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Short communication

Calcium modifies the accumulation and retention of daunorubicin by Ehrlich ascites carcinoma

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Summary. *Verapamil restores daunorubicin sensitivity to daunorubicin resistant Ehrlich ascites carcinoma but is without effect when used with daunorubicin in daunorubicin sensitive parental Ehrlich ascites tumor. Energy dependent daunorubicin efflux is more active in drug resistant than in drug sensitive cells. However, daunorubicin retention decreases equivalently in drug resistant and sensitive cells with increasing calcium levels in the presence of both intact and interrupted outward transport. Therefore, (1) daunorubicin accumulation and retention in Ehrlich ascites carcinoma cells is influenced by at least two independent mechanisms and (2) it is likely that verapamil modifies daunorubicin activity in drug resistant tumor variants by mechanisms beyond calcium inhibition.*

Introduction

Tsuruo et al. initially reported that verapamil enhances vincristine-induced cytotoxicity in vincristine-sensitive and vincristine-resistant P388 leukemia in vitro [3]. Verapamil also enhances anthracycline antibiotic-induced cytotoxicity in resistant P388 leukemia in vitro and in Ehrlich ascites carcinoma in vivo [2, 4]. Verapamil can also potentiate the cytotoxic effects of vincristine and adriamycin against human leukemic cells [5]. Since verapamil is a calcium-influx inhibitor we expected that calcium might play an important role in transport of vincristine and/or anthracycline antibiotics by tumor cells. We therefore investigated the influence of calcium on daunorubicin accumulation and retention in Ehrlich ascites carcinoma.

Methods

Ehrlich ascites (EA) carcinoma was maintained as an ascitic tumor in BALB/c mice. A daunorubicin-resistant subline was developed as previously described [2]. In vitro the uptake of daunorubicin in daunorubicin-resistant (DR) cells is approximately 75% of that in daunorubicin-sensitive (DS) cells. After removal of exogenous daunorubicin, DS cells retain 40% more daunorubicin at steady state than do DR cells. Our cell lines show similar energy-dependent efflux characteristics to those described by others, since the addition of glucose after 30 min of drug uptake to DS and DR cells preincubated with sodium azide caused an immediate drop in ^{3}H-daunorubicin uptake [1]. The efflux in DR cells was two to three times that in DS cells.

For the determination of daunorubicin accumulation, DS or DR cells were resuspended at 2×10^6 cells/ml in RPMI 1640 with 0–2.1 m*M* calcium. After 15 min in ice daunorubicin was added to give a final total concentration of 0.5 μg/ml ^{3}H-daunorubicin (New England Nuclear, sp. act. 2.5 Ci/mmol) and 4.5 μg/ml unlabeled daunorubicin. The cell suspensions were incubated for 120 min at 37 °C. Triplicate 200-μl aliquots were plated into microtiter plates at times indicated, and harvested immediately on glass fiber filters. For daunorubicin

Table 1. Calcium-dependent daunorubicin accumulation and retention in sensitive and resistant EA cells[a]

Calcium concentration (m*M*)	Accumulation				Retention			
	RPMI with glucose without azide		RPMI with 5 m*M* azide without glucose		RPMI with glucose without azide		RPMI with 5 m*M* azide without glucose	
	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)
0.0	100 ± 5.6	100 ± 6.0	100 ± 5.0	100 ± 1.3	100 ± 3.5	100 ± 4.0	100 ± 3.2	100 ± 3.2
0.42	85.4 ± 2.0	83.0 ± 6.8	98.7 ± 3.5	89.9 ± 2.7	86.4 ± 5.3	90.3 ± 4.6	90.0 ± 1.5	96.9 ± 3.3
0.84	–	–	86.0 ± 1.8	77.7 ± 0.6	–	–	76.7 ± 4.9	71.7 ± 4.6
2.1	63.1 ± 2.7	67.6 ± 2.6	69.3 ± 1.1	62.3 ± 3.9	69.9 ± 4.3	55.4 ± 11.8	61.7 ± 2.0	61.3 ± 3.8

[a] Results are expressed as percent retention (± SD) of ^{3}H-daunorubicin by cells maintained in calcium-containing RPMI compared with cells maintained in calcium-free RPMI at plateau levels (accumulation, 60 min; retention, 90 min)

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retention, 6-ml aliquots were removed from the calcium-free cell suspension after 45 min, centrifuged, washed, and resuspended in buffers containing 0–2.1 m*M* calcium. Suspensions were further incubated and harvested as described.

Calcium and glucose-free RPMI 1640 with 5 m*M* sodium azide (an oxidative phosphorylation inhibitor) was used to determine modifications in daunorubicin transport of DS and DR cells due to calcium without the influence of active outward transport [1]. This concentration of sodium azide causes a 30% loss of cellular viability which is independent of calcium. For drug accumulation, DS and DR cells were preincubated for 10 min in glucose-free RPMI with 5 m*M* sodium azide and calcium concentrations ranging from 0–2.1 m*M*. Daunorubicin retention was determined in the presence of sodium azide as described above.

Results and discussion

Table 1 shows that daunorubicin retention varies equivalently with calcium concentration in drug-sensitive and drug-resistant EA cells in the presence of intact and interrupted active outward transport. Table 1 also presents the effect of calcium concentration on ^{3}H-daunorubicin accumulation; an equivalent alteration can be seen in DS and DR cells in the presence of intact or interrupted active outward transport. Since drug efflux controls daunorubicin accumulation in these cell lines the decreased accumulation of daunorubicin observed with increasing calcium concentration is expected [1]. We have also noted that the addition of glucose and/or calcium to DR cells maintained in the 5-m*M* sodium azide buffer causes an equivalent and additive decrease in daunorubicin accumulation, again indicating independence of the calcium-related and energy-requiring transport mechanisms. Daunorubicin accumulation and retention in EA carcinoma cells is influenced by at least two independent mechanisms. Energy-dependent efflux is more active in drug-resistant than in drug-sensitive cells and may be inhibited by preincubation in buffers containing sodium azide without glucose [1]. Our results show that the second component is calcium-dependent and is equivalent in DS and DR cells. However, since verapamil has a greater effect on adriamycin and daunorubicin cytotoxicity in drug-resistant than in drug-sensitive tumors [2, 4], it probably modifies anthracycline antibiotic activity in these drug-resistant variants by mechanisms beyond calcium inhibition.

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