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Effect of cyclosporin A on daunorubicin accumulation in multidrug-resistant P388 leukemia cells measured by real-time flow cytometry

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Summary. We investigated the mode of action of cyclosporin A (Cy-A) as a modifier of multidrug resistance in P388 mouse leukemia cells. A fluorescence-activated flow cytometer (FCM) was modified with a flow-through cuvette to allow continuous on-line monitoring of daunorubicin uptake in vitro. The addition of Cy-A to multidrug-resistant P388/R cells at steady-state daunorubicin uptake, led to a dose-dependent increase in cellular daunorubicin accumulation, as measured by FCM and high-performance liquid chromatography (HPLC). A linear relationship was found between the daunorubicin concentration in the incubation medium and the Cy-A concentration required for optimal stimulation of cellular anthracycline accumulation. The results of a cytotoxicity assay indicated that Cy-A completely restored the chemosensitivity of the P388/R cells. Intracellular Cy-A measurements in P388/S and P388/R cells showed that P388/R cells accumulated significantly less Cy-A than P388/S cells. Relatively high daunorubicin concentrations could not restore that accumulation defect. These results suggest that Cy-A promotes cellular anthracycline accumulation by competing for an outward drug-transport system that operates in multidrug-resistant cells.

Introduction

Studies aimed at the elucidation of the mechanism of drug resistance have led to the development of cell lines that show a multidrug-resistant phenotype; that is, the cells are resistant to a variety of anticancer drugs, including DNA intercalating agents (e.g., anthracyclines, actinomycin D), mitotic spindle poisons (e.g., Vinca alkaloids, colchicine), and other structurally unrelated compounds [1, 2, 7, 17, 27]. The functional change in multidrug resistance is a decrease in drug accumulation, often apparently due to enhanced drug efflux [3, 7, 8, 17]. A variety of agents, among which are calcium influx blockers and calmodulin inhibitors, have been shown to overcome resistance to anthracyclines in both murine and human multidrug-resistant tumor cells in vitro by increasing the intracellular anthracycline accumulation due to interference with the outward drug-transport system [4, 14, 20, 21].

Clinical studies have been initiated in an attempt to overcome drug resistance in cancer patients by the simultaneous use of verapamil (VRP), a calcium channel blocker, and cytotoxic agents [11, 13]. However, it appears that the relatively high levels of VRP required in vitro to reverse multidrug resistance cannot be achieved in vivo without severe toxicity problems [12].

It has recently been shown that the immunomodulating agent cyclosporin A (Cy-A) can also reverse vincristine and daunorubicin resistance in a multidrug-resistant human leukemia cell line in vitro [18, 22]. Due to the relatively low toxicity of Cy-A even at high doses [15], this finding is potentially of clinical interest. In the present study we investigated the mode of action of Cy-A as a modifier of the multidrug-resistant phenotype.

Materials and methods

Cell cultures. Murine leukemic P388 daunorubicin-sensitive (P388/S) and -resistant (P388/R) cells were maintained as suspension cultures in RPMI-1640 medium (Gibco, Paisley, Scotland) supplemented with 10% horse serum (Sera-Lab, Sussex, England). The P388/S and P388/R cells were kindly provided by Dr. B. W. Fox (Paterson Laboratories, Manchester, England). Southern and Northern blotting analysis with an mdr-specific cDNA probe demonstrated highly amplified mdr genes and mRNA overexpression in P388/R cells (unpublished data). The doubling times of P388/S cells is 15.6 h and that of P388/R cells, 24 h. The P388/R cells were grown continuously in 200 n*M* daunorubicin. Prior to flow cytometric analysis, the P388/R cells were cultured in daunorubicin-free medium for 24 h; thereafter, they were centrifuged and resuspended in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid-buffered Hanks' balanced salt solution (pH 7.4).

Measurements of intracellular daunorubicin content by laser flow cytometry. The daunorubicin content of individual cells was measured in real time using the RELACS-3, our home-built flow cytometer (FCM) [25], which was modified to allow on-line measurements. This method makes use of the fluorescent properties of the anthracyclines. Briefly, the cells traverse the light of a 5-W argon ion laser (Coherent, Palo Alto, Calif) tuned at 488 nm (0.6 Watt);

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Abbreviations: Cy-A, cyclosporin A; FCM, flow cytometry; HPLC, high-performance liquid chromatography; ID_{50}, drug concentration that inhibits DNA synthesis by 50%; VRP, verapamil

this wavelength is close to the absorption maximum of daunorubicin. Illumination of the sample stream with the laser beam occurs inside a flow-through quartz cuvette. The fluorescence emitted by the cells on excitation by the laser light is registered on a photomultiplier; the scatter light is blocked by a KV-550 filter. The signals from the photomultiplier are amplified and the peak values are processed by the data-acquisition system. The forward and perpendicular light-scatter signals from the cells are measured on separate detectors and analyzed in a similar manner; the intensity of the scatter signals are related to cell size and structure, respectively [24], and can be used to discriminate between the different leukocyte subpopulations in peripheral blood and bone marrow [26]. The cells are analyzed at a rate of 500–1000/s.

Four signals (forward and perpendicular light scatter, time of flight, and fluorescence) per cell are digitized and stored in the data list mode in a Hewlett Packard 9000 series desktop computer. At predetermined times (e.g., 2, 4, 8, 16, 32, and 64 s) the data of 2000 cells are stored on disks. In addition to the normal pulse-height processing electronics, the system contains an internal clock that is read periodically (10 times/s), which can also be used to monitor the sampling time because the time markers are part of the accumulated list mode data. The means and SE of the peak of the frequency distribution in fluorescence intensity of specific cell populations, characterized by scatter parameters, can be computed.

The RELACS-3 was modified to allow on-line measurements of cellular daunorubicin uptake in the following way: the cells were kept at 37° C in a reaction vessel surrounded by a water jacket, which was connected to the flow cuvette of the RELACS-3. By means of air pressure, the medium containing the cells was forced through the flow cuvette. An extra inlet in the reaction vessel allowed the addition of drugs during monitoring of the fluorescence intensity of the cells.

Quantification of anthracyclines by HPLC. Intracellular daunorubicin and daunorubicinol concentrations were determined by high-performance liquid chromatography (HPLC) as previously described [10].

Quantification of Cy-A by RIA. Intracellular Cy-A concentrations were determined with the aid of a commercially available radioimmunoassay (RIA) (Sandoz, Basel).

Cytotoxicity assay. The cytotoxic effect of daunorubicin on P388 cells was estimated by means of a [^{3}H]-thymidine incorporation assay [19]. P388 cells (2×10^6) obtained from exponentially growing cultures were incubated for 1 h at 37° C in 1 ml RPMI-1640 medium to which a 10-fold serial dilution of daunorubicin and Cy-A (final concentration, 3 μ*M*) or VRP (final concentration, 5 μ*M*) were added. Thereafter, the cells were washed twice, resuspended, replated into microtiter plates, and incubated with [^{3}H]-thymidine (final concentration, 1 mCi/ml) for 1 h at 37° C in 5% CO_2. Samples were collected on glass fiber filters and the radioactivity was counted. The experiments were carried out in quadruplicate.

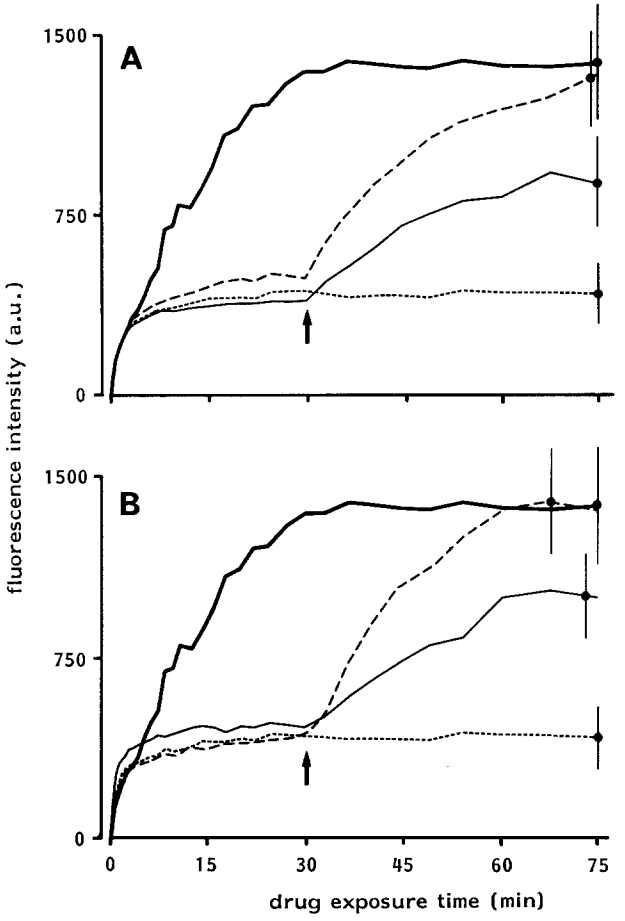

Fig. 1. Fluorescence intensity (arbitrary units, a.u.) of P388/S and P388/R cells vs drug exposure time, corrected for background fluorescence. The cells were incubated with 2 μ*M* daunorubicin (DAU) at 37° C. The *arrows* indicate the times of addition of VRP (**A**) and Cy-A (**B**). Bars: SD. **A:** P388/S + DAU, ——; P388/R + Dau, ...; P388/R + DAU + 1 μ*M* VRP, ——; P388/R + DAU + 5 μ*M*, VRP, -----. **B:** P388/S + DAU, ——; P388/R + DAU, ...; P388/R + DAU + 1 μ*M* Cy-A, ——; P388/R + DAU + 3 μ*M*, Cy-A, -----

Results

Flow cytometry

We have previously shown that after in vitro incubation there is a linear relationship between the intracellular daunorubicin content and the drug dose in the incubation medium [9]. In the present study, the anthracycline uptake by cells in vitro was measured with a modified FCM that enables uninterrupted monitoring of fluorescence signals. Figure 1 A shows the daunorubicin uptake curves for the anthracycline-sensitive P388/S and resistant P388/R lines after the addition of 2 μ*M* daunorubicin. The abscissa and ordinate represent the time after drug addition and the relative fluorescence intensity of the cells, respectively. The daunorubicin uptake by the resistant P388/R cells was about 3 times lower than that of the parent P388/S cells. However, the addition of 5 μ*M* VRP to the incubation medium completely restored the daunorubicin uptake of the P388/R cells. The addition of an excess of VRP (10 μ*M*) had no further enhancing effect (not shown), and lower concentrations (1 μ*M*) led to only partial restoration of daunorubicin uptake by the resistant cells.

Figure 1 B shows the effects of Cy-A on the daunorubicin uptake by the anthracycline-resistant P388/R cells. The effect of Cy-A was comparable with that of VRP: the addi-

Table 1. Intracellular drug concentrations in P388 cells

Incubation[b]		Drug concentrations[a] P388/S		P388/R	
DAU	Cy-A	DAU	Cy-A	DAU	Cy-A
2 μ*M*	–	527 ± 78	–	131 ± 25	–
–	3 μ*M*	–	371 ± 86	–	152 ± 46
2 μ*M*	3 μ*M*	464 ± 43	406 ± 72	510 ± 96	185 ± 21
10 μ*M*	3 μ*M*	ND	ND	ND	169 ± 15

[a] Expressed as μg ± SD/10^9 cells
[b] P388 cells (2×10^6/ml) were incubated at 37°C for 60 min with either DAU, Cy-A, or both. Thereafter, the cells were spun down and washed and the intracellular DAU and Cy-A concentrations were determined. The experiments were carried out in quadruplicate
DAU, daunorubicin; Cy-A, cyclosporin A; ND, not done

tion of 3 μ*M* Cy-A to the incubation medium completely restored drug uptake; an excess of Cy-A (15 μ*M*) had no further enhancing effect (not shown), and lower concentrations (1 μ*M*) led to only partial restoration of daunorubicin uptake. Neither VRP nor Cy-A affected the anthracycline uptake by P388/S cells.

No differences in the stimulation of anthracycline uptake by P388/R cells could be observed, whether the cells were stimulated by VRP or Cy-A before, at the time of, or after the addition of daunorubicin (data not shown). The increased daunorubicin accumulation induced by Cy-A in P388/R cells could not be further stimulated by VRP, and Cy-A could not further stimulate that induced by VRP (data not shown).

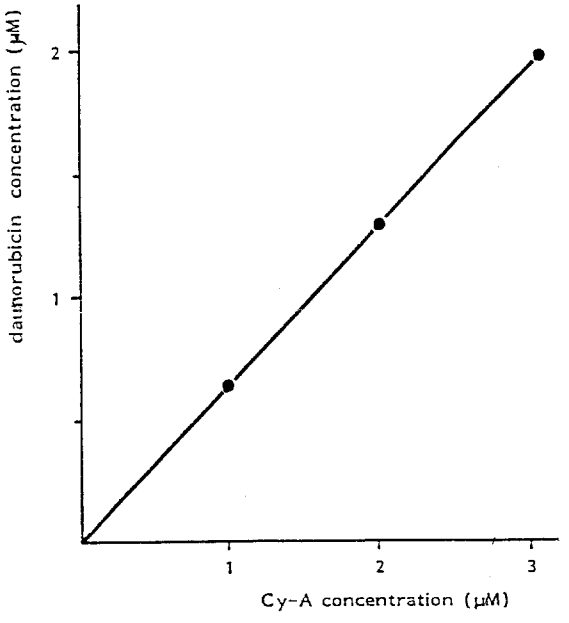

Fig. 2. Relationship between the daunorubicin concentration in incubation medium and the Cy-A concentration required for optimal stimulation of intracellular daunorubicin accumulation in multidrug-resistant P388 cells

Table 2. The ID_{50}[a] of P388/R and P388/S cells for daunorubicin

	DAU	DAU + Cy-A[b]	DAU + VRP[c]
P388/S	3.0 ± 1.0 μ*M*	3.5 ± 0.6 μ*M*	2.9 ± 0.7 μ*M*
P388/R	36.0 ± 7.2 μ*M*	3.8 ± 1.4 μ*M*	3.1 ± 1.2 μ*M*

[a] The ID_{50} was estimated by [^{3}H]-thymidine incorporation and is expressed as the mean ± SD of four separate experiments
[b] Cy-A was added to the incubation medium to a final concentration of 3 μ*M*
[c] VRP was added to the incubation medium to a final concentration of 5 μ*M*
DAU, daunorubicin; Cy-A, cyclosporin A; VRP, verapamil

Intracellular drug concentrations

The Cy-A data obtained by FCM correlated with those obtained by HPLC. Table 1 shows the intracellular daunorubicin concentrations determined by HPLC in sensitive and resistant P388 cells, with and without the addition of Cy-A. Again, the addition of Cy-A to the incubation medium led to a dramatic increase in intracellular daunorubicin concentrations in drug-resistant cells but had no significant effect on intracellular anthracycline concentrations in sensitive cells. No metabolites of daunorubicin were detected by HPLC in P388/R or P388/S cells.

Table 1 also shows the intracellular Cy-A concentrations determined by RIA. The P388/R cells accumulated significantly less Cy-A than did the P388/S cells; the addition of an excess of daunorubicin (10 μ*M*) could not restore the Cy-A accumulation defect in P388/R cells.

Optimal Cy-A concentrations

When P388/R cells were incubated with daunorubicin at a concentration of 2 μ*M*, the extracellular Cy-A concentration required for optimal stimulation of the anthracycline uptake appeared to be 3 μ*M* (see Fig. 1); however, when these cells were incubated with a lower concentration of daunorubicin, the optimally stimulating extracellular Cy-A concentration could be reduced proportionally. Figure 2 shows the relationship between the daunorubicin concentration used for incubation and the Cy-A concentration required for complete restoration of the intracellular daunorubicin concentration, as measured by on-line FCM.

Cytotoxicity

The cytotoxicity of daunorubicin to P388 cells was estimated by means of a [^{3}H]-thymidine incorporation assay. The daunorubicin concentration that inhibited DNA synthesis by 50% (ID_{50}) was 36.0 ± 7.2 μ*M* and 3.0 ± 1.0 μ*M* for P388/R and P388/S cells, respectively (Table 2). The addition of VRP or Cy-A to the incubation medium had no effect on the daunorubicin ID_{50} of P388/S cells; however, the sensitivity of the P388/R cells was completely restored. The VRP and Cy-A concentrations used were not toxic for the P388 cells in the absence of daunorubicin.

Discussion

The present study demonstrates that on-line FCM is a useful tool for monitoring the stimulation of anthracycline ac-

cumulation by multidrug-resistant cells. The advantage of on-line FCM over traditional FCM is that the whole-drug accumulation curve can be monitored; in this way, fluctuations in anthracycline accumulation will not lead to misinterpretation of the effects of agents such as VRP and Cy-A.

The present results, obtained by FCM and confirmed by HPLC, show that Cy-A increases the intracellular daunorubicin concentration in resistant P388 cells and that this effect is dose-dependent. The multidrug-resistant cell also accumulated less Cy-A than did the parent line, and this accumulation defect could not be restored by daunorubicin (in the concentration range tested).

From a study by Slater and co-workers [18] using radioactively labelled anthracyclines, it was concluded that Cy-A does not alter the uptake or efflux of daunorubicin by multidrug-resistant cells in vitro. However, it did correct the daunorubicin resistance of multidrug-resistant cells, as determined by a cytotoxicity assay. These authors thus concluded that the restoration of daunorubicin responsiveness by Cy-A must depend on mechanisms independent of modified anthracycline membrane transport. However, the present study shows Cy-A to behave exactly like VRP; even in the cytotoxicity assay, Cy-A and VRP were equally effective in restoring chemosensitivity in P388/R cells.

The molecular basis of multidrug resistance appears to involve an overexpression of a membrane-bound 170-kDa glycoprotein (P-170) [6, 16, 23]. Most likely, P-170 functions as a drug efflux pump [5] and overexpression of P-170 leads to increased drug extrusion. A current hypothesis for explaining the reversal of multidrug resistance by drugs such as verapamil suggests that their affinity for P-170 results in competition for outward transport [5]. The present data indicate that Cy-A may act in a similar way. If so, the fact that relatively high concentrations of daunorubicin could not restore the Cy-A accumulation defect in P388/R cells suggests that the affinity of Cy-A for the outward transport system is much stronger than that for daunorubicin. This would make Cy-A an interesting candidate for the reversal of typical multidrug resistance in refractory cancer patients.

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