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Effect of verapamil on daunorubicin accumulation in Ehrlich ascites tumor cells*

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Summary. Previous studies have demonstrated that verapamil may overcome resistance to anthracyclines. In vitro and in vivo experiments were performed on wild-type and resistant Ehrlich ascites tumor cells.

Verapamil in concentrations of 25–50 μM enhances the accumulation of daunorubicin (DNR) in resistant cells to the same level as in wild-type cells. No significant effect of verapamil on influx or nuclear binding could be demonstrated, indicating that verapamil enhances DNR uptake by blocking active drug extrusion. Exposure of cells to a high concentration of Ca^{2+} did not influence the effect of verapamil on DNR accumulation, suggesting a different mode of verapamil action apart from the Ca^{2+}-blocking effect. Attempts to circumvent acquired resistance to DNR in vivo with verapamil showed that the combination of the two drugs was more toxic than DNR given alone. The LD_{10} of DNR was determined as 3 mg/kg and the LD_{10} of the combination, as 2.5 mg/kg. The therapeutic effect of verapamil at a dose of 50 mg/kg and DNR of 2.5 mg/kg increased the life span of the mice by 50%. No difference was seen in the wild-type tumor in vivo.

These data lead us to conclude that verapamil can reverse DNR resistance completely, but that verapamil at non-toxic dosage only reduces DNR resistance by 50% in vivo.

Introduction

A major obstacle to the succesful use of anthracyclines and other cytostatics in cancer chemotherapy is the development of clinical drug resistance. Several studies have confirmed that in non-human systems acquired resistance to DNR and other anthracyclines [4, 5, 13] is accompanied by reduced cellular uptake. Attempts to overcome resistance in vivo have therefore been directed at increasing cellular drug accumulation [12]. Verapamil is one of the compounds that have been found to have the ability to increase the uptake of vinca alkaloids and anthracyclines in resistant tumor cells [14].

The aim of this study was the closer characterization of the influence of verapamil on DNR transport in DNR-sensitive and DNR-resistant Ehrlich ascites tumor cells. Furthermore, the study demonstrates the ability of verapamil to overcome DNR resistance in Ehrlich ascites tumor in vivo.

Material and methods

Chemicals. DNR as hydrochloride was obtained from Farmitalia Company (Milan, Italy). Verapamil was kindly supplied by Meda AS (Copenhagen, Denmark), and sodium azide was obtained from Merck (Darmstadt, FRG). All chemicals were of analytic grade.

Mice. First-generation hybrids of female random-bred Swiss mice and inbred DBS mice (N/D mice) were used throughout the study.

Tumor cells. The original wild-type tumor line (EHR 2) has been described previously [2]. The minimum dose that results in significant tumor inhibition corresponds to a total dose of 0.4 mg/kg. The resistant tumor line (EHR 2/DNR+) was developed in vivo by treatment with increasing doses of DNR during weekly i.p. tranplantation of the tumor [2]. The resistance of the tumor line was maintained, by daily i.p. injections for 4 concecutive days with a dosage corresponding to 1.6 mg/kg × 4 (LD_{10}). At this dosage no significant inhibition was observed; that is, compared with the wild-type tumor, the subline had at least 16-fold resistance. Ascites fluid was removed 6–8 days after inoculation of the tumor. No DNR was administered in the last passage of the DNR-resistant tumor before the in vitro experiments.

Medium. Standard medium was a phosphate buffer, 60 mM (pH adjusted to 7.45) containing NaCl, 57.0 mM; KCl, 5.0 mM; $MgSO_4$, 1.3 mM; NaH_2PO_4, 9.0 mM; Na_2HPO_4, 51.0 mM; and glucose 1.0 mM [10]. In suspensions containing Ca^{2+} the medium consisted of a Tris buffer (pH adjusted to 7.45) containing: Tris-HCL, 50 mM; KCL, 5 mM. Dialyzed calf serum, 5% v/v, was added to the medium in all trials. Experiments on the initial rate of uptake were performed in standard medium without glucose, containing 10 mM sodium azide. No calf serum was added in these experiments.

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Experimental. A viability test with methylene blue demonstrated that 93% of the tumor cells were intact. Details of the preparation of lysed cells have been described previously [11]. Microscopic control confirmed that all cells were lysed and the nuclei remained intact. Lysed cells were incubated in a buffer containing 250 m*M* sucrose, 5 m*M* $CaCl_2$, 0.1% (v/v) Nonidet P-40, and 25 m*M* Tris-HCl (pH adjusted to 7.45). No washing procedure was used in these experiments. Determination of the initial rate was performed as previously described [11]. Packed cell volume was determined by centrifugation of samples at 5500 *g* for 8 min in a hematocrit centrifuge, and the final cell suspension was adjusted to 0.5% (v/v). Serial samples of 2000 µl were withdrawn at varying intervals. The pellets were washed twice with ice-cold Ringer solution to remove the extracellular drug.

Determination of cellular DNR content. The drug content of intact cells or isolated nuclei was extracted with 0.3 *N* HCl – 50% ethanol solution [1]. The total fluorescence of the supernatant solution after centrifugation at 5000 *g*/min for 10 min was determined in an Aminco-Bowman spectrofluorometer (excitation 470 nm: fluorescence 585 nm), and the drug concentration was determined by comparison with spectrophotometrically adjusted standards [10].

The drug recovery in cell extracts and culture fluid was more than 90% (average 93%) in all experiments.

Animal experiments. For acute toxicity experiments, groups of 10 mice were given a single dose based on mouse weight and observed for a period of 60 days after the injection. The LD_{10} with 95% confidence limits was calculated as described previously [2]. For therapy experiments mice had 15×10^6 cells inoculated i.p. on day zero and the mice were randomized. The drug was administered as a single dose i.p. 24 h after inoculation. Deaths among the mice were recorded daily. Mice in the control group were treated with 0.9% NaCl. The geometric mean survival time was calculated and compared with the corresponding values in control groups. Calculation of 95% confidence limits was based on Wilcoxon's two sample test (non-parametric test). Each group consisted of 15 mice.

Results

Figure 1 shows the effect of increasing the dose of verapamil on the steady-state level of DNR in whole cells. It appears that verapamil increased steady-state uptake in whole cells of both wild-type and resistant lines. The maximum effect of verapamil on steady-state uptake was obtained with 10 µ*M* verapamil for the wild-type and with 50 µ*M* for the resistant cells. The maximum enhancement in level of uptake was 15% for the wild-type tumor cells and 540% for the resistant cells.

The time-course of uptake in sensitive and resistant whole cells is shown in Fig. 2. The data demonstrate that adding 25 µ*M* verapamil to resistant cells increased the uptake of DNR to the levels of sensitive cells. The cell-to-medium ratio of DNR at equilibrium (60 min) in sensitive cells was 466:1, and that in resistant cells, 34:1. After the addition of verapamil the cell-to-medium ratio was increased to 630:1 in sensitive cells and 502:1 in resistant cells.

To determine the speed of verapamil action, verapamil

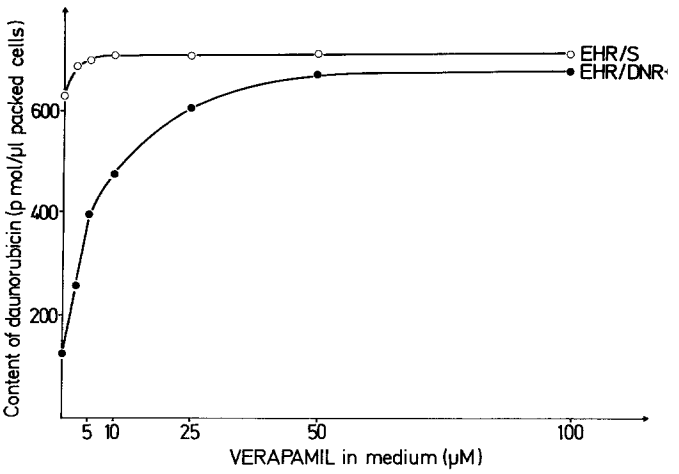

Fig. 1. Effect of verapamil on steady-state uptake of DNR in whole cells from the wild-type tumor EHR/S and from the resistant subline EHR/DNR+. Suspensions corresponding to 5 µl packed cells/ml were incubated for 60 min in standard medium at 37 °C with 5 µ*M* DNR and verapamil in the concentrations indicated. Each *point* represents the mean of three determinations

was added at different times in relation to the addition of DNR. Figure 3 shows that the sequence of administration did not influence the level at steady state. However, steady state was increasingly more rapidly obtained with reduction of the time verapamil was added in the uptake process. This finding indicates that the intracellular binding of DNR slows the action of verapamil on the outward transport mechanism.

To investigate whether the effect of verapamil was a result of an effect on the binding site of DNA, experiments were performed on nuclei, which account for 80% of cellular DNR [10]. It is seen in Fig. 4 that verapamil did not affect the nuclear binding to DNR in either sensitive or resistant cells. This finding indicates that the effect of verap-

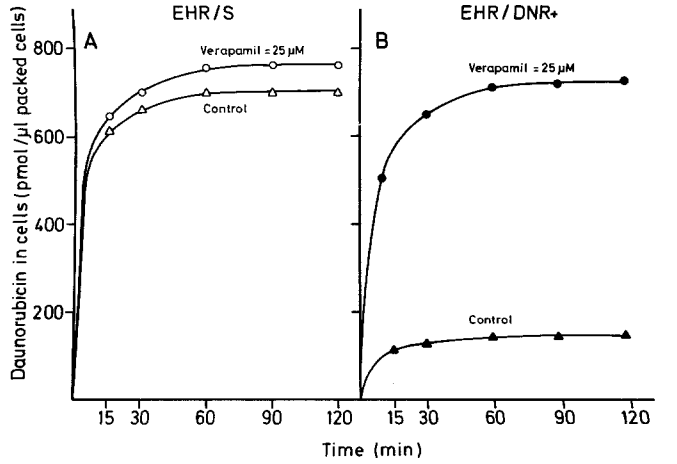

Fig. 2. Effect of verapamil on time-course of DNR uptake in whole cells. Wild-type cells of Ehrlich ascites tumor and cells from the DNR-resistant subline (5 µl packed cells/ml) were incubated in standard medium, pH 7.45 at 37 °C. DNR (5 µ*M*) and verapamil (25 µ*M*) were added at time zero. At the time indicated the cellular content of DNR was determined by measuring the total drug fluorescence extracted from the cells

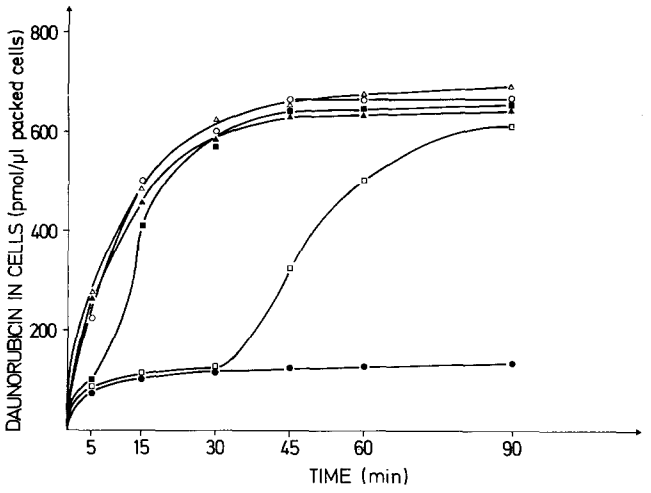

Fig. 3. The influence of the order of verapamil addition on uptake of DNR in resistant cells. Verapamil was added in a final concentration of 25 µ*M* 30 min before DNR (0), 5 min before DNR (▲) at time zero (Δ) immediately before DNR, and 5 min (■) and 30 min (□) after addition of DNR to the cells. In one set no verapamil was added (●). Each *point* represents the mean of three determinations

amil can most probably be ascribed to the level of membrane transport of DNR.

The effect of verapamil on the membrane transport of DNR could be a result of facilitation of influx, of reduction of active outward transport, or of both mechanisms.

To elucidate the influence of verapamil on unidirectional influx the effect of increasing concentrations of verapamil on initial rate of DNR uptake in resistant cells was determined (Fig. 5). To prevent active outward transport during the uptake process, energy metabolism was abolished by omission of glucose and addition of sodium azide.

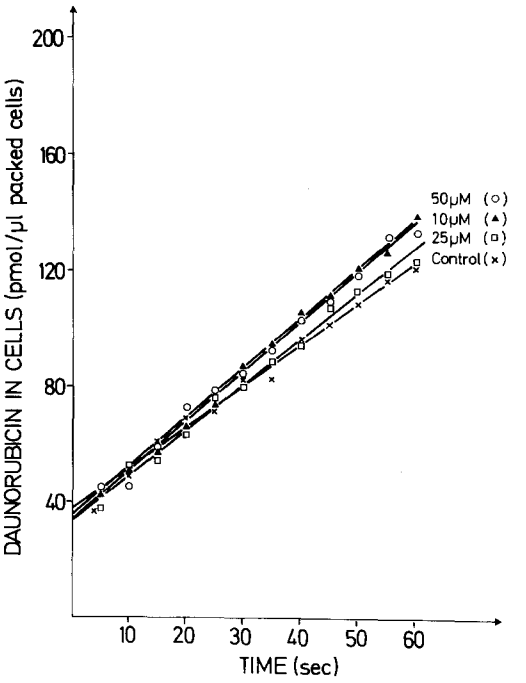

Fig. 5. Effect of verapamil on initial uptake of DNR in cells from a subline resistant to DNR. Verapamil in varying concentrations and DNR to a concentration of 5 µ*M* were added at time zero to the cell suspension (0.5% v/v) preincubated for 10 min in standard medium without glucose but containing 10 m*M* sodium azide. Serial samples of 2000 µl were withdrawn at 5-s intervals, and cellular content was determined by measuring the total drug fluorescence

These data show that verapamil has no significant influence on the initial rate of DNR uptake.

To elucidate the effect of verapamil on efflux of DNR, sensitive and resistant cells were loaded with DNR by the

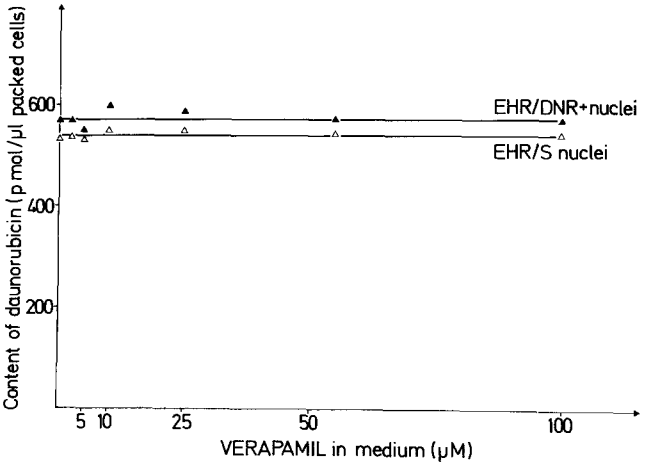

Fig. 4. Effect of verapamil on uptake of DNR in nuclei from the wild-type tumor (Δ) and the resistant subline (▲). Lysate was obtained as described in *Materials and methods.* Samples consisting of 2000 µl suspensions of lysate originating from a cell suspension containing 5 µl packed cells per ml were incubated with 5 µ*M* DNR and varying verapamil concentrations as indicated. Incubation time was 30 min. Content of DNR was determined by fluorometric measurements. Each *point* represents the mean of three determinations

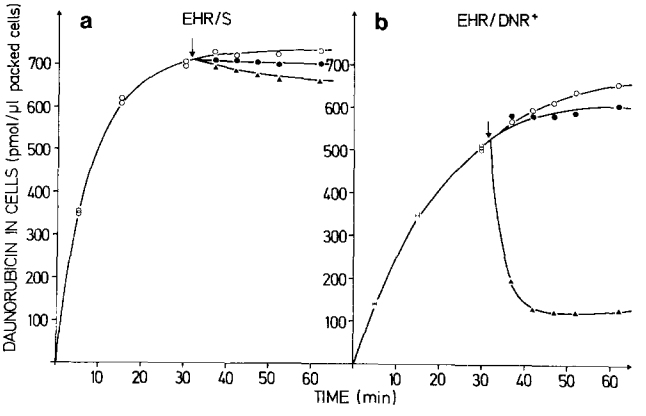

Fig. 6a, b. Efflux of DNR induced by glucose in wild-type Ehrlich ascites tumor cells **(a)** and a subline resistant to DNR **(b)** in suspensions with a concentration of 5 µl packed cells per ml. The suspensions were incubated in standard medium without glucose but containing 10 m*M* sodium azide. At time zero DNR was added to give a final concentration of 5 µ*M*. At time indicated by *arrow*, 10 m*M* glucose (▲) or 10 m*M* glucose plus 25 µ*M* verapamil (●) was added to the suspensions. In controls a corresponding volume of 0.9% NaCl was added (○). Cellular content was determined at the times indicated

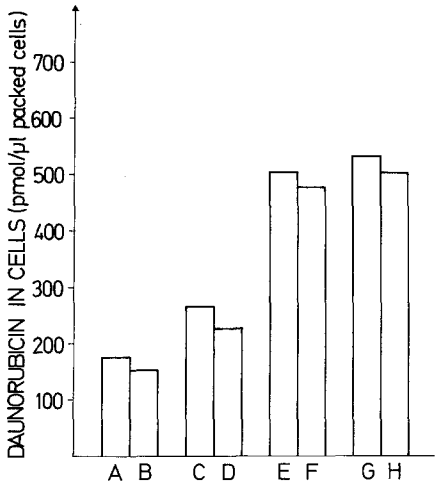

Fig. 7. Dose–response diagram showing the effect of verapamil alone or in combination with Ca^{2+} on uptake of DNR in daunorubicin-resistant Ehrlich ascites tumor cells. An 0.5% v/v suspension of cells was incubated at 37 °C with 5 µ*M* DNR in standard medium for 60 min. Ca^{2+} at 10 m*M* and verapamil in varying concentrations were added as follows: *A*, control; *B*, Ca^{2+}; *C*, 2.5 µ*M* verapamil; *D*, 2.5 µ*M* verapamil and Ca^{2+}; *E*, 25 µ*M* verapamil; *F*, 25 µ*M* verapamil and Ca^{2+}; *G*, 50 µ*M* verapamil; *H*, 50 µ*M* verapamil and Ca^{2+}. The cellular drug content was determined fluorometrically on cell extracts. Data shown represent the means of three determinations in each case

omission of glucose and addition of sodium azide to inhibit the energy metabolism (Fig. 6). In this medium nearly equal accumulation was obtained in sensitive and resistant cells. Activation of glycolysis by the addition of glucose results in a pronounced outward transport of DNR from resistant cells. However, when verapamil was added together with glucose, hardly any outward transport could be demonstrated. These findings indicate that the mechanism of verapamil in drug accumulation is a result of inhibition of the active extrusion of DNR.

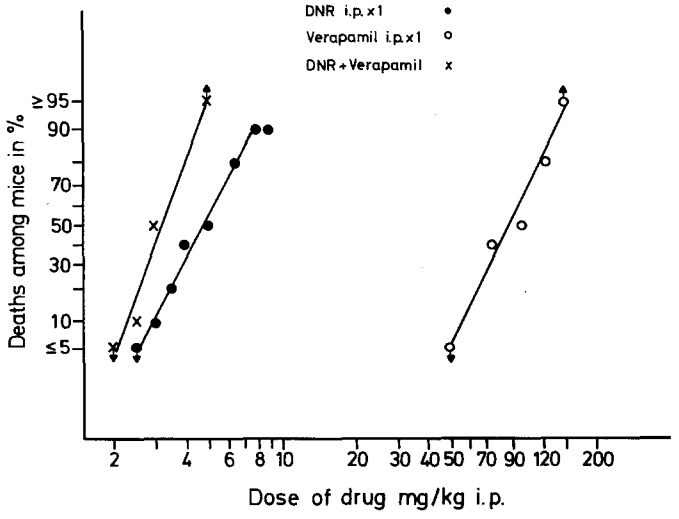

Fig. 8. Log-probit plot of toxicity of DNR (●), verapamil (○) and DNR and verapamil in combination (×). Mice in groups of ten each received one i.p. injection of the drugs. In the combination, verapamil was administered 10 s before DNR. *Arrows* pointing down and up indicate that either no mice died or all mice in the group died. Mice were observed for 60 days

Table 1. Comparison of effect of DNR and DNR plus verapamil on the life span of mice bearing resistant Ehrlich ascites tumor cells or wild-type cells

Treatment[a]		Tumor	
Drugs	Dose (mg/kg i.p. × 1)	EHR/S MST (days) (% ILS)[b]	EHR/DNR$^+$ MST (days) (% ILS)[b]
Untreated controls		12 (10–15)	8 (7–21)
DNR	2.5	19 (14–23) 58	8 (6–21) 0
Verapamil	50	12 (10–14) 0	9 (9–17) 12
DNR + Verapamil	2.5 + 50	20 (17–28) 66	12 (8–21) 50

[a] Drugs were administered i.p. 24 h after inoculation of 15×10^6 cells i.p. Each group had 15 mice

[b] MST, median survival time; ILS, percentage increase in life span relative to untreated controls

Most effects of verapamil are thought to be mediated by its ability to block the slow inward Ca^{2+} transport. Figure 7 shows that addition of Ca^{2+} (10 m*M*) reduces the accumulation of DNR in resistant cells by about 11%. When Ca^{2+} is added (10 m*M*) together with verapamil at different concentrations, DNR uptake was reduced by 14%, 4%, and 4%, depending on the concentration. Thus, a high concentration of Ca^{2+} can only counteract the effect of verapamil to a very small degree.

To evaluate the value of verapamil as chemosensitizer to daunorubicin, toxicity and therapy experiments were performed. Figure 8 shows a log probit plot of the results of toxicity experiments for DNR given as a single drug, for verapamil given as a single drug, and for the combination of DNR and verapamil. In the drug combination, verapamil was given at a dose level of 50 mg/kg and administered immediately before DNR. The LD_{10} of DNR was determined as 3 mg/kg, and the LD_{10} of the combination as 2.5 mg/kg. Thus, addition of verapamil resulted in a moderate increase in the toxicity of DNR.

Table 1 shows the therapeutic effect of a combination of DNR and verapamil compared with DNR alone on the life span of mice bearing a wild-type tumor and a resistant subline of Ehrlich ascites tumor, respectively. It appears that EHR/DNR$^+$ was completely resistant to DNR at a dose of 2.5 mg/kg, but if verapamil was administered at a dose of 50 mg/kg together with DNR the life span was increased by 50%. No increase in life span was seen in mice bearing the wild-type tumor.

Discussion

The present study demonstrated that verapamil enhances the accumulation of anthracyclines in resistant cells. The effect of verapamil is concentration-dependent, reaching a maximum effect at 25–50 µ*M*, at which level the uptake of DNR in resistant cells corresponds to that of wild-type cells. However, in wild-type cells verapamil influences the accumulation of DNR to only a modest degree.

During further attempts to characterize the mechanism of action of verapamil it was not possible to demonstrate any significant effect of verapamil on influx or nuclear binding.

However, when cells were loaded with DNR as a result of energy deprivation, the resumption of active outward transport induced by initiating glycolysis was completely blocked in both wild-type and resistant cells following verapamil addition. This finding indicates that verapamil increases DNR uptake by blocking active drug extrusion.

This mechanism is compatible with the previous findings of Tsuruo et al. [16, 17], Kessel and Wilberding [7], and Rogan et al. [9], who demonstrated that verapamil inhibited the cellular release of the drug in a drug-free medium.

Our study gave no indication that the verapamil effect was due to the blocking of the transmembrane Ca^{2+} flux, since an increase in the external Ca^{2+} had no effect on the verapamil action. Results consistent with this were obtained by Kessel and Wilberding [6, 7].

The effect of verapamil on anthracycline uptake in resistant cells in vitro provides the rationale for attempts to circumvent acquired resistance to DNR in vivo. Our toxicity experiments demonstrated that the combination of DNR and verapamil was more toxic than the individual compounds. This finding might suggest that verapamil increases daunorubicin accumulation to some degree in normal tissues. However, at the maximum tolerated dose of verapamil a significantly increased therapeutic effect against the resistant tumor, was obtained by treatment with the combination, but it did not reach the therapeutic level obtained with anthracycline treatment against the wild-type tumor. Thus, even if verapamil is capable of increasing drug uptake in resistant cells compared with that in wild-type cells in vitro, our in vivo experiment indicates that verapamil at a tolerated dose can only partially overcome drug resistance. Corresponding results have been described in other tumor systems [15, 16]. The in vitro–in vivo discrepancies in results are probably due entirely to the toxicity limitations of dosage, which permit only an insufficient concentration of verapamil during the phase of DNR uptake. Another possible explanation is that other factors apart from drug uptake determine the acquired resistance to anthracyclines [3].

Thus, even if the use of verapamil is one of the most promising ways of trying to circumvent acquired resistance to anthracyclines and vinca alkaloids, the dosage limitations make it unsuitable for clinical use as a chemosensitizer.

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