Molecular Pharmacology Fast Forward. Published on May 7, 2007 as doi:10.1124/mol.107.035592

MOL Manuscript # 35592

Multidrug resistance protein 2 and 3 provide alternative routes for hepatic excretion of morphine-glucuronides.

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Molecular Pharmacology Fast Forward. Published on May 7, 2007 as DOI: 10.1124/mol.107.035592
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MOL Manuscript # 35592

Running title: MRP2 and MRP3 transport M3G

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number of text pages:21
number of figures:5
number of references:38
number of words in the abstract:151
number of words in the introduction: 529
number of words in the discussion: 1288

abbreviations:

MRP, Multidrug Resistance Protein; M3G, morphine-3-glucuronide; UGT, UDP-glucuronosyl transferase; M6G, morphine-6-glucuronide; Sf9, *Spodoptera frugiperda*; HEK293 cells, human embryonic kidney cells 293; i.p., intraperitonealy; s.c., subcutaneously; LC-MS/MS, liquid chromatography-tandem mass spectrometry; K_m, Michaelis-Menten constant; V_{max}, maximal velocity; GSH, glutathione; WT, wild type; GI, gastrointestinal.

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Abstract

Glucuronidation is a major hepatic detoxification pathway for endogenous and exogenous compounds, resulting in the intracellular formation of polar metabolites that require specialized transporters for elimination. Multidrug Resistance Proteins (MRPs) are expressed in the liver and can transport glucuronosyl-conjugates. Using morphine as a model aglycone we demonstrate that morphine-3-glucuronide (M3G), the predominant metabolite, is transported *in vitro* by human MRP2 (ABCC2), a protein present in the apical membrane of hepatocytes. Loss of biliary M3G secretion in *Mrp2*$^{(-/-)}$ mice results in its increased sinusoidal transport that can be attributed to Mrp3. Combined loss of Mrp2 and Mrp3 leads to a substantial accumulation of M3G in the liver, from which it is transported across the sinusoidal membrane at a low rate resulting in the prolonged presence of M3G in plasma. Our results show that murine Mrp2 and Mrp3 provide alternative routes for the excretion of a glucuronidated substrate from the liver *in vivo*.

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The liver is the major organ responsible for the metabolism and excretion of xenobiotic and endobiotic substances. For many of these potentially toxic compounds, glucuronidation is a major route for inactivation and subsequent elimination from the body. Glucuronidation, catalyzed by a specific set of UDP-glucuronosyl transferases (UGTs), results in the formation of more hydrophilic and usually less toxic metabolites (Tukey and Strassburg, 2000). The increased polarity of glucuronides relative to the parent aglycone limits their diffusion through biological membranes. Hence, specific transporters are required to transport glucuronides across the sinusoidal and canalicular membranes of hepatocytes (Milne *et al.*, 1997;O'Brien *et al.*, 1996).

MRPs belong to the ATP-binding cassette family of membrane transporters and transport their substrates across biological membranes at the expense of ATP hydrolysis (Borst and Oude Elferink, 2002). MRP1-4 are expressed in the liver and are thought to contribute to the disposal of various metabolites from this organ (Scheffer *et al.*, 2000;Aleksunes *et al.*, 2006). The closely related MRP2 (ABCC2) and MRP3 (ABCC3) have an overlapping substrate specificity that includes several sulfate-, glutathione- and glucuronate-conjugates (Keppler and Konig, 2000;Borst *et al.*, 2005), but differ in their cellular localization in polarized cells. Whereas MRP2 is in the apical (canalicular) membrane, MRP3 is found in the basolateral (sinusoidal) membrane (Borst *et al.*, 2003;Borst *et al.*, 2005;Kruh and Belinsky, 2003). Experiments with rats that naturally lack Mrp2 (TR^-/EHBR) (Jansen *et al.*, 1993;Xiong *et al.*, 2000;Morikawa *et al.*, 2000) and more recently with *Mrp2*$^{(-/-)}$ mice show the critical role of MRP2 in the biliary excretion of organic anions (Chu *et al.*, 2006;Vlaming *et al.*, 2006). Similarly, loss of Mrp3 impairs sinusoidal secretion of organic anions (*e.g*. morphine-glucuronides) (Manautou *et al.*, 2005;Zelcer *et al.*, 2005;Zelcer *et al.*, 2006;Zamek-Gliszczynski *et al.*, 2006).

Morphine is a potent analgesic that acts by binding to specific opioid-receptors present in the central nervous system as well as in the periphery (Inturrisi, 2002). Morphine is extensively

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metabolized by glucuronidation and membrane transport systems have been shown to modulate its pharmacokinetics (Thompson *et al.*, 2000;King *et al.*, 2001;Bourasset *et al.*, 2003;Schinkel *et al.*, 1994;Zelcer *et al.*, 2005). In humans UDP-glucuronosyl transferase 2B7 (UGT2B7) converts morphine into morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (Coffman *et al.*, 1997), whereas mice only form M3G (Kuo *et al.*, 1991). The pharmacological effects of M3G and M6G differ profoundly. M3G has no analgesic properties and is even thought to antagonize some of the effects of unmodified morphine (Moran and Smith, 2002). In contrast, M6G is more potent than morphine itself and contributes to the analgesic effect of morphine (Lotsch and Geisslinger, 2001). After their formation in the hepatocyte, morphine-glucuronides are transported over the canalicular membrane into bile and over the sinusoidal membrane into the circulation (Milne *et al.*, 1997). We have previously shown that Mrp3 is the major sinusoidal transporter of morphine-glucuronides in mouse liver (Zelcer *et al.*, 2005): in mice lacking Mrp3 basolateral secretion of M3G is severely impaired. A substantial fraction of the intra-hepatically formed morphine-glucuronides is also excreted over the canalicular membrane into bile by a yet unidentified transporter(s). In this paper we further explore the contribution of members of the MRP-family to the disposal of morphine-glucuronides *in vitro* and *in vivo*.

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Materials and Methods

Materials - Morphine was from the pharmacy of The Netherlands Cancer Institute. [^{3}H]morphine (80 Ci/mmol) was purchased from Biotrend (Köln, Germany). Creatine phosphate and creatine kinase were from Roche (Almere, The Netherlands). All other chemicals and reagents were from Sigma (St. Louis, MO).

Cell lines and transfections - *Spodoptera frugiperda* (Sf9) insect cells and HEK293 cells overproducing UGT2B7 (Coffman *et al.*, 1997) were grown as described by Zelcer et al. (Zelcer *et al.*, 2003) and Coffman et al. (Coffman *et al.*, 1997), respectively. HEK293 cells expressing UGT2B7 and human MRP1 (hMRP1), or human MRP2 (hMRP2) were constructed by transfecting HEK293-UGT2B7 cells with pBabeCMVpuro-MRP1, pBabeCMVpuro-MRP2 or empty vector control by calcium phosphate precipitation. After 24 hours the cells were split and puromycin was used to select for clones expressing the transporter of interest. Puromycin resistant clones were analyzed for hMRP1 and hMRP2 expression by immunoblot analysis, using the mouse monoclonal antibody MRPr1 (Scheffer *et al.*, 2000) and M_2III-5 (Scheffer *et al.*, 2000), respectively, as previously described (not shown). Using the same antibodies, plasma membrane localization was confirmed by immunofluorescence (not shown). Efflux experiments were performed as described in (Zelcer *et al.*, 2005).

Preparation of [^{3}H]labeled M3G - [^{3}H]M3G was prepared by incubation of 9 μCi [^{3}H]morphine with 1 mg of microsomal protein from mouse liver in 100 mM phosphate buffer (pH 7.4) containing 5 mM UDP-glucuronic acid at 37°C for 16 h. M3G was extracted from the incubation mixture by solid phase extraction on an Oasis HLB cartridge (Waters, Milford, MA) and eluted with 5% methanol in 100 mM KCl and 50 mM Hepes (pH 7.4). The purity of the [^{3}H]M3G preparation was checked by high pressure liquid chromatography (not shown)

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Preparation of membrane vesicles and vesicular transport assays - ATP-dependent transport of [^{3}H]M3G into inside-out membrane vesicles prepared from Sf9 cells overproducing human MRP1-4 and control Sf9 cells was measured using the rapid filtration technique as described previously (Zelcer et al., 2003), with modifications: vesicles were filtrated using a MultiScreen$_{HTS}$ vacuum manifold in combination with Multiscreen$_{HTS}$ FB 96-well filter plates (Millipore, Bedford, MA). Membranes were washed 4 times with 200 µl ice cold PBS and the radioactivity retained on the membranes was counted by liquid scintillation counting.

Animals - The generation of *Mrp2*$^{(-/-)}$ (Vlaming *et al.*, 2006), *Mrp3*$^{(-/-)}$ (Zelcer *et al.*, 2006) and *Mrp4*$^{(-/-)}$ (Leggas *et al.*, 2004) mice has been described. *Mrp2Mrp3*$^{(-/-)(-/-)}$ and *Mrp3Mrp4*$^{(-/-)(-/-)}$ mice were generated by cross-breeding the corresponding strains. The new double knockouts were healthy and fertile. All animals 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.

Pharmacokinetic experiments - Morphine pharmacokinetics were determined as described (Zelcer *et al.*, 2005). For the determination of the biliary excretion of morphine and M3G, the gall bladder of 4 to 5 mice of each genotype was cannulated as described (Jonker *et al.*, 2001) and morphine (15 mg/kg body weight) was injected subcutaneously (s.c.). Subsequently, bile was collected in fractions of 15 min for 2 hours and stored at - 80 °C until analysis.

Analysis of morphine and its metabolites in cell culture medium, plasma, and urine was done as described by Rook *et al.* (Rook *et al.*, 2005). Bile was diluted in running buffer and directly injected into the liquid chromatography-tandem mass spectrometry apparatus. Liver tissue was homogenized in 5 ml human plasma using a polytron tissue homogenizer (Glenn Mills, Clifton, NJ) and a sample of 50 µl of the homogenate was treated like the plasma samples.

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RESULTS

Transport of M3G into MRP1-4 containing vesicles - To study transport of M3G by MRPs we used vesicular transport assays with membrane vesicles generated from Sf9 cells infected with baculoviruses encoding hMRP1-4. In MRP2-containing vesicles we found a time- and ATP-dependent accumulation of M3G in vesicles, which was absent in vesicles prepared from Sf9 cells infected with WT baculovirus (Fig 1A). hMRP2 transported M3G with apparent K_m and V_{max} values of 50 ± 5 μM and 1400 ± 30 pmol/mg/min, respectively (Fig 1B). human MRP3 (hMRP3) transports M3G in a time- and ATP dependent manner as well (Fig 1C), as we have shown previously (Zelcer et al., 2005). In contrast, membrane vesicles containing hMRP1 did not accumulate more M3G than control vesicles, irrespective of the presence of ATP and /or glutathione (GSH) (Fig 1D). Similar negative results were obtained for human MRP4 (hMRP4) (not shown). M3G does also not act as a competitive inhibitor of transport by hMRP1 or hMRP4, as we did not detect any effect of 400 μM M3G on the transport of 1 μM [^{3}H]$E_2$17βG by these MRPs (data not shown), independent of the presence of GSH.

Efflux of M3G and M6G from HEK293-cells co-expressing UGT2B7 and hMRP1 or hMRP2 - We have previously shown that transient co-expression of UGT2B7 and MRP3 in HEK293 cells results in increased efflux of intracellularly formed M3G and M6G (Zelcer *et al.*, 2005). We have now constructed HEK293 cells stably expressing UGT2B7 in combination with either hMRP1 or hMRP2. Incubation of these cells with morphine (10 μM) resulted in a gradual decrease in the morphine levels in the cell culture medium, that was hardly influenced by the presence of either hMRP1 or hMRP2 (Fig. 2A). As expected from the results in Fig. 1, hMRP2 increased the efflux of M3G as reflected by its earlier appearance and higher overall levels in the cell culture medium (Fig. 2B). However, efflux was even higher in the cells containing hMRP1, even though hMRP1 was unable to transport M3G in the vesicular transport experiments in Fig. 1. We return to this

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discrepancy below. Interestingly, only the presence of hMRP1 resulted in increased M6G levels in the cell culture medium over those found in the control cells (Fig. 2C). we speculate that the high rate of endogenous transport of morphine glucuronides in the control cells (Fig. 2B,*C*) could be due to the substantial endogenous levels of hMRP1 present in HEK293 cells, as efflux in the control cells could be completely blocked by MK571 ((Zelcer *et al.*, 2005), not shown). In experiments similar to those done with hMRP3 (Zelcer *et al.*, 2005) we did not find any contribution of hMRP4 to transport of M3G/M6G in this assay, consistent with our vesicular transport assays (not shown).

The intracellular levels of morphine and morphine-glucuronides mirrored the efflux patterns: morphine was not affected, M3G levels were lowered both in hMRP1 and hMRP2 expressing cells, and M6G levels only in the MRP1 cells (Fig. 2D). The higher levels of M3G than M6G in the medium and in the cells (note the difference in Y-axis between Figs 2B and C), reflect the preferential formation by UGT2B7 of the 3-glucuronide over the 6-glucuronide (Coffman *et al.*, 1997). The overall mass balance of the cellular efflux experiments varied between 90-110% (data not shown).

Pharmacokinetics of morphine in Mrp2-4$^{(-/-)}$ mice - To assess the contribution of cannalicular Mrp2 to the transport of M3G *in vivo* we performed bile duct cannulation experiments. Because we have previously shown that hepatic Mrp3 transports M3G over the sinusoidal membrane (Zelcer *et al.*, 2005) we tested the disposal of morphine and M3G in bile of WT, *Mrp2$^{(-/-)}$* and *Mrp2Mrp3$^{(-/-)(-/-)}$* mice (Fig. 3). WT mice have high levels of M3G in their bile after the s.c. administration of morphine (Fig. 3A), which were even higher in the *Mrp3$^{(-/-)}$* mice, consistent with impaired basolateral efflux leading to increased canalicular excretion. The absence of Mrp2 reduced M3G excretion into bile to <1% of that found in WT controls, showing that Mrp2 is the major, if not the only, canalicular M3G transporter. Only low levels of morphine were found in bile (Fig 3B), which were higher in WT and *Mrp3$^{(-/-)}$* mice than in *Mrp2$^{(-/-)}$* and *Mrp2Mrp3$^{(-/-)(-/-)}$*

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mice. Although small changes in the expression of transporters able to handle morphine (*e.g.* Mdr1a/b) could account for this increase, we think that it is more likely to be the result of some deglucuronidation of the very high levels of biliary M3G in bile samples of these mouse strains.

The pharmacokinetics of morphine and M3G in plasma after the i.p. administration of morphine (15 mg/kg) in wild type (WT), *Mrp2*$^{(-/-)}$, *Mrp3*$^{(-/-)}$, *Mrp3Mrp4*$^{(-/-)(-/-)}$ and *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice are shown in Fig. 4. Plasma morphine levels were unaltered by the absence of Mrp2 and/or Mrp3 (Fig. 4A). M3G levels, however, differed between the genotypes tested (Fig. 4B). In the absence of Mrp2, plasma M3G levels were increased, consistent with impaired canalicular transport leading to increased transport over the sinusoidal membrane. In contrast, Fig. 4C shows that 24 hours after the administration of morphine, plasma levels of M3G were lower in *Mrp2*$^{(-/-)}$ than in WT mice, presumably due to reduced enterohepatic circulation (EHC) of morphine/M3G in the absence of Mrp2. We have previously found that *Mrp3*$^{(-/-)}$ mice have impaired transport of M3G over the sinusoidal membrane (Zelcer *et al.*, 2005), relative to WT animals. *Mrp2Mrp3*$^{(-/-)(-/-)}$ and *Mrp3*$^{(-/-)}$ had a similar sluggish increase in M3G plasma levels in the first 30 min after morphine administration. However, M3G remained detectable in the plasma of *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice for at least 4 hours after the administration of morphine, in contrast to the results with the *Mrp3*$^{(-/-)}$ mice (Fig. 4B, the 4 hour time point is not shown). To study whether the residual basolateral transport of M3G in the *Mrp3*$^{(-/-)}$ and *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice could be attributed to Mrp4 we also determined morphine and M3G levels in plasma of *Mrp3Mrp4*$^{(-/-)(-/-)}$ mice, 30 min after morphine administration (Fig . 4E). M3G levels were indistinguishable from those found in *Mrp3*$^{(-/-)}$ mice, suggesting that Mrp4 does not contribute to the residual basolateral M3G transport in the *Mrp3*$^{(-/-)}$ mice. This also fits our vesicular transport and cellular efflux experiments in which we did not find any effect of MRP4 on the transport of M3G (not shown).

The increased plasma concentrations of M3G in the absence of Mrp2 resulted in an increased urinary M3G excretion (Fig. 4D). This is in contrast to the results obtained with the

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Mrp3$^{(-/-)}$ mice, which have low urinary excretion of M3G (Fig. 4D, (Zelcer *et al.*, 2005)). Interestingly, we detected substantial urinary M3G excretion in *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice, being only slightly lower than that found in WT animals (Fig. 4D). This indicates that the low but persistent presence of M3G in their plasma (Fig 4B and C) eventually results in near normal urinary excretion of M3G (Fig. 4D).

Livers were also analyzed for M3G content. 30 min after the administration of morphine, M3G levels in livers of *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice were 13-fold increased and they remained high for many hours (Fig. 5). This is consistent with severely impaired cellular efflux of M3G from hepatocytes in the absence of both the major canalicular and sinusoidal transporters of M3G. This result clearly demonstrates the cooperative involvement of Mrp2 and Mrp3 in the excretion of M3G from the liver. In livers of *Mrp2*$^{(-/-)}$ mice the M3G levels were 3-fold lower than in livers of WT animals. We return to this result in the Discussion.

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DISCUSSION

Glucuronidation, catalyzed by specific UGTs, represents a major detoxification pathway of endogenous and exogenous compounds (Tukey and Strassburg, 2000). Morphine is a model compound used to study glucuronidation (Coffman *et al.*, 1997), which occurs predominantly in the liver. In humans, morphine glucuronidation is catalyzed by UGT2B7, resulting in the formation of M3G and M6G (Coffman *et al.*, 1997). In mice a different UGT isoform is involved and only M3G is formed (Kuo *et al.*, 1991;Milne *et al.*, 1996). To prevent the intracellular accumulation of the hydrophilic morphine-glucuronides after their formation, they must be transported out of the hepatocytes by specific transport proteins.

Our vesicular uptake and cellular efflux experiments show that MRP2 transports M3G. The rate at which M3G was transported in vesicular uptake assays was high. Some caution is required, however, in directly extrapolating these data to the *in vivo* situation, as endogenous and exogenous compounds can modulate MRP2 activity (Zelcer *et al.*, 2003;Bodo *et al.*, 2003). Humans and guinea pigs form both M3G and M6G (Milne *et al.*, 1997) and only a small fraction of intrahepatically formed M6G is transported into bile (Milne *et al.*, 1997). Our cellular efflux experiments suggest that this is at least partly due to the fact that M6G is not an MRP2 substrate. Our results indicate that MRP1 transports both M3G and M6G, as shown by the increased efflux of both compounds from cells co-expressing UGT2B7 and MRP1. We have seen the same MRP1-dependent efflux in cells transiently transfected with MRP1 (data not shown), supporting the interpretation that it is MRP1 that transports M3G/M6G out of the cells. It is therefore remarkable that we do not find any MRP1-mediated transport of M3G in vesicular transport experiments, either directly using [^{3}H]M3G as substrate, or indirectly using M3G as competitor in transport experiments with [^{3}H]$E_2$17βG as substrate. This negative result is not due to the use of Sf9 insect cells to prepare the MRP1-containing vesicles as membrane vesicles prepared from

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MRP1-expressing HEK293 cells did not transport [^{3}H]M3G either (data not shown). Addition of GSH, sometimes required as co-substrate for MRP1 (Deeley and Cole, 2006) did not change these negative results. An intriguing possibility is that MRP1-mediated transport of M3G requires a different co-substrate than GSH, but there is no precedent for this.

Bile duct cannulation experiments showed that Mrp2 is the major, if not the only, canalicular transporter of M3G in mice. Increased levels of Mrp3 have been reported for the *Mrp2*$^{(-/-)}$ mice (Vlaming *et al.*, 2006;Chu *et al.*, 2006) and the higher levels of M3G in plasma of *Mrp2*$^{(-/-)}$ mice can be explained by increased transport by Mrp3, the major sinusoidal transporter of M3G (Zelcer *et al.*, 2005). By transport into bile, MRP2/Mrp2 contributes to the enterohepatic circulation (EHC) of its substrates (Borst and Oude Elferink, 2002). The lower M3G plasma levels in the *Mrp2*$^{(-/-)}$ mice 24 hours after morphine administration are therefore probably due to reduced EHC of morphine/M3G in these mice. 24 hours after morphine administration, *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice have M3G plasma levels similar to those found in WT mice. This is most likely caused by the prolonged, but low capacity transport into plasma of the M3G accumulated in the liver. This also explains the near normal urinary excretion of M3G in these mice: mice have a glomerular filtration rate of 250 μl/min (Qi *et al.*, 2005). The average M3G plasma concentration in the *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice in the first 4 hours after administration of morphine is about 1000 ng/ml. This means that the *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice excrete about 250 ng M3G/min (equals 15 μg/h). The average urinary excretion of M3G in *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice is 212 ± 18. This means that M3G concentration needs to remain on average 1000 ng/ml for 14 h (212 (μg) /15 (μg/h)) in order to account for the urinary excretion of 212 μg/24/h that we find in the *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice.

In the absence of both the major canalicular (Mrp2) and major sinusoidal (Mrp3) transporters of M3G, this morphine metabolite accumulates in the liver. In contrast, the absence of Mrp2 alone lowers the liver M3G content. Although the 2-fold upregulation of Mrp3 in livers

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of *Mrp2*$^{(-/-)}$ mice (Vlaming *et al.*, 2006) might have contributed to this decrease, the M3G levels the intra-hepatic bile are a much more important factor in our opinion. In the case of *Mrp2*$^{(-/-)}$ mice the intra-hepatic bile contains almost no M3G, whereas the biliary tree of WT mice contains very high levels of M3G. Hence, the paradoxical result found in livers of the *Mrp2* $^{(-/-)}$ mice (*i.e.* lower levels of M3G in the liver despite an important hepatic route for the excretion of M3G being blocked) is, therefore, readily explained by the loss of the very high levels of M3G present in bile of WT mice. Apparently, most of the M3G in liver samples comes from the bile present in the intrahepatic biliary tree and the loss of this M3G in the *Mrp2*$^{(-/-)}$ mice, which has at least 100-fold lower biliary M3G than WT mice, explains the low M3G in the *Mrp2*$^{(-/-)}$ liver. This means that the concentration of M3G present intracellularly in hepatocytes is probably best estimated from the concentration found in livers of the *Mrp2*$^{(-/-)}$ mice. 60 min after the administration of morphine, *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice have 36-fold higher M3G levels in their livers than *Mrp2*$^{(-/-)}$ mice. This appears not to result in end product inhibition of the murine UGT involved in the glucuronidation of morphine, as levels of morphine in plasma are unaltered. The high M3G levels did not result either in increased liver damage as assessed by both histological analysis and determination of the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (data not shown), indicating that morphine-glucuronides are not very hepatotoxic.

The fact that there is significant transport of M3G over the basolateral membrane in the absence of murine Mrp3, shows that there is an additional transport system for M3G in the sinusoidal membrane of the hepatocyte. As the liver is known to express many different transport proteins (Faber *et al.*, 2003), this does not come as a surprise. The additional transport system shows up most prominently in the *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice, in which it is responsible for the near normal urinary excretion of M3G. Two efflux transporters present at the basolateral membrane of hepatocytes are MRP1/Mrp1 and MRP4/Mrp4, which both have been shown to transport

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glucuronosyl-conjugates (Kruh and Belinsky, 2003). However, HEK293-UGT2B7 cells transiently transfected with hMRP4 do not efflux more M3G or M6G (data not shown). Moreover, 30 min after the administration of morphine, *Mrp3Mrp4*$^{(-/-)(-/-)}$ mice have similar M3G plasma levels as *Mrp3*$^{(-/-)}$ mice (Fig. 4E), indicating that MRP4/Mrp4 does not contribute to basolateral transport of morphine-glucuronides from the liver. In contrast, the M3G efflux experiments with UGT2B7/hMRP1 co-expressing cells suggest that MRP1/Mrp1 might be responsible for the sinusoidal M3G backup system. Mrp1 is known to be present at low levels in the sinusoidal membrane of murine hepatocytes (Roelofsen *et al.*, 1997;Aleksunes *et al.*, 2006), which might explain the low capacity of M3G transport in the absence of Mrp3. By immunoblot analysis we confirmed this for the mouse strains used in our study and these low levels of Mrp1 did not vary between the genotypes tested (data not shown).

In conclusion, our results show that MRP2/Mrp2 and MRP3/Mrp3 are the major hepatic transporters involved in the extrusion of M3G into bile and plasma, respectively. There is, however, a low capacity backup transport system present for M3G in the sinusoidal membrane of the hepatocyte, possibly MRP1/Mrp1. These backup systems may have evolved to deal with the wide spectrum of endogenous and exogenous anionic substrates that the hepatocyte faces and may also provide the liver with escape mechanisms in case one or more of the transport systems are blocked or overtaxed.

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FOOTNOTES

Our research was supported by grants of the Dutch Cancer Society (NKI-2001-2473, NKI-2001-2474 and NKI-2003-2940) and the Netherlands Organization of Scientific Research (MW PGS 912-02-073) to R. Oude Elferink, A.S. and P.B.

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Molecular Pharmacology Fast Forward. Published on May 7, 2007 as DOI: 10.1124/mol.107.035592
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Molecular Pharmacology Fast Forward. Published on May 7, 2007 as DOI: 10.1124/mol.107.035592
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FIGURE LEGENDS

Fig. 1. Transport of M3G by human MRP1-3 in vesicular uptake experiments. (**A**, **C**, **D**). Time course experiments of membrane vesicles from Sf9 cells infected with human MRP1-3 containing baculoviruses (●,○) or WT baculovirus (■, □) incubated with 1 μM [^{3}H]M3G. For human MRP1 (**D**) transport was also determined in the presence of 3 mM GSH (▲,△). Uptake was determined in the presence (●, ■, ▲) or absence (○, □, △) of 4 mM ATP. (note that due to the low background transport, the curves describing transport in WT-vesicles and in human MRP2-containing vesicles in the absence of ATP overlap). (**B**) Concentration-dependent uptake of M3G in human MRP2-containing vesicles. Values are corrected for transport determined in absence of ATP. Each data point and error are mean ± SD of an experiment performed in triplicate.

Fig. 2. Cellular efflux of M3G and M6G by HEK-293 cells co-expressing UGT2B7 and human MRP1 or human MRP2. (**A**) Amount of morphine in the cell culture medium as a function of incubation time. (**B**,**C**) Cumulative amounts of M3G (**B**), and M6G (**C**) secreted in the cell culture medium (note that during the first 30 min to 1 hour of the experiments, M6G concentrations were below the detection limit of the assay Fig. 2**C**). (**D**) Amounts of morphine, M3G and M6G in the cell pellet at the end of the experiment. Values shown are mean ± SD of an experiment in triplicate.

Fig. 3. Biliary excretion of morphine and M3G after the administration of morphine (15 mg/kg, s.c.) in WT, *Mrp2*$^{(-/-)}$, *Mrp3*$^{(-/-)}$ and *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice . After cannulation of

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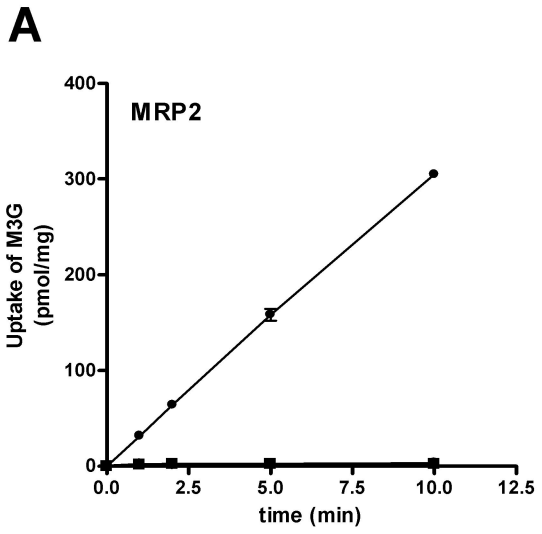
the common bile duct, groups of mice (n=4 or 5/time point) received a dose of 15 mg morphine/kg body weight s.c. and the cumulative excretion of M3G (**A**) and morphine (**B**) was determined at the indicated time points after injection. Values are expressed as % of the given dose and shown as means ± SD.

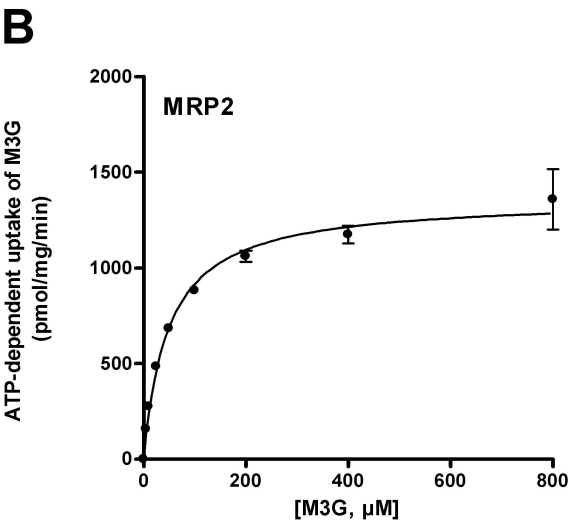
Fig. 4. Pharmacokinetics of morphine in WT, *Mrp2*$^{(-/-)}$, *Mrp3Mrp4*$^{(-/-)(-/-)}$ *and Mrp2Mrp3*$^{(-/-)(-/-)}$ mice. Groups of mice (n=3 or 4/ time point) received a dose of 15 mg morphine/kg body weight i.p.. Plasma concentrations of morphine (**A**) and M3G (**B**) were determined at the indicated time points. (**C**) Plasma concentrations of M3G 24-h after administration of morphine. N.D., non-detectable (**D**) Urinary excretion of morphine and M3G over 24 hours, expressed as percentage of the given dose. (**E**) plasma concentrations of morphine and M3G 30 min after administration of morphine in WT, *Mrp3*$^{(-/-)}$ and *Mrp3Mrp4*$^{(-/-)(-/-)}$ mice. Values shown are means ± SD.

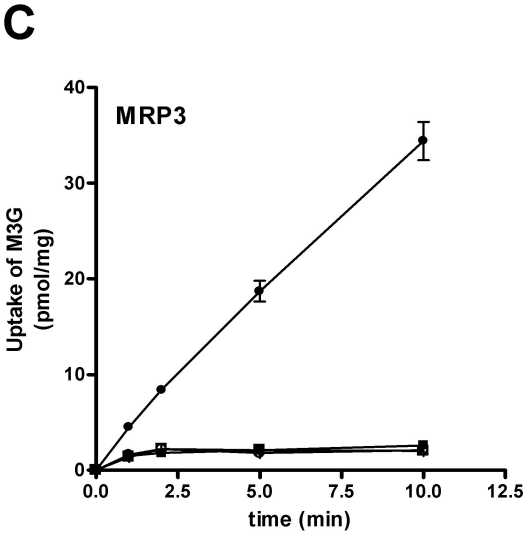
Note: the results shown for the *Mrp3*$^{(-/-)}$ mice are taken from (Zelcer et al., 2005) and have been added to be able to directly compare morphine pharmacokinetics in *Mrp2Mrp3*$^{(-/-)(-/-)}$ and *Mrp3*$^{(-/-)}$ mice.

Fig. 5. Intrahepatic M3G concentrations in WT, *Mrp2*$^{(-/-)}$ and *Mrp2Mrp3*$^{(-/-)(-/-)}$ mice after morphine administration (15mg/kg i.p.). Groups of mice (n=3/time point) received a dose of 15 mg morphine/kg body weight i.p. and intrahepatic concentrations of M3G were determined at the indicated time points. Values shown are means ± SD.

Figure 1







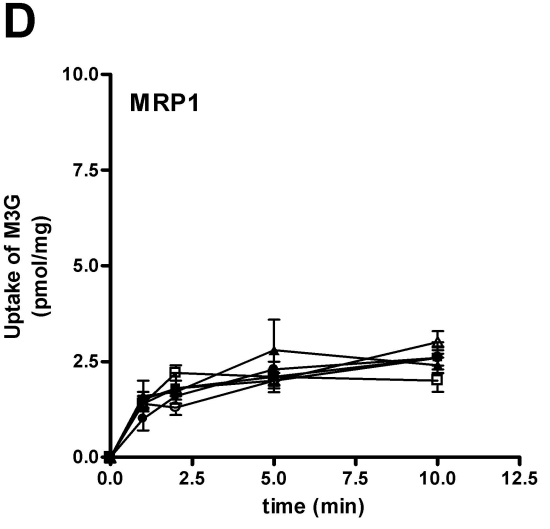


Figure 2

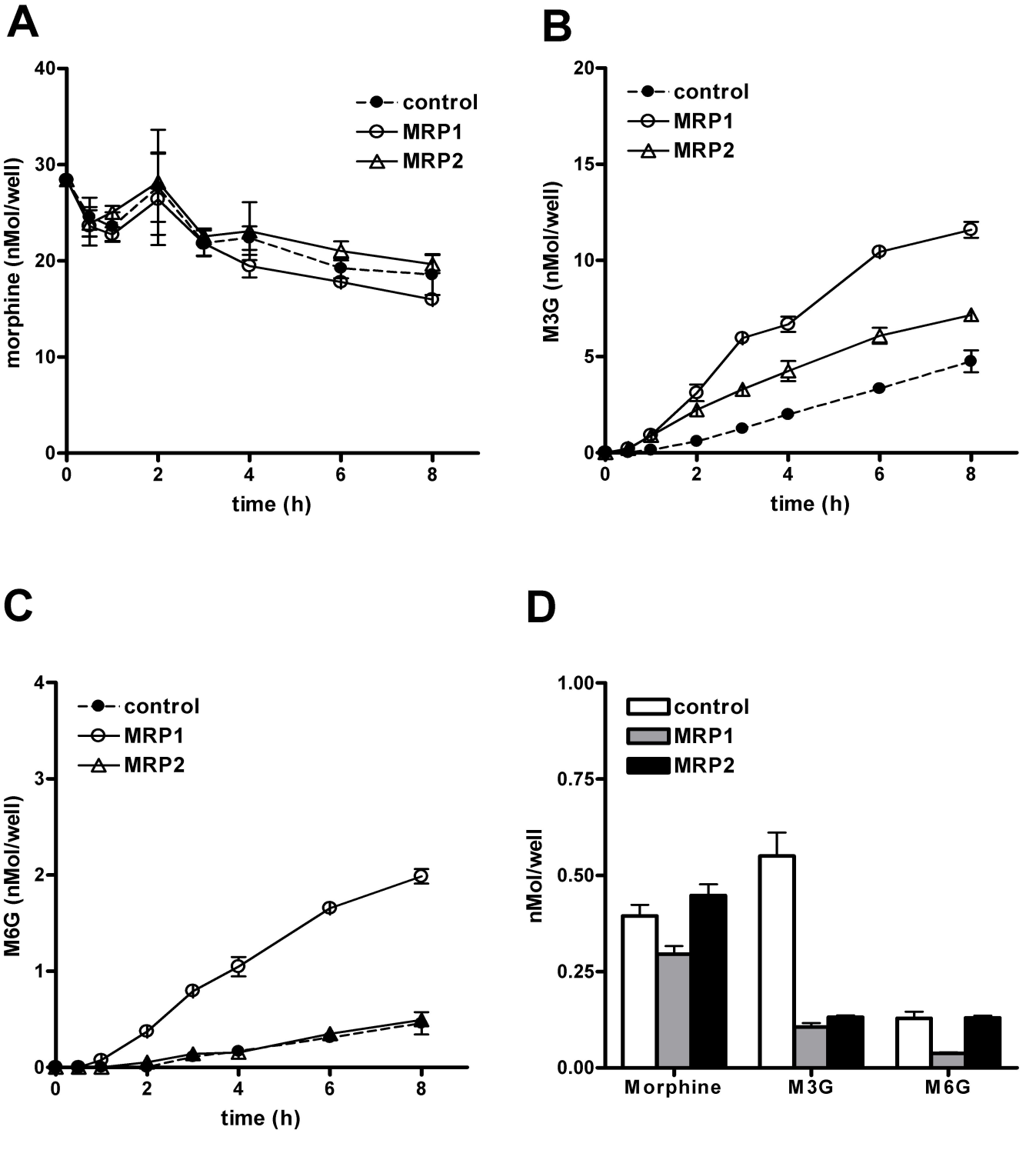

A
morphine (nMol/well)
time (h)
control
MRP1
MRP2
B
M3G (nMol/well)
time (h)
control
MRP1
MRP2
C
M6G (nMol/well)
time (h)
control
MRP1
MRP2
D
nMol/well
control
MRP1
MRP2
Morphine
M3G
M6G

Figure 3

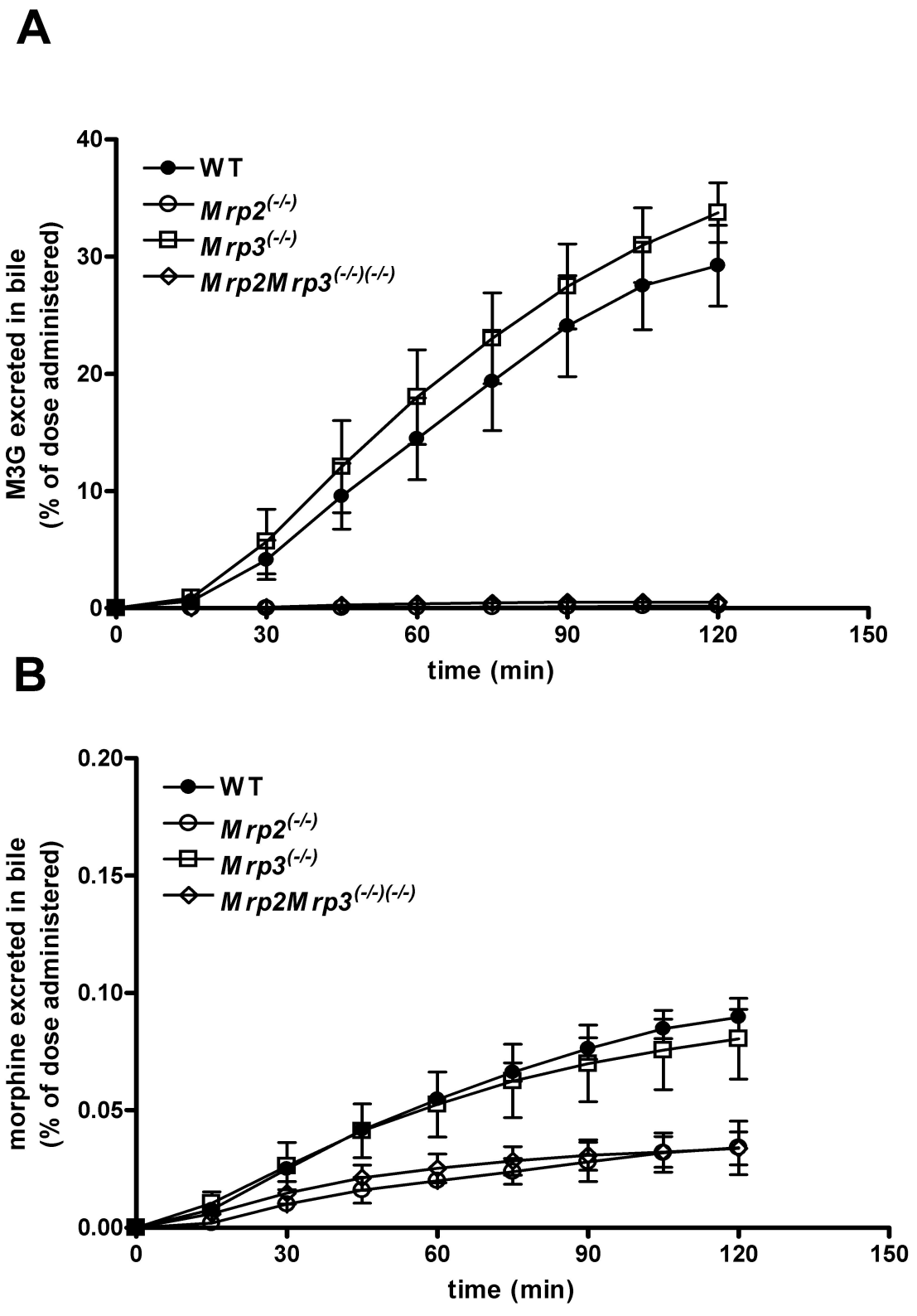

A
WT
Mrp2(-/-)
Mrp3(-/-)
Mrp2Mrp3(-/-)(-/-)
M3G excreted in bile
(% of dose administered)
40
30
20
10
0
0
30
60
90
120
150
time (min)
B
WT
Mrp2(-/-)
Mrp3(-/-)
Mrp2Mrp3(-/-)(-/-)
morphine excreted in bile
(% of dose administered)
0.20
0.15
0.10
0.05
0.00
0
30
60
90
120
150
time (min)

Figure 4

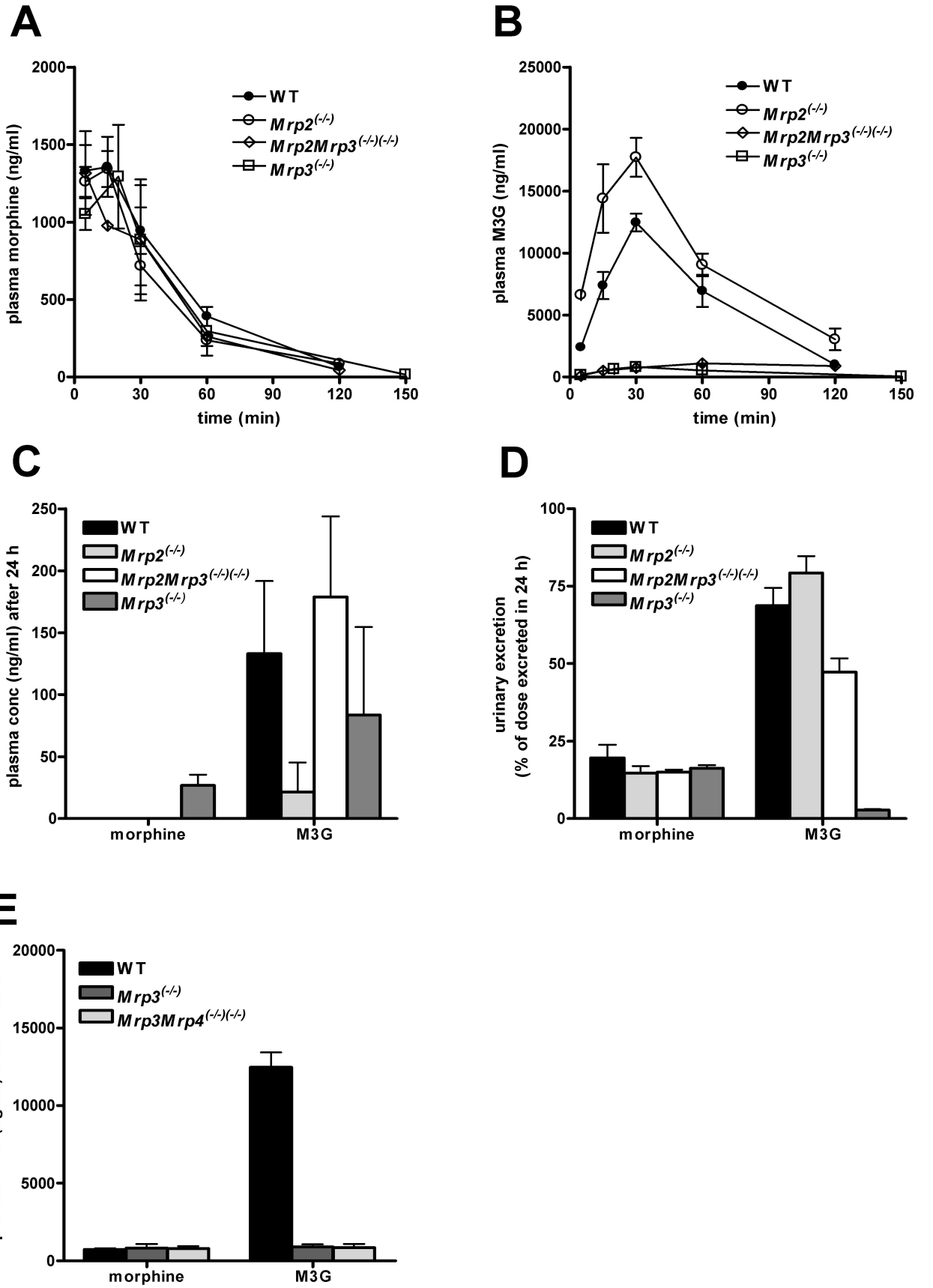
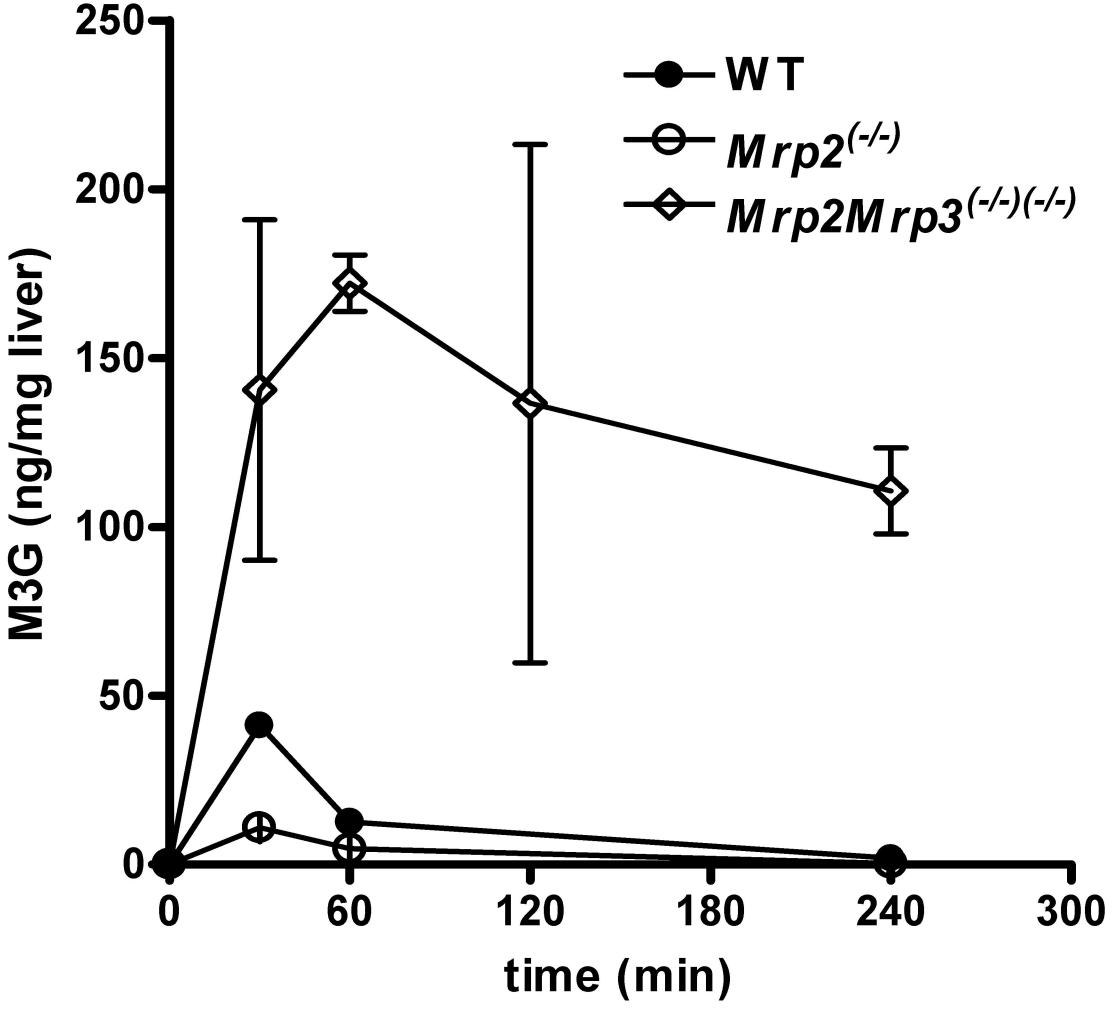

A
plasma morphine (ng/ml)
time (min)
WT
Mrp2(-/-)
Mrp2Mrp3(-/-)(-/-)
Mrp3(-/-)
B
plasma M3G (ng/ml)
time (min)
C
plasma conc (ng/ml) after 24 h
morphine
M3G
D
urinary excretion (% of dose excreted in 24 h)
E
plasma conc (ng/ml) after 30 min
Mrp3Mrp4(-/-)(-/-)

Figure 5


250
200
150
100
50
0
M3G (ng/mg liver)
0
60
120
180
240
300
time (min)
WT
Mrp2(-/-)
Mrp2Mrp3(-/-)(-/-)