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Effect of allyl alcohol on hepatic transporter expression: Zonal patterns of expression and role of Kupffer cell function

Sarah N. Campion^a, Cristina Tatis-Rios^a, Lisa M. Augustine^b, Michael J. Goedken^c, Nico van Rooijen^d, Nathan J. Cherrington^b, and José E. Manautou^{a,*}

^a Toxicology Program, Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, 69 North Eagleville Road, Unit 3092, Storrs, CT 06269-3092, USA ^b Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ, USA ^c Department of Pathology, Schering Plough Research Institute, Lafayette, NJ, USA ^d Department of Molecular Cell Biology, VU Medical Center, Amsterdam, The Netherlands

Abstract

During APAP toxicity, activation of Kupffer cells is critical for protection from hepatotoxicity and up-regulation of multidrug resistance-associated protein 4 (Mrp4) in centrilobular hepatocytes. The present study was performed to determine the expression profile of uptake and efflux transporters in mouse liver following treatment with allyl alcohol (AIOH), a periportal hepatotoxicant. This study also investigated the role of Kupffer cells in AIOH hepatotoxicity, and whether changes in transport protein expression by AIOH are dependent on the presence of Kupffer cells. C57BL/6J mice received 0.1 ml clodronate liposomes to deplete Kupffer cells or empty liposomes 48 h prior to dosing with 60 mg/kg AIOH, i.p. Hepatotoxicity was assessed by plasma ALT and histopathology. Hepatic transporter mRNA and protein expression were determined by branched DNA signal amplification assay and Western blotting, respectively. Depletion of Kupffer cells by liposomal clodronate treatment resulted in heightened susceptibility to AIOH toxicity. Exposure to AIOH increased mRNA levels of several Mrp genes, while decreasing organic anion transporting polypeptides (Oatps) mRNA expression. Protein analysis mirrored many of these mRNA changes. The presence of Kupffer cells was not required for the observed changes in uptake and efflux transporters induced by AIOH. Immunofluorescent analysis revealed enhanced Mrp4 staining exclusively in centrilobular hepatocytes of AIOH treated mice. These findings demonstrate that Kupffer cells are protective from AIOH toxicity and that induction of Mrp4 occurs in liver regions away from areas of AIOH damage independent of Kupffer cell function. These results suggest that Kupffer cell mediators do not play a role in mediating centrilobular Mrp4 induction in response to periportal damage by AIOH.

Keywords

Allyl alcohol; Multidrug resistance protein; Hepatotoxicity; Kupffer cell; Mrp4/Abcc4

Introduction

The expression profile of hepatic uptake and efflux transport proteins in mice in response to centrilobular hepatotoxicant exposure (acetaminophen, APAP and carbon tetrachloride, CCl₄) has been well-characterized (Aleksunes et al., 2005, 2006; Campion et al., 2008). We have demonstrated that mice exposed to toxic doses of these hepatotoxicants exhibit increased levels of Mrp3 and Mrp4 localized to centrilobular hepatocytes in regions with damage (Aleksunes et al., 2005, 2006). Mrp4, which is the most significantly up-regulated efflux transporter in mouse liver after APAP administration, was also found to be increased in livers of patients following ingestion of toxic APAP doses (Barnes et al., 2007). The up-regulation of efflux transporters and decreased expression of uptake transporters in response to chemical injury appears to be a compensatory response to limit the intracellular accumulation of potentially toxic chemicals in the liver as the organ attempts to repair the damage.

Preliminary studies have begun to provide clues on the regulatory mechanisms behind these alterations in hepatic transporter expression. In addition to the transcription factor NFE2-related factor 2, activation of Kupffer cells has been implicated in the regulation of hepatic transporters during drug-induced liver injury (Aleksunes et al., 2008b; Campion et al., 2008). Recent studies in our laboratory utilizing liposomal clodronate to eliminate Kupffer cells revealed that these cells participate in the up-regulation of hepatic Mrp4 protein during APAP-induced liver injury (Campion et al., 2008). These studies have not only shown that Kupffer cells play a significant role in the regulation of efflux transporter expression in response to APAP, but that they also confer protection against APAP hepatotoxicity. Of note, APAP toxicity targets hepatocytes in centrilobular regions of the liver, where Kupffer cells are functionally different from periportal Kupffer cells.

Morphological and functional heterogeneity in Kupffer cell populations within the liver lobule are well known. Kupffer cells are more abundant in periportal regions where they are larger in size and exhibit greater phagocytic activity as compared to those in centrilobular regions (Armbrust and Ramadori, 1996; Kono et al., 2002). Periportal Kupffer cells also produce more superoxide and TNF- α in comparison to Kupffer cells in centrilobular regions. The latter have a greater capacity for IL-6 production (Armbrust and Ramadori, 1996). It is unknown if toxicants that target different regions of the liver lobule may differentially activate distinct Kupffer cell populations, thus affecting the ultimate outcome of toxicity. Additionally, selective activation of functionally different Kupffer cell populations may also influence the expression profile of hepatobiliary transporters.

To date, the effect of periportal hepatotoxicants on liver transport protein expression and zonal localization has not been characterized. The goal of this study was to determine: a) how hepatic transporter expression may change in response to periportal hepatocyte damage, and b) the role of Kupffer cells in periportal hepatotoxicity and in regulation of hepatobiliary transporters. In the present study, Kupffer cells were depleted by treatment of C57BL/6J mice with liposome encapsulated clodronate prior to administration of the periportal hepatotoxicant allyl alcohol (AIOH) (Badr et al., 1986; Belinsky et al., 1986; Badr, 1991). These experiments revealed that AIOH treatment resulted in up-regulation of efflux

transport proteins with a concomitant down-regulation of uptake transport proteins. These changes were independent of the presence of Kupffer cells. Interestingly, the same selective centrilobular induction of Mrp4 that we previously reported with APAP treatment is also observed with AIOH despite the fact that periportal hepatocytes are the preferential targets for injury.

Methods

Animal care and treatment

Male 10–12 week old C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Upon arrival, mice were acclimated for 1 week and maintained in a 12-h dark/light cycle, temperature- and humidity-controlled environment. Clodronate was a gift from Roche Diagnostics GmbH (Mannheim, Germany) and clodronate liposomes were prepared as previously described (Van Rooijen and Sanders, 1994). Clodronate or empty liposomes (100 μ l, i.v.) were administered to mice 48 h prior to AIOH treatment. Animals were fasted for 16–18 h prior to challenge with AIOH. Groups of mice ($n=4-14$) were administered AIOH (60 mg/kg; 10 ml/kg, i.p.) or vehicle control (saline). Livers were collected 12, 24, 48, or 72 h after AIOH treatment. A portion of the liver was fixed in 10% zinc formalin and the remaining tissue was snap-frozen in liquid nitrogen and stored at -80°C until assayed. All animal studies were conducted in accordance with National Institutes of Health standards and the *Guide for the Care and Use of Laboratory Animals*.

ALT activity assay

Plasma ALT activity was measured as a biochemical indicator of hepatocellular necrosis. Infinity ALT Liquid Stable Reagent (Thermotrace, Melbourne, Australia) was used to determine ALT activity according to the manufacturer's protocol.

Histopathology

Liver samples were fixed in 10% zinc formalin followed by paraffin embedding. Liver sections (5 μ m) were stained with hematoxylin and eosin. Sections were examined by light microscopy for the presence and severity of hepatocellular degeneration and necrosis. A grading system similar to one described previously for APAP-induced histopathology (Manautou et al., 1994) was used. Liver injury was scored as follows: no injury=grade 0; minimal injury involving less than 10% injury/death=grade 1; mild injury affecting 10–25% of hepatocytes=grade 2; moderate injury affecting 25–40% of hepatocytes=grade 3; marked injury affecting 40–50% of hepatocytes=grade 4; or severe injury affecting more than 50% of hepatocytes=grade 5. Images of liver sections were captured using a Zeiss Axioplan 2 microscope equipped with a Sony DXS-S500 digital camera.

RNA isolation and branched DNA (bDNA) signal amplification assay

RNA Bee reagent (Tel-test Inc., Friendswood, TX) was used to extract total liver RNA according to the manufacturer's protocol. Mouse Oatp1a1 (*Slco1a1*), Oatp1a4 (*Slco1a4*), Oatp1b2 (*Slco1b2*), Ntcp (*Slc10a1*), and Mrp1-6 (*Abcc1-6*) mRNA were measured using the bDNA signal amplification assay (Quantigene® Screen Assay Kit, Panomics, Fremont, CA)

as previously described (Hartley and Klaassen, 2000; Aleksunes et al., 2005). The data are reported as relative light units (RLU) per 10 µg total RNA.

Preparation of crude membrane fractions

Liver plasma membrane fractions were prepared as previously described (Aleksunes et al., 2006). The resulting pellets were resuspended in sucrose-Tris buffer and protein concentrations were determined using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis

Membrane proteins were electrophoretically resolved using polyacrylamide gels and transblotted overnight at 4 °C onto polyvinylidene fluoride membrane (Micron Separations, Westboro, MA). Immunochemical detection of Oatp1a1, Oatp1a4, Oatp1b2, Ntcp, and Mrp1-6 was performed as previously described (Aleksunes et al., 2006; Campion et al., 2008). The detection of Oatp1a1, Oatp1a4, Oatp1b2, and Ntcp was performed using KS 219, KS 221, KS 223, and K4 antibodies, respectively. Antibodies for the detection of Oatps were provided by Curtis Klaassen and the K4 polyclonal antibody was provided by Bruno Stieger. Detection of Mrp1-6 was performed using MRPr1, M₂III-5, M₃II-2, M₄I-10, M₅I-10 and M₆II-68 antibodies, respectively. All Mrp antibodies were provided by George Scheffer. Equal protein loading was confirmed using β-actin (ab8227, Abcam) as a loading control. Quantification of the intensity of protein bands was performed using Quantity One® 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA).

Immunofluorescence staining and confocal laser scanning microscopy

Mouse liver cryosections (5 µm) were thaw-mounted onto Superfrost glass slides (Fisher Scientific, Pittsburgh, PA) and stored at –80 °C until use. Detection and immunofluorescent staining of Mrp4 was performed as previously described (Aleksunes et al., 2006) using the M₄I-10 primary antibody and Alexa488-labeled secondary antibody to rat IgG (Invitrogen). Tissue sections were mounted in Prolong Gold (Invitrogen, Carlsbad, CA). Images were acquired on a Leica SP2 laser scanning confocal microscope (Leica Microsystems Inc., Exton, PA) equipped with an Argon laser which allowed excitation at 488 nm for detection of Alexa 488. Negative control staining was performed by incubating sections with rat IgG control antibody (Vector Laboratories, Burlingame, CA).

Statistical analysis

mRNA data are presented as mean RLU/10 µg total RNA±standard error. For protein data, quantitative results are expressed as mean protein expression±standard error, and the data are normalized to pooled control values (0 h). Data were analyzed using a one-way analysis of variance followed by Neuman–Keuls multiple range test. *p* values<0.05 were considered significant.

Results

Effect of Kupffer cell depletion on AIOH-induced hepatotoxicity

The efficacy of clodronate liposome treatment in depleting Kupffer cells was assessed by immunostaining for the macrophage marker F4/80. Clodronate liposome pretreatment was effective at eliminating F4/80-positive Kupffer cells throughout the liver within 48 h (data not shown). The time course for Kupffer cell repopulation is similar to previous studies in which a small number of F4/80-positive macrophages began to reappear 5 days after clodronate treatment (Yamamoto et al., 1996; Campion et al., 2008). AIOH treatment by itself resulted in ALT elevations of 433 ± 255 U/L at 12 h and 264 ± 109 U/L at 24 h (Fig. 1). Plasma ALT levels returned to control values (39 ± 7 U/L) by 72 h. Kupffer cell depletion prior to AIOH exposure resulted in significantly higher ALT levels at both 12 h (1837 ± 609 U/L) and 24 h (2777 ± 1284 U/L), as compared to empty liposome pretreated mice. Histopathological analysis confirmed the enhanced AIOH hepatotoxicity in Kupffer cell-depleted mice (Table 1). A larger percentage of clodronate liposome pretreated mice had a histopathological grade of 2 or greater in comparison to empty liposome pretreated mice. Tissues with scores equal to or higher than 2 are considered to have significant hepatotoxicity. Representative images of histopathological changes are shown in Fig. 2. No hepatocellular damage was noted in control livers (Fig. 2A), while mild necrosis was observed after AIOH treatment (Fig. 2B). The severity of AIOH hepatocyte damage was greater in mice pretreated with clodronate liposomes (Fig. 2C). While histopathological analysis of AIOH-treated livers revealed some periportal areas with severe and bridging necrosis, other portal regions within the same tissue section showed minimal damage. This pattern was consistent among all animals treated with AIOH; and as previously stated, the intensity of the damage was less in mice in which Kupffer cells are present.

Gene expression of uptake transporters

qDNA analysis of hepatic transporter mRNA levels revealed reduced uptake transporter gene expression following AIOH exposure (Fig. 3). AIOH treatment resulted in decreased mRNA levels of Oatp1a1 at 24 h (15% of control). Oatp1b2 and Ntcp mRNA levels were decreased at 12 and 24 h to approximately 40–70% of control values. No change in Oatp1a4 mRNA levels was observed (Fig. 3). Clodronate liposome treatment had no effect on AIOH-induced changes in uptake transporter mRNA expression.

Western blot analysis of uptake transport proteins

Protein expression of Oatp1a1 was reduced by AIOH to 73%, 55% and 49% of control levels at 24, 48 and 72 h, respectively (Fig. 4A). Elimination of Kupffer cells resulted in a greater reduction of Oatp1a1 at 24 h as compared to empty liposome pretreated mice. Oatp1a4 protein levels were increased approximately 1.7-fold at 24 and 48 h, while Oatp1b2 protein decreased up to 61% by 24 h in clodronate liposome pretreated mice. Empty liposome pretreated mice exhibited a trend towards reduced hepatic Oatp1b2 levels which was not statistically significant. Ntcp protein was equally reduced by AIOH treatment in both empty and clodronate liposome pretreated mice at 24 and 48 h. Representative 24 h Western blots are shown in Fig. 4B. This is the time point where the most pronounced changes in transport protein levels were seen.

Gene expression of efflux transporters

mRNA levels of several Mrp transport proteins were increased following toxic AIOH exposure. Mrp1 gene expression was 2-fold higher at 24 h after AIOH exposure (Fig. 5). In the absence of Kupffer cells the increase in Mrp1 was even greater (3-fold). Increased mRNA levels of both Mrp3 (40-fold) and Mrp4 (8-fold) were detected at 12 h. Mrp5 mRNA was elevated by approximately 4-fold at both 12 and 24 h after AIOH treatment. Depletion of Kupffer cells resulted in a slightly greater increase in Mrp5 at 12 h in comparison to non-depleted mice. Mrp6 was the only efflux transporter showing reduced mRNA levels in response to AIOH treatment. mRNA levels of Mrp6 were decreased to 45% of control at 24 h (Fig. 5). Hepatic gene expression of Mrp2 was unaltered by AIOH treatment.

Western blot analysis of efflux transport proteins

AIOH increased Mrp1 protein 3.7-fold in mouse liver at 24 h (Fig. 6A). Mrp1 remained elevated through 48 h in empty liposome pretreated mice and through 72 h in clodronate liposome pretreated mice. Enhanced expression of Mrp2 was detected at 48 h (2.3-fold) after AIOH treatment in both clodronate and empty liposome treated mice. While no significant changes in Mrp3 protein were observed, AIOH administration increased hepatic Mrp4 by 8.8-fold at 24 h. Mrp4 remained elevated at 48 and 72 h, regardless of Kupffer cell status. In fact, mice lacking Kupffer cells exhibited higher Mrp4 expression between 24–72 h. Similarly, elevated Mrp5 protein was observed at all time points beyond 12 h. AIOH did not alter Mrp6 protein expression (Fig. 6A). Overall, the lack of Kupffer cells did not influence changes in efflux transporter protein expression by AIOH. For Mrp4, protein expression was significantly higher in mice depleted of Kupffer cells. Representative 24 h Western blots for each efflux transport protein are shown in Fig. 6B.

Immunofluorescent localization of Mrp4 after AIOH treatment

Zonal patterns of Mrp4 protein in response to AIOH treatment were determined by immunofluorescent staining of frozen liver sections. The images shown are from regions of the liver with minimal to mild necrosis, due to the fact that regions with severe portal damage showed intense autofluorescence. Minimal immuno-fluorescent staining of Mrp4 was seen in control mouse liver (Fig. 7A) as previously observed (Aleksunes et al., 2006). AIOH administration induced strong sinusoidal staining of Mrp4 in centrilobular hepatocytes at 24 h (Fig. 7B). Notably, enhanced Mrp4 protein was detected only in cells immediately surrounding the central vein. Kupffer cell-depleted mice exhibited a similar Mrp4 staining pattern (Fig. 7C). Consistent with the protein levels detected by Western blot analysis, centrilobular Mrp4 immunostaining remained strong at 48 and 72 h (data not shown).

Discussion

The purpose of the current study was to determine the effect of a periportal hepatotoxicant on the expression of liver uptake and efflux transport proteins. In addition, the role of Kupffer cells in AIOH hepatotoxicity and their contribution to alterations in hepatic transporter expression was also investigated. AIOH administration resulted in hepatocyte damage localized primarily to periportal regions of the liver lobule. Histological semi-

quantitative scores of liver lesions indicated that the dose of AIOH used (60 mg/kg) resulted in significant, but not overt portal damage. Mice lacking Kupffer cells experienced greater hepatotoxicity as compared to control mice with Kupffer cells present. This novel finding is analogous to previous reports indicating that Kupffer cells are protective against APAP toxicity (Ju et al., 2002; Campion et al., 2008). This contrasts with previous studies investigating the role of Kupffer cells in AIOH toxicity (Przybocki et al., 1992; Ganey and Schultze, 1995; Sneed et al., 1997; Panuganti et al., 2006). Previous findings show that Kupffer cell activation by lipopolysaccharide (LPS) treatment or immobilization stress exacerbates AIOH hepatotoxicity (Sneed et al., 1997; Sneed et al., 2000; Panuganti et al., 2006). Alternatively, inactivation of Kupffer cells by gadolinium chloride treatment in two separate studies either had no effect or attenuated AIOH-induced toxicity (Przybocki et al., 1992; Ganey and Schultze, 1995). Conflicting outcomes between previous and the present study are likely due to differences in the mode of action and specificity of Kupffer cell depleting agents and activators. Treatment with clodronate liposomes has been shown to be more effective than gadolinium chloride in inactivating Kupffer cells (Ju et al., 2002). Nearly complete depletion of Kupffer cells from all regions of the liver lobule has been reported with clodronate liposomes, while gadolinium chloride preferentially targets and inactivates the larger Kupffer cells in periportal regions (Hardonk et al., 1992; Ju et al., 2002; Kono et al., 2002). Furthermore, LPS treatment and immobilization stress are non-specific methods to activate Kupffer cells.

Gene and protein expression of uptake transport proteins Oatp1a1, Oatp1b2 and Ntcp was decreased in mouse liver after AIOH treatment. Increases in Mrp1, Mrp2, Mrp4 and Mrp5 protein expression were also noted. While Mrp3 mRNA was significantly increased at 12 h, there were no changes in Mrp3 protein at any time point. We have previously reported increases in Mrp3 protein in response to mild APAP hepatotoxicity. However during more severe damage by CCl₄, Mrp3 protein expression is reduced (Aleksunes et al., 2005). We postulated that the lower expression of Mrp3 (or absence of changes) when mRNA levels are elevated is possibly due to differential turnover or enhanced protein degradation when tissue damage is more severe. It is worth pointing out that the degree of injury produced by AIOH in the present study is comparable to that observed for CCl₄ in our previous study (Aleksunes et al., 2005). Similar discrepancies in mRNA and protein expression for some of the uptake transporters (Oatp1a1 and Oatp1b2) were also observed.

The transporter results reported here mirror those previously observed with APAP. APAP targets centrilobular hepatocytes due to the higher Cyp450 levels, and therefore, greater APAP bioactivation in centrilobular regions, while AIOH toxicity is localized to periportal hepatocytes due to the higher oxygen levels in this region (Badr et al., 1986; Badr, 1991). It is very intriguing that toxicants with such dissimilar mechanism of action and zonal selectivity for injury produce global changes in liver transport protein expression that are very similar. This suggests that increases in Mrp efflux proteins and decreases in uptake transporter expression might be a general response to toxic insult and not a direct response to the specific toxicant producing the injury. In contrast to APAP-induced toxicity, the presence of Kupffer cells is not required for any of these changes in transporter expression produced by AIOH exposure.

We have previously hypothesized that changes in transport proteins, specifically up-regulation of Mrp4 protein, may play a role in development of resistance to hepatotoxicant re-exposure (autoprotection). Our laboratory has demonstrated a correlation between enhanced Mrp4 protein levels and APAP autoprotection. Enhanced Mrp4 staining localized in proliferating hepatocytes at centrilobular regions was observed in mice that developed resiliency to APAP toxicity, suggesting that this zonal induction is critical for development of tolerance (Aleksunes et al., 2008a).

Zonal localization of Mrp4 was also investigated in the current study by immunofluorescent staining of frozen liver sections. Mrp4 was selected due to its documented association with acquired resistance and enhanced expression in humans exposed to APAP (Barnes et al., 2007; Aleksunes et al., 2008a). Our expected outcome was that Mrp4 would be localized to hepatocytes adjacent to areas of hepatocyte damage in periportal regions. We were surprised to find that Mrp4 was instead confined to centrilobular hepatocytes following AIOH-induced toxicity.

The zonal expression pattern of Mrp4 after AIOH exposure suggests that a unique characteristic(s) of centrilobular hepatocytes is responsible for their ability to selectively up-regulate Mrp4 protein in response to hepatic damage either adjacent to, or distant from this region. Zonal expression gradients have been well characterized for cytochrome P450s (Cyp450s), with induction restricted to perivenous regions where constitutive expression is greatest (Jungermann and Katz, 1989; Buhler et al., 1992). Other metabolic processes, such as glycolysis, gluconeogenesis and amino acid metabolism also exhibit hepatic zonation (Jungermann and Katz, 1989; Gebhardt, 1992; Jungermann and Kietzmann, 1996). Factors which govern the regulation of zonal constitutive or induced expression of proteins are unknown; however recent investigations have begun to provide some clues. The transcription factors constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AHR) are preferentially expressed in centrilobular hepatocytes, and may participate in conferring their centrilobular phenotype (Lindros et al., 1997; Braeuning et al., 2006). More recent data supports the hypothesis that zonal gene expression patterns are regulated by Ras- and β -catenin-dependent signaling pathways, with activation of Ras/ERK signaling stimulating periportal gene expression, while β -catenin signaling induces a centrilobular hepatocyte phenotype (Sekine et al., 2006; Braeuning et al., 2007a, 2007b). Further investigation is required to elucidate if a signaling pathway(s) with functions exclusive to centrilobular areas is responsible for induction of Mrp4 protein by APAP and AIOH.

While Kupffer cells are required for up-regulation of Mrp4 protein in response to APAP toxicity (Campion et al., 2008), elimination of Kupffer cells prior to AIOH exposure did not prevent Mrp4 induction. In fact, Mrp4 induction by AIOH is greater when Kupffer cells are not present. As seen with APAP toxicity, some of the changes in transport protein expression reported here are more pronounced when liver injury is greater (Aleksunes et al., 2005; Campion et al., 2008). This confirms our previously postulated concept that the degree of liver injury dictates the magnitude of changes in protein expression for some transporters. In addition to Mrp4 protein, Mrp1 and Mrp5 gene levels were more significantly altered by AIOH in mice lacking Kupffer cells.

One could speculate that mediators originating from Kupffer cells normally repress hepatic Mrp4 expression and possibly other transporters. This would explain the higher levels of Mrp4 protein in the absence of Kupffer cells when AIOH hepatotoxicity is higher. However, mRNA and protein expression of Mrp4 is the same in empty-and clodronate liposome-treated mice receiving no hepatotoxicant. This argues against a negative regulatory role for Kupffer cells in Mrp4 expression as the main reason for heightened Mrp4 levels in clodronate-treated mice exhibiting higher AIOH toxicity.

In summary, this study demonstrates that exposure of mice to a hepatotoxic dose of AIOH induces a reduction in uptake transporter expression with a concomitant increase in efflux transporters, independent of Kupffer cell function. While Kupffer cells do not contribute to these changes in hepatic transporters, they are required for hepatoprotection from AIOH toxicity. The selective up-regulation of Mrp4 in hepatocytes surrounding the central vein warrants further investigation into the specific mechanism of Mrp4 induction in this subset of hepatocytes. Given that Mrp4 is up-regulated in centrilobular hepatocytes, distant from the region of AIOH-induced hepatocyte damage, it would be interesting to determine if pretreatment of mice with AIOH would protect from a subsequent, higher AIOH dose. This may help provide clues as to the role of enhanced Mrp4 expression in development of tolerance to hepatotoxicant exposure.

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Fig. 1.

Plasma ALT activity after AIOH treatment. Plasma was isolated from empty or clodronate liposome pretreated mice 12, 24, 48, and 72 h following dosing with AIOH (60 mg/kg) or vehicle. The data are presented as mean plasma ALT (U/L) $\pm$ SE ($n=4-14$ animals). Asterisks (*) represent a statistical difference ($p<0.05$) from pooled control mice of the same liposome treatment (0 h) and daggers (†) represent a statistical difference ($p<0.05$) from empty liposome AIOH treated mice.

Fig. 2. Liver histopathology after AIOH treatment. Livers were collected from mice treated with vehicle (A), empty liposomes followed by 60 mg/kg AIOH (B), or clodronate liposomes followed by 60 mg/kg AIOH. Formalin-fixed, paraffin-embedded liver sections were prepared and stained with hematoxylin and eosin. Images were captured at 10× magnification.

Fig. 3.

Gene expression of hepatic uptake transporters. Total RNA was isolated from the livers of mice pretreated with either empty or clodronate liposomes and challenged with AIOH (60 mg/kg) or vehicle. RNA was analyzed by bDNA assay for expression of Oatp1a1, Oatp1a4, Oatp1b2, and Ntcp. The data are presented as mean RLU ± SE (n=4-12). Asterisks (*) represent a statistical difference ($p<0.05$) from pooled control mice of the same liposome treatment (0 h).

A

■ Empty Liposomes
 ■ Clodronate Liposomes

B **Fig. 4.**

Western blot analysis of hepatic uptake transport proteins. Western blots were performed using liver membrane fractions from liposome pretreated mice at 12, 24, 48, and 72 h after AIOH (60 mg/kg) or vehicle treatment (control). The data are presented as mean relative Oatp1a1, Oatp1a4, Oatp1b2, or Ntcp protein expression $\pm$ SE ($n=4-9$) (A) and as representative blots at 24 h with each lane representing an individual mouse (B). β -actin was used as a loading control. Asterisks (*) represent a statistical difference ($p<0.05$) from pooled control (0 h) mice of the same liposome treatment, and daggers (†) represent a statistical difference ($p<0.05$) from empty liposome AIOH treated mice.

Fig. 5. Gene expression of hepatic efflux transporters. Total RNA was isolated from the livers of mice pretreated with either empty or clodronate liposomes and challenged with AIOH (60 mg/kg) or vehicle. RNA was analyzed by bDNA assay for expression of Mrp1, Mrp2, Mrp3, Mrp4, Mrp5 and Mrp6. The data are presented as mean RLU $\pm$ SE ($n=4-12$). Asterisks (*) represent a statistical difference ($p<0.05$) from pooled control mice of the same liposome treatment (0 h) and daggers (†) represent a statistical difference ($p<0.05$) from empty liposome AIOH treated mice.

Fig. 6.

Western blot analysis of hepatic efflux transport proteins. Western blots were performed using liver membrane fractions from liposome pretreated mice at 12, 24, 48, and 72 h after AIOH (60 mg/kg) or vehicle treatment (control). The data are presented as mean relative Mrp1, Mrp2, Mrp3, Mrp4, Mrp5, or Mrp6 protein expression $\pm$ SE (A) and as representative blots at 24 h with each lane representing an individual mouse (B). β -actin was used as a loading control. Asterisks (*) represent a statistical difference ($p < 0.05$) from pooled control

(0 h) mice of the same liposome treatment, daggers (†) represent a statistical difference ($p < 0.05$) from empty liposome APAP treated mice.

Fig. 7. Immunofluorescent analysis of Mrp4 after AIOH treatment. Immunofluorescent staining of Mrp4 was conducted on liver cryosections obtained 24 h after vehicle treatment (A) and AIOH (60 mg/kg) treatment of empty (B) or clodronate (C) liposome pretreated mice. Representative images are shown, with CV indicating central veins and PV indicating portal veins. Scale bar: 71.56 µm.

Table 1

Histopathological grading of liver injury after AIOH treatment

Treatment	Histopathology grade						
	0	1	2	3	4	5	% 2
12 h							
Empty liposome control	4						0% (0/4)
Empty liposome AIOH *	4	3	3	1		1	42% (5/12)
Clodronate liposome control	4						0% (0/4)
Clodronate liposome AIOH *	4	2	3	2			45% (5/11)
24 h							
Empty liposome control	4						0% (0/4)
Empty liposome AIOH *	3	6	4	1			36% (5/14)
Clodronate liposome control	4						0% (0/4)
Clodronate liposome AIOH *		5	3	5		1	64% (9/14)
48 h							
Empty liposome control	4						0% (0/4)
Empty liposome AIOH	6	1	3				30% (3/10)
Clodronate liposome control	4						0% (0/4)
Clodronate liposome AIOH *	1	3	3	1	1		55% (5/9)
72 h							
Empty liposome control	4						0% (0/4)
Empty liposome AIOH	5	3	1				11% (1/9)
Clodronate liposome control	4						0% (0/4)
Clodronate liposome AIOH *	5	3	1			1	20% (2/10)

Livers were removed from mice pretreated with empty or clodronate liposomes at 12, 24, 48, and 72 h after AIOH (60 mg/kg) or vehicle treatment. Following fixation in 10% zinc formalin, livers were paraffin-embedded and stained with hematoxylin and eosin. Sections were evaluated for severity of degenerative and necrotic changes in periportal regions.

* Asterisks represent a statistical difference ($p < 0.05$) from control mice of the same liposome treatment.