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Analysis of Expression of *cMOAT (MRP2), MRP3, MRP4,* and *MRP5,* Homologues of the Multidrug Resistance-associated Protein Gene (*MRP1*), in Human Cancer Cell Lines[1]

Marcel Kool, Marcel de Haas, George L. Scheffer, Rik J. Scheper, Michiel J.T. van Eijk, Jenneke A. Juijn, Frank Baas, and Piet Borst[2]

Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam [M. K., M. d. H., P. B.]; Department of Pathology, Free University Hospital, 1081 HV Amsterdam [G. L. S., R. J. S.]; Department of Pediatric Gastroenterology and Laboratory for Metabolic Diseases, Wilhelmina Children's Hospital, 3501 CA Utrecht [M. J. T. v. E., J. A. J.]; and Department of Neurology, Academic Medical Center, 1105 AZ Amsterdam [F. B.], the Netherlands

ABSTRACT

By screening databases of human expressed sequence tags, we have identified three new homologues of *MRP1*, the gene encoding the multidrug resistance-associated protein, and *cMOAT* (or *MRP2*), the canalicular multispecific organic anion transporter gene. We call these new genes *MRP3, MRP4,* and *MRP5*. *MRP3,* like *cMOAT,* is mainly expressed in the liver. *MRP4* is expressed only at very low levels in a few tissues, and *MRP5,* like *MRP1,* is expressed in almost every tissue tested. To assess a possible role of these new *MRP* homologues in multidrug or cisplatin resistance, a large set of resistant cell lines was examined for the (over)expression of *MRP1, cMOAT, MRP3, MRP4,* and *MRP5*. We find that even in cells selected for a low level of resistance, several *MRP*-related genes can be up-regulated simultaneously. However, *MRP4* is not overexpressed in any of the cell lines we analyzed; *MRP3* and *MRP5* are only overexpressed in a few cell lines, and the RNA levels do not seem to correlate with resistance to either doxorubicin or cisplatin. *cMOAT* is substantially overexpressed in several cell lines, and *cMOAT* RNA levels correlate with cisplatin but not doxorubicin resistance in a subset of resistant cell lines. Our results emphasize the need for gene-specific blocks in gene expression to define which transporter contributes to resistance in each resistant cell line.

INTRODUCTION

After selection for resistance to a single cytotoxic drug, cells may become cross-resistant to a whole range of drugs with different structures and cellular targets, a phenomenon called MDR.[3] In human cancer cells, MDR can be caused by enhanced drug efflux mediated by transporter proteins such as the MDR1 P-glycoprotein (reviewed in Refs. 1 and 2) and MRP1 (reviewed in Refs. 3 and 4). Both are members of the ABC family of transporter proteins (5). In contrast to P-glycoprotein, MRP1 can act as a GS-X pump, *i.e.,* it can transport drugs conjugated with GSH, because: (*a*) the rates of ATP-dependent transport of several GSH-conjugated compounds have been shown to correlate with the level of expression of *MRP1* in several cell preparations (6, 7); (*b*) overexpression of *MRP1* in human cancer cells resulted in an increased ATP-dependent transport of GSH *S*-conjugates into isolated plasma membrane vesicles and from intact cells (7–9); (*c*) depletion of GSH in cells overexpressing *MRP1* reversed resistance (10, 11); and (*d*) MRP1 complemented yeast cells with a disrupted cadmium resistance factor (*YCF1*) gene (12), a known organic anion transporter (13, 14).

GS-X pumps have also been reported to be involved in the detoxification of heavy metals (15–20). The cell lines described in these papers were all selected for resistance against CDDP (cisplatin), and all showed an increased ATP-dependent transport of GS-platinum complexes across the plasma membrane. Cisplatin is known to interact with cellular GSH, and a complex with a 2:1 molar ratio of GSH:cisplatin has been detected in L1210 murine leukemia cells (15). Transport of the GSH/cisplatin complex across the cell membrane was found to be ATP dependent, inhibited by vanadate, DNP-SG, LTC_4, and oxidized glutathione (GSSG). Only in the human leukemia CDDP-resistant HL60/R-CP cell line was an increased GS-X activity correlated with an increased expression of *MRP1* (16, 17). It is, therefore, likely that other GS-X pumps exist, besides MRP1. Moreover, several MDR cell lines selected with natural product drugs display a MDR phenotype that cannot easily be explained by the overexpression of *MDR1* or *MRP1,* also suggesting that other drug transporters might exist (20–28).

A GS-X pump that might contribute to drug resistance is cMOAT. The cDNAs encoding cMOAT have recently been identified in rats and humans (29–33). This homologue of *MRP1* (49.0% identity with human MRP1 at the protein level), also known as *MRP2,* is mainly expressed in the canalicular membrane of hepatocytes. The identification of transport-deficient mutant rat strains, the TR^- or GY/TR^- rats (34) and the Eisai hyperbilirubinemic rats (35), has contributed to the functional characterization of cmoat (36). These rats have an autosomal recessive defect in the hepatobiliary excretion of bilirubin glucuronides and other multivalent organic anions, including GSH *S*-conjugates (for example LTC_4) and 3-OH-glucuronidated and -sulfated bile salts. Paulusma *et al.* (29) showed that a 1-bp deletion in the *cmoat* gene is responsible for the absence of cmoat in the TR^- rats. TR^- rats have a similar phenotype as patients with the Dubin-Johnson syndrome, characterized by mild chronic conjugated hyperbilirubinemia (37). Recently, we and others have shown that the cMOAT protein is also absent in canalicular membranes of hepatocytes of patients with the Dubin-Johnson syndrome (30, 38). In the one patient analyzed thus far, the absence of cMOAT was caused by a mutation in the *cMOAT* gene (30).

In addition to *MRP1* and *cMOAT* (*MRP2*), other *MRP* homologues encoding GS-X pumps might be present in the human genome, considering that there are at least four *MRP* homologues expressed in *Caenorhabditis elegans* (39). We, therefore, searched the EST library (40) for putative human *MRP* homologues and found three more *MRP* homologues expressed in humans. We call these new *MRP* homologues *MRP3, MRP4,* and *MRP5*.

To investigate a possible role of MRP homologues in drug resistance, we examined a large set of (multi)drug-resistant cell lines for the (over)expression of *cMOAT, MRP3, MRP4,* and *MRP5*. We find that especially *cMOAT* expression is elevated in several cell lines, selected for cisplatin resistance, and also in some sublines of the human

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[2] To whom requests for reprints should be addressed. Phone: 31-20-5122880; Fax: 31-20-6691383.

[3] The abbreviations used are: MDR, multidrug resistance; MRP, multidrug resistance-associated protein; ABC, ATP-binding cassette; GSH, glutathione; CDDP, *cis*-diamminedichloroplatinum(II); DNP-SG, *S*-(2,4-dinitrophenyl)-glutathione; LTC_4, leukotriene C_4; GSSG, glutathione disulfide; cMOAT, canalicular multispecific organic anion transporter; EST, expressed sequence tag; SUR, sulfonylurea receptor; Pgp, P-glycoprotein; Mab, monoclonal antibody; BRIC, benign recurrent intrahepatic cholestasis; PFIC, progressive familial intrahepatic cholestasis.

non-small lung cancer cell line SW1573/S1, selected for doxorubicin resistance. The expression level of *cMOAT* correlates with the cisplatin but not the doxorubicin resistance of these cell lines. Although *MRP3* and *MRP5* were overexpressed in some resistant cell lines, no clear correlation between drug resistance and the expression levels of *MRP3, MRP4,* and *MRP5* has emerged from these initial studies.

MATERIALS AND METHODS

Cell Lines. All cell lines used in this study have been described in the literature before: the drug-sensitive and doxorubicin-selected MDR sublines of the non-small cell lung cancer cell lines SW1573/S1 and COR-L23 (41–44); the small cell lung cancer cell line GLC_4 (45); the lung adenocarcinoma cell line MOR/P (44); the leukemia cancer cell line HL60 (46); the T24 bladder carcinoma cell line and three CDDP-resistant sublines (26); the 2008 ovarian carcinoma cell line, two CDDP-resistant sublines, and a Cd^{2+}-resistant subline (21, 22); the A2780 ovarian carcinoma and the HCT8 colon carcinoma cell lines and CDDP-resistant sublines of both (20, 28); the PXN94 ovarian carcinoma and the tetraplatin-resistant subline PXN94tetR (23); the GCT27 testicular carcinoma cell line and the CDDP-resistant subline GCT27cisR (25); and the KB-3–1 epidermoid carcinoma cell line and a CDDP-resistant subline KCP-4 (18, 19). All cells were grown in DMEM or RPMi 1640 (Life Technologies, Inc.), supplemented with 10% FCS, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). All cells were free of *Mycoplasma* as tested by the use of the Gene-Probe rapid *Mycoplasma* detection system (Gen-Probe, San Diego, CA).

Clonogenic Survival Assays. The drug sensitivity of cells was determined in clonogenic survival assays in the continuous presence of drugs. Five hundred cells/well were seeded in 24-well plates and incubated for 24 h at 37°C. Drugs, of which concentrations were varied in 2-fold steps, were added, and cells were incubated for 5–6 days at 37°C. After this, the cells were stained with 0.2% crystal violet in 3.7% glutaraldehyde, and colonies containing more then 50 cells were counted. The relative resistance was calculated as the ratio of IC_{50} (inhibitory concentration where 50% of the cells survive) of the resistant cell line to the IC_{50} of the parental cell line.

Cloning and Sequencing of *MRP3, MRP4,* and *MRP5* cDNA. For the isolation of *MRP3, MRP4,* and *MRP5* cDNA, human cDNA clones were obtained from the I.M.A.G.E. consortium (47). Additional *MRP3* cDNA clones were isolated by screening a human liver 5′ stretch plus cDNA library, oligo(dT) and random primed (Clontech, Palo Alto, CA), using a 1-kb *Eco*RI-*Sac*I fragment of a human cDNA clone (no. 84966, Stratagene liver cDNA library 937224) as probe. Several overlapping cDNAs were isolated and sequenced. For *MRP4,* the insert of a human cDNA clone (no. 38089, Soares infant brain 1NIB cDNA library) was sequenced, containing the 3′-terminal end of the gene. *MRP5* cDNA clones were isolated by screening a fetal brain cDNA library (Clontech), using the insert of human cDNA clone (no. 50857, Soares infant brain 1NIB cDNA library) as probe.[4] Several overlapping cDNAs were isolated and sequenced. For sequencing, the ABI 377 automatic sequencer was used. Sequence analysis was done using the GCG package of Wisconsin University, version 9.0 (48). All of the sequences have been deposited with GenBank (*MRP3* accession number U83659; *MRP4* accession number U83660; and *MRP5* accession number U83661).

RNA. Cytoplasmic RNA from cell lines was isolated by a NP40 lysis procedure (49). Total cellular RNA from tissue samples obtained during surgery or at autopsy was isolated by acid guanidinium isothiocyanate-phenol-chloroform extraction (50).

RNase Protection Assays. By PCR amplification of human *cMOAT* cDNA, a 241-bp fragment corresponding to nucleotides 4136–4376 (Ref. 30; GenBank accession number U49248) was generated. The primers used for amplification were 5′-CTGCCTCTTCAGAATCTTAG-3′ (forward primer) and 5′-CCCAAGTTGCAGGCTGGCC-3′ (reverse primer). For *MRP3, MRP4,* and *MRP5* RNA detection, the following fragments were generated by PCR amplification: (*a*) for *MRP3,* a 262-bp fragment was generated using the primers 5′-GATACGCTCGCCACAGTCC-3′ (forward primer) and 5′-CAGTTGGCCGTGATGTGGCTG-3′ (reverse primer); (*b*) for *MRP4,* a 239-bp fragment was generated using the primers 5′-CCATTGAAGATCTTCCTGG-3′ (forward primer) and 5′-GGTGTTCAATCTGTGTGC-3′ (reverse primer); (*c*) for *MRP5,* a 381-bp fragment was generated using the primers 5′-GGATAACTTCTCAGTGGG-3′ (forward primer) and 5′-GGAATGGCAATGCTCTAAAG-3′ (reverse primer). All of the fragments were cloned into pGEM-T (Promega Corp., Madison, WI), resulting in the plasmids hcMOAT-241, MRP3-262, MRP4-239, and MRP5-381, and the sequences were confirmed. For RNase protection assays, α-^{32}P-labeled RNA transcripts were transcribed from *Not*I-linearized DNA of hcMOAT-241 and MRP3–262, using T7 RNA polymerase, or from *Nco*I-linearized DNA from MRP4-239 and MRP5-381, using Sp6 RNA polymerase. For *MDR1* RNA detection, a 301-bp *MDR1* cDNA fragment was used [nucleotide positions 3500–3801 (51)], and for *MRP1* RNA detection, a 244-bp *MRP1* cDNA fragment was used [nucleotide positions 239–483 (52)]. RNase protection assays were carried out according to Zinn *et al.* (53), modified by Baas *et al.* (41). Protected probes were visualized by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. In all experiments, a probe for *γ-actin* (54) was included as control for RNA input. The amount of *MDR1, MRP1, cMOAT, MRP3, MRP4,* or *MRP5* RNA relative to the amount of *γ-actin* was calculated using a phosphorimager (Fuji BAS 2000, TINA 2.08b).

Protein Analysis. Total cell lysates were made by lysing harvested cells in 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), and 0.5% (w/v) SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml), pepstatin (1 μg/ml), and aprotinin (2 μg/ml). DNA was sheared by sonication, and samples containing 40 μg of protein were fractionated by SDS/7.5% PAGE and then transferred onto a nitrocellulose filter by electroblotting. After blotting, the filters were blocked for at least 2 h in Blotto (PBS containing 1% BSA, 1% milk powder, and 0.05% Tween 20), followed by incubation for 2 h with the primary antibody in Blotto. cMOAT protein was detected with mouse monoclonal antibodies M_2III-5 or M_2III-6, generated against a bacterial fusion protein containing the 202-amino acid COOH-terminus of rat cmoat (29). Immunoreactivity was visualized with peroxidase-conjugated rabbit antimouse immunoglobulins (Dako, Copenhagen, Denmark) followed by enhanced chemiluminescence detection (Amersham, Buckinghamshire, United Kingdom).

Fusion Proteins of cMOAT, MRP3, and MRP5. To test the cross-reactivity of the cmoat monoclonal antibodies with human cMOAT and other MRP homologues, fusion proteins were made of the *Escherichia coli* maltose-binding protein with COOH-terminal ends of human cMOAT, MRP3, and MRP5, respectively, using the plasmid vector pMal-c (55). The expression plasmids encoded, respectively, for cMOAT the 202-amino acid COOH-terminal end, for MRP3 the 190-amino acid COOH-terminal end, and for MRP5 the 169-amino acid COOH-terminal end. The fusion proteins were produced in *E. coli* DH5α and purified by amylose resin affinity chromatography (55).

Glutathione Assay. Cells ($1–2 \times 10^6$ per well) were plated in triplicate in six-well plates in medium with or without drugs. Forty-eight h after plating, the cells were washed with PBS and scraped in 10% perchloric acid. Precipitated protein was removed by centrifugation, and the supernatant was neutralized by adding 0.5 M 4-morpholinepropanesulfonic acid, 5 M KOH. The concentration of total glutathione (GSH and GSSG) was determined according to the recycling method of Tietze (56).

Chromosome Localizations. For the chromosome localization of *MRP3, MRP4,* and *MRP5,* radiation hybrid mapping was performed with *MRP3*-, *MRP4*-, and *MRP5*-specific primers and two different cell panels, Stanford G3 (StG3; Ref. 57) and Genebridge 4RH (Gb4RH; Ref. 58). The primers used for amplification were: (*a*) for *MRP3* 5′-CTCAATGTGGCAGACATCGG-3′ and 5′-GGGAGCTCACAAACGTGTGC-3′; (*b*) for *MRP4* 5′-CCATTGAAGATCTTCCTGG-3′ and 5′-GGTGTTCAATCTGTGTGC-3′; and (*c*) for *MRP5* 5′-CCTGTTTGGGAAGGAATATGA-3′ and 5′-GGGTCGTCCAGGATGTAGAT-3′. For the PCR reactions, 25 ng DNA, 2 ng/μl of each specific primer, 0.8 unit Goldstar polymerase (Eurogentec, Seraing, Belgium; *MRP3* and *MRP4*) or 1.5 units Amplitaq Gold polymerase (*MRP5*) were used in a total volume of 25 μl with 1.5 mM $MgCl_2$ and 100 μM of each deoxynucleotide triphosphate at final concentrations. The PCR conditions were: initial denaturation for 5 min at 94°C (*MRP3* and *MRP4*) or 12 min at 95°C (*MRP5*), followed by 42 cycles of 15 s at 94°C, 30 s at 58°C, and 45 s at 72°C. Final extension was for 10 min at 72°C. PCR products were resolved by 1% agarose gel electrophoresis, and the cell line scored positive, negative, or ambiguous for presence of the gene. Data files were submitted to the Stanford Human

[4] J. Wijnholds, C. Mol, and P. Borst, unpublished results.

Genome Center or Whitehead Institute radiation hybrid mapping databases for placing of the *MRP* genes in context of the respective radiation hybrid map framework markers.

Microsatellite Repeat Analysis. To confirm identity of cell lines and subclones, nine highly polymorphic microsatellite markers were used (*D1S1649, D2S434, D2S1384, D3S2427, D9S301, D9S934, D12S2070, D14S611,* and *D17S969*). PCR conditions were as described in the Genome Database. One primer of each set was labeled with a fluorescent dye, and PCR products were visualized by electrophoresis on an ABI 377 automatic sequencer. Data were analyzed with Genetyper software version 1.1.1 (Perkin-Elmer, Norwalk, CT). Allele sizes were within expected range.

RESULTS

Database Search for *MRP* Homologues. We searched human EST databases (dbEST and TIGR) for *MRP* homologues other than *MRP1* and *cMOAT*. Alignment and comparison of EST sequences with homology specific to the 3′-terminal ends of *MRP1* and *cMOAT*, including the coding sequence for the second ATP-binding domain, revealed that there are at least four more *MRP* homologues expressed in humans. One of these homologues is the human *SUR* gene (59). The other three *MRP* homologues had not been identified before and were designated *MRP3, MRP4,* and *MRP5*.

Cloning and Sequencing of *MRP3, MRP4,* and *MRP5* cDNA. Additional cDNA clones for *MRP3* and *MRP5* were isolated from a human liver and a fetal brain cDNA library, respectively. *MRP3* and *MRP5* cDNA clones were sequenced as well as the *MRP4* cDNA clone obtained from the I.M.A.G.E. consortium. Both *MRP3* and *MRP5* encode four domain proteins, *i.e.,* proteins with two ATP-binding domains and two domains with transmembrane regions.[5] More sequence data are required to determine whether this is also the case for *MRP4*. Fig. 1 shows the protein alignment for the COOH-terminal ends of the various members of the human MRP family, including the recently identified sixth *MRP* gene, called *MRP6* (see "Discussion"), and human SUR. The alignment includes the Walker A and B motifs and the signature sequence (C) of the second ATP-binding domain. The percentages of homology for the COOH-terminal 124 amino acids are shown in Table 1. The highest homology is found between MRP1 and MRP3 (83% similarity) and the lowest between SUR and any of the MRPs (≤59% similarity).

Chromosome Localization of *MRP3, MRP4,* and *MRP5*. The *MRP1* gene has been mapped to chromosome 16 at band p13.13–13.12 (60), and recently the *cMOAT* gene was mapped to chromosome 10, band q24 (33, 61). We mapped the other *MRP* homologues on the Gb4RH and StG3 radiation hybrid mapping panels, using *MRP3*-, *MRP4*-, or *MRP5*-specific primers. *MRP3, MRP4,* and *MRP5* are located on chromosomes 17, 13, and 3, respectively. The most closely linked markers were *D17S797* (Gb4RH) and *D17S1989* (StG3) for *MRP3, WI-9265* (Gb4RH) and *D13S281* (StG3) for *MRP4,* and *WI-6365* (Gb4RH) and *D3S4205* (StG3) for *MRP5*. These results are consistent between the radiation hybrid mapping panels and demonstrate that the new *MRP* homologues are indeed new genes and not splice variants of *MRP1* or *cMOAT*.

Human Tissue Distribution of *cMOAT, MRP3, MRP4,* and *MRP5* RNA. RNase protection assays were performed to determine the tissues that express *cMOAT* and *MRP3, MRP4,* and *MRP5*. The results are summarized in Table 2. Both *cMOAT* and *MRP3* are highly expressed in liver and to a lower extent also in duodenum. Low expression of *cMOAT* was found in kidney and peripheral nerve. For *MRP3,* substantial expression, similar to expression in duodenum, was also detected in colon and adrenal gland. *MRP4* is expressed at a low level in only a few tissues tested. *MRP5* RNA was detected in substantial amounts in every tissue tested, with relatively high expression in skeletal muscle and brain.

Expression of *MRP* Homologues in Resistant Cell Lines. In view of their homology with *MRP1, cMOAT* and the three new *MRP* homologues might encode transporter proteins involved in drug resistance. We, therefore, screened a large set of human cell lines derived from various tissues and their resistant sublines selected with either doxorubicin, cisplatin, tetraplatin, or $CdCl_2$. Only resistant lines showing decreased cellular accumulation of drugs were analyzed. All cell lines were analyzed by RNase protection assays for levels of *MDR1, MRP1, cMOAT, MRP3, MRP4, MRP5,* and *γ-actin* RNA. The results are summarized in Tables 3 and 4, and an example of each probe is shown in Fig. 2.

High *MDR1* overexpression was detected only in two sublines of the human non-small cell lung cancer cell line SW1573/S1, both selected for high level doxorubicin resistance (2R160 and 1R500). The low level of *MDR1* RNA in the other cell lines is not remarkable, because most of the cell lines selected for our panel were known to have a non-Pgp MDR phenotype. Low *MDR1* overexpression was found in the 2R120, a subline of the SW1573/S1, and in three cisplatin-selected sublines of the bladder carcinoma cell line T24. Interestingly, a decrease rather than an increase in *MDR1* RNA was seen in two cisplatin-selected sublines of the ovarian carcinoma cell line 2008 (Table 4). This phenomenon has been reported earlier in the SW1573/S1 sublines 1R50b, 2R50, and 3R80, selected for low level doxorubicin resistance (Refs. 41 and 42; Table 3).

MRP1 RNA is only highly overexpressed in the four non-Pgp MDR cell lines GLC_4/ADR, MOR/R, COR-L23/R, and HL60/ADR, all selected for high level doxorubicin resistance (52, 62, 63). The doxorubicin selected cell lines, derived from the SW1573/S1 cell line, showed no or only a minor increase in *MRP1* RNA, as reported before (52, 64). In the cell lines, selected for cisplatin resistance, we detected no major changes in *MRP1* RNA. Only in two sublines of the T24 cell line, T24/DDP7 and T24/DDP10, and in HCT8/DDP, a subline of the colon carcinoma HCT8 cell line, a slight (less than 2-fold) increase in *MRP1* RNA was found.

Expression of *cMOAT* varied greatly between the cell lines. Most parental cell lines did not express *cMOAT* or only at very low levels. Only the MOR/P and the KB-3–1 parental cell lines showed substantial *cMOAT* RNA levels. Overexpression of *cMOAT* was found in several doxorubicin-resistant sublines of SW1573/S1 (30.3M, 1R50b, 2R120, 2R160, and 1R500) and some cisplatin-selected cell lines (2008/C13*5.25, 2008/A, A2780/DDP, and HCT8/DDP).

Similar to *cMOAT*, most parental cell lines either did not express *MRP3* or only at very low levels. The only two parental cell lines, which show high expression of *MRP3,* the MOR/P and the KB-3–1, also show high expression of *cMOAT*. Overexpression of *MRP3* in resistant lines was only found in several doxorubicin-resistant sublines of the SW1573/S1 cell line and the cisplatin-resistant HCT8/DDP cell line.

MRP4 is expressed only at low or very low levels in the cell lines we analyzed, and no overexpression of *MRP4* was detected in resistant sublines.

MRP5 is expressed in every cell line we analyzed, with the highest levels in MOR/P and 2008, but in none of the resistant sublines is *MRP5* highly overexpressed. Only in three cisplatin-resistant cell lines, T24/DDP10, HCT8/DDP, and KCP-4(−), was a minor increase in *MRP5* RNA detected.

cMOAT Protein in Resistant Cell Lines. To investigate whether the increased *cMOAT* RNA levels in the resistant cell lines were accompanied by increased cMOAT protein levels, total cell lysates were tested on Western blot with the monoclonal antibodies M_2III-5 and M_2III-6, generated against amino acids 1340 to 1541 of the rat

[5] M. Kool and J. Wijnholds, unpublished results.

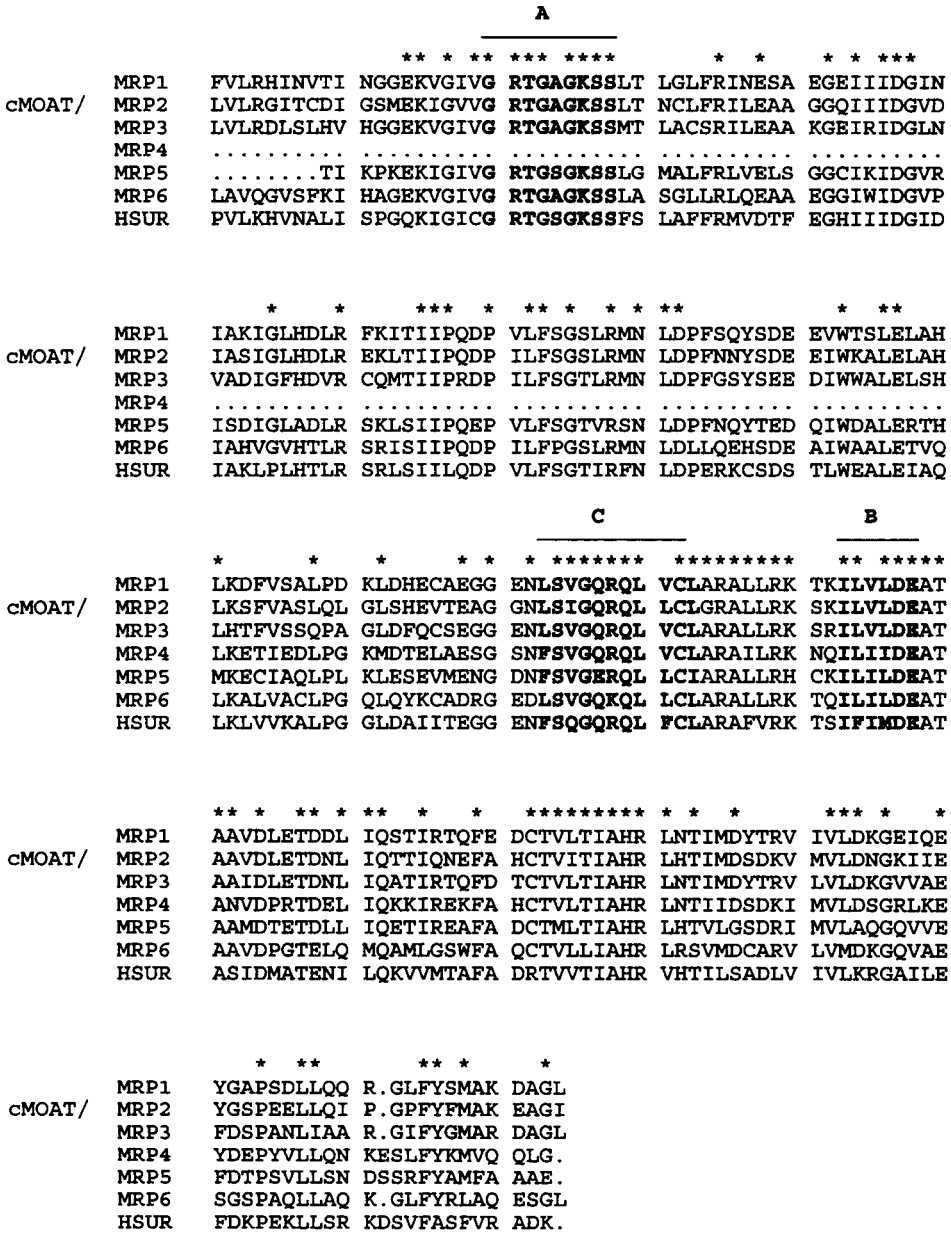

Fig. 1. Protein alignment of COOH-terminal ends of the six human MRP homologues and human SUR. The alignment was performed with the PILEUP program of GCG (48). The GenBank accession numbers for the proteins used in this comparison are the following: MRP1, L05628; cMOAT/MRP2, U49248; MRP3, U83659; MRP4, U83660; MRP5, U83661; MRP6, U91318; and SUR, L78207. The Walker A and B motifs and the nucleotide binding domain-specific signature sequence (C) are indicated. *Asterisks* above the alignment indicate identical amino acids in at least five of the six MRP proteins.

cmoat protein (29). To test the specificity for human proteins of the Mabs generated against rat cmoat, fusion proteins containing COOH-terminal ends of human cMOAT, MRP3, and MRP5 were made. Both cMOAT Mabs, M_2III-5 and M_2III-6, recognize human cMOAT. M_2III-5 also reacts with the MRP5 fusion protein, and M_2III-6 also reacts with the MRP3 fusion protein on Western blot. No cross-reaction was detected for both Mabs with MRP1 (data not shown).

Protein analysis of the cell lines with the cMOAT Mabs showed the presence of a M_r 190,000–200,000 protein in several lines (Fig. 3). Similar results were obtained with M_2III-5 and with M_2III-6 (data not shown), indicating that the protein detected is cMOAT. The level of cMOAT protein in each cell line correlated very well with the level of *cMOAT* RNA, even for the cell lines with only a marginal increase in *cMOAT* RNA, such as 2008/C13*5.25 and 2008/A. The only exception was the cisplatin-resistant subline of KB-3-1, KCP-4(−). The Western blot shows that the cMOAT protein level was about 2–3-fold higher in the KCP-4(−) cell line than in the KB-3-1, whereas the RNA levels were the same in parental and resistant cells. Mab M_2III-5

Table 1 *Homology between the COOH-terminal 124 amino acids of the six human MRP homologues and human SUR*

Percentages of identity and similarity were determined using the GAP program of GCG (48).

	MRP1	cMOAT	MRP3	MRP4	MRP5	MRP6	HSUR
MRP1	100/100						
cMOAT	73/67	100/100					
MRP3	83/75	73/62	100/100				
MRP4	69/60	65/56	64/52	100/100			
MRP5	66/55	65/52	62/51	66/57	100/100		
MRP6	69/58	64/51	67/56	62/49	57/45	100/100	
HSUR	59/48	57/46	57/46	58/47	57/45	46/40	100/100

Table 2 *Tissue distribution of human MRP gene transcripts*[a]

Levels of RNA transcripts of *MRP1*, *cMOAT* (*MRP2*), *MRP3*, *MRP4*, and *MRP5* in human tissues. RNA expression levels were determined by RNase protection assays with 10 μg of total RNA from various human tissues per probe. Expression of *τ-actin* was taken as control for total RNA input. Data for *MRP1* RNA levels are from Zaman *et al.* (52). The relative expression level is indicated by filled circles and very low or undetectable RNA levels by open circles.

	MRP1	cMOAT	MRP3	MRP4	MRP5
Lung	●●●●	○	●	●	●●
Kidney	●●●	●	●	●	●●
Bladder	●●●●	○	●	●	●●
Spleen	●●●●	○	●	○	●●
Mammary gland	ND	○	○	ND	ND
Salivary gland	ND	○	○	○	●●
Thyroid	●●●●	○	○	○	●
Testis	●●●●	○	○	○	●●
Nerve	●	●	○	○	●●
Stomach	●●●	○	●	○	●●
Liver	○	●●●●	●●●●	○	●
Gall bladder	●●●	ND	ND	●	●●
Duodenum	●●	●●	●●●	ND	ND
Colon	●●●	○	●●●	○	●●
Adrenal gland	●●●●	○	●●●	○	●
Skeletal muscle	●●	○	○	○	●●●●
Heart	●	○	○	○	●●
Brain	●	○	○	○	●●●
Placenta	●●	○	○	○	●
Ovary	●●	○	○	○	●
Pancreas	●	○	●	○	●
Tonsil	ND	○	●	●	●●

[a] ND, not determined; ○, no expression; ●–●●●●, low to high expression.

also reacts with MRP5, and MRP5 RNA is raised in the KCP-4(−) cells, but a similar result was obtained with Mab M_2III-6, which does not cross-react with MRP5.

All cell lines with no or only very low levels of *cMOAT* RNA also contained no detectable cMOAT protein (Fig. 3). The small amount of cMOAT detected in the parental A2780 cell line migrated faster in the gel than the cMOAT protein present in the cisplatin-resistant A2780/DDP cell line or the protein detected in the HCT8, HCT8/DDP, KB-3-1, and KCP-4(−) cells. The varying mobility of cMOAT in the gel could be caused by different degrees of posttranslational modification of cMOAT protein in each cell line, as we have observed for MRP1 (64),[6] but this remains to be verified.

GSH Assays. In view of the proposed role of cMOAT as a GS-X pump, intracellular GSH levels were measured for the cell lines in Table 4. GSH levels were elevated in all resistant cell lines (Table 4) and were not detectably different in cells cultured with or without drugs (data not shown).

Drug Resistance of the Cell Lines Analyzed. To determine whether there is a correlation between the elevation of expression of putative transporters and resistance pattern, we have extended the existing information on these cell lines with a more complete survey of resistance against either cisplatin or doxorubicin (Table 5). Interestingly, all of the doxorubicin-selected SW1573 cell lines with overexpression of *cMOAT* are also cross-resistant against cisplatin, and the level of *cMOAT* expression correlates quite well with the level of cisplatin resistance (Tables 3 and 5). Cytotoxicity analysis of the KCP-4(−) cell line showed that the IC_{50} for cisplatin for this cell line was much lower than reported [700 nM, RF 1.8 (Table 5) instead of 25.000 nM, RF 62.5; Ref. 18], suggesting that this cell line was a revertant or contaminated with another low level cisplatin-resistant cell line. When these KCP-4(−) cells were cultured in the presence of 6.7 μM cisplatin, more than 99% of the cells died. The surviving population, KCP-4(+), was highly cisplatin resistant again (IC_{50} 22.400, RF 59; Table 5), but surprisingly, did not express *cMOAT* anymore (Fig. 3). Microsatellite repeat analysis showed that both cell lines, KCP-4(−) and KCP-4(+), were derived from the parental KB-3-1, indicating that the KCP-4(−) is most likely a revertant.

All cell lines selected for resistance against cisplatin, tetraplatin, or $CdCl_2$ are not cross-resistant against doxorubicin (Table 5), with two exceptions: the KCP-4(−) cell line and the PXN94/tetR cell line. Cross-resistance did not correlate with *cMOAT* expression.

DISCUSSION

The *MRP* Gene Family. Our database search of ESTs has revealed that at least five homologues of *MRP1* are expressed in humans. Two of these were already known. *cMOAT* or *MRP2* encodes the major organic anion transporter in the canalicular membrane of hepatocytes (29–33, 38). The product of another homologue, *SUR*, plays a role in the regulation of insulin secretion (59). The other three homologues, *MRP3–5*, are novel and are all more related to MRP1 than SUR (Table 1). Identity is highest between MRP1 and MRP3 (75%). Because the region taken for comparison is small and one of the most conserved parts of the protein, the overall identity between the MRP homologues will probably be lower than the percentages in Table 1.

Very recently, the complete sequence of another MRP homologue appeared in the database (GenBank accession no. U91318).[7] This new MRP-like gene, located on chromosome 16, band 16p13.11 and next to *MRP1*, is different from the five *MRP* genes we have described in this report; hence, we have called it *MRP6*. *MRP6* is predicted to encode a protein of 1401 amino acids with 45% identity to human MRP1. In comparing the sequence of MRP6 with other sequences in the database, we found that the 3′ end of MRP6 is almost 100% identical with the recently identified MRP-like half transporter called ARA, described by Longhurst *et al.* (65). *ARA* is, therefore, possibly a splice variant of *MRP6*. Whether the complete *MRP6* gene is also transcribed in tissues and cell lines is not known yet and we will investigate this.

The newly identified *MRP* homologues *MRP3–5* are all located on other chromosomes than *MRP1* and *cMOAT*. This confirms that *MRP3*, *MRP4*, and *MRP5* are indeed new genes and not alternative splice products of *MRP1* or *cMOAT*. Klugbauer and Hofmann (66)

[6] M. Kool, unpublished results.

[7] M. D. Adams, B. J. Loftus, L. Zhou, C. Phillips, R. Brandon, J. Fuhrmann, U. J. Kim, A. R. Kerlavage, and J. C. Venter. Complete sequence of a chromosome 16p BAC, unpublished results.

Table 3 *Characteristics of the doxorubicin (DOX)-selected MDR cell lines analyzed*[a]

Resistant cell lines were selected by chronically exposing them to the concentrations of doxorubicin as shown. RNA levels were determined as in Fig. 2. The relative expression level is indicated by filled circles, very low expression by ⊕, and undetectable RNA levels by open circles.

Cell line	Drug used for selection (nM)	RNA levels					
		MDR1	MRP1	cMOAT	MRP3	MRP4	MRP5
Non-small cell lung cancer							
S1		⊕	●	⊕	⊕	●	●
30.3M	Dox (30)	⊕	●●	●●●	●●●	●	●
1R50b	Dox (50)	○	●	●●	●	●	●
2R50	Dox (50)	○	●●	⊕	⊕	●	●
3R80	Dox (80)	○	●	⊕	●●	●	●
2R120	Dox (120)	●	●●	●●	●●	●	●
2R160	Dox (160)	●●●●	●●	●●●	●	●	●
1R500	Dox (500)	●●●●	●	●●	⊕	●	●
COR-L23		○	●	○	○	●	⊕
COR-L23/R	Dox (368)	○	●●●●	○	○	●	⊕
Small cell lung cancer							
GLC_4		○	●	○	○	●	●
GLC_4/ADR	Dox (1160)	○	●●●●	○	○	●	●
Adeno lung carcinoma							
MOR/P		○	●	●●●●	●●●	●	●●●
MOR/R	Dox (368)	○	●●●●	●●●●	●●●	●	●●●
Leukemia							
HL60		○	●	○	○	●	⊕
HL60/ADR	Dox (186)	○	●●●●	○	○	●	⊕

[a] ○, no expression; ⊕, very low expression; ●–●●●●, low to high expression.

recently cloned another ABC transporter (*ABC-C*), located in the same chromosomal band as *MRP1*, but this is not a *MRP* homologue, because the identity between these two proteins is only 18%. After our work was completed Allikmets *et al.* (67) reported the identification of 21 new ABC genes also based on a search of the human EST database, and they mapped the identified partial sequences. Their new ABC genes also include *cMOAT, MRP3, MRP4, MRP5,* and *MRP6*. *cMOAT* corresponds with EST number 172291, *MRP3* with EST number 90757, *MRP4* with EST number 205858, *MRP5* with EST number 277145, and *MRP6* with EST number 349056. EST number 205858 (*MRP4*) was not mapped by them, but another EST sequence, number 170205, was mapped in the same region as *MRP4* and could, therefore, also be *MRP4*. Their data indicate that *MRP4* also encodes a protein with two ATP-binding domains.

Possible Physiological Functions of the MRP Family Members. The physiological role of these new MRP proteins is still unknown, but it is possible that they all play a role in cellular detoxification processes by exporting GSH *S*-conjugates or other organic anions, as has been suggested also for MRP1 and cMOAT. GSH *S*-conjugate carriers have been described in many mammalian cells, including liver, heart, lung, and mast cells and erythrocytes (36, 68). Kinetic studies indicate that both liver canaliculi and erythrocytes contain two different ATP-dependent transport activities for organic anions (69–72). cMOAT is localized in the canalicular membranes of hepato-

Table 4 *Characteristics of the cell lines selected for resistance to cisplatin, tetraplatin, or* $CdCl_2$[a]

Resistant cell lines were selected by chronically exposing them to the concentrations of drugs as shown. Only A2780/DDP and HCT8/DDP were selected by challenging them 1 h weekly with 50 μM cisplatin. RNA levels were determined as in Fig. 2. The relative expression level is indicated by filled circles, very low expression by ⊕, and undetectable RNA levels by open circles. Data for total intracellular GSH concentrations were obtained from three independently isolated cell extracts assayed in three independent experiments using the recycling method of Tietze (56) and presented as the mean GSH ± SD.

Cell line	Drug used for selection (μM)	RNA levels						GSH (nmol/mg protein)
		MDR1	MRP1	cMOAT	MRP3	MRP4	MRP5	
Bladder carcinoma								
T24		○	●	⊕	⊕	●	●	11.4 ± 2.4
T24/DDP5	CDDP (3.3)	●	●	⊕	⊕	●	●	26.8 ± 0.1
T24/DDP7	CDDP (4.7)	●	●●	⊕	⊕	●	●	65.5 ± 1.8
T24/DDP10	CDDP (6.7)	●	●●	⊕	⊕	●	●●	59.5 ± 3.3
Ovarian carcinoma								
2008		●	●●	⊕	⊕	⊕	●●●	33.5 ± 1.5
2008/C13*5.25	CDDP (5.0)	○	●●	●	⊕	⊕	●●●	113.2 ± 16.6
2008/MT	$CdCl_2$ (25)/$ZnCl_2$ (200)	●	●●	⊕	⊕	⊕	●●●	48.5 ± 5.4
2008/A	CDDP (0.5)	○	●●	●	⊕	⊕	●●●	124.7 ± 18.6
A2780		○	●	⊕	○	⊕	●●	13.3 ± 0.9
A2780/DDP	CDDP (50.0)	○	●	●●●●	○	⊕	●●	26.5 ± 2.4
PXN94		⊕	●●	○	○	●	●●	40.2 ± 5.6
PXN94/tetR	Tetraplatin (2.0)	⊕	●●	○	○	●	●●	91.8 ± 8.8
Colon carcinoma								
HCT8		●●●●	●	●	●	●	●	17.7 ± 2.2
HCT8/DDP	CDDP (50.0)	●●●●	●●	●●●	●●	●	●●	70.2 ± 6.8
Testis carcinoma								
GCT27		○	●	⊕	○	⊕	●●	5.2 ± 0.2
GCT27cisR	CDDP (4.0)	○	●	⊕	○	⊕	●●	9.2 ± 3.5
Epidermoid carcinoma								
KB-3-1		○	●●	●●●	●●●	⊕	●	36.7 ± 0.9
KCP-4(−)	CDDP (23.3)	○	●●	●●●	●●●	⊕	●●	72.7 ± 8.9
KCP-4(+)	CDDP (6.7)	ND	ND	⊕	ND	ND	ND	158.4 ± 13.3

[a] ○, no expression; ⊕, very low expression; ●–●●●●, low to high expression; ND, not determined.

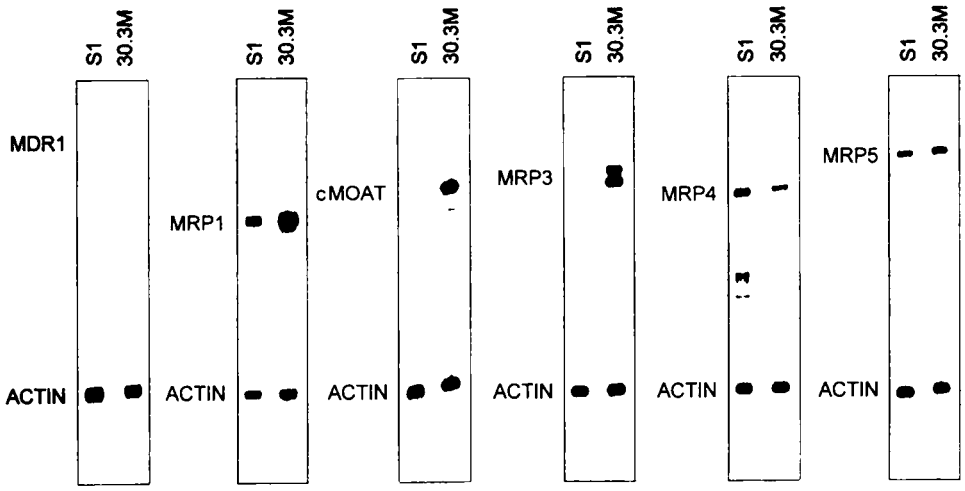

Fig. 2. RNase protection assays of RNA transcript levels of *MDR1, MRP1, cMOAT (MRP2), MRP3, MRP4,* and *MRP5* in the human non-small cell lung cancer cell line SW1573/S1 and its doxorubicin-selected subline 30.3M. Ten μg of total cytoplasmic RNA from each cell line were used per probe. The positions of the protected fragments of *MDR1, MRP1–5,* and *τ-actin* are indicated.

cytes, and the absence of this protein in the TR^- rats as well as in a patient with the Dubin-Johnson syndrome strongly suggests a role for the cmoat/cMOAT proteins in the transport of non-bile acidic organic compounds from liver to bile (29, 30, 38). The other ATP-dependent transport activity in liver canaliculi, responsible for transport of bile acids from liver to bile, is not attributable to cMOAT, because studies with TR^- rats and Dubin-Johnson patients showed that bile acid transport was not affected (36).

Two other congenital liver diseases characterized by a conjugated hyperbilirubinemia, like the Dubin-Johnson syndrome, are BRIC and PFIC (Byler disease; Refs. 73 and 74). The clinical and biochemical features of BRIC and PFIC are suggestive of a defect in primary bile acid secretion (75, 76). BRIC and PFIC have both been mapped to the same region on chromosome 18, 18q21-q22 (77, 78).

In view of the high expression of *MRP3* in the liver (Table 2), *MRP3* could be the bile salt transporter and possibly the *BRIC/PFIC* gene. However, the mapping of *MRP3* to chromosome 17 excludes MRP3 from being the *BRIC/PFIC* gene, although it may still be the bile acid transporter. Because none of the human ABC transporter genes identified thus far maps to chromosome 18 (Ref. 67 and this study), it is unlikely that BRIC/PFIC is caused by a defect in a readily recognizable ABC transporter gene.

GS-X activity has also been found in erythrocytes. Several studies have shown that human and rat erythrocytes contain a low- and a high-affinity DNP-SG transporter (72, 78, 79). The high-affinity DNP-SG transporter is most likely MRP1, because the presence of this protein and its binding to LTC_4 have been shown for erythrocytes (80, 81). The other transporter with low affinity for DNP-SG but high affinity for glucuronides and mercapturates (72) is not cMOAT or the bile salt transporter because: (*a*) no major alterations in DNP-SG transport in erythrocytes from TR^- rats and Dubin-Johnson patients were detected (36); and (*b*) erythrocytes transport DNP-SG and GSSG but no bile salts (71). It has to be seen whether this second transporter is encoded by one of the other MRP homologues or another, yet unidentified, gene.

Expression of *MRP* Homologues in Resistant Cell Lines. We screened a large set of cell lines and their resistant sublines to see whether *MRP1, cMOAT,* or one of the other *MRP* homologues is overexpressed. *MRP4* was not overexpressed in any of the lines. *MRP3* RNA was only found to be elevated in the cisplatin-resistant HCT8/DDP cell line and several SW1573/S1 sublines selected for doxorubicin resistance. However, overexpression did not correlate with the level of doxorubicin resistance. For *MRP5,* only low overexpression was found in three cell lines selected for cisplatin resistance (T24/DDP10, HCT8/DDP, and KCP-4(−); Table 4), but many other cisplatin-selected cell lines showed no overexpression; therefore, it is questionable whether this low level of *MRP5* overexpression has anything to do with cisplatin resistance.

Table 3 shows that the classical non-Pgp cell lines selected for high level doxorubicin resistance and known to highly overexpress the *MRP1* gene do not significantly overexpress other members of the *MRP* family. This is compatible with the interpretation that MRP1 is the transporter responsible for MDR in these cell lines. In the non-Pgp derivatives of the SW1573/S1 cell line presented in Table 3, a more complex situation is found, and the contribution of MRP1, cMOAT, MRP3, and the major vault protein, also present at increased levels in some of these cell lines (82), remains to be sorted out.

The Possible Involvement of Organic Anion Transporters in Cisplatin Resistance. Whereas Pgps do not transport small or highly charged molecules, organic anion transporters, such as MRP1 and cMOAT, have been speculatively linked to resistance to oxyanions (arsenite and antimonite) and cisplatin. These compounds can form complexes with GSH, and there is now considerable evidence that these complexes are substrates for organic anion transporters. Resistance caused by increased export of these complexes is bound to be complex, as pointed out by Ishikawa (68) and by us (8, 11, 83). Increased levels of pump or GSH, increased GSH synthesis, or a combination may be required, depending on the rate-limiting step in drug export.

In the protozoal parasite *Leishmania,* resistance to arsenite and antimonite can be associated with both a 40-fold increase in the *Leishmania* GSH homologue trypanothione (84) and an increase in the MRP-related ABC-transporter PgpA (85). Cancer cells selected for high levels of cisplatin may sometimes also contain extremely high concentrations of GSH (86), and the GSH synthesis in these cells is up-regulated (86–88). All of the cisplatin-resistant cell lines studied by us have elevated GSH levels as well, albeit not as high as the cell lines isolated by Godwin *et al.* (86). In contrast to published data, we also find raised GSH levels in the T24 sublines, the GCT27cisR and PXN94/tetR cell lines (23, 25, 26). We find no clear correlation, however, between the degree of cisplatin resistance and GSH levels, as observed by Godwin *et al.* (86). Moreover, all of the cell lines studied by us show a decreased accumulation of cisplatin, and an organic ion pump may, therefore, be involved in resistance.

Ishikawa *et al.* (17) showed that *MRP1* is overexpressed in the cisplatin-resistant human leukemia cell line HL60/R-CP. They concluded that an increased GSH synthesis in combination with raised MRP1 levels can cause cisplatin resistance. Active cisplatin efflux has

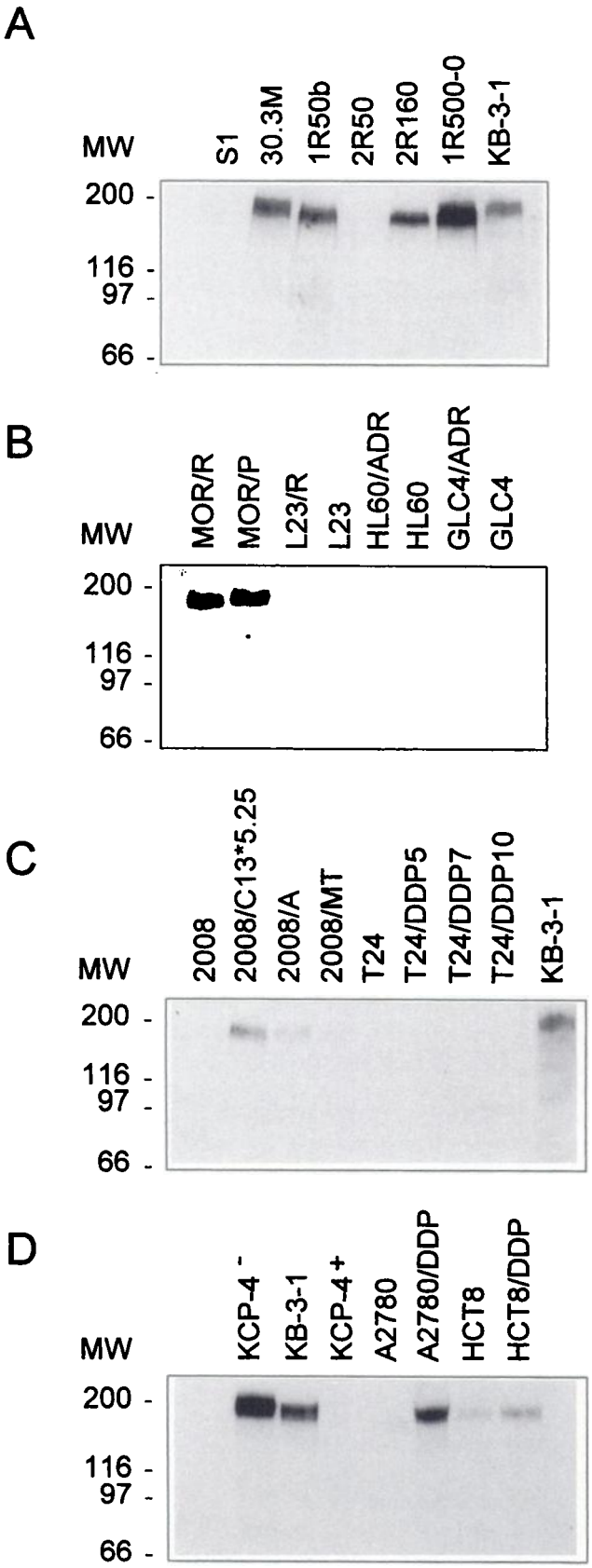

Fig. 3. Immunoblot detection of cMOAT protein in the doxorubicin-selected cell lines (*A* and *B*) and cisplatin- or $CdCl_2$-selected cell lines (*C* and *D*) analyzed in this paper. Total cell lysates were size fractionated (40 μg/lane) in a 7.5% polyacrylamide gel containing 0.5% SDS. The fractionated proteins were transferred to a nitrocellulose membrane, and cMOAT protein was detected by incubation with monoclonal antibody M_2III-5. *Left*, sizes (in thousands) and positions of molecular weight markers.

been described in three of the cell lines in Table 4: KCP-4, A2780/DDP, and HCT8/DDP (18–20, 89). The ATP-dependent efflux was inhibited by DNP-SG, indicating that it was catalyzed by a GS-X pump. In addition, the membrane vesicles of the KCP-4 cell line were shown to catalyze an increased uptake of LTC_4 (18, 19), known to be the substrate with the highest affinity for MRP1. However, data from these papers and our study show that *MRP1* is not overexpressed in these cisplatin-resistant cell lines, suggesting that MRP1 is not the major pump responsible for cisplatin resistance. This is supported by transfection studies with *MRP1*, which showed no cisplatin resistance of the transfected cells (4, 90). Nevertheless, it remains possible that transport of cisplatin conjugates by MRP1 is efficient and that the low levels of MRP1 present in parental cells suffice for resistance, if formation of cisplatin conjugates in resistant cells is increased, *e.g.*, by an increase in GSH synthesis.

An organic anion pump that could be important in cisplatin resistance is cMOAT. Especially striking is the correlation between cisplatin resistance and *cMOAT* expression in the non-Pgp MDR cell lines derived from the SW1573/S1 cell line (Table 5). These lines were selected for doxorubicin resistance, and it is, therefore, unlikely that other mechanisms of cisplatin resistance are activated in these lines. It should be noted, however, that these non-Pgp MDR lines, selected for low level doxorubicin resistance, contain multiple alterations in the expression of ABC transporters. Besides up-regulation of *MRP1*, *cMOAT*, and *MRP3* (Table 3), down-regulation of *MDR1* has occurred in these lines (Ref. 42, this study, and Table 3).

Some other cisplatin-resistant lines contain increased levels of *cMOAT* as well, notably 2008/C13*5.25, 2008/A, A2780/DDP, and HCT8/DDP (Fig. 3; Tables 4 and 5). Clearly, however, cMOAT is not indispensable for cisplatin resistance because some resistant lines do not contain detectable levels of cMOAT (*e.g.*, PXN94/tetR), whereas the KCP-4(+) line even lost its cMOAT when it became highly resistant.

The combination of cisplatin with doxorubicin resistance in resistant cell lines has been reported before (87, 91) and is also present in two other cisplatin-selected lines studied here, PXN94/tetR and KCP-4(−) (Table 5). All other cisplatin-selected lines in Table 5 are doxorubicin sensitive, however. There is also no clear correlation between doxorubicin resistance and *cMOAT* overexpression. This is unexpected. The substrate specificity of the organic anion pumps in the liver canalicular membrane (cMOAT) and in erythrocytes (presumably mainly if not exclusively MRP1) is very similar (92). We, therefore, expect both pumps to confer similar resistance spectra. We have recently succeeded in obtaining stably transfected kidney cells in which cMOAT is properly routed to the plasma membrane.[8] These cells should allow a direct test of the drug resistance spectrum that can be associated with *cMOAT* overexpression.

Overexpression of *cMOAT* in cisplatin-resistant cell lines was recently also reported by Taniguchi *et al.* (33). However, in contrast to our results (Table 4), they detected raised *cMOAT* RNA levels in the KCP-4 and T24/DDP10 cell lines. We do not find this. The level of *cMOAT* RNA was even decreased in the highly cisplatin-resistant KCP-4(+) cells, and in the T24/DDP10 cell line *cMOAT* RNA is hardly detectable by RNase protection assay. We also detected no cMOAT protein in these cell lines (Fig. 3). Cross-hybridization of the *cMOAT* probe used by Taniguchi *et al.* (33), which contains the coding sequence of the first ATP-binding domain, with RNA transcribed from the other *MRP* homologues might explain the discrepancy. This underlines the importance of the use of gene-specific probes to determine expression of *MRP* homologues.

[8] R. Evers, M. Kool, and P. Borst, unpublished data.

Table 5 *Cisplatin and doxorubicin resistance of characterized cell lines*[a]

IC_{50} values and relative resistance factors (RF) of the cell lines analyzed for cisplatin and doxorubicin. IC_{50} data were obtained from clonogenic survival assays with continuous exposure to drugs. The relative resistance factor was determined by dividing the IC_{50} of each resistant cell line by the IC_{50} of the corresponding parental cell line. Also shown are the levels of RNA transcripts of *MRP1* and *cMOAT*, taken from Tables 3 and 4.

	Cisplatin		Doxorubicin		RNA levels	
Cell line	IC_{50} (nM)	RF	IC_{50} (nM)	RF	MRP1	cMOAT
S1	144		13.8		●	⊕
30.3M	500	3.5	51.1	3.7	●●	●●●
1R50b	194	1.5	84.2	6.1	●	●●
2R50	115	0.9	69.0	5.0	●●	⊕
3R80	ND	ND	75.9	5.5	●	⊕
2R120	313	2.2	345	25	●●	●●
2R160	600	4.2	1380	100	●●	●●●
1R500	260	1.7	3450	250	●	●●
T24	825		6.5		●	⊕
T24/DDP5	2200	2.7	5.5	0.8	●	⊕
T24/DDP7	1800	2.2	3.0	0.5	●●	⊕
T24/DDP10	8000	9.7	6.5	1.0	●●	⊕
2008	340		57.6		●●	⊕
2008/C13*5.25	3000	8.8	43.2	0.8	●●	●
2008/MT	210	0.6	54.4	0.9	●●	⊕
2008/A	680	2.0	36.8	0.6	●●	●
A2780	430		5.2		●	⊕
A2780/DDP	4300	10.0	6.2	1.2	●	●●●●
PXN94	580		4.2		●●	○
PXN94/tetR	2400	4.1	11.0	2.6	●●	○
HCT8	1900		90.0		●	●
HCT8/DDP	4800	2.5	95.0	1.1	●●	●●●
GCT27	288		3.0		●	⊕
GCT27cisR	2100	7.3	3.1	1.0	●	⊕
KB-3-1	380		11.0		●●	●●●
KCP-4(−)	700	1.8	43.0	3.9	●●	●●●
KCP-4(+)	22400	58.9	8.0	0.7	ND	⊕

[a] ○, no expression; ⊕, very low expression; ●–●●●●, low to high expression; ND, not determined.

In conclusion, our data and those recently published by Ishikawa *et al.* (16, 17), Fujii *et al.* (18, 19), Goto *et al.* (20), Chuman *et al.* (89), and Taniguchi *et al.* (33) provide indirect evidence that an organic anion pump, notably cMOAT, could contribute to cisplatin resistance by exporting the cisplatin-GSH complex. Elevated GSH levels and synthesis may be required to drive formation of the complex if contact with cisplatin is extended, as is usually the case for cell lines selected for resistance *in vitro*. However, even in the limited set of cell lines analyzed by us, all chosen for lowered cisplatin accumulation, there was no simple quantitative correlation between cisplatin resistance and the combination of raised GSH and cMOAT. The picture is, therefore, far from complete. Other resistance mechanisms may contribute to resistance, or the levels of MRP1 and cMOAT deduced from RNase protection assay or immunoblot may insufficiently reflect the ability of these pumps to export the cisplatin-GSH complex.

Concluding Remarks. Our analysis underlines the complexity of the alterations that may occur in cells selected for drug resistance. The range of ABC-type transporters that can be (over)expressed in tumor cells is already impressive, and the end is not in sight (67). Although it remains to be shown that transporters such as MRP3–6 can, in principle, contribute to drug resistance, it is already clear that simple correlations between overexpression of a protein and resistance are not sufficient to establish a causal relation. Even gene transfection studies may not always be sufficient to assess the significance of multicomponent systems in resistance. This appears to be the case in resistance mediated by organic anion transporters, as pointed out by Zaman *et al.* (11) and Borst and Ouelette (83).

An additional complication is the possibility that transporters are rerouted in the cell. Flens *et al.* (80) have shown that MRP1 is exclusively found in the endosomal compartment of normal epithelial cells, whereas it is mainly in the plasma membrane of cells that overproduce MRP1 (93). Rerouting of MRP1 from endosomes to plasma membrane has even been observed in non-Pgp MDR cells without substantial increase of cellular MRP1 (64).[9] Such rerouting might also contribute to resistance, although this remains to be proven.

In view of this complexity, it is clear that specific inhibitors will be required to further dissect resistance mechanisms in cells selected for resistance *in vitro* or in patients. Methods for gene-specific inhibition, *e.g.*, using antisense techniques or ribozymes, seem indispensable to get a complete picture.

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