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P-gp, Mrp2, Cyp3a, and carboxylesterase affect the oral availability and metabolism of vinorelbine

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Abbreviations:

ABCC, ATP-binding cassette transporter family C; BNPP, bis(4-nitrophenyl) phosphate; CYP3A, cytochrome P450 3A; MDCK, Madin-Darby canine kidney; MRP, multidrug-resistance associated protein; HPLC, high performance liquid chromatography; P-gp, P-glycoprotein; PK, pharmacokinetics; AUC, under the plasma concentration-time curve.

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ABSTRACT

We investigated the interactions of the anticancer drug vinorelbine with drug-efflux transporters and Cytochrome P450 3A (CYP3A) drug-metabolizing enzymes. Vinorelbine was transported by human MRP2, and Mrp2 knockout mice displayed increased vinorelbine plasma exposure after oral administration, suggesting that Mrp2 limits the intestinal uptake of vinorelbine. Using P-gp-, Cyp3a-, and P-gp/Cyp3a knockout mice, we found that absence of P-gp or Cyp3a resulted in increased vinorelbine plasma exposure, both after oral and i.v. administration. Surprisingly, P-gp/Cyp3a knockout mice displayed markedly lower vinorelbine plasma concentrations than wild-type mice upon i.v. administration, but higher concentrations upon oral administration. This could be explained by highly increased formation of 4'*O*-deacetylvinorelbine, an active vinorelbine metabolite, especially in P-gp/Cyp3a knockout plasma. Using wild-type and Cyp3a knockout liver microsomes, we found that 4'*O*-deacetylvinorelbine formation was 4-fold increased in Cyp3a knockout liver, and not mediated by Cyp3a or other P450 enzymes. *In vitro* incubation of vinorelbine with plasma revealed that vinorelbine deacetylation in Cyp3a- and especially P-gp/Cyp3a knockout mice, but not P-gp-deficient mice, was strongly upregulated. Metabolite formation in microsomes and plasma could be completely inhibited with the non-specific carboxylesterase inhibitor bis(4-nitrophenyl) phosphate and partly with the CES2-specific inhibitor loperamide, indicating that carboxylesterase Ces2a, which was appropriately upregulated in Cyp3a- and especially P-gp/Cyp3a knockout liver, was responsible for the 4'*O*-deacetylvinorelbine formation. Such compensatory upregulation can complicate the interpretation of knockout mouse data. Nonetheless, P-gp, Mrp2, Cyp3a, and Ces2a clearly restricted vinorelbine availability in mice. Variation in activity of their human homologues may affect vinorelbine pharmacokinetics in patients as well.

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INTRODUCTION

Vinorelbine is a semi-synthetic *Vinca* alkaloid that is indicated in the treatment of non-small cell lung cancer and advanced breast cancer (Domenech and Vogel, 2001; Gralla et al., 2007). In current clinical practice, vinorelbine is mainly intravenously applied despite the development of an oral vinorelbine formulation, which has been approved in several European countries. This may be explained by the relatively high inter-individual variability in drug exposure that was observed for oral vinorelbine administration in numerous clinical studies (Delord et al., 2009; Hirsh et al., 2007; Gralla et al., 2007; Rossi et al., 2007). For clinical practice, it is thus important to identify interactions of vinorelbine with drug efflux transporters and drug metabolizing enzymes because these systems might affect the exposure and toxicity of vinorelbine, especially upon oral administration. Moreover, drug efflux transporters may also directly contribute to resistance of tumors against vinorelbine.

It has been well established by various groups that the drug efflux transporter P-glycoprotein (MDR1/ABCB1) transports vinorelbine *in vitro* and mediates resistance against vinorelbine (Adams and Knick, 1995; Shepard et al., 2003; Takara et al., 2002). However, data about the *in vivo* interaction of vinorelbine with P-gp are inconclusive. For instance, Press et al. (2006) found no significant changes in oral or i.v. vinorelbine area under the plasma concentration-time curves (AUCs) between wild-type (WT) or P-gp-deficient (Mdr1a/1b$^{-/-}$) mice, although interindividual variation in these experiments was very high, potentially obscuring modest shifts. Furthermore, in a phase I study with 26 patients with advanced solid tumors the P-gp inhibitor tariquidar was combined with intravenously applied vinorelbine (20 mg/m^2), but alterations in the pharmacokinetics of vinorelbine were not observed (Abraham *et al.*, 2009). On the other hand, Wong et al (2006) found in a cohort of 41 patients receiving i.v. vinorelbine that low hepatic [^{99m}Tc]-MIBI clearance (used as a

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measure for P-gp-mediated clearance) significantly correlated with low vinorelbine clearance. Additionally, a phase I trial with 19 patients with advanced solid tumors revealed increased exposure and decreased clearance when intravenously vinorelbine (22.5 mg/m^2) was combined with the potent P-gp inhibitor zosuquidar (Le *et al.*, 2005). A more extensive analysis of *in vivo* vinorelbine-P-gp interactions thus seemed worthwhile.

Thus far, interactions of vinorelbine with the drug efflux transporter MRP2 (ABCC2) have not been reported. However, cellular resistance to the *Vinca* alkaloid vincristine has been associated with MRP2 expression (Kawabe et al., 1999; Chen et al., 1999) and *in vitro* transport of vinblastine by MRP2 has also been demonstrated (Evers et al., 1998; Evers et al., 2000a; Huisman et al., 2005). It thus seemed possible that vinorelbine might also be transported by MRP2, which could affect vinorelbine pharmacokinetics, toxicity and resistance of tumors.

Vinorelbine is thought to be mainly metabolized by CYP3A4 to inactive metabolites (Kajita et al., 2000; Beulz-Riche et al., 2005). However, to some extent vinorelbine is also converted to a pharmacodynamically active metabolite: 4'*O*-deacetylvinorelbine. In fact, metabolic profiling after oral vinorelbine administration revealed that 4'*O*-deacetylvinorelbine is the predominant metabolite in human blood with an elimination half-life of at least 100 hr (Marty *et al.*, 2001). This deacetylation reaction is believed to be catalyzed by carboxylesterase enzymes, which are formed in the liver and reside in blood, although specific enzyme(s) responsible for this activity have not been identified yet (Delord et al., 2009; Beulz-Riche et al., 2005).

The aim of this study was to obtain better insight into the potential impact of P-gp, MRP2, and CYP3A on the pharmacokinetics of vinorelbine, utilizing *in vitro, ex vivo,* or *in vivo* assays and P-gp-, Mrp2-, and Cyp3a-deficient mouse strains.

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MATERIALS AND METHODS

Chemicals. Vinorelbine 10 mg/ml concentrate for solution for infusion (Navelbine, 10 mg/ml) originated from Pierre Fabre Medicament, Boulogne, France. Vinorelbine tartrate was purchased from Sequoia Research Products (Pangbourne, UK). Deacetylvinblastine sulphate was obtained from the faculty of Pharmacy (University of Utrecht, The Netherlands). Loperamide hydrochloride and bis(4-nitrophenyl) phosphate (BNPP) were from Sigma-Aldrich (Steinheim, Germany). Methoxyflurane (Metofane®) was from Medical Developments Australia (Springvale, Victoria, Australia). Heparin (5000 IE/ml) was from Leo Pharma BV (Breda, the Netherlands). The organic solvents methanol, acetonitrile (both HPLC grade), and diethyl ether were from Merck (Darmstadt, Germany). Drug-free human plasma was from healthy volunteers. GlaxoSmithKline (Uxbridge, UK) kindly provided elacridar (GF120918). All other chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Transport assays. Polarized canine kidney MDCK-II cell lines were used in transport assays. MDCK-II cells transduced with human MRP2 were described previously (Evers *et al.*, 1998). Transepithelial transport assays using Transwell plates were carried out as described previously with minor modifications (Schinkel *et al.*, 1995). Experiments were performed in the presence of 5 μM of elacridar, to inhibit any endogenous P-glycoprotein activity. Elacridar at 5 μM does not affect MRP2 activity (Evers *et al.*, 2000b). Elacridar was present in both compartments during 2 hr preincubation and during the transport experiment. After preincubation, experiments were started ($t = 0$) by replacing the medium in either the apical or basolateral compartment with fresh OptiMEM medium, either with or without 5 μM Elacridar and containing 5 μM of vinorelbine. Cells were incubated at 37°C in 5% CO_2, and 50 μl aliquots were taken at t = 2 and 4 h. The samples were diluted with 450 μl drug-free human plasma, 50 μl of internal standard (100 ng/ml deacetylvinblastine) was added and

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the samples were extracted with 3 ml diethyl ether. Vinorelbine was quantified by HPLC, as previously described (van Tellingen *et al.*, 1992). Transport was calculated as the fraction of drug found in the acceptor compartment relative to the total amount added to the donor compartment at t = 0. Transport is given as mean percentage ± SD (n = 3). Membrane tightness was assessed in parallel, using the same cells seeded on the same day and at the same density, by analyzing transepithelial [^{14}C]Inulin (approx. 3 kBq /well) leakage. Leakage had to remain <2% of the total added radioactivity per 2 hour. Active transport across MDCK-II monolayers was expressed by the relative transport ratio (r), defined as: r = percentage apically directed translocation divided by percentage basolaterally directed translocation, after 4 hr (Huisman et al., 2005). The apparent permeability coefficient (P_{app}) was calculated using the equation: P_{app} (cm/s) = dC/dt * 1/A * V/C_o [cm/s], where dC/dt ($\mu M.sec^{-1}$) represents the flux across the monolayer (permeability rate), A (cm^2) the surface area of the monolayer, V (cm^3) the volume of the receiver chamber and C_o (µM) the initial concentration in the donor compartment (Irvine *et al.*, 1999).

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were female *Mdr1a/1b*$^{-/-}$ (Schinkel *et al.*, 1997), *Mrp2*$^{-/-}$ (Vlaming *et al.*, 2006), *Cyp3a*$^{-/-}$ (van Herwaarden *et al.*, 2007), *Mdr1a/1b/Cyp3a*$^{-/-}$ (van Waterschoot *et al.*, 2009) and wild-type (WT) mice, all of a >99% FVB genetic background, between 9 and 12 weeks of age. Animals were kept in a temperature-controlled environment with a 12-hour light / 12-hour dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum.*

Plasma pharmacokinetics. For oral studies, Navelbine (10 mg/ml) was 10-fold diluted with saline and dosed at 10 mg/kg bodyweight (10 ml/kg). To minimize variation in absorption, mice were fasted for 3 hours, before vinorelbine was administered by gavage into the stomach, using a blunt-

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ended needle. Multiple blood samples (~40 µl) were collected from the tail vein at 15 and 30 min and 1, 2, 4, 8 and 24 h, using heparinized capillary tubes (Oxford Labware, St. Louis, USA). Blood samples were centrifuged at 2,100 $\times g$ for 6 min at 4°C, and the plasma was completed to 200 µl with drug-free human plasma and stored at -20°C until analysis. For intravenous studies, Navelbine (10 mg/ml) was diluted 5-fold with saline and injected as single bolus at 10 mg/kg (5 ml/kg) into the tail vein. Blood samples were collected by cardiac puncture under methoxyflurane anesthesia at 7.5 and 30 min and 1, 2, 4, 8 and 24 hr, with 4 animals per time-point. Samples were processed as describe above and stored at -20°C until analysis.

Drug analysis in plasma. Plasma concentrations of vinorelbine and 4'*O*-deacetylvinorelbine were determined by LC-MS/MS as previously described (Damen *et al.*, 2009).

Pharmacokinetic calculations and statistical analysis. The AUC was calculated using the trapezoidal rule. The peak plasma concentration (C_{max}) and the time of maximum plasma concentration (T_{max}) were estimated from the original data. Plasma clearance (CL) after i.v. administration was calculated by the formula CL = Dose/AUC. The oral bioavailability (*F*) was calculated by the formula $F = AUC_{oral}/AUC_{i.v.} \times 100\%$. To assess statistical significance, the two-sided unpaired Student's *t*-test (two groups) or one-way ANOVA followed by Dunnett's multiple comparison test (> 2 groups) was performed. Data obtained with single- and combination knockout mice were compared to data obtained with WT mice, unless stated otherwise. Data are presented as means ± SD and differences were considered statistically significant when $P < 0.05$.

Microsomal incubations. Microsomes from the livers of WT and *Cyp3a*$^{-/-}$ mice were prepared as described previously (Emoto *et al.*, 2000). Incubations were carried out in triplicate at 37°C in a total volume of 200 µl, containing 100 mM potassium phosphate buffer (pH 7.4), NADPH Regenerating System (BD Biosciences) and 0.5 mg protein/ml liver microsomes. Reactions were started by adding

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vinorelbine (18.5 μM), allowed to proceed for 20 minutes and terminated by adding 100 μl of ice-cold acetonitrile. Control incubations omitting cofactor NADPH were performed to establish whether the metabolism was CYP-dependent. For inhibition experiments, the non-specific CES inhibitor BNPP (100 μM), the CES2-specific inhibitor loperamide (100 μM) or vehicle were preincubated for 5 minutes at 37°C, and reactions were started by adding vinorelbine (18.5 μM). Incubation were carried out without NADPH, allowed to proceed for 30 minutes and terminated by adding 100 μl of ice-cold acetonitrile.

Concentrations of vinorelbine and 4'*O*-deacetylvinorelbine in supernatant (6,800 × *g* for 10 minutes) were determined by LC-MS/MS (Damen *et al.*, 2009).

Plasma incubations. Blood was collected from WT, *Mdr1a/1b*$^{-/-}$ (Schinkel *et al.*, 1997), *Cyp3a*$^{-/-}$ (van Herwaarden *et al.*, 2007) and *Mdr1a/1b/Cyp3a*$^{-/-}$ (van Waterschoot *et al.*, 2009) mice (n = 4 per strain) by cardiac puncture under methoxyflurane anesthesia. Blood samples were immediately centrifuged at 2,100 × *g* for 6 min at ambient temperature, and plasma was collected. Plasma samples (390 μl) were warmed to 37°C and incubations were started by adding 10 μl of vinorelbine stock solution (260 μM) to obtain a final concentration of 6.5 μM. Samples of 25 μl were collected at 0, 15, 30, 60 and 120 minutes. For inhibition experiments, the non-specific CES inhibitor BNPP (100 μM), the CES2-specific inhibitor loperamide (100 μM) or vehicle were preincubated for 5 minutes at 37°C. Reactions were started by adding vinorelbine (6.5 μM) and allowed to proceed for 120 minutes. Samples were immediately completed to 100 μl with ice-cold acetonitrile to stop the deacetylation reaction and subsequently stored at -20°C until analysis. Concentrations of vinorelbine and 4'*O*-deacetylvinorelbine were determined by LC-MS/MS (Damen *et al.*, 2009).

RNA isolation, cDNA synthesis and real-time RT PCR. Livers of mice between 9-11 weeks of age (n = 3 per genotype) were excised and immediately placed in an appropriate volume of RNAlater

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(QIAGEN, Venlo, The Netherlands). They were stored at 4°C until RNA was extracted using the RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. Subsequently, cDNA was generated using 5 µg of total RNA in a synthesis reaction using random hexamers (Applied Biosystems, Foster City, CA) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the supplier's protocols. The reverse transcription reaction was performed for 60 min at 42°C with a deactivation step of 15 min at 70°C. cDNA was stored at -20°C until use. Real-time RT-PCR was performed using specific primers (QIAGEN) on an Applied Biosystems 7500 real-time cycler system as previously described (van Waterschoot *et al.*, 2008). Analysis of the results was done by the comparative C_t method as described (Schmittgen *et al.*, 2008) and statistical analysis was performed on ΔCt values as previously described (Yuan *et al.*, 2006).

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RESULTS

In vitro transport of vinorelbine by MRP2.

To determine whether vinorelbine (Fig. 1A) is transported by MRP2, we used polarized MDCK-II cells transduced with human MRP2 cDNA. The MDCK-II-Neo cell line was used as a control, because it contains very little endogenous canine Mrp2 (Evers *et al.*, 1998). Since vinorelbine is a well-established P-gp substrate, 5 μM elacridar was added to inhibit any transport by endogenous canine P-gp. In MDCK-II-Neo cells no active polarized transport of vinorelbine was observed, as evident from roughly equal basolateral-to-apical and apical-to-basolateral translocation rates (Fig. 1B). In contrast, in MRP2-transduced cells apically directed translocation was markedly increased and basolaterally directed translocation markedly decreased (Fig. 1C). The transport ratio *r*, which is a measure for active transport, was 3.5-fold increased (1.3 for Neo versus 4.5 for MRP2-transduced cells; Fig. 1). Vinorelbine is thus a transported substrate of human MRP2.

Impact of Mrp2 and P-gp on the oral AUC of vinorelbine.

To test whether Mrp2 and P-gp restrict the oral uptake of vinorelbine, we orally administered 10 mg/kg vinorelbine to WT, *Mrp2*$^{-/-}$ and *Mdr1a/1b*$^{-/-}$ mice. Oral absorption in all strains was very rapid and maximal plasma concentrations were reached before 15 min, the first sampling time point (Fig. 2). The oral $AUC_{0\text{-}24}$ in *Mrp2*$^{-/-}$ mice was 1.5-fold higher than in WT mice ($P < 0.05$; Fig. 2; Table 1) and the C_{max} (earliest measured time point) was 3.3-fold increased ($P < 0.05$). The oral $AUC_{0\text{-}24}$ in *Mdr1a/1b*$^{-/-}$ mice was 3.6-fold higher than in WT mice ($P < 0.05$; Fig. 2; Table 1) and the C_{max} (earliest measured time point) was 7.0-fold increased ($P < 0.05$). These results show that Mrp2 can modestly, and P-gp markedly restrict the oral availability of vinorelbine. The effect of the absence of these transporters was particularly strong shortly after drug administration (15 and 30 min), suggesting a direct impact on the intestinal uptake of vinorelbine.

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Impact of P-gp and Cytochrome P450 3A on vinorelbine plasma pharmacokinetics.

Because we had found that P-gp markedly affected the oral AUC of vinorelbine, and since it is known that CYP3A4 can mediate metabolism of vinorelbine (Beulz-Riche *et al.*, 2005), it was of interest to establish the separate and combined impact of these two detoxifying systems on vinorelbine pharmacokinetics. We previously showed that for docetaxel, also a shared P-gp and CYP3A substrate, simultaneous disruption of both systems caused a drastic increase in oral AUC (van Waterschoot *et al.*, 2009). We therefore compared vinorelbine oral and i.v. plasma pharmacokinetics in WT, *Mdr1a/1b*$^{-/-}$, *Cyp3a*$^{-/-}$ and combination *Mdr1a/1b/Cyp3a*$^{-/-}$ mice. In line with the independent results described above, *Mdr1a/1b*$^{-/-}$ mice had a 3.4 -fold higher oral $AUC_{0\text{-}24}$ than WT mice ($P < 0.05$; Fig. 3A; Table 2) and the C_{max}, reached within 15 min after administration, was ~10-fold higher than in WT mice ($P < 0.01$). For *Cyp3a*$^{-/-}$ mice, the oral $AUC_{0\text{-}24}$ and C_{max} were 2.2-fold and 2.5-fold higher than in WT mice, respectively ($P < 0.05$ for both parameters; Fig. 3A; Table 2). These results indicate that both P-gp and Cyp3a reduce the oral AUC of vinorelbine. While the effect of P-gp deficiency was most obvious very shortly after drug administration (15 min), for Cyp3a deficiency the strongest effect was seen only after 1 hour. Unexpectedly, oral administration of vinorelbine to *Mdr1a/1b/Cyp3a*$^{-/-}$ mice did not result in an additive effect of the combined deficiencies on vinorelbine AUC. In fact, the $AUC_{0\text{-}24}$ and the C_{max}, while still higher than in WT mice ($P < 0.05$), were not significantly different from those observed for *Mdr1a/1b*$^{-/-}$ mice (Fig. 3A and Table 2).

After i.v. administration of 10 mg/kg vinorelbine, the $AUC_{0\text{-}24}$ for *Mdr1a/1b*$^{-/-}$ and *Cyp3a*$^{-/-}$ mice were 2.3- and 1.5-fold higher than for WT mice, respectively ($P < 0.01$, Table 2). However, combined deficiencies of P-gp and Cyp3a resulted in a 1.4-fold lower (rather than higher) $AUC_{0\text{-}24}$ than in WT mice ($P < 0.05$, Fig. 3B, insert; Table 2). These results suggest that an additional

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important parameter affecting vinorelbine plasma pharmacokinetics had changed in the *Mdr1a/1b/Cyp3a*$^{-/-}$ mice.

Increased formation of 4'*O*-deacetylvinorelbine in *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice.

We have previously observed that the metabolism of midazolam, one of the most widely used probe drugs to assess CYP3A activity, was not reduced in *Cyp3a*$^{-/-}$ mice (van Waterschoot *et al.*, 2008). This apparent discrepancy could be attributed to compensatory midazolam metabolism by mouse Cyp2c enzymes, which were substantially upregulated in *Cyp3a*$^{-/-}$ mice (van Waterschoot *et al.*, 2008). We therefore hypothesized that the lower plasma concentrations of vinorelbine in *Mdr1a/1b/Cyp3a*$^{-/-}$ mice might also be explained by increased metabolic conversion of vinorelbine, obviously via (an)other metabolic route(s) than Cyp3a. Because 4'*O*-deacetylvinorelbine is a major vinorelbine metabolite in mice and humans (van Tellingen et al., 1993; Puozzo et al., 2007), we analyzed the plasma samples from the i.v. experiment for the presence of this metabolite (for the oral experiment, due to the applied multiple sampling approach, the sample size was too small to additionally measure this metabolite). In stark contrast to the parental vinorelbine, plasma concentrations of 4'*O*-deacetylvinorelbine were highly increased in *Mdr1a/1b/Cyp3a*$^{-/-}$ mice as compared to WT mice (Fig. 3C; Table 2). Notably, also single *Cyp3a*$^{-/-}$ mice displayed markedly elevated plasma concentrations of this metabolite. The i.v. $AUC_{0\text{-}24}$ for 4'*O*-deacetylvinorelbine was 3.6-fold higher in *Mdr1a/1b*$^{-/-}$ mice, 5.1-fold increased in *Cyp3a*$^{-/-}$ mice and 11.2-fold higher in *Mdr1a/1b/Cyp3a*$^{-/-}$ mice, compared to WT mice, whereas the equivalent changes in parental vinorelbine $AUC_{0\text{-}24}$ were 2.3-, 1.5-, and 0.72-fold, respectively (Table 2). Especially the *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice thus displayed a profound increase in 4'*O*-deacetylvinorelbine over vinorelbine concentration ratios. This increased vinorelbine conversion might lead to an underestimation of the effect of the knocked out proteins on vinorelbine clearance.

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To determine the additive plasma exposure to vinorelbine and 4'*O*-deacetylvinorelbine after i.v. vinorelbine, we plotted the combined plasma-concentration-time curves and calculated the combined AUC_{0-24} and C_{max} values (Fig. 3D and Table 2). This indicated that the combined AUC_{0-24} values were 2.2- to 2.8-fold increased in each of the three knockout strains compared to the WT mice, illustrating a markedly reduced elimination of vinorelbine/4'*O*-deacetylvinorelbine in each of the knockout strains. Still, the overall effect seen in the combination knockout strain was less than expected from the effects seen in the separate knockout strains. 4'*O*-deacetylvinorelbine/vinorelbine exposure ratios increased from 0.25 in WT mice to 0.39, 0.81, and 3.8 in *Mdr1a/1b*$^{-/-}$, *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice, respectively, suggesting pronounced changes mainly in the latter two strains.

4'*O*-deacetylvinorelbine formation is upregulated in *Cyp3a*$^{-/-}$ liver microsomes.

These results raised the question to what extent vinorelbine in mice is cleared by Cyp3a, perhaps Cyp2c, or by conversion to 4'*O*-deacetylvinorelbine, and/or by some other metabolic pathway. Since metabolic drug clearance after i.v. administration is often mostly mediated by the liver, we incubated vinorelbine with liver microsomes from WT and *Cyp3a*$^{-/-}$ mice. We assessed Cyp-mediated metabolism by comparing vinorelbine concentrations in the presence and absence of the cofactor NADPH, which is essential for Cyp activity. In addition, we measured 4'*O*-deacetylvinorelbine formation. Unexpectedly, we observed only little disappearance of vinorelbine in microsomes from WT mice, and absence or presence of NADPH did not affect this process, indicating that this process was not Cyp-mediated (Fig. 4A). A small amount of 4'*O*-deacetylvinorelbine was formed, and this was again not NADPH-dependent (Fig. 4B). In the *Cyp3a*$^{-/-}$ microsomes, however, we observed much more extensive disappearance of vinorelbine, correlating with a higher production of 4'*O*-deacetylvinorelbine (Fig. 4A and B). Again, these conversions were independent of the presence or

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absence of NADPH, indicating that they were not Cyp-mediated. The sum of the remaining vinorelbine and 4'*O*-deacetylvinorelbine amounts in the various incubations (Fig. 4C) yielded total drug amounts that were not substantially below the originally applied amount of vinorelbine (18.5 μM), suggesting that there was little metabolism to other compounds under these conditions.

Increased 4'*O*-deacetylvinorelbine formation in *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ plasma.

Since the abundant 4'*O*-deacetylvinorelbine formation in the *Cyp3a*$^{-/-}$ liver microsomes was not Cyp-mediated, it might be catalyzed by (partly) soluble proteins that could also be secreted into the blood. We therefore incubated vinorelbine (6.5 μM or about 5000 ng/ml, i.e. of the same order as expected shortly after i.v. administration of vinorelbine) *in vitro* with plasma freshly collected from WT, *Mdr1a/1b*$^{-/-}$, *Cyp3a*$^{-/-}$, and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice, and measured disappearance of vinorelbine and appearance of 4'*O*-deacetylvinorelbine over time. In WT and *Mdr1a/1b*$^{-/-}$ plasma there was a slow disappearance of vinorelbine, associated with a low production of 4'*O*-deacetylvinorelbine over time (Fig. 5A and B). Interestingly, however, in *Cyp3a*$^{-/-}$ plasma vinorelbine disappearance and 4'*O*-deacetylvinorelbine appearance were clearly increased, and both processes were markedly further enhanced in the *Mdr1a/1b/Cyp3a*$^{-/-}$ plasma. These results indicate that there is a substantial upregulation of enzyme(s) forming 4'*O*-deacetylvinorelbine in *Cyp3a*$^{-/-}$ and especially *Mdr1a/1b/Cyp3a*$^{-/-}$ plasma, but not in *Mdr1a/1b*$^{-/-}$ plasma. Adding up the vinorelbine plus 4'*O*-deacetylvinorelbine values in plasma for each strain (Fig. 5C) indicated that most of the drug was still detectable in these two forms after 120 min, suggesting only little metabolism to alternative vinorelbine metabolites.

Upregulation of carboxylesterases in livers of *Mdr1a/1b*$^{-/-}$, *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice.

The conversion of vinorelbine to 4'*O*-deacetylvinorelbine is essentially an esterase reaction, so we considered that one or more (carboxyl)esterases or paraoxonases produced in the liver and secreted

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into the blood might be upregulated in the *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ strains, but not in the *Mdr1a/1b*$^{-/-}$ mice. We therefore tested gene expression of all obvious candidate hepatic carboxylesterases (Holmes *et al.*, 2010) and paraoxonases in liver of the different mouse strains using RT-PCR (Supplemental Figure 1 & Supplemental Table 1). The paraoxonase enzymes (Pon1, Pon2 and Pon3) were not differentially expressed in the WT and knockout strains. In contrast, 4 of the 6 tested Ces1 enzymes (Ces1b, Ces1c, Ces1d and Ces1e) were found to be highly upregulated in all three knockout strains. However, because these genes were also upregulated to the same extent in *Mdr1a/1b*$^{-/-}$ mice, whereas metabolite formation in *Mdr1a/1b*$^{-/-}$ mice was similar to that in WT mice (Fig. 5), these Ces1 enzymes cannot be responsible for the (increased) formation of 4'*O*-deacetylvinorelbine. Interestingly, however, the carboxylesterase Ces2a (formerly named Ces6) was 2.7-fold upregulated in *Cyp3a*$^{-/-}$ and 3.9-fold in *Mdr1a/1b/Cyp3a*$^{-/-}$ mice, whereas its expression was not altered in *Mdr1a/1b*$^{-/-}$ mice (Fig. 6D, Supplemental Figure 1 and Supplemental Table 1). Based on this Ces2a expression pattern (Fig. 6D) and its similarity to the pattern of metabolite formation in the knockout strains (Fig. 5 and 6A-C), Ces2a is the only plausible carboxylesterase candidate for the increased formation of 4'*O*-deacetylvinorelbine.

Increased 4'*O*-deacetylvinorelbine formation can be inhibited by BNPP and loperamide.

To further corroborate the involvement of carboxylesterases and especially Ces2a, we tested whether the formation of 4'*O*-deacetylvinorelbine in plasma could be inhibited using the non-specific carboxylesterase inhibitor BNPP and the (human) CES2-specific inhibitor loperamide (Wang et al, 2011; Williams et al., 2011). We incubated vinorelbine (6.5 μM) with BNPP (100 μM), loperamide (100 μM) or vehicle for 120 minutes *in vitro* with plasma freshly collected from WT, *Mdr1a/1b*$^{-/-}$, *Cyp3a*$^{-/-}$, and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice, and we measured disappearance of vinorelbine and appearance of 4'*O*-deacetylvinorelbine over time. Loperamide inhibited the formation of 4'*O*-

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deacetylvinorelbine about 2-3-fold in all four strains, whereas BNPP completely inhibited metabolite formation (Fig. 6A & 6B). Adding up the vinorelbine plus 4'*O*-deacetylvinorelbine values in plasma for each strain (Fig. 6C) suggested little metabolism to alternative vinorelbine metabolites. Moreover, we obtained similar inhibition data with liver microsomes of WT and Cyp3a knockout mice (Supplemental Figure 2). In microsomes of Cyp3a$^{-/-}$ mice, loperamide inhibited the formation of 4'*O*-deacetylvinorelbine ~3-fold, whereas BNPP completely inhibited metabolite formation. Collectively, these inhibition results combined with the RNA expression data indicate that a carboxylesterase is responsible for the formation of 4'*O*-deacetylvinorelbine in liver and plasma, and that this carboxylesterase is most likely Ces2a.

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DISCUSSION

In this study we demonstrated that vinorelbine is a transported substrate for MRP2, and that loss of Mrp2 in mice resulted in a 1.5-fold elevated plasma exposure upon oral vinorelbine administration. Absence of P-gp in mice resulted in a marked (~3.5-fold) increase in plasma exposure after oral vinorelbine, and a 2.3-fold increase after i.v. vinorelbine. The absence of Cyp3a alone or combined absence of P-gp and Cyp3a also resulted in increased plasma exposure levels (2.2-fold and 3.4-fold, respectively) of oral vinorelbine. However, these latter values are likely an underestimate, as in both strains, especially the *Mdr1a/1b/Cyp3a*$^{-/-}$ mice (and in contrast to the single P-gp-deficient strain), there was a substantial upregulation of plasma enzyme(s) that convert vinorelbine to its major metabolite 4'*O*-deacetylvinorelbine. The impact of this upregulation was even more apparent after i.v. vinorelbine administration. Inhibition experiments together with RT-PCR expression data indicated that upregulation of carboxylesterase 2a (Ces2a) is most likely responsible for the (increased) formation of 4'*O*-deacetylvinorelbine in the *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice.

Thus far, interactions of vinorelbine with MRP2 have not been reported. However, cellular resistance to the *Vinca* alkaloid vincristine has been associated with MRP2 expression (Kawabe et al., 1999; Chen et al., 1999), and transport of vinblastine by MRP2 has been demonstrated *in vitro* (Evers et al., 1998; Evers et al., 2000a; Huisman et al., 2005). In this study we show that human MRP2 can efficiently transport vinorelbine *in vitro*. This very likely means that tumor cells that express this protein will to some extent be protected from the cytotoxicity of vinorelbine. Moreover, *Mrp2*$^{-/-}$ mice had significantly higher plasma vinorelbine concentrations than WT mice, especially at early time points (15 and 30 min) after oral administration, suggesting that Mrp2 can limit the intestinal uptake of vinorelbine. This could mean that also in patients MRP2 activity and variations in

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its expression or activity level due to genetic polymorphisms, or induction or inhibition by other co-administered drugs, may affect the therapeutic efficacy of vinorelbine.

The marked (3.5-fold) increase in plasma exposure after oral vinorelbine in *Mdr1a/1b*$^{-/-}$ mice indicates that P-gp is an important determinant of oral availability of vinorelbine. The increase in plasma levels was especially evident shortly after vinorelbine administration, suggesting that the intestinal uptake was improved. Although vinorelbine is an established P-gp substrate (Adams and Knick, 1995;Shepard et al., 2003), in contrast to our results it was previously reported that *Mdr1a/1b*$^{-/-}$ mice did not have a statistically significantly increased plasma AUC upon oral vinorelbine administration at 10 mg/kg (Press *et al.*, 2006). However, variation in plasma levels in those experiments was extremely high, hampering reliable comparisons between strains. We have since then found that the reproducibility of plasma levels upon oral drug administration to mice can be markedly improved by including a short (3 hour) fasting period before administration, and this was applied in the vinorelbine experiments described in the current study. Press et al. (2006) did find a clear and highly significant reduction in fecally excreted vinorelbine in *Mdr1a/1b*$^{-/-}$ mice (from 34% to 6% of the dose), in line with a role of P-gp in limiting intestinal uptake of vinorelbine (Press *et al.*, 2006). Altogether, P-gp appears to substantially limit the oral availability of vinorelbine in mice. Since vinorelbine is also a good substrate of human P-gp, this might contribute to the variable oral availability of vinorelbine in patients as well. The previously reported association in patients of low i.v. vinorelbine clearance and low apparent P-gp clearance activity (Wong *et al.*, 2006) is also in line with our findings in *Mdr1a/1b*$^{-/-}$ mice. Possibly inhibiting P-gp with a dedicated modulating drug might therefore improve the oral and intravenous availability and overall therapeutic efficacy of vinorelbine in patients.

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The interpretation of vinorelbine experiments in the *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice is complicated by the marked upregulation of the plasma and liver carboxylesterase enzyme that converts vinorelbine to 4'*O*-deacetylvinorelbine. This almost certainly means that the vinorelbine AUCs measured in these strains for vinorelbine are an underestimate of the values that would have occurred without this upregulation. In spite of this, the oral and i.v. AUCs of vinorelbine were still significantly (2.2- and 1.5-fold, respectively) increased in *Cyp3a*$^{-/-}$ mice relative to WT mice. This strongly suggests that Cyp3a-mediated metabolism does substantially reduce the effective availability of vinorelbine. This would be consistent with the previous demonstration that CYP3A4 and, to a lesser extent, CYP3A5 are the primary vinorelbine-metabolizing enzymes in human liver microsomes, although the various metabolites formed have not been structurally defined yet (Kajita et al., 2000; Beulz-Riche et al., 2005). This, in combination with our mouse data, would also suggest that CYP3A4 and CYP3A5 may affect the oral and i.v. availability of vinorelbine in humans. Since these enzymes can display substantial inter- and intra-individual variation in expression, and since they are involved in many drug-drug interactions by either gene induction or direct inhibition, this could affect the therapeutic efficacy of vinorelbine.

We note that metabolism of vinorelbine by soluble (plasma) enzymes was thus far not tested (Beulz-Riche et al., 2005; Kajita et al., 2000). However, 4'*O*-deacetylvinorelbine is a major vinorelbine metabolite found in both mouse and human plasma, and this metabolite is also pharmacodynamically active (van Tellingen et al., 1993; Briasoulis et al., 2009). It is therefore interesting to find that one (or more) plasma enzyme(s) can mediate substantial formation of this metabolite. It has previously been suggested that 4'*O*-deacetylvinorelbine is formed by a carboxylesterase, but its identity has thus far not been resolved (e.g. Delord et al., 2009). In this study we have been able to identify mouse Ces2a as the most likely candidate for 4'*O*-deacetylvinorelbine

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formation. We note that loperamide is a specific CES2 inhibitor with $IC_{50} \leq 1$ µM in humans, IC_{50} ~100 µM for dog and IC_{50} ~35 µM for monkey (Williams et al., 2011). For mouse Ces2a, IC_{50} values for loperamide have thus far not been reported, but our results of ~50% inhibition at a concentration of 100 µM are well in line with the IC_{50} values obtained in other animal species. Future experiments with recombinant mouse Ces2a, which is currently not available to us, could clarify this further.

The upregulation of Ces2a makes it difficult to interpret the combined effect of P-gp and Cyp3a deficiency in the *Mdr1a/1b/Cyp3a*$^{-/-}$ mice on vinorelbine availability, after either oral or i.v. drug administration. Presumably, without this upregulation, the AUC of vinorelbine would have been considerably higher than that in the separate *Mdr1a/1b*$^{-/-}$ or *Cyp3a*$^{-/-}$ mice. This problem can not be solved by considering the sum of vinorelbine and 4'*O*-deacetylvinorelbine, since the further disposition and metabolism of either compound might be quite different. Any statement on the possible interaction between Cyp3a and Mdr1a/1b P-gp affecting vinorelbine availability therefore remains speculative. These findings once again illustrate that great caution should be exercised when assessing pharmacokinetic and related effects in knockout mouse strains, as there can sometimes be important changes in alternative detoxification pathways. This seems to be especially a risk with Cyp3a-deficient mouse strains (van Waterschoot et al., 2008 and the present study), although in the present study we also found upregulation of some carboxylesterase genes in *Mdr1a/1b*$^{-/-}$ mice.

In summary, we have demonstrated a substantial *in vivo* impact of P-gp, Mrp2, and Cyp3a on vinorelbine availability and clearance in mice, as well as the involvement of a plasma carboxylesterase, most likely Ces2a. Variation in the activity of their human homologues is likely to affect vinorelbine pharmacokinetics and therapeutic efficacy in patients as well.

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Authorship Contribution

Participated in research design: Lagas, Damen, van Waterschoot, Beijnen and Schinkel.

Conducted experiments: Lagas, Damen, van Waterschoot and Iusuf.

Contributed new reagents or analytic tools: Beijnen.

Performed data analysis: Lagas, Damen, Iusuf and Schinkel.

Wrote or contributed to the writing of the manuscript: Lagas and Schinkel.

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Legends for figures

Figure 1. Molecular structures of vinorelbine (R^* = $COCH_3$) and 4'*O*-deacetylvinorelbine (R^* = H) (***A***) and transepithelial transport of vinorelbine (5 μM) in MDCK-II-Neo cells (***B***) or MDCK-II-MRP2 cells (***C***), in the presence of elacidar (5 μM). At t = 0 h, vinorelbine was applied in one compartment (apical or basolateral), and the percentage translocated to the opposite compartment at t = 2 and 4 h was plotted (n = 3). Translocation from the basolateral to the apical compartment (■); translocation from the apical to the basolateral compartment (□). *r* represents the relative transport ratio (*i.e.* the apically directed translocation divided by the basolaterally directed translocation) at t = 4 hr. Data represent means ± SD. At t = 4 h, 1% of transport is approximately equal to an apparent permeability coefficient (Papp) of 0.30 x 10^6 cm/s.

Figure 2. Plasma concentration-time curves of vinorelbine in WT (■), *Mdr1a/1b*$^{-/-}$ (▲) and *Mrp2*$^{-/-}$ (○) mice, after oral administration of 10 mg/kg vinorelbine. Data represent mean concentrations ± SD, n = 5.

Figure 3. Plasma concentration-time curves of vinorelbine (***A*** and ***B***), 4'*O*-deacetylvinorelbine (*C*) and vinorelbine + 4'*O*-deacetylvinorelbine (combined; ***D***) in WT (■), *Mdr1a/1b*$^{-/-}$ (▲), *Cyp3a*$^{-/-}$ (Δ) and *Mdr1a/1b/Cyp3a*$^{-/-}$ (□) mice, after oral (***A***) and i.v. (***B***, ***C***, *and* ***D***) administration of vinorelbine at a dose of 10 mg/kg. Data represent mean concentrations ± SD, n = 4-6 for oral and n = 4 for i.v. administration. Inserts in B, C, and D show semilog plots of the data.

Figure 4. Concentrations of vinorelbine (***A***), 4'*O*-deacetylvinorelbine (***B***) and vinorelbine + 4'*O*-deacetylvinorelbine (***C****;* Combined) after incubation of liver microsomes from WT or *Cyp3a*$^{-/-}$ mice (Cyp3a KO) with 18.5 μM vinorelbine for 20 minutes in the absence or presence of NADPH. Data represent mean concentrations ± SD, n = 3. Dashed lines in the 3 panels represent the applied vinorelbine concentration of 18.5 μM at t = 0.

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Figure 5. Concentration-time curves of vinorelbine (***A***), 4'*O*-deacetylvinorelbine (***B***) and vinorelbine + 4'*O*-deacetylvinorelbine (***C;*** Combined) after ex-vivo incubation of 6.5 μM vinorelbine in plasma of WT (■), *Mdr1a/1b*$^{-/-}$ (▲), *Cyp3a*$^{-/-}$ (Δ) and *Mdr1a/1b/Cyp3a*$^{-/-}$ (□) mice. Data represent mean concentrations ± SD, n = 4.

Figure 6. Concentrations of vinorelbine (***A***), 4'*O*-deacetylvinorelbine (***B***) and vinorelbine + 4'*O*-deacetylvinorelbine (***C;*** Combined) after ex-vivo incubation of 6.5 μM vinorelbine for 120 minutes in plasma of WT, *Mdr1a/1b*$^{-/-}$, *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice in the absence or presence of the non-specific carboxylesterase inhibitor BNPP (100 μM) or the CES2-specific inhibitor loperamide (100 μM). Data represent mean concentrations + SD, n = 3. Panel D depicts the expression levels of Ces2a in livers of WT, *Mdr1a/1b*$^{-/-}$, *Cyp3a*$^{-/-}$ and *Mdr1a/1b/Cyp3a*$^{-/-}$ mice, as determined by real-time RT-PCR. Data are normalized to GAPDH expression. Values represent mean fold change + SD, compared to WT mice; n = 3. *** $P <$ 0.001, compared to WT ΔCt values.

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Table 1. Plasma pharmacokinetic parameters after oral administration of vinorelbine at 10 mg/kg.

	Strain		
	WT	*Mdr1a/1b*$^{-/-}$	*Mrp2*$^{-/-}$
$AUC_{0\text{-}24}$, hr.mg/l	0.27 ± 0.04	1.00 ± 0.17 *	0.41 ± 0.10 *
C_{max}, mg/l	0.06 ± 0.03	0.43 ± 0.09 **	0.20 ± 0.08 *
T_{max}, hr	0.25	0.25	0.25

$AUC_{(0\text{-}24)}$, area under plasma concentration-time curve up to 24 hr; C_{max}, maximum plasma levels; T_{max}, time of maximum plasma concentration. Data are means ± SD, n = 5. * $P < 0.05$ and ** $P < 0.01$, compared to WT mice.

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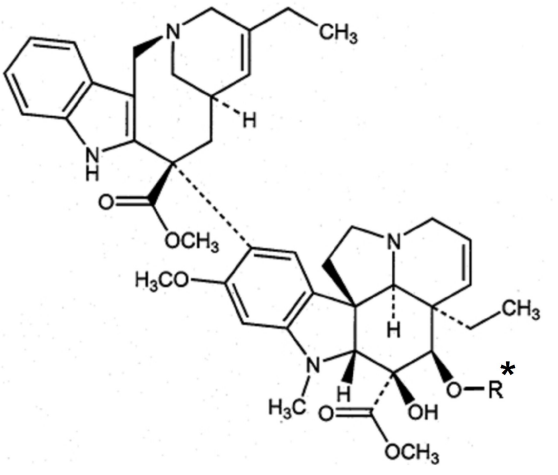
Table 2. Plasma pharmacokinetic parameters after oral or i.v. administration of vinorelbine at 10 mg/kg.

	Strain			
	WT	*Mdr1a/1b*$^{-/-}$	*Cyp3a*$^{-/-}$	*Mdr1a/1b/Cyp3a*$^{-/-}$
Oral vinorelbine				
$AUC_{0\text{-}24}$, hr.mg/l	0.25 ± 0.10	0.86 ± 0.37 *	0.54 ± 0.15 *	0.84 ± 0.22 *
C_{max}, mg/l	0.04 ± 0.02	0.45 ± 0.07 **	0.11 ± 0.03 *	0.33 ± 0.13 *
T_{max}, hr	0.25	0.25	1	0.25
i.v. vinorelbine				
$AUC_{0\text{-}24}$, hr.mg/l	1.62 ± 0.27	3.70 ± 0.35 **	2.50 ± 0.14 **	1.17 ± 0.04 *
CL, l/hr.kg	6.30 ± 0.97	2.72 ± 0.24 **	4.01 ± 0.23 **	8.55 ± 0.31 **
F(%)	15.4 ± 6.7	23.2 ± 10.2	21.6 ± 6.1	71.8 ± 19.1 *
i.v. 4'O-deacetylvinorelbine				
$AUC_{0\text{-}24}$, hr.mg/l	0.40 ± 0.05	1.45 ± 0.14 **	2.02 ± 0.02 **	4.46 ± 0.31 **
i.v. combined				
$AUC_{0\text{-}24}$, hr.mg/l	2.01 ± 0.30	5.15 ± 0.46 **	4.52 ± 0.14 **	5.63 ± 0.29 **
$AUC_{i.v.}$ 4'O-deacetylvinorelbine / $AUC_{i.v.}$ vinorelbine				
Exposure ratio	0.25	0.39	0.81	3.8

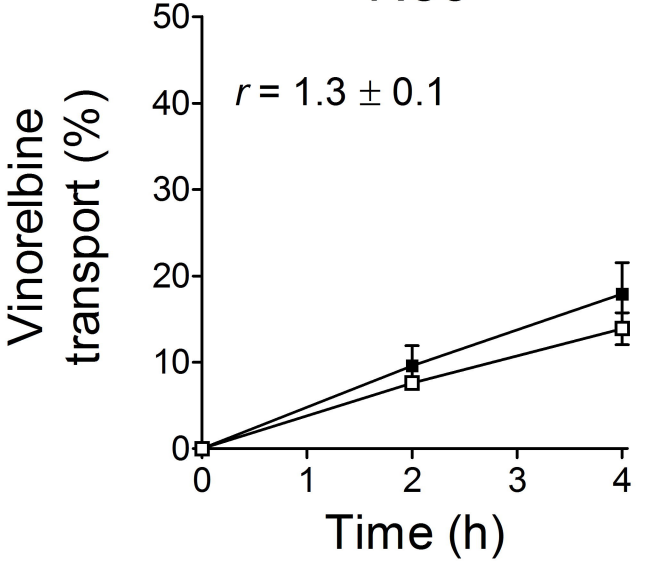
$AUC_{(0\text{-}24)}$, area under plasma concentration-time curve up to 24 hr; C_{max}, maximum plasma levels; T_{max}, time of maximum plasma concentration; CL, plasma clearance; *F*, oral bioavailability. Data are means ± SD, n = 4-6 for oral and n = 4 for i.v. administration. * $P < 0.05$ and ** $P < 0.01$, compared to WT mice.

Figure 1

A


N
CH3
H
N
H
O
OCH3
H3CO
N
CH3
H
N
H
H3C
O
OH
O–R*
OCH3

B


Neo
r = 1.3 ± 0.1
Vinorelbine transport (%)
50
40
30
20
10
0
0
1
2
3
4
Time (h)

C

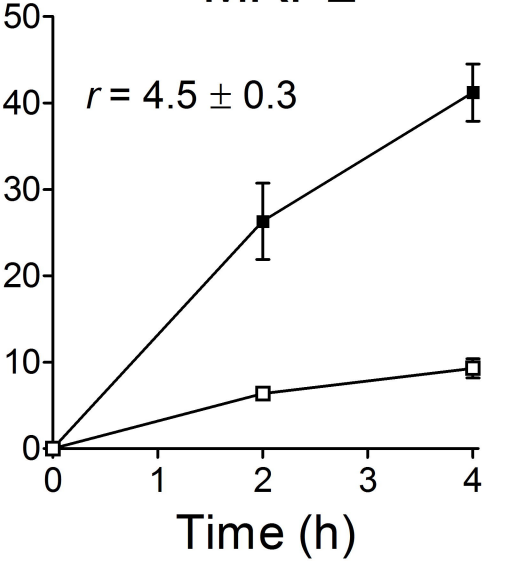

MRP2
r = 4.5 ± 0.3
50
40
30
20
10
0
0
1
2
3
4
Time (h)

Figure 2

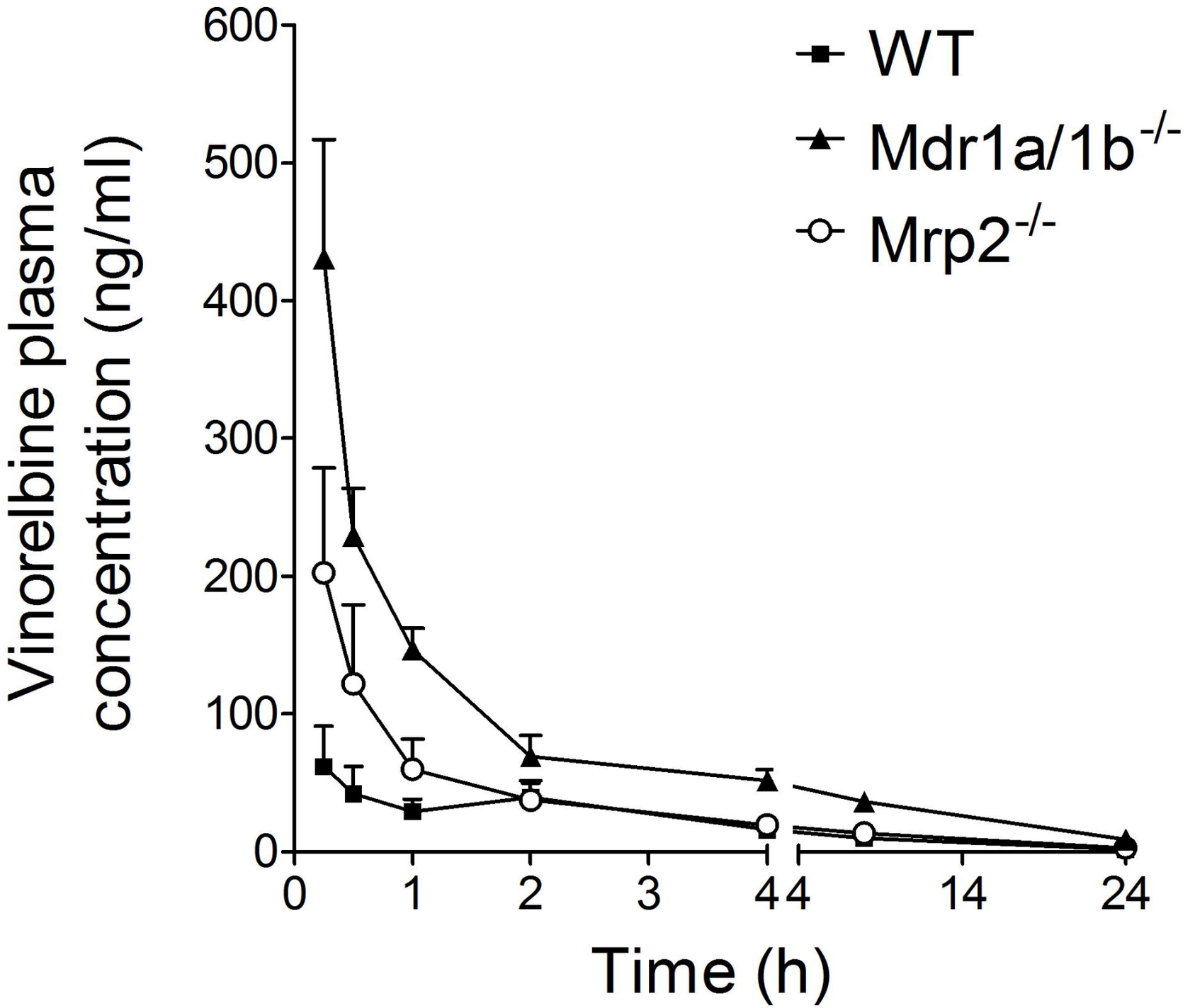

Vinorelbine plasma concentration (ng/ml)
600
500
400
300
200
100
0
0
1
2
3
4
4
14
24
Time (h)
WT
Mdr1a/1b-/-
Mrp2-/-

Figure 3

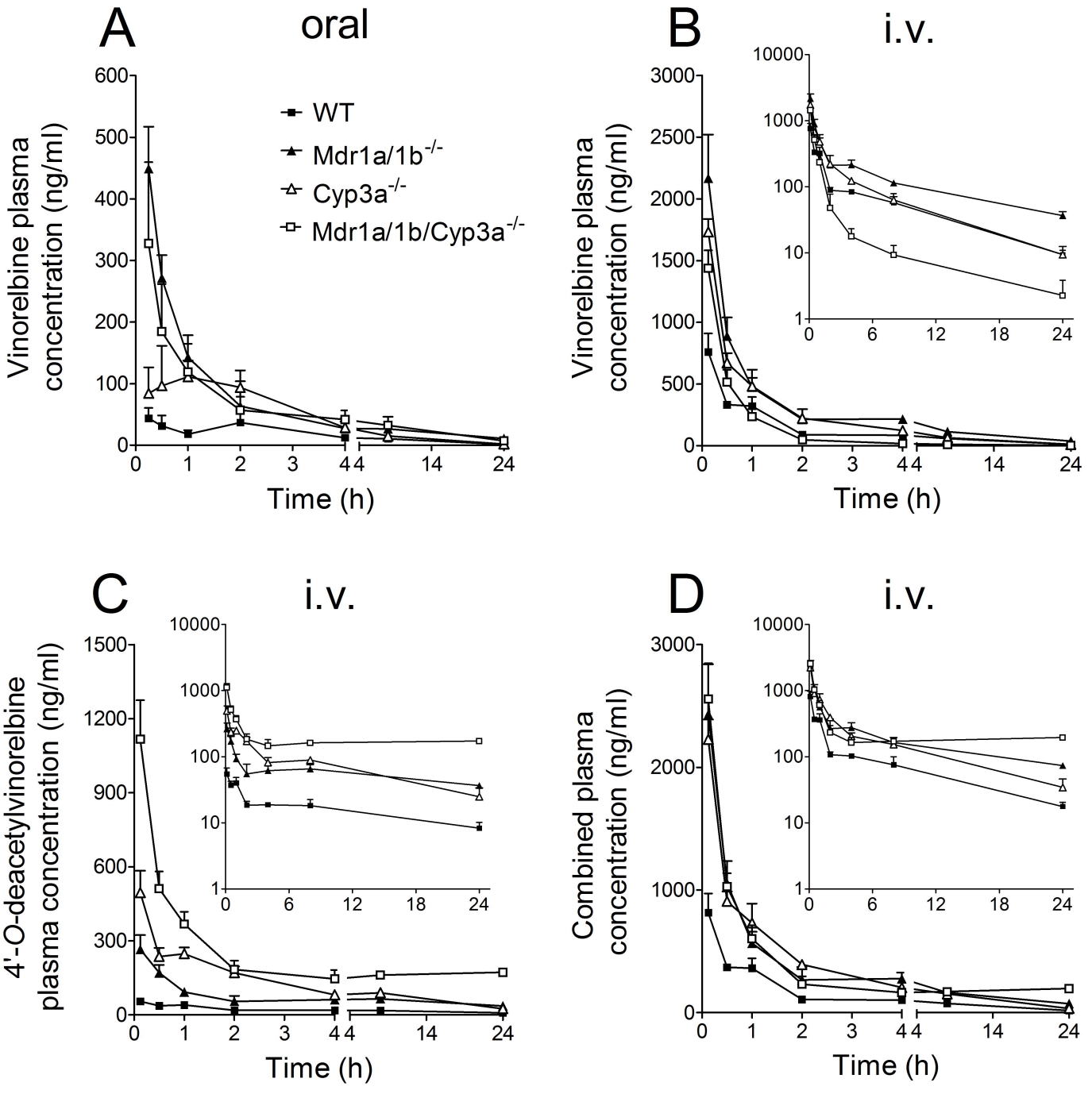

A
oral
B
i.v.
C
i.v.
D
i.v.
WT
Mdr1a/1b-/-
Cyp3a-/-
Mdr1a/1b/Cyp3a-/-
Vinorelbine plasma concentration (ng/ml)
4'-O-deacetylvinorelbine plasma concentration (ng/ml)
Combined plasma concentration (ng/ml)
Time (h)

Figure 4

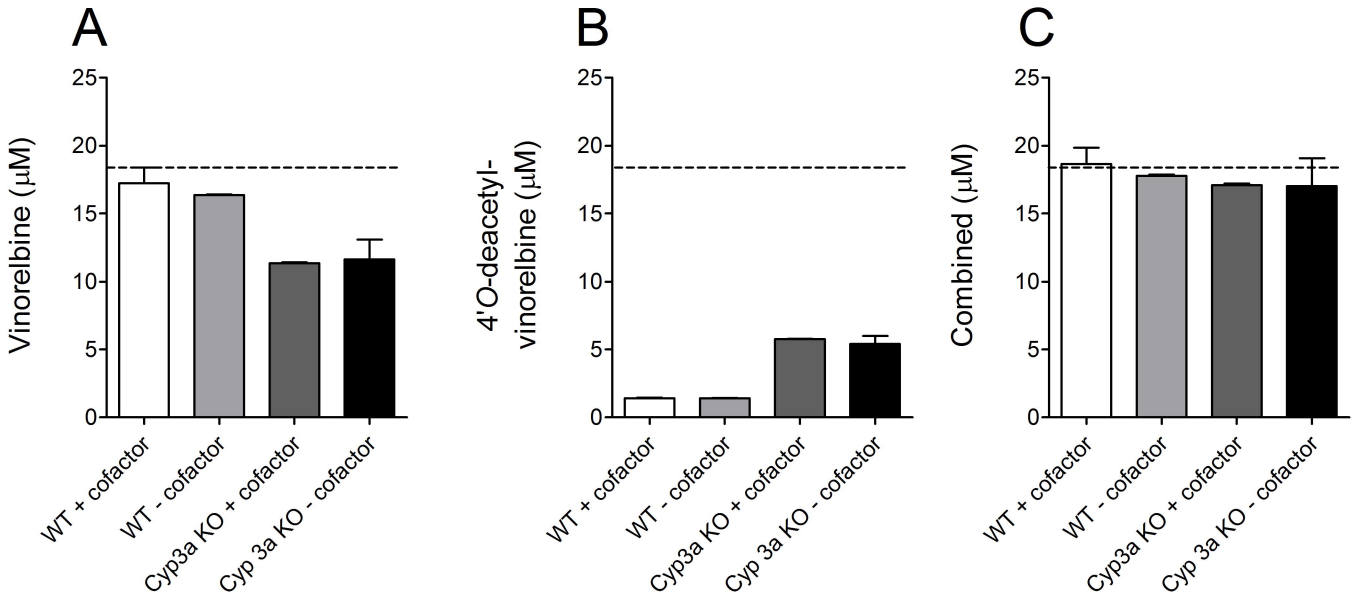

A
B
C
Vinorelbine (μM)
4'O-deacetyl-vinorelbine (μM)
Combined (μM)
25
20
15
10
5
0
WT + cofactor
WT - cofactor
Cyp3a KO + cofactor
Cyp 3a KO - cofactor

Figure 5

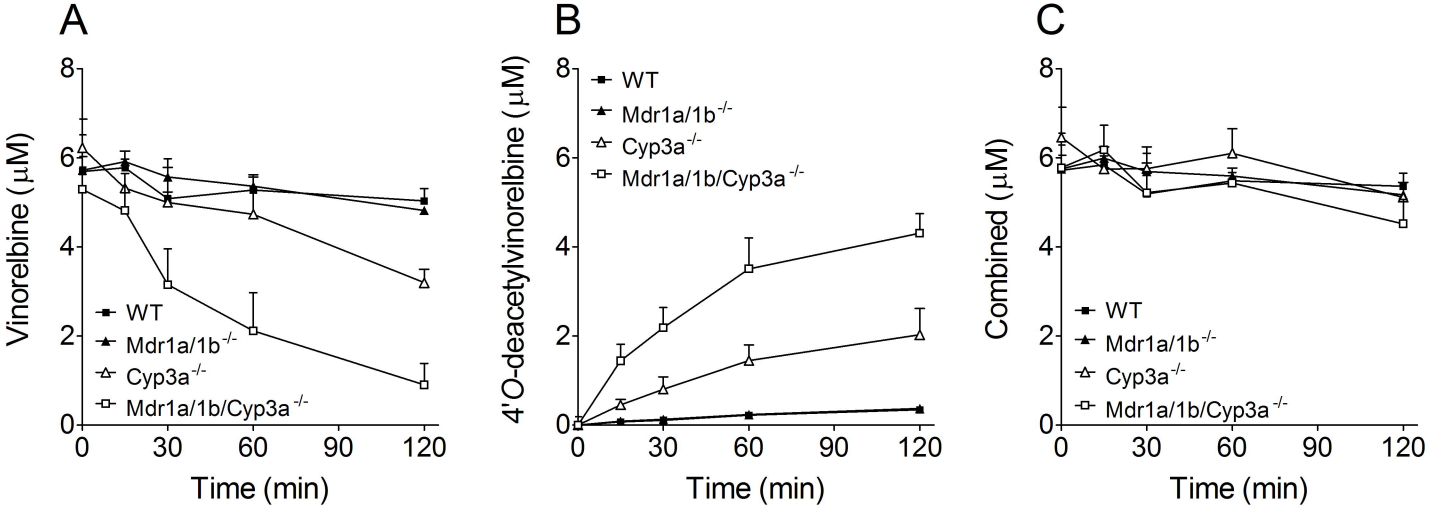
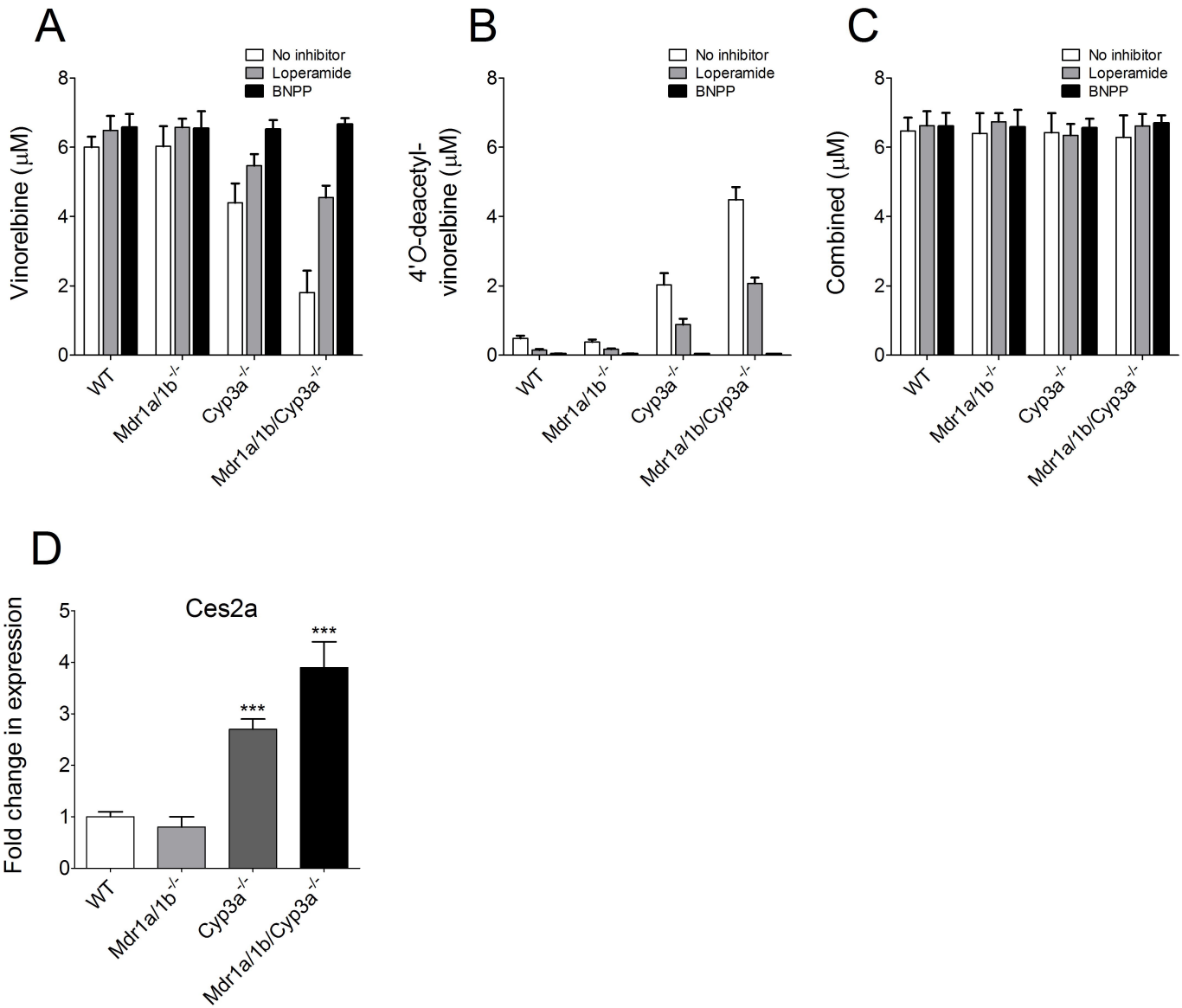

A
Vinorelbine (μM)
8
6
4
2
0
0
30
60
90
120
Time (min)
WT
Mdr1a/1b-/-
Cyp3a-/-
Mdr1a/1b/Cyp3a-/-
B
4'O-deacetylvinorelbine (μM)
8
6
4
2
0
0
30
60
90
120
Time (min)
WT
Mdr1a/1b-/-
Cyp3a-/-
Mdr1a/1b/Cyp3a-/-
C
Combined (μM)
8
6
4
2
0
0
30
60
90
120
Time (min)
WT
Mdr1a/1b-/-
Cyp3a-/-
Mdr1a/1b/Cyp3a-/-

Figure 6


A
No inhibitor
Loperamide
BNPP
Vinorelbine (μM)
0
2
4
6
8
WT
Mdr1a/1b -/-
Cyp3a -/-
Mdr1a/1b/Cyp3a -/-
B
No inhibitor
Loperamide
BNPP
4'O-deacetyl-vinorelbine (μM)
0
2
4
6
8
WT
Mdr1a/1b -/-
Cyp3a -/-
Mdr1a/1b/Cyp3a -/-
C
No inhibitor
Loperamide
BNPP
Combined (μM)
0
2
4
6
8
WT
Mdr1a/1b -/-
Cyp3a -/-
Mdr1a/1b/Cyp3a -/-
D
Ces2a
Fold change in expression
0
1
2
3
4
5
***
***
WT
Mdr1a/1b -/-
Cyp3a -/-
Mdr1a/1b/Cyp3a -/-