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The modulation of transcriptional expression and inhibition of multidrug resistance associated protein 4 (MRP4) by analgesics and their primary metabolites

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ABSTRACT

During the course of a toxic challenge, changes in gene expression can manifest such as induction of metabolizing enzymes as a compensatory detoxification response. We currently report that a single 400 mg/kg acetaminophen (APAP) dose to C57BL/6J mice led to an increase in multidrug resistance-associated (Mrp) 4 (*Abcc4*) mRNA 12 h after administration. Alanine aminotransferase, as a marker of liver injury, was also elevated indicating hepatotoxicity had occurred. Therefore, induction of Mrp4 mRNA was likely attributable to APAP-induced liver injury. Mrp4 has been shown to be upregulated during oxidative stress, and it is well-established that APAP overdose causes oxidative stress due to depletion of glutathione. Given the importance of Mrp4 upregulation as an adaptive response during cholestatic and oxidative liver injury, we next investigated the extent by which human MRP4 can be inhibited by the analgesics, APAP, diclofenac (DCF), and their metabolites. Using an *in vitro* assay with inside out human MRP4 vesicles, we determined that APAP-cysteine inhibited MRP4-mediated transport of leukotriene C_4 with an apparent IC_{50} of 125 μM. APAP-glutathione also attenuated MRP4 activity though it achieved only 28% inhibition at 300 μM. Diclofenac acyl glucuronide (DCF-AG) inhibited MRP4 transport by 34% at 300 μM. The MRP4 *in vitro* inhibition occurs at APAP-cysteine and DCF-AG concentrations seen *in vivo* after toxic doses of APAP or DCF in mice, hence the findings are important given the role that Mrp4 serves as a compensatory response during oxidative stress following toxic challenge.

1. Introduction

Injury following overdose with analgesics is relatively common and can result in extensive damage to multiple organs that may ultimately lead to death in severe cases (Jones, 2002). Mild to moderate toxicity may induce changes vis-à-vis regulation of genes. Such changes involve modulation of transcription factors or induction of drug transporters as a compensatory response (Blazka et al., 1995; Barnes et al., 2007). Intoxication with acetaminophen (APAP), for example, has been demonstrated to result in upregulation of the efflux transporter multidrug resistance-associated protein (Mrp) 4 as well as the metabolic enzyme flavin containing monooxygenase (Fmo) 3, a particularly novel finding given that Fmo3 was previously thought to be non-inducible (Campion et al., 2008; Rudraiah et al., 2014a; Cashman and Zhang, 2002). The mechanism of Mrp4 induction has been demonstrated to be linked to activation of nuclear factor erythroid 2-like 2 (Nrf2, *Nfe2l2*) as evidenced by lack of Mrp4 upregulation in Nrf2 knockout mice (Ghanem et al., 2015). Furthermore, Mrp4 induction by APAP was found to be dependent on Kupffer cell activation (Campion et al., 2008). Mrp4 expression also was induced after administration of clofibrate *via* activation of peroxisome proliferator-activated receptor α (Moffit et al., 2006). In a mouse model, the elevated expression of Mrp4 after an initial toxic APAP insult results in mice becoming refractory

Abbreviations: ALT, alanine aminotransferase; AMP, adenosine monophosphate; APAP, acetaminophen; APAP-CYS, acetaminophen cysteine; APAP-GLU, acetaminophen glucuronide; APAP-NAC, acetaminophen *N*-acetylcysteine; APAP-SUL, acetaminophen sulfate; ATP, adenosine triphosphate; DCF, diclofenac; DCF-AG, diclofenac acyl glucuronide; Fmo, flavin containing monooxygenase; IS, internal standard; LTC4, leukotriene C4; MRP, multidrug resistance-associated protein; OH-DCF, 4′-hydroxy diclofenac; PGE_2, prostaglandin E_2.

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to injury following subsequent toxic doses, an effect termed autoprotection (Aleksunes et al., 2008a).

In the previously cited work by Campion and colleagues, protein induction became evident two to three days after administration of toxic doses of APAP (Campion et al., 2008). Measurement of ALT as an indicator of hepatocellular injury has been utilized in human and rodents, and there is a clear relationship between ALT and manifestations of hepatic injury (Singhal et al., 2012; McGill et al., 2012). Furthermore, Rudraiah et al. (2014b) reported elevations in ALT and Fmo3 mRNA following toxic APAP treatment leading the authors to conclude that induction of Fmo3 was likely a protective mechanism in response to APAP-induced hepatotoxicity. To date, transporter expression as a function of changes in ALT has not been thoroughly investigated.

As expected, administration of xenobiotics results in the generation of metabolites that can be eliminated through biliary or urinary excretion. Work from our group has previously shown that metabolites of APAP and diclofenac (DCF) (Fig. 1) can be generated in high concentrations, with respect to the parent compound, after administration of toxic doses (Scialis et al., 2015; Manautou et al., 2005; Chen et al., 2003). Though metabolite disposition has been characterized, and in some instances the affinity for an uptake or efflux transporter to mediate metabolite clearance quantified (Zamek-Gliszczynski et al., 2006; Zelcer et al., 2003; Scialis and Manautou, 2016; Scialis et al., 2019), there has not been systematic examination as to the potential for these metabolites to interfere with normal transporter function. Despite the extensive pharmacokinetic and toxicological investigations on APAP and DCF as well as characterizations on their metabolites (such as metabolizing enzymes, formation rates, reactivities, etc.), there is still a lack of knowledge on how metabolites of these two important analgesics can perturb critical cellular functions. Considering the importance MRP4 serves as a compensatory response following toxicant challenge, it is crucial to understand the nature of MRP4 activity in the presence of metabolites at concentrations that are known to occur *in vivo*. The work presented herein addresses the aforementioned knowledge gap and represents hitherto unreported findings as it relates to MRP4 function.

Thus, the primary objective of our current work was to first assess the relationship, if one exists, between elevations in ALT and transcriptional regulation of Mrp4. To accomplish this goal, a toxic dose of APAP was given to C57BL/6J mice with measurement of ALT and Mrp4 mRNA carried out at several time points post-administration. The secondary objective of our inquiry focuses on the inhibition of MRP4 by APAP, DCF, and their primary metabolites. This objective was deemed relevant to better understand how MRP4 activity, following its induction after toxic challenge, could be modulated by physiological levels of xenobiotics and their derivatives. The MRP4 inhibition assays were conducted using APAP and DCF metabolite concentrations that reflect those observed during *in vivo* studies from our laboratory.

2. Materials and methods

2.1. Chemicals and reagents

APAP, AMP, ATP, DCF, formic acid, indomethacin (used as the internal standard, IS), KCl, $MgCl_2$, MOPS, 4′-hydroxy diclofenac (OH-DCF), and Tris-HCl, were purchased from Sigma-Aldrich Corporation. (St. Louis, MO). Diclofenac acyl glucuronide (DCF-AG) was purchased from Toronto Research Chemicals Incorporated (Toronto, Canada). Acetominophen

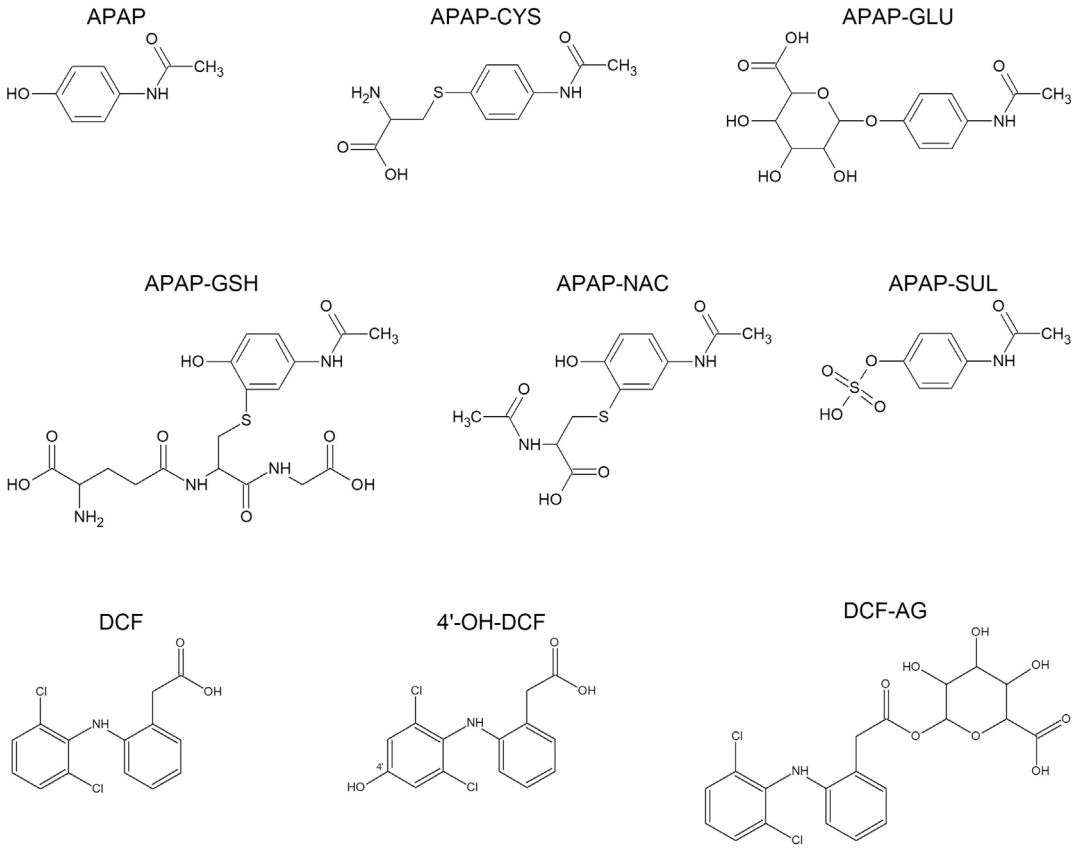

Fig. 1. Structures of APAP, DCF, and their major metabolites. The various conjugated metabolites of APAP as the result of Phase II metabolism or spontaneous adduction to the CYP450-mediated reactive intermediate NAPQI. APAP: acetaminophen, APAP-CYS: acetaminophen cysteine, APAP-GLU: acetaminophen glucuronide, APAP-GSH: acetaminophen glutathione, APAP-NAC: acetaminophen *N*-acetylcysteine, APAP-SUL: acetaminophen sulfate, DCF: diclofenac, 4′-OH-DCF: 4′-hydroxy diclofenac, DCF-AG: diclofenac acyl glucuronide.

cysteine (APAP-CYS), acetominophen glucuronide (APAP-GLU), acetominophen glutathione (APAP-GSH), acetominophen *N*-acetylcysteine (APAP-NAC), and acetominophen sulfate (APAP-SUL) were obtained from McNeil-PPC Incorporated (Fort Washington, PA). Leukotriene C4 (LTC_4) and MK-571 were purchased from Santa Cruz Biotechnology (Dallas, TX). MRP4 vesicles were purchased from GenoMembrane Corporation (Kanazawa, Japan). All LC-MS/MS solvents were of high analytical grade and were purchased from Burdick & Jackson (Muskegon, MI).

2.2. Animal studies

Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in an American Animal Associations Laboratory Animal Care accredited facility at the University of Connecticut under a standard temperature-, light-, and humidity-controlled environment. Mice had free access to Harlan Teklad 2018 chow (Harlan, Madison, WI) and drinking water. Mice, aged 2–3 months, received a single IP dose of 400 mg/kg APAP in 0.9% saline and were sacrificed by decapitation at 0, 6, 12, and 24 h after APAP administration. Blood samples were collected and subsequently centrifuged at 1200 $\times g$ for 15 min to yield plasma. Livers were harvested, rinsed in ice-cold saline, and quickly frozen in liquid nitrogen prior to storage at −80 °C. Untreated 2 month male mice C57BL/6J mice were sacrificed by decapitation for liver harvesting. Livers were quickly harvested, rinsed with phosphate buffer, and snap-frozen in liquid nitrogen. All animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols reviewed and approved by the local Institutional Animal Care and Use Committee at the University of Connecticut.

2.3. Transporter expression

Isolation of RNA and mRNA quantification for major hepatic transporters were conducted as previously reported (Campion et al., 2009). Briefly, RNA was extracted using RNA Bee reagent (Tel-Test, Friendswood, TX), pooled, and quantified with branched DNA (bDNA) method using a QuantiGene® Screen Assay Kit (Panomics, Freemont, CA) following the manufacturer's respective protocols. Probes against mouse uptake Oatps and Ntcp transporters and mouse Mrps and Mdr1 efflux transporters were utilized. Transporter expression was expressed as relative light units (RLU) per 10 μg of RNA.

2.4. RNA isolation and RT-PCR

Total liver RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm, and the integrity was evaluated by agarose gel electrophoresis. RNA (1 μg) was reverse transcribed to cDNA using an M-MLV Reverse Transcriptase kit (Thermo Fisher Scientific). mRNA expression of *Abcc4* was quantified using specific primers (Table 1) by the ΔΔCT method and normalized to the housekeeping gene *Actb*. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA). Amplification was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) and carried out in a 20 μL reaction volume containing diluted cDNA, Fast SYBR Green PCR Master Mix (Thermo Fisher Scientific), and 1 μM of each primer.

Table 1
Primer sequences for quantitative RT-PCR.

Gene (protein)	Primer sequence
Actb (β-actin)	Forward: 5′-GCA ACG AGC GGT TCCG-3′
	Reverse: 5′-GCA GAC AGC CAA GGA GCC CAA AGA CC-3′
Abcc4 (Mrp4)	Forward: 5′-ACC TCT GCT CGC GCG TGT TCT-3′
	Reverse: 5′-CCA GTA CCG TTG AAG CTC CTC TCC-3′

2.5. Clinical chemistry

Plasma samples were analyzed for alanine aminotransferase (ALT) using a kit purchased from Thermo Fisher Scientific as per the manufacturer's recommendations. A BioTek UV/Vis microplate spectrophotometer (BioTek Instruments Incorporated, Winooski, VT) was used to measure assay endpoints.

2.6. In vitro transport

Commercially available MRP4 inside-out vesicles from GenoMembrane were quickly thawed from storage and placed on ice. Incubation reactions consisted of uptake buffer at pH 7.0 (50 mM MOPS-Tris, 70 mM KCl, and 7.5 mM $MgCl_2$), 25 μg vesicle protein, 5 mM of AMP or ATP, and 2.5 mM GSH. After a 5 min pre-incubation period of reaction mixture, incubations were commenced by addition of increasing concentrations of LTC_4 for substrate studies. For inhibition studies, the concentration of LTC_4 was fixed while increasing concentrations of inhibitor was added. Incubations were conducted at 37 °C in a total volume of 75 μL. Reactions were quenched by the addition of 100 μL ice-cold stopping buffer (40 mM MOPS-Tris and 70 mM KCl), and the quenched mixtures were quickly transferred to a 96-well glass-fiber filter plate (EMD Millipore, Billerica, MA). The plate was subjected to vacuum filtration followed by 5 rapid washes of 100 μL/well ice-cold stopping buffer. The filter plate was allowed to dry before extraction of samples. Once the filter plate was dry, LTC_4 was extracted by filling each well of the filter plate with 200 μL of 80:20 (v/v) methanol: water. Plates were shaken for 15 min on ice, and the filtrate was collected *via* centrifugation at 3000 $\times g$ for 10 min and 4 °C. The filtrate was evaporated to dryness under warm N_2 at 40 °C. The resulting residue was reconstituted with 100 μL water and 100 μL IS in methanol, vigorously vortex-mixed, and injected onto the LC-MS/MS. The accumulation of LTC_4 was quantified against a standard curve, and the data were expressed as pmol uptake normalized to mg vesicle protein.

2.7. LC-MS/MS method

Chromatographic separation of analytes was performed on a 2.6 μm Kinetex XB-C18 30 × 2 mm column (Phenomenex Incorporated, Torrance, CA). The system front end consisted of a HTC PAL Autosampler (LEAP Technologies Incorporated, Carrboro, NC), a CBM-20A system controller, two LC20ADvp pumps, and a DGU-14A degasser (Shimadzu Scientific Instruments, Columbia, MD). Analytes of interest were eluted using a gradient program that began with 10% solvent B for the first 0.5 min, which was then increased to 90% solvent B at 1.25 min using a linear gradient and held at this mixture for 0.25 min before reverting back to initial solvent conditions for 0.5 min to re-equilibrate the column. The flow rate for was 0.3 mL/min, and the column effluent was directed to waste for the initial 0.5 min before switching to the mass spectrometer. Analytes were detected using an AB Sciex API™ 4000 LC-MS/MS triple quad mass-spectrometer with a TurboIonSpray® probe and Analyst version 1.5.2 software (AB Sciex, Framingham, MA) that was operated in multiple reaction monitoring mode. Ion spray voltage was −4250 V, and the source temperature was set to 400 °C. The mass transitions in negative ion mode for monitoring LTC_4 and IS were m/z 626.4 → 189.5 and 356.0 → 311.8, respectively. The retention times of LTC_4 and IS were 1.33 and 1.47 min, respectively. Concentrations of analytes in the samples were determined by comparing the peak area ratios (analyte/IS) to those in the standard curve using a linear regression model. The criterion of acceptance for standards was defined to be ±20% of nominal concentration.

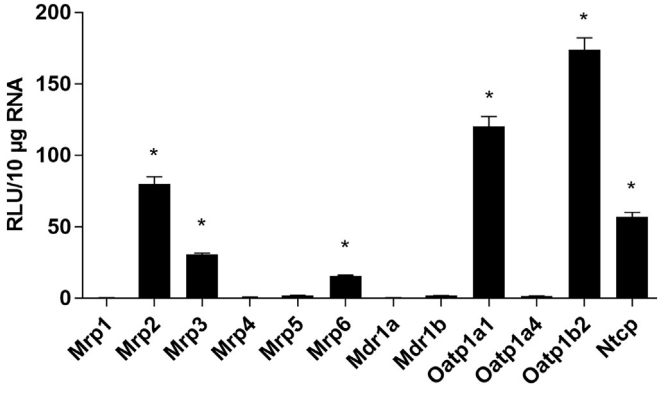

Fig. 2. Transporter expression in C57BL/6J male mouse liver. The basal transporter expression in the livers of naïve male C57BL/6J mice is shown. Expression profiles were detected using a bDNA assay. Data are expressed as relative light units (RLU) per 10 µg RNA. Each bar reflects the mean ± standard error of the mean of n = 4 subjects. *$P < 0.05$ *versus* all other groups.

2.8. Data analysis

Where IC_{50} estimates could be determined for Mrp4 inhibition, K_i values for the inhibitors were calculated according to the following equation (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \quad (1)$$

where K_i is the affinity of the inhibitor, IC_{50} is the inhibitor concentration producing 50% inhibition, [S] is the substrate concentration in the incubation, and K_m is the Michaelis constant representing substrate affinity for the transporter.

2.9. Statistical analysis

Data are expressed as mean ± standard error of the mean. *P* values ≤ 0.05 were considered as statistically significant. Statistical analysis of data was performed using R version 3.5.3 (R Core Team, 2015). Two groups were compared by Student's *t*-test, and multiple groups were compared by an analysis of variance followed by Tukey's *post hoc* test. GraphPad Prism version 6.0 (GraphPad Software Incorporated, La Jolla, CA) was used to calculate kinetic parameters (V_{max} and K_m) as well as the IC_{50} inhibition parameter.

3. Results

3.1. Basal transporter expression

Livers from naïve C57BL/6J male mice were harvested and assessed for transporter expression using a bDNA assay. The liver specific uptake transporter Oatp1b2 (*Slco1b2*) had the highest transcriptional expression of all the transporters investigated (Fig. 2). After Oatp1b2, Oatp1a1 (*Slco1a1*) and Ntcp (*Slco10a1*) had the second and third highest expression, respectively, for uptake transporters. In terms of efflux transporters, Mrp2 (*Abcc2*), which is expressed at the bile canaliculi, was highest while also being the third most abundant of all transporters. The basolateral transporters of Mrp3 (*Abcc3*) and Mrp6 (*Abcc6*) constituted the second and third most abundant of the efflux transporters. The expression of the six aforementioned transporters was statistically significant compared to each other ($P < 0.05$). Mrp4, along with Mrp1 (*Abcc1*) and Mdr1a (*Abcb1a*), had among the lowest basal hepatic transporter expression.

3.2. In vivo study

C57BL/6J male mice were administered a single IP dose of 400 mg/kg APAP in 0.9% saline. Animals were sacrificed 0, 6, 12, and 24 h post administration, and livers were harvested. Plasma was used to assess liver injury using ALT as a marker. The mean ALT values at 0, 6, 12, and 24 h were 41.4, 1140, 760, and 2400 U/L, respectively (Fig. 3A). Transcriptional changes of Mrp4 were assessed at the same time points used for ALT analysis (Fig. 3B). An increase in Mrp4 mRNA was only observed at 12 h, however this increase was statistically significant ($P < 0.05$). Taken together, the data suggest that APAP-induced liver injury, evidenced by high ALT values, resulted in the induction of Mrp4 mRNA.

3.3. LTC_4 kinetics

Human MRP4 was characterized for transport of LTC_4 prior to conducting inhibition studies. The linearity of MRP4-mediated transport for 0.1 µM LTC_4 was evaluated and determined to be time-dependent (Fig. 4A). The dynamic response reached a maximal value of 19-fold (ATP response divided by AMP response) by 20 min. Next, LTC_4 was characterized for concentration-dependent transport. Kinetic parameters of V_{max} and K_m for MRP4 LTC_4 transport were determined to be 245 pmol/min/mg and 4.25 µM, respectively (Fig. 4B). Kinetic data were fitted using a single K_m model without assuming cooperativity.

3.4. MRP4 inhibition

The potential for MRP4 inhibition was carried out in the presence of parent compounds and their metabolites. MK-571 was used as an inhibitor

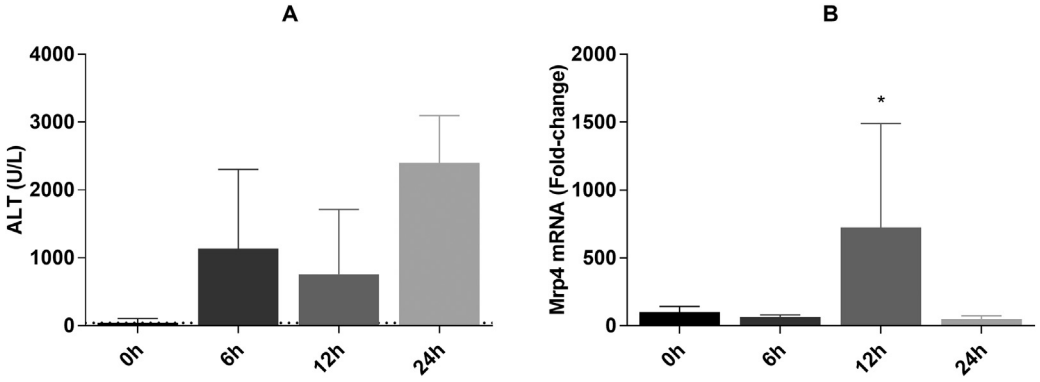

Fig. 3. Relationship between ALT values and Mrp4 gene expression following acute exposure to APAP. Male C57BL/6J mice were administered 400 mg/kg APAP and sacrificed 0, 6, 12, or 24 h after administration. A) ALT was determined from plasma. The horizontal dotted line reflects the ALT upper limit of normal value of 40 U/L. B) Mrp4 mRNA expression was determined *via* RT-PCR using RNA extracted from whole liver homogenate. Data are expressed as the mean ± standard error of the mean for n = 5–7 subjects/group. *$P < 0.05$ *versus* all other groups.

control yielding an IC_{50} of 14.2 μM (Fig. 5), a value that is close to its reported IC_{50} against other probes (Reid et al., 2003a; Rius et al., 2003). APAP-CYS inhibited transport with an IC_{50} of 125 μM. As only MK-571 and APAP-CYS could be fitted to yield IC_{50} values, none of the other compounds are shown with fitted lines. APAP, APAP-GLU, APAP-NAC, and APAP-SUL did not manifest any significant inhibition. APAP-GSH decreased MRP4 transport, but only at the two highest concentrations resulting in 29% inhibition at 300 μM. DCF and its metabolites likewise inhibited MRP4, with DCF-AG showing the greatest inhibition reaching a maximal inhibition of 34% at the highest tested concentration of 300 μM. The apparent K_i values using Eq. (1) for MK-571 and APAP-CYS were calculated to be 13.9 and 122 μM, respectively.

4. Discussion

The objective of our current work was to evaluate the regulation and function of Mrp4 following a toxic challenge by APAP. Analysis of major hepatic transporters in untreated C57BL/6J mouse livers showed that Mrp4 has one of the lowest expression levels in a comparative profile panel of uptake and efflux transporters. These data are comparable to the observations for C57BL/6J mice reported by Slitt et al. and Aleksunes et al. who independently demonstrated Oatp1b2 and Oatp1a1 have the highest and second highest basal expression for hepatic uptake transporters while Mrp2 and Mrp3 dominate efflux transporter expression relative to the other Mrps (Slitt et al., 2007; Aleksunes et al., 2005). The present work is advantageous in that it provides the reader a more thorough assessment of basal hepatic transporter expression in naive mouse liver. Despite its seemingly low hepatic expression during homeostatic conditions, Mrp4 is quite inducible and plays a major role after intoxication by xenobiotics. As an example, work by Campion et al. (2008) showed that among the Mrp class of transporters, Mrp4 was the most inducible after a 500 mg/kg IP APAP dose to C57BL/6J mice, and its protein expression increased 10- and 33-fold by 48 and 72 h, respectively. Furthermore, the mechanism of induction was shown to be dependent on the functional presence of hepatic macrophages known as Kupffer cells. Loss of Kupffer cell viability, *via* targeted cell death by the agent clodronate, resulted in loss of Mrp4 induction. Moreover, induction of Mrp4 following APAP challenge was also found to be dependent on the activation of transcription factor Nrf2, as mice lacking Nrf2 did not show any changes to Mrp4 mRNA or protein levels in response to APAP (Aleksunes et al., 2008b). During the course of our current study, we observed that Mrp4 mRNA was significantly upregulated 12 h, with respect to Mrp4 RNA at 6 and 24 h, after a 400 mg/kg APAP challenge. Curiously, the Mrp4 mRNA levels were essentially the same at 6 and 24 h suggesting that Mrp4 transcriptional activation may be transient. However, given the prior protein expression data, we postulate that the transcriptional upregulation at 12 h was sufficient to be the driving factor for Mrp4 protein levels to reach maximal expression at 48 h.

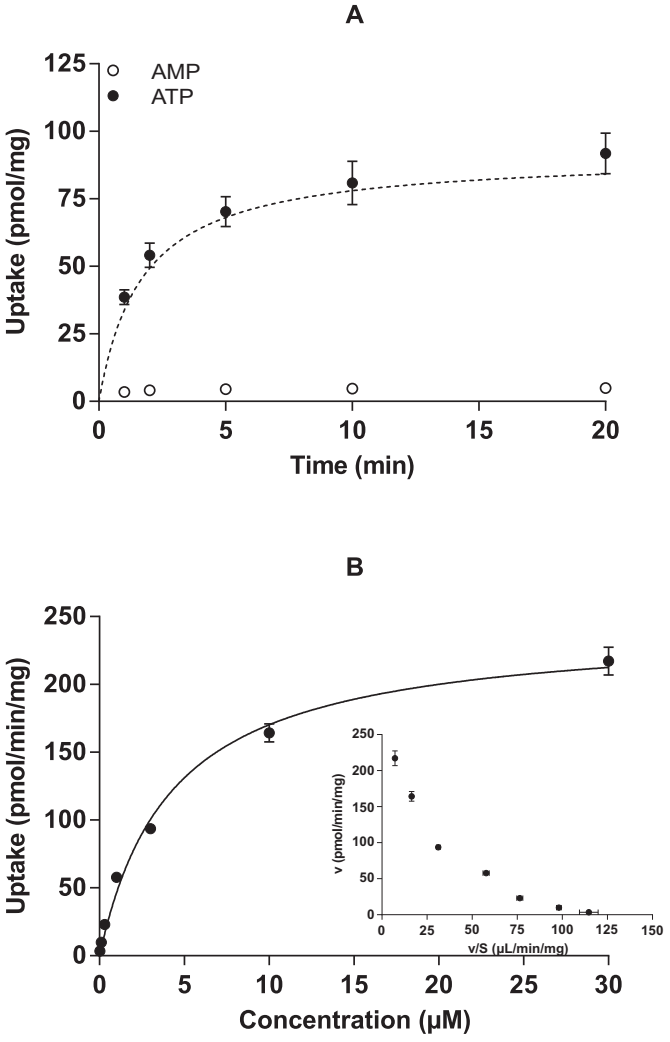

Fig. 4. Time-dependent and concentration-dependent transporter kinetics of LTC_4 with MRP4 vesicles. MRP4 vesicles were incubated with LTC_4 in the presence of either AMP or ATP. (A) The time-dependent transport of 0.1 μM LTC_4 in the presence of 5 mM AMP (○) or 5 mM ATP (●) at 37 °C. The dotted line represents the nonlinear fit of LTC_4 active transport and was calculated by subtracting the AMP values (background and passive uptake) from the ATP response. (B) The concentration-dependent transport of LTC_4 at 37 °C. The fitted line indicates the ATP-dependent transport assuming typical Michaelis-Menten kinetics. Inset: Eadie-Hofstee plot. Each data point reflects the mean ± the standard error of the mean for n = 3 measurements per time point or concentration.

We next sought to draw correlation between liver injury and Mrp4 mRNA. Serum ALT has been used a reliable indicator for liver injury, and we detected ALT levels that were consistent with APAP toxicity. ALT levels were elevated by nearly 20-fold or greater compared to the upper limit of normal at all the time points examined, while Mrp4 mRNA reached maximal induction 12 h post-dose. None of the other hepatic *Abc* transporters showed any significant increase compared to control following APAP administration (data not shown) suggesting that only Mrp4 induction occurs as a result of APAP toxic challenge. Ghanem et al. (2015) similarly reported that ALT and Mrp4 mRNA levels in mouse brain were significantly increased 24 h following toxic challenge by APAP. The sustained ALT in the current study from 6 to 24 h was a clear indication that liver injury was not merely a transient occurrence. Indeed, Rudraiah et al. (2014b) observed an association between ALT and flavin containing monooxygenase-3 (*Fmo3*) mRNA where both reached maximal values 48 h after a 400 mg APAP dose. Despite the fact that ALT and Mrp4 mRNA induction were not correlative across all three measured time points, the results suggest that liver injury following toxic APAP administration, as evidenced by high ALT levels, may have acted as trigger for the induction of Mrp4 mRNA. Interestedly, though it has been shown that perturbation of Mrp4 induction led to increased toxicity after APAP challenge, a recent study demonstrated that wild-type and Mrp4-null mice were equally susceptible to APAP-induced hepatotoxicity at the same 400 mg/kg dose used in the current study (Donepudi et al., 2019). However, it should be noted that Mrp4-null mice have decreased expression of Phase I enzymes involved in APAP metabolism, hence the injury parity between WT and Mrp4-null mice may have been due to decreased generation of reactive metabolites in the Mrp4-null mice. Lastly, the maximal Mrp4 mRNA induction observed at 12 h mimics that observed by Campion et al. (2009) who reported that both Mrp3 and Mrp4 mRNA peaked at 12 h before returning to baseline from 24 to 72 h following toxic challenge to allyl alcohol and that the induction led to increased protein expression of both efflux transporters. For further insight on Mrp3 induction after xenobiotic exposure, the reader is directed to a recent review by Ghanem and Manautou (2019).

Given the importance Mrp4 has as an apparent compensatory protective response during APAP toxicity, we investigated the potential for Mrp4

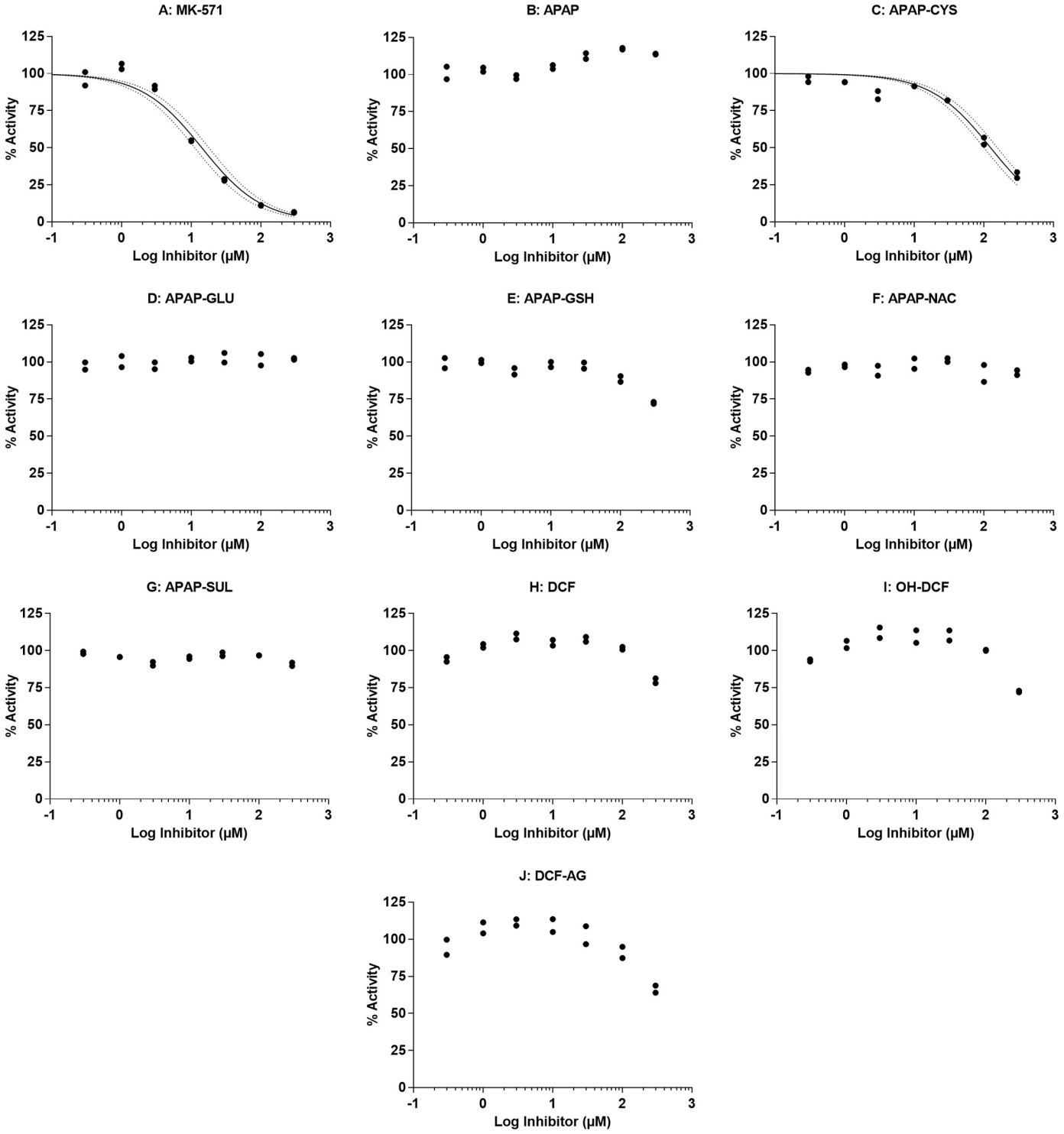

Fig. 5. Inhibition of MRP4 activity by APAP, APAP metabolites, DCF, and DCF metabolites. *In vitro* inhibition was conducted using MRP4 vesicles with 0.1 μM LTC_4 in the presence of 5 mM ATP at 37 °C for 5 min. The ATP-dependent transport of LTC4 in the absence of inhibitor was set to 100%. Inhibition data were fit with a three parameter model to calculate IC_{50}. Dotted lines reflect the 95% confidence intervals of the fit. Only compounds achieving >50% inhibition were fitted. Each data point reflects individual values (n = 2 per inhibitor concentration).

inhibition by the analgesics APAP, DCF, and their metabolites. The rationale for investigating Mrp4 inhibition is based on the finding that Mrp4 is upregulated as a compensatory mechanism during oxidative stress (Xu et al., 2010). Considering APAP and DCF metabolism can cause oxidative stress that can lead to hepatotoxicity (Lauer et al., 2009; Gao et al., 2017), inhibition of Mrp4 by APAP or its metabolites may further exacerbate liver injury and/or hamper tissue recovery. In lieu of mouse Mrp4, we chose to use vesicles containing human MRP4 as a surrogate. Our first course of action was to characterize the assay for performance. Among the substrates for MRP4 are estradiol-17β-glucuronide, furosemide, and methotrexate (Chen et al., 2001; Chen et al., 2002; Hasegawa et al., 2007). The choice of estradiol-17β-glucuronide as a probe can result in

potentially misleading findings as it was shown to be stimulated by numerous compounds that were able to inhibit the vesicular transport for other substrates (Kidron et al., 2012; Gilibili et al., 2017). Hence, we decided to use Leukotriene C_4 as our probe based on the work of Rius et al. (Rius et al., 2008) that identified LTC_4 as a substrate for MRP4. LTC_4 as a measure of MRP4 activity is advantageous since it is a naturally occurring derivative of arachidonic acid and plays a role during pro-inflammatory responses (Dahlen et al., 1983; Zipser et al., 1987). Using inside-out MRP4 vesicles, we determined that the transport of LTC_4 was time-dependent. Furthermore, the transport of LTC_4 was concentration-dependent with a V_{max} and K_m of 253 pmol/min/mg and 4.25 μM, respectively.

We next proceeded to conduct MRP4 inhibition assays, and expanded our assay to include the analgesics APAP and DCF as well as their major metabolites. Our results show that APAP has no effect on MRP4, and neither did APAP-SUL, APAP-GLU, or APAP-NAC. However, we observed that APAP-CYS had concentration-dependent inhibition of MRP4 with an apparent IC_{50} of 125 μM. Based on the *in vitro* parameters from our assays, we calculate a K_i of 122 μM for APAP-CYS. Bearing in mind the low LTC_4 substrate concentration relative to its K_m and ratio of IC_{50} to K_i, it is likely the inhibition of MRP4 by APAP-CYS was of a competitive nature (Burlingham and Widlanski, 2003). This is perhaps suggestive that APAP-CYS may be a substrate of MRP4 rather than an allosteric modulator of transporter activity. Indeed, cysteine derivatives have previously been shown to be transporter substrates. For example, *N*-acetylcysteine (NAC) and cisplatin *N*-acetylcysteine conjugate were separately determined to be substrates for human organic anion transporters (OAT) 1 and 3, however NAC was found to be negative for MRP 1- and 4-mediated transport (Hagos et al., 2017; Hu et al., 2017). That APAP-NAC did not manifest inhibition was surprising considering the structural resemblance it shares with APAP-CYS. Yet, APAP-GSH, which has a longer side chain compared to APAP-NAC and would potentially have greater steric hindrance, was able to inhibit LTC_4 transport by 29% at 300 μM. The inhibition by APAP-GSH may also have been due to competitive transport as it has been shown that glutathione itself is a substrate for MRP4 when tested at millimolar concentrations (Rius et al., 2003; Rius et al., 2006). The results show the high specificity and discretionary recognition by MRP4 of closely related chemical entities. Inhibition by APAP-GSH is intriguing in light of the fact that it is a major metabolite stemming from sequestration of the highly reactive APAP intermediate *N*-acetyl-p-benzoquinone imine (Moore et al., 1985; Mitchell et al., 1973).

A limitation of our study was that we were not able to measure either APAP-CYS or APAP-GSH MRP4-mediated transport due to limited metabolite supply. Follow-up investigation should be conducted to determine if APAP-CYS and APAP-GSH can in fact be transported by MRP4. Overall the results demonstrate that although metabolism can generally be regarded as a detoxification pathway, subsequent metabolites may possess untoward inhibitory potency against mechanisms, such as transporters, that would normally expedite elimination of said toxicants or reactive intermediates. Thus within the realm of discovery toxicology for compounds in preclinical development, it will be important to not only ascertain up- or down-regulation of important clearance mechanisms, but to also determine the extent by which these mechanisms may be impacted by either parent and/or metabolites present at the site of interest. This statement is underscored by the novel finding of MRP4 inhibition by APAP metabolites that were tested at concentrations known to occur following high dose APAP administration.

The interest in DCF as an MRP4 inhibitor comes from numerous observations that DCF, similar to APAP, generates reactive metabolites that may act as contributors to drug-induced liver injury following repeated DCF exposure. Akin to APAP-GSH, DCF and two of its main metabolites produced inhibition of MRP4, with DCF-AG achieving 34% inhibition. The DCF inhibition was not surprising as a previous investigation established that DCF was able to inhibit the MRP4-mediated uptake of methotrexate (El-Sheikh et al., 2007). In that study, DCF was found to possess a two-site affinity with IC_{50} values of 326 and 0.006 μM for the high and low affinity site, respectively. Since DCF only achieved 22% for the current study, the difference in potency is likely reflective of substrate selection. MRP4 inhibition by DCF parent and metabolites may have physiological ramifications. Studies point to MRP4 being an efflux transporter for prostaglandin E2 (PGE_2) (Reid et al., 2003b), which like LTC4 is also derived from arachidonic acid. In light of PGE_2's role in promoting cell survival and repair (Takeuchi, 2014), abrogation of its efflux could interfere with localized signaling in response to DCF-induced injury. It should be noted that the inhibitor concentrations chosen for the *in vitro* MRP4 inhibition assay were physiologically relevant as they reflect the *in vivo* metabolite concentrations observed in prior animal studies (Scialis et al., 2015; Manautou et al., 2005; Chen et al., 2003; Scialis et al., 2019).

5. Conclusions

To summarize our findings, we report that Mrp4 mRNA expression was induced after a single toxic dose of APAP, and that induction was maximal at 12 h. ALT, as a marker of hepatic injury, was also elevated. Also, we show that human MRP4 function was inhibited by APAP metabolites with APAP-CYS achieving an apparent IC_{50} that is physiologically relevant. We believe these findings demonstrate the intricate balance between compensatory transporter upregulation due to xenobiotic intoxication and the possible deleterious effects that xenobiotics continue to exert in the form of their circulating metabolites.

Declaration of competing interest

The authors state no conflict of interests.

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Authorship contributions

Participated in research design: Scialis, Ghanem, Manautou

Conducted experiments: Scialis, Ghanem

Performed data analysis: Scialis, Ghanem

Wrote or contributed to the writing of the manuscript: Scialis, Manautou.

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