Advances in Brief

Anti-CD19 Antibodies Inhibit the Function of the P-gp Pump in Multidrug-resistant B Lymphoma Cells

Maria-Ana Ghetie, Victor Ghetie, and Ellen S. Vitetta

The Cancer Immunobiology Center, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-8576

Abstract

After chemotherapy, tumor cells with multidrug resistance (MDR) often emerge. MDR is attributable to the expression of membrane transport proteins that inhibit the cellular influx and increase the efflux of many chemotherapeutic drugs. One such protein is P-glycoprotein (P-gp), which functions as an ATP-dependent active transporter. Recently, an anti-P-gp monoclonal antibody (MAb) that inhibits P-gp has been described. Previous studies from our laboratory using the anti-CD19 B-cell lymphoma-reactive MAb, HD37, have suggested that HD37 may also influence MDR. To test this directly, we used Namalwa/MDR1 cells to study the effect of HD37 on the efflux of rhodamine 123 from these cells. We found that HD37 and three other anti-CD19 MAbs inhibited the efflux of rhodamine 123 from Namalwa/MDR1 cells with ~50% of the efficiency of the well-known chemosensitizer, verapamil. In contrast, MAbs against seven other molecules expressed on these cells were ineffective. The inhibitory activity of HD37 did not require an Fc portion; F(ab')2 fragments were effective, but Fab fragments were not, suggesting that higher avidity binding and/or cross-linking of CD19 are necessary. We could find no evidence that HD37 recognizes a cross-reactive epitope on P-gp, modulates P-gp from the cell surface, or enhances the ATPase activity of membranes from treated cells.

Introduction

After chemotherapy, MDR tumor cells often emerge and, because of this, patients with relapsed cancers have a poor prognosis. The phenomenon of MDR is often attributable to the expression of membrane transport or “pump” proteins, which inhibit the cellular influx and increase the efflux of many chemotherapeutic drugs (1, 2). One such protein is P-gp, which functions as an ATP-dependent active transporter. A variety of agents have been developed to prevent MDR, including analogues of transported drug substrates (e.g., verapamil), inhibitors of ATP binding/utilization (e.g., oligomycin), and MAbs that recognize epitopes on the P-gp molecules (e.g., MRK16; Ref. 3) and block its activity (e.g., UIC2; Ref. 4). Although agents such as verapamil and oligomycin have side effects in humans, many MAbs are well tolerated, although anti-P-gp MAbs have not yet been used in humans.

CD19 is a B cell-specific glycoprotein that is expressed on virtually all normal and neoplastic cells of the B-cell lineage and which plays a role in signaling through the B cell receptor complex and other cell surface molecules (e.g., CD72; Ref. 5). Previous studies have shown that immunotoxins prepared with anti-CD19 MAbs render Namalwa/MDR1 tumor cells more sensitive to chemotherapeutic agents and reduce the levels of expression of P-gp on surviving cells (6, 7). Because immunotoxins enter cells through routes that avoid the P-gp pump and kill them by inhibiting protein synthesis, they could also result in a decrease in P-gp synthesis in surviving cells and, hence, decrease MDR.

Our previous studies have shown that a dimer of the “naked” anti-CD19 MAbs, HD37, can both signal CCA in lymphoma cells and potentate the cytotoxic effects of several chemotherapeutic agents in severe combined immunodeficiency mice with human lymphoma xenografts (8). These data suggested that HD37 itself, even without a toxin component, might be a chemosensitizer.

In the present study, we explored this possibility by determining whether anti-CD19 MAbs would inhibit the efflux of rhodamine 123 from the P-gp− Namalwa/MDR1 cells. We compared the effect of HD37 and other anti-CD19 MAbs to those of both verapamil and a unique anti-P-gp MAb, UIC2, which has been reported to inhibit P-gp activity (4, 9). We found that HD37 and three other anti-CD19 MAbs, used at 100-fold lower concentrations than those required to signal CCA (10), decreased the efflux of rhodamine 123 from the Namalwa/MDR1 cells, and that this effect did not occur using MAbs against seven other non-CD19 molecules expressed on the Namalwa/MDR1 cells. We have excluded the possibility that the HD37 MAb recognizes an epitope on P-gp, that it modulates P-gp from the cells, or that it alters ATP levels in cells. By exclusion, we hypothesize that it alters another biochemical function of P-gp and/or that the signaling pathways used by CD19 and P-gp share a common intermediary. If this should be the case in lymphoma cells from patients, HD37 might be useful as an antitumor agent, not only because of its ability to deliver toxins and (as a dimer) induce CCA, but also because it acts as a chemosensitizer.

Materials and Methods

Cell Lines, Antibodies, and Reagents. The human Burkitt’s lymphoma cell line, Namalwa, was infected with a human MDR1 gene-containing retrovirus (Namalwa/MDR1) and was a gift from Dr. R. O’Connor at ImmunoGen (Boston, MA; Refs. 6 and 7). Both the parental P-gp+ Namalwa cells and the transfected P-gp− Namalwa/MDR1 cell lines were maintained in culture in complete RPMI 1640 containing 10% heat-inactivated FBS.
supplemented with 20 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 mM L-glutamine. A-498 cells (a human kidney carcinoma cell line from American Type Culture Collection) were used as a P-gp control. Cells were cultured in Eagle’s MEM with 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 90% Eagle’s balanced salt solution, supplemented with 10% FBS. The cells were detached from culture flasks by treatment with a 0.25% trypsin solution, washed two to three times with medium, and phenotyped by immunofluorescence and FACS analysis. These cells were also used in the rhodamine 123 efflux assay as described below. Cells were grown in a humidified atmosphere of 5% CO2 and air, and viability was determined by trypan blue exclusion. Cell lines were maintained in culture for 6 weeks and were then replaced with frozen stock. The cellular phenotype was determined using a panel of MAbs.

MAbs and Other Reagents

The following MAbs or hybridomas were used: (a) anti-CD19: HD37 from Dr. D. Dorken, Germany (purified in our laboratory); BU12 from Dr. D. Flavell, University of Southampton, Southampton, United Kingdom; (4G7) from Dr. R. Levy, Division of Oncology, Stanford University, Stanford, CA; and FMC63 from Dr. M. LeTarte at the University of Alberta, Alberta, Canada; (b) anti-CD20: 1F5 from Bristol Meyers, Seattle, WA and the chimera anti-CD20 (IDE-C2B8 or Rituxan) from Genentech, San Francisco, CA; (c) anti-CD21: G28-5 from American Type Culture Collection, Rockville, MD; (d) anti-CD22: RFB4 from Dr. G. Janossy, Royal Free Hospital, London, United Kingdom; (e) anti-CD40: G29-5 from Bristol Myers, Seattle, WA; (f) anti-CD79a, CD79b: ZL9-1, ZL7-4 from Dr. M. Glennie, Tenovus Research Laboratory, Southampton General Hospital, Southampton, United Kingdom; (g) goat anti-human IgM (\(\mu\) chain) from Sigma, St. Louis, MO; (g) 3F12, an IgG1 isotype-matched control MAb, was a gift from Dr. E. Hansen, University of Texas Southwestern Medical School, Dallas, TX; (h) anti-P-gp: 4E3, from Signet Laboratories, Dedham, MA; MKR16 from Kamya Biochemical Co., Seattle, WA; UIC2 from Immunotech, Miami, FL; and (i) FITC-goat anti-mouse IgG (H+L); GAM IgM from Kirkegaard & Perry Laboratory, Inc., Gaithersburg, MD. All antibody preparations used in this study were extensively dialyzed against PBS, and concentrations were normalized in RPMI 1640. Verapamil and rhodamine 123 were determined in RPMI 1640. Verapamil and rhodamine 123 min. Cells were then washed with RPMI 1640 plus 0.1% sodium azide, incubated with FITC-GAM Ig in 30 min at 4°C, and then washed and analyzed on the FACS. For the evaluation of P-gp and CD19, a direct assay was also performed using PE-UIC2 for P-gp and FITC-HD37 for CD19.

Cytotoxicity Assays

The cytotoxicity of doxorubicin, the UIC2 and HD37 MAbs, either alone or in combination, was evaluated using a \([1^\text{H}]\)thymidine incorporation assay. Cells were treated in 96-well microtiter plates for 72 h at 37°C, followed by a pulse with \([1^\text{H}]\)thymidine for 18 h as described by O’Connor et al. (6, 7).

Flow Cytometric Assays

Indirect immunofluorescence assays were carried out using the panel of MAbs described above and FITC-GAM Ig. Cells (106) were treated with 1–10 \(\mu\)g of MAb for 30 min on ice, washed twice with complete medium containing 0.1% sodium azide, resuspended in 100 \(\mu\)l of medium, and treated with 2–3 \(\mu\)l of FITC-GAM Ig for 30 min on ice. The cells were washed twice, resuspended in medium, and analyzed on the FACS

<table>
<thead>
<tr>
<th>Antigenic marker</th>
<th>MAb</th>
<th>% positive cells</th>
<th>MFIa</th>
<th>% positive cells</th>
<th>MFI</th>
</tr>
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<tr>
<td>CD19</td>
<td>HD37</td>
<td>94.2</td>
<td>387</td>
<td>95.3</td>
<td>363</td>
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<tr>
<td>CD20</td>
<td>2H7</td>
<td>68</td>
<td>105</td>
<td>84</td>
<td>70</td>
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<td>G29-5</td>
<td>74.3</td>
<td>43</td>
<td>80.8</td>
<td>46</td>
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<tr>
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<td>RFB4</td>
<td>96.7</td>
<td>70</td>
<td>96.3</td>
<td>47</td>
</tr>
<tr>
<td>CD40</td>
<td>G28-5</td>
<td>93.2</td>
<td>108</td>
<td>96.1</td>
<td>91</td>
</tr>
<tr>
<td>CD79a</td>
<td>ZL7-4</td>
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<td>130</td>
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<td>ZL-9-1</td>
<td>18.7</td>
<td>161</td>
<td>15.8</td>
<td>114</td>
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<tr>
<td>(\mu) chain</td>
<td>Goat anti-human (\mu)</td>
<td>96.1</td>
<td>213</td>
<td>92.1</td>
<td>517</td>
</tr>
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<td>P-gp</td>
<td>4E3</td>
<td>89</td>
<td>684</td>
<td>4.3</td>
<td>17</td>
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<td>MRK16</td>
<td>90</td>
<td>550</td>
<td>3.5</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>UIC2</td>
<td>97</td>
<td>600</td>
<td>2.1</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>IgG1 control</td>
<td>3F12</td>
<td>3.5</td>
<td>21</td>
<td>4.5</td>
<td>24</td>
</tr>
</tbody>
</table>

a MFI, mean fluorescence intensity.

Cellular Influx and Efflux Assays

To study the function of the P-gp pump on Namalwa/MDR1 cells, rhodamine 123 was used in an efflux assay (4). We also used DIOC2 as an alternative P-gp-specific reagent (12). Namalwa/MDR1 cells (5 \(\times 10^6\)) were washed and resuspended in 1 ml of serum-free RPMI 1640. Cells were incubated with 10–6 to 10–8 \(\mu\)M MAbs and 1.3 \(\mu\)M rhodamine 123 or 0.55 \(\mu\)M DIOC2 for 1 h at 37°C to determine influx. In each experiment, 10 \(\mu\)M verapamil and 1.3 \(\mu\)M rhodamine 123 or 0.55 \(\mu\)M DIOC2 were used as positive controls. In addition, the parental P-gp– Namalwa cells were used as a control for P-gp-independent retention of rhodamine 123. After 1 h, the cells were washed twice with serum-free medium to remove excess rhodamine 123 or DIOC2, resuspended in 1 ml medium, and recultured at 37°C for 2 h to determine efflux. Verapamil (10 \(\mu\)M) was again added to the cells treated in the influx portion of the protocol with Verapamil. After 2 h of culture, cells were washed and analyzed on the FACS by accumulating events in the FL1 channel. The efflux rates of both rhodamine 123 and DIOC2 were determined. After 2 h, efflux was complete (data not shown). We, therefore, chose 2 h as the standard conditions for the assay. To quantitate the effect of treatment with different MAbs on rhodamine 123 efflux from Namalwa/MDR1 cells, we measured the shift of the histogram to the right as compared with the control (rhodamine 123 only). The effect of verapamil (10 \(\mu\)M) was taken as 100%, and the effect of each MAb was calculated as a percentage of the change induced by verapamil.
Cross-Blocking Assay

$5 \times 10^5$ cells in 100 µl of complete RPMI 1640 containing 0.1% sodium azide were incubated on ice with 0.1–10 µg of HD37 or UIC2 for 30 min. Excess MAbs was removed by centrifugation and washing, and cells were resuspended in 100 µl of complete medium containing sodium azide. Cells were treated on ice with secondary MAbs (either FITC-HD37 or PE-UIC2) for 30 min and then washed and analyzed on the FACScan (the FITC-MAbs in FL1 and the PE-MAbs in FL2).
at the same concentration. Significant differences (P < 0.05) between HD37 and UIC2 when compared at the same concentration.

Table 2 Dose-dependent effects of HD37 and UIC2 on the rhodamine 123 efflux from Namalwa/MDR1 cells

<table>
<thead>
<tr>
<th>MAb concentration (m)</th>
<th>% of the effect induced by verapamil (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HD37</td>
</tr>
<tr>
<td>10^-6</td>
<td>23.3 ± 7.4</td>
</tr>
<tr>
<td>10^-7</td>
<td>42.8 ± 19.6</td>
</tr>
<tr>
<td>10^-8</td>
<td>30.5 ± 7.4</td>
</tr>
<tr>
<td>10^-9</td>
<td>23.0 ± 0.8</td>
</tr>
<tr>
<td>10^-10</td>
<td>11.9 ± 6.1</td>
</tr>
</tbody>
</table>

\(^a\)The effect of verapamil was estimated by measuring the area under the portion of the curve that was shifted to the right. This value was taken as 100%, and the other values represent the percentage of the verapamil effect.

\(^b\)Data represent the mean ± SD of three to four separate experiments. Using the Student t test, statistical analysis did not show significant differences (P > 0.05) between HD37 and UIC2 when compared at the same concentration.

\(^c\)ND, not determined.

Fig. 3 The efflux of rhodamine 123 alone (peak 1) or with HD37 as an intact IgG (peak 2), F(ab') \(_2\) (peak 3), or Fab' (peak 4), or with rhodamine 123 plus verapamil (peak 5). The conditions for this assay are as described in Fig. 2. The histogram depicts one representative experiment of three performed.

Modulation Assay

10^6 cells per ml of complete RPMI 1640 with 10% FBS were incubated on ice for 30 min with 10 μg of HD37 or UIC2. Excess MAb were washed, and cells were cultured in complete RPMI 1640 at 37°C for 16 h. The cells were stained with FITC-HD37 or PE-UIC2 and analyzed on the FACScan.

Cellular ATP Assay

Intracellular ATP was measured by the Bioluminescent Somatic Cell Assay kit (Sigma). Namalwa/MDR1 cells (10^6) were treated with 10^-8 m HD37, UIC2, or an irrelevant MAb of the same subclass. After 1 h at 37°C, the cells were pelleted and resuspended in RPMI 1640 without FBS, and the levels of ATP were determined. Light emission was measured using the Biolumat LB 9500C Universal Luminometer (Berthold, Wildbad, Germany) at 25°C. The levels of ATP per cell were determined from an ATP standard curve.

Table 3 The effect of different anti-CD19 MAbs on the efflux of rhodamine 123 from Namalwa/MDR1 cells versus antibody affinity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ka (X 10^-8 m)</th>
<th>% of verapamil (^b) effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>HD37</td>
<td>3.6</td>
<td>41.6 ± 5.7</td>
</tr>
<tr>
<td>FMC63</td>
<td>3.6</td>
<td>20.2 ± 2.8</td>
</tr>
<tr>
<td>4G7</td>
<td>11.0</td>
<td>19.4 ± 3.8</td>
</tr>
<tr>
<td>BU12</td>
<td>2.5</td>
<td>38.7 ± 8.6</td>
</tr>
</tbody>
</table>

\(^b\)Cells were treated with 10 μM verapamil or with 10^-6 m MAbs. The effect of verapamil, estimated as described in Table 2, was taken as 100%. The effects of MAbs were calculated as a percentage of the verapamil effect.

\(^c\)Data represent the mean ± SD of three separate experiments. Using the Student t test, statistical analysis shows a significant difference (P ≤ 0.01) between the effect of HD37 and FMC63 or 4G7 but not between HD37 and BU12 (P ≥ 0.05).

Plasma Membrane Preparation and ATPase Assay

The procedure for isolating plasma membranes from Namalwa/MDR1 cells was that recommended by Naito et al. (13) for other MDR cell types. The ATPase activity of the isolated cell membranes was estimated by measuring the liberation of inorganic phosphate from ATP as recommended by Sarkadi et al. (14). The ATPase medium contained EGTA, sodium azide, and ouabain at concentrations inhibiting all nonrelevant ATPase activities of the membrane preparation (14).

The presence of P-gp and CD19 on the Namalwa/MDR1 membrane preparation was determined by flow cytometry using PE-anti-P-gp (UIC2; Immunotech) and by RIA using 125I-labeled anti-CD19 (HD37). Treatment of the membrane preparations with MAbs or verapamil was performed as follows. To 70 μl membrane suspension (1–2 mg/ml protein), 30 μl of MAb (100 μg/ml) or verapamil (100 μM) was added, and after a 5-min incubation at room temperature, aliquots of 30 μl of the mixture were added in triplicates to 200 μl of ATPase medium containing 5 mEq MgATP (Sigma) and further incubated at 37°C for 30 min. The mixtures were centrifuged to discard the membranes, and the presence of inorganic phosphate was determined.

Results

Phenotyping of the Namalwa and Namalwa/MDR1 Cells.

As shown in Table 1, the Namalwa/MDR1 and Namalwa cells express a variety of markers typical of the human B-cell lineage. The Namalwa cells were slightly more positive for all of the markers tested, with the exception of P-gp, which was lacking on these cells but present on 89–97% of the Namalwa/MDR1 cells. Preliminary experiments had established that the expression of these markers remained relatively constant over 6 weeks of culture.

Sensitivity of Namalwa and Namalwa/MDR1 Cells to Doxorubicin in the Presence and Absence of HD37 or UIC2.

As shown in Fig. 1A, the Namalwa cells were 5–6-fold more sensitive to the cytotoxic effects of doxorubicin than the Namalwa/MDR1 cells. As shown in Fig. 1B, the HD37 MAbs had a synergistic effect on the cytotoxicity of doxorubicin on Namalwa/MDR1 cells, decreasing the IC50 of doxorubicin by 3–4-fold.

The effect of the HD37 MAb on the cytotoxicity of doxorubicin on Namalwa/MDR1 cells was comparable with that of the UIC2 MAb (Fig. 1B). HD37 MAb alone did not have a cytotoxic effect at the concentrations used in this study (data not shown). The combination index (15) was <1.0, indicating that...
the effects were synergistic rather than additive. Synergistic effects were not observed using the P-gp− Namalwa cell line (Fig. 1C). The results using the HD37 MAb at a noncytotoxic concentration strongly suggested that HD37 alters the drug sensitivity of the Namalwa/MDR1 cells in a manner similar to that of the UIC2 MAb, which is an efficient P-gp-mediated MDR-reversing agent (4). The similar effect of two MAbs with different specificities pointed to several possibilities: (a) HD37

Fig. 4 Cross-blocking of HD37 and UIC2 MAbs on Namalwa/MDR1 cells. Cells in RPMI 1640 were incubated for 30 min on ice with the 10 μg/ml HD37 or UIC2. Excess MAb was washed out with the same medium containing 0.1% sodium azide. Cells were then incubated with the secondary antibodies, FITC-HD37 or PE-UIC2, for 30 min on ice, washed, and analyzed on the FACScan. The histograms were compared after: A, staining with FITC-HD37 before (peak 1) and after (peak 2) treatment with HD37; B, staining with FITC-HD37 before (peak 1) and after (peak 2) treatment with UIC2; C, staining with PE-UIC2 before (peak 1) and after (peak 2) treatment with UIC2; D, staining with PE-UIC2 before (peak 1) and after (peak 2) treatment with HD37. The histogram depicts one experiment of six performed.

Fig. 5 The modulation of CD19 and P-gp on Namalwa/MDR1 cells. Cells were incubated with 10 μg/ml HD37 or UIC2 for 16 h at 37°C and then washed twice with RPMI 1640 containing 10% FBS, resuspended in 100 μl of the same medium, and phenotyped by staining with: A, FITC-HD37 before (peak 1) and after (peak 2) incubation with HD37; B, PE-UIC2 before (peak 1) and after (peak 2) incubation with UIC2; C, PE-UIC2 before (peak 1) and after (peak 2) incubation with HD37; D, FITC-HD37 before (peak 1) and after (peak 2) incubation with UIC2. The histogram depicts one experiment of six performed.
temperature, aliquots of 30 μl of membrane suspensions were treated with 30 μl of MAb (100 μg/ml) or verapamil (100 μM), and after a short incubation at room temperature, aliquots of 30 μl were added to 200 μl of ATPase assay medium and further incubated at 37°C for 30 min. The mixture was centrifuged, and the supernatant was analyzed for the inorganic phosphate. Column 1, no treatment; Column 2, irrelevant antibody (RFT5); Column 3, anti-CD19 antibody (HD37); Column 4, anti-P-gp antibody (UIC2); Column 5, verapamil. Three experiments are shown in Columns 1–4, and two experiments are shown in Column 5. Bars, SD.

Effect of MAbs against P-gp and CD19 on the Efflux of Rhodamine 123.

As shown in Fig. 2A, rhodamine 123 is retained by Namalwa cells but not by Namalwa/MDR1 cells, although the efflux of rhodamine 123 from Namalwa/MDR1 cells can be blocked by a “classical” inhibitor such as verapamil (Fig. 2B). In addition, at a concentration of 10^{-8} M, both the anti-P-gp (UIC2) and anti-CD19 (HD37) MAbs blocked efflux similarly, albeit less effectively than verapamil (Fig. 2, C and D). Two other anti-P-gp MAbs, 4E3 and MRK16, had no effect (data not shown). The relative effects of the MAbs and verapamil were determined by calculating the shift to the right of the rhodamine efflux curves, taking the verapamil value as 100%. As shown in Table 2, at 10^{-7} M, HD37 was 42% as effective as 10 μM verapamil, and the effect was dose related. Similar results were obtained using the anti-P-gp antibody, UIC2. The results of several different experiments using the two MAbs demonstrated that they were virtually identical in their ability to decrease efflux, and both were approximately half as effective as verapamil. We also used DIOC2 as an alternative P-gp-specific reagent and obtained similar results (data not shown). We also determined whether the effect of HD37 was related to its divalency and/or presence of the Fc fragment. To this end, we prepared both F(ab')2 and Fab' fragments of HD37 and repeated the experiments. As shown in Fig. 3, the F(ab')2 fragments were as effective as IgG, but the Fab' fragments had no effect. These results indicate that the Fc receptor is not involved in blocking the rhodamine 123 efflux and that either cross-linking and/or higher avidity binding of the HD37 MAbs are required to decrease the efflux of rhodamine 123.

Effect of Other anti-CD19 MAbs on the Efflux Rhodamine 123. To determine whether the HD37-mediated blocking of the rhodamine 123 efflux was unique to this particular anti-CD19 MAb, three other anti-CD19 MAbs were also evaluated. These MAbs recognize the same epitope on CD19 but have different affinities. As shown in Table 3, the three MAbs also inhibited the efflux of rhodamine 123 from the Namalwa/MDR1 cells, but to different degrees. Thus, BU12 was as effective as the HD37, whereas FMC63 and 4G7 were approximately half as effective. Because FMC63 has the same affinity constant (K_a) as HD37, its decreased effectiveness cannot be attributed to an affinity difference. Thus, subtle differences in the epitopes recognized by a particular anti-CD19 MAb may be important, because the cross-blocking assay defines proximal, but not necessarily identical, epitopes.

Effect of MAbs against Other Surface Molecules on the Efflux of Rhodamine 123. To determine whether anti-CD19 was unique in its ability to affect the rhodamine efflux, we also used antibodies against CD20, CD21, CD22, CD40, CD79a, CD79b, and IgM. None of these MAbs inhibited efflux (data not shown), indicating that CD19 is relatively unique in its ability to alter the function of the P-gp pump.

HD37 Does Not Recognize P-gp, as Determined by Cross-Blocking Experiments. One possibility to explain the effect of HD37 on the P-gp pump was that it recognizes a cross-reactive epitope on the P-gp molecule that interferes with its function. Because UIC2 is the only anti-P-gp MAb thus far described that blocks the effect of the pump (4), we carried out cross-blocking experiments to determine whether HD37 and UIC2 recognized the same epitope. This was accomplished using a standard cross-blocking protocol described previously (10). As shown in Fig. 4, each MAb effectively blocked its own binding but failed to block the binding of the other MAb. These experiments demonstrated that the two MAbs do not recognize the same epitope on P-gp. They did not, however, exclude the possibility that HD37 recognizes another epitope on P-gp that also modulates its function. To exclude this possibility, we stained the CD19^-P-gp^+ cell line A-498 with HD37 and could not demonstrate any binding, suggesting a complete lack of cross-reactivity between HD37 and P-gp. In addition, the efflux of rhodamine 123 from the CD19^-P-gp^+ A-498 cells was not affected by HD37, further demonstrating that anti-CD19 does not have an effect on the P-gp pump in the absence of coexpression of CD19 (data not shown).

Anti-CD19 Does Not Modulate Expression of P-gp. Another possibility to explain the effect of the anti-CD19 MAb is that the P-gp and CD19 molecules interact on the membrane of the Namalwa/MDR1 cells to inhibit efflux, and that the binding of a MAb against either molecule can alter the activity of P-gp. To determine whether these molecules interact on the surface of the cells, Namalwa/MDR1 cells were incubated for 24 h at 37°C with either HD37 or UIC2. At the end of this incubation period, HD37 but not UIC2 induced the complete modulation of its corresponding antigen (Fig. 5). Even secondary cross-linking UIC2 with anti-mouse immunoglobulin did not enhance its modulation (data not shown). Nevertheless, there was no evidence of modulation of P-gp after complete modulation of HD37. Although this does not exclude the possibility that a small fraction of the HD37 and P-gp molecules interact and that this interaction is critical, measurable cross-modulation could not be demonstrated.
Anti-CD19 Antibodies Inhibit the P-gp Pump

Discussion

In these experiments, we compared the ability of a variety of MAbs to alter the efflux of rhodamine 123 from Namalwa/MDR1 cells. We first demonstrated that the Namalwa/MDR1 cells were 5–6-fold less sensitive to doxorubicin than the Namalwa cells and that rhodamine 123 was not retained by Namalwa/MDR1 cells. Furthermore, verapamil, a classic inhibitor of the P-gp pump, inhibited the efflux of rhodamine 123. Using this model, we investigated the effect of MAbs against several different molecules on the P-gp+ Namalwa/MDR1 cells for their ability to alter the efflux of rhodamine 123. The major findings to emerge from these studies are: (a) four MAbs against CD19 reduced the efflux of rhodamine 123 from the Namalwa/MDR1 cells. Seven other MAbs against molecules expressed on these cells, i.e., CD20, 21, 22, 40, CD79a, CD79b, and IgM, had no effect; (b) HD37, the most effective anti-CD19 efflux inhibitor, was as effective as the previously described anti-P-gp MAb UIC2, which also inhibits the P-gp pump. Both MAbs were approximately half as effective as 10 μM verapamil; (c) the HD37-mediated inhibition of efflux did not require the Fc portion of the HD37 MAb, suggesting that Fc receptors are not involved. Fab' fragments had no effect, suggesting that either higher avidity binding and/or cross-linking of CD19 are required; (d) HD37 did not recognize a cross-reactive epitope on P-gp; (e) anti-CD19 did not modulate P-gp from the surface of the Namalwa/MDR1 cells but rendered these cells as drug-sensitive as the anti-P-gp MAb UIC2; and (f) HD37 did not alter levels of ATP or ATPase in Namalwa/MDR1 cells.

Previous studies by O'Connor et al. (6) and Liu et al. (7) demonstrated that an anti-CD19 IT increased the drug sensitivity of MDR lymphoma cells. However, because the IT itself is cytotoxic, it is unclear whether the anti-CD19 MAb, in the absence of its toxin, was responsible. We therefore explored the possibility that this was the case. One measurement of an effective P-gp pump is its ability to cause agents such as rhodamine 123 to efflux from MDR cells.

Minderman et al. (12) demonstrated that despite the fact that rhodamine 123 is a substrate for both P-gp and MRP, the efflux of rhodamine 123 from MRP+/P-gp- cells takes place at a much slower rate than that from MRP+/P-gp+ cells. We found that the HD37 MAbs inhibited the efflux of both rhodamine 123 and DIOC2 from MDR cells at a comparable rate, suggesting that HD37 is specific for P-gp and not the MRP. The effect of HD37 on the rhodamine 123 efflux was also comparable with that of a unique anti-P-gp MAb, UIC2, which recognizes an extracellular epitope on the human P-gp molecule and which also inhibits efflux. However, neither UIC2 nor HD37 was as effective as 10 μM verapamil, even when used together. This suggests that verapamil, which is a small molecule, acts more rapidly and hence inhibits the efflux completely in a 2-h assay. Considering the fact that verapamil and other drugs that act as chemosensitizers are toxic in humans, nontoxic MAbs that are even 50% as active as verapamil might be of clinical value.

The ability of HD37 to inhibit rhodamine 123 efflux from Namalwa/MDR1 tumor cells appears to be attributable to its specificity for CD19, because three other anti-CD19 MAbs were also effective. Moreover, MAbs against several other cell surface molecules were ineffective. The varying degrees of effectiveness of the different anti-CD19 MAbs could not be uniquely attributed to different affinities or cross-blocking abilities. In addition, because HD37 had a similar effect on rhodamine 123 efflux whether used as an intact IgG or as F(ab')2 fragments, the Fc receptor plays no role. Because Fab' fragments had no effect, either cross-linking and/or higher avidity binding of CD19 are required. The concentrations of HD37 MAb that inhibited the P-gp pump did not induce apoptosis or cell cycle arrest (data not shown). Recently, it has been demonstrated that P-gp can protect MDR tumor cells from caspase-dependent apoptosis (16) but not from caspase-independent cell death (17). However, because HD37 MAb does not induce apoptosis, its antitumor activity does not involve a caspase-dependent pathway. Recently, it has been shown that three different inhibitors of the P-gp pump (e.g., SDZ PSC 833) can induce cytoxic failure and apoptosis of MDR variants of two different cell lines (18). Our results suggest that the HD37 MAbs inhibit the P-gp pump by a different, as yet to be defined, mechanism.

The fact that both HD37 and UIC2 inhibited efflux to a similar degree suggested that HD37 might cross-react with P-gp. However, both cross-blocking experiments and the use of CD19- P-gp+ cells excluded this possibility. We also considered the possibility that CD19 associates with P-gp on the cell membrane and thereby inhibits its activity. However, we found that the complete modulation of CD19 had no effect on the expression of P-gp. Unfortunately, the reverse experiment was not possible, because P-gp cannot be modulated by the UIC2 antibody. Nevertheless, the one-way modulation experiment argues against interactions between these two molecules but does not formally exclude minor interactions not measurable in this assay.

The effect of HD37 on the function on P-gp pump appears to involve a cytoplasmic pathway because, in contradistinction to UIC2 and verapamil, the anti-CD19 antibody did not effect the ATPase activity of membranes from treated cells. The enhancing effect of verapamil on the ATPase activity of the membrane of the MDR cells has been reported previously (14, 19). UIC2 has been cited in a personal communication to be an inhibitor of the ATPase activity of membrane from P-gp-transfected insect cells (9).

The lack of activity of HD37 on cell membranes suggests that its inhibitory effect on intact cells is mediated by intracellular signaling. These signals could modulate the regulatory signals of other receptors. The molecular mechanisms by which cross-linking or hypercross-linking of CD19 generates a series of biochemical signals (20) have not been elucidated. Because HD37 can also negatively signal cells via the Lyn kinase pathway (21), this pathway may intersect with a P-gp-related signaling pathway. Multiple kinases may also be involved in regulating P-gp expression and/or activity, depending on the cell type and state of differentiation.
(2). However, information concerning the role of these different protein kinases in P-gp-mediated MDR is lacking.

Mutations that inactivate both nucleotide-binding domains of P-gp also inhibit its function, suggesting that signaling through P-gp may not involve modifications of the extracellular portions of P-gp. It has been reported that P-gp can exist in different transmembrane orientations, resulting in the exposure of different portions of the molecule on the exterior of the cell (22). Thus, P-gp might form dimers or oligomers (1, 22). In a recent study (9), it was suggested that P-gp can exist in different conformations and that trapping P-gp in a transient conformation could be the mechanism underlying MAb-mediated inhibition of P-gp. Because both the HD37 and the UIC2 MAbs have similar effects on P-gp function, it is possible that signaling through CD19 might indirectly induce changes in P-gp that result in increased retention or slower elimination of drugs from MDR cells.

P-gp belongs to the ABC group of proteins that require ATP for functional activity (2, 23–25). Our failure to demonstrate any effect of UIC2 (or HD37) on ATP levels in Namalwa/MDR1 cells might be attributable to the fact that the ATP used by the P-gp pump represents only a small fraction of the cellular pool of ATP. Further studies in other experimental systems may therefore be required to fully elucidate the mechanisms of action of HD37 versus UIC2. Regardless of the mechanisms involved, however, we will determine whether HD37 can reverse MDR in vivo in severe combined immunodeficient/Namalwa/MDR1 mice. If this should be the case, HD37 might be useful as a chemosensitizer in patients with MDR lymphomas.

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Maria-Ana Ghetie, Victor Ghetie and Ellen S. Vitetta


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