a-amino and carboxyl group (Uchino et al., 2002; Hawkins et al., 2006; Geiera et al., 2013). This has been capitalized for transport of drugs to brain (Bauwens et al., 2007; Hellwig et al., 2008; Kersemans et al., 2005; Gabathuler 2010; Pardridge 2012; Peura et al., 2013). Additionally, Na-dependent glutamine transporters (systems A and N) also facilitate permeation of glutamine across BBB (Ennis et al., 1998). Besides, glutamine is also a substrate to other amino acid transporters expressed at BBB including ATB0,+ (Umapathy et al., 2008; Czeredys et al., 2008). Thus, these multiple transportation systems may help transportation of glutamine conjugate of MTX across BBB to deliver MTX in brain. Glutamine is also known to reduce complications of chemotherapy including cytotoxicity and neurotoxicity (Stubblefield et al., 2005; Gourav et al., 2012).

Taking these facts into consideration, we tried to use structural features of glutamine and developed reversible conjugate with MTX (MTX–GLU) to improve its brain permeability.

2.5.2 Rationale of selection of MTX-LYS

Brain cannot synthesise adequate amounts of L-lysine and L-arginine. Hence these essential cationic amino acids are required as a constant supply from circulation for optimum brain function. The cationic amino acid transporter system y+ at BBB is believed to facilitate their transport (Smith Q R. 2000). System y+ is expressed at both luminal and abluminal membranes, but predominantly on the abluminal side. Lysine shares a common cerebrovascular cationic transporter (y+L system) which is estimated in situ with Km values of 70 μM (O’Kane et al., 2006). The affinity of this transporter for lysine has the scope of using lysine as substrate for conjugations to drugs for their brain transport. Accordingly L-lysine was conjugated to ketoprofen for enhancement in brain uptake of ketoprofen. Although neither lysine nor ketoprofen is a substrate of LAT1-their conjugate was surprisingly found to bind to LAT1, suggesting contribution of both neutral amino acid and cationic amino acid
transporters in the brain uptake (Gynther et al., 2010). Encouraged by this we developed L-lysine-MTX conjugate to enhance brain uptake of MTX.

2.5.3 Rationale of selection of MTX-TYR

The large neutral amino acid transporters (LAT1) are expressed on the luminal and abluminal membrane of the brain capillary endothelial cells (Duelli et al., 2000). L-Tyrosine in the plasma is transported into the brain across the blood–brain barrier by this system. Amino acids show higher affinity for this system expressed at BBB than those expressed peripherally which may account for its selective brain delivery (Pardridge 1983; Sanchez del Pino et al., 1995; Smith & Stoll 1998). However some structural features including free carboxylic, α-primary amino group and L-configuration are considered to be important in substrate identification of this transporter (Smith Q R 1987). Several molecules with similar features including L-dopa, α-methyldopa and gabapentin are transported capitalizing this LAT1 transporter. Tyrosine has been used to conjugate with nipecotic acid (a potent inhibitor of GABA uptake), ketoprofen and phosphono formate for their respective brain delivery (Bonina et al., 1999; Walker et al., 1994; Gynther et al., 2008). These findings have validated LAT1 as an effective transporter at BBB. Its selective expression at BBB has the potential to allow selective brain delivery of anticancer drugs like MTX. This can reduce toxicity and undesirable effects of MTX. Besides these studies shows the substrate flexibility of this transporters which can be capitalized for brain transport of MTX. Since L-Tyrosine can be esterified to MTX while retaining requisite substrate features of LAT1, MTX-TYR reversible conjugate was developed to enhance brain permeability of MTX across BBB.

2.5.4 Rationale of selection of MTX-PAL and MTX-LEU

Like L-tyrosine, L-Phenyl alanine and L-Leucine are good substrates of LAT1. As these transporters show some flexibility and accepts larger molecules as substrates efforts have been made to use this for transport of drugs to brain which otherwise do not cross BBB. Melphalan, the nitrogen mustard derivative of L-phenylalanine is reported to use this transporter for brain transport (Greig et al., 1987; Cornford et al., 1992; Chastain et al., 1990). Leucine modulates peptide transport system and facilitates transport across BBB (Banks et al., 1991). Besides its conjugates including leucine-enkephalin cross BBB facilitated by these transporters (Zlokovic et al., 1987; Zlokovic et al., 1985). Amino acids and drugs lacking exact substrate specificity are also shown to have some affinity for this transporter (Gynther et al., 2010). Considering this substrate flexibility, MTX-PAL and MTX-LEU were developed as reversible conjugates of L-Phenyl alanine and L-Leucine with MTX.
For development reversible conjugates, peripheral stability and ease of reconversion are essential factors for brain delivery. To minimize peripheral hydrolytic degradation amide conjugates were developed for MTX-GLU, MTX-LYS, MTX-PAL and MTX-LEU. This is also expected to slow down the reconversion to MTX in brain by enzymatic activity. This slow release may lead to sustained action which is desirable in chemotherapy. Although the amide conjugates retain substrate specificity of transporters including ATB 0,+, PEPT1, LAT1 and LAT2, in principle, they lack exact substrate specificity of LAT1. So an ester conjugate of L-Tyrosine with MTX was studied for enhancing brain availability of MTX. Besides most of these amino acids including L-Glutamine, L-Lysine, L-Phenyl alanine, L-Tyrosine and L-Leucine have CNS protective properties (Stubblefield et al., 2005; Gourav et al., 2012; Young SN. 2007; Maurois et al., 2008). This may modulate the anticancer effect of MTX and reduce associated CNS toxicity of MTX.