Identification and Functional Characterization of a GSH Conjugate Efflux Pathway in the Rat Lens

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Glutathione (GSH) is the principal antioxidant in the lens and is present at unusually high concentrations exceeding those found in any other ocular tissue.1 In the lens, GSH plays a vital role in scavenging reactive oxygen species and maintaining lens protein thiols in a reduced state.2 It is important in the nonenzymatic recycling of ascorbic acid3 and as a cofactor for several enzyme systems that are involved in protecting redox homeostasis including repair enzymes such as thioredoxinase.2 Glutathione also plays a role in the detoxification of reactive xenobiotic compounds and/or endogenous metabolites through GSH conjugation, which is followed by export of the GSH-conjugated compound from the cell.4 These conjugation reactions not only play an important role in xenobiotic metabolism, but also in normal physiological processes through the formation of important biological mediators such as leukotriene C4. However, very little is known about the identity of transport systems that export GSH conjugates from the lens.

Studies in the kidney and liver, the two major sites of GSH synthesis and metabolism in the body, have identified members of the multidrug resistance-associated proteins (Mrp) and/or organic anion transporting polypeptide (Oatp) families as transporters involved in the release of glutathione (GSH) conjugates from the rat lens.5,6 The mechanism of Mrp-mediated export of GSH conjugates involves the coupling of ATP binding and hydrolysis to substrate binding, translocation, and release. From the human genome, eight human MRP isoforms have been reported to be involved in GSH conjugate removal (MRP1-8).7 However, rat Mrp3, 6, and 7 have been reported to be poor transporters of GSH conjugates8–10 and no orthologous Mrp8 gene has been found in rodents.11 In contrast, the Oatp family of transporters function independently of ATP and sodium gradients, and were originally identified as organic anion uptake transporters, although some act to mediate export from the cell.12 Two members of the rat Oatp family, Oatp1a113 and 1a4,14 have been shown to mediate GSH conjugate export.

In this paper, we used a combination of RT-PCR and Western blotting to identify members of the Mrp and Oatp families in the lens at the transcript and protein level. Immunohistochemistry was then used to localize members of the Mrp family to the different regions of the lens with Mrp1 and 5 identified as being ideally positioned to the lens epithelial membrane to mediate GSH conjugate release. To test for Mrp function, we

Key words: lens, detoxification, GSH conjugate release, multidrug resistance–associated proteins

Purpose. To identify and functionally characterize transporters involved in the release of glutathione (GSH) conjugates from the rat lens.

Methods. Polymerase chain reaction and Western blotting were used to screen for the presence of multidrug resistance–associated protein (Mrp) and organic anion transporting polypeptide (Oatp) isoforms, and immunohistochemistry used to localize Mrp isoforms. To test for Mrp function, lenses were loaded with 5-chloromethylfluorescein diacetate and monochlorobimane to form the fluorescent GSH conjugates glutathione methylfluorescein (GSH-MF) and glutathione bimane (GSH-B), respectively, and cultured in artificial aqueous humour (AAH) in the presence or absence of MK571, an Mrp-specific inhibitor, or benz bromarone, a nonspecific organic anion transporter inhibitor. Glutathione-MF and GS-B fluorescence were measured in the AAH media and lenses.

Results. Multidrug resistance–associated proteins 1, 4, 5, and Oatp1a4 were present at the transcript level, but only Mrp1, 4, and 5 were detected at the protein level. Multidrug resistance–associated proteins 1 and 5 localized to the epithelium and peripheral fiber cells, whereas Mrp4 strongly labeled the nuclei. Glutathione-MF and GS-B efflux was significantly decreased and accumulation in the lens significantly increased in the presence of MK571, indicating that the Mrps are the predominant transporters involved in GSH conjugate release from the lens. Glutathione-B conjugate efflux was further inhibited in the presence of benz bromarone, suggesting that alternative organic anion pathways were involved in mediating GS-B efflux.

Conclusions. Multidrug resistance–associated proteins are present in the lens and may be used to remove endogenous and exogenous compounds from the lens via GSH conjugation. This may represent an important pathway of detoxification required to minimize oxidative stress and maintain lens homeostasis.

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used two nonfluorescent probes, 5-chloromethylfluorescein diacetate (CMFDA) and monochlorobimane (mCB) which upon conjugation to GSH form the fluorescent compounds glutathione methylfluorescein (GSH-MF) and glutathione bimane (GSH-B), respectively. We demonstrate that the lens is capable of releasing both fluorescent GSH conjugates and that this release is significantly inhibited by the Mrp-specific inhibitor, MK571, suggesting that the Mrps are the predominant pathway for the removal of GSH conjugates from the lens.

**Materials and Methods**

**Reagents**

Phosphate-buffered saline (PBS) was prepared from PBS tablets (Sigma-Aldrich Corp., St. Louis, MO, USA). The Mrp1 (A23) antibody was purchased from Enzo Life Sciences (Farmingdale, NY, USA). The C-terminus Mrp5 (C-17) antibody and its corresponding control peptide, and the C-terminal organic anion transporting polypeptide Oatp1a4 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The C-terminal Mrp4 antibody (ab32550) and its corresponding control peptide were purchased from Abcam (Cambridge, UK). The goat anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor 488 secondary antibodies, and the membrane marker wheat germ agglutinin (WGA) conjugated to Alexa Fluor 594 were obtained from Life Technologies (Carlsbad, CA, USA). Monochlorobimane (mCB) and benzbromarone were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich Corp.

**Animals**

All animals were treated according to the ARVO Statement for the use of Ophthalmic and Vision Research and the University of Auckland (Auckland, NZ) Animal Ethics Committee. Eighteen- to 21-day-old Wistar rats were killed by CO2 asphyxiation. The eyes were removed and lenses immediately extracted from the globe and placed in PBS.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Kidney and brain tissue (positive controls), and whole lenses (epithelial and fiber cells) were extracted from 18- to 21-day-old Wistar rats and total RNA isolated using Trizol according to the manufacturer’s protocol (TRizol reagent; Life Technologies). Genomic DNA was removed from total RNA before complementary DNA (cDNA) synthesis by incubation with 10 U/µl recombinant DNase I (Roche Diagnostics, Basel, Switzerland). Kidney, brain, and total lens cDNA were synthesized from 5 µg total RNA mixed with 50 µM oligo(dT)20 in 8 µl final reaction volumes. The RNA was denatured at 65°C for 5 minutes, immediately placed on ice to cool, and then combined with 2X First-Strand Reaction Mix and SuperScript III/RNaseOUT Enzyme Mix (Life Technologies) for cDNA amplification. A control reaction (no cDNA synthesis) was also conducted in the absence of SuperScript III/RNaseOUT enzyme. Synthesized cDNA or control reaction (0.5–1 µl) were added to separate PCR mixtures containing final concentrations of 1X PCR buffer, 2.5 mM MgCl2, 0.25 mM dNTPs, 0.05 U/µl Platinum Taq Polymerase (Life Technologies), and 0.25 µM sense and antisense primers15,16 (Table). All primers were synthesized and purified by Life Technologies (Auckland, NZ).

The DNA polymerase was heat activated at 94°C for 3 minutes prior to PCR cycling. Polymerase chain reaction amplification was performed as follows: denaturation at 94°C for 1 minute, annealing at 55°C (Mrp1, 2, 4, 5) or 57°C (Oatp1a1, Oatp1a4) for 1 minute, extension at 72°C for 1 minute for 36 cycles, and a final extension at 72°C for 10 minutes. Amplified PCR products were analyzed by electrophoresis on a 1.5% wt/vol agarose gel containing SYBR Safe (Life Technologies, Carlsbad, CA, USA) and visualized using an UV illuminator. Polymerase chain reaction bands were subsequently extracted and DNA sequenced. The primer sets and the expected sizes of PCR products are listed in the Table.

**Immunohistochemistry**

Whole lenses were fixed in 0.75% wt/vol paraformaldehyde, cryoprotected, and cryosectioned in an equatorial orientation using standard protocols developed in our laboratory.17 Sections were washed three times and incubated in blocking solution (3% wt/vol bovine serum albumin and 3% vol/vol normal goat/donkey serum) for 1 hour to reduce nonspecific labeling. The sections were then labeled with either Mrp1 (1:50), 4 (1:400), or 5 (1:200) antibodies diluted in blocking solution, followed by the goat anti-rabbit Alexa Fluor 488 (1:100) or donkey anti-goat Alexa Fluor 488 (1:400) for 2 hours each. Peptide control experiments for Mrp4 and 5 followed identical procedures except that the primary antibodies were incubated with at least 15-fold excess of their antigenic peptide for 2 hours at 37°C, followed by overnight incubation at 4°C with gentle rotating. The complexes were then pelleted by centrifuging at 12,000g for 15 minutes and the supernatant added to the sections. To highlight cell morphology, cell membranes were labeled with WGA Alexa Fluor 594 (1:50) in PBS and to highlight epithelial and fiber cell nuclei, sections were stained with DAPI (1:10,000). Sections were then washed and mounted with VECTASHIELD Hardset aqueous mountant (Vector Laboratories, Burlingame, CA, USA) and viewed using an Olympus FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan). To facilitate comparison between data sets, the same pinhole size was used. Specific emission filter sets were used to detect signals from Alexa Fluor 488, WGA Alexa Fluor 594, and DAPI fluorophores.

**Crude Membrane Protein Preparation**

Total lens crude membrane protein fractions (~60 µg protein/lane) were prepared from 8 to 18 whole lenses pooled together. Lens epithelial membranes were prepared by peeling away and pooling the capsule and adherent epithelial cells of 16 to 26 lenses. Membranes from the outer cortex, inner cortex, and core regions were prepared from eight to nine decapsulated lenses using a microscope and a pair of sharpened tweezers, and dissected into their distinct zones based on their physical properties. The superficial layers of the lens comprising of differentiating fiber cells were pooled away and pooled as the outer cortex fraction. The inner cortex was obtained by peeling away the subsequent sticky inner layers of the lens to leave behind a hard mass of tissue which was retained as the core fraction. Total kidney (~18–45 µg protein/lane) and brain (~10 µg protein/lane) crude membrane protein fractions were prepared by careful excision of these tissues. All samples were homogenized in 5 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 5 mM EGTA, pelleted, and washed four times at 13,000g for 20 minutes in storage buffer (5 mM Tris [pH 8.0], 0.2 mM EDTA, and 100 mM NaCl). The resultant crude membrane fractions containing both membranous and cytoplasmic pools of proteins were stored in storage buffer at ~80°C. Concentrations of lens, kidney, and brain membrane samples were...
TABLE. Polymerase Chain Reaction Primer Sets and Predicted Product Sizes for Mrp1, 2, 4, 5, Oatp1a1, and 1a4

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>GenBank Access No.</th>
<th>Expected PCR Product Size, kb</th>
<th>Sense 5’–3’</th>
<th>Antisense 5’–3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mrp1</td>
<td>AJ277881</td>
<td>511</td>
<td>GCACTGGCTTCTAAGTATTG</td>
<td>TCTCAATGGAGTGTAGC</td>
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<tr>
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<td>808</td>
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<tr>
<td>Mrp4</td>
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<td>229</td>
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<tr>
<td>Mrp5</td>
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<td>310</td>
<td>GGAACGGCAGTTGTATT</td>
<td>CTGGCCACCCACTTG</td>
</tr>
<tr>
<td>Oatp1a1</td>
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<td>1025</td>
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<tr>
<td>Oatp1a4</td>
<td>U95011</td>
<td></td>
<td>GAGTACCTTCTGGTTTCTTAGCTA</td>
<td>AACATACGCCAATCTGGGTTAACCAAG</td>
</tr>
</tbody>
</table>


determined using the Pierce 660 nm protein assay kit (Rockford, IL, USA).

Western Blotting

Proteins were first separated on a 4% to 15% vol/vol precast gradient polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA), or a 10% and 7.5% vol/vol acrylamide separating gel with 4% vol/vol stacking gel, and then transferred onto the Immuno-Blot PVDF membrane (Bio-Rad Laboratories) by electroforesis for 60 minutes at 170 mA. After transfer, membranes were incubated with blocking solution (5% wt/vol) for 1 hour. Labeled protein was visualized using enhanced chemiluminescence (ECL Prime; GE Healthcare, Buckinghamshire, UK) at room temperature for 1 hour and then incubated with primary antibodies (Mrp1 [1:200], 4 [1:200], 5 [1:200], and Oatp1a4 [1:200]) diluted in 1× TBS-T buffer containing 2% vol/vol skim milk powder or 1× Tris-buffered saline with Tween 20, [TBS-T]: 0.1% vol/vol Tween-20, 20 mM Tris, and 137 mM NaCl (pH7.6) at room temperature for 1 hour and then incubated with primary antibodies (Mrp1 [1:200], 4 [1:200], 5 [1:200], and Oatp1a4 [1:200]) diluted in 1× TBS-T buffer containing 2 mM EDTA (pH 8.0) and 1% wt/vol BSA overnight at 4°C. Peptide control experiments for Mrp4 and 5 were also performed as previously described above for immunohistochemical peptide control experiments to determine antibody specificity. After rinsing twice with milliQ water and washing three times for 10 minutes in 1× TBS-T buffer, the membranes were then incubated with horseradish peroxidase conjugates (donkey anti-rabbit, 1:10,000; rabbit anti-goat, 1:10,000; GE Healthcare, Buckinghamshire, UK) at room temperature for 1 hour. Labeled protein was visualized using enhanced chemiluminescence detection (ECL Prime; GE Healthcare) and developed using the Fujifilm Luminescent Image Analyser LAS-4000 System (GE Healthcare).

Mrp Functional Assays

Whole lenses were dissected and cultured in media containing 1 mL of prewarmed isosmotic artificial aqueous humour (AAH; 125 mM NaCl, 0.5 mM MgCl2, 4.5 mM KCl, 10 mM NaHCO3, 2 mM CaCl2, 5 mM glucose, 20 mM HEPES, pH 7.2–7.4, 300 ± 5 mOsm) containing 1% vol/vol penicillin/streptomycin/neomycin and loaded with glucose by centrifugation at 13000g for 20 minutes at 4°C and analysis of the supernatant. Isosmotic AAH alone or 5 mM Tris (pH 8.0) were used as background fluorescence controls and these values subtracted from media and lens sample values, respectively. Fluorescence was measured using the SpectraMax M2 Multi-Mode Microplate reader ( Molecular Devices, Sunnyvale, CA, USA) at the wavelengths specified. Glutathione-MF is presented using raw fluorescence values, whereas GS-B concentration is quantified using a GS-B standard curve, which was prepared by incubating 1 mM GSH and increasing concentrations of mCB (0–100 μM), shaking in the dark for two hours at 37°C. All data were analyzed and presented in two ways. Firstly, the percentage efflux of each fluorescent probe was calculated as the concentration/raw fluorescence of the probe in the media as a proportion of the total concentration/raw fluorescence of the probe in the lens and media. Secondly, the accumulation of the fluorescent probe within the lens was calculated by normalizing concentration/raw fluorescence of the probe in the lens by lens wet weight. Data were expressed as the mean ± SE.

Lactate Dehydrogenase (LDH) Assay

In order to ensure that GSH conjugate efflux was not a result of release from damaged membranes, an LDH assay (Roche Applied Science, Roche Diagnostics) was used to screen for assessing lens viability. A fresh LDH standard dilution series was prepared using Type III bovine heart L-LDH. In brief, 100 μL reaction solution containing diaphorase/NAD+ mixture, iodotetrazolium chloride, and sodium lactate was mixed with either 100 μL LDH standard solution or 100 μL samples (appropriately diluted as required) in a 96-well plate for 15 minutes at room temperature. Absorbance was measured at a wavelength of 490 nm using a Synergy 2 Multi-Detector Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Sample LDH absorbance was calculated as an average of triplicate values, and activity determined using the standard curve. Data is presented as percentage LDH release and is calculated based on the LDH activity detected in the media as a proportion of the total LDH activity in the lens and media. Prior to LDH analysis, a predetermined LDH “cut-off” value was calculated to distinguish between controlled transport and membrane leakage as a result of loss of lens integrity. This involved measuring the release of fluorescent GSH conjugates (expressed as %fluorophore efflux) plotted against the corresponding %LDH release values (Supplementary Fig S1). It can be seen that for GS-MF and GS-B fluorescence, the data clusters tightly below 0.1% LDH release. This is likely to represent basal LDH release for individual lenses that are loaded with exogenous compounds. Therefore, individual
lenses that exhibited LDH release greater than 0.1% when loaded with CMFDA or mCB were excluded from the study.

Data Analysis

Statistical analyses were done with GraphPad Prism, version 5.02 (GraphPad Software, Inc., San Diego, CA, USA). Standard curves for GS-B were prepared by fitting data to a linear regression equation and for LDH by fitting data to a nonlinear quadratic equation. In all analyses, a P value less than 0.05 was deemed to be statistically significant. The Mann-Whitney U test was used to detect significance between vehicle- and inhibitor-treated lenses. All data are presented as the mean ± SEM, with statistical significance displayed as *P less than 0.05, **P less than 0.01, ***P less than 0.001, or ****P less than 0.0001.

Results

Molecular Identification of GSH Conjugate Efflux Transporters

In tissues such as the liver, brain, and kidney, GSH conjugate removal is predominantly mediated by Mrp1, 2, 4, 5,7 Oatp1a1, and 1a4.15,14 To determine which, if any, of these transporters were present in the rat lens at the transcript level, RT-PCR was performed using Mrp and Oatp isomorph-specific primers (Table).

Polymerase chain reaction products of the predicted size were obtained from the control tissues, kidney (K+) or brain (B+), thereby verifying primer specificity (Fig. 1). However, PCR products for Mrp1, 4, 5, and Oatp1a4 were only obtained for total lens mRNA (L+). The products were sequenced and found to be identical to the sequences contained in GenBank confirming Mrp1, 4, 5, and Oatp1a4 to be present at the transcript level in the rat lens.

We next wanted to confirm that the four putative GSH conjugate efflux transporters identified at the transcript level were also expressed at the protein level by Western blotting. The same positive control tissues used in our PCR analysis were also used for Western blotting with kidney used for Mrp1, 4, and Oatp1a4 validation and brain for Mrp5 validation. For each transporter, a blot of the control tissue crude membrane protein was shown alongside total lens crude membrane protein. The protein bands that represent the transporter of interest are indicated by black arrowheads. Where control peptides (+P) were available to test antibody specificity, these blots are shown alongside the blots with antibody only and no control peptide.

A strong single band for Mrp1 was observed at approximately 170 kDa in the kidney (K) and in the lens (L; Fig. 2A). There was no control peptide commercially available to confirm the specificity of this single band. However, the size of this single band is consistent with the reported size of rat Mrp1 being approximately 170 to 190 kDa in the liver,18,19 kidney,20 and brain.21–23 It has been reported that in these tissues, the 170-kDa band represents the deglycosylated form, whereas the 190-kDa band represents the glycosylated form.24,25 Therefore, the 170-kDa band in the rat lens most likely represents the deglycosylated form of Mrp1.

Several bands for Mrp4 were detected in the rat kidney and lens (Fig. 2B), which is not surprising, given that intertissue variations in Mrp4 protein size have been reported as a result of differential glycosylation.26,27 In the kidney, of the five bands observed, a band at 140 and 37 kDa were completely knocked down by preabsorption of the Mrp4 antibody with its antigenic peptide. Although the 140-kDa band size is lower than the reported band size of 150 to 170 kDa for Mrp4 in rat kidney,28–30 studies have also reported a band for human MRP4 at 140 kDa in human kidney tissue and human embryonic kidney (HEK293) cells.31–33 In addition, the size of the 140-kDa band is consistent with the two known transcripts of Mrp4 from the Ensembl Protein database.34 It has been reported that the 140- to 150-kDa band represents the deglycosylated form of Mrp4.35–37 It is unclear what the 37-kDa band represents but may be a degraded/truncated form of Mrp4. The additional bands at approximately 75, 50, and 45 kDa were only partially knocked down when the Mrp4 antibody was preabsorbed with its antigenic peptide (Fig. 2B). In Western blot analysis of human kidney lysates, bands at 53, 42, and 32 kDa have also been noted by Abcam, the company that manufactures the Mrp4 antibody, although the identity of these bands could not be confirmed. Interestingly, these bands are of similar size to the bands seen in our blot and may represent degraded forms of Mrp4. In the lens, multiple bands were shown to be specific for Mrp4 following complete knockdown of bands in control peptide experiments (Fig. 2B). These bands were consistently detected at approximately 130, 70, 55, 45, and 37 kDa. It is likely that the 130-kDa band represents full length Mrp4 and that the lower molecular weight bands may represent degradation products, similar to what was seen in the kidney.

In the brain, multiple bands were detected for Mrp5 (Fig. 2C). Of these, only a band at approximately 140 kDa appeared to be completely knocked down following preabsorption of the Mrp5 antibody with its antigenic peptide. However, this band is lower than the reported size of approximately 155 kDa for rat Mrp5 in the Ensembl Protein database.34 Additional bands were also detected that were partially knocked down at 220 and 60 kDa. The 220-kDa band may represent the glycosylated form of Mrp5 because studies have shown that human Mrp5 is able to be deglycosylated in the presence of the glycosidase PNGase F.30 However, one study has suggested that protein bands above 200 kDa for Mrp5 may be a result of incomplete separation of protein aggregates.35 It is unclear what the strong 60-kDa band in the brain represents but it was interesting to note that it was brain-specific as it was absent in the lens. This band size has also been observed in...
human MRP5-overexpressing HEK293 cells where it has been
that suggested to represent a breakdown product. In the
lens, two major bands were detected for Mrp5 with the 230-
and 150-kDa bands completely knocked down following
preabsorption of Mrp5 with its antigenic peptide (Fig. 2C).
The 150-kDa band in the lens is slightly lower than the
reported size for Mrp5 in the brain, but it is similar to the
predicted size of Mrp5 in the Ensembl Protein database.
The difference in sizes may represent differential processing of the
Mrp5 protein as has been noted for Mrp1 and 4. Multidrug
resistance–associated protein 5 has eight potential glycosyla-
tion sites indicating that the 230-kDa band in the lens may
represent the glycosylated form of the Mrp5 protein.

In the kidney, two bands were detected for Oatp1a4 at
approximately 73 and 100 kDa (Fig. 2D). While no control
peptide was available to confirm specificity, previous studies
have shown Oatp1a4 to be detected at 65 to 75 and 115 kDa in
the rat kidney and between 68 and 92 kDa in the rat brain,
retina, and ciliary body. Because Oatp1a4 has five
potential glycosylation sites, it is believed that the variation
in molecular sizes for Oatp1a4 is most likely a result of
differential glycosylation of the Oatp1a4 protein, which may
explain the additional bands seen in the blot. No bands were
detected for Oatp1a4 in the rat lens with the antibody
employed in this study, indicating the absence of Oatp1a4
protein in this tissue.

![Figure 2](https://arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/934287/)
A regional distribution of GSH conjugate efflux transporters in the rat lens.

**Figure 3.** Regional expression of Mrp1, 4, and 5 proteins in the rat lens. Western blot analysis using crude membrane protein fractions prepared from the epithelium (Epi), outer cortex (OC), inner cortex (IC), and core (C) were probed with Mrp isoform-specific antibodies. Only bands representing full length proteins are shown with the protein size noted on the left. (A) Multidrug resistance–associated proteins 1 and (B) 4 are detected in the epithelium, outer and inner cortical regions, whereas (C) Mrp5 is detected in the epithelium, outer and inner cortex, and core regions.

Taken together, Western blot analysis indicates that out of the four candidate transporters screened, Mrp1, 4, and 5 were detectable at the protein level in the rat lens.

**Regional Distribution of GSH Conjugate Efflux Transporters in the Rat Lens**

In order to determine the regions of the lens in which Mrp1, 4, and 5 were expressed, we dissected lenses into epithelium (epi), outer cortex (OC), inner cortex (IC), and core (C) regions. We then prepared crude membrane preparations (containing total proteins from the plasma membrane as well as proteins stored in cytoplasmic/membrane vesicles) from each region for Western blot analysis (Fig. 3). It should be noted that although we would have preferred to load equal amounts of protein from each region for consistency, this was not possible for a number of reasons. Firstly, to obtain a protein concentration for the epithelium comparable to any of the other regions in the lens, an excessive number of lenses would have to be pooled. As a result, we loaded the maximum amount of protein sample from the epithelium (~2 μg protein/lane), which was sufficient to obtain a signal. Secondly, we always loaded a higher concentration of protein from the core region (~60–100 μg protein/lane) relative to the outer cortex and inner cortex (~10–15 μg protein/lane). The lens core contains a higher protein concentration, largely due to a greater amount of crystallin proteins, compared with the outer regions of the lens and as a result, the amount of membrane proteins per sample is often underestimated in core fractions necessitating the need to load a higher protein concentration to obtain a signal from this region. Therefore, these findings do not provide quantitative information on the relative expression levels of the various Mrp isoforms in the different regions; but instead only provide verification of their expression in that region. For Mrp1 (Fig. 3A) and Mrp4 (Fig. 3B), bands were detected in the epithelium, outer cortex, and inner cortex but were absent from the core. For Mrp5 (Fig. 3C), bands were detected in the epithelium, outer cortex, inner cortex, and core regions; although the bands were faint in intensity in the epithelium and core fractions.

In order for a transporter to mediate export of GSH conjugates from the lens, we would expect these proteins to be localized to the membranes of the epithelium and cortical fiber cells. To investigate this, immunohistochemistry was performed on equatorial lens cryosections labeled with the membrane marker WGA (red) and Mrp isoform-specific antibodies (green; Fig. 4). Multidrug resistance–associated protein 4 labeling was most intense in the lens periphery with labeling predominantly associated with the membranes of the lens epithelium (Figs. 4B, 4F) and the cytoplasm of cortical fiber cells (Figs. 4C, 4G). Multidrug resistance–associated protein 4 labeling was most notable in the nuclei of epithelial cells and cortical fiber cells (Figs. 4D–E, 11–I). In addition to the nuclei labeling, some cytoplasmic staining for Mrp4 could also be detected in the epithelium (Fig. 4H) and cortical fiber cells (Fig. 4I). Multidrug resistance–associated protein 5 labeling was localized predominantly to the lens periphery and associated with the membranes of the lens epithelium (Figs. 4J, 4O) and within the cytoplasm of cortical fiber cells (Figs. 4K, 4Q). For both Mrp4 and Mrp5, we were able to verify these labeling patterns as sections were also labeled with Mrp4 or Mrp5 antibodies preabsorbed with their respective antigenic control peptides. In both cases, there was a complete knock down of signal (Figs. 4L, 4M, 4P, 4Q).

It appears that all Mrp isoforms were present in the lens periphery placing them in an ideal position to mediate efflux of GSH conjugates from the lens. However, the membranous labeling of Mrp1 and 5 in the epithelium would tend to indicate that these isoforms are most likely to mediate GSH conjugate efflux from the lens.

**GSH Conjugate Release is Mediated by Mrp Transporters**

In order to measure GSH conjugate release from the lens, individual lenses were loaded with either CMFDA or mCB, which
are membrane-permeable probes that are nonfluorescent. However, upon preferential conjugation to GSH within the cell through a process catalyzed by glutathione-S-transferase, they form membrane-impermeable and highly fluorescent conjugates GS-MF and GS-B, respectively.\textsuperscript{53–55} As a lipophilic ester, CMFDA is additionally hydrolyzed by intracellular nonspecific esterases in order to generate the final fluorescent product. To confirm that members of the Mrp family were involved in GSH conjugate efflux, lenses were loaded in the presence or absence of the Mrp-specific inhibitor, MK571. MK571 is known to inhibit all members of the Mrp family\textsuperscript{56} and is widely used at concentrations less than or equal to 50 \mu M for attributing GS-B and GS-MF conjugate transport, in a variety of cell types, to members of the Mrp family.\textsuperscript{57–62}

To assess the efficacy of transport inhibition, we measured two parameters; firstly, GSH conjugate efflux which is the amount of GSH conjugate in the media as a percentage of total lens and media GSH conjugates and secondly, GSH conjugate accumulation in the lens. If the Mrps were involved in mediating GSH conjugate release, we would expect that in the presence of an Mrp inhibitor, GSH conjugate efflux would decrease and GSH conjugate accumulation in the lens would increase relative to control lenses.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Localization and subcellular distribution of Mrp1, 4, and 5 in the lens periphery. (A) Montage of extended confocal images of a lens equatorial cryosection double labeled with WGA (\textit{red}) and DAPI (\textit{blue}), extending from the capsule to the outer cortex region. The \textit{boxes} indicate the areas from which high magnification images were taken (B–E, J, K). Equatorial cryosections were labeled with Mrp1 (B, C), 4 (D, E), and 5 (J, K) antibodies (\textit{green}) and the membrane marker, WGA (\textit{red}) in the lens epithelium (B, D, J) or lens cortex (C, E, K). (F–I, N, O) The same images seen in (B–E, J, K) but with Mrp1 (F, G), 4 (H, I), and 5 (N, O) labeling only (\textit{green}) in the lens epithelium (F, H, N) or lens cortex (G, I, O). (L–M, P, Q) Control sections labeled with WGA (\textit{red}) and Mrp antibodies preabsorbed with their corresponding control peptide. An absence of \textit{green} labeling indicates complete knockdown of Mrp4 labeling in the epithelium (L) and outer cortex (M), and Mrp5 labeling in the epithelium (P) and outer cortex (Q). (A) \textit{Scale bar:} 25 \mu m; (B–Q) \textit{scale bar:} 5 \mu m.}
\end{figure}
Lenses were first loaded with CMFDA in the presence of 50 μM MK571 or 0.5% DMSO (vehicle control) for 1 hour, washed, and then incubated in fresh isosmotic AAH for another hour. First, LDH activity was measured in the media and lenses to calculate %LDH release and only lenses that released less than 0.1% LDH were included in the data set to ensure that release of GS-MF or GS-B caused by membrane damage was minimal (Fig. 5A). Although the %LDH release value for MK571-treated lenses loaded with CMFDA appears higher than control lenses, this increase was not statistically significant (P = 0.08, Fig. 5A) and was well below our 0.1% LDH cut-off value. Glutathione-MF fluorescence was then measured in the media and lenses to assess GS-MF efflux and lens accumulation. In the absence of MK571, GS-MF efflux was 40.7 ± 2.5% of total loaded GS-MF, however in the presence of MK571, GS-MF efflux was reduced to 11.1 ± 1.3% of total lens GS-MF (Fig. 5B). This resulted in an approximately 73% inhibition of GS-MF efflux, which was statistically significant. In terms of GS-MF accumulation, it can be seen that in the presence of MK571, GS-MF accumulation in the lens was dramatically increased relative to control (Fig. 5C) and this increase was statistically significant.

Lenses were loaded with mCB in the presence of 50 μM MK571 or 0.5% DMSO (vehicle control) for 1 hour. The lenses were washed and then incubated in fresh isosmotic AAH for another hour. Lactate dehydrogenase activity and GS-B concentration were then measured in the media and the lens. Any pathological leakage of GS-B conjugates caused by membrane damage was considered to be minimal because only lenses that exhibited LDH release values below our 0.1% cut-off value were included in the data set and the %LDH release values between control and MK571-treated lenses were similar (P = 0.72, Fig. 5D). In the absence of MK571, lenses extruded 13.6 ± 0.8% of total loaded GS-B into the media (Fig. 5E). However, in the presence of MK571, %GS-B export was
Benzbromarone is used to block organic anion uptake or efflux by organic anion transporters. Rat lenses were preloaded with 100 μM mCB in the presence of 50 μM benzbromarone or 0.25% vol/vol DMSO (vehicle control) for 1 hour. Lenses were then washed and incubated in fresh isosmotic AAH for another hour following which LDH activity and GS-B concentration was measured in the media and in the lens (Fig. 6). Only lenses that exhibited LDH release values less than 0.1% were included in the data set and it can be seen that these %LDH release values were similar between control and benzbromarone-treated lenses (Fig. 6A). In the absence of benzbromarone, lenses extruded 8.9 ± 0.4% of total loaded GS-B into the media (Fig. 6B). However, in the presence of benzbromarone, lenses extruded 1.6 ± 0.3% of total loaded GS-B into the media. This represents an 82% inhibition of GS-B efflux and is a higher level of inhibition than seen in lenses treated with MK571. As for MK571, a higher concentration of benzbromarone (100 μM) did not increase the degree of inhibition (data not shown). In terms of lens GS-B accumulation in the presence or absence of benzbromarone, it can be seen that GS-B accumulation is greater in lenses treated with benzbromarone, compared with control lenses (Fig. 6C). This accumulation was approximately 21% higher in benzbromarone-treated lenses compared with control lenses and was statistically significant.

Taken together, these results indicate that although the Mrps appear to be the predominant pathway for GS-B release, other pathways are also likely to be involved. These include, a benzbromarone-sensitive pathway that is not Mrp-mediated but likely to be alternative organic anion transporters, and a benzbromarone-insensitive pathway that does not involve organic anion transporters (Fig. 7).

**FIGURE 6.** Glutathione-B conjugate efflux from the rat lens is mediated by organic anion transporters. Rat lenses were preloaded with 100 μM mCB in the presence of 50 μM benzbromarone (n = 7) or 0.25% vol/vol DMSO (vehicle control, n = 11) for 1 hour. Lenses were then washed and incubated in fresh media lacking mCB or benzbromarone for another hour. (A) Lactate dehydrogenase activity was measured in the media and lenses, and expressed as %LDH release. (B) Glutathione-B concentration was measured in the media and lenses, and expressed as %GS-B efflux. (C) Total lens GS-B concentration was measured and normalized by lens wet weight. All data are shown as mean ±SEM. ('P < 0.05, ****P < 0.0001 Mann-Whitney U test).

Reduced to 5.3 ± 0.5%, resulting in a statistically significant 61% inhibition of GS-B efflux (Fig. 5E). In addition to a decrease in GS-B efflux, GS-B accumulation was greater in lenses treated with MK571 compared with control lenses (Fig. 5F). This increase was approximately 28% higher than that seen in control lenses and was statistically significant. A higher concentration of MK571 (100 μM) did not increase the degree of inhibition (data not shown).

Because we were not able to completely inhibit GS-MF or GS-B release in the presence of MK571, this suggested that other transport pathways were likely to be involved in the release of GSH conjugates from the lens. Therefore, we examined the effects of using a nonspecific inhibitor of organic anion transport, benzbromarone, on GS-B release. Benzbromarone is used to block organic anion uptake or efflux mediated by the Mrps, Oatps, Oat1/4 (organic anion transporters), and Urat1 (urate transporter), but not organic cation transporters. Lenses were loaded with mCB in the presence of 50 μM benzbromarone or 0.25% DMSO (vehicle control) for 1 hour. The lenses were then washed and incubated in fresh isosmotic AAH for another hour following which LDH activity and GS-B concentration was measured in the media and in the lens (Fig. 6). Only lenses that exhibited LDH release values less than 0.1% were included in the data set and it can be seen that these %LDH release values were similar between control and benzbromarone-treated lenses (Fig. 6A). In the absence of benzbromarone, lenses extruded 8.9 ± 0.4% of total loaded GS-B into the media (Fig. 6B). However, in the presence of benzbromarone, lenses extruded 1.6 ± 0.3% of total loaded GS-B into the media. This represents an 82% inhibition of GS-B efflux and is a higher level of inhibition than seen in lenses treated with MK571. As for MK571, a higher concentration of benzbromarone (100 μM) did not increase the degree of inhibition (data not shown). In terms of lens GS-B accumulation in the presence or absence of benzbromarone, it can be seen that GS-B accumulation is greater in lenses treated with benzbromarone, compared with control lenses (Fig. 6C). This accumulation was approximately 21% higher in benzbromarone-treated lenses compared with control lenses and was statistically significant.

**DISCUSSION**

In this study, we show that putative GSH conjugate transporters Mrp1, 4, 5, and Oatp1a4 are present in the rat lens at the transcript level. Of these four candidates, Mrp1, 4, and 5 were identified at the protein level with Mrp1 and 5 localizing to the lens epithelial membrane, indicating that these isoforms are most likely to be involved in mediating GSH conjugate release from the lens. We demonstrate that the lens is able to form GSH conjugates, GS-MF and GS-B, and in addition release these conjugates primarily by Mrp transporters. However, because GSH conjugate release was not completely blocked with MK571, a nonspecific organic anion inhibitor, benzbromarone was used to determine whether GS-B efflux could be further inhibited. Here, it was shown that GS-B release could be inhibited further and suggests that alternative organic anion efflux transporters may also be involved in mediating GS-B release.

Multidrug resistance-associated protein isoforms have previously been found in the blood–retina barrier and the blood–aqueous barrier and are known to play important roles in regulating the entry and accumulation of potentially toxic compounds. Pelis and colleagues have shown that Mrp2 expression in cultured porcine nonpigment epithelial cells of the ciliary body reflects a role in preventing the entry of systemic hydrophobic drugs from entering the aqueous humour or mediating the removal of endogenously generated intraocular compounds from the aqueous humour. Although we have used exogenous compounds to form GSH conjugates, it is known that the lens can form endogenous GSH conjugates of 4-HNE under oxidative stress conditions. Therefore, in this study, having shown that Mrps are functional in the lens and capable of GSH conjugate release, these transporters are most likely used as a...
However, in contrast to Sugamo et al.,73 we did not detect a Mrp1 but not Oatp1a1 transcript. To our knowledge, only one other study has attempted to elucidate the molecular identities of efflux transporters in the rat lens, albeit in a different strain of rat.73 In this analysis, the expression of major transporter genes reportedly involved in removal of xenobiotics from the liver was screened in the rat eye. Of those transporters involved in GSH conjugate release (Oatp1a1, Mrp1, 2), Mrp1 and 2 but not Oatp1a1 were shown to be detected at the transcript level in the lens. In our study, we also detected the Mrp1 but not Oatp1a1 transcript. However, in contrast to Sugamo et al.,73 we did not detect a transcript for Mrp2 (Fig. 2). To confirm our finding, we performed immunohistochemistry on lens tissue using an Mrp2 isoform-specific antibody and showed that Mrp2 was not detectable (data not shown), confirming in our hands that Mrp2 was absent in the lens.

Visualization of the location and subcellular distribution of the Mrps within the lens periphery indicated that Mrp1 and 5 were localized to the epithelial membranes suggesting that these transporters are functionally active. On the other hand, in the same region, Mrp4 labeling was predominantly restricted to the nuclei raising some interesting possibilities about Mrp4 function in the lens. Although studies have shown some Mrp isoforms, such as Mrp1 through 3, to be associated with the nuclei,74–76 we could not find any prior evidence of Mrp1 labeling of the nucleus. If Mrp4 functions as a GSH conjugate transporter in the lens, its nuclear localization indicates that it may play a role in removing toxins/reactive metabolites from the cell nucleus. On the other hand, Mrp4 is known to be involved in the transport of cyclic nucleotides like cAMP and cGMP77 and ADP transport,78 and so its intracellular localization may infer a role that does not involve GSH conjugate release but rather a role in the regulation of intracellular cyclic nucleotide levels and cell signalling within the lens.

In the outer cortex, labeling for all the Mrp isoforms was predominantly cytoplasmic, with some membranous labeling evident for Mrp1 and 5 (Figs. 4G, 4O). However, because the majority of transporters were present in the cytoplasm, this may indicate the presence of a functionally inactive pool of transporters. We have previously proposed that young fiber cells in the outer cortex produce a cytoplasmic store of membrane proteins, which can be inserted into the membranes at discrete stages of fiber cell differentiation to compensate for the inability of mature anucleate fiber cells to perform de novo protein synthesis.79 In addition to this, we have also shown that transporter insertion can be stimulated by changes in environmental conditions. For example, Chee and colleagues80 showed that exposure of the lens to hypotonic conditions induced fiber cell swelling and the recruitment to the membrane of the volume-sensitive KCC isoforms 1 and 4, but not the volume-insensitive isoform, KCC3. Suzuki-Kerr et al.81 have also revealed that in response to hypertonic stress, purinergic receptor isoforms P2X1 and P2X4 become inserted into broad sides of fiber cells, while under hypotonic conditions P2X4 and P2X6 isoforms associate with the narrow side membranes. In a similar manner, it is possible that in the outer cortex, Mrp transporters could be triggered to be redistributed from a cytoplasmic pool into the membrane as a response to cellular stress. This potentially could act to enhance the elimination and clearance of xenobiotics and/or other cellular stresses from the lens.

Having established that Mrp isoforms were most likely to mediate GSH conjugate release, we then carried out functional assays to demonstrate Mrp functionality. Although we were able to demonstrate that the lens was capable of GS-MF and GS-B release, and that this release could be significantly inhibited in the presence of MK571, it is important to note that the percentage of GS-MF and GS-B conjugates released from the lens, in the absence of inhibitors, was low when compared with the proportion of GS-MF/GS-B conjugates retained within the lens. The reason for this is most likely 2-fold. Firstly, the lens possesses a number of antioxidant defence systems to protect itself from oxidative stress and harmful reactive oxygen species (e.g., glutathione peroxidase, catalase, superoxide dismutase, GSH, ascorbic acid), that under our culturing conditions, may be sufficient to maintain redox balance without a need to expedite the removal of a GSH conjugate. Secondly, the GSH conjugate we are monitoring is not necessarily a “toxic” conjugate and could be viewed by the lens as a source of GSH, which does not require removal. However, under conditions of oxidative stress and/or advancing age, where antioxidant defence systems may be weakened,82 the contribution by the Mrps in the lens to enhance clearance of toxic compounds from the lens and out of the anterior chamber via the trabecular meshwork may be more critical.

In conclusion, we have shown for the first time shown that Mrp isoforms are present in the rat lens and are functionally active in mediating GSH conjugate release from the lens. Current methods for the delivery of drugs to the eye involve bypassing the blood–ocular barrier to increase drug bioavailability,83 resulting in the exposure of tissues such as the lens to...
higher levels of foreign compounds. Therefore, identifying these removal pathways is becoming increasingly important in providing a greater understanding of the molecular mechanisms used by the lens to detoxify and clear unwanted compounds to minimize oxidative stress, maintain lens homeostasis, and preserve lens transparency.

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**References**


