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Generation of multidrug resistant lymphoma cell lines stably expressing P-glycoprotein

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Abstract. The objective of this study was to generate new P-glycoprotein (P-gp)-expressing multidrug resistant (MDR) cell lines by drug selection. Since our previous studies have been carried out with cells infected with a P-gp-containing vector, it was important to confirm our findings in cells generated by drug selection. In this report, we describe three B-lymphoma cell lines which became drug-resistant by stepwise exposure to vincristine (VCR): Raji cells resistant to 18 nM VCR (R18V), Namalwa cells resistant to 21 nM VCR (N21V) and DHL-4 cells resistant to 12 nM VCR (DHL-4/12V). Cells overexpressed P-gp and continued to express CD19, CD20 and CD22, all of which are targets for monoclonal antibody (MAb) therapy. The P-gp pump in these new cells was functional as determined by the efflux of Rhodamine 123 and DIOC2, and the three cell lines were resistant to several chemotherapeutic drugs. We further determined that their P-gp phenotype was stable in xenografted SCID mice and that the tumors were also resistant to chemotherapy. We will now use these new MDR cells to determine whether monoclonal antibodies against CD19 and -20 can reverse P-gp, as we previously demonstrated using Namalwa cells infected with a human *mdr1* gene-containing retrovirus.

Introduction

Patients who develop multidrug resistant (MDR) tumors after chemotherapy have a poor prognosis. MDR is generally attributed to the expression of membrane transport or pump proteins, such as P-glycoprotein (P-gp), which inhibit the cellular influx and/or increase the efflux of a variety of chemotherapeutic drugs (1-3). Fifty percent of human cancers (e.g. leukemia, myeloma, lymphomas, breast and ovarian cancer) express P-gp at levels sufficient to confer MDR. Preliminary results with P-gp inhibitors have demonstrated improved responses to chemotherapy in some patients (3-5). However, tumors that expressed P-gp at the time of diagnosis (e.g. colon, kidney, pancreas and liver) did not respond to P-gp inhibitors since they utilized other mechanisms of resistance (6). In order to study mechanisms underlying MDR and therapeutic agents to overcome MDR, stable cells are needed. Since there is no commercially available human *mdr1* gene, obtaining MDR cells by transfection is very costly. Very few MDR tumor cell lines obtained by drug-selection have been described (7-10). To the best of our knowledge, drug-resistant B-lymphoma cells are not available.

Materials and methods

Cell lines. The following cell lines were used: two Burkitt's lymphoma cell lines, Namalwa and Raji, and the diffuse hystiocytic lymphoma, DHL-4, as well as our standard MDR cells, Namalwa/MDR1, which was a gift from Dr R. O'Connor at ImmunoGen (Boston, MA) (11). Namalwa/MDR1 derived from Namalwa cells were infected with a human *MDR1* gene-containing retrovirus. The cells were cultured in RPMI-1640 medium supplemented with 20 mM HEPES (Invitrogen, CA), 10% heat-inactivated fetal bovine serum (FBS) (HyClone, UT), 1% penicillin-streptomycin and 1% L-glutamine (Invitrogen). Cells were grown at 37˚C in a humidified incubator with 5% CO_2. Viability was determined by trypan blue exclusion. The media were changed every 48 h and the cells were passaged three times a week. Cell lines were maintained in culture for 6 weeks and were then discarded and replaced with frozen stock.

Generation of new MDR cells. Three cell lines (Namalwa, Raji and DHL-4) were exposed to vincristine (VCR) at a starting concentration of 3 nM. VCR was increased every 10 days by 3 nM increments until cells became resistant to 12-21 nM VCR. The cells were named by the parental cell names and the final concentration of VCR to which they were resistant (e.g. N21V, R18V and DHL-4/12V). The cells were routinely tested for P-gp expression and activity.

Antibodies and reagents. The following MAbs or hybridomas were used: a) UIC2 anti-P-gp from Immunotech, Miami, FL, or purified in our laboratory from the hybridoma that was a gift from Dr E.B. Mechetner, Oncotech Inc., Irvine, CA and MRK16 anti-P-gp from Kamya Biochemical Co. Seattle,

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WA; b) HD37 anti-CD19 from Dr D. Dorken, Germany (purified in our laboratory); c) Rituximab, RTX anti-CD20 from Genentech, San Francisco, CA; d) RFB4 anti-CD22 from Dr G. Janossy, Royal Free Hospital, London, UK and purified in our laboratory; e) fluorescein isothiocyanate (FITC) - goat anti-mouse IgG (H+L) (GAMIg) from Kirkegaard and Perry Laboratory Inc., Gaithersburg, MD. Verapamil and Rhodamine 123 were purchased from Sigma, St. Louis, MO; DIOC2 from Molecular Probes (Eugene, OR) and Valspodar (PSC 833) was a gift from Novartis Pharma AG, Basel, Switzerland. Macro and micro bicinconinic acid (BCA) protein assay reagent kits were purchased from Pierce Biotechnology, Rockford, IL and were used to measure the protein concentrations of cell lysates.

Chemotherapeutic drugs. The following drugs were used: vincristine sulfate (injection, USP) from Faulding Pharmaceutical Co. (a Mayne Group Company, Paramus, NJ); vinblastine from Bedford Laboratories, Bedford, OH; doxorubicin hydrochloride (injection, USP) from Gensia Sicor Pharmaceuticals, Inc., Irvine, CA; doxorubicin, etoposide and cyclophosphamide monohydrate (cytoxan) from Sigma.

Flow cytometry. Indirect immunofluorescence assays were carried out to evaluate the binding of UIC2 anti-P-gp, HD37 anti-CD19 and RFB4 anti-CD22 to cells using FITC-GAMIg as the secondary antibody (12,13). The cells were analyzed on a FACScan (Becton-Dickison, Mountain View, CA). Direct immunofluorescence assays for CD20 were performed using FITC-Rituximab (FITC-RTX prepared in our laboratory) and PE-UIC2 (purchased from Immunotech, Miami, FL) or FITC-UIC2 (prepared in our laboratory) for P-gp.

Cellular efflux assays. The activity of P-gp was investigated using two reagents, Rhodamine 123 and DIOC2, as previously described (12). Cells ($5x10^5$) were washed and resuspended in 1 ml of serum-free RPMI-1640, then incubated with 1.3 μM Rhodamine 123 or 0.55 μM DIOC2 in the presence or absence of P-gp inhibitors (10 μM Verapamil or Valspodar) for 1 h at 37˚C to determine influx. After the influx step, the cells were washed twice with serum-free medium to remove the excess Rhodamine 123 or DIOC2, resuspended in 1 ml medium, and recultured for 2 h at 37˚C to determine efflux. After 2 h, the cells were washed and analyzed on the FACScan by accumulating events in the FL1 channel.

Cytotoxicity assays. The cytotoxic activity of different drugs was tested by a colorimetric method using the CellTiter 96® AQ_{ueous} One solution cell proliferation assay, which was purchased from Promega Corporation, Madison, WI. The kit is composed of the novel tetrazolium compound which is reduced to a formazan compound, absorbance at 490 nm. The manufacturer's protocol was used. A 20 μl reagent was added per well in 96-well plates, which were incubated for 4 h at 37˚C. The data were analyzed using a SpectraFluor Plus (TECAN, Austria) microplate reader.

Western blot (WB) analysis. Ten million cells were lysed on ice with lysis buffer consisting of 0.25 M Tris pH 7.5 containing 1% Triton X-100. The protein concentration of the lysates was determined using macro and micro BCA kit protein assays and equal amounts of proteins were used for WB analysis as previously described (13,14). For the detection of P-gp, rabbit IgG anti-P-gp was used (Santa Cruz Biotechnology, Santa Cruz, CA) followed by HRP-anti rabbit IgG antibody and visualized with chemiluminiscent substrate ECL Western blotting detection reagents (Amersham Biosciences, UK). The bands on the WB corresponding to 170 kDa (the MW of P-gp) were scanned using a personal densitometer SI (Amersham Biosciences) and the data were analyzed quantitatively using image analysis software.

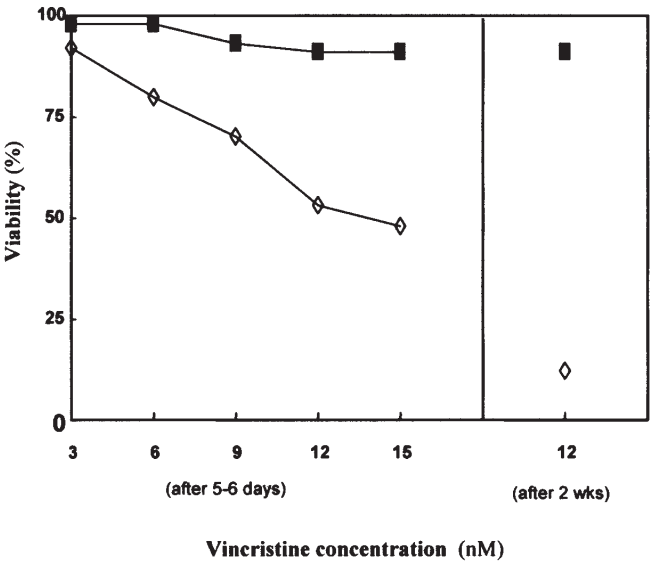


Figure 1. The effect of VCR on the viability of Raji and Raji-MDR cells. Cells were cultured in complete RPMI media ± VCR (3-21 nM), counted daily and the viability was determined by trypan blue exclusion. Raji cells cultured for 5-6 days with VCR gradually died. After two weeks in culture with the drug, all Raji cells (◇) were dead whereas Raji cells that were resistant to VCR (R18V) (▮) survived and grew well in the culture.

The growth of MDR tumors in SCID mice. Female CB17-SCM SCID mice (6-8 weeks old) (Taconic Farms, Germantown, NY) were housed and cared for according to the NIH Guidelines on Laboratory Animal Welfare and the guidelines of our Animal Resource Center (ARC). The mice were injected on day 0 *via* the lateral tail vein with $5x10^6$ tumor cells in 200 μl saline solution (0.9% sodium chloride, Braun Medical Inc., CA). The mice were treated with a total dose of 50 μg of VCR divided into 5 equal doses administered on days 2, 4, 8, 10 and 12 by i.p. injection in 100 μl saline solution. Tumor development in the spinal canal, resulting in hind-leg paralysis, was considered the end point (15). The animals were sacrificed with CO_2 asphyxiation as recommended by our institutional ARC protocol.

Results

The viability of drug-selected cells improved over time when they were continuously exposed to the drug. As shown in Fig. 1, whereas Raji cells that were resistant to 12 nM VCR recovered in culture in ~2 weeks and reached >90% viability the parental Raji cells continued to die as the concentration

The progressive adaptation to increasing concentrations was necessary to select drug-resistant cells.

VCR concentration increase	Viability after 24 h (%)	Growth factor[a]
Raji-VCR		
9→12 nM	90.9	9.75
9→15 nM	89.9	10.25
9→21 nM	90.4	6.60
9→24 nM	86.4	5.55
Raji		
12 nM	53.6	0.38

[a]The growth factor represents the ratio between the cell concentration after 24 h in culture and the initial concentration of $2x10^5$ cells/ml. The data in the table depict one experiment of two performed.

Table II. The phenotype of MDR vs. parental cells.[a]

Cell line	Percent of positive cells CD19	CD20	CD22	P-gp
Namalwa	95.7	83.5	95.5	7.1
NMDR1	92.7	90.9	91.7	90.0
N21V	93.3	90.4	93.3	91.9
Raji	92.0	98.1	91.9	1.8
R18V	92.0	97.3	94.3	85.7
DHL-4	77.8	99.7	76.4	4.1
DHL-4/12V	89.6	98.8	89.6	76.3

[a]FACS analysis was performed using saturating concentrations of HD37 and RFB4 and a FITC-GAMIg as a secondary antibody. Direct FACS analysis was performed for CD20 (using FITC-RTX) and P-gp (using either PE-UIC2 or FITC-UIC2). The results depict one representative experiment of 2-5 performed.

of the drug increased. Progressive adaptation was necessary to obtain the surviving cells and selection was achieved using increments of 3 nM VCR. When the drug concentration was increased by >1 increment of 3 nM, all the cells died (Table I). Even though the viability was not affected immediately (after 24 h), the cells grew more slowly and only the adapted cells survived. When the Raji cells were cultured directly in 12 nM VCR, 50% died. The surviving cells grew much more slowly and the selection of MDR cells failed. To enrich the P-gp$^+$ population of the cells we also carried out cell-sorting using FACS analysis after staining the cells with UIC2 anti-P-gp antibody. The sorted P-gp$^+$ population was further grown in the culture in the presence of VCR and the phenotype remained stable. The working batches of the cells were cultured for 6-8 weeks, at which time new cells were thawed from frozen stocks. The best batches of selected MDR cells were frozen and stored.

The phenotype of drug-selected MDR cells. The MDR cells were evaluated for the expression of P-gp, CD19, CD20 and CD22 using direct and indirect immunofluorescence and FACS analysis. The results are presented in Table II and indicate that none of the parental cells (Namalwa, Raji or DHL-4) expressed P-gp whereas the drug-selected MDR cells expressed P-gp at different densities. However, 80-90% of drug-selected cells (N21V, R18V and DHL-4/12V) were P-gp$^+$ and the expression of P-gp remained unchanged when the cells were grown in complete RPMI media without VCR. The expression of CD19, CD20 and CD22 on the surface of the MDR cells was not changed after drug-selection (Table II). The mean fluorescence intensity (MFI) of CD20 was highly variable among the cell lines. The Namalwa cells expressed low amounts of CD20, whereas the Raji and DHL-4 expressed higher amounts (data not shown). The MDR cells did not express multidrug resistant protein, another MDR specific marker (data not shown).

The cytotoxic effect of different drugs on the drug-selected MDR cells. The results of drug-killing assays (Table III) indicate that the three parental cells were killed to varying degrees not only by VCR but also by similar drugs (e.g. vinblastine), or different drugs (e.g. doxorubicin, daunorubicin, etoposide and cyclophosphamide). The most toxic drug was VCR and its toxicity was ~5 times higher on the Raji than on the Namalwa cells. The other drugs were significantly less toxic than VCR, but Raji cells were always killed by smaller amounts of drugs (e.g. doxorubicin and daunorubicin) than were the Namalwa cells. The new MDR cells as well as the Namalwa/MDR1 cells were resistant to all of the drugs. These results demonstrate that the new drug-resistant cells have a typical MDR phenotype. Fig. 2 illustrates that VCR killed the two parental cells (Namalwa and Raji) whereas the three MDR cells were resistant to the drug.

The P-gp pump is functional as demonstrated by the efflux of Rhodamine 123 and DIOC2. To determine whether P-gp was active, the cells were incubated with Rhodamine 123 or DIOC2 and the efflux of the dyes was determined. The three parental cells retained the two dyes whereas the MDR cells effluxed them. The P-gp activity was blocked specifically by the well-known P-gp substrate, Verapamil as demonstrated by the Rhodamine 123 (Fig. 3A) or DIOC2 efflux assays (Fig. 3B). The use of Valspodar as a P-gp blocker had a similar effect and the cells retained the dyes (Fig. 3C).

The overexpression of P-gp in MDR cells was confirmed by WB analysis. Cells ($4x10^7$) were lysed on ice with lysis buffer containing 1% Triton X-100 and the concentration of proteins was determined. Equal amounts of proteins from each sample were analyzed by WB. The gels were then scanned and the amount of P-gp was determined. As shown in Fig. 4, Namalwa/MDR1 and N21V cells expressed comparable amounts of P-gp, whereas the R18V cells expressed more P-gp and the DHL-4/12V less than Namalwa/MDR1 for the same amount of protein loaded, which is consistent with the FACS analysis (Table II). Despite the quantitative differences among the cell lines, this result demonstrates that the new MDR cells over-expressed P-gp while the parental cells did not.

Table III. The cytotoxic effect of different drugs on MDR cells vs. parental cells.

Cells	Drugs/IC50 (nM) Vincristine	Vinblastine	Doxorubicin	Daunorubicin	Etoposide	Cyclophosphamide
Namalwa	1.0	0.6	140	112	330	370
Namalwa/MDR1	No killing[a]	8.7	>400	>355	No killing	>400
Namalwa 21 nM VCR	No killing	6.3	>400	>1000	2500	No killing
Raji	0.2	0.3	24	5.5	350	No killing
Raji 18 nM VCR	No killing	15.0	No killing	No killing	No killing	No killing

[a]No killing means that the MDR cells were not killed even at concentrations of drug 10-50 times higher than its IC_{50} for parental cells. Other drugs may eventually kill MDR cells but above the high concentrations shown in the table whereas the parental cells were always killed by much lower concentrations of drugs. The data depict one representative experiment of 2-3 performed.

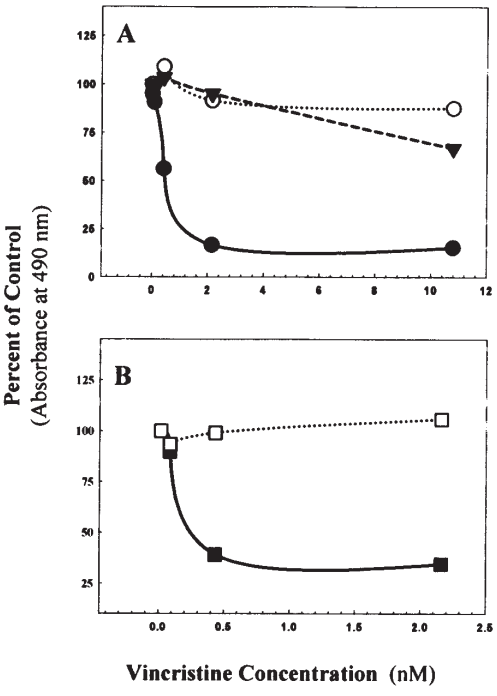
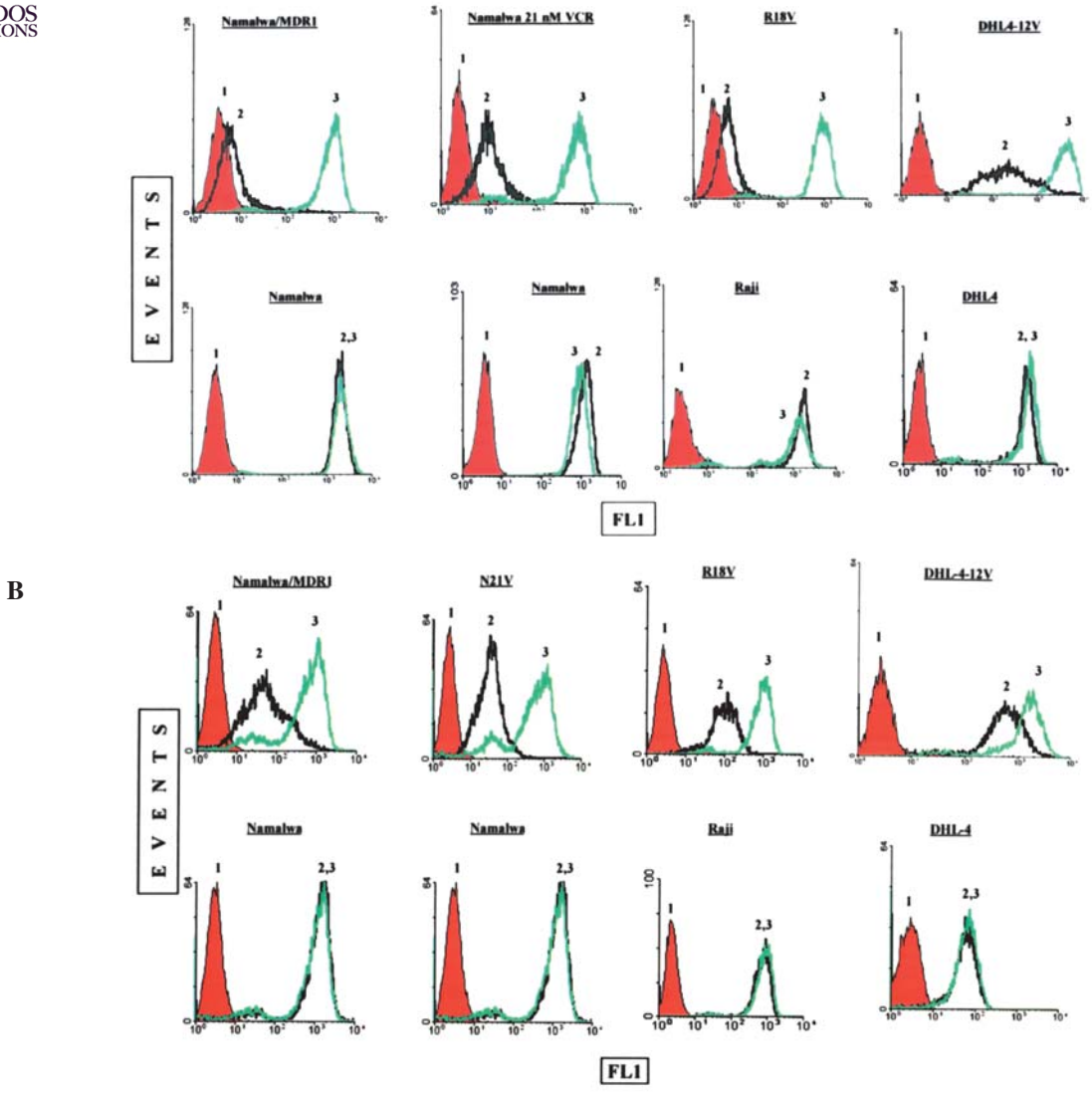


Figure 2. The cytotoxic effect of VCR on MDR vs. their parental tumor cells. 1.2 million cells/ml were cultured in 96-well plates either in media or in the presence of different concentrations of VCR (0.1-12 nM). After 72 h of incubation at 37˚C the cytotoxic effect was tested by a colorimetric method using the CellTiter 96® AQ_{ueous} One solution cell proliferation assay. The absorbance of the non-treated cells was taken as 100% and the percent reduction of the absorbance of the treated cells was calculated. (A) Namalwa (●), Namalwa/MDR1 (○) and Namalwa 21 nM VCR (N21V) (▼); (B) Raji (■) and Raji 12 nM VCR (R12V) (□). This is one representative experiment of three performed.

The MDR cells grow in SCID mice and are drug-resistant. N21V, R18V and Namalwa/MDR1 cells were inoculated into SCID mice and the tumor take was 100%. All mice were consistently paralyzed 20 days after tumor cell inoculation. The treatment of SCID mice with VCR demonstrated that all the MDR tumors were resistant, whereas the parental counterparts responded to VCR and xenografted SCID mice survived significantly longer than the untreated controls (Fig. 5 and Table IV). VCR was less effective in mice with Namalwa than with Raji tumors, which is consistent with the cytotoxic effect of the drug on these cells in the culture as shown in Table III.

Discussion

We have previously reported that two MAbs, HD37 anti-CD19 and RTX anti-CD20, chemosensitized Namalwa/MDR1 cells and inhibited the activity of P-gp (13,14). To confirm these results it was important to use more than one cell line. Moreover, we needed stable MDR cells generated in a manner similar to that developed by patients after chemotherapy. For this reason, we generated three new drug-resistant cell lines by progressive adaptation to 3 nM increments of VCR. We chose to generate: i) N21V cells, as an alternative to Namalwa/MDR1 cells; ii) R18V cells since Raji cells express more CD20 than Namalwa cells; and iii) DHL-4/12V cells, which are derived from diffuse hystiocytic lymphoma cells, whereas the others are Burkitt's lymphomas. The new MDR cells expressed P-gp but at a lower density than the Namalwa/MDR1 cells. However, the P-gp pump was as active in the new cells as in the Namalwa/MDR1 cells despite the lower density of P-gp. The selected VCR-resistant cells remained $P\text{-}gp^+$ and VCR-resistant even after several passages without VCR in the media. The same observation was made by another group who generated L1219/VCR-resistant cells (16). Moreover, P-gp was blocked by Verapamil and Valspodar suggesting that the pump was functional. The activity of P-gp was also demonstrated by the lack of a cytotoxic effect, not only of the drug used for selection (VCR) but also of other drugs. The fact that these cells are resistant to other drugs demonstrates a typical MDR phenotype when overexpression of the membrane efflux pumps (e.g. P-gp) is involved in MDR. Other studies indicate that drug resistance may be very specific for the drug used, due to abnormal genetic machinery such as gene amplification within the tumor cells (17). A study performed with a different cell line (MOLT-4) showed that the acquisition of the resistance was relatively reagent-specific (18). However, it is well known that P-gp, a member of the ATP-binding cassette transport family, can confer resistance against a large number of functionally and chemically distinct cytotoxic compounds. Several investigations suggest that P-gp contains multiple drug-binding sites rather



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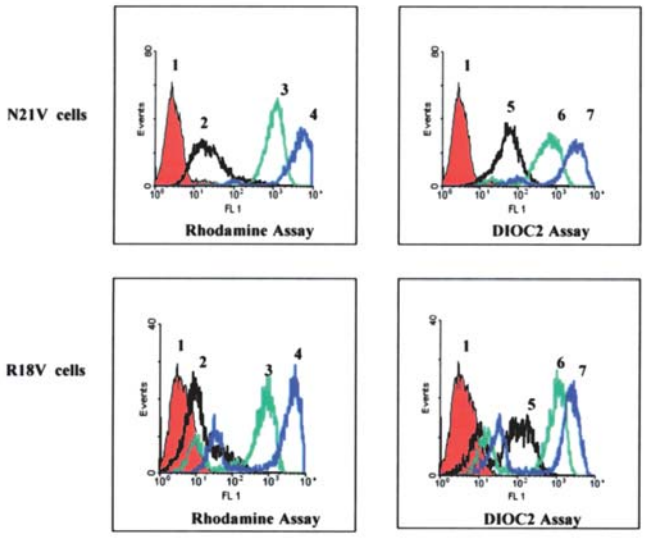

Figure 3. The P-gp pump is functional in MDR cell lines. (A) A Rhodamine 123 efflux assay. Cells ($5x10^5$/ml) were treated for 1 h at 37˚C (influx step) with 3 μM Rhodamine alone or in combination with the P-gp substrate/blocker Verapamil (10 μM). Cells were then washed twice with RPMI-1640 without FBS, resuspended in FBS-free media and incubated for 2 more h at 37˚C to determine the efflux of the dye. The histograms (top panel) represent the MDR cells and the parental counterparts (bottom panel). (1) Cells + media, (2) cells + Rhodamine 123 only and (3) cells + Verapamil + Rhodamine 123. Each histogram is one representative experiment of 3-5 performed. (B) A DIOC2 efflux assay. The protocol was essentially the same as the one described in (A). DIOC2 was used at 0.55 μM. The top histograms represent the MDR cells and the bottom the parental counterparts. (1) Cells + media, (2) cells + DIOC2 only and (3) cells + Verapamil + DIOC2. This is one representative experiment of 2 performed. (C) Verapamil and Valspodar specifically block the efflux of Rhodamine and DIOC2. The top histograms show the Namalwa 21 nM VCR (N21V) cells and the bottom histograms the Raji 18 nM VCR (R18V). (1) Cells + media, (2) cells + Rhodamine only, (3) cells + Verapamil + Rhodamine, (4) cells + Valspodar + Rhodamine, (5) cells + DIOC2, (6) cells + Verapamil + DIOC2 and (7) cells + Valspodar + DIOC2. This is one experiment of two performed.

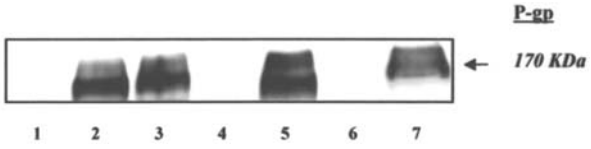

Figure 4. WB analysis of P-gp overexpression in the new MDR cells (N21V, R18V and DHL-4/12V) and Namalwa/MDR1 vs. P-gp$^-$ parental cells. Equal amounts of protein from each cell lysate were loaded and the gels were scanned and analyzed quantitatively to compare the amount of P-gp expressed by each MDR cell line: (1) Namalwa, (2) Namalwa/MDR1, (3) Namalwa 21 nM VCR (N21V), (4) Raji, (5) Raji 18 nM VCR (R18V), (6) DHL-4 and (7) DHL-4/12 nM VCR (DHL-4/12V).

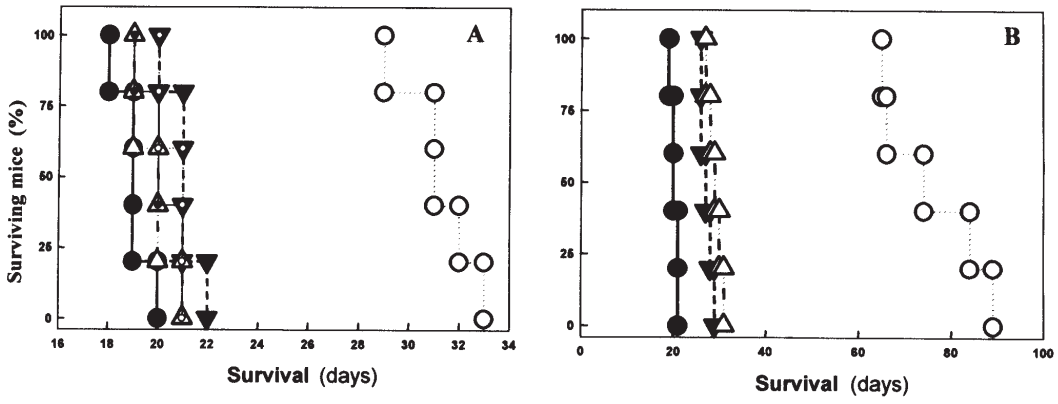

Figure 5. Survival of SCID mice xenografted with parental tumors (Namalwa and Raji) or MDR tumors (Namalwa/MDR1, N21V and R18V) before and after treatment with VCR. The mice were inoculated with $5x10^6$ tumor cells and treated with VCR as described in Materials and methods. The paralysis time is shown in the figure. This is one representative experiment of two performed. In each experiment, 5 mice were used per group. (A) shows the survival of SCID mice xenografted with Namalwa cells ± VCR (●, control and ○, VCR); Namalwa/MDR1 ± VCR (▼, control and △, VCR) and N21V ± VCR (●, control and ○, VCR). (B) shows the survival of SCID mice xenografted with Raji ± VCR (●, control and ○, VCR) or R18V ± VCR (▼, Control and △, VCR).

Table IV. The survival of tumor-bearing SCID mice treated with VCR vs. non-treated (control).

Cell line	Mean paralysis time (MPT)[a] (days) Control	VCR	p[b] VCR vs. control
Namalwa (N)	20.2±1.1	42.8±6.4	0.03
N MDR1	21.0±0.7	20.0±0.8	0.10
N 21 V	19.0±0.0	20.0±1.1	0.09
Raji	20.2±0.8	75.6±10.7	0.02
R18V	27.2±1.3	29.0±1.6	0.10

[a]Groups of 5 SCID mice were sacrificed when they developed hind-leg paralysis. The data are the mean of 2-3 experiments performed. [b]p is the coefficient of significance as determined by the log-rank test to compare the survival time of mice.

than a single site of broad substrate specificity. Martin *et al* (19) found that a minimum of four binding sites can be assigned to P-gp and these multiple sites display complex allosteric interactions. These data support our finding that P-gp induced by selection to VCR confers resistance to other drugs.

The WBs showed that the total amount of P-gp in the new MDR cells differs. The N21V and Namalwa/MDR1 cells contain a comparable amount of P-gp whereas R18V contain more and the DHL-4/12V cells contain less P-gp than the Namalwa/MDR1. Importantly, the P-gp phenotype was stably expressed not only *in vitro* but also in tumors grown in SCID mice.

In conclusion, we have generated three different B-lymphoma tumor cells which stably express P-gp and have an active pump that is responsible for drug efflux from the MDR tumor cells. We did not find significant differences between our MDR cells obtained by drug-selection and the Namalwa/MDR1 generated by retroviral infection with the human *mdr1* gene. Importantly, these new MDR cells express three other B-lymphoma markers (CD19, CD20 and CD22) at levels similar to those expressed by their parental counterparts and with the Namalwa/MDR1 cells, suggesting that they can be used for MAb therapy. Studies carried out with other drug-resistant cells obtained by transfection with the *mdr1* gene have demonstrated that drug resistance conferred by this procedure is not as robust as that of drug-selected MDR cell lines (20,21). Yu *et al* (21) found that when MCF-7 breast carcinoma cells were transfected with the human *mdr1* gene, they expressed levels of P-gp equivalent to those of cells selected for resistance to doxorubicin but exhibited 10-50-fold less resistance to doxorubicin and vinblastine. The fact that the cells described in this report stably express P-gp *in vitro* as well as *in vivo* suggests that we have induced a typical MDR phenotype characterized by the overexpression of P-gp. Moreover, drug resistance was similar to that observed in the Namalwa/MDR1 cells. These new cell lines should be useful for a variety of applications and will be provided upon request.

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