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CYCLOSPORINS AS DRUG RESISTANCE MODIFIERS

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Abstract—Cyclosporin A (CsA), a cyclic peptide of 11 amino acids isolated from the fungus *Tolypocladium inflatum* Gams, is the principle drug used for immunosuppression in organ transplant patients. It is known to have a very specific effect on T-cell proliferation although the precise mechanism remains unclear. Following internalization, CsA binds to a cytosolic protein, cyclophilin, which has been shown to possess peptidyl-prolyl *cis–trans* isomerase activity. CsA is an effective modifier of multidrug resistance in human and rodent cells at doses in the range of 1 to 5 μg/mL. Although it reverses the drug accumulation deficit associated with multidrug resistance in some cell types, this is not always the case. CsA has P-glycoprotein binding activity but less specific membrane effects and inhibition of protein kinase C may also be involved in its resistance modifier action. A number of non-immunosuppressive analogues of CsA have been shown to have resistance modifier activity and some are more potent than the parent compound. One analogue from Sandoz, PSC-833, has been shown to be approximately 10-fold more potent than CsA and is expected to enter clinical trial in the near future. The use of such agents may allow a full test of the hypothesis that reversal of multidrug resistance will prove a useful clinical strategy.

It is now well established that cells with *in vitro*-derived acquired resistance to certain cytotoxic drugs generally show cross-resistance to other drugs within the group [1, 2]. Such cells are said to possess a multidrug resistant (MDR†) phenotype and frequently hyperexpress a 170-kD membrane glycoprotein (P-glycoprotein) which is believed to act as a drug efflux "pump" [2]. MDR cells are generally able to accumulate lower levels of the relevant drugs than their sensitive counterparts. Agents involved in the group of "MDR-type" drugs include doxorubicin (Adriamycin®), daunorubucin, vincristine, vinblastine, colchicine, actinomycin D and etoposide (VP16). Detection of P-glycoprotein in a variety of tumour types in patients has led to the belief that the MDR phenotype may be of significance in clinical oncology [3]. It has, therefore, been considered important to discover chemical agents with the ability to restore drug sensitivity to MDR cells.

Although it is now 10 years since Professor Tsuruo first described the RM properties of verapamil [4], a clear clinical benefit of the approach remains to be demonstrated. Indications that addition of verapamil to chemotherapy treatment protocols may lead to improved response in myeloma or lymphoma [5, 6] are difficult to interpret in view of the small patient numbers and the modified chemotherapy regimes used in these studies. In general, attempts to use verapamil in the clinic have been hampered by the difficulty of achieving adequate plasma levels of the compound due to the cardiotoxic effects of the compound alone. Studies using other RMs such as quinidine or amioderone are not yet sufficiently advanced to allow any conclusions to be drawn.

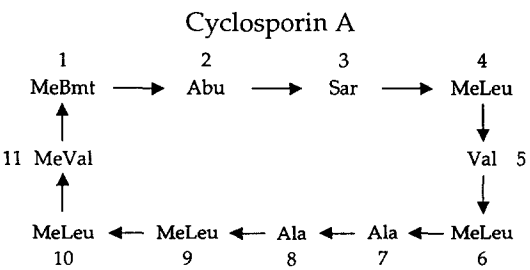

Fig. 1. Amino acid sequence of cyclosporin A. Redrawn from Ref. 74. MeBmt (previously known as C9) is the hitherto unknown amino acid *N*-methyl-4-butenyl-4-methyl-threonine. Abu, L-α-amino butyric acid; MeVal, *N*-methyl-L-valine; MeLeu, *N*-methyl-L-leucine.

At the time of writing, certain non-immunosuppressive analogues of CsA, because of their extreme potency as RMs, are leading candidates for the clinical proof or disproof of the concept. It appears likely that it will be possible to administer such agents to patients so as to achieve plasma levels found in *in vitro* studies to be adequate for full chemosensitisation, without unacceptable toxicity from the compound alone. It may be that problems will arise due to increased toxicity of the cytotoxic drugs towards P-glycoprotein-expressing normal tissues but that remains to be seen. In this article, I will review developments to date concerning cyclosporins as RMs.

CsA: background

CsA is a cyclic peptide of 11 amino acids isolated from the fungus *Tolypocladium inflatum* Gams. Its structure is shown in Fig. 1. An impure compound, "ovalicin", was shown in 1968 to have both antimitotic and immune suppressive properties [7].

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† MDR, multidrug resistant; RM, resistance modifier; CsA, cyclosporin A.

The pure compound, CsA, was subsequently isolated in cystalline form and found to have highly specific immunosuppressive properties [8]. Following extensive development, CsA is now widely used to prevent immune rejection of transplanted tissues in human subjects. The immunosuppressive action of CsA is based on its specific inhibition of T-cell proliferation at an early stage following activation. This results in a failure of T-cells to express receptors for IL-2 or to secrete IL-2 [9, 10]. A greater effect on cytotoxic than suppressor T-cells has been reported [11].

The basic mechanism underlying these events remains to be elucidated. It is known that CsA is internalized following binding to a specific cell surface receptor and subsequently binds to cyclophilin, a cytosolic protein [12]. This cytosolic binding protein was originally thought to be identical, or closely related, to calmodulin but has now been shown to have distinct properties [13]. Although CsA does bind to calmodulin as well as to cyclophilin, equal calmodulin-binding ability is demonstrated by cyclosporin H, an analogue lacking immunosuppressive properties [14]. It seems unlikely therefore that calmodulin binding is of mechanistic significance. Interaction between CsA and nuclear proteins involved in the transcription of genes involved in T-cell activation has also been reported [15]. Cyclophilin has been shown to possess peptidyl-prolyl *cis–trans* isomerase activity [16], as have FK506 and other immunosuppressive fungal products also [17]. It may be, therefore, that CsA may act, at least in part, through inhibition of peptidyl-prolyl *cis–trans* isomerase function.

A number of studies have also found effects of CsA on the physio-chemical properties of the plasma membrane. The agent has been shown to bind to phospholipid vesicles [18], interfere with the incorporation of fatty acids into membrane phospholipids [19] and depolarise cytoplasmic membrane potentials [20].

CsA as a resistance modifier

When it became clear that neither verapamil nor trifluoperazine, the compounds originally described by Tsuruo *et al.* [4, 21], would prove to be optimal for clinical use, the search for other compounds began. Interest in CsA arose for two distinct reasons. Firstly, its ability to bind to and possibly inhibit the function of calmodulin was a property shared by other calmodulin inhibitors, such as trifluoperazine, known to possess RM ability [21]. Secondly, following their clinical observation of an interaction between CsA and etoposide resulting in a lethal bone marrow depletion [22], Osieka *et al.* [23] in Germany had confirmed such an interaction in laboratory studies. In peripheral blood mononuclear cells from either healthy donors or leukaemia patients, DNA damage due to etoposide was enhanced by 2 μg/mL CsA. A similar dose of CsA also increased clonogenic cell kill in L1210 murine leukaemia cells by etoposide or doxorubicin but not by cisplatin or ionising radiation. Reduced effects in this system were also produced by lower doses of CsA [23]. *In vivo* experiments with human embryonic cancer or testicular cancer xenografts showed increased growth delays by the addition of CsA to etoposide or doxorubicin [23, 24]. Host toxicity was also increased but by a smaller factor than antitumour effect. Although these studies did not specifically address the question of drug-resistant versus sensitive tumour cells, it was apparent that both drugs shown to be potentiated (i.e. etoposide and doxorubicin) are involved in the MDR phenotype.

The first report of a preferential effect of CsA in resistant versus sensitive tumour cells was by Slater *et al.* [25] in 1986. They took a human T-cell acute lymphatic leukaemia cell line and developed a resistant variant by continuous exposure to vincristine. In addition to being 60-fold resistant to vincristine, the variant cell line also displayed 5-fold cross-resistance to daunorubicin. In the resistant line, complete restoration of vincristine or daunorubicin sensitivity was achieved by the addition of 13.2 μg/mL CsA with a reduced effect at lower doses (Fig. 2). There were essentially no effects of CsA upon the sensitivity to these drugs of the parent cell line. A second publication by the same group described *in vivo* studies using a 2-fold daunorubucin-resistant Ehrlich ascites carcinoma in mice [26]. Enhancement of the survival time of host mice bearing the resistant tumour and treated with daunorubicin was achieved by the addition of CsA at doses of 5–25 mg/kg. It should be noted that neither of the resistant cell lines studied by Slater *et al.* [25] were shown to hyperexpress P-glycoprotein. As, however, the cellular accumulation of ^{3}H-labelled daunorubicin was similar between resistant and parent lines in both systems, this appears unlikely. The first report of CsA effects in a human cell line of solid tumour origin was from our laboratory in 1987 [27]. We used the H69/LX4 subline of the human small cell lung cancer line NCI-H69. This subline was derived by *in vitro* growth in doxorubicin; is cross-resistant to colchicine, vincristine and etoposide; shows a clear doxorubicin accumulation defect and hyperexpresses P-glycoprotein due to amplification of the *mdr*1 gene [28, 29]. The 100-fold resistance to doxorubicin seen in the H69/LX4 line (Fig. 3) could be reduced to approximately 50, 15 and 2-fold by the addition of 1, 2 or 5 μg/mL of CsA, respectively. There were only minimal effects of CsA, even at 5 μg/mL, in the parent line. Resistance to vincristine in H69/LX4 was similarly reduced from 1000- to 20-fold by 5 μg/mL CsA whereas there was only a 2-fold sensitization of the parent line.

Less pronounced differential sensitization between parental and P-glycoprotein hyperexpressing resistant cell lines was reported in a number of later studies. In a comparison of Chinese hamster parental cell line $AuxB_1$ and its MDR subline CH^RC_5, the reductions in doxorubicin IC_{50} caused by 1 μg/mL CsA were 11- and 60-fold, respectively [30]. Indeed, for a doxorubicin analogue, 4′-epidoxorubicin, the effects of 1 μg/mL CsA were greater in the parent than in the resistant cells. The same cell lines studied in a different laboratory also gave 7, 16 and 11-fold reductions in IC_{50} for daunorubicin, vincristine and colchicine, respectively, in the parent line; compared with 32, 37 and 9-fold reductions, respectively, in the resistant line when 1 μg/mL CsA was added to the cytotoxic drug treatment [31]. It became clear

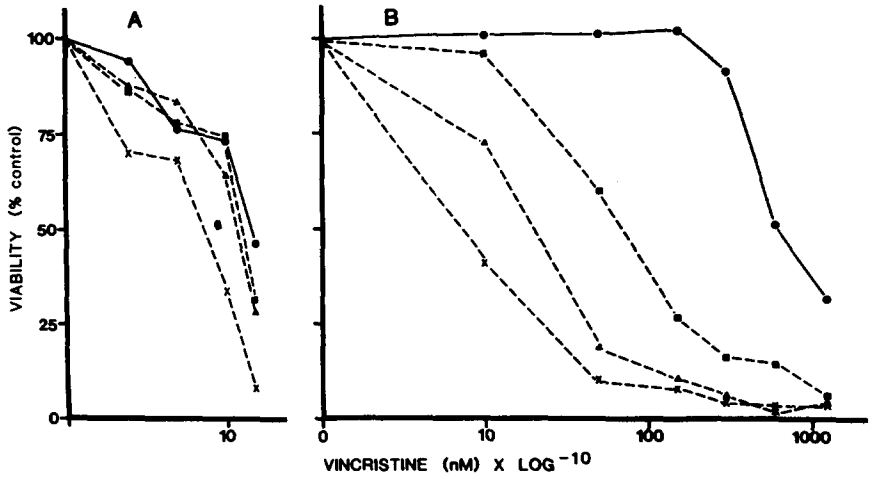

Fig. 2. Effects of CsA and vincristine in combination on the viability of drug-sensitive (panel A) or resistant (panel B) acute lymphatic leukaemia cells. Cell viability determined by vital dye exclusion after 3 days continuous drug exposure. Concentrations of CsA are: (●) 0 μg/mL; (■) 3.3 μg/mL; (▲) 6.6 μg/mL; (×) 13.2 μg/mL. Reproduced from *J Clin Invest* 77: 1405–1408, 1986 [25] with permission.

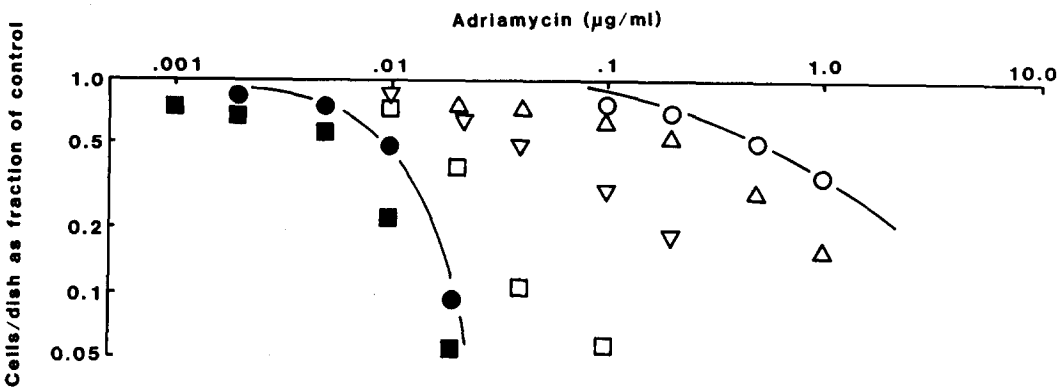

Fig. 3. Number of cells/dish following continuous incubation for 6 or 7 days with various doses of Adriamycin®. Solid symbols, human small cell lung cancer parent line NCI-H69/P; open symbols, multidrug resistant subline H69/LX4. (●, ○) Adriamycin alone; (△) Adriamycin plus 1 μg/mL CsA; (▽) Adriamycin plus 2 μg/mL CsA; (■, □) Adriamycin plus 5 μg/mL CsA. Reproduced from *Br J Cancer* **56**: 55–57, 1987 [27] with permission.

from studies in our laboratory that the relative sensitization of parental and resistant cell lines is highly dependent upon, *inter alia*, the dose of CsA [32, 33]. Sensitization of EMT6 mouse tumour parental cells to doxorubicin, colchicine and vincristine begins at 0.1 μg/mL and reaches a maximum at 1 μg/mL. In MDR sublines with high level expression of P-glycoprotein, however, little or no sensitization is seen below 1 μg/mL and most of the effect is seen between 2 and 5 μg/mL. As a consequence, at doses of CsA below 1 μg/mL there may be less relative sensitization of the MDR cells than of the parental cell line. Support for the concept that lower doses of RM may be needed for optimal sensitization of cells expressing lower rather than higher levels of P-glycoprotein was provided by the results of drug accumulation studies carried out in Chinese hamster cells [34]. In both the Chinese hamster $AuxB_1$ and EMT6 parental cell lines, P-glycoprotein is expressed at low, but detectable, levels and hence the results seen may be dependent upon relative competition between CsA and the different cytotoxic drugs for binding to this molecule. In the parental cell line NCI-H69, however, P-glycoprotein cannot be detected and this could account for the absence of sensitization of this cell line even at 5 μg/mL of CsA.

We have also studied two human lung cancer cell lines where the MDR sublines have a drug accumulation deficit but do not hyperexpress P-glycoprotein (Refs 28 and 35, and unpublished data). The MDR sublines COR-L23/R and MOR/R are 24- and 10-fold resistant to doxorubicin compared to their parental lines, and cross-resistant to colchicine and vincristine. In each of these sublines only modest (<3-fold) sensitization to doxorubicin

is caused by CsA at 5 μg/mL and sensitization is also seen at this dose in the parental line (unpublished data). However, a recent study in a human thyroid cancer cell line which does not express P-glycoprotein has demonstrated clear sensitization to vincristine, daunorubicin and etoposide by CsA (1 μg/mL) [36].

We have carried out a series of studies in our laboratory aimed at the identification of anthracyclines which, to a lesser or greater extent, retain activity in MDR cell lines with high levels of resistance to doxorubicin [37–39]. Compounds with an alkyl group at the 9 position of the A ring or certain substitutions (including a morpholinyl ring) in the sugar have been found to possess this property. Investigation of the effects of CsA in combination with such anthracyclines showed that modest sensitization could be achieved, suggesting that such a combination of resistance modification approaches may be useful therapeutically [38, 39]. For example, the small cell lung cancer MDR line was 100-fold resistant to doxorubicin but only 6-fold resistant to aclacinomycin A when compared to its parent line. Sensitization by 5 μg/mL CsA reduced these factors to 5- and 1.2-fold, respectively [38].

Mechanism of action: (A) accumulation

Considerable confusion exists regarding the mechanism(s) by which CsA brings about increased drug effects in resistant (and parent) cells. In some examples, increased effect is apparently associated with increases in intracellular drug accumulation but, in other examples, this does not appear to be the case. Even where accumulation is affected, it is unclear whether this results from a specific interaction between CsA and P-glycoprotein or whether less specific effects on the plasma membrane are also involved. The available data are of limited value in addressing these issues as there are relatively few studies of the effects on chemosensitivity and drug accumulation in parallel. In addition, several of the earlier studies were carried out in cell lines where the P-glycoprotein status had not been determined.

In most situations where the drug accumulation ratio and the drug sensitization ratio have been obtained in the same pair of sensitive and resistant cell lines, the former has often been considerably lower than the latter. In the original study by Slater *et al.* [25], a 5-fold resistance to daunorubicin was associated with only a 20% reduction in drug accumulation. In the resistant line, a 3-fold sensitization to daunorubicin was achieved without any effect on daunorubicin accumulation. Similarly, a much larger sensitization to doxorubicin by CsA was seen in MDR line P388/DOX than in the parent P388, although the small effect on doxorubicin accumulation was similar in both lines [40].

In our human small cell lung cancer cell line pair, the resistant line H69/LX4 is 100-fold resistant to doxorubicin and yet shows only a 2.5-fold lower doxorubicin accumulation as measured at 4 hr (when drug levels have plateaued) [39]. When CsA (5 μg/mL) is added to this system, 20-fold sensitization in the resistant line is accompanied by almost complete restoration of drug accumulation levels to parent value. In the parent line there is little if any effect of CsA on either sensitivity or accumulation.

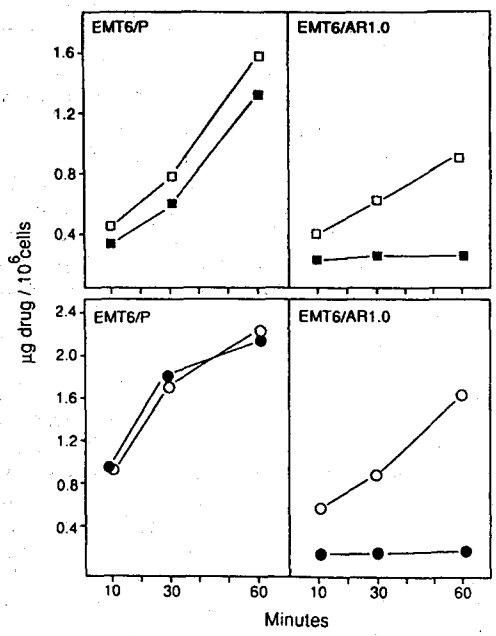

Fig. 4. Accumulation of Adriamycin® (10 μg/mL) in EMT6/P (left hand panels) or EMT6/AR1.0 (right hand panels) in the presence (□) or absence (■) of verapamil (3.3 μg/mL), or in the presence (○) or absence (●) of cyclosporin A (5 μg/ml) at 37°. Data points are the mean values of duplicate samples from each of two independent experiments. Reproduced from *Biochem Pharmacol* **38**: 4467–4475, 1989 [39] with permission.

Similarly, a drug accumulation deficit of approximately 6-fold is associated with a 34-fold resistance to doxorubicin in our mouse tumour MDR cell line EMT6/AR1.0 in comparison with the EMT6/P parent line. However, in this latter system, although the parent cell line shows considerable sensitization to doxorubicin by 5 μg/mL CsA, there is essentially no effect on doxorubicin accumulation. In the resistant line, however, sensitization is accompanied by major changes in drug accumulation [39] (Fig. 4). However, it should be noted that in this latter system, similar results were obtained for verapamil, so discrepancies between sensitization and drug accumulation effects do not necessarily demonstrate different bases for sensitization by verapamil and CsA.

These few examples illustrate the point that drug accumulation cannot account for all cases of CsA effect on sensitivity to MDR-type drugs. A variety of preliminary evidence suggests that MDR may be characterized by changes in intracellular drug distribution in addition to changes in gross cellular accumulation [41, 42]. Clearly, if CsA is able to modify intracellular drug distribution, then this could account for at least some of the discrepancies observed between gross accumulation and sensitization. It also appears possible, however, that other mechanisms not involving cellular pharmacokinetics may be involved. The ability of CsA to interfere with membrane structure [19, 20] together with the identification of the cell membrane as one of the

targets of anthracycline cytotoxicity [43] provides at least one possible basis for interaction.

Mechanism of action: (B) binding to P-glycoprotein

The use of photoactive, labelled analogues of vinblastine enables two different groups of workers to demonstrate that verapamil and cytotoxic drugs involved in the MDR phenotype bind competitively to P-glycoprotein [44, 45]. A similar technique was subsequently used to demonstrate interaction between CsA and P-glycoprotein [46]. A more direct confirmation was provided by a study in which a photoactive CsA analogue was used to label viable MDR Chinese hamster ovary cells [47]. Although a number of proteins were labelled, clear differential labelling of a protein of 170 kDa was observed between resistant and parent cells. The differential labelling could be inhibited by excess cold CsA or by verapamil, and furthermore, labelling of the 170 kD protein with [^{3}H]azidopine could be inhibited by cold CsA. Taken together, therefore, these data indicate strongly the ability of CsA to bind to P-glycoprotein. Using a less specific mechanism in which [^{3}H]vincristine binds to isolated membrane vesicles, CsA was found to have a particularly high binding coefficient in comparison with verapamil or a range of cytotoxic drugs including doxorubicin [48]. This observation, therefore, supports the generally higher potency of CsA compared with verapamil seen in experimental systems *in vitro*. In a separate study carried out using [^{3}H]CsA, it was shown that MDR Chinese hamster cells CH^RC_5 accumulate only 50% of the agent compared to the parent $AuxB_1$ cell line and that this difference could be overcome by concurrent treatment with excess verapamil [49]. This result is again compatible with the notion that CsA acts directly by interaction with P-glycoprotein. However, it should be noted that [^{3}H]CsA accumulation in an MDR variant of the P388 leukaemia cell line was similar to that in the parent line [40] and, in our EMT6/AR1.0 MDR subline which hyperexpresses P-glycoprotein and shows reduced accumulation of MDR-type drugs, there was again similar accumulation of [^{3}H]CsA to that seen in the parent line (unpublished). The data on this aspect of the question are, therefore, conflicting.

Mechanism of action: (C) other studies

A few investigations over the last few years have examined novel aspects of the differential response of parent and MDR cells to CsA. Following a report that doxorubicin and actinomycin D promote the translocation of nucleolar phosphoprotein B23 from the nucleoli to the nucleus of P388 parent murine leukaemic cells but not of the cells of an MDR subline [50], the effects of CsA were studied [51]. The initial observation was confirmed in two further pairs of parent and MDR lines and it was found that addition of CsA enhanced the translocation in both parent and resistant cells. There was, again, a lack of correlation between effects on this translocation process and chemosensitization by CsA in the various lines studied, and it is unclear to what extent the observations are dependent upon CsA-mediated changes in cellular drug levels.

An interesting study of intracellular pH found that MDR cells had consistently higher (by 0.10–0.17 units) intracellular pH values than their corresponding parent cells, possibly due to increased Na^+/H^+ antiport activity [52]. Although CsA was both able to reduce the level of resistance and lower the intracellular pH in one resistant line, this was not true in another resistant line. Furthermore, verapamil had no effect on intracellular pH whilst reversing resistance whereas for amiloride, the opposite was true. Effects of modifying agents on intracellular pH do not, therefore, appear to be generally implicated in their mode of action. It has also been reported that MDR cells have lower membrane potentials than their parental counterparts and that the potentials can be restored by CsA or verapamil [53].

In a comparison of the effects of CsA and verapamil on energy metabolism in MDR and parent cells, it was found that whereas verapamil can modify the ATP/ADP ratio in MDR but not in parental cells, no effects of CsA were seen in either cell type [54]. As CsA is the more effective RM in this system, at the doses used, it again does not appear that this is the primary mechanism of resistance modification, at least by CsA. A report that CsA inhibits protein kinase C activity [55] is of potential significance with respect to MDR reversal.

Investigation of analogues

Many of the early investigations of CsA as an RM *in vitro* used doses of 5 μg/mL or more in order to produce maximum effects. Clinical pharmacokinetic data were available to suggest that such levels were not clinically achievable without unacceptable renal and/or hepatic toxicity [56]. In addition, the potent immunosuppressive properties of CsA were considered to be disadvantageous for its potential administration to cancer patients as an RM. Examination of analogues was, therefore, undertaken in the hope that cyclosporins with increased potency as RMs and/or reduced immunosuppression would be found. In my laboratory, we began by examining three naturally occurring analogues supplied by Sandoz, each of which had a single amino acid difference from CsA [27]. The results are summarized in Table 1. It may be seen that, disappointingly, there was a close agreement between the immunosuppressive and RM properties of this series of compounds. A further series of analogues, either synthetic or chemically derived from naturally occurring cyclosporins was, however, subsequently examined and these produced more encouraging results [57]. Several compounds with little or no immunosuppressive ability were found to be highly effective modifiers. One compound in particular, B3-243 (*O*-acetyl $MeBmt^1$ CsA), was found to be not only non-immunosuppressive but approximately 4-fold more potent than CsA in potentiating the effects of doxorubicin in our H69/LX4 human small cell lung cancer MDR line (Table 1). In an independent study, a further two analogues, $MeLeu^{11}$ CsA and $MeAla^6$ CsA, were also identified as non-immunosuppressive but possessing RM properties [30]. Studies at Sandoz confirmed that many non-immunosuppressive analogues of CsA possess RM

Table 1. Immunosuppressive and resistance modification abilities of cyclosporin analogues

Compound	Structure	Immunosuppressive ability	Resistance modification	Reference
CsA	See Fig. 1	+++	+++	27
CsC	Thr^2CsA	++	+	27
CsG	L-$NorVal^2$ CsA	+++	+++	27
CsH	D-$MeVal^{11}$ CsA	—	—	27
B3-243	*O*-acetyl Bmt^1 CsA	—	+++	57
PSC-833	3'keto-Bmt^1-Val^2-CsA	—	+++++	59–62

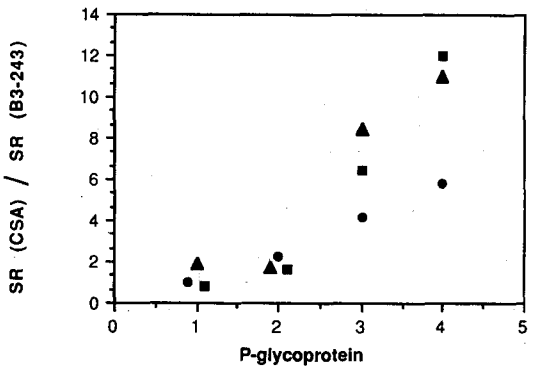

Fig. 5. Relative sensitization ratios [SR(CsA)/SR(B3-243)] in cell lines with differing P-glycoprotein content. The P-glycoprotein content is an arbitrary scale based on intensity of bands in autoradiographs of Western blots as described in Table 1. (●) Adriamycin®; (▲) vincristine; (■) colchicine. Reproduced from *J Cell Pharmacol* **1**: 26–34, 1990 [58] with permission.

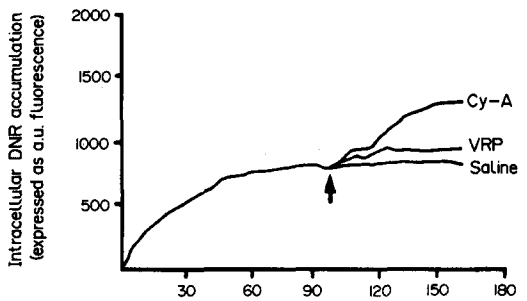

Fig. 6. Daunorubucin (DNR) accumulation, expressed as fluorescence intensity in arbitrary units (a.u.) by leukaemic cells *in vitro*, obtained from the peripheral blood of an acute myeloid leukaemia patient. At t = 0, DNR was added to the cell suspension (2×10^5 cells/mL) to a final concentration of 2 μM. The arrows indicate time points of addition of cyclosporin A (Cy-A) (final concentration 3 μM), verapamil (VRP) (final concentration 10 μM) or physiological saline. Reproduced from *Int J Cancer* **45**: 263–268, 1990 [64] with permission.

properties and, additionally, demonstrated for the first time that some immunosuppressive cyclosporins could be ineffective with regard to resistance modification [31]. Interestingly, in this study the sensitization to doxorubicin of a resistant cell line could not be attributed to either increased drug accumulation or to increased DNA single strand breaks. Further studies in my laboratory were directed towards a comparison of CsA and *O*-acetyl CsA as RMs in a panel of mouse tumour cell lines with a wide range of P-glycoprotein expression [58]. We found that the relative efficacy of the two compounds varied with the extent of P-glycoprotein expression and with the particular cytotoxic drug. In mouse lines with high levels of P-glycoprotein, CsA at 2.5 μg/mL was 4–12-fold more effective than *O*-acetyl CsA, whereas in lines with low levels, only a 1–2-fold ratio was seen (Fig. 5). In almost all cases, the effectiveness of *O*-acetyl CsA was considerably lower at 0.5 than at 2.5 μg/mL. The advantage of *O*-acetyl CsA seen in the earlier study using human cells did not, therefore, pertain in this series of experiments.

During 1988–1990 a large programme was carried out at Sandoz to investigate in detail the structure–activity requirements for cyclosporins with regard to RM properties. A paper describing the best compound emerging from this investigation has appeared recently [59]. The compound PSC-833 (3'-keto'-[Bmt^1]-[Val^2]-CsA) was at least 10-fold more active than CsA in reversing resistance to doxorubicin, daunorubicin, vincristine, etoposide and colchine in a number of MDR cell lines whilst being approximately equal to CsA in terms of cytotoxicity of compound alone.

Subsequent studies, currently in press and kindly made available to me by Professor Francis Loor [60, 61], have confirmed the effectiveness of PSC-833 as an RM for *in vivo* tumours in mice and established that the compound causes an increase in daunomycin accumulation in MDR cells *in vivo*. Work in my laboratory, also in press, has confirmed the approximately 10-fold superiority of PSC-833 over CsA as an RM in human and murine cells *in vitro* [62].

Clinical studies

A series of papers from the Netherlands has described effects of CsA on the accumulation of daunorubicin in cells taken directly from leukaemia patients [63–66]. The authors used a flow cytometer to measure by native fluorescence the time course of daunorubicin accumulation in single cells and the effect of adding CsA to the cell suspension (Fig. 6).

In various type of leukaemias, the ability of CsA to increase daunorubicin accumulation was proportional to the level of expression of either the *mdr*1 gene (coding for P-glycoprotein) or the related *mdr*3 gene which codes for a similar protein believed previously not to confer drug resistance. In one patient with acute myeloid leukaemia, studied in great detail throughout the course of his disease, changes in daunorubicin responsiveness *in vivo* correlated with changes in cellular accumulation measured *in vitro*. Furthermore, positive effects of CsA on accumulation *in vitro* predicted clinical benefit from co-administration of the modifier to the patient [66].

We have also been examining cells taken from leukaemia patients and determining whether or not CsA at 1 μg/mL is able to alter their *in vitro* chemosensitivity. Clear sensitization has been seen in a variety of cases, particularly chronic lymphoid leukaemia lymphocytes treated with vincristine [67].

In a Phase I clinical trial of the combination of CsA and vinblastine, a blood level of 1.5 μg/mL of CsA was achieved [68]. Higher doses of CsA resulted in unacceptable renal toxicity. It was reported that unspecified toxicity was produced by the drug combination in "tissues that express P-glycoprotein". A Phase II trial of CsA in combination with epidoxorubicin in colon cancer has been reported recently [69]. Twenty four patients were treated and the median CsA peak level achieved was 6.2 μg/mL, and the median 18 hr level 1.0 μg/mL. No CsA toxicity was seen apart from "flushing" during infusion, attributed to the effects of the vehicle. There was an unexpectedly high incidence of leukocytopenia and only one partial tumour response was seen.

Clinical trials of CsA in combination with chemotherapy are also ongoing at Stanford University and at the University of California, Irvine, but results have not been reported to date. It would appear from the available data that CsA levels of 1–2 μg/mL are achievable in cancer patients without undue toxicity. On the basis of *in vitro* experiments, such levels would be expected to produce some (but not optimal) sensitization of cells expressing high levels of P-glycoprotein. Cells expressing lower levels may, however, be fully sensitized by this dose of CsA. The potent non-immunosuppressive compound Sandoz PSC-833 is likely to enter the clinic in late 1991.

Other studies

A few reports have described the effects of CsA as a "chemosensitizer" in circumstances where conventional modification of MDR would not appear to be involved. A report from Japan described sensitization of human small cell and non small cell lung cancer cell lines to cisplatin by CsA [70]. We do not, however, see any comparable sensitization in a panel of similar cell lines established in my laboratory (unpublished data).

In ovarian cancer cells with acquired resistance to cisplatin, a short treatment with cisplatin resulted in increased transcription of the c-fos and c-H-ras oncogenes together with other genes involved in DNA synthesis and repair [71]. If, however, the cells were pretreated with CsA, no such increased transcription occurred, and the sensitivity of the cells to cisplatin was increased. Additionally, weekly exposure of the resistant cell line to CsA alone resulted in a loss of resistance to cisplatin which did not occur in the absence of CsA. The mechanism underlying these interesting observations remains to be clarified.

Little attention has been paid to the question of whether a combination of two or more RMs is likely to produce better effects than a single modifier. However, recent studies in human leukaemia cell lines have claimed significant synergy between CsA and verapamil when used at clinically achievable concentrations [72, 73].

Conclusions

CsA is one of the most widely studied *in vitro* modifiers of MDR. Despite this, its mechanism of action remains unclear. In many cases, its restoration of sensitivity is associated with increased cytotoxic drug accumulation but this is not universally true. It seems likely that there is no single mechanisms and that CsA acts, at least in part, by binding to P-glycoprotein and hence modifying drug accumulation but also through a less specific interaction with the plasma membrane, and possibly also by inhibition of protein kinase C. There is no doubt that CsA is able to enhance daunorubicin accumulation *in vitro* in leukaemia cells taken directly from patients. It is too early to predict whether or not CsA is likely to prove of benefit to patients receiving chemotherapy. Non-immunosuppressive analogues of CsA also possess RM ability and can be considerably more potent. The lead compound Sandoz PSC-833 may provide the vehicle for a true test of the clinical usefulness of a "resistance-modification" strategy.

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