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

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4-Hydroxynonenal (4HNE) is the most prevalent toxic lipid peroxidation product formed during oxidative stress. It exerts its cytotoxicity mainly by the modification of intracellular proteins. The detection of 4HNE-modified proteins in several degenerative disorders suggests a role for 4HNE in the onset of these diseases. Efficient protection mechanisms are required to prevent the intracellular accumulation of 4HNE. The toxicity of 4HNE was tested with the small cell lung cancer cell lines GLC_4 and the multidrug-resistance-protein (MRP1)-overexpressing counterpart GLC_4/Adr. In the presence of the MRP1 inhibitor MK571 or the GSH-depleting agent buthionine sulphoximine, both cell lines became more sensitive and showed decreased survival. Transport experiments were performed with the ^{3}H-labelled glutathione S-conjugate of 4HNE ([^{3}H]GS-4HNE) with membrane vesicles from GLC_4-derived cell lines with different expression levels of MRP1. [^{3}H]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 decreased [^{3}H]GS-4HNE uptake. MRP1-specific [^{3}H]GS-4HNE transport was demonstrated with membrane vesicles from High Five insect cells overexpressing recombinant MRP1. Kinetic experiments showed an apparent K_m of $1.6 \pm 0.21\ \mu$M (mean $\pm$ S.D.) for MRP1-mediated [^{3}H]GS-4HNE transport. In conclusion, MRP1 has a role in the protection against 4HNE toxicity and GS-4HNE is a novel MRP1 substrate. MRP1, together with GSH, is hypothesized to have a role in the defence against oxidative stress.

Key words: ATP-dependent transport, detoxification, glutathione, oxidative stress.

INTRODUCTION

Lipid peroxidation is a degenerative process affecting cell membranes and other lipid-containing structures and is associated with pathological implications [1]. It is initiated by reactive oxygen species (ROS) generated under conditions of oxidative stress. The conversion of polyunsaturated fatty acids by short-lived ROS results in the 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 polyunsaturated fatty acids such as arachidonic acid and linoleic acid [1,2].

4HNE shows a variety of cytotoxic effects such as the inhibition of DNA, RNA and protein synthesis, cell cycle arrest, mitochondrial dysfunction, the 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's disease [8], deficiency in mitochondrial complex I [9], Alzheimer's disease [10] 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 the accumulation and the subsequently toxic effects of 4HNE. Enzymes primarily known for the metabolism and/or detoxification of 4HNE are aldehyde dehydrogenases, aldo–keto reductases and glutathione S-transferases (GSTs). These enzymes convert 4HNE into 4-hydroxynoneic acid, 1,4-dihydroxynonene or the GSH conjugate of 4HNE (GS-4HNE) [2,13–15]. Of these metabolites, GS-4HNE is found predominantly when heart, liver or kidney is perfused with 4HNE [13,16,17]. This showed that GSH has 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 GSTs, for example human GST A4-4 [15,18] and rat GST A5-5 [19].

The GSH/GST system is a well-known mechanism in the cellular defence against oxidative stress. The excretion of GSH conjugates is important in decreasing intracellular concentrations of toxins because conjugation reactions are reversible. In addition, some metabolites become toxic on conjugation with GSH [20]. GS-4HNE seems to be a product inhibitor for GST [18] and therefore the accumulation of GS-4HNE might lead to decreased detoxification capacities for 4HNE and other toxic metabolites. The extrusion of GS-4HNE is required for preventing these effects; the existence of a transport mechanism has been proposed [21]. One of the best candidates for the transport of GS-4HNE across the cell membrane is the multidrug resistance protein

Abbreviations used: BSO, buthionine sulphoximine; GS-4HNE, glutathione S-conjugate of 4-hydroxynonenal; GST, glutathione S-transferase; 4HNE, 4-hydroxynonenal; LTC_4, leukotriene C_4; MDR1, multidrug resistance P-glycoprotein 1; MRP1, multidrug resistance protein; PGA, prostaglandin A; PNGase F, peptide N-glycosidase F; p[CH_2]ppA, adenosine 5′-[β,γ-methylene]triphosphate; ROS, reactive oxygen species.

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MRP1. Together with GSH, MRP1 is expressed ubiquitously in the human body [20,22]. Although MRP1 is primarily known for conferring multidrug resistance (MDR) on cancer cells by transporting anti-cancer drugs [23], the best substrates identified for MRP1 so far have been endogenous GSH conjugates [24]. By analogy with MRP1, MRP2 is also a transporter with the highest affinity for GSH conjugates [24] and might also be a transporter for GS-4HNE [25]. MRP2 is predominantly found in liver and kidney, where MRP1 expression is relatively low [26]. In the present study we focused 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 [27]. We hypothesize that GS-4HNE is a novel substrate for MRP1 and that MRP1, together with GSH, has a role in the defence against oxidative stress in the human body.

EXPERIMENTAL

Materials

4HNE-diethylacetal was a gift from the late Dr H. Esterbauer (University of Graz, Graz, Austria). The pJ3Ω-MRP1 vector was kindly provided by Dr P. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and the multidrug resistance P-glycoprotein 1 (MDR1) virus was a gift from Dr U. S. Rao (University of Nebraska, Omaha, NE, U.S.A.). The Bac-to-Bac baculovirus expression system was obtained from Life Technologies (Paisley, Renfrewshire, U.K.). *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, Groningen, The Netherlands). [*glycine*-2-^{3}H]Glutathione ([^{3}H]GSH) (1620.6 GBq/mmol) was purchased from NEN (Boston, MA, U.S.A.) and MK571 was purchased from Biomol (Plymouth Meeting, PA, U.S.A.). All other chemicals were obtained from Sigma (St Louis, MO, U.S.A.) unless stated otherwise.

Generation of 4HNE

To obtain an aqueous solution of 4HNE, the precursor 4HNE-diethylacetal 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 GLC_4 and its doxorubicin-selected multidrug-resistant counterparts GLC_4/10x and GLC_4/Adr were cultured as described [28]. 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 × penicillin/streptomycin/neomycin (PSN) and 5% (v/v) foetal calf serum (Life Technologies). HF insect cells were cultured in Insect-XPRESS™ medium supplemented with 0.5 × PSN.

Cell survival assay

To determine the cytotoxicity of 4HNE, the GLC_4 and GLC_4/Adr cells were seeded at 100000 cells/ml and grown overnight in either the absence or the presence of 25 μM of the γ-glutamylcysteine synthetase inhibitor buthionine sulphoximine (BSO). GLC_4/Adr cells contain approximately twice as much GSH as do GLC_4 cells; treatment with BSO decreased the GSH contents by approx. 75–80% in both cell lines (results not shown) [29,30]. Cells were collected by centrifugation (15 min at 1000 ***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 the addition of 4HNE. This MK571 concentration was not toxic to 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, then grown for a further 66 h. Cell survival was measured with 100 μl cell suspensions by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide ('MTT') assay.

Production of recombinant MRP1 and MDR1

The production of recombinant MRP1 and MDR1 protein was performed by the baculovirus expression system. A *Not*I–*Sal*I fragment encoding the complete human MRP1 sequence was excised from the pJ3Ω-MRP vector and cloned into pFastBac1. Recombinant bacmids were generated in accordance with the manufacturer's instructions. Purified bacmids were transfected into Sf21 insect cells by using CellFECTIN transfection reagent (Life Technologies). After 5 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 titre. Titres were determined by plaque assays; 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. HF insect cells were used for the production of recombinant MRP1 and MDR1 proteins. (HF cells are supposed to have a higher protein expression yield than Sf21 cells.) HF cells were seeded at a density of 3.5×10^5 cells/ml and infected with a multiplicity of infection of 5.0 and 7.0 plaque-forming units per ml for MRP1 and MDR1 respectively. 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 GLC_4, GLC_4/10x and GLC_4/Adr cells as described previously [31]. Membrane vesicles from the HF insect cells were isolated with 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 instead of 1 mM $NaHCO_3$. Deglycosylation of membrane proteins was performed with a peptide N-glycosidase F (PNGase F) kit from New England Biolabs (NEB) (Beverly, MA, U.S.A.). Membrane vesicles (5–20 μg of protein) were incubated for 16 h at 37 °C in 50 mM sodium phosphate buffer, pH 7.5, containing 3 mM PMSF in the absence or the presence of 500 NEB-defined units of PNGase F. Immunoblot analysis of MRP1 and MDR1 expression in the membrane vesicles was performed essentially as described [31].

Generation of GS-4HNE

Dithiothreitol was removed from the [^{3}H]GSH solution by extraction with ethylacetate. The [^{3}H]GSH conjugate of 4HNE ([^{3}H]GS-4HNE) was formed by the incubation of a 10-fold molar excess of 4HNE with [^{3}H]GSH in the presence of 100 mM Tris/HCl, pH 7.2, and 2 units of rat liver GST. The reaction was performed at 37 °C for 1 h with gentle shaking. Unlabelled

GS-4HNE was generated by incubating 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 propan-1-ol/water/acetic acid (10:5:1, by vol.). The GS-4HNE conjugates were extracted from the plates and eluted in 70% (v/v) ethanol. The extracts were dried in a Speed-Vac, dissolved in a small volume of 70% (v/v) ethanol and stored at −20 °C. The concentration of [^{3}H]GS-4HNE was determined by scintillation counting. The concentration of GS-4HNE was measured by a colorimetric assay with ninhydrin [32]. Nonyl-glutathione was used as a standard.

Transport studies

The uptake of [^{3}H]GS-4HNE into membrane vesicles was measured as described [31]. In brief, membrane vesicles (10–25 μg of protein) were rapidly thawed and added to a buffer containing either 4 mM ATP or the non-hydrolysable ATP analogue p[CH$_2$]ppA (adenosine 5′-[β,γ-methylene]triphosphate) at 4 mM, with 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase, 10 mM Tris/HCl, pH 7.4, and 250 mM sucrose. After prewarming for 1 min at 37 °C, [^{3}H]GS-4HNE was added (110 μl final volume). Samples of 25 μl were taken at indicated time points and diluted in 1 ml of ice-cold stop solution (10 mM Tris/HCl/250 mM sucrose). The stop solutions were filtered through 0.45 μm pore-size nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). Filters were washed with 5 ml of ice-cold 10 mM Tris/HCl/250 mM sucrose, dried in air and counted by liquid scintillation. ATP-dependent transport was calculated by the subtraction of pp[CH$_2$]pA values from ATP values. pp[CH$_2$]pA did not stimulate uptake, demonstrating that ATP hydrolysis was required.

RESULTS

Cytotoxicity of 4HNE in GLC$_4$ and GLC$_4$/Adr cells

First we studied the toxicity of 4HNE in GLC_4 small cell lung cancer cells and its MRP1-overexpressing multidrug-resistant counterpart GLC_4/Adr. The IC_{50} (mean ± S.D.) for GLC_4 cells was 10 ± 2.0 μM and for GLC_4/Adr cells 9.3 ± 1.5 μM. Modulation with the GSH-depleting agent BSO resulted in 4.3-fold and 1.9-fold decreases in the IC_{50} values for GLC_4 and GLC_4/Adr cells respectively. Incubation with MK571, an inhibitor for MRP1, caused 1.7-fold and 2.0-fold decreases in the respective IC_{50} values (Table 1).

Table 1 Sensitivity of GLC$_4$ and GLC$_4$/Adr cells to 4HNE

Cells were incubated with 4HNE (0–40 μM) for 4 h; cell survival was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide ('MTT') assay. BSO (25 μM) was added 16 h, and MK571 (50 μM) 1 h, before incubations with 4HNE started. Results are IC_{50} values (means ± S.D.) for at least three experiments with quadruplicate determinations. *$P < 0.05$ (one-way analysis of variance) compared with 4HNE alone (control).

Addition	IC_{50} values (μM)	
	GLC_4	GLC_4/Adr
Control (none)	10.0 ± 2.0	9.3 ± 1.5
BSO	2.3 ± 1.4*	5.0 ± 1.7*
MK571	6.0 ± 1.4*	4.7 ± 1.2*

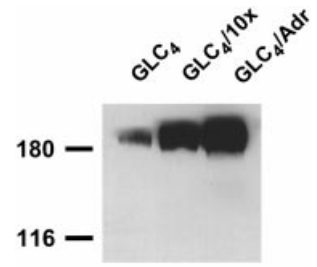

Figure 1 MRP1 expression in GLC$_4$, GLC$_4$/10x and GLC$_4$/Adr membrane vesicles

Protein (10 μg) was separated by SDS/PAGE [7.5% (w/v) gel] and transferred to nitrocellulose by electroblotting. MRP1 protein levels were analysed with the monoclonal antibody MRPr1 (1:500). The primary antibody was detected by enhanced chemiluminescence. The positions of molecular mass markers are indicated (in kDa) at the left.

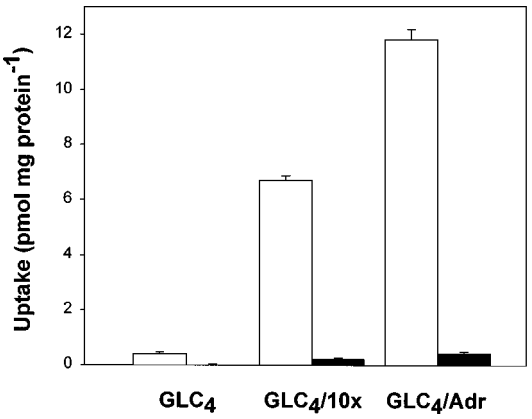

Figure 2 ATP-dependent uptake of [^{3}H]GS-4HNE into membrane vesicles with different MRP1 expression

ATP-dependent uptake of [^{3}H]GS-4HNE (10 nM) into GLC_4, GLC_4/10x and GLC_4/Adr membrane vesicles (20 μg protein) was measured over 5 min. MK571 was omitted (open bars) or added to a final concentration of 10 μM (filled bars). ATP-dependent uptake was calculated by subtraction of pp[CH$_2$]pA levels from ATP levels. Results are means ± S.D. for three experiments with quadruplicate determinations.

Uptake of [^{3}H]GS-4HNE into membrane vesicles with different MRP1 expression

We hypothesized that MRP1 is a transporter for GS-4HNE. We therefore isolated membrane vesicles from cells with different levels of MRP1 expression and measured the uptake of [^{3}H]GS-4HNE. MRP1 levels in the isolated membrane vesicles were analysed by immunoblotting (Figure 1). GLC_4, GLC_4/10x and GLC_4/Adr membrane vesicles showed respectively low, intermediate and high expressions of MRP1. ATP-dependent uptake of [^{3}H]GS-4HNE into membrane vesicles from these three cell lines was measured for 5 min (Figure 2): the uptake of [^{3}H]GS-4HNE was 0.4, 6.7 and 11.8 pmol/mg of protein respectively for GLC_4, GLC_4/10x and GLC_4/Adr cells. This ATP-dependent uptake was completely inhibited by MK571 (10 μM final concentration). The uptake rate of [^{3}H]GS-4HNE was correlated with levels of MRP1 expression. As control, membrane vesicles from A2780 ovarian tumour cells and their MDR1-overexpressing counterpart A2780AD, both with basal MRP1 expression [31], showed only marginal ATP-dependent [^{3}H]GS-4HNE uptake (results not shown).

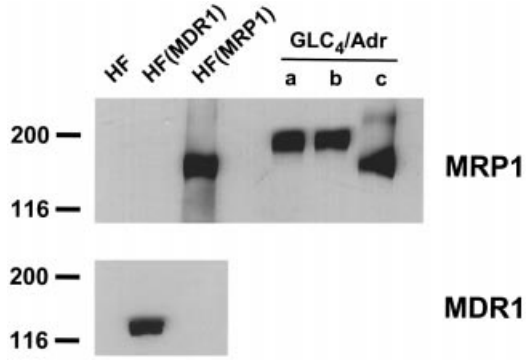

Figure 3 Immunoblot analysis of recombinant MRP1 and MDR1 expression in High Five (HF) insect cells

Protein (20 μg) was separated by SDS/PAGE [7.5% (w/v) gel] and transferred to nitrocellulose by electroblotting. Membrane vesicles from GLC_4/Adr cells (5 μg of protein) served as positive control for MRP1 expression (lane a). GLC_4/Adr membranes (5 μg of protein) were incubated with deglycosylation buffer in the absence (lane b) or the presence (lane c) of PNGase F. Protein levels were analysed with monoclonal antibody MRPr1 (1:500) for MRP1 and with monoclonal antibody C219 (1:300) for MDR1. The primary antibodies were detected by enhanced chemiluminescence. The positions of molecular mass markers are indicated (in kDa) at the left.

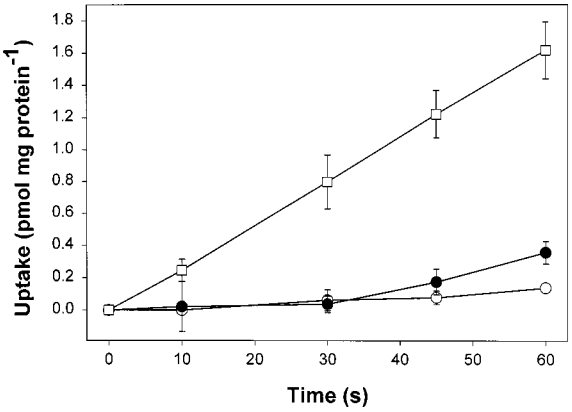

Figure 4 ATP-dependent uptake of [^{3}H]GS-4HNE into membrane vesicles from High Five (HF) insect cells overexpressing recombinant MRP1 and MDR1

Time course of ATP-dependent uptake of [^{3}H]GS-4HNE (10 nM) into HF (○), HF(MRP1) (□) and HF(MDR1) (●) membrane vesicles (20 μg of protein). ATP-dependent uptake was calculated by subtraction of pp[CH_2]pA levels from ATP levels. Results are means ± S.D. for three experiments with quadruplicate determinations.

Production of recombinant MRP1

To demonstrate MRP1-specific [^{3}H]GS-4HNE transport, recombinant MRP1 was produced by HF insect cells with the baculovirus system. Parental HF cells and HF cells infected with an 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 approx. 20 kDa less than that in GLC_4/Adr cells. Treatment of membrane vesicles from GLC_4/Adr cells with PNGase F, which cleaves the N-linked glycosylation, decreased the molecular mass of MRP1 from GLC_4/Adr membrane vesicles to a mass similar to that from MRP1 expressed in untreated HF(MRP1) membrane vesicles (Figure 3). Treatment of HF(MRP1) membrane vesicles with PNGase F did not further decrease the molecular mass of MRP1 (results not shown). These results suggest that MRP1 expressed in HF cells is not, or is only partly, glycosylated.

Table 2 Effect of inhibitors of MRP1-mediated transport on ATP-dependent uptake of [^{3}H]GS-4HNE

ATP-dependent uptake of [^{3}H]GS-4HNE (10 nM) into HF(MRP1) membrane vesicles (20 μg of protein) was measured for 1 min in the absence or the presence of each of the indicated compounds. ATP-dependent uptake was calculated by subtraction of pp[CH_2]pA levels from ATP levels. Results are means ± S.D. for at least two experiments with quadruplicate determinations. *$P < 0.05$ (one-way analysis of variance) compared with the control.

Compound	Concentration (μM)	[^{3}H]GS-4HNE transport (% of control)
None	–	100
Methyl-glutathione	1	102 ± 3.4
	10	111 ± 3.7
Propyl-glutathione	1	109 ± 7.9
	10	102 ± 5.4
Hexyl-glutathione	1	83 ± 4.9
	10	52 ± 3.4*
Nonyl-glutathione	1	47 ± 8*
	10	3 ± 10*
GSH	5000	94 ± 1
GSSG	100	61 ± 7.8*
	500	18 ± 13*
MK571	0.5	95 ± 6.9
	1	80 ± 8.3*
	5	42 ± 12.8*

Characterization of [^{3}H]GS-4HNE transport by recombinant MRP1

ATP-dependent uptake of [^{3}H]GS-4HNE into HF(MRP1) membrane vesicles was linear for 1 min and was increased by up to 11-fold compared with that of HF or HF(MDR1) membrane vesicles (Figure 4). To characterize further the ATP-dependent uptake of [^{3}H]GS-4HNE into HF(MRP1) membrane vesicles, we studied the effect of several inhibitors of MRP1-mediated transport (Table 2). Inhibition of [^{3}H]GS-4HNE uptake increased with the length of the alkyl chain of the GS-derivative, and varied from undetectable (methyl-glutathione and propyl-glutathione) to highly effective (nonyl-glutathione). GSH did not inhibit [^{3}H]GS-4HNE uptake, whereas GSSG was a weak inhibitor. MK571 inhibited [^{3}H]GS-4HNE uptake in a dose-dependent manner. Together with the results presented in Figure 2, these results demonstrate that [^{3}H]GS-4HNE is transported by MRP1.We examined the kinetics of MRP1-mediated [^{3}H]GS-4HNE transport and determined an apparent K_m of 1.6 ± 0.21 μM and a V_{max} of 804.5 ± 28.8 pmol/min per mg of protein (Figure 5).

DISCUSSION

ROS are formed under physiological conditions in eukaryotic cells as a consequence of aerobic metabolism. ROS can cause lipid peroxidation and the formation of potentially toxic aldehydes such as 4HNE [2]. Endogenously formed adducts of 4HNE with deoxyguanosine have been detected and are supposed

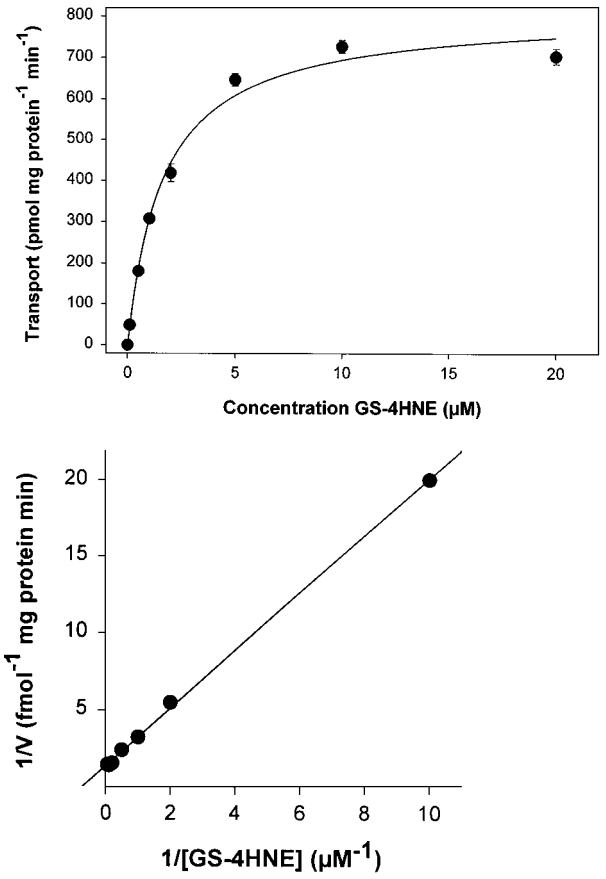

Figure 5 Kinetics of MRP1-mediated [^{3}H]GS-4HNE transport

Upper panel: ATP-dependent transport of [^{3}H]GS-4HNE into HF(MRP1) membrane vesicles (25 μg) was measured over 1 min. ATP-dependent uptake was calculated by subtraction of pp[CH_2]pA levels from ATP levels. Results are means $\pm$ S.D. for two experiments with quadruplicate determinations. Lower panel: double-reciprocal Lineweaver–Burk plot. Curve fitting and the determination of K_m and V_{max} values were performed with the Sigma Plot 3.03® program.

to be involved in ROS-mediated tumour promotion [33]. In addition, at low concentrations, 4HNE seems to have a role in cell proliferation and differentiation [34–36]. 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 [1,2]. 4HNE causes cellular damage mainly by the modification of intracellular proteins [6,37]. The detection of 4HNE-modified proteins in early stages of degenerative diseases implies that 4HNE could have a role in the onset of these disorders. Efficient protection mechanisms preventing the accumulation of 4HNE are therefore required.

GSH is present in all eukaryotic cells and serves as a protection mechanism against ROS-induced toxic intermediates, either by reduction or by conjugation [20]. 4HNE is conjugated with GSH and in this study we have shown that MRP1 is a transporter for GS-4HNE and thus can function as an export mechanism. This is consistent with the idea of a cardiac export system for GS-4HNE that might in fact be MRP1 [21]. Factors that have a role in this detoxification pathway for 4HNE are GSH ([5], and this study), GST [18] and MRP1 (this study). GLC_4 and GLC_4/Adr differ greatly in the expression of MRP1 but this is not correlated with sensitivity to 4HNE in these cells. Thus under basal conditions GSH and GST seem to be the dominant factors in the protection against 4HNE. However, on inhibition of MRP1, GLC_4 and GLC_4/Adr cells become more sensitive to 4HNE showing that MRP1 does indeed have 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 has a role in defence against 4HNE toxicity, it does so in conjunction with GSH and GST. For example, inhibition of MRP1 leads to the accumulation of GS-4HNE; this might cause product inhibition of GST and thus an increase in the intracellular concentration of 4HNE.

In the present study we have 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 with Sf21 insect cells, this does not influence the transport capacity of MRP1 [38]. Compared with the K_m values of other MRP1 substrates, the K_m that we found for MRP1-mediated GS-4HNE transport suggests that GS-4HNE is an MRP1 substrate with a relatively high affinity [24]. N-alkyl GSH conjugates are potent inhibitors of GSTs [39]. For example, 200 μM n-octyl-glutathione results in an approx. 80 % inhibition of the GSTs. The efficient removal of such products from GST is necessary to prevent product inhibition [21]. Because of the low K_m of MRP1 for GS-4HNE, we conclude that MRP1 will effectively contribute to a rapid diminution in the inhibitory effects of GSH conjugates such as the oxidative stress product GS-4HNE.

MRP1 shows a substrate specificity highly similar to that of MRP2 [24]. In contrast with MRP2, MRP1 is expressed at low levels in normal liver and is localized at basolateral membranes, whereas MRP2 is located at canalicular membranes. The detection of GS-4HNE in bile after the administration of 4HNE [40] suggests that GS-4HNE might also be a substrate for MRP2 and that MRP2 might have a role in the detoxification of 4HNE in the liver by extrusion of GS-4HNE into bile.

MRP1 was originally identified in drug-resistant tumour cells; research has focused on its capacity to confer drug resistance [41]. Overexpression of MRP1 is associated with resistance against the anthracycline doxorubicin [42]. Although MRP1 is a transporter for anthracyclines [29,31], doxorubicin resistance levels from MRP1-overexpressing cells often are not correlated with a decrease in its intracellular accumulation [43,44]. Doxorubicin is known to generate ROS; increased 4HNE formation has been observed after doxorubicin administration [45]. In addition, transfection with a GST isoenzyme with a high affinity for 4HNE resulted in resistance to doxorubicin [46]. MRP1 might therefore also have a role in doxorubicin resistance by the export of endogenously formed GS-4HNE.

Leukotriene C_4 (LTC_4) and prostaglandin A-glutathione (PGA-GS) are two other endogenously formed GSH conjugates that are efficiently transported by MRP1 [47,48]. LTC_4 is important in the inflammatory response [49] and PGA causes cell cycle arrest and apoptosis [50,51]. A tight regulation of the intracellular concentrations of LTC_4 and PGA is required to retain the response to inflammatory stimuli and to prevent toxic effects. MRP1-mediated GSSG transport suggested that MRP1 might also be involved in protection against oxidative stress [52]. The oxidative-stress-mediated induction of the genes encoding MRP1 and γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, supports this [53]. GSSG is generated by the GSH-mediated reduction of ROS; MRP1, as GSSG transporter, prevents intracellular GSSG accumulation and might

function in maintaining the cellular redox state [20]. In this study we have demonstrated that MRP1 protects against the toxicity of one of the most important metabolites formed during oxidative stress. On the basis of its ubiquitous expression and its substrate specificity, the physiological functions of MRP1 seem to be the cellular extrusion of GSH-dependent detoxification products and involvement in the regulation of the cellular redox homoeostasis [27].

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

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