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Tudor Bîrsan
Camille Dambrin
Derrick G. Freitag
Randall W. Yatscoff
Randall E. Morris

# The novel calcineurin inhibitor ISA247: a more potent immunosuppressant than cyclosporine in vitro

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T. Bîrsan · C. Dambrin
Transplantation Immunology Laboratory, Department of Cardiothoracic Surgery, Stanford University School of Medicine, Stanford, California, USA

D. G. Freitag · R. W. Yatscoff
Isotechnika Inc., Edmonton, Alberta, Canada

R. E. Morris (✉)
Corporate Headquarters, Novartis Institutes for BioMedical Research, Novartis Pharma AG, WSJ-386.610, 4002 Basel, Switzerland
E-mail: randall.morris@pharma.novartis.com
Tel.: +41-61-3244713
Fax: +41-61-3243537

**Abstract** ISA247 is a novel cyclosporine analog. In this study we compare, in vitro, the effects of ISA247 on immune function with those of cyclosporine. Whole blood from cynomolgus monkeys ($n = 5$) was incubated with different concentrations of ISA247 or cyclosporine and stimulated with different mitogens in culture medium. Lymphocyte proliferation was assessed by [$^3$H]-TdR incorporation assay and by flow cytometry. Flow cytometry was also used to assess production of intracellular cytokines by T cells and expression of T cell activation surface antigens. The concentration of drug necessary to attain 50% of the maximum effect ($EC_{50}$) was subsequently calculated. $EC_{50}$ values for ISA247 were lower than for cyclosporine, and the differences were statistically significant for lymphocyte proliferation, T cell cytokine production, and expression of all T cell activation surface antigens but one. We conclude that ISA247 suppresses diverse immune functions more potently than cyclosporine in vitro.

**Keywords** Calcineurin inhibitors · Cyclosporine · FACS · Lymphocytes

## Introduction

The majority of solid-organ transplant recipients receive a calcineurin inhibitor—cyclosporine (CsA) or tacrolimus—as life long maintenance therapy. These drugs prevent allograft rejection by suppressing T cell activation through inhibition of calcineurin. However, their use is commonly associated with side effects, mainly nephrotoxicity and neurotoxicity. It is generally accepted that many toxic side effects are directly related to inhibition of calcineurin [1, 2, 3, 4]. Recent studies suggest that CsA toxicity may be also attributable to other factors, such as induction of TGF$\beta$ [5] or inhibition of mitochondrial high-energy phosphate metabolism [6, 7], implying that there might be a disassociation between calcineurin inhibition and toxicity. Therefore, it may be theoretically possible to develop calcineurin inhibitors that are less toxic but are effective immunosuppressants.

ISA247 is a CsA analog (US patents 6613739 and 6605593) and, in initial in vitro experiments, it inhibited calcineurin activity in human whole blood at lower drug concentrations than CsA. Furthermore, with the same doses, heterotopic rat heart grafts survived longer in recipients treated with ISA247 than in those treated with CsA. In rabbits, ISA247 showed less toxicity than CsA at similar drug exposures [8].

The aim of our in vitro study was to characterize more completely the immunosuppressive effects of ISA247 on non-human primate T cell functions and to determine whether ISA247 inhibited immune functions at lower concentrations than CsA in such primate species, before initiating a transplant study comparing the two drugs in this animal model.

## Materials and methods

CsA was purchased from Sigma (St. Louis, Mo., USA). ISA247 was a gift from Isotechnika, (Edmonton, Alberta, Canada). The molecular weight of ISA247 is within ±5% of that of CsA, of which ISA247 is a derivative (personal communication, R. Yatscoff, Isotechnika). Stock solutions of both drugs (500 µg/ml in methanol, stored at −20°C) were prepared independently and their identities remained unknown to the investigators until the experiments and statistical analysis were completed.

To compare the immunosuppressive effects of ISA247 and CsA, we used whole blood mitogen-stimulated lymphocyte proliferation and T cell function assays developed in our laboratory and optimized for use with whole blood from cynomolgus monkeys [9].

Whole blood was collected from cynomolgus monkeys ($n=5$) under light ketamine sedation and anti-coagulated with sodium heparin. We diluted stock solutions of ISA247 and CsA with culture medium [CM: RPMI 1640 (Life Technologies, Rockville, Md., USA) supplemented with 100 U/ml of penicillin, 100 mg/ml streptomycin, and 2 mmol/l L-glutamine (all from Sigma)] to obtain different concentrations of drug. To generate final drug concentrations of 50, 100, 250, 500, 1,000, 5,000, and 10,000 ng/ml, we added 12 µl of each drug dilution to whole blood. For unstimulated control and stimulated untreated control samples, we added an equal amount of CM. Once added to whole blood, the test compounds were allowed to equilibrate for 30 min at 37°C, to ensure homogenous distribution within the blood. The blood was then stimulated with different mitogens as follows:

- For assessment of lymphocyte proliferation and T cell surface antigen expression: concanavalin A (Con A, Vector Laboratories, Burlingame, Calif., USA), 7.5 µg/ml diluted whole blood
- For assessment of intracellular T cell cytokine production: phorbol 12-myristate 13-acetate (PMA, Sigma) and ionomycin (ICN Biomedical, Costa Mesa, Calif., USA), 150 ng/ml and 7.5 µg/ml, respectively; brefeldin A (Sigma) was added (10 µg/ml diluted whole blood) 30 min later

Lymphocyte proliferation was assessed by [$^3$H]-TdR incorporation as well as by flow cytometry, by methods previously described [10]. Furthermore, expression of the following surface activation antigens on T cells after stimulation with Con A for 72 h was quantified by flow cytometry: transferrin receptor (CD71), IL-2 receptor α chain (CD25), LFA-1 α chain (CD11a), Fas (CD95), CD40 ligand (CD154). To perform three-color and two-color flow cytometric analysis, respectively, we used the following monoclonal antibodies (PharMingen, San Diego, Calif., USA):

- FITC-labeled anti-CD71 + PE-labeled anti-CD3ε + Cy-chrome-labeled anti-CD25
- FITC-labeled anti-CD11a + PE-labeled anti-CD95 + PerCP-CY5.5-labeled anti-CD3ε
- FITC-labeled anti-CD3ε + PE-labeled anti-CD154

After incubation of the blood with the monoclonal antibodies in the dark for 30 min, the red blood cells were lysed. Leukocytes were pelleted, washed with PBS, and then resuspended in 500 µl PBS containing 0.5% formaldehyde.

Emitted light of the fluorochromes was collected through 525 nm (FITC), 575 nm (PE) and 675 nm (PerCP-CY5.5 and Cy-chrome) band-pass filters. Forward and side scatter were used to differentiate lymphocytes from debris, dead cells, and other leukocytes. Five thousand light scatter gated lymphocytes were analyzed per sample. Unstimulated and stimulated diluted whole blood cultures were incubated with isotype control antibodies (PE-labeled mouse $IgG_{1,\kappa}$, PharMingen) and used as specificity controls. To distinguish between fluorescence positive and negative cell populations, we set up analysis regions, using isotype controls to achieve non-specific binding of < 1% within the positive event regions. For all studied activation antigens, the entire population of lymphocytes was light scatter gated and CD3 positive cells were subsequently subgated. The percentages of $CD3^+$ lymphocytes expressing CD71, CD25, CD11a, CD95 and CD154 were calculated thereafter.

For the assessment of intracellular T cell cytokine production, the blood was incubated with mitogens at 37°C for 5 h. It was then incubated with PerCP-CY5.5-labeled monoclonal anti-human CD3 antibodies for 15 min at room temperature in the dark. Subsequently, the cells were fixed and permeabilized with a standard fixation–permeabilization reagent kit under the manufacturer's immunofluorescence staining protocol (Intra-Prep, Immunotech, Marseilles, France). For staining of intracellular cytokines, FITC-labeled anti-IL-2, FITC-labeled anti-IFNγ, and PE-labeled anti-TNFα(all from PharMingen) were used. Flow-cytometric analysis was performed as described above, and percentages of $CD3^+$ lymphocytes positive for IL-2, IFNγ, and TNFα were calculated thereafter.

We quantified the immunosuppressive effect of both drugs by expressing the immune function of interest (i.e., lymphocyte proliferation, T cell activation surface anti-

gen expression or cytokine production) normalized to 100%, using the following formula:

Immune function (% ) = (treated/not - treated) × 100.

"Not-treated" represents the results obtained from stimulated blood without addition of drug, while "treated" represents the results obtained from stimulated blood after addition of a specific concentration of either drug.

ISA247 and CsA concentrations causing 50% of the maximum effect ($EC_{50}$) were calculated for each experiment (i.e., for each animal) after fitting the concentration–effect curves in a sigmoid pharmacodynamic model using WinNonlin software, version 1.1 (Scientific Consulting, Cary, N.C., USA). $EC_{50}$ values obtained for the two drugs were compared by Wilcoxon signed rank test using SPSS software, version 10.0 (SPSS, Chicago, Ill., USA).

## Results and discussion

ISA247 inhibited multiple immune functions at lower concentrations than CsA (Fig. 1, Fig. 2). $EC_{50}$ values for ISA247 were lower than for CsA, and the differences were statistically significant for lymphocyte proliferation, T cell cytokine production, and expression of all T cell activation surface antigens but CD11a (Table 1).

We used the versatility of flow cytometry to determine the effects of ISA247 and CsA on different T cell functions. We were thus able to characterize the spectrum of their immunological effects more completely than by using only traditional proliferation assays. We demonstrate that, in vitro, ISA247 inhibits diverse immune functions in whole blood from non-human primates at concentrations significantly lower than those of CsA. Our findings show that ISA247 is a more potent immunosuppressant than CsA in vitro.

The exact mechanism of this higher potency is not currently known. However, it cannot be due to higher molar concentrations of ISA247, as its molecular weight is ± 5% that of CsA. In addition, we believe it is not due to ISA247 metabolites, since the drugs were not exposed to metabolic enzymes in these in vitro assays. Furthermore, availability of the drugs to lymphocytes probably does not account for the difference in potencies, since protein binding and partitioning between plasma and blood cells are similar for both compounds (personal communication, R. Yatscoff, Isotechnika).

ISA247 is not the first calcineurin inhibitor that is more potent than CsA: tacrolimus, which has been in clinical use for more than a decade, inhibits immune functions at concentrations 10–100 times lower than CsA [11]. CsA and tacrolimus, which are structurally different substances, form complexes with immunophilins (cyclophilins and FKBPs, respectively), and the active drug–immunophilin complexes bind and inhibit calcineurin. The CsA–cyclophilin binding site on calcineurin overlaps to a certain extent the tacrolimus–FKBP binding site, and it is generally assumed that both drugs have a similar mechanism of action [12, 13, 14]. Immunophilin concentration may limit inhibition of calcineurin phosphatase activity at high concentrations of CsA or tacrolimus, but this limitation does not have an impact on immune events downstream of calcineurin activity and is present in drug ranges much higher than the ones achieved in vivo in clinical situations [15]. While all immunophilins form drug–immunophilin complexes, not all drug–immuno-

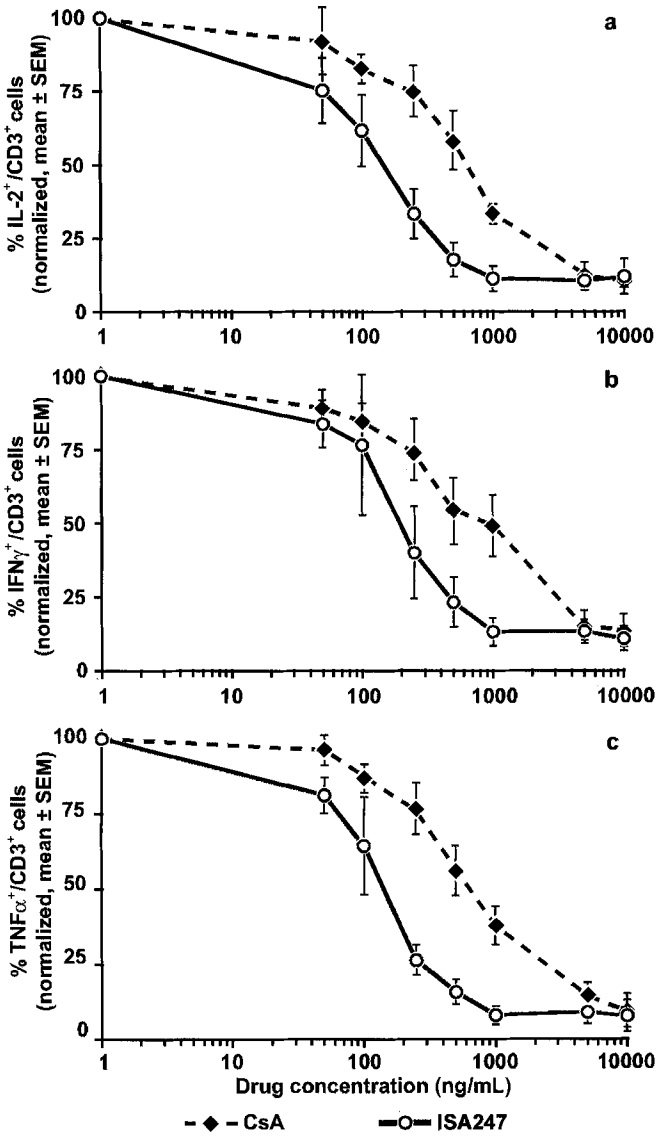


**Fig. 1a–c** Inhibition of intracellular cytokine production by ISA247 and cyclosporine. Production of cytokines IL-2 (**a**), IFN-γ(**b**) and TNF-α (**c**) is shown as normalized to values (100%) after mitogen stimulation without drug. Values are means ± SEM. Drug concentrations are represented on a logarithmic scale

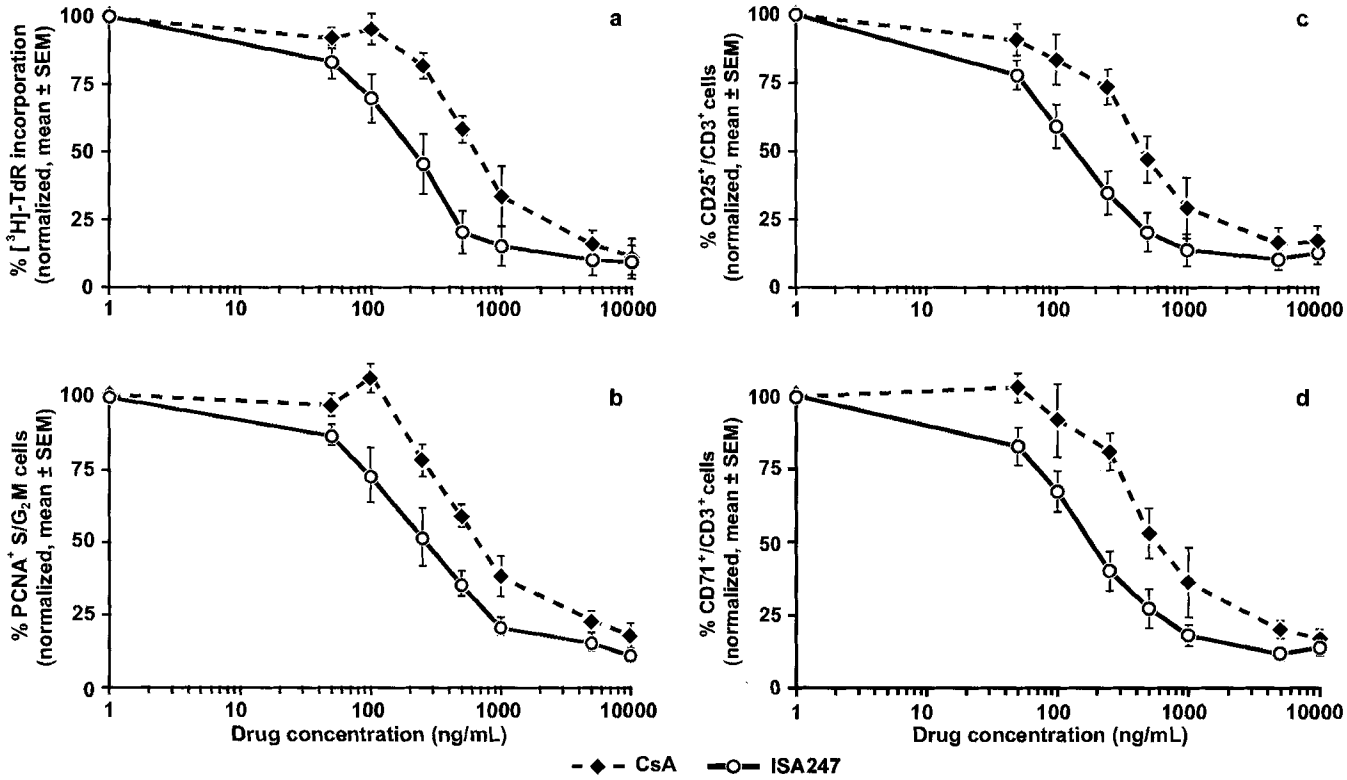


**Fig. 2a–d** Inhibition of lymphocyte proliferation and T cell surface antigens by ISA247 and cyclosporine. **a** Lymphocyte proliferation assessed by [$^3$H]-TdR incorporation assay; **b** flow-cytometric analysis of the percentage of S/$G_2$M phase cells positive for proliferating cell nuclear antigen (PCNA); **c,d** expression of activation antigens IL-2 receptor α chain—CD25 (**c**) and transferrin receptor—CD71 (**d**). Lymphocyte proliferation and T cell surface antigen expression are shown as normalized to values (100%) after mitogen stimulation without drug. Values are means ± SEM. Drug concentrations are represented on a logarithmic scale

philin complexes are active and inhibit calcineurin [16, 17, 18, 19]. ISA247 may form active drug–immunophilin complexes with more or different types of cyclophilins than CsA, or it may simply have a higher affinity for calcineurin than CsA. Furthermore, protein–protein interactions may also play a role in intracellular partitioning of calcineurin and differential regulation of different subsets of calcineurin [15, 20]. The higher potency of ISA247 may thus be due to inhibition of either a larger number of intracellular subsets of calcineurin or of subsets of calcineurin that are more relevant for immune function. However, the design of our experiments does not allow us to determine why ISA247 exerts a more pronounced immunosuppressive effect than CsA.

*ISA247 is a more potent immunosuppressant than* CsA in vitro. Future studies will determine whether this novel drug is also more potent in vivo and whether its increased potency will also produce a wider therapeutic index than to that of the currently used calcineurin inhibitors.

**Table 1** Comparison of $EC_{50}$ of ISA247 and cyclosporine. Values are means ± SEM

| Immune function | $EC_{50}$ (ng/ml) | | *P* |
|---|---|---|---|
| | Cyclosporine | ISA 247 | |
| [$^3$H]-TdR incorporation assay | 766.03 ± 257.81 | 202.79 ± 59.44 | 0.043 |
| PCNA expression on S/$G_2$M cells | 494.96 ± 49.48 | 240.57 ± 73.04 | 0.043 |
| IL-2 production in CD3$^+$ cells | 537.29 ± 107.06 | 135.32 ± 30.43 | 0.043 |
| IFN-γ production in CD3$^+$ cells | 680.83 ± 198.50 | 177.11 ± 58.93 | 0.043 |
| TNF-α production in CD3$^+$ cells | 541.81 ± 117.82 | 138.56 ± 27.48 | 0.043 |
| CD71 expression on CD3$^+$ cells | 568.55 ± 228.10 | 169.75 ± 57.12 | 0.043 |
| CD25 expression on CD3$^+$ cells | 431.93 ± 132.91 | 137.66 ± 38.11 | 0.043 |
| CD11a expression on CD3$^+$ cells | 583.80 ± 86.10 | 266.58 ± 105.81 | 0.068 |
| CD95 expression on CD3$^+$ cells | 422.36 ± 67.88 | 190.98 ± 98.78 | 0.043 |
| CD154 expression on CD3$^+$ cells | 638.34 ± 201.56 | 248.15 ± 84.82 | 0.043 |

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