

Multidrug Resistance Proteins 2 and 3 Provide Alternative Routes for Hepatic Excretion of Morphine-Glucuronides

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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.

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 (O'Brien et al., 1996; Milne et al., 1997).

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 to -4 are expressed in the

liver and are believed 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 König, 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, 2005; Kruh and Belinsky, 2003). Experiments with rats that naturally lack Mrp2 (TR⁻/EHBR) (Jansen et al., 1993; Morikawa et al., 2000; Xiong 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). Likewise, loss of Mrp3 impairs sinusoidal secretion of organic anions (e.g., morphine-glucuronides) (Mantou et al., 2005; Zelcer et al., 2005, 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 and in the periphery (Inturrisi, 2002). Morphine is extensively metabolized by glucuronidation, and membrane transport systems have been shown to modulate its pharmacokinetics (Schinkel et al., 1994; Thompson et al., 2000; King

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ABBREVIATIONS: UGT, UDP-glucuronosyl transferase; MRP, multidrug resistance protein; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; Sf9, *Spodoptera frugiperda* insect cells; HEK, human embryonic kidney; EHC, enterohepatic circulation; GSH, glutathione; WT, wild type; UGT2B7, UDP-glucuronosyl transferase 2B7.

et al., 2001; Bourasset et al., 2003; 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 even is believed 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 shown previously 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 intrahepatically formed morphine-glucuronides is also excreted over the canalicular membrane into bile by a yet-unidentified transporter(s). In this article, we further explore the contribution of members of the MRP family to the disposal of morphine-glucuronides *in vitro* and *in vivo*.

Materials and Methods

Materials. Morphine was from the pharmacy of The Netherlands Cancer Institute (Amsterdam, the Netherlands). [^3H]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. (2003) and 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 h, 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 antibodies MRPr1 (Scheffer et al., 2000) and M₂III-5 (Scheffer et al., 2000), respectively, as described previously (data not shown). Using the same antibodies, plasma membrane localization was confirmed by immunofluorescence (data not shown). Efflux experiments were performed as described in Zelcer et al. (2005).

Preparation of ^3H -Labeled M3G. [^3H]M3G was prepared by incubation of 9 μCi of [^3H]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 [^3H]M3G preparation was checked by high-pressure liquid chromatography (data not shown).

Preparation of Membrane Vesicles and Vesicular Transport Assays. ATP-dependent transport of [^3H]M3G into inside-out membrane vesicles prepared from Sf9 cells overproducing human MRP1 to -4 and control Sf9 cells was measured using the rapid filtration technique as described previously (Zelcer et al., 2003) with

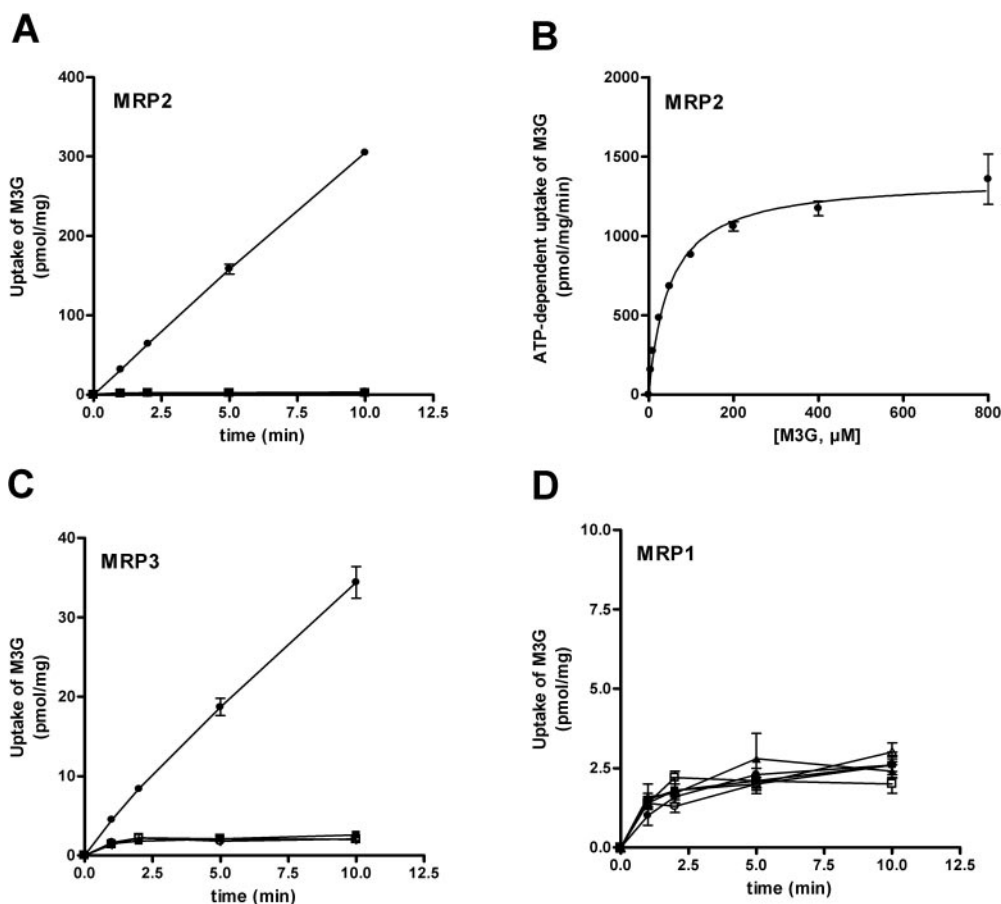


Fig. 1. Transport of M3G by human MRP1–3 in vesicular uptake experiments. A, C, and D, time course experiments of membrane vesicles from Sf9 cells infected with human MRP1 to -3 containing baculoviruses (●, ○) or WT baculovirus (■, □) incubated with 1 μM [^3H]M3G. For human MRP1 (D), transport was also determined in the presence of 3 mM GSH (▲, △). Uptake was determined in the presence (●, ■, ▲) or absence (○, □, △) of 4 mM ATP. (note that because of 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 \pm S.D. of an experiment performed in triplicate.

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 four times with 200 μ l of ice-cold phosphate-buffered saline, 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 previously. *Mrp2*^{-/-}*Mrp3*^{-/-} and *Mrp3*^{-/-}*Mrp4*^{-/-} 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-h light/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 previously (Zelcer et al., 2005). For the determination of the biliary excretion of morphine and M3G, the gallbladder of four to five mice of each genotype was cannulated as described previously (Jonker et al., 2001), and morphine (15 mg/kg body weight) was injected subcutaneously. Subsequently, bile was collected in 15-min fractions for 2 h 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. (2005). Bile was diluted in running buffer and injected directly into the liquid chromatography-tandem mass spectrometry apparatus. Liver tissue

was homogenized in 5 ml of human plasma using a Polytron tissue homogenizer (Kinematica AG, Littau-Lucerne, Switzerland), and a sample of 50 μ l of the homogenate was treated like the plasma samples.

Results

Transport of M3G into MRP1 to -4-Containing Vesicles. To study the transport of M3G by MRPs, we used vesicular transport assays with membrane vesicles generated from Sf9 cells infected with baculoviruses encoding hMRP1 to -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 wild-type (WT) baculovirus (Fig. 1A). hMRP2 transported M3G with apparent K_m and V_{max} values of 50 ± 5 μ M and 1400 ± 30 pmol/mg/min, respectively (Fig. 1B). 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 hMRP4 (data not shown). M3G also does not act as a competitive inhibitor of transport by hMRP1 or hMRP4, because we did not detect

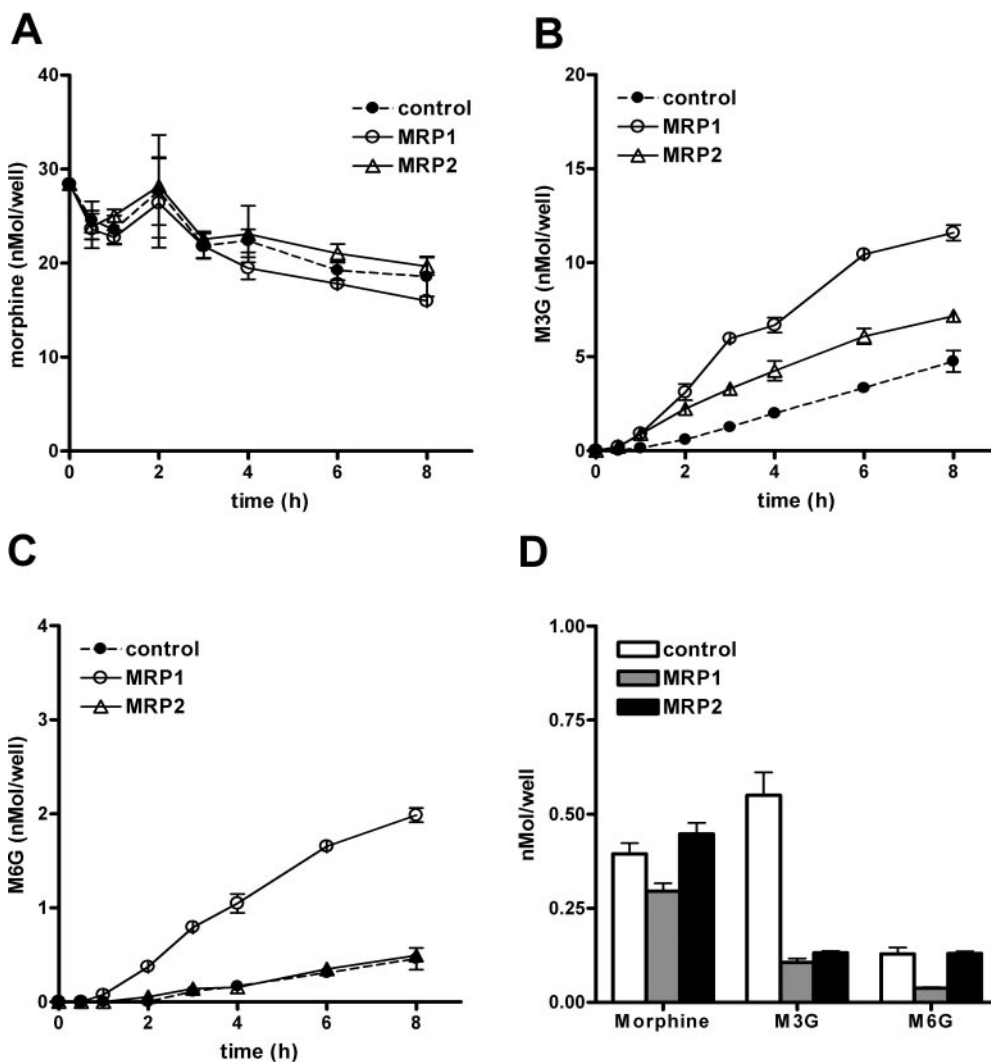


Fig. 2. Cellular efflux of M3G and M6G by HEK293 cells coexpressing UGT2B7 and human MRP1 or human MRP2. A, amount of morphine in the cell culture medium as a function of incubation time. B and C, cumulative amounts of M3G (B) and M6G (C) secreted in the cell culture medium (note that during the first 30 min to 1 h of the experiments, M6G concentrations were lower than the detection limit of the assay (C)). D, amounts of morphine, M3G, and M6G in the cell pellet at the end of the experiment. Values shown are mean \pm S.D. of an experiment performed in triplicate.

any effect of 400 μM M3G on the transport of 1 μM [^3H]E $_2$ 17 β G by these MRPs (data not shown), independent of the presence of GSH.

Efflux of M3G and M6G from HEK293 Cells Coexpressing UGT2B7 and hMRP1 or hMRP2. We have shown previously that transient coexpression 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 discrepancy below. It is interesting that 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. 2, B and C) could be caused by the substantial endogenous levels of hMRP1 present in HEK293 cells, because efflux in the control cells could be completely blocked by MK571 (Zelcer et al., 2005; data 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 (data 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 were lowered 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 Fig. 2, B 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 and 110% (data not shown).

Pharmacokinetics of Morphine in *Mrp2*^(-/-) Mice. To assess the contribution of canalicular *Mrp2* to the transport of M3G in vivo, we performed bile duct cannulation

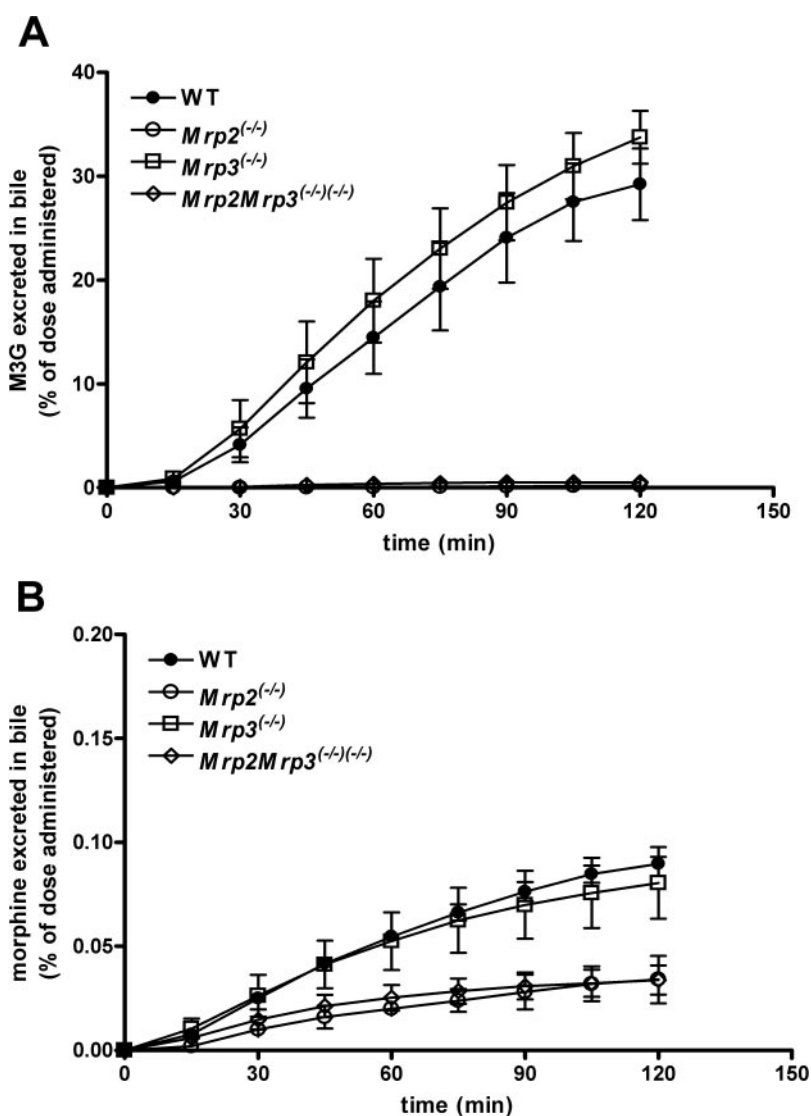


Fig. 3. Biliary excretion of morphine and M3G after the administration of morphine (15 mg/kg s.c.) in WT, *Mrp2*^(-/-), *Mrp3*^(-/-), and *Mrp2*^(-/-)*Mrp3*^(-/-) mice. After cannulation of the common bile duct, groups of mice ($n = 4$ or 5 per time point) received a dose of 15 mg of morphine per kilogram of body weight subcutaneously, and the cumulative excretion of M3G (A) and morphine (B) was determined at the indicated time points after injection. Values are expressed as a percentage of the given dose and shown as means \pm S.D.

experiments. Because we have shown previously 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*^(-/-), *Mrp3*^(-/-), and *Mrp2*^(-/-)*Mrp3*^(-/-) mice (Fig. 3). WT mice have high levels of M3G in their bile after the subcutaneous 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 *Mrp2*^(-/-)*Mrp3*^(-/-) mice. Although small

changes in the expression of transporters able to handle morphine (e.g., Mdr1a/b) could account for this increase, we believe 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 i.p. administration of morphine (15 mg/kg) in WT, *Mrp2*^(-/-), *Mrp3*^(-/-), *Mrp3*^(-/-)*Mrp4*^(-/-), and *Mrp2*^(-/-)*Mrp3*^(-/-) 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 mem-

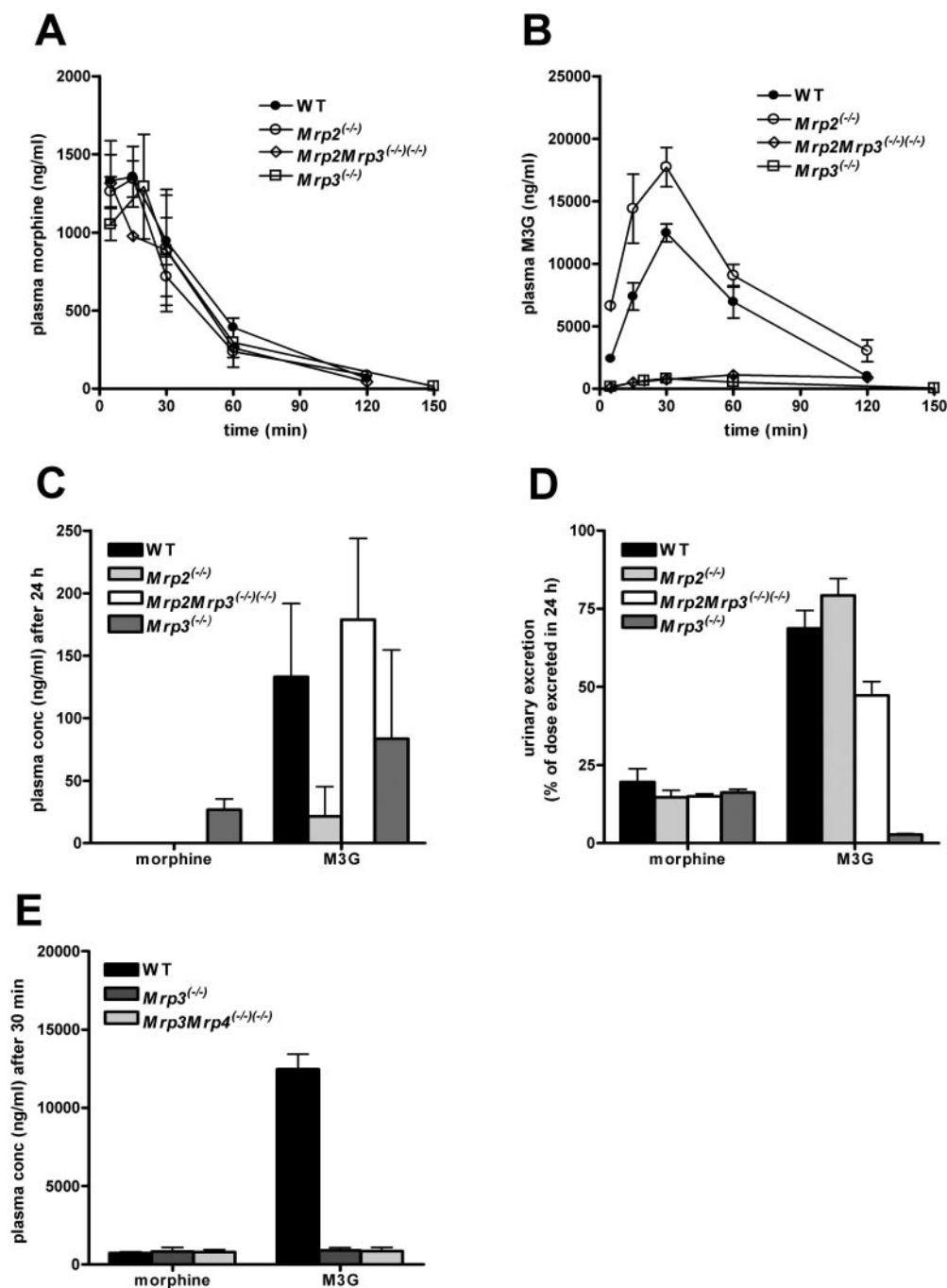


Fig. 4. Pharmacokinetics of morphine in WT, *Mrp2*^(-/-), *Mrp3*^(-/-), *Mrp3*^(-/-)*Mrp4*^(-/-), and *Mrp2*^(-/-)*Mrp3*^(-/-) mice. Groups of mice ($n = 3$ or 4 per time point) received a dose of 15 mg of morphine per kilogram of body weight i.p. Plasma concentrations of morphine (A) and M3G (B) were determined at the indicated time points. C, plasma concentrations of M3G 24 h after administration of morphine. N.D., nondetectable. D, urinary excretion of morphine and M3G over 24 h, expressed as a percentage of the given dose. E, plasma concentrations of morphine and M3G 30 min after administration of morphine in WT, *Mrp3*^(-/-), and *Mrp3*^(-/-)*Mrp4*^(-/-) mice. Values shown are means \pm S.D. Note that 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 *Mrp2*^(-/-)*Mrp3*^(-/-) and *Mrp3*^(-/-) mice.

brane. In contrast, Fig. 4C shows that 24 h after the administration of morphine, plasma levels of M3G were lower in *Mrp2*^(-/-) than in WT mice, presumably because of reduced enterohepatic circulation (EHC) of morphine/M3G in the absence of Mrp2. We have found previously that *Mrp3*^(-/-) mice have impaired transport of M3G over the sinusoidal membrane (Zelcer et al., 2005) relative to WT animals. *Mrp2*^(-/-)*Mrp3*^(-/-) 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 *Mrp2*^(-/-)*Mrp3*^(-/-) mice for at least 4 h after the administration of morphine in contrast to the results with the *Mrp3*^(-/-) mice (Fig. 4B; the 4-h time point is not shown). To study whether the residual basolateral transport of M3G in the *Mrp3*^(-/-) and *Mrp2*^(-/-)*Mrp3*^(-/-) mice could be attributed to Mrp4, we also determined morphine and M3G levels in plasma of *Mrp3*^(-/-)*Mrp4*^(-/-) 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 (data 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 *Mrp3*^(-/-) mice, which have low urinary excretion of M3G (Fig. 4D; Zelcer et al., 2005). It is interesting that we detected substantial urinary M3G excretion in *Mrp2*^(-/-)*Mrp3*^(-/-) 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. 4, B and C) eventually results in near normal urinary excretion of M3G (Fig. 4D).

Livers were also analyzed for M3G content. Thirty minutes after the administration of morphine, M3G levels in livers of *Mrp2*^(-/-)*Mrp3*^(-/-) 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 excre-

tion 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 under the *Discussion*.

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, because endogenous and exogenous compounds can modulate MRP2 activity (Bodo et al., 2003; Zelcer 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 due, at least in part, 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 coexpressing 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 [³H]M3G as substrate or indirectly using M3G as competitor in transport experiments with [³H]E₂17βG as substrate. This negative result is not caused by the use of Sf9 insect cells to prepare the MRP1-containing vesicles, because membrane vesicles prepared from MRP1-expressing HEK293 cells did not transport [³H]M3G either (data not shown). The addition of GSH, sometimes required as cosubstrate 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 cosubstrate 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 (Chu et al., 2006; Vlaming 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 EHC of its substrates (Borst and Oude Elferink, 2002). The lower M3G plasma levels in the *Mrp2*^(-/-) mice 24 h after morphine administration are therefore probably caused by reduced EHC of morphine/M3G in these mice. Twenty-four hours

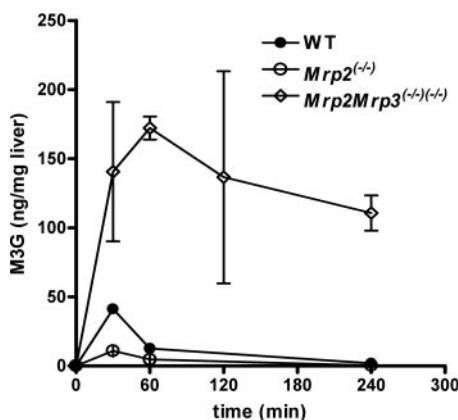


Fig. 5. Intrahepatic M3G concentrations in WT, *Mrp2*^(-/-), and *Mrp2*^(-/-)*Mrp3*^(-/-) mice after morphine administration (15 mg/kg i.p.). Groups of mice (*n* = 3 per time point) received a dose of 15 mg of morphine per kilogram of body weight i.p. and intrahepatic concentrations of M3G were determined at the indicated time points. Values shown are means ± S.D.

after morphine administration, $Mrp2^{-/-}Mrp3^{-/-}$ 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 $\mu\text{L}/\text{min}$ (Qi et al., 2005). The average M3G plasma concentration in the $Mrp2^{-/-}Mrp3^{-/-}$ mice in the first 4 h after administration of morphine is approximately 1000 ng/mL. This means that the $Mrp2^{-/-}Mrp3^{-/-}$ mice excrete approximately 250 ng of M3G per minute (equal to 15 $\mu\text{g}/\text{h}$). The average urinary excretion of M3G in $Mrp2^{-/-}Mrp3^{-/-}$ mice is 212 ± 18 . This means that M3G concentration needs to remain on average 1000 ng/mL for 14 h ($212 \mu\text{g}/15 \mu\text{g}/\text{h}$) to account for the urinary excretion of 212 $\mu\text{g}/24$ h that we find in the $Mrp2^{-/-}Mrp3^{-/-}$ 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 up-regulation of Mrp3 in livers of $Mrp2^{-/-}$ mice (Vlaming et al., 2006) might have contributed to this decrease, the M3G levels in the intrahepatic bile are a much more important factor in our opinion. In the case of $Mrp2^{-/-}$ mice, the intrahepatic 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. It is apparent that 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. Sixty minutes after the administration of morphine, $Mrp2^{-/-}Mrp3^{-/-}$ mice have 36-fold higher M3G levels in their livers than $Mrp2^{-/-}$ mice. This seems not to result in end-product inhibition of the murine UGT involved in the glucuronidation of morphine, because 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 and alanine aminotransferase (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. Because 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 $Mrp2^{-/-}Mrp3^{-/-}$ 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 glucuronosyl-conjugates (Kruh and Belinsky, 2003). However, HEK293-UGT2B7 cells transiently trans-

ected with hMRP4 do not efflux more M3G or M6G (data not shown). Moreover, 30 min after the administration of morphine, $Mrp3^{-/-}Mrp4^{-/-}$ mice have M3G plasma levels similar to those of $Mrp3^{-/-}$ 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 coexpressing 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 (Aleksunes et al., 2006; Roelofs et al., 1997), 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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