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Review

In Silico Methods to Assess CNS Penetration of Small Molecules

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Abstract: In CNS drug discovery, estimation of brain exposure of lead compounds is critical for their optimization. Compounds need to cross the blood-brain barrier (BBB) to reach the pharmacological targets in the CNS. The BBB is a complex system involving passive and active mechanisms of transport and efflux transporters such as P-glycoproteins (P-gp) and breast cancer resistance protein (BCRP), which play an essential role in CNS penetration of small molecules. Several *in vivo*, *in vitro* and *in silico* methods are available to estimate human brain penetration. Preclinical species are used as *in vivo* models to understand the unbound brain exposure by deriving the Kp,uu parameter, the brain/plasma ratio of exposure corrected with the plasma and brain free fraction. MDCK-mdr1 (Madin Darby canine kidney cells transfected with the MDR1 gene encoding for the human P-gp) assay is the most commonly used *in vitro* assay to estimate compound permeability and human efflux. The *in silico* methods to predict brain exposure, such as CNS MPO, CNS BBB scores and various Machine Learning models, help save costs and speed up compound discovery and optimization at all stages. These methods enable the screening of virtual compounds and building of a CNS penetrable compounds library as well as optimization of lead molecules for CNS penetration. Therefore, it is crucial to understand these methods' reliability and ability to predict CNS penetration. We review the *in silico*, *in vitro* and *in vivo* data and their correlation with each other and assess published *in silico* approaches to predict the BBB penetrability of compounds.

Keywords: The Blood-Brain Barrier (BBB); CNS drug discovery; passive diffusion; active transport; efflux transporters; influx transporters; P-glycoproteins (P-gp); breast cancer resistance protein (BCRP); in silico models

1. Introduction

In central nervous system (CNS) drug discovery, estimating brain exposure of lead compounds is critical for their optimization. Compounds need to cross the blood-brain barrier (BBB) to reach the pharmacological targets in the CNS. The BBB is a complex physical barrier that surrounds most of the blood vessels in the brain and prevents the permeation of harmful molecules from circulating blood into the brain (see Figure 1 (a)). The tight junctions of the BBB severely restrict paracellular transport, whereas specialized transporters, pumps, and receptors regulate the transcellular transport of metabolic nutrients and other essential molecules. Small lipophilic molecules can passively diffuse across the lipid bilayer but are often returned to the blood by efflux pumps [1,2]. Due to the impermeability of the CNS, it is a challenge for most molecules to gain access to the brain, although several molecules do transfer from the blood to the brain. Several mechanisms are potentially involved in this process [3]. While passive diffusion is a major mechanism of penetration of drugs into CNS, efflux by several transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and members of the multidrug resistance protein (MRP) family at the BBB limit concentration of drugs in the CNS [4-6]. Influx transporters such as OCT1 and OCT2 surrogate penetration of bulky and charged molecules across BBB (see Figure 1 (b)). P-gp and BCRP are relatively well characterized among these efflux transporters, with considerable overlap among their substrates. P-gp (also known as MDR1 (multidrug resistance protein 1) and ABCB1 (ATP-binding

cassette sub-family B member 1)) is widely expressed at BBB. In the last 20 years, with the availability of the P-glycoprotein (P-gp) knockout mouse model, numerous studies conducted in P-gp knockout versus wild-type mice observed significant P-gp efflux of drugs [7]. Thus, the efflux of drugs by P-gp has been regarded as an essential factor determining the drug concentration in the brain. Further, over the past few decades, it has also become clear that reliance on total drug level in the brain is often misleading and that unbound drug concentration is more predictive of target occupancy and, ultimately, *in vivo* efficacy [8]. These developments led to the use of MDR1-MDCK *in vitro* assay to estimate the permeability and efflux of lead molecules and *in vivo* (rat or mouse) models to determine unbound brain exposure of lead molecules.
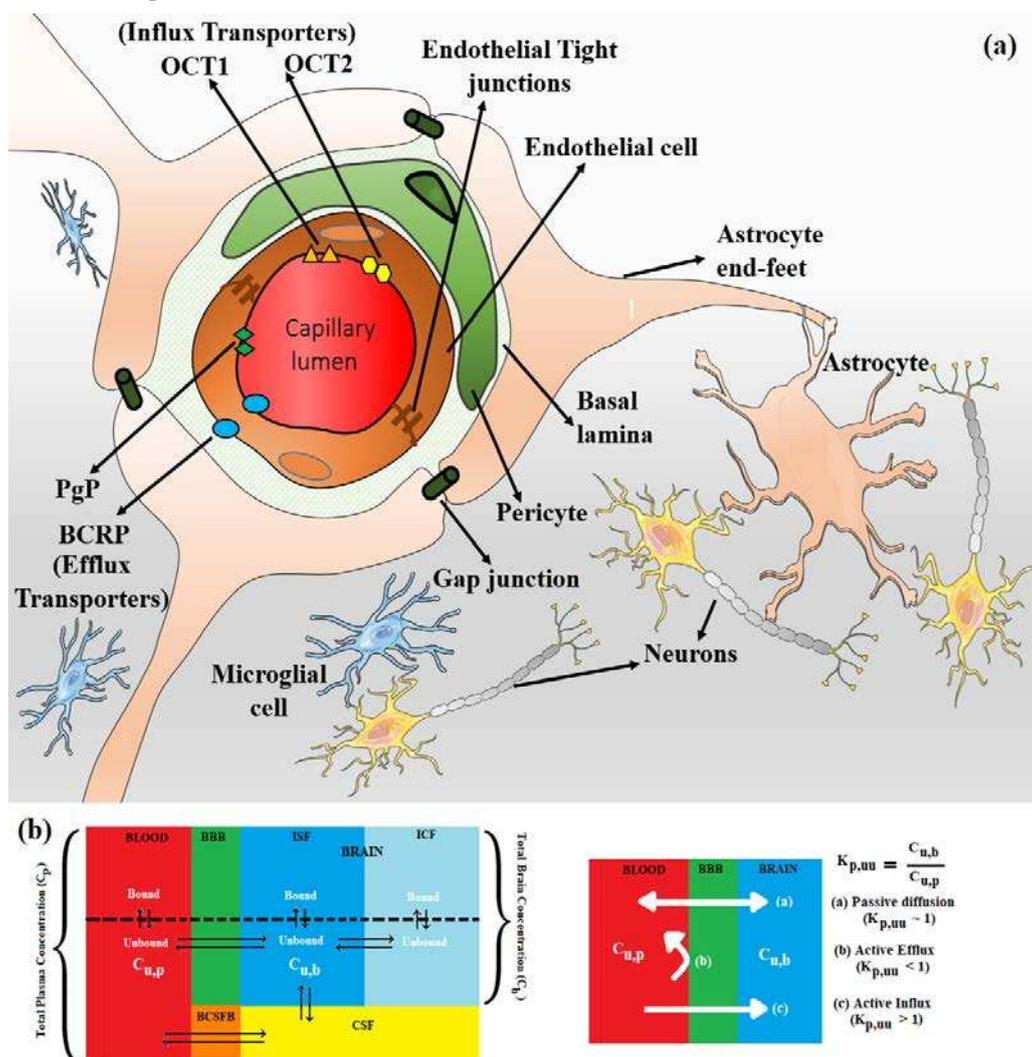

Figure 1. (a) The endothelial tight junctions of the BBB (shown in brown) severely restrict paracellular transport, whereas specialized transporters, e.g., P-gp (green diamond) and BCRP (blue oval) (efflux transporters); OCT1 (orange triangle) and OCT2 (yellow hexagon) (influx transporters) regulate the transcellular transport of metabolic nutrients and other essential molecules across BBB. Enclosed in the basal lamina, pericyte cells partially surround these BBB endothelial cells. The complex tight cellular network of BBB is further maintained by astrocytes' end feet. Astrocytes maintain the cellular link between neurons and microglial cells. The transport across BBB involves concentration gradient-driven passive diffusion and active transport employing various efflux and influx transporters in the endothelial cell membrane. (b) Schematic of plasma and brain compartments presenting different modes of transport across BBB, i.e., passive diffusion and active transport using efflux (e.g., P-gp, BCRP) and influx (e.g., OCT1, OCT2) transporters. Kp,uu represents the unbound brain to unbound plasma drug concentration ratio where $C_{u,b}$ and $C_{u,p}$ represent unbound drug concentration in brain and plasma, respectively. Different brain compartments, i.e., Blood BBB, CSF, BCSFB, ISF and ICF,

correspond to blood, blood-brain barrier, cerebrospinal fluid, blood-cerebrospinal fluid barrier, interstitial fluid and intracellular fluid, respectively.

A reliable *in silico* method for predicting the brain penetration of lead compounds would provide significant value and acceleration to drug discovery programs, save precious *in vivo* resources, and prioritize leads for *in vivo* assessment. The challenges faced in developing such *in silico* models arise from the complexity of the BBB involving multiple transporters and the influence of multiple pharmacokinetic parameters. Also, the available datasets for training models are small and do not cover the entire drug space. Also, only a subset of compounds in these datasets has both *in vitro* and *in vivo* data. Nevertheless, prediction models are being built as they are crucial for assessing the CNS penetrability of compounds in commercial libraries, virtual libraries and molecules generated by AI-enabled de novo design methods. Numerous *in silico* methods have been developed to predict brain exposure using different classification and/or regression algorithms. The type of experimental data used to build prediction models changed from the simple classification of BBB+ (for penetrating compounds) and BBB- (for non-penetrating compounds) to Kp (logBB, the brain-to-plasma ratio of the total drug concentration) to more recent Kp,uu (the unbound brain-to-unbound plasma concentration ratio). In many publications, the easily accessible and abundant classification data (BBB+ for penetrating and BBB− for non-penetrating compounds), often estimated by the presence or absence of CNS activity, are used. Earlier QSAR and Machine Learning prediction models utilized LogBB (logarithm of the ratio of total steady-state concentration in the brain to that in blood at a given time, also referred to as Kp) and LogPS (logarithm of the permeability surface area product) data. LogBB lacks information regarding the free drug concentration available for transport across the BBB, and LogPS does not incorporate BBB transporter-mediated efflux. These models were extensively reviewed elsewhere [9] and are not discussed in this review as there has been a paradigm shift away from optimizing Kp toward Kp,uu in CNS drug discovery. Free tissue drug concentration, Kp,uu, is considered to be the therapeutically relevant metric for estimating free drug concentration at the receptor site over the time course of its action, not the total drug concentration, Kp, based on the free drug hypothesis [8,10,11]. From a compartmentalized CNS drug distribution model (see Figure 1(b)), steady state Kp,uu can be presented in terms of passive diffusion ($PassDiff$), active influx clearance (Cl_{influx}), active efflux clearance (Cl_{efflux}), brain interstitial fluid bulk flow clearance (Cl_{bulk}), and brain metabolic clearance ($Cl_{metabolism}$). Cl_{bulk} and $Cl_{metabolism}$, become insignificant and can be disregarded for molecules having high permeability and low metabolic clearance. Kp,uu parameter presents efflux and influx permeation across BBB relative to passive diffusion. Passive diffusion, active efflux and active influx correspond to values of unity, below unity and above unity, respectively. Kp,uu can be understood as a measure of lateral efficacy of various efflux and influx transporters independent of the extent of brain or plasma tissue binding (eq 1).

$$K_{p,uu} = \frac{PassDiff + Cl_{influx}}{PassDiff + Cl_{efflux} + Cl_{bulk} + Cl_{metabolism}} \qquad (1)$$

The closer the Kp,uu value is to 1, the less peripheral body burden is required to achieve efficacious free concentration in the brain. Generally, a Kp,uu >0.3 in the rat is considered adequate, although this value depends on the drug's potency and other ADME properties. Therefore, this review will focus on the models that utilize the preclinical *in vivo* Kp,uu data and the MDR1-MDCK *in vitro* data in validation and training sets, as well as the physicochemical properties, different multiparameter scores and the prediction models that distinguish CNS and non-CNS drugs. The applicability and limitations of different *in silico* methods will also be discussed.

2. Physicochemical Properties of CNS Drugs

Despite significant challenges in designing compounds that cross the BBB, multiple classes of drugs cross BBB as they are known to treat CNS diseases, and many more CNS drugs are in clinical development [12]. The principal medicinal chemistry strategy in drug discovery has been to optimize the physicochemical properties of compounds to maximize CNS penetration.

Since the publication of Lipinski's rule of 5 in 1997 that defined desirable physicochemical properties (MW < 500 Da, log P < 5, HBD < 5, and HBA < 10) for oral bioavailability of a drug candidate [13], several groups attempted to map the physicochemical space of CNS drugs employing different approaches. Hansch *et al.* [14] studied a dataset of 201 barbiturates having preclinical *in vivo* efficacy data and found the *in vivo* efficacy of the drug to have a parabolic dependency on LogP and suggested LogP = 2 optimal for *in vivo* activity. The improved chance of CNS penetration was predicted for the following desirability ranges: MW < 450, PSA < 90 $Å^2$ and Log D [1,4] for a dataset of 125 CNS and non-CNS drugs analyzed by Van der Waterbeemd *et al.* [15]. In a study of 776 CNS and 590 non-CNS oral drugs that reached at least phase 2 clinical trials, Kelder *et al.* [16] suggested an upper polar molecular surface area (PSA) limit of < 60-70 $Å^2$ for most CNS drugs. Doan *et al.* [17] have indicated that physiochemical properties of CNS drugs differ substantially from non-CNS drugs having CNS dataset mean values of cLogP (3.43), cLogD (2.08), HBD (0.67), PSA (40.5 $Å^2$) for a dataset containing 48 CNS and 45 non-CNS drugs. Norinder *et al.* [18] based on a literature review, have suggested that a molecule having O + N < 5, or cLogP − (O + N) > 0, has an improved chance of CNS penetration. The physiochemical property space suggested by Didziapetris *et al.* [19] for better CNS penetration while avoiding P-gp efflux liability: MW <400, pKa <8, N+O < 4. Leeson *et al.* [20] suggested mean values of 310 (MW), 4.32 (O + N), 2.12 (HBA), and 4.7 (RB) for CNS drug molecules based on a review of a dataset of 329 oral drugs marketed during 1983-2002. The recommended attributes of successful CNS drugs suggested by Pajouhesh *et al.* [12] for a study of a dataset of marketed CNS drugs were MW < 450; H-bonds < 8; pKa 7.5–10.5; HBD < 3; HBA < 7; RB < 8; cLogP < 5; PSA < 60–70 $Å^2$. Based on a medicinal chemistry literature review, Hitchcock *et al.* [21] recommended physicochemical property ranges for improving BBB penetration: MW < 500, PSA < 90 Å2, cLogD (pH 7.4) [2,5], cLogP [2,5], and HBD < 3.

It was realized that CNS drugs occupy considerably smaller chemical space than oral drugs designed for peripheral targets [22]. Indeed, CNS drugs tend to be smaller with higher lipophilicity and lower polar surface area (PSA) than non-CNS drugs (see Table 1).

Table 1. Mean (Range) of Physical Chemical Properties of CNS and Non-CNS Drugs (copied from Pajouhesh *et al.* [12]).

Physical Chemical Properties	CNS	Non-CNS
Molecular weight	319 (151–655)	330 (163–671)
ClogP	3.43* (0.16–6.59)	2.78* (−2.81–6.09)
ClogD	2.08 (−1.34–6.57)	1.07 (−2.81–5.53)
PSA	40.5 (4.63–108)	56.1 (3.25–151)
Hydrogen bond donors	0.85* (0–3)	1.56* (0–6)
Hydrogen bond acceptors	3.56 (1–10)	4.51 (1–11)
Flexibility (rotatable bonds)	1.27* (0–5)	2.18* (0–4)
Aromatic rings	1.92 (0–4)	1.93 (0–4)

In the broadest sense, moderately lipophilic drugs cross the BBB by passive diffusion, and the hydrogen bonding properties of drugs can significantly influence their CNS uptake profiles. Polar molecules are generally poor CNS agents unless they undergo active transport across the CNS. Size, ionization properties, and molecular flexibility are other factors observed to influence the transport of a compound across the BBB. The design of CNS drug candidates with intracellular targets may benefit from increased basicity and/or the number of hydrogen bond donors [23]. However, it should be noted that the "older" CNS drugs modulate ion channels, monoamine GPCR and transporter gene families with common pharmacophoric features of small lipophilic amines [24]. This scenario is rapidly changing as CNS drug discovery efforts have been shifting towards emerging therapeutic areas such as neurodegeneration and neuro-oncology with novel "non-traditional" CNS targets. The compounds targeting these new targets are relatively larger and more polar ligands with wider chemical diversity. It is, therefore, possible that the current understanding of the allowed physicochemical properties space of CNS drugs may expand.

3. BBB Penetration Scoring Schemes for Predicting Brain Penetrance across BBB Primarily by Passive Diffusion

Analyzing the physicochemical properties of CNS (CNS+) and non-CNS (CNS-) drugs led to the formulation of different scoring schemes to design CNS drugs. Recently, multiple algorithms have been proposed to improve RO5 for drug discovery of CNS target space. Wager *et al.* [25,26] developed an algorithm called the "multiparameter optimization (MPO)" based on a study of 119 CNS drugs and 108 CNS clinical candidates to suggest the optimal range of property space for different physicochemical properties of drug molecules. For each of these calculated properties, a range of values is identified as more favorable (score = 1) or less favorable (score = 0) for a CNS candidate. The algorithm comprises six physiochemical properties with median values for CNS drugs: MW 305.3 Da, PSA 44.8 Å^2, HBD = 1, cLogP 2.8, cLogD 1.7, and pKa = 8.4. This scoring method showed that 74% of marketed CNS drugs and Pfizer CNS candidates displayed a high CNS MPO score (MPO desirability score ≥ 4, using a scale of 0-6). However, a follow-up study involving re-examining the MPO score by the authors suggested that MPO score can vary substantially depending on the computational software and method used to calculate physiochemical properties comprising the MPO score (mainly LogD and pKa) [27]. Also, MPO score is congenitally biased toward lipophilicity parameters. MPO score also poses the risk of populating the chemical space with small molecules with very low molecular weights as MPO score does not apply lower limits (e.g., clogP, clogD, MW and pKa) but only apply upper limits to physiochemical properties used. These very low molecular weight small molecules may not bind to certain targets of interest with sufficient binding potency. MPO also does not characterize non-CNS drugs as it is based on CNS drugs (119) and CNS candidates (108); this could potentially make MPO less adequate to capture the physiochemical nature of BBB. A separate study assessing 616 compounds with measured unbound concentrations in the brain confirmed that a higher CNS MPO score correlated with a higher unbound concentration in the brain [22]. A probabilistic MPO scoring function, designated as pMPO, is based on defining the physiochemical properties of a dataset of 299 CNS penetrant and 366 non-CNS penetrant molecules. pMPO physiochemical descriptors, along with their weighing, are as follows: TPSA (0.33), HBD (0.27), MW (0.16), clogD (0.13) and basic pKa (0.12) [28]. Ghose *et al.* [29] studied a dataset of 317 CNS and 626 non-CNS oral drugs and have proposed property ranges for CNS penetration: TPSA < 76 Å^2 (25–60 Å^2), 740–970 Å^3 volume, N [1,2], linear chains outside of rings <7 [2,4], HBD <3, (0,1), and SAS (460–580) Å^2; the ranges given in the parentheses are preferred. They optimized the relative weights of these parameters by comparing the physiochemical property distribution of CNS versus non-CNS oral drugs. Rankovic *et al.* [30] have mapped the physiochemical properties of a diverse corporate dataset from Eli Lilly based on brain-penetrant and peripherally confined molecules. They developed an algorithm termed MPO_V2, which contained five descriptors. They dropped LogD descriptor from MPO and included double weight for the HBD descriptor MPO_V2: ∑T0 (clogP, MW, TPSA, pKa, 2 HBD). However, this article was retracted[31]. Recently, Gupta *et al.* [32] proposed the Blood–Brain Barrier (BBB) Score that is composed of stepwise and polynomial piecewise functions with five physicochemical descriptors: number of aromatic rings, heavy atoms, MWHBN (a descriptor comprising molecular weight, hydrogen bond donor, and hydrogen bond acceptors), topological polar surface area, and pKa. The BBB Score outperformed (AUC = 0.86) the CNS MPO approach (AUC 0.61).

The ease of calculation of the CNS MPO, CNS MPO_V2, CNS pMPO and CNS   BBB scores and their capability to predict BBB penetration of compounds can aid in mapping the property space of large commercial and virtual compound libraries to help lead optimization. CNS MPO and CNS BBB scores are widely utilized for CNS drug discovery programs. Figures 2 and 3 represent 100% stacked bar graphs for low to high CNS MPO, MPO_V2, pMPO and BBB scores for a dataset of CNS and non-CNS drugs. Ideally, a CNS MPO, MPO_V2, and BBB score in the range of (4,6] should correlate to a CNS drug, and a CNS MPO, MPO_V2 and BBB score of (0,4] should correspond to a non-CNS drug. pMPO outputs a score in the range of (0,1], which has been scaled to (0,6] to compare against other scores. However, the CNS BBB score performs better in identifying a higher percentage of CNS compounds than other scores.

In addition to scoring methods, Quantitative Structure-Activity Relationship (QSAR) and Machine Learning (ML) algorithms were successfully applied to predict BBB permeability. The derivation of QSAR and ML models involves calculating molecular descriptors, fitting them to experimental values using a statistical algorithm on a training dataset, and predicting experimental values of the test dataset. ML methods such as support Vector Machine (SVM), Decision Tree (DT) and K-Nearest Neighbor (KNN) that combine property-based descriptors with molecular fingerprints of compounds predict the classification of CNS and non-CNS drugs with high accuracy [33]. Chen et al.[34] and Varadharajan et al.[35] employed machine learning algorithms (Random Forest (RF) and Support Vector Machine (SVM)) to develop direct and indirect regression models based on Kp,uu, Kp,brain, Vu,brain and fu,pl using. For 173 compounds in the training set [34], their model used a total of 196 descriptors. Their model predicted Kappa2 descriptor, i.e. the measure of molecular linearity being strongly correlated while poor reliability on lipophilicity of a molecule as predicted by Fridén et al. [36]. Similarly, Saxena et al. [37] published accurate classification models using different ML algorithms using physicochemical properties, Molecular ACCess Systems keys fingerprint (MACCS) [38] and substructure fingerprints. A Deep Learning method was shown to achieve better accuracy than the ML methods on three different datasets [39]. Alsenan et al. [40] published a highly accurate deep-learning model based on a recurrent neural network. Zhang and Ding [41] deployed SVM and Greedy Algorithms to identify key features of CNS Drugs. An excellent review of classification models using different datasets and ML algorithms is published by Saxena et al. [42]. These qualitative classification models are helpful for the quick screening of large compound databases at early-stage drug discovery.
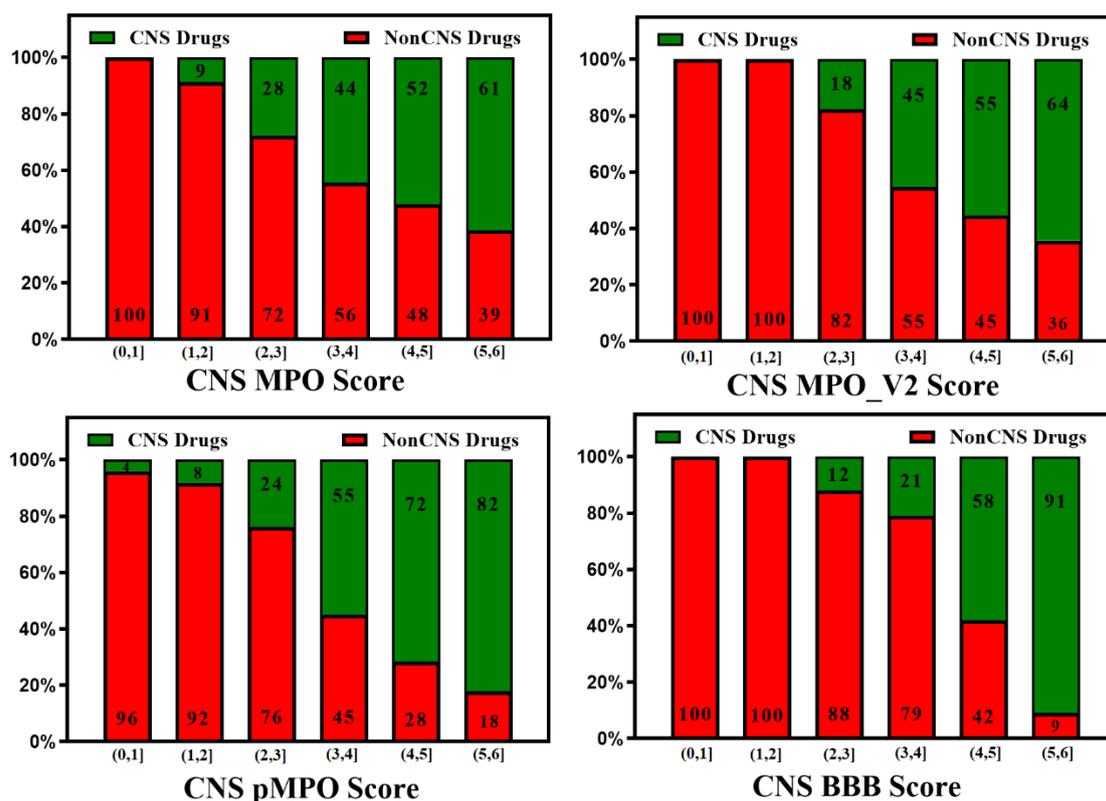

Figure 2. Relative distribution of CNS class of compounds: CNS+ (green) and non-CNS- (red). The MPO, MPO_V2, pMPO and BBB scores range from 0 to 6 (a score within a range of (4,6] means better CNS penetration). Original pMPO scores range between 0 to 1. To be consistent with MPO scores, we scaled the pMPO scores from 0 to 6.

Figure 3. Relative distribution of CNS class of compounds: CNS+ (green) and non-CNS- (red). The MPO, MPO_V2, pMPO and BBB scores range from 0 to 6 (a score in the range of (4,6) means better CNS penetration). Original pMPO scores range between 0 to 1. To be consistent with MPO scores, 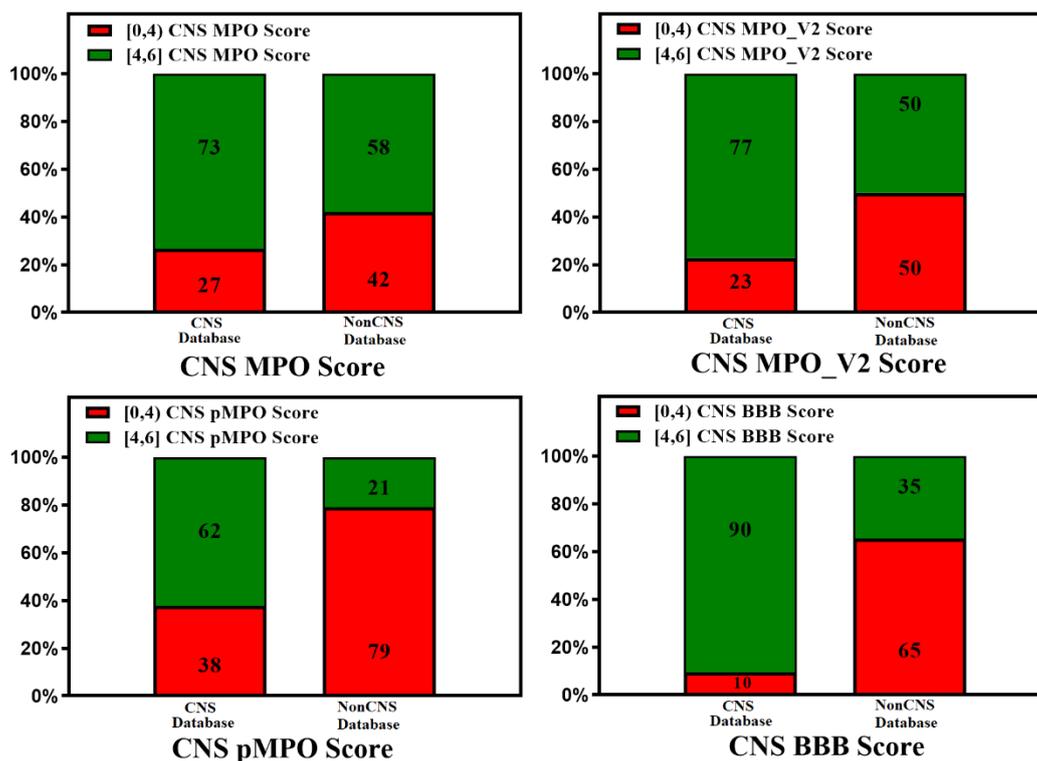we scaled the pMPO scores from 0 to 6. Percentage of CNS drugs and non-CNS drugs correctly identified (for CNS: MPO, MPO_V2, pMPO, BBB Score [4,6]; for non-CNS: MPO, MPO_V2, BBB Scores [0,4)) in their respective CNS and non-CNS database is plotted on 100% stacked bar graph for MPO, MPO_V2, pMPO and BBB Scores.

3. Active Transport across BBB (Efflux Transporters, Influx Transporters and Kp,uu)

Kp,uu (the unbound brain-to-unbound plasma concentration ratio) is an important parameter to estimate the unidirectional or bidirectional active transport of drugs across BBB via specified influx and efflux transporters. As discussed in equation 1 above, Kp,uu presents a measure of lateral efficacy of various efflux and influx transporters independent of the extent of brain or plasma tissue binding. Quantitative prediction of Kp,uu by QSAR and ML methods has been challenging [43,44]. The limited size of the training sets of compounds combined with highly variable (fivefold) experimental Kp,uu data is the main reason for the moderate performance of the models. Three experimental techniques are usually employed to estimate experimental Kp,uu: (a) microdialysis, (b) brain homogenate and (c) brain slice method. Each method has advantages and challenges; the variability within experimental results exists even within the same method based on the different experimental set-up, preclinical species and protocol used. A detection probe is implanted into the brain by surgery to estimate the unbound concentration of a molecule in the microdialysis method, which is considered an in vivo gold standard for measuring Kp,uu [45-47]. However, this method poses many technical challenges, including recovery of microdialysis probe while working with lipophilic drugs, high resource and time demanding, involves risk of brain injury and subsequent increase in BBB permeability; additionally, it necessitates use of lots of animals leading to ethical concerns, making it less applicable in the initial phases of drug discovery [48-50]. The brain homogenate method introduced by Kalvass et al. [51] involves dialyzing into a 96-well equilibrium dialysis apparatus a small sample of brain homogenate infused with the molecule. This method is used for high throughput screening of CNS drugs; unbound brain concentration is calculated from total steady-state brain concentration and free fraction [52]. One drawback of this method is that binding

properties of brain tissue could be changed during brain homogenization, unfolding new binding sites and resulting in underrepresenting available free fraction [53]. In this review, we have compiled Kp,uu values from Summerfield et al. [54,55] and Culot et al. [56]. In the brain slice method of calculating Kp,uu, animal brain slices (usually rat or mouse) are infused with test molecules incubated at 37°C in either plasma or buffer solution. The test amount of buffer or plasma solution at designed time points is withdrawn. Kp,uu is calculated as a ratio of in vivo total brain to plasma concentration (Kp) and in vitro unbound brain volume of distribution (Vu, brain) and the unbound fraction of drug in plasma (fu, plasma) in the incubated brain slices. The brain slice method has the advantage over other methods as the cell structure in brain tissue is maintained in brain slices, and this method could be developed as a high throughput screening method [53,57,58].

Frieden et al.[36] and Culot et al.[56] have calculated Kp,uu for a dataset of 41 molecules, including substrates of various efflux transporters P-gp, BCRP, multidrug resistance-associated proteins (MRPs)) and influx transporters (organic anion transporters (OATs), organic anion transporting polypeptide (OATPs), and organic cation transporters (OCTs)), making the selection important for BBB penetration study.

Accurate in silico prediction of Kp,uu using various in silico methods has been challenging as limited experimental data for Kp,uu is available in the literature, and only a few in silico models of Kp,uu with moderate accuracy have been reported [23,34-36,59-61]. Poor performance of global models of Kp,uu is understandable as (a) the training datasets do not have good coverage of chemical space, and (b) models need to account for multiple factors that affect brain penetration, e.g., experimental protocols and animal species, as explained above. In some cases, the higher-than-expected accuracy of ML models may be due to model overfitting [62]. Two pioneer Log Kp,uu QSAR models employing the PLS method for a training set of 41 marketed drugs having experimental (brain slice method) Kp,uu range of 300-fold have been developed by Fridén et al. [36]. The first model had 16 molecular descriptors, and the second only had a number of hydrogen bond acceptor descriptor with R^2 of 0.45 and 0.43, respectively. The indirect regression model employing Fridén et al. [36] Kp,uu dataset showed reasonable accuracy with an R^2 of 0.74 [59]. However, when validated against the dataset given by Summerfield et al. [44], this model resulted in poor performance. Loryan et al. [23] have trained a PLS regression QSAR model on a dataset of 79 Kp,uu values using two molecular descriptors (vsurf_Cw8 and TPSA) having moderate accuracy (R2 = 0.82 and RMSE = 0.31). However, this model's performance was unsatisfactory when validated against the Fridén et al. [36] dataset. Zhang et al. [61] developed a binary Kp,uu classification model on a dataset containing 677 and 169 molecules in training and test sets, respectively, which showed similar accuracy (R2 ~ 0.75).

Integrating Kp,uu in silico models with the knowledge of molecule interactions in BBB efflux and influx transporters [63], which influence brain permeability, can improve Kp,uu model performance [64]. Of the many influencing factors such as active uptake, brain metabolism, bulk flow, passive permeability, etc., efflux by several membrane transporters, such as P-gp has a dominant role [61,65]. P-gp is widely expressed at BBB. It transports molecules against a concentration gradient utilizing the energy of ATP hydrolysis (See Figure 4).
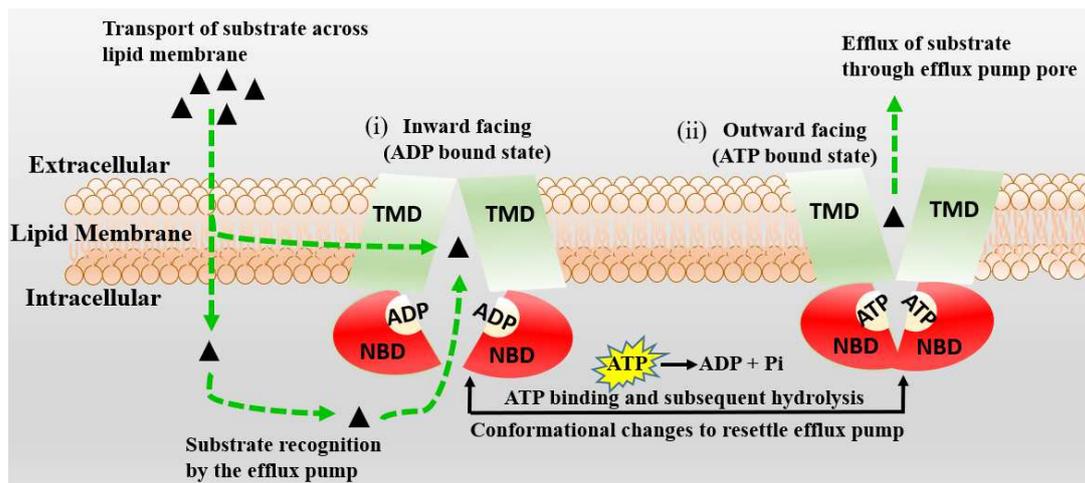
Figure 4. The schematic diagram of the proposed mechanism of P-gp (MDR1) is represented. Transmembrane (TBDs) and nucleotide (NBDs) binding domains of P-gp are presented in green and red, respectively. The P-gp substrates are shown by black triangles, which cross the BBB membrane by passive diffusion or active transport. The inward-facing, ADP-bound state structure (i) changes conformation, the NBDs dimerize, and the TMDs re-orientate to extracellular space to adopt an outward-facing (ATP-bound state) (ii). The extracellular segment's transmembrane helices in the outward-facing conformation of P-gp reorient to release the substrate. Upon ATP hydrolysis, the transporter is reoriented to the inward-facing structure, and two phosphate molecules are released.

Dolgikh et al. [60] incorporated the P-gp efflux ratio in direct and indirect regression QSAR models for Kp,uu. The performance of the Kp,uu model improved significantly by adding P-gp efflux data (R^2 enhanced from 0.39 to 0.53). For understanding the quantitative correlations between the structure of P-gp and the various molecular descriptors, different computational algorithms accompanying structure and ligand-based approaches [66,67], pharmacophore models [68,69] and machine learning methods [70] have been studied. With the availability of high-throughput P-gp efflux data using MDCK-MDR1 assays, there is an increasing effort to measure the efflux of a large number of compounds experimentally [71]. These data enabled better predictive models for P-gp efflux. Ohashi et al. [72] constructed regression models to predict the value of P-gp-mediated efflux using 2397 data entries with an extensive data set collected under the same experimental conditions. Most compounds in the test set fell within two- and three-fold errors in the random forest regression model. Available literature P-gp transporter efflux data have considerable variability as molecules tested using different protocols, cell lines and biological assays [73]. Broccatelli et al. [74] tested a dataset of 187 compounds in the Borst-derived MDCK-MDR1 cell lines to calculate P-gp Efflux Ratios (ERs). ER presents the ratio of the apparent permeability from the basolateral to the apical direction (excretory) to the apparent permeability from the apical to the basolateral direction (intake) in an overexpressing P-gp cell line. Molecules having ER ≥ 2 are typically considered P-gp substrates [75]. Available P-gp efflux data (measured in MDCK-MDR1 cells) for CNS (CNS+) and non-CNS (CNS-) drugs show that most CNS drugs have P-gp efflux ratio below 10. In cases where brain metabolism and uptake effects are negligible, it has been shown that compounds with higher efflux generally have lower Kp,uu values. (See Figure 5). As CNS distribution of a compound does not depend only on P-gp efflux, a significant percentage of compounds with lower efflux do not distribute into CNS, and a considerable percentage of compounds with higher efflux distribute into CNS. It is important to note that the analysis involves a small number of drugs (128 CNS+ and 39 CNS-) that have MDR1-MDCK efflux data and provide qualitative guidance to utilize efflux data for selecting CNS penetrant compounds.
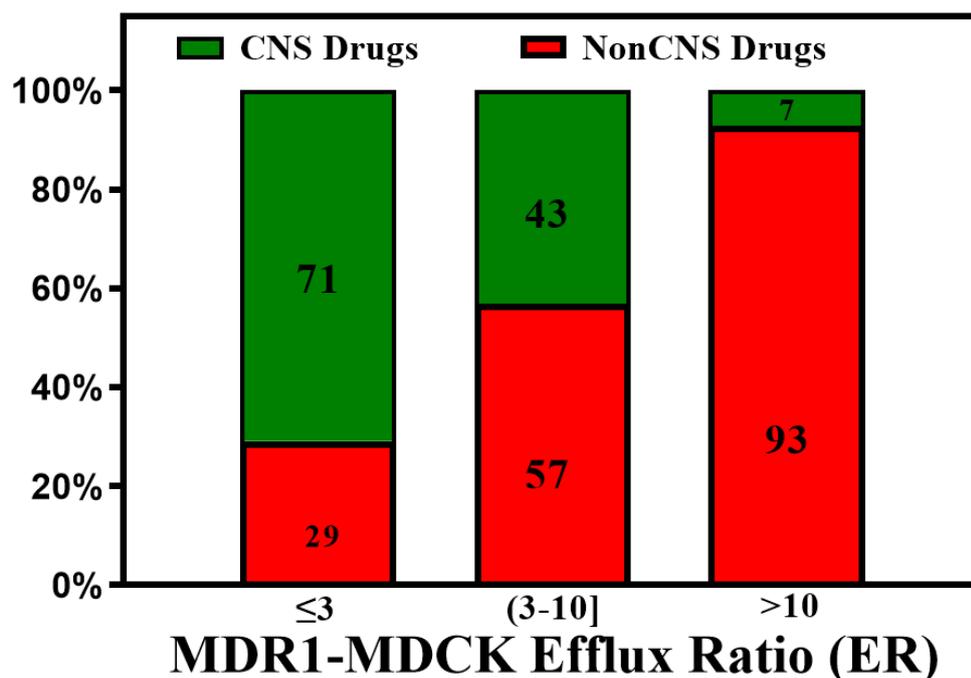
Figure 5. A plot of the CNS class of compounds CNS+ (green) and CNS- (red) against their measured efflux ratios. CNS+ compounds with good brain exposure have a higher probability of having lower efflux.

These efflux transporters modulate the brain exposure of a drug without affecting systemic exposure. It was recognized that passive permeability and P-gp efflux impact the extrusion of drugs from the brain and that in vitro Efflux Ratios (ER) can predict in vivo brain penetration [17,76]. Incorporating in vitro P-gp efflux information into the computational models improved the predictive performance of a QSAR model, as explained above [77]. Recently, Gupta et al. [78] have augmented the previous Kp,uu models by incorporating the Kp,uu model with various efflux (P-gp, BCRP) and influx (Organic Cation Transporters (OCT1, OCT2) and Organic Anion Transporting Polypeptides (OATP2B1). The model is termed the Brain Exposure Efficiency (BEE) Score. The BEE algorithm is devised based on a comprehensive series of QSAR calculations and molecular modeling simulations and implemented as an open-source calculator for predicting the unidirectional or bidirectional active transport of molecules across the BBB via specified transporter proteins. BEE score is also implemented in MOE software as an SVL utility to predict Kp,uu and Cu,b (unbound concentration of the molecule in the brain) as a function of various efflux and influx transporters, experimental methods (i.e., Kp,uu microdialysis, brain homogenate, brain slice). Kp,uu QSAR model based on the brain slice method incorporating efflux and influx transporters data performed better than models based on literature data from microdialysis and brain homogenate methods [78]. More recently, Kosugi et al. [79] reported improvement in the predictivity and coverage of application by machine learning approaches for Kp,uu prediction by incorporating in vitro P-gp and BCRP activities.

In vitro MDR1-MDCK represents a valid assay for predicting human P-gp efflux. It generally correlates well with in vivo Kp,uu of preclinical species, although other transporters (like BCRP) may cause a disconnect. Consistent Kp,uu and P-gp efflux data are available for only a limited number of drugs [55,80-82] and are plotted in Figure 6, showing limited data coverage of the drug space [83].
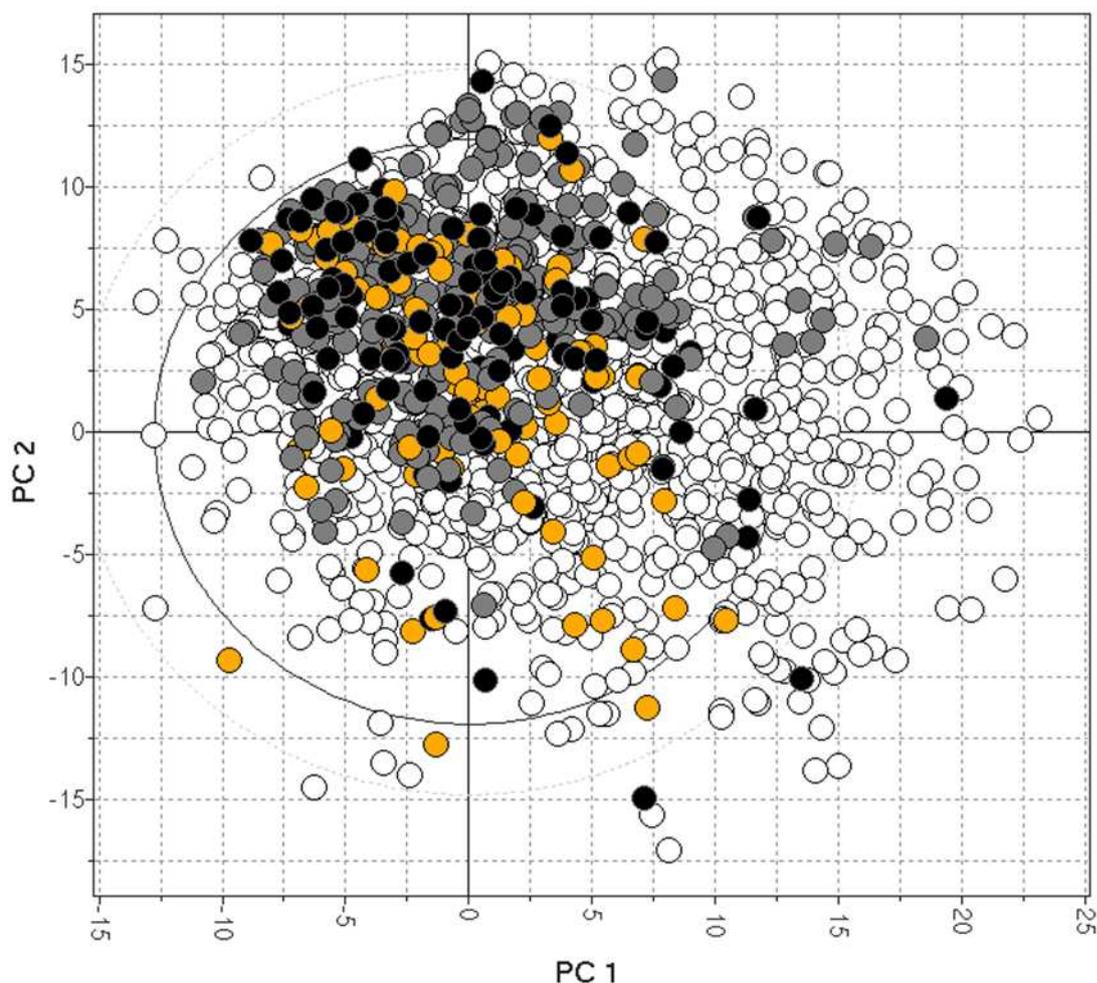
Figure 6. The bias of available drugs with MDCK (orange) and Kp,uu data (black). The grey and white dots represent CNS+ and CNS- compounds, respectively.

4. In Silico, In Vitro and In Vivo Correlations

A reliable in silico prediction method for CNS penetration can provide several advantages for discovering small molecule drugs for neurological diseases, but only if prediction results correlate with in vitro and in vivo measurements. Various CNS scoring schemes are fast and easy to apply to screen libraries at the exploratory stage of drug discovery; however, these scores correlate to some extent with in vitro efflux and animal Kp,uu as illustrated by Figure 7 and 8 that plot the relative distribution of CNS and non-CNS drugs based on P-gp efflux ratio and rat Kp,uu corresponding to different CNS scoring schemes (CNS MPO, MPO_V2, pMPO and BBB Score). Ideally, CNS drugs, i.e., the range of scores (4,6], should be more populated with drugs having ER ≤ 3, and non-CNS drugs should be heavily populated in the lower ranges of scores, i.e., (0,4]. It is encouraging to see in Figure 7 that all scoring methods do segregate the drugs with lower ER towards a higher score range (4,6], and the drugs with higher ER towarders lower range (0,3], but the segregation is not perfect. There is a lot of room for improvement. Figure 8 presents 100% stacked bar graphs for low to high MPO, MPO_V2, pMPO and BBB Scores for the rat Kp,uu dataset. Ideally, any molecule with an MPO, MPO_V2, pMPO and BBB Score in the range of (4,6] should correspond to a CNS drug. Most CNS drugs have Kp,uu in a moderate range (i.e., Kp,uu of (0.1,0.3] or < 0.3). Ideally, the in silico scoring schemes plotted in Figure 8 could be interpreted as a probability of a molecule attaining a score between 4 and 6 to have decent Kp,uu. MPO and MPO_V2 predict 33% and 32% of drugs having Kp,uu > 0.3, respectively, in the range (5,6], which is very low compared to the 62% and 68% predicted by the pMPO and BBB Score, respectively. For MPO_V2 and pMPO, drugs in range (3,4] have poor predictability (nearly 50%) in differentiating between high and low Kp,uu exposure. The MPO and

BBB Score predict that 69% and 61% of drugs score in the (3,4] range having Kp,uu ≤ 0.1, which is an improvement over the MPO_V2 and pMPO scores.
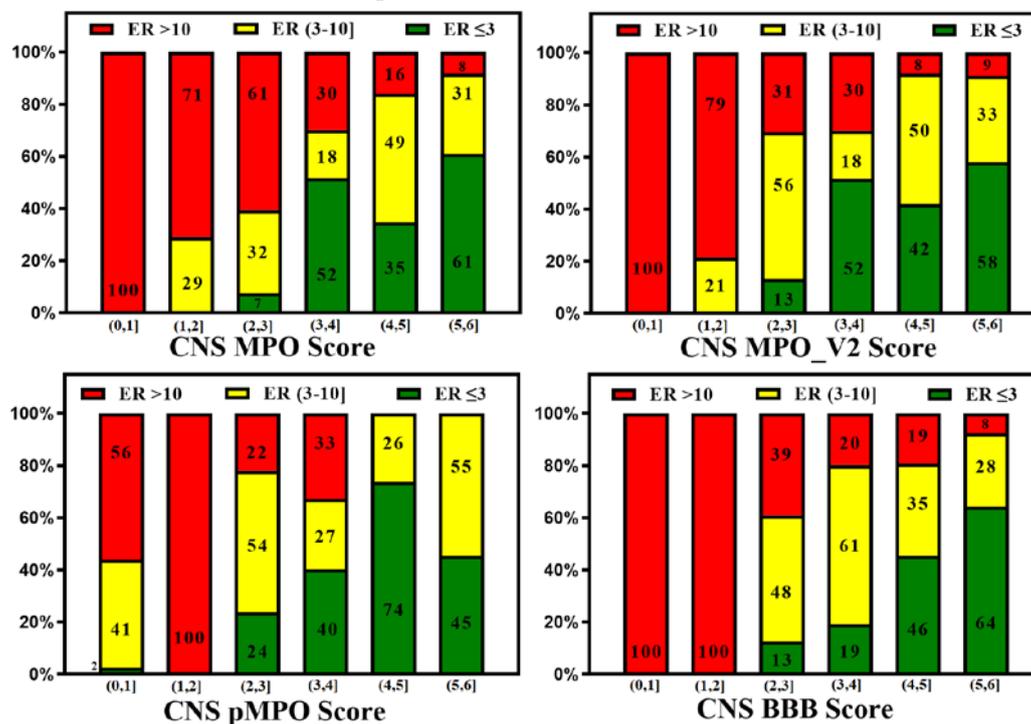

Figure 7. In silico methods (CNS MPO, MPO_V2, pMPO and BBB Score) segregate low vs. high efflux compounds, but there is much room for improvement.
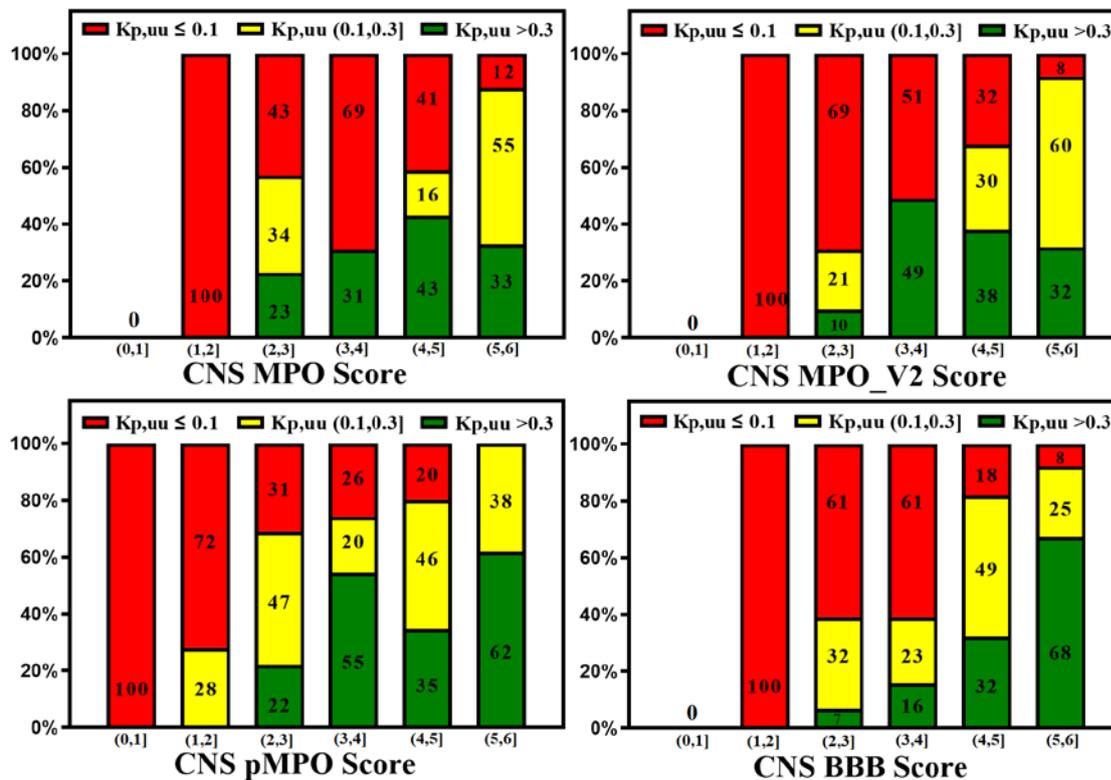

Figure 8. 100% stacked bar graphs for low to high MPO, MPO_V2, pMPO and BBB Scores for rat Kp,uu dataset. Compounds with a higher score tend to show higher unbound brain exposure.

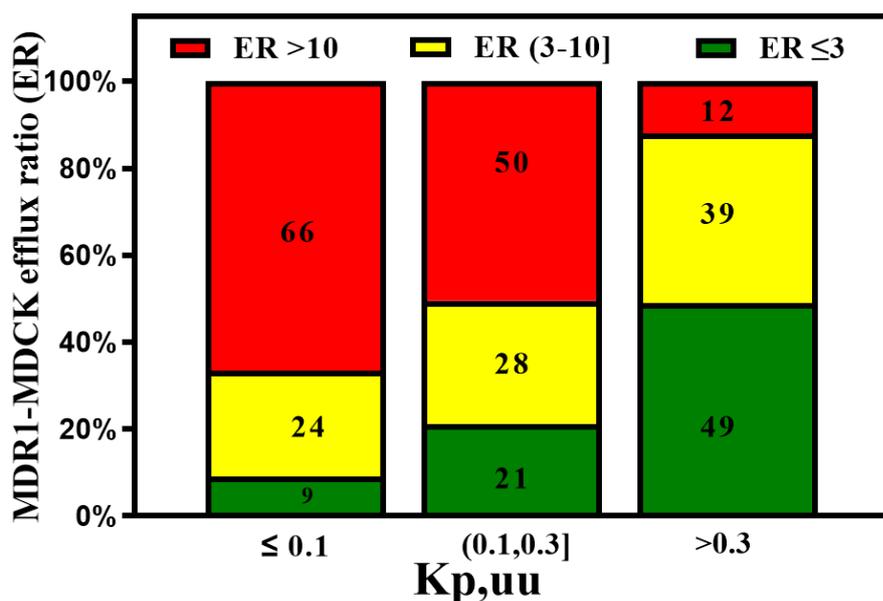On the other hand, the efflux ratio correlates well with animal Kp,uu (Figure 9).

Figure 9. The MDR1-MDCK in vitro assay predicts good in vivo Kp,uu when ER < 3. However, compounds with medium efflux (3-10) also show moderate in vivo brain exposure.

Similarly, high rat Kp,uu correlate well with human BBB penetrability. Zhang et al. [61] found the vast majority (>85%) of the CNS drugs show rat Kp,uu over 0.3, which is consistent with our analysis of the available data shown in Figure 10.
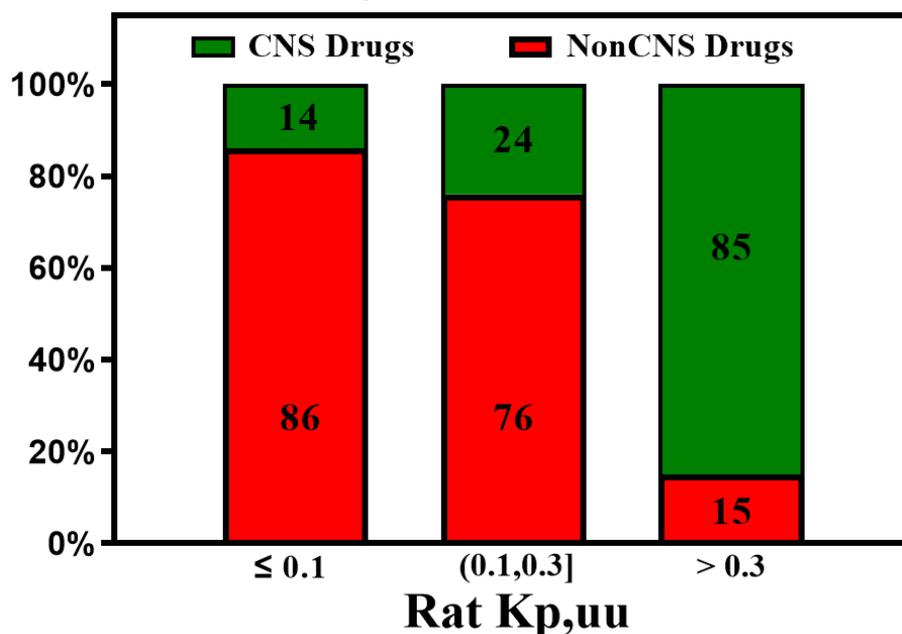

Figure 10. The Kp,uu of preclinical species is an important parameter for predicting human brain exposure. Most of the CNS drugs show rat Kp,uu over 0.3.

Animal Kp,uu measurements are also nontrivial, expensive, and time-consuming. Such measurements are made for compounds at the lead optimization of drug discovery. On the other hand, in vitro measurements are faster, less expensive, and utilized extensively at earlier stages of discovery. In silico methods that rely only on chemical structure information (like the novel Kp,uu prediction method proposed by Watanabe et al. [83]) are highly useful at the Hit identification and Hit expansion stages.

The ultimate purpose of predictive models is to improve the odds of success of drug candidates for CNS diseases. Patel et al. [84] outline a parallel analysis of previously published models for

predicting brain penetration that utilizes MDR1-MDCK efflux data as a better predictor of brain penetration. They demonstrate the ability to harness lower species preclinical data to predict human brain availability. Sato et al. [85] described a translational CNS steady-state drug disposition model to predict Kp,uu across rats, monkeys, and humans using only in vitro and physicochemical data. This model can potentially minimize animal use and improve CNS drug discovery.

5. Conclusions

The successful discovery of small-molecule drugs to treat neurological disorders requires CNS penetration of these drugs [86]. The design of CNS penetrable molecules was initially guided by the physicochemical properties of a limited set of known CNS active drugs and drugs that cause side effects in the CNS. The CNS MPO scores and later improvements were useful in simultaneously optimizing multiple properties of molecules for CNS targets. However, these scores do not always correlate well with in vitro (P-gp efflux) and animal in vivo (Kp,uu) data. The Machine Learning and Deep Learning models that classify CNS and non-CNS drugs achieve higher accuracy and are successfully applied to screen vast chemical libraries in the early phase of drug discovery but are found to be less beneficial for lead optimization. The need for higher accuracy in silico models with broader applicability is recognized, but such efforts require much more in vitro and in vivo data. These findings have prompted a generation of in vivo animal Kp,uu data and in vitro efflux data on a large number of compounds covering the available drug-like molecule space. Biogen scientists recently released in vitro efflux data on 3521 compounds [71]. Such expansion of datasets will improve the accuracy and coverage of in silico models. Accurate assessment of a drug molecule's ability to cross BBB is determined by in vivo measurements. It has been established that Kp,uu data measured in preclinical animal studies predict human brain availability. A threshold of 0.3 for in vivo Kp,uu of mouse and rat are used for estimating good human brain exposure. Moreover, there is a good correlation between animal Kp,uu data and in vitro MDR1-MDCK efflux data, which represents a valid assay for the prediction of human P-gp efflux, although other species-specific transporters may cause a disconnect. A recent publication of the physiologically-based pharmacokinetic (PBPK) model shows that in vitro efflux data can be used to predict the degree of brain penetration across species accurately.

Recent efforts to develop in silico models to predict CNS penetration of small molecules focused on applying machine and deep learning methods using animal Kp,uu and in vitro MDR1-MDCK efflux data has been less successful. Available Kp,uu data is still limited, and in silico models to predict these data with high accuracy have not yet been achieved. In addition to smaller data sets, higher variability of the measured Kp,uu values that are influenced by multiple factors such as pharmacokinetics and transporters appear to limit the accuracy of published models. Better accuracy is achieved for in silico models to predict in vitro efflux data that are measured accurately on larger sets of compounds. These in silico models are deployed to prioritize molecules with higher probability of success to cross BBB.

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