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Title:

In vitro P-gp efflux ratio can predict the in vivo brain penetration regardless of BDDCS class

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Running Title:

In vitro-in vivo correlation of P-gp efflux ratio

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Abbreviations: P-gp, P-glycoprotein; BBB, blood-brain barrier; CNS, central nervous system; ER, efflux ratio; BDDCS, Biopharmaceutics Drug Disposition Classification System; NME, new molecular entity; NIH, National Institute of Health; NKI, Netherlands Cancer Institute; BCS, Biopharmaceutics Classification System; CSF, cerebrospinal fluid;

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Abstract

P-glycoprotein (P-gp) is expressed at the blood-brain barrier (BBB) and restricts the penetration of its substrates into the central nervous system (CNS). In vitro substrate assessment for P-gp is frequently used to predict the in vivo relevance of P-gp-mediated efflux at the BBB. We have conducted a comprehensive review of literature focusing on the in vitro-in vivo correlation of P-gp efflux ratio (ER), and demonstrated that in vitro substrates of P-gp are also in vivo substrates at the BBB. It was of note that the in vitro ER in MDCK-MDR1 cell line from National Institute of Health was found to be a better predictor of in vivo ER compared with that from Netherlands Cancer Institute with r^2 values of 0.813 and 0.531, respectively. Recently, Broccatelli and coworkers have proposed that 98% of Biopharmaceutics Drug Disposition Classification System (BDDCS) class 1 drugs can penetrate the brain even when those compounds are shown as P-gp substrates in vitro (Broccatelli et al., 2012). However, our data analysis suggested that in vitro ER can predict the in vivo brain penetration regardless of the class in BDDCS. Considering that very few marketed CNS drugs are in vivo substrates for P-gp, the in vitro substrate assessment of P-gp should be used in the early stages of drug discovery to select compounds which most likely penetrate the CNS to exert their pharmacological action.

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Introduction

Drug disposition in the central nervous system (CNS) is important to understand its pharmacological effect if the target resides in the CNS, or its undesired CNS side effect for non-CNS indications. It is therefore one important attribute to characterize a new molecular entity (NME) regardless of its therapeutic area. Extensive efforts have been made in the prediction of drug entry into the CNS, which include the development of various in silico models (Norinder and Haeberlein, 2002; Clark, 2003; Ecker and Noe, 2004; Pajouhesh and Lenz, 2005). These models base their prediction on the molecular structural information of a compound including drug lipophilicity (cLogP), molecular weight, and the tendency to form hydrogen bonds quantified as polar surface area or as a function of nitrogen and oxygen counts. Although these models have yielded reasonable success in predicting the brain penetration of the NMEs, they do not take into consideration the physiological factors affecting the CNS disposition of drugs such as the blood-brain barrier (BBB).

It is widely recognized that the CNS penetration of xenobiotics is predominantly restricted by the BBB (Pardridge, 1999). BBB is formed by brain capillary endothelial cells, which are characterized by highly developed tight junctions between adjacent cells and a paucity of fenestra and pinocytotic vesicles. Due to these anatomical features, the BBB works as a static wall between the brain parenchyma and circulating blood. Therefore, the transcellular route is the major pathway for the exchange of compounds between the two compartments. In addition, cumulative evidence suggests the role of multiple efflux transporters located either on the luminal or abluminal membrane of the brain capillary endothelial cells in the active extrusion of their substrates from the CNS, providing another barrier function to the BBB (Sun et al., 2003; Kusuhara and Sugiyama, 2005; Urquhart and Kim, 2009).

P-glycoprotein (P-gp) is one of the most well characterized efflux transporters expressed on the luminal side of the BBB. In vivo studies using P-gp deficient mice such as Mdr1a single-knockout ($Mdr1a^{-/-}$) or Mdr1a/1b double-knockout mice ($Mdr1a/1b^{-/-}$) highlighted its importance in limiting the CNS entry of a range of xenobiotics (Schinkel, 1999). Indeed, it is believed that the brain penetration of the second generation

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antihistamines are restricted by P-gp-mediated efflux at the BBB, which explains the lack of sedative effect, compared with the first generation antihistamines which are non-substrates of P-gp (Chen et al., 2003). The transcellular transport assay in P-gp overexpressing cell lines is frequently used to predict the in vivo relevance of P-gp-mediated efflux at the BBB. Efflux ratio (ER) is defined as the ratio of flux from the basal to apical compartment (Papp B-to-A) to that from apical to basal compartment (Papp A-to-B) in the cell monolayer. Comparing the in vitro ER, it has been demonstrated that CNS drugs have less incidence of being P-gp substrates compared with non-CNS drugs, which reinforces the importance of early assessment of P-gp substrate liability in drug discovery (Mahar Doan et al., 2002; Feng et al., 2008). In order to elucidate the in vivo relevance of in vitro substrate assessment, we have conducted a comprehensive review of literature focusing on in vitro-in vivo correlation of P-gp ER.

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Predictive utility of in vitro P-gp efflux ratio

P-gp deficient mice such as Mdr1a$^{-/-}$ or Mdr1a/1b$^{-/-}$ mouse models are powerful tools to understand the impact of P-gp-mediated efflux at the BBB on the brain disposition of drugs. The in vivo ER, which is the ratio of brain/plasma partition coefficient ($K_{p,brain}$) values between Mdr1a$^{-/-}$ or Mdr1a/1b$^{-/-}$ and wildtype mice, is often used to represent the degree to which P-gp-mediated efflux attenuates the CNS penetration. In vitro experiments using Caco-2 cells that endogenously express P-gp or P-gp overexpressing cells such as MDCK-MDR1 or LLC-PK1-MDR1 have been successfully used to predict the in vivo relevance of P-gp mediated efflux at the BBB. It has been demonstrated that the in vivo ER correlates well with the in vitro ER (Adachi et al., 2001; Yamazaki et al., 2001; Feng et al., 2008), proving these in vitro tools suitable in the prediction of brain penetration at early stages in drug discovery.

Recently, Broccatelli and coworkers have proposed a new framework to predict the brain disposition of NMEs, which is a simple 3-step classification tree comprising in silico permeability, in vitro P-gp ER and the class in the Biopharmaceutics Drug Disposition Classification System (BDDCS) (Broccatelli et al., 2012). The model could accurately predict CNS disposition for more than 90% of 153 drugs in their data set. They also noted that 98% of BDDCS class 1 drugs can penetrate the brain even when those compounds are shown as P-gp substrates in cellular systems (Table 1, Figure 1). In order to investigate the validity of their argument, we have conducted an extensive literature search to pull together as many in vivo P-gp ER as we could and compared them to the in vitro ER compiled by Broccatelli and coworkers (Table 2). In vivo ER was not available for BDDCS class 4 drugs. The in vitro ER summarized by Broccatelli and coworkers was obtained either in MDCK-MDR1 cell line from National Institute of Health (NIH) or that from Netherlands Cancer Institute (NKI; Borst cell line). In order to compare the predictive utility of in vitro ER in two different cell lines, compounds with in vitro ER reported in both cell lines were used for analysis (Figure 2). Even though all data in this analysis were obtained from literature and represented data across many different laboratories, a strong correlation was observed between in vivo and in vitro ER. It is of note that the in vitro ER in NIH cell line predicts the in vivo ER better than that in

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Borst cell line (overall r^2, 0.813 vs 0.531). This could be ascribed to the higher sensitivity in the former cell line than the latter to detect potential P-gp substrate, which is in good agreement with the different expression level of P-gp in these two cell lines; the protein expression of P-gp appears to be higher in MDCKI-MDR1 cells from NIH compared with MDCKII-MDR1 cells from NKI (Park et al., 2011). Considering the potential cell-dependent variability in the in vitro ER, each laboratory is encouraged to calibrate their experimental system using compounds with varying degree of brain penetration and build in vitro-in vivo correlation first, which may increase the precision of the prediction of CNS penetration during drug discovery. Furthermore, the in vitro ER predicts the in vivo ER of compounds equally well between BDDCS class 1 and the other classes (class 2 and 3). The r^2 values for BDDCS class 1 and class 2-3 drugs are 0.893 and 0.761, respectively, in NIH cell line (Figure 3A and B), and those in the Borst cell line are 0.504 and 0.515, respectively (Figure 3C and D). When a simple cut-off value in the in vitro (NIH cell line; 4, and Borst cell line; 2) and in vivo ER (3) was adopted, 15 of the 16 class 1 compounds (94%) and 11 of the 14 class 2-3 compounds (79%) were correctly classified using the data in NIH cell line (Figure 3A and B), and similarly, 20 of the 27 class 1 compounds (74%) and 17 of the 21 class 2-3 compounds (81%) were correctly classified using the data in Borst cell line (Figure 3C and D). There was similar level of concordance between BDDCS class 1 and the other classes, suggesting that in vitro P-gp ER can predict the in vivo brain penetration regardless of BDDCS class. It is well accepted that there is an overlap between Biopharmaceutics Classification System (BCS) and BDDCS class assignment for many drugs, where BDDCS class 1 drugs that are characterized by showing extensive metabolism and high solubility are also highly permeable compounds. Permeability of a compound is inherently assessed in the in vitro efflux assay using P-gp overexpressing cells, i.e. the reduction in the impact of P-gp mediated efflux due to high passive permeability is also captured in the in vitro read-out (i.e. efflux ratio) (Kalvass and Pollack, 2007). These data mining and theoretical considerations strongly suggest that an in vitro substrate of P-gp will most likely be an in vivo substrate at the BBB regardless of the class in BDDCS or BCS.

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Criteria for BBB class assignment

Our data analysis suggested that in vitro P-gp ER can predict the in vivo brain penetration regardless of the class in BDDCS, which is in direct contrast to the conclusion by Broccatelli and coworkers (Broccatelli et al., 2012). This could be explained by difference(s) in the criteria for BBB class assignment.

Broccatelli and coworkers have classified drugs either as brain-penetrating (BBB+) or having restricted ability to cross the BBB (BBB-) based on the following criteria (Broccatelli et al., 2012):

1) *Marketed CNS agents were directly assigned to the BBB+ class without further investigation*;
2) *When the unbound drug brain/plasma ratios in humans were available, these values were used for defining the BBB class; drugs with values greater than or equal to 0.1 were assigned as BBB+, otherwise, they were assigned as BBB-*; and
3) *When the unbound brain/plasma ratio of a drug in humans was unavailable, then either the unbound CSF/plasma ratio in humans or brain penetration data from other species was used*.

In the third criteria, human CSF/plasma ratio was used for assigning the BBB class over any other species data including brain/plasma ratios when more than one of these values was available.

A data set for 153 drugs and their P-gp class (+ or -), BDDCS class (1,2,3 or 4), and BBB classification (+ or -) suggested by Broccatelli and coworkers are summarized in Table 1 and Figure 1. With their BBB classification, 89% of P-gp substrates in class 1 were BBB+ (17 of 19 drugs) meaning that these are “false P-gp positive” (Figure 1A, upper-right quadrant), while 75% of P-gp substrates in class 2, 3, and 4 were BBB- (21 of 28 drugs), which are thus considered as “true P-gp positive” (Figure 1B, lower-right quadrant). In other words, most (71%, 17 of 24) of the drugs in “false P-gp positive” are BDDCS class 1, while only 9% (2 of 23 drugs) are class 1 in “true P-gp positive”. This indicated that BDDCS class 1 drugs are not substrates of clinical relevance for P-gp at the BBB regardless of the data in cellular systems. However, there are strong caveats on the BBB classification applied by the authors.

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1) Not all CNS agents require good brain penetration to show their efficacy. For example, antimigraine triptans such as rizatriptan, sumatriptan, eletriptan, and zolmitriptan are thought to work on vascular system. Furthermore, some marketed CNS agents are known to have poor brain penetration including risperidone and metoclopramide (Doran et al., 2005).
2) Broccatelli and coworkers used a cut-off value of 0.1 in the unbound brain/plasma ratio in humans to distinguish the BBB-penetrating (BBB+) drugs from the others (BBB-). This represents the 10-fold brain impairment which is defined as a steady-state unbound plasma/unbound brain concentration ratio ($[plasma]_u/[brain]_u$). This cut-off value (unbound brain/plasma ratio of >0.1) to assign BBB+ drugs seems to be too low and not appropriate. Indeed, Kalvass and coworkers previously proposed that brain impairment, i.e. $[plasma]_u/[brain]_u$, being greater than 3, or in other words, unbound brain/plasma ratio less than 0.33, is considered meaningful to define poor brain penetration (BBB-) because such ratio is sufficiently different from unity and most CNS drugs (29 of 32) have been shown to exhibit in vivo P-gp ER less than 3 (Doran et al., 2005; Kalvass et al., 2007).
3) In the BBB classification by Broccatelli et al., human unbound CSF/plasma ratio data trumped brain penetration data from other species when the unbound brain/plasma ratio in humans was unavailable. This could potentially lead to a misclassification in the BBB assignment because unbound CSF/plasma ratio is recognized as a poor surrogate for brain penetration for compounds which are actively extruded from the brain by efflux transporters (Kodaira et al., 2011), i.e. a drug concentration in the CSF may severely overestimate the unbound concentration in the brain for P-gp substrates because P-gp is not expressed on the plasma membrane of choroid plexus epithelial cells facing the blood unlike the BBB.

Taking these points into account, we propose the use of a different criteria for BBB class assignment, which is, unbound brain/plasma ratio being greater than 0.33 or in vivo P-gp ER less than 3.0 to assign drugs as BBB+ (Table 3). With our criteria, many BDDCS class 1 drugs are classified as BBB-, which were originally classified as BBB+,

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including eletriptan, quinidine, risperidone, rizatriptan, and verapamil. The in vivo ER is significantly greater than 3 for eletriptan (47), quinidine (24), risperidone (10), rizatriptan (4.3), and verapamil (7.7), suggesting that these compounds are brain impaired by P-gp-mediated efflux (Doran et al., 2005; Kalvass et al., 2007). However, Broccatelli and coworkers argued that verapamil is a BBB+ drug, for example, based on the human total brain/plasma ratio ($K_{p,brain}$) of 0.55 (Sasongko et al., 2005), ignoring the unbound ratio ($K_{p,uu,brain}$) of 0.053 in rat (Friden et al., 2009) and 0.10 in human. Note $K_{p,uu,brain}$ in human is estimated using $K_{p,brain}$ of 0.55 (Sasongko et al., 2005), unbound fraction in human plasma (0.099) (Giacomini et al., 1984), and unbound fraction in rat brain (0.0185) (Friden et al., 2009) since it has been demonstrated that brain tissue binding is species-independent (Di et al.). Moreover, (R)-[11C]verapamil is used as a clinical probe for P-gp efflux at the BBB in positron emission tomography studies (Syvanen and Hammarlund-Udenaes, 2010). Careful evaluation of literature data is necessary in the BBB classification.

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Conclusion

Our data analysis focusing on the in vitro-in vivo correlation of P-gp ER suggested that an in vitro substrate of P-gp will most likely be an in vivo substrate regardless of the class in BDDCS or BCS. It was noted that the relationship and quality of P-gp in vitro-in vivo correlation is dependent on the original source of MDCK-MDR1 cells, where the in vitro ER in MDCK-MDR1 cell line from NIH was found to be a better predictor of in vivo ER compared with that from NKI. Considering that very few marketed CNS drugs are in vivo substrates for P-gp (Mahar Doan et al., 2002; Doran et al., 2005), the in vitro substrate assessment of P-gp should be used in the early stages of drug discovery to select compounds which most likely penetrate the CNS to exert their pharmacological action. Moreover, the bidirectional transcellular transport assay using the MDCK or LLC-PK1 cells overexpressing human P-gp continues to be the most reliable in vitro tool to assess P-gp liability such as brain penetration.

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Authorship Contributions

Participated in research design: Kikuchi, de Morais, and Kalvass

Performed data analysis: Kikuchi and Kalvass

Wrote or contributed to the writing of the manuscript: Kikuchi, de Morais, and Kalvass

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Footnotes

Author Disclosure

AbbVie provided no support outside of Ryota Kikuchi, J. Cory Kalvass, and Sonia M. de Morais being employees of AbbVie. The presentation contains no proprietary AbbVie data.

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Legends for Figures

Figure 1

A data set for 153 drugs in terms of P-gp class (+ or -), BDDCS class (1,2,3 or 4), and BBB classification (+ or -) suggested by Broccatelli and coworkers are summarized; the criteria for BBB class assignment is listed in Table 3. The distribution of class 1 (A) and class 2-4 drugs (B) in each category is visualized. Gray squares, white circles, black diamonds, and gray triangles represent class 1, 2, 3 and 4 drugs, respectively. Note that most (71%) of the drugs in "false P-gp positive" (upper-right quadrant; panel A and B) are BDDCS class 1 while only 9% are BDDCS class 1 in the "true P-gp positive" category (lower-right quadrant; panel A and B).

Figure 2

In vitro-in vivo correlation of P-gp efflux ratio. The in vitro P-gp efflux ratio obtained in MDCKI-MDR1 cells (NIH cell line; A) and MDCKII-MDR1 cells (Borst cell line; B) are compared with in vivo P-gp efflux ratio for the same set of compounds. Gray squares, white circles, and black diamonds represent class 1, 2, and 3 drugs, respectively. A linear regression analysis was conducted using the data for all BDDCS classes of drugs (class 1, 2 and 3). The dotted lines represent the 2-fold window of linear regression. The overall r^2 value of correlation was 0.813 (A) and 0.531 (B).

Figure 3

In vitro P-gp ER can predict the in vivo brain penetration regardless of BDDCS class. The in vitro P-gp efflux ratio obtained in MDCKI-MDR1 cells (NIH cell line; A and B) and MDCKII-MDR1 cells (Borst cell line; C and D) are compared with in vivo P-gp efflux ratio. The data were obtained from Table 2, and the graphs were separated between BDDCS class 1 (A and C) and class 2-3 drugs (B and D). Gray squares, white circles, and black diamonds represent class 1, 2, and 3 drugs, respectively. A linear regression analysis was conducted for each data set. A simple cut-off value in the in vitro (NIH cell line; 4, and Borst cell line; 2) and in vivo ER (3) was adopted. Quadrants highlighted in gray represent the correct classification.

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Tables

Table 1

The number of drugs in each category among the 153 drugs analyzed by Broccatelli et al. (Broccatelli et al., 2012).

	P-gp +		P-gp -	
	Class 1	Class 2-4	Class 1	Class 2-4
BBB+	17	7	63	34
BBB-	2	21	0	9

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Table 2

The in vitro and in vivo P-gp efflux ratio of drugs and their classification in BDDCS.

Drug	BDDCS Class	Average ER (Borst)	Average ER (NIH)	in vivo P-gp ER
Amprenavir	2	27.4		13.9
Bupropion	1		1.4	1.3
Caffeine	1	0.6	1.1	1.1
Carbamazepine	2	0.9	1.1	1.1
Cetirizine	3	7.2	63.0	4.0
Chlorpromazine	1	2.4	1.7	1.3
Cimetidine	3	3.5		0.9
Citalopram	2	1.6	20.1	1.9
Clozapine	2	2.5	1.3	1.6
Cyclobenzaprine	1	1.4		1.4
Desloratadine	2	9.1		14.0
Dexamethasone	1	12.4		2.3
Diazepam	1	0.7	1.0	1.2
Digoxin	3	9.0	35.0	18.8
Diphenhydramine	1	0.9	3.7	1.0
Eletriptan	1	32.9		46.7
Ethosuximide	1	0.8	0.8	1.0
Fexofenadine	3		3.9	1.8
Fluoxetine	1	1.9	2.0	1.5
Fluvoxamine	1	1.7		2.3
Haloperidol	2	0.8	1.3	1.4
Hydrocodone	1	1.1		2.1
Hydroxyzine	1	0.6	1.4	1.3
Indinavir	2	22.5		9.6
Lamotrigine	2	0.9	1.6	1.0
Loperamide	3	9.0	237.0	59.4
Loratadine	2	1.4	7.4	2.1
Meprobamate	1	1.0	3.3	1.7
Methylphenidate	1	1.0		1.6
Metoclopramide	3	1.1	13.2	6.6
Morphine	1	1.9		1.5
Nelfinavir	2	15.6		30.2
Nortriptyline	1	1.4		1.8
Paroxetine	1	3.1		2.2
Phenytoin	2	1.0	2.8	1.2
Prazosin	1	3.8		2.0
Quinidine	1	16.8	338.0	24.0
Ranitidine	3	2.1	5.4	1.8
Risperidone	1	1.8	20.8	10.0
Ritonavir	2	38.8		13.5
Rizatriptan	1		8.4	4.3

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Table 2, continued

Drug	BDDCS Class	Average ER (Borst)	Average ER (NIH)	in vivo P-gp ER
Saquinavir	2	162.9		6.8
Selegiline	1	0.8	0.8	1.1
Sertraline	1	7.4	2.6	1.1
Sulpiride	3	0.9		1.9
Sumatriptan	1	1.4	2.9	1.7
Trazodone	2	0.9	1.1	0.9
Venlafaxine	1	1.0	4.7	1.8
Verapamil	1	2.2		8.3
Zolmitriptan	1	2.5		2.2
Zolpidem	1	1.0		1.4

The data for BDDCS class and average ER in Borst and NIH cell lines were extracted from Broccatteli et al., 2012. The data for in vivo P-gp ER were extracted from literature (Doran et al., 2005; Maurer et al., 2005; Kalvass et al., 2007).

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Table 3

Criteria for BBB class assignment

	Criteria for BBB+ drugs (i.e. "good" CNS penetration, CNS drug like, etc.)	
	Broccatelli et al	Kikuchi et al
Marketed CNS agents	Yes	Can be misleading
Unbound brain-to-plasma ratio	>0.1	>0.33
Unbound CSF-to-plasma ratio	>0.1	Can be misleading
In vivo P-gp ER	Not considered	<3.0

Comparison of criteria for BBB class assignment (BBB+ drugs). See text for details.

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Figure 1

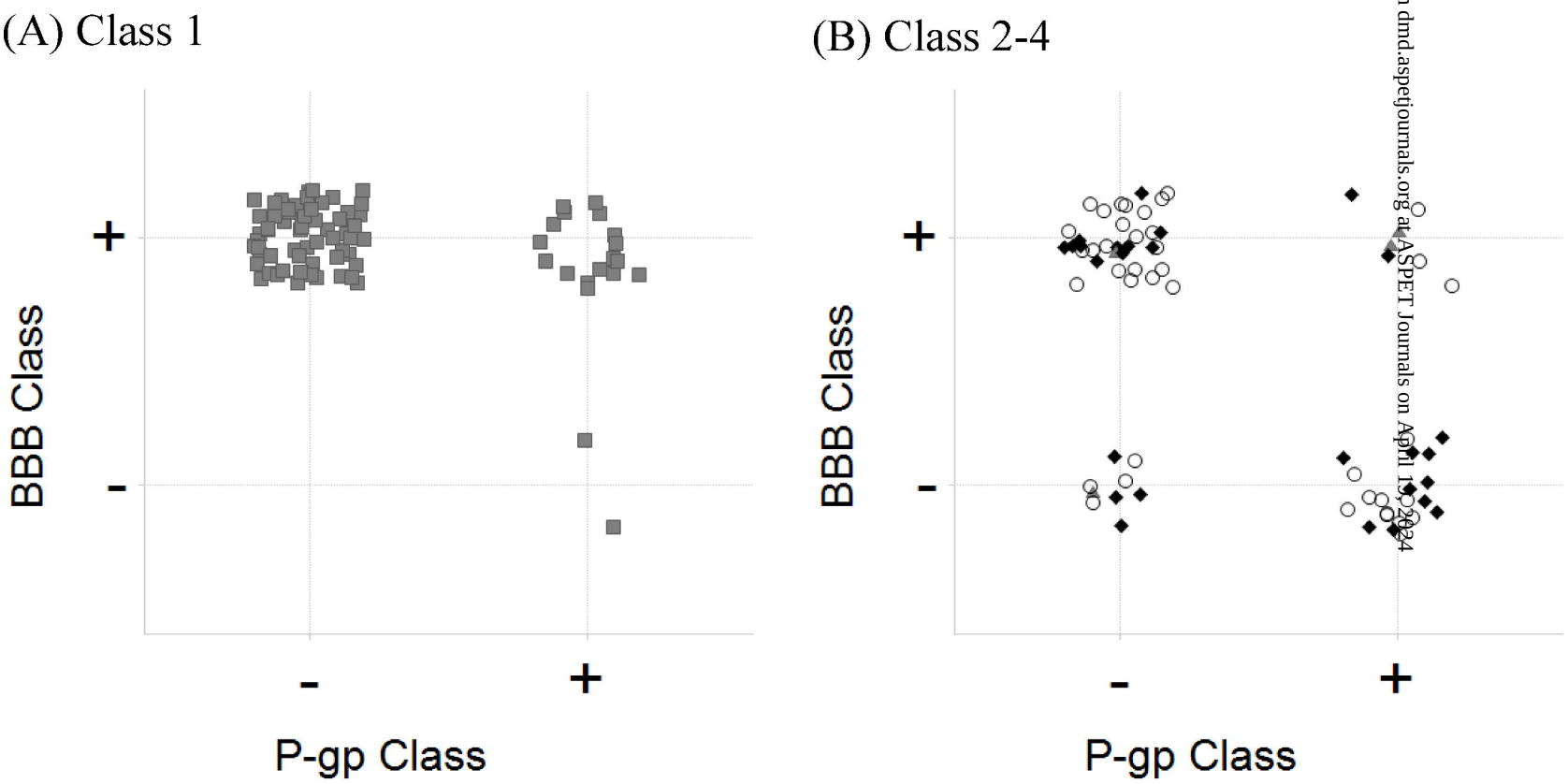

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Figure 2

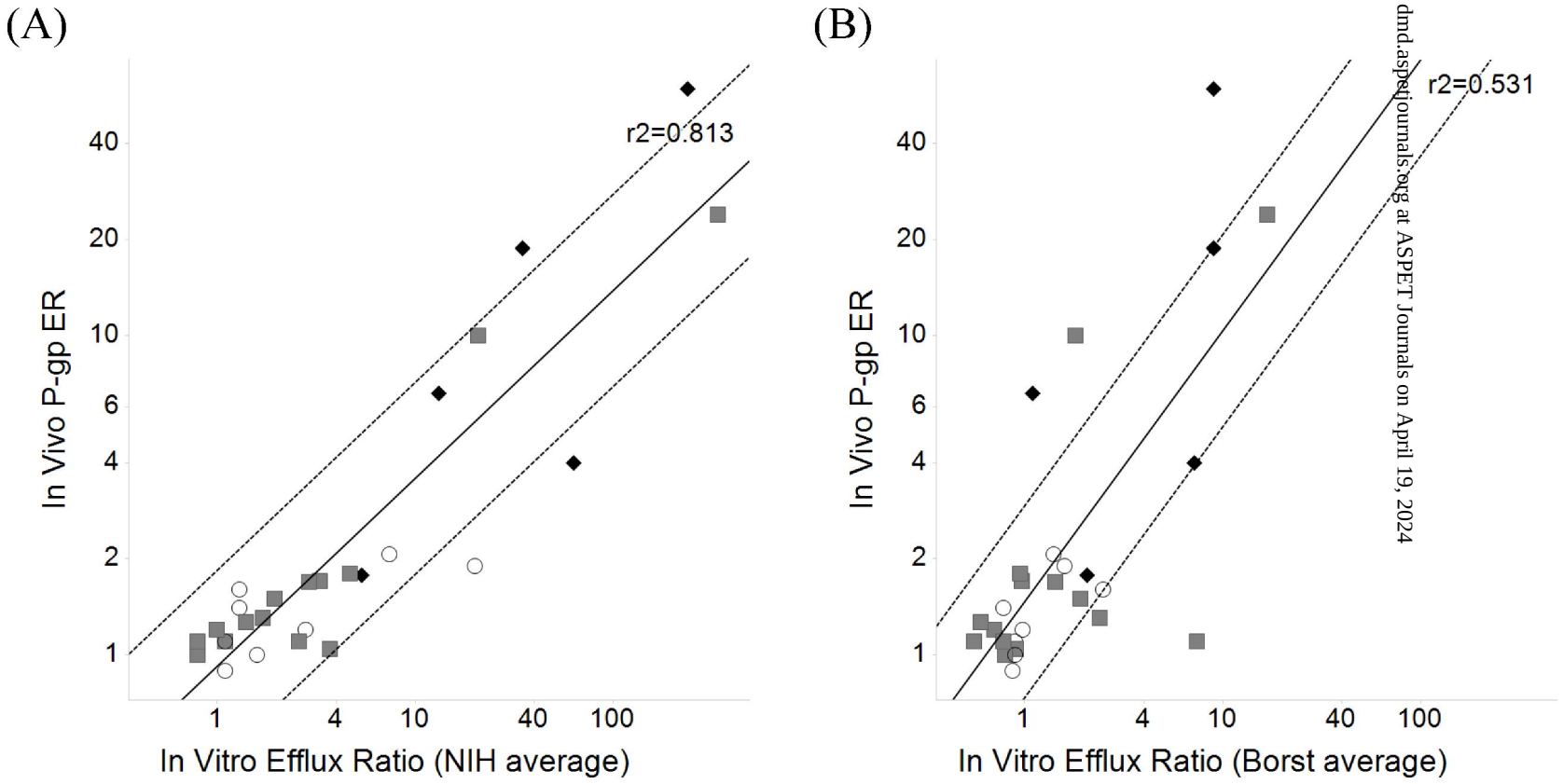

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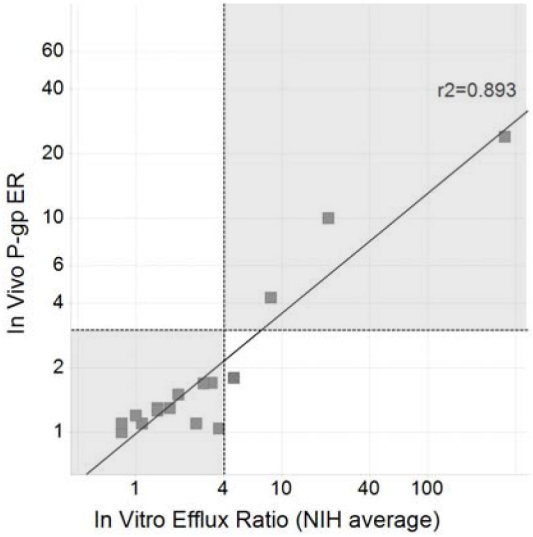
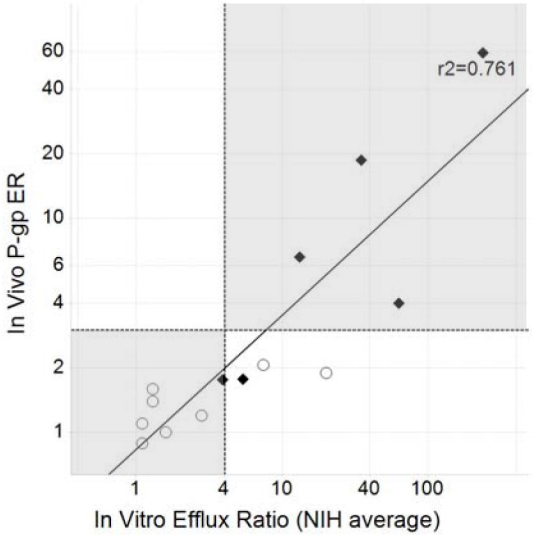
Figure 3

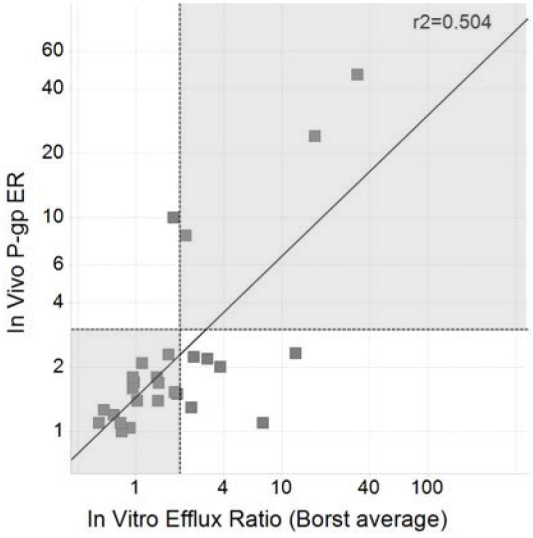
(A) NIH – class 1



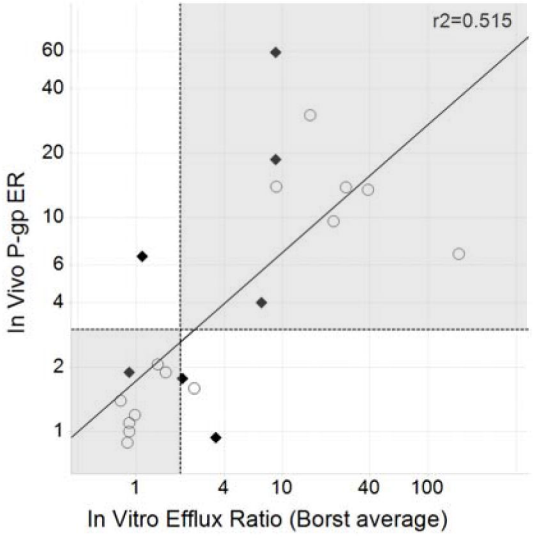
(B) NIH – class 2-3



(C) Borst – class 1



(D) Borst – class 2-3



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