Cancer Chemother Pharmacol (1988) 21: 251–254

Cancer Chemotherapy and Pharmacology

Lack of cross-resistance of a doxorubicin-resistant B16 melanoma line with 4'-deoxy-4'-iodo-doxorubicin

Rosanna Supino[1], Mariangela Mariani[1], Ennio Prosperi[2], and Giorgio Parmiani[1]

[1] Division of Experimental Oncology B, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy
[2] Centro di Studi per l'Istochimica del CNR, Dipartimento di Biologia Animale, University of Pavia, Italy

Summary. A B16 melanoma cell line in which resistance to doxorubicin (Dx) had been induced by in vitro exposure to the drug, was found not to be cross-resistant with 4'-deoxy-4'-iodo-doxorubicin (4'-I-Dx), a new Dx derivative. Dx was 200 times less active in resistant than in sensitive cells, whereas the iodo derivative compound had the same level of activity in both cell lines. Cytotoxicity of Dx was dependent on concentration and on length of treatment, whereas that of 4'-I-Dx was correlated only with drug concentration. In an effort to explain this different behavior, intracellular retention and distribution of the two drugs was examined. Uptake and efflux of 4'-I-Dx in sensitive and resistant cells were similar, and cellular retention of the drug was 5–25 times higher than that of Dx. In addition, intracellular distribution of the iodo-derivative compound was similar in both cell lines, whereas more nuclear Dx was found in sensitive than in resistant cells. These differences may explain not only the lack of cross-resistance, but also the different cytotoxic behavior, of 4'-I-Dx.

Introduction

Doxorubicin (Dx) is an anticancer drug effective against a wide spectrum of experimental [9] and clinical tumors [3, 6]. Unfortunately, as for anticancer drugs in general, tumors often become resistant to the treatment subsequent to selection or induction of a resistant cell subpopulation [12, 18, 19].

Tumor cells resistant to Dx are generally cross-resistant to other anthracycline analogues [17, 18] and also to other anticancer agents, such as vinca alkaloids [14, 23]. The mechanisms responsible for resistance are still under study, but some of them have been identified in gene amplification [16, 22], alterations of plasma membrane [11, 14], reduced intracellular accumulation of the drug due to higher efflux [8, 13], energy-dependent drug transport [2, 10] and altered drug-DNA interactions [4, 15].

In our laboratory a subline of B16 melanoma cells with elevated levels of resistance to Dx was selected by continuous in vitro exposure to increasing concentrations of Dx [20]. This line showed cross-resistance with vincristine and other anthracyclines, such as daunorubicin and 4'-epi-Dx, but a lower resistance index (RI) was found with 4'-deoxy-Dx (RI = 30) and 4-demethoxy-daunorubicin (RI = 3) [20]. In the same study, however, no cross-resistance was obtained with a new Dx derivative, 4'-deoxy-4'-iodo-Dx, whereas collateral sensitivity was found with *cis*-diamminedichloroplatinum.

In the present paper we report studies performed with the objective of elucidating the mechanisms responsible for the lack of cross-resistance with 4'-deoxy-4'-iodo-Dx in B16 melanoma cells. A difference in the uptake, efflux and intracellular distribution of the two drugs was found; however, a different mechanism of toxicity is suggested by our results as an explanation for the lack of cross-resistance.

Offprint requests to: R. Supino, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1. I-20133 Milano, Italy

Material and methods

Drugs. Dx and 4'-deoxy-4'-iodo-Dx (4'-I-Dx) were a gift from Farmitalia-Carlo Erba (Milan, Italy). Dx was dissolved in 0.9% NaCl solution immediately before use, while 4'-I-Dx was melted with methanol and diluted in 0.9% NaCl solution. Drug concentrations were determined spectrophotometrically by using the coefficient $E^{1\%}_{480}$ = 173 for Dx and $E^{1\%}_{478}$ = 198 for 4'-I-Dx.

Cell lines. The B16 melanoma cell line (B16V) and its resistant variant cell line (B16VDXR) were obtained as already reported [20] and maintained in RPMI 1640 medium (Flow Laboratories, Irvine, Ayrshire, UK) supplemented with 10% fetal calf serum (FCS; Flow Laboratories), 0.03 m*M* $Fe(CN)_6K_3$ and antibiotics.

Cytotoxicity evaluation. Exponentially growing cells were treated with Dx for 1 or 72 h at 37° C. After 1 h of treatment, cells were washed with saline solution and cultured in drug-free medium for 72 h. After harvesting with trypsin-EDTA, cells were counted in a Coulter counter (ZBI Electronics, Luton, UK). Cell viability was determined by the Trypan Blue exclusion test. The resistance index (RI) was calculated as the ratio between the ID_{50} of the drug in resistant cells and the ID_{50} of the same drug in sensitive cells.

Drug uptake studies. For drug uptake experiments, 1×10^6 exponentially growing cells were seeded into dishes 5 cm in diameter (Falcon, Becton Dickinson, Calif) with 4 ml complete medium and incubated at 37° C in a humidified 5% CO_2 atmosphere. After 24 h, the culture medium was

replaced with fresh medium containing Dx or 4'-I-Dx; the concentration used for both drugs was 1 μg/ml. In each experiment three dishes were used for each point. The intracellular drug content was evaluated according to Finkel [7]. Influx was stopped at different points during incubation; the medium was removed, cells were washed with 2 ml ice-cold phosphate-buffered solution (PBS), detached with trypsin-EDTA and harvested in 2 ml distilled water. Then, 3 ml 1-butanol was added to each sample, vortex-mixed twice for 1 min and centrifuged at 2000 rpm for 10 min. The butanolic phase was analyzed in a fluorescence spectrophotometer (Perkin-Elmer MPF-44A) at 500 nm excitation and 589 nm emission. The drug concentration in each sample was determined from a calibration curve.

Drug efflux studies. For drug efflux experiments, 1×10^6 exponentially growing cells were seeded into 75-cm^2 flasks (Corning Glass Work, Corning, NY) with 4 ml complete medium. After 24 h, the culture medium was replaced with fresh medium containing Dx or 4'-I-Dx (1 μg/ml). Efflux was evaluated after 1 h of drug uptake. At this time the medium was removed, cells were washed with cold PBS, and 50 ml of drug-free medium was added. At different times medium was removed and cells were washed and detached as described for the studies of drug uptake.

Intracellular distribution of the drugs. Intracellular distribution of Dx and 4'-I-Dx was studied in cell monolayers grown on coverslips, incubated with the drug for 0.5, 24 or 48 h. At the end of the incubation, the coverslips were rinsed with PBS and mounted upside-down on microscopic slides. The fluorescence intensity of anthracycline was analyzed subsequently in the nucleus and in the cytoplasm of each cell by performing spot measurements with a diaphragm 2 μm in diameter. Measurements were performed with a Leitz MPV2 microscope photometer, as previously reported [20].

Results

Cytotoxic activity

The cytotoxic activity of Dx and 4'-I-Dx on B16V and B16VDXR cells after 72 h of treatment has already been reported [20], but it is included again in Fig. 1 A to allow comparison of the cytotoxicity of the two drugs after 1 h of treatment (Fig. 1 B). Although a dose-response effect was evident in all the experiments, Dx, even at very high concentrations, failed to cause a 100% cell killing both with 1 and 72 h exposures, whereas no surviving B16V and B16VDXR cells can be found after treatment with 5 μg/ml of 4'-I-Dx. No differences were observed in the

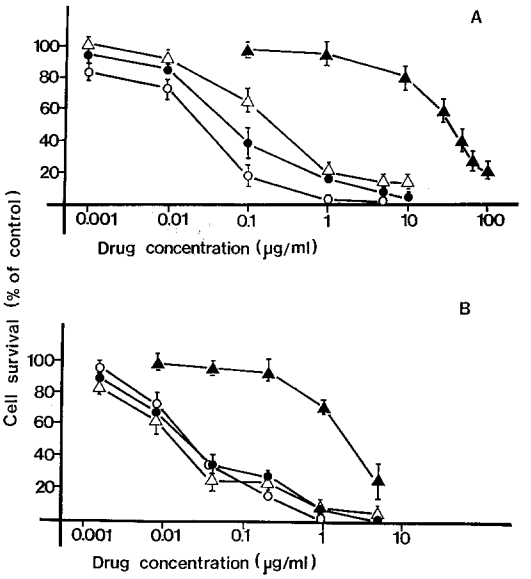

Fig. 1 A, B. Cytotoxic activity of Dx and 4'-I-Dx on sensitive and resistant cells after **A** 1 h or **B** 72 h of treatment. △, Dx on B16V; ▲, Dx on B16VDXR; ○, 4'-I-Dx on B16V; ● 4'-I-Dx on B16VDXR. Data shown are the means ± SD of six determinations in three independent experiments

ID_{50} of 4'-I-Dx for the two cell lines, suggesting the absence of a cell subpopulation resistant to this drug.

A comparison between the ID_{50} and the resistance indexes of the two drugs tested on the sensitive and resistant lines is reported in Table 1. The RI for each drug remained essentially unchanged after treatments for 1 or 72 h (180–200 for Dx and 2.1–1.2 for 4'-I-Dx). However, shortening the time of treatment from 72 to 1 h increased the ID_{50} of Dx on B16V cells 20 times, while the ID_{50} of 4'-I-Dx was not dependent on the time of exposure. Thus, whereas after long-term treatment the two drugs had similar ID_{50} values in B16V cells (10 vs 15 ng/ml), after 1 h of treatment a Dx concentration about 8 times higher (200 vs 27 ng/ml) was needed to kill 50% of cells. In addition, Dx was about 100 and 600 times less active than 4'-I-Dx in B16VDXR cells after 72 and 1 h of treatment, respectively. Taken together, these data suggest different mechanisms of action of the two drugs on the two cell lines.

Drug uptake and efflux studies

In order to explain the different behavior of the two drugs, we have evaluated their uptake in sensitive and resistant cell lines. Figure 2 shows that the uptake of Dx in B16V and B16VDXR cells was about 5 and 15 times lower than

Table 1. Cytotoxic activity of Dx and 4'-I-Dx on B16V and B16VDXR cells evaluated as ID_{50} (ng/ml) after 1 or 72 h of treatment

Duration of treatment (h)	Dx			4'-I-Dx		
	B16V	B16VDXR	RI[a]	B16V	B16VDXR	RI[a]
1	200	36000	180	27	58	2.1
72	10	2000	200	15	18	1.2

[a] Resistance index = $\frac{ID_{50} \text{ on B16VDXR}}{ID_{50} \text{ on B16V}}$

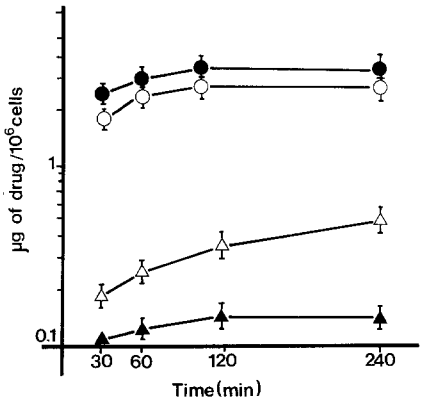

Fig. 2. Dx and 4'-I-Dx uptake in sensitive and resistant cells. Cellular drug content was evaluated spectrofluorometrically in butanolic cell extracts. △, Dx in B16V; ▲, Dx in B16VDXR; ○, 4'-I-Dx in B16V; ●, 4'-I-Dx in B16VDXR. Data are the means ± SD of four determinations in two independent experiments

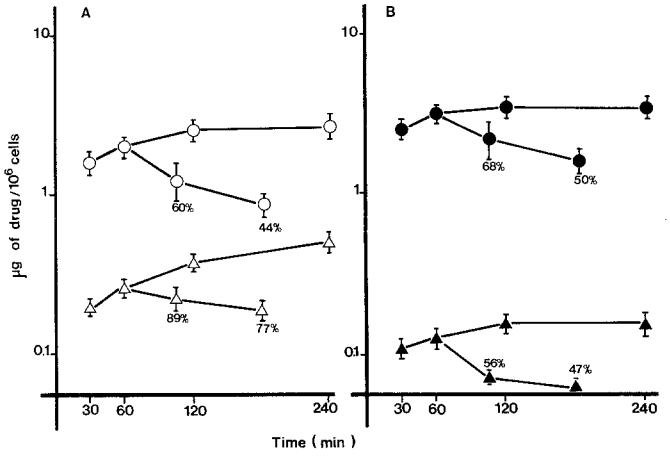

Fig. 3A, B. Uptake and efflux of Dx and 4'-I-Dx in **A** B16V and **B** B16VDXR. Cellular drug content was evaluated spectrofluorometrically in butanolic cell extracts. *Triangles*, Dx; *circles*, 4'-I-Dx. Data are the means ± SD of four determinations in two independent experiments. Figures in *parentheses*, percentage cellular drug retention

that of 4'-I-Dx, respectively. However, while the content of Dx in B16V cells increased at least until 4 h in the presence of the drug, the cellular content of 4'-I-Dx peaked in 2 h and no significant amount of drug seems to enter the cells with further exposure. In contrast in B16VDXR cells the plateau was reached with both Dx and 4'-I-Dx after 2 h of exposure; in these cells the difference between cellular content of the two drugs subsequently remained constant.

In B16V cells (Fig. 3A) significant differences were observed in the rate and in the amount of efflux of the two drugs, the percentage efflux of 4'-I-Dx being twice that of Dx; then the amount of cellular drug content after 2 h of efflux was five-fold higher for the iodo-derivative than for Dx. In B16VDXR cells (Fig. 3B), in contrast, where the uptake of 4'-I-Dx was 10 times higher than that of Dx, the percentage efflux of the two drugs was similar; in both cases the release of the drug was about 50%. Comparison of the effluxes in the two cell lines [21] revealed a higher amount of Dx release in resistant than in sensitive cells, while the efflux of 4'-I-Dx was similar in B16V and

Table 2. Intracellular distribution of DX and 4'-I-Dx in B16V and B16VDXR cells

Drug	Treatment		Nucleus/cytoplasm ratio[a] (± SD)	
	Dose (µg/ml)	Time	B16V	B16VDXR
Dx	10	30 min	4.83 ± 1.50**	2.55 ± 0.78
	1	24 h	2.44 ± 0.68*	1.56 ± 0.46
	1	48 h	0.87 ± 0.08	0.82 ± 0.13
4'-I-Dx	5	30 min	2.31 ± 0.43	2.18 ± 0.18
	1	24 h	0.86 ± 0.05	1.08 ± 0.24
	1	48 h	0.60 ± 0.21	1.05 ± 0.27

[a] Mean of at least 10 samples for each point
* $P < 0.05$; ** $P < 0.01$ (Student's *t*-test)

B16VDXR cells. As a consequence of these differences, the intracellular content of 4'-I-Dx was 5 and 25 times higher in B16V and B16VDXR, respectively, than that of Dx.

It was interesting that the 4'-I-Dx uptake and efflux were so high that when the drug efflux was evaluated in the same volume of culture medium as drug uptake only 30% was found to be released in the first 45 min and no further release was observed (data not shown). This was due to the high extracellular concentration reached by this drug in these conditions. In contrast, Dx release, which was minimal in comparison with 4'-I-Dx, was not affected by the volume of culture medium. These experiments, therefore, show that a different amount of drug efflux, and consequently of intracellular drug, may be one of the mechanisms responsible for the lack of cross-resistance with 4'-I-Dx.

Intracellular drug distribution

Since a difference in the intracellular distribution of Dx in the sensitive and resistant cells has been reported [20], we also examined this parameter for the anthracycline derivative. Since the Dx uptake was lower than that of 4'-I-Dx, the latter was determined at lower concentrations. Results of this experiment are reported in Table 2, which shows that the ratio of nucleus/cytoplasm distribution of Dx was significantly higher in B16V (4.83) than in B16VDXR (2.55) and that this difference was maintained with longer treatment durations (24 and 48 h). In contrast, intracellular distribution of 4'-I-Dx after 30 min of incubation was two-fold in the nucleus compared with cytoplasm, but this ratio was similar in sensitive and resistant cells. After 24 and 48 h of treatment this ratio fell to 1 in both cell lines, whereas this value was not reached in cells treated with Dx until after 48 h of exposure. The observation that the intracellular distribution of 4'-I-Dx is similar in the two cell lines is a possible explanation for the equal cytotoxicity of the drug in B16V and B16VDXR cells.

Discussion

Cellular resistance to DX in leukemic and solid tumors is usually associated with cross-resistance to other anthracyclines [6, 12] as well to other antitumor agents, such as vinca alkaloids [14, 23]. Several studies have been performed to explain the resistance to Dx, which seems to be due to a reduced accumulation of the drug in the cell [8, 10] and/or

to a gene amplification [16, 22] and/or to a different interaction of the drug with DNA [4, 15]. Cross-resistance, which occurs among drugs having different molecular structures, has been correlated with an alteration of membrane permeability, which would lead to a reduced cellular accumulation of drugs [8, 13].

Dx and its derivative 4'-I-Dx were not cross-resistant in B16 melanoma cells. In fact treatments for both 1 and 72 h could define an ID_{50} for Dx that was 200 times higher in resistant than in sensitive cells, whereas that of 4'-I-Dx was similar in both cell lines. In addition, our data show that while the ID_{50} of Dx is dependent on the time of exposure being 20 times lower at 72 than at 1 h of treatment, the ID_{50} of 4'-I-Dx is similar with 72-h or 1-h treatments. These differences can be explained by a faster uptake of the Dx derivative or by a different mechanism of toxicity.

The lack of cross-resistance between the two drugs seems to be due to similar 4'-I-Dx uptake and efflux in both Dx-sensitive and Dx-resistant cell lines; these data, together with a similar intracellular distribution of the drug in both cell lines, indicates no differences in the nuclear and cytoplasmic membrane transport of 4'-I-Dx in Dx-sensitive and -resistant cells. The higher uptake of 4'-I-Dx than of Dx in B16V cells, probably due to the higher lipophilicity of the drug [1], is responsible for the 10 times higher cytotoxic activity of the anthracycline derivative when the exposure to the drug lasts for only 1 h. This difference in the amount of drug uptake could also explain the non-time-dependent cytotoxicity of 4'-I-Dx: in fact, in 1 h it reaches intracellular drug levels sufficient to induce the same activity as with a longer duration of exposure. The differences observed in drug uptake, efflux and distribution and in the ability to induce DNA strand breaks [5] could be responsible for the lack of cross-resistance between the two compounds. However, in order to explain their different cytotoxic behavior, a different stability of the linking to DNA or a different interaction with the DNA repair system must be postulated.

Acknowledgements. The skillful technical help of Mr A. Colombo is gratefully acknowledged. The work has been partially supported by grant no. 86.02610.44 from CNR (Rome), Finalized Project Oncology.

References

1. Arcamone F (1985) Properties of antitumor anthracyclines and new development in their application: Cain memorial award lecture. Cancer Res 45: 5995
2. Bates DA, Fung H, Mackillop WJ (1985) Adriamycin uptake, intracellular binding and cytotoxicity in Chinese hamster ovary cells. Cancer Lett 28: 213
3. Bonadonna G, Beretta G, Tancini G, Brambilla C, Bajetta C, De Palo GM, De Lena M, Fossati-Bellani F, Gasparini M, Valagussa P, Veronesi U (1975) Adriamycin studies at the Istituto Nazionale Tumori, Milan. Cancer Chemother Rep 6: 231
4. Capranico G, Dasdia T, Zunino F (1986) Comparison of doxorubicin-induced DNA damage in doxorubicin-sensitive and -resistant P388 murine leukemia cells. Int J Cancer 37: 277
5. Capranico G, Riva A, Tinelli S, Dasdia T, Zunino F (1987) Markedly reduced levels of anthracycline-induced DNA strand breaks in resistant P388 leukemia cells and isolated nuclei. Cancer Res 47: 3752
6. Davis HL, Davis TE (1979) Daunorubicin and adriamycin in cancer treatment: an analysis of their roles and limitations. Cancer Treat Rep 63: 809
7. Finkel JM, Knapp KT, Mulligan LT (1969) Fluorometric determination of serum levels and urinary excretion of daunomycin (NSC-82151) in mice and rats. Cancer Chemother Rep 53: 159
8. Fojo A, Akiyama S, Gottesman MM, Pastan I (1985) Reduced drug accumulation in multiple drug-resistant human KB carcinoma cell lines. Cancer Res 45: 3002
9. Goldin A, Venditti JM, McDonald JS, Muggia FM, Henney JE, De Vita VT (1981) Current results of the screening program at the division of Cancer Treatment, National Cancer Institute. Eur J Cancer 17: 129
10. Inaba M, Kobayashi H, Sakurai Y, Johnson RK (1979) Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines P388 leukemia. Cancer Res 39: 2200
11. Kartner N, Evernden-Porelle D, Bradley G, Ling V (1985) Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. Nature 316: 820
12. Kaye S, Merry S (1985) Tumor cell resistance to anthracyclines. A review. Cancer Chemother Pharmacol 14: 96
13. Merry S, Ferherston CA, Kaye SB, Freshney RI, Plumb JA (1986) Resistance of human glioma to adriamycin "in vitro". The role of membrane transport and its circumvention with verapamil. Br J Cancer 53: 129
14. Peterson RH, Meyers MB, Splengler BA, Biedler JL (1983) Alteration of plasma membrane glycopeptides and gangliosides of Chinese hamster cells accompanying development of resistance to daunorubicin and vincristine. Cancer Res 43: 222
15. Pommier Y, Zwelling LA, Kao-shan CS, Whang-Peng J, Bradley MO (1985) Correlations between intercalator-induced DNA strand breaks and sister chromatid exchanges, mutations, and cytotoxicity in Chinese hamster cells. Cancer Res 45: 3143
16. Roninson IB, Abelson HT, Housman DE, Howell N, Varshavsky A (1984) Amplification of specific DNA sequences correlates with multidrug resistance in Chinese hamster cells. Nature 309: 626
17. Seeber S, Osieka R, Schmidt CG, Achterrath W, Crooke ST (1982) In vivo resistance towards anthracyclines, etopside, and *cis*-diamminedichloro-platinum (II). Cancer Res 42: 4719
18. Shoemarker RH, Curt GA, Carney DN (1983) Evidence for multidrug-resistant cells in human tumor cell population. Cancer Treat Rep 67: 883
19. Smith BJ, Kundu D (1986) Acquired resistance to daunorubicin in a patient with acute myelogenous leukemia. Br J Cancer 34: 53
20. Supino R, Prosperi E, Formelli F, Mariani M, Parmiani G (1986) Characterization of a doxorubicin-resistant murine melanoma line: studies on cross-resistance and its circumvention. Br J Cancer 54: 33
21. Supino R, Mariani M, Capranico G, Colombo A, Parmiani G (1988) Doxorubicin cellular pharmacokinetics and DNA breakage in a multi-drug resistant B16 melanoma cell line. Br J Cancer (in press)
22. Van der Bliek AM, Van der Velde-Koerts T, Ling V, Borst P (1986) Overexpression and amplification of five genes in a multidrug-resistant Chinese hamster ovary cell line. Mol Cell Biol 6: 1671
23. Wilkoff LJ, Dulmadge EA (1978) Resistance and cross-resistance of cultured leukemia P388 cells to vincristine, adriamycin, adriamycin analogs, and actinomycin D. J Natl Cancer Inst 61: 1521

Received May 25, 1987/Accepted December 10, 1987