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**SPONTANEOUS REVERSAL OF P-GLYCOPROTEIN EXPRESSION IN  
MULTIDRUG RESISTANT CELL LINES**

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**Running title:** P-glycoprotein Time Study

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## **ABSTRACT**

Increased expression of P-glycoprotein encoded by the *mdr-1* gene is a well-characterised mechanism for resistance to cancer chemotherapeutic drugs in cell lines. However, the P-glycoprotein expression after removal of the selection pressure has not fully been elucidated. The stability of P-glycoprotein expression in the presence (+) and absence (-) of vincristine (30 or 150 nM) was studied in multidrug resistant K562 cell lines (VCR30+, VCR150+, VCR30- and VCR150-) for 11 months. The P-glycoprotein protein and *mdr-1* mRNA levels were determined at regular intervals using flow cytometry and real-time PCR, respectively. Chemosensitivity to a panel of antineoplastic drugs was measured using an MTT assay. The presence of vincristine (VCR30+ and VCR150+) resulted in high and stable levels of P-glycoprotein and *mdr-1* mRNA during the whole period compared to wild type. As for the VCR30- and VCR150- subcultures, the expressions of P-glycoprotein and *mdr-1* mRNA were stable for five months, and then the levels decreased rapidly. Concomitantly, the sensitivity to drugs known as P-glycoprotein substrates was restored. In conclusion resistant cells growing in the presence of the inducing drug have a stable P-glycoprotein expression and resistance level, but removing the inducing drug may result in a sudden and rapid lowering of P-glycoprotein and *mdr-1* mRNA levels as long as five months after drug withdrawal.

## **INTRODUCTION AND BACKGROUND**

The development of multidrug resistance during cancer chemotherapy is a major obstacle to successful treatment of cancer patients. Increased expression of P-glycoprotein encoded by the *mdr-1* gene is a well-characterised mechanism for cancer cells in culture to avoid the action of chemotherapeutic agents. P-glycoprotein is a 170 kDa plasma membrane protein that functions as an ATP-driven drug export pump. Cytotoxic drugs of natural origin with very different chemical structures and mechanisms of action, such as vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes, can be extruded by P-glycoprotein through the cell membranes of resistant cells and cross-resistance occurs (Gottesman & Pastan 1993; Germann 1996). The level of P-glycoprotein varies in different cells and tumours, and has also been shown to differ in the same patient before and after chemotherapy (Gruber et al. 1992; De Moerloose et al. 2000).

More recently, P-glycoprotein has proved to be a major determinant for the intestinal absorption of drugs such as protease inhibitors,  $\beta$ -blockers, cyclosporin A and digoxin (Fricker & Miller 2002). It has also been shown that drug efflux due to P-glycoprotein is important in the distribution of various substances to the CNS, and may therefore not only be of importance for the therapeutic effect of psychopharmacological drugs, but also the neurotoxicity of chemotherapeutic agents and pesticides (Sun et al. 2003). However, many questions about the regulation and stability of P-glycoprotein expression remain.

Variable levels of P-glycoprotein have been observed in different multidrug resistant cell lines and an increased expression of P-glycoprotein generally correlates with an increase of drug resistance (Fujimaki et al. 2002), however the opposite might not be true due to the presence of other resistance factors (Sonneveld 2000). The induction of the multidrug resistance P-glycoprotein phenotype has been studied extensively, but the maintenance of the expression is less well characterised. In vitro studies have revealed that expression of the *mdr-1* gene is induced by a variety of toxic agents (Kohno et al. 1989; Chaudhary & Roninson 1993), UV radiation (Uchiumi et al. 1993) and heat shock (Miyazaki et al. 1992; Vilaboa et al. 2000). From a biochemical point of view, nuclear factor- $\kappa$ B and stimulating protein-1 interact with the promoter region of *mdr-1* and these two transcription factors cooperate in controlling the basal *mdr-1* promoter activity (Sundseth et al. 1997). Recently, an important role was demonstrated for both nuclear factor- $\kappa$ B and stimulating protein-1 in the transcriptional activation of the *mdr-1* gene after genotoxic stress (Hu et al. 2000). However, activation has also been shown to be regulated in a two-step post-transcriptional process, mediated by changes in mRNA stability and translation (Yague et al. 2003).

Since the discovery of P-glycoprotein, chemical inhibitors have been designed to reverse the drug efflux. Many compounds such as verapamil (Tsuruo et al. 1983), cyclosporins (Twentyman et al. 1992) etc, have been demonstrated to antagonize the multidrug resistant phenotype. Decreasing or reversing of the *mdr-1* gene expression level has proved to be more difficult, but down-regulation in human cells has been accomplished using antisense RNA (Chan et al. 2000) and by raising intracellular reactive oxygen species by depletion of glutathione

(Wartenberg et al. 2001). Recently, curcumin an inhibitor of the AP-1 transcription factor was shown to decrease the *mdr-1* mRNA and P-glycoprotein levels as well as to chemosensitize P-glycoprotein expressing cells (Anuchapreeda et al. 2002). On the other hand, activation of c-jun, a part of the AP-1 complex, has been shown to repress *mdr-1* transcription (Miao & Ding 2003). The biochemical mechanism behind the reduction in P-glycoprotein expression has still not been fully elucidated, but one or several protein kinase C's (Castro et al. 1999) and p53 (Thottassery et al. 1997; Zhan et al. 2001) seem to be involved.

The aim of this study was to determine the stability of P-glycoprotein levels in two previously established vincristine-resistant cell lines (Gruber et al. 1994) during prolonged culturing of the cells in the presence or absence of the P-glycoprotein inducing drug.

## MATERIALS AND METHODS

### *Drugs, Chemicals and Reagents*

The commercially available formulations of etoposide (Vepesid<sup>®</sup>) and paclitaxel (Taxol<sup>®</sup>) from Bristol-Myers Squibb, daunorubicin (Cerubidin<sup>®</sup>) from Rhône-Poulenc Rorer, idarubicin (Zavedos<sup>®</sup>) from Pharmacia & Upjohn and vincristine (Oncovin<sup>®</sup>) from Lilly were used. 9-β-D-arabinofuranosylguanine was from R.I. Chemical Inc. (Orange, CA, USA).

The FITC conjugated mouse IgG<sub>2</sub> anti human P-glycoprotein clone 4E3 antibody was purchased from Alexis Biochemicals (Lausen, Switzerland). The FITC conjugated mouse IgG<sub>2</sub> anti-human P-glycoprotein clone 17F9 and the isotype control antibody (FITC-conjugated mouse IgG<sub>2</sub> anti-dansyl) were purchased from PharMingen (San Diego, CA, USA). RPMI 1640 medium, fetal calf serum, L-glutamine and penicillin-streptomycin were all from Gibco (Life Technologies, Paisley, UK). All reagents for PCR reactions were purchased from Applied Biosystems (Foster City, CA). All other reagents were from Sigma.

### *Cell Lines*

Cells were subcultured twice weekly in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37°C in a humidified air atmosphere containing 5% CO<sub>2</sub>. The cells were routinely tested for *Mycoplasma* contamination. The K562/wt, a human chronic myelogenous leukaemia cell line (Lozzio & Lozzio 1975), was grown in the absence of vincristine. Established resistant K562 cell lines were grown in the presence of 30 nM or 150 nM of vincristine (Gruber et al. 1994). At the start of the experiment, each of the two resistant cell lines were subcultured into two new subclones, growing in the presence of the drug (the VCR30+ cell line and VCR150+ cell

line) and in the absence of the drug (the VCR30- cell line and VCR150- cell line). The day when the drug was withdrawn from the VCR30- and VCR150- cell lines was denoted day 0.

### ***Chemosensitivity Assay***

The chemosensitivities to vincristine, daunorubicin, idarubicin, paclitaxel, etoposide and 9- $\beta$ -D-arabinofuranosylguanine were assessed using the MTT assay (Mosmann 1983). In brief, the 96 well plates were set up with cells (100  $\mu$ l/well) at the initial density of  $2 \times 10^5$  cells/ml and were incubated at 37°C for 72 hours in an atmosphere of 5% CO<sub>2</sub> in the absence and presence of nine different concentrations of each drug in triplicate. After incubation, 10  $\mu$ l of MTT solution (5 mg/ml tetrazolium salt) was added to each well and the plates were reincubated for 4 hours at 37°C. The formazan salt crystals formed were dissolved by adding 100  $\mu$ l of 10% SDS in 10 mM HCl solution and incubating overnight at 37°C. The absorbance was measured at 540 nm with a reference at 650 nm by a 96-well ELISA plate reader (Labsystems Multiscan RC, Helsinki, Finland). The chemosensitivity was expressed as IC<sub>50</sub>, which is the concentration of drug causing 50% cell survival compared to control cells grown without drug. The chemosensitivity was measured at the start (drugs added day 0) and repeatedly after a change could be identified in the P-glycoprotein expression in the VCR30- and VCR150- cell lines.

### ***RNA Isolation and Real-time Quantitative PCR***

The Taqman technology was used and samples were analysed by means of the ABI Prism 7700 instrument (Applied Biosystems, Sweden), as previously described (Lotfi et al. 2002b). RNA was extracted from  $3 \times 10^6$  cells from each of the cell lines using QIAshredder<sup>®</sup> homogeniser spin column technology (QIAGEN, VWR International, Sweden) and a RNeasy<sup>®</sup> mini-kit (QIAGEN) according to the manufacturer's instructions. cDNA was

synthesised using the Reverse-IT RT-PCR kit (ABgene, VWR International, Sweden). To remove any secondary structures, the samples were heated briefly at 70°C for 5 minutes. The thermal conditions in the RT-PCR reaction were 42°C for 1 hour and 75°C for 10 minutes. Primers and probes were designed using Primer Express™ (Applied Biosystems) with some manual modifications and purchased from Scandinavian Gene Synthesis AB (Köping, Sweden). The forward primer for mdr-1 was 5'-TCC ATG CTC AGA CAG GAT GT -3' and the reverse primer was 5'-CTG GAA CCT ATA GCC CCT TT-3'. The sequence of the probe was 5'-FAM-AA CAC CAC \*GG AGC ATT GAC TAC CAG GT -3' (\* = dark quencher), with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5' end and with an internal dark quencher. Cyclophilin, huCYC (Applied Biosystems), was used as an internal endogenous control. VIC was used as a reporter dye for cyclophilin. The PCR reaction mixture (50 µl) contained TaqMan® mastermix (Applied Biosystems), in combination with designed forward and reverse primers (300 nM each), a probe (25 nM) and 10 ng of cDNA. The reactions were performed for 2 min at 50°C, 10 min at 95°C and then 40 cycles of amplification for 15 s at 95°C and for 1 min at 60°C.

### ***Flow Cytometric Analysis of P-glycoprotein***

The flow cytometry analysis of P-glycoprotein was set up in our laboratory according to the recommendations by Beck et. al (Beck et al. 1996). The cells were counted in a Coulter multisizer (Coulter Electronics, Luton, UK), 10<sup>6</sup> cells were centrifuged for 10 min at 600g and then resuspended in PBS. FITC conjugated anti-P-glycoprotein antibodies at 1µg/10<sup>6</sup> cells (clone 4E3 (Arceci et al. 1993), Alexis Biochemicals or clone 17F9 (Shi et al. 1995), Pharmingen), an isotypic antibody at 1µg/10<sup>6</sup> cells (Pharmingen) or PBS was added to yield a final volume of 100 µl. The samples were incubated for 30 min at room temperature. The cells were then centrifuged for 5 min at 600g and washed with 200 µl PBS. After

centrifugation, the cells were resuspended in 1 ml PBS and 15 000 cells were analysed on a FACScalibur (Becton Dickinson, San Jose, CA, USA). Before and after analysis, the flow cytometer was calibrated using Quantum Beads (medium range, Dako, Denmark).

### *Statistics and Calculations*

Data from the real-time PCR analysis was further processed in the Sequence Detection Software (Applied Biosystems) to create amplification plots and to determine CT-values (threshold cycle). The mean CT value of each triplicate was calculated. cDNA from a resistant cell line was diluted to create standard curves (range over three decades) for both *mdr-1* ( $R^2 > 0.996$ ) and cyclophilin ( $R^2 > 0.993$ ). The corresponding amount of cDNA for each triplicate was calculated from the standard curves. The ratio between *mdr-1* and cyclophilin was then calculated and denoted the *mdr-1* mRNA level. The between-days coefficient of variation calculated for ng *mdr-1*/ng CYC was <20%.

The CellQuest programme (version Becton Dickinson, San Jose, CA, USA) was used to analyse the flow cytometry data. Histograms were created for the viable cell population of each sample. The median fluorescence intensity (MFI) for the fluorescence channel corresponding to the P-glycoprotein expression was calculated for the cell lines and for the beads. For each run, a standard curve was created from the MFI of the beads and the known number of fluorochromes corresponding to each bead ( $R^2 > 0.999$ ). The MFI values for each cell sample were converted to the number of molecules of equivalent soluble fluorochromes (MESF). The MESF value from the isotypical control of each sample was subtracted from the value obtained when using the specific antibodies, in order to eliminate the influence of non-specific binding. The specific MESF was then considered to be a measurement of P-glycoprotein expression. The within-day and between-days variations for the instrument,

calculated using data from the beads, were  $CV < 0.5\%$  and  $CV < 13\%$ , respectively. The between-day coefficient of variation of the analyses, calculated from five consecutive analyses on the drug-resistant cell line, was  $< 28\%$  and  $< 12\%$ , respectively, for the clone 4E3 and clone 17F9 antibodies.

In the MTT assay, the mean of each triplicate was calculated and normalised to the control cells growing without drug. The  $IC_{50}$  value was calculated using linear regression analysis on two to five points around the value in question using Microsoft Excel.

## RESULTS

All five subcultures, K562/wt, VCR30+, VCR150+, VCR30- and VCR150- cell lines (grown in presence + or absence - of 30 or 150 nM vincristine) were followed for 11 months. The *mdr-1* levels and P-glycoprotein expression were analysed at regular intervals. The chemosensitivity pattern of the K562 cells to a panel of antineoplastic drugs is shown in table 1. The IC<sub>50</sub> values for the K562/wt cell line were in the same range as previously reported for that cell line (Gruber et al. 1994). The K562/wt cell line had no detectable P-glycoprotein expression on comparing the specific antibodies with the isotypical control. The *mdr-1* mRNA level of the K562/wt cell line was not detectable after 40 cycles in the real-time PCR throughout the study period.

### *Flow Cytometry Analysis of P-glycoprotein Expression*

The VCR30+ and VCR150+ cell lines showed a high and stable P-glycoprotein level during the study period compared to the K562/wt cell line (Figures 1a and 1b). During the first five months, the VCR30- and VCR150- cell lines also showed a stable expression. Thereafter (at 185 days), a rapid decrease in the protein level could be noted (figures 1a and 1b). At 185 days, the expression was slightly higher in the VCR30- cell line compared to VCR150- cell line (figures 1a and 1b), which is consistent with higher IC<sub>50</sub> values for vincristine, paclitaxel and daunorubicin at day 189 (table 1). The P-glycoprotein level in the VCR30- cell line was, during the last six months, less than 0.6% (clone 17F9) and 1.3 % (clone 4E3) compared to the average during the first five months and, for the VCR150- cell line, it was less than 3.3% (clone 17F9) and 3.9% (clone 4E3) (figures 1a and 1b). The expression of P-glycoprotein on these cell lines did not reach the same low level as for the wild type, however. On the contrary, a slight increase could be noticed during the last months.

On analysing the distribution of the cellular P-glycoprotein expression, it could be seen that by 150 days some of the cells in the VCR30- and VCR150- cell lines had lost their P-glycoprotein expression and two populations were present (figure 2, column 2). After a further 35 days, the non-expressing population was dominant (figure 2, column 3) and by 214 days, the P-glycoprotein-positive cell population was almost non-detectable (figure 2, column 4). However, the expression did increase a little during the last months of the study and at 343 days the VCR150- cell line had a small fraction of P-glycoprotein-positive cells (figure 2, column 5).

#### ***Real-Time PCR Analysis of *mdr-1* mRNA Levels***

In the resistant cell lines, grown in the presence of vincristine, the *mdr-1* mRNA expression was stable during the whole period (figure 3). For the VCR30- and VCR150- cell lines, the levels were in the same range as the resistant subclones during the first five months and then they decreased concomitantly with the P-glycoprotein expression (figure 3). After the decrease, the expression remained less than 5.2% and less than 34% for the VCR30- and VCR150- cell lines, respectively, compared to the average of the first five months.

#### ***In Vitro Drug Chemosensitivity***

MTT assays were performed at the beginning of the study (day 0) and again as soon as a change in the P-glycoprotein expression could be detected. The K562/wt cell line had low  $IC_{50}$  values for all the drugs tested in this experiment (table 1). The resistant cell lines, VCR30+ and VCR150+, had high  $IC_{50}$  values for P-glycoprotein substrates such as daunorubicin, vincristine, idarubicin, paclitaxel and etoposide. All subcultures except K562/wt were highly resistant to 9- $\beta$ -D-arabinofuranosylguanine and retained their resistance to the nucleoside analogue throughout the study period (table 1). At the same time as the P-

glycoprotein and *mdr-1* mRNA expressions decreased in the VCR30- and VCR150- cell lines, they started to lose their resistance to P-glycoprotein transported drugs i.e. daunorubicin, vincristine, paclitaxel and, to some extent, also etoposide (table 1). They were, however, still less sensitive to these drugs than the wild type was. After another month, the  $IC_{50}$  values for the P-glycoprotein-mediated drugs were even lower. They maintained a slight resistance even after the P-glycoprotein level had decreased and it was not until the final month that the chemosensitivity to most of the drugs was in the same range as for the wild type (table 1).

## **DISCUSSION**

The levels of P-glycoprotein and *mdr-1* mRNA in K562/wt, VCR30+, VCR150+, VCR30- and VCR150- cell lines were followed for 11 months. The VCR30+ and VCR150+ cell lines had stable levels of P-glycoprotein and *mdr-1* mRNA and showed similar chemoresistance patterns for a variety of drugs during the whole period. In the VCR30- and VCR150- subcultures, the levels of P-glycoprotein and *mdr-1* mRNA were stable for the first five months and then they rapidly lost a large portion of their expression. They also became more sensitive to P-glycoprotein substrates, but they still remained highly resistant to 9- $\beta$ -D-arabinofuranosylguanine.

In the VCR30- and VCR150- cell lines, some cells lost their P-glycoprotein expression after five months while others retained the expression a bit longer, giving rise to two distinct cell populations; hence the decrease in the expression of P-glycoprotein and *mdr-1* mRNA was not successive. As the half-life of P-glycoprotein is between 14 and 17 h under normal conditions (Muller et al. 1995) the loss of transcription in part of the population should give rise to two populations, P-glycoprotein expressing and non-expressing cells, if the reversal was not an immediate response in all cells at once. The results show that when working with P-glycoprotein-expressing cell lines in the absence of inducing drug for a long period of time, it is essential to determine the presence of P-glycoprotein and its stability as well as analysing the distribution of the cellular P-glycoprotein expression.

The development of resistance by induction demands the continuous presence of drug, whereas drug resistance created by mutations occurs at random. Several studies have shown that P-glycoprotein can be induced by certain anticancer drugs (Kohno et al. 1989; Chaudhary & Roninson 1993; Nielsen et al. 1998) and that spontaneous mutations can confer P-

glycoprotein-mediated drug resistance (Chen et al. 1994; Dumontet et al. 1996; Nielsen et al. 1998). Licht et al. found that P-glycoprotein was dose-dependently induced in the absence of proliferation, thus supporting the hypothesis that increased P-glycoprotein is associated with induction rather than resulting from selection of a pre-existing drug-resistant subpopulation (Licht et al. 1991). Whether or not the decrease in P-glycoprotein expression in our experiments is due to a spontaneous mutation or reversal due to a loss of stimuli is not known. The cell culture data does not indicate a change in growth rate or cell morphology (not shown). The P-glycoprotein expression does not completely disappear 11 months after the withdrawal of the drug and it is easily re-induced if the drug is added again (data not shown). This would indicate that the signal pathway is still present in the cells and the expression can be induced, which might explain why these cells still showed some chemoresistance although the P-glycoprotein expression had disappeared. These facts and the fact that the VCR30- and VCR150- cell lines also lose their expression at the same time might indicate a reversal and not a spontaneous mutation. However, the biochemical changes behind the reversal have not been investigated.

The MTT assay gives rise to rather large variation between different analyses when determining the chemosensitivity. The  $IC_{50}$  values of the K562/wt, VCR30+ and VCR150+ cells were, however, in the same range during the whole period. When the P-glycoprotein levels decrease in the VCR30- and VCR150- cell lines, the chemosensitivity to P-glycoprotein substrates increase. The chemosensitivity to the specific P-glycoprotein substrate such as vincristine, daunorubicin and paclitaxel increases remarkably when they lose their *mdr-1* expression. Idarubicin, which is not as good a P-glycoprotein substrate as vincristine and daunorubicin (Hargrave et al. 1995; Roovers et al. 1999), shows the same pattern as the specific P-glycoprotein substrates. The resistance of the VCR30- and VCR150- cell lines to

these drugs decreases from day 0 to day 189 and even further to day 217. However, the VCR30- cell line seems to have a slightly higher resistance than the VCR150- cell line at day 189, which is consistent with its slightly higher P-glycoprotein expression at the same time. During the last period of the study (day 189 to 343), the protein level seems to be stable or even increases slightly, on the contrary the chemoresistance seems to decrease. This might indicate that just after a loss of P-glycoprotein expression the cells may still have an upregulated signalling pathway and undergo a rapid induction of P-glycoprotein, e.g. during the 72h incubation in the MTT assay, and therefore show a resistant phenotype although the P-glycoprotein expression has disappeared. The VCR30- and VCR150- cells also lose their resistance to etoposide and show the same tendencies so that they are still slightly resistant at day 189, and it is not until the final MTT assays that the IC<sub>50</sub> values reach the same chemosensitivity as that of the K562/wt cell line. However, etoposide resistance seems to be more dependent on the topoisomerase activity than on P-glycoprotein expression (Zhou et al. 1999; Lotfi et al. 2001).

9-β-D-arabinofuranosylguanine is selectively cytotoxic to leukaemic cells from patients with T-lymphocytic leukaemia (Shewach & Mitchell 1989). The K562/wt cell line has an IC<sub>50</sub> value of ~4μM for 9-β-D-arabinofuranosylguanine. However, the vincristine induced resistant cell line shows a very high resistance to this nucleotide analogue. 9-β-D-arabinofuranosylguanine has also been shown to induce P-glycoprotein (Lotfi 2001), indicating a common factor in this kind of cross-resistance. Data on the mechanisms of resistance to 9-β-D-arabinofuranosylguanine are limited and, for these cell lines, the mechanisms remain unknown. However, lowering of the active metabolite of 9-β-D-arabinofuranosylguanine, 9-β-D-arabinofuranosylguanine-5'-triphosphate, has been demonstrated in 9-β-D-arabinofuranosylguanine resistant cell lines (Lotfi et al. 2002a). The

fact that the VCR30<sup>-</sup> and VCR150<sup>-</sup> cell lines show the same phenotype concerning 9- $\beta$ -D-arabinofuranosylguanine resistance as the VCR30<sup>+</sup> and VCR150<sup>+</sup> cell lines indicates that these cell lines are, in fact, subcultures from the cell lines grown with drug.

The results of this study show that P-glycoprotein-expressing cells might lose their expression even after showing stable expression for several months. This indicates that P-glycoprotein expression is a dynamic process. A drug resistant cell line should not be considered stable when grown without the inducing drug. Checking that a resistant phenotype is stable for i.e. two months is not sufficient since, as shown here, the expression might change as long as five to six months after a change in drug pressure. This study also shows that the use of flowcytometry has an advantage of being able to detect one or several subpopulations in a cell line. The results also indicate that if the cells have lost their expression and are transformed into low-level expressing cells, they might still have a resistant phenotype, probably due to the presence of an up-regulated signal pathway or a heterogeneous subpopulation. The fact that we can not detect a P-glycoprotein expressing clone although the cell population shows a slightly resistant phenotype, might be an explanation to why P-glycoprotein expression analysis has not been the success in the clinical environment that we once hoped for. One complicating factor might be the presence of other drug transporting proteins such as lung resistance related protein and multidrug resistance-associated proteins, that can be co-over expressed with P-glycoprotein (Lehne et al. 1998), which also affect the efflux of drugs and may explain some of the discrepancies when just studying one resistance factor.

As demonstrated by us and others (Chen et al. 1994; Nielsen et al. 1998; De Moerloose et al. 2000), P-glycoprotein expression should be regarded as a changing phenotype. The kinetics of the protein should always be considered and the presence or absence of a protein should be

correlated with the phenotype at the same time. These data may explain some of the discrepancies in results obtained when analysing P-glycoprotein and mdr-1 gene expression in tumours and cell lines growing in vivo or in vitro, as well as why P-glycoprotein measurements and reversal agents have not been the great clinical success they once were hoped to be.

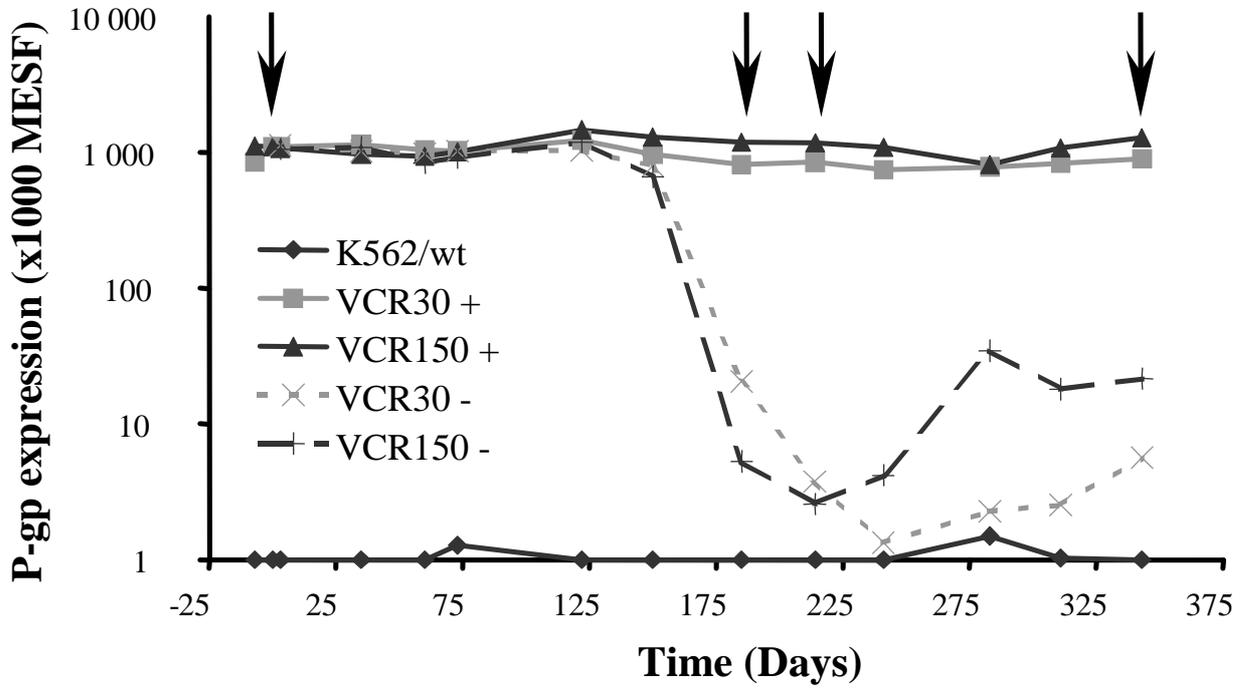
## **ACKNOWLEDGEMENTS**

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FIGURE 1.

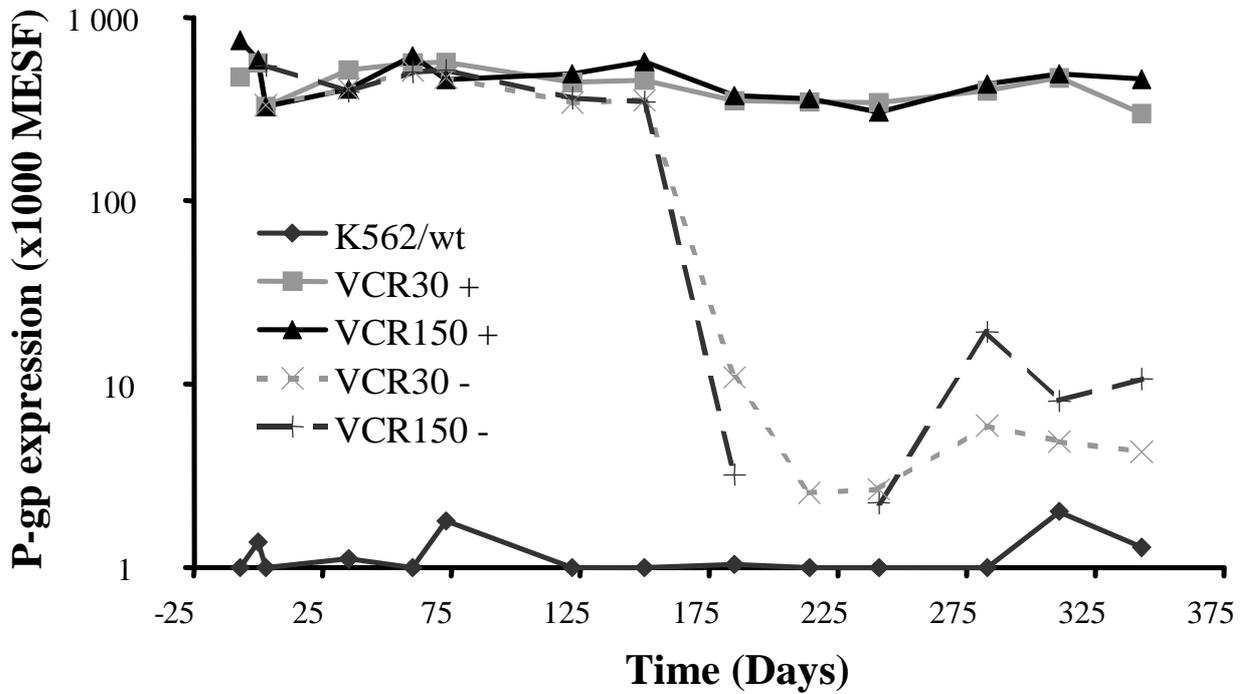
A

P-glycoprotein expression using the  $\alpha$ -P-gp antibody, clone 17F9



B

P-glycoprotein expression using the  $\alpha$ -P-gp antibody, clone 4E3



**FIGURE 2.**

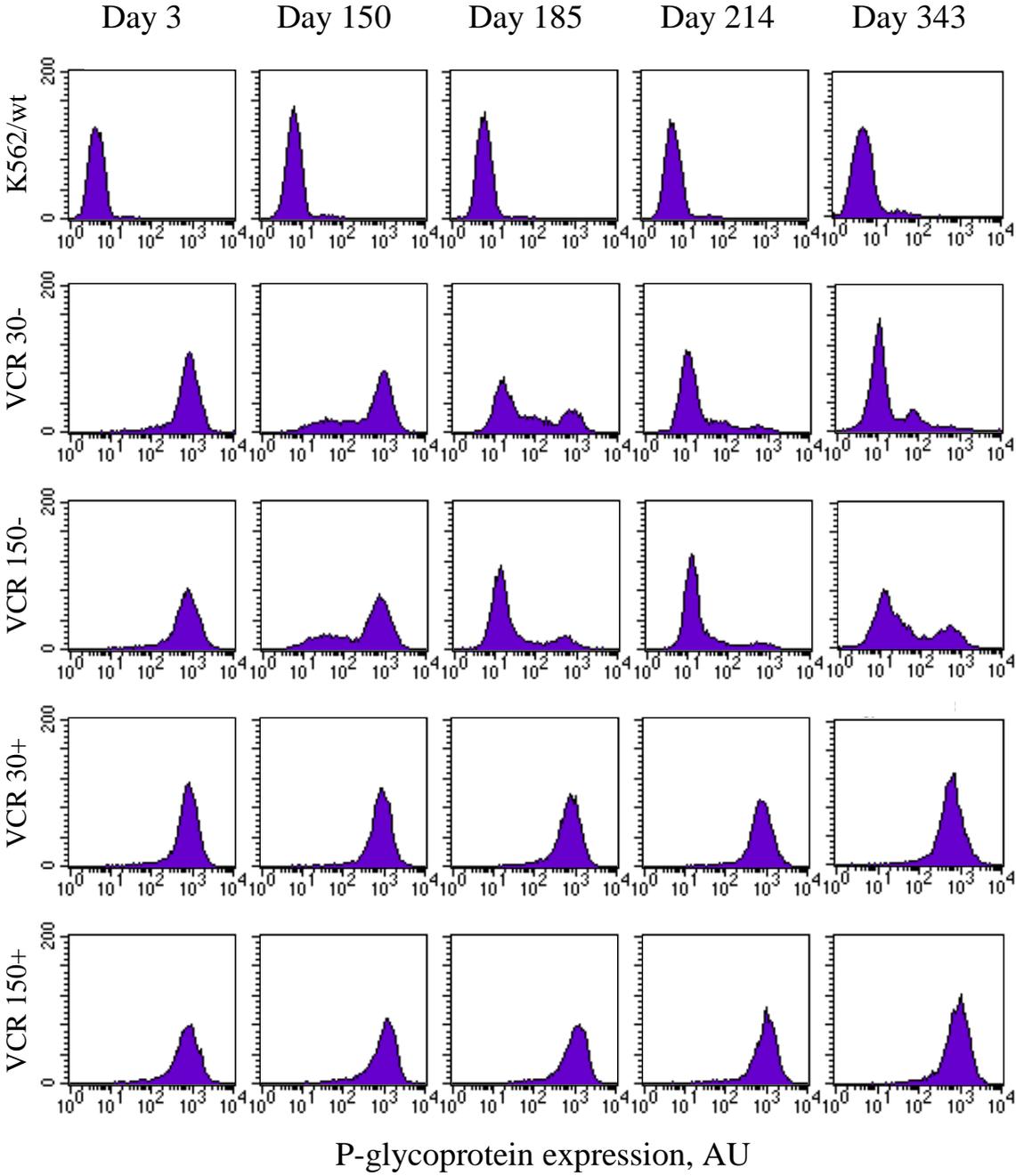
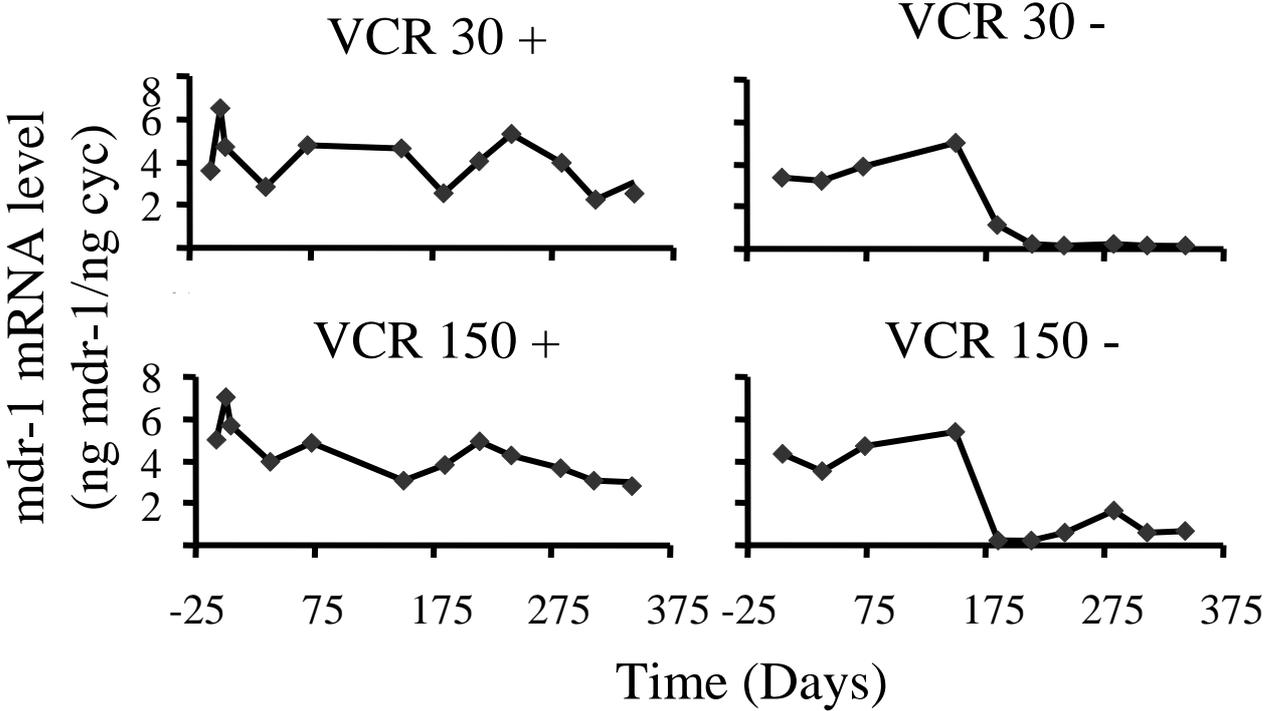


FIGURE 3.



**TABLE 1.**

The IC<sub>50</sub> values (μM) for six different drugs at four different time points during the study.

Day	Vincristine					Paclitaxel				
	wt	VCR30-	VCR150-	VCR30+	VCR150+	wt	VCR30-	VCR150-	VCR30+	VCR150+
0	0.05	>9.52	3.5	>9.52	3.5	0.007	>=9.5	>=9.5	>=9.5	>=9.5
189	0.11	1.46	0.19	1.9	2.2	0.017	7.51	0.058	6.2	5.1
217	0.12	0.23	0.11	1.8	3.7	0.008	n.d.	0.026	8.0	7.9
343	0.03	0.17	0.22	3.8	1.6	0.008	0.1-2.3	0.27	22.8	5.1
Daunorubicin						Idarubicin				
	wt	VCR30-	VCR150-	VCR30+	VCR150+	wt	VCR30-	VCR150-	VCR30+	VCR150+
0	0.30	16.6	22.8	16.6	22.8	0.21	1.1	1.1	1.1	1.1
189	0.37	4.97	1.12	13.6	23.2	0.20	0.88	0.91	1.3	1.5
217	0.66	3.65	2.94	21.3	34.7	0.26	0.72	0.44	1.2	1.6
343	0.44	0.78	0.90	15.2	8.9	0.39	0.66	0.59	1.8	1.1
Etoposide						Ara-G				
	wt	VCR30-	VCR150-	VCR30+	VCR150+	wt	VCR30-	VCR150-	VCR30+	VCR150+
0	4.7	>4.76	>4.76	>4.76	>4.76	1,9	>305	>305	>305	>305
189	6.2	12.1	14.9	25.7	n.d.	1,3	>305	>305	>305	>305
217	8.2	4.2	3.5	15.4	28.4	8,7	>305	>305	>305	>305
343	8.7	8.9	2.5-5.0	37.0	23.0	4,0	>610	>610	>610	>610

The chemosensitivity for the five K562 subcultures, wildtype and vincristine (VCR) induced multidrug resistant cells grown in the presence (+) of 30 nM or 150 nM of vincristine or in the absence of drug (-). The chemosensitivity was measured at the start and end of the study as well as during two points in time just as the VCR30- and VCR150- cell lines lost their P-glycoprotein expression; see also arrows in figure 1. The indicated days are the days when the drugs were added. The MTT and SDS were added 3 days later. Some IC<sub>50</sub> values were not reached in the MTT assay; the values are then denoted as greater than the highest drug concentration used in the assay. n.d. = not determined.

## LEGENDS

**Figure 1.** A - The P-glycoprotein (P-gp) expression as determined by the clone 17F9 antibody (Pharmingen). B - The P-glycoprotein expression as determined using the clone 4E3 antibody (Alexis). The vincristine (VCR) induced multidrug resistant K562 cell lines were grown in the presence (+) of 30 nM or 150 nM of vincristine or in the absence of drug (-). The x-axis represents the number of days after the drug was withdrawn from the VCR30- and VCR150- subclones and the y-axis shows the P-glycoprotein expression converted to specific molecules of equivalent soluble fluorochromes (MESF). The arrows indicate the points in time when the chemosensitivity was determined. Symbols: K562/wt (◆), VCR30+ (■), VCR150+ (▲), VCR30- (×), VCR150- (+).

**Figure 2.** Flow cytometry histograms showing the P-glycoprotein expression on five of the subclones. The fluorescence intensity (AU - Arbitrary Unit) is shown on the x-axis and the counts on the y-axis. The K562/wt cell line were grown in the absence of vincristine. The vincristine (VCR) induced multidrug resistant K562 cell lines were grown in the presence (+) of 30 nM or 150 nM of vincristine or in the absence of drug (-). The days indicate is the number of days since the drug was withdrawn from the VCR30- and VCR150- subclones.

**Figure 3.** The *mdr-1* mRNA levels expressed as ng *mdr-1*/ng cyclophilin for the four resistant subclones, grown in the presence (+) of 30nM or 150 nM of vincristine or in the absence of drug (-). The x-axis represents the number of days after the drug was withdrawn from the VCR30- and VCR150- cell lines. No *mdr-1* mRNA was detected in the K562/wt cell line.

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