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An Insect-Based Ex Vivo Blood Brain Barrier Efflux Assay

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Abstract

Drug efflux activity of ABC transporters, at the human blood brain barrier (BBB), constitutes a crucial challenge for central nervous system (CNS) drug development. Accordingly, early screening of CNS drug candidates is pivotal to sort out those whose brain uptake is substantially affected by efflux activity. In this context, affordable, simple, high-throughput and predictive screening models are required. It has recently been proposed that the grasshopper (locust) could be exploited as an ex-vivo model for drug BBB permeability assessment, as it has shown some similarities to vertebrate models. The p-glycoprotein (p-gp), encoded by the ABCB1 gene, is described as the most potent efflux pump that modulates drug brain disposition, so identification and characterization of such a transporter in the locust model is essential to demonstrate its utility and validity for drug development. The present work entails transcriptomic profiling followed by amino acid-based homology analysis of locust genes, in parallel to functional investigations using rhodamine 123 as a selective p-gp substrate. A protein with high sequence similarity to ABCB1 was found in the locust brain transcriptome, which indicates a conserved mechanism of brain efflux activity between insects and vertebrates. Functionally, the developed locust model showed a kinetic behavior comparable to those obtained from in vitro cell models such as the MDCKII cells expressing p-gp. Overall, the locust ex-vivo BBB model holds promise as a cheap model with a high-throughput screening potential in the early discovery phase of CNS drugs.

Keywords: Blood brain barrier, P-gp efflux transporter, Functional characterization, Transcriptome and homology analysis, Ex-vivo model.

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Introduction

ATP-driven efflux transporters have been described as the major challenge for central nervous system (CNS) drug development (Glavinas et al., 2004; Franke et al., 2010; Shawahna et al., 2011; 2013). More specifically, the p-glycoprotein transporter (p-gp, mdr1), encoded by the ABCB1 gene, is increasingly recognized as a key molecular determinant of drug permeability through the blood brain barrier (BBB), due to its multi-specificity and potency, thus limiting the clinical success of drug candidates (Glavinas et al., 2004; Franke et al., 2010). Consequently, there have been different in vitro and in vivo models established to primarily test new CNS drugs for restricted entry into the brain. Nonetheless, the available in vitro models have not been satisfactory, as a realistic representation of the BBB, for accurate prediction of kinetic behaviors of drugs targeted to the brain. This can be explained by the fact that in vitro models lack the structural and functional complexity of the BBB in vivo (Naik et al., 2012; Geldenhuys et al., 2012; Weksler et al., 2013). Although in vivo models are better suited for this task, ethical acceptability and technical inconveniences as well as high cost complicate their use. Consequently, there have been ongoing efforts to develop other physiologically based pharmacokinetic models, bearing in mind that important attributes of an acceptable BBB model include reduced expenditure, simplicity, high-throughput, reproducibility and good predictivity of pharmacometrics. In this respect, the grasshopper (locust) has been proposed as an insect-based ex vivo model for BBB permeability screening (Nielsen et al., 2011; Andersson et al:, 2013), relying on the hypothesis that basic physiological mechanisms have been conserved between insects and vertebrates (Giacomotto et al., 2010; Matsumoto et al., 2011; Zhang, et al., 2012).

The newly developed ex vivo BBB screening model, by Andersson et al. (2013), affords a high throughput screening capability, besides being inexpensive, and relies on the use of an intact and viable brain of locust under controlled in vitro-like exposure conditions. As evidenced in vertebrate models, preliminary ex vivo uptake studies have interestingly revealed that the locust BBB model permitted differentiation between low and high permeability compounds as well as those with and without a p-gp substrate character. Furthermore, the generated ex vivo uptake data strongly correlated with in situ perfusion data (Andersson et al., 2013).

In addition to gaining information on transporter's functional and molecular interactions with drugs, it is also essential to investigate gene expression patterns of transporters, during drug testing model development (Volpe, 2010), in order to elucidate conserved machineries among species and, hence understand the physiological basis of drug brain pharmacokinetics. Such characterizations will thereby endow in-depth insights into our model and certainly render it reliable for drug screening. In this report we present the gene sequence for a putative p-gp protein, and provide data that suggest a p-gp activity which is the major player of brain defense mechanism at the BBB. At gene level, transcriptome analysis was performed followed by a homology

study. Functionally, rhodamine 123 (Rhod) was utilized as a selective p-gp substrate, in order to determine the kinetic parameters, including the substrate affinity constant (K_m) and maximal transport velocity (V_{max}).

Materials and Methods

Animals

Two locust species; Desert locusts, Schistocerca gregaria (Sg) and Locusta migratoria (Lm) were obtained from a commercial animal breeder (Petra Aqua, Prague, Czech Republic). After arrival, the locusts were housed under crowded conditions in an insect room and adapted to a 12:12 hour dark/light cycle. They were also maintained in colonies at a local terrarium temperature of 30^{o}C to 34^{o}C and fed Chinese cabbage and oat. All experiments were carried out on fifth instar locusts and 2 to 3 weeks after adult emergence.

Chemicals

Rhodamin 123 (Rhod) and HEPES were purchased from Sigma-Aldrich (Copenhagen, Denmark). RNeasy® mini kit for total RNA extraction was supplied by Qiagen (Hilden, Germany). All other chemicals were analytical reagent grade.

Locust Brain Dissection

Using fine forceps, a cut was made through the frontal part of the head separating the brain with its nerve connections to the compound eyes, antenna and ocelli, existing in the cuticle. Afterwards, the interior of the cuticle was stained with Evans blue and the brain was immediately dissected out in locust buffer, under the light microscope. For the transciptome analysis, the neural lamella enclosing the brain was further removed, while it was retained for the ex vivo studies.

Transcriptome and Bioinformatic Analysis

Total RNA was isolated from brain tissue of 22 Lm locusts using the RNeasy Mini Kit according to the manufacturer´s instructions with DNAse I treatment. Extracted RNA was assessed for purity and integrity using spectrophotometry (Nano drop 1000) and agarose gel electrophoresis, respectively, and subsequently kept at -80°C until use. Transcriptome sequencing and data processing was performed by BGI-Hong Kong. Briefly, Poly(A) mRNA was isolated from total RNA using magnetic Oligo (dT) beads and fragmented by nebulization. The fragmented mRNA was then used as template for making double stranded cDNA, which was subjected to end reparation and single nucleotide A (adenine) addition, and equipped with adapters. The fragments were then size selected to 200 bp and PCR amplified. Finally, the library was sequenced on an Illumina HiSeq™ 2000 system. Clean reads were assembled into transcripts using the Trinity software package (Grabherr et al., 2011). Transcripts belonging to the ABCB gene subfamily

were identified by reciprocal BLAST searches using known ABCB genes as queries. Protein alignments were performed in MEGA 6 (Tamura et al., 2013)

using Clustal W with default parameters, and the alignment file was processed in BoxShade (http://www.ch.embnet.org/software/BOX_form.html) to make it into a publishable figure. Protein domains were predicted using the Simple Modular Architecture Research Tool (SMART) web interface (http://smart.embl.de/ (Letunic et al., 2012)).

Ex vivo Kinetic Studies

Eight concentrations of Rhod in HEPES insect buffer (pH 7.2), in the range of 0.3-500 uM, were prepared by diluting DMSO stock solutions. The kinetic experiments were started by loading the dissected brains of Sg locusts with different Rhod concentrations via incubation in a 96-well plate containing 150 µL solutions cooled to 4°C in a block thermostat for 30 minutes. Next, brains were removed from the wells and washed 3 times with ice-cold locust buffer. Subsequently, secretory phase was initiated by transferring Rhod-loaded brains to new microplate wells containing 250 µl of blank locust buffer heated to 30°C or cooled to 4°C for control brains. Afterwards, 30 µL aliquots from the incubation media were withdrawn at different time points (1-60 minutes) and transferred into vials until analysis.

Analysis by LC-MS

Samples were assayed by liquid chromatography–mass spectrometry (LC-MS), using an Agilent 1200 HPLC coupled to a MSD 1100 detector (Agilent Technologies, Walbronn, Germany). Three microliters of sample solution were injected via an autosampler to be eluted, using a Kinetex™ 2.6 µm C8 100 Å column (50 x 4.6 mm) (Phenomenex®, Denmark) and, then directed to the mass spectrometer. The mobile phase [A: 0.1% formic acid in water; B: 0.1% formic acid in methanol] was pumped at a flow rate of 0.5 mL/min, with a gradient that increased linearly from 5 to 90% B over 5 min and held at 90% B for 1 min, followed by a linear decrease to 5% B for 0.1 min. MS analysis was performed by electrospray ionization, operating at a spray voltage and temperature of 4000 V and 300°C, respectively. The $[M+H]^+$ ion of Rhod was detected in positive single ion monitoring mode at 345.2 m/z, and quantification was performed with a standard calibration curve in the linear range of 1-100 nM.

Data Analysis

K_m and V_{max} values of Rhod transport were determined from the ex vivo studies. To this end, sampling at different time points was performed, at both 30°C and 4°C (control brains), following incubation of preloaded brains in fresh buffer media. Correction for active transport, at each time point, was carried out by subtracting the Rhod amount passively transported at 4°C from the total flux (secretory flux) of preloaded brain, at 30°C. The time course of active transport component, at each concentration, was monitored to determine

the initial linear phase wherein the transporter pumps Rhod out of the brain at a constant rate, denoted as the initial velocity (Vo) and represented as the linear regression slope. The graphical representation of Lineweaver-Burk equation (reciprocals of Vo versus Rhod concentration) resulted in linear correlations ($R^2 \geq 0.95$), where the y-intercept and the slope represent $1/V_{max}$ and K_m/V_{max}, respectively. In this study, all data are expressed as the average of 3-5 independent experiments (each was done in 3-4 replicates) ± standard deviation. Statistical analysis was performed by Student´s t-test, as well as one-way analysis of variance (ANOVA) with the post hoc test (Tukey) used for multiple comparison analysis (SPSS program, USA) and differences were considered significant at a level of $p < 0.05$.

Results and Discussion

In this study, our goal was to investigate the existence and potential similarities in efflux activity between locust and human BBB, from a gene and functional standpoint.

Determination of Sequence Identity of Locust Gene Transcripts Showing Best Matches with Human ABCB1

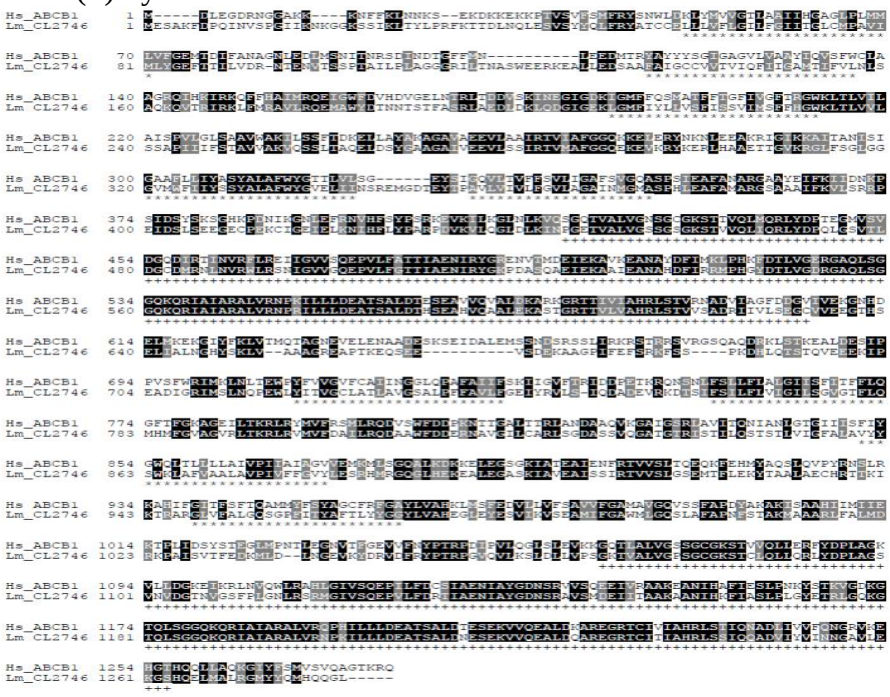
Sequence conservation is broadly believed to reflect similar functionality, and in order to identify putative orthologs of the p-gp in locusts we initially annotated all ABCB genes found in the locust brain transcriptome, using reciprocal BLAST searches. Using this method, we could identify five transcripts showing high similarity to human ABCB genes (Table 1). This suggests the presence of a conserved BBB efflux mechanism between insects and vertebrates (Mayer et al., 2009; Broehan et al., 2013; Dermauw et al., 2013). Figure 1 shows the alignment of the human p-gp protein with the most closely related locust protein (CL2746) encoded by the brain transcriptome. The overall similarity between ABCB1 and this locust transcript was 48 % amino acid sequence identity (Table 1). Sequence analysis predicts the presence of two conserved ABC transporter transmembrane domains, represented by asterisk (*) symbols in the figure, and two conserved ATP-binding sites, represented by plus (+) symbols in the figure. A previous study identified a Drosophila ortholog (mdr65) of the human ABCB1 protein, and found that flies mutated in the gene encoding this protein showed impaired xenobiotic efflux activity at the BBB (Mayer et al., 2009). Thus, it is likely that a similar function is mediated by the locust ABCB1 ortholog (CL2746). Even though the ABCB1 is considered the most important mediator of xenobiotic efflux activity at the BBB (Shawahna et al., 2011), other ABCB transporters may also contribute to this process, as both ABCB8 and ABCB10 have been shown to be present in brain microvessels of five mammal species including human (Kim et al., 2006).

Interestingly, inhibition of orthologues of the p-gp in insects, like H.virescens and D.melanogaster, using the respective p-gp inhibitors; quinidine and verapamil, increased pesticide toxicity in resistant strains, which functionally suggests a highly conserved efflux activity (Lanning et al., 1996; Strycharz et al., 2013). Importantly, the human ABCB 6, 7, 8 and 10 genes have been also proved to contribute in drug efflux, as illustrated by their relatively altered expression in multidrug-resistant cell lines (Gillet et al., 2004; Liu et al., 2005; Elliott and Al-Hajj., 2009). In particular, the ABCB8 has been associated with melanomas, exhibiting resistance to a broad spectrum of chemo-therapeutics (Chen et al., 2009).

Table 1. *Human ABCB Genes and Predicted Hits in Locusta Migratoria, with their Amino Acid Identity, Protein Length, as well as Number of ATP-binding and Transmembrane Domains*

Gene	Length	ATPase domains	TM domains	Human Homolog	Length	% Identity
CL2746	1282	2	2	ABCB1	1280	48
CL3788	835	1	1	ABCB6	842	51
CL4845	784	1	1	ABCB7	752	54
CL5917	598	1	1	ABCB8	630	56
Unigene21195	660	1	1	ABCB10	595	54

Figure 1. Multiple Sequence Alignment of the Human ABCB1 and the Locust Transcript (Lm2746). Identical Amino Acids are Highlighted in Black, While Similar Amino Acids are Highlighted in Grey. Transmembrane Regions are Marked with Asterisk (*) Symbols, and ATP-binding Domains are Marked with Plus (+) Symbols

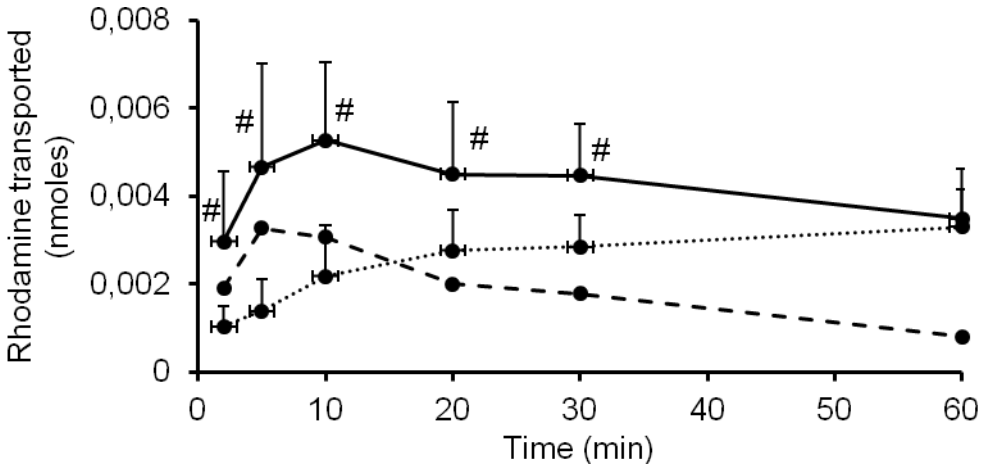


Kinetic Analysis of Rhod Transport across Locust BBB

Rhod transport at a concentration of 1 uM was examined, as a function of time at both 4°C and 30°C (Figure 2). Incubating Rhod-preloaded brains at both temperatures depicted a temperature-dependent transport of Rhod and, accordingly a combination of passive diffusion and active transport which is a saturable process. At low temperatures (e.g., 4°C), cellular energy levels are decreased and, hence Rhod flux is merely driven by passive diffusion, which is different from active transport that particularly occurs at elevated temperatures in the presence of cellular energy input. So, at 30°C, total Rhod flux, observed in the incubation media, corresponds to both passive and active transport mechanisms. On the other hand, both transport mechanisms are time-dependent, as Rhod flux, at both temperatures, increased over the incubation time (Ott et al., 2010). It is noteworthy that Rhod flux at 30°C is significantly higher than that at 4°C, which suggests the important role of active transport in its excretion from locust brains. Contrary to passive transport, Rhod flux started to gradually decline after a maximum increase at 10 min of post-incubation, which might indicate a decreased activity of the efflux transporter as a consequence of decrease in ATP consumption (Jutabha et al., 2009) or decrease in the pool of Rhod inside the brain. We thereby can infer that Rhod transport characteristics across locust BBB, based on time, temperature and mechanism, are in accordance with the results reported for other traditionally

used systems (Troutman and Thakker., 2003; Annaert and Brouwer., 2005; Forster et al., 2012).

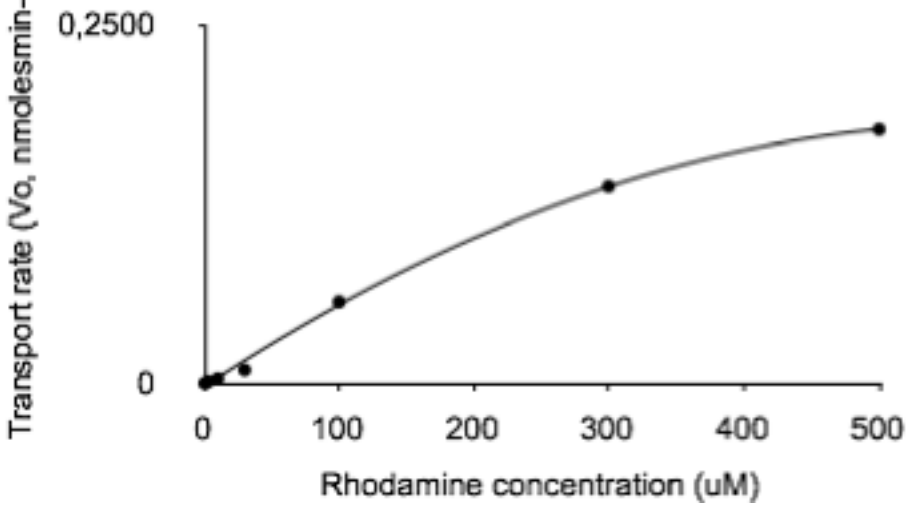
Figure 2. Time Course of Rhodamine 123 Flux across Locust Brain Ex-vivo. Brains, Preloaded with 1 uM Rhodamine 123, Were Incubated in Blank Buffer at 30°C and 4°C for Increasing Periods of Time (1-60 min). Active Contribution (Dashed Line) Was Calculated by Subtracting Passive Contribution (Dotted Line) from the Total Flux (Solid Line) Observed in the Bathing Medium at 4 °C and 30°C, Respectively.



From a kinetic point of view, involvement of transporters e.g., efflux pumps, at biological barriers, is referred to as saturable transport mechanism

and can be evaluated and/or expressed by kinetic parameters. For describing Rhod export from preloaded brains in terms of saturable transport mechanism, experiments were carried out at 30°C, using different Rhod concentrations and under linear conditions of transport activity, which ultimately enabled the calculation of K_m and V_{max} values. At all concentrations, data suggested that Rhod export into the incubation medium, via the active transport component, was achieved at a constant velocity (Vo) within the first 2 minutes and, then declined mostly after 5 min. The values of Vo progressively increased, as a function of Rhod concentration, until a sigmoidal pattern was attained (Figure 3), suggesting a single saturable transport component that fits with Michaelis-Menten equation. Fitting data with the reciprocal plot of this equation (Lineweaver-Burk plot) resulted in the respective K_m and V_{max} values: 17.3 ± 14.2 uM and 0.0022 ± 0.0023 nmoles.min^{-1}.

Figure 3. Rates of Rhodamine Flux across Locust Brain Ex-vivo, at 30°C. The Line Represents Fitted Data Based on Michaelis-Menten Equation. Data Points Correspond to Efflux Rates Derived from Linear Regressions of Active Transport Components of Each Concentration Over 2 min at 30°C. Reciprocal Plots Resulted in K_m and V_{max} Equal to 17.3 ± 14.2 uM and 0.0022 ± 0.0023 nmoles.min^{-1}, respectively, with Linear Coefficient Values (R^2) ≥ 0.95



In general, the measured K_m value for a particular substrate relies on the model used and, hence inter-system variations in such parameters are usually reported. Interestingly, the calculated K_m value of Rhod, using the locust model, is close to that estimated (17.5±2.8 uM) in mdr1-expressing MDCKII cells (Forster et al., 2012), which are widely used as a non-cerebral BBB penetration model (Wang et al., 2005; Hellinger et al., 2012), besides that in HEK293 cells (16.58 ±23.01 uM), which express mdr1, as well (Huang et al., 2013). Other efflux assays have also reported close Rhod K_m values like 13.5 uM in membrane vesicles (Shapiro and Ling., 1997) and 8.4 uM in sandwich-cultured rat hepatocytes (Annaert and Brouwer., 2005).

Conclusion

The strong correlation of Rhod transport characteristics and kinetic behavior, with those observed in in vitro/ex vivo settings specifically established for investigating P-gp efflux activity, strikingly demonstrates the existence of a comparable drug efflux pump across the locust BBB. These functional results are in agreement with the gene data which further support a conserved machinery of chemo-protection at the locust BBB, mediated by a protein with high sequence similarity to human ABCB1. Our findings demonstrate the capability of locust ex vivo model to categorize or screen new CNS drugs, as potential p-gp substrates and, thereby could be viewed as an alternative permeability assay.

References

Annaert, P. P., Brouwer, K. L. 2005. Assessment of drug interactions in hepatobiliary transport using rhodamine 123 in sandwich-cultured rat hepatocytes. Drug. Metab. Dispos. 33(3):388-94.

Andersson, O., Hansen, S.H., Hellman, K., Olsen, L.R., Andersson, G., Badolo, L., Svenstrup, N., Nielsen, P.A. 2013. The grasshopper: a novel model for assessing vertebrate brain uptake. J. Pharmacol. Exp. Ther. 346(2):211-8.

Broehan, G., Kroeger, T., Lorenzen, M., Merzendorfer, H. 2013. Functional analysis of the ATP-binding cassette (ABC) transporter gene family of Tribolium castaneum. BMC. Genomics. 16;14:6.

Chen, K. G., Valencia, J. C., Gillet, J. P., Hearing, V. J., Gottesman, M. M. 2009. Involvement of ABC transporters in melanogenesis and the development of multidrug resistance of melanoma. Pigment. Cell. Melanoma. Res. 22(6):740-9.

Dermauw, W., Osborne, E. J., Clark, R. M., Grbić, M., Tirry, L., Van Leeuwen, T. 2013. A burst of ABC genes in the genome of the polyphagous spider mite Tetranychus urticae. BMC. Genomics. 10;14:317.

Elliott, A and M., Al-Hajj, M. A. 2009. ABCB8 mediates doxorubicin resistance in melanoma cells by protecting the mitochondrial genome. Mol. Cancer. Res. 7(1):79-87.

Forster, S., Thumser, A. E., Hood, S. R., Plant, N. 2012. Characterization of rhodamine-123 as a tracer dye for use in in vitro drug transport assays. PLoS. One. 7(3):e33253.

Franke, R.M., Gardner, E.R., Sparreboom, A. 2010. Pharmacogenetics of drug transporters. Curr. Pharm. Des. 16(2):220-30.

Geldenhuys, W.J., Allen, D.D., Bloomquist, J.R. 2012. Novel models for assessing blood-brain barrier drug permeation. Expert. Opin. Drug. Metab. Toxicol. 8(6):647-53.

Giacomotto, J., Ségalat, L. 2010. High-throughput screening and small animal models, where are we?. Br. J. Pharmacol. 160(2):204-16.

Gillet, J. P., Efferth, T., Steinbach, D, Hamels, J., de Longueville, F., Bertholet, V., Remacle, J. 2004. Microarray-based detection of multidrug resistance in human tumor cells by expression profiling of ATP-binding cassette transporter genes. Cancer. Res. 64(24):8987-93

Glavinas, H., Krajcsi, P., Cserepes, J., Sarkadi, B. 2004. The role of ABC transporters in drug resistance, metabolism and toxicity. Curr. Drug. Deliv. 1(1):27-42.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhur, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29(7):644-52. doi: 10.1038/nbt.1883.

Hellinger, E., Veszelka, S., Tóth, A. E., Walter, F., Kittel, A., Bakk, M. L., Tihanyi, K., Háda, V., Nakagawa, S., Duy, T. D., Niwa, M., De, M. A., Vastag, M. 2012. Comparison of brain capillary endothelial cell-based and epithelial (MDCK-MDR1, Caco-2, and VB-Caco-2) cell-based surrogate blood-brain barrier penetration models. Eur. J. Pharm. Biopharm. 82(2):340-51. doi: 10.1016/ j.ejpb.2012.07.020.

Hung, C. C., Chiou, M. H., Teng, Y. N., Hsieh, Y. W., Huang, C. L., Lane, H. Y. 2013. Functional impact of ABCB1 variants on interactions between P-glycoprotein and methadone. PLoS. One. 2013;8(3):e59419.

Jutabha, P., Wempe, M. F., Anzai, N., Otomo, J., Kadota, T., Endou, H. 2009. Xenopus laevis oocytes expressing human P-glycoprotein: probing trans- and cis-inhibitory effects on [3H]vinblastine and [3H]digoxin efflux. Pharmacol. Res. 61(1):76-84. doi: 10.1016/j.phrs.2009.07.002.

Kim, W. S., Guillemin, G. J., Glaros, E. N., Lim, C. K., Garner, B. 2006. Quantitation of ATP-binding cassette subfamily-A transporter gene expression in primary human brain cells. Neuroreport. 17(9):891-6.

Lanning, C. L., Fine, R. L., Corcoran, J. J., Ayad, H. M., Rose, R. L., Abou-Donia, M. B. 1996. Tobacco budworm P-glycoprotein: biochemical characterization and its involvement in pesticide resistance. Biochim. Biophys. Acta. 1291(2):155-62.

Letunic, I., Doerks, T., Bork, P. 2012. SMART 7: recent updates to the protein domain annotation resource. Nucleic. Acids. Res. 40(Database issue):D302-5. doi: 10.1093/nar/gkr931.

Liu, Y., Peng, H., Zhang, J. T. 2005. Expression profiling of ABC transporters in a drug-resistant breast cancer cell line using AmpArray. Mol. Pharmacol. 68(2):430-8.

Matsumoto, Y., Sumiya, E., Sugita, T., Sekimizu, K. 2011. An invertebrate hyperglycemic model for the identification of anti-diabetic drugs. PLoS. One. 6(3):e18292.

Mayer, F., Mayer, N., Chinn, L., Pinsonneault, R. L., Kroetz, D., Bainton, R. J. 2009. Evolutionary conservation of vertebrate blood-brain barrier chemoprotective mechanisms in Drosophila. J Neurosci. 29(11):3538-50.

Naik, P., Cucullo, L. 2012. In vitro blood-brain barrier models: current and perspective technologies. J. Pharm. Sci. 101(4):1337-54.

Nielsen, P. A., Andersson, O., Hansen, S. H., Simonsen, K. B., Andersson, G. 2011. Models for predicting blood-brain barrier permeation. Drug. Discov. Today. 16(11-12):472-5. 12.

Ott, M., Huls, M., Cornelius, M. G., Fricker, G. 2010. St. John's Wort constituents modulate P-glycoprotein transport activity at the blood-brain barrier. Pharm. Res. 27(5):811-22.

Shapiro, A. B., Ling, V. 1997. Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. Eur. J. Biochem. 1997 Nov 15;250(1):130-7.

Shawahna, R., Uchida, Y., Declèves, X., Ohtsuki, S., Yousifm, S., Dauchy, S., Jacob, A., Chassoux, F., Daumas-Duport, C., Couraud, P.O., Terasaki, T., Scherrmann, J. M. 2011. Transcriptomic and quantitative proteomic analysis of transporters and drug metabolizing enzymes in freshly isolated human brain microvessels. Mol. Pharm. 8(4):1332-41.

Shawahna, R., Decleves, X., Scherrmann, J. M. 2013. Hurdles with using in vitro models to predict human blood-brain barrier drug permeability: a special focus on transporters and metabolizing enzymes. Curr. Drug .Metab. 14(1):120-36.

Strycharz, J. P., Lao, A., Li, H., Qiu, X., Lee, S. H., Sun, W., Yoon, K. S., Doherty, J. J., Pittendrigh, B. R., Clark, J. M. 2013. Resistance in the highly DDT-resistant 91-R strain of Drosophila melanogaster involves decreased penetration, increased metabolism, and direct excretion. Pestic. Biochem. Physiol. 107(2):207-217

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30(12):2725-9. doi: 10.1093/molbev/mst197. Epub 2013 Oct 16.

Troutman, M. D., Thakker, D. R.2003. Rhodamine 123 requires carrier mediated influx for its activity as a P-glycoprotein substrate in a Caco-2 cells. Pharm. Res. 2003; 20: 1192–1199

Volpe, D.A. 2010. Application of method suitability for drug permeability classification. AAPS. J. 12(4):670-8.

Wang, Q., Rager, J. D., Weinstein, K., Kardos, P. S., Dobson, G. L., Li, J., Hidalgo, I.J. 2005. Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier. Int J Pharm. 288(2):349-59.

Weksler, B., Romero, I.A., Couraud, P.O. 2013. The hCMEC/D3 cell line as a model of the human blood brain barrier. Fluids. Barriers. CNS. 26;10(1):16.

Zhang, X., Xue, R., Cao, G., Pan, Z., Zheng, X., Gong, C. 2012. Silkworms can be used as an animal model to screen and evaluate gouty therapeutic drugs. J. Insect. Sci. 2012;12:4. 12:4. doi: 10.1673/031.012.0401.