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Cross Resistance and Cellular Uptake of 4′-O-Methyldoxorubicin in Experimental Tumors with Acquired Resistance to Doxorubicin

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Summary. *A new analog of doxorubicin, 4′-O-methyldoxorubicin, was previously reported to have a pronounced activity against L1210 leukemia, which shows a natural partial resistance to doxorubicin itself. In the present study, lines of P388 leukemia and Ehrlich ascites tumor with acquired resistance to doxorubicin were found to be cross-resistant to 4′-O-methyldoxorubicin, indicating that the natural and the acquired resistance to doxorubicin involve different mechanisms. In vitro studies on the uptake of 4′-O-methyldoxorubicin in the Ehrlich ascites tumor cells indicated that the observed cross-resistance was partly due to a decreased drug uptake in the resistant cells because of an increased extrusion of the drug, in accordance with previous findings on the mechanism of acquired resistance to doxorubicin.*

Introduction

The outstanding antineoplastic properties of the anthracycline antibiotic doxorubicin [5] have stimulated the search for new derivatives, with the aim of reducing the dose-limiting toxic side-effects and of improving the antineoplastic activity against sensitive tumors or against tumors with natural or acquired resistance.

Cassinelli et al. [1] have recently synthesized a new derivative of doxorubicin, 4′-O-methyldoxorubicin, in which the amino sugar moiety is modified by methylation (Fig. 1). Among a variety of analogs, this compound distinguished itself by having a pronounced activity against L1210 leukemia, which shows a natural partial resistance to doxorubicin [1].

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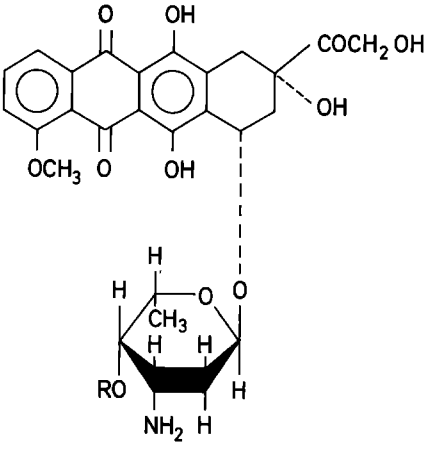
In the present study, we have tested the activity of 4′-O-methyldoxorubicin against lines of Ehrlich and P388 ascites tumors in which resistance to doxorubicin was acquired after treatment with the drug. In both cases, we found cross-resistance to 4′-O-methyldoxorubicin. Previous studies have indicated that resistance to anthracyclines in Ehrlich ascites tumors is related to a decreased cellular drug uptake at steady-state conditions, caused by an increased active outward transport of the drugs [3, 4, 7]. In this paper, we present the results of in vitro studies of the cellular uptake of 4′-O-methyldoxorubicin, which indicate that a similar mechanism is involved in the observed cross-resistance to this drug.



Doxorubicin, R = H
4′-O-methyldoxorubicin, R = CH_3

Fig. 1. The chemical structures of doxorubicin and 4′-O-methyldoxorubicin [1]

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Materials and Methods

Materials. Doxorubicin and 4'-O-methyldoxorubicin hydrochlorides were gifts from Dr G. Cassinelli, Farmitalia, Carlo Erba, Milano. For in vivo experiments, the drugs were dissolved in 0.9% NaCl or in distilled water so that the dose per gram mouse-weight was contained in 0.01 ml, and the appropriate volume was administered intraperitoneally (IP). Calf serum was from Grand Island Biological Co., USA. All other reagents were of the best commercially available grade.

Tumors and Animals. A wild-type Ehrlich ascites tumor (EHR2) and a subline in which resistance was developed and maintained by treatment with doxorubicin (EHR2/Dx+ = EHR/AM+) [2] was maintained in first-generation hybrids of random-bred Swiss mice and inbred DBA mice (N/D mice) by weekly IP transplantation, as described previously [2]. The mice used for maintenance of the EHR2/Dx+ tumor received 2.5 mg doxorubicin per kg body weight IP daily for 4 consecutive days [2]. When experiments were performed with the EHR2/Dx+ tumor the maintenance treatment was interrupted during the passage prior to the experiment. A wild-type P388 leukemia and a subline in which resistance to doxorubicin was developed and maintained by treatment with the drug (P388/Dx+, obtained from F. M. Shabel, Southern Research Institute, Birmingham, Alabama, USA) were maintained in first-generation hybrids of inbred C57Bl/6 and DBA/2 mice (BDFl mice) by weekly IP transplantation of 10^7 cells per mouse. The mice used for maintenance of P388/Dx+ were treated with 6 mg doxorubicin per kg body weight IP 2 days after transplantation.

Toxicity Experiments. Five to eight groups of 10–20 mice each were treated daily for 4 days with IP injections of drug doses causing lethality (as observed within 60 days after first injection) ranging from 0 to 100%, and the LD_{10} with 95% confidence limits was calculated as described previously [2].

Evaluation of Antitumor Activity. For therapy experiments with the Ehrlich ascites tumors, N/D mice were inoculated with 15×10^6 cells IP, as described previously [2]. Drug administration IP was started 24 h after inoculation and continued with daily treatments for a total of 4 consecutive days. Deaths among mice were recorded daily and the median survival time was calculated for the treated groups and for parallel control groups which were treated with 0.9% sodium chloride IP. P388 leukemia cells were inoculated IP (10^6 cells per mouse) in BDFl mice 2–3 months old and weighing 20–30 g. The mice were treated with drugs IP as indicated in each experiment, and deaths among mice were recorded daily until day 60 after inoculation. The geometric mean survival time and the percentage of long-term survivors (> 60 days) were calculated and compared with the corresponding values in parallel mock-treated control groups. Calculation of 5% confidence limits was based on a normal distribution of the logarithm of the survival times. Each treated group consisted of at least six mice and the control groups of larger numbers.

Determination of Cellular Drug Uptake. As described previously [7], uptake of doxorubicin and 4'-O-methyldoxorubicin in Ehrlich ascites tumor cells in vitro was determined by incubation of cell suspensions (5 µl packed cells per ml suspension) in a phosphate-buffered medium (pH 7.45) containing 10 m*M* glucose, 5% calf serum, and the drugs, as indicated in each experiment. After incubation, 2 ml suspension was added to 8 ml Ringer solution at 0° C and pelleted by centrifugation at 3,000 *g* for 1 min. The cells were washed twice with a Ringer solution at 4° C, and extracted with 50% ethanol, 0.3 *M* HCl. Drug content in the extracts and in the culture fluid (after addition of one tenth [v/v] 1.0 *M* HCl) was determined by fluorometry (excitation 485 nm/emission 585 nm for 4'-O-methyldoxorubicin and excitation 470 nm/emission 585 for doxorubicin). The recovery of drugs in cell extracts and culture fluid was more than 90% in all experiments.

Results

Activity of 4'-O-methyldoxorubicin against P388 Leukemia and a Doxorubicin-resistant Subline

The effect of doxorubicin and 4'-O-methyldoxorubicin on mice inoculated with wild-type P388 leukemia cells was tested at various doses of the drugs given in three different schedules (Table 1). At their optimal doses, both drugs achieved significant and pronounced prolongation of the median survival time, including the survival of a significant number of mice for more than 60 days. In none of the schedules were there any pronounced differences in the antitumor activity of the two drugs at their respective optimal doses. The effect of the two drugs was also tested on the doxorubicin-resistant subline of P388 leukemia, P388/Dx+. When given at the dose and schedule resulting in optimal effect on the wild-type P388 leukemia, both drugs had only a very slight effect on the survival of the mice, and no mice were found to survive more than 60 days (Table 2). With regard to both increase in median survival time and induction of long-term survivors, the effect of both drugs on the P388/Dx+ leukemia was significantly smaller than their respective effects on the wild-type P388 leukemia. The P388/Dx+ leukemia thus showed cross-resistance to 4'-O-methyldoxorubicin.

Activity of 4'-O-methyldoxorubicin against a Wild-type Ehrlich Ascites Tumor and a Doxorubicin-resistant Subline

To allow comparison of the therapeutic activity of doxorubicin and 4'-O-methyldoxorubicin at equitoxic doses, toxicity experiments were performed with the two drugs. Mice were given daily doses IP for 4 days and observed for 60 days after the first injection. The LD_{10} for 4'-O-methyldoxorubicin was 2.0 mg/kg (95% confidence limits 1.7–2.3), as against an LD_{10} for doxorubicin of 2.9 mg/kg (95% confidence limits 2.5–3.4).

Table 3 shows the response to therapy with doxorubicin and 4'-O-methyldoxorubicin of mice inoculated with a wild-type Ehrlich ascites tumor and a subline in which resistance to doxorubicin had been developed by treatment with the drug [2]. It appears

Table 1. Effect of doxorubicin and 4′-O-methyldoxorubicin on survival of mice with wild-type P388 leukemia

Days of treatment[a]	Drug	Daily dose (mg/kg)	Number of mice in treated group	Mean survival time (% of control)[b]	Mice surviving more than 60 days[c]	Number of toxic deaths
1	Doxorubicin	4.4	10	236 (200–279)	0	0
		6.6	10	283 (212–378)	3	0
		10.0	10	344 (254–464)	5	0
	4′-O-Methyl-doxorubicin	2.9	10	271 (209–352)	2	0
		4.4	10	236 (166–334)	2	0
		6.6	10	403 (333–488)	3	3
5–9	Doxorubicin	2.0	10	179 (153–208)	0	0
		2.6	10	179 (154–209)	0	0
		3.3	10	191 (164–222)	0	0
	4′-O-Methyl-doxorubicin	1.2	10	170 (146–198)	0	0
		1.5	10	185 (159–216)	0	0
		2.0	10	191 (164–223)	0	0
5, 9, 13	Doxorubicin	2.2	10	152 (112–207)	1	0
		3.3	20	144[d] (131–158)	0	2
		5.0	20	164[d] (145–185)	0	3
		7.5	10	197 (172–226)	0	2
		11.2	10	172 (150–197)	0	9
	4′-O-Methyl-doxorubicin	1.4	10	131 (112–153)	0	0
		2.2	20	176[d] (146–212)	2	0
		3.3	20	182[d] (162–205)	0	0
		5.0	19	213[d] (197–230)	0	4
		7.5	10	137 (119–157)	0	7
		11.2	9	113 (98–130)	0	9

[a] IP treatment, at the indicated days after IP inoculation of 10^6 cells/mouse
[b] Geometric mean survival time of treated mice/geometric mean survival time of controls, × 100. Long-term survivors were included as surviving 60 days. The geometric mean survival time of controls was 11 days in all experiments. In brackets, 5% confidence limits
[c] There were no long-term survivors in any of the control experiments
[d] Data from two separate experiments

Table 2. Effect of doxorubicin and 4′-O-methyldoxorubicin on survival of mice inoculated with cells from a subline of P388 leukemia in which resistance to doxorubicin was developed by treatment with the drug (P388/Dx+)

Drug	Dose (mg/kg)[a]	Number of mice in treated group	Mean survival time (% of control)[b]	Mice surviving more than 60 days[c]	Number of toxic deaths
Doxorubicin	10	10	119 (101–140)	0	2
4′-O-Methyl-doxorubicin	2.9	10	107 (91–126)	0	0
	4.4	10	123 (104–144)	0	0
	6.6	10	117 (100–138)	0	0

[a] Treatment IP 24 h after IP inoculation of 10^6 cells/mouse
[b] Figures given show geometric mean survival time of treated mice/geometric mean survival time of controls, × 100. The geometric mean survival time of controls was 12 days in all the experiments. In brackets, 5% confidence limits
[c] There were no long-term survivors in any of the control experiments

that at drug doses of approximately LD_{10} the antitumor activity of 4′-O-methyldoxorubicin was comparable to that of doxorubicin in the wild-type tumor, whereas no activity could be demonstrated against the tumor line selected for resistance to doxorubicin. This line was thus cross-resistant to 4′-O-methyldoxorubicin.

Uptake of 4′-O-methyldoxorubicin in Wild-Type and Doxorubicin-Resistant Ehrlich Ascites Tumor Cells in vitro

Previously, we found that the development of resistance to doxorubicin in Ehrlich ascites tumor was accompanied by reduced cellular drug uptake [4]. To

Table 3. Effect of doxorubicin and 4'-O-methyldoxorubicin on survival of mice inoculated with cells from a wild-type Ehrlich ascites tumor (EHR2) and a subline in which resistance to doxorubicin was developed by treatment with the drug (EHR2/Dx+)

Drug	Dose (mg/kg, daily × 4)[a]	Mean survival time (percent of control)[b]	
		EHR2 ascites tumor	EHR2/Dx+ ascites tumor
Doxorubicin	2.5	267 (239–287)	113 (96–147)
4'-O-Methyldoxorubicin	1.8	244 (220–264)	94 (80–121)

[a] Drugs were administered IP. Treatment was started 24 h after IP inoculation of 15×10^6 cells

[b] Geometric mean survival time of treated mice/geometric mean survival time of controls, × 100. Numbers in brackets indicate 5% confidence limits. The geometric mean survival time was 10 and 16 days in control mice inoculated with wild-type and doxorubicin-resistant tumor cells, respectively

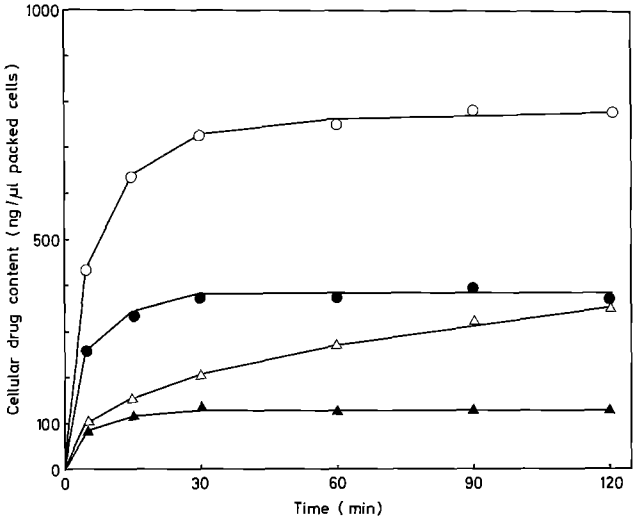

Fig. 2. Time course of the uptake of doxorubicin (△/▲) and 4'-O-methyldoxorubicin (○/●) in cells from a wild-type Ehrlich ascites tumor and from a subline resistant to doxorubicin, respectively. Suspensions of cells (5 µl packed cells/ml) were incubated in a phosphate-buffered medium containing 10 m*M* glucose and 5% calf serum at 37° C and pH 7.45. Drugs (5 µg/ml) were added at time zero and serial samples were withdrawn. The cellular drug content after two washings was determined by fluorometry on cell extracts

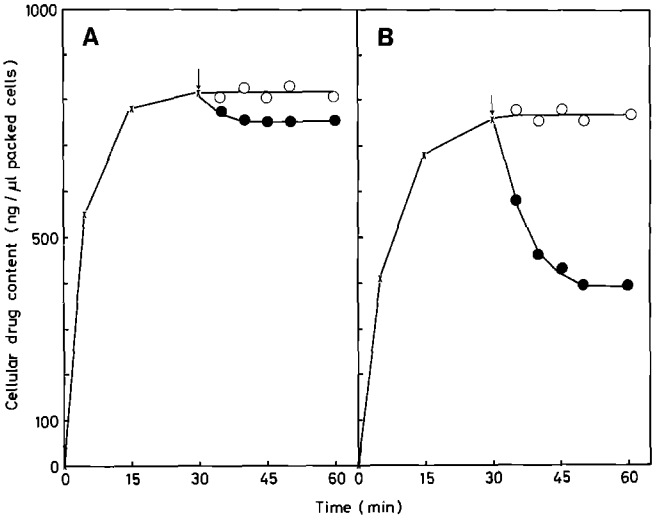

Fig. 3 A and B. Efflux of 4'-O-methyldoxorubicin induced by glucose in cells from a wild-type Ehrlich ascites tumor (**A**) and from a subline resistant to doxorubicin (**B**). Cell suspensions were incubated as described in Fig. 2, except that the medium contained sodium azide (10 µg/ml), while glucose was omitted (×———×). 4'-O-methyldoxorubicin was added at time zero. After 30 min glucose in a final concentration of 10 m*M* (●———●) or the same volume of 0.9% NaCl (○———○) was added. Serial samples were withdrawn at the times indicated and cellular drug content was determined as described in Fig. 2

elucidate the role of the cellular drug uptake of 4'-O-methyldoxorubicin for the mechanism of cross-resistance, some in vitro experiments were performed. Figure 2 shows the time course of uptake of 4'-O-methyldoxorubicin and doxorubicin in the wild-type tumor cells and in cells from the subline selected for resistance to doxorubicin. In both cell types, considerably more 4'-O-methyldoxorubicin than doxorubicin was taken up in the 120-min incubation period. In the resistant cells, the uptake of both drugs reached a steady state within 60 min. In the wild-type cells this was also the case for the uptake of 4'-O-methyldoxorubicin, while the uptake of doxorubicin was still increasing after 120 min. At steady-state conditions considerably less 4'-O-methyldoxorubicin was taken up in the doxorubicin-resistant than in the wild-type cells. The mean cellular steady-state concentration of 4'-O-methyldoxorubi-

cin which was not washed out by two washings at 4° C was 760 μg/ml in wild-type cells, as against a concentration of 1.2 μg/ml in the surrounding medium (ratio 633). For the resistant cells the corresponding figures were 380 μg/ml and 3.1 μg/ml (ratio 22). This indicates that a decreased drug uptake plays a role in the observed cross-resistance to 4′-O-methyldoxorubicin.

Previous studies have indicated that a lower drug uptake in Ehrlich ascites tumor cells with acquired resistance to various anthracyclines is related to an increased active efflux of daunorubicin and doxorubicin [3, 4, 7]. The effect of this outward transport mechanism can be illustrated by loading the cells with the drugs under conditions in which the energy metabolism has been inhibited by omission of glucose from the medium and addition of an inhibitor of the oxidative phosphorylation, e.g., sodium azide, and thereafter restoring the energy metabolism by the addition of glucose [7]. Figures 2 and 3 show that when the energy metabolism was inhibited the steady-state uptake of 4′-O-methyldoxorubicin in the doxorubicin-resistant cells was strongly increased and nearly equal to that in cells from the wild-type tumor line. Glucose induced an efflux from cells of both tumor lines, but the efflux component was much more pronounced in the resistant cells.

Discussion

These studies show that with IP administration, 4′-O-methyldoxorubicin is significantly more toxic, on a weight basis, than doxorubicin. At equitoxic doses the two drugs had approximately the same effect on a wild-type Ehrlich ascites tumor and, in agreement with a previous report [1], on a wild-type P388 leukemia. Sublines of the P388 leukemia and the Ehrlich ascites tumor, which had developed resistance to doxorubicin by treatment with the drug, showed cross-resistance to 4′-O-methyldoxorubicin in both cases. This finding is in good agreement with a variety of reports on cross-resistance between anthracycline antibiotics [reviews see 4, 8], but it is in contrast to the report that the naturally partly doxorubicin-resistant L1210 tumor [1] was much more sensitive to 4′-O-methyldoxorubicin than to doxorubicin. This probably signifies that the mechanism of acquired resistance to doxorubicin to some extent differs from the mechanism of natural resistance to this drug.

In Ehrlich ascites tumor cells, the acquired cross-resistance to 4′-O-methyldoxorubicin was related to a decreased steady-state uptake of the drug, as measured in vitro. Similar observations have previously been reported for daunorubicin and doxorubicin in resistant sublines of Ehrlich ascites tumors and other experimental tumors [reviews see 4, 8]. We have previously discussed the fact that in resistant Ehrlich ascites tumor cells the decreased steady-state uptake of daunorubicin and doxorubicin seems related to an active extrusion of the drugs [3, 4, 7]. This hypothesis is based on a variety of observations on the steady-state uptake of the drugs; e.g., that less of the drugs was taken up in intact resistant cells than in isolated nuclei; that analogs to the drugs increased their steady-state uptake in resistant cells; and that the steady-state uptake in the resistant cells was reversibly increased by inhibition of energy metabolism. Similar findings have recently been reported for doxorubicin-resistant P388 leukemia cells [6]. The present study shows that inhibition of energy metabolism increased the steady-state uptake of 4′-O-methyldoxorubicin in resistant Ehrlich ascites tumor cells, and that the uptake was decreased again by restoration of the energy metabolism. These findings are similar to those previously reported for daunorubicin and doxorubicin in the same cell type [3, 4, 7, 8] and indicate that the mechanism of the decreased uptake of 4′-O-methyldoxorubicin in the resistant cells is similar to that of daunorubicin and doxorubicin, and is therefore probably related to an increased extrusion of 4′-O-methyldoxorubicin from these cells.

The insight into the mechanism of acquired resistance to doxorubicin, 4′-O-methyldoxorubicin, and other anthracyclines raises several possibilities for counteracting the resistance. One approach is to combine treatment with cytotoxic anthracycline with non-cytotoxic analogs that decrease the extrusion of the cytotoxic drug by competition. This approach has recently shown its validity in the combined treatment with daunorubicin and the non-cytotoxic analog N-acetyl-daunorubicin, which rendered a daunorubicin-resistant subline of Ehrlich ascites tumor sensitive to the drug [8]. An alternative approach to counteracting resistance to anthracyclines would be the synthesis of analogs that are not transported by the supposed outward-directed transport mechanism, but are still cytotoxic. The present study shows a correlation between decreased uptake of 4′-O-methyldoxorubicin as measured in resistant cells in vitro and the finding of cross-resistance with doxorubicin, as measured in vivo. This correlation illustrates that simple in vitro measurements of drug uptake may be predictive for whether cross-resistance exists between doxorubicin and new analogs of this drug.

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