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ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · JUNE 2005

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Int. J. Cancer: **115,** 393–402 (2005)
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Protection of platinum–DNA adduct formation and reversal of cisplatin resistance by anti-MRP2 hammerhead ribozymes in human cancer cells

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Resistance to platinum-containing antineoplastic drugs is the major limitation in their clinical use. To elucidate the role of the ABC transporter MRP2 in platinum drug resistance, its expression was analyzed in human cisplatin-resistant cell lines: the ovarian carcinoma line A2780RCIS, the adrenocortical carcinoma line D43/86RCIS and the melanoma line MeWoCIS1. All these cells showed overexpression of MRP2. For reversal of platinum resistance, 2 anti-MRP2 hammerhead ribozymes were introduced into A2780RCIS cells. Both ribozymes showed gene-silencing activities and reversed the drug-resistant phenotype. Moreover, formation of platinum-induced intrastrand cross-links was measured in DNA. The level of DNA platination corresponded inversely to the level of MRP2 expression and was accompanied by increased caspase-3-dependent apoptosis. Kinetics of formation and elimination of platinum–DNA adducts suggest that the DNA repair capacity was not altered; the decrease in platinum–DNA adduct formation was rather a reflection of the protecting activity of MRP2. In conclusion, functional inhibition of MRP2 might be a promising strategy in the reversal of resistance to platinum-based anticancer drugs. This was reflected by the specific inhibition of MRP2 by ribozyme technology, indicating that this gene therapeutic approach may be applicable as a specific means to overcome platinum resistance in human neoplasms.
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Key words: ABC transporter; ribozyme; drug resistance; cisplatin; DNA adduct

Cisplatin [*cis*-diamminedichloroplatinum (II)] is one of the most active and widely used anticancer drugs. It has proven efficacy against various cancers, including ovarian carcinoma,[1] adrenocortical carcinoma[2] and malignant melanoma.[3] The cisplatin analog carboplatin [1,1-cyclobutanedicarboxylate platinum (II)] has also been used increasingly in the last 2 decades. Therapeutic use of these platinum-containing compounds is limited by their dose-dependent side effects, including nephrotoxicity, myelosuppression, neurologic damage and strong nausea. Furthermore, the antineoplastic efficiency of cisplatin and its analogs is often unsatisfactory due to primary nonresponsiveness or the development of a secondary, acquired drug resistance of the platinum-treated neoplasm. Acquired drug resistance of tumor cells occurs rapidly both *in vitro* and *in vivo* and is the major reason for failure of platinum-based therapy.[4]

After the entry of the neutral square–planar platinum (II) complex cisplatin into the cell, binding of the 2 chloride ligands is destabilized. One of the chlorides is displaced by a water molecule. As a consequence, the drug becomes a monoaquated, charged electrophile that can react with nucleophilic sites of intracellular macromolecules such as DNA. The second reactive site of cisplatin can lead, with lower kinetics, to the formation of several types of bifunctional platinum adduct with DNA bases. Although only about 1% of intracellular cisplatin reacts with nuclear DNA, this is presumably the critical event in cisplatin-mediated cytotoxicity.[5] The 2 major adducts are Pt-d(GpG) and Pt-d(ApG) intrastrand cross-links, representing about 90% of DNA platination. Other lesions include Pt-(dG) monoadducts, Pt-d(GpNpG) intrastrand cross-links and $Pt(dG)_2$ interstrand cross-links.[6] Formation of these platinum–DNA adducts *per se* may not be sufficient to cause cell death, whereby the exact cascade of the downstream events leading to cell death is not clear. However, it is generally accepted that formation of platinum–DNA adducts and the subsequent triggering of cellular signal-transduction pathways leading to apoptosis may be the primary cytotoxic mechanism of platinum-containing drugs.[7] Among the mechanisms conferring cellular resistance to platinum-based anticancer drugs is enhanced expression of the ABC transporter[8] MRP2,[9] also called cMOAT[10] or, according to the Human Gene Nomenclature Committee, ABCC2.

To improve the efficacy of chemotherapy, circumvention of resistance against platinum-based anticancer drugs is of huge clinical interest. The classical strategy to overcome antineoplastic drug resistance is the development of low m.w. pharmacologically active compounds, designated as chemosensitizers or drug-resistance modulators.[11,13] An obstacle in applying classical compounds that inhibit MRP2 *in vitro, e.g.,* MK-571[14] or cyclosporin A,[15] arises from their intrinsic toxicity at doses necessary for activity and their unspecificity. Moreover, in the case of the MDR1/P-glycoprotein-mediated classical MDR phenotype, improved so-called second-generation chemosensitizers were demonstrated to induce enhanced activity of liver enzymes of the family of cytochrome P_{450} mixed-function oxidases, resulting in increased pharmacokinetic turnover of the applied anticancer drugs. Additionally, tumor cells can acquire resistance against the applied chemosensitizers, a so-called tertiary resistance. Consequently, it is necessary to develop alternative, less toxic and more efficient strategies to overcome drug resistance. Such an alternative procedure for circumvention of cisplatin resistance is the selective blocking of MRP2-specific mRNA expression by gene therapeutic technologies. This approach is aimed at increasing the efficiency and specificity of chemosensitization of drug-resistant cancer cells while at the same time reducing toxicity and undesirable side effects. Thus, antisense oligonucleotides,[16] siRNAs and hammerhead ribozymes can potentially be used to modify the expression of the MRP2 encoding gene. One considerable advantage of hammerhead ribozymes and siRNAs over antisense oligonucleotides is their property of endoribonucleolytic cleavage of the target molecule. Hammerhead ribozymes can be designed to cleave in *trans* a specific mRNA molecule at an NUX (N = any nucleotide; X = A, C or U) motif.[17] Ribozymes have been reported to be successful at reversing MRP2-independent drug-

Abbreviations: ABC, ATP binding cassette; BER, base excision repair; cMOAT, canalicular multispecific organic anion transporter; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPC3, glypican-3; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; IC_{50}, 50% inhibitory concentration; ICA, immunocytologic assay; MAb, monoclonal antibody; MDR, multidrug resistance; MMR, mismatch repair; MRP, multidrug resistance protein; NER, nucleotide excision repair; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; SRB, sulforhodamine B; TC, transcription-coupled.

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant numbers: LA 1039/1-3, LA 1039/2-3; Grant sponsor: Berliner Krebsgesellschaft.

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Received 24 June 2004; Accepted after revision 15 November 2004
DOI 10.1002/ijc.20899
Published online 1 February 2005 in Wiley InterScience (www.interscience.wiley.com).

resistant phenotypes.[18,22] Modulation of *MRP2* gene expression in cisplatin-resistant cancer cells may decrease transporter-mediated cisplatin efflux and thus increase the formation of platinum–DNA adducts. The cytotoxic effects of platinum-based anticancer drugs would be restored and the cisplatin-resistant phenotype, reversed.

Accordingly, we previously constructed 2 hammerhead ribozymes, RzM1 and RzM2, directed against MRP2 mRNA and characterized the kinetic parameters in a cell-free system demonstrating high endoribonucleolytic cleavage activity.[23] In the present work, we report the insertion of these 2 ribozymes into a eukaryotic expression vector system; its implementation in MRP2-overexpressing, cisplatin-resistant human ovarian carcinoma cells; and the effects on DNA platination and cisplatin resistance.

Material and methods

Cell lines and cell culture

Human cancer cells were grown in Leibovitz L-15 medium (BioWhittaker, Walkersville, MD) supplemented by 10% FCS (GIBCO BRL, Grand Island, NY), 1 mM L-glutamine, 6.25 mg/l fetuin, 80 IE/l insulin, 2.5 mg/ml transferrin, 0.5 g/l glucose, 1.1 g/l $NaHCO_3$, 1% minimal essential vitamins and 20,000 kIE/l trasylol in a humidified atmosphere of 5% CO_2 at 37°C. Cisplatin-resistant cell lines were established by continuous exposure to increasing concentrations of cisplatin for 12–24 months. The cisplatin-resistant cell line A2780RCIS was derived from the ovarian carcinoma cell line A2780,[24] whereas the cisplatin-resistant variant D43/86RCIS was selected from the adrenocortical carcinoma cell line D43/86, which was established in our laboratory (not published). The malignant melanoma cell line MeWo[25] and its cisplatin-resistant subline MeWoCIS1[26] were described earlier. To ensure maintenance of the cisplatin-resistant phenotypes, medium was supplemented with cisplatin (A2780RCIS, 10 μg/ml [33.3 μM]; D43/86RCIS, 2 μg/ml [6.66 μM]; MeWoCIS1, 1 μg/ml [3.33 μM]). In addition, medium used for cultivation of transfected cell clones contained 800 μg/ml (2.668 mM) G418 (Invitrogen, San Diego, CA).

Drugs

The following antineoplastic agents were used: cisplatin and vincristine (GRY-Pharm, Kirchzarten, Germany), carboplatin and etoposide (Bristol-Myers, Munich, Germany) and daunorubicin (Farmitalia Carlo Erba, Freiburg, Germany).

Cell proliferation assay

Chemoresistance was tested using a proliferation assay based on SRB staining, as described previously.[21,22,27,28] Cells were seeded in 96-well plates in triplicate. After 24 hr attachment, cytostatic drugs were added for a 5-day incubation before SRB staining was performed. IC_{50} values were calculated from multiple (at least 3) independent experiments in triplicate for each cell line.

Northern blot

Total RNA was prepared using TRIZOL reagent (GIBCO BRL) according to the manufacturer's instructions. Total cellular RNA (10 μg) was resolved on 1% agarose-formaldehyde gels and transferred onto a Hybond-N+ membrane (Amersham, Aylesbury, UK). Hybridization probes specific for MRP2 and GAPDH as control were generated by RT-PCR, as described previously.[29] Blots were incubated with 25 ng randomly primed [^{32}P]dCTP-labeled (Amersham) cDNA probes in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) at 58°C for at least 16 hr. Finally, the membrane was washed under high-stringency conditions (0.1 × SSC, 0.1% SDS) at 58°C.

Quantitative RT-PCR

For quantitative RT-PCR of MRP2 and GAPDH mRNA, real-time RT-PCR was carried out using a LightCycler instrument and SYBR-green fluorescent dye (Roche, Mannheim, Germany), as described previously.[27,29] The copy number was quantified by serial dilutions of cDNA fragments, cloned in the vector pCR2.1 (Invitrogen; 10^0–10^7 cDNA copies). Total RNA (2 μg) was reverse-transcribed by Superscript II enzyme (GIBCO BRL, Gaithersburg, MD) using arbitrary hexamers as random primers. The reaction mixture was incubated at 42°C for 50 min, followed by incubation at 72°C for 15 min. All samples were subjected to real-time RT-PCR with oligonucleotide primers specific for MRP2 and GAPDH, as described previously.[29] Cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 55°C for 5 sec and 72°C for 10 sec. Fluorescence was measured at 83°C for MRP2 and 87°C for GAPDH. Specificity of amplification products was confirmed by melting curve analysis.

Western blot

Extracted membrane proteins (30 μg) were separated on 4% stacking and 7.5% resolving SDS-PAGE gels and transferred to a 0.2 μm cellulose nitrate membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked in 5% nonfat dry milk and 0.05% Tween-20 in TBS overnight. Subsequently, membranes were incubated with mouse MAb directed against MRP2 (clone M2I-4; Sanbio, Uden, the Netherlands) diluted 1:100 for 2 hr. As control for equivalent protein loading, membranes were simultaneously incubated with a mouse MAb directed against actin (clone C4; Chemicon, Temecula, CA) diluted 1:4,000. Protein–antibody complexes were visualized using horseradish peroxidase–conjugated rabbit antimouse IgG (1:10,000; Dianova, Hamburg, Germany) and chemoluminescence (ECL system, Amersham) according to the manufacturer's protocol.

Measurement of intracellular glutathione

For measurement of intracellular glutathione, cells were seeded in 6-well plates and allowed to grow until 80% confluence. Cells were harvested, counted, centrifuged, washed in PBS, suspended in 100 μl of 6% trichloroacetic acid and mixed vigorously at once. Cell lysates were centrifuged (4 min, 11,000*g*) to remove particulate elements. Supernatants were used to assay GSH or total glutathione consisting of GSH and GSSG. Experiments were performed in triplicate and repeated to ensure reproducibility. Concentrations of GSH and GSSG were measured based on a photometric procedure for animal tissue[30] modified for cell culture.[31] GSH was measured directly; GSSG content was determined by subtracting GSH levels from total glutathione levels (GSH + GSSG).

Construction of ribozyme and control expression vector systems

Anti-MRP2 hammerhead ribozymes RzM1 and RzM2, encoding sense and antisense oligodeoxynucleotides (RzM1 sense, 5′-CTG CTG TGG CTG ATG AGT CCG TGA GGA CGA AAC ATA GGC T-3′; RzM1 antisense, 5′-CTA GAG CCT ATG TTT CGT CCT CAC GGA CTC ATC AGC CAC AGC AGG TAC-3′; RzM2 sense, 5′-CGA ATC CAG CTG ATG AGT CCG TGA GGA CGA AAC TGC TGT T-3′; RzM2 antisense, 5′-CTA GAA CAG CAG TTT CGT CCT CAC GGA CTC ATC AGC TGG ATT CGG TAC-3′) were synthesized chemically with overhanging ends for site-directed ligation into the KpnI and XbaI restriction sites of the eukaryotic expression vector pcDNA3.0 (Invitrogen). The coding and corresponding strands (each at 50 μM) were hybridized for 8 min at 70°C and ligated using T4 DNA ligase FPLCpure (Pharmacia, Uppsala, Sweden). Controls, RzM1/mut and RzM2/mut, contain a point mutation that

TABLE I – CROSS-RESISTANCE PATTERN OF CISPLATIN-RESISTANCE TUMOR CELLS AND ANTI-MRP2 RIBOZYME RzM1- AND RzM2-TREATED CISPLATIN-RESISTANT OVARIAN CARCINOMA CELLS

Cell lines	IC_{50}[1] (RR[2])				
	Cisplatin (μM)	Carboplatin (μM)	Daunorubicin (nM)	Etoposide (nM)	Vincristine (pM)
Cancer cell lines					
A2780	2.6 ± 0.3 (1.0)	10.8 ± 2.4 (1.0)	5.0 ± 0.6 (1.0)	28.5 ± 5.8 (1.0)	832 ± 69 (1.0)
A2780RCIS	43.7 ± 10.0 (16.8)***	233.0 ± 128.8 (21.6)***	19.1 ± 3.4 (3.8)***	493.5 ± 257.7 (17.3)***	724 ± 176 (0.9)$^{n.s.}$
D43/86	8.2 ± 0.2 (1.0)	49.8 ± 2.0 (1.0)	8.8 ± 2.8 (1.0)	179.5 ± 32.0 (1.0)	877 ± 39 (1.0)
D43/86RCIS	27.6 ± 1.6 (3.4)***	168.1 ± 12.2 (3.4)***	7.1 ± 1.3 (0.8)$^{n.s.}$	147.4 ± 28.2 (0.8)$^{n.s.}$	825 ± 58 (0.9)$^{n.s.}$
Mewo[3]	5.5 ± 0.7 (1.0)	55.3 ± 8.5 (1.0)	12.1 ± 0.9 (1.0)	195.7 ± 25.1 (1.0)	793 ± 66 (1.0)
MeWoCIS1[3]	20.3 ± 4.8 (3.7)***	158.4 ± 35.8 (2.9)***	16.2 ± 2.6 (1.3)**	313.9 ± 157.8 (1.6)*	778 ± 55 (1.0)$^{n.s.}$
Anti-MRP2 ribozyme					
A2780RCIS/RzM1 clone 1	16.1 ± 3.1 (6.2)***	203.5 ± 43.7 (18.8)$^{n.s.}$	14.9 ± 2.9 (3.0)*	210.7 ± 52.2 (7.4)**	737 ± 95 (0.9)$^{n.s.}$
A2780RCIS/RzM1 clone 2	32.1 ± 4.0 (12.3)*	139.2 ± 18.8 (12.9)*	14.6 ± 8.7 (2.9)$^{n.s.}$	189.2 ± 27.1 (6.6)**	841 ± 84 (1.0)$^{n.s.}$
A2780RCIS/RzM1 clone 3	18.3 ± 3.0 (7.0)***	181.7 ± 42.0 (16.8)$^{n.s.}$	13.2 ± 2.9 (2.6)**	328.3 ± 68.2 (11.5)$^{n.s.}$	837 ± 97 (1.0)$^{n.s.}$
A2780RCIS/RzM1 clone 4	24.1 ± 6.4 (9.2)**	90.4 ± 23.6 (8.4)**	14.8 ± 11.0 (3.0)$^{n.s.}$	134.7 ± 53.9 (4.7)**	902 ± 75 (1.1)$^{n.s.}$
A2780RCIS/RzM1 clone 5	19.0 ± 4.4 (7.3)***	155.3 ± 69.2 (14.4)$^{n.s.}$	12.9 ± 8.7 (2.6)$^{n.s.}$	86.7 ± 21.0 (3.0)***	493 ± 126 (0.6)*
A2780RCIS/RzM2 clone 1	20.3 ± 4.2 (7.8)***	126.2 ± 20.4 (11.7)*	9.7 ± 2.0 (1.9)***	145.5 ± 29.4 (5.1)**	871 ± 235 (1.0)$^{n.s.}$
A2780RCIS/RzM2 clone 2	23.2 ± 4.5 (8.9)**	190.1 ± 42.4 (17.6)$^{n.s.}$	16.6 ± 8.8 (3.3)$^{n.s.}$	391.9 ± 117.1 (13.7)$^{n.s.}$	340 ± 74 (0.4)**
A2780RCIS/RzM2 clone 3	21.7 ± 3.3 (8.3)***	136.3 ± 26.4 (12.6)*	12.6 ± 1.8 (2.5)**	222.5 ± 45.7 (7.8)**	423 ± 79 (0.5)**
A2780RCIS/RzM2 clone 4	24.5 ± 3.3 (9.4)**	98.5 ± 48.4 (9.1)*	13.7 ± 8.5 (2.7)$^{n.s.}$	139.6 ± 55.0 (4.9)**	294 ± 107 (0.4)**
A2780RCIS/RzM2 clone 5	17.9 ± 3.1 (6.2)***	174.5 ± 42.4 (16.2)$^{n.s.}$	11.1 ± 2.8 (2.2)**	109.3 ± 22.9 (3.8)***	300 ± 48 (0.4)***
A2780RCIS/RzM2 clone 6	24.9 ± 5.0 (9.6)**	242.3 ± 45.8 (22.4)$^{n.s.}$	12.4 ± 2.7 (2.5)**	238.9 ± 41.4 (8.4)**	445 ± 54 (0.5)**
A2780RCIS/vec clone 1	40.4 ± 5.4 (15.4)$^{n.s.}$	207.7 ± 44.6 (19.2)$^{n.s.}$	n.d.	n.d.	778 ± 75 (0.9)$^{n.s.}$
A2780RCIS/vec clone 2	45.6 ± 7.7 (17.5)$^{n.s.}$	267.9 ± 30.9 (24.8)$^{n.s.}$	n.d.	n.d.	643 ± 52 (0.8)$^{n.s.}$
A2780RCIS/RzM1-mut	25.4 ± 3.4 (9.8)**	141.2 ± 26.2 (13.1)*	10.3 ± 3.4 (2.1)***	251.7 ± 46.1 (8.8)*	998 ± 125 (1.2)*
A2780RCIS/RzM2-mut	27.1 ± 3.8 (10.4)**	72.5 ± 11.2 (6.7)**	17.7 ± 5.1 (3.5)$^{n.s.}$	220.7 ± 55.0 (7.7)**	399 ± 68 (0.5)**

[1]IC_{50} and SD values were calculated from multiple (at least 3) independent experiments in triplicate.–[2]RR, relative resistance (x-fold).–[3]IC_{50} values of MeWo and MeWoCIS1 were taken from Liedert *et al.*[29] p values calculated by 2-tailed Student's t-test with regard to corresponding parental cells (cancer cell lines) or to A2780RCIS (anti-MRP2 ribozyme clones).–*$p < 0.05$;–**$p < 0.01$;–***$p < 0.001$; n.s., no significant difference; n.d., not determined.

completely blocks the ribozyme's cleavage activity (A14→C).[17] Correct sequence and insert orientation of each ribozyme within the vector were confirmed by sequencing using an ABI-373 sequencer (Perkin-Elmer, Foster City, CA).

Plasmid transfection and selection of stably transfected cell clones

Cisplatin-resistant ovarian carcinoma A2780RCIS cells were transfected with 2 μg plasmid DNA containing RzM1, RzM2, RzM1/mut or RzM2/mut sequence using DMRIE-C reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's recommendations. G418 (800 μg/ml [2.668 mM], Invitrogen) was used for selection of positively transfected cell clones. Anti-MRP2 hammerhead ribozyme RzM1- or RzM2-expressing clones, clones of each control variant expressing RzM1/mut or RzM2/mut and vector control clones were isolated and expanded. Each of these clones was analyzed separately, and representative clones were chosen and used for the detailed characterization of the cellular effects. Expression of the ribozymes in transfected clones was confirmed by RT-PCR using vector-specific oligonucleotide primers as described previously.[21,22]

Caspase-3 activity assay

For the specific quantitative *in vitro* determination of cisplatin-induced caspase-3-like activity, a fluorometric immunosorbent enzyme assay (Roche) was used according to the manufacturer's recommendations. Briefly, active caspase-3 was bound by a mouse anti-caspase-3 MAb to a microtiter plate. After washing, the fluorogenic caspase-3-specific substrate peptide DEVD-AFC was added and the caspase-3-catalyzed cleavage rate measured by a fluorescent microplate reader after 1 hr with a 405 nm excitation filter and a 535 nm emission filter. Data were normalized against the untreated control of cell line A2780 and interpreted as a factor of fold induction.

Detection of platinum–DNA adducts

Cisplatin-induced Pt-d(GpG) intrastrand cross-links in the nuclear DNA of single cells were visualized and measured by ICA using the Pt-d(GpG) adduct-specific MAb 18G10, a double sandwich procedure with FITC-labeled antibodies and quantitative image analysis, as described previously.[29,32]

Results

Drug cross-resistance pattern in cisplatin-resistant cancer cell lines

The cytotoxicity of 5 different anticancer drugs was examined in drug-sensitive parental ovarian carcinoma (A2780), adrenocortical carcinoma (D43/86) and malignant melanoma (MeWo) cells and in derived cisplatin-resistant cell variants (A2780RCIS, D43/86RCIS, MeWoCIS1) by an SRB-based cell proliferation assay. The duplication time for untreated cells under these conditions was identical for drug-sensitive and cisplatin-resistant cell line pairs. IC_{50} values for the 5 anticancer drugs and relative resistance factors are shown in Table I. All cisplatin-resistant cell lines exhibited high cross-resistance to carboplatin. In addition, cisplatin-resistant A2780RCIS ovarian carcinoma cells showed cross-resistance against the anthracycline daunoblastin and the DNA topoisomerase II inhibitor etoposide. D43/86RCIS and MeWoCIS1 cells exhibited no or only weak cross-resistance against those anticancer agents. In all cisplatin-resistant cell lines, no change in chemosensitivity was observed against the tubulin-interacting vinca alkaloid vincristine.

MRP2 mRNA and protein levels in cisplatin-resistant cancer cells

Levels of MRP2-encoding mRNA were determined in parallel by Northern blotting and quantitative real-time RT-PCR. Northern blot analysis demonstrated overexpression of MRP2 in cisplatin-resistant cell lines A2780RCIS and MeWoCIS1 compared to parental cell lines A2780 and MeWo (Fig. 1*a*). MRP2 mRNA expression levels of cell lines D43/86 and D43/86RCIS were too low for convincing evaluation by this method. By applying a more sensitive, quantitative real-time RT-PCR protocol, the differences in mRNA expression levels could be confirmed and completed. After normalization to the constitutively expressed housekeeping gene *GAPDH,* a very low basic MRP2 mRNA expression level could be measured in drug-sensitive cell lines. The MRP2-specific transcript was overexpressed in A2780RCIS (20.2-fold), MeWoCIS1 (3.7-fold),[29] and D43/86RCIS (1.5-fold) cells compared to the corresponding parental cell variant (Fig. 1*b*). According to these observations on mRNA expression, Western blot analysis demonstrated overexpression of MRP2 protein in cisplatin-resistant A2780RCIS and MeWoCIS1 cells (Fig. 1*c*). Unfortunately, this technique was not as sensitive for exposing the expected small difference in MRP2 protein expression between D43/86 and D43/86RCIS.

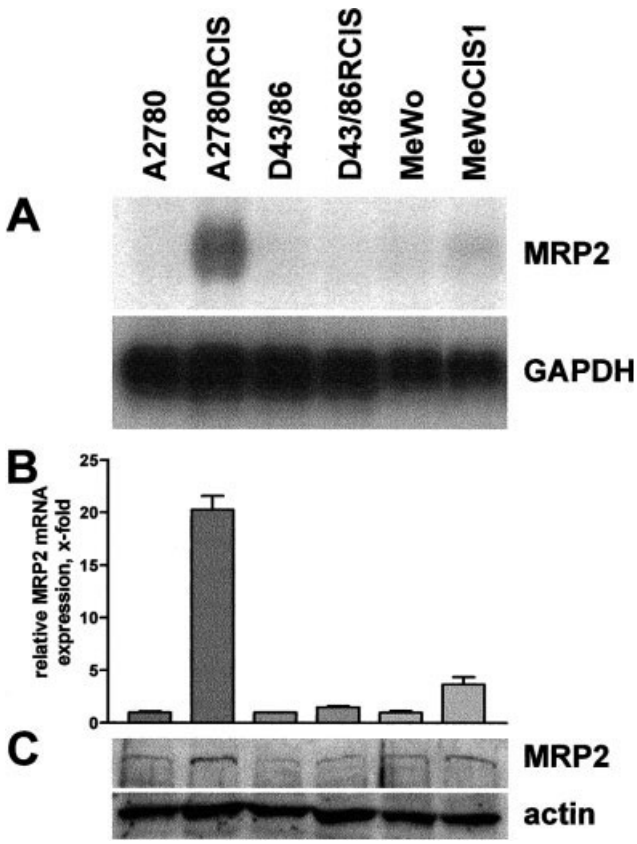

Figure 1 – (*a*) Northern blot analysis of MRP2 mRNA expression in human cancer cells. Total cellular RNA was hybridized with a ^{32}P-labeled cDNA probe encoding MRP2. As control, equivalent amounts of RNA were analyzed and blots probed with a cDNA probe specific for GAPDH. (*b*) Relative MRP2 mRNA expression level after normalization against GAPDH expression was determined by quantitative real-time RT-PCR. A2780RCIS cells showed 20.2-fold overexpression of MRP2 in comparison to the parental variant; MeWoCIS1 cells exhibited 3.7-fold overexpression; whereas 1.5-fold MRP2 overexpression was measured in D43/86RCIS cells. (*c*) Western blot analysis using mouse MAb M2I-4 directed against MRP2. As control, filters were incubated with a mouse MAb directed against the cytoskeleton protein actin.

Intracellular concentrations of glutathione

Since MRP2 transports many of its substrates, including cisplatin, as glutathione conjugates, intracellular glutathione concentrations were measured in all cancer cell lines. As shown in

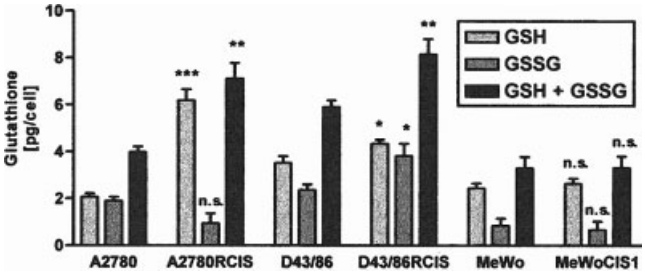

FIGURE 2 – Cellular glutathione content in drug-sensitive and cisplatin-resistant human cancer cells. GSH and total glutathione (GSH + GSSG) were experimentally determined in a photometric assay. Cellular GSSG content was calculated from these values. Displayed values represent the mean of 2 independent measurements in triplicate; error bars depict SD (n.s., not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ in comparison to the corresponding parental cell line).

Figure 2, enzymatic determination of glutathione in cellular extracts demonstrated that total cellular glutathione (GSH + GSSG) was significantly increased in cisplatin-resistant ovarian carcinoma A2780RCIS cells (3.9 pg/cell in A2780, 7.1 pg/cell in A2780RCIS) as well as in the cisplatin-resistant adrenocortical carcinoma cell line D43/86RCIS (5.9 pg/cell in D43/86, 8.1 pg/cell in D43/86RCIS) compared to their drug-sensitive counterparts. In A2780RCIS cells, the cellular content of GSH was particularly elevated (2.1 pg/cell in A2780, 6.2 pg/cell in A2780RCIS). Similar but contradictory levels of 3.3 ng/cell total cellular glutathione were detected in both malignant melanoma variants, parental MeWo and cisplatin-resistant MeWoCIS1 cells. Moreover, the distributions of GSH and GSSG showed no significant differences in cisplatin-sensitive and cisplatin-resistant melanoma cell lines.

Expression of the anti-MRP2 ribozymes RzM1 and RzM2 in A2780RCIS cells

Since the platinum-resistant phenotype of A2780RCIS cells is accompanied by considerable overexpression of MRP2, the effects of reducing the level of the encoding mRNA were analyzed in those cells. The cell line was transfected with a cytomegalovirus promoter-driven eukaryotic expression vector carrying either the anti-MRP2 ribozyme RzM1 or RzM2 (Fig. 3). A previous study revealed high catalytic activity of the ribozymes RzM1 and RzM2 in a cell-free system.[23] As controls, an empty expression vector (vec) and vectors directing the expression of hammerhead ribozymes containing an inactivating point mutation (A_{14}→C) were used (Fig. 3). Stable expression of the hammerhead ribozymes in transfected clones was confirmed by RT-PCR (data not shown). Names of the cell lines were as follows: A2780, parental, nonresistant ovarian carcinoma cell line; A2780RCIS, cisplatin-resistant variant; A2780RCIS/RzM1 and A2780RCIS/RzM2, cisplatin-resistant A2780RCIS cells expressing either anti-MRP2 ribozyme RzM1 or RzM2; A2780RCIS/RzM1-mut and A2780RCIS/RzM2-mut, A2780RCIS-derived control clones each expressing a catalytic inactive mutated ribozyme; A2780RCIS/vec, A2780RCIS-derived vector control clones.

Ribozyme effects on MRP2 mRNA and protein expression

Northern blot and quantitative real-time RT-PCR experiments were performed to analyze the expression levels of the MRP2-encoding mRNA. Both analyses demonstrated distinct downregulation of the MRP2-specific transcript in RzM1- and RzM2-treated clones, some to an expression level that was comparable to that observed in platinum-sensitive A2780 cells (Fig. 4). By Northern blot analysis, none of the controls exhibited a decreased MRP2 mRNA expression level. In contrast, control clones A2780RCIS/RzM1-mut and A2780RCIS/RzM2-mut showed lower MRP2

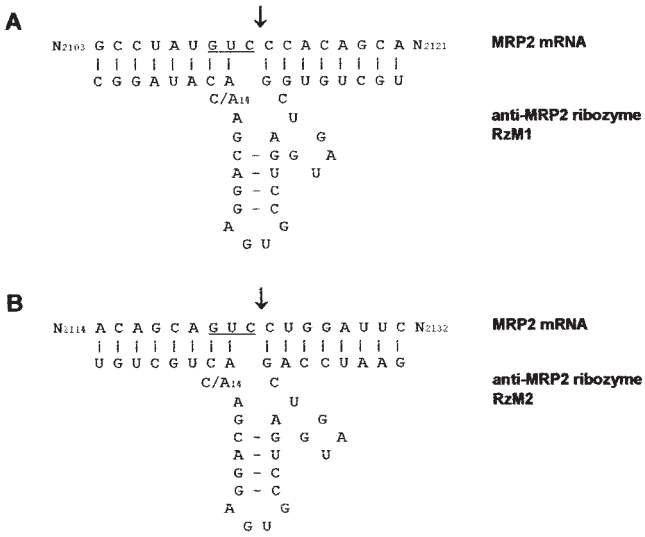

FIGURE 3 – Schematic representation of hammerhead ribozymes (*a*) RzM1 and (*b*) RzM2 directed against MRP2-encoding mRNA. The cleavage site in the target mRNA is indicated by an arrow; the GUC recognition triplet is underlined. Numbering of the hammerhead ribozyme is according to the system suggested by Hertel *et al*.[53] For construction of a noncatalytically active control ribozyme, a point mutation was introduced in the sequence, A_{14}→C. The MRP2-encoding sequence and nucleotide positions were derived from the MRP2 cDNA sequence (GenBank accession NM 000392). Nucleotide position +1 is assigned to the A residue of the ATG translation start codon.

mRNA expression than nontransfected A2780RCIS cells using quantitative real-time RT-PCR, whereas A2780RCIS/vec control clones did not show any alterations in MRP2 mRNA expression. This effect, detected by the more sensitive PCR-based method, might be the result of putative antisense effects of the 5′ and 3′ arms of the catalytically inactive ribozyme sequence in A2780RCIS/RzM1-mut and A2780RCIS/RzM2-mut cells.

Western blot analysis was performed to confirm the results for MRP2 on the protein level. As shown in Figure 5, distinct MRP2 protein expression was detected in drug-resistant A2780RCIS cells and the A2780RCIS/vec control clone. In contrast, the nonresistant cell line A2780 and the catalytically active anti-MRP2 ribozyme–treated A2780RCIS-RzM2 cell clones displayed considerably decreased expression of MRP2 protein. Likewise, the second control clone, A2780RCIS/RzM2-mut, treated with an endoribonucleolytic inactive mutated anti-MRP2 hammerhead ribozyme, also showed a decrease in MRP2 protein compared to the drug-resistant cell line A2780RCIS. Accordingly, this observation might be the result of putative antisense effects of the catalytically inactive ribozyme.

Sensitivity of anti-MRP2 ribozyme RzM1- and RzM2-treated cells to anticancer drugs

The anti-MRP2 ribozyme-treated cell clones A2780RCIS/RzM1 and A2780RCIS/RzM2 showed a decreased resistance level against cisplatin, to approximately 50% (ranging 73–37%), of the untransfected cell line A2780RCIS (Table I). Similar changes were found for the modulation of resistance against carboplatin, daunorubicin and etoposide, though not all clones showed IC_{50} values with a statistically significant decrease. Drug resistance against vincristine was modulated in approximately 50% of the A2780RCIS/RzM transfectants (1/5 and 5/6 of the A2780RCIS/RzM1 and A2780RCIS/RzM2 clones, respectively). These results suggest a putative additional factor changing the sensitivity against vincristine in a subpopulation of the cisplatin-resistant variant A2780RCIS. Additional controls were generated by treatment

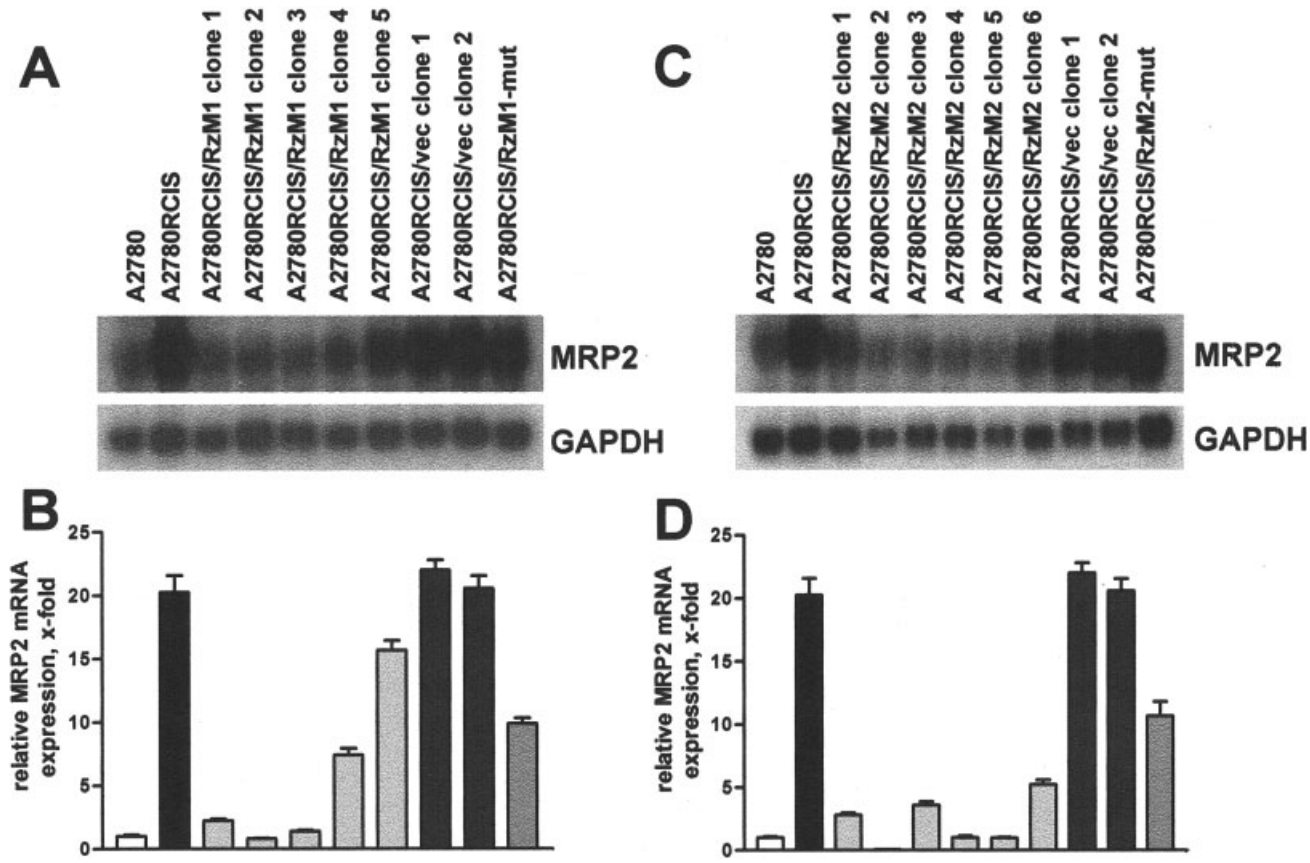

FIGURE 4 – Analysis of (*a,b*) anti-MRP2 ribozyme RzM1- and (*c,d*) RzM2-mediated silencing of MRP2 mRNA expression. (*a,c*) For Northern blot analysis, total RNA was hybridized with ^{32}P-labeled cDNA probes encoding MRP2, with GAPDH as control. (*b,d*) Relative MRP2 mRNA expression level after normalization against GAPDH expression level determined by quantitative real-time RT-PCR. (*b*) The 20.2-fold MRP2 mRNA expression in A2780RCIS was decreased to 2.2-fold in A2780RCIS/RzM1 clone 1, 0.8-fold in A2780RCIS/RzM1 clone 2, 1.4-fold in A2780RCIS/RzM1 clone 3, 7.4-fold in A2780RCIS/RzM1 clone 4 and 15.7-fold in A2780RCIS/RzM1 clone 5. Control clones transfected with empty expression vector exhibited no significant alterations of the relative MRP2 expression level, *i.e.*, 22.0-fold in A2780RCIS/vec clone 1 and 20.6-fold in A2780RCIS/vec clone 2. The clone treated with catalytically inactive ribozyme A2780RCIS/RzM1-mut decreased MRP2 expression to 9.8-fold. (*d*) The 20.2-fold MRP2 mRNA expression in A2780RCIS was decreased to 2.8-fold in A2780RCIS/RzM2 clone 1, 0.1-fold in A2780RCIS/RzM2 clone 2, 3.6-fold in A2780RCIS/RzM2 clone 3, 1.0-fold in A2780RCIS/RzM2 clone 4, 1.0-fold in A2780RCIS/RzM2 clone 5 and 1.0-fold in A2780RCIS/RzM2 clone 6. Control clones transfected with empty expression vector exhibited no significant alterations in MRP2 expression, *i.e.*, 21.8-fold in A2780RCIS/vec clone 1 and 20.6-fold in A2780RCIS/vec clone 2. The clone treated with catalytically inactive ribozyme A2780RCIS/RzM2-mut decreased MRP2 expression to 10.7-fold.

of parental A2780 cells with the anti-MRP2 ribozyme RzM1 and the control ribozyme RzM1-mut. As expected, no effect on the cisplatin-specific IC_{50} value was observed.

Activation of caspase-3 following cisplatin treatment

For monitoring the effects of MRP2 expression on apoptosis triggered by cisplatin exposure, the catalytic activity of caspase-3 was measured. Caspase-3 is one death protease, among others, that can be activated as a consequence of upstream apoptotic signal transduction and catalyzes the cleavage of several cellular components related to DNA repair and regulation.[33] As demonstrated in Figure 6, caspase-3-mediated cleavage activity increased obviously in drug-sensitive A2780 cells following cisplatin treatment (100 μM, 24 hr) to 13.9-fold. Cisplatin-resistant A2780RCIS cells and the control clone A2780RCIS/vec exhibited distinctly diminished caspase-3 cleavage activity under the same conditions (4.9- and 3.7-fold, respectively). By treatment of A2780RCIS cells with the anti-MRP2 ribozyme RzM2 (A2780RCIS/RzM2 clone 1), the cellular capability to induce caspase-3 cleavage activity was restored to 24-fold. In the anti-MRP2 ribozyme-treated clone, activation of caspase-3 was more pronounced than in the drug-sensitive cell variant A2780. This effect may be the result of clonal variability.

Formation and repair of platinum–DNA adducts

Augmented export of cisplatin from cells with high MRP2 expression levels should result in reduced interaction of the drug with intracellular targets and, hence, lower DNA platination. To test this correlation, we determined the nuclear concentrations of Pt-d(GpG) intrastrand cross-links, the major product of cisplatin-induced DNA adducts at the single-cell level, employing adduct-specific MAbs in an ICA. Cell lines with different MRP2 expression levels were exposed to various doses of cisplatin and analyzed by ICA, the parental drug-sensitive A2780 cells, the platinum-resistant derivative A2780RCIS and the anti-MRP2 ribozyme-treated A2780RCIS/RzM1 clone 1. Quantitative image analysis of Pt-d(GpG) adduct fluorescence signals revealed dose-dependent adduct formation in all cell lines and maximal adduct formation at all 3 doses (10, 20, 50 μM) 6 hr after treatment (Fig. 7). In comparison to the sensitive A2780 cells, the relative adduct burden after 50 μM cisplatin at this time point was lowered by 54% and 68% in the more resistant A2780RCIS/RzM1 and A2780RCIS cells, respectively. A similar ranking of the cell lines

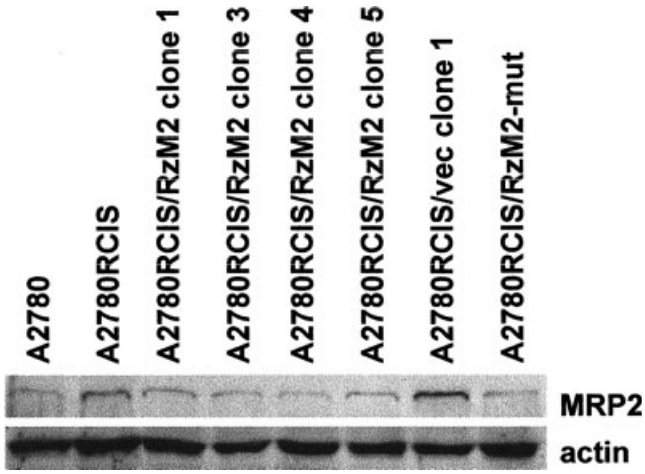

FIGURE 5 – Effect of the anti-MRP2 ribozyme RzM2 on MRP2 protein expression determined by Western blot analysis using mouse MAb M2I-4 directed against MRP2. MRP2-specific bands of the membrane protein fraction were visualized by chemoluminescence. As control, filters were incubated with a mouse MAb directed against the cytoskeleton protein actin.

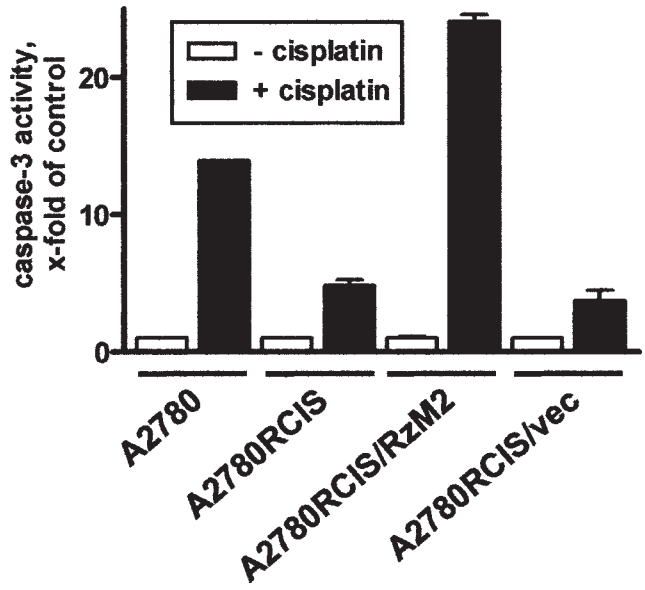

FIGURE 6 – Activation of caspase-3-like activity in platinum-sensitive A2780 cells, cisplatin-resistant cell line A2780RCIS, anti-MRP2 ribozyme-treated A2780RCIS/RzM2 clone 1 and as control A2780RCIS/vec clone 1 after 24 hr exposure to 100 μM cisplatin. Columns represent relative caspase-3-like activity induction compared to untreated A2780 cell line. Bars = SD.

in peak adduct formation was seen at both other cisplatin doses. Between 6 and 48 hr postexposure, Pt-d(GpG) cross-links were efficiently removed by DNA repair from the genome of all tested cell lines. The remaining adducts at this time point were rather resistant to further repair within the next 24 hr. Both measures, peak cross-link formation and integrated adducts under the curve values, were in good concordance with cellular cisplatin sensitivity and strongly suggest significantly reduced DNA platination in MRP2-expressing cells in correlation to their respective expression levels.

Discussion

Primary and secondary resistance to platinum-based antineoplastic drugs are major limitations to their use in cancer chemotherapy. Improved understanding of the biologic mechanisms leading to platinum resistance will provide molecular targets for therapeutic intervention and may facilitate prediction of response and therewith the basis for individually tailored therapy. In the last few years, various *in vitro* models for cisplatin resistance derived from different tumor entities were analyzed to identify the underlying molecular mechanisms. In this context, alterations in drug uptake and efflux,[34,35] including enhanced expression of the ABC transporter MRP2,[10] detoxification by methallothioneins,[36] increased glutathione level as well as increased activity of GST,[37] have been described. Furthermore, increased capacity for platinum–DNA adduct tolerance,[35,38] reduced apoptotic response to cisplatin exposure,[39,40] alterations in regulatory proteins (*e.g.,* oncogene or tumor-suppressor gene encoded factors such as c-fos[41] or p53[39]) and changes in DNA repair status[42] are possible causes of cisplatin resistance. These DNA repair pathways include enhanced NER[43] and decreased activity of the DNA MMR system.[43] Even though these mechanisms have been identified as contributing to cisplatin resistance *in vitro,* hitherto the problem of clinical resistance against platinum drugs was not vanquished because chemotherapeutic treatment of any cancer cell population selects for more than one mechanism of drug resistance, resulting in a multifactorial resistance phenotype.

Earlier studies demonstrated that a cisplatin-resistant phenotype could be associated with overexpression of the ABC transporter MRP2. In these studies, carcinoma-derived cells with acquired cisplatin resistance[10,45] or kidney cells transfected with MRP2-encoding cDNA[46,47] were used. The conclusiveness of these studies, however, was hampered by the lack of functional assays. Physiologically, MRP2 mediates the ATP-dependent transport of a broad range of substrates, including glutathione, glucuronide and sulfate conjugates of many endogenous and xenobiotic compounds across the apical canalicular membrane of hepatocytes.[48] The gene, encoding the 190 kDa ABC-transporter protein, is localized in chromosomal region 10q24. Mutations in this gene cause Dubin-Johnson syndrome,[49] a rare autosomal recessive disorder characterized by chronic conjugated hyperbilirubinemia due to impaired hepatobiliary transport of nonbile salt organic anions.

In the present study, expression analyses revealed that MRP2 was overexpressed in the complete panel of cisplatin-resistant cancer cell lines. This overexpression was also associated with increasing drug resistance against carboplatin and, in some cases, cross-resistance against the anthracycline daunoblastin and the epipodophyllotoxin etoposide. In contrast to studies with MRP2 cDNA–transfected human HEK293 kidney cells,[46] no cross-resistance was observed against the vinca alkaloid vincristine in any of the MRP2-overexpressing cells. Interestingly, the resistance against cisplatin and carboplatin in D43/86RCIS cells, exhibiting merely a weak 1.5-fold increased MRP2 mRNA expression level, was comparable to that in MeWoCIS1 cells, indicating that additional factors are involved in the platinum-resistant phenotype of this cell line.

Previous studies have shown that some cisplatin-resistant cells contain increased levels of glutathione[37] and that MRP2 is able to transport glutathione conjugates.[48] Cisplatin-resistant ovarian carcinoma A2780RCIS cells and adrenocortical carcinoma D43/86RCIS cells exhibited a distinct elevated cellular glutathione content compared to the drug-sensitive parental cells, whereas the MeWoCIS1 cell line has not. Not only the total cellular glutathione content (GSH + GSSG) but also the GSH level, the relevant glutathione form for the synthesis of glutathione conjugates, was elevated. These observations support the idea that in these cell models enhanced MRP2-dependent platinum–glutathione conjugate extrusion could take place. Since the MRP2 expression data demonstrated very weak overexpression of MRP2 in D43/86RCIS cells, in this cell line the level of GSH may be rate-limiting; therefore, the increased cisplatin resistance in D43/86RCIS cells may

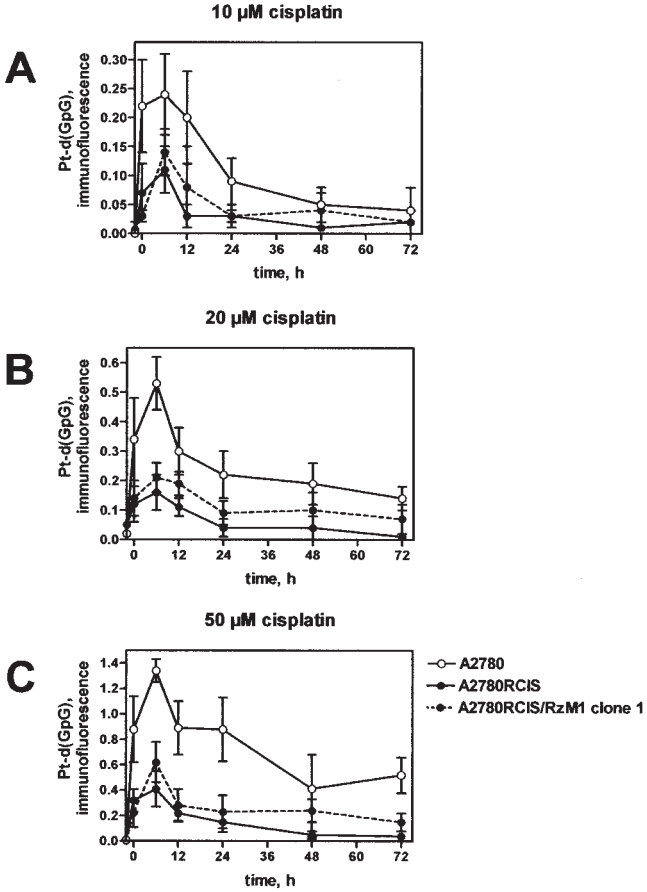

FIGURE 7 – Time kinetics of cisplatin-induced Pt-d(GpG) adduct formation in the drug-sensitive ovarian carcinoma cell line A2780, its cisplatin-resistant derivative A2780RCIS and the anti-MRP2 ribozyme-treated A2780RCIS/RzM1 clone 1, as measured by ICA. Cisplatin was added at $t = -2$ hr. Cells were exposed to (*a*) 10, (*b*) 20 or (*c*) 50 µM cisplatin for 2 hr; and nuclear Pt-d(GpG) adduct content was determined after different time periods by fluorescence microscopy using the rat anti Pt-d(GpG) adduct MAb 18G10 and a FITC-based double sandwich staining procedure. Displayed values represent the mean. Vertical error bars = SD.

be mainly the result of upregulation of GSH and not of MRP2 overexpression. However, in the MeWo cell line, export of cisplatin may be rate-limiting; and the increased cisplatin resistance in MeWoCIS1 is predominantly the result of MRP2 upregulation. In A2780RCIS, which is also the most resistant cell line, there may be upregulation of both GSH and MRP2.

Two anti-MRP2 hammerhead ribozymes, previously shown to have high endoribonucleolytic cleavage activity,[23] were applied to inhibit expression of the MRP2-specific mRNA in cisplatin-resistant A2780RCIS cells. Distinct reduction of MRP2 mRNA and protein levels was accompanied by reversion of cisplatin resistance to approximately 50%. Gene expression analyses also revealed decreased MRP2 mRNA and protein expression in control clones treated with ribonucleolytically inactive ribozymes (RzM1/mut, RzM2/mut). Moreover, this reduction in MRP2 expression was accompanied by a decrease in corresponding drug resistance, probably due to antisense effects of the 5′- and 3′-flanking sequences of the ribozyme. This effect is in line with the observation that such antisense activities of catalytically inactivated hammerhead ribozymes were found in human cancer cells treated with ribozymes directed against the ABC transporter BCRP (ABCG2)[21] or the mitoxantrone resistance–associated GPC3.[22] However, there was no absolute 1:1 correlation between MRP2 expression levels in the ribozyme-treated clones and the corresponding resistance factors. This phenomenon may be achieved by clonal variability of each of the cell variants. The fact that all ribozyme-treated clones showed considerable effects of MRP2 inhibition and reversal of drug resistance strongly supports the important role of MRP2 in cisplatin resistance of the ovarian carcinoma cell line A2780RCIS. As expected, control clones transfected with empty expression vector did not show any alterations in MRP2 mRNA or protein expression level, indicating no nonspecific side effects of the gene therapeutic procedure.

The kinetics of formation and elimination of nuclear platinum–DNA adducts were measured in anti-MRP2 ribozyme-treated A2780RCIS cells compared to untransfected A2780RCIS and A2780 cells. During the first hour after cisplatin exposure, nuclear amounts of Pt-d(GpG) adducts in all cell lines reflected the balance between ongoing formation of platinum–DNA intrastrand cross-links and the beginning of adduct elimination by activated cellular DNA repair. After reaching the maximum DNA platination at 6 hr following cisplatin withdrawal, adduct levels started to drop, first caused predominantly by prevailing DNA repair and later supported by adduct dilution due to ongoing cell proliferation. Experiments with different cell systems revealed that cisplatin resistance could be associated with elevated NER,[50] in particular TC-NER.[51] However, measurements of platinum–DNA adduct formation and elimination kinetics did not elucidate which DNA repair pathways are activated in the ovarian cancer cell lines. Experimental data from the cisplatin-resistant melanoma cell line MeWoCIS1 imply that neither increased NER nor increased BER is involved in the platinum-resistant phenotype of this cell line.[29]

At each time point after cisplatin exposure, nuclear levels of platinum–DNA adducts were lower in drug-resistant cells compared to the cisplatin-sensitive cell line, whereas the anti-MRP2 ribozyme-treated cells exhibited intermediate platinum–DNA adduct levels. Cisplatin-resistant cells did not show faster or more efficient removal of Pt-d(GpG) cross-links in comparison to the parental drug-sensitive or anti-MRP2 ribozyme-treated cells, indicating that in all cell variants similar DNA repair activities were triggered by these lesions. Adduct levels were already extensively lower in cisplatin-resistant cells as early as 2 hr after cisplatin exposure, resulting in more convenient conditions for cell proliferation under cisplatin treatment and less induction of apoptosis, determined by caspase-3 activity measurements. These data support the idea that the drug-extrusion activity mediated by MRP2 protects cisplatin-resistant ovarian carcinoma cells from undergoing caspase-3-dependent programmed cell death after DNA damage by platinum–DNA adducts.

In summary, our data indicate that the cisplatin-resistant phenotypes exhibited by A2780RCIS ovarian carcinoma cells, D43/86RCIS adrenocortical carcinoma cells and MeWoCIS1 melanoma cells are mediated by the cooperative action of different biologic mechanisms. The following hypothesis of cisplatin resistance in these cell systems is offered: (*i*) enhanced drug-extrusion activity of MRP2 decreases the cellular platinum concentration, (*ii*) this results in decreased formation of platinum–DNA adducts and (*iii*) in consequence, cellular triggering of apoptotic pathways is reduced. However, this postulated cisplatin resistance–mediating pathway does not exclude that additional mechanisms may contribute to this phenotype. Thus, increased MRP2-dependent drug efflux may be supported by enhanced levels of glutathione in A2780RCIS and D43/86RCIS cells. However, the data indicate that MRP2 appears to be a key molecule in cellular cisplatin resistance.

Whether accelerated export of cisplatin by the MRP2 transporter is an important feature not only in cell culture systems but also in resistance of primary tumors has to be confirmed. Our data may be important for clinical management of human cancers since they emphasize the formation of platinum–DNA adducts as the crucial effect mediating the cytotoxicity of platinum-based anticancer drugs. Thus, drug-efflux prevention leading to increased

DNA platination appears to be a very promising strategy to overcome nonresponsiveness to cisplatin because all consecutive cellular events are merely direct consequences of platinum–DNA adduct formation. Accordingly, MRP2 appears to be an excellent candidate both for the diagnosis of clinical platinum resistance and for gene-therapeutic strategies to overcome platinum resistance in human cancers.

Since the available low m.w. pharmacologic inhibitors[11,13] for ABC transporters, such as the leukotriene LTD_4 receptor antagonist MK-571[14] and the immunosuppressive agent cyclosporin A,[15] are less specific for MRP2, the gene-silencing ribozyme approach is an excellent strategy for the specific inhibition of this membrane pump. Here, we describe the successful ribozyme-dependent downregulation of MRP2-specific transcript and protein expression in human cancer cells. The extent of MRP2 downregulation achieved MRP2 expression levels comparable to those of the parental nonresistant control cells. Both anti-MRP2 ribozymes, RzM1 and RzM2, were highly active in a cellular environment and showed their potential as valuable laboratory tools for investigating the relationship between MRP2 expression and drug resistance. In addition, ribozyme technology has implications for the prevention and reversal of drug resistance in humans and other animals by gene therapeutic approaches. Gene-silencing RNAi technology[52] has become available, and this technique has already been applied to reverse the ''classical'' MDR phenotype mediated by the ABC transporter MDR1/P-glycoprotein (ABCB1) using chemically synthesized siRNAs[27] or vector-based systems expressing shRNAs.[28] The experimental system (A2780 *vs.* A2780RCIS) described here will be used to test whether the RNAi approach can be successfully applied also for the functional inhibition of MRP2. A direct comparison between ribozyme and RNAi technology would help to develop a better system for clinical applications.

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