

DMD #11866

Interactions of Cyclosporin A with Breast Cancer Resistance Protein

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DMD #11866

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ABBREVIATIONS: Cyclosporin A (CsA), P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), canalicular multispecific organic anion transporter (cMOAT)

DMD #11866

ABSTRACT

The objective of this study was to investigate if cyclosporin A (CsA) is a modulator for BCRP. The interactions between CsA and BCRP were evaluated by using both membrane and cell-based assays. CsA inhibited BCRP or BCRP R482T mutant associated ATPase with an IC_{50} of 26.1 and 7.3 μM (31388 and 8779 ng/mL), respectively, indicating that CsA is a modulator for BCRP and its R482T mutant. The apparent permeability (P_{app}) of CsA was not affected by the BCRP specific inhibitor Ko143 in both apical-to-basolateral (A-to-B) and basolateral-to-apical (B-to-A) directions in hBCRP- or mBcrp- transfected MDCKII cells, whereas CsA at 50 μM significantly increased the A-to-B transport and decreased B-to-A transport of BCRP substrates, [^3H]-E3S and [^3H]-MTX, in hBCRP- and mBcrp1-transfected MDCKII cells. Similar to cellular transport studies, CsA did not exhibit ATP dependent uptake in BCRP expressed membrane vesicles but inhibited the ATP mediated E3S and MTX uptake in the same vesicles. The inhibitory constant (K_i) of CsA toward BCRP was 6.7 μM (8507 ng/mL) and 7.8 μM (9380 ng/mL) when using E3S or MTX as a BCRP substrate, respectively. The inhibitory potency of CsA on BCRP wild type or its R482T mutant was lower than that on P-gp. The present studies demonstrate that CsA is an inhibitor but not a substrate for BCRP, and has low potential to cause drug-drug interactions with BCRP substrate drugs due to its weak inhibitory effect on BCRP and BCRP R482T mutant at its normal therapeutic blood concentrations (200 to 400 ng/mL) (Emilia et al., 1998).

DMD #11866

INTRODUCTION

Cyclosporin A (CsA) is a noncytotoxic immunosuppressant that was first discovered in 1970. It is an antibiotic produced by the fungus *Tolypocladium Inflatum* Gams. CsA initially was used for immunosuppression following organ and marrow transplantation. Subsequently, it has been applied in virtually all branches of medicine where autoimmune or inflammatory processes play a role in the pathology (Laupacis et al., 1982).

CsA is a substrate and inhibitor of P-glycoprotein (P-gp) (Coley et al., 1989; Silbermann et al., 1989; Saeki et al., 1993). It has been used as one of the first-generation multidrug resistance (MDR) modulators to reverse MDR and improve chemotherapy (Tan et al., 2000). As a P-gp inhibitor, CsA is involved in drug-drug interactions (Sparreboom and Nooter, 2000). It can increase the plasma concentration and decrease the clearance of P-gp substrates, such as digoxin and etoposide (Carcel-Trullols et al., 2004; Englund et al., 2004; Shibayama et al., 2004), and it can also increase the brain penetration of P-gp substrates such as nimodipine and verapamil (Liu et al., 2003; Sasongko et al., 2005).

Breast cancer resistance protein (BCRP), also known as MXR, ABCP and ABCG2, was initially cloned from highly doxorubicin-resistant MCF7 AdVp human breast cancer cells and mitoxantrone-resistant S1-M1-80 human colon carcinoma cells in 1998 (Doyle et al., 1998; Miyake et al., 1999). Subsequently, it has been demonstrated to confer the resistance to quite a few anticancer drugs such as mitoxantrone, doxorubicin, daunorubicin, topotecan, irinotecan, etoposide, flavopiridol, methotrexate (MTX) and imitinib and other therapeutic agents, such as zidovudine, pantoprazole, cimetidine

DMD #11866

sulfasalazine, nitrofurantoin, and several statins (Xia et al., 2005b). BCRP mutations have been found in some cancer cell lines (Honjo et al., 2001). The mutation has either threonine (T) or glycine (G), instead of arginine (R), at the amino acid position 482. The R482 of BCRP locates in the third transmembrane domain and may alter the substrate specificity upon mutation. The mutant BCRP confers resistance to daunorubicin, doxorubicin, epirubicin and idarubicinol (Xia et al., 2005b). Interestingly, these mutations were only found in drug resistant human tumor cell lines (Honjo et al., 2001), but not in human individuals (Honjo et al., 2002; Zamber et al., 2003). To date, BCRP has become one of the three major ATP-binding cassette (ABC) membrane efflux transporters besides P-glycoprotein (P-gp) and multi-drug resistance associated protein (MRP) conferring drug resistance in cancer and inflammation chemotherapies (Silbermann et al., 1989; Litman et al., 2001; Merino et al., 2004; van der Heijden et al., 2004a; van der Heijden et al., 2004b). Besides being present in drug-resistant cancer and T-cells, BCRP is also endogenously expressed at high level in human placenta and to a lesser extent in liver, small intestine and colon, ovary, veins, capillaries, kidney, adrenal and lung, with little to no expression in brain, heart, stomach, prostate, spleen and cervix (Doyle et al., 1998; Litman et al., 2001; Maliepaard et al., 2001; Scheffer and Scheper, 2002). In addition, BCRP is expressed in the human jejunum at levels considerably higher than many other ABC transporters (Taipalensuu et al., 2001). BCRP has been demonstrated to be present on the apical membrane of intestinal epithelium and to limit the oral absorption of topotecan in mouse and human (Jonker et al., 2000; Kruijtzter et al., 2002a). Given the liver and intestinal localization pattern, BCRP, similar to P-gp, may act as a barrier to uptake and limit the oral bioavailability of

DMD #11866

drugs as well as mediating hepatobiliary excretion of drugs (Jonker et al., 2000; Jorritsma et al., 2002; Kruijtzter et al., 2002b).

Since CsA is a commonly used drug for treating autoimmune or inflammatory diseases and reversing the MDR of anti-cancer reagents (Laupacis et al., 1982), and BCRP has broad substrate specificity (Xia et al., 2005b), it is essential to clearly understand the interactions between CsA and BCRP to better interpret the enhanced cytotoxicity of anticancer drugs and drug-drug interactions caused by CsA clinically. The effect of CsA on BCRP has been debatable (Wierdl et al., 2003; Xia et al., 2004; Ejendal and Hrycyna, 2005; Qadir et al., 2005). CsA has been demonstrated as a broad spectrum modulator for multidrug resistance proteins such as P-gp, BCRP, multi-drug resistance protein 1 (MRP1) and lung resistance protein (LRP), by cell based uptake and cytotoxicity studies (Qadir et al., 2005). Gupta A. et al. have demonstrated that CsA is not a BCRP substrate but inhibit BCRP substrate efflux with an IC_{50} of $4.3 \pm 1.9 \mu\text{M}$ ($5171 \pm 2285 \text{ ng/mL}$) using flow cytometric efflux assay in BCRP-expressing HEK293 cells (Pawarode et al.). Wierdl et al. demonstrated that CsA was a weak BCRP inhibitor and its inhibitory potency depended upon the amount of BCRP expression. CsA showed higher IC_{50} value in high protein expressing cells than in the low expressing ones (Wierdl et al., 2003). However, Ejendal and Hrycyna showed that CsA was not an inhibitor or substrate of BCRP by ATPase assay and cell accumulation studies of [^3H]-CsA (Ejendal and Hrycyna, 2005). All the controversial results could be due to the different assay systems and the chosen substrates. In the present studies, we characterized the modulations of BCRP by CsA using both cellular transport studies and membrane based assays. We demonstrated that CsA was not a substrate of BCRP in BCRP-overexpressed

DMD #11866

membrane vesicles, human BCRP (hBCRP) or murine Bcrp1 (mBCRP1) transfected MDCKII cells. We also demonstrated that CsA reduced the ATPase activities of both wild type BCRP and BCRP R482T mutant with an IC_{50} of 26.1 and 7.3 μ M (31388 and 8779 ng/mL), respectively, and inhibited BCRP-mediated efflux of estron-3-sulfate (E3S) and methotrexate (MTX) with a K_i of 6.7 and 7.8 μ M (8507 and 9380 ng/mL), respectively. K_i was measured to avoid the potentially different inhibitory potency values caused by experimental conditions as K_i is an intrinsic constant, which reflects the affinity of the inhibitor for the functional protein (such as transporter, enzyme or receptor) and is independent of substrate and incubation conditions (protein concentration or incubation time etc.). Compared with the effect on daunorubicin-stimulated P-gp ATPase activity, CsA had less potency on the inhibition of daunorubicin-stimulated BCRP R482T mutant ATPase activity. Knowledge on the inhibitory potency of CsA on BCRP is valuable to understand the DDI caused by CsA and assist clinic dose regimen of CsA as a MDR reverse reagent.

MATERIALS AND METHODS

Human BCRP or BCPR R482T mutant expressed cell membranes and human BCRP expressing and control membrane vesicles were obtained from Solvo Biotechnology, Inc. (Budaörs, Hungary). The control membranes were not used in the assays since the BCRP expressing membranes have specific BCRP activities (personal communication with Solvo). MDR1 (P-gp) expressed membrane was obtained from BD Gentest (Woburn, MA). All chemicals were analytical grade and purchased from Sigma Aldrich (St. Louis, MO). [3 H]-CsA, [3 H]-MTX and [3 H]-estrone-3-sulfate were obtained

DMD #11866

from American Radiolabeled Chemicals (St. Louis, MO), Morevek Biochemicals (Brea, CA) and Perkin Elmer Life Sciences (Boston, MA), respectively.

ATPase activity assay

BCRP, BCRP R482T mutant or P-gp associated ATPase activities were measured according to the method by Sarkadi et al. (Sarkadi B., 1992). The liberation of inorganic phosphate from ATP was quantified using a sensitive colorimetric assay originally described by Chifflet et al (Chifflet, 1988). Cell membrane expressing human BCRP (5 $\mu\text{g}/\mu\text{L}$) was thawed rapidly in 37 °C water bath before diluting to a concentration of 1 $\mu\text{g}/\mu\text{L}$ in ice cold ATPase assay medium (KCl, 50 mM; dithiotheritols, 2 mM; MOPS-Tris, 50 mM, pH 7.0) 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. Cell membrane (20 μL of 1 $\mu\text{g}/\mu\text{L}$ solution), with or without 360 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 adding 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 dodecylsulfate (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 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 (Pavek et al.,

DMD #11866

2005). The SDS containing samples were supplemented with 160 μ L 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 Plus³⁸⁴ Spectrophotometer (Molecular Device, Sunnyvale, CA). 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 BCRP mediated ATPase activity (Ozvegy et al., 2001). The IC₅₀ of CsA on BCRP or Mutant BCRP ATPase activities was calculated by XL-Fit (ID Business Solutions Ltd., Cambridge, MA).

Membrane Vesicle Transport Assay

ATP-dependent transport of [³H]-CsA, [³H]-E3S, or [³H]-MTX into inside-out BCRP membrane vesicles was measured by a modified rapid filtration method that was adapted to a 96-well plate format (List et al., 2001). The assay was conducted at 37°C in a total volume of 50 μ L assay buffer (50 mM KCl, 7 mM MgCl₂ and 50 mM MOPS-Tris, pH 7.0) containing 10 μ g of membrane vesicle protein, test compounds and 10 mM of Mg-ATP or 10 mM of Mg-AMP. BCRP-mediated uptake was measured for 4 min, and stopped by 200 μ L ice-cold washing buffer (70 mM KCl and 40 mM MOPS-Tris, pH 7.0). The uptake buffer mixture was then transferred to a Perkin Elmer unifilter GF/B plate and followed by five more 250 μ L washes using the cell harvest (Perkin Elmer Life Sciences, Boston, MA). Tritium was counted on a Perkin Elmer Top Count NXT Microplate Scintillation and Luminescence Counter (Perkin Elmer Life Sciences, Boston, MA). ATP-dependent uptake of the test compound was calculated by subtracting the uptake in the presence of Mg-AMP from the uptake measured in the presence of Mg-

DMD #11866

ATP. The uptake rate was calculated based on the total protein content of the membrane vesicles. K_i values were calculated using a computer generated curve-fitting program, Sigma Plot 8.0 (SYSTAT Software Inc, Chicago, IL).

Cell Culture

Polarized MDCKII cells stably expressing human BCRP (hBCRP) or murine *Bcrp1* (*mBcrp1*) (*ABCG2*) cDNA were kindly provided by Dr. Alfred Schinkel (Netherlands Cancer Institute) (Jonker et al., 2000). The MDCKII cells were cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO₂ under humidifying conditions. Cells were seeded on microporous polycarbonate membrane filters (0.33 cm² growth area and 0.4 μ pore size Transwell; Costar, Corning, NY) at a density of 1 x 10⁵ cells/well in 0.2 ml of complete medium. MDCKII cells were grown for 3 days with medium replacements every day.

Transport Studies

Bi-directional transport studies were performed at 37°C in air. Prior to each experiment, the confluent cell monolayers on Transwell™ inserts were washed and equilibrated for 30 minutes with transport media (Hank's balanced salt solution (HBSS) containing 10 mM of N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) and 10 mM of glucose, pH 7.4). The experiment was initiated by adding a solution containing the test compound to either the apical (for A-to-B transport) or basolateral (for B-to-A transport) compartment. When applicable, inhibitors were present in the transport medium of the donor side from the preincubation period throughout the permeability study. At 15, 30, 45 and 60 minutes, the sample aliquots of receiving solutions were

DMD #11866

withdrawn from the basolateral side (for A-to-B transport) or from the apical side (for B-to-A transport), and replaced immediately with an equal amount of fresh transport media except at the 60 min time point (the end of the incubation). The samples were mixed with 5 mL of scintillation cocktail and the radioactivity was determined in a liquid scintillation spectrophotometer (Beckman, Fullerton, CA). The cell integrity was monitored by transepithelial electric resistance (TEER).

Transport Calculation

The cumulative amount of drug (Q) on the receiver side was plotted as a function of time. The steady-state flux J was then estimated from the slope (dQ/dt). The apparent permeability coefficient (P_{app}) of unidirectional flux for the test compound was estimated by normalizing the flux J (mole/sec), against the nominal surface area A (0.33 cm^2) and the initial drug concentration in the donor chamber C_0 (mol/mL), or $P_{app} = J/(A \cdot C_0)$. The B/A ratio equals to the P_{app} value for B-to-A transport ($P_{app, B-to-A}$) divided by the P_{app} value for A-to-B transport ($P_{app, A-to-B}$).

All the data are expressed as mean \pm standard deviation (SD) of three individual incubations. Test of significance of difference between mean values were made using a two-tailed unpaired Student's *t* test. A probability of less than 0.05 ($p < 0.05$) was considered to be statistically significant.

Results

Effects of CsA on BCRP ATPase activities.

CsA reduced vanadate sensitive ATPase activities of wild type BCRP and BCRP R482T mutant with the IC_{50} of 26.1 and 7.3 μM (31388 and 8779 ng/mL), respectively

DMD #11866

(Figure 1). The inhibitory effects of CsA on BCRP and BCRP R482T mutant associated ATPase activities indicate that CsA is a modulator for both BCRP and BCRP R482T mutant.

Effects of CsA on daunorubicin-stimulated P-gp or BCRP R482T mutant ATPase activities

To compare the inhibitory potency of CsA on P-gp and mutant BCRP, the effects of CsA on daunorubicin-stimulated P-gp or BCRP R482T mutant ATPase activities were evaluated. The basal ATPase activities of P-gp and BCRP mutant were 3.8 and 37.4 nmole/min/mg. Daunorubicin at the concentration of 200 μ M could stimulate P-gp or BCRP R482 mutant ATPase activities by 2.6 and 2.4 fold, respectively (Figure 2A). CsA inhibited daunorubicin-stimulated P-gp or BCRP mutant ATPase in a concentration dependent manner. The solubility of CsA allowed using 50 μ M as the highest concentration in these assays. The IC_{50} of CsA on daunorubicin-stimulated BCRP mutant ATPase activity was 29.2 μ M (35116 ng/mL) which is 3.0 fold lower than that on daunorubicin-stimulated P-gp ATPase activity (Figure 2B), indicating that CsA may have less inhibition potency on BCRP mutant than on P-gp.

CsA is not a Substrate for BCRP

The known BCRP substrate, E3S (Suzuki et al., 2003), and BCRP highly selective inhibitor, Ko143 (Allen et al., 2002; van der Heijden et al., 2004a) were used to confirm the activity of BCRP in the transfected membrane vesicles. There were no difference of E3S uptake among the BCRP expressing membrane vesicles in the presence of AMP and the control membrane vesicles in the presence of AMP or ATP (Figure 3A, $p > 0.05$). The uptake of E3S in the BCRP expressing membrane vesicles was significantly different

DMD #11866

in the presence of ATP and AMP (Figure 3A, $p < 0.05$). The ATP-dependent uptake of E3S was saturable (Figure 3A, $K_m = 2.4 \mu\text{M}$ and $V_{\text{max}} = 50.4 \text{ pmole/mg/min}$). The BCRP selective inhibitor, Ko143, only inhibited E3S uptake in BCRP expressing membrane vesicles in the presence of ATP but not in the membranes in the presence of AMP or in the control membrane vesicles (Figure 3B). The IC_{50} of Ko143 on the ATP-mediated E3S uptake in BCRP expressing membrane vesicles was about $0.013 \mu\text{M}$ (Figure 3B inset). All these results demonstrated that BCRP function well in the BCRP expressing membrane vesicles. There was no difference of $[^3\text{H}]\text{-CsA}$ uptake in BCRP over-expressed cell membrane vesicles in the presence or absence of ATP (Figure 3C), suggesting that CsA is not a substrate of BCRP. To further prove this observation, the transport of CsA in hBCRP, mBcrp and vector control-transfected MDCKII cells was also assessed. Ko143 did not significant increase A-to-B transport and decrease B-to-A transporter of CsA in vector control, hBCRP, or mBcrp1-transfected MDCKII cells (Figure 4, $p > 0.05$) indicating that CsA is not a substrate for BCRP or mBcrp. The lower P_{app} values of CsA from A-to-B direction than those from B-to-A direction in all three transfected MDCKII cells (Figure 4) could be caused by the endogenous P-gp and MRP efflux functions. The different P_{app} values of CsA from B-to-A directions among three transfected-MDCKII cells (Figure 4) were probably due to the expression level differences of other transporters, such P-gp, MRP and OATP (Su et al., 2004).

CsA is an inhibitor of BCRP

The P_{app} value of E3S from A-to-B direction was 4.5 fold less than that from A-to-B direction in both hBCRP and mBcrp transfected MDCKII cells while they did not show any significant difference in vector control transfected MDCKII cells ($p > 0.05$),

DMD #11866

indicating the hBCRP and mBcrp function well in the transfected cell lines. At a concentration of 50 μM , CsA diminished the directional transport of E3S in both hBCRP and mBcrp-transfected MDCK cells, suggesting that CsA inhibited BCRP-mediated E3S efflux (Figure 5A).

MTX has been shown as a wild-type BCRP substrate in membrane vesicle assays (Volk and Schneider, 2003), cell accumulation studies (Volk et al., 2002), and in the present transport studies (Figure 6). In vector control-transfected MDCKII cells, MTX did not show any significant difference of transports from both A-to-B and B-to-A (Figure 6). However, in hBCRP and mBcrp1-transfected MDCKII cells, the P_{app} value of MTX from A-to-B direction was 7.9-fold and 12.5 fold less than that from B-to-A direction, respectively, indicating that BCRP mediates MTX efflux in transfected cells. With 50 μM CsA co-incubation in hBCRP and mBcrp1-transfected MDCKII cells, the A-to-B transport rate of MTX was significantly increased and the B-to-A transport rate was significantly decreased (Figure 5B, $p < 0.05$).

In order to further investigate the inhibition potency of CsA on BCRP, we exploited BCRP-expressing membrane vesicular transport assay by using E3S and MTX as BCRP substrates. The K_m of E3S and MTX to BCRP was 2.5 μM and 1.9 mM, respectively (Figure 6A and 6B). CsA was an uncompetitive inhibitor of BCRP and the inhibition constant (K_i) of CsA for BCRP-mediated E3S and MTX efflux was 6.7 μM (8507 ng/mL) and 7.8 μM (9380 ng/mL), respectively, which was calculated by Eadie-Hofstee plot (Figures 6A and 6B).

Discussion

DMD #11866

BCRP, one of the ABC transporter family members, demonstrated a baseline level of ATPase activity in BCRP over-expressed mammalian or insect cell membranes, which is 2~3 fold higher than that of P-gp (Ozvegy et al., 2001; Ozvegy et al., 2002). Similar to the P-gp ATPase activity, both BCRP and BCRP R482T mutant ATPase activities were vanadate sensitive and could be stimulated or inhibited by its substrates or inhibitors (Ozvegy et al., 2001; Ozvegy et al., 2002; Xia et al., 2004). Although the BCRP R482T mutant was only found in the drug resistant human tumor cell lines (Honjo et al., 2001) but not in human individuals (Honjo et al., 2002; Zamber et al., 2003), it is still of interest to know if CsA can modulate this mutant because CsA is commonly used MDR reversing reagent in anti-cancer therapy. CsA reduced vanadate sensitive ATPase activities of wild type BCRP and BCRP R482T mutant with the IC_{50} of 26.1 and 7.3 μ M (31388 and 8779 ng/mL), respectively (Figure 1). Because the ATPase assay is not a transport functional assay and can not distinguish substrates from inhibitors, the inhibitory effects of CsA on BCRP ATPase activities (Figure 1) indicate that CsA is a modulator of BCRP and BCRP R482T mutant activity. The lower IC_{50} of CsA on the BCRP R482T mutant ATPase activity than that on the wild type BCRP ATPase activity (Figure 1) suggest that CsA binds to the BCRP R482T mutant stronger than to the wild type BCRP. However, it does not mean that CsA has more potent inhibitory effect on the efflux function exerted by the BCRP R482T mutant than by the wild type BCRP because it is still contentious whether the ATPase can reflect transport functions (Polli et al., 2001).

The functional interactions between CsA and BCRP transporter were evaluated using both membrane vesicle and cellular based assays. BCRP membrane vesicular

DMD #11866

transport assay is a high throughput assay to identify BCRP substrates or inhibitors. Because BCRP-mediated transport needs ATP as an energy source and ATP can not pass through the lipid membrane due to its hydrophilicity, only inside-out membrane vesicles can bind to ATP and pump a substrate into the vesicles. By rapid filtration, the membrane vesicles can stay in a filter membrane and the substrate trapped inside the vesicles can be measured by sensitive analytical techniques such as LC/MS/MS, fluorescent spectrometer and scintillation spectrophotometer. The difference of the uptake in the presence or absence of ATP is attributed to BCRP-mediated transport. The membrane vesicular assay is an effective *in vitro* system to assess kinetic constant such as K_m for substrates and K_i for inhibitors (Xia et al., 2004). CsA did not show any ATP-dependent uptake in BCRP expressed membrane vesicles (Figure 3C) indicating that CsA is not a substrate of BCRP. Since the membrane vesicular transport assay may give a false negative result for compounds with high passive permeability and high lipophilicity, hBCRP-MDCKII, and mBcrp1-MDCKII cells were chosen to further confirm the findings from membrane vesicular transport studies (Figures 4). Combined with BCRP highly selective inhibitor, Ko143 (IC_{50} is 0.033 μ M for BCRP, > 50 μ M for P-gp, > 100 μ M for MRP2 and 61.3 μ M for OATP2 (Xia et al., 2005a)), we have demonstrated that CsA is not a substrate for mBcrp and BCRP in both mBcrp1 and hBCRP transfected MDCKII cells (Figure 4) and is consistent with the published observations (Saeki et al., 1993; Gupta et al., 2006).

CsA has been used to reverse the MDR by abrogating P-gp in cancer treatments (List et al., 2001). CsA also inhibits BCRP or mBcrp1-mediated E3S and MTX efflux hBCRP-MDCKII, and mBcrp1-MDCKII cells (Figures 5 and 6). As an inhibitor for

DMD #11866

efflux pumps, CsA can be potentially applied in clinic to alleviate MDR function in cancer cells and improve the drug absorption and disposition of efflux pump substrates. In order to get clinical benefits from the combination chemotherapy by overcoming MDR with CsA, determination of the inhibition potency is valuable for the dose regimen. Since the IC_{50} values of CsA on BCRP inhibition are dependent on BCRP protein expression (Wierdl et al., 2003), we measured the K_i of CsA on BCRP using a low affinity substrate (MTX) and a high affinity substrate (E3S) in membrane vesicular transport assays. The K_i of CsA for wild type BCRP was $6.7 \mu\text{M}$ (8507 ng/mL) and $7.8 \mu\text{M}$ (9380 ng/mL) determined by using human BCRP expressing membrane vesicles and E3S and MTX as BCRP substrates, respectively (Figure 6). The Southwest Oncology Group reported (List et al., 2001; Doyle and Ross, 2003) that CsA administered by 72-hour continuous intravenous infusion at a dosage of 5 mg/kg per day concurrently with daunorubicin has no clinical benefit in the daunorubicin chemotherapy for relapsed acute myeloid leukemia, whereas, CsA at a dosage of 16 mg/kg significantly improved median survival and overall survival rates in P-gp positive AML but not in P-gp negative AML patients. It is suspected that the lack of survival benefits in the P-gp negative may be due to a presence of other efflux transporter, such as BCRP, in AML patients (Doyle and Ross, 2003). A possible reason could be that the median whole blood CsA concentration of 1774 ng/mL, when CsA was i.v. infused at 16 mg/kg/day in AML patients, was higher than CsA K_i for P-gp of $1.1 \mu\text{M}$ (1311 ng/mL) (Shiraki et al., 2000) but lower than the K_i of $6.7 \mu\text{M}$ (8507 ng/mL) or $7.8 \mu\text{M}$ (9380 ng/mL) for BCRP (Figure 6). Daunorubicin is known to be a more selective substrate for BCRP R482T mutant (Honjo et al., 2001) than wild type BCRP. The inhibitory effect of CsA on daunorubicin-simulated BCRP R482T

DMD #11866

mutant ATPase activity was 3.0 fold less than that on P-gp ATPase activity (Figure 2B), indicating that CsA has less inhibition potency on BCRP mutant than on P-gp as well.

In conclusion, we have used BCRP-expressing membrane and cell lines to investigate the inhibitory mechanism and potency of CsA on BCRP. CsA is not a substrate but an uncompetitive inhibitor of BCRP. CsA can modulate both wild type BCRP and BCRP R482T mutant activity with K_i of 6.7 ~ 7.8 μM (8507-9380 ng/mL) for the wild type BCRP using E3S and MTX as substrates. Therefore, CsA may not cause BCRP-related drug-drug interactions at its normal therapeutic blood concentrations (200 to 400 ng/mL, $[I]/K_i < 0.04$) (Emilia et al., 1998). In order to exploit CsA-mediated BCRP inhibition for reversing the MDR in cancer chemotherapy, the pharmacological concentration of CsA may need to be higher than 6.7-7.8 μM (8507-9380 ng/mL) in order to obtain therapeutic benefits upon coadministration with BCRP substrate drugs.

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DMD #11866

LEGENDS FOR FIGURES

Figure 1. Effects of CsA on BCRP (A) or BCRP R482T mutant (B) associated ATPase activities. The vanadate-sensitive ATPase activity of BCRP expressed cell membrane was determined as described under “Materials and Methods”. The maximum concentration of tested CsA was 50 μ M in the assays because of its low solubility. The IC_{50} of CsA on BCRP or BCRP mutant associated ATPase activities was calculated by XL-Fit. Each data point represents the mean of triplicate determinations (\pm STD).

Figure 2. The effect of CsA on the daunorubicin-stimulated P-gp and BCRP R482T mutant ATPase activity. (A) P-gp and BCRP R482 mutant ATPase activities in the absence or presence of 200 μ M of Daunorubicin. The basal ATPase activities of P-gp and BCRP mutant were 3.8 and 37.4 nmole/min/mg, respectively. In the presence of 200 μ M of daunorubicin, the ATPase activities of P-gp and BCRP mutant were increased to 9.8 and 90.4 nmole/min/mg. (B) The inhibitory effects of CsA on daunorubicin-stimulated P-gp and BCRP mutant ATPase activities. Daunorubicin was tested at 200 μ M and the maximum concentration of tested CsA was 50 μ M in the assays because of its low solubility. The IC_{50} s of CsA on daunorubicin-stimulated P-gp and BCRP mutant ATPase activities were 9.8 and 29.2 μ M (11785 and 35116 ng/mL), respectively. The IC_{50} of CsA on P-gp or BCRP mutant associated ATPase activities was calculated by XL-Fit. Each data point represents the mean of triplicate determinations (\pm STD).

Figure 3. (A) E3S uptake in BCRP-expressing or control membrane vesicles. The K_m of E3S was about 2.0 μ M. **(B) The inhibitory effect of Ko143 on E3S uptake in BCRP-expressing or control membrane vesicles.** E3S was tested at 2.0 μ M, which was close to K_m . The inset is the determination of the IC_{50} of Ko143 on BCRP-mediated

DMD #11866

E3S uptake in BCRP expressing membrane vesicles. The IC₅₀ curve was fitted by XL-Fit. **(C) CsA uptake in BCRP-expressing membrane vesicles.** [³H]-CsA at different concentrations was incubated with BCRP membrane vesicles (10 μg) in the assay buffer containing 10 mM ATP or AMP for 4 min. Each data point represents the mean of triplicate determinations (± STD).

Figure 4. Transport of CsA in hBCRP or mBcrp1-transfected MDCKII cells. CsA and Ko143 were used at the concentration of 0.22 μM and 1 μM respectively. Each bar represents the mean of triplicate determinations (± STD).

Figure 5. The effect of CsA on the transport of E3S and MTX in vector control, hBCRP or mBcrp1-transfected MDCKII cells. [³H]-E3S, [³H]-MTX and CsA were used at the concentration of 35 nM, 60 nM and 50 μM, respectively, during the transport study. Each bar represents the mean of triplicate determinations (± STD).

Figure 6. The inhibitory effect of cyclosporin A on the BCRP-mediated membrane vesicular uptake of estrone-3-sulfate (A) and methotrexate (B). [³H]-estrone-3-sulfate (1.0, 1.5, 3.0, 6.0 μM) or [³H]-methotrexate (0.375, 0.75, 1.5, 3.0 mM) was incubated with BCRP membrane vesicles (10 μg) in the assay buffer containing 10 mM ATP or AMP for 4 min at 37°C in the presence or absence of cyclosporin A (0, 5, 10, 20 μM). ATP-dependent uptake of the test compound was calculated by subtracting the uptake in the presence of Mg-AMP from the uptake measured in the presence of Mg-ATP. Each data point represents the mean of triplicate determinations (± STD). The data points were fitted by Eadie-Hofstee Plot from SigmaPlot (version 8.0).

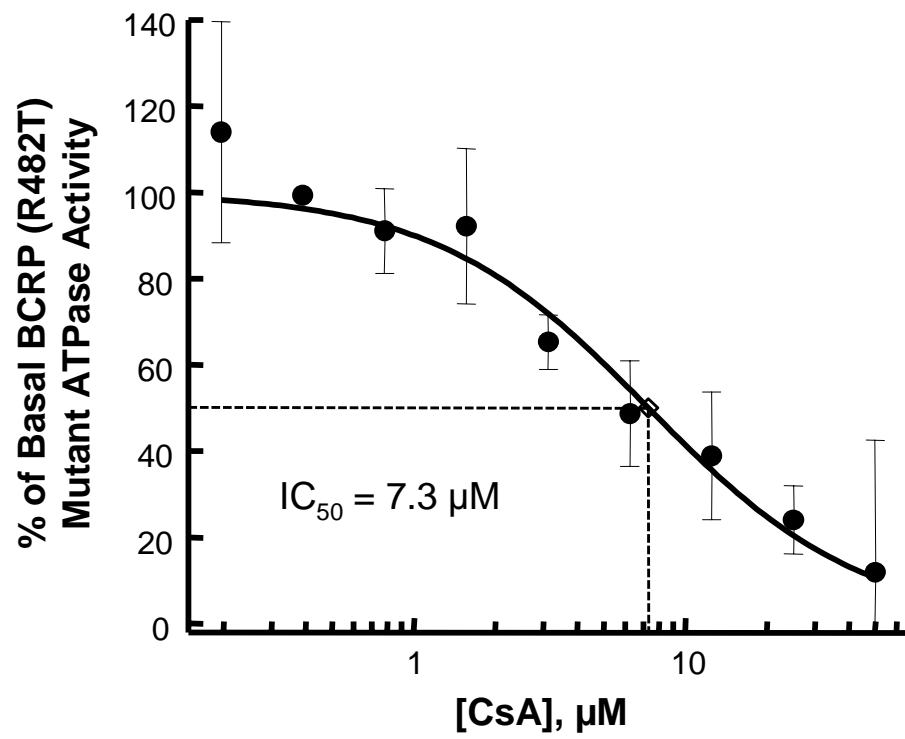
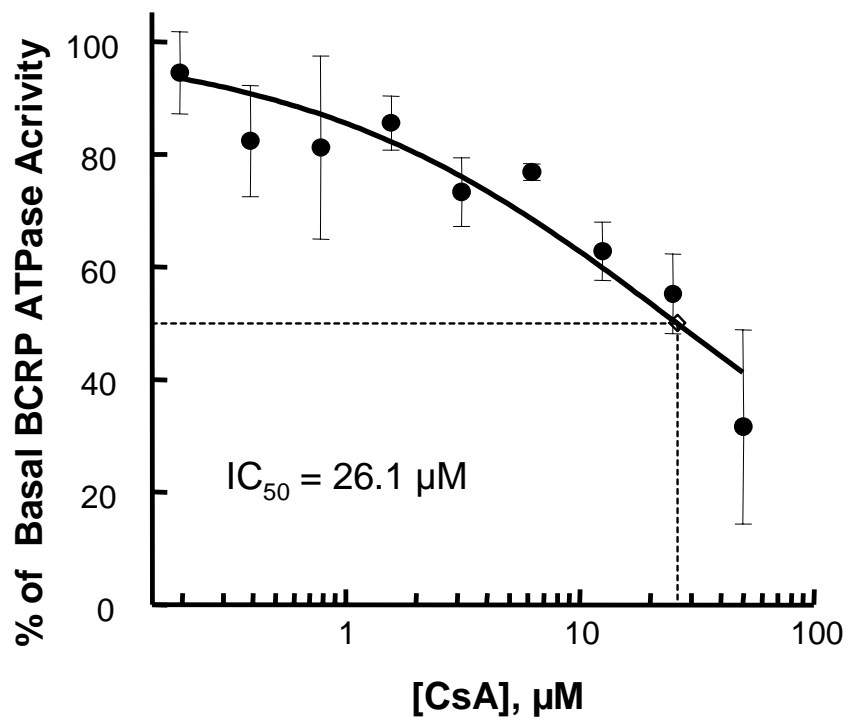
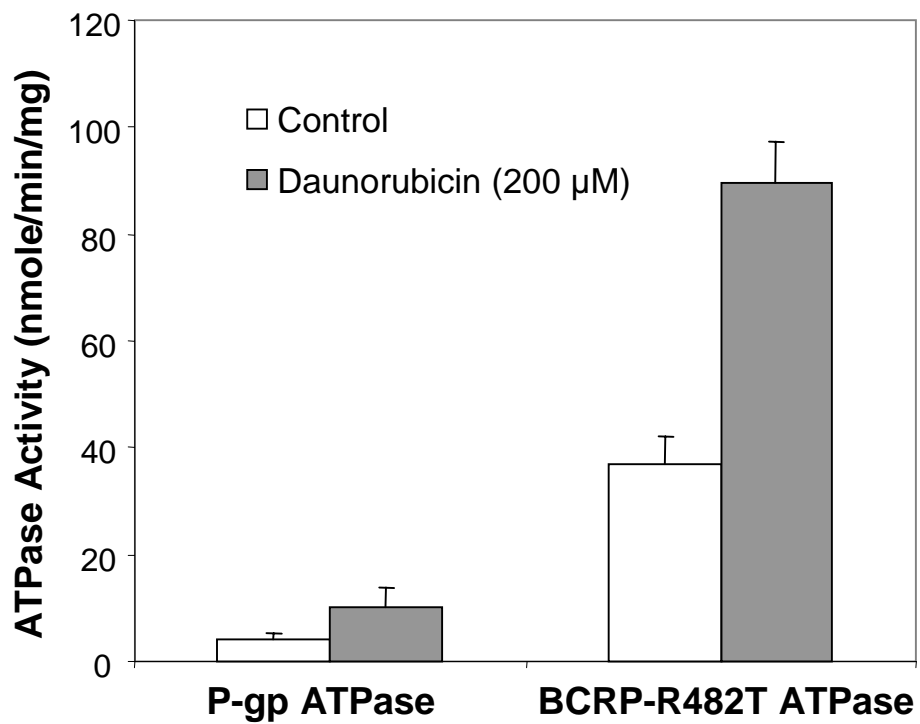
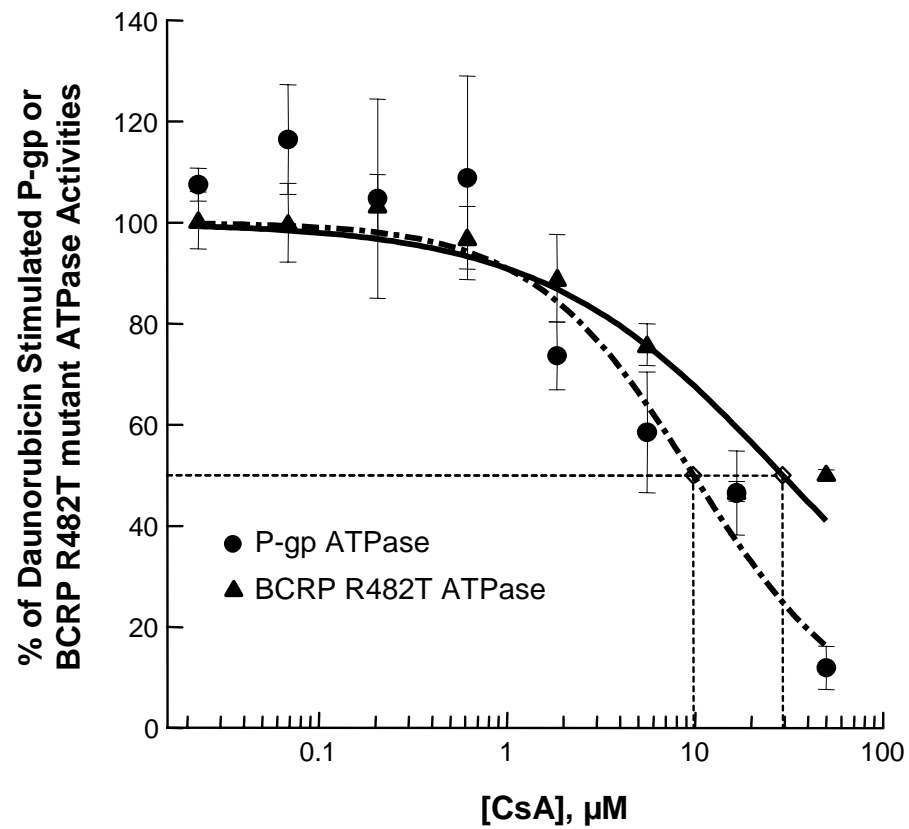


Figure 1



(A)



(B)

Figure 2

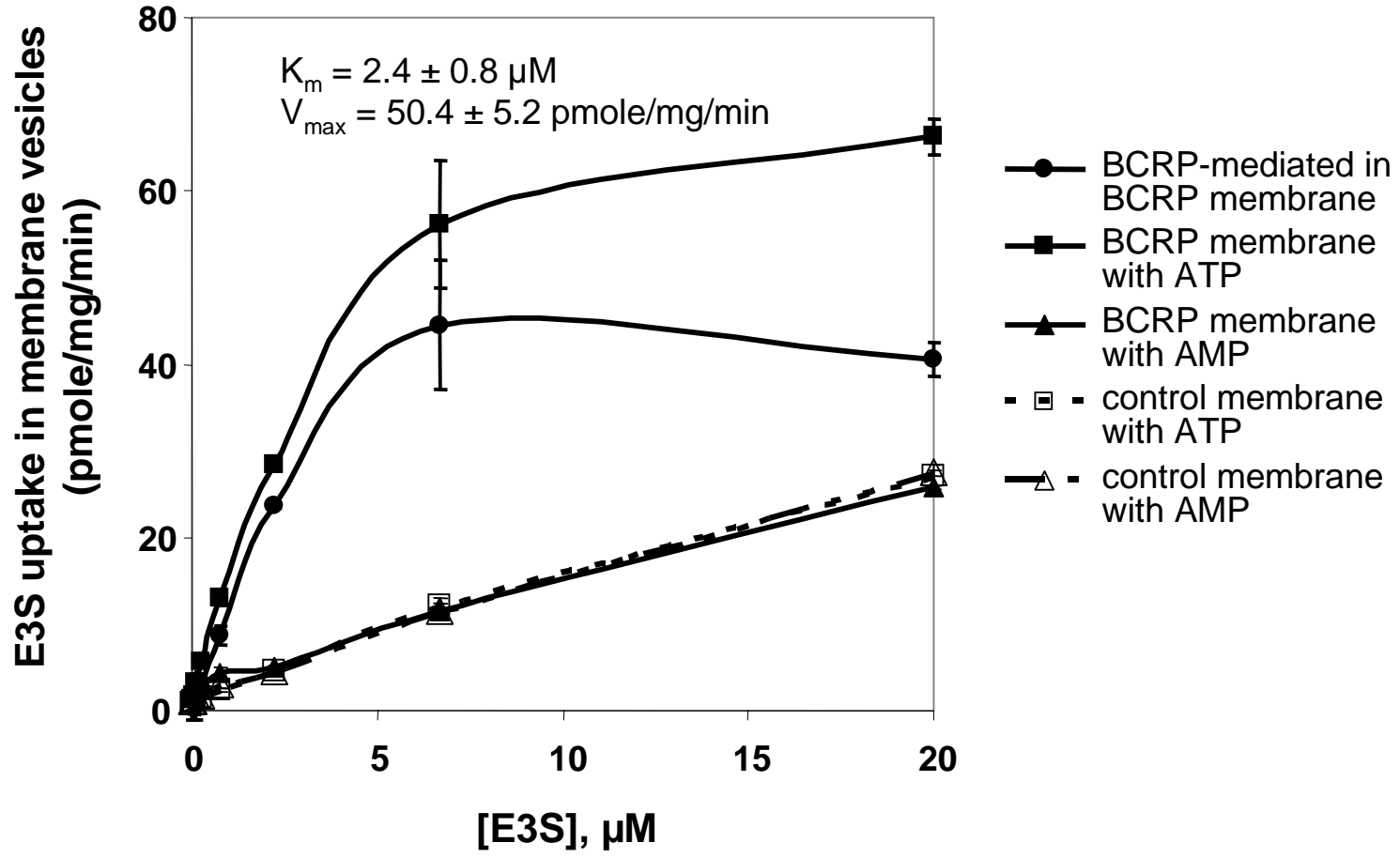


Figure 3A

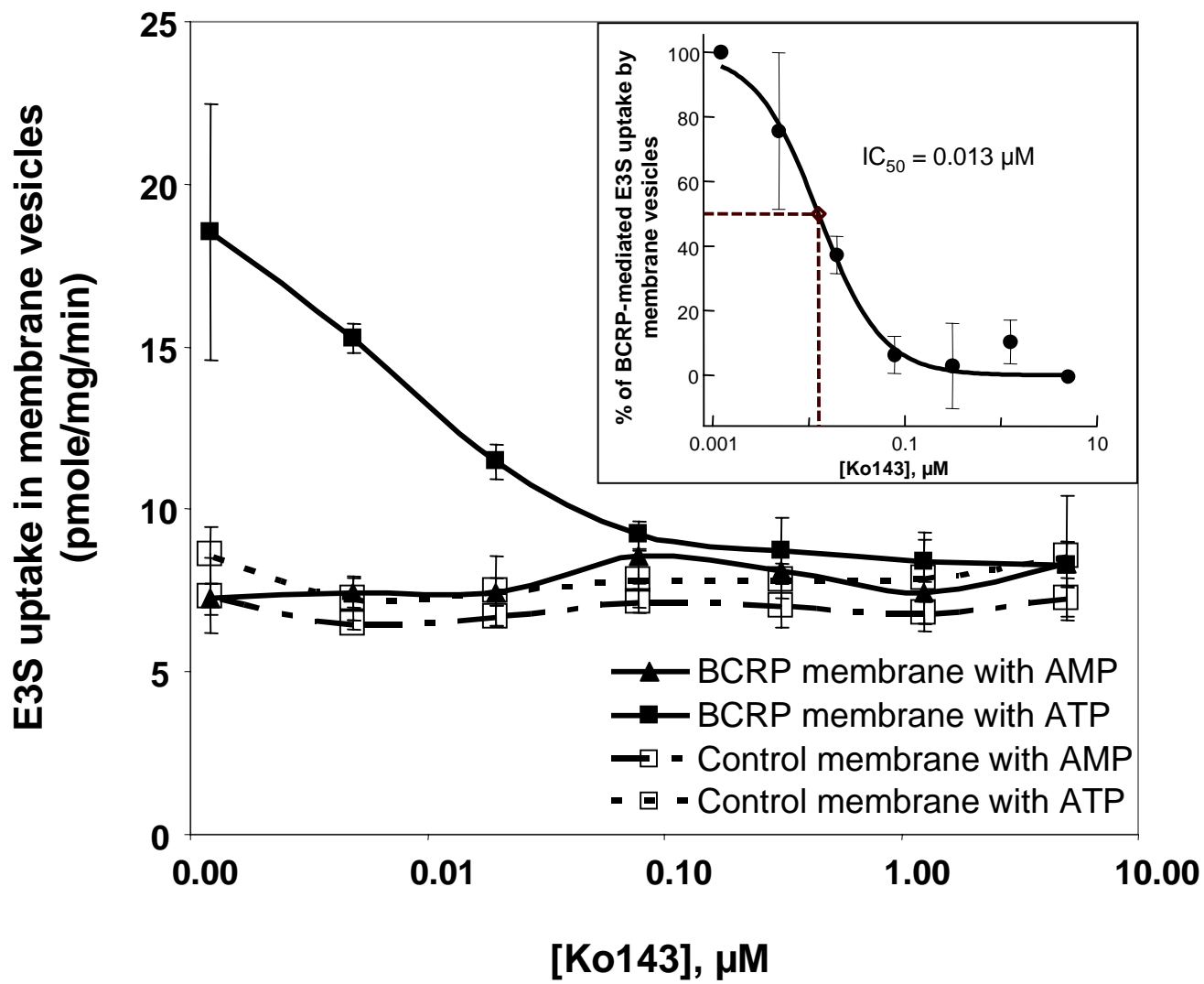


Figure 3B

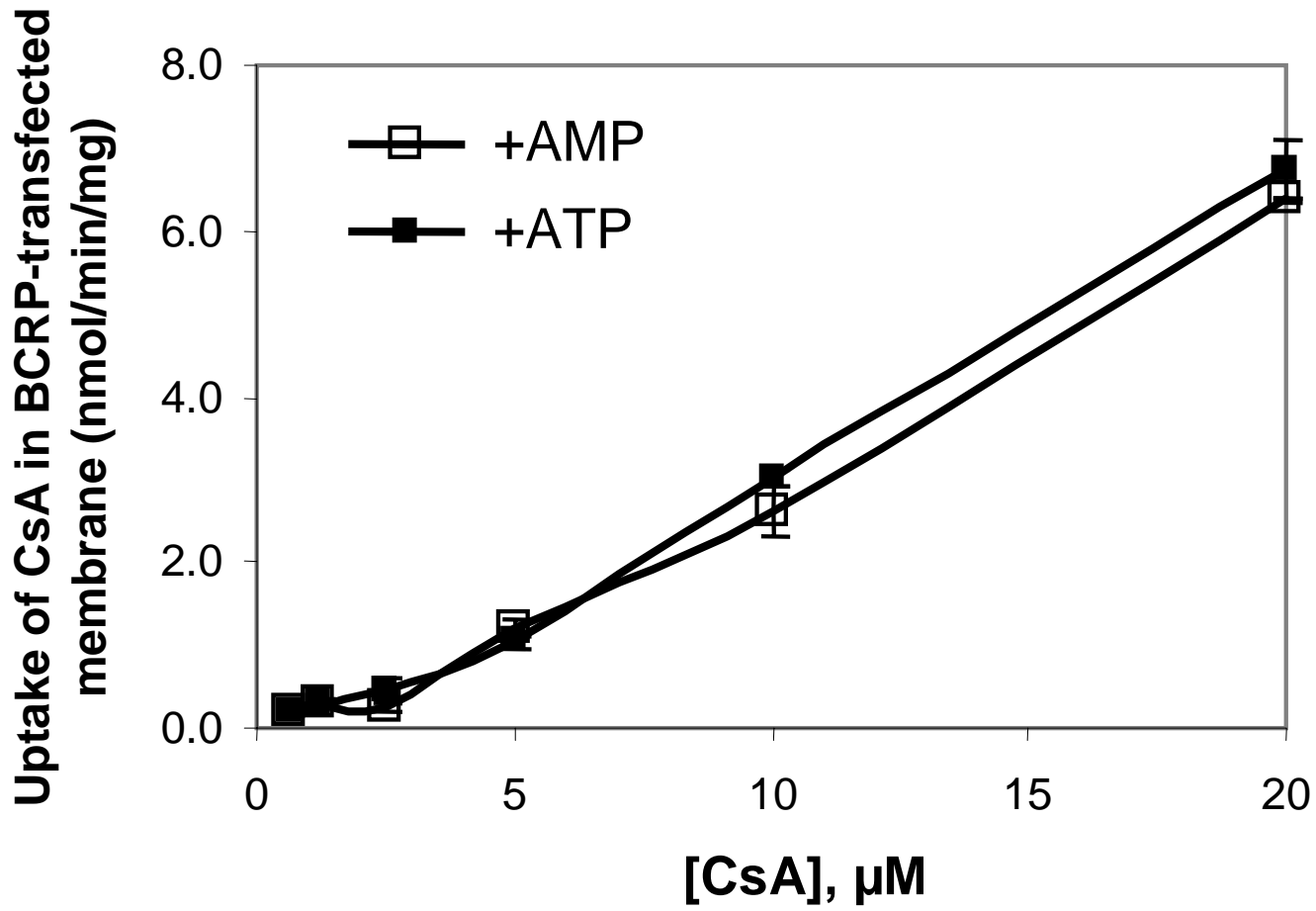


Figure 3C

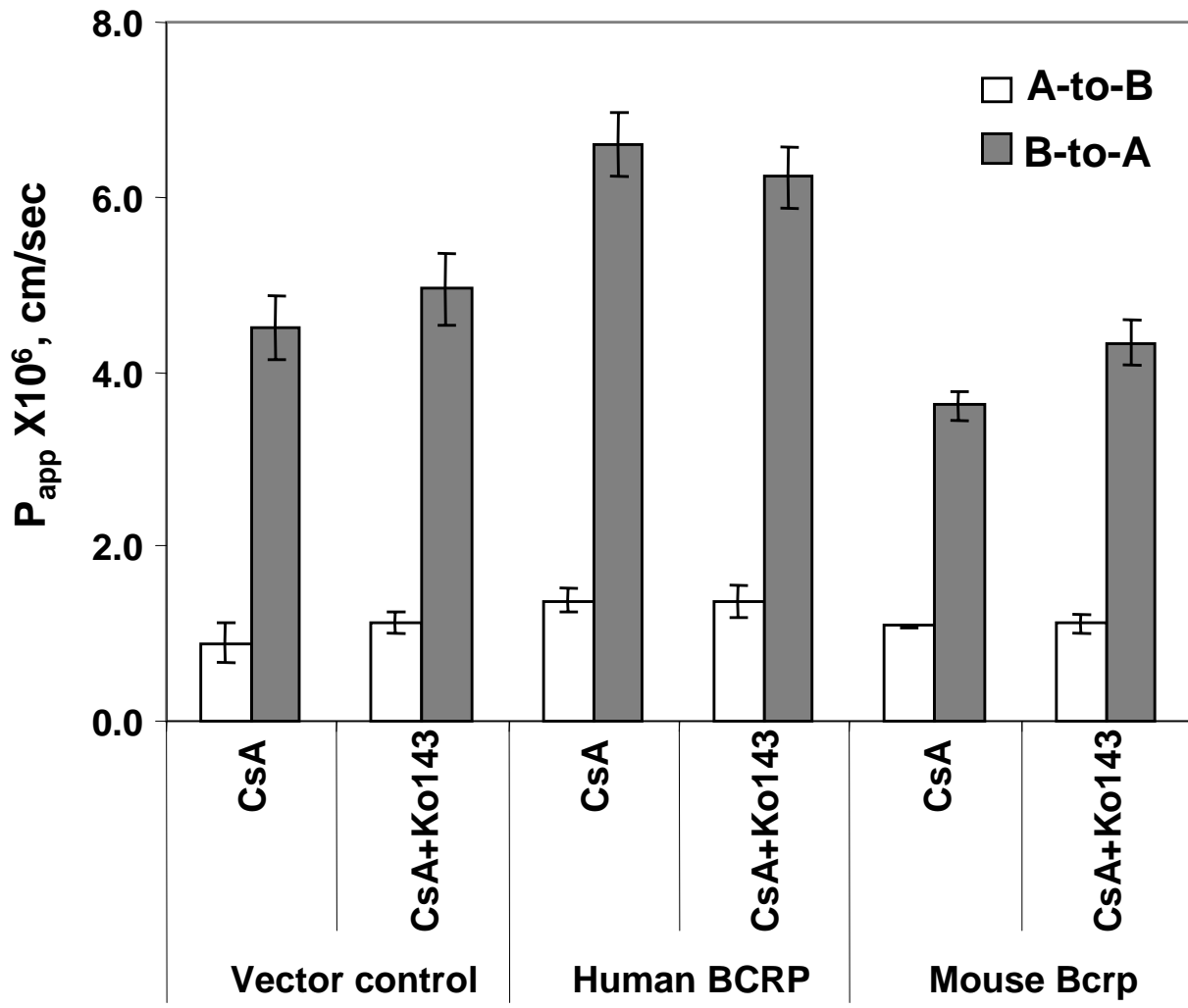


Figure 4

CsA=0.222uM

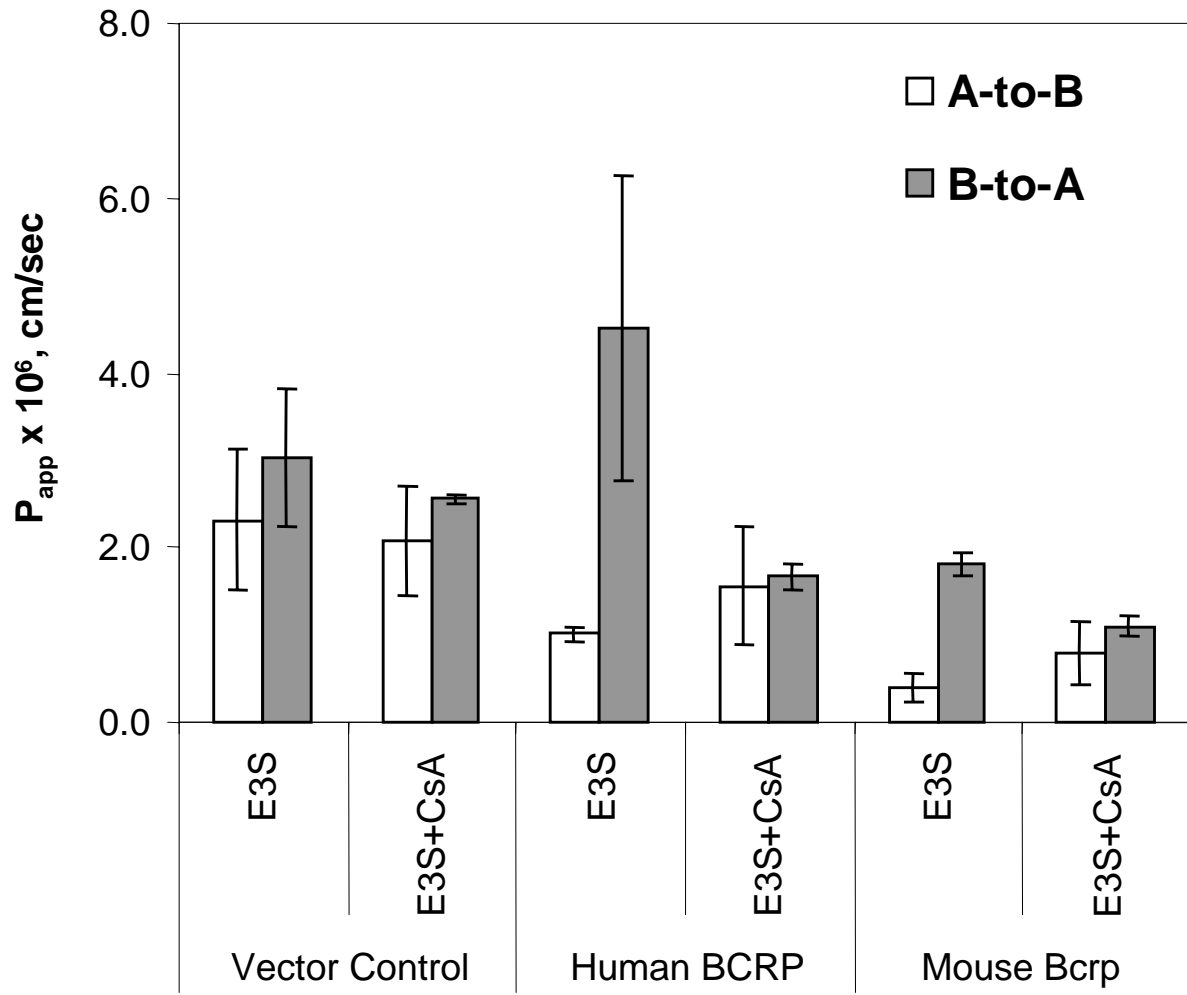


Figure 5A

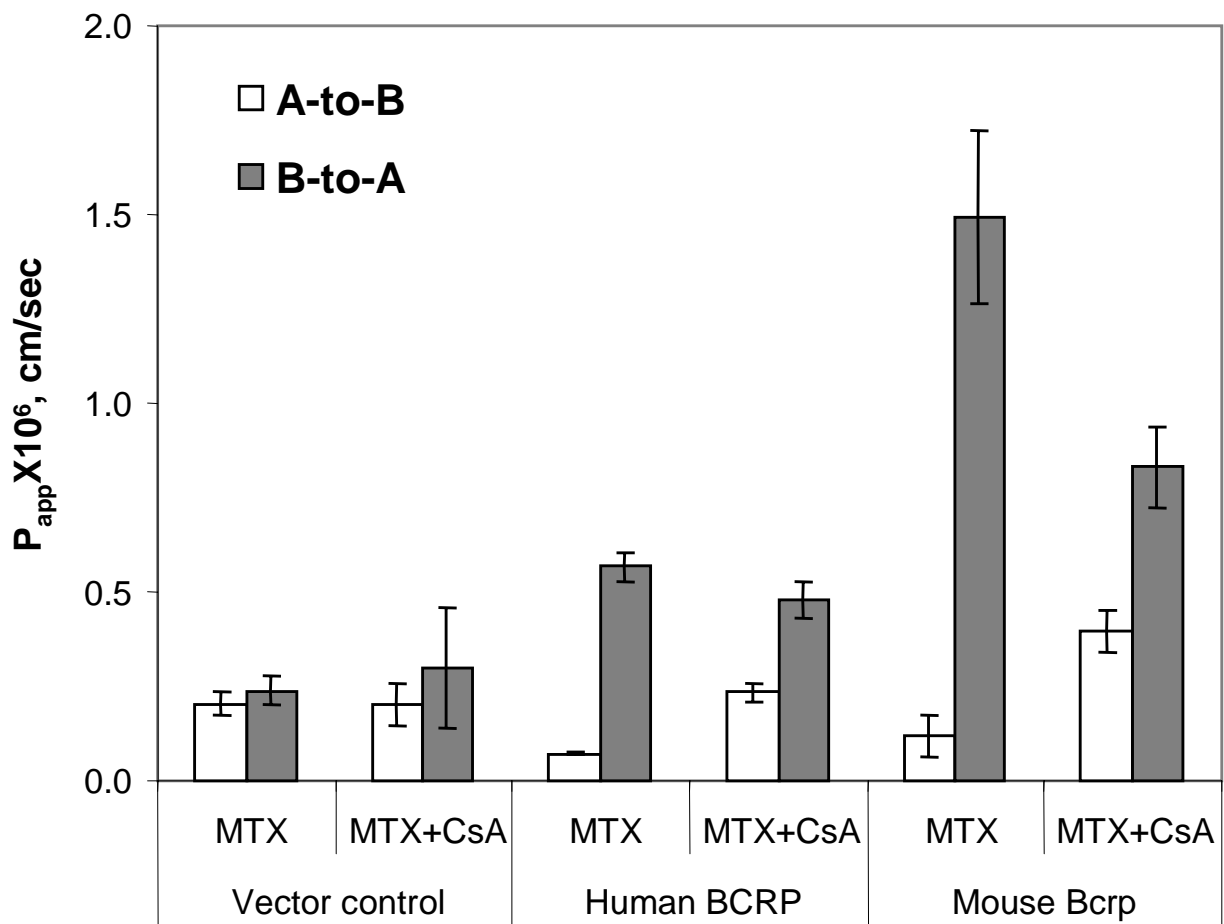


Figure 5B

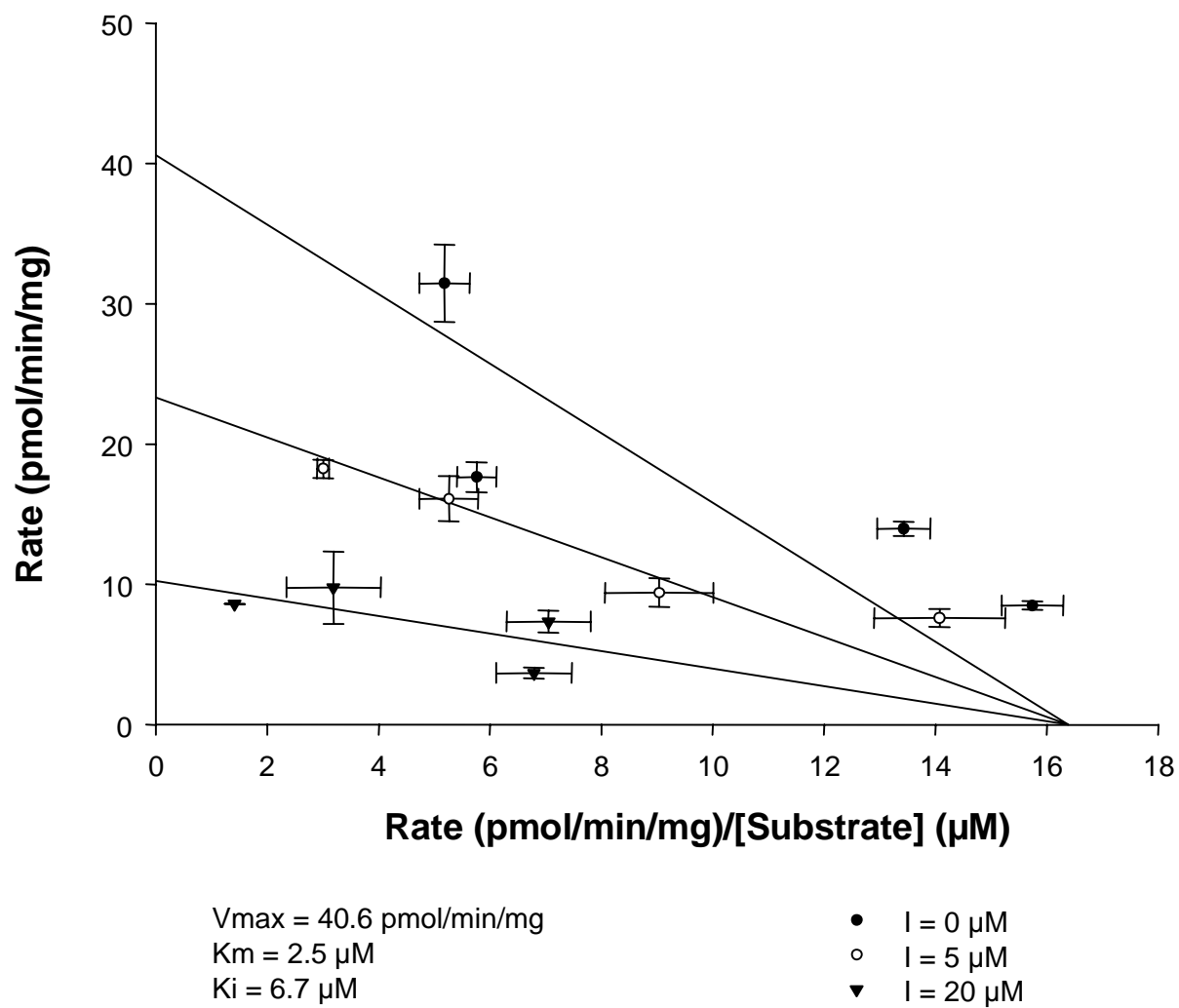


Figure 6A

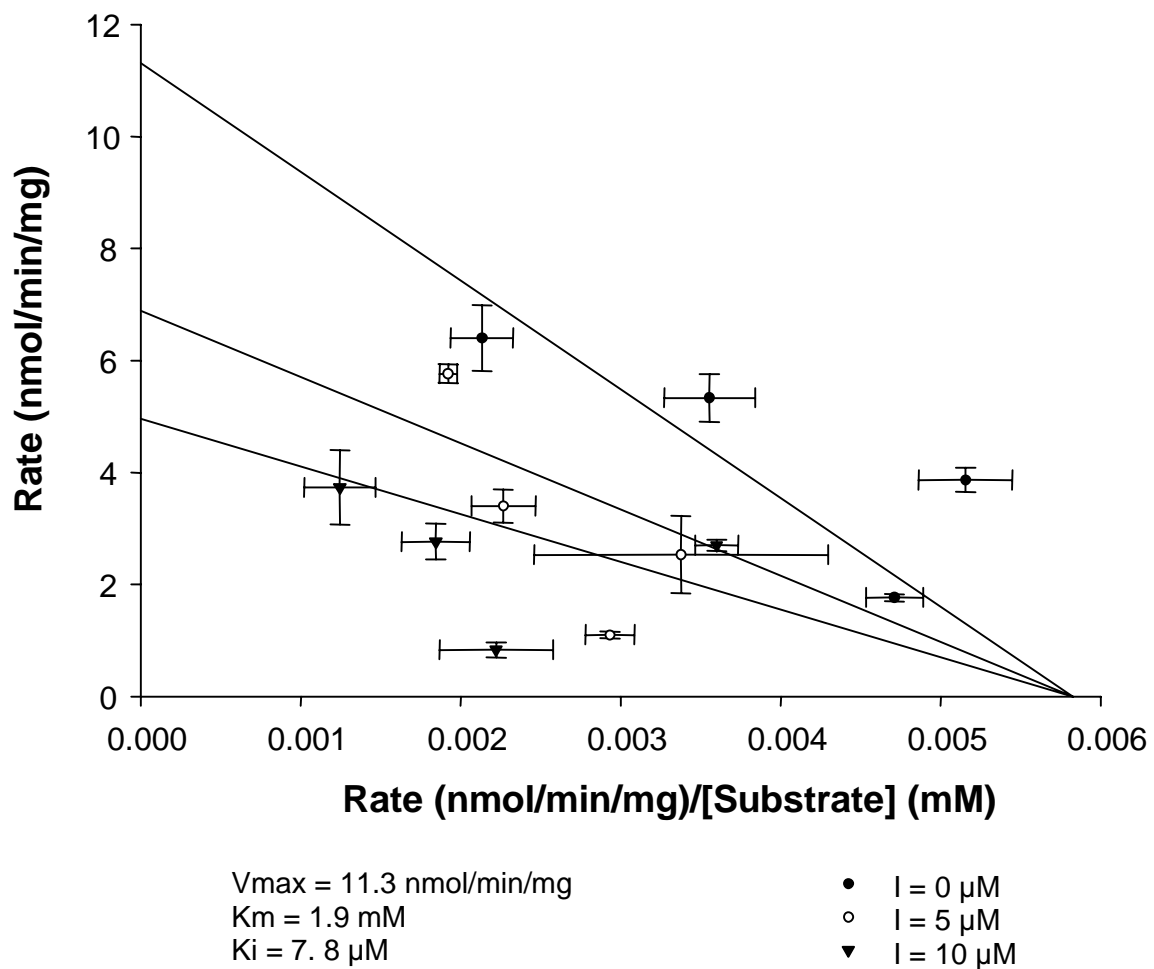


Figure 6B