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Comparison of Species Differences of P-Glycoproteins in Beagle Dog, Rhesus Monkey, and Human Using ATPase Activity Assays

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Abstract: P-glycoprotein (P-gp) is a transmembrane efflux transporter which possesses many important functions in drug absorption, disposition, metabolism, and toxicity. The ultimate goal of investigating drug interactions between P-gp and drug molecules in early drug discovery is to understand the contribution of P-gp to the pharmacokinetic and pharmacodynamic properties of drug candidates and to project drug−drug interaction (DDI) potentials in humans. Understanding species differences in P-gp activities further helps the prediction of P-gp-mediated drug disposition and DDI in humans from preclinical pharmacokinetics data. The objective of the present study is to investigate the species difference in P-gp activities, via P-gp ATPase assays, using rhesus monkey Mdr1, beagle dog Mdr1, and human MDR1 expressed insect cell membranes. Twenty-one compounds with diverse chemical structures and different P-gp binding sites were chosen for the ATPase assays. P-gp ATPase binding affinities (αKa) and fold increases in P-gp ATPase activities (β) of P-gp substrates were determined. Consistent with the gene and amino acid similarity, the binding affinities of test compounds to rhesus monkey P-gp were much closer to those of human P-gp than beagle dog P-gp. This is the first study which investigates the ligand affinities of P-gp from three different species. The result of this study provides an example of how to use membrane P-gp ATPase assays to evaluate interspecies P-gp differences.

Keywords: P-glycoprotein; species difference; ATPase activity; binding affinity; rhesus monkey Mdr1; beagle dog Mdr1; human MDR1

Introduction

P-glycoprotein (P-gp), encoded by the multidrug resistance protein 1 (MDR1) gene in humans and the Mdr1 gene in animals, is a member of the ATP binding cassette (ABC) superfamily of transport proteins (also known as traffic ATPase protein).[1] As an efflux transporter, P-gp plays a major role in the multidrug resistance of anticancer chemotherapy.[2] In addition, P-gp has many important functions in drug absorption, disposition, metabolism, and toxicity. The goal of investigating interactions between P-gp and drug molecules in early drug discovery is to understand the

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contribution of P-gp to pharmacokinetic/pharmacodynamic properties of drug candidates and to predict drug−drug interaction (DDI) potentials in humans.[3,4] Species differences have been observed in drug metabolism enzymes.[5,6] However, little is known about species differences in P-gp protein. To date, studies of P-gp have been conducted primarily in mouse Mdr1 and human MDR1 transfected cell lines[7−9] and rarely in other species. In the pharmaceutical industry, dogs and monkeys are the nonrodent species commonly used in early metabolism and toxicology studies of drug candidates. In order to extrapolate pharmacokinetic behaviors of the drug candidate observed in dogs or monkeys to those in humans, it is important to investigate the species differences or similarities of P-gp activities in these preclinical animal models.

P-gp is composed of two homologous halves, each contains six transmembrane domains and an ATP binding/utilization domain, separated by a flexible linker polypeptide. ATP binding and hydrolysis appear to be essential for the proper function of P-gp, including drug transport.[10] Scarborough and co-workers[11] have demonstrated that P-gp substrates stimulated the ATPase activity of human P-gp expressed in Sf9 insect cell membranes. Several other laboratories have subsequently shown that P-gp substrates also stimulated ATPase activities in mammalian cells that constitutively express P-gp.[12−14] Although P-gp ATPase assay has been mainly used for identifying the P-gp ligands in a high throughput mode, it can give the false negative results of P-gp ligands.[15] The profile of the P-gp substrate stimulated ATPase activity has been demonstrated to reflect the nature of interactions between P-gp and substrates.[16,17] Thus, the drugs which show the stimulation of P-gp ATPase can be characterized with respect to their affinities to P-gp ATPase activities.

We report here the species differences kinetically in terms of affinities (αKa) and activity stimulation folds (β) of P-gp ATPase activities for 21 test compounds via the use of Hi5 insect cell membranes expressing rhesus monkey Mdr1, beagle dog Mdr1, and human MDR1. The P-gp ligands are chosen on the basis of their chemical structures, drug classes, and reported P-gp binding sites. This comparative study on the interaction between P-gp ligands and P-gp ATPases from different species has not been reported. It provides an example of how to use animal membrane ATPase assays to evaluate interspecies differences in P-gp affinities.

Materials and Methods

Insect cell membranes expressing human, rhesus monkey, and beagle dog P-gp were obtained from BD Biosciences, Discovery Labware (Woburn, MA).

Chemical and Biochemical Materials. All chemicals were analytical grade and purchased either from Moravek Biochemicals (Brea, CA) or from Sigma Aldrich (St. Louis, MO). C219 murine monoclonal antibody was obtained from Signet (Dedham, MA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and SDS−PAGE Fast-Safe-Stain were gifts from Huatesheng Biotechnology (Liaoning, China). All supplies for Western blot and SDS−PAGE studies were obtained from Invitrogen Life Technology (Carlsbad, CA).

SDS−PAGE and Western Blot Assays. Proteins of the insect cell control membranes and membranes expressing human, rhesus monkey, and beagle dog P-gp were separated on 4−12% gradient polyacrylamide gels and subsequently stained by the Fast-Safe-Stain method (Huatesheng Biotech-

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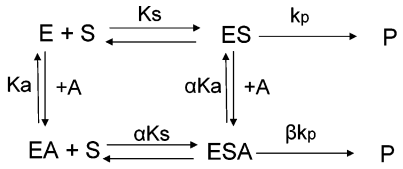
nology, Liaoning, China) or transferred electrophoretically to PVDF membrane. Proteins in the PVDF membrane were hybridized using murine monoantibody C219 (1:150) and HRP-conjugated goat anti-mouse IgG (1:1000), and further visualized by ECL (Amersham Life Science, Piscataway, NJ). The protein bands from fast-safe-staining gel were semiquantified by NIH image software.

ATPase Activity Assay. The P-gp associated ATPase activity was measured according to the method by Sarkadi et al.[11] The liberation of inorganic phosphate from ATP was quantified using a sensitive colorimetric assay originally described by Chifflet et al.[18] Hi5 insect cell membrane expressing human MDR1 or animal Mdr1 (5 μg/μL) was thawed rapidly in 37 °C water bath before diluting to a concentration of 1 μg/μL in ice cold ATPase assay medium (KCl, 50 mM; dithiothreitols, 2 mM; Tris-Mes, 50 mM, pH 6.8) containing 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM ouabain, and 5 mM sodium azide. The experiment was carried out in a 96-well microtiter plate in triplicate. Hi5 insect cell membrane (20 μL of 1 μg/μL solution), with or without 300 μM of sodium orthovanadate, was mixed with 20 μL of test compound serially diluted in the assay medium and preincubated at 37 °C for 5 min. The reaction was initiated by addition of 20 μL of 15 mM MgATP. The final protein amount in the assay was 0.02 mg, and the ATP concentration was 5 mM. The assay plate was placed in a 37 °C incubator for 20 min after shaking at room temperature for 2 min. The reaction was terminated by the addition of 30 μL of stopping medium (10% sodium dodecyl sulfate (SDS) with 1 drop of antifoam). The phosphate standards were constructed by mixing 20 μL of KH_2PO_4 standard (0.0, 0.15, 0.45, 1.5, 3.0, 4.5, 6.0, and 7.5 mM) with 20 μL of assay buffer, following addition of 30 μL of stopping medium and 20 μL of 15 mM of MgATP. The released phosphate or phosphate standards were measured by a modified colorimetric reaction assay described previously.[19] The SDS containing samples were supplemented with 160 μL of the detection reagent (5 mL of 35 mM ammonium molybdate in 15 mM zinc acetate, pH 5.0, mixed with 20 mL of 10% ascorbic acid, pH 5.0) for 20 min at 37 °C and the reaction product was measured by absorbance at 800 nm with a SpectraMax Plus[384] spectrophotometer (Molecular Devices, Sunnyvale, CA).

Data Analysis. The binding affinities (αKa) of test compounds to the P-gp ATPase and the stimulation folds of ATPase activities (β) by test compounds were calculated on the basis of a nonessential enzyme activation model (Scheme 1).[20]

Scheme 1



Where
S = substrate (MgATP in P-gp ATPase assay)
E = enzyme (P-gp ATPase membrane)
A = enzyme activator (test compounds)
P = product
Ka = a constant reflecting the binding affinity of activator with enzyme
Ks = a constant reflecting the binding affinity of substrate with enzyme
αKa = a constant reflecting the binding affinity of activator with enzyme-substrate complex
For activation, β>1 (β is activation fold)

The velocity equation is

$$v = k_p[\mathrm{ES}] + \beta k_p[\mathrm{ESA}] \quad (1)$$

The ATPase substrate ATP used in the assay was 5 mM, at which concentration the ATPase has reached the maximal hydrolysis velocity. Therefore, at any activator concentration,

$$\frac{v_{max,app}}{v_{max}} = \frac{\alpha\mathrm{Ka} + \beta[\mathrm{A}]}{\alpha\mathrm{Ka} + [\mathrm{A}]} \quad (2)$$

where [A] is a fixed activator concentration, αKa is a constant reflecting the binding affinity of activator with enzyme−substrate complex, β is the maximal stimulation fold by an activator, $v_{max,app}$ is the observed maximal ATP hydrolysis velocity in the presence of an activator, and v_{max} is the maximal velocity in the absence of any activator. The kinetic parameters for P-gp ATPase were estimated using a nonlinear regression with a method of least-squares fitting (eq 2, GraphPad, San Diego, CA).

Results

Characterization of the P-gp ATPase Activities. The ATPase activity of Hi5 insect cell membrane expressing MDR1 or Mdr1 could be inhibited by 2 mM EGTA (a Ca^{2+} ATPase inhibitor), 2 mM ouabain (a Na^+−K^+ ATPase inhibitor), 5 mM sodium azide (a mitochondrial ATPase inhibitor), and 100 μM sodium vanadate (a potent inhibitor of P-gp ATPase[21,22]) (Figure 1). The difference of ATPase activity in the presence or absence of sodium vanadate in the assay buffer containing EGTA, ouabain, and sodium azide was the P-gp mediated ATPase activity (Figure 1). The vanadate-sensitive basal ATPase activity in MDR1 (Figure 2) or Mdr1 (data not shown) was linear with respect to time

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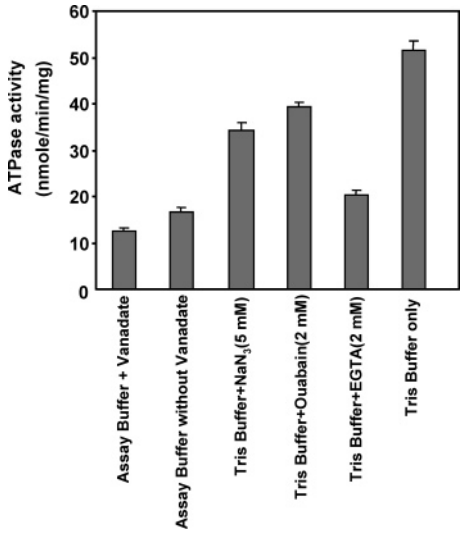

Figure 1. Effect of ATPase inhibitors on the ATPase activity present in the human P-gp expressed Hi5 insect cell membranes. Tris buffer contained 50 mM KCl, 2 mM dithiotheritols, and 50 mM Tris-Mes (pH 6.8). The assay buffer is the Tris buffer containing 2 mM EGTA, 2 mM ouabain, and 5 mM sodium azide (NaN_3). The released phosphate or phosphate standards were measured by a modified colorimetric reaction assay described previously.[19] Each bar expressed as mean ± SD ($n = 3$).

for up to 60 min at 37 °C with 100 μg of membrane protein in the assay buffer (Figure 2A), and it was linear with respect to membrane concentrations between 100 and 800 μg during 20 min incubation (Figure 2B). Therefore, 20 min incubation and 1 μg/μL (200 μg) membrane protein were used in all other experiments. To ensure the robustness of the ATPase assay, we evaluated interday and intraday variations of determined αKa and β by using the prototypical P-gp ATPase stimulant verapamil and human P-gp membranes (Table 1A,B). Coefficients of the variation (CVs) of αKa obtained from inter- and intradays were lower than 30%. However, the P-gp ATPase activity without any stimulant (background) had the CV higher than 30%. Although the CV of β measurement seemed to be lower from interday experiments, the CV obtained from different plates determined on the same day was higher than 40%. All experiments were carried out with the same lot of P-gp membrane (rhesus monkey, Lot 001; beagle dog, Lot 001; and human, Lot 021) to make sure that there was no P-gp expression difference within the same species.

As shown in Figure 3, the P-gp ATPase activity was dependent on the concentration of MgATP and followed Michaelis−Menten kinetics, with Ks of 1.6 ± 0.6 mM and v_{max} of 7.1 nmol mg^{-1} min^{-1}. In the presence of 20 μM verapamil, a substrate and inhibitor of P-gp, the maximal P-gp ATPase activity increased 3-fold, from 7.1 nmol mg^{-1} min^{-1} to 23 nmol mg^{-1} min^{-1}. However, the binding affinity (apparent Ks (Ks_{app})) of MgATP to the P-gp slightly but not significantly decreased to 1.2 ± 0.2 mM.

P-gp Expression in Hi5 Insect Membranes. The protein band locations of human, rhesus monkey, and beagle dog

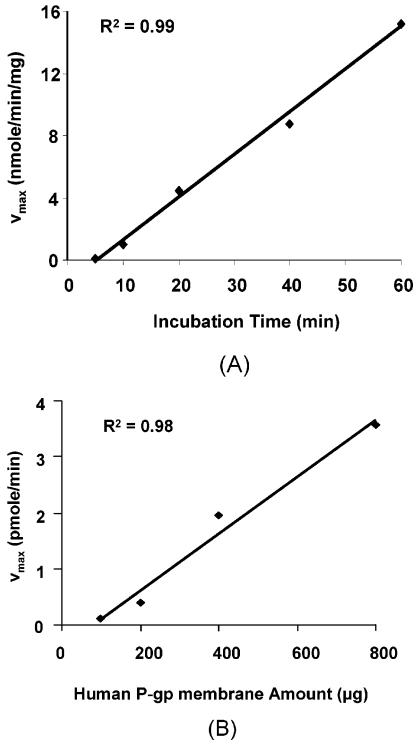

Figure 2. The maximal velocity (v_{max}) of human P-gp ATPase activity with respect to incubation time (A) and membrane protein concentrations (B). Incubation conditions: (A) One microgram of P-gp membrane was used in all assays. (B) Incubation time was 20 min. The assay buffer is the Tris buffer containing 2 mM EGTA, 2 mM ouabain, and 5 mM sodium azide (NaN_3). MgATP was tested at 5 mM in all the assays, which is 3-fold higher than Ks (1.6 mM). The released phosphate or phosphate standards were measured by a modified colorimetric reaction assay described previously.[19] The v_{max} was not normalized by total protein amount.

Table 1. Inter- and Intraday Variations of P-gp ATPase Assay by Using Verapamil as a Stimulant

(A) Interday Variations

	day 1	day 2	day 3	mean	SD[a]	CV[b] (%)
background (nmol min^{-1} mg^{-1})	3.3	4.3	7.1	4.9	2.0	40.2
αKa (μM)	27.8	29.5	17.3	24.9	6.6	26.6
fold of activation (β)	6.5	8.6	8.6	7.9	1.2	15.3

(B) Intraday Variations

	plate 1	plate 2	plate 3	mean	SD[a]	CV[b] (%)
background (nmol min^{-1} mg^{-1})	9.1	8.0	4.2	7.1	2.6	36.2
αKa (μM)	15.4	19.9	16.7	17.3	2.3	13.4
fold of activation (β)	5.3	7.3	13.2	8.6	4.1	47.8

[a] SD: standard deviation. [b] CV: coefficient of the variation.

P-gp in Hi5 insect membranes were identified by Western blot assay (Figure 4B), and the protein levels were semi-quantified from the corresponding fast-safe-staining SDS−PAGE gel (Figure 4A) because the murine monoantibody C219 may have different binding affinities to human, monkey, and dog. From fast-safe-staining SDS−PAGE

Table 2. Kinetic Parameters (αKa and β) of 21 Test Drugs for P-gp Associated ATPase Activities

	human		beagle dog		rhesus monkey		P-gp binding sites[a]	category
	αKa (μM)	β	αKa (μM)	β	αKa (μM)	β		
CsA	0.01	3.6	no stimulation		2.8	4.4		immunosuppressants
physostigmine	0.02	2.5	no stimulation		no stimulation			indo alkaloids
ritonavir	0.1	4.7	0.04	5.4	0.4	4.36	both R- and H-sites	anti-HIV protease inhibitors
saquinavir	0.6	5.4	0.9	2.8	0.6	7.1		
etoposide	1.2	2.0	no stimulation		2.5	2.4	both R- and H-sites	DNA topoisomerase II inhibitors
paclitaxel	1.6	2.1	no stimulation		no stimulation		H-site	disrupt microtubule
vinblastine	1.6	4.8	1.7	2.2	1.4	3.2	both R- and H-sites	vinca alkaloids
quercetin	2.3	3.5	3.2	2.5	4.9	17.5	H-site	flanovonoids
amiodarone	10.0	21.6	no stimulation		3.2	12.7		cardio vasodilators
verapamil	17.3	8.6	12.6	5.2	11.0	8.9		calcium channel blockers
tamoxifen	22.0	2.6	no stimulation		21.3	2.3		anti-estrogens
erythromycin	25.3	6.3	no stimulation		no stimulation			antibacterials
digoxin	42.3	17.1	44.1	2.0	79.1	5.3		cardiac glycoside
progesterone	48.5	28.4	35.9	28.5	63.8	3.8	third binding site	hormones
thioridazine	58.0	4.1	no stimulation		47.0	9.4		calmodulin antagonists
Rhodamine123	78.4	4.7	104.1	2.3	>200	ND[b]	R-site	dye
dexamethasone	89.8	11.4	2.2	2.4	no stimulation			hormones
propranolol	224.6	4.7	65.1	2.3	>200	ND		β-blockers
daunorubicin	no stimulation		no stimulation		no stimulation		R-site	anthracyclines
chloroquine	no stimulation		no stimulation		1.3	5.7		quinolines
Hoechst 33342	no stimulation		no stimulation		no stimulation		H-site	dye

[a] References 27−29. [b] Not determined.

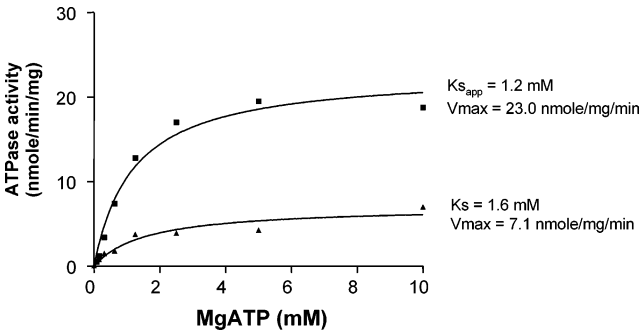

Figure 3. Affinity of P-gp for binding ATP in the presence (■) or absence (▲) of verapamil (20 μM). The P-gp ATPase assay was executed for 20 min with 1 μg of P-gp membranes. MgATP was chosen at concentrations ranging from 5 μM to 10 mM. The kinetic parameters for ATP binding were estimated using a nonlinear regression with a method of least-squares fitting (GraphPad, San Diego, CA)

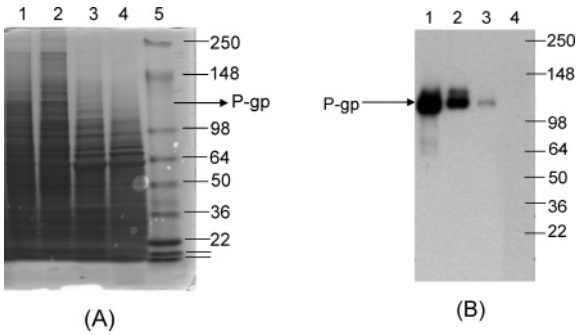

Figure 4. SDS−PAGE and Western blot assays of Hi5 insect membranes transfected with control vector, human MDR1, rhesus monkey Mdr1, and beagle dog Mdr1. (A) SDS−PAGE: One hundred micrograms of total membrane protein was loaded in each lane. The gel was stained by Fast Safe Stain (HTS Biotech, Inc. Liaoning, China). (B) Western blot: Two micrograms of total membrane protein was loaded in each lane. P-gp were hybridized using murine monoantibody C219 (1:150) and HRP-conjugated goat anti-mouse IgG2a (1:1000), and further visualized by ECL (Amersham Life Science, Piscataway, NJ). Lane 1: membrane expressing human P-gp. Lane 2: membranes expressing rhesus monkey P-gp. Lane 3: membranes expressing beagle dog P-gp. Lane 4: control membranes. Lane 5 (only in SDS−PAGE): protein marker.

assay, the rhesus monkey P-gp and beagle dog P-gp were expressed 1.2-fold higher or 0.5-fold lower than human P-gp, respectively.

P-gp ATPase Activity Profiles for 21 Drugs. The maximal velocity (υ_{max} or $\upsilon_{max,app}$) of P-gp ATP activity was calculated on the basis of total protein amount. Since the binding affinity of activator with enzyme−substrate complex (αKa) and the maximal stimulation fold by an activator (β) were calculated by the ratio of $\upsilon_{max,app}$ over υ_{max} (eq 2), the P-gp expression level has little impact on αKa and β calculation.

On the basis of chemical structures, drug classes, and reported P-gp binding sites (Table 2), 21 compounds were chosen to characterize their interactions with P-gp ATPase activities in human MDR1, rhesus monkey Mdr1, and beagle dog Mdr1 expressed Hi5 insect cell membranes. The results indicated that these compounds have different activation profiles (Table 2). Most of the test compounds such as ritonavir, saquinavir, vinblastine, quercetin, verapamil, digoxin, progesterone, rhodamine123, and propranolol stimulated

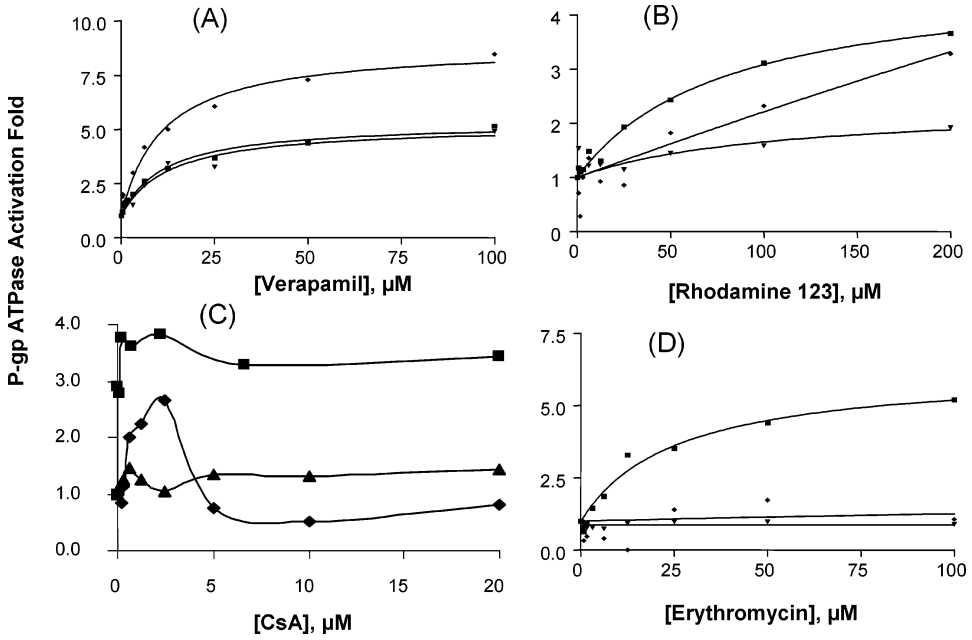

Figure 5. Activation of P-gp ATPase activities in human MDR1 (■), beagle dog Mdr1 (▲), and rhesus monkey Mdr1 (◆) expressed Hi5 insect cell membranes by verapamil, rhodamine 123, CsA, and erythromycin. The P-gp ATPase assay was executed for 20 min with 1 μg of P-gp membranes. MgATP was used at 5 mM in all assays. The released phosphate or phosphate standards were measured by a modified colorimetric reaction assay described previously.[19]

Table 3. Comparison of P-gp in Rhesus Monkeys or Beagle Dogs with the P-gp in Humans

	rhesus monkey P-gp	beagle dog P-gp
Gene Bank	AF537133	AF536758
amino acids	1283	1278
(human 1280)	96% identical to human MDR1	90% identical to human MDR1
	46 substitutions, 3 aa[a] insertion	120 substitutions, 4 insertions/deletions
	1 aa substitution (G to K) at a position (TM6) known to affect function	3 substitutions at positions (TM1, IC1, TM6) known to affect human MDR1 function
	2 of 3 aa changes in TM4; could affect function	

[a] Amino acid.

P-gp ATPase activities in all three species, although the αKa values of these compounds are different in these three species. Compounds such as cyclosporin A (CsA), etoposide, amiodarone, tamoxifen, and thioridazine activated both human and rhesus monkey P-gp ATPases, but not the beagle dog P-gp ATPase, while dexamethasone activated both human and beagle dog P-gp ATPases but not the rhesus monkey P-gp ATPase. Physostigmine, paclitaxel, and erythromycin stimulated only the human P-gp ATPase, and choloroquine stimulated only the rhesus monkey P-gp ATPase. A few compounds such as daunorubicin and Hoechst 33342 did not interact with P-gp ATPase activities in all three species at the concentrations tested. Thus, the ATPase activation pattern depends not only on the species but also on the structure and the concentration of test compounds. Examples of different activation patterns are presented in Figure 5. Rhodamine 123 had different maximal stimulation fold (β) and αKa values toward human, rhesus monkey, and beagle dog P-gp ATPase activities (Figure 4B and Table 2), whereas verapamil showed different β but similar αKa values in these three species (Figure 4A and Table 2). CsA had high αKa and β values to the human P-gp ATPase at concentrations up to 20 μM. However, its activation on the rhesus monkey P-gp ATPase decreased at concentrations greater than 2.5 μM. CsA did not stimulate the beagle dog P-gp ATPase at the tested concentration range (Figure 4C). Erythromycin stimulated only the human P-gp ATPase, but not rhesus monkey and beagle dog P-gp ATPase (Figure 4D and Table 2).

At the protein level, rhesus monkey P-gp and beagle dog P-gp are 96% and 90% identical to human P-gp, respectively (Table 3). In order to investigate if there are any correlations between protein similarities and P-gp ATPase activities, we compared αKa and β of P-gp ligands to P-gp ATPase activities in these three species. The correlation coefficient of αKa was 0.83 between human and rhesus monkey (with 12 known ligands which have αKa and β values from both human and rhesus monkey P-gp ATPase assays), and was only 0.38 between human and beagle dog (with 10 known ligands which have αKa and β values from both human and beagle dog P-gp ATPase assays) (Figure 6). Consistent with the protein identities, our results demonstrated that the binding affinities of the P-gp ligands to the rhesus monkey P-gp ATPase were closer to those of the human P-gp ATPase

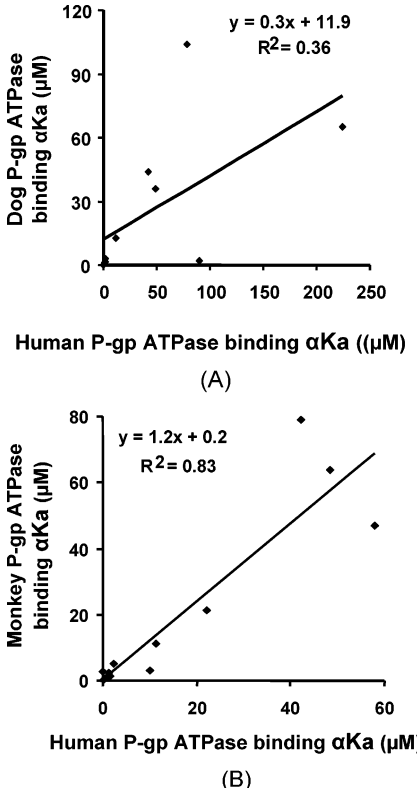

Figure 6. Correlations of αKa between human MDR1 and beagle dog Mdr1 (A), and between human MDR1 and rhesus monkey Mdr 1 (B). The αKa values were taken from Table 2. Ten compounds which have αKa values from both human and beagle dog P-gp ATPase assays in part A and 12 compounds which have αKa values from both human and rhesus monkey P-gp ATPase assays in part B were used in the correlation curve.

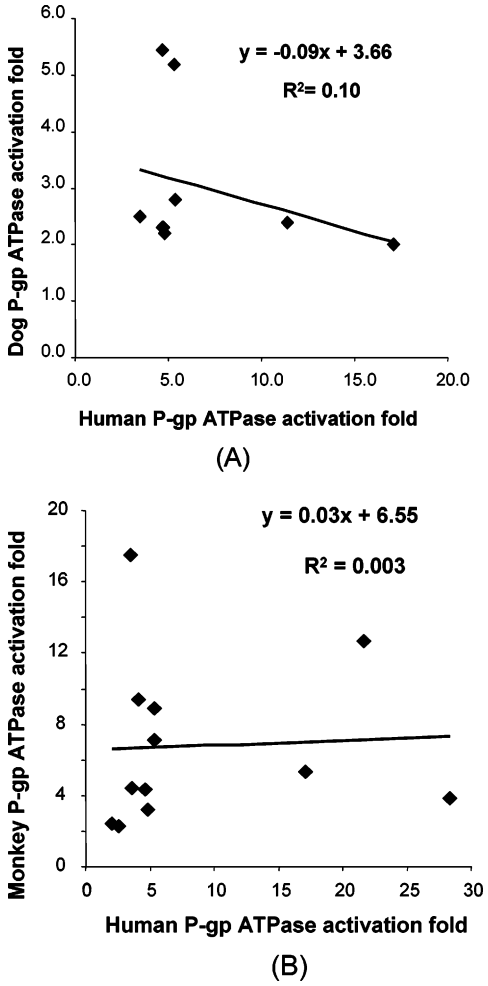

Figure 7. Correlation of the maximal activation fold of P-gp ATPase stimulated by the test compounds between (A) human MDR1 and beagle dog Mdr1, and between (B) human MDR1 and rhesus monkey Mdr1. The β values were taken from Table 2. Ten compounds which have β values from both human and beagle dog P-gp ATPase assays in part A and 12 compounds which have β values from both human and rhesus monkey P-gp ATPase assays in part B were used in the correlation curve.

than those of the beagle dog ATPase (Figure 6). There were no good correlations of maximal stimulation folds to P-gp ATPase activities in humans, rhesus monkeys, and beagle dogs (Figure 7).

Discussion

Information on the species difference in drug transporters is important for predicting human pharmacokinetics from preclinical studies. In the present study, we addressed the interspecies difference by analyzing P-gp ligand-induced ATPase activities in human, rhesus monkey, and beagle dog P-gp expressed baculovirus infected insect cells. The term "ligand" was used in the present study since P-gp ATPase assay cannot differentiate P-gp substrates from inhibitors.

P-gp, like many other ATPases, can be trapped in an intermediate state pathway through the catalytic cycle by reaction with inorganic vanadate (V_i) and ATP.[23] After one round of ATP hydrolysis, P_i dissociates and V_i moves in, leading to trapping of the complex $ADP{\cdot}V_i{\cdot}M^{2+}$ (where M^{2+} is a divalent cation such as Mg^{2+}, Mn^{2+}, or Co^{2+}) in one of the nucleotide binding domains. The conformation of the trapped state is thought to resemble that of the catalytic transition state. Vanadate trapping at one nucleotide binding domain of P-gp completely inhibits ATPase activities at both active sites.[23] Senior and co-workers have demonstrated that vanadate is a potent inhibitor of the ATPase activity in purified reconstituted P-gp proteins. In their experiments, the concentration of vanadate required for the 50% inhibition was 9 μM, and total inhibition was observed at 100 μM.[21,22] To achieve the maximal inhibition of P-gp ATPase activity, 100 μM vanadate was used in all present assays. Inhibitors of other ion-translocating ATPases, such as sodium azide, oligomycin, ouabain, and EGTA, have been demonstrated not to inhibit P-gp ATPase activity,[12] and they were added

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in the assay buffer to inhibit non-P-gp-related ATPase activities. In the presence of EGTA, ouabain, and sodium azide, the difference of phosphate released from P-gp membranes with and without vanadate treatment reflected the P-gp associated ATPase activity (Figure 1).

The preferred substrate for hydrolysis by P-gp is MgATP. For MgATP, P-gp ATPase activity followed simple Michaelis−Menten kinetics with an apparent Ks of 1.6 mM (Figure 3), which is close to the reported value of 1 mM.[21,22] Several groups have shown that P-gp displays a constitutive ATPase activity even in the absence of substrate,[2,22,24,25] and the full catalytic cycle of ATP hydrolysis can clearly proceed when the substrate binding sites are unoccupied. However, since substrate transport is driven by ATP hydrolysis, occupation of the substrate binding site can alter the affinity of the catalytic sites for nucleotide. Sharom and co-workers demonstrated that binding of ATP to P-gp was favored if the substrate binding site is also occupied, since the presence of both transport substrate and ATP would be expected to promote both transport cycle and ATPase catalytic cycles. Our current results (Figure 3) also showed that, in the presence of 20 μM verapamil, the Ks of MgATP slightly decreased from 1.6 mM to 1.1 mM whereas the maximal ATPase catalytic activity (ν_{max}) increased from 7.1 to 23 nmol mg^{-1} min^{-1}, suggesting that verapamil increased the binding affinity of ATP to P-gp and stimulated the P-gp ATPase hydrolysis activity.

The nonessential enzyme activation model (Scheme 1)[20] was applied to our data analysis for estimating the constant of substrate binding affinity to the P-gp−ATP complex (αKa) and the maximal P-gp ATPase stimulation fold (β), since occupation of the substrate and nucleotide binding sites occurs in a random order,[26] and P-gp possesses both constitutive and drug-stimulated ATPase activities.

Since profiling drug-stimulated ATPase activities can reflect the nature of interaction between P-gp and drug ligands, the P-gp ATPase assay on P-gp activities from different species may be used as a tool to assess the interspecies P-gp difference. We selected 21 drugs to characterize the differences of P-gp ATPase activities in human MDR1, rhesus monkey Mdr1, and beagle dog Mdr1 expressed insect cell membranes (Table 2) in order to understand the species differences in P-gp activities. These 21 drugs cover a variety of structures and different P-gp binding sites.[27−29] The R-site is the rhodamine123 binding site which is located on transmembrane domains TM6, TM9, and TM12,[30,31] and the H-site is the Hoechst 33342 binding site which is on TM5 and TM11.[30] The third binding site is a P-gp binding site which can positively stimulate either R-site or H-site specific substrate transport.[27] The test compounds stimulated ATPase in human, rhesus monkey, and beagle dog P-gp membranes with different patterns (selectively shown in Figure 5). The inhibitory effect on the rhesus monkey P-gp ATPase by CsA, at high concentrations, may be caused by more regulatory binding sites present in the rhesus monkey P-gp membranes. The binding affinities (αKa) of these test compounds toward the rhesus monkey P-gp membranes are much closer to those in the human P-gp than those in the beagle dog P-gp (Table 2). The correlation coefficient (R^2) of αKa between human P-gp and monkey P-gp was 0.83, and the slope was 1.2 (Figure 6A). However, the correlation coefficient of (R^2) of αKa between human P-gp and beagle dog P-gp was 0.36, and the slope was only 0.3 (Figure 6B). At the protein level, rhesus monkey P-gp and beagle dog P-gp are 96% and 90% identical to the human P-gp, respectively (Table 3). Thus, the correlation of ligand binding affinities to P-gp agreed well with P-gp protein similarities among these species. Although the rhesus monkey Mdr1 is close to the human MDR, the binding affinity of CsA to the rhesus monkey Mdr1 is more than 200-fold lower than that to the human MDR1. This huge difference could be due to the binding of CsA to TM4 or TM6 domains, where the amino acid substitutions occurred in rhesus monkey Mdr1 (Table 3). Several groups have demonstrated that a single mutant present in P-gp cytoplasmic loops, transmembrane segments, and linker regions could change the cross-linking between nucleotide binding domains (NBDs), and thus change the P-gp ATPase activities.[32−34] Rhodamine 123, which binds to TM6, TM9, and TM12 of the P-gp,[31] exhibited a lower binding affinity to the rhesus monkey Mdr1 than to the human MDR1, likely for the same reason. However, it is difficult to conclude that the change

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in binding affinity constants among three species is merely due to P-gp binding sites, since little information on the binding sites of test compounds is known.

Interestingly, although there is a good correlation between P-gp substrate binding affinity constants in humans and those in rhesus monkeys, there were no good correlations of the maximal ATPase activation fold stimulated by the test substrates among human, rhesus monkey, and beagle dog P-gp activities. Besides the high interplate variation of β values (Table 1), the more important reason could be the unknown degree of the ATP hydrolysis activity coupling to the P-gp efflux function since the rate-limiting step of P-gp-mediated drug transport is the carrier reorientation step.[30]

In conclusion, the present studies demonstrated that rhesus monkeys are much closer to humans than beagle dogs with respect to the P-gp ligand binding affinities. The maximal hydrolysis velocity of P-gp ATPase, however, cannot directly reflect the P-gp efflux transport rate, and, therefore, the correlation of drug transport velocity among different species needs to be further investigated using transport assays in MDR1/Mdr1 transfected cells or membrane vesicles.

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