In vivo characterization of a doxorubicin resistant B16 melanoma cell line

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Summary A doxorubicin-resistant line of B16 melanoma (B16VDXR) was obtained in vitro by continuous exposure to increasing concentrations of doxorubicin of an in vitro line (B16V) derived from the in vivo transplanted B16 melanoma. When injected s.c. into mice, B16VDXR exhibited histological features, metastatic behaviour, doubling time and tumourigenic potential similar to those of the parental B16V line. Tumours obtained by implantation of B16VDXR, however, had longer latency and permitted a longer survival time than B16V and had, as in vitro, a higher DNA content. After i.v. inoculation, B16VDXR cells had lower lung colonizing capability compared to B16V. B16V and B16VDXR had significantly lower metastatic potential compared to the B16 melanoma from which they derived. Doxorubicin treatment significantly delayed the growth of B16 and B16V transplanted s.c. and increased the life span of animals bearing B16V. B16VDXR was resistant to doxorubicin treatment when the in vitro resistance index was >100. While the doxorubicin-resistance phenotype was stable in vitro for 50 passages, in vivo the resistance phenotype was lost in 5 passages and tumours grown from s.c. inocula of mixtures of similar percentages of sensitive and resistant cells behaved as sensitive tumours. Cis-diaminedichloroplatinum (II), although marginally active in animals bearing B16V, was highly effective in B16VDXR bearing animals, suggesting a collateral cis-diaminedichloroplatinum (II) sensitivity of the B16VDXR line. After a single i.v. administration, doxorubicin reached initially, in the B16VDXR line, levels similar to those found in the B16 and B16V lines, but its release was faster from the resistant line in comparison with the sensitive ones. Doxorubicin-resistance was not overcome by more frequent treatments with doxorubicin. This doxorubicin-resistant tumour line obtained in vitro and used as a first in vivo transplant, may be a suitable metastasizing model for in vivo study of the mechanisms of resistance and of collateral sensitivity and for screening new drugs.

Doxorubicin (DX) is one of the most widely used antineoplastic drugs because it exhibits considerable activity against a broad spectrum of solid tumours and leukaemias. Unfortunately, as for anticancer drugs in general, tumours often are either resistant from the outset or become so after chemotherapy. This phenomenon, together with metastatic spread, represents the most important obstacles which limit the success of chemotherapy. In order to understand the mechanisms involved in anthracycline resistance, several experimental systems have been developed both in vitro and in vivo (Biedler et al., 1983; Dane, 1972; Inaba et al., 1979). Most of the in vitro studies, however, have been performed either on leukaemias or sarcomas grown in ascitic form and treated with i.p. administration of the drugs to be tested, i.e. by an assay which mimics the in vitro situation (Biedler et al., 1983; Seeber et al., 1982), or on solid tumours whose sensitivity and resistance to DX were tested only in in vitro assays (Giavazzi et al., 1983). Such experimental systems, although very helpful for an understanding of specific molecular events associated with acquired resistance, may not be suitable for examining other important factors which may influence the response of the tumour to chemotherapy such as metastatic spread, immunological sensitivity in addition to the pharmacokinetics and the metabolism of the drug.

Our aim was to develop a DX-resistant solid metastasizing tumour as a potentially useful model for defining the nature of resistance to DX in vivo, for investigating means of circumventing DX-resistance and for screening new non-cross resistant drugs. We describe here the main in vivo biological properties and the sensitivity of a B16 melanoma line, made resistant to DX in vitro, in comparison with those of the original tumour and with those of an in vitro DX-sensitive line.

Materials and methods

Animals

Adult (8–10 weeks old) C57BL/6NCrl and (C57BL/6NCrl x DBA/2NCrl)F1 (B6D2F1) male mice were supplied by Charles River Breeding.

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Laboratories (Calco, Como, Italy). Eight to 10 animals per group were used in each experiment.

**Tumour and tumour cell lines**

The B16 melanoma, obtained from the Division of Cancer Treatment of the National Cancer Institute (Bethesda, MD, USA) was maintained by s.c. implantation of a tumour homogenate in C57BL/6 mice (Geran et al., 1972). Detailed description of the in vitro lines is reported in a previous paper (Supino et al., 1986). The schematic representation of the obtained lines is given in Figure 1. A cell line obtained by trypsinization of B16 melanoma and designated B16V, was maintained in medium RPMI 1640 (Flow Laboratories, Irvine, Ayrshire, UK) supplemented with 10% foetal calf serum (Flow Laboratories) and Fe(CN)$_6$K$_3$ 0.03 mM and routinely subcultured twice a week. The DX-resistant cell line, designated B16VDXR, was obtained by continuous exposure of B16V cells to increasing concentrations of DX starting from 5 ng ml$^{-1}$ up to 420 ng ml$^{-1}$ in about 60 transplant generations. These cells were cultured further either in medium containing 420 ng ml$^{-1}$ (B16VDXR) or in drug-free medium (B16VR) for 20 passages. B16VDXR cells, continuously exposed to 420 ng ml$^{-1}$ DX were subcultured in drug free medium for 24 h before being tested.

**Drugs**

DX (Farmitalia-Carlo Erba, Milan, Italy) was dissolved in distilled water immediately before use. Cis-diamminedichloroplatinum (II) (cis-DDP) (Farmitalia-Carlo Erba, Milan, Italy) was dissolved in 0.9% NaCl.

**Index of resistance (RI) in vitro**

Detailed description of the in vitro assays of levels of resistance has been reported (Supino et al., 1986). Briefly, cells were treated at cell seeding with different concentrations of DX. After 72 h, cells were harvested by trypsinization and counted in a model ZBI Kontron Coulter Counter. The RI was evaluated as the ratio between the graphically determined concentration causing a 50% decrease in cell number at this time point (ID50) on the B16VDXR cells and the ID50 on the B16V cells.

**In vivo studies**

All the in vivo studies were performed in B6D2F1 mice, since chemosensitivity studies on B16 melanoma were performed in this strain according to standard, accepted procedures (Geran et al., 1972). No differences in takes and growth rate for this tumour were found in syngeneic C57BL/6 as compared to semi-syngeneic B6D2F1 animals. One million cells from B16 melanoma, B16V, B16VDXR and B16VR lines were implanted s.c. in a 0.2 ml of RPMI 1640 in the right flank of B6D2F1 mice. For B16 melanoma, tumour homogenates were obtained 10 days after tumour transplant and for the lines grown in vitro tumour cells were obtained by brief exposure of 24 h
monolayer cultures to 0.25% trypsin and resuspension in serum-free medium. Only suspensions containing single cells with >95% viability, as determined by the trypan blue exclusion, were injected. B16V cells were used at about the 30th in vitro transplant, B16VDXR at about the 70th and B16VR at about the 90th transplant generation, the last 20 ones having been done without DX. DX and cis-DDP were administered i.v. once a week for 3 weeks starting 1 day after tumour implant. The longest and the shortest tumour diameters were measured by caliper twice a week and tumour weight was estimated according to Geran et al. (1972). Each animal was checked until death; at autopsy lungs were removed and the number of metastases per lung were counted with the aid of a dissecting microscope. Three end points were used to assess the antitumour activity.

1. The tumour growth delay (T-C), i.e. the difference in number of days required for the tumours to reach 1g between treated mice and controls; the significance (Student's t test) of the difference of the tumour weights of treated and control mice at 1 week after the last treatment was also evaluated; usually a T-C >4 days corresponded to a significant reduction of tumour weight.

2. The percentage increase of median survival time of treated mice compared to controls (% ILS) (statistical significance evaluated by Student's t test).

3. The metastasis efficiency i.e. the average number of metastases of treated mice/average number of metastases of control mice (statistical significance evaluated by Mann–Whitney U test).

For colonization potential assay, cells harvested from tissue culture, as previously described, were injected i.v. in a 0.2 ml volume in a lateral tail vein of B6D2F1 mice. Viable tumour cells were also mixed with 10^6 irradiated (100 Gy) cells in order to obtain more reproducible and meaningful results for quantitative analysis of experimental metastatic capacity (Hart et al., 1983) and heparin was also added to reduce intravascular cell clumping (Stackpole et al., 1985a). Three weeks after tumour cell injection, mice were killed, the lungs removed and the number of colonies per lung were counted with the aid of a dissecting microscope.

**Flow cytometric determination of DNA content**

The tumours (5–10 mm diameter) were removed, washed in 0.9% NaCl and minced with scissors to remove the necrotic part from the vegetal part which was used for DNA analysis. Cells were then harvested with trypsin-EDTA, washed in PBS and resuspended in a solution of 0.1% sodium citrate containing 50 μg ml⁻¹ propidium iodide (Calbiochem-Behring Corp., La Jolla, CA, USA), 50 U ml⁻¹ RNase A (Sigma, St. Louis, MO, USA) and 0.05% triton X-100 (Calbiochem-Behring Corp.). Mouse thymocytes used as a reference for the diploid value were processed in the same way. Flow cytometric measurements were performed with a microscope-based flow cytofluorimeter (Leitz, Wetzlar, West Germany), equipped with a 100 W Hg lamp as the source of excitation light. Excitation and emission wavelengths were selected by BG38 and BG12 excitation filters, a 580 nm chromatic beam splitter and a 610 nm barrier filter. Fluorescence intensity, proportional to DNA content, was recorded by a multichannel analyzer (Spectroscope Modular 8000, Laben, Milan, Italy) and displayed as fluorescence histograms.

**Pharmokinetics studies**

B6D2F1 mice with s.c. implanted cells were treated i.v. with DX 6.6 mg kg⁻¹ when the tumours were palpable (5–10 mm diameter). Three animals per point were killed with ether at different times after treatment. Tumours were removed, rinsed in cold saline and stored at −70°C until drug analysis. DX was assayed from the tumours as already reported (Formelli et al., 1986). Briefly, tumour homogenates were deproteinized by CH₃CN-phosphate buffer and drug released from DNA by AgNO₃ and analyzed by high performance liquid chromatography (HPLC) into a C₁₈ reverse phase column (Perkin Elmer HS-5) with CH₃CN:0.01 M KH₂PO₄ pH 3.8 (34:66) as mobile phase. Detection was by fluorometry on a Perkin Elmer MFP44A spectrofluorometer at 570 nm excitation and 590 nm emission wavelengths.

**Results**

**Dx-sensitivity of B16 melanoma lines**

The activity of DX administered to mice transplanted s.c. with 10⁶ cells of the original B16 melanoma and of the in vitro obtained lines was tested in parallel in several experiments in order to have a ‘head-to-head’ comparison. DX was administered i.v. at a dose of 6.6 mg kg⁻¹ starting from day 1 after tumour implant once a week for 3 weeks. This dose was chosen as the maximal tolerated dose with this schedule of treatment on non tumour-bearing B6D2F1 mice, and as the optimal therapeutic dose against B16 melanoma from a dose-response study (data not shown).

The results are reported in Table I; only one experiment was performed with the B16VR line.
Table I Antitumour activity of DX in mice bearing B16 melanoma lines

| Line      | DX* (ng ml⁻¹) | RI² | Tumour growth delay (T-C)³ | % ILS (T/C)⁴ | Metastasis efficiency (T/C)⁵ | Latency (days)⁶ |
|-----------|----------------|-----|---------------------------|--------------|-------------------------------|-----------------|
| B16       | —              | —   | 17 (14-23)                | +24 (12-30)  | 0.44 (0.04-0.90)⁶            | 8 (7-12)        |
| B16V      | —              | —   | 11 (8-12)                 | +44 (15-73)⁷ | 6.00 (0.50-20.00)            | 9 (7-12)        |
| B16VDXR   | 50             | 15  | 9                         | +13          | 2.80                          | 12              |
|           | 100            | 69  | 9.7                       | +42²         | 9.30¹                         | 11              |
|           | 420            | 137 | 2                         | -3           | 0.80                          | 18              |
|           | 420            | 275 | 4                         | +4           | 1.28                          | 24              |
|           | 420            | 260 | 0                         | +16          | 0.31                          | 24              |
|           | 420            | 310 | 2                         | -9           | 1.20                          | 19              |
| B16VR     | 0              | 428 | 0                         | +18          | 3.7/0                         | 17              |

For B16 and B16V the average and the range (in parenthesis) of the results of 5 experiments are reported. *DX concentrations in which cells were cultured in vitro. B16VR was cultured without DX during 20 transplants. ¹IV50 on B16VDXR/ID50 on B16V. ²Difference in number of days required for tumours to reach 1 g between treated mice and controls. T-C > 4 days corresponds to a significant tumour growth reduction. ³Percentage increase of median survival time of treated mice compared to controls. ⁴Average number of metastases of treated mice/Average number of metastases of control mice. ⁵Average time for tumours to become palpable (±0.1 g) in non-treated animals. ⁶The results were significant for P<0.05 in 4/5 experiments and for P<0.01 in 1/5 experiments by Student's t test. ¹The results were significant for P<0.05 in 1/5 experiments by Mann–Whitney U test. ²P<0.01 by Student's t test. ³P<0.01 by Mann–Whitney U test.

DX treatment caused a significant delay of the growth of B16 melanoma and of the B16V line. This growth delay resulted in a significant increase of survival time only in animals bearing the in vitro derived B16V line, possibly due to the fact that this line has a lower spontaneous metastasizing capacity compared to B16 (see Table II). DX treatment caused a reduction of lung metastases in animals bearing melanoma B16 while in animals bearing the B16V line an increase in metastasis efficiency was observed most likely as a consequence of their increase in life span.

The B16VDXR line, at different in vitro RI values during induction of resistance was also tested in the same experiments, to assess the relationship between in vitro and in vivo resistance to DX. The B16VDXR line was sensitive to DX treatment when the in vitro RI was as high as 69 while at higher RI values, DX treatment had no effect either on tumour growth or on survival time. The B16VR line, derived from B16VDXR after 20 in vitro passages in the absence of DX, was found more resistant in vitro than the original B16VDXR line and it was also resistant in vivo. An increase in metastasis efficiency was sometimes observed in animals bearing the two resistant lines which was not associated with the increase in survival time. In Table I the latency periods of non-treated mice injected with the four tumour lines are also reported. The two DX-resistant lines had a longer latency compared to the two sensitive lines and, in particular, the latency of B16VDXR was longer than the latency of B16V, but only when the tumour was resistant to DX treatment (in vitro RI higher than 69).

All the experiments reported in this paper refer to the B16VDXR line were run with cells grown in vitro in medium containing 420 ng ml⁻¹ and showing an in vitro RI of ~200.

Biological properties of B16 melanoma lines

The main biological properties after s.c. inoculum in B6D2F1 male mice of B16 melanoma and of the three lines obtained from it are summarized in Table II. The resistant lines, compared to the in vitro sensitive one (B16V), led to the appearance of tumours with longer latency and with higher variability among the single animals in all the experiments. As a consequence of the longer tumour-free interval, the survival time of mice bearing the resistant line was longer and they died with slightly larger tumours (12.8±3.7 vs. 9.5±3.6 g). The doubling time of the B16VDXR line was slightly higher, with a higher standard deviation, and the metastasizing capacity, evaluated at death, slightly lower compared to the sensitive line. All the in vitro obtained lines had lower metastasizing capacity compared to the original tumour and this difference does not seem to be due to survival duration. The two in vitro obtained lines had a histological pattern similar to the original tumour formed by epithelial cells with a minimal percentage of spindle cells. The tumourigenic dose 50 of the resistant line was slightly lower compared to the sensitive line.
**IN VIVO DOXORUBICIN-RESISTANT B16 MELANOMA LINE**

**Table II** Biological properties

|                | B16  | B16V | B16VDXR | B16VR |
|----------------|------|------|---------|-------|
| After s.c. inoculum of 10^6 cells* |      |      |         |       |
| — Latency (days)* | 7.9 ± 2.1 | 9.3 ± 1.8 | 19.5 ± 4.8*** | 17.1 ± 9.3 |
| — Doubling time (days)* | 3.0 ± 2 | 1.7 ± 0.8 | 2.8 ± 1.3 | 1.7 ± 0.7 |
| — Lung metastasis (%)* | 70 | 48 | 28 | 0 |
| — Lung metastasis (No.)* | 8 (0–30) | 0 (0–20) | 0 (0–22) |       |
| — Median survival time (days) | 34 (22–39) | 28 (14–40) | 41.5 (26–69)*** | 28 (13–>62) |
| Histology | Epithelioid | Epithelioid | Epithelioid | ND |
| Tumorigenic dose 50’ | ND | 0.97 x 10^5 | 1.3 x 10^5 | ND |

*The results are the average ± s.d. and the median with, in parenthesis, the range obtained from control groups each one consisting of 8–10 animals of 5, 5, 4 and 1 experiments, respectively of B16, B16V, B16VDXR and B16VR. Student’s t test has been applied to the data of the different groups of B16V and B16VDXR when there was homogeneity of variances (latency and survival time). *Time for tumours to become palpable (±0.1 g). *Calculated from single tumour growth curves from 500 to 1000 mg. *Percentage of animals with metastasis. *Median number of metastases per animal and, in parenthesis, range. *Evaluated from the number of mice developing tumours on the total number of mice injected s.c. with different cell inocula.

**Lack of cross-resistance of the B16VDXR line with cis-DDP**

To see whether there was cross-resistance between DX and another anticancer agent known to have a different mechanism of action, the activity of cis-DDP administered i.v. at the maximal tolerated dose, (4 mg kg⁻¹) starting on day 1 after tumour implant once a week for 3 weeks was tested in animals transplanted s.c. with 10⁶ cells of the B16V and the B16VDXR lines (Table IV). Cis-DDP caused a significant delay of the growth of both tumour lines, but it prolonged survival only in animals bearing the resistant line, a finding which resulted in an increase in metastases. In the experiments carried out with animals bearing the resistant line, cis-DDP treatment caused a very high percentage increase of the median survival time (107%) in one experiment and 3 long term survivors out of 10 in the other one. These data suggest that the B16VDXR line is not cross-resistant but, on the contrary, is collaterally sensitive with cis-DDP.

**Stability of the resistance phenotype**

B16VDXR cells have been shown to maintain resistance to DX during 50 in vitro passages in absence of the drug (Supino et al., 1986). To check the in vivo stability of DX-resistance phenotype, the two resistant lines B16VDXR and B16VR were transplanted in vivo when the tumour diameter was 5–10 mm and their sensitivity after s.c. transplant of 10⁶ cells to DX treatment (6.6 mg kg⁻¹ from day 1 once a week for 3 weeks) was checked at different transplant generations. Table V shows that the two
Figure 2  Distribution of fluorescence intensity of DNA-propidium iodide in B16, B16V, B16VDXR tumour. (a) B16: peaks at channels 124 and 243; (b) Mouse thymocytes: peaks at channels 129 and 249; (c) B16V: peaks at channels 123 and 240; (d) B16VDXR: peaks at channels 127, 253 and 370.

lines became very quickly sensitive to DX: DX treatment significantly increased the tumour growth delay and the survival time of animals bearing the fifth transplants of both lines. Among the checked parameters of growth of the two lines, the latency of the tumour varied in the serial transplants in vivo and in particular it became shorter and similar to that of the DX-sensitive line with the increase in the number of transplants.

To check the outcome of DX chemotherapy in mice bearing both sensitive and resistant tumours and how resistant and sensitive cells in the same tumours influence the growth and the sensitivity of the whole tumours, mice were transplanted s.c. either with B16V cells in one flank and B16VDXR on the other flank or with 50% B16V cells and 50% B16VDXR cells in the same flank and treated with DX (Table VI). In animals bearing separate tumours, each tumour behaved as if it was in separate animals i.e. the resistant tumour had a longer latency, compared to the sensitive one, and, differently from the sensitive one, its growth was not delayed by DX. As a whole it should be noted that DX treatment caused a significant increase of survival time with a consequent increase in metastasis incidence. In mice bearing tumours consisting of 50% sensitive and 50% resistant cells, the latency was similar to that of the sensitive line and this may be due to the fact that the growth of B16V cells, which have shorter latency, masks the latency of B16VDXR cells. In animals treated with DX, the growth of the tumour was delayed 5 days and the survival time was significantly longer compared to non-treated animals.

Therefore, these results suggest that DX-resistant cells do not influence the sensitivity of sensitive cells when present in two separate tumours or in similar percentages in the same tumour.
### Table III  Titration of colonization capacity

| Cell inoculum | Pulmonary colonies | Extrapulmonary colonies | Pulmonary colonies | Pulmonary colonies |
|---------------|-------------------|------------------------|-------------------|-------------------|
|               | Incidence | Median | Range  | Incidence | Median | Range  | Incidence | Median | Range  |
| 5 x 10^4      | ND       |        |        |           |         |        | 2/5      | 0      | 0–31   |
| 1 x 10^3      | 10/10   | 67     | 30–100 | 8/8      | 105     | 35–187 |           |         |        |
| 1 x 10^3 +    |          |        |        |           |         |        | 2/8 Ovary| 0/5    | 0–21   |
| 1 x 10^6 X    | ND       |        |        |           |         |        |          | 0/7    | 0–27   |
| cells         |          |        |        |           |         |        |          | 6/8    | 6      |
|               |          |        |        | 1/6 Ovary|         |        |          | 6/8    | 0–1    |
|               |          |        |        | 1/6 Kidney|        |        |          |        |        |
|               |          |        |        | 1/6 i.p.  |        |        |          |        |        |

*1 x 10^6 irradiated cells (100 Gy) were admixed to the viable cells. Inoculum of 1 x 10^6 irradiated cells in 5 animals for each line gave no colony; ND = not detected.
### Table IV  Antitumour activity of cis-DDP in B16V and B16VDXR bearing mice

| Line       | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|
| B16V       | 12     | 4      | -25    | -4     | ND     | 0/7.1  | 0/10   | 0/10   |
| B16VDXR    | 8.5    | 6      | +107** | +40**  | ND     | 5.25   | 0/10   | 3/10   |

*Difference in average number of days required for tumours to reach 1 g between treated mice and controls; 
^bPercentage increase of median survival time of treated mice compared to controls; 
^cAverage number of metastases of treated mice/Average number of metastases of control mice; 
^dLong term survivors at 3 months; 
**P<0.01 Student's t test; ND=not detected.

### Table V  Antitumour activity of DX against in vivo serial transplantation of B16VDXR and B16VR

| Line       | In vivo transplants | Tumour growth delay (T - C)^a | % ILS (T/C)^b | Metastasis efficiency (T/C)^c | Latency (days)^d |
|------------|---------------------|-------------------------------|--------------|-------------------------------|-----------------|
| B16VDXR    | 1st                 | 1                             | +13          | 10.6†                        | 12              |
|            | 2nd                 | 3.5                           | +32*         | 2.24                         | 13              |
|            | 5th                 | 11                            | +119*        | 43.5†                        | 9               |
| B16VR      | 1st                 | 0                             | +18          | 3.7/0                        | 14              |
|            | 3rd                 | 2.5                           | +9           | 1.04                         | 9               |
|            | 5th                 | 4                             | +42*         | 5.43†                        | 8               |

^a,b,c See Table IV; 
^dAverage time for tumours to become palpable (\(\approx 0.1 \text{ g}\)) in non-treated animals; 
^P<0.05 Student's t test; †P<0.05 Mann–Whitney U test.

### Table VI  Antitumour activity of DX in mice bearing both B16V and B16VDXR lines

| Lines      | Exp. | Tumour growth delay (T - C)^a | % ILS (T/C)^b | Metastasis efficiency (T/C)^c | Latency (days)^d |
|------------|------|-------------------------------|--------------|-------------------------------|-----------------|
| B16V       | 1    | 8                             | +32**        | 2.3                           | 12              |
| and B16VDXR| 2    | 8                             |              |                               | 18              |
| 50% B16V   | 2    | 5                             | +62***       | 1.17/0                        | 12              |
| plus 50% B16VDXR| 2 | 5                      |              |                               | 12              |

In experiment 1, 10^6 cells B16V were inoculated s.c. in one flank and 10^6 cells B16VDXR were inoculated s.c. in the other flank of the same animal. In experiment 2, 5 \times 10^5 cells B16V were admixed with 5 \times 10^5 cells B16VDXR and inoculated s.c. in one flank. DX treatment: 6.6 mg kg^{-1} i.v. from day +1, 1 wk \times 3 wks. 
^a,b,c,d See Table IV. 
**P<0.01; ***P<0.001 Student's t test.
In order to understand whether DX-resistance was due to different pharmacokinetics in the tumour, DX concentrations were evaluated in B16 and the two tumour lines at different times after i.v. administration (Table VII). No DX metabolites were found in the two lines at all the time points examined. A great variability in DX concentrations during the first 6h after treatment was found both in the B16 and the B16VDXR lines. In spite of this variability, drug levels were rather similar. Then the release of the drug was quicker from the resistant line compared to the two sensitive ones since drug concentrations were significantly lower.

**Effect of the increase in frequency of DX administration**

The retention of DX being lower in the resistant as compared to the sensitive line, we checked if a more frequent treatment would also be effective against the B16VDXR line. No increase in DX activity against the resistant line was obtained by increasing the frequency of DX administration (4mg kg\(^{-1}\) on days 1, 4, 7, 9 vs. 6.6mg kg\(^{-1}\) on days 1, 8, 15) (data not shown).

**Discussion**

The resistant line of B16 melanoma described in this paper shows, when first transplanted in vivo, resistance to DX treatment which is effective, as assessed from the growth of the tumour and the survival time, on B16 melanoma and on the line obtained in vitro (B16V), from which the resistant line was derived.

As reported for other murine tumours (Schabel et al., 1983), no cross-resistance was found for this tumour line with cis-DDP. The 2-fold greater in vitro cis-DDP sensitivity of the resistant line compared to the sensitive one (Supino et al., 1986) and the marked increase in survival time together with the presence of 3/10 long term survivors after cis-DDP treatment in animals bearing the DX-resistant line, suggest a collateral sensitivity of the B16VDXR line to cis-DDP. The reduction in survival time after cis-DDP treatment in animals bearing the B16V line, in spite of the effect on tumour growth, suggests a different toxicity of cis-DDP in mice bearing different tumours, a finding which might be due to a higher toxic – may be cachetic – effect of the sensitive line on the host compared to the resistant one. Similar higher toxicities have been reported for cis-DDP and other anticancer drugs in mice bearing the ovarian reticular cell sarcoma M5076 compared to the cyclophosphamide resistant line (D’Incalci et al., 1983).
The main biological properties that characterize the B16VDXR line in vivo compared to the parent line B16V are the longer latency period with consequent longer survival time and the lower colonization capacity, all features which go along with lower malignancy as reported for other drug-resistant tumours (Biedler et al., 1983). The longer latency of the B16VDXR line correlates with the in vitro longer doubling time (25 vs. 15 h) (Supino et al., 1986) and this might be responsible for the different sensitivity to both DX and cis-DDP. In fact it has been reported that while rapidly growing tumours are more sensitive to DX than slowly growing tumours, the opposite is true for cis-DDP (Mattern et al., 1981). The only slight differences found in the growth rates of the two tumour lines after they had become palpable is probably due to the very short doubling times of the two lines, and to the short experimental range time of tumour measurements. Extrapolation of the experimental data by Gompertz analysis is in progress.

The fact that B16VDXR showed after in vivo growth the same higher DNA content compared to the sensitive line B16V found in vitro (Supino et al., 1986), indicates that no cell selection occurred during in vivo growth. Similarly no selection seems to have occurred in the sensitive line B16V obtained by growing the parental B16 melanoma in vitro. Karyotype analyses of the two lines are in progress in order to better understand if this modification is directly related to the drug-resistant phenotype since double minute chromosomes and homogenously staining regions have been found in different multidrug resistant cell lines and have been associated with gene amplification (Riordan & Ling, 1985). As far as the DNA content in resistant lines compared to the sensitive ones is concerned, increase (Parsons & Morrison, 1978) or decrease (McMillan et al., 1985) in chromosome number and similar DNA content (D'Incalci et al., 1983) have been reported.

The finding that B16V and B16VDXR lines have similar spontaneous metastatic behaviour but different colonization capability extends similar observations of lack of coincidence of the two properties as reported for B16 melanoma (Stackpole, 1981) and other murine tumours (Price et al., 1984). The lower colonization potential of the resistant line is directly related to the cloning efficiency which has been found to be 10 times lower for the resistant line (Supino et al., 1986) and confirm similar findings on B16 melanoma subclones (Stackpole et al., 1985b). Such a correlation does not exist between the cloning efficiency and the tumourogenicity of these two lines as already reported for B16 melanoma cells in culture (Kreider & Schomayer, 1975). In addition it should be noted that, at variance with what has been reported for other resistant tumour lines (Biedler et al., 1975; Biedler et al., 1983) only a slight decrease in tumourogenic potential is associated with development of resistance in our line.

The enhancement in metastasis formation not associated with an increase in survival time, sometimes observed in DX treated mice bearing the resistant lines, is an interesting finding with potentially important clinical implications. This unfavourable result might be due to the immuno-suppressive effect of chemotherapy on moderately or markedly antigenic tumours (Elbe et al., 1973; Nowak et al., 1973). In fact, it has been reported that treatment with anticancer drugs may lead to induction of new antigens on tumour cells (Nicolin et al., 1972).

The analysis of the relationship between the degree of resistance in vitro and the sensitivity to DX treatment in vivo, show that the B16VDXR line retained significant responsiveness in vivo in spite of a R1 value of 69 and this argues about the relationship between in vitro and in vivo resistance results. From the results obtained in our study this discrepancy might be explained by the latency differences between sensitive and resistant cells with consequent in vivo predominance of sensitive cells, with shorter latency, probably still present in the resistant line at that particular R1. This possibility is supported by the fact that if resistant cells were inoculated together with similar percentages of sensitive cells, the resultant tumour behaved as if it was sensitive both in terms of latency and sensitivity.

The loss also of the resistant phenotype during few in vivo transplants might be due to latency differences between sensitive and resistant cells and to the procedure used for tumour propagation since each transplant has been performed few days after the tumours had become palpable, i.e. when most likely sensitive cells with shorter latency were replicating.

Our results on DX pharmacokinetics on the resistant line confirm the data obtained in vitro on several rodent and human tumour cell lines (Giavazzi et al., 1983; Dane, 1983; Inaba et al., 1979; Howell et al., 1984; Rogan et al., 1984) in which the induction of DX-resistance is associated with decreased DX retention. The lack of activity of DX against the resistant line even if administered more frequently, suggests that decreased drug retention is probably only one component of resistance. In fact, B16VDXR cells have also shown when incubated with DX in vitro, a different drug intracellular distribution with lower nucleus/cytoplasm ratio compared to the sensitive cells (Supino et al., 1986).

In conclusion, our results obtained with this cell
line, which when first transplanted in vivo shows resistance to DX treatment, indicate this model to be suitable for in vivo studies of the mechanisms of resistance to DX and for selecting non-cross-resistant drugs and drugs able to circumvent DX-resistance.

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