Effects of cyclosporin A and a non-immunosuppressive analogue, O-acetyl cyclosporin A, upon the growth of parent and multidrug resistant human lung cancer cells in vitro

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Summary We have studied the ability of cyclosporin A (CsA) and a non-immunosuppressive analogue, O-acetyl cyclosporin A (OACsA), B3-243) to inhibit the growth of human lung cancer cells in vitro. Using continuous drug exposure and the MTT colorimetric assay to determine cell growth we found that CsA produced partial growth inhibition at doses ranging from 0.5 to 3.0 μg ml⁻¹ (0.4-2.4 μM). At progressively higher doses, complete growth inhibition and in situ cell lysis were seen. The P-glycoprotein expressing multidrug resistant (MDR) variant H69 LX4 of the small cell line H69 P was less sensitive to cyclosporins than the parent line, but this was not true of the non-P-glycoprotein expressing MDR variants of large cell line COR-L23 or adenocarcinoma line MOR. Sensitivity to OACsA was approximately 2-fold higher than that to CsA in most of the lines although not in the most sensitive line, COR-L88. Even in COR-L88, exposed to CsA or OACsA for 24 h, clonogenic cell survival was reduced only to 50% of that in controls. There was no reduction in polyamine content of COR-L23 or COR-L88 cells following 48 h of exposure to CsA or OACsA. The effects on cell growth could not be inhibited by the addition of exogenous putrescine, nor could they be enhanced by the addition of α-difluoromethyloroticine. It does not appear therefore that inhibition of polyamine synthesis is the basis of the observed growth inhibition.

Cyclosporin A (CsA), a cyclic peptide of 11 amino acids, has received much attention as an immunosuppressive drug used in organ transplantation (Borel et al., 1976). Whereas its precise mechanism of action is not fully evaluated, it is known to bind to a cytoplasmic receptor, cyclophilin (Handschumacher et al., 1984) which has peptidyl-prolyl cis-trans isomerase activity (Fischer et al., 1989). A specific inhibition of T-cell proliferation occurs at an early stage following activation. This results in a failure of T-cells both to express receptors for IL-2 or to secrete IL-2 (Elliott et al., 1984; Prince & John, 1986). The effect is greater on cytotoxic T-cells than on suppressor T-cells (Hess et al., 1981).

Inhibition by CsA of rodent and human leukaemic T-cell proliferation in vitro has been reported by a number of laboratories (Foa et al., 1981; Totterman et al., 1982; Yama gihara & Adler, 1983) and clinical therapy of T-cell disease has been attempted (Puttick et al., 1983; Morland et al., 1985). There have also been reports of antiproliferative effects of CsA in tumour cells of non-T-cell origin, although these are generally observed at higher doses than found effective in T-cell studies. In two recent studies of the effect of CsA in hamster pancreatic and mouse colon cancer cell lines, it was found that the effects could be blocked by the addition of the polyamine, putrescine, and enhanced by the addition of the polyamine biosynthesis inhibitor α-difluoromethyloroticine (Sadyari et al., 1986, 1987).

An additional potential role for CsA in cancer therapy has recently been developed in that the compound has been found to act as an effective modifier of multidrug resistance (MDR) both in vitro and in vivo (Slater et al., 1986; Meador et al., 1987; Twentyman et al., 1987). Addition of CsA to an agent such as adriamycin or vincristine can, at least partially, restore sensitivity to cells in which MDR results from hyperexpression of P-glycoprotein. This property is also possessed by some, but not all, analogues of CsA including non-immunosuppressive analogues (Twentyman, 1988; Chambers et al., 1989). Clinical trials of CsA as a resistance modifier are in progress (Sonneveld & Nooter, 1990).

In our studies of CsA and various analogues as resistance modifiers in human lung cancer cells, we observed antiproliferative effects at doses of 5 μg ml⁻¹ or less. This paper describes a series of experiments designed to study these effects in more detail and to determine whether or not inhibition of polyamine synthesis is involved.

Materials and methods

Cell lines and culture conditions

The human small cell lung cancer cell line H69 P was originally supplied by Drs A. Gazdar and D. Carney, NCI Navy Medical Oncology Branch, Bethesda, MD, USA. An MDR subline H69 LX4 was derived in our laboratory by in vitro growth in adriamycin (Twentyman et al., 1986; Reeve et al., 1989). This subline hyperexpresses P-glycoprotein and demonstrates an MDR drug accumulation deficiency. The large cell lung carcinoma line COR-L23 P was derived in our laboratory (Bailie-Johnson et al., 1985) and an MDR subline COR-L23 R obtained by growth in adriamycin (Twentyman et al., 1986). Adeno carcinoma line MOR was originally supplied by Dr M. Ellison, Ludwig Institute, Sutton. MDR subline MOR R was also derived by in vitro growth in adriamycin. Both L23 R and MOR R whilst exhibiting an MDR phenotype and reduced drug accumulation do not hyperexpress P-glycoprotein (Twentyman et al., 1986; Reeve et al., 1990 and unpublished). The small cell lung cancer line COR-L88 was derived in this laboratory (Bailie-Johnson et al., 1985).

All cells were grown in RPMI 1640 medium ( Gibco Biocult Ltd) with 10% foetal calf serum (Seratal Ltd) and penicillin and streptomycin (100 U ml⁻¹ and 100 μg ml⁻¹ respectively, Gibco Biocult Ltd). The small cell lines grew as floating aggregates in 75 cm² tissue culture flasks (Falcon Plastics) whilst non-small cell lines grew as attached monolayers in similar flasks. All lines were passaged weekly. Routine tests for mycoplasma contamination were carried out and gave negative results throughout the period of these studies.

Disaggregation of small cell cultures was achieved by repeated pipetting of aggregates. For attached cultures,
15 min incubation with 0.5% trypsin and 0.02% versene solution in PBS was used.

**Drugs**

Cyclosporin A (CsA) and O-acetyl cyclosporin A (OACsA, B3-243) were kindly supplied by Sandoz (Basle). Putrescine was obtained from Sigma and a-difluoromethyl ornithine (a-DFMO) was a gift from Merrell Dow (Cincinnati, USA).

Cyclosporins were dissolved in absolute ethanol at 5 mg ml⁻¹ and stored at 4°C. Dilution in medium was carried out immediately before use in experiments and the final ethanol concentration did not exceed 0.1%. Putrescine and a-DFMO were dissolved in PBS. Appropriate solvent controls were used in all experiments.

**Drug response assays**

To determine the sensitivity of the various cell lines to continuous cyclosporin exposure we used the MTT colorimetric assay (Mosmann, 1983) as adapted in our laboratory for use with human lung cancer cell lines (Twentyman, 1988). Cells were inoculated into wells on 96 well microtitre plates at between 10^2 and 10^4 cells well in a volume of 200 µl. Cyclosporins were added 1 h later in a volume of 10 µl. After the required incubation period (1–7 days) 20 µl of a 5 mg ml⁻¹ solution of MTT (Sigma) in PBS was added to each well. The plates were then re-incubation for 5 h. At the end of this time the bulk of the medium was removed from each well by aspiration. For plates containing small cell lines, it was necessary to centrifuge the plates before aspiration in order to pack the floating aggregates on the bottom of the wells. Two hundred µl of DMSO was then added to each well and the plates agitated for 10 min on a plate shaker. The optical densities of the wells were then read on a Titertek Multiskan MCC 340 plate reader at 540 nm.

Clonogenic cell survival assays were also carried out on cells exposed to cyclosporins for 4 or 24 h. Drugs were added in a small volume to 25 cm² flasks of cells in the exponential phase of growth. At the end of the exposure period COR-L23 cells were rinsed three times in PBS and then reduced to a single cell suspension using a combination of trypsin and versene as previously described. Aggregates of small cell line COR-L88 were also dispersed for clonogenic assay using trypsin versene. Following cell counting using haemocytometers, appropriate dilutions were made and survival was assayed using our previously described version (Walls & Twentyman, 1986) of the Courtenay and Mills (1978) soft agar assay. This assay uses incubation in low oxygen (5% O₂, 5% CO₂, 90% air) and rat red blood cells as a source of nutrient to stimulate growth of clonogenic cells. Colonies containing more than 50 cells were counted after an incubation period of 3 weeks.

**Polyamine determination**

Cells for polyamine assay were inoculated into 9 cm plastic petri dishes at numbers such that control cells would be in late exponential phase at the time of assay, 3 days later. Cyclosporins or putrescine were added to the dishes 2 h after inoculation. At the time of assay, cells were washed twice with PBS and reduced to a single cell suspension using trypsin versene. Cells were resuspended in PBS, counted and polyamines were then extracted using 0.2 M perchloric acid as previously described (Wallace et al., 1984). The extracts were stored at −70°C and thawed immediately prior to polyamine assay. This was carried out by the hplc method of Wallace et al. (1988). Results were corrected according to the previously determined cell numbers and expressed as nmoles 10⁵ cells.

**Results**

**Cell growth**

Typical cell growth curves as determined using the MTT assay are shown in Figure 1. In this example it is seen that,
with increasing dose of either cyclosporin, the effect increased from partial growth inhibition to complete growth inhibition to in situ cell destruction. In wells treated with 10 μg ml⁻¹ CsA for >3 days, the total absence of intact cells was confirmed by visual examination under the inverted microscope. Similar data for the various cell lines are summarised as ID₉₀ values in Table I.

It may be seen that ID₉₀ values for both drugs were clearly higher in MDR small cell line H69 LX4 than in the parent line H69 P. This was not, however, true for the non-P-glycoprotein expressing MDR lines L23 R and MOR R compared with their respective parent lines. ID₉₀ values for OACsA were generally somewhat lower than those for the parent compound except in small cell line COR-L88. The sensitivity of the small cell line COR-L88 was clearly higher than that of the other lines studied.

Timing of administration

Experiments were carried out using a 6-day MTT assay on COR-L23 and COR-L88 cells in which exposure to CsA was for different intervals within the 6 day period. In these experiments, the medium on all wells was changed to fresh medium immediately before MTT addition in order to eliminate any artefacts due to different medium conditions in different wells during the MTT reduction process (Jabbar et al., 1989). Data for COR-L88 are shown in Table II. Continuous exposure (throughout the 6 days) was the most effective treatment. Clear effects were, however, seen for shorter treatments given at the early part of the 6 day period whereas relatively little effect was seen at later times. In the second experiment, the control optical density was monitored throughout the period of the experiment and was 0.36, 0.57, 0.82, 1.10, 1.44 and 1.57 on days 1–6 respectively. The relatively small effects at later times are, therefore, compatible with inhibition of increases in optical density seen over these periods. It is clear, however, that effects of exposure at early times are greater than would be expected from growth inhibition only during the period of drug exposure. This presumably reflects a 'recovery time', perhaps due to residual bound drug, following the exposure period.

Cell survival

The effect of treating cultures of COR-L23 P or COR-L88 cells with either CsA or OACsA for 4 or 24 h was determined using clonogenic assay. The results are shown in Table III. It may be seen that the effects were quite modest. Although significant effects were seen at 24 h, the reduction in cell survival was never more than 2-fold.

Effects of putrescine

To test the hypothesis that growth inhibitory effects of cyclosporins may be due to polyamine depletion, the effect of adding exogenous putrescine was studied. In preliminary experiments with H69 P and COR-L88 cells it was found that, in a 6 day MTT assay, concentrations of putrescine up to 160 μg ml⁻¹ (1 mM) had little if any effect on cell growth. We therefore tested the effect of adding a range of putrescine doses to either CsA or OACsA in the MTT assay. A typical data set for H69 P cells is shown in Figure 2. There was clearly no protective effect produced by adding putrescine to either cyclosporin. Similar experiments with H69 LX4 and COR-L88 cells produced similar data and conclusions.

Effect of α-DMFO

Preliminary experiments carried out with this compound in the MTT assay indicated that, at 0.1 mM, there was no effect on the growth of H69 P, COR-L88 or COR-L23 P cells. At 0.2 and 0.5 mM there were detectable effects, the final optical densities being between 60 and 80% of control at 0.5 mM. The effect of combining α-DMFO with CsA in COR-L88 is shown in Figure 3. There was clearly no potentiation of the effect of CsA alone. Similar results were obtained in experiments with H69 P and COR-L23 P cells.

Polyamine levels

Data for the effects of cyclosporins on polyamine levels in COR-L23 and COR-L88 cells after 72 h treatment are shown in Tables IV and V. It may be seen that no reductions in polyamine levels resulted from any of these treatments.

Discussion

Antiproliferative and or cytotoxic effects of CsA on human T cell leukaemia cells were reported by Foa et al. (1981) and by Totterman et al. (1982). The effective doses of CsA in these studies were 5 and 0.1 μg ml⁻¹ respectively with the drug being continuously present. A study by Yanagihara and Adler (1983) found that, in mouse lymphoreticular cell lines, treated continuously with 5 μg ml⁻¹ CsA, a total loss of viability occurred in T-cell lines, whereas only a small degree of growth inhibition was seen in non-T cell lines. The first

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### Table I Growth inhibition by CsA and OACsA

| Cell line       | Type       | Parent | MDR | ID₉₀ (μg ml⁻¹) | CsA | OACsA |
|-----------------|------------|--------|-----|----------------|-----|-------|
| NCI-H69 P       | Small cell | P      |     | 4.5 ± 3.7      | 2.0  | 1.9   |
| NCI-H69 LX4     | MDR        |        |     | 8.6 ± 6.1      | 3.6  | 2.4   |
| COR-L23 P       | Large cell | P      |     | 8.0 ± 5.5      | 4.8  | 4.1   |
| COR-L23 R       | MDR        |        |     | 3.9 ± 5.1      | 3.4  | 6.5   |
| MOR P           | Adeno-     | P      |     | 10.5 ± 7.2     | 4.5  | 2.8   |
| MOR R           | Small cell | MDR    |     | 9.3 ± 8.1      | 5.5  | 3.4   |
| COR-L88         | Small cell | P      |     | 2.1 ± 1.0      | 1.7  | 1.5   |

* MDR = multidrug resistant subline. ID₉₀ = drug concentration to reduce the optical density in the MTT assay to 50% of control at the time at which the control optical density is at its highest value during the 7 day assay period. Each value is taken from a set of dose-response curves obtained in an independent experiment.

### Table II Effect of different protocols of cyclosporin A exposure on growth of COR-L88 cells

| Exposure conditions | Cyclosporin A (μg ml⁻¹) | 5 | 10 |
|---------------------|------------------------|---|----|
| Continuous          | 0.45 ± 0.40            | 0.10 ± 0.12 |
| day 0–day 1         | 0.55 ± 0.60            | 0.38 ± 0.42 |
| day 0–day 2         | 0.53 ± 0.45            | 0.27 ± 0.19 |
| day 0–day 3         | 0.49 ± 0.37            | 0.26 ± 0.20 |
| day 3–day 6         | ND ± 0.71              | ND ± 0.54 |
| day 4–day 6         | 0.86 ± 0.75            | 0.73 ± 0.71 |
| day 5–day 6         | 0.98 ± 1.00            | 0.90 ± 1.01 |

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### Table III Clonogenic cell survival after cyclosporin treatment

| Cell line | Agent | Dose (μg ml⁻¹) | Surviving fraction |
|-----------|-------|---------------|--------------------|
|           |       |               | 4 h  | 24 h |
| COR-L23 P | CsA   | 0.96 ± 0.05   | 0.83 (0.15)       |
|           | OACsA | 1.13 ± 0.22   | 0.84 (0.14)       |
|           |       | 0.97 ± 0.17   | 0.62 (0.06)       |
| COR-L88   | CsA   | 0.81 ± 0.09   | 0.74* (0.14)      |
|           | OACsA | 0.78 ± 0.13   | 0.58* (0.16)      |

Values are means (s.e.) of three or five independent determinations, each based on colony counts in triplicate tubes.
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Figure 2 Effect of putrescine on the response of H69 P cells to (a) cyclosporin A or (b) O-acetyl cyclosporin A as determined by the MTT assay after 6 days growth. \( \bullet \), control; \( \circ \), putrescine 20 \( \mu \)g ml\(^{-1} \); \( \triangle \), 80 \( \mu \)g ml\(^{-1} \); \( \Delta \), 160 \( \mu \)g ml\(^{-1} \).

Table IV Polyamine content of COR-L23 P cells

| Treatment | Putrescine | Spermidine | Spermine | Total | % Control |
|-----------|------------|------------|----------|-------|-----------|
| Control   | 0.2        | 1.1        | 4.4      | 5.7   | 100       |
|           | 0.4        | 2.2        | 4.0      | 6.6   |           |
| CsA       | 0.3        | 1.4        | 5.7      | 7.4   | 128       |
| 1 \( \mu \)g ml\(^{-1} \) | 0.3        | 2.6        | 3.8      | 6.9   | 104       |
| 2 \( \mu \)g ml\(^{-1} \) | 0.2        | 1.2        | 5.0      | 6.4   | 112       |
| 4 \( \mu \)g ml\(^{-1} \) | 0.5        | 2.7        | 5.6      | 8.8   | 133       |
| OACsA     | 0.8        | 3.1        | 6.1      | 10.0  | 152       |
| 0.5 \( \mu \)g ml\(^{-1} \) | 0.8        | 1.2        | 4.7      | 6.1   | 105       |
| 1.0 \( \mu \)g ml\(^{-1} \) | 0.5        | 2.4        | 4.9      | 7.8   | 118       |
| 2.0 \( \mu \)g ml\(^{-1} \) | 0.6        | 2.3        | 7.4      | 10.3  | 180       |

Expressed as nmoles per 100\(^6\) cells. Each value is the mean of two replicate determinations. Data for two independent experiments are shown.

Figure 3 Effect of DFMO on the response of COR-L88 cells to cyclosporin A as determined by the MTT assay after 6 days growth. \( \bullet \), control; \( \circ \), DFMO 0.1 \( \mu \)g ml\(^{-1} \); \( \triangle \), 0.2 \( \mu \)g ml\(^{-1} \); \( \Delta \), 0.5 \( \mu \)g ml\(^{-1} \).

Table V Polyamine content of COR-L88 cells

| Treatment | Putrescine | Spermidine | Spermine | Total | % Control |
|-----------|------------|------------|----------|-------|-----------|
| Control   | 0.1        | 0.7        | 1.1      | 1.9   | 100       |
|           | 0.2        | 0.9        | 0.7      | 1.8   | 100       |
| CsA       | 0.25 \( \mu \)g ml\(^{-1} \) | 0.2        | 1.0      | 0.6   | 1.8       |
| 0.3 \( \mu \)g ml\(^{-1} \) | 0.1        | 2.0        | 2.4      | 4.3   | 226       |
| 0.5 \( \mu \)g ml\(^{-1} \) | 0.2        | 1.3        | 0.8      | 2.3   | 127       |
| 1.0 \( \mu \)g ml\(^{-1} \) | 0.0        | 1.0        | 1.3      | 2.3   | 121       |
| OACsA     | 0.25 \( \mu \)g ml\(^{-1} \) | 0.2        | 1.2      | 0.9   | 2.3       |
| 0.5 \( \mu \)g ml\(^{-1} \) | 0.0        | 1.1        | 1.6      | 2.7   | 142       |
| 0.25 \( \mu \)g ml\(^{-1} \) | 0.2        | 1.3        | 1.3      | 2.8   | 156       |
| 1.0 \( \mu \)g ml\(^{-1} \) | 0.0        | 0.9        | 1.1      | 2.0   | 105       |
|           | 0.2        | 0.8        | 1.1      | 2.1   | 117       |

Expressed as nmoles per 100\(^6\) cells. Each value is the mean of two replicate determinations. Data for two independent experiments are shown.

The results reported in this paper indicate that effects of CsA and OACsA are seen in human lung cancer cells only when the agents are present for prolonged periods of time and that the doses required to produce these effects are in the range 0.5–10 \( \mu \)g ml\(^{-1} \). Even in the relatively sensitive cell line COR-L88, a 24 h exposure to 10 \( \mu \)g ml\(^{-1} \) of either cyclosporin produced no more than a 2-fold reduction in clonogenic cell survival. These results are very different to those of Totterman et al. (1982) who observed extensive cytolyis within 24 h of human leukaemic T cells treated with 5 \( \mu \)g ml\(^{-1} \) CsA. However, our results in the MTT assay, by the standard endpoint of formazan reduction and confirmed by visual observation of the wells indicates that, at higher doses (>5 \( \mu \)g ml\(^{-1} \)) for longer periods of time (>72 h), complete loss of cell viability occurs. In the clinical use of CsA as an immunosuppressive agent, a rapid initial phase of plasma clearance occurs following the attainment of peak levels of 1–2 \( \mu \)g ml\(^{-1} \) (Kahan et al., 1983). Levels during the prolonged second plateau phase are very much lower (~0.1 \( \mu \)g ml\(^{-1} \)). It appears unlikely therefore that the results which we report here in which continuous exposure to at least 1.0 \( \mu \)g ml\(^{-1} \) was required for growth inhibition are of significance for the use of CsA as an anticancer agent. Nevertheless there is clearly a heterogeneity of sensitivity amongst lung cell lines and furthermore, OACsA in most of the lines studied is approximately 2-fold more potent than CsA.

Based on a report by Fidelius et al. (1984) that CsA could act as an inhibitor of ornithine decarboxylase and therefore
of polyamine synthesis. Saydari et al. (1986, 1987) investigated the in vitro effects of combining CsA with α-DFMO, a known inhibitor of polyamine synthesis. They studied the growth of hamster pancreatic and mouse colon carcinoma cells in vitro and at CsA doses of 1 and 5 μg ml⁻¹. In both series of experiments, a small inhibition of cell growth by CsA was reported which was both reversible by the addition of exogenous putrescine (0.05 mM) and synergistic with the effects of α-DFMO. In one of the studies the effects of CsA (1μg ml⁻¹) upon polyamine levels were studied after 72 h exposure. Three and 2-fold reductions in levels of spermidine and spermine respectively were seen (Saydari et al., 1986). In contrast to these data, McLachlan et al. (1991) have shown that in MOLT-4 lymphoblastoid cells, where a transient decrease in ODC activity was produced by exposure to CsA (2.5 or 5 μg ml⁻¹), there was no significant change in polyamine concentrations after 48 or 96 h of exposure. Moreover, growth inhibitory effects of CsA were unaffected by the addition of putrescine at concentrations between 0.1 and 10 mM. These data are therefore in agreement with the results which we present in this paper.

Following a description of its ability to act as a modifier of multidrug resistance (Slater et al., 1986; Twentymam et al., 1987) CsA was shown to bind to P-glycoprotein, the putative drug efflux pump molecule involved in the MDR phenotype (Foxwell et al., 1989). It has also been demonstrated that MDR Chinese hamster cells accumulate less tritium-labelled CsA than the corresponding parent cells (Goldberg et al., 1988). The clear reduction in CsA sensitivity seen in H69 LX4 (P-glycoprotein positive) compared with H69 P (P-glycoprotein negative) may therefore be accounted for by a similar differential CsA accumulation in this pair of cell lines. In our pairs of cell lines where the MDR phenotype occurs in the absence of P-glycoprotein, no such differential was seen. The experiments which we have carried out do not clarify the mechanism whereby cyclosporins can inhibit cell growth. It is known that CsA binds to a specific cytosolic protein, cyclophilin (Hardschumacher et al., 1984) and that this protein has peptidyl-prolyl cis-trans isomerase activity (Fischer et al., 1989). It has been recently suggested that such interactions may interfere with the activity of protein kinase C and cell signal transduction (Tropschug & Hoffman, 1991). A number of groups have reported effects of CsA on the physico-chemical properties of the plasma membrane. The drug binds to phospho-lipid vesicles, thereby disrupting membrane architecture (Haynes et al., 1985) and interferes with the incorporation of fatty acids into the plasma membrane phospholipids of activated T-cells (Szamel et al., 1986). It also depolarises cytoplasmic membrane potentials (Matys et al., 1986).

In conclusion, therefore, we have shown that CsA and OA-CsA will each inhibit growth of human lung cancer cells in vitro, but only at doses in excess of those which are clinically achievable. OA-CsA is, however, more potent than the parent compound and as further analogues enter clinical trial as possible resistance modifiers therefore, the possibility that antitumour effects of the cyclosporin alone may occur should be borne in mind.

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