**Peptide Transport by the Multidrug Resistance Protein MRPI**

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**ABSTRACT**

Small hydrophobic peptides were studied as possible substrates of the multidrug resistance protein (MRP)-1 (ABCC1) transmembrane transporter molecule. As observed earlier for P-glycoprotein- (Pgp; ABCB1) overexpressing cells, MRP1-overexpressing cells, including cells stably transfected with the MRPI cDNA, showed distinct resistance to the cytotoxic peptide N-acetyl-Leu-Leu-norleucinal (ALLN). Resistance to this peptide and another toxic peptide derivative, which is based on a Thr-His-Thr-Nle-Gly backbone conjugated to butyl and benzyl groups (4A6), could be reversed by MRPI inhibitors. The reduced toxicity of 4A6 in MRP1-overexpressing cells was found to be associated with lower accumulation of a fluorescein-labeled derivative of this peptide. Glutathione (GSH) depletion had a clear effect on resistance to ALLN but hardly affected 4A6 resistance. In a limited structure-activity study using peptides that are analogous to 4A6, MRP1-overexpressing cells were found to be resistant to these peptides as well. Remarkably, when selecting A2780 ovarian cancer cells for resistance to ALLN, even in the absence of Pgp blockers, resulting cell lines had up-regulated MRPI, rather than any of the other currently known multidrug resistance transporter molecules including Pgp, MP2 (ABC2), MP3 (ABC3), MP5 (ABC5), and the breast cancer resistance protein ABCG2. ALLN-resistant, MRPI-overexpressing cells were found to be cross-resistant to 4A6 and the classical multidrug resistance drugs doxorubicin, vincristine, and etoposide. This establishes MRPI as a transporter for small hydrophobic peptides. More extensive structure-activity relationship studies should allow the identification of clinically useful peptide antagonists of MRPI.

**INTRODUCTION**

A major problem in the treatment of cancer patients with chemotherapeutics is the occurrence of drug resistance. At least two proteins are well-known for causing MDR, which is the resistance of tumor cells to structurally and functionally unrelated drugs such as the anthracyclines, the Vinca alkaloids, and the epipodophyllotoxins. Both proteins, the MDR1 gene encoded-Pgp (reviewed in Ref. 1) and MRPI (reviewed in Ref. 2), are members of the ATP binding cassette transporter superfamily, which includes proteins in organisms varying from bacteria to man (reviewed in Ref. 3). 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.

Despite their common involvement in MDR, there are clear differences in function and substrate specificity of Pgp and MRPI. Pgp binds and transports neutral, or positively charged, hydrophobic compounds. In contrast, MRPI transports conjugated organic anions such as the leukotriene C4 and GSH S-conjugates of prostaglandin A2 and aflatoxin B1. Therefore MRPI is known as multispecific organic anion transporter (MOAT; Ref. 4) or GS-X pump (5). For transport by MRPI of cytoxicostatics such as the anthracyclines, GSH is required (6), but GSH is not necessarily conjugated to the drug. Instead, GSH may be cotransported (7).

In humans, a well-known ABC protein involved in peptide transport is the transporter associated with antigen presentation. It plays a pivotal role in MHC class I restricted antigen presentation by mediating peptide translocation across the endoplasmic reticulum membrane (8). Regarding Pgp, among the diverse range of substrates, peptides have also been reported to interact with this protein. Sharma et al. (9) selected CHO cells with the cytotoxic tripeptide ALLN, which resulted in a cell line that showed the classical MDR phenotype with overexpression of Pgp. Also, hydrophobic peptides stimulate Pgp ATPase activity (10) and a radiolabeled tripeptide (Nac-Lyl-amide) was shown to be transported by Pgp into both membrane vesicles and reconstituted proteoliposomes (11). Furthermore, several peptides can reverse the Pgp-mediated resistance to cytostatic drugs. Well-known cyclic chemosensitizing peptides are cyclosporin A and its nonimmunosuppressive derivative, PSC833, but linear peptides are also able to antagonize Pgp-mediated drug transport (10, 12).

We decided to investigate the potential role of MRPI as a transmembrane transporter for peptides. To this goal, we selected cytotoxic peptides of different origins and structures for study as possible substrates of MRPI (Table 1). Both ALLN and valinomycin are known substrates of Pgp (9). 4A6 is a synthetic peptide derivative [Ac-Thr(Bzl)-His(Bzl)-Thr(Bzl)-Nle-Glu(OBu)-Gly-Bza; for structure see Fig. 1] that was discovered by us in a cytotoxicity-based screen for potential MDR antagonists. The present results show that MRPI is an effective peptide transporter. This may give a lead to the design of peptide-based selective and potent reverting agents.

**MATERIALS AND METHODS**

**Chemicals.** For 4A6 peptide synthesis, we used: Fmoc-Ala-OH, Fmoc-Gly-Sar-resin, Fmoc-Glu(OBu)-OH, Fmoc-Lys(Z)-OH, Fmoc-Thr(Bzl)-OH, Fmoc-His(Bzl)-OH, and Fmoc-Thr(Bzl)-OEt (all from Bachem, Bubendorf, Switzerland); fluorescein (98%), pyrrole-2-carboxylic acid, and 2-pyrazinecarboxylic acid (all from Aldrich, Zwijndrecht, the Netherlands); Bza (99.5%; Acros, Geel, Belgium); acetic anhydride and DMF (both from Merck, Amsterdam, the Netherlands); N-methylpyrrolidone (peptide synthesis grade), trifluoroacetic acid (peptide synthesis grade), DIEA (peptide synthesis grade), HOBt, 1-hydroxybenzotriazole, BHT, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylenuronium hexafluorophosphate; BSO, d-(+)-thiolactic acid-5-sulphonic acid; DCM, dichloromethane; HOBr, 1-hydroxybenzotriazole; HTBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylenuronium hexafluorophosphate; BSO, d-(+)-thiolactic acid-5-sulphonic acid; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphonylphenyl)-2H-tetrazolium-5-carboxanilide); RF, resistance factor; VP-16, etoposide; CHO, Chinese hamster ovary; IL, interleukin.

Received 5/11/00; accepted 1/16/01.

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1 Supported by Dutch Cancer Society Grant KWF-VU96-1256.

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3 The abbreviations used are: MDR, multidrug resistance; MRP, MDR protein; Pgp, P-glycoprotein; ALLN, N-acetyl-Leu-Leu-norleucinal; GSH, glutathione; OEtBu, O-(1-tert-butyl), Bzl, benzyl; Bza, benzyamine; Z, benzyloxycarbonyl; DMF, N,N-dimethyl formamide; DCM, dichloromethane; HOBr, 1-hydroxybenzotriazole; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylenuronium hexafluorophosphate; BSO, d-(+)-thiolactic acid-5-sulphonic acid; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphonylphenyl)-2H-tetrazolium-5-carboxanilide); RF, resistance factor; VP-16, etoposide; CHO, Chinese hamster ovary; IL, interleukin.

All of the other chemicals and drugs used, including the peptide ALLN, were from Sigma Chemical Co. (St. Louis, MO) except for doxorubicin, which was purchased from Farmitalia Carlo Erba (Brussels, Belgium), trichloroacetic acid, which was from ICN Biomedicals Inc. ( Aurora, OH), and MK571 (L-660,711), obtained from Dr. Robert Zamboni (Mercck-Frost, Pointe-Claire, Quebec, Canada).
**Peptide Synthesis.** For 4A6 synthesis, DMF (1 ml) was added to 100 mg of Fmoc-Gly-Sar-resin (0.69 mmol Fmoc-Gly per g resin) and mixed (1 h). After washing with DMF, the Fmoc-group was removed using 2 ml of 20% piperidine in DMF (10 min). The first amino acid Fmoc-Glu(OrBu)-OH (0.5 mmol in 1 ml DMF) was coupled to the resin using DIEA (1.25 mmol in 0.5 ml DMF) and HBTU/HOBt (0.5 mmol in 1.1 ml DMF). The same procedure was used for coupling of the second to the fifth amino acids, i.e., Fmoc-NeuOH, Fmoc-Thr(Bzl)-OH, Fmoc-His(Bzl)-OH, and Fmoc-Thr(Bu)-OH. To obtain acetyl-4A6, the peptide-resin was acetylated, after Fmoc cleavage and washing, using acetic anhydride/DMF/DIEA 1/10/0.1 (30 min). For fluorescein-labeled 4A6 the peptide-resin was, after Fmoc cleavage and washing, incubated with a solution of fluorescein (0.14 mmol in 290 μl of DMF), DIEA (0.36 mmol in 143 μl of DMF), and HBTU/HOBt (0.14 mmol in 350 μl of DMF). After washing with DMF, the peptide-resin was treated with 1% trifluoroacetic acid in DMF, which cleaved the protected peptide from the resin. To the free COOH-terminus of the protected peptide (0.069 mmol in 690 μl of DMF) benzylamine (0.1 mmol) was coupled using DIEA (0.175 mmol in 70 μl of DMF). Finally the protected peptides were purified on HPLC (Waters 2690 Alliance with a reverse phase C8-column) and subsequently, the protected peptide was precipitated from the previous solution using water. Then the peptides were centrifuged and washed two times with ice-cold PBS. The free peptide was dissolved in 50% DMSO and probenecid. Total amount of DMSO in the final solution was always less than 20% of the total volume.

**Results.** Cross-Resistance of MRPl-overexpressing Cells to ALLN and 4A6, but not Valinomycin. Characteristics of the three toxic peptides selected for the present study are shown in Table 1. Both Pgp-overexpressing cell lines SW-1573/2R160 and 2780AD were resistant to ALLN and the K⁺ ionophore valinomycin, confirming that these peptides are substrates of Pgp, which was reported previously (9). Also the newly synthesized toxic peptide 4A6 appeared to be a Pgp substrate (Table 2). Subsequent experiments with MRPl-overexpressing SW-1573/2R120 and HL60/ADR cell lines revealed that these cells also show distinct resistance to ALLN (RFs, 4.4 and 5.1, respectively) and 4A6 (RFs, 8.3 and 10.8, respectively; Table 2). The ability of MRPl to transport these peptides was confirmed with two independent clones of MRPl-transfected 2008 cells. These cell lines also showed a resistant phenotype with resistance factors varying from 3.4 and 2.5 for ALLN and 3.8 and 2.9 for 4A6 (Table 2). In MRPl-overexpressing cells, however, no resistance was found to valinomycin (Table 2).

**Reversal of Peptide Resistance by MRPl Antagonists.** The role of MRPl in the resistance to the peptides ALLN and 4A6 was confirmed in subsequent experiments in which we studied the effects of the MRPl antagonists probenecid and MK571 on HL60 and HL60/ADR cytotoxicity. Both agents have previously been shown to reverse the resistance to cytostatic drugs in MRPl but not in Pgp.

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**Table 1 Characteristics of peptides used in the study**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
<th>Charge/structure</th>
<th>Toxic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALLN</td>
<td>Microbial</td>
<td>Neutral, linear</td>
<td>Proteases and proteasome inhibition</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>Microbial</td>
<td>Neutral, cyclic</td>
<td>K⁺ ionophore</td>
</tr>
<tr>
<td>4A6</td>
<td>Synthetic</td>
<td>Neutral, linear</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

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* M. Kool, M. de Haas, M. van der Linden, F. Baas, and P. Borst. MRPl: Localization, drug resistance and GSH transport in comparison to MRPl and MRPl3, manuscript in preparation.

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![Fig. 1. Chemical structure of 4A6.](image)
overexpressing cells in a concentration-dependent way (22). Probenecid (at 0.5 mM) and, even more effectively, MK571 (at 30 μM) almost fully restored the cytotoxicity of the peptides ALLN and 4A6 in the HL60/ADR cells to the levels observed in the parental cells (Fig. 2). The small, but consistent chemosensitizing effects in the HL60 cells are most likely related to a low level of MRP1 in the latter cells.

Defective Accumulation of Labeled 4A6 in MRP1-overexpressing Cells. A hallmark of the classical MDR phenotype caused by transmembrane transporter molecules like MRP1 is the existence of a drug accumulation defect attributable to the enhanced cellular efflux of drug (23). Using a fluorescein-conjugated derivative of the 4A6 peptide (Flu-4A6) we determined peptide accumulation in cells by flow cytometry. Cells were incubated with Flu-4A6 overnight because the peptide appeared to accumulate in the cells only slowly (data not shown). Indeed, in comparison with the HL60 cells, the HL60/ADR cells accumulated approximately only one-third of the amount of Flu-4A6 (Fig. 3). The addition of MK571 restored the accumulation defect of 4A6-Flu in these cells to the levels observed in HL60 cells, in line with the finding that MK571 reversed the 4A6 resistance in HL60/ADR cells (Fig. 3). Although a contribution of the fluorescein moiety of Flu-4A6 to these results cannot be excluded, these findings support the conclusion reached above that MRP1 can mediate peptide resistance.

Potentiation of ALLN but not 4A6 Cytotoxicity by BSO. Next, we studied whether, and to which extent, GSH might contribute to the observed peptide resistance. BSO is an inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyzes the first step in GSH synthesis (24). Depletion of cellular GSH by BSO potentiates the toxicity of a broad variety of drugs, including vincristine, VP-16, and doxorubicin, to MRP1-, but not Pgp-, overexpressing cells (6), which indicated that GSH is needed for the export of these drugs by MRP1. On the other hand, efflux of some MRP1 substrates, for instance calcein (25), is not affected by GSH depletion. Here, we found that BSO restores the cytotoxicity of ALLN in HL60/ADR cells almost to the parental level but has only a small effect on the cytotoxicity of 4A6 (Fig. 4). These results indicate that, depending on the peptide, GSH can contribute to peptide transport by MRP1.

Resistance to 4A6 Analogous Peptides in MRP1-overexpressing Cells. To study the structural requirements for peptide transport by MRP1, we synthesized a small peptide library of 17 peptides that are analogous to 4A6. Changes were made replacing one of five residues Thr(tBu), His(Bzl), Thr(Bzl), Nle, or Glu(OtBu) by Lys(Z) or Ala and replacing the NH$_2$-terminal acetyl group by either pyrrole-2-carbox-
reported that the selection of CHO cells for resistance to ALLN resulted in a cell line with up-regulated Pgp. Therefore, we decided to select cells both in the absence and the presence of 10 μM verapamil, the latter to prevent up-regulation of Pgp. Using the A2780 ovarian cancer cell line for these experiments within 2–3 months of culturing, we obtained ~10 ALLN-resistant cell lines. Interestingly, none of the resistant cell lines that were obtained showed overexpression of Pgp, independent from coculturing with or without verapamil. Instead, most of the cell lines showed low, but distinct, up-regulation of MRP1. When cells were selected for higher resistance levels to ALLN, MRP1 was not further up-regulated, possibly because other resistance mechanisms emerged. Immunocytochemical staining for other MDR-associated proteins [Pgp, MRP2/cMOAT (multispecific organic anion transporter), MRP3, MRP5, BCRP (breast cancer resistance protein); Ref. 26] and the lung resistance protein (LRP)/major vault protein (MVP; reviewed in Ref. 27) remained, however, negative (results not shown). One of the ALLN/verapamil coselected cell lines (A2780/ALLN1) was chosen for further analysis. These cells were obtained in a single-step selection by culturing A2780 cells with 10 μM ALLN and 10 μM verapamil continuously. The cell line was found to be 6.4-fold more resistant to ALLN than the parental A2780 cells (Table 4). Cytosin stainings of A2780/ALLN1 cells showed up-regulated MRP1 but not Pgp (Fig. 5). In cytotoxicity experiments, A2780/ALLN1 cells were found to be cross-resistant to the 4A6 peptide (RF, 7.3), and the cytotoxic drugs VP-16 (RF, 4.7), vincristine (RF, 3.4), and doxorubicin (RF, 2.7; Table 4). No resistance could be detected to the Pgp substrate valinomycin (RF, 1.2). This cross-resistance pattern is consistent with an MDR phenotype caused by overexpression of MRP1.

**DISCUSSION**

This report provides several lines of evidence to show that small hydrophobic peptides can be substrates of MRP1. MRP1-overexpressing cells, including cells stably transfected with MRP1 cDNA, were resistant to the cytotoxic peptides ALLN and 4A6, and this resistance could be reversed by MRP1-specific inhibitors. The reduced toxicity of 4A6 in MRP1-overexpressing cells was found to be associated with lower accumulation of a fluorescein-labeled derivative of this peptide in these cells. Furthermore, cells that were selected for resistance to ALLN, even in the absence of Pgp blockers, appeared to have up-

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**Table 3 Resistance to 4A6 analogous peptides in MRP1-overexpressing cells**

Cells were cultured with ~20 μM of peptide for 96 h. Results are shown as mean percentage survival of duplicate measurements.

<table>
<thead>
<tr>
<th>Peptide*</th>
<th>SW-1573</th>
<th>SW-1573/2R120</th>
<th>2008</th>
<th>2008-M1-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₂ terminus: acetyl ac-Thr(Bu)-His(Bz)-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza(4A6)</td>
<td>7</td>
<td>76</td>
<td>16</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>ac-Lys(Z)-His(Bz)-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza</td>
<td>10</td>
<td>95</td>
<td>46</td>
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<td></td>
<td>ac-Thr(Bu)-Lys(Z)-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza</td>
<td>94</td>
<td>101</td>
<td>100</td>
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<td></td>
<td>ac-Thr(Bu)-His(Bz)-Lys(Z)-Nle-Glu(OBU)-Gly-Bza</td>
<td>89</td>
<td>102</td>
<td>100</td>
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<td>ac-Thr(Bu)-His(Bz)-Thr(Bz)-Lys(Z)-Glu(OBU)-Gly-Bza</td>
<td>23</td>
<td>101</td>
<td>85</td>
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<tr>
<td></td>
<td>ac-Thr(Bu)-His(Bz)-Thr(Bz)-Nle-Lys(Z)-Gly-Bza</td>
<td>91</td>
<td>101</td>
<td>94</td>
</tr>
<tr>
<td>NH₂ terminus: pyrrole-2-carboxylic acid (P-2-CA) P-2-CA-Thr(Bu)-His(Bz)-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza</td>
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<td>99</td>
<td>81</td>
<td>100</td>
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<tr>
<td></td>
<td>P-2-CA-Thr(Bu)-Ala-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza</td>
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<td>104</td>
<td>88</td>
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<td>106</td>
<td>102</td>
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<tr>
<td></td>
<td>P-2-CA-Thr(Bu)-His(Bz)-Thr(Bz)-Ala-Glu(OBU)-Gly-Bza</td>
<td>100</td>
<td>106</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>P-2-CA-Thr(Bu)-His(Bz)-Thr(Bz)-Nle-Ala-Gly-Bza</td>
<td>41</td>
<td>106</td>
<td>74</td>
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<tr>
<td></td>
<td>P-2-CA-Thr(Bu)-His(Bz)-Thr(Bz)-Nle-Ala-Gly-Bza</td>
<td>33</td>
<td>70</td>
<td>46</td>
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<tr>
<td>NH₂ terminus: 2-pyrazine carboxylic acid (2-PCA) 2-PCA-Thr(Bu)-His(Bz)-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza</td>
<td>5</td>
<td>45</td>
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<td>66</td>
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<td>2-PCA-Thr(Bu)-Ala-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza</td>
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<td>86</td>
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<td>2-PCA-Thr(Bu)-Ala-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza</td>
<td>12</td>
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<td>2-PCA-Thr(Bu)-Ala-Thr(Bz)-Nle-Glu(OBU)-Gly-Bza</td>
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<td>2-PCA-Thr(Bu)-His(Bz)-Thr(Bz)-Ala-Glu(OBU)-Gly-Bza</td>
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<td>102</td>
<td>103</td>
<td>98</td>
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</table>

* Amino acids replacing the original residues are printed in italics.
regulated MRP1 and were cross-resistant to 4A6 and the classical MDR drugs doxorubicin, vincristine, and VP-16; the latter experiments illustrate the heterogeneity of cells in responding to cytostatic drug pressure. Selection of CHO cells with ALLN led to Pgp overexpression (9), whereas Pgp was not detected in any of the presently selected human tumor cell lines, even after extended selection, despite the proven ability of A2780 cells to overexpress Pgp (27).

Until now, only few, indirect, data have become available about possible interactions of MRP1 with peptides. Of course, GSH, which has been described as a required cosubstrate for transport of substrates by MRP1 (7, 28) and may even be transported on its own (29), is a tripeptide itself, consisting of glutamate, cysteine, and glycine residues. In this study, we set out to further investigate putative peptide transport by MRP1, and the results obtained confirm that two neutral peptides, ALLN and 4A6, are efficiently transported both by Pgp and MRP1. This suggests that, at least for peptides, a negative charge is not a required factor in determining MRP1 substrate properties. This view is supported by the experiments with BSO-pretreated cells showing that MRP1-mediated resistance to 4A6, a neutral toxic peptide, was largely independent of intracellular levels of the anionic tripeptide GSH. Because, in contrast, MRP1-mediated resistance to the other neutral, toxic peptide, ALLN, was found to be strongly GSH dependent, cotransport of GSH may facilitate transport of certain neutral substrates but is not an absolute requirement for MRP1 function. Alternatively, certain substrates such as 4A6 may need relatively low concentrations of GSH compared with other substrates (ALLN).

Pgp can interact with circular peptides, because both Pgp overexpressing cell lines showed resistance to the peptide K+ ionophore valinomycin. This is in line with the well-known potencies of the circular peptides cyclosporin and its congener PSC833 as Pgp chemosensitizers (30). We did not, however, find resistance to valinomycin. This is in line with the well-known potencies of the ionophore

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 ALLN (µM)</th>
<th>IC50 4A6 (µM)</th>
<th>IC50 VP-16 (µM)</th>
<th>IC50 vincristine (nM)</th>
<th>IC50 doxorubicin (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>11.8 ± 2.1</td>
<td>1.1 ± 0.5</td>
<td>0.6 ± 0.1</td>
<td>3.8 ± 2.7</td>
<td>0.3 ± 0.2</td>
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<tr>
<td>A2780/ALLN</td>
<td>75.9 ± 20.1</td>
<td>8.0 ± 3.5</td>
<td>2.8 ± 0.1 (4.7)</td>
<td>13.1 ± 8.8 (3.4)</td>
<td>0.8 ± 0.3 (2.7)</td>
</tr>
</tbody>
</table>

Fig. 5. Up-regulation of MRP1, but not Pgp, in ALLN selected cells. Cytocentrifuge preparations of A2780 and A2780/ALLN1 tumor cells were stained with the monoclonal antibodies JSB-1 for Pgp and MRPr1 for MRP1.

**REFERENCES**


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