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

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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 in gene expression as a compensatory response. Such changes occur in response to APAP overdose, 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 due to 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 to 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 C4 with an apparent IC50 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 that Mrp4 serves as a compensatory response during oxidative stress following toxic challenge.

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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, MgCl2, 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). Acetaminophen

![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.](image-url)
cysteine (APAP-CYS), acetaminophen glucuronide (APAP-GLU), acetaminophen glutathione (APAP-GSH), acetaminophen N-acetylcyesteine (APAP-NAC), and acetaminophen sulfate (APAP-SUL) were obtained from McNeil-PPC Incorporated (Fort Washington, PA). Leukotriene C4 (LTC4) 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 decapititation at 0, 6, 12, and 24 h after APAP administration. Blood samples were collected and subsequently centrifuged at 1200 × g for 15 min to yield plasma. Livers were harvested, rinsed in ice-cold saline, and quickly 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.

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 MgCl2), 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 LTC4 for substrate studies. For inhibition studies, the concentration of LTC4 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, LTC4 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 × g for 10 min and 4 °C. The filtrate was evaporated to dryness under warm N2 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 LTC4 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 HTIC 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 was for 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 LTC4 and IS were m/z 626.4 → 189.5 and 356.0 → 311.8, respectively. The retention times of LTC4 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.

| Table 1 |
| --- |
| Primer sequences for quantitative RT-PCR. |
| Gene (protein) | Primer sequence |
| Actb (β-actin) | Forward: 5′-GCA AGC AGC GGT TCCG-3′  
Reverse: 5′-GAC GAG CAC GGA GCC CCA AGA CC-3′ |
| Abcc4 (Mrp4) | Forward: 5′-ACC TCT GCT GCC GGG TGT TCT-3′  
Reverse: 5′-CCA GTA CCG TTT AAG CTC TCT TCC-3′ |
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):

\[ K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \]

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 canalici, 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. LTC4 kinetics

Human MRP4 was characterized for transport of LTC4 prior to conducting inhibition studies. The linearity of MRP4-mediated transport for 0.1 \( \mu \)M LTC4 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, LTC4 was characterized for concentration-dependent transport. Kinetic parameters of V_{max} and K_{m} for MRP4 LTC4 transport were determined to be 245 pmol/min/mg and 4.25 \( \mu \)M, 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...
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 Ki 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 naïve 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.

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 monoxygenase-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 correlated 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. Interestingly, 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...
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

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 LTC4 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 IC50. 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).
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; Glibili et al., 2017). Hence, we decided to use Leukotriene C4 as our probe based on the work of Riis et al. (Riis et al., 2008) that identified LTC4 as a substrate for MRP4. LTC4 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 LTC4 was time-dependent. Furthermore, the transport of LTC4 was concentration-dependent with a Vmax and Km 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 analogues 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 IC50 of 125 μM. Based on the in vitro parameters from our assays, we calculate a Ks of 122 μM for APAP-CYS. Bearing in mind the low LTC4 substrate concentration relative to its Ks and ratio of IC50 to Ks, 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, surprisingly considering the structural resemblance it shares with APAP-CYS.

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 IC50 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 (PGE2) (Reid et al., 2003b), which like LTC4 is also derived from arachidonic acid. In light of PGE2’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 IC50 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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