Chapter 2

Literature Review

2. Literature review on CNS targeting

2.1 Limitations of basic criteria

2.1.1 Molecular weight

Histamine, a small molecule of 111 Da with relative lipid solubility, have readily navigated into the porous capillary of all organs, whereas showed no significant penetration into the CNS, in an autoradiogram of radiolabeled histamine studied in the whole body of an adult mouse (Pardridge et al. 1986; Pardridge, 2007). Quinidine, of 324 Da, is reported to exhibit significantly restricted distribution in the brain (Terasaki and Hosoya, 1999).

Erythrosine B, a fluorescent dye, and 2′7′ -bis(2-carboxyethyl)-5(6)-carboxyl fluorescein tetraacetoxymethyl ester (BCECF-AM) are of 880 Da and of 809 Da, respectively. The former shows the *in vivo* BBB permeability-surface area product (BBB PS) of 39 µl/min/g brain, and the later, 295±48 µl/min/g brain by brain perfusion method. The results suggest that the BBB does not restrict the permeation of substrates with molecular weight of >500 Da. The observations alone are sufficient to invalidate this widely held misconception about MW (Levitan et al., 1984; Hirohashi et al., 1997).

2.1.2 Lipophilicity (log P)

Log Po/w is considered to be a key parameter for CNS penetration (Lipinski et al., 1997). Lipophilicity constant (log k) of 28 structurally different compounds, both CNS^{+} and CNS^{-}, for which the CNS permeation tendency had already been established, was

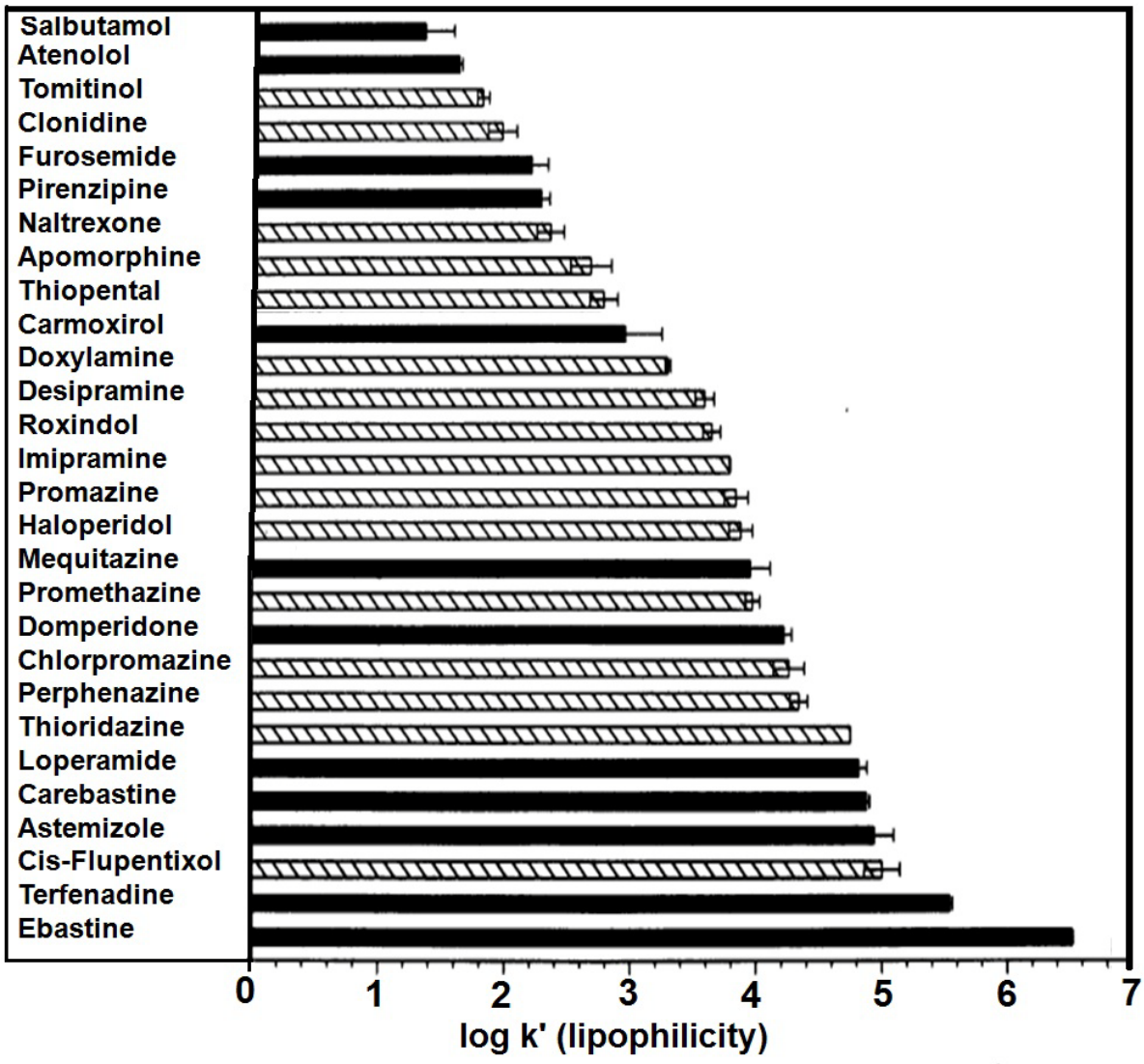

Figure 2.1. Non-linear relationship between CNS permeation tendency and lipophilicity. CNS+ compounds are indicated by hatched bars, and CNS– compounds by black bars. Reproduced from Seelig et al., 1994.

determined by polycratic reversed-phase HPLC. CNS permeation tendency was irrelevant to their order of lipophilicity (**Figure 2.1**). This study showed that even some CNS^{-} compounds exhibit high lipophilicity; For example, terfenodine and ebastine showed the highest log k among the 28 compounds studied. In contrast, some CNS^{+} compounds such as tomitinol and clonidine exhibit low lipophilicity (Seelig et al., 1994).

Cyclosporin A is more lipophilic than diazepam. Diazepam crosses the BBB while cyclosporin does not cross. Anticancer agents, vincristine, vinblastin, doxorubicin and epipodophylotoxin, limited permeability across the BBB, though they are highly lipophilic in nature (Tsuji and Tamai, 1999).

New quinolone antibacterial agents are poorly distributed into the brain. When log P nearing two, a molecule exhibits maximum transportation across the BBB as per lipinski's "Rule of 5" (Lipinski et al., 1997). Olamufloxacin (HSR-903), a newly synthesized quinolone antibacterial agent of highly lipophilic nature (the octanol-water partition coefficient at pH 7.4 is 2.58), also showed a very limited brain distribution (Griffiths et al., 1994). Clonidine, a hydrophilic compound, reaches the brain even if administered in small concentration. Homovanilic acid, a metabolite of cerebral transmitters and hydrophilic in nature, crosses the BBB without any obstacle (Seelig et al., 1994).

Glycosylation further decreases the lipophilicity of peptides, according to Lipinski's ''Rule of Five'' (Lipinski et al., 1997). Introduction of glucose and galactose via *O*-linkage, however, has enhanced the BBB transportation as well as antinociceptive activity of a series of enkephalins analgesics *viz.* *D*-Met2-enkephalin, Hyp5-enkephalin, Leu-enkephalin, dermorphins, endomorphin-1 & 2 (Polt et al., 2005). Many a times it is just amazing that there are no restrictions for the free movements of essential nutrients such as glucose. Amino acids like *L*-phenylalanine and *L*-leucine are regularly transported into the brain even though they are polar in nature (Ohtsuki and Terasaki, 2007; Celia et al., 2010).

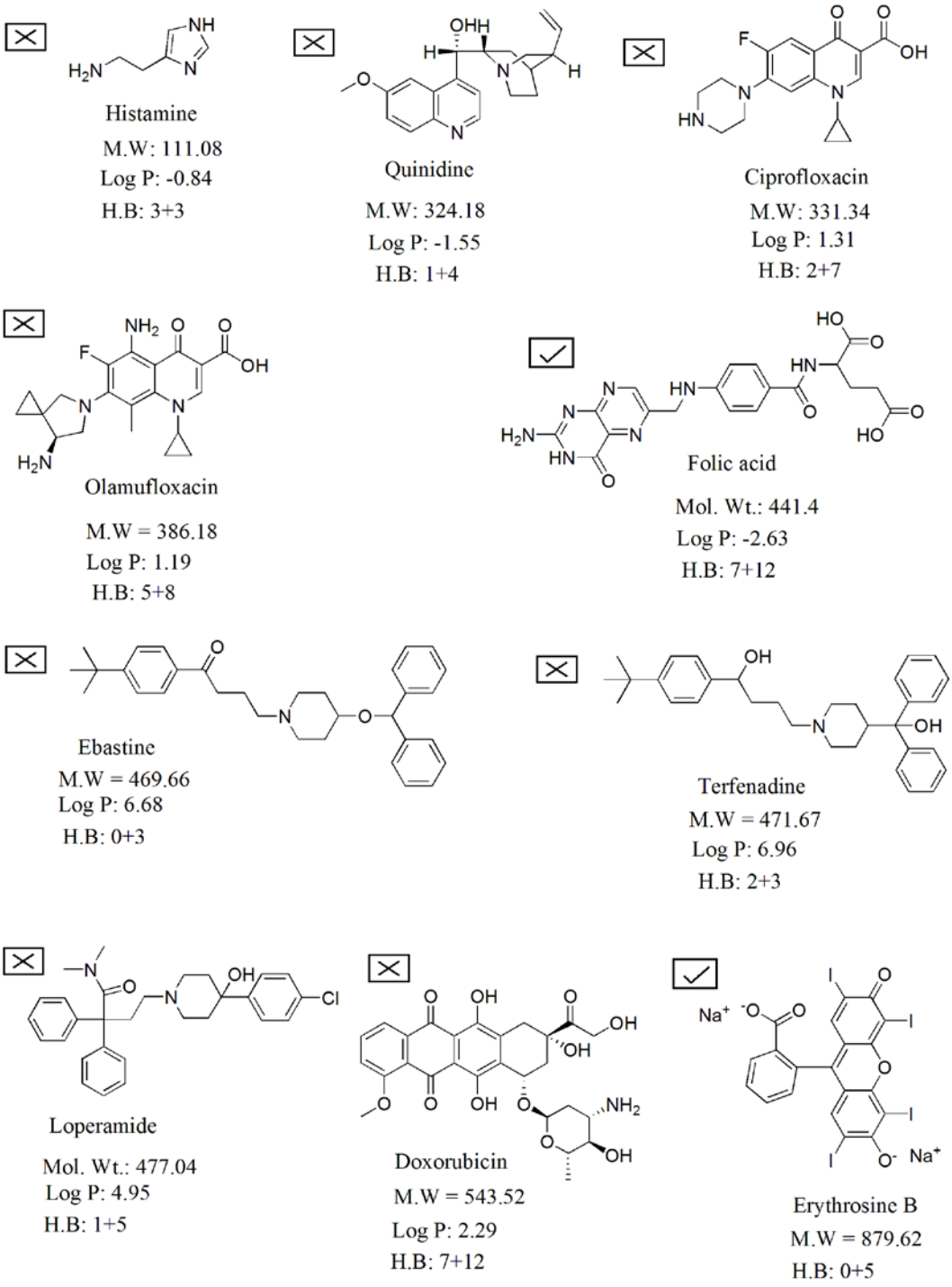

Figure 2.2. Insignificance of molecular weight, lipophilicity and hydrogen bonds on BBB penetration. No. of hydrogen bonds have been presented in 'A + B' form. 'A' denotes possible no. of hydrogen bonds that could occur between 'H' atom of molecule with electronegative atom 'O' present in the surrounding medium; and 'B' denotes possible no. of hydrogen bonds that could occur between electronegative atom 'O', 'N', 'S', 'F', 'I' of molecule with 'H' atom present in the surrounding medium. Molecules with 'Tick' mark cross the BBB; and molecules with 'Cross' marks do not cross the BBB.

Further, increasing lipid solubility would increase the volume of distribution (V_d) to all possible organs, including the BBB, and would result in reduced area under concentration curve and minimal transport of drug to the brain (Kaur et al., 2008). Moreover, increasing lipid solubility of a drug may enhance plasma proteins binding, which could offset the enhanced membrane permeation caused by the lipid solubility. Increasing lipophilicity also increases the rate of oxidative metabolism by cytochrome P450 (Lewis and Dickins, 2002). Insignificance of molecular weight and lipophilicity of molecules on BBB permeation tendency has been figured (**Figure 2.2**).

2.1.3 Polar surface area

Next to molecular weight and lipophilicity, there comes polar molecular surface area. It is defined as the surface area (Å²) occupied by nitrogen and oxygen atoms, and polar hydrogen atoms bonded to these heteroatoms. Molecules of <60 Å² show better brain penetration. Dynamic polar surface area of 45 drug molecules was investigated for their brain penetration. A linear relationship between dynamic polar surface area and brain penetration, however, was not found with all tested molecules; For example, Org 12962, a compound of 23.45 Å², shows better log(Cbrain/Cblood) than amitriptyline, a compound of 4.19 Å² (Table 1). It suggests that polar surface area alone is not a satisfactory predictor of the brain penetration tendency. However, this important descriptor influences drugs that are undergoing passive transportation (Kelder et al., 1999).

Table 2.1. Brain penetration data and dynamic polar surface areas.

S. No.	Compounds	Log (C_{brain}/C_{blood})	Dynamic polar surface area (Å^2)
1	Org 12962	1.64	23.45
2	Imipramine	1.05	5.21
3	Mianserin	0.99	6.55
4	Amitriptyline	0.98	4.19
5	Org 4428	0.82	24.19
6	Org 30526	0.39	19.97

Extracted from (Kelder et al., 1999).

2.1.4 Hydrogen bonds

Lipophilicity of a drug or compound is inversely related to the degree of hydrogen bond formation that occurs with surrounding medium. It has been reported that, for a compound to be transported through the BBB, the cumulative number of hydrogen bonds should not go beyond 8-10 (Lipinski et al., 1997). Increasing lipophilicity i.e. decreasing hydrogen bonds works for small therapeutics, and on molecules of above 500 Da or on those with strong polarity, the increased lipophilicity has no effect (Kaur et al., 2008). Even peptide lipidization has been useful for small peptides, and has no effect on peptides of more than five amino acids.

2.1.5 Miscellaneous

Kp describes the ratio of brain to blood (or plasma) concentration. A study conducted by Doran et al. measured the Kp of 34 structurally diverse CNS drugs including two active

metabolites. The values of kp were in the range of 0.06 to 24. This suggests that it is not necessary for a CNS drug to exhibit a high Kp value i.e. “good CNS penetration” (Doran et al., 2005; Jeffrey and Summerfield, 2010).

2.2. Transport mechanisms present in the BBB

Apart from basic criteria, expression of transporters on BBB limits the BBB permeation of molecules. With the help of positron emission tomography, the *in vivo* activity of human BBB transport systems has been evaluated. Multiple transporters are expressed in the brain microvessel endothelial cells (BMEC) that line cerebral capillaries of the BBB, and the BMEC has been equipped with three different specialized mechanisms of solute transfer (Begley, 2003; Kaur et al., 2008; Ohtsuki and Terasaki, 2007). Briefly, *blood-to-brain influx transport system* that supplies nutrients, including glucose, amino acids and nucleotides, to the brain. Accordingly, xenobiotics recognized by influx transporters are expected to have high permeability across the BBB. *Brain-to-blood efflux transport system* that functions to eliminate metabolites and neurotoxic compounds from brain interstitial fluid; and molecules recognized by this efflux transporter are expelled from the brain parenchyma. *Drug efflux pump* that prevents entry of xenobiotics into the brain by pumping them out. Blood-to-brain influx and brain-to-blood efflux transporters have been delineated in several reviews, and readers are referred to these reviews for a comprehensive background view (Tsuji and Tamai, 1999; Ohtsuki and Terasaki, 2007, Celia, et al. 2010). Impact of the third transporter has been documented in the following section.

2.3 ABC transporters

Adenosine triphosphate (ATP)-binding cassette (ABC) transporter is a chief member of efflux pump transporters. Prevention of intercalation and diffusion of xenobiotics into cell membranes is carried out by these transporters as protective means. They are transmembrane protein transporters situated in BBB as well as other parts of the body (Chang, 2003; Schinkel and Jonker, 2003). These transporters are named after a biochemical process that involves hydrolysis of ATP upon exporting substrates. Nucleotide binding domains (NBDs), also referred as ABC site, is a hallmark feature of these transporters. Recognition of substrates and their passage across the cell membrane is through transmembrane domains (TMDs). Each transporter has at least two NBDs and two TMDs; and its substrate specificities are owing to the overall divergence in their TMDs. This ABC family of transporters consists of A to G (ABCA-ABCG) subfamilies, and 48 subtypes (Chang, 2003; Dean et al., 2001)

Among the subfamilies, ABCB (MDR1/P-gp/ABCB1), ABCC (MRP) and ABCG (BCRP/ABCG2) are the main efflux transporters. BCRP/ABCG2, of 655 amino acids, is a most recently discovered efflux transporter; and it was named as breast cancer resistance protein (BCRP), as this protein was identified from breast cancer cell line for the first time (Schinkel and Jonker, 2003; Chen et al., 1990). BCRP has been found to be a main active drug efflux transporter of mouse and rat brain capillary endothelial cells. However, the presence of BCRP in human BBB has not been clarified.

2.3.1 P-glycoprotein

P-glycoprotein (P-gp/MDR1/ABCB1), of 1280 amino acids, is a well known active efflux transporter of ABCB family and of subtype ABCB1. This 170 to 180-kDa plasma membrane glycoprotein (P-gp) was discovered for the first time in Chinese hamster ovary cell mutants by Juliano and Ling (1976). However, discovery of this intrinsic membrane protein in the luminal plasma membrane of BMEC is dated back to 1989 by Cordon-Cardo et al (1989). It is also the first drug efflux transporter detected in the endothelial cells of the BBB.

In 1994, for the first time, the mdr1a (also called as mdr3) gene knock-out mouse was produced by Schinkel et al; and the role of mdr1a gene product in restricted apparent permeation across the BBB was proved with ivermectin, a centrally neurotoxic pesticide, and vinblastine, a carcinostatic agent (Schinkel et al., 1994).

Human P-gp is encoded by MDR1 and rodent P-gps by Mdr1a and Mdr1b (Smyth et al., 1998). The presence of P-gp has been evidenced in choroid plexus (CP) along with MRP and active organic acid transporter (Rao et al., 1999). P-gp is an efflux transporter of highly hydrophobic molecules, and its most of the substrates are quite hydrophobic in nature. It actively efflux both lipids and structurally diverse hydrophobic drugs; therefore, during 1970s, it was known as “hydrophobic vacuum cleaner” [Chang, 2003; Sharom, 1997). [Note: It exhibits a higher basal ATPase activity in the presence of cholesterol. A role of drug floppase or lipid flip-floppase has been assigned for P-gp (MDR1), and it has 75% homology with a mouse P-gp (mdr2) which is a phosphatidylcholine flippase (Garrigues et al., 2002)]. It has been chiefly known for its efflux actions in tumor cells,

and confers high level of resistance to paclitaxel (Ohtsuki and Terasaki, 2007; Schinkel and Jonker, 2003; Smyth et al., 1998).

Verapamil , a calcium channel blocker, and cyclosporin A, an immunosuppressive agent, are competitive and nonspecific inhibitors of P-gp (Tsuruo et al., 1981). Valspodar (SDZ PSC 833 or PSC 833), GF120918 (also called as GG918), LY335979, and XR9576 are specific inhibitors of P-gp. Valspodar is a cyclosporin A analogue with no immunosuppressive effect of cyclosporin A (Schinkel and Jonker, 2003).

P-gp exhibits an apparently null basal ATPase activity, which is unusual for an active transporter, in the absence of any identified substrate, and multidrug resistance activity when overproduced in the plasma membrane of tumor cells (Sharom, 1997; Garrigues et al., 2002). In addition to drug efflux transport function, P-gp may generally inhibit caspase-dependent tumor cell apoptosis; and it could be reversed by inhibition of P-gp function by using P-gp-specific mAbs (Smyth et al., 1998).

2.3.2 Multidrug resistance-associated protein (MRP) transporters

Endothelial cells of the BBB also express a transmembrane glycoprotein called multidrug resistance-associated protein (MRP/ABCC), which is another active efflux drug transporter expressed at the BBB to the same extent of P-gp. As P-gp in BBB, this 190-kDa protein has mainly expressed in blood-CSF barrier which is localized to the epithelium of the CP– a site of elimination of xenobiotics and endogenous waste. MRP1 to 5 have been identified till date (Rao et al., 1999).

MRP1 was first identified in a cell line made highly resistant to a cytotoxic drug namely doxorubicin, and subsequent analysis showed that it confers multidrug resistance against a range of anticancer drugs (Cole et al., 1992). In contrast to P-gp which is an apical-to-basal trans epithelial permeation barrier, MRP1 functions as a basal-to-apical drug-permeation barrier. Alike P-gp, MRP is an efflux transporter of amphiphilic molecules and confers high level of resistance to doxorubicin. General organic anion transporter inhibitors, such as sulfinpyrazone, benzbromarone and probenecid are used as MRP inhibitors; however, they lack in specificity and suitability (Schinkel and Jonker, 2003).

MRP4, of 1325 amino acids, involves in the efflux transport of prostaglandins, PGE1 and PGE2 (autocrine- or paracrine-signaling molecules) from within cells, whereas MRP1–3 and MRP5 do not. In the absence of MRP4, prostaglandins get accumulated. Recent studies have revealed that several nonsteroidal anti-inflammatory drugs (NSAIDs) are potent inhibitors of MRP4 at concentrations that did not inhibit MRP1-3 and MRP5; and NSAIDs might act by inhibiting this MRP4 mediated prostaglandins release along with inhibition of prostaglandin synthesis (Reid et al., 2003).

2.3.3 Miscellaneous

Other efflux transporters are multidrug resistance protein (MDRP), Organic anion and organic cationic transporters. MDRP transporters are also transmembrane protein transporters present in BBB as well as other part of the body to the same extent of P-gp and MRP (Chang, 2003; Schinkel and Jonker, 2003). Organic anion transporters, such as Oatp3 and Oat3, and organic cation transporters, such as Oct2 and/or Oct3, play a major role in the uptake of amphiphilic and hydrophilic organic anions, and hydrophilic organic

cations, respectively, at the brush border surface epithelia of the CP (Kusuhara and Sugiyama, 2004).

2.4 Intervention of P-gp in CNS targeting

The impact of P-gp on BBB penetration of therapeutics was first demonstrated by using mdr1a knockout mice that lack the mouse homologue of P-gp. In mdr1a knockout mice, the brain-to-plasma (B/P) concentration ratios of ivermectin and vinblastine were increased by 100- and 3-fold, respectively, compared with those of wild-type mice (Schinkel et al., 1994).

Pgp substrate profiles of marketed and successful 48 CNS and 45 non-CNS drugs, totally 93, were determined by Doan et al. using monolayer efflux method. The CNS drugs had a three-fold (14.6%) lower incidence of Pgp-mediated efflux than the non-CNS drugs (42.2%). This owes to the unintended trial-and-error drug discovery process of olden days used in drug selection, and instinctively resulted in drugs of lower incidence of Pgp for the treatment of CNS disorders. Doan et al found that Pgp-mediated efflux to be a second most discriminating factor between CNS and non-CNS drugs (Doan et al., 2002).

However, later, a study conducted by Doran et al. has revealed that P-gp is an important determinant in the CNS penetration of the molecules. Differences in B/P ratios and cerebrospinal fluid/plasma (CSF/P) ratios between wild-type and knockout animals were studied for 34 structurally diverse CNS drugs including two active metabolites and 8 non-CNS drugs, to know the importance of P-gp profile. Intriguingly, a majority of the CNS drugs (27 of 34, 79.4%) demonstrated a significantly increased B/P ratios in knockout

mice when compared to wild type. The B/P ratios of metoclopramide (6.6-fold), risperidone (10-fold), and 9-hydroxyrisperidone (17-fold) among CNS drugs, and quinidine (36-fold), loperamide (9.3-fold), verapamil (17-fold), amiodarone(21-fold) among non-CNS drugs showed marked alterations in P-gp profile of knockout mice (Doran et al., 2005).

Olamufloxacin (HSR-903), a newly synthesized quinolone antibacterial agent of high lipophilicity (the octanol-water partition coefficient at pH 7.4 is 2.58), has been reported to show a very limited brain distribution owing to the efflux action of P-gp. Nearly eight-fold higher B/P ratio of HSR-903 was observed in mdr1a gene-deficient mice (mdr1a (2/2)) than that in normal mice (Tsuji and Tamai, 1999; Griffiths et al., 1994). New quinolone antibacterial agents, including ciprofloxacin, norfloxacin, and sparfloxacin have also been reported to show highly restricted BBB transportation, while comparing with their peripheral transportation, owing to the efflux action of P-gp. List of P-gp substrates and their inhibitor has been reviewed, and readers are referred to these reviews (Ohtsuki and Terasaki, 2007; Celia et al., 2010). Studies suggest that both P-gp and MRP have to be blocked for better CNS penetration (Rao et al., 1999).

2.5 Receptor mediated transport (RMT)

Amidst the presence of effective efflux transporters, BBB transportation of endogenous substrates and even therapeutics that mimic endogenous substrates occurs with no restrictions. BBB is bestowed with many such endogenous transport systems. For example, glucose, a major energy source for the brain, is transported via glucose

transporter-1 (GLUT-1), and amino acids via L-type amino acid transporter 1 (LAT1) (Ohtsuki and Terasaki, 2007; Celia et al., 2010).

The uptake of essential compounds, such as thiamine, biotin, folic acid, vitamin B_{12}, transferrin and neuropeptides, takes place through their specific receptor (Kaur et al., 2008). Even the presence of endogenous receptors for insulin, insulin-like growth factors namely IGF-I and IGF-II, and leptin have been located in the brain vascular endothelium which forms the BBB (Celia et al., 2010; Boado, 2005). It has been reported that genetically engineered chimeric HIR mAb, a ligand to human insulin receptor, has permeated the human BBB, and it is nearly ten times more active than anti-TfR mAb in primates (Coloma et al., 2000).

2.6 Transportation through chimeric peptide technology

Transportation of drugs that are not recognized by receptor on their own accord could be achieved through chimeric peptide technology. Herein, a non-transportable drug is conjugated to a BBB transport vector/ ligand which has its receptor expression in the BBB, and undergoes transcytosis (Kaur et al., 2008; Pardridge, 1997; Wu and Pardridge, 1999). The transport vectors could be conjugated either directly to the drug compounds or to the surface of particulate colloidal carriers *viz*. nanoparticles, liposomes, etc. through covalent or noncovalent linkage (Kaur et al., 2008; Pardridge, 2002b).

2.7 Epilepsy

Among the CNS disorders, the prevalence of epilepsy is as high as other abreast CNS disorders (**Table 2.2**). The epilepsies are common and have frequently devastating

influences, affecting approximately 2.5 million people in the United States alone and about 4 % of individuals (50 million) over their lifetime worldwide (Godman & Gilman, 2006; Browne and Holmes, 2001). More than 40 distinct forms of epilepsy have been identified. People with epilepsy are well known to be at increased risk of sudden death. A survey conducted by Department of Neurology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum in Kerala says it is also subsidizing for unemployment among the suffer of epilepsy. It is surprising, among the unemployed population, 58 % of persons are of epilepsy compared with 19% of the general population (Varma et al., 2007).

Table 2.2**.** Prevalence of various CNS disorders

S.No	**Disorder**	**Prevalence (world wide)**	**Reference**
1	Epilepsy	4 %	Browne and Holmes, 2001
2	Depression	2.9 %	Copeland et al.,1987
3	Alzheimer's	2 %	Ferri et al., 2005
4	Parkinson's Disease	1 %	Zhang Z, Roman G. 1993
5	Schizophrenia	< 0.4 - 1 %	Sullivan et al., 2003

2.8 Phenytoin sodium

Sodium phenytoin is chemically known as 5,5-diphenyl-2,4,imidazolidinedione sodium salt, with a formula $C_{15}H_{11}N_2NaO_2$. Phenytoin is an excellent anticonvulsant and because of its putative mechanisms of action, it masters three different conditions like Grandmal, Partial & status epileptics (Aaron et al., 1999). It increases the seizure threshold by its stabilizing effect on all neuronal membrane and reduces the paroxysmal depolarization shift (PDS) by limiting the neuronal sodium concentration (Godman &

Gilman, 2006). Due to its weakly acidic nature (p*K*a 8.31) and poor aqueous solubility (100 mcg:ml) phenytoin often it shows erratic & slow absorption after oral route (Robinson et al.,1975).

It is also a good candidate of P-glycoprotein (P-gp), Mrp1, Mrp2, and BCRP (Van vliet et al., 2005, Van vliet et al., 2006, Prasad et al., 2011). Recent studies have suggested that overexpression of P-glycoprotein in the hippocampal region affects brain uptake of phenytoin in epileptic rats and causes a decrease of local PHT levels in the rat brain (Van Vliet et al., 2007). Even, it has been observed that expression of multidrug resistance–associated proteins MRP1 and MRP2 and breast cancer–resistance protein (BCRP) was upregulated shortly after status epilepticus, during the Latent Period, and in Chronic Epileptic Rats, and affects distribution of phenytoin (PHT) in the brain (Van vliet et al., 2005).

2.9 Folic acid

Folic acid (pteroylglutamic acid), also known as Vitamin B_9, and its double-reduced form tetrahydrofolate are cofactors of several enzymes and a notable endogenous substrate. Folic acid, being an essential compound, its uptake is takes place through a specific saturable transport system exists at the BBB. Folate is relatively easy to ligate to any therapeutics, and retains its ability to bind to its receptor with normal affinity when attached via its γ-carboxylate, and thereby enter receptor-bearing cell by endocytosis (Zhang et al., 2010; Majoros et al., 2005). Further, its receptor has wildly been expressed in the cells of brain and CP (Wu and Pardridge, 1999). FR is a glycopolypeptide with a high affinity (KD<10_ 9 M) for folic acid and the physiological form of circulating

vitamin, N5-methyltetrahydrofolate (Elnakat and Ratnam, 2004). Folic acid has shown considerable promise as a potential means of delivering a wide variety of novel therapeutic agents. Till date, folate or FR antibody conjugates of cytotoxics, and radiopharmaceuticals, folate-coated liposomes containing antisense oligonucleotides, genes or cytotoxics, and folate-linked nanoparticle carriers for therapeutic drugs and genes has been implicated (Elnakat and Ratnam, 2004). Thus folate mediated targeting may be interesting for drug delivery to CNS.

2.10 Gelatin

Gelatin is a natural, inexpensive, low immunogenic, non-toxic, and good biodegradable macromolecule which is registered as excipient for pharmaceutical formulations. As a protein-based product, gelatin possesses several functional groups which are available for covalent modifications for drug or ligand binding and useful in targeted drug delivery (Balthasar et al., 2005). Characteristic features of gelatin are a high content of the amino acids glycine, proline (mainly as hydroxyproline) and alanine which occur in repeating sequences and which confer on gelatin its triple helical structure. Commercial gelatin is a heterogeneous protein mixture of polypeptide chains and has a wide range of molecular weight ranging from a few thousand to several hundred thousand, even up to a few million Daltons (Ofokansi et al., 2010).

Furthermore, the high physiological tolerance of gelatin, its biocompatibility, and its biodegradability are well established for years. The US Food and Drug Administration (FDA) classified gelatin as a “Generally Recognized as Safe” excipient. Gelatin has been

used for decades in parenteral formulations and as an approved plasma expander (Schwick and Heide, 1969 & Zwiorek et al., 2008).

Characteristic features of gelatin are a high content of the amino acids glycine, proline (mainly as hydroxyproline) and alanine which occur in repeating sequences and which confer on gelatin (Balthasar et al., 2005). it is a polypeptide with a nonuniform distribution of at least 18 amino acids, of which glycine (32-35%), proline (11-13%), alanine (10-11 %), hydroxyproline (9- lo%), glutamic acid (7-8%), arginine (5%), and aspartic acid (4-5%) are the most numerous (Miller et al., 1994).