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


Departments of Pathology [M. C. d. J., G. L. S., R. J. S.], Medical Oncology [H. J. B.], and Pediatric Hematology/Oncology [J. H. H.], VU University Medical Center, 1081 HV Amsterdam, the Netherlands; Department of Molecular Recognition, Institute for Animal Science and Health (ID-DLO), 8200 AB Lelystad, the Netherlands [J. W. S., R. H. M.]; Laboratory of Molecular Biology, Division of Basic Sciences, National Cancer Institute, NIH, Bethesda, Maryland 20892 [R. J. K.]; and Laboratory of Molecular Tumor Biology, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892 [S. R. H., B. H. J., R. K. P.]

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–MRP5; ABCCI–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 *Pseudomonas* exotoxin (PE) [IL-4(38–37)-PE38KDEL]. Ninety-six-h cytotoxicity assays and 10-day clonogenic assays showed that drug-selected multidrug resistant (MDR) tumor cells that overexpress P-glycoprotein or breast cancer resistance proteins are still sensitive to IL-4 toxin. Also, tumor cells transfected with cDNA for MRP2–5 showed no resistance, or marginal resistance, only to the toxin as compared with the parent cells. In contrast, MRP1-overexpressing cells, both drug selected and *MRP1* transfected, are clearly resistant to IL-4 toxin with resistance factors of 4.3 to 8.4. MRP1-overexpressing cells were not resistant to PE itself. IL-4 toxin resistance in MRP1-overexpressing cells could be reversed by the MRP1 inhibitors probenecid or MK571 and were not affected by glutathione depletion by DL-buthionine-S,R-sulfoximine. In a transport assay using plasma membrane vesicles prepared from MRP1-overexpressing cells, IL-4 toxin and IL-4, but not PE, inhibited the translocation of the known MRP1 substrate 17β-estradiol 17-(β-n-glucuronide) (E217βG). These data suggest that MRP1-overexpressing 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.

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; ABCCI–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

Received 10/18/02; revised 5/12/03; accepted 5/13/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Supported by Grant KWF-VU96-1256 of the Dutch Cancer Society.

1 To whom requests for reprints should be addressed, at Department of Pathology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam. Phone: 31-20-444-4031; Fax: 31-20-444-2964; E-mail: rj.scheper@VUmc.nl.

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; E217βG, 17β-estradiol 17-(β-n-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 Farmatilia Carlo Erba (Brussels, 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 Zamboni (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 amino acids 38–37 via a GGNGG linker and then fused to truncated toxin PE38KDEL, consisting of amino acids 253–364 and 381–608 of PE, was from bovine (Merck-Frosst, Pointe-Claire, Quebec, Canada).

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. Reed (Netherlands Cancer Institute, Amsterdam, the Netherlands). The 2008 ovarian carcinoma cell line was obtained from the American Type Culture Collection. The 2008 ovarian carcinoma cell line was obtained from Dr. Reed (Netherlands Cancer Institute, Amsterdam, the Netherlands).

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 and 2008-M1-6 and 2008-M3-4 and 2008-M3–8) and cultured for 24 h. Trypsin treatment facilitated the most accurate plating of the cells, whereas such short exposure never resulted in a detectable loss of IL-4R. 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 sulforhodamine B method for the adherent cell lines (SW-1573, MCF7, and 2008 and sublines) and the XTT method for the semiaherent 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_{50} (concentration of the compound that inhibits growth of the cells by 50%) of the resistant cells to the IC_{50} 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]E17βG 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 MgCl₂, 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% sucrose cushion (KC1/HEPES buffer) and was centrifuged at 100,000 × g for 60 min. The interface was removed and washed in the buffer described above. The final membrane preparations were stored at -80°C at a protein concentration of 4 mg/ml.

A rapid filtration method (27) was used to measure the transport of [3H]E17βG 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 MgCl₂, 2 mM ATP, and 10 nM [3H]E17βG (specific activity, 40.5 Ci/mmol; Perkin-Elmer Life Science, Boston, MA). 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 filtrated through O/E67 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 to IL-4 toxin as the non-MDR tumor cells with RFs <2 (Table 1; Fig. 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; Fig. 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 Probenecid, 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. Probenecid 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 probenecid or MK571, in concentrations routinely used to antagonize MRP1 (1 mM and 30 μM, respectively; Refs. 29, 30), reversed IL-4 toxin resistance in MRP1-transfected 2008-M1− cells (Fig. 2). This is in line with the involvement of MRP1 in IL-4 toxin resistance. The smaller, but consistent, effect of probenecid and MK571, seen in 2008 parental cells, has been attributed to the basal expression of different concentrations of IL-4 toxin the uptake of [3H]E217βG. Importantly, also IL-4 in-

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 (IC50 of 50.3 ng/ml versus 82.4 ng/ml; Tables 1 and 3). This is probably because of high expression of the α2 macroglobulin receptor, which binds PE and internalizes PE into cells. This receptor is widely expressed in different normal human tissues, and replacement of the α2 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−, were slightly resistant [SW-1573, RF 1.9 (P < 0.01) and 2008-M1−, RF 1.4; Table 3] or even more sensitive to PE [2008-M1−, 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 α2 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 [3H]E217βG 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 E217βG (34, 35) by IL-4 toxin. Vesicles prepared from MRP1-overexpressing GLC4/ADR cells were used for these experiments. At a concentration of 10 nM, the uptake of [3H]E217βG was 2.1 ± 0.2 pmol/mg protein/min. In the presence of different concentrations of IL-4 toxin the uptake of [3H]E217βG was inhibited (Fig. 4). Importantly, also IL-4 in-

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>
</tr>
</thead>
<tbody>
<tr>
<td>Non-small cell lung carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW-1573</td>
<td>Parental</td>
<td>13.4 ± 9.8 (n = 2)</td>
<td>82.4 ± 48.7</td>
</tr>
<tr>
<td>SW-1573/2R120</td>
<td>dox− selected, MRP1</td>
<td>21.9 ± 18.0 (n = 2)</td>
<td>718.3 ± 83.0</td>
</tr>
<tr>
<td>SW-1573/2R160</td>
<td>dox selected, Pgp</td>
<td>11.9 ± 5.1 (n = 2)</td>
<td>120.0 ± 21.2</td>
</tr>
<tr>
<td>Small cell lung carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLC4</td>
<td>Parental</td>
<td>10.0 ± 7.7 (n = 3)</td>
<td>273.3 ± 99.4</td>
</tr>
<tr>
<td>GLC4/ADR</td>
<td>dox selected, MRP1</td>
<td>18.2 ± 8.1 (n = 3)</td>
<td>1033.0 ± 628.2</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF7</td>
<td>Parental</td>
<td>7.4 ± 0.3 (n = 2)</td>
<td>1.4 ± 1.0</td>
</tr>
<tr>
<td>MCF7/D40</td>
<td>dox selected, Pgp</td>
<td>9.4 ± 1.6 (n = 2)</td>
<td>2.0 ± 1.2</td>
</tr>
<tr>
<td>MCF7/DR</td>
<td>Mitox selected, BCRP</td>
<td>7.1 ± 0.9 (n = 2)</td>
<td>2.6 ± 2.3</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8226</td>
<td>Parental</td>
<td>16.5 ± 10.0 (n = 3)</td>
<td>603.0 ± 188.8</td>
</tr>
<tr>
<td>8226/D40</td>
<td>dox selected, Pgp</td>
<td>23.9 ± 15.6 (n = 3)</td>
<td>99.4 ± 56.8</td>
</tr>
<tr>
<td>8226/MR20</td>
<td>Mitox selected, BCRP</td>
<td>17.8 ± 13.1 (n = 2)</td>
<td>603.2 ± 137.9</td>
</tr>
</tbody>
</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 E217βG 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–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$_2^{17\beta}$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

Results are shown as means ± SD (in ng/ml and nM) of two to four experiments. Respective RFs and number of measurements ($n$) are shown within parentheses.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>IL-4R</th>
<th>$IC_{50}$ IL-4 toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFI</td>
<td>ng/ml, nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>Parental</td>
<td>5.7 ± 2.3 ($n$ = 3)</td>
</tr>
<tr>
<td>2008-M1-4</td>
<td>MRP1</td>
<td>9.0 ± 6.0 ($n$ = 2)</td>
</tr>
<tr>
<td>2008-M1-6</td>
<td>MRP1</td>
<td>7.0 ± 6.3 ($n$ = 2)</td>
</tr>
<tr>
<td>2008-cM-1</td>
<td>MRP2/COAT</td>
<td>4.5 ± 1.0 ($n$ = 2)</td>
</tr>
<tr>
<td>2008-cM-23</td>
<td>MRP2/COAT</td>
<td>6.2 ± 4.4 ($n$ = 2)</td>
</tr>
<tr>
<td>2008-M3-4</td>
<td>MRP3</td>
<td>4.3 ± 1.0 ($n$ = 2)</td>
</tr>
<tr>
<td>2008-M3-8</td>
<td>MRP3</td>
<td>4.2 ± 0.6 ($n$ = 2)</td>
</tr>
<tr>
<td>HEK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293</td>
<td>Parental</td>
<td>ND*</td>
</tr>
<tr>
<td>HEK293/MRP4</td>
<td>MRP4</td>
<td>ND</td>
</tr>
<tr>
<td>HEK293/MRP5</td>
<td>MRP5</td>
<td>ND</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.
- ND, not determined.

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 mM), 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 mM). 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.

- E$_2^{17\beta}$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 3  *Pseudomonas* (P.) exotoxin A cytotoxicity to MRP1-overexpressing cells

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>IC₅₀ P. exotoxin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW-1573 Parental</td>
<td>50.3 ± 2.1 ng/ml</td>
</tr>
<tr>
<td>SW-1573/2R120 MRP1, dox* selected</td>
<td>95.3 ± 4.7 ng/ml</td>
</tr>
<tr>
<td>2008 MPR1 transfected</td>
<td>219.0 ± 9.9 ng/ml</td>
</tr>
<tr>
<td>2008-M1-4 MPR1 transfected</td>
<td>313.0 ± 103.2 ng/ml</td>
</tr>
<tr>
<td>2008-M1-6 MPR1 transfected</td>
<td>142.5 ± 7.8 ng/ml</td>
</tr>
</tbody>
</table>

*a* dox, doxorubicin.  
*b* *p* < 0.05, **p** < 0.01 statistically different from parent cell line 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 E₂<sub>17</sub>G 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 toxic to our tumor cell lines, but MRP1-mediated E₂<sub>17</sub>G 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 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. Alternatively, 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.

![Fig. 4](image-url)  
*Fig. 4* Uptake of E₂<sub>17</sub>G is inhibited by IL-4 toxin and IL-4, but not by PE. Plasma membrane vesicles of MRP1-overexpressing GLC4/A DR cells were incubated with the known MRP1 substrate [³H]E₂<sub>17</sub>G in the presence or absence of different concentrations of IL-4 toxin, IL-4, or PE for 2 min. The amount of [³H]E₂<sub>17</sub>G transported into the vesicles was measured as the radioactivity associated with the vesicles. Results shown are the means ± SDs of three experiments. Where present, SD error bars are hidden within the symbol. *s*, statistically significant (*P* < 0.05) compared with controls in unpaired two-tailed Student’s *t* test.

**REFERENCES**


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

Mariska C. de Jong, George L. Scheffer, Henk J. Broxterman, et al.


Updated version  Access the most recent version of this article at:  
http://clincancerres.aacrjournals.org/content/9/13/5009

Cited articles  This article cites 44 articles, 26 of which you can access for free at:  
http://clincancerres.aacrjournals.org/content/9/13/5009.full.html#ref-list-1

Citing articles  This article has been cited by 2 HighWire-hosted articles. Access the articles at:  
/content/9/13/5009.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.