Mechanisms of resistance to combinations of vincristine, etoposide and doxorubicin in Chinese hamster ovary cells

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Summary We have isolated from Chinese hamster ovary cells, 30 sublines resistant to vincristine, doxorubicin or etoposide and 43 sublines evading treatment with a pair of these drugs. Isolated in one step and under low selective pressure, sublines were 3- to 25-fold more resistant to their selecting drug(s) than the parental cells. Possible P-glycoprotein-associated multidrug resistance was investigated through pgp gene copy number and mRNA expression level. DNA topoisomerase II alteration was evaluated from the ability of nuclear extracts to form cleavable complexes. Vincristine (all sublines) and doxorubicin (67 sublines) preferentially selected for pgg gene amplification and mRNA overexpression, whereas selection with etoposide resulted in a decrease of cleavable complex formation in 11 out of 13 sublines. A common pgg gene-mediated resistance was found in the 13 doxorubicin plus vincristine-selected sublines, whereas all but one of the 12 etoposide plus vincristine-resistant sublines displayed both pgg mRNA overexpression and decreased ability to form cleavable complexes. Among the 18 doxorubicin plus etoposide selected sublines, five exhibited a decreased ability to form cleavable complexes only, six exhibited pgg mRNA overexpression only and six exhibited both alterations. Overall, drug resistance may not be attributed to either mechanism in three of the 73 sublines. We conclude that under low selective pressure it is possible to find a combination of drugs which require simultaneous selection of more than one resistance mechanism; such cells emerge with very low frequency.

Keywords: drug resistance; combined alterations; multidrug resistance; DNA topoisomerase II; pgg genes

Drug resistance is, beside the poor selectivity of antitumoral agents, the main limitation of cancer chemotherapy (Goldie and Coleman, 1984). Regimens usually involve at least two drugs, but resistance often develops simultaneously to several agents. Typically, the frequency of resistance to combined drugs is much higher than the product of the mono-resistant frequencies (Giulotto et al., 1987; Rice et al., 1987; Souès and Charcosset, 1993). Multiple immunities may result from a specific phenotype, characterised by DNA amplification. For instance, the high frequency of simultaneous resistance to methotrexate and N-phosphonacetyl-L-aspartate is due to gene amplification of both target enzymes: dihydrofolate reductase and polypeptide having carbamyl phosphate synthetase, aspartate transcarbamylase and dihydrooroticase activities (CAD) (Giulotto et al., 1987). Similarly, concurrent treatment with methotrexate and doxorubicin (DOX) induces amplification of the dihydrofolate reductase and of the multidrug resistance-associated genes (Rice et al., 1987). Multiple drug resistance (MDR) may also follow overexpression of the multidrug efflux pump P-glycoproteins (PGP), coded by three classes of genes (Georges et al., 1990; Gottesman and Pastan, 1993). Transfection of hamster ppg1 (class I genes) and of mouse mdr1 gene, homologous to the hamster ppg2 gene (class II genes), has been shown to induce the classical MDR phenotype (Gottesman and Pastan, 1993; Devine and Meiera, 1994). Modification of DNA topoisomerase II, or a nuclear decrease in this enzyme, may also confer resistance to several of its poisons (Charcosset et al., 1988; Fernandes et al., 1990; Schneider et al., 1990; Harker et al., 1991; Hinds et al., 1991; Rappa et al., 1992). The phenotype being referred to as atypical MDR (at MDR) (Danks et al., 1988).

Etoposide (ETO) is an epipodophyllotoxin derivative which select cells with quantitative and/or qualitative alterations of DNA topoisomerase II (Ferguson et al., 1988; Long et al., 1991; Takano et al., 1991; Webb et al., 1991; Danks et al., 1993; Patel and Fisher, 1993; Sullivan et al., 1993; Ritke et al., 1994). To date, only one cell line exhibited mdr1 mRNA overexpression, without topoisomerase II modification, after selection by teniposide (Long et al., 1991). Vincristine (VCR) is a vinca alkalaid which selects for modified tubulin, its target (Houghton et al., 1985; Pain et al., 1988), or for PGP overexpression (Georges et al., 1990). Finally, DOX is a DNA intercalating drug, which selects for DNA topoisomerase II modification (De Jong et al., 1990; McPherson et al., 1993) as well as for the MDR phenotype (Georges et al., 1990; Slapak et al., 1990).

In previous studies, we reported that the frequencies of resistance to combinations of DOX, VCR and ETO suggest that both common and independent mechanisms of resistance can be selected in Chinese hamster ovary (CHO) cells (Souès and Charcosset, 1993). In this paper, we document that VCR and DOX preferentially select CHO cells with amplification and overexpression of the pgg genes, whereas DNA topoisomerase II alteration almost systematically accounts for ETO resistance. We also provide evidence that amplification of the pgg genes develops without DNA topoisomerase II modification after treatment with VCR plus DOX, whereas both pgg gene amplification and DNA topoisomerase II alteration occur in VCR plus ETO-selected sublines. When the selection is achieved with a combination of DOX plus ETO, drug resistance is ensured by either or both mechanisms. We conclude that cells selected with two drugs tend to exhibit a mechanism that allows them to resist both drugs simultaneously. Alternatively cells need two, independent, mechanisms of resistance.

Materials and methods

Cell lines and cell culture

The Chinese hamster ovary (CHO) cells AA8 (Thompson et al., 1980) were used as parental cell line and grown as previously described (Muller et al., 1992). Chinese hamster ovary cell line AuxB1 and its colchicine-resistant subline CHPC5 (Kartner et al., 1985) were kindly provided by Dr RM Baker (RPMI, Buffalo, NY, USA).

Drugs

Aqueous solution of VCR (Oncovin) was purchased from Lilly France (Saint Cloud, France). DOX (Adriablastine) was
purchased from Laboratoires Roger Bellon (Neuilly-sur-Seine, France) and stored at −20°C after dissolution in water at 1 mg ml⁻¹. ETO was kindly provided by Drs HM Holava and JD Matiskella (Bristol Myers, Wallingford, CT, USA) or by Dr C Durnay (Laboratoires Sandax, Rueil-Malmaison, France), and 10 mM aliquots in dimethylsulphoxide were stored at −20°C.

Selection of resistant cells and dose-dependent survival analysis

Selection of AA8 cells (10⁵ to 10⁶ cells in 100 mm Petri dishes) was achieved in 13–15 days in the continuous presence of the selecting drug concentration. Care was taken to avoid cell-to-cell contact for possible metabolic cooperation (Hooper and Subak-Sharpe, 1981). Colonies of various morphologies were isolated, and expanded in the presence of the selecting drug(s) for up to 2 months, before constitution of frozen stocks. Each selected subline is defined according to its parental cells (AA8), followed by the selecting drug(s) used (DOX, VCR and/or ETO) and a clone number (i.e. AA8/DOX + VCR B6 stands for clone B6 selected from AA8 cells by DOX plus VCR).

Drug cytotoxicity was determined by colony formation assay after 6–8 days of continuous exposure to the drug, as previously (Muller et al., 1992). No inoculum effect was observed with either parental or selected cells (Ohnuma et al., 1986). Each cell line was characterised by the concentration of drug resulting in 10% survival (D10) and by its resistance index (RI), defined as the ratio of the D10 value for a particular subline over that for the parental cell line.

Northern and Southern blots

RNA and DNA were extracted by the guanidine thiocyanate method, separated using a cesium chloride cushion (Chirgwin et al., 1979; Muller et al., 1992) and purified by phenol–chloroform extraction. Four micrograms of total RNA was resolved in 1.2% agarose gel containing 2.2 M formaldehyde, and Northern blots were performed as described by Dautry et al. (1988). The amount of RNA loaded was quantified by ethidium bromide staining (Muller et al., 1992), and that of actin mRNA was probed, using a transcript of pBACT 5 plasmid containing the 600 bp Tago/PstI fragment of the mouse gene (Dautry et al., 1988), after labelling with [³²P]UTP. A 1.2 kb cDNA fragment encompassing from nucleotide 3051 to the poly-A tail of the human mdr1 gene (kindly provided by Dr T Tsuruo, Tokyo, Japan) was labelled with [³²P]CTP, and used to probe the pgg hamster mRNA. The amount of pgg mRNA in each cell line was quantified by densitometry of the autoradiograms, using a double-beam microdensitometer (MIIIIIC, Joyce, Loeb, UK), and averaged from a minimum of two RNA extractions. Ten micrograms of total cellular DNA was developed in 0.8% agarose gel after complete digestion by EcoRI. Southern blots were probed using the 1.2 kb human mdr1 cDNA insert, which hybridized to eight EcoRI fragments, covering the three pgg genes (Riordan et al., 1985; Ng et al., 1989; Muller et al., 1992). DNA and RNA from CHO CH⁺C5 and AuxB1 cells were extracted, and analysed as positive and negative controls respectively.

Preparation of the nuclear extracts and DNA topoisomerase II assay

Nuclear extracts were prepared according to Glisson et al. (1986) with minor modifications as described by Charcosset et al. (1988). Briefly, 4–6 × 10⁶ exponentially growing cells were trypanosised, washed with phosphate-buffered saline, and the nuclei were extracted at 4°C by Dounce homogenisation in a swelling buffer containing 0.3% Triton X-100 (v/v). Nuclei were further purified by centrifugation through a sucrose cushion, and the concentration of sodium chloride was raised to 0.35 M to extract DNA topoisomerase II. For the purpose of comparison, AA8 cells were processed systematically with resistant sublines. In preliminary experiments we determined that the protein content in nuclear extracts was directly proportional to the initial number of cells used for the extraction. Therefore, the total protein concentration was used to normalise the nuclear extracts.

Etoposide-stimulated DNA cleavage activity was assayed using the ability of the 0.35 M sodium chloride nuclear extract to linearise supercoiled pBR 322 DNA, as described by Charcosset et al. (1988). Samples were analysed by gel electrophoresis in 0.8% agarose gel in the presence of ethidium bromide and pictures were taken under UV light. The percentage of form III plasmid (linear) was estimated by scanning negative films. With all sublines, the DNA cleavage activity of the nuclear extracts increased with the concentration of ETO added and plateaued to concentrations higher than 64 μM. The percentages of cleavable complexes resolved in this study correspond to that measured for a given subline, relative to that for the parental cell line, in the presence of a saturating concentration of ETO (128 μM).

Results

Selection of AA8 colonies resistant to DOX, VCR and/or ETO

We have isolated from CHO-AA8 cells, under low selective pressure and in a single step, 30 sublines resistant to either DOX, VCR or ETO, and 43 sublines resistant to combinations of these drugs. D10 values for DOX, VCR and ETO of the AA8 cells were 59 ± 2.6 μM, 23 ± 4.5 μM and 630 ± 70 μM respectively (mean ± s.d. of 4–10 determinations, each in triplicate). Selecting concentrations, frequency of surviving cell colonies, number of colonies obtained and plating efficiencies are summarised in Table I. To select single drug-resistant colonies, we used 3 × 10⁶, which induced a frequency of surviving cells of about 10⁻³ to 10⁻⁴. To obtain doubly resistant colonies, we combined 0.28 μM DOX with 0.05 μM VCR or 1.6 μM ETO (or VCR and ETO at the same concentrations). These combinations induced a frequency of surviving cells of 10⁻⁷ to 10⁻⁸. The frequency of surviving cells was 700-fold greater than the product of each single drug resistance frequency with the association DOX plus VCR, approximately equal to this product with VCR and ETO, and intermediate with DOX plus ETO (Souès and Charcosset, 1993).

The plating efficiency of each subline was determined in the absence as well as in the presence, of the selecting drug (Table I). Without drug, plating efficiencies were in the range

Table I  Sensitivity of AA8 cells to DOX, VCR or ETO, selective conditions and plating efficiencies of sublines resistant to DOX, VCR or ETO or to a combination of these drugs

| Subline | Selecting concentrations (μM) | Selecting frequency | Number of colonies | No drug | DOX | VCR | ETO |
|---------|-------------------------------|---------------------|--------------------|---------|-----|-----|-----|
|         | DOX | VCR | ETO |                 |        |     |     |     |        |     |     |     |
| AA8/DOX | 0.18 | -    | - | 5 × 10⁻⁵ | 7     | 42–57* | 30–46 | -   | -      |     |     |     |
| AA8/VCR | -    | 0.07 | - | 2 × 10⁻⁵ | 10    | 67–89 | -     | 44–66 | -      |     |     |     |
| AA8/ETO | -    | -    | 2.00 | 2 × 10⁻⁴ | 13    | 44–88 | -     | -     | 34–51  |     |     |     |
| AA8/DOX + VCR | 0.28 | - | 0.05 | 2 × 10⁻⁷ | 13    | 48–88 | 30–75 | 25–60 | -      |     |     |     |
| AA8/VCR + ETO | - | - | 0.05 | 2 × 10⁻⁴ | 12    | 38–82 | -     | 30–72 | 30–73  |     |     |     |
| AA8/DOX + ETO | 0.28 | - | 1.60 | 8 × 10⁻⁷ | 18    | 40–70 | 25–61 | -     | 26–63  |     |     |     |

*Plating efficiency was determined by colony-forming assay, under continuous exposure to the drug. *Range: each value is the mean of 2–5 determinations (each in triplicate). **The plating efficiencies of 2 of the 18 sublines were 4% and 6%.
of 40–90%. In the presence of the selecting drug, cells retained 51–91% of this plating efficiency. Thus most sublines effectively resisted their selecting drug (Thompson and Baker, 1973). However, sublines exhibited relatively low level of resistance to the selecting drug: RI values extended from 3- to 25-fold (Figures 1–6). In an attempt to identify possible PGP overexpression and/or topoisomerase II alteration, we characterised these sublines for pgp gene copy number, pgp mRNA overexpression and the ability of the nuclear extracts to form cleavable complexes in the presence of ETO.

**Characterisation of the DOX-resistant sublines**

Among the DOX-selected sublines, six out of seven exhibited pgp gene amplification as well as pgp mRNA overexpression, whereas formation of cleavable complexes was similar to that of the parental cells (Figure 1). Amplification of the pgp genes appeared homogeneous in sublines AA8/DOX.A1, A3, A4 and A5 (i.e. the relative intensities of the EcoRI DNA fragments were similar to that of the AA8 cells). In contrast, in sublines AA8/DOX.A6 and B6, the most amplified EcoRI fragments were those corresponding to the pgp2 gene, whereas the bands of pgp3 gene were the least amplified. The level of mRNA overexpression was not related to the gene amplification pattern or level. For instance, sublines AA8/DOX.A1 and A6 had the highest level of mRNA detectable, but different pgp gene amplification pattern and level. In addition, there was no correlation between the sensitivity to DOX and the level of pgp gene amplification or mRNA overexpression: RI values were between 5 and 10 with all

![Figure 1](image1.png)

**Figure 1** Characterisation of the DOX-resistant sublines. Top: resistance indexes to DOX of the selected sublines. Middle: Southern blot analysis. The amount of DNA loaded on the gel was 10 µg for the CHO cells and 3 µg for the CH³C5 cells (used as positive control). The EcoRI fragments which hybridise to the hamster *mdr1* probe are each attributed to one of the hamster pgp genes, according to their size in kilobases. Sublines A6 and B6 presented a preferred pgp2 amplification pattern. In the other sublines, the relative intensity of each band was similar to that of the AA8 parental cells. The lower panel indicates the fold increase in the pgp mRNA expression level (relative to the average obtained with the AA8 cells). There was no pgp gene amplification in subline A2, which was the only one to exhibit a decreased ability to form cleavable complexes (not shown).

![Figure 2](image2.png)

**Figure 2** Characterisation of the VCR-resistant sublines. Top: resistance indexes to VCR. Middle: Southern blot analysis. Bottom: fold increase in pgp mRNA level (as in Figure 1). All sublines exhibited both pgp gene amplification and mRNA overexpression. Sublines B16, C13 and C14 exhibited a homogeneous pgp gene amplification pattern, the other sublines a specific pgp1 amplification. As expected with VCR-selected cells, no decrease in the ability to form cleavable complexes was detected (not shown).
sublines (Figure 1). In the seventh subline (AA8/DOX.A2), neither *ppg* gene amplification nor mRNA overexpression was detected, but *in vitro* drug-dependent DNA cleavage was only 50% that of the AA8 cells, suggesting a decrease in the ability to stabilise cleavable complexes. Topoisomerase II alteration, and *ppg* gene amplification can both confer cross-resistance to several drugs. Therefore we examined the resistance of four sublines to VCR and ETO. Sublines AA8/DOX.A4, A5 and A6, with *ppg* gene amplification, presented a cross-resistance to VCR and ETO (RI values were between 4 and 6, Table II). Subline AA8/DOX.A2, with a reduced ability to form cleavable complexes, retained sensitivity to VCR (RI value of 0.9), but was 3-fold cross-resistant to ETO.

Thus, DOX preferentially selects AA8 cells with *ppg* gene amplification, resulting in VCR and ETO cross-resistance. Modification of topoisomerase II (without *ppg* gene amplification) accounted for DOX resistance and ETO cross-resistance in one subline only.

Characterisation of the VCR-resistant sublines

Compared with the parental cells, *ppg* genes were amplified in all VCR-selected sublines, whereas *ppp* mRNA was moderately overexpressed (Figure 2). In sublines AA8/VCR.B16, C13 and C14, the relative intensities of the DNA fragments were similar to that of the parental cells, but in sublines AA8/VCR.A12, B11, B12, B14, C12 and C16, *ppp* gene was preferentially amplified. The *ppp* gene was particularly amplified in subline AA8/VCR.A11, but the intensity of the 4.2 kb fragment of *ppp* gene was also increased. Here the RI values were related to the level of *ppp* amplification. Sublines with preferential *ppp* gene amplification had RI values higher than 11, and when there was homogeneous amplification the RI was still higher for sublines with high *ppp* copy number. In fact, subline AA8/VCR.C14, with the lowest *ppp* amplification, had the lowest RI value (4.8). Three sublines with representative amplification pattern (AA8/VCR.B14, B16 and C13) exhibited cross-resistance to both DOX and ETO (RI values higher than 5.3, Table II), whereas subline AA8/VCR.C14 was both DOX and ETO sensitive (RI values of 1.1 and 1.5 respectively). It is unlikely that topoisomerase II modification had been co-selected with *ppg* amplification. Indeed, the capacity to form cleavable complexes was similar to that of the AA8 parental cells in sublines AA8/VCR.B14, B16, C12, C13 and C14. Taken together, these data suggest that VCR typically selects AA8 cells with amplification and overexpression of *ppp* genes resulting in cross-resistance to DOX and ETO.

Characterisation of the ETO-resistant sublines

A decreased ability to form cleavable complexes was observed in 11 out of the 13 ETO-resistant sublines, whereas none exhibited *ppg* gene amplification (Figure 3). The percentages of drug-stimulated DNA cleavage were 2- to 20-fold lower than that of the parental cells, and RI values varied between 3.5 and 19. Among these sublines, AA8/ETO.C6, with a high RI value (11.9), exhibited a slight *ppp* mRNA overexpression. Sublines AA8/ETO.B4 and C3 are potentially interesting, because neither *ppg* gene amplification, mRNA overexpression nor decreased cleavable complex formation could explain their high RI values (10.8 and 18.9 respectively). ETO and DOX are both topoisomerase II poisons, and VCR, while a tubulin binder, preferentially selects PGP-associated multidrug resistance. Possible cross-resistance to DOX or VCR of sublines AA8/ETO.A5, B1 and B2 was tested. Cross-resistance to DOX was only about
were topoisomerase 2-fold, and cells retained sensitivity to VCR (RI values were between 0.9 and 1.2; Table II).

Therefore, ETO preferentially selects AA8 cells through topoisomerase II modification. Yet, the highest RI values were obtained with a concomitant pgp gene overexpression in one subline, and through neither mechanism in two sublines.

**Characterisation of the DOX plus VCR-resistant sublines**

All the sublines selected with a combination of DOX and VCR exhibited pgp gene amplification and pgp mRNA overexpression, but, in contrast to the VCR-selected cells, there was no evidence of specific pgp1 amplification (Figure 5).

![Figure 5](image)

**Figure 5** Characterisation of the ETO plus VCR-resistant sublines. Top: resistance indexes to ETO (■) and to VCR (□). Middle: Southern blot analysis. Bottom: fold increase in pgp mRNA level (as in Figure 1) and ability to form cleavable complexes (as in Figure 3). Seven sublines exhibited a homogeneous pgp gene amplification pattern (A3, B2, B3, C3, C6, F2 and G2), the other sublines (A6, B1, B4, E3 and F3) a preferred pgp2 amplification pattern. All sublines overexpressed to various extents the pgp mRNA, and all but subline E3 had a reduced ability to form cleavable complexes.

![Figure 6](image)

**Figure 6** Characterisation of the DOX plus ETO-resistant sublines. Top: resistance indexes to DOX (■) and to ETO (□). Middle: Southern blot analysis and Northern blot analysis [total RNA (4 μg) was hybridised to the human mdr1 probe; the arrows indicate the position of 28S rRNA]. Bottom: fold increase in pgp mRNA level (as in Figure 1) and ability to form cleavable complexes (as in Figure 3). Sublines A5, C3, D5, E4, G5 and H6 exhibited pgp mRNA overexpression only, sublines A3, C5, D2, G1 and G4 exhibited a decreased ability to form cleavable complexes only and sublines A2, A4, B1, B4, C6 and G3 exhibited both alterations. In subline E5 neither pgp gene overexpression nor topoisomerase II alteration could account for the resistance to DOX and ETO.
Table II  Resistance (bold) and cross-resistance to DOX, VCR or ETO of selected sublines

| Resistant subline       | DOX  | VCR  | ETO  |
|-------------------------|------|------|------|
| AA8/DOX.A2              | 8.1  | 0.9  | 3    |
| AA8/DOX.A4              | 10.2 | 6.2  | 4    |
| AA8/DOX.A5              | 10.2 | 5.9  | 5.4  |
| AA8/DOX.B6              | 9.7  | 5.3  | 6.3  |
| AA8/VCR.B14             | 5.3  | 11.3 | 6    |
| AA8/VCR.B16             | 10.4 | 8.3  | 7    |
| AA8/VCR.C13             | 10.2 | 7.4  | 5.4  |
| AA8/VCR.C14             | 1.1  | 4.8  | 1.5  |
| AA8/ETO.A5              | 1.9  | 1.0  | 8.9  |
| AA8/ETO.B1              | 2.1  | 0.9  | 5.1  |
| AA8/ETO.B2              | 2.0  | 1.2  | 7.5  |

*P-values for comparison of the mean D10 subline and D10 AA8 were 0.0012–0.032 (significant differences) when R1s were equal or above 1.9 and 0.848–0.46 (not significant differences) when R1s were between 0.9 and 1.5.

4). Compared with the parental cells, the relative intensities of the DNA fragments were similar in all but one subline. Interestingly, the pattern of the 13th subline (AA8/DOX + VCR.B3) was similar to that of the DOX-selected cells AA8/DOX.A6 and B6: pgpl fragments were the most intense and the pgp3 gene was less amplified than the pgp1 gene. Subline AA8/DOX + VCR.B3 also had the highest level of pgp mRNA overexpression among the doubly resistant cells. In spite of the higher selective drug concentration, the resistance to DOX of the DOX plus VCR-selected cells was similar to that obtained with the DOX-selected sublines. In contrast, possibly because of the lack of specific pgp1 gene amplification, R1 values to VCR followed the selective drug concentration: values obtained with a lower selective drug concentration in the doubly resistant sublines were lower than that observed with the VCR-selected cells. mRNA overexpression and pgp gene amplification are potentially sufficient to evade DOX plus VCR treatment, but topoisomerase II modification could also induce resistance to DOX. Three sublines (AA8/DOX + VCR.B3, C1 and D1) were tested for their ability to form cleavable complexes, but all extracts had the same ability as the AA8 parental extract to induce cleavable complex formation.

Taken together, these results suggest that, rather than DNA topoisomerase II alteration, increased pgp gene copy number and mRNA overexpression account for the double resistance to VCR and DOX.

Characterisation of the VCR plus ETO-resistant sublines

All but one of the 12 sublines which resisted the combination of ETO plus VCR had a reduced ability (up to 30%) to form cleavable complexes (Figure 5). These 12 sublines also exhibited pgp gene amplification as well as pgp mRNA overexpression. No subline presented a specific pgp1 amplification pattern. Compared with the parental cells, the relative intensities of the DNA fragments were similar in seven sublines (AA8/ETO + VCR.A3, B2, B3, C3, C6, F2 and G2). The five other sublines (AA8/ETO + VCR.A6, B1, B4, E3 and F3) exhibited a pattern similar to that of AA8/DOX + VCR.B3, AA8/DOX.A6 and B6 cells (i.e. the pgp2 gene was more amplified than the pgp1 gene, itself more amplified than the pgp3 gene). As for AA8/DOX + VCR cells, R1 values obtained with VCR were lower (mean value of 6) than for the cells selected with VCR only (mean value of 11.6).

Therefore, two, independent mechanisms of resistance seem to be required to ensure survival to a combination of ETO and VCR.

Characterisation of the DOX plus ETO-resistant sublines

All combinations of resistance mechanisms were found within the 18 DOX plus ETO-selected sublines (Figure 6).

Amplification of the pgp genes ranged from very high to non-detectable and ability to form cleavable complexes ranged from 10% to indistinguishable from that of the parental cells. Five sublines exhibited only a decreased ability to form cleavable complexes (AA8/DOX + ETO.A3, C5, D2, G1 and G4), six displayed pgp mRNA overexpression only (AA8/DOX + ETO.A5, C3, D5, E4, G5 and H6), whereas both mechanisms were present in sublines AA8/DOX + ETO.A2, A4, B1, B4, C6 and G3. In one subline (AA8/DOX + ETO.E5), neither pgp gene amplification nor decreased ability to form cleavable complexes could account for the resistance. As for most of the DOX-selected sublines, and for all but one of the DOX plus VCR-selected sublines, when pgp gene amplification was observed the relative intensities of the EcoRI fragments were similar to that of the AA8 parental cells. Various levels of pgp mRNA expression were observed, but, as previously, no correlation between pgp gene amplification and mRNA expression could be drawn. Most high R1 values to ETO were among the cells presenting a decreased ability to form cleavable complexes, but a 30% decrease in the ability to stabilise cleavable complexes was associated with both the greatest R1 value to ETO (25 in subline AA8/DOX + ETO.G4), and the R1 value of 11 in subline AA8/DOX + ETO.A3. Furthermore, subline AA8/DOX + ETO.H6, with pgp amplification only, had a R1 value to ETO of 24.

Our results suggest that either of the two mechanisms, pgp mRNA overexpression or decreased ability to form cleavable complexes, can induce resistance to DOX plus ETO.

Discussion

In this study, we verify that DOX, VCR and ETO preferentially select one mechanism of resistance in CHO-AA8 cells, and we investigate whether there is common or independent resistance to combinations of these antitumoral drugs. We show that DOX and VCR preferentially select amplification of the pgp gene-associated multidrug resistance, whereas ETO resistance results primarily from topoisomerase II alteration. We unveil common resistance mechanisms to combination of these drugs, except for the VCR plus ETO association, which requires selection of two independent mechanisms.

Clinically used concentrations of antitumoral drugs induce emergence of resistant cells. We observed, however, that a low selective pressure results in a moderate level of resistance in CHO-AA8 sublines. The alterations conferring resistance to the antitumoral agents could not result from a mutagenic response owing to a long exposure to drug or from pretreatment with a mutagen (Singh and Gupta, 1983). Therefore, it is likely that the selection resulted from a threshold effect: there were pre-existing mutants which emerged from the parental population. Consistent with our observations, a low concentration of vinblastine or colchicine with human melanoma cells (Lemontt et al., 1988), or of DOX with murine erythroleukaemia cells (Slapak et al., 1990) also induce low levels of resistance.

Overexpression of the pgp genes occurred in most CHO-AA8 sublines selected with VCR or DOX; only one of the DOX-resistant sublines exhibited a modified ability to form cleavable complexes. Typically, cells selected with DOX (or VCR) and featuring pgp gene overexpression were cross-resistant to ETO and VCR (or DOX). The ETO-selected cells exhibited primarily a modified drug-stimulated ability to stabilise cleavable complexes; this includes the only subline which slightly overexpressed pgp mRNA. Cells selected with ETO (or DOX) and featuring topoisomerase II alteration were cross-resistant to DOX (or ETO), but retained their selectivity to VCR. Consistent with our data, VCR selects for pgp overexpression, and ETO selects for topoisomerase II alteration, whereas both mechanisms of resistance may apply with DOX. The three drugs are potentially recognised by PGP, but only ETO and DOX can stabilise the complexes.
between DNA and topoisomerase II (Liu, 1989; Georges et al., 1990; Corbett et al., 1993; Gottesman and Pastan, 1993). Alterations of topoisomerase II, without overexpression of PGP, have been observed in P388 murine leukemia cells and human small-cell lung carcinoma cells selected with DOX (De Jong et al., 1990; McPherson et al., 1993). In addition, very few cell lines exhibited the classical MDR phenotype after selection with ETO or teniposide (Long et al., 1991; Hosking et al., 1994). There was no correlation between pgp gene amplification or mRNA overexpression and level of resistance. Regulation of the transcription and/or mRNA stabilisation may control PGP expression. Overexpression of PGP, as determined by either pgp mRNA or protein in immortalised tumour cells resistant to vinca alkaloids (Bradley et al., 1989; Hill et al., 1990; Biedler, 1994). Resistance to VCR could result from tubulin alteration (Houghton et al., 1985; Sirionak et al., 1986; Pain et al., 1988). Such a mechanism possibly accounts for two of the VCR-resistant sublines exhibiting only a slight increase in pgp mRNA. Alternatively, point mutations in PGP could affect the transporter affinity for a particular drug (Gottesman and Pastan, 1993). Finally, specific drug immunity may be associated with a particular pgp gene (Georges et al., 1990; Gottesman and Pastan, 1993).

Three patterns of pgp gene amplification were established in the CHO-AA8 cells. The most common pattern was a general amplification of all three pgp1, pgp2 and pgp3 genes. A specific pgp1 amplification pattern was found in the VCR-selected cells, and a preferential amplification of pgp2 gene characterised some of the DOX-selected cells. In sublines displaying a general amplification pattern, the RIs to DOX were higher than those to VCR, and the resistance to ETO the lowest. In sublines with specific pgp1 amplification, the highest RIs were to VCR, and a relationship seemed to link the extent of pgp1 amplification to the level of VCR resistance. Similarly, a preferred pgp2 amplification may improve the resistance to DOX. We did not find a specific pgp3 gene amplification, but transfection of the human mdr3 gene (homologous to the hamster pgp3 gene) failed to induce the MDR phenotype in RBO melanoma cells (Gottesman and Pastan, 1993). While DOX and ETO are both topoisomerase II poisons, they appear to select preferentially different mechanisms of resistance. Topoisomerase II modifications obtained through DOX selection were far less frequent than those emerging through ETO selection. Actually, the unique DOX-selected subline exhibiting topoisomerase II alteration was only 3-fold cross-resistant to ETO. Conversely, the ETO-selected subline exhibiting expression of cleavable complexes were at most 2-fold cross-resistant to DOX. However, the DOX- (or VCR) resistant sublines (overexpressing the pgp genes) were cross-resistant to ETO, suggesting that pgp gene overexpression may have been sufficient to confer ETO resistance. Again, independently from the pgp gene amplification pattern, cross-resistance to ETO was lower than resistance to DOX (or VCR). Consistent with our data, PGP does not recognise ETO as efficiently as DOX (Politi et al., 1990; Long et al., 1991). While Giaccone et al. (1992) found a direct relationship between sensitivity to topoisomerase II poisons and expression of topoisomerase II, the sensitivity to ETO or teniposide does not always correlate with reduction in topoisomerase II level and/or activity (Danks et al., 1988; Ferguson et al., 1988; Matsuo et al., 1990; Ritke et al., 1994). In our study, cleavable complex formation was not directly related to the level of the drug resistance. It has been suggested that 0.35 μM sodium chloride may not fully extract the topoisomerase II. Nevertheless, different mutations in topoisomerase II, which would induce various sensitivity to the selecting drug, may also reconcile these observations (Bugg et al., 1991; Danks et al., 1993). Cleavable complex formation accounts for both qualitative and quantitative modifications of either topoisomerase II target or drug (Drabkin et al., 1989; Van der Velden et al., 1994). In addition, other cellular alterations may confer resistance to ETO. Two of the ETO-selected sublines exhibited neither pgp RNA overexpression nor reduced drug-stimulated DNA cleavage. Possible alternative mechanisms of resistance include overexpression of another multidrug transporter associated with the MDR phenotype (Grant et al., 1994; Schneider et al., 1994), inducible P450 dependent drug-metabolising activity (Sinha et al., 1988) and alteration of an enzyme involved in the glutathione metabolism (Sinha and Myers, 1984; Haim et al., 1987).

With all the DOX plus VCR-resistant sublines, there was a common mechanism of resistance, which corresponded to the one preferentially selected by each drug alone. Simultaneous resistance to both drugs may have resulted from overexpression of a single PGP (which would recognise both drugs). Alternatively, concurrent amplification of distinct pgp genes, coding for different proteins, has been observed in cross-resistant sublines (Ng et al., 1989; Gottesman and Pastan, 1993). While the general pgp gene amplification observed supports the latter hypothesis, both are in agreement with the high frequency of cells surviving DOX plus VCR treatment, which is similar to the frequency resulting from selection with DOX or VCR alone (Soués and Charcosset, 1993). In contrast, the frequency observed with the ETO plus VCR-selected cells was rather low, suggesting that two mutational events may be necessary to ensure complete MDR phenotype. Indeed, we observed, in all but one of the ETO plus VCR-selected sublines, both pgp mRNA overexpression and decreased ability to form cleavable complexes. While cells selected with ETO retained sensitivity to VCR, cells selected with VCR alone were cross-resistant to ETO. Two mechanisms of resistance were selected, yet resistance to VCR alone alleviated the sensitivity to ETO. It is conceivable that cross-resistance to VCR in cells selected with VCR alone rationalises this apparent contradiction: the need for a higher resistance to ETO would exclude PGP as a major source of protection in the doubly selected sublines. With the DOX plus ETO-selected cells, either one or both mechanisms were observed. Here, each mechanism could account for the double resistance: pgp overexpression (preferentially selected by DOX) procured cross-resistance to ETO and topoisomerase II alteration (almost systematically selected by ETO) accounted for the resistance of one of the DOX-selected sublines. PGP overexpression seemed, however, to be required for high DOX resistance, and topoisomerase II modification was associated with high resistance to ETO. This observation is compatible with the low cross-resistance to DOX of the ETO-selected sublines and with the moderate cross-resistance to ETO of the DOX-resistant cells. DOX plus ETO combination selected a heterogeneous population with either or both conferring mechanisms. The selection of single and double mutants probably accounted for the intermediate frequency of resistant cells observed: higher than the product of single resistance frequencies (as with the VCR plus ETO-resistant cells), but lower than either of the single resistance frequencies, as with the VCR plus DOX-selected cells (Soués and Charcosset, 1993). Finally, in one of the ETO plus DOX-resistant sublines, neither pgp overexpression nor topoisomerase II alteration was detected. In addition, a high resistance to ETO was observed in some of the doubly resistant sublines, despite an unchanged ability to form cleavable complexes. While an increased level of PGP without mRNA overexpression cannot be ruled out (Bradley et al., 1989; Hill et al., 1990), other modifications, as above, may have conferred resistance to ETO (Sinha and Myers, 1984; Haim et al., 1987; Sinha et al., 1988); Schneider et al., 1994) and DOX (Sinha and Chignell, 1979; Berlin and Haseltine, 1981; Zamboni et al., 1981; Bar- rand et al., 1994). Resistance to both drugs could also result from alteration in the lethal processing of the cleavable complexes (Schneider et al., 1990; Glisson et al., 1992).

Abbreviations: PGP, P-glycoprotein; MDR, multidrug resistance; mdr, MDR-associated genes; pgp, MDR-associated genes in hamster species; VCR, vincristine; ETO, etoposide; DOX, doxorubicin (Adriamycin); RI, resistance index; D10, 10% survival dose; CAD, polypeptide having carbamyl phosphate synthetase, aspartate transcarbamylase and dihydrolorotate activities.
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