Selection of non-P-glycoprotein mediated high-level etoposide resistant cell lines by adriamycin with P-gp inhibitors

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Abstract. In murine erythroleukemia (MEL) A20 cells (grown in 20 ng/ml adriamycin), mutation(s) producing 10-fold adriamycin (doxorubicin) resistance emerged via an unknown mechanism. Exposure of A20 cells to further stepwise increasing concentrations of ADR in combination with MDR modulators (PSC833 and verapamil) aimed to amplify the undetermined A20 mechanism while controlling P-glycoprotein (P-gp) overexpression. The growth of the derived cell lines A30P, A40P and A60P (grown in 30, 40 and 60 ng/ml ADR with PSC833 and verapamil) was initially slow, but eventually reached near WT rates. The new cell lines A30P and A40P were only 1.3- and 1.6-fold more resistant to adriamycin than PC4 A20. Resistance to vincristine was unchanged, but resistance to etoposide (VP-16) was 3.7-fold higher in A40P than A20 (itself 97-fold higher than wild-type). Expression of mdr3 and mpr mRNA tested by RT-PCR showed no increase. Daunorubicin and etoposide accumulation was not different among the cell lines, and no changes were detected in the number of daunorubicin fluorescent lysosomes. In comparison to WT, reduced topoisomerase IIα activity (20%) and protein expression (80%) was similar to the parental A20 cells. No mutations in the coding sequence of topoisomerase IIα could be located to account for the high etoposide resistance levels. The inhibitor combination of verapamil and PSC833 prevented the emergence of transporter mediated MDR, but not ADR selection of cell lines highly resistant to etoposide.

Introduction

The development of drug resistance in cancer cells is a major problem in clinical oncology. Although observations of a large number of cell lines indicate that resistance emerges in steps from low, through intermediate, to high levels, the genetic and/or biochemical basis for early drug resistance is unclear. The in vitro multidrug resistance (MDR) phenotype, a late form of acquired resistance, is characterized by reduced susceptibility to structurally dissimilar antineoplastic agents including anthracyclines, Vinca alkaloids and epipodophyllotoxins (reviewed in refs. 1 and 2). The predominant mechanism of anthracycline induced cellular MDR is activation of efflux proteins P-glycoprotein (P-gp, MDR1) and multidrug resistance protein (MRP), which results in the transport of xenobiotics to the outside of the cell. However, resistance to anthracyclines can also be attributed to topoisomerase IIα (EC 5.99.1.3) alterations (3), subcellular redistribution into lysosomes (4,5), enhanced drug detoxification (6,7) and abrogation of apoptosis (8).

There has been a relatively less effort to identify low-level mechanisms of resistance in cancer cells prior to the acquisition of high-level mechanisms, such as the P-gp pump (9,10). Low to intermediate resistance mechanisms may be more clinically relevant than those of highly resistant cell lines, as only 5-fold resistance may be all that is necessary to overcome the effects of cytotoxic agents in vivo. Further, because of the toxicity of many chemotherapy drugs, there are restrictions on higher dosing regimes, limiting the concentration of drug acting upon cancerous tissues.

Previously, our laboratory generated and described a series of MDR murine erythroleukemia (MEL-PC4) cell lines selected by exposure to ADR in sequentially increasing amounts. Resistance emerged in a stepwise fashion (11) from low-level resistance to high-level (P-gp mediated) resistance to multiple classes of drugs (12). The initial adaptation of PC4 cells to increasing doses of ADR (5-10-20 ng/ml) was associated with reduced topoisomerase IIα and IIβ enzyme levels (13), and increased c-Fos expression (14). Only in A40 cells (grown in
40 ng/ml ADR) was P-gp expression up-regulated. The low-level mutation(s) associated with A20 cells (derived in 20 ng/ml ADR) are, as yet, unknown. The resistance phenotype in A20 cells is not modulated by MDR inhibitors, nor is it accompanied by alterations in glutathione/glutathione S-transferase levels, internal pH, or overexpression of mdr3 (murine homologue of MDR1), mrp, or mvp (murine homologue of LRP) genes (13).

We describe the development and characterization of new cell lines selected by ADR, along with P-gp inhibitors, designed to amplify the unknown mechanism for the A20 cell line. The new cell lines showed a small increase in ADR resistance but exhibited high levels of etoposide resistance without the up-regulation of ABC type efflux pumps.

Materials and methods

Materials. All drugs were purchased from Sigma-Aldrich (Milwaukee, WI, USA) or Invitrogen (Carlsbad, CA, USA). PSC833 (Valspodar) was obtained from Novartis Pharma AG (Basel, Switzerland). SYBR Green PCR Master Mix and Multiscribe Reverse Transcriptase were purchased from Applied Biosystems (Foster City, CA, USA).

Cell viability assay. Cell viability was measured by the MTT assay after 48 h, as described previously (15). Ninety-six well plates were read using a Spectramax 250 plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The IC_{50} is the concentration of drug that results in a 50% decrease in cell viability. Assays were typically performed after 25 and 30 passages in culture conditions.

Growth of cell lines. The original selected PC4 series of drug resistant cell lines were named to reflect the highest concentration of ADR in which they were selected, i.e. PC4 A20 were selected by 20 ng/ml ADR (13). Cells were cultured in Basal Medium Eagle (BME), 10% FBS, L-glutamine and 100 μg/ml gentamicin. Cell counts were made using a model ZBI Coulter Counter.

The A30P, A40P and A60P cell lines were derived by exposure of A20 cells to 30, 40 and 60 ng/ml of ADR, respectively, in the presence of PSC833 0.2 μg/ml and verapamil 1 μM. A new control cell line (A30co) was derived for the present study by repeated passage of A20 cells in the presence of ADR (30 ng/ml) without any inhibitors. Inhibitor concentrations were chosen so as not to adversely affect cell growth, but to inhibit pump mediated ADR resistance by >90% in P-gp overexpressing PC4 A80 cells.

Cell line growth under adriamycin selection in the presence of efflux protein inhibitors. To inhibit expression of ABC type efflux proteins, we used various combinations of PSC833 and verapamil. PSC833 is a cyclosporin A derivative which exerts a higher MDR reversal activity and lacks the toxic or immuno-suppressive side effects of cyclosporin A (16-19). Cell viability assays were performed over a range of inhibitor concentrations to determine the maximum concentration that supported 90% cell growth compared to control cultures (data not shown). Subsequent assays then used this concentration of PSC833 in combination with ADR in the highly P-gp expressing A80 cell line to determine if the concentration was sufficient to reduce the IC_{50} of ADR to that of the A20 cell line. The fold resistance to ADR of the A80 cell line decreased from 71.1 to 9.7 and 8.3 in the presence of 0.2 μg/ml PSC833. These values matched the resistance of the A20 cells, determined both in our hands (10.0±2.3, data not shown), and in previous published work [17-fold resistance; (11)]. Thus, the concentration of inhibitors used to develop these cell lines was sufficient to inhibit the efflux proteins.

Cell lines meant to amplify the uncharacterized mechanism present in A20 were initiated by exposure to increasingly higher concentrations of ADR in the presence of MDR1 inhibitors as described in the methods. On exposure to 30 ng/ml ADR in the presence of inhibitors, growth was initially slow, with doubling times of 17.1 (A30co) and 22.1 (A30P) h, compared with a doubling time of 10.5 h for A20. The experimental cell lines stabilized at doubling times of 14.3 h (A30P) passage 22; A40P and A60P showed growth rates similar to A30P.

Fluorescence RT-PCR. Quantitative one-step fluorescent RT-PCR was performed using an ABI PRISM 7500 Sequence Detector. Total cellular RNA was extracted using Trizol Reagent (GibcoBRL, Grand Island, NY, USA), followed by DNase treatment, phenol-chloroform extraction, and ethanol precipitation. A non-specific DNA-intercalating dye contained within the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and Multiscribe™ Reverse Transcriptase were used in RT-PCR experiments according to product specifications. The total volume of a one-step reaction was 25 μl including 12.5 μl of 2X Master Mix, 5 μl primers, 5 μl tRNA/water (1 mg/ml), and 2.5 μl of RNA template (0.2 mg/ml). Thermocycling parameters were 30 min at 48˚C for reverse transcription (RT), 10 min at 95˚C for RT inactivation and DNA polymerase activation, 94˚C denaturation (30 sec), 53˚C annealing (1 min) and 72˚C synthesis (1 min). Product dissociation curves were analyzed to ensure appropriate product size and absence of template contamination in controls.

Relative standard curves were generated using amplicon-containing plasmids to enable comparison of mRNA expression among cell lines. All samples were performed in triplicate over a minimum of 2 separate experiments. Primers used were mdr1/mdr3: forward, AAGGGGCTCT ACCTGAAAGT (nucleotides 1355-1374) and reverse, CCTGAGAGGCA CTAGG (nucleotides 1550-1531), product size 196 bp; murine mrp1: forward, ATG GGCATGAAAGCACAGAC (nucleotides 1450-1469) and reverse, CTTGCCG AGTGCAGATTT (nucleotides 1642-1623), product size 193 bp; β-actin: forward, TGACATCCGTAAGAC (nucleotides 782-799) and reverse, CTTGCTGATTCCACTG (nucleotides 999-982), product size 217 bp.

Fluorescence microscopy. Cells (1x10^6 /ml) were incubated in BME containing 500 ng/ml daunorubicin (DNR) for 1 h. Subsequently, cells were washed with ice-cold phosphate-buffered saline (PBS), resuspended in PBS containing 5 mM glucose, and kept on ice until viewing with a Leica TCS SP2 confocal microscope or an upright Leica DMRXE microscope using a x63 (1.32 NA) apochromatic lens. The DNR was excited at the 488 nm wavelength with an argon laser.
Table I. Cell viability assay results in transport protein inhibitor selected MEL cell lines.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Drug</th>
<th>A20\textsuperscript{b}</th>
<th>A40\textsuperscript{c}</th>
<th>A80\textsuperscript{c}</th>
<th>A30\textsuperscript{d}</th>
<th>A30P\textsuperscript{d}</th>
<th>A40P\textsuperscript{d}</th>
<th>A60P\textsuperscript{d}</th>
</tr>
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<tr>
<td>ADR\textsuperscript{b}</td>
<td>10.0±2.3</td>
<td>19.2±4.5</td>
<td>61.5±17.1</td>
<td>58.2±19.6</td>
<td>13.3±3.2</td>
<td>15.5±0.1</td>
<td>-</td>
</tr>
<tr>
<td>VCR\textsuperscript{b}</td>
<td>1.6±0.5</td>
<td>3.5±1.1</td>
<td>11.0±6.5</td>
<td>12.3±0.6</td>
<td>2.3±0.6</td>
<td>2.2±0.4</td>
<td>-</td>
</tr>
<tr>
<td>VP-16\textsuperscript{b}</td>
<td>97.0±9.0</td>
<td>nt\textsuperscript{e}</td>
<td>209.0±26.0</td>
<td>nt</td>
<td>nt</td>
<td>371.0±9.0</td>
<td>nt</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Measured by MTT assay; experiment performed minimum of 3 times. Values are reported as fold resistance compared to PC4-WT calibrator. Baseline IC\textsubscript{50} concentrations for WT are 10.8±3.2, 1.3±0.4 and 35±5.0 ng/ml for ADR, VCR and VP-16, respectively. \textsuperscript{b}ADR, adriamycin; VCR, vincristine; VP-16, etoposide. \textsuperscript{c}Selected in adriamycin at 20, 40 and 80 ng/ml (reviewed in ref. 11). \textsuperscript{d}Cell lines selected in ADR 30 or 40 ng/ml with (P) or without (co) efflux protein inhibitors (see text). \textsuperscript{e}nt, not tested.

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Results

Determination of multidrug resistance phenotype in cell lines grown in the presence of ADR and efflux protein inhibitors. Cell viability (MTT) assays were performed on the newly-derived cell lines to determine the degree of resistance to ADR, vincristine, and etoposide (Table I). Using the WT cell line as a calibrator, results showed an incremental increase in resistance to ADR from 10 in A20 to 13.3- and 15.5-fold-resistance for A30P and A40P cells, respectively. Resistance profiles of the MDR expressing A80 cell line and the newly derived control line A30co were elevated and comparable to previous work (11-13). The data for vincristine susceptibility were similar to A20 cell lines. However, higher etoposide resistance was found in the A20 derived cell lines as compared to A20 (Table I).

Lack of expression of mdr3 and mrp genes in cell lines developed in the presence of efflux protein inhibitors.
Quantitative fluorescent RT-PCR was used to ensure that
*mdr1*, *mdr2* and *mdr3* (the murine genes homologous to human
*mdr1*) and the *mrp* gene were not overexpressed. No increase
in *mdr3* or *mrp* expression was seen in A30P or A40P cells,
while A30co cells showed a 4-fold increase in *mdr3* expression,
compared to WT (Table II). PC4 V40, a cell line obtained
through exposure of WT cells to 40 ng/ml vincristine serves
as a positive control for the *mrp* expression RT-PCR assays
(21). As expected, there was an 18-fold increase in *mdr3*
expression in A80 cells, serving as a positive control for *mdr3*.

**Table II. Fluorescence RT-PCR results for *mdr* and *mrp* mRNA expression in single drug and transport protein inhibitor
selected MEL cell lines.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>A20</th>
<th>A40</th>
<th>A80</th>
<th>V40</th>
<th>A30co</th>
<th>A30P</th>
<th>A40P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mdr</em></td>
<td>1.4±0.1</td>
<td>1.5±0.4</td>
<td>17.7±8.6</td>
<td>nt</td>
<td>4.3±1.3</td>
<td>1.2±0.2</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td><em>mrp</em></td>
<td>3.1±1.0</td>
<td>1.7±0.6</td>
<td>1.2±0.1</td>
<td>43.6±10.1</td>
<td>2.1±0.4</td>
<td>3.2±1.2</td>
<td>3.6±2.5</td>
</tr>
</tbody>
</table>

*pValues are reported as fold increase compared to PC4-WT calibrator. *mdr*, 200 bp amplicon found in both *mdr1* and *mdr3* murine isoforms of
the multidrug resistance gene; *mrp*, multidrug resistance-related protein; *PC4-V40* was the positive control cell line used in the *mrp* RT-PCR.
Selected previously with 40 ng/ml vincristine (13). *Selected in adriamycin at 20, 40 and 80 ng/ml (11). *Cell lines selected in Adriamycin 30 or
40 ng/ml with (P) or without (co) efflux protein inhibitors (see text). *nt, not tested.

**Figure 1.** Daunorubicin (DNR) subcellular distribution in PC4 cells. Cells were
incubated for 1 h with 500 ng/ml DNR, washed and resuspended in PBS
before viewing at 63X with a Leica TCS SP2 confocal microscope. DNR was
excited at 420 nm with emission of 600 nm. The number of acidic vesicles in
each cell line was: WT, 17±6.2; A30co, none detected; A20, 21±3.7; A40P,
18.8±4.6. Experiments were repeated a minimum of three times.

**Figure 2.** Accumulation of [3H]-etoposide into MEL-PC4 cells. Cells were
incubated with 4 μM [3H]-etoposide for 1 h at 37°C. A 200 μl sample was
layered over silicone oil over concentrated formic acid. The tube was centri-
fuged and then frozen at -80°C. The frozen formic acid layer containing the
cell pellet was cut-off and counted by liquid scintillation. Experiments were
repeated a minimum of three times.

**Daunorubicin and etoposide accumulation in cell lines
developed in the presence of inhibitors.** The subcellular dauno-
rubicin distribution within selected cell lines was examined
by confocal microscopy. Daunorubicin accumulation into cyto-
plasmic vesicles detected by fluorometric methods (5,11)
was observed in all cell lines, except for the control cell line
A30co (data not shown). This finding indicated that the efflux
pump was up-regulated and functional in the control cells
under experimental conditions. No changes were detected in
the number of fluorescent lysosomes (Fig. 1), confirming that
the observed increase in resistance in the selected cell lines
was not due to an altered intracellular distribution pattern of
DNR.

We measured the accumulation values of [3H]-labeled
VP-16 for the drug and inhibitor selected cell lines (Fig. 2).
The results indicated no change in cellular uptake or efflux
for the ADR and PSC833 + verapamil selected cells. There-
The resistance phenotype of A20 cells includes a decrease in both topoisomerase IIα and IIβ gene and protein expression. In order to investigate the underlying mechanism(s) of resistance contributing to the A20 phenotype, we attempted to amplify the ADR resistance of this cell line in the presence of combinations of transporter protein inhibitors, namely PSC833, and verapamil, which have been shown to inhibit activity of key ABC transporters (13-22). In our study, the inhibitor combination prevented up-regulation of mdr3 or mrp mRNA expression, and resulted in only a small increase in ADR resistance but a large increase in etoposide resistance. The latter was not associated with a change in daunorubicin or topoisomerase IIα intracellular accumulation. These data point out the usefulness of PSC833 as an inhibitor of increased expression of efflux pump proteins.

Most studies have focused on the use of inhibitors in cell lines that already express P-gp (16-19). However, PSC833 (3,23,24), or verapamil (25), have previously been used to inhibit the emergence of MDR, with varying success. In one report, following selection with PSC833 and ADR in MES-SA human sarcoma cells, a mutant P-gp variant with decreased affinity for PSC833 and Vinca alkaloids was derived (24). In another study, after a brief 3-week exposure to the same drugs, the only alterations detected were a decrease in topoisomerase IIα mRNA and protein, with an associated drop in catalytic activity (3). Other investigators found that a combination of both PSC833 and CSA was required to prevent MDR1 up-regulation caused by exposure to the drug epirubicin (23). A third group successfully used verapamil and ADR co-selection to derive drug resistant human multiple myeloma cells with decreased topoisomerase II levels and activity, and no P-gp overexpression (25). None of these mechanisms is likely to account for the high etoposide specific phenotype of our derived cell lines.

The resistance phenotype of A20 cells includes a decrease in both topoisomerase IIα and IIβ protein and activity. This type of multidrug resistance, associated primarily with altered topoisomerase IIα, has been reported in cases where a single topoisomerase II interacting drug was used to select the resistance phenotype (20,26-28). A recent study of the multidrug-resistant K562 human leukemia cell line after exposure to the anthracycline derivative MX2 reported reduced topoisomerase IIα protein levels and catalytic activity. Interestingly, resistance was attributed to reduced topoisomerase IIα gene expression and increased methylation of the gene which could be overcome in the presence of a demethylating agent (29). A previous study of the CCRF-CEM cell line, and two teniposide (VM-26) resistant sublines, showed that topoisomerase IIα in nuclear extracts from resistant cells
required a higher concentration of ATP than sensitive cell extracts to achieve equivalent activity in a DNA unknotting assay (20). This study confirmed through DNA sequencing the presence of a G to A base change and corresponding amino acid substitution at a critical ATP interaction site. A number of other resistance causing mutations in the sequence of topoisomerase II alpha have been reported in the literature (27,30-32). Alterations in the phosphorylation status of the topoisomerase IIalpha enzyme have been implicated as causing a resistance phenotype (33). Changes in topoisomerase II can have other effects in the cell that can then lead to chemoresistance. For example, altered topoisomerase II function can lead to an increase in DNA damage, which is a critical signal for transcription factor NF-kB activation. Altered NF-xB activation has been shown to contribute to anthracycline resistance in a human ovarian carcinoma cell line (34), and gemcitabine resistance in pancreatic carcinoma cell lines (35). This and other anti-apoptotic proteins such as IAPs and BCL-2 have been implicated in cellular resistance to cytotoxic agents (36,37).

In summary, our results support a growing body of evidence showing that treatment with multidrug resistance modulators, such as cyclosporin A, PSC833 and verapamil, together with multidrug resistance cytotoxins, can suppress the emergence of ABC transporter mediated resistance. Our results also indicate that even when the efflux pump is effectively inhibited, an extremely high level of resistance can emerge, as observed in the A40P cell line that has an etoposide resistance level 371-fold higher than that of the WT cell line. It should be noted that a topoisomerase II enzyme alteration contributes, at least in part, to the resistant phenotype observed in the A20 cells. Possible explanations are an increase or decrease in phosphorylation of the enzyme, or a redistribution of the drug to outside the nucleus into the cytoplasmic compartment (33,38). However, these changes would not account for an etoposide specific resistance phenotype. Thus, while resistance to etoposide is 3-fold greater in A40P than A20, topoisomerase IIA activity and topoisomerase IIA protein levels are similar among all of the A20 derived cell lines, and reduced as compared to wild-type. Furthermore, in this cell line, there was no evidence of a change in subcellular redistribution of daunorubicin into increasing numbers of lysosomes, and prior work from this group showed no changes in glutathione levels, cellular pH, or expression of the major vault protein (13) in A20 cells. These findings suggest that at least one other mechanism may be accounting for the increased resistance to etoposide in A40P and A60P cells. Given that multiple apoptotic mechanisms are at play which may be distinct for different cytotoxic agents, drug-specific resistance to programmed cell death may be contributing to the phenotype of the new cell lines. Future work will focus on microarray transcription analysis to look at differential gene expression across this new series of cell lines.

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