Cyclosporin A and verapamil have different effects on energy metabolism in multidrug-resistant tumour cells

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Summary Cyclosporin A (Sandimmune®) rapidly induced an increase in daunorubicin accumulation in multidrug-resistant human ovarian carcinoma cells (2780AD) and was more potent than verapamil. Steady-state \(^{14}\text{C}\)-cyclosporin A accumulation at 37°C in 2780AD cells was 60–70% of that in the sensitive A2780 cells. A rapid increase of ATP consumption and lactate production was induced in 2780AD cells by verapamil, but not by cyclosporin A. These results suggest that the interactions of cyclosporin A and verapamil with P-glycoprotein, which leads to inhibition of drug transport, have a different mechanistic basis.

The development of resistance of tumour cells to a wide range of natural product derived anticancer agents is generally called multidrug resistance. One type of multidrug resistance is characterised by an increased energy-dependent outward cellular transport of the anti-cancer drugs (Peterson et al., 1980; Skovsgaard & Nissen, 1982; Inaba & Johnson, 1978). This transport is related to overexpression of a plasmamembrane glycoprotein, termed P-glycoprotein (Bradley et al., 1988), which has been shown to have ATPase activity (Hamada & Tsuruo, 1988). The discovery of this drug export system has triggered research to find drugs which specifically would block its activity, since such drugs might be potentially useful to increase the efficacy of chemotherapy. A number of classes of compounds, which increase the accumulation of cytostatic drugs, such as anthracyclines and vinca alkaloids, in P-glycoprotein expressing cell lines, have now been identified (Helson, 1984; Kessel, 1986; Schuurhuis et al., 1987). Specific binding of such drugs to P-glycoprotein (Safa et al., 1987), stimulation of ATPase activity of P-glycoprotein (Hamada & Tsuruo, 1988) and increase of ATP hydrolysis in multidrug resistant cells (Broxterman et al., 1988b) by such drugs has been shown. However, the exact molecular mechanism of action of these drugs has not been elucidated (Huet & Robert, 1988; Gruber et al., 1988) nor is it known whether they all act via the same mechanism (Akiyama et al., 1988). Structure-activity relationships are now being determined for these drugs (Zamora et al., 1988; Ramu & Ramu, 1989).

Recently it has been found that the immunosuppressive drug cyclosporin A is able to reverse P-glycoprotein-dependent multidrug resistance (Slater et al., 1986; Osieka et al., 1986; Twenteman et al., 1987; Twenteman, 1988; Vayvugula et al., 1988), but the mechanism by which cyclosporins modulate the sensitivity to chemotherapeutic agents has not been elucidated (Chambers et al., 1989). In order to delineate further the mechanism of action of cyclosporin A as a resistance modifier we compared its effect with verapamil on daunorubicin accumulation and energy metabolism in multidrug-resistant cells and found evidence that these agents represent categories of drugs which differ in their interaction with P-glycoprotein.

Materials and methods

Chemicals

Verapamil hydrochloride was from Sigma (St Louis, MO, USA) and daunorubicin hydrochloride was from Specia (Paris, France). Sandimmune®, which is cyclosporin A, dissolved in cremophor EL/ethanol and pure cyclosporin A (which we dissolved in ethanol) were gifts from Sandoz (Basle, Switzerland). \(^{14}\text{C}\)-daunorubicin (spec. act. 45 Ci mol\(^{-1}\)) and [\(^{3}\text{H}\)cytosine] cyclosporin A (10.5 Ci mmol\(^{-1}\)) were from Amersham (UK).

Cells

Human ovarian carcinoma cells A2780 and 2780AD and culture conditions have been described (Rogan et al., 1984; Broxterman et al., 1987). 2780AD cells were cultured in the presence of 2 \(\mu\)M doxorubicin (Adriablastina, Farmitalia Carlo Erba, Milan, Italy) until 4–7 days before experiments.

Drug accumulation experiments

Cellular steady-state accumulation of \(^{14}\text{C}\)-daunorubicin and \(^{3}\text{H}\)-cyclosporin A was determined by incubation of cells in complete growth medium, including 10% fetal calf serum and buffered by 20 mM Heps, pH 7.45. After incubation cells were washed twice in ice-cold phosphate-buffered saline and cell-associated radioactivity was determined with liquid scintillation counting (Broxterman et al., 1987). Values are expressed as pmol per cell associated drug per 10\(^{6}\) cells after correction for 0°C direct binding to the cells.

\(\text{ATP and ADP measurements}\)

Ribonucleoside di- and triphosphates were measured in cellular trichloroacetic acid extracts with an ion exchange high-performance liquid chromatography system (Leyva et al., 1982). Cells were incubated with verapamil, cyclosporin A or vehicle in complete medium (medium A) or medium without glucose but with 10% fetal calf serum and 10 mM sodium azide (medium B) (Broxterman et al., 1988b). Cellular lactate formation was measured as described (Broxterman et al., 1989).

Results and discussion

We first examined the potency of cyclosporin A (Sandimmune) to induce an increase in daunorubicin accumulation in 2780AD cells, because such an effect has been reported (Nooeter et al., 1989), while others have found no evidence for correction of daunorubicin accumulation in multidrug resistant cells (Vayvugula et al., 1988). As shown in Figure 1, Sandimmune is a more potent inducer of daunorubicin accumulation than verapamil in 2780AD cells: e.g. 2 \(\mu\)M Sandimmune had a similar effect as 8 \(\mu\)M verapamil, while 8 \(\mu\)M Sandimmune was more potent than 8 \(\mu\)M verapamil (Table I). Since we found that the vehicle Cremophor EL, when used in the same concentration as present in a 2 \(\mu\)M Sandimmune solution (i.e. 0.003% vol./vol. or 33 \(\mu\)g ml\(^{-1}\))

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itself caused a small, but significant increase of daunorubicin accumulation in 2780AD cells, we have checked in a separate experiment that 8 μM cyclosporin A (dissolved in ethanol) had a similar effect as 8 μM Sandimmune (Table I). The effects of Cremophor EL on doxorubicin and vincristine cytotoxicity in multidrug resistant cells have been further analysed (Schaauw et al., submitted).

Since 2780AD is a typical multidrug resistant cell line, in which the defect of daunorubicin transport is related to overexpression of the mdr1 gene (Van der Bliek et al., 1988; Broxterman et al., 1988a), cyclosporin A apparently is a potent modulator of P-glycoprotein mediated drug transport. A number of agents that reverse multidrug resistance have been suggested to interact competitively with antitumour agents such as the vinca alkaloids for the P-glycoprotein associated transport mechanism (Horio et al., 1988). Therefore, to find evidence for P-glycoprotein dependent transport of cyclosporin A itself, we measured the accumulation of cyclosporin A in A2780 and 2780AD cells and found (Table II) that the accumulation of 3H-cyclosporin A in 2780AD cells was 60-70% of that in A2780 cells. Although, the solubiliser Cremophor EL tended to cause a higher cyclosporin A accumulation in both cell types, a difference in accumulation between parent and resistant cells was present, whether Cremophor EL was present in the medium (in Sandimmune) or not, although in the latter case no statistical significance was reached (Table II). We found similar accumulation differences for AUXB1 and CHF/C5 cells, another couple of sensitive and multidrug resistant cells (not shown). These data, together with the recent finding that cyclosporin A binds specifically to P-glycoprotein (Safa et al., 1989; Foxwell et al., 1989) would suggest that cyclosporin A itself is a substrate for the P-glycoprotein related transport mechanism.

We did find a small effect of 16 μM verapamil on the accumulation of cyclosporin A in 2780AD cells (legend Table II), while it had a large effect on daunorubicin accumulation in 2780AD cells (Figure 1). Goldberg et al. (1988) found a more pronounced effect of verapamil on cyclosporin A accumulation in CHF/C5 cells at a higher verapamil/cyclosporin concentration ratio than we used. Therefore some interaction between these drugs at P-glycoprotein level may still be present.

To delineate further the mechanism of reversal of resistance by cyclosporin A we investigated the effect of the drug on energy metabolism in 2780 cells. We have previously shown that resistance modifiers such as verapamil, bepridil, trifluoperazine and diltiazem under appropriate conditions can decrease the ATP/ADP ratio in P-glycoprotein over-expressing cells (Broxterman et al., 1988b), and in addition we showed that the same compounds induced a specific increase in lactate formation rate in multidrug-resistant, but not in sensitive cells (Broxterman et al., 1989). Together with data showing that these drugs are potent inhibitors of ATP-dependent vinblastine binding and transport in P-glycoprotein containing plasmamembrane vesicles (Horio et al., 1988), these results suggested that resistance modifying agents themselves may be transported by the P-glycoprotein dependent pump system. Table III, however, shows that 4 μM (and 8 μM, not shown) Sandimmune, which more effectively reversed daunorubicin accumulation than 8 μM verapamil (Figure 1), did not affect the ATP/ADP ratio in 2780AD cells, while 8 μM verapamil decreased this ratio to 48%. In a separate experiment we have checked that the onset of cyclosporin's effect on daunorubicin accumulation was very rapid, that is within 2.5 min. Furthermore a steady-state accumulation of cyclosporin A was already reached in 2 min (legend of Table II). Thus if a transport-related energy effect of cyclosporin A would be present, it should have a rapid onset too. Furthermore we found that cyclosporin A in contrast to verapamil (Broxterman et al., 1989), did not induce an increase in glycolysis during 60 min of drug exposure in three multidrug resistant cell lines, 2780AD (Table IV), H134AD and MCF-7/Adr® (not shown). A small effect of cremophor EL on lactate formation was seen in A2780 only.

In conclusion, we present evidence that cyclosporin A is a very effective inhibitor of the P-glycoprotein related dauno-

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**Table II** Cyclosporin A accumulation in 2780 cells

| Drug       | Accumulation (pmol per 10⁶ cells) |
|------------|----------------------------------|
|            | A2780          | 2780AD          |
| Sandimmune | 88 ± 17        | 56 ± 7*         |
| Cyclosporin| 46 ± 8         | 33 ± 6*         |

About 1,000,000 cells were incubated in medium A at 37°C with 3H-cyclosporin A, diluted with Sandimmune® or cyclosporin A (stock in ethanol) to 2 μM cyclosporin A final concentration. From time-curves it appeared that a steady-state was reached in 2 min incubation. Data are from 60 min incubations, means ± s.d. from 5 (Sandimmune) or 2 (cyclosporin A) separate experiments. *P < 0.01; †P > 0.05, compared to A2780 (Student's t test). In three experiments the effect of 16 μM verapamil on cyclosporin A accumulation in 2780AD cells was measured; a small increase (122 ± 13% of controls) was measured.

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**Table III** Effect of cyclosporin A and verapamil on ATP/ADP ratio in 2780 cells

| Drug       | Concentration (μM) | A2780          | 2780AD          |
|------------|--------------------|----------------|-----------------|
| Verapamil  | 8                  | 98 ± 11%*      | 48 ± 10%        |
| Sandimmune| 4                  | 98 ± 16%       | 97 ± 6%         |

Cells were incubated in medium with sodium azide (medium B, as described in Broxterman et al., 1988b) during 7.5 min. *Data are expressed as percent of control samples (no drug added; mean ± s.d. of 2 experiments) incubated for 7.5 min in medium B. In these control cells ATP levels decreased to about 40% compared to cells incubated in complete growth medium.
rubin transport across the plasmamembrane, but apparently does not increase the cellular energy demand for its interaction with P-glycoprotein. Evidence for a direct interaction of cyclosporin A with P-glycoprotein comes from the observation that this drug competitively inhibits the binding of a photoaffinity-labelled vinblastine analogue to P-glycoprotein and of ATP-dependent vincristine binding to plasma membranes of multidrug resistant cells with an apparent inhibition constant of 0.1 μM (Safa et al., 1989). The present study suggests that there are differences between the mechanism of interaction of cyclosporin A and verapamil with P-glycoprotein.

Cyclosporin A is a hydrophobic peptide and has been shown to partition into phospholipid vesicles and to increase membrane fluidity (Haynes et al., 1985). We have also found, using 1,6-diphenyl-1,3,5-hexatriene fluorescence polarisation, that cyclosporin increased plasmamembrane fluidity of A2780 and 2780AD cells (unpublished observations). Thus by disrupting membrane architecture cyclosporin may affect P-glycoprotein function (Arsenault et al., 1988). Alternatively, inhibition of protein kinase C activity by cyclosporin A (Walker et al., 1989) might interfere differentially with the energizing of P-glycoprotein.

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