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Intestinal BCRP/Bcrp1 and MRP3/Mrp3 are involved in the pharmacokinetics of resveratrol.

Koen van de Wetering, Alexander Burkon, Wouter Feddema, Alice Bot, Hugo de Jonge, Veronika Somoza and Piet Borst

Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands (K.v.d.W., W.F. and P.B.), German Research Center of Food Chemistry, Garching, Germany (Alexander Burkon and V.S.) and Department of Biochemistry, Erasmus University Medical Center, Rotterdam, The Netherlands (Alice Bot and H. d. J.).

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Running title: BCRP and MRP3 transport resveratrol-metabolites

* Corresponding author: Piet Borst, Division of Molecular Biology (P2), Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, Tel: +31-20-5122880, Fax: +31-20-6691383
Email: p.borst@nki.nl

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Abbreviations used in this paper:

ABC, ATP-binding cassette; BCRP, Breast Cancer Resistance Protein; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; DAD, diodearray; $E_2$17βG, Estradiol-17β-D-glucuronide; EHC, enterohepatic circulation; FCS, fetal calf serum; GI, gastrointestinal; GSH, glutathione; HEK, human embryonic kidney; HPLC, high-pressure liquid chromatography; K_i, inhibitory constant; K_m, Michaelis-Menten constant; LSC, liquid scintillation counting; MDCK, Madin-Darby canine kidney; MLM, mouse liver microsomes; MRP, Multidrug Resistance Protein; MTX, methotrexate; PAS, periodic acid Schiff; PBS, phosphate buffered saline; Res-3-G, resveratrol-3-glucuronide; Res-3-S, Resveratrol-3-sulfate; Res-di-S, resveratrol-di-sulfate; Sf9, sphodoptera frugiperda 9; V_{max}, maximal velocity; WT, wild type.

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Abstract

The phyto-estrogen resveratrol has putative health promoting effects and is present in several dietary constituents. Resveratrol is extensively metabolized in the gut epithelium resulting in the formation of hydrophilic glucuronic acid- and sulfate-conjugates. These polar resveratrol-conjugates need specific transporters to cross the cell membrane. We show here that vectorial transport of some of these metabolites is mediated by Multidrug Resistance Protein 3 (MRP3, ABCC3) and/or Breast Cancer Resistance Protein (BCRP, ABCG2) located in the basolateral and apical membranes of enterocytes, respectively. In vitro, MRP3 transports resveratrol-glucuronide (Res-3-G). The absence of Mrp3 in mice results in altered disposition of Res-3-G and its parent compound resveratrol, leading to a reduced percentage of resveratrol being excreted via the urine in *Mrp3*$^{(-/-)}$ mice. Circumstantial evidence suggests that circulating resveratrol is formed by deglucuronidating Res-3-G in vivo, providing a possible explanation for the health beneficial effects of resveratrol in vivo, despite its rapid and extensive conjugation. BCRP transports Res-3-G and resveratrol-sulfates in vitro and its absence in mice results in high plasma levels of resveratrol-di-sulfate (Res-di-S), a resveratrol metabolite hardly detectable in plasma of WT mice, and in an increased disposal of resveratrol via the urine. The profound effects of ABC-transporters on the disposal of resveratrol may be representative for the handling of several other polyphenols of dietary origin.

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Introduction

Resveratrol is a phyto-estrogen with postulated health beneficial effects present in food like grapes and peanuts (Baur and Sinclair, 2006). The mammalian gastrointestinal (GI) tract provides a barrier for the uptake of resveratrol and other xenobiotics of dietary origin (Kaminsky and Zhang, 2003) and an important component of this barrier are the phase I and phase II metabolizing enzymes, together with associated transporters (phase III metabolism) (Xu *et al.*, 2005). Oral resveratrol is metabolized in the gut wall (Kuhnle *et al.*, 2000;Andlauer *et al.*, 2000) and found in plasma and urine mainly as sulfo- and glucuronosyl-conjugates (Wenzel *et al.*, 2005;Walle *et al.*, 2004).

Many of the health-promoting effects attributed to resveratrol have been found in experiments with lower organisms and/or test tubes (Howitz *et al.*, 2003;Baur and Sinclair, 2006), in which its actions were attributable to unmodified resveratrol. Modifications such as glucuronidation and sulfation normally result in compounds that have less pharmacological activity (Tukey and Strassburg, 2000;Kaminsky and Zhang, 2003;Rimbach *et al.*, 2004). The extensive modification of resveratrol in the gut wall has therefore led to skepticism about its therapeutic potential in humans after oral dosing (Wenzel *et al.*, 2005;Wenzel and Somoza, 2005;Walle *et al.*, 2004;Baur and Sinclair, 2006). Recent studies indicate, however, that dietary resveratrol can have beneficial effects in mice (Baur *et al.*, 2006;Lagouge *et al.*, 2006). It is therefore possible that the resveratrol metabolites that enter the circulation retain (some) pharmacological activity or that de-conjugation of these metabolites occurs, thereby releasing the pharmacologically active parent compound. A better understanding of the factors involved in the

tissue distribution of resveratrol and its metabolites would therefore help to identify the target organs for resveratrol and allow a better prediction of its pharmacological potency.

Following their intracellular formation in enterocytes, sulfo- and glucuronic acid conjugates of resveratrol are too hydrophilic to passively diffuse over the plasma membrane and specific transporters are implied in their extrusion from the cell (Kuhnle *et al.*, 2000;Andlauer *et al.*, 2000). Recently, hepatic Mrp2 was shown to mediate the canalicular efflux of resveratrol-glucuronides from rat liver (Maier-Salamon *et al.*, 2007). Resveratrol-glucuronides are excreted over the basolateral membrane of hepatocytes as well (Maier-Salamon *et al.*, 2007), but the responsible transporter remains to be identified. The transporters involved in the cellular extrusion of resveratrol-sulfates are also not yet known.

Two transport proteins that have previously been shown to mediate transport of phase II metabolites are Multidrug Resistance Protein 3 (MRP3, ABCC3) and Breast Cancer Resistance Protein (BCRP, ABCG2), both belonging to the ATP-binding cassette (ABC) superfamily of membrane transporters (Borst and Oude Elferink, 2002). Both proteins have a similar tissue distribution and are expressed in liver, kidney and gut, organs known for their high phase II metabolizing capacity (Tukey and Strassburg, 2000). In polarized cells MRP3 and BCRP route to the basolateral and apical membrane, respectively, as also found in the enterocytes of the gut (Scheffer *et al.*, 2002;Haimeur *et al.*, 2004). In vitro studies have shown that MRP3 and BCRP are typical organic anion transporters (Kool *et al.*, 1999;Borst and Oude Elferink, 2002;Haimeur *et al.*, 2004;Kruh and Belinsky, 2003). Substrates of both transporters include compounds conjugated to sulfate, glucuronate, or glutathione (GSH) (*i.e.* phase II conjugation products) (Borst and Oude Elferink, 2002;Kruh and Belinsky, 2003). MRP3 seems to have a preference for glucuronidated substrates (Kruh and Belinsky, 2003;Borst *et al.*, 2003;Zamek-Gliszczynski *et*

al., 2006;Manautou *et al.*, 2005;Zelcer *et al.*, 2006), whereas the substrate spectrum of BCRP is broader and includes many compounds containing a sulfate moiety (Schinkel and Jonker, 2003;Adachi *et al.*, 2005;Haimeur *et al.*, 2004;Borst and Oude Elferink, 2002;Schinkel and Jonker, 2003). Hence, MRP3 and BCRP are involved in the protection of the body against xenobiotics by transporting xenobiotic conjugates out of the cell for subsequent urinary (MRP3) or fecal (BCRP) excretion (Haimeur *et al.*, 2004;Borst *et al.*, 2007;Schinkel and Jonker, 2003).

Intestinal BCRP limits the systemic exposure to several xenobiotics (including many important drugs) and xenobiotic-conjugates after oral exposure (Schinkel and Jonker, 2003;Adachi *et al.*, 2005). Less is known about the function of intestinal MRP3 but its basolateral localization implies that it transports its substrates towards the circulation for subsequent urinary excretion. Indeed, hepatic MRP3 is involved in the transport of several glucuronidated compounds to the circulation for subsequent urinary excretion (Zelcer *et al.*, 2005;Zelcer *et al.*, 2006;van de Wetering *et al.*, 2007;Manautou *et al.*, 2005;Zamek-Gliszczynski *et al.*, 2006).

Since intestinal BCRP and MRP3 are optimally placed to handle sulfo- and glucuronic acid conjugates of resveratrol, we have used vesicular transport experiments to test the interaction of these transporters with resveratrol metabolites and studied the effect of a complete loss of Bcrp1 and Mrp3 on resveratrol pharmacokinetics and pharmacodynamics in mice.

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Materials and methods

[^{3}H]Resveratrol (3.6 Ci/mmol) was from Moravek Biochemicals (Brea, CA). [^{3}H]Estradiol-17β-D-glucuronide ($E_2$17βG) and [^{3}H]methotrexate (MTX) were from Perkin Elmer (Waltham, MA). 96-wells MultiScreen$_{HTS}$ MSFB filter plates and the MultiScreen$_{HTS}$ vacuum manifold were from Millipore (Amsterdam, the Netherlands). Oasis HLB solid phase extraction cartridges were from Waters (Milford, MA). The Luna C18(2) reversed phase column was from Phenomenex (Torrance, Ca). Dulbecco's modified eagles medium, Sf-900 II SFM medium, Opti-MEM, penicillin/streptomycin and fetal calf serum (FCS) were from Invitrogen (Breda, the Netherlands). Transwell plates were from Costar (Corning, NY). All other chemicals and reagents were from Sigma Chemicals (St Louis, MO).

Synthesis of resveratrol metabolites

Non radiolabeled resveratrol metabolites were synthesized as previously described (Wenzel *et al.*, 2005). [^{3}H]Resveratrol-glucuronide was synthesized from [^{3}H]resveratrol using mouse liver microsomes (MLMs). Preparation of MLMs and subsequent *in vitro* generation of [^{3}H]resveratrol-glucuronide was done as previously described for morphine (van de Wetering *et al.*, 2007). Completeness of conversion to [^{3}H]resveratrol-glucuronide was determined by high pressure liquid chromatography (HPLC) and liquid scintillation counting (LSC). Conversion of [^{3}H]resveratrol to [^{3}H]resveratrol-glucuronide after glucuronidation was complete as no radioactivity corresponding to the aglycon was detected and apart from resveratrol-glucuronide no additional peaks were present in the radiochromatogram (data not shown). Recovery of resveratrol-glucuronide after synthesis was 95 %. [^{3}H]Resveratrol-3-sulfate (Res-3-S) and

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[^{3}H]resveratrol-di-sulfate (Res-di-S) were generated by incubation of 10 µCi [^{3}H]resveratrol with 450 µg cytosolic protein from mouse liver in the presence of 100 µM ’3-phosphoadenosine-‘5-phosphosulfate (PAPS) in 100 mM phosphatebuffer (pH 7.4) for 18 h at 37 ºC. [^{3}H]Resveratrol-sulfates were extracted from the reaction mix by solid phase extraction on Oasis HLB cartridges and [^{3}H]Res-3-S and [^{3}H]Res-di-S were purified by preparative HPLC on a Luna C18(2) 3.0 µm column (150 mm x 4.6 mm) (isocratic elution in 19% acetonitrile in 10 mM ammonium formate pH 8.2 for 25 min. Purified [^{3}H]resveratrol-sulfates were subsequently concentrated by freeze-drying.

Cell lines and Culture conditions

Sf9 insect cells in suspension were grown in Sf-900 II SFM medium containing 10% FCS. HEK293 cells expressing hMRP3 were generated by transfecting HEK293 cells with pCMVneoMRP3 (Kool *et al.*, 1999) using calcium phosphate precipitation. After 48 hours, cells were split and G418 (800 µg/ml) was used to select for clones that express hMRP3. G418 resistant clones were tested for MRP3 expression by immunoblot analysis using the M_3II9 antibody (Scheffer *et al.*, 2002). Plasma membrane localization was confirmed by immunofluorescence microscopy using the same antibody (not shown). HEK293 parental and HEK293-MRP3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS and 100 units of penicillin/streptomycin/ml.

Preparation of membrane vesicles

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Membrane vesicles from Sf9 cells were obtained after infection with a BCRP (Breedveld *et al.*, 2007) or control cDNA containing baculovirus at a multiplicity of infection of 1. After incubation at 27 ˚C for 3 days, cells were harvested by centrifugation at 500 g for 5 min. The pellet was resuspended in ice cold hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.4) supplemented with a protease inhibitor cocktail (Roche, Basel Switzerland) and incubated at 4°C for 90 min. The suspension was centrifuged at 100,000 x *g* for 40 min and the pellet was homogenized in ice-cold TS buffer (50 mM Tris-HCl, 250 mM sucrose, pH 7.4) using a tight fitting Dounce homogenizer. After centrifugation at 500 x *g* at 4 °C for 10 min, the supernatant was collected and centrifuged at 4 °C at 100,000 x *g* for 40 min. The pellet was resuspended in TS buffer and passed through a 27-gauge needle 25 times. The vesicles were dispensed in aliquots, snap frozen in liquid nitrogen and stored at -80°C until use.

Vesicles of HEK293-MRP3 and HEK293-control cells were prepared as described for the Sf9 vesicles with 1 modification: the final homogenisation buffer was 10 mM Tris-HCl pH 7.4 instead of the 50 mM Tris-HCl/250 mM sucrose pH 7.4 used for the Sf9 vesicles.

Vesicular transport assays

The time- and concentration-dependent transport of various substrates into membrane vesicles was studied using the rapid filtration method as described (van de Wetering *et al.*, 2007). K_i's were calculated using the following equation: $K_m' = K_m * (1 + [I]/K_i)$, with K_m' being the apparent K_m determined in the presence of inhibitor [I] and the concentration of the inhibitor in µM.

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Animals

Bcrp1$^{(-/-)}$ (Jonker *et al.*, 2002) and *Mrp3*$^{(-/-)}$ (Zelcer *et al.*, 2006) mice have been described, were on a 99% FVB background, received food and water *ad libitum* and were housed in constant temperature rooms with a 12-hour light/12-hour dark cycle. Mouse handling and experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

Analysis of resveratrol pharmacokinetics in mice

Male age and weight matched mice were used throughout the study and were fasted for 4 hours before the onset of the experiments. Resveratrol (50 mg/kg body weight, vehicle: 0.5% hydroxypropylmethyl cellulose) was administered intragastrically by oral gavage feeding. For the time course experiments 4 to 5 mice of each group were sacrificed at each time point by cardiac puncture and blood sampling in heparinized syringes under methoxyflurane anesthesia. Livers and kidneys were collected and immediately stored at -80 ˚C until analysis. Plasma was obtained after centrifugation for 10 minutes at 1500 g at 22 °C and stored at -80 ˚C until analysis. To analyze the urinary excretion of resveratrol and its metabolites mice were placed in metabolic cages, resveratrol was administered as described above and urine was collected for 24 hours. At the end of the experiment mice were sacrificed as mentioned above.

Quantification of resveratrol and its metabolites.

Resveratrol and resveratrol metabolites in plasma, urine and tissues were determined as

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described previously (Burkon and Somoza, 2008) .

Tissue distribution of [^{3}H]resveratrol

[^{3}H]resveratrol (50 mg/kg; 1.5-2 μCi per mouse) was administered as described above and after 24 h, mice were sacrificed as described above, tissues collected and solubilized in 0.5 M NaOH/0.1% Triton-X-100. After solubilization, samples were bleached using 30% H_2O_2. The level of radioactivity in tissue homogenates was determined by LSC, using Ultima Gold scintillation fluid (Packard Biosciences, Groningen, The Netherlands). Urine was counted by directly adding LSC fluid without processing.

Statistical analysis

In Figures 1 and 2, non-linear regression and statistical analysis were performed using Graphpad Prism software (La Jolla Ca). Statistical analysis was done by testing the null hypothesis that the fits of the curves obtained in the presence of inhibitor did not significantly differ from the curve obtained in the absence of inhibitor. For statistical analysis of the pharmacokinetic experiments One Way Anova was used including a Dunnett's multiple comparison test. Statistically significant differences from control values are indicated in the figures with * $p<0.5$, ** $p<0.01$ and *** $p<0.001$.

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Results

Effect of resveratrol metabolites on MRP3- and BCRP-mediated transport in vesicular transport experiments

In vesicular transport experiments, competition with a known substrate is often used to screen for new substrates of membrane transporters: competitive inhibition is an indication that both compounds compete for the same binding/transport site on the transporter. We therefore studied the effects of resveratrol metabolites on MRP3-mediated transport of $E_2$17βG, a prototype MRP3 substrate. At a concentration of 10 μM, only resveratrol-3-glucuronide (Res-3-G) inhibited transport of $E_2$17βG (Fig. 1A). Inhibition could be overcome by increasing the substrate concentration, compatible with competitive inhibition. In addition, Lineweaver-Burke transformations also indicated that inhibition of MRP3-mediated $E_2$17βG transport by Res-3-G was competitive (Fig 1B), which was confirmed by non-linear regression analysis yielding apparent K_m's of 16.9 μM and 60.0 μM in the absence and presence of Res-3-G, respectively, without significant alteration of the V_{max}. The K_i is approximately 8 μM suggesting that Res-3-G is a relatively high affinity substrate of MRP3. Interestingly, both resveratrol sulfo-conjugates tested, resveratrol-3-sulfate (Res-3-S) and resveratrol-di-sulfate (Res-di-S), moderately stimulated MRP3-mediated transport of $E_2$17βG by increasing the V_{max} without significantly altering the K_m (Fig. 1A). We will come back to this stimulatory effect of the resveratrol-sulfates in the discussion section.

[^{3}H]MTX was used as substrate to study the ability of resveratrol-metabolites to interact with BCRP. At a concentration of 10 μM, both Res-3-S and Res-di-S inhibited BCRP-mediated

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transport of MTX, whereas higher concentrations of Res-3-G were needed to inhibit transport (Figure 1C and not shown). Because we can not saturate MTX transport by BCRP, we were unable to determine whether inhibition of BCRP-mediated transport by resveratrol-metabolites is competitive. Lineweaver-Burke-transformation, however, suggested that competition by Res-3-S and Res-di-S was competitive as their curves crossed the y-ax at the same position as the curve obtained in the absence of inhibitor, whereas the position at which the x-ax was crossed differed, indicative of competitive inhibition (Fig. 1D).

Transport of Res-3-G into MRP3- and BCRP- containing vesicles

As our inhibition experiments indicated that Res-3-G was a substrate of MRP3 we enzymatically synthesized [^{3}H]Res-3-G and tested whether this could serve as a substrate for this transporter. MRP3 indeed transported Res-3-G in a time- and ATP-dependent manner (Fig 2A). Moreover, transport followed Michaelis-Menten kinetics (Fig. 2C) and non-linear regression analysis yielded K_m and V_{max} values of 14 ± 2.2 μM and 72 ± 4.5 pmol/mg/min, respectively. The K_m is in good agreement with the K_i we found for this compound in competition experiments.

Despite the fact that Res-3-G only weakly inhibited BCRP-mediated transport of MTX (Fig. 1C), BCRP readily transported [^{3}H]Res-3-G in an ATP- and time-dependent way (Fig. 2B). Although we could not completely saturate BCRP-mediated transport of Res-3-G at the concentration range tested (1-200 μM), the approximate K_m and V_{max} values were roughly 120 ± 15 μM and 340 ± 22 pmol/mg/min, respectively. This low affinity explains why Res-3-G only weakly inhibited BCRP-mediated transport of MTX.

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Transport of Res-3-S and Res-di-S by BCRP

As inhibition experiments indicated that Res-3-S and Res-di-S are substrates of BCRP we tested whether there was direct transport of the purified [^{3}H]Res-3-S and Res-di-S. At 37 ºC the reaction rate for BCRP-mediated transport of both resveratrol-sulfates into the vesicles was too fast to reliably determine initial rates of transport (not shown) and to overcome this problem we carried out all reactions at 21 ºC. Both Res-3-S and Res-di-S, were transported by BCRP in a time- and ATP-dependent manner (Figs 3A and B). Transport of the resveratrol-sulfates by BCRP followed Michaelis-Menten kinetics (Figs 3C and D) with Res-3-S transport having a K_m of 5.0 ± 1.3 µM and a V_{max} of 1060 ± 69 pmol/mg/min and Res-di-S transport a K_m of 10 ± 2.0 µM and a V_{max} of 1600 ± 94 pmol/mg/min. This shows that both resveratrol-sulfates are transported by BCRP with high affinity and high capacity. In vivo transport rates at 37 ºC will probably be even higher, as the in vitro reactions were carried out at 21 ºC.

Pharmacokinetics of resveratrol and its metabolites in wild type, Bcrp1$^{(-/-)}$ and Mrp3$^{(-/-)}$ mice.

We chose a dose of 50 mg resveratrol per kg body weight as resveratrol has been shown to have health beneficial effects in mice given at daily doses ranging from approximately 40-400 mg/kg (Baur *et al.*, 2006;Lagouge *et al.*, 2006). After the oral administration of resveratrol, its main circulating metabolites in WT mice were found to be Res-3-G and Res-3-S, whereas Res-di-S was not detected in plasma and only present in low amounts in 24-h urine samples. Plasma levels of resveratrol and its metabolites followed a biphasic elimination pattern, suggestive of

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enterohepatic circulation of resveratrol (Fig. 4).

In the absence of Mrp3, plasma levels of the main resveratrol metabolite Res-3-G were reduced 10-fold (Fig. 4B) and this was accompanied by a similar reduction in its urinary excretion (Fig. 5B). The plasma and urinary excretion levels of the other resveratrol metabolites were unaltered (Res-di-S) or slightly reduced (Res-3-S) in *Mrp3*$^{(-/-)}$ mice relative to wild type mice (Figs 4 and 5). Unexpectedly, the absence of Mrp3 in mice also resulted in a 10-fold reduction in the levels of unconjugated resveratrol in urine (Fig. 5A). Although plasma levels of resveratrol were similar in WT and *Mrp3*$^{(-/-)}$ mice the first 30 min after resveratrol administration, subsequent time points after dosing showed lower resveratrol plasma levels in the absence of Mrp3 (Fig. 4A). These lower levels of resveratrol can not be due to a direct effect of Mrp3 on the transport of resveratrol, as we have been unable to demonstrate transport of resveratrol by human MRP3 in MDCKII cells (Suppl. Fig. 3B). Neither did resveratrol inhibit $E_2$17βG transport by human (Suppl. Fig. 2B) or mouse (not shown) MRP3/Mrp3 in vesicular transport experiments.

BCRP is present at the apical membrane of the enterocyte and limits the uptake of its substrates from the gut (van Herwaarden and Schinkel, 2006). The absence of Bcrp1 also altered the pharmacokinetics of resveratrol metabolites in mice, resulting in slightly increased plasma concentrations of Res-3-G and Res-3-S (Fig. 4.). However, the most salient finding in *Bcrp1*$^{(-/-)}$ mice was the dramatically increased plasma level of Res-di-S, a metabolite undetectable in plasma samples of WT and *Mrp3*$^{(-/-)}$ mice (Fig. 4D). This led to a more than 20-fold increase in the urinary excretion of Res-di-S in *Bcrp1*$^{(-/-)}$ mice (Fig. 5D) and an increase in the fraction of the administered resveratrol dose excreted via urine (Fig. 5E). These results indicate that Res-di-S is a transported BCRP/Bcrp1 substrate, in line with our in vitro studies showing that BCRP

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transports Res-di-S (Fig. 3B and D) and that Res-di-S acts as an inhibitor of BCRP-mediated MTX transport (Fig. 1C). The elevated plasma levels of Res-3-G and Res-3-S in the *Bcrp1*$^{(-/-)}$ mice were not accompanied by higher urinary excretion of these compounds. We will return to this result in the discussion. Although BCRP is known to transport unconjugated resveratrol (Breedveld *et al.*, 2007), we did not detect increased circulating levels of resveratrol in *Bcrp1*$^{(-/-)}$ mice, nor the increased urinary excretion of non-metabolized resveratrol. On the contrary, we found somewhat reduced amounts of resveratrol excreted via the urine and resveratrol was below the limit of detection in plasma samples of *Bcrp1*$^{(-/-)}$ mice.

Levels of resveratrol and its metabolites were also determined in livers and kidneys 1 hour after the administration of resveratrol (Fig. 6). Although *Mrp3*$^{(-/-)}$ mice had about 10-fold lower plasma levels of Res-3-G than WT mice 1 h after resveratrol administration, Res-3-G levels in their livers were only 2-fold reduced (Fig. 6C). Liver levels of resveratrol were similar in WT and *Mrp3*$^{(-/-)}$ mice (Fig. 6A), even though the absence of Mrp3 led to 50% lower plasma concentrations of resveratrol 1h after administration (Fig. 4A). No differences in liver concentrations of Res-3-S were found either (Fig. 6E). In kidneys the concentration of resveratrol and its metabolites reflected levels found in plasma 1 h after resveratrol administration with the parent compound resveratrol being undetectable in kidney samples of *Mrp3*$^{(-/-)}$ mice.

Excretion of [^{3}*H]resveratrol in wild type, Bcrp1*$^{(-/-)}$ *and Mrp3*$^{(-/-)}$ *mice.*

We also administered [^{3}H]resveratrol intragastrically to mice and determined the amount of radiolabel in 24-h urine and feces samples of *Mrp3*$^{(-/-)}$, *Bcrp1*$^{(-/-)}$ and WT mice (Fig. 7). As

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expected, *Mrp3*$^{(-/-)}$ mice excreted less radiolabel via the urine, whereas urinary excretion rates of resveratrol(metabolites) in *Bcrp1*$^{(-/-)}$ mice were increased (Fig. 7A). These alterations were mirrored by the changes in excretion via the feces: increased fecal excretion in the absence of Mrp3 and reduced fecal excretion in *Bcrp1*$^{(-/-)}$ mice (Fig. 7B). This shows that the absence of Mrp3 results in a shift from urinary to fecal excretion of resveratrol(metabolites), whereas in *Bcrp1*$^{(-/-)}$ mice the opposite occurs: higher urinary excretion rates and lower fecal excretion. These results are in line with analysis of the urinary resveratrol-(metabolite) profile by HPLC/DAD. Total recovery of resveratrol and its metabolites in urine was somewhat lower when analyzed by HPLC/DAD (Fig. 5E) than by LSC. We attribute this to our inability to detect some minor resveratrol metabolites by HPLC/DAD analysis such as sulfo-resveratrol-glucuronide and resveratrol-di-glucuronide. The latter was recently identified as one of the resveratrol metabolites present in human plasma (Burkon and Somoza, 2008).

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Discussion

Our experiments identify BCRP and MRP3 as two major determinants of the pharmacokinetics of the phytoestrogen resveratrol and its metabolites. BCRP was already known to transport resveratrol itself (Breedveld *et al.*, 2007). We show here that it also transports resveratrol-sulfates and Res-3-G, albeit the latter substrate with low affinity. Res-3-G is a high affinity substrate for MRP3, however, in line with the known preference of this transporter for glucuronidated substrates (Borst *et al.*, 2007).

Liver, kidney and the gastrointestinal tract are organs with a high phase II metabolizing activity (Tukey and Strassburg, 2000;Gamage *et al.*, 2006). In male mice, Mrp3 is present in liver and gastrointestinal tract but absent in kidney (Zelcer *et al.*, 2006). After oral administration, resveratrol is predominantly detected in plasma and urine conjugated to glucuronic acid. Intestinal perfusion (Andlauer *et al.*, 2000) and Ussing chamber experiments (Kuhnle *et al.*, 2000)have shown that resveratrol is extensively glucuronidated in the gut wall. In addition, in humans, sulfotransferase activity towards resveratrol was higher in duodenum than in liver (De Santi *et al.*, 2000). This indicates that after oral administration, the gastrointestinal tract is the major site for resveratrol metabolism.

The absence of Mrp3 or Bcrp1 has pronounced effects on the disposition of resveratrol in vivo. Mice lacking Mrp3 had up to 10-fold lower levels of Res-3-G in plasma and urine than control mice. Because resveratrol appears to be mainly glucuronidated in the gut, the decreased plasma Res-3-G must be due to the absence of Mrp3 in the basolateral membrane of the enterocytes. Absence of Bcrp1, normally located in the apical membrane of enterocytes leads to elevated plasma levels of Bcrp1 substrates (van Herwaarden and Schinkel, 2006), as we also found for all resveratrol-metabolites in *Bcrp1*$^{(-/-)}$ mice, with the most pronounced effect on Res-di-S. The

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plasma concentrations of Res-3-G were also elevated. This could be due to stimulation of Mrp3-mediated transport of Res-3-G by the resveratrol-sulfates that accumulate within the enterocyte in the absence of Bcrp1. We have found that the sulfated resveratrol metabolites (Res-3-S and Res-di-S) stimulate MRP3-mediated transport of $E_2$17βG by increasing the V_{max}. Stimulation has also been seen for other substrate combinations (Chu *et al.*, 2004) and this stimulation of MRP3-mediated transport might therefore be a general feature of sulfo-conjugates.

In the *Bcrp1*$^{(-/-)}$ mice the fraction of a dose resveratrol excreted via the urine was increased due to the increased excretion of Res-di-S. The higher plasma levels of Res-3-G and Res-3-S in *Bcrp1*$^{(-/-)}$ mice were not accompanied by their higher urinary excretion, however. This apparent discrepancy might be explained by the presence of Bcrp1 in cells of the proximal tubule of the kidney (van Herwaarden and Schinkel, 2006) where it might actively transport Res-3-G and Res-3-S towards the urine within the tubules, a process missing in *Bcrp1*$^{(-/-)}$ mice.

Potentially handling of resveratrol could be altered in the knockout mice by changes in the level of conjugating enzymes secondary to the absence of transporters. No such changes were found, however, in microarray gene expression profiles of the knockout mice livers (not shown)

Unmodified resveratrol was found to be substantially reduced in plasma and urine of *Mrp3*$^{(-/-)}$ mice. Five explanations can be considered for this result: 1) Resveratrol is a substrate of MRP3. It does not act as a competitive inhibitor in vesicular transport experiments using vesicles containing MRP3, however (Suppl. Fig. 2B) and in transwell experiments we did not detect transport either (Suppl. Fig. 3), clearly showing that resveratrol is not a substrate of MRP3/Mrp3. 2) The unmodified resveratrol transporter BCRP/Bcrp1 (Suppl. Fig. 3C and (Breedveld *et al.*, 2007)) is more active in *Mrp3*$^{(-/-)}$ than in WT mice, resulting in less uptake of resveratrol from the gut. A major effect of Bcrp1 on uptake is improbable, however, as concentrations of

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resveratrol in plasma and urine of *Mrp3*$^{(-/-)}$ and *Bcrp1/Mrp3*$^{(-/-)(-/-)}$ mice were similar (not shown). 3) Decreased enterohepatic circulation (EHC) of resveratrol(metabolites), as less Res-3-G is available for EHC in the *Mrp3*$^{(-/-)}$ mice. If this were the case, we should also find substantially lower levels of Res-3-S and Res-di-S in plasma and/or urine of *Mrp3*$^{(-/-)}$ than of WT mice and this is not the result obtained (Figs 4 and 5). An additional argument against reduced EHC is that the absence of Mrp3 in enterocytes is expected to result in more Res-3-G being secreted towards the intestinal lumen, where it is available for deglucuronidation by the bacteria present in the large intestine. This should result in increased uptake of resveratrol and elevated plasma levels, not reduced levels as we find. 4) *Mrp3*$^{(-/-)}$ mice have altered levels of an as yet unidentified transport protein capable of transporting unmodified resveratrol. Against this hypothesis argue our microarray data of WT and *Mrp3*$^{(-/-)}$ livers (not shown) where we did not find altered expression of transport proteins. Of course this does not exclude the possibility that in other organs, including the gut, such alterations could be present. 5) Plasma levels of resveratrol decreased in the *Mrp3*$^{(-/-)}$ mouse, because it is mainly derived from deglucuronidation of Res-3-G, which is 10-fold lower in the *Mrp3*$^{(-/-)}$ mouse. This explanation is supported by several arguments: First, we find similar Res-3-G/resveratrol ratios in WT and *Mrp3*$^{(-/-)}$ mice, indicative of a similar percentage of circulating Res-3-G being deglucuronidated. Secondly, *Bcrp1*$^{(-/-)}$ mice do not show altered pharmacokinetics of unmodified resveratrol, even though BCRP/Bcrp1 readily transports resveratrol in vitro (Breedveld *et al.*, 2007). This indicates that intracellular levels of unmodified resveratrol within enterocytes are too low for substantial amounts to be transported by Bcrp1 towards the gut lumen. This is in line with a study of Kuhnle et al. (Kuhnle *et al.*, 2000) who have shown in Ussing chamber experiments that almost all resveratrol absorbed by the rat jejunum comes out basolaterally as resveratrol conjugated to

glucuronic acid. We have determined whether Res-3-G is hydrolyzed in mouse plasma, but found no significant resveratrol formation (data not shown). Hydrolysis might, however, occur intra-cellularly, followed by export of the resveratrol formed into the circulation by passive diffusion (Maier-Salamon *et al.*, 2006) or via an as yet unidentified transporter. Intracellular deglucuronidation is not without precedent as HepG2 cells can form quercetin from quercetin-glucuronide (O'Leary *et al.*, 2003), moreover diflunisal-glucuronides are deglucuronidated by rat liver during perfusion experiments (Brunelle and Verbeeck, 1997).

The fact that the major resveratrol metabolite, Res-3-G, is transported by BCRP and MRP3, is another illustration of the overlap in the substrate spectrum of BCRP and the members of the ABCC subfamily (Borst and Oude Elferink, 2002). MRP2 is probably a third player in the disposal of resveratrol, as we have found in vesicular uptake experiments that MRP2 transports Res-3-G as well (Suppl. Fig. 1). Indeed, experiments with Mrp2-deficient rats have shown that Mrp2 is the main transporter of Res-3-G into bile (Maier-Salamon *et al.*, 2007). This overlap in substrate specificity of BCRP, MRP2 and MRP3 provides the cell with alternative excretion routes for the disposal of xenobiotics in case one or more transporters are blocked or overtaxed. We think that under normal conditions most Res-3-G formed in the enterocytes is transported by MRP3 with high affinity towards the circulation for urinary excretion. This will keep Res-3-G concentrations inside the enterocyte low and will prevent the transport of Res-3-G by BCRP or MRP2 over the apical membrane towards the lumen of the gut, where the Res-3-G would be deglucuronidated by resident bacteria thereby releasing resveratrol. This would result in a futile cycle of resveratrol/Res-3-G. When transport by MRP3 is hampered, however, BCRP and MRP2 might come into play transporting Res-3-G towards the lumen of the gut thereby preventing the intracellular accumulation of Res-3-G.

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The presence of Res-3-S and Res-di-S in urine samples after oral resveratrol administration implies that these metabolites are transported over the basolateral membrane of enterocytes and/or liver. The carriers responsible for this transport still need to be identified. Possible candidates that could do the job include MRP1/Mrp1 and MRP4/Mrp4 which both transport sulfo-conjugates (Qian *et al.*, 2001;Zelcer *et al.*, 2003)and are present in enterocytes (Maher *et al.*, 2005).

An intriguing observation was that *Mrp3*$^{(-/-)}$ mice develop diarrhea after oral administration of resveratrol, whereas WT mice and *Bcrp1*$^{(-/-)}$ mice do not (data not show). The diarrhea in mice lacking Mrp3 does not appear to develop through acute effects of resveratrol on NaCl excretion or absorption, as we found no differences in Ussing chamber and ligated intestinal loop experiments between WT and *Mrp3*$^{(-/-)}$ mice (Supplementary Figs 4 and 5). Although we have currently no explanation for the resveratrol induced diarrhea in the absence Mrp3 it might be related to the accumulation of resveratrol metabolites in colon and cecum that we detect in *Mrp3*$^{(-/-)}$ mice, 24 h after resveratrol administration (data not shown).

In conclusion, our results show that the ABC-transporters MRP3 and BCRP transport resveratrol metabolites in vitro and that their absence in mice results in large alterations of the pharmacokinetics of major resveratrol metabolites after an oral dose of resveratrol. It is now clear that several ABC transporters are involved in the tissue distribution and subsequent elimination of resveratrol from the body.

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FOOTNOTES

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For reprint requests contact: Piet Borst, Division of Molecular Biology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, Tel: +31-20-5122880, Fax: +31-20-6691383 Email: p.borst@nki.nl.

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FIGURE LEGENDS

Fig. 1. Inhibition of MRP3- and BCRP-mediated transport by resveratrol metabolites. (**A**) concentration-dependent transport of [^{3}H]$E_2$17βG in MRP3-containing membrane vesicles in the presence or absence of 10 μM Res-3-G, Res-3-S or Res-di-S as indicated. (**C**) Concentration dependent transport of [^{3}H]MTX by BCRP in the presence or absence of 10 μM Res-3-G, Res-3-S or Res-di-S. Values are corrected for transport in the absence of ATP. Each data point and error are the mean ± SD of a representative experiment of 2 independent experiments each done in triplicate. (**B**) and (**D**) are Lineweaver-Burk transformations of the data presented in panel (**A**) and (**C**), respectively.

Fig. 2. Transport of Res-3-G by BCRP and MRP3 in vesicular transport experiments. Time course experiments of membrane vesicles containing human MRP3 (**A**) or BCRP (**B**) with 1 μM [^{3}H]Res-3-G and control vesicles as indicated. Transport was determined in the presence or absence of 4 mM ATP as indicated. Concentration-dependent transport of Res-3-G in MRP3- (**C**) and BCRP-containing (**D**) vesicles. Values are corrected for transport determined in absence of ATP. Each data point and error are the means ± SD of an experiment performed in triplicate.

Fig 3. Transport of Res-3-S and Res-di-S by BCRP in vesicular transport experiments. Time course experiments of membrane vesicles containing human BCRP using 140 nM [^{3}H]Res-3-S (**A**) or [^{3}H]Res-di-S (**B**). Transport was determined in the presence or absence of 4 mM ATP as indicated. Concentration-dependent transport of Res-3-S and Res-di-S by BCRP is shown in (**C**) and (**D**), respectively. Values are corrected for transport determined in absence of ATP. Each

data point and error are the means ± SD of an experiment performed in triplicate.

Note: transport was determined at 21 ºC.

Fig. 4. Pharmacokinetics of resveratrol in WT, *Mrp3*$^{(-/-)}$ *and Bcrp1*$^{(-/-)}$ mice. Groups of mice (n=4 or 5/ time point) received a dose of 50 mg resveratrol/kg body weight via oral gavage feeding. Plasma concentrations of resveratrol (**A**), Res-3-G (**B**), Res-3-S (**C**) and Res-di-S (**D**) were determined at the time points indicated. Values shown are means ± SD.

Fig 5. Urinary excretion of resveratrol and its metabolites in WT, *Mrp3*$^{(-/-)}$ and *Bcrp1*$^{(-/-)}$ mice. Groups of mice (n=5-9 /genotype) received an oral dose of 50 mg resveratrol/kg body weight and the excretion of resveratrol (**A**), Res-3-G (**B**), Res-3-S (**C**) and Res-di-S (**D**) over 24-h was determined. In (**E**) the summarized excretion of resveratrol and its metabolites is shown. Values are expressed as % of the dose of resveratrol administered and are mean ± SD.

Fig. 6. Concentration of resveratrol and its metabolites in liver and kidney of WT and *Mrp3*$^{(-/-)}$ mice. Groups of mice (n = 4 or 5/genotype received an oral dose of 50 mg resveratrol/kg body weight and the concentrations of the indicated resveratrol (metabolites) were determined in liver (**A**, **C**, **E**) and kidney (**B**, **D**, **F**). Values shown are means ± SD.

Fig. 7. Determination of the urinary and fecal excretion of resveratrol in WT, *Mrp3*$^{(-/-)}$ and *Bcrp1*$^{(-/-)}$ mice. Groups of mice (n = 4 or 5/genotype received an oral dose of 50 mg [^{3}H]resveratrol/kg body weight and 24 hours later the presence of [^{3}H]label was determined in urine (**A**) and feces (**B**). In (**C**) summarized excretion via urine and feces is shown. Values are

expressed as % of the dose of [^{3}H]resveratrol administered and are mean ± SD.

Figure 1

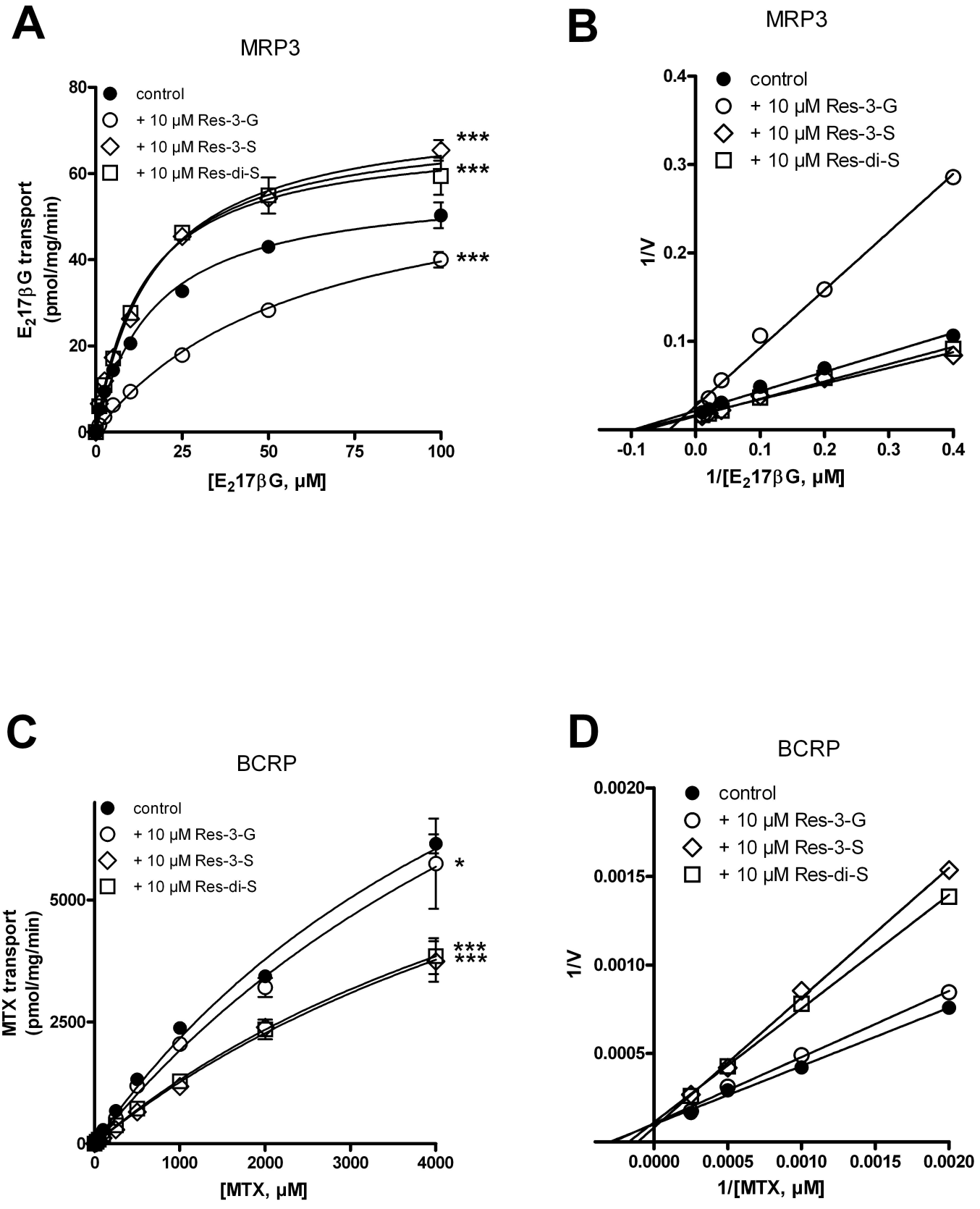
A
MRP3
control
+ 10 μM Res-3-G
+ 10 μM Res-3-S
+ 10 μM Res-di-S
E2 17βG transport (pmol/mg/min)
[E2 17βG, μM]
***
***
***
B
MRP3
control
+ 10 μM Res-3-G
+ 10 μM Res-3-S
+ 10 μM Res-di-S
1/V
1/[E2 17βG, μM]
C
BCRP
control
+ 10 μM Res-3-G
+ 10 μM Res-3-S
+ 10 μM Res-di-S
MTX transport (pmol/mg/min)
[MTX, μM]
*
***
***
D
BCRP
control
+ 10 μM Res-3-G
+ 10 μM Res-3-S
+ 10 μM Res-di-S
1/V
1/[MTX, μM]

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Figure 2

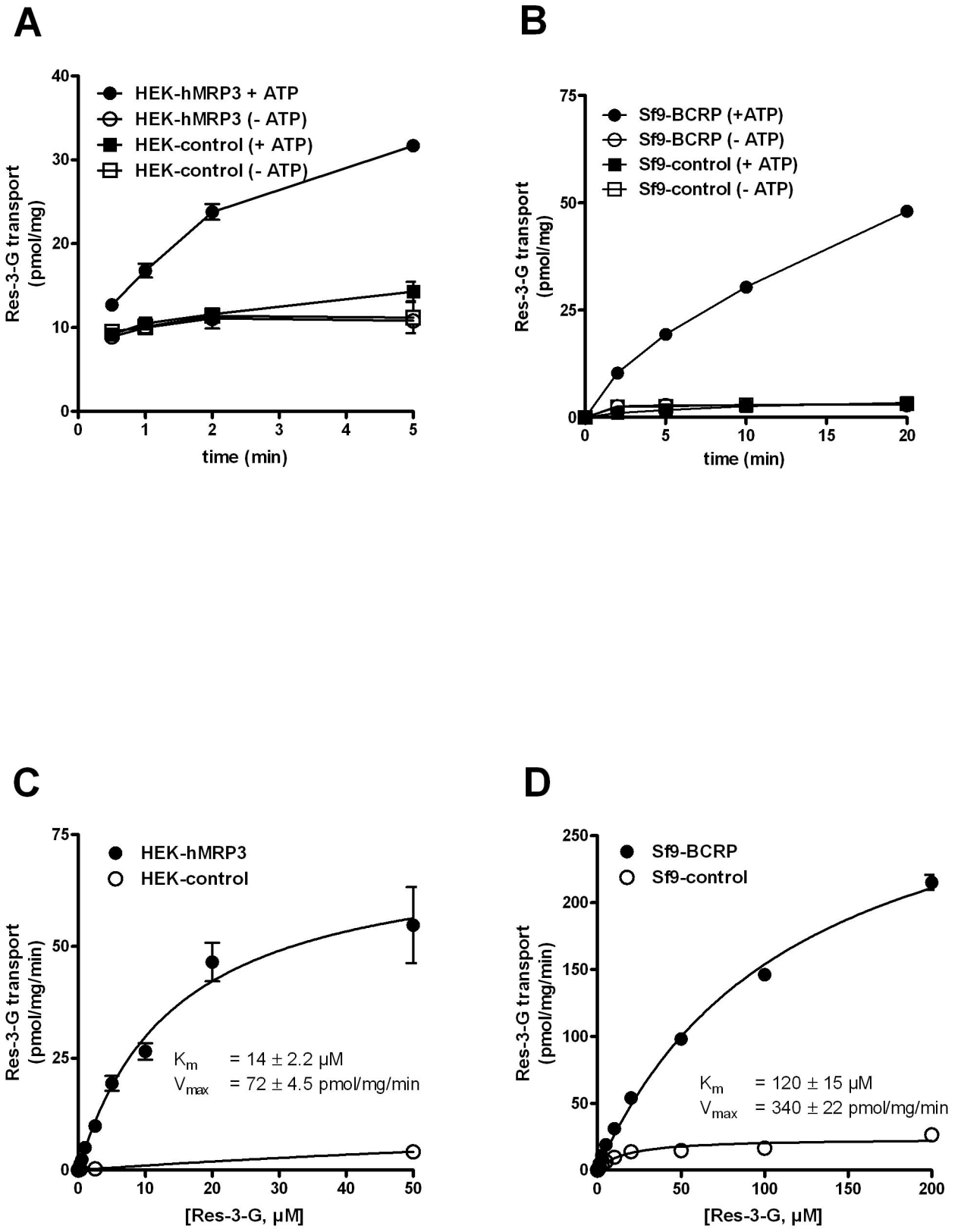
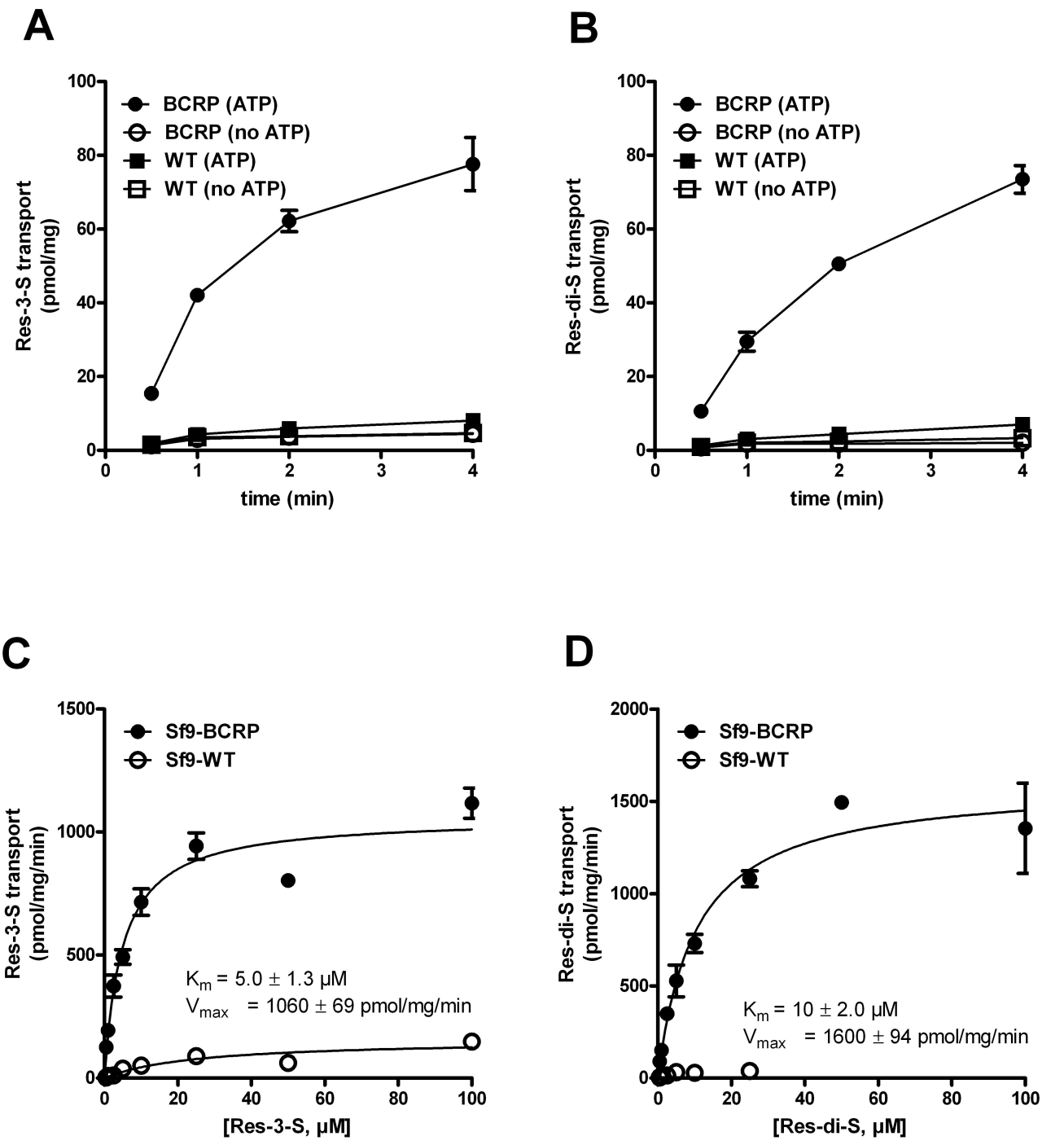


Figure 3


A
BCRP (ATP)
BCRP (no ATP)
WT (ATP)
WT (no ATP)
Res-3-S transport (pmol/mg)
time (min)
B
BCRP (ATP)
BCRP (no ATP)
WT (ATP)
WT (no ATP)
Res-di-S transport (pmol/mg)
time (min)
C
Sf9-BCRP
Sf9-WT
Res-3-S transport (pmol/mg/min)
Km = 5.0 ± 1.3 µM
Vmax = 1060 ± 69 pmol/mg/min
[Res-3-S, µM]
D
Sf9-BCRP
Sf9-WT
Res-di-S transport (pmol/mg/min)
Km = 10 ± 2.0 µM
Vmax = 1600 ± 94 pmol/mg/min
[Res-di-S, µM]

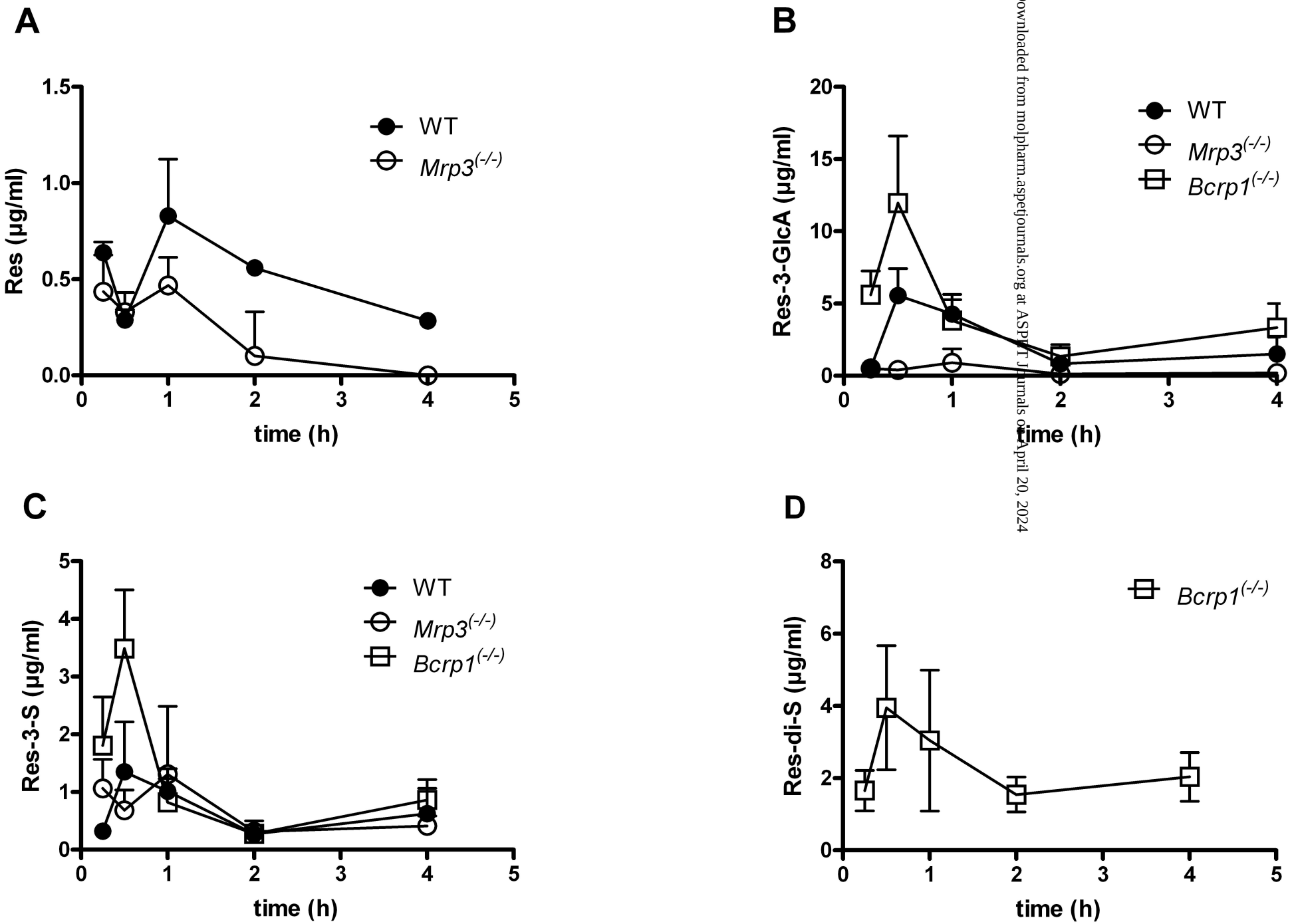


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Figure 5

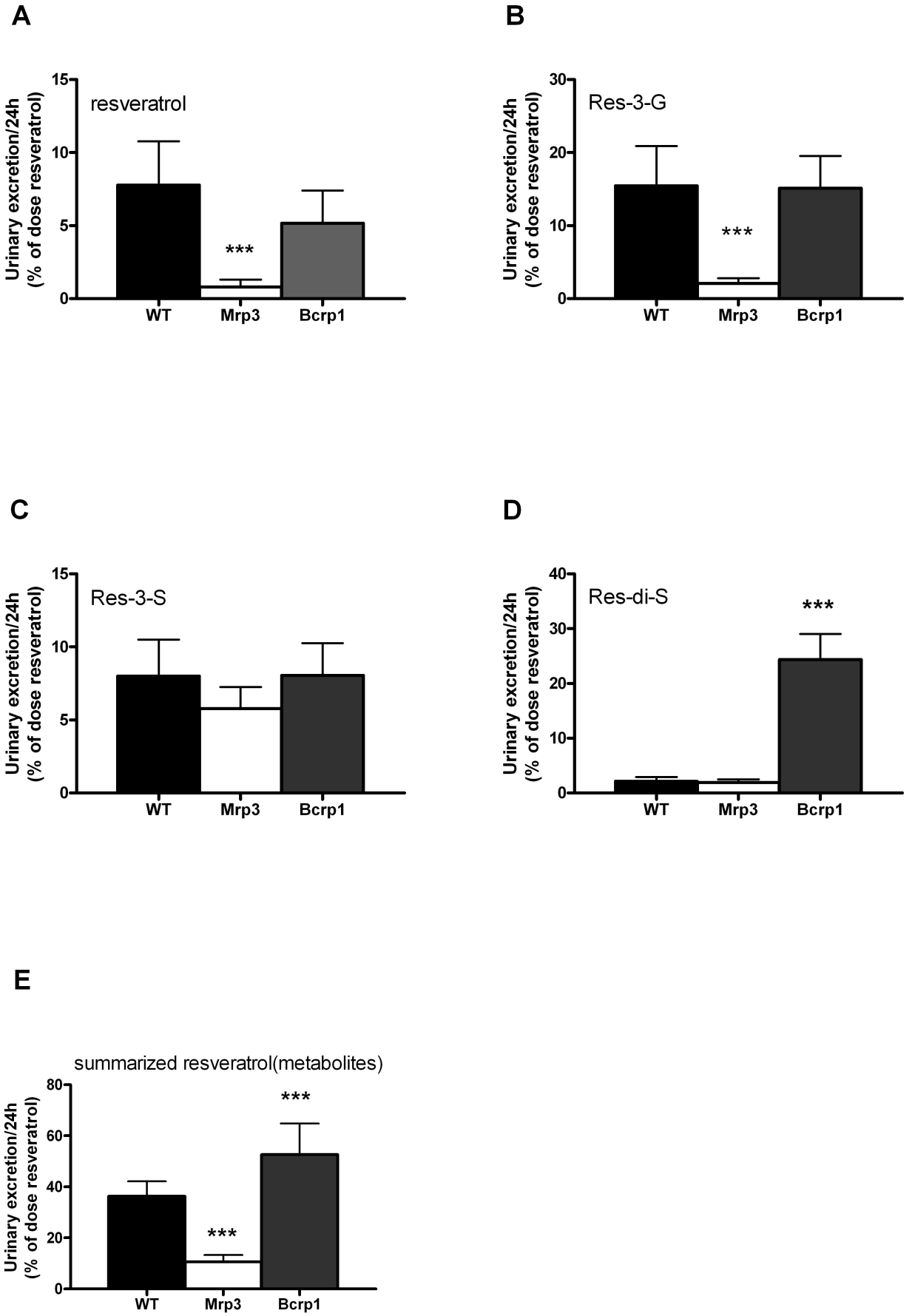

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Figure 6