(Patho)physiological function of the Multidrug Resistance protein MRP1
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Chapter 7

The multidrug resistance protein MRP1 protects against the toxicity of the major lipid peroxidation product 4-hydroxynonenal

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Abstract

4-Hydroxynonenal (4HNE) is the most prevalent toxic lipid peroxidation product formed during oxidative stress. It exerts its cytotoxicity mainly by modification of intracellular proteins. The detection of 4HNE-modified proteins in several degenerative disorders suggests a role of 4HNE in the onset of these diseases. Efficient protection mechanisms are required to prevent intracellular accumulation of 4HNE. Toxicity of 4HNE was tested with the GLC₄ and the MRP1-overexpressing counterpart GLC₄/Adr small cell lung cancer cell lines. In the presence of the MRP1-inhibitor MK571 or the GSH-depleting agent buthionine sulfoximine, both cell lines became more sensitive and showed decreased survival. Transport experiments were performed with the tritium-labelled glutathione S-conjugate of 4HNE ([³H]GS-4HNE) using membrane vesicles from GLC₄-derived cell lines with different expression levels of MRP1. [³H]GS-4HNE was taken up in an ATP-dependent manner and the transport rate was dependent on the amount of MRP1. The MRP1 inhibitor MK571 reduced [³H]GS-4HNE uptake. MRP1-specific [³H]GS-4HNE transport was demonstrated using membrane vesicles from High Five insect cells overexpressing recombinant MRP1. Kinetic experiments showed an apparent $K_m$ value of $1.6 \pm 0.21 \mu$M for MRP1-mediated [³H]GS-4HNE transport. In conclusion, MRP1 plays a role in the protection against 4HNE toxicity and GS-4HNE is a novel MRP1 substrate. MRP1, together with GSH, are supposed to play a role in the defence against oxidative stress.
Introduction

Lipid peroxidation is a degenerative process affecting cell membranes and other lipid-containing structures and is associated with pathological implications. It is initiated by reactive oxygen species (ROS) generated under conditions of oxidative stress. Conversion of polyunsaturated fatty acids (PUFA) by short lived ROS results in formation of relatively stable aldehydes that can diffuse from their site of origin and affect targets distant from the initial free radical attack. One of the major toxic products generated during lipid peroxidation is the α,β-unsaturated aldehyde 4-hydroxynonenal (4HNE) which is derived from ω-6-PUFA’s like arachidonic acid and linoleic acid.1,2

4HNE shows a variety of cytotoxic effects such as inhibition of DNA, RNA and protein synthesis, cell cycle arrest, mitochondrial dysfunction, induction of cataracts of the lens and neuronal apoptosis.2-4 Intracellularly, 4HNE reacts rapidly with thiol groups of GSH and cysteine and with lysine and histidine residues of proteins.5,6 4HNE-modified proteins have been detected in pathological disorders such as chronic liver diseases,7 Parkinson disease,8 mitochondrial complex I deficiency,9 Alzheimer’s disease10 and atherosclerotic lesions.11 Furthermore, 4HNE plasma levels are increased in patients with rheumatoid arthritis.12

It is obvious that efficient protection mechanisms are required to prevent accumulation and subsequently toxic effects of 4HNE. Enzymes primary known for metabolism and/or detoxification of 4HNE are aldehyde dehydrogenases, aldo-keto reductases and glutathione S-transferases (GST’s). These enzymes convert 4HNE either to 4-hydroxynoneic acid, 1,4-dihydroxynonene or the GSH-conjugate (GS S-conjugate) of 4HNE (GS-4HNE).2,13-15 Among these metabolites, GS-4HNE is predominantly found when heart, liver or kidney are perfused with 4HNE.13,16,17 This showed that GSH plays a major role in the metabolism of 4HNE. GSH can react rapidly and spontaneously with 4HNE,5 but the reaction is far more efficient when it is catalysed by glutathione S-transferases (GST’s), for example human GST A4-415,18 and rat GST A5-519.

The GSH/GST system is a well-known mechanism in the cellular defence against oxidative stress. Excretion of GS S-conjugates is important for reducing intracellular concentrations of toxins because conjugation reactions are reversible. In addition, some metabolites become toxic upon conjugation with GSH.20 GS-4HNE appears to be a product inhibitor for GST18 and therefore accumulation of GS-4HNE might lead to decreased detoxification capacities for 4HNE and other toxic metabolites. Extrusion of GS-4HNE is required to prevent these effects and existence of a transport mechanism has been proposed.21 One of the best candidates for transport of GS-4HNE across the cell membrane is the multidrug resistance protein MRP1. Together with GSH, MRP1 is ubiquitously expressed in the human body.22,20 Although MRP1 is primary known for conferring multidrug resistance (MDR) to cancer cells by transporting anticancer drugs,25 the best substrates identified for MRP1 thus far are endogenous GS S-conjugates.24 In analogy with MRP1, MRP2 is also a transporter with highest affinity for GS-S conjugates24 and may also be a transporter for GS-
4HNE. MRP2 is predominantly found in liver and kidney where MRP1 expression is relative low. In this study we focussed on MRP1.

In view of its ubiquitous expression and its substrate specificity, the putative physiological substrates of MRP1 seem to be GSH-dependent detoxification products. We hypothesise that GS-4HNE is a novel substrate for MRP1 and that MRP1 together with GSH play a role in the defence against oxidative stress in the human body.

Materials and Methods

Materials
4-hydroxynonenal-diethylacetal (4HNE-DEA) was a kind gift of the late Dr. H. Esterbauer (University of Graz, Austria). The pJ3Ω-MRP1 vector was kindly provided by Dr. P. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and the MDR1 virus was a gift from Dr. U.S. Rao (University of Nebraska, Omaha, NE, USA). The Bac-to-Bac baculovirus expression system was obtained from Life Technologies (Paisley, UK). Spodoptera frugiperda Sf21 cells were from Invitrogen (Groningen, The Netherlands) and the Trichoplusia ni High Five™ (HF) cells were a gift from Dr. M. Harmsen (University of Groningen, The Netherlands). [glycine-2-³H]-glutathione (¹³H]GSH) (1620.6 GBq mmol⁻¹) was purchased from NEN (Boston, MA, USA) and MK571 was purchased from Biomol (Plymouth Meeting, PA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

Generation of 4HNE
To obtain an aqueous solution of 4HNE, the precursor 4HNE-DEA was hydrolysed by 1 mM HCl for 1 h at 37°C. The concentration of 4HNE was determined by spectrophotometry at 224 nm.

Cell culture
The human small cell lung cancer cell line GLC₄ and its doxorubicin selected multidrug resistant counterparts GLC₄/10x and GLC₄/Adr were cultured as described. These cells were used because of their different MRP1 expression levels. Sf21 insect cells were cultured in Insect-XPRESS™ medium (Biowhittaker, Verviers, Belgium), supplemented with 0.5 x penicillin/streptomycin/neomycin (PSN) and 5% foetal calf serum (Life Technologies). HF insect cells were cultured in Insect-XPRESS™ medium supplemented with 0.5 x PSN.

Cell survival assay
To determine the cytotoxicity of 4HNE, GLC₄ and GLC₄/Adr cells were seeded at 100.000 cells per ml and grown overnight either in the absence or in the presence of 25
µM of the γ-glutamylcysteine synthetase (γ-GCS) inhibitor buthionine sulfoximine (BSO). GLC4/Adr cells contain about twice as much GSH as GLC4 cells and treatment with BSO decreased the GSH contents with about 75-80% in both cell lines (data not shown and 29,30). Cells were collected by centrifugation (15 min at 1000 x g), washed with Hanks’ buffered salt solution and seeded at similar densities in serum free medium. Part of the cells was preincubated with 50 µM MK571 for 1 h before addition of 4HNE. This MK571 concentration was not toxic for the cells. Cells were incubated with 4HNE (0 – 40 µM) for 4 h, collected by centrifugation and resuspended in a similar amount of serum supplemented medium and grown further for 66 h. Cell survival was measured with 100 µl cell suspensions using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Production of recombinant MRP1 and MDR1
Production of recombinant MRP1 and MDR1 protein was performed by the Bac-to-Bac baculovirus expression system. A NotI – SalI fragment encoding the complete human MRP1 sequence was excised from the pJ3ω-MRP vector and cloned into pFastBac1. Recombinant bacmids were generated according to the manufacturer’s instructions. Purified bacmids were transfected into Sf21 insect cells using CellFECTIN transfection reagent (Life Technologies). After five days, the culture medium was used for a plaque assay. Single plaques were isolated, eluted into medium and used to infect fresh Sf21 cells to increase the virus titer. Titters were determined by plaque assays and virus stocks were stored at 4°C. The MDR1 virus stock, provided by Dr. U.S. Rao, was further amplified in Sf21 cells to obtain larger amounts of virus stock. For production of recombinant MRP1 and MDR1 proteins HF insect cells were used. HF cells are supposed to have a higher protein expression yield compared to Sf21 cells. HF cells were seeded at a density of 0.35 x 10^6 cells ml^-1 and infected with a multiplicity of infection of 5.0 and 7.0 plaque forming units per ml, respectively, for MRP1 and MDR1. After 62-64 h, cells were collected and membrane vesicles were isolated.

Isolation of membrane vesicles, deglycosylation and immunoblot analysis
Membrane vesicles used for transport studies were isolated from the GLC4, GLC4/10x and GLC4/Adr cells as described before.31 Membrane vesicles from the HF insect cells were isolated by the same protocol, except that the cells were lysed in 1 mM Tris-HCl, pH 7.2/0.1 mM EDTA/0.1 mM PMSF in stead of 1 mM NaHCO3. Deglycosylation of membrane proteins was performed using a PNGase F kit from New England Biolabs (NEB), (Beverly, MA, USA). Membrane vesicles (5-20 µg protein) were incubated during 16 h at 37°C in a 50 mM sodium phosphate buffer, pH 7.5 containing 3 mM PMSF in the absence or in the presence of 500 NEB-defined units PNGase F. Immunoblot analysis of MRP1 and MDR1 expression in the membrane vesicles was performed essentially as described.31
Dithiotreitol (DTT) was removed from the [3H]GSH solution by extraction with ethylacetate. The [3H]GSH-conjugate of 4HNE ([3H]GS-4HNE) was formed by incubation of a 10-fold molar excess of 4HNE with [3H]GSH in the presence of 100 mM Tris, pH 7.2 and 2 units of rat liver GST. The reaction was performed at 37°C for 1 h with gentle shaking. Unlabeled GS-4HNE was generated by incubation of freshly prepared GSH with 4HNE in a 1:1 molar ratio in the presence of 20 mM potassium phosphate buffer, pH 6.8. This reaction was performed for 2.5 h at 37°C with gentle shaking. The reaction mixtures were spotted on TLC plates (Merck, Darmstadt, Germany) and developed in a chamber containing 1-propanol/water/acetic acid (10:5:1, by volume). The GS-4HNE conjugates were extracted from the plates and eluted in 70% ethanol. The extracts were dried in a Speed-Vac and dissolved in a small volume of 70% ethanol and stored at –20°C. The concentration of [3H]GS-4HNE was determined by scintillation counting. The concentration of GS-4HNE was measured by a colorimetric assay using ninhydrin. Nonyl-GS was used as a standard.

**Table 1.** Sensitivity of GLC4 and GLC4/Adr cells for 4HNE.

<table>
<thead>
<tr>
<th></th>
<th>IC50 values (µM)</th>
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<tbody>
<tr>
<td></td>
<td>GLC4</td>
</tr>
<tr>
<td>4HNE</td>
<td>10.0 ± 2.0</td>
</tr>
<tr>
<td>+ BSO</td>
<td>2.3 ± 1.4 *</td>
</tr>
<tr>
<td>+ MK571</td>
<td>6.0 ± 1.4 *</td>
</tr>
</tbody>
</table>

Cells were incubated with 4HNE (0-40 µM) for 4 h and cell survival was measured with the MTT assay. BSO (25 µM) was added 16 h and MK571 (50 µM) was added 1 h before incubations with 4HNE started. Data represent IC50 values (means ± S.D.) of at least three experiments with quadruplicate determinations. *P < 0.05 (one-way ANOVA) compared to 4HNE alone.

**Transport studies**

Uptake of [3H]GS-4HNE into membrane vesicles was measured as described. Briefly, membrane vesicles (10-25 µg protein) were rapidly thawed and added to a buffer containing 4 mM ATP or 4 mM of the non-hydrolyzable ATP analogue AMP-PCP, 10 mM MgCl2, 10 mM creatine phosphate, 100 µg/ml creatine kinase, 10 mM Tris pH 7.4 and 250 mM sucrose. After 1 min prewarming at 37°C, [3H]GS-4HNE was added (110 µl final volume). Samples of 25 µl were taken at indicated time points and diluted in 1 ml ice cold stop solution (10 mM Tris/250 mM sucrose). The stop solutions were filtered through 0.45 µm nitrocellulose filters (Schleicher & Schuell, Dassel, Germany). Filters were washed with 5 ml ice cold 10 mM Tris/250 mM sucrose, air dried and counted by liquid scintillation. ATP-dependent transport was calculated by subtraction of AMP-PCP values from ATP values. AMP-PCP did not stimulate uptake, demonstrating that ATP hydrolysis was required.
Results

4HNE cytotoxicity in GLC4 and GLC4/Adr cells
First we studied the toxicity of 4HNE in GLC4 small cell lung cancer cells and its MRP1-overexpressing multidrug resistant counterpart GLC4/Adr. The IC50 value for GLC4 cells was 10 ± 2.0 µM and for GLC4/Adr cells 9.3 ± 1.5 µM. Modulation with the GSH-depleting agent BSO resulted in a 4.3- and 1.9-fold decrease of the IC50 values for GLC4 and GLC4/Adr cells, respectively. Incubation with MK571, an inhibitor for MRP1, caused a 1.7-fold and a 2.0-fold decrease of the respective IC50 values (Table 1).

Figure 1. MRP1 expression in GLC4, GLC4/10x and GLC4/Adr membrane vesicles. Ten µg of protein was separated on 7.5% SDS-PAGE and transferred to nitrocellulose by electroblotting. MRP1 protein levels were analysed with the monoclonal antibody MRPr1 (1:500). The primary antibody was visualised by enhanced chemiluminescence. Molecular masses of markers are indicated in kDa.

Uptake of [3H]GS-4HNE into membrane vesicles with different MRP1 expression
We supposed that MRP1 is a transporter for GS-4HNE. Therefore we isolated membrane vesicles from cells with different levels of MRP1 expression and measured the uptake of [3H]GS-4HNE. MRP1 levels in the isolated membrane vesicles were analysed by immunoblotting (Figure 1). GLC4, GLC4/10x and GLC4/Adr membrane vesicles showed respectively low, intermediate and high MRP1 expression levels. ATP-dependent uptake of [3H]GS-4HNE into membrane vesicles from these three cell lines was measured during 5 min (Figure 2). The uptake of [3H]GS-4HNE was 0.4, 6.7 and 11.8 pmol mg protein⁻¹, respectively, for GLC4, GLC4/10x and GLC4/Adr. This ATP-dependent uptake was completely inhibited by MK571 (10 µM final concentration). The uptake rate of [3H]GS-4HNE correlated with levels of MRP1 expression. As control, membrane vesicles from A2780 ovarian tumour cells and its MDR1-P-glycoprotein (MDR1) overexpressing counterpart A2780AD, both with basal MRP1 expression, showed only marginal ATP-dependent [3H]GS-4HNE uptake (data not shown).
Production of recombinant MRP1
To demonstrate MRP1-specific [3H]GS-4HNE transport, recombinant MRP1 was produced by HF insect cells using the baculovirus system. Parental HF cells and HF cells infected with a MDR1 construct (HF(MDR1)) served as control. Membrane vesicles from HF, HF(MDR1) and HF(MRP1) cells were isolated and protein expression was determined by immunoblot analysis.

MRP1 expression was detected in HF(MRP1), but not in HF or HF(MDR1) membrane vesicles. MDR1 was present only in HF(MDR1) membrane vesicles (Figure 3). The molecular mass of MRP1 in HF(MRP1) cells is about 20 kDa less than in GLC4/Adr cells. Treatment of membrane vesicles from GLC4/Adr cells with PNGase F that cleaves the N-linked glycosylation reduced the molecular mass of MRP1 from GLC4/Adr membrane vesicles to a similar mass as from MRP1 expressed in untreated HF(MRP1) membrane vesicles (Figure 3, top panel). PNGase F treatment of HF(MRP1) membrane vesicles did not further decrease the molecular mass of MRP1 (data not shown). These results suggest that MRP1 expressed in HF cells is not or only partial glycosylated.

Characterisation of [3H]GS-4HNE transport by recombinant MRP1
ATP-dependent uptake of [3H]GS-4HNE into HF(MRP1) membrane vesicles was linear during 1 min and was increased up to 11-fold compared to HF or HF(MDR1) membrane vesicles (Figure 4). To further characterise ATP-dependent uptake of [3H]GS-4HNE into HF(MRP1) membrane vesicles, we studied the effect of several inhibitors of MRP1-mediated transport (Table II). Inhibition of [3H]GS-4HNE uptake increased with the length of the alkyl chain of the GS-derivative, and varied from not detectable (methyl-GS and propyl-GS) to highly effective (nonyl-GS). GSH did not
inhibit \[^{3}\text{H}]\text{GS-4HNE}\) uptake while GSSG was a weak inhibitor. MK571 inhibited \[^{3}\text{H}]\text{GS-4HNE}\) uptake in a dose-dependent manner. Together with the results presented in figure 1, these results demonstrate that \[^{3}\text{H}]\text{GS-4HNE}\) is transported by MRP1. We examined the kinetics of MRP1-mediated \[^{3}\text{H}]\text{GS-4HNE}\) transport and determined an apparent \(K_m\) value of 1.6 ± 0.21 \(\mu\text{M}\) and a \(V_{\text{max}}\) value of 804.5 ± 28.8 pmol mg protein\(^{-1}\) min\(^{-1}\) (Figure 5).

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**Figure 3.** Immunoblot analysis of recombinant MRP1 and MDR1 expression in High Five insect cells. Twenty \(\mu\text{g}\) of protein was separated on 7.5% SDS-PAGE and transferred to nitrocellulose by electroblotting. Membrane vesicles from GLC\(_4\)/Adr cells (5 \(\mu\text{g}\) protein) served as positive control for MRP1 expression (lane a). GLC\(_4\)/Adr membranes (5 \(\mu\text{g}\) protein) were incubated with deglycosylation buffer in the absence (lane b) or in the presence (lane c) of PNGase F. Protein levels were analysed with the monoclonal antibody MRPPr1 (1:500) for MRP1 and with the monoclonal antibody C219 (1:300) for MDR1. The primary antibodies were visualised by enhanced chemiluminescence. Molecular masses of markers are indicated in kDa.

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**Figure 4.** ATP-dependent uptake of \[^{3}\text{H}]\text{GS-4HNE}\) into membrane vesicles from High Five insect cells overexpressing recombinant MRP1 and MDR1. Time course of ATP-dependent uptake of \[^{3}\text{H}]\text{GS-4HNE}\) (10 nM) into HF, HF(MRP1) and HF(MDR1) membrane vesicles (20 \(\mu\text{g}\) protein). ATP-dependent uptake was calculated by subtraction of AMP-PCP levels from ATP levels. Data represent means ± S.D. from three experiments with quadruplicate determinations.

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**Figure 5.** Time course of ATP-dependent uptake of \[^{3}\text{H}]\text{GS-4HNE}\) into membrane vesicles from High Five insect cells overexpressing recombinant MRP1 and MDR1. \[^{3}\text{H}]\text{GS-4HNE}\) uptake was measured at different time points (0-60 s). Data represent means ± S.D. from three experiments with quadruplicate determinations.
Table 2. Effect of inhibitors of MRP1-mediated transport on ATP-dependent uptake of [3H]GS-4HNE.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>% [3H]GS-4HNE transport</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Methyl-GS</td>
<td>1</td>
<td>102 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>111 ± 3.7</td>
</tr>
<tr>
<td>Propyl-GS</td>
<td>1</td>
<td>109 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>102 ± 5.4</td>
</tr>
<tr>
<td>Hexyl-GS</td>
<td>1</td>
<td>83 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>52 ± 3.4*</td>
</tr>
<tr>
<td>Nonyl-GS</td>
<td>1</td>
<td>47 ± 8*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3 ± 10*</td>
</tr>
<tr>
<td>GSH</td>
<td>5000</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>GSSG</td>
<td>100</td>
<td>61 ± 7.8*</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>18 ± 13*</td>
</tr>
<tr>
<td>MK571</td>
<td>0.5</td>
<td>95 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>80 ± 8.3*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>42 ± 12.8*</td>
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ATP-dependent uptake of [3H]GS-4HNE (10 nM) into HF(MRP1) membrane vesicles (20 µg protein) was measured during 1 min in the absence or in the presence of the indicated compound. ATP-dependent uptake was calculated by subtraction of AMP-PCP levels from ATP levels. Data represent means ± S.D. of at least two experiments with quadruplicate determinations. *P < 0.05 (one-way ANOVA) compared to control.

Discussion

Reactive oxygen species (ROS) are formed under physiological conditions in eukaryotic cells as a consequence of aerobic metabolism. ROS can cause lipid peroxidation and formation of potentially toxic aldehydes such as 4HNE. Endogenously formed adducts of 4HNE with deoxyguanosine have been detected and are supposed to be involved in ROS-mediated tumour promotion. In addition, at low concentrations, 4HNE seems to play a role in cell proliferation and differentiation. Under conditions of oxidative stress the formation of ROS is strongly increased and this leads to a propagated generation of lipid peroxidation products with increased levels of 4HNE. 4HNE causes cellular damage mainly by modification of intracellular proteins. The detection of 4HNE-modified proteins in early stages of degenerative diseases implies that 4HNE could play a role in the onset of these disorders. Efficient protection mechanisms preventing accumulation of 4HNE are thus required.
MRP1-mediated protection against 4-hydroxynonenal

GSH is present in all eukaryotic cells and serves as a protection mechanism against ROS-induced toxic intermediates, either by reduction or by conjugation.\(^\text{20}\) 4HNE is conjugated with GSH and in this study we showed that MRP1 is a transporter for GS-4HNE and thus can function as export mechanism. This is consistent with the idea of a cardiac export system for GS-4HNE that may in fact be MRP1.\(^\text{21}\) Factors that play a role in this detoxification pathway for 4HNE are GSH,\(^\text{5}\) this study, GST\(^\text{18}\) and MRP1 (this study). GLC\(_4\) and GLC\(_4/\text{Adr}\) greatly differ in MRP1 expression but this is not correlated with 4HNE sensitivity in these cells. Thus under basal conditions GSH and GST seem to be the dominant factors in the protection against 4HNE. However, upon inhibition of MRP1, GLC\(_4\) and GLC\(_4/\text{Adr}\) cells become more sensitive for 4HNE showing that MRP1 indeed plays a role in the defence against 4HNE toxicity but again little difference is seen between the two cell lines. We therefore conclude that GSH, GST and MRP1 are interdependent factors and that although MRP1 clearly plays a role in defence against 4HNE toxicity, it does it in conjunction with GSH and GST. For example, inhibition of MRP1 will lead to accumulation of GS-4HNE and this may cause product inhibition of GST and thus increase in the intracellular concentration of 4HNE.

In this study we demonstrated that GS-4HNE is a novel MRP1 substrate. We used a baculovirus expression system to demonstrate MRP1-specific GS-4HNE transport. MRP1 expressed in HF insect cells is not glycosylated. Consistent with a previous study using Sf21 insect cells, this does not influence the transport capacity of MRP1.\(^\text{38}\) Compared to the K\(_m\) values of other MRP1 substrates, the K\(_m\) value we found for MRP1-mediated GS-4HNE transport suggests that GS-4HNE is a MRP1 substrate with a relatively high affinity.\(^\text{24}\) N-alkyl GS S-conjugates are potent inhibitors of GST’s.\(^\text{39}\) For example, 200 µM of n-octyl-GS results in an approximately 80% inhibition of the GST’s. Efficient removal of such products from GST will be necessary to prevent product inhibition.\(^\text{21}\) Because of the low K\(_m\) value of MRP1 for

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**Figure 5.** Kinetics of MRP1-mediated [\(^3\)H]GS-4HNE transport. ATP-dependent transport of [\(^3\)H]GS-4HNE into HF(MRP1) membrane vesicles (25 µg) was measured during 1 min. ATP-dependent uptake was calculated by subtraction of AMP-PCP levels from ATP levels. Data represent means ± S.D. of two experiments with quadruplicate determinations. Inset, double-reciprocal Lineweaver-Burk plot. Curve fitting and determination of K\(_m\) and V\(_{\text{max}}\) values was performed with the Sigma Plot 3.03\(^\text{40}\) program.
GS-4HNE we conclude that MRP1 will effectively contribute to a rapid diminishing in the inhibitory effects of GS S-conjugates such as the oxidative stress product GS-4HNE.

MRP1 shows a high similarity in substrate specificity compared to MRP2.\textsuperscript{24} In contrast to MRP2, MRP1 is expressed at low levels in normal liver and is localised at basolateral membranes, while MRP2 is located at canalicular membranes. The detection of GS-4HNE in bile after administration of 4HNE\textsuperscript{40} suggests that GS-4HNE may also be a substrate for MRP2 and that MRP2 might play a role in the detoxification of 4HNE in the liver by extrusion of GS-4HNE into bile.

MRP1 has originally been identified in drug resistant tumour cells and research has focussed on its drug resistance conferring capacity.\textsuperscript{41} Overexpression of MRP1 is associated with resistance against the anthracycline doxorubicin.\textsuperscript{42} Although MRP1 is a transporter for anthracyclines,\textsuperscript{29,31} doxorubicin resistance levels from MRP1-overexpressing cells often do not correlate with the decrease in intracellular accumulation.\textsuperscript{33,44} Doxorubicin is known to generate ROS and increased 4HNE formation has been observed after doxorubicin administration.\textsuperscript{45} In addition, transfection with a GST isoenzyme with high affinity for 4HNE resulted in resistance to doxorubicin.\textsuperscript{46} MRP1 may thus play a role in doxorubicin resistance also by export of endogenously formed GS-4HNE.

LTC\textsubscript{4} and prostaglandin A-GS (PGA-GS) are two other endogenously formed GS S-conjugates that are efficiently transported by MRP1.\textsuperscript{47,48} LTC\textsubscript{4} is important in the inflammatory response\textsuperscript{49} and PGA causes cell cycle arrest and apoptosis.\textsuperscript{50,51} A tight regulation of the intracellular concentrations of LTC\textsubscript{4} and PGA is required to retain the response against inflammatory stimuli and to prevent toxic effects. MRP1-mediated GSSG transport suggested that MRP1 also could be involved in protection against oxidative stress.\textsuperscript{52} The oxidative stress-mediated induction of the genes encoding MRP1 and γ-GCS, the rate limiting enzyme in GSH-synthesis, supports this.\textsuperscript{53} GSSG is generated by GSH-mediated reduction of ROS and MRP1, as GSSG transporter, prevents intracellular GSSG accumulation and may function in maintaining the cellular redox state.\textsuperscript{20} In this study we demonstrated that MRP1 protects against the toxicity of one of the most important metabolites formed during oxidative stress. Based on its ubiquitous expression and its substrate specificity, the physiological function of MRP1 seems to be cellular extrusion of GSH-dependent detoxification products and maintenance of cellular homeostasis.\textsuperscript{27}

In conclusion, we showed that GS-4HNE is a novel MRP1 substrate and that MRP1 protects against 4HNE toxicity, most likely by extrusion of its GS S-conjugate. We propose that MRP1, in conjunction with GSH, functions as a ubiquitous protection mechanism against oxidative stress.

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MRP1-mediated protection against 4-hydroxynonenal

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