Regulation of P-glycoprotein 1 and 2 gene expression and protein activity in two MCF-7/Dox cell line subclones

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Summary The MCF-7 doxorubicin-resistant cell line MCF-7/Dox has been used extensively for studies of the multidrug resistance phenomenon. Using fluorescence-activated cell sorting (FACS), these cells were separated into two populations on the basis of rhodamine 123 (R123) accumulation. We designated these as low P-glycoprotein (LP-gp) and high P-gp (HP-gp) cells on the basis of their P-gp content. Using the reverse transcriptase-polymerase chain reaction technique controlled by homologous internal standards, we analysed levels of MDR1 and MDR2 mRNA in each cell type. LP-gp and HP-gp cells had MDR1 mRNA levels of 2.17 ± 0.17 and 6.65 ± 2.29 amol ng⁻¹ total RNA respectively, compared with 0.0008 ± 0.00005 amol ng⁻¹ in wild-type MCF-7 cells (MCF-7/WT). MCF-7/WT cells additionally contained 0.023 ± 0.016 amol ng⁻¹ of MDR2 mRNA, which was unchanged in LP-gp cells, but lower in HP-gp cells, which contained 0.42 ± 0.08 amol ng⁻¹. Both LP-gp and HP-gp cells contained increased copies of the MDR1 gene. However, the degree of gene amplification did not correlate with the changes in MDR1 mRNA levels, indicating further regulatory levels of gene expression. The level of P-gp detected by MRK16 correlated with R123 accumulation. HP-gp cells expressed a 10-fold higher level of P-gp than LP-gp cells. However, there was only a 3-fold increase in MDR1 mRNA level in HP-gp cells compared with LP-gp cells. These data suggest that some regulation of P-gp expression also occurred at the post-translational level. Phosphorylation of P-gp by protein kinase C (PKC)α is necessary for its activity. Our analysis of PKC-α, β and ε isoform levels and subcellular distribution, shows a co-regulation of expression with P-gp, suggesting a necessary role for PKC in P-gp regulation.

Keywords: multidrug resistance; gene expression; MCF-7; flow cytometry; protein kinase C

Multidrug resistance (MDR) caused by overexpression of the membrane ATPase pump P-glycoprotein (P-gp) can be induced in cells in culture by exposure to cytotoxic agents at progressively increasing concentrations (Riordan and Ling, 1979; Kartner et al., 1983a,b; Chan et al., 1988). In vitro levels of resistance to the inducing agent of greater than 1000-fold compared with wild-type cells have been achieved (Beck et al., 1979; Shen et al., 1986a). P-gps are coded for by the MDR gene family, the number of members of which varies between species. Humans possess two MDR genes, MDR1 and MDR2 (Chin et al., 1989; Ng et al., 1989). Of these, only MDR1 codes for a P-gp (P-gp 1) that can confer a drug resistance phenotype when transfected into drug-sensitive cells (Ueda et al., 1987; Choi et al., 1991). Multidrug-resistant cells, particularly those which display high levels of resistance, often possess an increased copy number of the MDR1 gene (Batist et al., 1986; Fairchild et al., 1987; Endicott and Ling, 1989; Van der Bieke and Borst, 1989). However, in clinical samples resistance levels due to P-gp expression appear to be lower than those induced in vitro, and gene amplification has rarely been observed (Efferth and Osieka, 1993). Additionally, cells selected in vitro for lower levels of drug resistance do not show MDR1 gene amplification (Shen et al., 1986b; Kohno et al., 1994).

One enzyme system that seems to be an important post-translational regulator of P-gp activity via phosphorylation is protein kinase C (PKC). The phosphorylation state and activity of P-gp can be augmented by PKC activators, such as tumour-promoting phorbol esters (Chambers et al., 1990, 1992; Ma et al., 1991) and inhibited by agents such as staurosporine (Chambers et al., 1990, 1992; Chaudhary and Roninson, 1992; Bates et al., 1993). Of the PKC isozymes, only PKC-α has to date been identified as important in the post-translational regulation of P-gp activity (Yu et al., 1991; Ahmad and Glazer, 1993; Ahmad et al., 1994). We analysed PKC expression levels and subcellular distribution to evaluate possible candidates that may have a role to play in phosphorylation and regulation of P-gp.

Doxorubicin-resistant MCF-7 cells (MCF-7/Dox) have been used frequently as a model for the study of MDR resistance and its modulation. These cells were initially derived by exposure of the MCF-7 breast carcinoma cell line to increasing concentrations of doxorubicin (Batist et al., 1986). MCF-7/Dox cells show a 192-fold increase in resistance towards doxorubicin and cross-resistance to a variety of other drugs such as actinomycin D and vinblastine (Batist et al., 1986). The resistance phenotype is stable for 3 months when the cells are maintained in drug-free medium. It is not clear how P-gp-mediated drug resistance in these cells is regulated at the genetic level and which molecular events are required to maintain it. The cells are characterised by a number of biochemical changes when compared with their wild-type counterparts, in particular elevated expression of P-gp, amplification of the MDR gene (Cowan et al., 1986; Fairchild et al., 1987), augmented expression of glutathione S-transferase (Batist et al., 1986), and increased expression of PKC (Fine et al., 1988).

During a study to validate P-gp activity using rhodamine-123 (R123) accumulation and efflux we detected the presence of two cell populations in our culture of MCF-7/Dox cells with differing rates of R123 efflux. We sorted these cells into two lines with different P-gp levels and used them to explore mechanisms of MDR1 and 2 gene regulation and the involvement of PKC isoenzymes in the regulation of P-gp activity.

Materials and methods

Cell culture and maintenance

MCF-7/WT and MCF-7/Dox cells were gifts from J Carmichael, City Hospital, Nottingham, and were originally obtained from K Cowan, NIH, Bethesda, MD, USA. The cells were maintained in phenol-red-free RPMI-1640 (Gibco/BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 μg ml⁻¹ gentamycin.
Flow cytometric analysis of rhodamine (R123) accumulation and retention

Cells (1 x 10⁶) were allowed to attach to 55 mm Petri dishes (Falcon) for 1.5 h in 3 ml of complete medium. Following cell attachment the medium was replaced with 5 ml of serum-free medium and cells were cultured for 10 or 20 min with 1.66 µM R123. After this incubation period cells were washed with phosphate-buffered saline (PBS) and either harvested or treated with 5 ml of fresh medium and maintained in culture for 1.5 h to allow for dye efflux to occur before cell harvest. Cells were detached by treatment with trypsin-EDTA at 4°C, washed and resuspended in 1 ml of ice-cold PBS, and 10 µM propidium iodide (PI) was added. Flow cytometric analysis of the cells was carried out using a Becton Dickinson FACScan flow cytometer with excitation wavelength set at 488 nm. R123 fluorescence was collected after passage through a 515–545 nm band pass filter, and PI fluorescence was collected after passing through a 546–600 nm band pass filter. Single and multiparameter measurements of R123 forward angle, side angle scatter, R123 and PI fluorescence were collected for 10 000 events. Fluorescence data were collected on a four decade log scale and analysed using Lysis 2 software. Only R123 fluorescence from viable cells (i.e. PI-excluding cells) were evaluated.

The effects of P-gp inhibitors on R123 accumulation and efflux were studied by co-incubating cells with R123 and inhibitor (5 µM) for 10 min. The efflux of R123 over a 1.5 h period was determined by incubating the cells in R123-free medium but containing the inhibitor (5 µM).

Simultaneous detection of R123 accumulation and P-gp levels

Cells (1 x 10⁶) were allowed to accumulate R123 for 20 min as described above, then harvested, resuspended in 250 µl PBS containing 10% normal goat serum (BSSG) and incubated with 10 µg of MRK16 antibody (Kamiya Biochemical Company, Thousand Oaks, CA, USA) at 4°C for 30 min. After washing with ice-cold PBS the cells were incubated with R-phycocerythrin (RPE)-conjugated F(ab')₂ fragment of affinity-purified goat anti-mouse IgG (1:10 dilution) (Dako) in PBSG for 30 min at 4°C, before being washed with 2 ml of PBS and resuspended in 1 ml PBSG before flow cytometric analysis. RPE fluorescence was collected after passage through a 464–606 nm band pass filter. Duplicate analyses were performed in which the RPE-conjugated antibody was replaced by PI (10 µg ml⁻¹) so that dead cells could be eliminated from the subsequent analysis.

Flow cytometric sorting of cells

Cells (4 x 10⁶) were allowed to take up R123 for 20 min before being harvested and resuspended in 4 ml of PBS at 4°C. The cells were sorted into high and low R123 accumulation populations using a FACSort Vantage flow cytometer. The cells were sorted at about 2000 per second which resulted in an accumulation of 0.9 x 10⁶ of both 'high' and 'low' R123 fluorescence cells during a 40 min sorting period. Only two populations were detectable by R123 accumulation in the MCF-7/Dox cell line. They were present at a ratio of approximately 6:4 (low–high R123 fluorescence). Cells were sedimented by centrifugation and cultured. After 2 h the culture medium was replaced.

Analysis of gene amplification

DNA was extracted from each of the cell lines by lysis and phenol–chloroform extraction. Three digestions of the DNA were made with EcoRI, HindIII and NcoI and 10 µg of each digest electrophoresed on a 1% non-denaturing agarose gel. After electrophoresis the DNA was transferred to Hybond N (Amersham) by capillary transfer as previously described (Sambrook et al., 1989). The DNA was cross-linked to the nylon by ultraviolet light (Stratagene UV crosslinker, 120 000 µJ) and hybridised to probes pHDR5A (Ueda et al., 1987) and MDR2-pvuII (Currier et al., 1992), which detected MDR1/2 and MDR2 gene fragments respectively. Hybridisation was performed as previously described for the use of these probes in Northern analysis for RNA expression (Gant et al., 1991, 1992). The blots were washed with 0.1 x SSC/0.1%SDS for 1 h at 42°C. Gels were visualised and quantitated using one specific band for MDR1 and MDR2 as described below for quantitation of the RT–PCR gels.

Determination of cytotoxicity

Cells were seeded at 2 x 10⁴ per 35-mm-diameter dish in 3 ml of medium. After a 4 h incubation period to allow cell attachment various concentrations of doxorubicin were added. The toxicity of each concentration was determined in duplicate dishes. The cells were kept for 96 h (four doubling times) and medium and doxorubicin was replenished at 48 h. The cells were trypsinised and counted using a Coulter Counter model ZM (Coulter Electronics, Luton, UK). Growth inhibition was calculated as a percentage of drug-free control. The concentration of doxorubicin causing a 50% growth reduction (IC₅₀) was determined for each cell line.

RT–PCR analysis of gene expression

RT–PCR was carried out using internal RNA standards. The internal standard was a modified sequence of the cellular RNA being amplified and which contained the same primer sites. The modification made the internal standard sequence slightly longer by duplication of a piece of sequence between the primer sites. Details of primer construction are given below. In the analysis between 20 and 300 ng of cellular RNA was mixed with between 0.005 and 20 µg of the internal standard RNA in a final reaction volume of 10 µl containing 20 mM Tris-HCl, pH 8.4, and 50 mM potassium chloride, Mg²⁺ 2.5 mM, RNAsin (Promega) 10 µl⁻¹, MMLV-reverse transcriptase (Gibco/BRL) 10 U µl⁻¹, dNTP 1 mM, hexamers (Pharmacia) approximately 15 pmol µl⁻¹ and dithiothreitol 1 mM. Hexamers were annealed at 23°C for 10 min, products extended at 42°C for 45 min and the reaction terminated by heating to 95°C before being quick chilled to 4°C (Futschek et al., 1993). The PCR stage was carried out by the addition of reagents (made as a master mix) to concentrations of 20 mM Tris-HCl, pH 8.4, 50 mM potassium chloride, 2.5 mM Mg²⁺, 1 pmol µl⁻¹ sense and antisense primer (see below for primers) of which 0.01 pmol µl⁻¹ sense primer was end labelled with ³²P, 0.05% w:1 detergent (Gibco/BRL) and 2.5 U Taq DNA polymerase in a final volume of 20 µl. The nucleotides were derived entirely from the original reverse transcriptase reaction and so were at a final concentration of 0.5 mM for each nucleotide. PCR was carried out for 28 cycles at an annealing temperature of 55°C for MDR1 and 59°C for MDR2. Denaturation was at 95°C for 1 min in each cycle except the first in which it was extended to 5 min. The extension time was 2 min (72°C) in each cycle except the last in which it was extended to 5 min. Annealing was for 2 min in each cycle. For each set of reactions a negative control was run that did not contain RNA. PCR products were not detectable in this reaction.

After PCR a 5 µl aliquot of each reaction was analysed on an 8% non-denaturing gel. After drying, the gel was exposed in a phosphorimagier screen (Molecular Dynamics, Sunnyvale, CA, USA). Expression of each RNA was analysed by volume analysis with a local background using Image Quant 3.3 software (Molecular Dynamics).

To calculate absolute RNA concentrations five reactions for each RNA sample were performed using the same amount of cellular RNA in each, but an increasing amount of the internal standard RNA. The ratio of the PCR band volume from cellular RNA over band volume due to internal standard RNA was plotted as a double log, plot against the amount of the internal standard. The amount of internal standard and cellular RNA are equal when the ratio of these
bands is 1 (log, = 0). Care was taken to ensure that the experimental data spanned a ratio of 1 so that linearity to a ratio of one was not assumed. For each sample three analyses were performed. Kinetics and reaction characteristics are published in Zhang et al. (1996).

Construction of the MDR internal standard RNAs

The piece of the MDR1 gene chosen for RT–PCR assay was the region between bases 1991 (numbered from the adenosine of the translation initiation codon) and 2416. The region was amplified using primers 5'-AAAAAGATCAACTCTCGTAGGAGTG-3' (sense strand) and 5'-GCACAAAATACACAACAAACAAATCG-3' (antisense strand). The internal standard was constructed by duplication of the region between base 2040 (HindIII site) and 2085 by PCR of this region using the sense primer described above and an antisense primer spanning bases 2065 to 2085 which had a HindIII site added at the 5' end. Following PCR the product was cut with HindIII and inserted into the HindIII site of the assay sequence. Thus the internal standard was 51 bp longer than the assay region allowing for inclusion of a second HindIII site. The whole construct was contained in the pGem T vector (Promega) and sequenced using a primer to the SP6 promoter region. The plasmid was linearised using PstI before transcription of sense RNA from the T7 promoter.

Construction of the MDR2 internal standard control was very similar to that of MDR1. The area chosen for RT–PCR assay spanned bases 1343 (numbered from the adenosine of the translation initiation codon) and 1570. The region was amplified using primers 5'-TGATGAGGCACAATTAACA-3' (sense) and 5'-GTGTCATTTTTCTGGAAT-3' (antisense). The internal standard was made by duplication of the sequence between bases 1408 and 1494 (NcoI site) using the antisense primer above and a primer between bases 1408 and 1427 which had an NcoI site added to the 5' end. The PCR product was cut with NcoI and inserted into the analysis region at the NcoI site. The whole construct was contained in the pGEM4Z plasmid (Promega). The insert was sequenced using a primer to the SP6 promoter and linearised using PstI before transcription of sense RNA from the SP6 promoter.

Following transcription the size and integrity of the RNA was checked on a denaturing agarose gel and concentration assessed by determining optical absorbance at 260 nm. The molecular weight of the construct was determined, taking into account the additional sequence derived from the vector. The RNA was aliquoted and stored at −80°C before use.

Western blot analysis of PKC isozymes

Cells were grown on 140 mm Petri dishes. When they approached confluence cytosolic, particulate (membrane) and nuclear fractions were prepared as previously described (Greif et al., 1992) with some modifications (Stanwell et al., 1994). The protein content of each sample was determined by the method of Bradford, (1976).

Western blot analysis was performed as described previously (Stanwell et al., 1994) loading 20 μg of protein per lane. Monoclonal antibodies to PKC-α (TCS, Botolph Claydon, UK) PKC-ε and -δ (Afinitti, Nottingham, UK) and a polyclonal antibody to PKC-ζ (Gibco, Paisley, UK) were used. Detection was by enhanced chemiluminescence using an ECL kit (Amersham, UK). Immunoreactivity was quantified using a Molecular Dynamics computing densitometer and Image Quant software.

Results

Identification of two subclones by R123 efflux

Accumulation and efflux of R123 was compared in MCF-7/WT and MCF-7/Dox cells. Whereas MCF-7/WT cells...

Figure 1 R123 uptake and retention in MCF-7/WT (a–c) or MCF-7/Dox cells (d–f), and effect of verapamil (g, h). Cells were incubated without (a, d) or with R123 for 10 min without (b, e) or with (c, f) a subsequent 1.5 h incubation period in the absence of the dye. MCF-7/Dox cells were exposed to R123 and verapamil 5 μM without (g) or with (h) a subsequent 1.5 h dye retention period in the continued presence of verapamil. Each value represents the mean fluorescence of the cell population.
accumulated and retained R123 efficiently accumulation was drastically reduced and efflux enhanced in MCF-7/Dox cells (Figure 1). In our MCF-7/Dox cells two populations were evident, both with a higher R123 efflux rate than that observed in the parent MCF-7/WT cells. The cells in the two populations were present at an approximately 6:4 (low–high R123 fluorescence) ratio, and together constituted the entire MCF-7/Dox population. When verapamil was present more R123 was accumulated and R123 efflux was substantially reduced. A similar result was obtained with reserpine, except that this inhibitor was more potent than verapamil (data not shown).

**Cell sorting and culture on the basis of R123 efflux**

The cells were sorted on the basis of their ability to efflux R123, and established in culture. After sorting two cell populations were obtained each of which was homogeneous for R123 accumulation and efflux (Figure 2). To ensure that the two separated cell lines were from the same source their DNA was profiled and fingerprinted. The results of the analysis (not shown) show that the low and high fluorescence cells were indistinguishable on the basis of their DNA.

**Analysis of P-gp levels using antibodies and FACS analysis**

With the MRK-16 antibody (Figure 3) two cell populations were evident, confirming that the MCF-7/Dox cell line is composed of two populations with differing P-gp levels. Correlation of P-gp content in MCF-7/Dox cells, detected by
MRK-16 antibody, with their R123 accumulation showed that the low R123 fluorescence cells had the higher P-gp content. Likewise, the high R123 fluorescence cells expressed the lower P-gp level. Thus, R123 accumulation was inversely correlated with P-gp content (Figure 3). Furthermore, the sorted high R123 fluorescence cell line expressed the lower P-gp levels (Figure 3) and will therefore be referred to in the following text as LP-gp (low P-gp) cells. The sorted R123 low fluorescence cells expressed higher P-gp levels and will therefore be referred to as HP-gp (high P-gp) cells. Both for MRK-16 binding and R123 accumulation there was a 10-fold difference between LP-gp and HP-gp cells.

Sensitivity of cells to doxorubicin cytotoxicity

Sensitivity of the cells against doxorubicin as reflected by IC_{50} values decreased in the order MCF-7/WT > LP-gp > MCF-7/Dox > HP-gp (Table I).

Gene amplification

MDR gene amplification was analysed in all four MCF-7 cell types (Figure 4). In LP-gp, MCF-7/Dox and HP-gp cells, we found MDR1 gene amplifications of 9-, 40- and 78-fold over the MCF-7/WT cells respectively. For MDR2 we found similar amplification values of 8-, 36- and 65-fold respectively.

Analysis of cellular mRNA levels

Using RT–PCR with primers specific for the MDR1 and MDR2 genes and RNA internal standard controls it was possible to detect and quantitate MDR1 and MDR2 mRNA in all four MCF-7 cell types. In the MCF-7/WT cells the MDR1 mRNA level was low at 0.00088 ± 0.00005 amol ng^{-1} RNA compared with the MDR2 mRNA level, which was 0.023 ± 0.016 amol ng^{-1} RNA (Figure 5). MDR2 gene expression occurs in many normal tissues (Brown et al., 1993; Lee et al., 1993). Therefore it was not surprising that MDR2 expression was higher than that of MDR1 in the

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Table I: Doxorubicin cytotoxicity in MCF-7/Dox cell subclones

| MCF-7 cell clone | IC_{50} (µM) |
|------------------|--------------|
| MCF-7/WT         | 0.04 ± 0.01  |
| LP-gp            | 0.61 ± 0.13  |
| MCF-7/Dox        | 1.35 ± 0.05  |
| HP-gp            | 1.99 ± 0.12  |

*Cells (2 x 10^5) were exposed to various doxorubicin concentrations for 96h with the medium being replenished at 48h. After 96h the cells were detached by trypsinisation and counted using a Coulter counter. Inhibition of growth was calculated as a percentage of drug free control.

Figure 4: Amplification of the MDR1 (■) and MDR2 (■) genes in MCF-7/Dox, LP-gp and HP-gp cells over MCF-7/WT cells. Using the pHDR5A probe, which detects the MDR1 and MDR2 genes, or the MDR2pnuR probe, which detects only MDR2, copy numbers of the MDR1 and 2 genes in each cell type were determined as described under Materials and methods. The results were quantitated using volume analysis on a phosphor-imager. Equal DNA loading was assessed by hybridisation to a probe for the GAPDH gene which spanned bases 2 to 1023 of the GAPDH cDNA (Fort et al., 1985).

Figure 5: mRNA levels of MDR1 (a) and MDR2 (b) in MCF-7/WT (■), LP-gp (■), MCF-7/Dox (■), and HP-gp (■) cells. Levels of mRNA for MDR1 and MDR2 were determined using internally controlled RT–PCR, as described in Materials and methods. Results are the means ± s.d. of three determinations, except for MDR1 in MCF-7/WT cells, which is the mean of two determinations.
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MCF-7/WT cells. In LP-gp cells *MDR1* mRNA levels were 2.17 ± 0.17 amol ng⁻¹ RNA, which constitutes a 2500-fold increase over the MCF-7/WT cells. HP-gp cells contained 6.63 ± 2.29 amol ng⁻¹ RNA of *MDR1* mRNA, a 7556-fold increase. In HP-gp cells *MDR2* mRNA levels were 0.42 ± 0.08 amol ng⁻¹ RNA, 18 times the level observed in MCF-7/WT and LP-gp cells. MCF-7/Dox cells expressed levels of *MDR1* and *MDR2* mRNA that were intermediate between the levels of LP-gp and HP-gp cells, consistent with the ratio of LP-gp and HP-gp subpopulations in the parent MCF-7/Dox cell line.

**Analysis of PKC isozymes**

As PKC-α has been shown to play a role in the regulation of P-gp activity (Yu et al., 1991; Ahmad and Glazer, 1993; Ahmad et al., 1994) levels and subcellular distribution of PKC isozymes were examined. All three MCF-7 doxorubicin-resistant cell lines expressed PKC-α, -ζ, -ε and -θ (Figure 6 and Table II). Other PKC isozymes could not be detected by Western blot analysis. Total PKC expression was similar between LP-gp and HP-gp cells, but the isozyme distribution pattern in these cells differed substantially from that seen in

![Figure 6](https://example.com/figure6.png)

*Figure 6* Western blot analysis (a) and distribution (b) of PKC isozymes in MCF-7/WT, LP-gp and HP-gp cells. Distribution is expressed as a percentage of total cellular expression for each individual cell type. Expression was determined by Western blot analysis with the appropriate antibodies in the cytosolic ( ), membrane ( ) and nuclear fractions ( ). Data for the MCF-7/Dox cells were very similar to those in LP-gp/HP-gp cells, and are not shown. Values are the means ± s.d. of three experiments, statistical significance was determined (*P ≤ 0.05, **P ≤ 0.001) by analysis of variance.
Table II PKC expression in MCF-7/WT cells and their drug-resistant counterparts

| PKC | MCF-7/WT* | MCF-7/Dox | LP-gp | HP-gp |
|-----|-----------|-----------|-------|-------|
| α   | +         | +         | +     | +     |
| β   | +         | +         | +     | +     |
| γ   | +         | +         | +     | +     |
| δ   | +         | +         | +     | +     |
| ε   | +         | +         | +     | +     |
| ζ   | +         | +         | +     | +     |

*Cells were harvested at near confluence and subcellular fractions were isolated. Analysis of protein levels in the cytosolic, membrane and nuclear fractions was by Western blotting with specific antibodies as described in Materials and methods. Antibody binding was detected using enhanced chemiluminescence and quantitated using a Molecular Dynamics densitometer. 

Discussion

The multidrug-resistant MCF-7/Dox cell line was originally derived in 1986 and has been widely used for studies of P-gp (Batist et al., 1986). The results presented above show that our MCF-7/Dox cells comprised two subclones that were separable according to their ability to efflux R123. The presence of two clones in the MCF-7/Dox cells has been shown previously by MRK-16 antibody binding, but was not discussed (Yu et al., 1991). In contrast another very recent analysis with MRK16 found only one cell clone (Molinari et al., 1994).

In the work outlined above we present six separate pieces of evidence that suggest differences in P-gp expression levels between LP-gp and HP-gp cells that constitute the MCF-7/ Dox cell line: (i) they accumulate and retain R123 differently; (ii) they demonstrate different levels of immunoreactivity with the anti-P-gp antibody MRK-16; (iii) they exhibit different degrees of MDR1 and MDR2 gene amplification; (iv) they display different sensitivity towards doxorubicin; (v) they possess different levels of MDR1 and MDR2 mRNA; and (vi) they are differentially susceptible towards verapamil.

MDR1 mRNA levels were 2500- and 7556-fold greater in the LP-gp and HP-gp cells respectively, than in MCF-7/WT cells. The difference in MDR1 mRNA levels between LP-gp and HP-gp cells was therefore only 3-fold. However, both R123 accumulation and MRK16 binding indicate a difference of approximately one order of magnitude in the amount of P-gp protein between LP-gp and HP-gp cells. Therefore a post-translational regulatory step for P-gp stability may be operative in HP-gp cells. Both MDR1 and 2 genes were amplified in LP-gp cells about 9- and 8-fold respectively, over MCF-7/WT cells. In LP-gp cells this amplification translated into an increase of MDR1 mRNA level of 2500, whereas MDR2 mRNA was not increased. This discrepancy indicates that the regulatory step was involved in the control of the MDR1 gene in which the rate of gene transcription or mRNA half-life was increased about 250-fold over MCF-7/WT cells to result in the observed mRNA level. The result with MDR2 is more difficult to analyse, given the possibility that the gene may not have been amplified intact. In the case of HP-gp cells the picture was similar. MDR1 and MDR2 gene amplification in HP-gp cells was 78- and 65-fold respectively, over MCF-7/WT cells. However, MDR1 and MDR2 mRNA levels were increased 7556- and 18-fold respectively. In analogy to LP-gp cells this difference indicates an increase in MDR1 gene transcription rate or mRNA half-life of about 100-fold over MCF-7/WT cells. An increase in MDR1 transcription rate has been previously observed in MCF-7/Dox, doxorubicin-resistant ovarian 2780 and human colon carcinoma SW620 cells (Morrow et al., 1992; Madden et al., 1993). These data suggest that regulation of mRNA level in the LP-gp and HP-gp cells may occur to a greater extent at the transcriptional rather than post-transcriptional level.

Correlation of the results obtained by fluorescence analysis using the MRK-16 antibody with those observed for R123 accumulation suggests that R123 efflux was mediated completely via P-gp. Furthermore, as MRK-16 is specific for the P-gp1 isofrom (Shinkel et al., 1991, 1993), we conclude that R123 efflux is driven by P-gp1 and not P-gp2, the mRNA for which was increased in HP-gp but not LP-gp cells. This result is consistent with that previously obtained by Ludescher et al. (1993) in B-cell chronic lymphocytic leukaemia. All MCF-7-derived cell clones had detectable MDR2 mRNA levels. The correlation between R123 accumulation and P-gp1 levels as detected by MRK 16 in LP-gp and HP-gp cells indicates that there is no additional post-translational regulation of P-gp protein activity in HP-gp in comparison with LP-gp cells.

A PKC-α has been shown to play an important role in the post-translational regulation of P-gp activity (Yu et al., 1991; Ahmad and Glazer, 1993; Ahmad et al., 1994), we analysed the level and distribution of PKC isozymes. In LP-gp and HP-gp cells overall levels of PKC isozymes α, γ, ξ, and θ, were similar. However, compared with MCF-7/WT cells the resistant cells expressed higher levels of cPKC-α and nPKC-θ and lower levels of nPKC-ε and aPKC-ζ. The subcellular distribution of nPKC-ε differed significantly between LP-gp, HP-gp and MCF-7/WT cells. The resistant cells possessed more nPKC-ε in the membrane, and especially the nucleus, than wild-type cells. The fact that this distribution pattern locates nPKC-ε more efficiently in close vicinity to P-gp is commensurate with a role for this enzyme in the post-translational regulation of P-gp activity. This finding is consistent with the report of Slapak et al., 1993, who observed decreased in overall PKC activity in the HL-60 cells with acquired vinblastine resistance and an increase in enzyme content in the membrane fractions compared with HL-60 WT cells. The results presented above show for the first time that PKC-θ is present in MCF-7/Dox cells and its subclones. Its absence in MCF-7/WT cells and expression in all three cell compartments, particularly in the membrane and nuclear fractions of LP-gp and HP-gp cells, render it another candidate for post-translational regulation of P-gp activity.

In conclusion we derived two cell lines from the widely used MCF-7/Dox cell line, of the same origin, which differed in their expression of both MDR1 and MDR2 genes. This difference translated into an altered level of P-gp1 expressed in the plasma membrane and a corresponding difference in the ability of each cell line to efflux R123. In both LP-gp and HP-gp cells the change in MDR2 mRNA over MCF-7/WT cells was smaller than the increase in MDR2 gene copy number indicating a decreased transcription rate or mRNA stability or defective ampolon. The opposite was the case for the MDR1 gene in which the increase in MDR1 mRNA level was greater than that which could be accounted for by increased gene copy number. This result indicates an increased rate of MDR1 gene transcription or mRNA stability. Comparison of MRK16 binding in R123 efflux retention in each cell type is consistent with the hypothesis that R123 efflux occurs only through P-gp1. Comparison of P-gp protein level and mRNA levels between LP-gp and HP-gp cells suggested that in addition to the transcriptional/ post-translational regulation of MDR1 expression some post-translational increase in protein stability occurred.
However, the identical 10-fold difference in R123 accumulation and P-gp levels between LP-gp and HP-gp cells indicated no change in post-translational regulation of protein activity. Analysis of PKC isozymes indicated a dramatic increase in nPKC-α and nPKC-θ levels in both LP-gp and HP-gp cells over MCF-7/WT cells. In comparison with MCF-7/WT cells nPKC-ε expression was reduced overall in the resistant cell types, however the enzyme was distributed differently. More nPKC-ε was located in the membrane and nucleus, which might indicate an important role for nPKC-ε in the regulation of P-gp1 activity.

Overall, the results highlight the complicated and multifaceted nature of regulation of the multidrug resistance phenotype in MCF-7/Dox cells. A better appreciation of how it is controlled will help to devise more efficacious therapies to overcome P-gp-mediated drug resistance.

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