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Models for predicting blood–brain barrier permeation

Peter Aadal Nielsen[1], Olga Andersson[1], Steen Honoré Hansen[2], Klaus Bæk Simonsen[3] and Gunnar Andersson[1,*], ga@entomopharm.dk

The endothelial blood–brain barrier (BBB) ensures an optimal environment for proper neural function in vertebrates; however, it also creates a major obstacle for the medical treatment of brain diseases. Despite significant progress in the development of various *in vitro* and *in silico* models for predicting BBB permeation, many challenges remain and, so far, no model is able to meet the early drug discovery demands of the industry for reliability and time and cost efficiency. Recently, it was found that the grasshopper (*Locusta migratoria*) brain barrier has similar functionality as the vertebrate BBB. The insect model can thus be used as a surrogate for the vertebrate BBB as it meets the demands required during the drug discovery phase.

Introduction

The complex neural function of the central nervous system (CNS) depends on a highly stable environment, enabling proper firing and communication of its neurons. In vertebrates, such environment is obtained by a sophisticated blood–brain barrier (BBB), consisting of a single layer of microvascular endothelial cells that prevent the free movement of molecules from the blood into the brain, thus contributing to the homeostasis of the microenvironment. Paracellular diffusion of hydrophilic compounds is prevented by the BBB endothelial cells as a result of lateral transmembrane proteins, which form the so-called 'tight junctions'. In addition, ATP binding cassette transporters are asymmetrically located at the humoral side of the endothelial cells, preventing the entry of the majority of invading xenobiotics by efflux mechanisms [1,2].

The preservation of a proper microenvironment in the vertebrate brain, including that of humans, by a BBB also therefore prevents therapeutic drugs from entering the brain. Consequently, the BBB is a major obstacle in the discovery of new drugs for efficient treatment of CNS-related diseases. For this reason, effective CNS drug discovery programs require early information about the ability of compounds to permeate the BBB and reach their intended brain targets. By contrast, programs targeting peripheral organs also need to investigate the BBB permeability of the compounds to avoid CNS side effects. Therefore, it is of the utmost importance to address the BBB permeability during the early drug discovery phases and this requires BBB models that are useful in guiding the drug discovery process, including drug design.

Status of BBB permeation models

Techniques used for the prediction of the BBB permeability of chemical compounds have been repeatedly scrutinized for their reliability and utility [3–6]. In 2004, Abbott reviewed the status of the most commonly used technologies for measuring or predicting the BBB permeation of drug discovery compounds in vertebrates [7]. In agreement with Abbott, we conclude that *in vivo* and *in situ* rodent models for studying BBB permeation are not time and cost efficient enough to be applied as screening models during the early drug discovery phases; we have therefore omitted these models from the following discussion.

Furthermore, Abbott pointed out that there is no simple *in vitro* model that sufficiently covers the brain uptake functionalities. There is, therefore, also a need for new, more reliable, yet simple models to test BBB permeation. No single model (cell-based or non-cell-based) is thus available that can meet the requirements of the drug discovery phase. Consequently, companies have to consider using results from various *in vitro* models and techniques that, when combined, fulfill the criteria required to substitute *in*

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vivo models. Today, *in silico* models, artificial membranes for studying passive diffusion and cell-based models are commonly used to support and facilitate the drug discovery process by guiding the drug design of compounds with improved properties to optimize BBB permeability [4–6,8].

Earlier evidence that glial cells regulate the establishment and function of the endothelial barrier has resulted in a more complex and very promising model, where primary brain endothelial cells are co-cultured with primary astrocytes [9,10]. This model shows a strong correlation to *in vivo* BBB permeability models [2] but because it is time and recourse consuming, the model has not been adopted as a common screening model in early drug discovery [11].

BBB models applied in the drug discovery phase

The most frequently used *in silico* models are based on quantitative structure–activity relations (QSAR). These models are limited by the restricted accessibility of high-quality *in vivo* data during the early drug discovery phase. However, owing to low costs and high throughput, *in silico* models are useful for the preliminary assessment of BBB permeability and classification of test compounds in the hit to lead phase. The parallel artificial membrane permeability assay (PAMPA) has been used for more than 10 years by the pharmaceutical industry. This model also meets the demand in early drug discovery for high throughput and it can be useful in supporting *in silico* models in determining the permeability classification of molecules.

During the lead optimization phase, there is a need to assess the structure-related effects of drugs on paracellular diffusion and transcellular efflux mechanisms. This requires cell-based models that include both these parameters. Isolated brain capillaries and primary endothelial cultures have, for a long time, been used in BBB studies [12,13]. Primary or low passage cultures of brain capillary endothelial cells, which are close to the *in vivo* phenotype, require too many resources to be used as a screening model and the leakiness and reproducibility are other disadvantages of such a model [3,5,14,15]. Cell-based models of non-brain origin [e.g. CaCo-2 and Madin-Darby Canine Kidney (MDCK)] frequently show low correlation with BBB permeability [9,16], probably because of the discrepancy between transporters in these

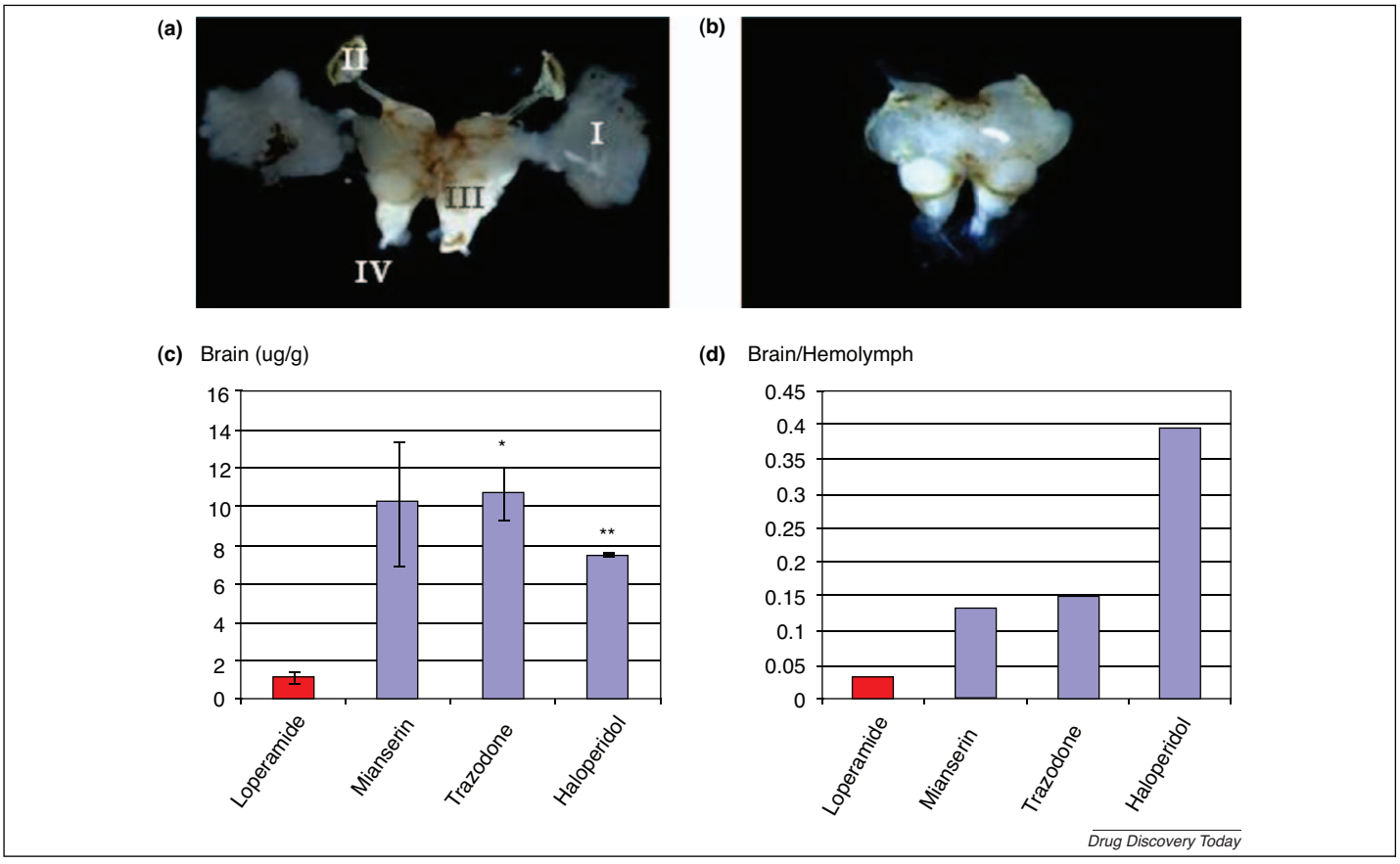

FIGURE 1

The locust brain: an association center receiving sensory input from the sense organs of the head and from posterior ganglia, with a barrier that determines the influx of molecules to the brain. **(a)** The bi-lobed locust brain with its lateral connections from the optic lobes and the compound eyes to the protocerebrum (I). Also is shown the lateral and median ocellus (II), the deutocerebral antennal (olfactory) lobes (III) and the circumesophageal connectives (IV). (For a comprehensive description of insect brain barrier structure and function and a comparison to vertebrate BBB, the readers are referred to [28,29].) **(b)** Brain prepared for analysis after elimination of the neural lamella that surrounds it. The integrity of the barrier is routinely checked using Evans Blue vital staining. **(c)** *In vivo* tests to investigate the ability of the locust brain barrier to discriminate between vertebrate CNS and non-CNS molecules. The test compounds were injected into the abdominal hemolymph and, after 15 min, the hemolymphs were collected using calibrated capillaries and the brains were dissected out and the presence of the test compounds was analyzed by liquid chromatography–mass spectrometry. The bars show that the hemolymph concentrations and the brain exposure of the CNS active compounds were significantly higher than for loperamide. **(d)** The brain:hemolymph ratios of the test compounds show the marked difference between the CNS drugs and the peripheral drug loperamide. Results are mean ± SEM. *$P < 0.05$, **<0.01 in two-tailed two-sampled Student's *t*-test against the non-CNS drug loperamide ($n = 4$).

models [14,17]. Therefore, there are concerns with the presently used *in vitro* models because most of these are of non BBB-cell origin and, thus, differ from endothelial cells in the expression of transporter proteins. Thus, there is a strong need for new models that more accurately mimic the mammalian BBB function while balancing the need from industry for high throughput and minimal time and human resource use [11,18].

Similarities between vertebrate and invertebrate brain barriers

We were inspired by the discussion of Bundgaard and Abbott [19] on the presence of a glial barrier in invertebrates (including insects) based on their prediction that ancestral vertebrates had a glial BBB that evolved into endothelial cells [19]. Furthermore, recent homologies have been shown between *Drosophila melanogaster* (*Dm*) septate junction and mammalian tight junction claudins (required for paracellular barrier function), which strongly indicates that there is solid evolutionary conservatism between these two barrier systems [20]. This notion is further supported by the observation of homologies between vertebrate claudins and paracellular barrier claudins in *Caenorhabditis elegans* [20,21]. Thus, the vertebrate and the insect brain barriers comprise different cell types. However, the fundamental molecular mechanisms for nervous system protection were established very early during the evolutionary process and they are probably a result of strong selection pressure during the very early evolution of complex organisms with complex nervous system functions.

These homologies between the insect and mammalian brain barrier structures and functions encouraged us to investigate the relevance of the insect brain barrier system as a model for the mammalian BBB. We focused on the migratory locust (*Locusta migratoria*) because this species has a brain size that is relevant for quantitative drug measurements. Furthermore, electron microscopy studies have revealed that the glial paracellular barrier in locusts consists of tight junctions, as occur in the mammalian BBB [22].

The insect model

Initially, we documented the *in vivo* brain uptake of a selected number of test compounds, which have been well characterized in terms of BBB permeability in humans. We selected the test compounds according to several physicochemical key characteristics. The CNS drugs were neuroleptics (haloperidol) and antidepressants (mianserin and trazodone). For a peripherally acting drug, we used the P-glycoprotein (Pgp) substrate loperamide. This compound is particularly interesting because it is not taken up in the vertebrate brain despite the fact that it displays structural and physicochemical properties that are characteristic of CNS active compounds (e.g. high lipophilicity, basic center as main functionality and relatively low molecular weight). All three psychotropic drugs (haloperidol, trazodone and mianserin) were present in the insect brain (measured as brain:hemolymph ratio) in amounts that were significantly higher than the non-CNS drug loperamide (Fig. 1). Mianserin has been reported to induce behavior effects in the locust [23] and our measurements of this drug in the locust brain could explain the behavior changes.

We developed an *ex vivo* model to expose the locust brains to a constant concentration of a test compound (Fig. 2). As expected from the *in vivo* study, there was a high uptake of the CNS drugs mianserin and trazodone. However, colchicine, which is a Pgp substrate, and the cationic hydrophilic drug atenolol both showed low uptake (Fig. 2a). Thus, the locust BBB excludes compounds that are vertebrate Pgp substrates and compounds that are too hydrophilic to permeate the vertebrate BBB. By contrast, vertebrate CNS drugs, including the antidepressant trazodone, cross the locust brain barrier.

Pgp transporter system

To reduce the risk of developing compounds that are Pgp substrates, the pharmaceutical industry uses *in vitro* models based on cells overexpressing the multidrug resistance protein 1 (mdr1) for extensive screening. The low brain uptake of loperamide in the insect *in vivo* study reflects what is seen for loperamide in rodents and humans. Here, loperamide is effluxed

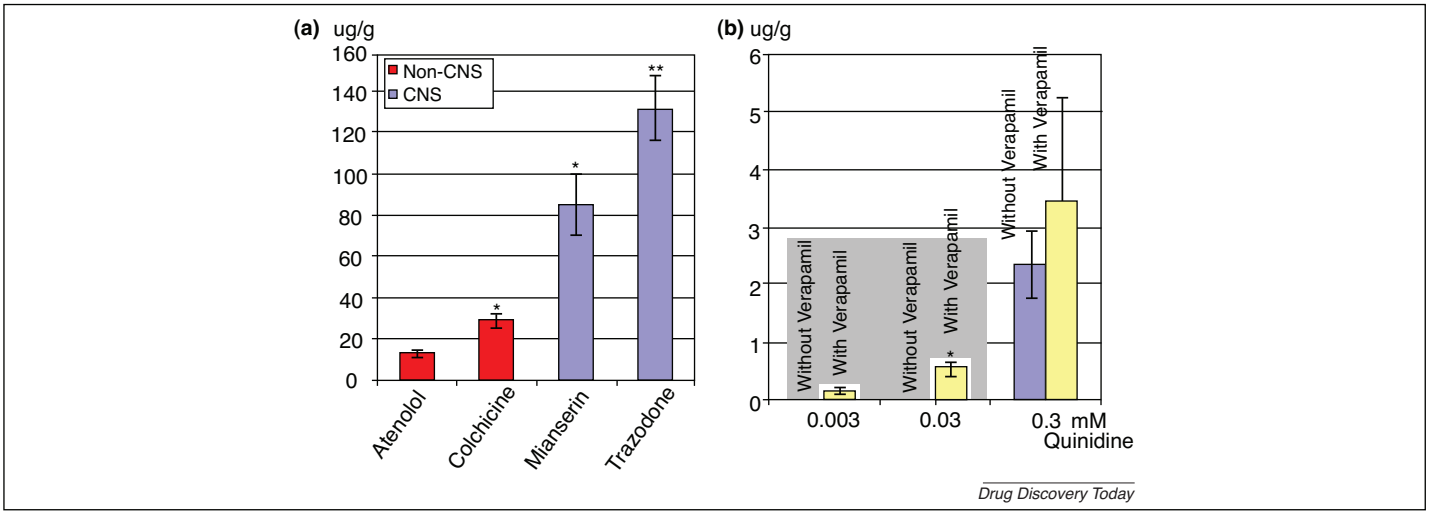

FIG. 2

Discrimination of the locust brain barrier between vertebrate CNS and non-CNS drugs *ex vivo* and inhibition of the locust Pgp efflux transporter by verapamil. A cut was made through the frontal part of the head, through the esophagus; this frontal cuticle shell with brain, eyes, antenna and nerve associations was then exposed (for 5 min) to the test compound in a 300 μl microtiter well. After exposure, the brain was dissected according to the description in Fig. 1. **(a)** Red bars, CNS drugs; blue bars, non-CNS drugs. **(b)** Locust brains were exposed to increasing concentrations of the Pgp substrate compound quinidine in an *ex vivo* model. Whereas no quinidine was detected in the brain samples at low exposures (0.003 and 0.03 mM), co-administration with the human Pgp inhibitor verapamil significantly increased the brain uptake of quinidine. The shaded background area shows the range of a progressive increase in Pgp transporter inhibition by quinidine in L-MDR1 mouse cells [30] indicating that the two-test systems exhibit the same sensitivity and thus operate within the same test concentration range ($n = 4$). Results are mean concentration per g locust brain tissue ± SEM. *P0.05, **0.01 in a two-tailed two-sampled Student's t-test.

by the Pgp transporter system and its uptake can be affected by Pgp transporter inhibition or elimination [24,25]. Given that Pgp-mediated drug efflux is a major hurdle in CNS drug discovery, it is important to prove the utility of the locust model by confirming the presence of the Pgp efflux mechanism in this model. To accomplish this, we exposed locust brains in the *ex vivo* model to various concentrations of the Pgp substrate inhibitor quinidine. At the lower concentrations (3 and 30 μM), there was no uptake of quinidine in the locust brain, but there was a dose-related increase at higher concentrations (Fig. 2). This suggests that the Pgp efflux transporter is saturated at higher concentrations, resulting in an increased uptake. Co-treatment with quinidine and the Pgp inhibitor, verapamil significantly increased the uptake of quinidine at the low dose range, whereas the uptake was unaffected at the high dose where the Pgp proteins were expected to be saturated. Our observation in the locust model is supported by a recent study showing that the Dm homolog (Mdr65) to the human ABC transporter MDR/Pgp is inhibited by the human Pgp transporter inhibitor cyclosporin A [26]. In addition to the Pgp transporter, several other transporters have been identified, although the *in vivo* relevance of these remains to be evaluated. Pgp is still currently considered to be the most important transporter in the mammalian BBB [27].

Conclusions

It has been shown that the locust *in vivo* and *ex vivo* models discriminate compounds from paracellular diffusion and transcellular transport over the hemolymph–brain barrier in the same way as in the vertebrate BBB. As such, these models could represent a markedly improved prediction of the vertebrate BBB permeability that can be used to support the early drug discovery process. The presence of both the mammalian ABC transporter homolog Mdr65 and the mammalian claudin homologs in the insect brain barrier strongly indicate that insect glial cells, similar to vertebrate vascular endothelial cells, have diffusion barriers and efflux transporters that are both structurally and functionally conserved during evolution [26].

The locust models have advantages at several levels. First, they are natural biological brain barriers that retain their biological integrity and control functions during the test procedure, similar to vertebrate *in vivo* BBB models. Second, the insect *in vivo* model enables screening of compounds with low solubility, requiring solubilizing agents that are not compatible with *in vitro* test systems (e.g. with a low pH). A third advantage of using insect models is the fact that only a small amount of compound material is needed for the insect models and resynthesis is seldom necessary.

During the drug discovery process, there is a need to use models (*in silico* or non-cell-based models) for the classification of the numerous chemical compounds in the hit identification phase. Later on, during the lead identification and optimization stages, there is a documented need for cell-based models with high reproducibility and reliability. The locust models fulfill these criteria and, in addition, they balance the commercial requirements compared with cell-based *in vitro* models. We propose, therefore, that insect models should be applied in the future as a compound selection filter during the early drug discovery process.

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Peter Aadal Nielsen[1]
Olga Andersson[1]
Steen Honoré Hansen[2]
Klaus Bæk Simonsen[3]
Gunnar Andersson[1,]

[1]*EntomoPharm ApS, Fruebjergvej 3, 2100 Copenhagen, Denmark*
[2]*Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark*
[3]*Division of Discovery Chemistry and Drug Metabolism and Pharmacokinetics, H. Lundbeck A/S, Ottiliavej 9, 2500 Copenhagen, Denmark*