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Biochemical Pharmacology 70 (2005) 500–510
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Interactions of mefloquine with ABC proteins, MRP1 (ABCC1) and MRP4 (ABCC4) that are present in human red cell membranes

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Received 2 March 2005; accepted 23 May 2005

Abstract

Human erythrocyte membranes express the multidrug resistance-associated proteins, MRP1, MRP4 and 5, that collectively can efflux oxidised glutathione, glutathione conjugates and cyclic nucleotides. It is already known that the quinoline derivative, MK-571, is a potent inhibitor of MRP-mediated transport. We here examine whether the quinoline-based antimalarial drugs, amodiaquine, chloroquine, mefloquine, primaquine, quinidine and quinine, also interact with erythrocyte MRPs with consequences for their access to the intracellular parasites or for efflux of oxidised glutathione from infected cells. Using inside-out vesicles prepared from human erythrocytes we have shown that mefloquine and MK-571 inhibit transport of 3 μM [^{3}H]DNP-SG known to be mediated by MRP1 (IC_{50} 127 and 1.1 μM, respectively) and of 3.3 μM [^{3}H]cGMP thought but not proven to be mediated primarily by MRP4 (IC_{50} 21 and 0.41 μM). They also inhibited transport in membrane vesicles prepared from tumour cells expressing MRP1 or MRP4 and blocked calcein efflux from MRP1-overexpressing cells and BCECF efflux from MRP4-overexpressing cells. Both stimulated ATPase activity in membranes prepared from MRP1 and MRP4-overexpressing cells and inhibited activity stimulated by quercetin or PGE_1, respectively. Neither inhibited [α-^{32}P]8-azidoATP binding confirming that the interactions are not at the ATP binding site. These results demonstrate that mefloquine and MK-571 both inhibit transport of other substrates and stimulate ATPase activity and thus may themselves be substrates for transport. But at concentrations achieved clinically mefloquine is unlikely to affect the MRP1-mediated transport of GSSG across the erythrocyte membrane.

Keywords: ATP hydrolysis; Mefloquine; Multidrug resistance-associated proteins 1 and 4; MK-571; Erythrocyte membranes

1. Introduction

Multidrug resistant-associated proteins (MRPs) mediate ATP-dependent transport of a wide range of compounds including glutathione conjugates and cyclic nucleotides. MRP1 (ABCC1) is a primary active transporter of organic anions including the oxidised form of glutathione, GSSG [1]. It is expressed in human erythrocytes where it accounts for the high affinity component of the transport of GSSG and dinitrophenyl-*S*-glutathione, DNP-SG [2–4]. In addition to MRP1 there are in red blood cells other efflux transporters, one or more of which account for the low affinity efflux of GSSG, DNP-SG and cGMP. The identities of these transporters have not been conclusively identified though MRP4 is thought to play a major role [4]. One of the most potent inhibitors of MRP-mediated transport is the LTD4 receptor antagonist MK-571, a quinoline derivative. Another quinoline-based compound, IAAQ, photolabels MRP1 and both chloroquine and MK-571 inhibit this labelling [5]. Thus it is probable that at least some quinolines in clinical use interact with MRPs. Quinoline-based antimalarials, especially chloroquine, have been used for decades to kill malaria parasites in the intraerythrocytic stage of their life cycle, If indeed these quinolines do

Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5-(6)-carboxyfluorescein; BCECF-AM, BCECF acetoxy-methyl ester; BeFx, Beryllium fluoride; calcein-AM, calcein acetoxy-methyl ester; DNP-SG, dinitrophenyl S-glutathione conjugate; GSH, reduced glutathione; GSSG, oxidised glutathione; MK-571, (3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl) ((3-(dimethyl amino-3-oxo propyl)thio)methyl)thio)propanoic acid; MRP, multidrug resistance-associated protein

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doi:10.1016/j.bcp.2005.05.022

interact with active transporters on erythrocytes, the possibility exists that they could affect access of drug to the parasite inside the cell. Interaction of mefloquine with *P*-glycoprotein, a mammalian ATP-binding cassette transporter has been documented [6,7] but, while it has been sought, *P*-glycoprotein has not been detected in human erythrocyte membranes by immunoblotting [8] (Wu and Woodcock personal communication). Little or no information is available regarding the interaction of mefloquine with other ABC transporters such as the MRPs that are present in red blood cells.

The aim of the present study was to explore how some widely used quinoline-based antimalarial drugs affect activities of some MRPs known to be present in human erythrocytes. Using inside-out vesicles prepared from human red blood cells, we show that MK-571 and mefloquine directly inhibit MRP-mediated uptake of DNP-SG or cGMP. Confirmation of these interactions was obtained using inside-out vesicles prepared from MRP1-overexpressing tumour cells or transfected HEK 293 cells overexpressing MRP4. Further evidence of their interactions was obtained by observing their effects on accumulation and efflux of MRP substrates at the whole cell level. The effects of quinolines on ATPase activities and ATP binding to MRP1 and MRP4 protein provide further insight into the nature of the quinoline-MRP interactions and suggest that MK-571 and mefloquine appear to be substrates for transport.

2. Materials and methods

2.1. Cell culture

Cells of the human large-cell lung tumour line L23/P and of its doxorubicin-selected MRP1-overexpressing multidrug-resistant variant L23/R [9,10] were cultured as previously described [4]. Parental 293 human embryonic kidney cells (HEK 293 cells) and the MRP4-overexpressing HEK 293/4.63 cells [11] were generous gifts of Prof P. Borst (Division of Molecular Biology and Center for Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands). HEK 293/4.63 cells were reported to express 75 times more MRP4 than the controls [12]. Parental HEK 293 cells and transfectants were grown in DMEM, supplemented with 10% fetal calf serum and 100 units of penicillin/streptomycin per millilitre (Invitrogen), at 37 °C under 5% CO_2-humidified air. Continued expression of MRP4 was confirmed using immunoblot analysis with anti-MRP4 as previously described [4].

2.2. Preparation of plasma membrane vesicles

Membrane vesicles from human erythrocytes were isolated as previously described [4] using the one-step, spontaneous vesiculation procedure [13,14]. The membrane vesicles formed and washed in a solution containing 2 mM Hepes and 0.1 mM EGTA (pH 7.5) were finally resuspended in 10 mM Tris–HCl (pH 7.4), frozen and stored at −80 °C until use.

Membrane vesicles from human lung tumour cells were prepared as described previously [4,15] in the presence of protease inhibitors (5 μg ml^{-1} leupeptin, 2 μg ml^{-1} aprotinin, 80 ng ml^{-1} pepstatin). Vesicles were formed, washed and stored in buffer containing 10 mM Tris–HCl, 250 mM sucrose (pH 7.4), and stored in aliquots at −80 °C.

Membrane vesicles from HEK 293 and HEK 293/4.63 were prepared as described previously [12]. Briefly, cells were harvested by centrifugation at 3000 rpm for 5 min. The pellet was resuspended in ice-cold hypotonic buffer containing 0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.4, supplemented with mixture of protease inhibitors containing 2 mM phenylmethylsulfonyl fluoride, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin and 10 μM pepstatin. The suspended mixture was incubated at 4 °C for 90 min before centrifuged at 4 °C at 100,000 × *g* for 40 min. The pellet was homogenized using a tight-fitting Dounce homogenizer in ice-cold TS buffer containing 50 mM Tris–HCl, 250 mM sucrose, pH 7.4. The homogenised mixture was centrifuged at 500 × *g* at 4 °C for 10 min before supernatant was centrifuged at 100,000 × *g* at 4 °C for 40 min. The pellet was resuspended in TS buffer and passed through a 27-gauge needle 25 times. The vesicles were dispensed, frozen in aliquots and stored at −80 °C until use.

The protein content of the membrane vesicles was determined using the BCA (Bicinchoninic acid) protein assay using bovine serum albumin as standard. Sidedness of the vesicles as assessed from the activity of glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase in the presence or absence of 0.05% Triton X-100 was generally around 30–37% of vesicles inside-out.

2.3. Vesicle Transport of [^{3}H]DNP-SG and of [^{3}H]cGMP

ATP-dependent transport of DNP-SG or cGMP into membrane vesicles was measured by a rapid filtration technique [4,15]. All standard transport assays were carried out at 37 °C. Vesicles (12.5–25 μg of membranes from L23/R, 50 μg of membranes from human erythrocytes, or 40 μg for HEK) were added to a buffer system (55 μl final volume) containing 1 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg ml^{-1} creatine kinase, 10 mM Tris–HCl (pH 7.4) and either 3 μM [^{3}H]DNP-SG or 3.3 μM [^{3}H]cGMP. For experiments with vesicles from L23/R cells or HEK cells, 250 mM sucrose was included in the efflux, stop and wash solutions. All transport data are presented as the difference of the values measured in the presence and absence of ATP. Inhibitors were added

to the uptake buffer solution immediately prior to the addition of the vesicles. Red blood cells possess phosphodiesterases [16,17], however the activity is low and cleavage products have not been detected even in prolonged incubations for transport experiments [18].

2.4. Fluorimetric determination of calcein and of BCECF efflux

Fluorescence for cells adherent to glass coverslips was measured as described previously [19]. Cells overexpressing MRP1 (L23/R) or MRP4 (HEK 293/4.63) were plated onto glass coverslips 24–48 h before use so that at the time of the experiment they would cover the illuminated area, about 2 mm × 8 mm. Cells were loaded with calcein or BCECF by exposure to 0.5 μM of the esterified precursors at 15 °C in 2.5 ml of the standard buffer containing 135 mM NaCl, 4 mM KCl, 0.3 mM $CaCl_2$, 0.6 mM $MgSO_4$, 10 mM HEPES, 1.1 mM Na_2PO_4, 0.6 mM KH_2PO_4 and 6 mM glucose (pH 7.4). Calcein/BCECF fluorescence was monitored at 37 °C using an Hitachi 2000 spectrofluorimeter with excitation 485 nm, emission 530 nm, 10 nm bandpass and corrected for background fluorescence by subtracting the value measured at the end of each experiment after releasing the remaining cytoplasmic fluorescent dye by permeabilising the cells with 0.1% (w/v) saponin. The rate constant for efflux was estimated by linear unweighted least-squares fitting to the steepest 400 s portion of a plot of ln[fluorescence (t)/fluorescence (t_0)] versus t where t is the time and t_0 is an arbitrary, convenient reference time. Fluorescent dye efflux assays were performed with and without the inhibitor present during the efflux period.

2.5. Preparation of crude membrane from High Five insect cells infected with recombinant baculovirus carrying the human MRP1 or MRP4 cDNA

High Five insect cells infected with the recombinant baculovirus carrying the human MRP1 cDNA with 10 histidine tag at the C-terminal end (BV-MRP1(H10)) or human MRP4 cDNA (pVL1393-MRP4 plasmid were provided by Dr. Gary Kruh, Fox Chase Cancer Center, PA) [20]. Crude membranes were prepared from these cells as described previously [21]. The protein content was determined by the amido black method [22].

2.6. ATPase assays

ATPase activities of MRP1 and MRP4 in crude membranes were measured by endpoint, P_i assay as previously described [23,24]. MRP-specific activity was recorded as BeFx-sensitive ATPase activity. The assay measured the amounts of inorganic phosphate released over 20 min at 37 °C in the ATPase assay buffer (50 mM MES–Tris, pH 6.8, 50 mM *N*-methyl-D-glucamine chloride, 5 mM NaN_3, 1 mM EGTA, 1 mM ouabain, 2 mM DTT and 10 mM $MgCl_2$) in the absence or presence of BeFx (2.5 mM NaF and 0.2 mM beryllium sulfate). The assay was initiated by the addition of 5 mM ATP in the presence and absence of chemicals or transport substrates and quenched with SDS (2.5% final concentration). The amount of P_i released was quantified using a colorimetric method [25].

2.7. Binding of [α-^{32}P]8-azidoATP

Crude membranes (50 μg) were incubated in the ATPase assay buffer containing 10 μM [α-^{32}P]8-azidoATP (10 μCi nmol^{-1}) in the dark at 37 °C for 5 min and then 4 °C for 5 min in the presence or absence of indicated concentrations of tested compounds. The samples were then illuminated with a UV lamp assembly (365 nm) for 10 min on ice (4 °C). Ice-cold ATP (12.5 mM) was then added to displace excess non-covalently bound [α-^{32}P]8-azidoATP. After SDS-PAGE on an 7% Tris–glycine gel at constant voltage, gels were dried and exposed to Bio-Max MR film at −70 °C for the required period of time (12 h) as described previously [26].

2.8. Materials

[Glycine-2-^{3}H]glutathione (GSH) (1.9 TBq mmol^{-1}) and [8-^{3}H]-guanosine-3′,5′-cyclic phosphate, NH_4 salt (cGMP) (0.559 TBq mmol^{-1}) were purchased from Perkin-Elmer LAS Ltd. and Amersham Biosciences, respectively. Amodiaquine, ATP, ATP-γ-S, 1-chloro-2,4-dinitrobenzene (cDNB), chloroquine, creatine phosphokinase, creatine kinase, 3′,5′-cyclic guanosine monophosphate (cGMP), doxorubicin, reduced glutathione (GSH), glutathione *S*-transferase, primaquine, prostaglandin E_1 (PGE_1), quercetin, quinine and quinidine were all obtained from Sigma Chemicals (Poole, Dorset, UK); (3-([[3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl]-[((3-(dimethylamino-3-oxopropyl)-thio]-methyl]thio) propanoic acid, MK-571, from SPI-BIO, Massy, France; the acetoxy-methyl esters of calcein (calcein-AM) and of BCECF (BCECF-AM) from Molecular Probes; NaF and $BeSO_4$ from Fluka (Sigma-Aldrich). Mefloquine was a gift of Dr P. Smith (Malaria Research Center, Department of Pharmacology, University of Cape Town, Cape Town, RSA). The monoclonal antibody anti-MRP4, which has been previously described [20] was a kind gift of Dr G. Kruh (Fox Chase Cancer Centre, Philadelphia, PA, USA). GSH stock solutions were freshly prepared on the day of each experiment. Stock solutions for the quinolines were prepared in DMSO except for mefloquine which was dissolved in methanol. BeFx was added to assays as 2.5 mM NaF plus 0.2 mM $BeSO_4$ (both Fluka Biochemical).

[^{3}H]DNP-SG was synthesised enzymatically as previously described [27,28]. The separated product was lyophilised, and stored at −20 °C until used. The purity

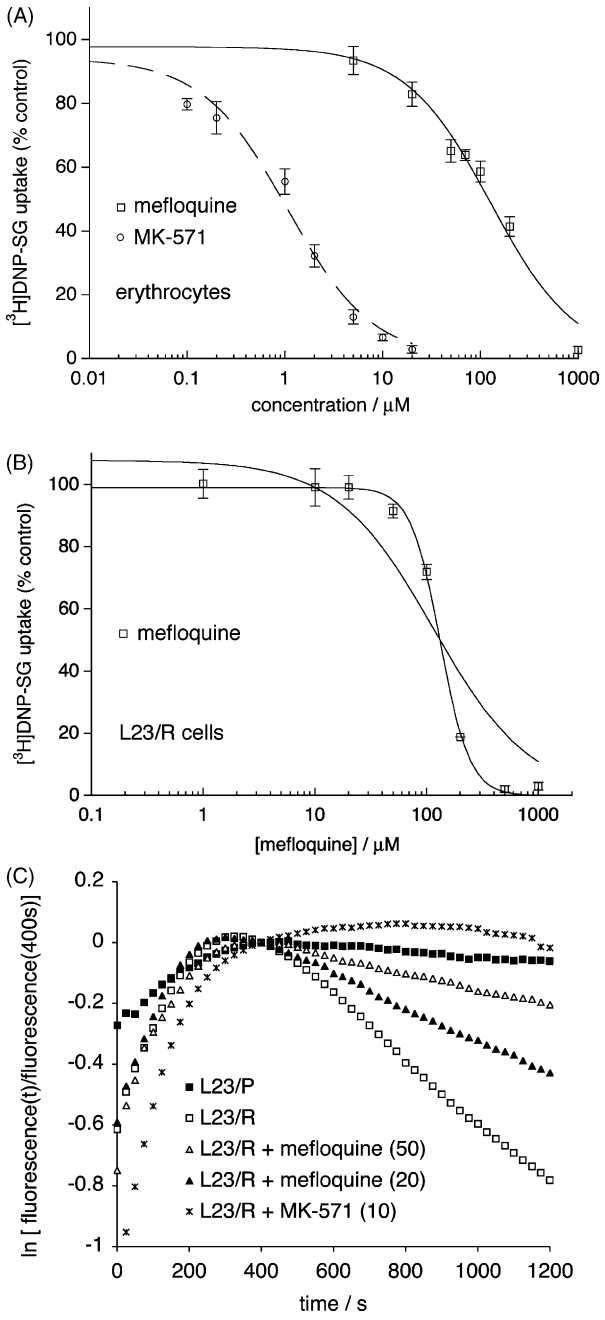

Fig. 1. MK-571 and mefloquine inhibit MRP1-mediated transport of 3 μM [^{3}H]DNP-SG into membrane vesicles prepared from human erythrocytes (A) or L23/R cells (B) and efflux of calcein from MRP1-overexpressing L23/R cells (C). (A) and (B) ATP-dependent uptake at 37 °C for 30 min is shown as a percentage of the mean of the controls with no inhibitor. The inhibition curves are fitted as described in the methods. The shallower curve in (B) is fitted assuming the inhibition is non-cooperative; the steeper curve allows for cooperativity. (C) calcein fluorescence vs. time for parent, L23/P, cells or for MRP1-overexpressing multidrug resistant L23/R cells in the absence of inhibitors or in the presence of 20 μM mefloquine, 50 μM mefloquine or 10 μM MK-571. Cells were loaded with calcein by exposure to 0.5 μM calcein-AM for 15 min at 15 °C and were transferred at time zero into dye-free buffer at 37 °C. The initial increase in fluorescence presumably reflects continuation of conversion of non-fluorescent ester inside the cells into its fluorescent de-esterified form. The rate constants for efflux are given in Table 1. Values are shown as mean values ($n = 4$, S.E.M. < 0.06 for all points).

of the [^{3}H]DNP-SG was determined by thin-layer chromatography on silica gel plates [(0.25 × 40 × 80) mm, Alugram® SIL G/UV254, Macherey-Nagel, Germany] using *n*-propanol:water (7:3, v/v) as solvent [29].

2.9. Data analysis

Fits to inhibition curves were to equations of the form:

$$U = \frac{(100\% - U_{\text{noninh}}) \times \text{IC}_{50}^{nh}}{\text{IC}_{50}^{nh} + I^{nh}} + U_{\text{noninh}}$$

where I is the concentration of the inhibitor, IC_{50}, the inhibitor concentration producing 50% inhibition of the inhibitable component, *nh*, the Hill slope which is 1 when the inhibition is non-cooperative and U_{noninh} is the percentage of the uptake which cannot be inhibited. $nh \neq 1$ was used only in fitting Fig. 1B. Data were fitted using KaleidaGraph® (unweighted least-squares). Fits with and without a non-inhibitable component were compared using an F-test on the ratio of the variance associated with the reduction of one degree of freedom to the variance remaining [30]:

$$\text{VR} = \frac{(\text{SSE}_2 - \text{SSE}_1)/1}{\text{SSE}_1/\text{df}}$$

where SSE_1 is the sum of squared residuals and df = number of data points − number of fitting parameters when U_{noninh} is allowed to vary and SSE_2 is the sum of squared residuals for the fit assuming $U_{\text{noninh}} = 0$. For Fig. 1B the fit with variable nh was compared with that assuming $nh = 1$ in the same manner. The improvement in fit is labelled "significant" if the probability, p, from the F-test is less than 0.05.

Values are shown as mean ± S.E.M.

3. Results

3.1. Effects of quinoline derivatives on high affinity DNP-SG transport in human erythrocyte vesicles

ATP-dependent uptake of DNP-SG was measured over the course of 30 min in the absence or presence of quinolines. It has previously been shown [31] that DNP-SG uptake into human erythrocyte vesicles is linear for at least 60 min. The rate of uptake was significantly inhibited by MK-571 and by mefloquine with IC_{50} values of 1.1 ± 0.2 μM ($n = 7$, maximum percentage uptake 94 ± 4%) and 127 ± 17 μM ($n = 4$, maximum percentage uptake 98 ± 3%), respectively (see Fig. 1A). Other quinoline antimalarial drugs (amodiaquine, chloroquine, primaquine, quinidine and quinine) used at concentrations between 200 and 1000 μM showed little or no inhibition of uptake rates (data not shown).

The high affinity DNP-SG transport in erythrocyte membranes is known to be mediated by MRP1 [2,4]. To

Table 1
Effect of selected quinolines on the rate constants for MRP1-mediated calcein efflux from L23/R cells

	Rate constant (10^{-4} s^{-1} mean ± S.E.M.)
L23/P control	0.9 ± 0.1
L23/R control	10.9 ± 0.4
+10 μM MK-571	1.2 ± 0.4
+5 μM mefloquine	7.8 ± 0.2
+10 μM mefloquine	6.7 ± 0.3
+20 μM mefloquine	5.3 ± 0.3
+50 μM mefloquine	2.3 ± 0.5
+50 μM amodiaquine	8.3 ± 0.9
+50 μM chloroquine	12 ± 2
+50 μM quinine	7.0 ± 1.7
+50 μM quinidine	11.0 ± 0.3
+50 μM primaquine	10 ± 1

Each value is the mean of at least three experiments.

verify that mefloquine can inhibit MRP1, its effects on DNP-SG transport were observed using vesicles made from L23/R lung tumour cells that highly overexpress MRP1 (>500-fold, Wu et al., in press) with only very low expression if any of *P*-glycoprotein and other MRP proteins [32] (Wu et al., in press) except for a (presumably non-functional) fragment of MRP6 [33]. It has been shown previously that MK-571 completely inhibits DNP-SG uptake into these vesicles [19,31]. As shown in Fig. 1B mefloquine also completely inhibited the DNP-SG uptake. The data were significantly better fitted allowing for cooperative inhibition ($nh = 3.3 \pm 0.2$) than with the assumption that inhibition was non-cooperative ($p < 0.001$) with an IC_{50} value of 131 ± 4 μM (maximum percentage binding 99 ± 1%). The cooperativity may indicate interaction between the MRP1 molecules at the high concentrations found in the strongly overexpressing L23/R cells.

Additional evidence of mefloquine interactions with MRP1 was obtained by observing its inhibitory effect on the efflux of calcein from L23/R (MRP1-overexpressing cells). Calcein is a known substrate for MRP1 and it has previously been shown that its efflux is blocked by MK-571 [19]. The IC_{50} for inhibition by mefloquine was between 10 and 20 μM (see Fig. 1C). The other quinolines tested at 50 μM produced either much less inhibition or no inhibition (see Table 1 showing rate constants for efflux in the presence of the various quinolines).

3.2. Effects of quinoline derivatives on cGMP transport in human erythrocyte vesicles

In human erythrocytes, efflux of cGMP is mediated by a transporter (or transporters) distinct from MRP1 [4]. In the present study, this cGMP transport was found to be inhibited (see Fig. 2A) by MK-571 ($IC_{50} = 0.41 \pm 0.03$ μM, maximum percentage uptake = 99 ± 2%, non-inhibitable uptake −0.3 ± 1.4%, n.s., $p = 0.8$)) and by mefloquine ($IC_{50} = 21 \pm 3$ μM, maximum percentage uptake = 102 ± 2%, non-inhibitable uptake 11 ± 3%, $p < 0.01$)

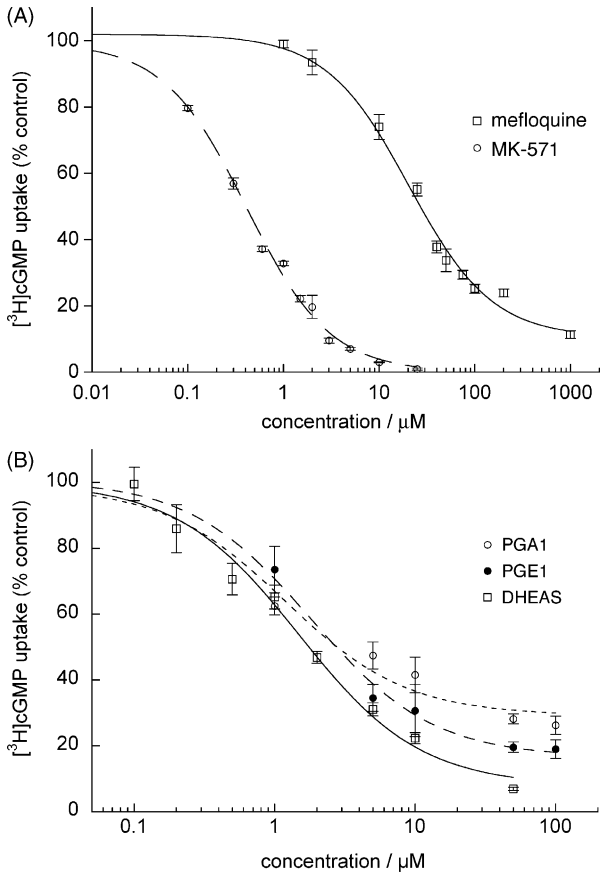

Fig. 2. MK-571, mefloquine, DHEAS, PGA_1 and PGE_1 inhibit uptake of 3.3 μM [^{3}H]cGMP into inside-out membrane vesicles prepared from human erythrocytes. ATP-dependent uptake at 37 °C for 30 min is shown as a percentage of the mean of controls with no inhibitor. The points are displayed as mean ± S.E.M (MK-571 $n = 4$; mefloquine $n = 4$; DHEAS, $n = 6$; PGE_1, $n \geq 4$; PGA_1, $n = 6$). The inhibition curves are fitted as described in the methods.

and at higher concentrations by all the other quinolines (see Table 2). It should be noted that whereas the uptake was totally inhibited by MK-571, for several quinolines including mefloquine there was a small non-inhibitable component (11–33%).

Table 2
Effect of quinoline-based drugs on uptake of 3.3 μM [^{3}H]cGMP into inside-out human erythrocyte membrane vesicles

	IC_{50} μM mean ± S.E.M.	Percent of uptake that is non-inhibitable mean ± S.E.M. (p)
MK-571	0.41 ± 0.03	−0.3 ± 1.4 (0.82)
Mefloquine	21 ± 3	11 ± 3 (0.01)
Quinine	67 ± 22	33 ± 5 (0.02)
Quinidine	56 ± 12	13 ± 5 (0.06)
Chloroquine	86 ± 14	[a]
Amodiaquine	52 ± 16	24 ± 5 (0.01)
Primaquine	127 ± 18	15 ± 3 (0.01)

Each value point is the mean of at least four experiments. p Values are for a two-sided t-test for the hypothesis that the non-inhibitable percentage is zero.

[a] The unconstrained fit for non-inhibitable uptake for chloroquine produced a negative value. To calculate the IC_{50} the data were refitted with this value set to zero.

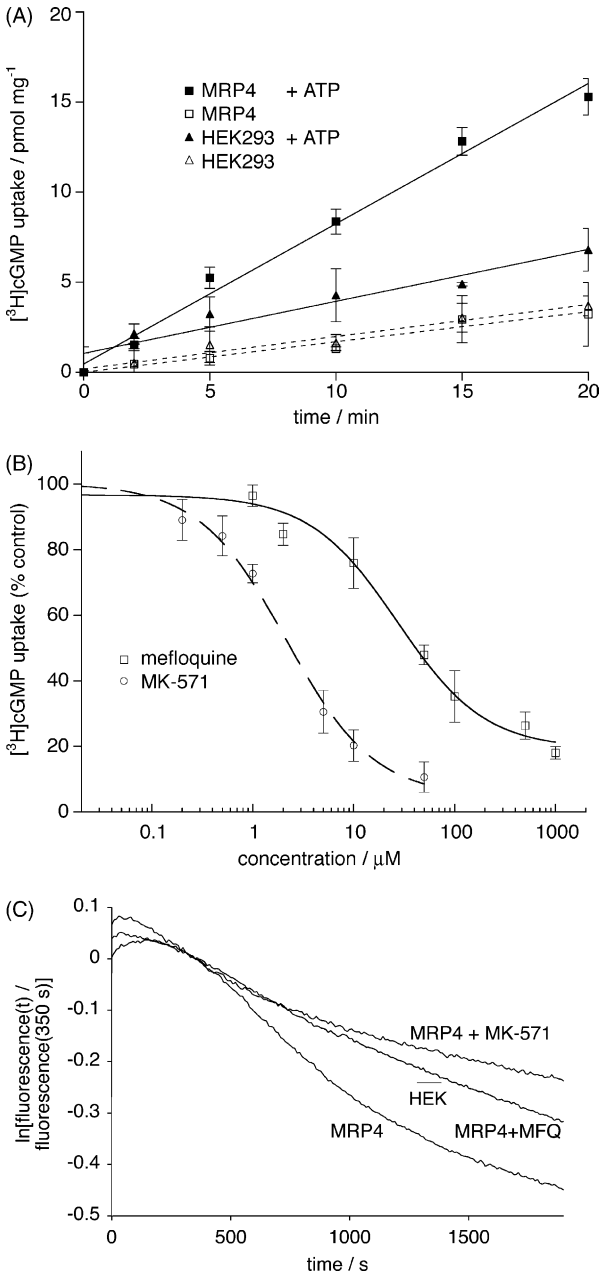

Fig. 3. MK-571 and mefloquine inhibit uptake of [^{3}H]cGMP into inside-out membrane vesicles prepared from HEK 293 cells expressing MRP4 and efflux of BCECF from MRP4-expressing cells. (A) Time dependent uptake of 3.3 μM cGMP into inside-out vesicles prepared from HEK 293 cells expressing MRP4 (HEK 293/4.63) (squares) or from parental HEK 293 cells (triangles) is shown in the presence of 3 mM ATP (filled symbols) or the non-hydrolysable analogue ATP-γ-s (open symbols). Values represent mean ± S.E.M. ($n = 3$). Similar curves for erythrocyte membrane vesicles have been reported previously, the rate of ATP-dependent uptake being more than 10 times greater than seen here. (B) 15 min uptake into membrane vesicles from HEK 293/4.63 cells (40 μg protein equivalent) in transport medium containing 3.3 μM [^{3}H]cGMP in the presence of increasing concentrations of MK-571 and mefloquine. Data points represent mean ± S.E.M. ($n = 3$). (C) BCECF fluorescence was monitored over time for HEK 293 cells (HEK) or for MRP4-expressing HEK 293/4.63 cells in the absence (MRP4) or presence of 50 μM mefloquine (MRP4 + MFQ) or 50 μM MK-571 (MRP4 + MK-571). The curves for HEK 293 cells and for HEK 293/4.63 in the presence of 50 μM mefloquine are almost superimposed. Cells were exposed to 0.5 μM BCECF-AM for 15 min at 15 °C to allow diffusional entry of the non-fluorescent ester and cleavage inside the cells to the fluorescent BCECF. Cells were transferred at time zero from 0.5 μM BCECF-AM at 15 °C to BCECF and BCECF-AM free buffer at 37 °C. Values are shown as means of at least three independent experiments (S.E.M. < 0.06).

3.3. Characterisation of cGMP transport in human erythrocyte vesicles using PGA_1, PGE_1 and dehydroepiandrosterone-3-sulfate (DHEAS)

A likely candidate for transport of cGMP in human erythrocyte membranes is MRP4. This is known to be expressed in erythrocytes and, when transfected into HEK 293 cells, to bring about transport of cGMP. The principal evidence that MRP4 is the transporter primarily responsible for cGMP uptake in erythrocytes comes from observations that substances that inhibit transport by MRP4 also inhibit cGMP uptake [4]. Since PGA_1, PGE_1 [34] and dehydroepiandrosterone-3-sulfate (DHEAS) [35] have been revealed as inhibitors or substrates of MRP4, these agents were used in the present study to explore further the identity of the transporter responsible for cGMP uptake into erythrocyte membrane vesicles. Clear inhibition was observed (see Fig. 2B) so providing further support for the idea that MRP4 is the principal cGMP transporter in erythrocyte membranes. With DHEAS the inhibition may have been complete ($IC_{50} = 1.5 \pm 0.3$ μM, maximum percentage uptake = 100 ± 3%, non-inhibitable uptake 8 ± 4%, $p = 0.08$) but with PGE_1 ($IC_{50} = 1.8 \pm 0.3$ μM, maximum percentage uptake = 101 ± 3%, non-inhibitable uptake 17 ± 2%, $p < 0.001$) and PGA_1 ($IC_{50} = 1.2 \pm 0.4$ μM, maximum percentage uptake = 99 ± 6%, non-inhibitable uptake 29 ± 4%, $n = 6$, $p < 0.001$) there was a small non-inhibitable component.

3.4. Effects of quinoline derivatives on transport from HEK 293 cells overexpressing MRP4

To verify that quinolines can indeed affect MRP4-mediated cGMP transport, their effects were observed using vesicles prepared from MRP4-overexpressing tumour cells. In these vesicles, ATP-dependent cGMP uptake was found (see Fig. 3A) to proceed at a rate significantly greater than in the controls but still at less than 1/10th the rate seen in vesicles prepared from erythrocytes [4]. The ATP-dependent transport was inhibited by MK-571 and mefloquine at concentrations similar to those that inhibit transport in the erythrocyte membrane vesicles (see Fig. 3B). The fitted inhibition curve for MK-571 yields $IC_{50} = 2.1 \pm 0.3$ μM, maximum percentage uptake = 100 ± 2% and non-inhibitable uptake 5 ± 3% (n.s. $p > 0.1$). The fitted inhibition curve for mefloquine corresponds to $IC_{50} = 27 \pm 6$ μM, maximum percentage uptake = 97 ± 3% and non-inhibitable uptake 19 ± 3% ($p < 0.01$).

Further evidence for quinoline interactions with MRP4 was seen from their inhibitory effects on the efflux of a known MRP4 substrate, BCECF, from MRP4-transfected

cells (see Fig. 3C). There was clearly more rapid efflux than from the HEK control cells (rate constants: parental HEK cells $3.16 \pm 0.34 \times 10^{-4}\ s^{-1}$ $n = 3$; HEK 293/4.63 (MRP4) cells $6.35 \pm 0.53 \times 10^{-4}\ s^{-1}$ $n = 3$). This efflux was reduced to the control rate in the presence of 50 μM MK-571 ($3.31 \pm 0.12 \times 10^{-4}\ s^{-1}$, $n = 3$) and 50 μM mefloquine ($2.91 \pm 0.45 \times 10^{-4}$, $n = 3$).

3.5. Effect of quinoline derivatives and MK-571 on ATP hydrolysis by MRP1 and MRP4

To investigate further the possible interactions of quinolines with MRPs 1 and 4, their effects on the ATPase activities associated with MRP1 and MRP4 were examined in crude membranes of High Five insect cells overexpressing these proteins to a level sufficient for ATPase activity

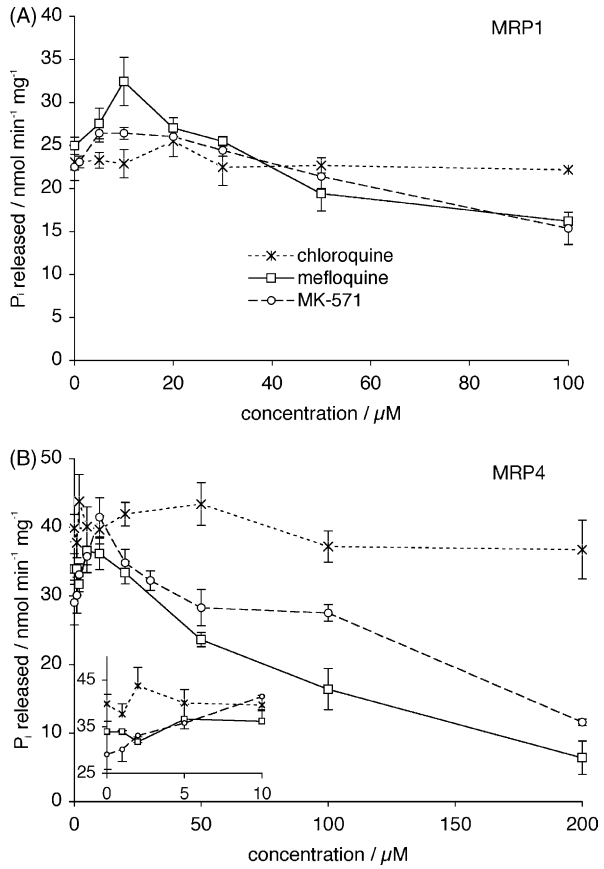

Fig. 4. Effect of quinolines on MRP1- and MRP4-associated ATPase activity. BeFx-sensitive ATP hydrolysis after inhibition of other ATPases with NaN_3, EGTA and ouabain was measured as the amount of inorganic phosphate, P_i, released over 20 min at 37 °C. (A) With MRP1, mefloquine ($n = 4$) and MK-571 ($n = 4$) both stimulate ATP hydrolysis at low concentrations and inhibit at higher concentrations. Chloroquine ($n = 3$) has little effect. (B) With MRP4, mefloquine ($n = 4$) inhibits at high concentrations, MK-571 ($n = 4$) produces a small stimulation at low concentrations and inhibits at high concentrations and chloroquine ($n = 4$) again has little effect. In the inset, which shows the values for low concentrations using enlarged scales, error bars are shown upwards for chloroquine and mefloquine and downwards for MK-571. Where not visible, the error bars are hidden by the symbols. Amodiaquine, quinine and quinidine gave results indistinguishable from those seen with chloroquine.

measurements. Chloroquine, amodiaquine, quinine and quinidine had no significant effects on the rate of ATP hydrolysis for either MRP1 or MRP4. Mefloquine and MK-571 were seen to have biphasic effects on MRP1-mediated ATP hydrolysis. At low concentrations they produced stimulation suggesting they may be substrates. At higher concentrations they caused inhibition as if high concentrations reduce the net rate of dissociation after the substrate translocation step of the transport cycle (see Fig. 4).

Sauna et al. [36] have shown previously that PGE_1 stimulates MRP4-mediated ATP hydrolysis and this result is confirmed here (see Fig. 5A). Furthermore it is shown here that quercetin at low concentrations markedly stimulates MRP1-mediated ATP hydrolysis (see Fig. 5B) (and to a similar extent MRP4-mediated ATP hydrolysis, data not shown). Using quercetin as a stimulant for MRP1 and PGE_1 for MRP4, both mefloquine and MK-571 significantly reduced substrate-stimulated MRP1- and MRP4-mediated ATP hydrolysis (see Fig. 5C and D).

To confirm the expectation that mefloquine and MK-571 do not interact with the ATP binding sites on MRP1 and MRP4, photoaffinity labelling of MRP1 and MRP4 was performed using the ATP analogue [α-^{32}P]8-azidoATP. This labelling (see Fig. 6) was effectively inhibited by the presence of excess cold ATP as was to be expected for labelling of the nucleotide binding site. Neither mefloquine nor MK-571 inhibited this labelling even at concentrations which blocked transport of known substrates and which affected ATPase activity of the transporters. This lack of effect suggests as expected that mefloquine and MK-571 produce their effects at the binding site(s) for substrates rather than at the nucleotide binding site.

4. Discussion

The purpose of this study was to investigate the interactions of mefloquine and other quinolines used in the treatment of malaria with MRPs present in red blood cell membranes. Of these, mefloquine was the most effective. It blocked the higher affinity DNP-SG transport observed in red blood cells and the MRP1-mediated DNP-SG transport in lung tumour cells as well as the principal cGMP transport in red blood cells and the cGMP transport measured in MRP4-transfected HEK cells with IC_{50} values of 126, 131, 20 and 27 μM, respectively.

Further confirmatory evidence of the inhibitory potential of mefloquine and MK-571 on MRP1 and MRP4 were obtained by observing their effects on MRP-mediated efflux of fluorescent substrates from whole cells. The results using MRP1-overexpressing L23/R cells demonstrate that mefloquine can inhibit calcein efflux in a concentration-dependent manner and this inhibition appears comparable to that seen with MK-571, an established inhibitor of MRP1 transport. Similarly mefloquine and

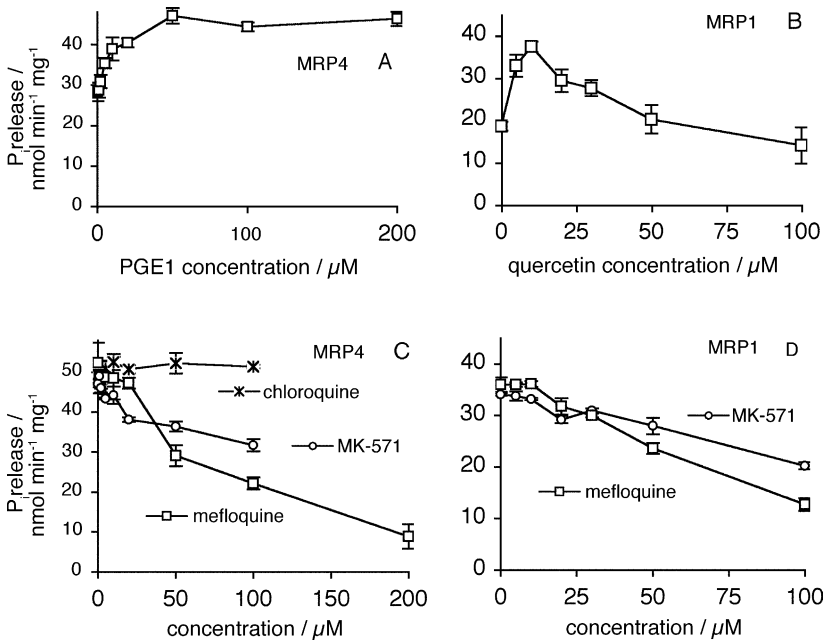

Fig. 5. Stimulation of MRP4-associated ATPase activity by PGE_1 (A) and of MRP1-associated ATPase activity by quercetin (B) and inhibition of these stimulated ATPase activities by mefloquine and MK-571 (C and D). BeFx-sensitive ATP hydrolysis was measured after inhibition of other ATPases with NaN_3, EGTA and ouabain. (A) PGE_1 stimulates the ATP hydrolysis by MRP4 with no apparent inhibition for PGE_1 concentrations up to 200 μM. (B) Quercetin produces nearly a doubling of the ATP hydrolysis by MRP1 at low concentrations but inhibits the activity at high concentrations. The clear stimulation of the ATP hydrolysis suggests that PGE_1 is a substrate for MRP4 and quercetin is a substrate for MRP1. Points are shown as mean ± S.E.M. ($n = 6$ for PGE_1, 4 for quercetin). (C) Inhibition of 20 μM PGE_1 stimulated MRP4 ATPase activity by increasing concentrations of mefloquine or of MK-571. (D) Inhibition of 10 μM quercetin stimulated MRP1 ATPase activity by increasing concentrations of mefloquine or of MK-571. The clear inhibition of the stimulated ATPase activity is consistent with competition between each of MK-571 and mefloquine and PGE_1 on MRP4 and quercetin on MRP1. Points are shown as mean ± S.E.M. ($n = 3$ except $n = 6$ for mefloquine and MRP4).

MK-571 both inhibited the efflux of BCECF from MRP4-overexpressing cell lines.

To assess the nature of the interactions of quinoline-based antimalarials with MRP1 and 4, the ATPase activities of these transporters were examined. ATP hydrolysis and transport are closely linked such that exposure to drug-substrates leads to stimulation or inhibition of ATPase activity of the transporter concerned [37,38]. Stimulation of ATP hydrolysis by transported substrates has previously been shown for both MRP1 [39,40] and MRP4 [36]. It is confirmed here that PGE_1 stimulates MRP4 ATP hydrolysis [36] and we have shown that the bioflavonoid quercetin stimulates both MRP1 and MRP4 ATP hydrolysis at low concentrations. Leslie et al. [41] have previously reported that quercetin at 10 μM inhibits the ATPase activity of MRP1 reconstituted into proteoliposomes. The reason for this discrepancy is not known. Mefloquine and MK-571 but not the other quinoline drugs tested were seen to have biphasic effects on MRP1-mediated ATP hydrolysis with stimulation at low concentrations and inhibition at higher concentrations.

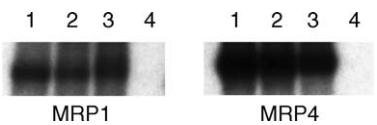

Fig. 6. MK-571 and mefloquine do not inhibit photoaffinity labelling of MRP1 or MRP4 by [α-^{32}P]8-azidoATP. Labelling was detected by phosphorimaging after gel electrophoresis. In each image, lane 1 is the control for membranes exposed to [α-^{32}P]8-azidoATP alone, lane 2 has in addition 100 μM mefloquine, lane 3 100 μM MK-571 and lane 4 10 mM ATP. While excess ATP inhibits the photoaffinity labelling (as is evident in lane 4), mefloquine and MK-571 at concentrations that inhibit transport and ATPase activity do not, indicating that their inhibitory effects are unlikely to be due to interactions at the nucleotide binding sites of the transporters.

Stimulation of ATP hydrolysis can be taken as evidence that the substances involved are substrates for transport linked to ATP hydrolysis. However, inhibition of hydrolysis can by produced by (relatively poor) substrates or pure inhibitors [21] and lack of obvious effect does not imply lack of interaction. An alternative strategy to demonstrate interaction of an agent with the substrate binding site is to look for inhibition of the clear stimulation of ATPase activity by another substrate [36]. PGE_1 has already been shown to produce such stimulation of ATPase associated with MRP4 and it is shown here that quercetin stimulates ATPase activity associated with MRP1. Both mefloquine and MK-571 were able to reduce substrate-stimulated MRP1- and MRP4-mediated ATP hydrolysis significantly suggesting competitive interactions at the substrate binding sites of these transporters. By contrast, these quinolines did not affect photoaffinity labelling of MRP1 and MRP4 by [α-^{32}P]8-azidoATP even at concentrations which inhibited ATPase activity or transport of known substrates. This lack of effect suggests that mefloquine and MK-571 do not

interact with MRP1 or MRP4 at their nucleotide binding sites.

Our results demonstrate unequivocally that mefloquine interacts with MRP1 and MRP4 and influences both DNP-SG and cGMP transport in red blood cells. The available evidence demonstrates that both mefloquine and MK-571 interact with these transporters and suggests that they may be transported substrates. It thus remains possible that the distribution of mefloquine in erythrocytes may be altered by MRP activities. Based on the concentrations required to produce inhibition, it seems unlikely that mefloquine exerts any of its antimalarial action by inhibiting transport of substrates such as oxidised glutathione or glutathione conjugates by MRP1 or MRP4. Substantial inhibition has been demonstrated but only at relatively high concentrations, 10–100 μM. The total plasma concentration of mefloquine during treatment for malaria is typically less than 1 μM with 98% of this bound to plasma proteins [42] though concentrations higher than this may be achieved within the red cells.

P. falciparum possesses an active efflux mechanism for GSSG [43]. It also expresses both mRNA and protein for an ABC protein that is homologous with the MRP transporters [44]. It remains an interesting hypothesis that mefloquine may interact with that transporter thus interfering with the export of GSSG from the parasite.

The results of this study also provide useful indicators about the identity of the principal cGMP transporter in red blood cells. Erythrocyte membranes express at least two transporters for glutathione conjugates [2,4,45], one with a high affinity for DNP-SG ($K_d \sim 2$ μM) and identifiable as MRP1 and another with a low affinity ($K_d > \sim 60$ μM) [4]. There are also cGMP transporters, one with a low affinity (K_d 50–80 μM) [4] and another with a high affinity ($K_d \sim 1$ μM) [46]. This latter probably contributes less, perhaps much less, than 20% of the transport even at the lowest concentrations [4]. Because the low affinity component (but not the high affinity component) of DNP-SG transport can be totally inhibited by cGMP and the low affinity transport of cGMP can be totally inhibited by DNP-SG, it is likely that these two transport systems are mediated by the same transporter. Indeed, for every inhibitor that has been tested against both types of transport, inhibition of one implies inhibition of the other.

Every inhibitor of the low affinity cGMP transport in red blood cells when tested against MRP4-mediated transport has brought about inhibition [4]. In keeping with these observations, it is shown in the present study that the MRP4 substrates PGA_1, PGE_1 and DHEAS all caused a reduction in cGMP uptake into erythrocyte membrane vesicles. The quinoline-based drugs, MK-571 and mefloquine exhibited similar behaviour, inhibiting both MRP4-mediated transport and red blood cell transport of cGMP. Interestingly while MK-571 and DHEAS appeared to inhibit all of the cGMP uptake seen in erythrocytes, there was a significant component of uptake that was not inhibited by mefloquine, PGA_1 and PGE_1. One possible explanation is the presence of two transporters with similar apparent affinities for cGMP, with the cGMP transport activity of both inhibited by MK-571 and DHEAS, but only the activity of the one with the larger maximum transport rate inhibited by PGA_1, PGE_1 and mefloquine. Red blood cells also express MRP5 [4,47], which is known to transport cGMP [34] and there is evidence that immunoprecipitation of MRP5 decreases the cGMP transport activity of reconstituted protein extracts from red blood cell membranes [18]. However, the observation that both MK-571 and PGE_1 inhibit the principal component of cGMP transport in the erythrocyte vesicles but do not inhibit MRP5-mediated transport [34,47] argues strongly against MRP5 as the transporter primarily responsible for cGMP transport in red blood cells. Its contribution to erythrocyte cGMP transport thus remains unclear. The present results provide further support for the earlier hypothesis that the principal transporter of cGMP in red blood cells is MRP4.

Acknowledgements

We thank Dr. Gary Kruh (Fox chase Cancer Centre) for the gifts of plasmid PVL1393-MRP4 and anti-MRP4 and Dr. Piet Borst (The Netherlands Cancer Institute) for HEK 294/4.63 cells. CPW is supported by the Cambridge Commonwealth Trust Fund and by a visiting preCRTA award from the NCI, NIH.

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