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ATP-dependent Transport of Rosuvastatin in Membrane Vesicles Expressing Breast Cancer Resistant Protein

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Running Title: BCRP-mediated transport of rosuvastatin

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A list of nonstandard abbreviations used in the paper: ABC, ATP-binding cassette transporter; BCRP, breast cancer resistance protein; $E_2$17G, estradiol-17ß--D-glucuronide; GSH, glutathione; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MDR1, multidrug resistance protein 1; MDR1-MDCK, Madin-Darby Canine Kidney cells transfected with the human *MDR1* gene; MRP2, multidrug resistance protein 2; MXR, mitoxantrone resistance protein; P_{app}, apparent permeability; Pgp, P-glycoprotein; OATP, organic anion transporting polypeptide; A→B apical to basolateral; B→A, basolateral to apical.

Abstract

MDR1/ABCB1, MRP2/ABCC2 and BCRP/ABCG2 are expressed in the liver and intestine and contribute to the disposition of many drugs. Rosuvastatin, an HMG-CoA reductase inhibitor for the treatment of patients with dyslipidemia, is primarily excreted via bile as unchanged drug. The present study was designed to determine if rosuvastatin is transported by MDR1, MRP2 and BCRP. The apparent permeability value for rosuvastatin across MDR1-MDCK cells was low (~ 8 nm/second) and no directional transport was observed. rosuvastatin uptake into control Sf9 membranes and membranes expressing MRP2 was similar in the presence or absence of GSH. In contrast, ATP dramatically stimulated rosuvastatin uptake into membranes expressing BCRP, but not control membranes. Rosuvastatin transport occurred into an osmotically sensitive space and was saturable. An Eadie-Hofstee analysis suggested that there were two transport sites in BCRP, with an apparent K_m of 10.8 μM for the high affinity site and 307 μM for the low affinity site. These data demonstrate that rosuvastatin is transported efficiently by BCRP and suggest that BCRP plays a significant role in the disposition of rosuvastatin.

A number of ATP-binding cassette transporters (ABC) are expressed at the canalicular membranes of hepatocytes and the brush border membranes of enterocytes including the 1 P-glycoprotein (MDR1/ABCB1), the multidrug resistance protein 2 (MRP2/ABCC2), and the breast cancer resistance protein (BCRP/ABCG2) which is also known as the mitoxantrone resistance protein (MXR). These efflux transporters have distinct, but overlapping, substrate specificity and have been shown to be involved in biliary excretion of many drugs (Sasabe et al., 1998; Jonker et al., 2000). MDR1 P-glycoprotein (Pgp), which is overexpressed in resistant tumor cells, transports a wide variety of hydrophobic drugs such as daunorubicin, vinblastine, and loperamide (Hochman et al., 2002). MRP2 has been shown to transport many glutathione-S-, glucuronid-, and sulfate-conjugates. In addition to conjugates, MRP2 transports some unconjugated organic anions including methotrexate, irinotecan, and ampicillin (Gerk and Vore, 2002). The BCRP gene has been cloned and the protein product was found to be a half-transporter, consisting of a single 70 kDa, six-transmembrane peptide. Structurally diverse compounds such as anthracyclines, mitoxantrone, methotrexate, the camptothecins and estrone-3-sulfate have been identified as BCRP substrates (Sarkadi et al., 2004).

Rosuvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor for the treatment of patients with dyslipidemia. The absolute bioavailability of rosuvastatin was estimated as 20% with an estimated hepatic extraction ratio of 0.63 (Martin et al., 2003a). Metabolism of rosuvastatin appears to be a minor route of elimination in humans and rosuvastatin is mainly excreted into bile as parent. In a human

mass balanced study, 90% of the dose was recovered in feces, primarily (92%) as unchanged drug (Martin et al., 2003b).

Statins including rosuvastatin, pitavastatin, cerivastatin and pravastatin are substrates for OATP-C (OATP1B1), an organic anion transporting polypeptide expressed exclusively in the basolateral membrane of hepatocytes (Hsiang et al., 1999; Shitara et al., 2003; Schneck et al., 2004; Hirano et al., 2004). It has been shown that statins are substrates for multiple efflux transporters. Atorvastatin, cerivastatin and simvastatin have been reported to be Pgp substrates (Hochman et al., 2004; Kivisto et al., 2004). Pravastatin appears to be an MRP2 substrate. ATP-dependent transport of pravastatin has been demonstrated in the canalicular membranes isolated from rats and the biliary excretion of pravastatin is decreased in Mrp2-mutant rats (Yamazaki et al., 1997). The transport of pravastatin in the basal-to-apical direction (B→A) is 2.5 times greater than that in the apical-to-basal (A→B) direction across Madin-Darby Canine kidney (MDCK) monolayer expressing both OATP-C and MRP2 (Sasaki et al., 2002). Recent studies indicate that several statins are also transported by BCRP in vitro (Matsushima et al., 2005; Hirano M et al., 2005).

The present study was designed to determine if rosuvastatin is a substrate for MDR1, MRP2 or BCRP. The results indicate that rosuvastatin is transported efficiently by BCRP in membrane vesicles, and suggest that BCRP may play a significant role in the disposition of rosuvastatin.

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Methods

Materials

Rosuvastatin was supplied by AstraZeneca Pharmaceuticals LP. GF120918 was obtained from GlaxoSmithKline under a material transfer agreement. [^{3}H]-estradiol-17-β-D-glucuronide ($E_2$17G) was purchased from Perkin Elmer (Boston, MA). [^{3}H]-rosuvastatin was supplied by Amersham Bioscience UK Limited (Little Chalfont Buckinghamshire, UK). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Madin-Darby Canine Kidney Cells transfected with human MDR1 (MDR1-MDCK) were obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands). Transwells (12-well, 11-mm diameter, 0.4-μm pores) were purchased from Corning Costar (Cambridge, MA)

MRP2-Sf9-VT membranes (membranes isolated from Sf9 cells expressing MRP2, ATP-dependent transport of 200 nM ^{3}H-LTC4 was 20.8 pmol/mg protein/min), CTRL-Sf9 membranes (control membranes isolated from Sf9 cells expressing β-gal), MXR-M-VT membranes (membranes isolated from mammalian cells expressing BCRP, ATP-dependent transport of 100 μM ^{3}H-methotrexate was 172.6 pmol/mg protein/min) and CTRL-M membranes (control membranes isolated from mammalian cells which have no BCRP expression) were purchased from Solvo Biotechnology (Budapest, Hungary).

Monolayer efflux studies

MDR1-MDCK cells were cultured and transport experiments were conducted as described by Polli et al (2001). Briefly, cells were grown in Dulbecco's Modified Eagle

Medium supplemented with 10% fetal bovine serum in the absence of any antibiotics or selection agents. Cells were seeded onto Transwell at a density of 300,000 cells/cm^2 and medium was replaced daily and 2 h before transport experiments were performed. Monolayers were used for transport studies 3 days after seeding. Compounds were dissolved in DMSO, and then diluted in Hank's balanced transport buffer (pH 7.4) (Mediatech). The amount of DMSO in the final transport solution was less than 0.5% (v/v). Rosuvastatin and reference compounds were tested in triplicate over 60 minutes in a 37°C shaking water bath.

Rosuvastatin concentrations were analyzed by LC/MS/MS using a Sciex API 3000 with turboion spray source (Applied Biosystems/MDS Sciex, South San Francisco, CA) (Schneck et al, 2004). Erythromycin and propranolol concentrations were determined by LC/MS/MS using a Micromass Quattro Ultima (Micromass UK Ltd, Cheshire, UK) equipped with an electrospray ionization interface. The samples were loaded (injection volume 10 to 20 μL) on columns by means of a HTS Pal autosampler (CTC Analytics AG, Switzerland). Chromatography was performed on columns of Synergi MAX-RP 30 × 2.0 mm, 4 micron (Phenomenex, USA) at a flow rate of 1.5 mL/minute. The mobile phase consisted of 2 solvents: 0.1% formic acid in water (A) and 10% methanol in acetonitrile (B). The gradient profile was 0 to 0.3 minutes 0% B, 0.3 to 1.3 minutes linear gradient to 95% B, 1.3 to 1.6 minutes 95% B and 1.6 to 2 minutes 0% B.

The apparent permeability (P_{app}) values were calculated using the equation $P_{app} = (1/AC_0) \times (dQ/dt)$, where A is the membrane surface area, C_0 is the donor drug concentration at t

= 0, and dQ/dt is the amount of drug transported within a given time period. Flux ratio = $P_{app\ (B\rightarrow A)}$ / $P_{app\ (A\rightarrow B)}$.

Rosuvastatin transport in membrane vesicles

ATP-dependent transport of [^{3}H]-rosuvastatin or [^{3}H]-$E_2$17G, a known substrate for MRP2, was measured using a rapid filtration technique (Huang et al, 2000). Briefly, membranes were quickly thawed at 37 °C. Transport was initiated by adding membranes (30-66 µg) to the preincubated buffer (50 mM Tris, 50 mM mannitol and 10 mM $MgCl_2$, pH 7.5) containing 5 mM ATP or AMP and [^{3}H]-rosuvastatin or [^{3}H]-$E_2$17G. Transport was terminated by the addition of 2.5 mL ice-cold transport buffer, and vesicle-associated [^{3}H]-rosuvastatin were separated from free [^{3}H]-rosuvastatin by rapid filtration through a GF/F filter (Whatman, Florham Park, NJ). Filters were further washed twice with 2.5 mL of ice-cold buffer and assayed for radioactivity in 10 mL of liquid scintillation cocktail. Because nonspecific binding to the filter was relatively minimal, the data were not corrected for the binding to the filter unless stated otherwise in the legend. ATP-dependent transport was determined as the difference in uptake between 5 mM ATP and 5 mM AMP.

Statistics

Statistical differences ($p < 0.05$) were determined by one way analysis of variance followed by a multiple comparison test or unpaired student's t test. Software Prism 3.0 (GraphPad Software Inc, San Diego, CA) was used to produce the best fitting curves.

Results

Pgp-mediated transport of rosuvastatin

Rosuvastatin transport across MDR1-MDCK monolayers was measured at concentrations of 10 and 50 μM in the presence or absence of 2 μM GF120918, a potent inhibitor for both Pgp and BCRP. Rosuvastatin transport in both B→A and A→B directions was similar, with a P_{app} value of ~ 8 nm/second, and was not affected by GF120918. In contrast, transport of the Pgp substrate erythromycin (10 μM) in the B→A direction was 35-fold greater than that in the A→B direction. (Table 1).

MRP2-mediated transport of rosuvastatin

No significant ATP-dependent transport of rosuvastatin was observed in either CTRL-Sf9-(control) or MRP2-Sf9-membranes in the absence of glutathione (GSH) (Figure 1A). Significant ATP-dependent transport of rosuvastatin was observed in the presence of 5 mM GSH in both MRP2-, and control-membrane vesicles (Figure 1B). However, ATP-dependent transport of rosuvastatin between control and MRP2-Sf9-membranes was not significantly different (p >0.05).

BCRP-mediated transport of rosuvastatin

ATP dramatically stimulated rosuvastatin uptake in MXR-M-VT membranes expressing high levels of BCRP in a time-dependent manner (Figure 2). An initial uptake rate of ATP-dependent transport of rosuvastatin (5 μM) was too rapid to be determined accurately, and the transport at 30 second was 239.7 pmol/mg protein. In contrast, no ATP-dependent transport of rosuvastatin was observed in CTRL-M membranes that did

not express BCRP. The difference in the uptake between ATP and AMP was sensitive to extravesicular osmolarity. As the concentration of extravesicular sucrose increased, rosuvastatin uptake decreased in the presence of ATP (p <0.05), whereas the uptake did not change significantly in the presence of AMP (p >0.05) (Figure 3).

Concentrations of rosuvastatin ranging from 0.2 to 1000 μM were used to determine if BCRP-mediated transport of rosuvastatin was saturable (Figure 4). An Eadie-Hofstee analysis suggested that there were two binding sites in BCRP for rosuvastatin with an apparent K_m of 10.8 ± 1.1 μM for the high affinity site, and 307 ± 89.4 μM for the low affinity site.

GF120918 (2 μM), an inhibitor of Pgp and BCRP, inhibited ATP-dependent transport of rosuvastatin by 89% (p <0.05) (Figure 5). Pravastatin up to 250 μM inhibited rosuvastatin transport by only 30%.

Discussion

The purpose of this study was to identify and characterize ATP-dependent transporters for rosuvastatin. Our results provide the direct evidence that rosuvastatin is a substrate for BCRP. Rosuvastatin transport in MXR-M-VT membranes was dramatically stimulated by ATP, and was saturable with an apparent K_m of 10.8 μM for the high affinity binding or transport site. This value is comparable to the Ki (15.4 ±2.4 μM) for inhibiting [^{3}H]-estrone-3-sulfate uptake by human BCRP-membrane vesicles (Hirano et al., 2005), suggesting rosuvastatin and estrone-3-sulfate may share the same transport or binding site. The uptake in the presence of ATP, but not in the presence of AMP was decreased as the intravesicular space was decreased by increasing extravesicular sucrose concentration (osmolarity). These data indicate that rosuvastatin transport occurred into the intravesicular space and the observed difference in uptake between ATP and AMP was mainly due to transport, not binding (Vore et al., 1996). Western blot analysis showed that high levels of BCRP, moderate levels of MRP4 and MRP5, very low levels of MRP1, and undetectable levels of Pgp, MRP2 and MRP3 were expressed in MXR-M-VT; and total MRPs expressed in MXR-M-VT were much lower than that in control membranes (personal communication with the chief scientist in Solvo Biotechnology). Moreover, rosuvastatin transport in MXR-M-VT membranes was inhibited by GF120918. Thus, the observed ATP-dependent transport in MXR-M-VT membranes is likely mediated by BCRP. In an attempt to determine if pravastatin is also transported by BCRP, the ability of pravastatin to inhibit BCRP-mediated transport of rosuvastatin was evaluated. However, pravastatin up to 250 μM inhibited rosuvastatin transport (1 μM) by only 30%, suggesting that pravastatin is not transported by BCRP, or possibly binds to a

different site. These data suggest that the transporter mechanisms for the first pass and biliary excretion between pravastatin and rosuvastatin may be different.

The Eadie-Hofstee analysis suggested that there were two binding or transport sites for rosuvastatin in MXR-M-VT membranes. Although multiple binding sites in BCRP have not been reported previously, and the presence of unknown transporters for rosuvastatin in these membranes cannot be excluded, our results are consistent with the findings of multiple binding sites in other ABC transporters. It has been proposed that MDR1 has three binding sites, including two transport sites and an allosteric site. Pgp-mediated drug transport was stimulated by prazosin and progesterone (Shapiro et al., 1999); Similarly, two transport or binding sites have been proposed for MRP2. Zelcer et al. (2003) postulated two MRP2 transport sites with positive cooperativity to explain the ability of sulfinpyrazone and indomethacin to stimulate MRP2-mediated transport.

Based on that rosuvastatin is an organic anion and that pravastatin is an MRP2 substrate, we had speculated that rosuvastatin was an MRP2 substrate. However, there was no ATP-dependent transport of rosuvastatin in MRP2-Sf9-membranes or control membranes in the absence of GSH, although significant ATP-dependent transport of $E_2$17G, a known MRP2 substrate, was clearly observed. Uptake of $E_2$17G measured over 5 minutes was 839 ± 64 and 146 ± 4 (pmol/mg protein) in the presence of 5 mM ATP and AMP, respectively. The high expression level of MRP2 in these membranes, but not in control membranes, was confirmed by Western blot analysis (data not shown). It has been shown that GSH stimulates MRP-mediated transport of vincristine (Loe et al., 1998). In the

presence of 5 mM GSH, significant ATP-dependent transport of rosuvastatin was observed in both control and MRP2-Sf9-VT membranes. However, there was no significant difference in ATP-dependent transport of rosuvastatin between control and MRP2-Sf9-VT membranes, suggesting that endogenous transporters in control Sf9 cells transport rosuvastatin in the presence of GSH. These data do not support our speculation that rosuvastatin is an MRP2 substrate. However, the possibility that MRP2 transports rosuvastatin in the presence of GSH cannot be excluded. It is also possible that the expression levels of endogenous MRP-like proteins in Sf9 cells expressing MRP2 are lower than that in control cells, and MRP2-mediated transport is masked by the high background transport in control membranes.

The role of Pgp in rosuvastatin efflux was assessed using MDR1-MDCK cells, a cell line widely used to identify Pgp substrates. No significant directional transport of rosuvastatin across MDR1-MDCK cells was observed. The apparent permeability of rosuvastatin was low, and was similar to mannitol, a marker for paracellular diffusion, suggesting that rosuvastatin may not be able to penetrate into cells without the activity of uptake transporters. . The lack of directional transport of rosuvastatin across MDR1-MDCK cells may reflect its inability to access Pgp rather than it not being transported by Pgp. . Therefore, rosuvastatin uptake into membrane vesicles isolated from wild-type and MDR1-MDCK cells was further examined. Marginal ATP-dependent transport of rosuvastatin was observed in membranes isolated from both wild-type and MDR1-MDCK cells. The ATP-dependent transport at 1 min was 11 and 9 (pmol/mg protein) for

wild-type and MDR1-MDCK membranes, respectively, ($p > 0.05$) (data not shown). These data do not support that rosuvastatin is a Pgp substrate.

Recently, Tiberg et al (2004) reported that rosuvastatin transport in the serosal to the mucosal (secretory) direction was higher than that in the mucosal to the serosal (absorptive) direction across rat intestinal segments mounted in Ussing chambers, with the highest efflux in the ileum. The efflux ratios were 8.2, 7.2, 15 and 1.7 across duodenum, jejunum, ileum and colon segments, respectively, which is consistent with the expression pattern of Bcrp in rat intestine (Tanaka et al., 2005). Bcrp mRNA levels are high throughout the intestinal tract with highest levels in the ileum, which differs from the limited expression of Mrp2 in proximal segments of rat small intestine (Mattino et al., 2000). Mrp2 protein is present mainly in brush border membranes of the proximal segments and gradually decreases from jejunum to the distal ileum. It has been shown that BCRP plays an important role in biliary excretion and oral absorption of topotecan (Jonker et al., 2000). The present data clearly demonstrated that rosuvastatin is a substrate for BCRP, and this transporter likely contributes to its biliary excretion. However, it is not known whether BCRP plays a significant role in limiting oral absorption of rosuvastatin. Rosuvastatin has poor permeability, and its flux across cell monolayers in vitro is mainly through the paracellular pathway. If this is the case in vivo, rosuvastatin may not be able to access BCRP for efflux without the involvement of uptake transporters. Recently, it was found that OATP-B is expressed at the apical membrane of human intestinal epithelial cells, and may involve pH-dependent absorption of pravastatin (Kobayashi et al., 2003). An uptake transport system for rosuvastatin at

the apical membranes of the intestines may exist and, therefore, BCRP may limit its oral absorption.

In summary, the present studies demonstrate that rosuvastatin is transported efficiently by BCRP in vitro. These data suggest that BCRP may play a significant role in the disposition of rosuvastatin.

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Footnotes

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Legends for figures

Fig 1. Transport of rosuvastatin in MRP2- or CTRL-Sf9-membrane vesicles. Transport was initiated by adding membranes (50 μg) to the preincubated mixtures containing 5 mM ATP or AMP and [^{3}H]-rosuvastatin. A, in the absence of GSH; B, in the presence of 5 mM GSH. Values are the means ± SD of triplicate or duplicate determinations from a single experiment.

Fig 2. Time course of rosuvastatin (5 μM) transport in MXR-M-VT (BCRP) and CTRL-M membrane vesicles. Transport was initiated by adding membranes (35 μg for MXR-M-VT, 60 μg for CTRL-M) to the preincubated mixtures containing 5 mM ATP or AMP and [^{3}H]-rosuvastatin. Data were corrected for the nonspecific binding of rosuvastatin to the filter and the values are the means ± SD of triplicate determinations from a single experiment.

Fig 3. Effect of osmolarity on ATP-dependent transport of rosuvastatin (25 μM) in MXR-M-VT membrane vesicles. Uptake was measured at 2 min in the presence of 5 mM ATP or AMP after prior incubation (37 °C, 10 min) of the vesicles in the buffer containing indicated sucrose concentrations. Transport was initiated by adding a mixture containing [^{3}H]-rosuvastatin and ATP or AMP to the preincubated membranes. The values are the means ± SD of triplicate determinations from a single experiment. *, $p < 0.05$.

Fig 4. Concentration dependence of ATP-dependent transport of rosuvastatin in MXR-M-VT membrane vesicles. ATP-dependent transport was determined as the difference in the uptake between 5 mM ATP and AMP at 30 seconds. An Eadie-Hofstee plot is shown in the inset. The values are the combined results from two independent experiment.

Fig 5. Effect of GF120918 and pravastatin on ATP-dependent transport of rosuvastatin (1 µM) in MXR-M-VT-membrane vesicles. Transport was measured at 30 seconds and was initiated by adding [^{3}H]-rosuvastatin and ATP or AMP to the preincubated mixtures containing membranes and test compounds. Data are expressed as percent of control (control values were 75.3 ± 5.7 or 63.2 ± 7.3 pmol/mg protein) and represent means ± SD of triplicate determinations from a single experiment. *, $p < 0.05$.

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Table 1. Transport of rosuvastatin across monolayers of MDR1-MDCK cells in the presence and absence of 2 μM GF120918. Values are the means ± SD of triplicate from a single experiment.

	$P_{app\ (A \to B)}$ (nm/sec)	$P_{app\ (B \to A)}$ (nm/sec)	Flux Ratio
10 μM	8.9 ± 0.5	8.5 ± 2.7	1.0
10 μM + GF120918	8.3 ± 0.0	8.4 ± 0.3	1.0
50 μM	7.5 ± 0.5	9.3 ±1.1	1.2
50 μM + GF120918	6.1 ± 0.7	8.6 ± 0.7	0.9
Erythromycin (10 μM)	8.5 ± 1.0	298 ± 9.6	35

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Figure 1

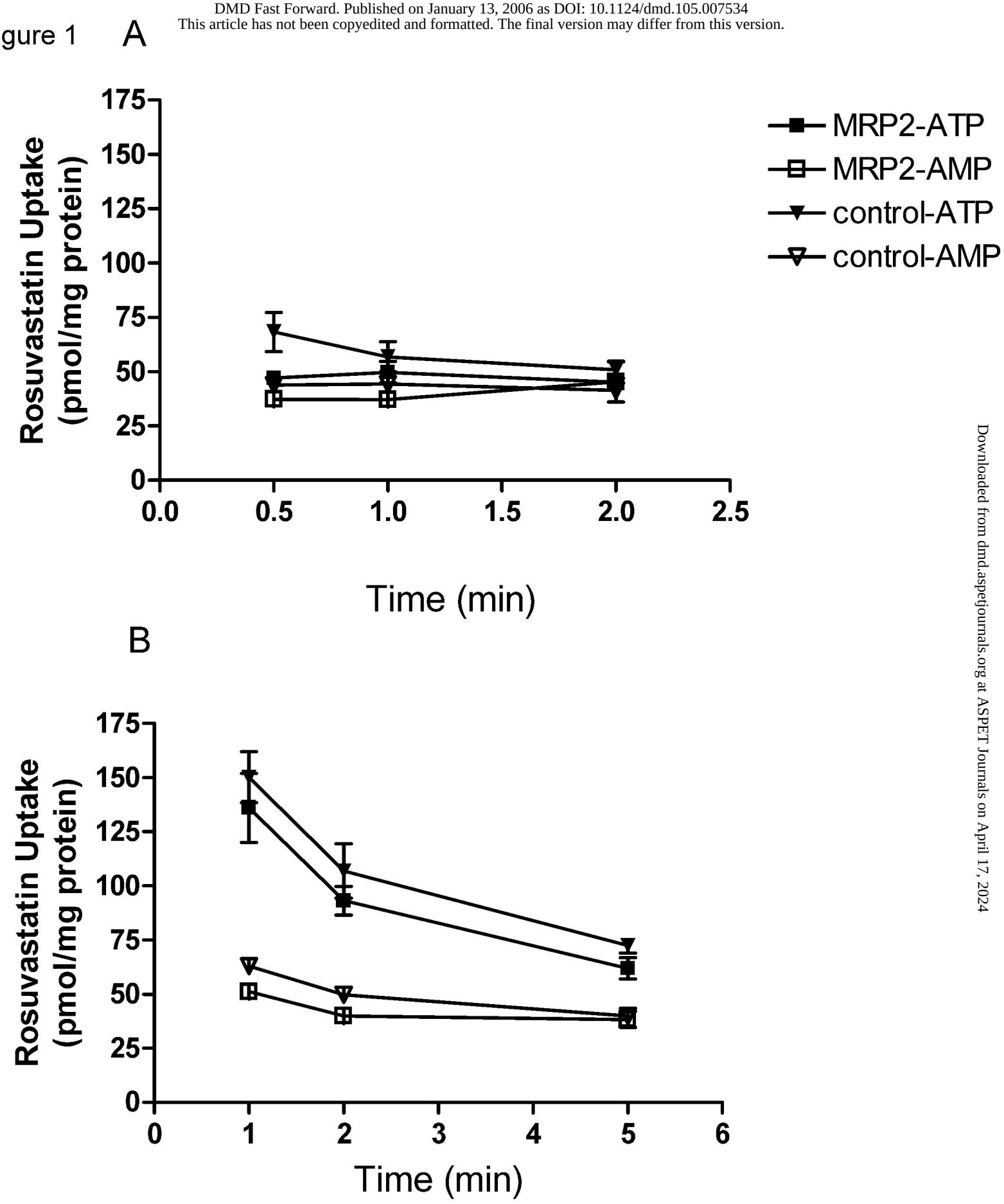

Figure 2

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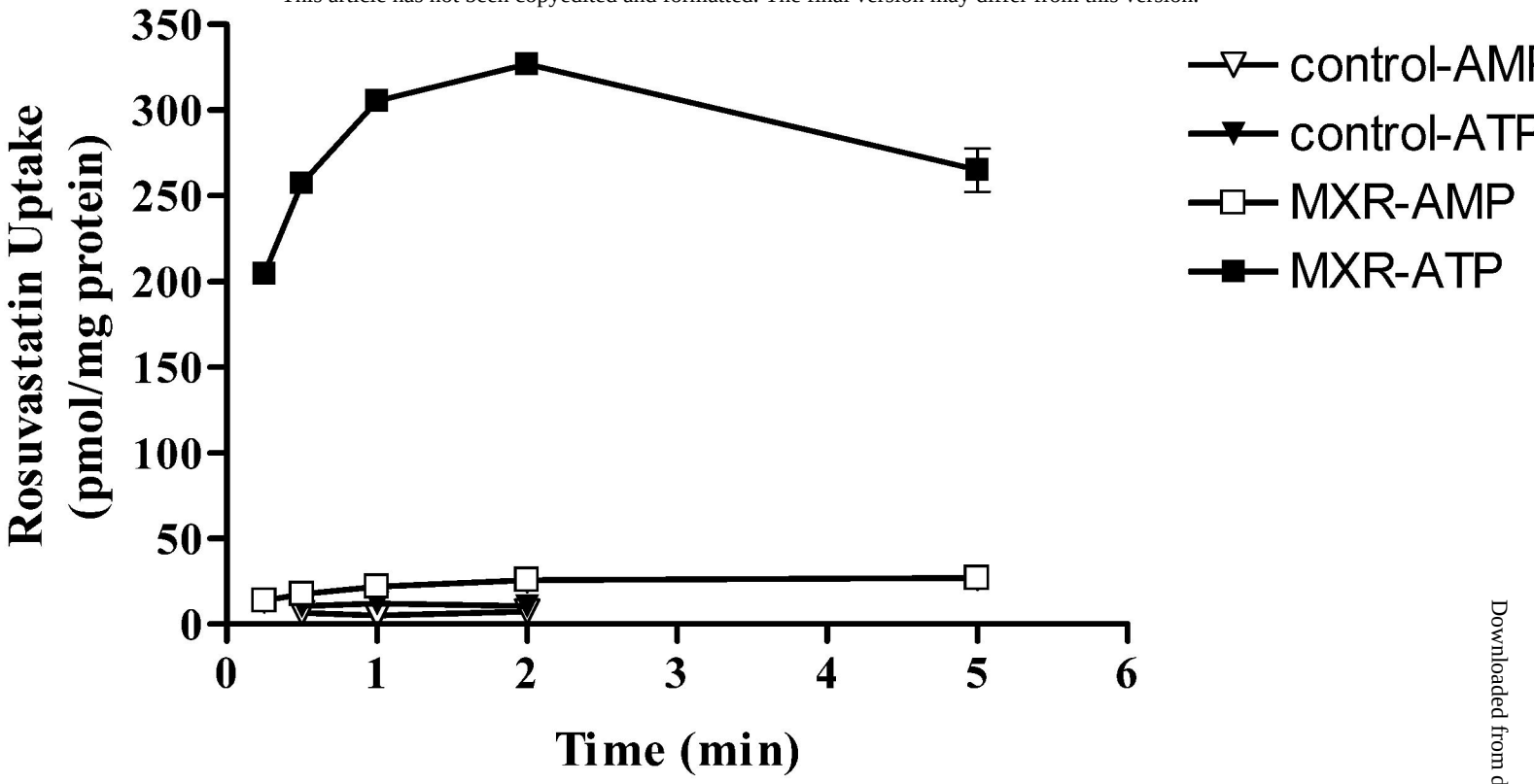


DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007534

Figure 3

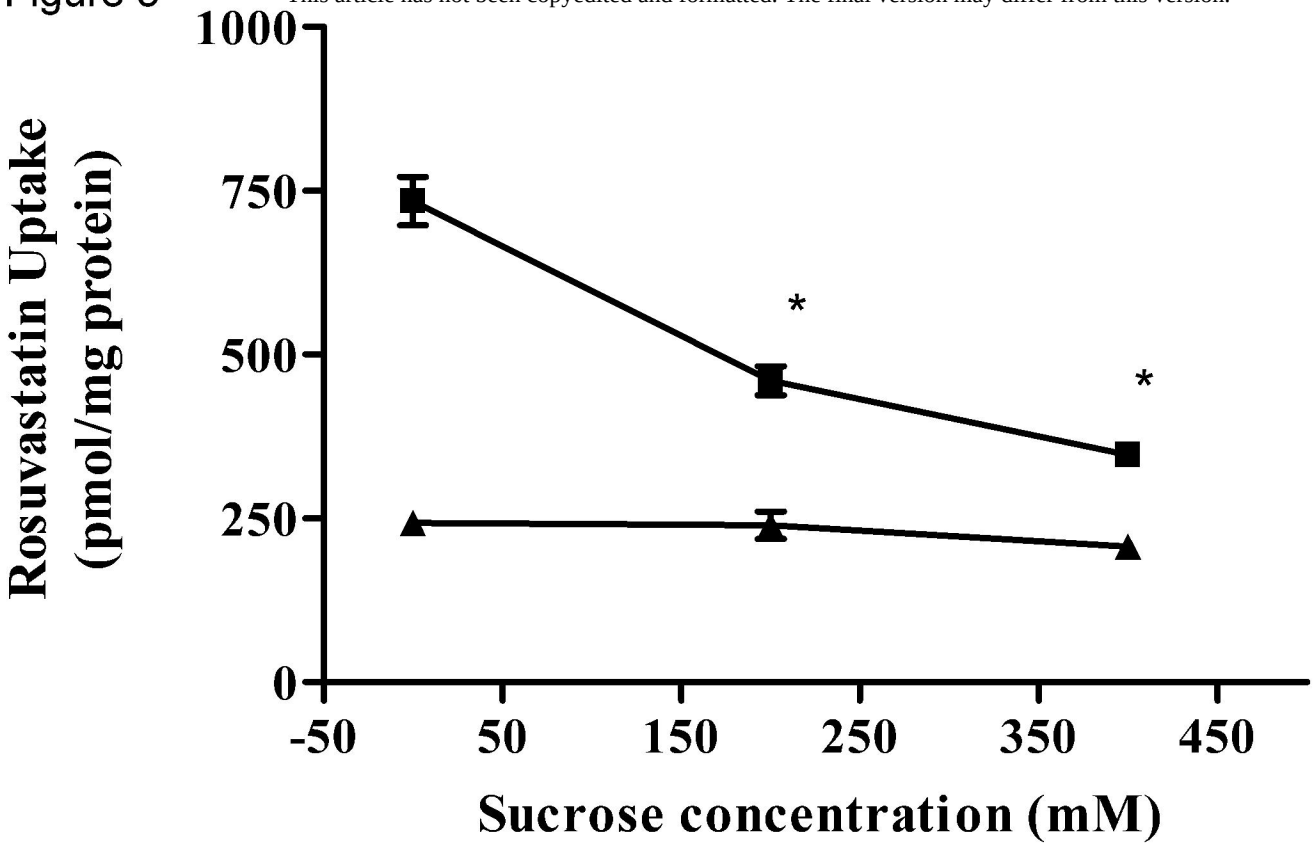
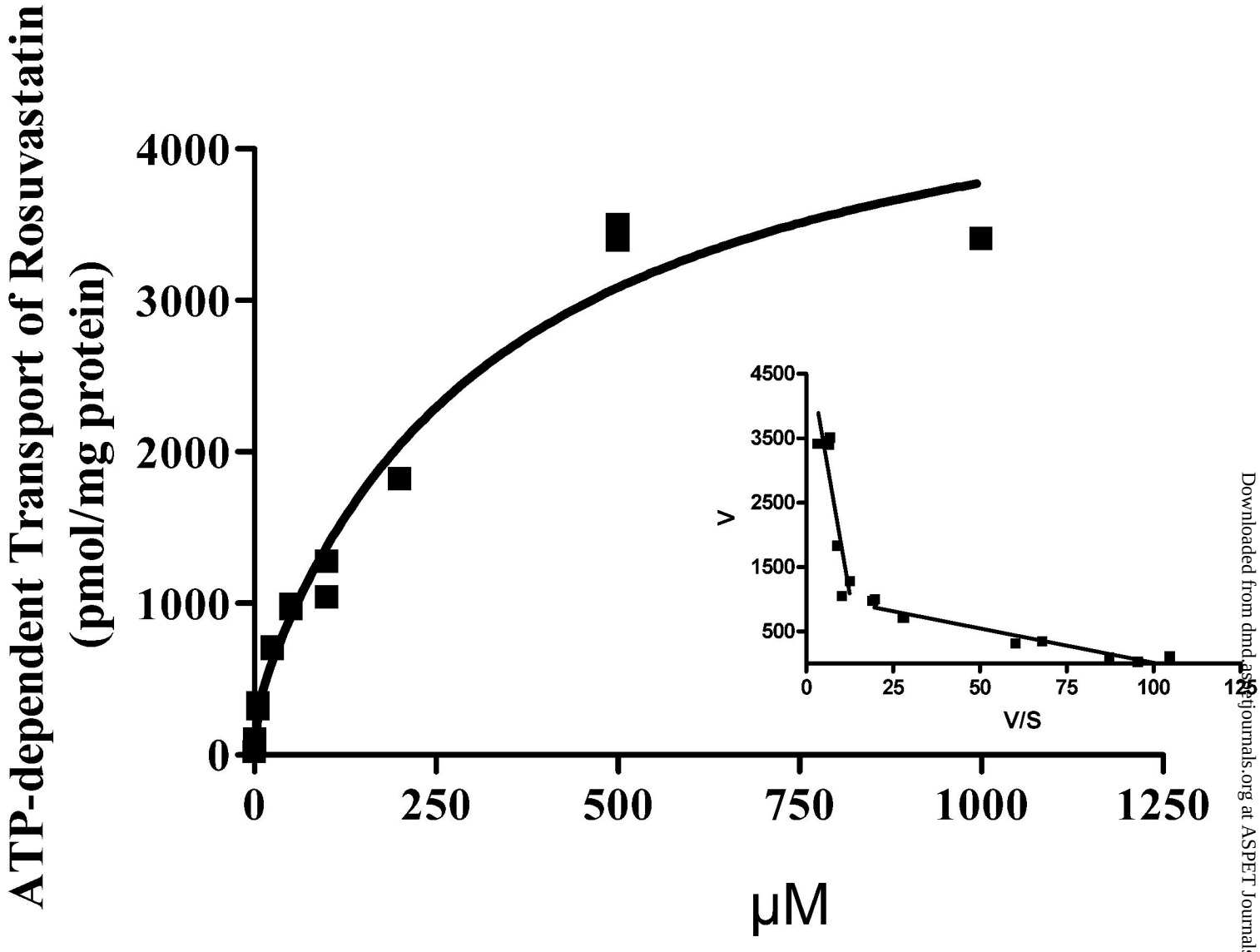


Figure 4

DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007534



DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.007534

Figure 5