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FK-506 (fujimycin) reverses the multidrug resistance of tumor cells *in vitro*

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Cyclosporin A (CsA) and FK-506 have similar immunosuppressive activity profiles and cyclophilin-like intracellular targets. Since CsA can reverse the multidrug resistance of tumor cells showing P-glycoprotein-mediated drug efflux, the possible resistance-modulating activity of FK-506 was evaluated *in vitro* with multidrug-resistant P388 cells and their sensitive parental controls. Higher concentrations of FK-506 than CsA were needed to achieve a similar degree of chemosensitization, suggesting that FK-506 might interact less efficiently than CsA with the P-glycoprotein expressed in multidrug-resistant tumor cells. However, FK-506 was active on a broader range of concentrations than CsA, particularly because of direct cytostatic effects of CsA which appeared at concentrations only slightly higher than those required to show a significant resistance-modulating activity.

Key words: CsA, FK-506, resistance-modifiers, multidrug resistance, P388 tumor cells.

Introduction

Innate or acquired resistance of tumor cells frequently impairs or makes inefficient treatment of cancer with potent anti-tumor chemotherapeutics. This resistance most often extends to several anticancer drugs (ACD) of unrelated structural classes and mechanisms of action, a phenomenon known as 'multidrug resistance' (MDR).[1]

A common mechanism making tumor cells multidrug-resistant (MDR) is the overexpression of a particular class of transmembrane glycoproteins called P-glycoproteins (Pgp).[2] Pgp molecules rapidly efflux ACD out of the cells as soon as they have passively diffused within the cells, thus decreasing the intracellular concentration of the ACD below their active thresholds.

This Pgp-pump-escaping mechanism can be overcome by increasing the ACD concentration in the medium surrounding the MDR cells. While this is possible *in vitro*, it is not *in vivo*. Indeed, any further increase of the chemotherapeutic agents in cancer patients would lead to severe toxicity sequels, since such treatments are already performed with ACD regimens which are close to the maximum tolerated dose.

A variety of agents able to restore the full ACD-sensitivity of MDR tumor cells, *in vitro*, or at least decrease their resistance, have been identified in several studies. Such chemosensitizers or resistance-modulating agents (RMA) are characterized by a variety of structures, and their mechanism of action seems to be the blocking of the effluxing function of Pgp.[3–5] They restore the normal accumulation and distribution of ACD within the cells and therefore their sensitivity.

FK-506 is a macrolide antibiotic of streptomyces origin. It has been reported so far to be a very potent immunosuppressive agent with a spectrum of action similar to cyclosporin A (CsA).[6,7] However, it binds to a peptidyl-prolyl-*cis-trans* isomerase (FKBP) distinct from the cyclophilin bound by CsA.[8] Since CsA was also reported to be active in the reversion of tumor-cell MDR,[9–11] FK-506 was tested for this indication on a couple of normally sensitive (Par-P388) and multidrug-resistant (MDR-P388) P388 murine leukemia cell lines *in vitro*. Hence we compared the capacity of structurally different RMA thought or known to be active in MDR such as amiodarone, verapamil,

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CsA, and FK-506 using an inactive Cs analog (Cs-NEG) as negative control, and using doxorubicin and vincristine as ACD.

Materials and methods

Cell lines

Parental (Par) and multidrug-resistant (MDR) murine monocytic leukemia P388 cells were obtained from Dr M. Grandi (Farmitalia, C. Erba Research Center, Milano, Italy). The MDR-P388 cell line has been selected by culture of the Par-P388 cells on doxorubicin.[12] In our culture conditions, its relative resistance to a variety of ACD (doxorubicin, daunomycin, etoposide, vincristine, colchicine) was about 200-fold in comparison with the Par-P388 cells.[13]

Both cell lines were grown in RPMI-1640 culture medium, supplemented by 2 mM glutamine, 1 mM pyruvate, 100 IU/ml streptomycin-penicillin, 5×10^{-5}M β-mercaptoethanol, 1% of a non-essential amino acid solution 100×, and 10% heat-inactivated fetal calf serum, partially buffered by 10 mM HEPES (all from Gibco). The medium for MDR-P388 cells was further supplemented with 300 ng/ml of doxorubicin in order to maintain their MDR properties. This doxorubicin was removed the day before experimental use.

Anti-cancer drugs (ACD) and resistance-modifying agents (RMA)

Doxorubicin and vincristine (Sigma Chemicals Company, St Louis, MO) were prepared as stock solution at 1 mg/ml in RPMI-1640 medium.

CsA (Sandoz, Basel, Switzerland) and FK-506 (Fujisawa Pharmaceutical Co., Osaka, Japan) were respectively dissolved at 1 mg/ml and 5 mg/ml in absolute ethanol (Prolabo, France). Cs-NEG (*N*-phenyl-amiothiocarbamoyl-CsA, Sandoz, Basel, Switzerland), verapamil (Sigma Chemicals Company, St Louis, MO), and amiodarone (Labaz, France) were prepared at 1 mg/ml, 10 mM and 10 mM in absolute ethanol, respectively.

RMA capacity assay by analysis of cell growth inhibition

Cultures were made in a final volume of 0.2 ml in 96-well microplates (COSTAR 3599). Cell suspensions (2×10^3 cells/well for the parental line and 5×10^3 cells/well for the MDR line) were put as 100 μl into plates containing ACD and RMA. Then 50 μl of doxorubicin or vincristine solutions were added in culture medium in triplicate to obtain final concentrations of 0, 0.03–10 μg/ml for the MDR line, and 0, 0.001–0.3 μg/ml for the parental line. A further down-extension of the dose range was performed when necessary, i.e. when the RMA used was strongly increasing the ACD sensitivity. Peaking concentrations of verapamil, amiodarone, Cs-NEG, CsA and FK-506 were distributed in triplicates as 50 μl/well, with controls being treated with the corresponding ethanol solvent dilutions.

After a 3-day incubation at 37°C, the final cell number was measured by a colorimetric assay using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma).[14] First, 100 μl of supernatant was removed from each well, and then 10 μl of the MTT solution (5 mg/ml in RPMI medium) was added to the remaining cell suspension and the plates incubated for 90 min at 37°C; 100 μl of solvent (butanol-2, isopropanol, HCl 1 N in volume ratio 16/8/1) was added per well and the plates shaken until complete dissolution of the formazan crystals. The absorbance was read at 540 nm on a plate reader (Titertek Multiskan). The extent of growth was represented as a function of the ACD concentration and led to calculation of IC_{50}.

The ACD IC_{50} values were determined from the concentration–response curves by plotting the measured growth versus the ACD concentration. The increases of ACD sensitivity or 'gain of sensitivity' brought by each RMA were given by the ratio [IC_{50} drug + solvent]/[IC_{50} drug + RMA]. Gains of sensitivity were then used to construct isobolograms by plotting them against the RMA concentrations. The difference of cell growth in presence of solvent and in presence of RMA, giving the growth inhibition rates, was represented as a function of each concentration of both CsA and FK-506. These measurements were performed for both cell lines (Par-P388 and MDR-P388).

Results

The possible activity of FK-506 in sensitizing a MDR state was first studied in comparison with structurally different molecules, including a cyclosporin analog as negative control (Cs-NEG) (Figure 1).

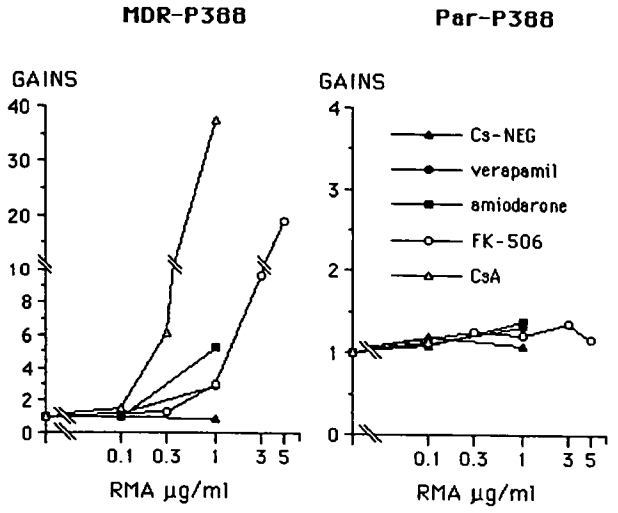

Figure 1 Effects of various RMA on the sensitivity of MDR-P388 and Par-P388 cells to doxorubicin. Results are expressed as gains: [IC_{50} drug + solvent]/[IC_{50} drug + RMA].

MDR-P388 cells were not sensitized to doxorubicin by Cs-NEG up to 1 µg/ml. CsA gave no detectable chemosensitization (gain of 1) at or below 0.1 µg/ml (not shown) but the gain of sensitivity rapidly increased from 6 at 0.3 µg/ml to 37.7 at 1 µg/ml. At this dosage however, CsA alone began to inhibit the cell growth (see below) so that the evaluation of its chemosensitizing activity was questionable. Lower levels of activity were found at 1 µg/ml for verapamil (gain of 2.9), amiodarone (gain of ±5), and FK-506 (gain of 3). We tested FK-506 in a larger range of concentrations and the gain rose to 19 at 5 µg/ml. Hence FK-506 was active in chemosensitizing MDR-P388 cells to doxorubicin though at higher concentrations than CsA.

No significant variation of sensitivity to doxorubicin was found for Par-P388 cells (gain of ±1) whichever the RMA added, which fits with their lack of expression of Pgp and suggests that the gains of sensitivity obtained with the MDR-P388 were linked to inhibition of Pgp function.

The resistance-modulating activities (i.e. on IC_{50} values) of CsA and FK-506 were compared over a larger range of doses using doxorubicin and vincristine as ACD (Figure 2). As expected neither FK-506 nor CsA could reduce the IC_{50} values for doxorubicin of Par-P388 cells (Figure 2a), showing no unspecific sensitization of the cells, i.e. no sensitization that might be unrelated to Pgp presence. The direct effects of both RMA on

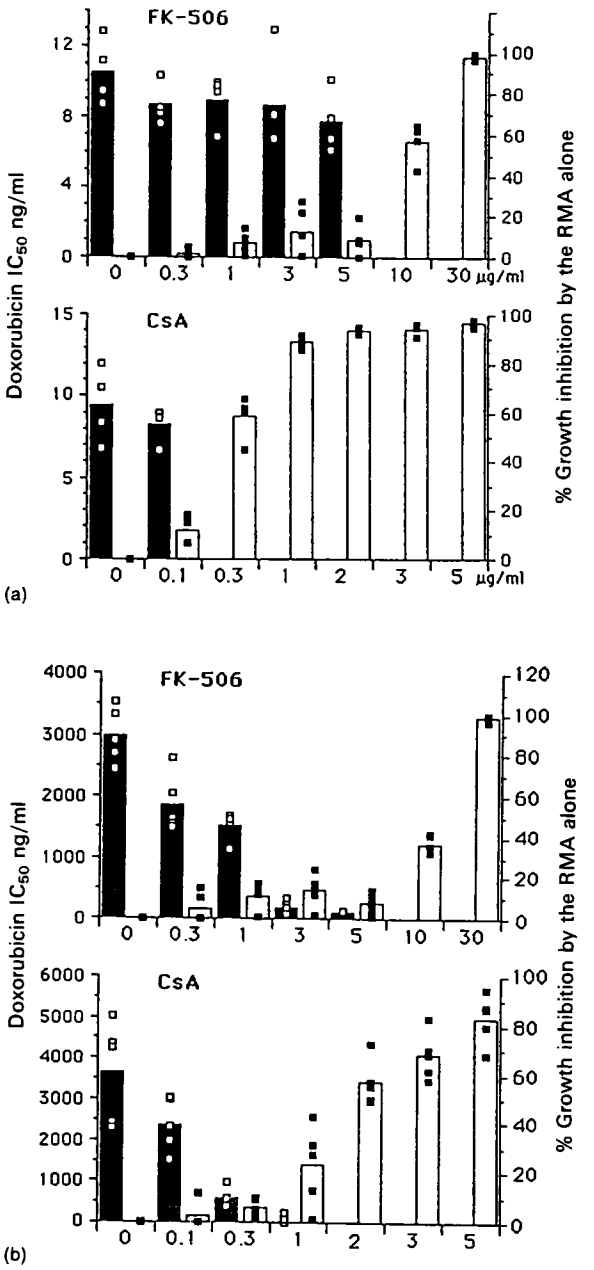

Figure 2 Effects of CsA and FK-506 on (a) Par-P388 and (b) MDR-P388 cells. Doxorubicin IC_{50} values (ng/ml) and growth inhibition rates are represented as means (columns) and individual experiments (squares) for each tested concentration of RMA. (■) Doxorubicin IC_{50} (ng/ml); (▣) % growth inhibition by RMA alone.

cell growth inhibition were also recorded. In absence of ACD growth inhibition appeared more rapidly for CsA since at 0.3 µg/ml CsA inhibited 58% of growth while FK-506 had no effect. With FK-506 high levels of Par-P388 cell growth inhibition occurred only from 10 µg/ml.

Thirty μg/ml FK-506 but only 1 μg/ml CsA were required completely to inhibit the growth of Par-P388 cells.

In the case of the MDR-P388 cell line (Figure 2b), increasing concentrations of both FK-506 and CsA allowed a marked sensitization of the cells to doxorubicin. The lowest doxorubicin IC_{50} values of MDR-P388 cells were reached with 5 μg/ml FK-506 and 1 μg/ml CsA. However, in the absence of ACD, the 5 μg/ml FK-506 concentration was not detectably inhibiting MDR-P388 cell growth, while the 1 μg/ml CsA concentration was at the borderline of inhibiting growth.

Similar data were obtained when the RMA activities of both CsA and FK-506 were tested in independent experiments using another ACD, vincristine (Table 1). CsA alone led to a significant growth inhibition from 0.3 μg/ml for Par-P388 and 1 μg/ml for MDR-P388, whereas this did not appear for FK-506 below 10 μg/ml for both cell types. The low vincristine IC_{50} values of Par-P388 were not decreased by CsA or FK-506. As regards the chemosensitizing activities of these two RMA on MDR-P388 cells, the lowest vincristine IC_{50} values could be obtained by use of 1 μg/ml CsA or 5 μg/ml FK-506 as chemosensitizer. However, the growth inhibition, caused by CsA alone at 1 μg/ml, made gain determination questionable, whereas this was not the case with FK-506 whose activity as RMA was clearly demonstrated.

Discussion

Neither CsA nor FK-506 sensitized the Par-P388 cells to doxorubicin or vincristine, two ACD with different structures, cellular targets and mechanisms of action, although both acted as RMA in the case of the MDR-P388 cells. This suggests that the CsA or FK-506-mediated sensitization of the MDR-P388 cell line was specifically Pgp-linked.

Therefore, although CsA was active as RMA at lower concentrations than FK-506, this was on a shorter range of concentrations: FK-506 left a broader *in vitro* 'therapeutic window', being specifically active as RMA for doxorubicin or vincristine from 0.3 μg/ml to 5 μg/ml (0.37–6.2 μM), while this was limited to the very narrow 0.1–0.3 μg/ml concentration range (0.08–0.25 μM) in the case of CsA.

At the *in vitro* 'maximal tolerated dose' of FK-506 however, there was no complete reversion of the MDR character of the MDR-P388 cells. Their relative resistance in comparison with Par-P388 (about 250-fold) is given by the ratio [IC_{50} without RMA for MDR-P388]/[IC_{50} without RMA for Par-P388]. The highest tolerated dose of FK-506, 5 μg/ml, allowed gains of sensitivity of the MDR-P388 cells of about 20 for doxorubicin and 70 for vincristine. Though quite high, such gains represent only a 10–25% attenuation of the resistant character of the MDR-P388 cells.

Although FK-506 is, on a molar basis, a stronger immunosuppressive agent than CsA, concentrations of FK-506 higher than those of CsA were required to achieve similar degrees of sensitization of the MDR-P388 cells. This confirms the lack of strict correlation between the immunosuppressive activity and the resistance-modulating activity. Since CsA binds Pgp,[4] it would be interesting to know whether FK-506 binds it too.

Table 1. Effects of various concentrations of CsA and FK-506 on P388 cell lines sensitivity[a] to vincristine

RMA (μg/ml)	Par-P388				MDR-P388			
	CsA		FK-506		CsA		FK-506	
	(IC_{50})	(GI%)	(IC_{50})	(GI%)	(IC_{50})	(GI%)	(IC_{50})	(GI%)
0	3.2 (2)	0(—)	3.8 (2.3)	0 (—)	1005 (231)	0 (—)	990 (341)	0 (—)
0.1	2.2 (1)	2.3 (2.9)			770 (392)	9.6 (8.8)		
0.3	—	53 (21)	3.3 (1.9)	4.4 (7.0)	130 (59)	9 (8.3)	680 (197)	2.5 (3.8)
1	—	85 (13.9)	2.4 (0.9)	4.6 (3.8)	35 (28)	30 (15.6)	530 (191)	17 (10)
2	—	90 (7.6)			—	64 (20.9)		
3	—	91 (8.3)	1.4 (0.5)	11 (10.5)	—	73 (2.8)	40 (44)	14 (7.0)
5	—	93 (5.4)	1.4 (0.5)	5.6 (6.3)	—	85 (14)	14.4 (6.0)	5.8 (8.7)
10			—	43 (11.6)			—	40 (17.7)
30			—	96 (3.0)			—	97 (3.1)

[a] Growth inhibition (GI%) is expressed as a percentage of control growth and IC_{50} in ng/ml as a mean (±SD) of at least four independent experiments.

Conclusion

A variety of RMA with widely different structures have already been identified, some of which are now entering clinical trials. FK-506, although known already for its immunosuppressive properties, is a novel structure for what concerns resistance-modifying activity. It is of course not conceivable to use the parent FK-506 molecule itself as a RMA in MDR cancer patients, since the high dosages of FK-506 required to achieve a significant MDR attenuation, would be both highly immunosuppressive and generally toxic for the patient. It is possible, however, that chemical modifications aimed at blocking the latter activities of FK-506 might preserve or even enhance its RMA properties, as has been done successfully in the case of cyclosporins.[13,14]

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References

1. Kartner N, Ling V. Multidrug resistance in cancer. *Sci Am* March 1989; 44–51.
2. Juranka PF, Zastawny RL, Ling V. P-glycoprotein: multidrug resistance and a superfamily of membrane-associated transport proteins. *FASEB J* 1989; **3**: 2583–2592.
3. Cornwell MM, Pastan I, Gottesman MM. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J Biol Chem* 1987; **262**: 2166–2170.
4. Foxwell BMJ, Mackie A, Ling V, *et al.* Identification of the resistance-related P-glycoprotein as a cyclosporine binding protein. *Mol Pharmacol* 1989; **36**: 543–546.
5. Safa AR. P-glycoprotein as a target for chemosensitizing agents. In: Tapiero H, Robert J, Lampidis TJ, eds. *Anticancer Drugs, Colloque INSERM.* John Libbey Eurotext Ltd 1989; **191**: 277–287.
6. Sawada S, Suzuki G, Kawase Y, *et al.* Novel immunosuppressive agent, FK506 *in vitro*: effects on the cloned T cell activation. *J Immunol* 1987; **139**: 1797–1803.
7. Lagodzinski Z, Gorski A, Wasik M. Effect of FK506 and cyclosporine on primary and secondary skin allograft survival in mice. *Immunol* 1990; **71**: 148–150.
8. Siekierka JJ, Hung SHY, Poe M, *et al.* A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* 1989; **341**: 755–757.
9. Slater LM, Sweet P, Stupecky M, *et al.* Cyclosporin A corrects daunorubicin resistance in Ehrlich ascites carcinoma. *Br J Cancer* 1986; **54**: 235–238.
10. Slater LM, Sweet P, Stupecky M, *et al.* Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphatic leukemia *in vitro*. *J Clin Invest* 1986; **77**: 1405–1408.
11. Twentyman PR. A possible role for cyclosporins in cancer chemotherapy. *Anticancer Res* 1988; **8**: 985–994.
12. Grandi M, Young C, Bellini O, *et al.* Pleiotropic multidrug resistant LoVo, P388 and I-407 cell lines have an increased tubulovesicular compartment. *Proc AACR* 1987; **28**: 279.
13. Gavériaux C, Boesch D, Jachez B, *et al.* PSC-833, a non immunosuppressive cyclosporin analog, is a very potent multidrug-resistance modifier. *J Cell Pharmacol* 1991; in press.
14. Gavériaux C, Boesch D, Boelsterli JJ, *et al.* Overcoming multidrug resistance in Chinese hamster ovary cells *in vitro* by cyclosporin A (Sandimmune) and non-immunosuppressive derivatives. *Br J Cancer* 1989; **60**: 867–871.

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