Verapamil Restoration of Daunorubicin Responsiveness in Daunorubicin-resistant Ehrlich Ascites Carcinoma

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ABSTRACT We have studied the influence of verapamil hydrochloride on the in vitro and in vivo effects of daunorubicin in Ehrlich ascites carcinoma. Daunorubicin-sensitive tumor was rendered resistant to daunorubicin by the continuous treatment of sequential generations of tumor-bearing BALB/c mice. The ability of daunorubicin to inhibit [^{3}H]uridine and [^{3}H]thymidine incorporation and the effect of daunorubicin on the mean survival time of host animals bearing daunorubicin-sensitive and daunorubicin-resistant Ehrlich ascites carcinoma were compared. The addition of verapamil to daunorubicin in vitro reduced the concentration of daunorubicin required to inhibit 50% of DNA and RNA synthesis in the daunorubicin-resistant tumor to that required in the daunorubicin-sensitive tumor, from 6 and 4.4 μg/ml to 1.5 and 1.3 μg/ml, respectively. Verapamil also restored drug sensitivity to daunorubicin-resistant Ehrlich ascites carcinoma in vivo. The 21.7±0.7 d mean survival time (MST) of BALB/c mice bearing daunorubicin-resistant tumor treated with daunorubicin alone rose to 44.0±0.7 d when the same tumor was treated with verapamil and daunorubicin, $P < 0.001$. This in vivo effect is specific for daunorubicin-resistant Ehrlich ascites carcinoma, since there is no alteration in MST of BALB/c mice bearing daunorubicin-sensitive or daunorubicin-resistant tumor when they are treated with verapamil alone or when BALB/c mice bearing daunorubicin-sensitive tumor are treated with daunorubicin and verapamil.

INTRODUCTION

Altered drug transport characterizes the resistance of experimental tumors to several classes of chemotherapeutic agents including the anthracycline antibiotics adriamycin and daunorubicin (1–3). Tsuruo et al. (4) recently reported that verapamil, the calcium influx inhibitor, enhanced the cellular level of vincristine in vincristine-sensitive P388 leukemia 2-fold, and in vincristine-resistant P388 leukemia 10-fold. Verapamil did not alter binding of vincristine to tubulin and it was felt that the enhanced accumulation of vincristine in these cell lines could be explained by the inhibition of vincristine efflux (4). Because anthracycline antibiotic resistance is also related to altered membrane transport by anthracycline-resistant tumor cells, (1–3) we have investigated the effect of verapamil on daunorubicin-resistant Ehrlich ascites carcinoma.

METHODS

Tumor lines and treatment regimens. Ehrlich ascites carcinoma (EA)[1] was maintained as an ascitic tumor in BALB/c mice. A daunorubicin-resistant subline was developed by sequential transfer of EA cells to subsequent generations of host mice with continuous daunorubicin treatment. The treatment regimen consisted of daunorubicin (N.C.I-BV-77-246) 0.2 mg/kg, i.p. daily for five doses, starting 24 h after the inoculation of 0.2 ml, i.p. of undiluted malignant ascites harvested from preterminal animals. The mean survival time±standard error of the mean (MST±SEM) of untreated BALB/c mice bearing EA is 18.6±0.3 d (n = 90). The MST±SEM of host mice bearing this tumor after initial treatment with daunorubicin is 25.7±0.7 d, whereas the MST of the ninth transfer generation of host mice is 19.6±0.7 d. After this degree of resistance had been developed the daunorubicin dose was increased to 0.4 mg/kg, which resulted in a 21.7±0.7 d MST. This tumor subline shows stable resistance to daunorubicin. When it was retreated with daunorubicin after 16 transfers over an 11-mo period, its MST was 21.8±1.4 d. Verapamil treatment regimens consisted of 25 or 50 mg/kg, i.p. of verapamil hydrochloride (kindly supplied by Knoll Pharmaceutical Co., Whippany, NJ) either given alone or simultaneously with

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[1] *Abbreviations used in this paper*: EA, Ehrlich ascites carcinoma; EA/DR, EA daunorubicin-resistant; EA/DS, EA daunorubicin-sensitive; IC_{50}, 50% inhibition; MST, mean survival time.

daunorubicin. Both drugs were reconstituted with 9.0 mg/ml NaCl. MST differences were calculated by Student's *t* test. Five mice were used in all experimental groups.

Nucleoside incorporation. 3 ml of ascitic fluid was aspirated from BALB/c host mice bearing EA, washed twice with 15 vol of chilled 0.17 M NH_4Cl, once with chilled phosphate-buffered saline (PBS) (130 mM NaCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, and counted. Nucleoside incorporation was measured by a modification of the methods of Inaba and Johnson (5) and Dano et al. (6). Aliquots of 4.5 $\times 10^6$ cells in 1.6 ml RPMI were incubated with 0.2 ml verapamil (final concentration 0–50 μg/ml) and 0.2 ml daunorubicin HCl (Sigma Chemical Co., St. Louis, MO, final concentration 0–10 μg/ml) for 1 h at 37°C. Cultures were washed twice and resuspended in 1.8 ml RPMI. Triplicate 180-μl aliquots were plated into microtiter plates and incubated with 20 μl of either [^{3}H]uridine (Amersham Corp., Arlington Heights, IL, sp act 16 Ci/mmol, final concentration 1 μCi/ml) or [^{3}H]thymidine (Amersham Corp., sp act 24 Ci/mmol, final concentration 1 μCi/ml) for 1 h at 37°C and 5% CO_2. Samples were collected on glass fiber filters with a Titertek multiple automated sample harvester unit, dried and counted in a PPO/POPOP/toluene liquid scintillation system.

RESULTS

It can be seen from Table I that the MST±SEM of untreated EA/daunorubicin-sensitive (DS) is 18.6±0.3 d, while that of untreated EA/daunorubicin resistant (DR) is 20.3±0.6 d. At the daunorubicin dose regimens of 0.2 and 0.4 mg/kg the MST±SEM of EA/DS is 25.7±0.7 and 37.4±5.4 d, whereas the MST±SEM of EA/DR is 19.6±0.7 and 21.7±0.7. These differences are significant at a P value of less than 0.02.

Fig. 1 defines the daunorubicin concentration required to inhibit incorporation of [^{3}H]thymidine and [^{3}H]uridine by EA/DS and EA/DR cells and the modification of this inhibition by verapamil. The concentration of daunorubicin required to inhibit 50% of DNA and RNA synthesis (IC_{50}) of daunorubicin-sensitive cells is 1.5 and 1.3 μg/ml, respectively, whereas 6 and 4.4 μg/ml of daunorubicin is required for equivalent inhibition in daunorubicin-resistant cells. The alteration of the daunorubicin [^{3}H]thymidine IC_{50} by 5, 20, and 50 μg/ml of verapamil in EA/DS and EA/

TABLE I
Mean Survival Time Responses of BALB/c Mice with Ehrlich Ascites Carcinoma to Daunorubicin or Daunorubicin-Verapamil Treatment

Tumor line	Drug regimen	MST±SEM	*P*
		d	
(a) EA/DS	—	18.6±0.3	
(b) EA/DS	Verapamil, 25 mg/kg	19.4±0.4	
(c) EA/DS	Verapamil, 50 mg/kg	17.6±0.7	
(d) EA/DS	Daunorubicin, 0.2 mg/kg	25.7±0.7	
(e) EA/DS	Daunorubicin, 0.4 mg/kg	37.4±5.4	
(f) EA/DS	Daunorubicin, 0.4 mg/kg Verapamil, 25 mg/kg	39.0±5.8	>0.10 vs. (e)
(g) EA/DR	—	20.3±0.6	
(h) EA/DR	Verapamil, 25 mg/kg	20.2±0.4	
(i) EA/DR	Verapamil, 50 mg/kg	20.0±0.6	
(j) EA/DR	Daunorubicin, 0.2 mg/kg	19.6±0.7	<0.02 vs. (d)
(k) EA/DR	Daunorubicin, 0.4 mg/kg	21.7±0.7	<0.02 vs. (e)
(l) EA/DR	Daunorubicin, 0.4 mg/kg Verapamil, 25 mg/kg	44.0±0.7	<0.001 vs. (k) >0.10 vs. (e) (f)
(m) EA/DR	Daunorubicin, 0.4 mg/kg Verapamil, 50 mg/kg	41.2±3.2	<0.01 vs. (k) >0.10 vs. (e)

EA/DS and EA/DR signify daunorubicin sensitive and resistant tumor lines, respectively. The daunorubicin-resistant subline was developed by sequential transfer of EA cells to subsequent generations of host mice with continuous daunorubicin treatment through nine transfer generations. The treatment regimen consisted of daunorubicin 0.2 mg/kg i.p. daily for five doses, starting 24 h after the inoculation of 0.2 ml i.p. of undiluted malignant ascites harvested from preterminal animals. Verapamil treatment regimens consisted of 25 or 50 mg/kg i.p. of verapamil hydrochloride either given alone or simultaneously with daunorubicin. Both drugs were reconstituted with 9.0 mg/ml NaCl. MST differences were calculated by the Student's *t* test. Five mice were used in all experimental groups.

DR can be seen in Figs. 1a and b. There is only slight modification of the daunorubicin IC_{50} in EA/DS compared to a dose-related daunorubicin IC_{50} alteration from 6 to 1.5 μg/ml in EA/DR. Similarly, Figs. 1c and d compare alteration of the daunorubicin modification of [^{3}H]uridine incorporation by 5, 20, and 50 μg/ml of verapamil in daunorubicin-sensitive and resistant EA. Again, the daunorubicin IC_{50} is only slightly modified in EA/DS compared with a verapamil dose-related daunorubicin IC_{50} alteration from 4.4 to 1.3 μg/ml in EA/DR. It should be noted that verapamil shifts the daunorubicin IC_{50} for uridine and thymidine incorporation of EA/DR to that characteristic of drug-sensitive Ehrlich ascites carcinoma. In a similar experiment using 1 and 5 μg/ml verapamil the alteration of daunorubicin IC_{50} for DNA was 4.5 and 5.8 μg/ml and for RNA 4.9 and 7.9 μg/ml, respectively. The viability of EA/DS and EA/DR cells maintained in culture for 2 h with 1, 5, and 20 μg/ml verapamil is >80%; determination of the verapamil IC_{50} for EA/DS and EA/DR over the same dose range (without daunorubicin) shows preservation of >83% of DNA and RNA synthesis.

Table I compares the in vivo effect of 25 and 50 mg/kg verapamil regimens. Verapamil alone is without effect in either EA/DS or EA/DR; it also fails to

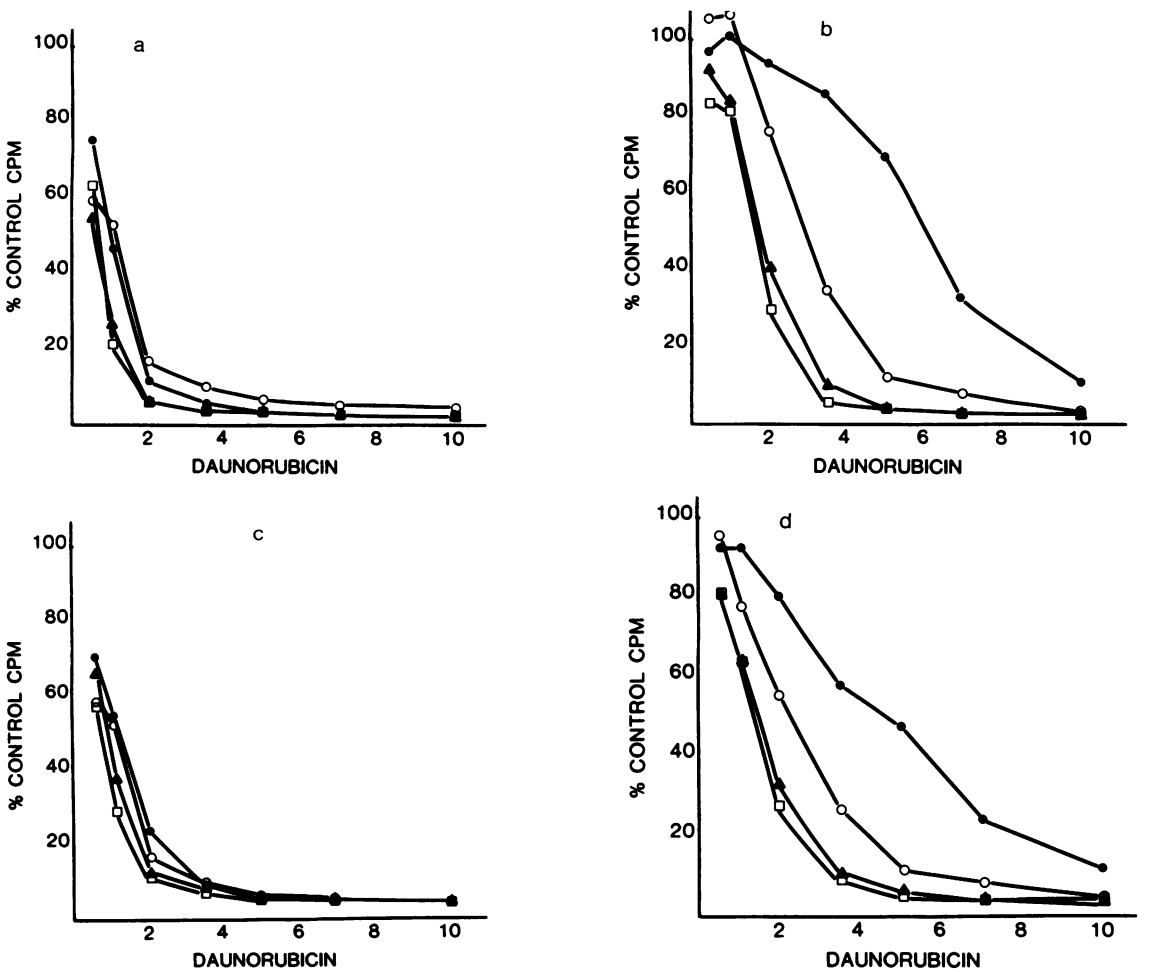

FIGURE 1 Alteration of the daunorubicin modification of [^{3}H]thymidine and [^{3}H]uridine incorporation (IC_{50}) by verapamil in EA/DS (a, c) and EA/DR (b, d). Cells were aspirated, washed, and counted. Aliquots were resuspended in RPMI at 2 × 10^6 cells/ml, preincubated with daunorubicin (0–10 μg/ml) for 1 h, washed twice, and incubated with [^{3}H]thymidine (a, b) or [^{3}H]uridine (c, d) for 1 h before harvesting. ●, 0 μg/ml verapamil; ○, 5 μg/ml verapamil; ▲, 20 μg/ml verapamil; □, 50 μg/ml verapamil. The maximum standard deviations in IC_{50} are < 0.05, 0.2, 0.05, and 0.3 μg/ml daunorubicin for a–d, respectively.

enhance the effect of daunorubicin in EA/DS. However the addition of verapamil, 25 mg/kg i.p. for five doses, to the 0.4 mg/kg daunorubicin regimen for the treatment of EA/DR results in a MST of 44.0±0.7 d compared to a MST of 21.7±0.7 d for daunorubicin treatment alone. This difference is highly significant at a P value of <0.001. This treatment regimen of verapamil-daunorubicin for daunorubicin-resistant EA is as effective as daunorubicin alone in drug-sensitive EA since the MST for BALB/c mice bearing EA/DS is 37.4±5.4 d when treated with 0.4 mg/kg daunorubicin, $P > 0.10$. The MST for EA/DR when treated with 50 mg/kg verapamil and daunorubicin is 41.2±3.2 d compared with 21.7±0.7 d for daunorubicin treatment alone. The MST differences between these groups is also significant, $P < 0.01$.

DISCUSSION

Our studies show that verapamil alters the daunorubicin inhibition of DNA and RNA synthesis in EA/DR. The concentration of daunorubicin required to inhibit 50% of DNA and RNA synthesis is 1–2 μg/ml in daunorubicin-sensitive tumor, whereas 4–6 μg/ml of daunorubicin is required to inhibit an equivalent amount of DNA and RNA synthesis in the daunorubicin-resistant variant. The addition of verapamil to daunorubicin shifts the daunorubicin IC_{50} of thymidine and uridine incorporation of EA/DR cells to levels that are characteristic of the drug-sensitive tumor.

These in vitro alterations of the daunorubicin effect correlate with the in vivo responses of host mice bearing EA/DR tumor to combined verapamil-daunorubicin treatment. Verapamil enhancement of the daunorubicin effect occurs in vivo only in daunorubicin-resistant tumor. Verapamil is without effect in either EA/DS or EA/DR when used alone and it also fails to enhance the response of EA/DS-bearing mice when added to the daunorubicin regimen. The MST when EA/DR-bearing mice are treated with daunorubicin alone is 21.7 d, whereas the mean survival times of mice treated with combined daunorubicin verapamil are >41 d. These differences are highly significant. Equivalent doses of daunorubicin are used in our matched sensitive and resistant experimental groups whereas Tsuruo et al. (4) reported that a tripling of vincristine dose was required to increase the survival of host mice with vincristine-resistant P388 leukemia to that of host mice with vincristine-sensitive leukemia.

The mechanisms by which verapamil modifies daunorubicin's nucleic acid synthesis inhibition and in vivo effect on Ehrlich ascites carcinoma are uncertain. Several observations suggest that these alterations may relate to the ability of verapamil to inhibit the slow membrane transfer of ionic calcium (7). Tumor cells resistant to daunorubicin or vincristine show enhanced active efflux of these respective drugs (2–4). Preliminary experiments from our laboratory show that extracellular calcium enhances the efflux of daunorubicin by Ehrlich ascites cells while Tsuruo et al. (4) relate the increased accumulation of vincristine by verapamil-treated leukemia cells to inhibition of vincristine efflux.

Our studies show that verapamil can restore daunorubicin responsiveness to EA/DR in vitro and in vivo. Studies are underway to determine if verapamil can prevent or delay the development of daunorubicin resistance by Ehrlich ascites tumor. Since a verapamil concentration of 1 to 3 μg/ml is in the clinical range (8), and since we have noted significant decrements in daunorubicin IC_{50} for thymidine and uridine incorporation in EA/DR by as little as 1 μg/ml of verapamil, our observations may be directly applicable to the therapy of human neoplasia.

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