Multidrug-Resistant Tumor Cells Remain Sensitive to a Recombinant Interleukin-4-Pseudomonas Exotoxin, Except When Overexpressing the Multidrug Resistance Protein MRP1

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ABSTRACT

Tumor cells may become resistant to conventional anticancer drugs through the occurrence of transmembrane transporter proteins such as P-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2), or members of the multidrug resistance-associated protein family (MRP1–MRP2, ABCB1–ABCC5). In this report, we studied whether tumor cells that are cytostatic drug resistant because of overexpression of one of the above mentioned proteins are sensitive to a new anticancer agent, interleukin-4 toxin (IL-4 toxin). IL-4 toxin is a fusion protein composed of circularly permuted IL-4 and a truncated form of a bacterial toxin called PE38KDEL. Ninety-six-hour cytotoxicity assays and 10-day clonogenic assays showed that drug-selected multidrug resistant (MDR) tumor cells that overexpress P-glycoprotein or breast cancer resistance protein (ABCG2), or members of the MRP family (MRP1–5; reviewed in Ref. 1), proteins of the MRP family (MRP1–5; ABCC1–ABCC5; reviewed in Ref. 2), and BCRP (ABCG2 (3)). They all are members of the ATP-binding cassette transporter superfamily and have different, but overlapping, drug specificities. Conventional anticancer drugs that are known to be transported by these transporters include the anthracyclines (Pgp, MRP1–2, BCRP), the Vinca alkaloids (Pgp, MRP1–2), epipodophyllotoxins (Pgp, MRP1–3), camptothecins (BCRP), mitoxantrone (Pgp, MRP1–2, BCRP), cisplatin (MRP2), methotrexate (MRP1–4), and the purine analogues 6-mercaptopurine and thioguanine (MRP4–5; Refs. 1–3).

A different class of anticancer therapeutics are the immunotoxins. IL-4 toxin is a fusion protein composed of circularly permuted IL-4 and a truncated form of a bacterial toxin called Pseudomonas exotoxin [IL-4(38–37)-PE38KDEL; Ref. 4]. We have shown before that various kinds of tumor cells express the IL-4R, including renal cell carcinoma, breast carcinoma, ovarian

INTRODUCTION

Cytostatic drug resistance in cancer patients may be attributable to a phenomenon called MDR. MDR is caused by the presence of drug pumps in the plasma membrane of cancer cells that transport structurally and functionally unrelated drugs out of the cell. Proteins best known for their cytostatic drug transport activity are the MDR1 gene-encoded Pgp (ABCB1; reviewed in Ref. 1), proteins of the MRP family (MRP1–5; ABCC1–ABCC5; reviewed in Ref. 2), and BCRP (ABCG2 (3)). These data suggest that MRP1-overexpressing cancer cells are resistant to IL-4 toxin because of extrusion of this agent by MRP1. Still, the results of this study demonstrate that IL-4 toxin effectively kills most MDR tumor cells and, therefore, represents a promising anticancer drug.

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2 The abbreviations used are: MDR, multidrug resistance/multidrug resistant; Pgp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP, multidrug resistance protein; IL-4, interleukin-4; PE, Pseudomonas exotoxin; GSH, glutathione; BSO, dL-buthionine-S,R-sulfoximine; E17BG, 17β-estradiol 17-(β-d-glucuronide); IL-4R, IL-4 receptor; HEK, human embryonic kidney; MPI, mean fluorescence index; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; RF, resistance factor.
carcinoma, melanoma, Kaposi sarcoma, head and neck cancer, glioma, and glioblastoma cells (5–10). These IL-4R-positive tumor cells could effectively and specifically be killed using IL-4 toxin, both in vitro (9–12) and in vivo (8, 13, 14). On the basis of these results, this IL-4 toxin was recently tested in nine patients with recurrent, malignant high-grade gliomas, which are tumors with very poor prognosis. It was shown that direct glioma injection was safe, without systemic cytotoxicity and, in seven patients, resulted in necrosis of tumor, but not brain tissue. One patient was still a complete responder 18 months after drug infusion (15).

Thus, IL-4 toxin appears to be a promising new drug in cancer treatment. It would be especially important if it shows itself to be active in tumor cells intrinsically resistant, or with acquired resistance, to conventional chemotherapy. Therefore, we investigated the activity of IL-4 toxin in tumor cells that are drug resistant because of overexpression of any of the above mentioned drug pumps.

MATERIALS AND METHODS

Chemicals. All chemicals and drugs were obtained from Sigma Chemical Co. (St. Louis, MO) except for doxorubicin, which was purchased from Farmitalia Carlo Erba (Brussel, Belgium), trichloroacetic acid and Pseudomonas aeruginosa

toxin A, which were from ICN Biomedicals Inc. (Aurora, OH), recombinant IL-4 (CLB, Amsterdam, the Netherlands), and MK571 (L-660,711), which was obtained from Dr. Robert Zam- 
boni (Merck-Frosst, Pointe-Claire, Quebec, Canada).

Recombinant IL-4 Toxin. The IL-4 toxin IL-4(38-37)-PE38KDEL, containing the circularly permuted IL-4 mutant in which amino acids 38–129 were linked to amino acids 1–37 via a GGNGG linker and then fused to truncated toxin PE38KDEL, consisting of amino acids 253–364 and 381–608 of PE, fol- 
lowed by KDEL, was expressed in Escherichia coli and purified as described previously (4, 11, 13).

Cell Lines. The SW-1573 non-small cell lung carcinoma, GLC4 small cell lung carcinoma, MCF7 breast carcinoma, and 8226 myeloma cell lines and their MDR sublines have been described previously (16–20). The HT-29 colon carcinoma cell line was obtained from the American Type Culture Collection. The 2008 ovarian carcinoma cell line was obtained from Dr. Howell from the University of California, San Diego, CA.

MRP1-transfected 2008-M1 and 2008-M1-6 cells, MRP2/ 
cMOAT-transfected 2008-cM-1 and 2008-cM-23 cells and MRP3-transfected 2008-M3-4 and 2008-M3-8 cells (21, 22) were kindly provided by M. Kool (Netherlands Cancer Institute, Amsterdam, the Netherlands). 293 HEK- and MRP5-transfected HEK293/ MRPS cells (23) were kindly provided by J. Wijnholds (Netherlands Cancer Institute, Amsterdam, the Netherlands). MRP4-transfected HEK293/MP4 were kindly provided by G. Reid (Netherlands Cancer Institute, Amsterdam, the Netherlands) and are described elsewhere (24). All of the transfected cell lines have been shown previously to overexpress functional drug pumps as reflected in their resistance to cytostatic drugs. In cytotoxicity assays, 2008-MRP1 cells were reported to be clearly resistant to VP-16 (RF, 20) and methotrexate in short-term exposure experiments (RF, 93; Ref. 21). Also, the 2008-MRP2 cells were found to be resistant to methotrexate in short-term assays, although to a lower extent than MRP1 cells (RF, 21.4; Ref. 25). 2008-MRP3 cells are low-level resistant to VP-16 (RF, 3.3 and 1.7 for 2008-M3-8 and 2008-M3-4, respectively), but highly resistant to short-exposure methotrexate (RF, 75 and 33 for 2008-M3-8 and 2008-M3-4, respectively; 21). HEK293/MRP5 cells were shown to be low-level resistant to the drugs 6-mercaptopurine (RF, 3.1) and thioguanine (RF, 2.1; Ref. 23). The same was found for HEK293/MRP4 cells, which are ~3–5-fold more resistant to 6-mercaptopurine and thioguanine than to HEK293 parental cells (24).

The cells were grown in RPMI 1640 or DMEM (both from Bio-Whittaker, Verviers, Belgium) supplemented with 10% FCS (Integro, Zaandam, the Netherlands), 2 mM l-glutamine (Life Technologies, Inc., Paisley, Scotland), 50 IU/ml penicil- 
lin and 50 μg/ml streptomycin. MDR cell lines SW-1573/2R120 (MRP1), SW-1573/2R160 (Pgp), GLC4/ADR (MRP1), MCF7/ D40 (Pgp), MCF7/MR (BCRP), 8226/Dox40 (Pgp), and 8226/ MR20 (BCRP) were cultured in the presence of either doxorubicin or mitoxantrone, until 3–10 days before experiments. Cell lines were routinely tested to ensure the absence of Myco-

plasma.

IL-4R Expression. IL-4R expression was determined using a Fluorokine human IL-4R detection kit (R&D systems, Minneapolis, MN) according to the manufacturer’s recommenda-
dentions. Adherent cells were washed with PBS and were de-
tached from the culture flask bottom by using 5 mM EDTA. The suspension cells were washed thoroughly with PBS to remove any residual growth factors that may have been present in the culture medium. Then 105 cells/sample were incubated with biotinylated IL-4 for 60 min and were stained using FITC- 
conjugated avidin. For negative controls, cells were incubated with an irrelevant protein that was biotinylated to the same degree as IL-4 (soybean trypsin inhibitor, delivered with the IL-4 detection kit). Fluorescence was analyzed on a FACS-star flow cytometer (Becton and Dickinson, San Jose, CA). The MFI was calculated as the ratio of the mean fluorescence of biotinyl-
ated IL-4 treated samples to the mean fluorescence of the negative control samples.

Cytotoxicity Assays. For cytotoxicity experiments, at day 0, exponentially growing cells were briefly trypsinized (<10 min) and plated in triplicate in 96-well plates (number of cells/well: 5,000 SW-1573; 6,000 SW-1573/2R120 and SW-
1573/2R160; 5,000 GLC4; 6,250 GLC4/ADR; 7,500 MCF7; 10,000 MCF7/D40 and MCF7/MR; 10,000 8226 and 8226/DOX40 and 8226/MR20; 5,000 2008; 6,250 2008-M1-4 and 2008-M1-6 and 2008-cM-23 and 2008-M3–4 and 2008-M3–8) and cultured for 24 h. Trypsin treatment fa-
militated the most accurate plating of the cells, whereas such short exposure never resulted in a detectable loss of IL-4R.3 Twenty-four h after plating the cells, toxins (IL-4 toxin, PE) were added in six to eight different concentrations, and, after another 72 h, cell survival was determined using the sulforho-
damine B method for the adherent cell lines (SW-1573, MCF7, and 2008 and sublines) and the XTT method for the semiadher-
ent GLC4 and GLC4/ADR and suspension 8226, 8226/DOX40,

and 8226/MR20 cells. GLC4 and GLC4/ADR cells are more efficient metabolizing XTT than the 8226, 8226/DOX40, and 8226/MR20 cells. Therefore, the optimal XTT concentrations were 25 μg of XTT mixed with 1.9 μg of phenazine methosulfate for the GLC4 and GLC4/ADR cells and 50 μg of XTT and 1.9 μg of phenazine methosulfate for the 8226, 8226/DOX40, and 8226/MR20 cells/well for 3–4 h. The RF was calculated as the ratio of the IC_{so} (concentration of the compound that inhibits growth of the cells by 50%) of the resistant cells to the IC_{so} of the parental cells. In some experiments, also MRP1 modulators (MK571 or probenecid) were added to the cultures. Appropriate controls were done to check for the cytotoxicity of DMSO that was used to dissolve the probenecid. The total amount of DMSO in the cell cultures never exceeded 0.5%. For MRP1 modulation through GSH depletion, cells were preincubated with BSO 24 h before the addition of the cytotoxic compound to be tested.

Clonogenic Assay. At day 0, SW-1573, SW-1573/2R120, and SW-1573/2R160 were plated in duplicate in 6-well plates to obtain ~200 colonies/well in the control (no drug) wells. Twenty-four h later, cells were exposed to different concentrations of IL-4–PE and were left to grow for another 9 days. Then cells were washed, fixed, and stained with crystal violet (0.1% crystal violet in 20% ethanol). Colonies of >30 cells were counted.

Inside-Out Plasma Membrane Vesicle [3H]E2-17βG Uptake Assay. Inside-out vesicles were prepared from plasma membranes of GLC4/ADR cells as described previously (26) with slight modifications. Cells were collected by centrifugation (275 × g, 5 min) and washed twice in ice-cold PBS (pH 7.4). The cell suspension (10^7 cells/ml) was incubated in a buffer containing 100 mM KCl, 5 mM MgCl_2, 1 mM phenylmethylsulfonyl fluoride, and 50 mM HEPES/KOH (pH 7.4) for 60 min at 0°C. Hereafter, cells were disrupted by sonication at 20% of the maximum power of an M.SE sonicator (Soniprep 150) for 3 bursts of 15 s each. The suspension was centrifuged at 1500 × g for 10 min. The supernatant was layered on top of a 46% protein, in the presence of 10 mM MgCl_2, 2 mM ATP, and 10 nM [3H]E2-17βG (specific activity, 40.5 Ci/mmol; Perkin-Elmer Life Science, Boston, MA). The reaction was started by adding the [3H]E2-17βG to a final concentration of 4 μM/ml. A rapid filtration method (27) was used to measure the transport of [3H]E2-17βG into isolated inside-out plasma membrane vesicles. Vesicles were incubated in a buffer containing 100 mM KCl/50 mM HEPES/KOH (pH 7.4) at 37°C (0.15 mg/ml protein), in the presence of 10 mM MgCl_2, 2 mM ATP, and 10 nM [3H]E2-17βG. The final reaction volume was 50 μl. The transport was stopped after 2 min by the addition of 2 ml of ice-cold KC1/HEPES buffer. Thereafter, the mixture was rapidly filtered through OE67 membrane filters (Schleicher & Schuell, Dassel, Germany). The filters were washed twice with 2 ml of KC1/HEPES buffer. The radioactivity associated with the filters was measured by liquid scintillation counting.

Statistical Analysis. Statistical analyses of the data were performed using the unpaired two-tailed Student’s t test. Differences were considered statistically significant when P < 0.05.

RESULTS

Drug-Selected MDR Tumor Cells Express IL-4R. The expression of IL-4R on MDR tumor cells was determined using biotinylated IL-4 in a flow cytometry assay. IL-4R expression was calculated as the ratio of mean fluorescence of cell bound, stained, IL-4 compared with the mean fluorescence of an irrelevant control protein, soybean trypsin inhibitor (MFI). For a colon carcinoma cell line, HT-29, previously reported to express ~6000 IL-4Rs/cell (28), which is similar to IL-4R numbers of other, IL-4 toxin sensitive, tumor cell lines (5, 7), we found positive staining with a MFI of 14.9 ± 7.6.

In general, the tested tumor cell lines, which were from different histogenetic origins, including lung carcinoma, breast carcinoma, and multiple myeloma, and their drug-selected MDR sublines, bound biotinylated IL-4 to a level similar to that of the HT-29 colon tumor cell line (Table 1). In some cases, however (for instance, the SW-1573/2R120 lung carcinoma cells), drug selection had resulted in more binding of IL-4 to the cells compared with the parental cells, suggesting up-regulation of the IL-4R (Table 1). On the basis of these data, and because IL-4 toxin sensitivity of cells is dependent on their IL-4R expression, MDR tumor cells would be expected to be as sensitive, or even more sensitive, to IL-4 toxin than the non-MDR, parental cells from which they were derived.

Drug-Selected MDR Tumor Cells Are Sensitive to IL-4 Toxin Except When Overexpressing MRP1. In 72-h cytotoxicity assays, tumor cells that are resistant to the established anticancer drugs doxorubicin or mitoxantrone because of overexpression of Pgp (SW-1573/2R160, MCF7/D40) or BCRP (MCF7/MR and 8226/MR20) were found to be almost as sensitive as the non-MDR tumor cells with RFs <2 (Table 1). Notably, the doxorubicin-selected and highly Pgp-overexpressing 8226/D40 myeloma cells were found to be five times more sensitive to the toxin than were the parental 8226 cells (P < 0.01; Table 1). In contrast, the doxorubicin-selected MRP1-overexpressing cells SW-1573/2R120 were significantly resistant to IL-4 toxin with a RF of 8.7 (P < 0.01). The MRP1-overexpressing GLC4/ADR cells also were resistant with RF 4.7, but this difference did not reach statistical significance (P = 0.09; Table 1). In a 10-day clonogenic assay using SW-1573 cells and the MRP1- and Pgp-overexpressing sublines, the colony formation of the Pgp-overexpressing SW-1573/2R160 cells was inhibited as well as the colony formation of the parental cells (Fig. 1). The MRP1 overexpressing SW-1573/2R120, again, showed resistance because tumor cell colonies were formed at higher doses of IL-4 toxin.

MRP1-Transfected Cells Are Resistant to IL-4 Toxin. Because these results with drug-selected cell lines suggested a possible role for MRP family members in IL-4 toxin resistance, we selected a panel of MRP transfecants and performed cytotoxicity assays. Two independent clones of MRP1-transfected cells, 2008-M1–4 and 2008-M1–6, showed clear resistance to IL-4 toxin with RFs of 5.9 and 4.3 (P < 0.01; Table 2). In contrast, the cells that were transfected with the full-length cDNA of other members of the MRP protein family, MRP2–5, were not (MRP3–5), or only slightly (MRP2), resistant to IL-4 toxin (Table 2). Similarly as seen for the drug-selected MRP1-
overexpressing cells, the resistance in the MRP1-transfected cell lines was not related to a decreased IL-4R expression (Table 2).

**IL-4 Toxin Resistance Can Be Reversed by MRP1 Antagonists MK571 and Prob恒ed, but not by BSO.** To test whether the mechanism leading to resistance to the IL-4 toxin fusion protein behaves similarly to the more classical MRP1 substrates, we tested two established strategies for MRP1-resistance reversal, by pump modulators and by GSH depletion. Prob恒ed and MK571 are known antagonists of MRP1. They were previously shown to reverse the resistance to cytostatic drugs in MRP1-, but not in Pgp-, overexpressing cells in a concentration-dependent way (29, 30). BSO inhibits the enzyme γ-glutamylcysteine synthetase, which catalyzes the first step in GSH synthesis. GSH depletion by BSO results in the reversal of MRP1-mediated resistance to drugs like doxorubicin and vincristine, indicating that GSH is needed for the efflux of these drugs from the cell (31).

In our experiments, the addition of prob恒ed or MK571, in concentrations routinely used to antagonize MRP1 (1 μM and 30 μM, respectively; Refs. 29, 30), reversed IL-4 toxin resistance in MRP1-transfected 2008-M1–4 cells (Fig. 2). This is in line with the involvement of MRP1 in IL-4 toxin resistance. The smaller, but consistent, effect of prob恒ed and MK571, seen in 2008 parental cells, has been attributed to the basal expression level of MRP1 in 2008 cells (22).

Because 2008, 2008-M1–4, and GLC4 cells showed high intrinsic sensitivity to BSO treatment (results not shown) GLC4/ADR cells were used to test the effect of GSH depletion on MRP1-mediated IL-4 toxin resistance. GLC4/ADR cells were incubated with 25 μM BSO for 24 h before the addition of IL-4 toxin. Previously, we reported that this procedure results in reduced GSH levels to 18% of control cultures of this cell line (31). This GSH depletion by BSO, however, did not affect the IL-4 toxin resistance of GLC4/ADR cells, whereas it did affect the doxorubicin resistance as expected (Fig. 3). This indicates that a physiological level of GSH is not required for MRP1-mediated IL-4 toxin resistance.

**MRP1-Expressing Cells Are Not Resistant to PE.** To define the structural requirements for resistance to IL-4 toxin in MRP1-overexpressing cells, cytotoxicity assays were performed with IL-4 and PE. IL-4 did not inhibit the growth of 2008 ovarian, and SW-1573 and GLC4 lung carcinoma cells up until a 100-ng/ml dose (results not shown), even though IL-4 at high doses (10–100 ng/ml) may be cytostatic to some tumor cell lines (5, 32). PE was highly toxic and, in the case of SW-1573 cells, was found to be even more toxic than the IL-4 toxin (IC_{50} of 50.3 ng/ml versus 82.4 ng/ml; Tables 1 and 3). This is probably because of high expression of the α_{2} macroglobulin receptor, which binds PE and internalizes PE into cells. This receptor is widely expressed in different normal human tissues, and replacement of the α_{2} macroglobulin receptor-binding fragment of PE by other ligands, e.g., IL-4, facilitates more specific targeting of the toxin to tumor cells only. Cells with overexpression of MRP1, like SW-1573/2R120 and 2008-M1–4 and 2008-M1–6, were slightly resistant [SW-1573, RF 1.9 (P < 0.01) and 2008-M1–4, RF 1.4; Table 3] or even more sensitive to PE [2008-M1–6, RF 0.6 (P < 0.05); Table 3]. This indicates that the main difference between PE and IL-4 toxin, the above mentioned replacement of the α_{2} macroglobulin receptor binding domain of PE (amino acids 1 to 252, of a total of 613 amino acids) by IL-4, is a structural requirement for resistance to the cytotoxic activity of IL-4 toxin mediated by MRP1.

**Inhibition by IL-4 Toxin and IL-4, but not by PE, of MRP1-Mediated [\(^{3}\)H]E_{2}β\(^{17}\)G Transport.** To further delineate the mechanism of MRP1-mediated IL-4 toxin resistance, we used an inside-out vesicle system, developed earlier for studying transmembrane transporter functions (26, 27, 33). We measured the inhibition of transport of the established MRP1 substrate E_{2}β\(^{17}\)G (34, 35) by IL-4 toxin. Vesicles prepared from MRP1-overexpressing GLC4/ADR cells were used for these experiments. At a concentration of 10 μM, the uptake of [\(^{3}\)H]E_{2}β\(^{17}\)G was 2.1 ± 0.2 pmol/mg protein/min. In the presence of different concentrations of IL-4 toxin the uptake of [\(^{3}\)H]E_{2}β\(^{17}\)G was inhibited (Fig. 4). Importantly, also IL-4 in-

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**Table 1** IL-4R expression and IL-4 toxin sensitivity of drug-sensitive and drug-selected MDR tumor cell lines

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>MFI IL-4R</th>
<th>ng/ml</th>
<th>nM</th>
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<tr>
<td><strong>Non-small cell lung carcinoma</strong></td>
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<td>SW-1573 Parental</td>
<td>13.4 ± 9.8 (n = 2)</td>
<td>82.4 ± 48.7</td>
<td>1.6 ± 1.0 (n = 3)</td>
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<td>SW-1573/2R120 dox selected, MRP1</td>
<td>21.9 ± 18.0 (n = 2)</td>
<td>718.3 ± 83.0</td>
<td>14.0 ± 1.6 (8.7) (n = 3)</td>
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<tr>
<td>SW-1573/2R160 dox selected, Pgp</td>
<td>11.9 ± 5.1 (n = 2)</td>
<td>120.0 ± 21.2</td>
<td>2.3 ± 0.4 (1.5) (n = 2)</td>
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<tr>
<td><strong>Small cell lung carcinoma</strong></td>
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<tr>
<td>GLC4 Parental</td>
<td>10.0 ± 7.7 (n = 3)</td>
<td>273.3 ± 99.4</td>
<td>5.3 ± 1.9 (n = 3)</td>
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<tr>
<td>GLC4ADR dox selected, MRP1</td>
<td>18.2 ± 8.1 (n = 3)</td>
<td>1033.0 ± 628.2</td>
<td>20.0 ± 12.2 (4.7) (n = 3)</td>
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<tr>
<td><strong>Breast carcinoma</strong></td>
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<td>MCF7 Parental</td>
<td>7.4 ± 0.3 (n = 2)</td>
<td>1.4 ± 1.0</td>
<td>0.027 ± 0.019 (n = 6)</td>
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<tr>
<td>MCF7/DO4 dox selected, Pgp</td>
<td>9.4 ± 1.6 (n = 2)</td>
<td>2.0 ± 1.2</td>
<td>0.039 ± 0.023 (1.4) (n = 4)</td>
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<tr>
<td>MCF7/MR Mitox selected, BCRP</td>
<td>7.1 ± 0.9 (n = 2)</td>
<td>2.6 ± 2.3</td>
<td>0.050 ± 0.044 (1.9) (n = 4)</td>
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<td><strong>Multiple myeloma</strong></td>
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<tr>
<td>8226 Parental</td>
<td>16.5 ± 10.0 (n = 3)</td>
<td>603.0 ± 188.8</td>
<td>11.7 ± 3.7 (n = 5)</td>
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<td>8226/D40 dox selected, Pgp</td>
<td>23.9 ± 15.6 (n = 3)</td>
<td>99.4 ± 56.8</td>
<td>1.9 ± 1.1 (0.2) (n = 3)</td>
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<tr>
<td>8226/MR20 Mitox selected, BCRP</td>
<td>17.8 ± 13.1 (n = 2)</td>
<td>603.2 ± 137.9</td>
<td>11.7 ± 2.7 (1.0) (n = 3)</td>
</tr>
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</table>

* a dox, doxorubicin; mitox, mitoxantrone.

* b Statistically significantly (P < 0.01) different from parent cell line in unpaired two-tailed Student’s t test.
IL-4 toxin and IL-4 inhibited MRP1-mediated E₂₁7G transport up to 75 and 55%, respectively, of the control. For comparison, in our hands, the known MRP1 substrate daunorubicin (500 nM) inhibited this transport to 51% of the control (result not shown).

In the presence of the unmodified PE toxin, no inhibition of MRP1-mediated transport could be observed, in line with the results showing that MRP1-overexpressing cells are resistant to IL-4 toxin, but not to PE.

**DISCUSSION**

The success rate of chemotherapy in cancer treatment is limited. Tumors can be intrinsically resistant or acquire resistance during treatment with cytostatic drugs. In these cases, other therapies like immunotherapy may be useful. Most immunotoxins, composed of either a monoclonal antibody or another tumor cell-binding ligand, and a toxin, like PE, are proteins of rather large size and do not resemble the classical chemical structures of substrates of drug pumps like Pgp and the MRP proteins. Therefore, they may be good alternatives or additives to conventional chemotherapy.

A number of studies reported on the effects of immunotoxins on drug-resistant, primarily Pgp-overexpressing, cells with some conflicting results. Some reported a beneficial effect of an immunotoxin alone or in combination with conventional drugs in cell kill of MDR tumor cells (36–38). Others argue for using immunotoxins in combination with primary conventional chemotherapy before drug resistance develops, because an additive effect of a saporin toxin-conjugated anti-CD138 immunotoxin on chemotherapy could be measured only in drug-sensitive, non-Pgp-overexpressing, cells (39). Notably, two independent studies reported on immunotoxins that specifically target Pgp-overexpressing cells (40, 41). Both compounds consist of an antibody (fragment) recognizing Pgp and (truncated) PE. In both reports, it was concluded that, independent of the question as to whether treating patients with such an immunotoxin will be feasible because of potential toxicity to normal tissues expressing Pgp, the sensitivity of MDR cells to the toxin was sufficient.

IL-4 toxin is an immunotoxin that takes advantage of the finding that, for an as-yet-unknown reason, many tumor cell types overexpress IL-4R (5–10). IL-4R is also expressed in several types of normal cells (e.g., immune cells and fibroblast and endothelial cells) but in lower amounts, and it, therefore, is an attractive feature to use for targeting an anticancer drug to tumor cells. After several years of preclinical testing, IL-4 toxin has reached clinical testing with promising first results in the treatment of high-grade glioma, a particularly deadly form of cancer (15).

In this report, we show the results of an *in vitro* study on the potential use of the IL-4 toxin in drug-resistant tumors. We show that the majority of MDR cell lines, that is, the Pgp-, MRP2–, and BCRP-overexpressing tumor cell lines, remain sensitive or almost just as sensitive to the toxin, as do the parental cells. Slight resistance cannot be completely ruled out in MRP2–5-overexpressing cells because the overexpression levels of functional protein have not been directly compared in these cells, but only MRP1-overexpressing tumor cells, both drug-selected and *MRP1*-transfected, show robust resistance to IL-4 toxin. Results of additional experiments with the MRP1 antagonists probenecid and MK-571 confirmed a role of MRP1-mediated transport in IL-4 toxin resistance.

An intriguing question is how MRP1 can confer IL-4 toxin resistance. The cytotoxicity experiments showing that MRP1-overexpressing cells are not resistant to unmodified PE indicate that MRP1 does not confer general resistance to PE-based toxins. For PE to become toxic, several processes are required, including internalization of the toxin into the endosome com-
partment, followed by cleavage of the toxin into two fragments and transport of the enzymatically active part to the endoplasmic reticulum, translocation of this part from the endoplasmic reticulum into the cytoplasm, and the inhibition of protein synthesis through ADP-ribosylating elongation factor 2 (42). These processes are likely to be the same for the natural PE and IL-4 toxin, and, therefore, MRP1 probably does not affect these processes.

Instead, it would be more likely that the resistance to IL-4 toxin is related to the transport function of MRP1. The inhibition of E_{2}^{17}G uptake into inside-out plasma membrane vesicles prepared from MRP1-overexpressing cells by IL-4 toxin supports this view. Previously, we reported that MRP1 can transport short (5-mer) peptides (43). Little is known, however, about the transport of such large-size proteins as this toxin, which has 484 amino acids and a $M_{r}$ of 52,000. Two reports have suggested that MRP1 may play a role in the secretion of basic fibroblast growth factor (at $M_{r}$ 16,000), which lacks a signal sequence (44, 45). Independent and definitive proof for that finding, however, is lacking.

Table 2  IL-4R expression and IL-4 toxin sensitivity of MRP-transfected cell lines

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>MFI IL-4R</th>
<th>$IC_{50}$ IL-4 toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml</td>
<td>nm</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2008 Parental</td>
<td>5.7 ± 2.3 (n = 3)</td>
<td>2.9 ± 0.6 0.056 ± 0.012 (n = 4)</td>
</tr>
<tr>
<td>2008-M1-4 MRP1</td>
<td>9.0 ± 6.0 (n = 2)</td>
<td>17.0 ± 2.1 0.329 ± 0.041 (5.9) (n = 3)***</td>
</tr>
<tr>
<td>2008-M1-6 MRP1</td>
<td>7.0 ± 6.3 (n = 2)</td>
<td>12.6 ± 4.4 0.244 ± 0.085 (4.3) (n = 2)***</td>
</tr>
<tr>
<td>2008-cM-1 MRP2/εMOAT</td>
<td>4.3 ± 1.0 (n = 2)</td>
<td>3.5 ± 0.3 0.068 ± 0.006 (1.2) (n = 2)</td>
</tr>
<tr>
<td>2008-cM-23 MRP2/εMOAT</td>
<td>6.2 ± 4.4 (n = 2)</td>
<td>6.0 ± 2.2 0.116 ± 0.043 (2.1) (n = 2)***</td>
</tr>
<tr>
<td>2008-M3-4 MRP3</td>
<td>4.3 ± 1.0 (n = 2)</td>
<td>2.5 ± 0.1 0.049 ± 0.002 (0.9) (n = 2)</td>
</tr>
<tr>
<td>2008-M3-8 MRP3</td>
<td>4.2 ± 0.6 (n = 2)</td>
<td>1.8 ± 0.4 0.034 ± 0.008 (0.6) (n = 2)</td>
</tr>
<tr>
<td>HEK Parental</td>
<td>ND°</td>
<td>7.6 ± 6.8 0.147 ± 0.132 (n = 2)</td>
</tr>
<tr>
<td>HEK293/MPR4</td>
<td>ND°</td>
<td>7.4 ± 6.9 0.144 ± 0.134 (1.0) (n = 2)</td>
</tr>
<tr>
<td>HEK293/MPR5</td>
<td>ND°</td>
<td>6.1 ± 7.9 0.118 ± 0.153 (0.8) (n = 2)</td>
</tr>
</tbody>
</table>

° Statistically significantly (*, P < 0.05; **, P < 0.01) different from parent cell line in unpaired two-tailed Student’s $t$ test.

Fig. 2  Reversal of IL-4 toxin resistance by MRP1 antagonists probenecid and MK-571. 2008- and MRP1-transfected 2008-M1-4 cells were cultured in the presence or absence of probenecid (1 mM) or MK-571 (30 μM), and survival was determined after 72 h by using the sulforhodamine B assay. Results shown are means ± SDs of triplicate measurements in a typical experiment. Where not visible, SD error bars are hidden within the symbol.

Fig. 3  BSO does not reverse resistance to IL-4 toxin in MRP1-overexpressing cells. GLC4/ADR cells were cultured in the presence or absence of BSO (25 μM). IL-4 toxin or doxorubicin was added after 24 h. Survival was determined after an additional 72 h by using the XTT assay. Results shown are means ± SDs of triplicate measurements in a typical experiment.
ADR cells were incubated with the known MRP1 substrate [3H]E217G by PE. Plasma membrane vesicles of MRP1-overexpressing GLC4/cytotoxicity experiments and in the E217G uptake was measured as the radioactivity associated with the vesicles.

Results shown are the means ± SDs of three experiments. Where not visible, SD error bars are hidden within the symbol. * Statistically significant (P < 0.05), ** Statistically significantly different from parent cell line in unpaired two-tailed Student’s t test.

![Graph](image)

**Fig. 4** Uptake of E217G is inhibited by IL-4 toxin and IL-4, but not by PE. Plasma membrane vesicles of MRP1-overexpressing GLC4/ADR cells were incubated with the known MRP1 substrate [3H]E217G in the presence or absence of different concentrations of IL-4 toxin, IL-4, or PE for 2 min. The amount of [3H]E217G transported into the vesicles was measured as the radioactivity associated with the vesicles. Results shown are the means ± SDs of three experiments. Where not visible, SD error bars are hidden within the symbol. * Statistically significant (P < 0.05) compared with controls in unpaired two-tailed Student’s t test.

Our results obtained with IL-4 toxin and PE, both in the cytotoxicity experiments and in the E217G transport experiments, indicate that the IL-4 part of the toxin, which is the major alteration in the toxin compared with natural PE, determines transport of this toxin by MRP1. We could not determine whether MRP1-overexpressing cells are also resistant to IL-4 because IL-4 was not toxic to our tumor cell lines, but MRP1-mediated E217G uptake was clearly inhibited by IL-4. For Pgp, it has previously been suggested that it is involved in the secretion of IL-4 by activated lymphocytes (46), but this was disputed in a more recent study showing that activated lymphocytes from Pgp knock-out mice secreted IL-4 equally as well as did lymphocytes from wild-type mice (47). Using human polyclonally activated T-cells, we tested whether MRP1 might be involved in the secretion of IL-4 from these cells, but we did not find clear MRP1 staining in these cells, nor did we find any influence of MK571 or probenecid on IL-4 secretion (data not shown). Thus, MRP1 is probably not required for IL-4 secretion, and IL-4 is, for the most part, secreted through the classical exocytosis pathway as facilitated by its signal sequence. This does not, of course, exclude the possibility that MRP1 is able to transport IL-4 toxin, as indicated by the present results.

natively, IL-4 toxin may interact with MRP1 in a noncatalytic fashion, *i.e.*, through binding rather than actual transport, still resulting in interference with substrate transport and IL-4 toxin resistance. In any case, this transport apparently does not require physiological levels of GSH as seen for some, but not all, other substrates (29, 43).

From a clinical point of view, our findings show that increased expression of MDR proteins, except for MRP1, in tumor cells does not, or only marginally, reduce the sensitivity to IL-4 toxin. Still, because MRP1 resistance may be reversed using MRP1 antagonists, IL-4 toxin may be an effective drug for cancer patients presenting with MDR tumors.

**REFERENCES**


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Multidrug-Resistant Tumor Cells Remain Sensitive to a Recombinant Interleukin-4- *Pseudomonas* Exotoxin, Except When Overexpressing the Multidrug Resistance Protein MRP1

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