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Dependence of erythromycin metabolism on ABCC2 (MRP2) transport function

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Abstract

The macrolide antiobiotic erythromycin undergoes extensive hepatic metabolism and is commonly used as a probe for CYP3A4 activity. Using a transporter screen, erythromycin was identified as a substrate for the transporter ABCC2 (MRP2) and its murine ortholog, Abcc2. Since these proteins are highly expressed on the biliary surface of hepatocytes, we hypothesized that impaired Abcc2 function may influence the rate of hepatobiliary excretion and thereby enhance erythromycin metabolism. Using Abcc2-knockout mice, we found that erythromycin metabolism was significantly increased, whereas murine Cyp3a protein expression and microsomal Cyp3a activity were not affected by Abcc2 deficiency. Next, in a cohort of 108 human subjects, we observed that homozygosity for a common reduced-function variant in *ABCC2* (rs717620) was also linked with increased erythromycin metabolism, but was not correlated with the clearance of midazolam. These results suggest that impaired ABCC2 function can alter erythromycin metabolism independently of changes in CYP3A4 activity.

Keywords

Erythromycin; midazolam; hepatic metabolism; ABCC2 (MRP2)

Erythromycin is the oldest and best known antibiotic agent in the class of macrolides that is effective against Gram-positive bacteria, with modest effects in some Gram-negative bacteria.[1] The hepatic metabolism of erythromycin by CYP3A isozymes has been extensively studied and was the basis for the development of the erythromycin breath test (ERMBT) in order to study the CYP3A activity phenotype in patients.[2, 3] However, several studies in recent years have demonstrated that membrane transporters may affect the rate of

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CONFLICT OF INTEREST

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erythromycin metabolism and have an influence on how the ERMBT is interpreted.[4–9] For example, a mouse model lacking Abcb1a (mdr1a P-glycoprotein) was previously shown to have increased metabolism of erythromycin, as determined by the ERMBT.[4] Recently, erythromycin was also shown to be a substrate for the rabbit transporter Abcc2 (Mrp2; cMoat), localized in the corneal epithelium.[10] Given the abundant expression of human ABCC2 along the bile canalicular membrane of hepatocytes,[11, 12] we hypothesized that impairment of ABCC2 function could also increase the metabolism of erythromycin by reducing the biliary secretion of the drug, increase hepatocellular residence time, and thus facilitate the interaction of the drug with metabolic enzymes. The aims of the current study were (*i*) to identify erythromycin as a substrate for human ABCC2 and evaluate affinity of the drug for other members of the ABCC family that are expressed in the liver; and (*ii*) to use preclinical and clinical models to confirm the *in vivo* relevance of ABCC2 in the metabolism of erythromycin.

RESULTS

In vitro transport studies

To assess the extent of erythromycin diffusion across cell membranes, a parallel artificial membrane permeability assay (PAMPA) was performed under steady-state conditions. The percent transfer of erythromycin was 27±1.7%, compared with 0.40±0.14% for methotrexate and 54±5.6% for midazolam, agents representative of those known to diffuse poorly or diffuse freely across cell membranes, respectively. The relatively low permeability supports the possibility that membrane transport of erythromycin is at least partially dependent on active carriers.

In order to identify efflux proteins involved in membrane transport of erythromycin, experiments were carried out using validated transfected inside-out vesicles expressing 7 different ABC transporters (Supplementary Figure 1). Following a 5-min incubation period with these vesicles, erythromycin was identified as a substrate for human ABCC2, mouse Abcc2, as well as the human transporter, ABCC3 (Figure 1A). This finding is consistent with the prior notion that ABCC2 is closely related to ABCC3 (MRP3), sharing 58% amino acid homology and a considerable overlap in substrate specificity. In contrast to ABCC2, however, ABCC3 is located to the basolateral membrane of hepatocytes.

The transport of erythromycin by ABCC2 and Abcc2 was sensitive to inhibition by the ABC transporter inhibitor MK571 (Figure 1A). Furthermore, using MDCKII cells transfected with ABCC2, increased basal-to-apical transport of erythromycin (2.7-fold, P=0.038, Figure 1B) was observed, which process was abolished in the presence of MK571 (Figure 1C). Whereas the affinity of erythromycin for human ABCC2 and mouse Abcc2 was similar (Km, 64.4±12.3 μM *versus* 81.3±12.4 μM, respectively), the maximum rate of transport was lower for human ABCC2 compared with mouse Abcc2 (Vmax, 45.6±4.32 pmol/mg/min *versus* 238±19.3 pmol/mg/min, respectively) (Supplementary Figure 2), although it cannot be excluded that this difference is due to a higher total expression of Abcc2 protein in the mouse cell line. Nonetheless, similar species differences in ABCC2-mediated transport have been reported for various agents, including saquinavir and docetaxel.[13]

Erythromycin was not transported by the hepatic ABC transporters ABCC1 (MRP1), ABCC4 (MRP4), ABCC11 (MRP8), and ABCG2 (BCRP) (Figure 1A). Different ABCC transporters may exhibit different time courses for erythromycin accumulation. Therefore, two time course studies (5 and 60 min) were performed to capture the effects of other ABC transporters, and similar results were observed with the short and long incubation periods (Supplementary Figure 3). As anticipated, midazolam, used as a negative control, was not a substrate for any of the studied ABC transporters (Figure 1A and Supplementary Figure 3).

Erythromycin transport in Abcc2-knockout mice

The *in vivo* role of Abcc2 in the transport of erythromycin was next evaluated in wildtype mice and age-matched Abcc2-deficient [Abcc2(−/−)] mice. To rule out potentially altered, compensatory expression of transporters in the liver of Abcc2(−/−) mice at baseline, real-time PCR arrays were used to evaluate differential expression profiles of 88 genes (Supplementary Table 1). Compared to levels in liver of wildtype mice, expression of 3 genes was altered in the Abcc2(−/−) mice (Figure 2A). In particular, transcripts of *Slco2b1* (encoding Oatp2b1) and *Vdac2* (encoding Voltage-dependent anion-selective channel protein 2; Vdac2) were increased in the Abcc2(−/−) mice, whereas expression of *Slc22a7* (encoding Oat2) was reduced in the Abcc2(−/−) mice. Since macrolide antibiotics such as erythromycin do not interact with mouse Oatp2b1,[14] and are not known to be substrates for Vdac2, these genetic alterations are unlikely to directly or indirectly influence erythromycin handling by the liver. It is noteworthy that erythromycin was previously identified as an inhibitor of cellular uptake of glutarate mediated by mouse Oat2, a multispecific organic anion transporter,[15] although the ability of Oat2 to transport erythromycin remains controversial.[16, 17] Besides *Slc22a7*, hepatic expression of other liver transporter genes of known or suspected relevance to erythromycin, including *Abcb1* (P-glycoprotein), *Abcc3* (Mrp3), *Slco1a4* (Oatp1a4) and *Slco1b2* (Oatp1b2), was not significantly altered in Abcc2(−/−) mice compared with wildtype mice (Figure 2B). Likewise, none of the 15 known *Cyp2c* genes was differentially expressed in livers of the Abcc2(−/−) mice (Figure 2C), including those that were previously reported to be involved in the murine metabolism of midazolam such as *Cyp2c29*, *Cyp2c39*, *Cyp2c55*, *Cyp2c65*, and *Cyp2c70*.[18]

Using samples from the same livers, we also found that expression of the Cyp3a11 protein, the main murine erythromycin-metabolizing enzyme, was not significantly changed in Abcc2(−/−) mice (Figure 3A and Figure 3B). Furthermore, there were no potentially compensatory changes in hepatic Cyp3a activity, since Abcc2-deficiency had no influence on the microsomal metabolism of the Cyp3a substrate midazolam *ex vivo* (Figure 3C) in the presence of absence of the Cyp3a inhibitor ketoconazole (wildtype, 89.1±8.30% inhibition vs Abcc2(−/−), 87.5±6.10% inhibition; P=0.39; Figure 3D), or *in vivo*, as measured by a midazolam sleep test (P=0.56) (Figure 3E).

The *in vivo* role of Abcc2 in the transport of erythromycin was next evaluated using an erythromycin breath test (ERMBT). Compared to wildtype mice, a more than 2-fold increase in the exhaled $^{14}CO_2$ was seen in the Abcc2(−/−) mice (P<0.0001) (Figure 3F), with cumulative levels significantly rising over time by 2.2- to 4.2-fold (Figure 3G). This suggests that the loss of Abcc2 in these mice leads to an increased metabolism of

erythromycin in the absence of changes in Cyp3a activity, and despite the decreased hepatic expression of *Slc22a7*, which could theoretically diminish the hepatocellular uptake of erythromycin. The observed increase in erythromycin metabolism in Abcc2(−/−) mice is consistent with an increased residence time of the drug in hepatocytes due to impaired biliary secretion by Abcc2.

Association of ABCC2 variants with erythromycin transport in humans

To provide preliminary evidence for a possible role of ABCC2 in the clinical pharmacology of erythromycin, a pharmacogenetic-association analysis was performed in human subjects with cancer undergoing an ERMBT prior to the administration of antineoplastic therapy.

ERMBT in cancer patients—Cancer-associated inflammation has previously been linked with impaired CYP3A4-mediated drug metabolism *in vivo*.[19] This suggests that the disease itself may have an impact on hepatic CYP3A4 activity, and this possibility may affect the ability to extrapolate our findings to other therapeutic areas and to healthy volunteers. Therefore, we assessed the potential influence of cancer on the ERMBT in a cohort of 134 patients with cancer,[20] and compared the results with literature data obtained in healthy volunteers.[21] After i.v. injection of [^{14}C-*N*-mehtyl]erythromycin to White cancer patients with normal liver function, the median %dose per hour recovery rate measured at 20 min, the time point equal to the maximum rate of $^{14}CO_2$ exhalation,[22] was 2.20%/hour (interquartile range, 1.50–2.99%/hour). As expected due to the presumed reduction in hepatic CYP3A4 activity, this value was slightly lower as compared to that observed at baseline in a cohort of 32 healthy volunteers of similar racial ancestry, where the median value was 2.57%/hour (interquartile range, 1.98–2.94%/hour).[21] In cancer patients, we observed a binomial relationship between the 20-min breath samples collected after the ERMBT, with breath collections sampled every 10 min for 1 hour to estimate the area under the curve for actual $^{14}CO_2$ production during the interval (Figure 4A). Furthermore, there was a strong correlation between the observed $^{14}CO_2$ production during 1 hour from the same cancer patients with predicted values obtained from the equation originally derived from data obtained in healthy volunteers (Figure 4B).[23] These findings suggest that the ERMBT is quantitatively and qualitatively similar in human subjects with and without cancer, even though in the former, erythromycin metabolism is slightly impared.

Genotyping results—Seven single-nucleotide polymorphisms (SNPs) in *ABCC2* were analyzed in DNA obtained from 108 cancer patients undergoing an ERMBT (Table 1). The relative frequencies of the variant alleles were comparable with previously reported estimates,[24] and the frequency distributions of all SNPs were in Hardy-Weinberg equilibrium. As observed previously,[25] significant linkage was observed between *ABCC2* -24C>T and the SNPs at the -1019A>G ($D' = 0.75$; $P > 0.01$) and 3972C>T loci ($D' = 0.89$; $P > 0.001$).

Genotype-ERMBT relationships—We specifically focused on variation in the *ABCC2* promoter at the -24C>T locus because of its known influence on functional properties of the encoded protein.[26] In a cohort of 108 subjects on whom DNA was available, homozygosity for this variant was associated with significantly increased metabolism of erythromycin

(*P*=0.013), as determined by the ERMBT parameter $1/T_{max}$ (Figure 5A), which was identified by Rivory et al[27] as the most informative value to estimate hepatic CYP3A4 activity. Patient demographic characteristics at baseline for the homozygous carriers of this SNP were similar to patients with the CC- or CT-genotype (Table 2). Individuals heterozygous for the variant were found to have an erythromycin metabolism rate that was not significantly different from individuals who were homozygous for the reference *ABCC2* allele (Figure 5B). None of the other investigated SNPs in *ABCC2* was found to be significantly associated with the ERMBT (Supplementary Figure 4). Furthermore, none of the observed common haplotypes in *ABCC2* was a predictor of the ERMBT, and similar results were obtained when the analysis was restricted to patients with the *CYP3A5*3C/*3C* genotype, i.e. CYP3A5 non-expressors (data not shown). Likewise, when excluding the 3 individuals carrying the *SLCO1B3* 334TT genotype (all had the *ABCC2* -24TT genotype), which is associated with decreased erythromycin metabolism,[28] the association between *ABCC2* -24C>T and ERMBT 1/Tmax remained significant at *P*=0.019 (data not shown). As anticipated based on our cellular transport and Abcc2(−/−) mouse data, the *ABCC2* -24C>T variant was not related with the clearance of midazolam in human subjects (Figure 5B).

DISCUSSION

The current study provides support for a growing body of knowledge that ABC transporters can impact the hepatic accumulation and metabolism of CYP3A substrates such as erythromycin. A variety of *in vitro* transport assays was utilized, including intracellular accumulation with inside-out vesicles and transcellular transport studies. We found that erythromycin is a substrate for human ABCC2 and mouse Abcc2, but it was not identified as a substrate for the ABCC1, ABCC4, ABCC11, and ABCG2 transporters.

Mice lacking Abcc2 had higher values of exhaled $^{14}CO_2$ following an ERMBT, while the activity of hepatic Cyp3a enzymes was unchanged, as determined by the midazolam sleep test. Differences in baseline Cyp3a activity would have directly impacted the metabolism of midazolam and altered the midazolam-induced sleep time.[19] From a mechanistic perspective it is plausible to postulate that when canalicular transport is inhibited, the increase in metabolism is due to the increase in the ratio of intracellular to extracellular concentrations. Importantly, this increase in metabolism is only an apparent increase because plasma concentration, rather than hepatic concentration, is used as the reference point.[29] Nonetheless, these findings add to the supposition that, unlike CYP3A activity determined by midazolam clearance, the results from the ERMBT are a net effect resulting from interactions between both CYP3A isozymes and membrane transporters.

Recently, it was found that an increased-function haplotype in *CYP3A4* and *CYP3A5* is related to altered midazolam clearance, but not associated with the ERMBT.[24] However, it should be noted that this patient population had functional variants in the gene encoding OATP1B3 (*SLCO1B3*), which was previously identified as an uptake carrier of erythromycin.[28] In recent years, the importance of uptake and efflux transporters has also been demonstrated using inhibitors in both the *in vitro* hepatic metabolism of erythromycin and in the ERMBT administered to humans.[9, 30] Interestingly, in our current study, we

found that a functional genetic variant in the promoter region of *ABCC2* (-24C>T) was associated with altered ERMBT results.

This *ABCC2* variant has previously been associated with decreased mRNA levels in normal tissues,[31] and shown to have decreased protein levels and decreased efflux capabilities.[32] For example, transplant recipients being treated with telmisartan had higher peak plasma levels if they carried the variant allele. Furthermore, women carrying this variant have a higher area under the curve (AUC) of methotrexate.[33] Interestingly, different studies have linked the *ABCC2* -24C>T variant with a decreased AUC of the anticancer agent irinotecan,[34] an increased AUC of the irinotecan metabolite SN-38,[35] increased tumor response rates,[36] and longer progression free survival in patients with non-small cell lung cancer receiving treatment with irinotecan.[36] The reported opposite associations of the ABCC2 variant with the AUCs of irinotecan and SN-38 suggest that the hepatic metabolism of irinotecan might be increased in carriers. However, it should be noted that the increase in SN-38 was not seen in all studies.[36, 37]

Our results, along with the previous studies mentioned above, suggest that genetic variation in *ABCC2* can contribute considerably to interindividual pharmacokinetic variability of multiple, clinically-important prescription drugs. Indeed, a decrease in ABCC2-mediated hepatobiliary secretion can increase the metabolism of agents like erythromycin, and perhaps irinotecan. It has been suggested that previously identified drug-drug interactions with erythromycin that were presumably due to alterations of CYP3A may rather be due to interactions at the level of efflux transporters, such as ABCC2.[38] While a re-evaluation of all previously identified erythromycin-drug interactions is not feasible, future studies should test for possible drug-drug interactions that may be due to inhibition and/or induction of hepatic ABCC2.

METHODS

Parallel artificial membrane permeability assay (PAMPA)

Diffusion was assessed using Millipore 96-well multi-screen filter plates with polyvinylidine fluoride membranes and transport receiver plates (Millipore, Billerica, MA). The artificial membrane was prepared by adding 5 μl of 5% lecithin in dodecane (Avanti Polar Lipids Inc., Alabaster, AL) to the donor well. Then 150 μl of PBS solution containing [^{14}C]erythromycin (2 μM), [^{3}H]midazolam (1 μM), or [^{3}H]methotrexate (500 μM), each containing 5% DMSO, at pH 7.4 was added to the lipid-treated donor wells. The donor plate was placed onto the acceptor plate, and incubation was carried out over 16 hours as directed in the Millipore assay protocol at room temperature with the wells covered, to prevent evaporation. After incubation, 75-μl samples from each donor well and 150-μl samples from each acceptor well were analyzed using liquid scintillation alongside theoretical equilibrium drug solutions containing 150 μl of each stock drug solution in 300 μl of the buffer. Membrane integrity was confirmed by determining the equilibrium transport of Brilliant Cresyl Blue and Lucifer Yellow. Results from the PAMPA analysis were expressed as a percentage of the drug accumulating in the acceptor wells, which values can vary between 0%, showing no diffusion, and 50%, showing free diffusion.

Inside-out vesicle accumulation studies

Inside-out vesicles of Sf9 cells expressing ABCC1, ABCC2, mAbcc2, ABCC3, ABCC4, ABCC11, or ABCG2 (Genomembrane, Yokohama, Japan) were used to determine uptake of either [^{14}C]erythromycin (2–8 μM) or [^{3}H]midazolam (440 nM) according to the manufacturer's protocol in the presence and absence of MK571 (100 μM). [^{3}H]Estradiol-17β-glucuronide (1–50 μmol/L) and [^{3}H]methotrexate (100 μM) were used as positive controls for the various transporters (Supplementary Figure 1). Briefly, tubes containing vesicles and tubes containing radiolabeled agents were separately pre-incubated at 37 °C for 10 min. Tubes were then combined and incubated for 5 min. Cold buffer (200 μL) was added to stop the reaction, and contents were transferred to a 96-well glass-fiber filter plate with a vacuum. Filters were washed 5 times with cold buffer, removed and then combined with scintillation fluid for measurement of total radioactivity.

In vitro transcellular transport

Transcellular transport assays were performed in MDCKII cells transfected with ABCC2 (kindly provided by Dr. Raymond Evers[39]). Briefly, cells were plated at 2×10^{6} density per well in Transwell dishes containing 2 mL DMEM with 10% fetal bovine serum. Medium was changed every 48 hours until cells were 100% confluent. Membrane integrity was confirmed by determining the equilibrium transport of Brilliant Cresyl Blue and Lucifer Yellow. Fresh serum free medium containing either [^{14}C]erythromycin (2 μM) or [^{3}H]midazolam (110 nM) was added to either the apical or the basal compartment and cells were incubated at 37°C in the presence and absence of MK571 (100 μM). At 1, 2, 3, and 4 h, 50-μl aliquots from the acceptor compartment were sampled, analyzed using a LS 6000IC scintillation counter (Beckman Coulter, Fullerton, CA). The cumulative amount of erythromycin (Q) on the receiver side was plotted as a function of time, and the steady-state flux (J) estimated from the slope (dQ/dt). The apparent permeability coefficient (P_{app}) of unidirectional flux for erythromycin was estimated by normalizing the flux J (mol/s), against the nominal surface area A (0.33 cm^2) and the initial drug concentration in the donor chamber C_0 (mol/ml).

Animal experiments

Adult (8–12 week old) male wildtype mice and Abcc2 knockout mice (Taconic, Germantown, NY) were housed in a temperature-controlled environment with a 12-hour light cycle, and given a standard diet and water *ad libitum*. Experiments were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital. Hepatic Cyp3a activity in mice was assessed by the midazolam sleep test, as described,[19] using a midazolam dose of 12 mg/kg (i.p.). After 2 weeks, mice underwent an erythromycin breath test (ERMBT), as described [4]. Briefly, mice received [^{14}C-*N*-methyl]erythromycin (1 μCi/100 g; i.v.) in 2.5% dextrose, and were placed in a water-sealed polyurethane breath chamber with air continuously drawn through a vapor trap (acetone and dry ice), bubbled through an acidic methanol solution, and finally through 3 gas trapping washes containing 30 mL of gas trapping solution, composed of 27% (vol/vol) methanol, 41% toluene, 5% Emulsifier-safe, and 27% phenethylamine. Collection of breath was performed at 15, 30, 60, 90, 120, 150, and 180 min, and duplicate

samples were analyzed by liquid scintillation counting. Values were used to calculate total $^{14}CO_2$ exhaled during the collection period as described.[4]

Gene expression analysis of mouse livers

RNA was extracted using the RNEasy mini kit (Qiagen, Valencia, CA). RNA samples were amplified from 3 animals per group and then analyzed using real-time PCR with primers for 15 Cyp2c isoforms (Qiagen, Valencia, CA) or with the Mouse Transporter RT^2 Profiles PCR array system on a 96-well format (SABiosciences, Frederick, MD). Each array contained a panel of primer sets for 88 transporter genes, including 27 ABC transporters and 49 solute carriers (SLCs). Relative gene expression was determined using the $\Delta\Delta C_t$ method, and normalized to the housekeeping gene, *Gapdh*.

Protein expression and microsomal incubations

Cyp3a11 protein expression in liver samples was assessed by Western blot.[40] Hepatic microsomes were prepared from wildtype mice and age-matched Abcc2(−/−) mice (n=4 each), as described previously.[40] Total protein concentrations were measures using a BCA protein assay kit (Pierce, Rockford, IL). Microsomal fractions were diluted to a final protein concentration of 4 mg/mL using 0.5 M potassium phosphate buffer (pH 7.4). To 137μL of water, in a 1.7 mL microcentrifuge tube, were added 40 μL of 0.5 M potassium phosphate buffer (pH 7.4), 12 μL of an NADPH regenerating reagent (BD Biosciences, Franklin Lakes, NJ), and 1 μL of 2.5 mM midazolam (Sigma-Aldrich, St. Louis, MO) in DMSO (final concentration, 12.5 μM; final DMSO concentration, 0.5% v/v). The tubes were pre-incubated for 5 min in a water bath at 37°C. Next, the reaction was initiated by the addition of 10 μL of each of the respective microsomal solutions (final protein concentration, 0.2 mg/mL). Reactions were carried out for 30 min before the addition of 100 μL acetonitrile containing 1 μg/mL lorazepam, used as an internal standard, to quench the reaction. Samples were vortex-mixed for 30 sec before centrifugation for 5 min at 13,000 rpm to remove precipitated protein, and the supernatant was transferred to a vial for analysis by liquid chromatography/mass spectrometry (see Supplementary Methods). An additional experiment was performed using ketoconazole to inhibit Cyp3a-mediated metabolism. Ketoconazole in DMSO (final concentration, 0.625 μM; final DMSO concentration, 0.5% v/v) was added to each reaction, followed by 10 uL of microsomes and a pre-incubation period of 15 min at 37°C. The reaction was then initiated by the addition of midazolam (final concentration, 12.5 μM; final DMSO concentration, 1% v/v), and was run for 30 min in at 37°C. Each of the microsome reactions was performed in duplicate.

Patient populations

Demographic characteristics and treatment protocols for the ERMBT[20] and midazolam clearance[41] in the human subjects have been reported elsewhere. Regarding liver function, appropriate criteria for eligibility in the context of the ERMBT have been reported previously.[20, 42] These criteria include: (i) total bilirubin always <1.5 × upper limit of normal (ULN); (ii) elevations in transaminases were allowed as follows: Aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) <1.5 × ULN if concurrent with alkaline phosphatase <2.5 ×ULN, or AST and/or ALT ≥1.5 to ≤5 × ULN if alkaline

phosphatase ≤1.0 × ULN, or AST/ALT ≤1.0 × ULN with alkaline phosphatase ≥2.5 to ≥5 × ULN, or isolated elevations in either AST or ALT or alkaline phosphatase (ie, without concurrent elevations of either AST and/or ALT ≥1.5 × ULN or alkaline phosphatase ≥2.5 × ULN). Written informed consent was obtained for all participants, the studies were approved by the local institutional review board, and Declaration of Helsinki recommendations for human subject biomedical research was followed. Briefly, a cohort of 134 white patients with cancer received 0.04–0.07 mg of [^{14}C-*N*-mehtyl]erythromycin (3–5 μCi) by i.v. bolus. Eight serial breath samples were collected and analyzed as described.[20] The parameter 1/T_{max} was used as a surrogate for erythromycin clearance.[27] To evaluate the possible impact of cancer on the ERMBT, data on 32 healthy volunteers were extracted from the literature.[21]

A separate cohort of 30 patients with cancer was administered 0.025 mg/kg midazolam by i.v. bolus prior to initiating treatment.[41] Eight serial blood samples were collected, processed to plasma and analyzed for midazolam using a validated liquid chromatography-tandem mass spectrometry method.[43] The intrinsic clearance of midazolam intrinsic clearance was calculated as described elsewhere.[44]

Genotyping procedures

DNA was isolated from either whole blood or plasma and then amplified[20] from 108 subjects undergoing the ERMBT and 30 subjects assessed for midazolam clearance. Variation in the *ABCC2* promoter at 7 different loci, including the -24C>T substitution (rs717620) in the promoter region, was determined using direct nucleotide sequencing, as described.[24] These variants were selected on the basis of their relatively high allelic frequency and/or the known or suspected influence on functional properties of the encoded proteins.[26]

Statistical considerations

Due to the exploratory nature of the clinical studies no power calculations for sample size were performed *a priori*. All experiments were analyzed using either one-way analysis of variance, followed by Dunnett's post-hoc test, or Student's *t*-test. Analysis was performed for erythromycin and midazolam independently and all *P* values are two-tailed. Statistical analyses were performed using Graphpad Prism version 5.00 (GraphPad Software, San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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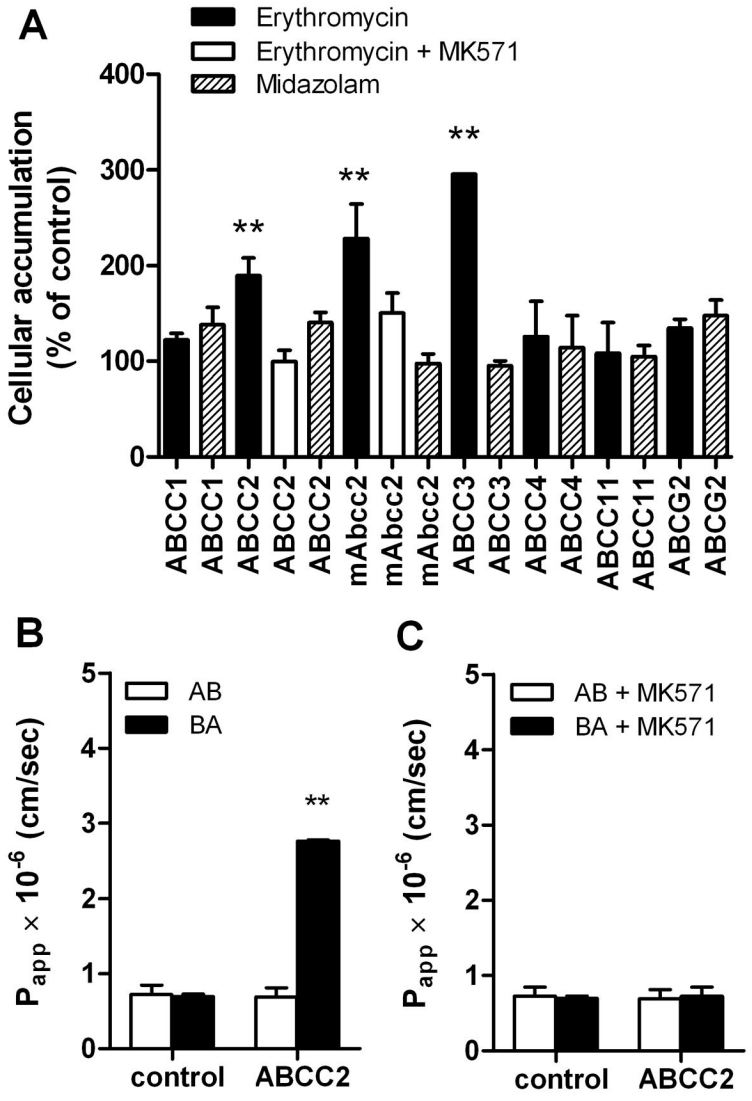

Figure 1.

Erythromycin is a substrate for human ABCC2 and mouse Abcc2, but midazolam is not. (**A**) Using inside-out vesicles, intracellular accumulation of erythromycin and midazolam was assessed in cells over-expressing a variety of ABC transporters (black bars) or in Sf9 control cells (open bars) (n=6/group) in the presence and absence of the ABC transporter inhibitor MK571 (100 μM). (**B**) Transcellular transport assays were performed for erythromycin in cells expressing ABCC2 by showing basal to apical (BA) and apical to basal (AB) transport (n=5–6/group) in the presence and absence of MK571. Data represent mean (bars) and SEM (error bars). ** $P<0.01$.

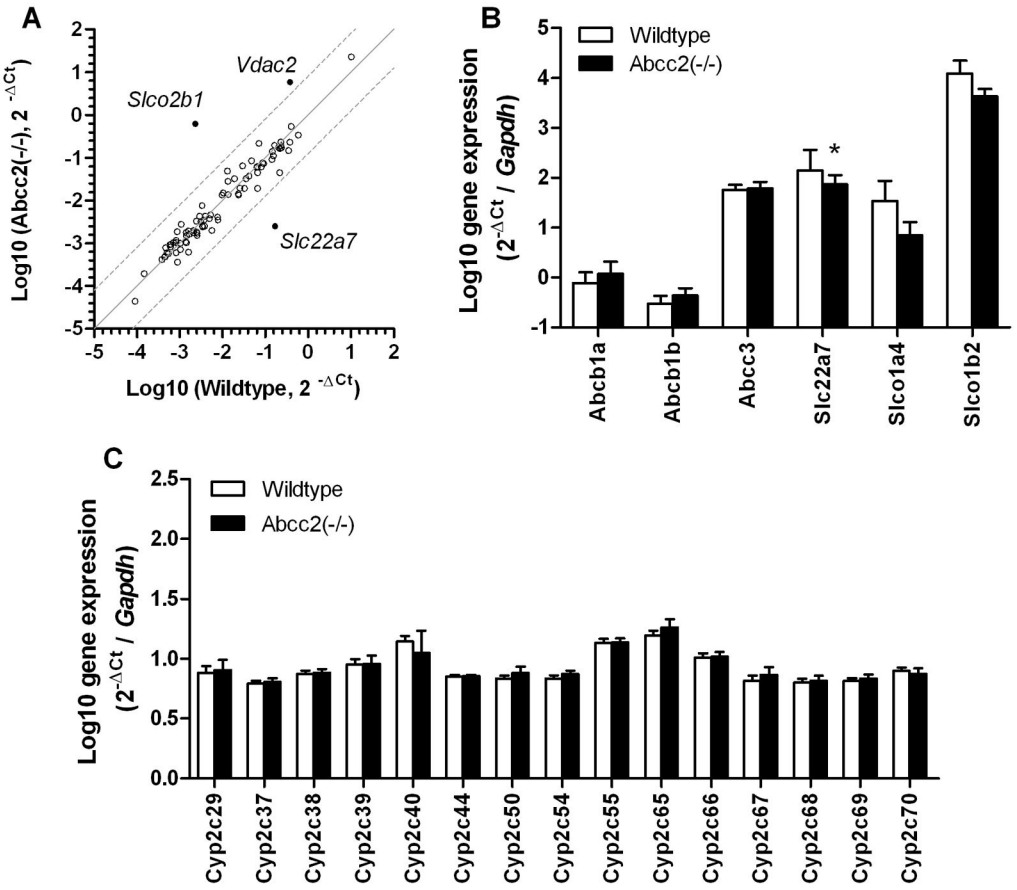

Figure 2.

(**A**) Comparative expression of 88 transporter genes at baseline in livers of wildtype mice and age-matched Abcc2(−/−) mice (n=3 each). Each symbol represents a single gene, the solid line is the line of identity, and the dotted lines are the 95% confidence intervals. Expression of *Slco2b1* (Oatp2b1) and *Vdac2* are increased in the Abcc2(−/−) mice, whereas expression of *Slc22a7* (Oat2) is reduced in the Abcc2(−/−) mice. (**B**) Pairwise comparison of select transporters genes of known or suspected relevance to erythromycin. Data represent mean (bars) and SEM (error bars). (**C**) Pairwise comparison of the 15 known Cyp2c genes. Data represent mean (bars) and SEM (error bars).*, $P<0.05$ vs wildtype mice.

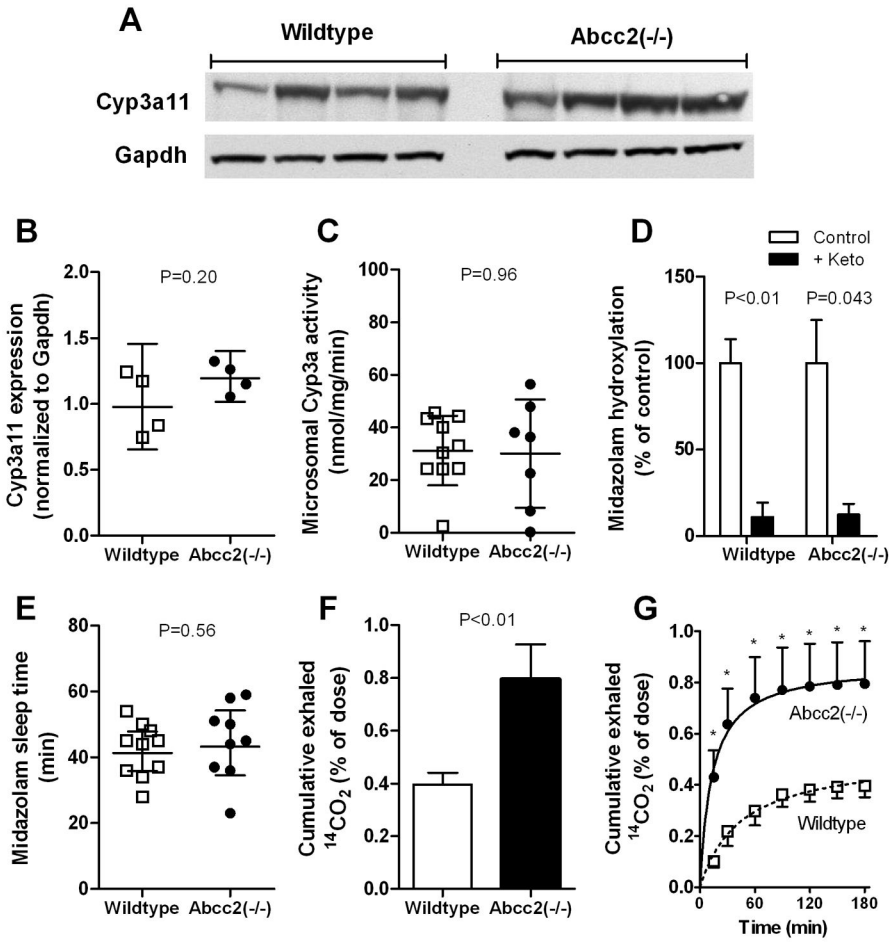

Figure 3.

Loss of Abcc2 alters erythromycin metabolism without impacting Cyp3a function. (**A**, **B**) Western blot of Cyp3a11 protein expression, along with Gapdh, used as a housekeeping gene, in livers of wildtype mice and age-matched Abcc2(−/−) mice (n=4 each). (**C**) Microsomal Cyp3a activity in the same livers, as determined by midazolam hydroxylation. (**D**) Influence of the Cyp3a inhibitor ketoconazole (keto) on the midazolam hydroxylation in the same livers. (**E**) Midazolam sleep test, (**E**) cumulative exhaled $^{14}CO_2$ following an erythromycin breath test (ERMBT), and (**F**) time course of cumulative exhaled $^{4}CO_2$ in wildtype mice Abcc2-deficient mice. n=7–10/group. Data represent individual values (symbols), mean (horizontal lines) and SEM (error bars).

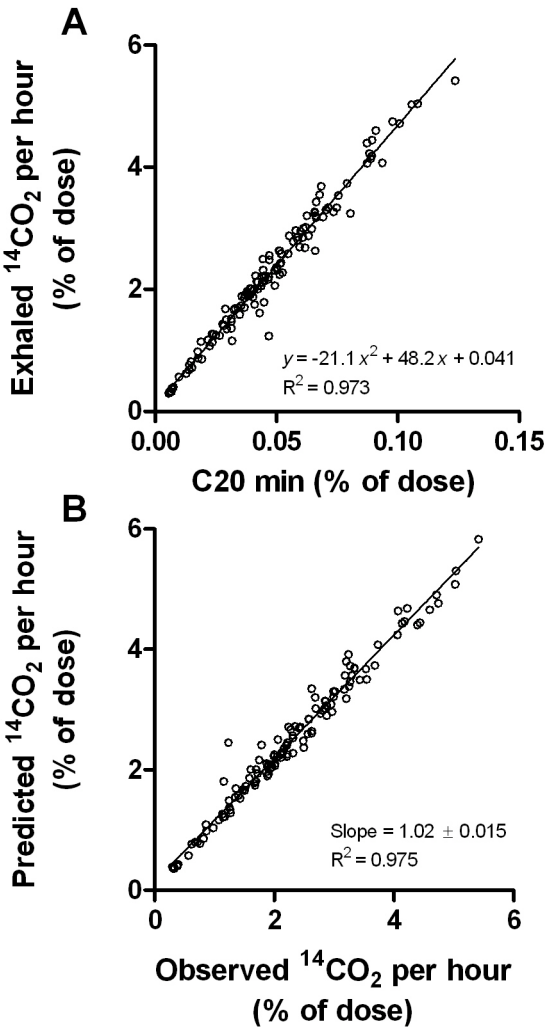

Figure 4.

Comparison of erythromycin breath test (ERMBT) results in cancer patients and healthy volunteers. (**A**) Correlation of a 20-min breath sample collected following the erythromycin breath test in 134 cancer patients with area under the curve for actual $^{14}CO_2$ production during a 1 hour interval. (**B**) Correlation of observed $^{14}CO_2$ production during 1 hour from the same cancer patients with predicted values obtained from a binomial equation originally derived from data obtained in healthy volunteers.[23]

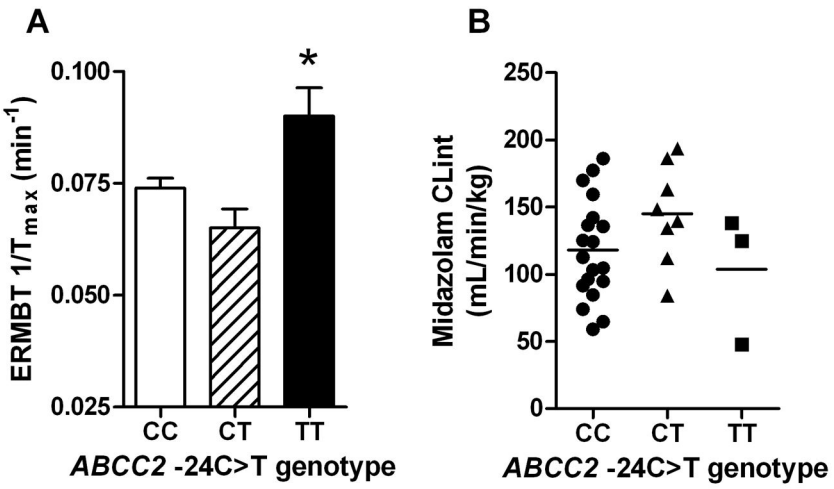

Figure 5.

ABCC2 -24C>T is associated with erythromycin metabolism (**A**) but not midazolam metabolism (**B**). Erythromycin metabolism was assessed using the ERMBT $1/T_{max}$ in 108 subjects, and midazolam metabolism was assessed from intrinsic midazolam clearance (Clint) in 30 subjects. Data represent mean (symbols) and SEM (error bars) for ERMBT and individual values (symbols), mean (horizontal lines) and SEM (error bars) for midazolam. * $P<0.05$.

Table 1

Description and allele frequencies of the investigated *ABCC2* variants[a]

***ABCC2* Genotype**	**Position**	**Effect[b]**	**Function[c]**	**NCBI ID**	***p***	***q***
ABCC2 -1549G>A	5′-flanking	-	Unknown	rs1885301	0.843	0.157
ABCC2 -1019A>G	5′-flanking	-	Unknown	rs2804402	0.617	0.383
ABCC2 -24C>T	5′-UTR	-	Decreased	rs717620	0.832	0.168
ABCC2 1249G>A	Exon 10	V417I	Unknown	rs2273697	0.791	0.209
ABCC2 IVS26 -34T>C	Intron 26	Exon 26	Unknown	rs8187698	0.939	0.061
ABCC2 3972C>T	Exon 28	I1324I	Unknown	rs3740066	0.640	0.360
ABCC2 4544G>A[d]	Exon 32	C1515Y	Unknown	rs8187710	0.971	0.029

[a] Hardy-Weinberg notation for allele frequencies;

[b] Number represent amino acid codon;

[c] Proposed functional activity of variant protein relative to reference protein;

[d] In complete linkage disequilibrium with the polymorphisms 3844-73A>G, 4146+11G>C, 4290G>T (V1430V), 4488C>T (H1469H), and 4508+12G>A.

Abbreviations: NCBI ID, National Center for Biotechnology Information identification number; *p*, frequency for reference allele and *q*, frequency for variant allele; 5′-UTR, five prime untranslated region.

Table 2

Patient demographic data by *ABCC2* -24C>T genotype[a]

Variable	CC genotype	CT genotype	TT genotype	*P*-value
Baseline screening				
Number of evaluable patients	76	26	6	
Age (years)	61 (24–79)	64 (52–83)	69 (59–77)	0.67
Sex (male/female)	50 (66)/26 (34)	11 (42)/15 (58)	3 (50)/3 (50)	0.075
Body-surface area (m^2)	1.88 (1.40–2.50)	2.08 (1.50–2.29)	2.12 (2.08–2.72)	0.90
ECOG performance status	1 (0–2)	0 (0–1)	1 (0–1)	0.27
Primary tumor site				
Breast	15 (20)	10 (38)	2 (33)	0.41
Prostate	26 (34)	5 (19)	0 (0)	
Lung	12 (16)	2 (8)	1 (17)	
Head and neck	5 (7)	6 (23)	0 (0)	
Hepatocellular	1 (1)	0 (0)	0 (0)	
Miscellaneous	17 (22)	3 (12)	3 (50)	
Pre-therapy chemistry				
Total bilirubin (× ULN)	0.40 (0.17–1.25)	0.42 (0.20–0.92)	0.58 (0.30–0.70)	0.70
Aspartate aminotransferase (× ULN)	0.73 (0.28–4.70)	0.89 (0.41–3.20)	0.70 (0.60–2.40)	0.44
Alanine aminotransferase (× ULN)	0.60 (0.18–6.61)	0.60 (0.10–2.20)	0.80 (0.18–1.70)	0.77
Alkaline phosphatase (× ULN)	0.80 (0.36–8.69)	0.80 (0.54–3.88)	1.21 (0.70–2.10)	0.70
α1-acid glycoprotein (mg/dL)	135 (60–254)	129 (81–257)	100 (79–170)	0.55

[a]Continuous data are given as median with range in parenthesis, and categorical data as number of patients with percentage of the total population in parenthesis.

Abbreviations: ECOG, Eastern Cooperative Oncology Group; ULN, upper limit of institutional normal.