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Effect of Verapamil on doxorubicin activity and pharmacokinetics in mice bearing resistant and sensitive solid tumors*

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Summary. The effect of the combined administration of verapamil (i.p. twice daily) and doxorubicin (i.v. once weekly) was tested in mice bearing the following: (a) a tumor with induced resistance to doxorubicin (B16VDXR melanoma line); (b) a tumor inherently resistant (MXT mammary carcinoma); and (c) four solid tumors sensitive to doxorubicin (B16 melanoma, B16V melanoma line, M5076 reticulum cell sarcoma, and Lewis lung carcinoma). Verapamil, given according to this treatment schedule, reached peak plasma concentrations of 3 μM. Such treatment did not enhance doxorubicin activity on either inherently or induced resistant tumors, whereas it significantly enhanced doxorubicin growth inhibition in all the sensitive tumors except the Lewis lung carcinoma. Doxorubicin pharmacokinetics after administration of the drug alone and in combination with verapamil was analyzed after the first and repeated treatments in animals bearing B16 melanoma or its resistant subline B16VDXR. The resistance of the B16VDXR line was associated with the ability of the tumor to retain less doxorubicin (AUC = 83 μg h/g) than the sensitive tumor B16 (AUC = 204 μg h/g) in spite of similar initial levels. The potentiating effect of doxorubicin activity by verapamil in B16 melanoma was not associated with increased doxorubicin levels or retention in the tumor, nor were differences in doxorubicin levels or retention found in the B16VDXR line. The combined treatment did not modify doxorubicin pharmacokinetics in plasma, heart, or spleen. These studies suggest that verapamil in vivo is ineffective in potentiating doxorubicin activity in tumors against which doxorubicin is inactive, that sensitive tumors are heterogeneous in their sensitivity to modulation by verapamil, and that this effect is not associated with modification of doxorubicin pharmacokinetics.

Introduction

Drug resistance is commonly encountered in cancer chemotherapy. The heterogeneity of a tumor cell population with respect to chemosensitivity may be responsible for the incurability of some tumors by chemotherapy and may explain the development of drug-resistant tumors during chemotherapy. Doxorubicin (DX) is widely used to treat human cancer, and many studies have been devoted to improving the therapeutic index of the drug and circumventing the resistance of tumor cells to it. Resistance to anthracyclines, as well as to vincristine and vinblastine, has been associated with the increased active efflux of the drug [3, 6, 13, 18, 28], and it has been reported that calmodulin inhibitors [10, 27, 29, 30] and calcium antagonists such as verapamil (VRP) increase anthracycline, vincristine, and vinblastine accumulation and cytotoxicity in several resistant sublines [23, 27–31].

In our laboratories it has been found that in vitro pretreatment with VRP and trifluoperazine, a calmodulin antagonist, partially overcomes the DX resistance of B16VDXR, a DX-resistant line obtained in vitro from B16 melanoma [26]. Most of the reported studies have been performed in vitro or in vivo on leukemias or sarcomas grown in the ascitic form and treated by i.p. administration of the drugs to be tested, i.e., by an assay which mimics the in vitro situation [27, 28, 31]. Since phase I–II studies of VRP combined with vinblastine [2] and with DX [22] are in progress, we think that results of such combined treatments on solid tumors in mice receiving systemic drugs may provide useful information for the interpretation of clinical findings.

Our aim was, therefore, to check the in vivo effects of the combined treatment of VRP with systemic DX on resistant and sensitive solid tumors and to evaluate whether VRP affects DX pharmacokinetics in plasma, tumor, or in target organs for anthracycline toxicity, such as the heart and spleen. Moreover, since it has been reported that VRP inhibits experimental and spontaneous metastases of some murine tumors [32], the effect of VRP on metastases was also evaluated. For these studies we chose the MXT mammary carcinoma, which is inherently resistant to DX, and the B16VDXR subline of B16 melanoma, which has in vitro-induced resistance to DX [26] and whose growth in vivo is not affected by DX treatment [8]. For comparison we chose the DX-sensitive tumors B16 melanoma, M5076 reticulum cell sarcoma, Lewis lung carcinoma, and B16V melanoma.

Materials and methods

Animals. Adult (8–10 weeks old) C57BL/6NCrl and (C57BL/6NCrl xDBA/2NCrl)F1 (B6D2F1) mice were supplied by Charles River Breeding Laboratories (Calco, Como, Italy). Eight to ten animals per group were used in

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each experiment and at least two experiments were performed for each tumor line.

Drugs. DX (Farmitalia-Carlo Erba, Milan, Italy) was dissolved in distilled H_2O and given i.v. at a dose of 6.6 mg/kg starting 1 day after tumor implant, once a week, for 3 weeks. This dose was chosen as the maximal tolerated dose with this treatment schedule in nontumor-bearing $B6D2F_1$ mice. VRP (Knoll AG Liestal, Switzerland), formulated for clinical use, was diluted in 0.9% NaCl and given i.p. twice daily at 8-h intervals starting from day 1 after tumor implant, 5 times a week, for 4–5 weeks; this schedule was chosen as the most feasible for achieving high constant plasma VRP levels. On the basis of preliminary experiments, the 10% lethal doses (LD_{10}) used were 25 mg/kg for B6D2F1 mice and 12.5 mg/kg for C57BL/6 NCrl mice. In combination experiments, VRP was given 3 h before DX. All drugs were freshly prepared, protected from light, and administered at a volume of 10 ml/kg body weight.

Tumors and tumor cell lines. The B16 melanoma and the Lewis lung carcinoma, obtained from the Division of Cancer Treatment of the National Cancer Institute (Bethesda, Md, USA), were maintained by s.c. implant in C57BL/6 NCrl mice according to the protocols of that institute [12]. The B16V line was obtained in vitro in our laboratories from B16 melanoma [26]: in vivo it has almost the same sensitivity to DX and a lower metastatic potential than the original B16 melanoma [8]. The B16VDXR line is DX-resistant and was obtained in vitro by continuous exposure of the B16V line to increasing concentrations of DX; its resistance index is equal to 200 [26]. In vivo its growth is not affected by DX treatment and, like the B16V line, it has a lower metastatic potential than the original B16 melanoma [8]. The MXT mammary carcinoma, obtained through the courtesy of Dr. A. Bogden (Mason Research Institute, Worcester, Mass, USA), was maintained in female B6D2F1 mice by serial s.c. transplantations of $3 \times 3 \times 3$ mm fragments. This hormone-independent line was selected from the original estrogen-dependent MXT tumor [33]. The M5076 reticulum cell sarcoma, obtained through the courtesy of Dr. M. D'Incalci (Mario Negri Institute, Milan, Italy), was maintained by i.m. implant of tumor cell suspensions in C57BL/6 NCrl mice.

For antitumor activity assays, 10^6 viable (by trypan blue exclusion) cells of each tumor were implanted as follows: B16, B16V, B16VDXR s.c. in B6D2F1 males; MXT s.c. in B6D2F1 females; M5076 s.c. in C57BL/6 NCrl females; and Lewis lung i.m. in C57BL/6 NCrl females. Tumor cells were obtained from in vivo-transplanted tumors by mechanical disruption in saline and from in vitro-grown lines by brief exposure of 24-h monolayer cultures to 0.25% trypsin and resuspension in serum-free medium. Maximal and minimal tumor diameters were measured by caliper three times a week, and tumor weight was estimated according to Geran et al. [12]. Each animal was checked until death, and lungs were removed at autopsy and analyzed under a dissecting microscope. The number of metastases per lung was counted, and the two diameters of individual metastases were measured and their weight estimated according to Geran et al. [12].

End points for assessing antitumor activity. The following end points were used to assess antitumor activity: the increase in survival time (% ILS), the percentage tumor weight of treated mice compared to controls (% T/C tumor weight), and the gross $\log_{10}$ kill. % ILS is the percentage increase in median survival time of treated mice compared to controls (statistical significance was evaluated by Student's *t*-test). % T/C tumor weight is the percentage of average tumor weight of treated mice compared to controls evaluated 1 week after the last treatment (a T/C $\leq 42\%$ is considered necessary to demonstrate activity [12]; statistical significance was evaluated by Student's *t*-test). Gross $\log_{10}$ cell kill was calculated according to Corbett et al. [5] from the tumor growth delay (T-C) and from the average tumor doubling time of controls (Td). Conversion of the T-C values to $\log_{10}$ cell kill was possible because the Td of tumors regrowing posttreatment approximated the Td values of the tumors in untreated control mice.

Pharmacokinetic studies. B6D2F1 mice with s.c. palpable (5–10 mm in diameter) B16 and B16VDXR tumors were treated i.v. with DX alone or in association with i.p. VRP given according to the same schedule used to evaluate antitumor activity, i.e., 3 h before DX treatment, twice daily for 5 days a week.

For VRP assay, three animals per group were killed with ether at 30 min, 1, 2, 3, and 8 h after the first VRP treatment, and 30 min after the second. Blood was collected from the retro-orbital plexus into cold heparin-coated glass tubes. After the blood had been centrifuged, the plasma was removed and stored at −70° C until analysis. VRP plasma concentrations were measured using the high-performance liquid chromatographic (HPLC) assay described by Harapat and Kates [15].

For DX assay, three animals per group were killed with ether at 10 and 45 min, 2, 3, 6, 24, 72, 120, and 168 h after the first DX treatment, and 6 h after the second and third. The tumor, heart, and spleen were removed, rinsed in cold saline, and stored at −70° C until drug extraction. Plasma was collected and stored as described for VRP. DX was assayed as previously reported [9], with slight modifications. Briefly, plasma was diluted 1:4 (v:v) in 4 *M* NaCl, and the tumor and tissues were homogenized 1:4 (w:v) in 4 *M* NaCl. Then, 100 µl 0.5 *M* H_3PO_4 + 500 µl CH_3CN:CH_3OH:H_2O (50:30:10) were added to 400 µl diluted plasma or to homogenates. The supernatants were analyzed by HPLC in a C8 reverse-phase column (5 µm; Perkin Elmer) with CH_3CN:0.05 *M* potassium phosphate buffer, pH 3.9 (36:64, v:v). Detection of DX and metabolites was carried out by a Perkin-Elmer MPF44A spectrofluorometer at 500 nm excitation and 590 nm emission wavelengths. Quantitative evaluation of DX was done by comparison with reference standard curves set up for plasma and tissues; the recoveries were 70% and 85% and the limits of detectability 10 ng/ml and 10 ng/g, respectively. The areas under the curves (AUC) were calculated by trapezoidal rule. Metabolites were identified by cochromatography with the standards doxorubicinol and doxorubicinone, kindly supplied by Ricerca Chimica, Farmitalia-Carlo Erba (Milan, Italy).

Results

Effect of VRP on DX activity in sensitive and resistant murine tumors

The results obtained by giving DX and VRP alone and in combination to mice bearing solid tumors with different sensitivity to DX are reported in Table 1. The data refer to

Table 1. Response of early stage tumors to DX alone and in combination with VRP[a]

Tumor	Drug	% ILS[b]	% T/C tumor weight[c]	Gross $\log_{10}$ kill[d]
B16				
	VRP	21	96	0
	DX	40**	24***	0.70
	DX+VRP	85***(*)	3***(***)	1.70
Lewis lung				
	VRP	−13	100	0
	DX	55**	30***	0.98
	DX+VRP	42**	32***	0.98
M5076				
	VRP	−4	98	0
	DX	22***	30***	0.75
	DX+VRP	22***	15***(*)	1.28
B16V				
	VRP	−12	87	0
	DX	5	34***	0.60
	DX+VRP	15	14***(**)	1.00
MXT				
	VRP	44	69	0.15
	DX	11	67	0.23
	DX+VRP	15	76	0.07
B16VDXR				
	VRP	−16	133	0
	DX	12	63	0.30
	DX+VRP	−2	49	0.43

[a] VRP was given i.p. starting on day 1, twice daily, 5 days/week for 4–5 weeks, at a dose of 25 mg/kg to B6D2F1 mice bearing B16, B16V, MXT, or B16VDXR tumors, and at a dose of 12.5 mg/kg to C57BL/6 NCrl mice bearing Lewis lung and M5076 tumors (see *Material and methods*). DX was given i.v. starting on day 1, 1 day/week for 3 weeks, at a dose of 6.6 mg/kg

[b] % increase in median survival time of treated compared to control mice

[c] % tumor weight of treated compared to control mice 1 week after the last treatment (day 22); evaluation of Lewis lung carcinomas was done at the end of the treatment (day 15), because at day 22 more than 50% of control mice were dead

[d] Gross $\log_{10}$ kill = (T-C)/(3.32xTd)

**, $P \leq 0.01$; ***, $P \leq 0.001$, Student's *t*-test compared to controls (*), $P \leq 0.05$; (**), $P \leq 0.01$; (***), $P \leq 0.001$, Student's *t*-test compared to DX alone

one of at least two experiments carried out with similar results. VRP, given at 25 mg/kg to B6D2F1 mice bearing B16, B16V, MXT, or B16VDXR tumors, and at 12.5 mg/kg to C57BL/6 NCrl mice bearing M5076 or Lewis lung tumors, did not affect the growth of any tumor tested. The tumors chosen exhibited a wide range of sensitivity to DX. B16, Lewis lung, M5076, and B16V tumors were sensitive to DX treatment, since the tumor weight of treated animals was significantly lower than that of controls. To give a quantitative evaluation of DX activity, the approximate gross log kill was calculated, assuming that the delay of tumor growth was only due to cell-killing effect. The effect of DX on these tumors corresponded to a 0.6–0.98 gross $\log_{10}$ kill. A significant increase in life span was also found for all the animals except those bearing the B16V line. In contrast, the MXT tumor and the B16VDXR line were resistant to DX, since the percentage tumor weight of treated animals compared to controls was higher than 42% [12], with only a 0.2–0.3 gross $\log_{10}$ kill. VRP given in combination with DX significantly enhanced the tumor growth inhibition of DX in three of the four sensitive tumors (B16, M5076, and B16V), causing a 1–1.7 $\log_{10}$ kill, and it also caused a significant increase in the survival time of B16 tumor-bearing animals, in which the potentiating effect on DX activity was particularly evident. On the two DX-resistant tumors (MXT and B16VDXR), the combined treatment did not increase the activity of DX alone and no effect on survival time was found. Figure 1 shows the growth curves of animals bearing the B16 melanoma and the resistant B16VDXR. It can be seen that the average tumor weights for animals treated with VRP in combination with DX were significantly lower than those treated with DX alone for B16 tumor-bearing animals, but not for those bearing the B16VDXR line.

Effect of VRP on spontaneous and experimental metastases

Since it has been reported that VRP can inhibit both spontaneous and experimental metastases [32], we checked the effect of this drug on the metastases of the three melanoma lines in the same animals in which the antitumor activity was evaluated. The results are reported in Table 2. As has previously been reported [8], the two lines obtained in vitro, B16V and B16VDXR, had a lower metastasizing potential than the parent B16 melanoma. VRP significantly inhibited the number and the weight of metastases of B16V, but not of the other lines, and only in one of two experiments in which the metastasizing potential was higher than usual. In fact, the average incidence of animals with metastases is $\leq 50\%$ [8]. To check whether the effect of VRP was dependent on the number of background metastases of control mice, two different doses of cells were injected i.v. to obtain different numbers of experimental lung metastases (Table 3). Animals treated with VRP according to the same treatment schedule used to evaluate antitumor activity did not show any reduction in the number of metastases with either tumor cell inoculum.

VRP plasma levels

The concentrations of VRP in plasma were determined to relate them to those reported to be effective in enhancing DX cytotoxicity in in vitro experiments [26, 31]. The maximal tolerated dose of VRP (25 mg/kg i.p.) was given according to the same schedule of treatment (twice daily at 8-h intervals) used to evaluate antitumor activity. Figure 2 shows VRP plasma levels found after the first and second treatments. Peak levels, found half an hour after injection, were 3 μM, and drug levels were 0.8 μM at 3 h, when DX was given. VRP was eliminated from plasma with a half life ($t_{1/2}$) of 1.3 h.

Effect of VRP on DX pharmacokinetics

To see whether the increase in DX activity against B16 melanoma was due to differences in DX pharmacokinetics, DX levels were determined up to 7 days after the first treatment, and 6 h after the second and third, in the tumors of mice treated with DX alone and in combination with VRP. The plasma, heart, and spleen of the same animals were also analyzed. In addition, to assess whether, in spite of the lack of an increase in DX activity, VRP influ-

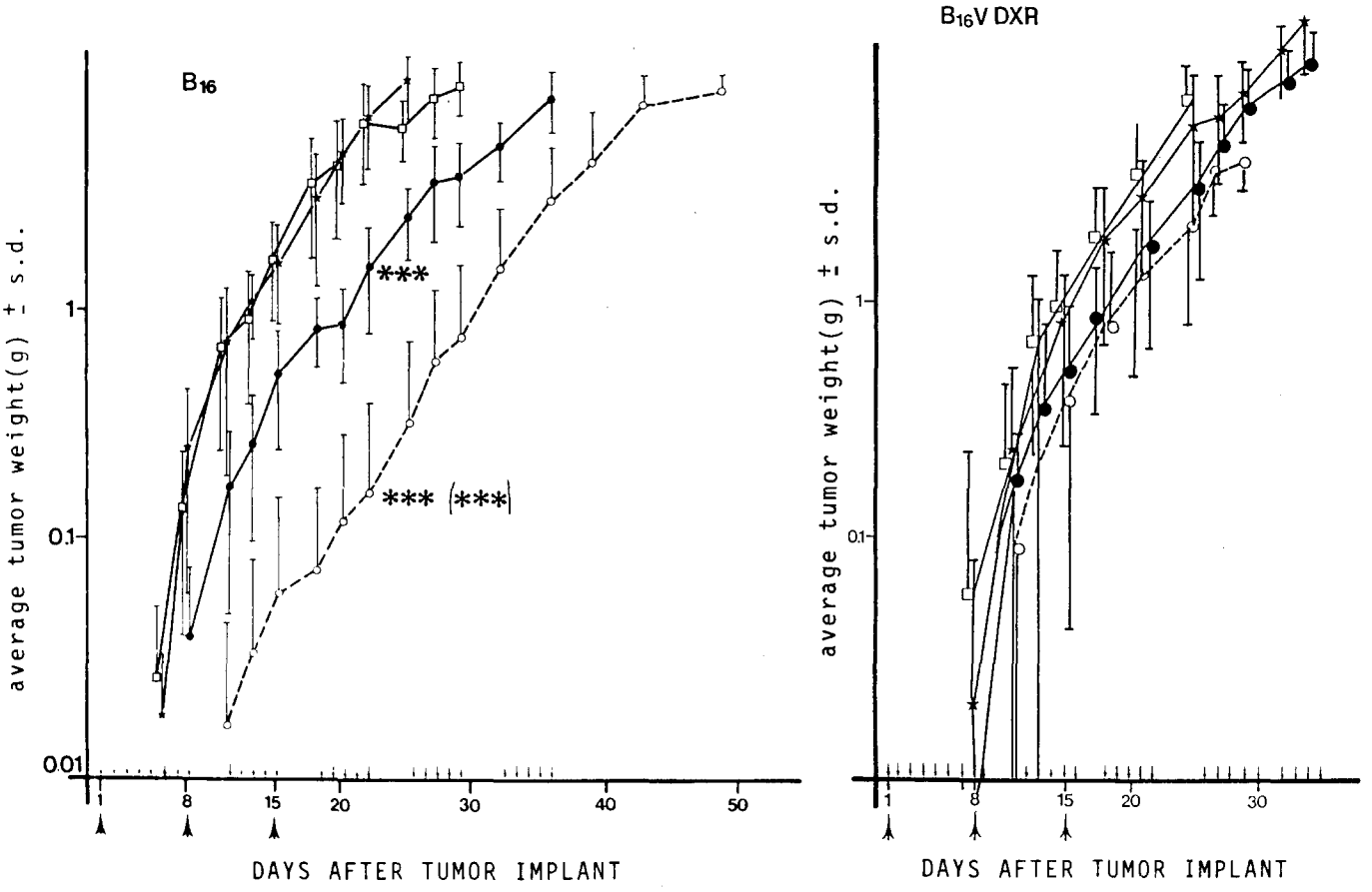

Fig. 1. Effect of DX and VRP alone and in combination on the growth of B16 and B16VDXR tumors. Mice were treated starting 1 day after tumor implant with DX (↑), 6.6 mg/kg i.v. once a week for 3 weeks (●), and with VRP, 25 mg/kg i.p. twice daily, 5 days a week, for 5 weeks (□). In combined treatments, each drug was given according to the same dose and the same schedule used for single treatments, and VRP was given 3 h before DX (○). Control mice (*). ***, $P \leq 0.001$, Student's *t*-test compared to controls; (***), $P \leq 0.001$, Student's *t*-test compared to DX alone

Table 2. Effect of VRP on metastases from s.c. implanted tumors[a]

Tumor	Treatment	No. of mice with metastases	Median no. of metastases (range)	Median weight (mg) of metastases (range)
B16	–	9/9	41 (6–200)	44.0 (1.3–543)
	VRP	7/7	25 (9–150)	61.5 (2.0–582)
	–	8/8	19 (5–100)	7.6 (0.6–148.4)
	VRP	10/10	8 (3–58)	3.3 (0.2–72)
B16V	–	4/10	0 (0–38)	0 (0–32.7)
	VRP	7/10	1.5 (0–7)	0.50 (0–3.7)
	–	7/8	10.5 (0–18)	0.73 (0–41.2)
	VRP	5/8	2.5 (0–9)*	0.06 (0–6.6)*
B16VDXR	–	2/10	0 (0–3)	0 (0–5.7)
	VRP	2/10	0 (0–60)	0 (0–917)
	–	4/6	1 (0–3)	0.13 (0–4.01)
	VRP	3/10	0 (0–5)	0 (0–0.85)

[a] The lungs of the same animals with s.c. tumors used to evaluate antitumor activity reported in Table 1 were analyzed at autopsy for the number and weight of metastases
*, $P \leq 0.05$, Mann-Whitney U-test

ences the drug's pharmacokinetics in resistant tumors, DX levels in B16VDXR tumors were also evaluated up to 7 days after treatment in animals treated with DX alone and in combination with VRP. DX and VRP were given at the same doses and according to the same schedules used to evaluate the antitumor effect. The results are reported in Figs. 3 and 4. In keeping with previous data [8], DX given alone reached similar initial levels in the sensitive and the resistant lines (Fig. 3); then it was more rapidly released from B16VDXR than from B16, particularly between 6 and 24 h, with consequently lower drug levels at late times and lower AUC (83 μg h/g in the resistant tumor com-

Table 3. Effect of VRP on experimental lung metastases of B16V[a]

No. of cells injected	Treatment	No. of mice with metastases	Median no. of metastases (range)	Median weight (mg) of metastases (range)
$5 \cdot 10^5$	–	10/10	42.5 (3 – > 200)	76.6 (0.2 – 901.5)
	VRP	10/10	68.0 (10 – > 200)	60.7 (0.9 – 1727)
$5 \cdot 10^4$	–	5/10	0.5 (0 – 32)	0.37 (0 – 24.9)
	VRP	5/10	0.5 (0 – 30)	0.02 (0 – 17.8)

[a] Cells were injected i.v. and animals were killed 3 weeks later. VRP was given i.p. at 25 mg/kg from day 1, twice daily, 5 days a week, for 3 weeks

pared to 204 μg h/g in the sensitive tumor). The administration of VRP did not modify DX pharmacokinetics in either the sensitive or resistant tumor. The differences in tumor DX concentrations found at some points were not significant due to the high variability of drug levels in these tumors. As can be seen from the results reported in Fig. 4, the combined treatment with VRP also did not modify DX pharmacokinetics in the plasma, spleen, or heart.

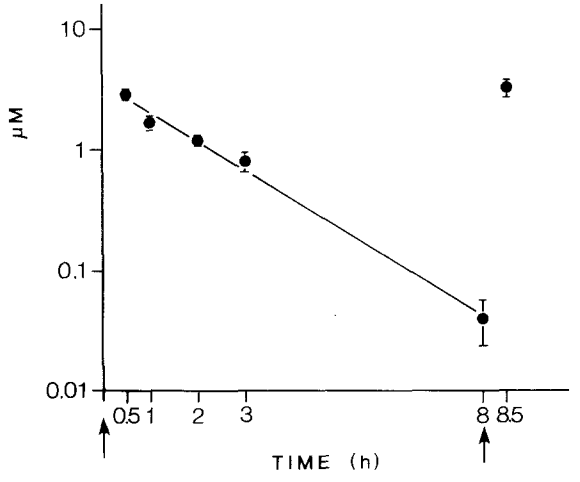

Fig. 2. VRP plasma levels in mice treated i.p. with 25 mg/kg twice at an 8-h interval. The values are the means of 3 values ± SD

No quantitative evaluation of the metabolites could be made since, as has been previously reported [9], DX is metabolized to a limited extent in mice. In both treated groups, only traces of doxorubicinol were found in the tumor, heart, and spleen. Doxorubicinone was detectable in small amounts in the plasma only at early times (up to 45 min) and in tumors from 3 h on, but not in all the animals in both groups.

Discussion

In the present study we have shown that the inhibition of in vivo growth of some DX-sensitive tumors by DX can be significantly enhanced by concomitant administration of VRP: such an effect has not been obtained in either inherently or induced resistant tumors. The in vitro studies on reversal of DX resistance by calcium-influx blockers or by calmodulin inhibitors apparently indicate opposite results, since it has been reported that these agents enhance DX cytotoxicity to a greater extent in resistant than in sensitive cells [27, 30, 31], although it should be pointed out that DX resistance is, in most cases, only partially antagonized.

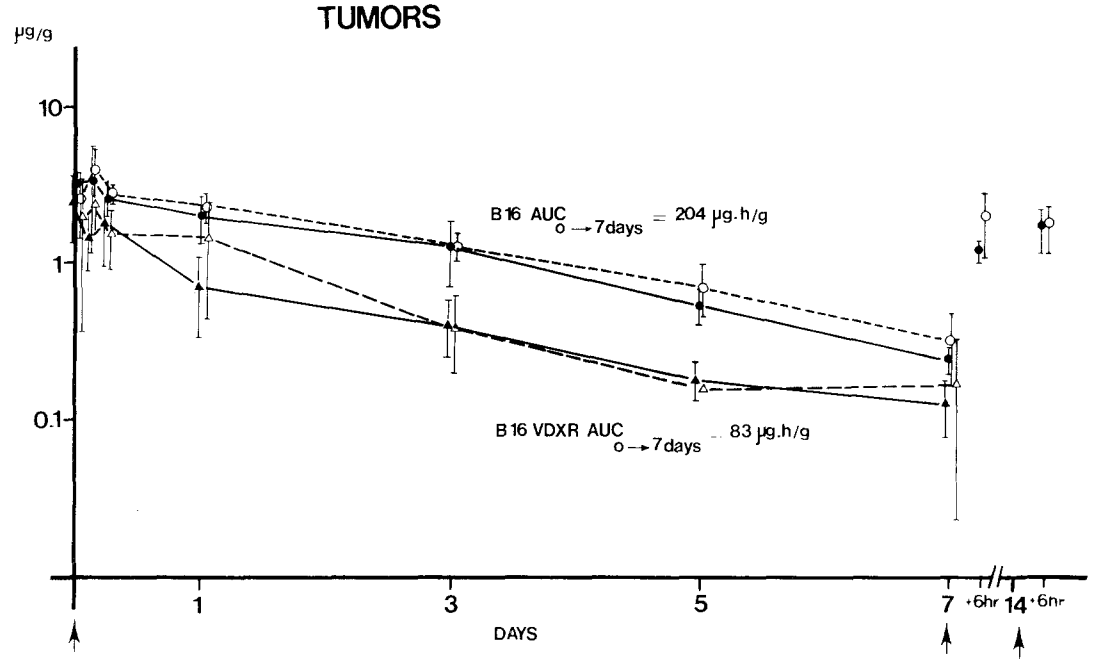

Fig. 3. DX levels in B16 tumor of mice treated with DX alone (●) and in combination with VRP (○), and in B16VDXR tumor of mice treated with DX alone (▲) and in combination with VRP (△). The values are the means of 3 values ± SD

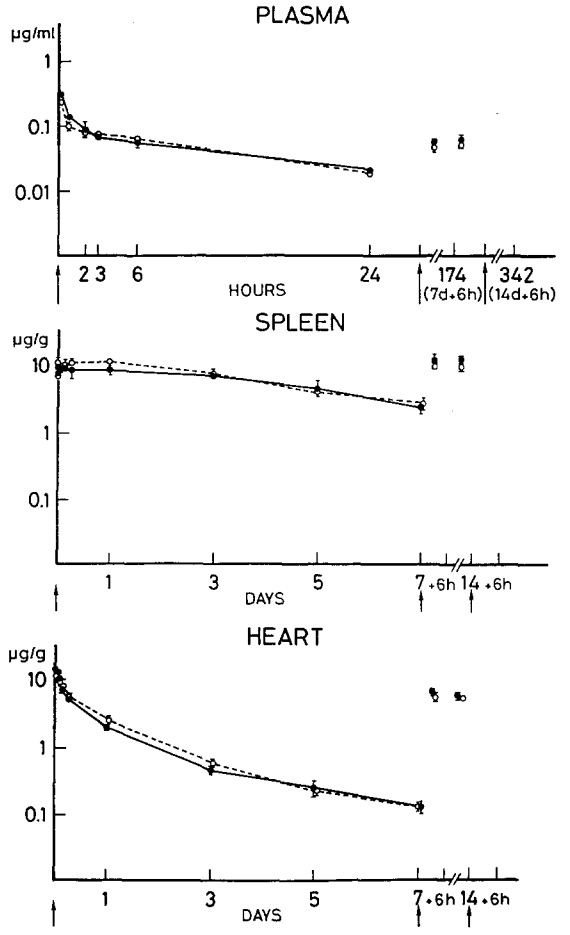

Fig. 4. DX levels in plasma, spleen, and heart of mice treated with DX alone (●) and in combination with VRP (○). The values are the means of 3 values ± SD; the bars for SD were omitted when their value was smaller than the size of the symbol used to indicate the mean value

In our experiments with the schedule of treatment employed, VRP plasma concentrations of 3 μ*M*, which decreased to 0.8 μ*M*, were achieved every time VRP was given. Continuous concentrations of 300–500 ng/ml, corresponding to 0.7–1 μ*M*, have been achieved in mice by other investigators [24] by delivering VRP from s.c. implanted Alzet mini osmotic pumps. However, even at this schedule, VRP enhanced DX activity only in sensitive tumors and not in the resistant ones. In clinical trials, by giving VRP as a loading dose followed by a maintenance infusion for 5.5 days [2], the mean plasma VRP concentration achieved was 0.45 μ*M*.

In in vitro studies [26] carried out on the same B16 melanoma lines studied here in vivo, the B16VDXR and the B16V lines, it has been found that at concentrations of 1 μ*M*, similar to the concentrations achieved in mice, VRP enhanced DX cytotoxicity in the sensitive line by 1.5 times and in the resistant line by 3 times. Even so, the resistant line was still 52 times more resistant to DX than the sensitive one. Similar results have been obtained with trifluoperazine, a calmodulin antagonist, in the same lines [26]. The lack of an antagonizing effect of VRP on DX resistance in animals bearing the B16VDXR line and the increase in DX activity in animals bearing the B16V line might be due to the fact that at doses tolerable to the host, effective DX concentrations already achieved in the sensitive or moderately resistant B16V line may be more effective, but they are virtually impossible to be achieved for the still resistant B16VDXR line. Similar considerations can be made for the previously reported lack of an in vivo effect of trifluoperazine on DX activity against B16VDXR and for the increase in DX activity against B16V [7]. Therefore, our results suggest that the low degrees of potentiation of antitumor activity found in vitro for sensitive lines might be more relevant for in vivo activity than the higher degrees found for resistant lines.

The lack of a potentiating effect of DX activity by VRP against a DX-sensitive tumor such as the Lewis lung tumor parallels in vitro results on some sensitive cells [21] and suggests that "sensitive" tumors are heterogeneous in their sensitivity to modulation by VRP.

Concerning the effect of VRP on metastases, we obtained rather negative results on the three tumor lines tested, and no antimetastatic effect against B16 was found, contrary to that reported by Tsuruo et al. [32] in animals with the primary tumor excised. The only antimetastatic effect was obtained on spontaneous metastases of the B16V line in one experiment, in which the metastasizing potential was higher than usual, and this result is difficult to explain. Moreover, since the antimetastatic effect of VRP seems to be due to inhibition of platelet aggregation induced by tumor cells [32], all the contradictory reports on the efficacy of antiplatelet agents in the prevention of metastases [11, 14, 16, 17, 19, 20, 34] suggest that many variables, including tumor type, host, and experimental conditions, still need to be clarified.

The mechanism of the potentiating effect of VRP on DX cytotoxicity in in vitro-growing cells has been associated with higher drug accumulation, which seems to be due to interference with the drug efflux, since anthracycline resistance has been attributed to enhanced drug efflux from resistant cells [21, 23, 27, 29]. Studies are in progress in our laboratories on the in vitro uptake and release of DX in the two lines, B16V and B16VDXR, incubated with or without VRP. In the B16 melanoma and the resistant B16VDXR line (the two solid tumors in which DX pharmacokinetics was studied in the present paper), initial drug-peak levels were similar and therefore not related to drug sensitivity, whereas the rate at which DX was eliminated was higher and the AUC consequently lower in the resistant line than in the sensitive line. From these results, we can conclude that in in vivo tumors DX sensitivity may also be related to the ability of the tumor to retain the drug. However, at variance with in vitro results, no evident modifications in DX levels in the in vivo-growing tumors were found following VRP treatment, either in the B16 melanoma, where a significant increase in DX activity was evident, or in the B16VDXR line, where VRP had no effect.

The lack of an increase in DX levels in the B16 melanoma, in spite of the potentiation of DX activity, might be due to the fact that the increase in activity was due to the presence of a small percentage of "nonsensitive" tumor cells (20% calculated from a gross $\log_{10}$ kill of 0.7). Even if these cells, with a lower drug retention than the sensitive ones, increase their drug accumulation following VRP treatment, this increase would be virtually impossible to evaluate, especially considering the high variability (about

20%) of DX levels in the tumors. It should be pointed out that a similar lack of an increase in DX uptake by VRP has been also reported in vitro in resistant human leukemic cells [1], and that other mechanisms of action could be responsible for the potentiating effect of VRP on DX antitumor activity. In fact, since it has been shown that B16VDXR cells, when incubated in vitro with DX, have a different intracellular drug distribution with a lower nuclear/cytoplasmic ratio than sensitive cells [26], it might also be that, as has been reported for resistant colonic cancer cells [4], VRP has inhibitory effects on DX transport from the nucleus of resistant cells. It has also been reported that treatment of resistant cells with VRP and daunomycin caused a partial synchronization with a G_2-M proliferation arrest [35], which may result in an increased cell kill in repeated treatments.

DX pharmacokinetics and metabolism were not modified by the combined treatment with VRP in the tumor, plasma, heart, or spleen. Different results have been reported in rabbits [25], where DX concentrations in cardiac muscle of animals treated with VRP were increased 2- to 5-fold 1 h after treatment, whereas after 24 h they were similar to those of rabbits treated with DX alone. We do not know whether these differences were due to the different species used or to the different VRP doses and schedules.

In conclusion, the present results indicate the lack of an effect of VRP on DX activity against highly resistant tumors in mice and a significant enhancement of DX activity in some sensitive tumors. The latter evidence suggests that the addition of VRP to DX therapy might prevent problems derived from intrinsic heterogeneity concerning chemosensitivity.

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