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The Potent Immunosuppressive Cyclosporin FR901459 Inhibits the Human P-Glycoprotein and Formyl Peptide Receptor Functions

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By sequestering cytosolic calcineurin into a molecular complex with cyclophilin and its consequent T-cell dysfunction, some cyclosporins, such as CsA and FR901459 ([Thr^2-Leu^5-Leu^{10}]-CsA), display potent immunosuppressive activity. Independently on this property, cyclosporins may display one or more other biological activities mediated by interaction with cell surface glycoproteins. Several cyclosporins inhibit the function of human *MDR1*-encoded P-glycoprotein (Pgp), a flippase known to cause cancer multidrug resistance, but also expressed by some normal immunocompetent cells and by normal epithelial cells which control drug bioavailability *in vivo*. CsA is known to be a potent Pgp inhibitor with a 3.2 μM IC_{50} in an assay where the most potent derivative SDZ PSC 833 gives a 0.49 μM IC_{50}. FR901459 is now shown to be a good Pgp inhibitor, being 2-fold weaker only (IC_{50} of 6 μM) than CsA. Some cyclosporins may also inhibit the function of the human FPR1-encoded formyl peptide receptor (FPR), a chemotactic receptor whose absence is known to impair antibacterial immunity. Yet this inhibition is very weak for all, but one of them, CsH, whose 0.15 μM IC_{50} makes it a much more potent FPR inhibitor than CsA (IC_{50} >10 μM in the same assay). FR901459 is now shown to be a very potent inhibitor of FPR function (IC_{50} of 0.6 μM). Since CsH shows little Pgp-inhibitory activity and has no known immunosuppressive activity, FR901459 displays a unique pharmacological profile: like CsA, it inhibits T-cell function; less than CsA, it can inhibit Pgp function on selected leukocyte subsets and on epithelial barriers known to control drug bioavailability; however, much more efficiently than CsA, it can inhibit the FPR function, a receptor involved in some leukocytic inflammatory responses to chemotactic peptides.

Cyclosporin A (CsA) is a neutral cyclic undecapeptide (cyclo-[MeBmt^1-Abu^2-Sar^3-MeLeu^4-Val^5-MeLeu^6-Ala^7-D-Ala^8-MeLeu^9-MeLeu^{10}-MeVal^{11}]) of fungal origin (*Tolypocladium inflatum* Gams)[1]. The sequestration of calcineurin by the cyclophilin-CsA complex which forms in the cytosol impairs T-cell proliferation, resulting in the large immunomodulatory (IM-) activity of CsA[2]. However, several other biological activities of CsA and other cyclosporin analogs (antifungal, antiparasitic or anti-inflammatory) cannot be interpreted by this unique intracellular [cyclophilin-calcineurin]-based mechanism[1]. As a matter of fact, CsA is a typical 'membrane-active' compound, displaying typically membrane-mediated biological activities. Thus, various cyclosporins, including CsA, could modulate the function of some members of the twelve transmembranous (12TM) ABC-transporter and of the seven transmembranous (7TM) G-protein-coupled receptor superfamilies[1,3], both of which may participate to the eventual immunosuppressive features shown by any given cyclosporin.

More specifically, CsA and some analogs are potent inhibitors of the human 12TM *MDR1* P-glycoprotein (Pgp), a membranous flippase with selectivity for some membrane phospholipids and xenobiotics, among which some of the most efficient cancer chemotherapeutics. By restricting anti-cancer drug uptake, the overexpression of *MDR1* Pgp in some tumor cells is known to be at the origin of their multidrug-resistant (MDR) phenotype and the failure of

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numerous anti-cancer therapies[1,3]. The mechanism of Pgp function impairement by cyclosporins is competitive inhibition, cyclosporins being slow substrates of the flippase, although other non-competitive mechanisms were also suggested[3,4].

The expression of cyclosporin IM-activity is not obviously related to the Pgp inhibitory potential since the most potent Pgp-inhibitory cyclosporin found so far (SDZ PSC 833, which is about 6-8-fold more potent than CsA) cannot sequester calcineurin on cyclophilin and is essentially devoid of IM-activity *per se*[3,4]. Yet, as the Pgp molecules transport the cyclosporin molecules[3,5], the Pgp molecules expressed by normal subsets of selected leukocytes and lymphocytes can decrease intracellular cyclosporin bioavailability[3,4,6~11], and therefore modulate its calcineurin sequestration potential. Moreover, the epithelial cells located at the absorbtion and clearance borders show a strong and polarized expression of Pgp molecules and they critically control *in vivo* features of cyclosporin pharmacokinetics; the bioavailability of various cyclosporins thus depends on their capacity to inhibit Pgp function[4,12].

With regards to the 7TM G-protein-coupled receptors, cyclosporins interfere with the binding of formylpeptides to the human *FPR1*-encoded 7TM-receptor for chemotactic *N*-formylated peptides of bacterial origins (FPR)[1,13~15]. Whether this reflects simple antagonism was recently challenged, and the current opinion is that it could be achieved by inverse agonism, the cyclosporins recruiting the FPR molecules into an inactive conformation[16]. However, only CsH, a [D-MeVal^{11}]-CsA analog which lacks IM-activity as it does not complex calcineurin to cyclophilin, was found to be a highly active and selective inhibitor of FPR functions triggered by formylpeptides (calcium transients, oxydative burst, chemotaxis, degranulation,...)[1,13~16]. In contrast, all other tested immunosuppressive cyclosporins such as CsA, CsB, CsC, CsD, CsE, and CsG were only weakly, if at all active as very large molarities were required to show only marginal inhibition of FPR responses[1,13~16]. An inflammatory response of leukocytes towards chemotactic peptides of bacterial origin plays a role in immune responses since mice with a disrupted FPR receptor gene display an impaired antibacterial immunity[17].

Until recently, CsH was thus the only obviously active cyclosporin for the inhibition of FPR function, being at least 100-fold more potent than CsA; but for Pgp inhibition, CsH was about 5-fold less potent than CsA. In contrast, SDZ PSC 833 (6-fold more potent than CsA for Pgp inhibition) lacked detectable FPR inhibitory capacity[1,4,18,19]. Thus, CsA, CsH and SDZ PSC 833 displayed largely different combinations of biological activities. These cyclosporins should display different effects for immunocompetent cells which display the Pgp flippase and the FPR chemotactic receptor. However, CsH and SDZ PSC 833 lacked obvious IM-activity when compared to CsA[1,4,18,19].

Here, we report the Pgp and FPR inhibitory activities of FR901459, a naturally occurring, highly immunosuppressive cyclosporin, which was isolated from the fermentation broth of *Stachybotrys chartarum* No. 19392[20]. This cyclo-[MeBmt^1-Thr^2-Sar^3-MeLeu^4-Leu^5-MeLeu^6-Ala^7-D-Ala^8-MeLeu^9-Leu^{10}-MeVal^{11}] which differs from CsA at the 2nd, 5th and 10th amino acid positions, shows a slightly lower Pgp inhibitory activity but a much enhanced FPR inhibitory activity, suggesting the potential expression of a distinct pharmacological profile.

Materials and Methods

Cell Lines

Human T-cell leukemia[18] either normally drug sensitive (Par-CEM [CEM 1.3], parental sensitive, Pgp-lacking cells) or showing pleiotropic resistance to anti-cancer drugs (MDR-CEM [CEM$^{VBL-100}$], highly resistant Pgp-expressing cells) were as described[1]. Pro-myelocytic human leukemia HL-60 cells[19] show little expression of membranous or functional markers of the granulocytic or monocytic lineages; they were differentiated towards the neutrophil lineage by growth for 48 hours in presence of 0.2 mM N^6,2′-*O*-dibutyryl-cAMP [Bt_2-cAMP] (neutrophilic HL-60 cells) as described[19].

Chemicals

The formylated peptide fMLF (formyl-Met-Leu-Phe, Sigma, St. Louis, MO, USA) was dissolved in DMSO and kept as stock solution at 1 mg/ml. For the assay, fMLF was used at a final concentration of 30 nM. Cytochalasin B (Calbiochem, San Diego, CA, USA) was prepared as stock solution at 5 mg/ml in DMSO; for the assay, it was used at 5 μM in the cell-containing medium. Paranitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) was prepared just before use at 10 mM in citrate buffer (125 mM, pH 4.6). Calcein-AM (Molecular Probes Europe, Leiden, The Netherlands) was kept as a 1 mg/ml stock solution ($\cong$1 mM) in DMSO; for the assay, it was used at a final concentration of 0.25 μM. The cyclosporins, CsA (a gift from Novartis, Basel, Switzerland) and FR901459 (a gift from Fujisawa, Osaka, Japan), were dissolved in DMSO as 10 mM stock

solutions, and diluted to obtain a range of (3 and 10 stepwise) concentrations in DMSO; for the assays, they were further diluted in the appropriate buffer just prior to the test to have a final 0.125% and 1% DMSO (for the FPR and Pgp inhibitory activities respectively).

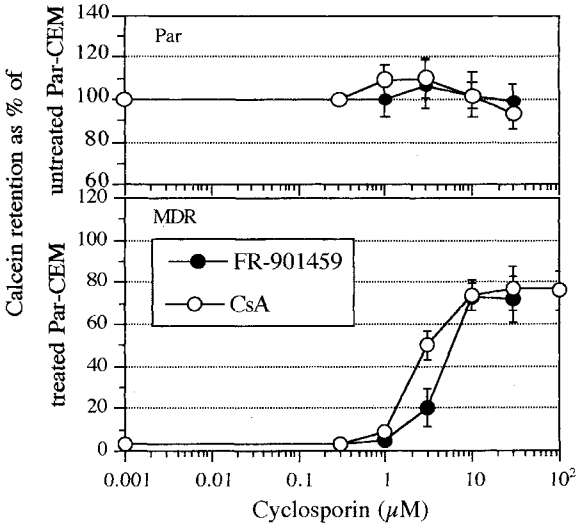
Assay for P-Glycoprotein (Pgp) Inhibitory Activity

The assays for Pgp activity and inhibition were performed by use of the human T-cell leukemia CEM cells and the standard calcein-AM efflux method reported in details earlier[18]. Briefly, highly resistant, *MDR1* Pgp-overexpressing MDR-CEM cells were first exposed to the Pgp-modulator for 15 minutes at 37°C; calcein-AM (0.25 μM final) was then added and the cells were kept at 37°C for further 15 minutes. After three washes by centrifugation, flicking and cell resuspension in the medium, inhibition of Pgp function in Pgp-expressing MDR-CEM cells was measured as calcein specific fluorescence and expressed as percentage of the calcein retention in Pgp-lacking parental CEM cells. Dose-response correlations were built with the cyclosporin concentrations on the X-axes (log scales) and the percentage of calcein retention in MDR-cells on the Y-axes (arithmetic scales). The Pgp-modulator IC_{50} values were defined as the concentrations (μM) which, in MDR-CEM cells, restored 50% of the calcein retention shown by similarly treated parental CEM cells. These IC_{50} values±standard deviations were calculated from IC_{50} measured in four independent experiments (each with duplicated microculture wells). For the compounds tested in the present paper, calcein retention by the parental CEM cells was not affected even by the highest tested compound concentrations.

Assay for Formyl Peptide Receptor (FPR) Inhibitory Activity

The FPR activity and its inhibition were assayed by ligand-induced granule enzyme release using neutrophilic HL-60 cells as reported earlier[19]. Briefly, the release of *N*-acetyl-β-D-glucosaminidase was measured upon stimulation of the neutrophilic HL-60 cells with fMLF in the presence of ranges of cyclosporin concentrations. The released enzyme was collected by filtration, using the MultiScreen® system (Millipore, Molsheim, France). Dose-response correlations were built with the cyclosporin concentrations on the X-axes (log scales) and the absorbance expressed as percentage of the normal response obtained with 30 nM fMLF on the Y-axes (arithmetic scales). To calculate those percentages, the spontaneous release (absolute absorbance in the absence of fMLF) was subtracted from all values, after checking it was not affected by the highest cyclosporin concentration. The IC_{50} values were measured on six to eight independent experiments (each with duplicated microculture wells) and they were expressed as mean±standard deviation.

Fig. 1. Comparison of CsA and FR901459 for their capacity to inhibit the human *MDR1* Pgp function.



The figure shows the cyclosporin dose-dependence of the restoration of calcein retention in Par- and MDR-CEM cells. The X-axis shows the cyclosporin concentrations as μM and the Y-axis shows the level of calcein-specific fluorescence which is expressed, in the case of Par-CEM cells (top part), as percentage of the untreated Par-CEM cell one, and in the case of MDR-CEM cells (bottom part), as percentage of similarly treated Par-CEM cell one. The data are shown as means±standard deviation from four independent experiments.

Results

Comparative Inhibition of Pgp Function by CsA and FR901459

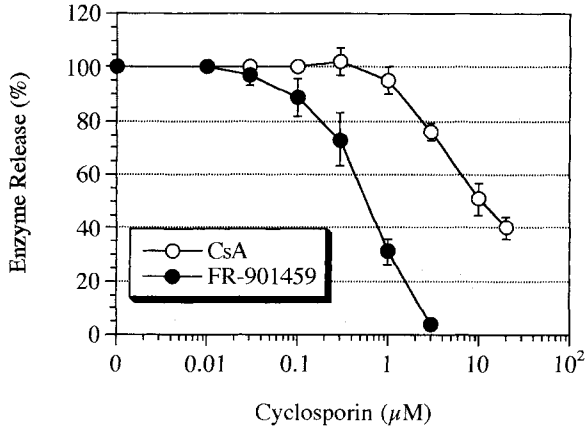
The inhibition of Pgp function is measured by the cyclosporin dose-dependent inhibition of cellular uptake of the Pgp probe calcein-AM by Pgp-expressing cells, using Pgp-lacking cells as controls. The interference with the efflux of calcein-AM which is entering the plasma membrane, reduces the amount of free calcein in the cytosol, the cellular fluorescence being the quantitative read-out[18]. CsA and FR901459 were thus compared for their capacity to inhibit the human *MDR1* Pgp function of

MDR-CEM cells, using Pgp-lacking Par-CEM cells as controls, with the resulting mean (±SD, n=4) dose-response curves (Fig. 1).

As earlier shown for CsA[18], FR901459 did not affect unspecifically the cellular uptake of the non-fluorescent Pgp-substrate calcein-AM and the level of the specific fluorescence signal of free calcein in the cytosol (control assays with Pgp-lacking Par-CEM cells). With the Pgp-expressing MDR-CEM cells, FR901459 (IC_{50}= 6.0±0.8 μM) was nearly two-fold less potent than CsA (IC_{50}=3.2±0.9 μM) for Pgp inhibition.

In comparison to FR901459, using the same [calcein-AM assay/MDR-CEM cell] assay system to measure Pgp inhibition, early characterized MDR-reversing agents such as verapamil, quinidine and others show IC_{50} of 20~30 μM and higher[3,18]. Thus, like CsA, FR901459 is a cyclosporin which is definitely endowed with a substantial Pgp inhibitory capacity. FR901459 (IC_{50}=6.0 μM) is actually equipotent to CsC, a [Thr^2]-CsA (IC_{50}=5.9 μM, unpublished). The most potent Pgp inhibitory cyclosporin SDZ PSC 833 (a [3′-keto-$MeBmt^1$-Val^2]-CsA) was 12-fold more potent (IC_{50}=0.49 μM)[18], whereas the most potent FPR inhibitory cyclosporin (CsH, a [D-$MeVal^{11}$]-CsA analog) was 2.5-fold less potent (IC_{50}=15.5 μM, unpublished).

Fig. 2. Comparison of CsA and FR901459 for their capacity to inhibit the [FPR-fMLF] interaction-induced response.



The figure shows the cyclosporin dose-dependence of the 30 nM fMLF-induced release of *N*-acetyl-β-D-glucosaminidase by neutrophilic HL-60 cells. The data are expressed as percentages (Y-axis) of the enzyme release responses as a function of the CsA or FR901459 cyclosporin concentrations (μM, X-axis). The data are shown as means±standard deviation from six (CsA) and eight (FR901459) independent experiments.

Comparative Inhibition of FPR Function by CsA and FR901459

The inhibition of FPR function is measured by the cyclosporin dose-dependent inhibition of granule enzyme release by fMLF-activated FPR-expressing cells, using untreated cells as controls. With FPR-expressing neutrophilic HL-60 cells, 30 nM fMLF regularly induces a substantial activation reflected by a large enzyme release[19]. The interference with the fMLF-induced enzyme release is quantitatively measured by use of adequate substrates[19].

Cyclosporins do not by themselves induce such enzyme release[19] and this was extended to FR901459 (not shown). CsA and FR901459 were thus compared for their capacity to inhibit the human FPR function of neutrophilic HL-60 cells. As shown here using the release of *N*-acetyl-β-D-glucosaminidase, CsA could detectably but not completely inhibit this fMLF-induced process, confirming earlier data[19], whereas FR901459 was a very potent inhibitor capable to fully inhibit the response (Fig. 2).

The comparison of the dose-response curves obtained with the reference CsA (n=6) and FR901459 (n=8) shows that a complete inhibition of FPR function could be achieved with FR901459 at 3 μM, whereas no complete inhibition was achieved with CsA even at 20 μM. A comparison of the mean IC_{50} values obtained with CsA (IC_{50}=11.1±3.3 μM), and with FR901459 (IC_{50}=0.6± 0.1 μM) clearly shows the higher potency of FR901459 for this inhibitory function.

In comparison with FR901459, using the same assay to measure FPR inhibition, the most potent FPR inhibitory cyclosporin CsH is 4-fold more potent (IC_{50}=0.15 μM)[19] whereas the most potent Pgp inhibitory cyclosporin SDZ PSC 833 gave no detectable inhibition (IC_{20}≫10 μM, not shown). FPR inhibition by CsC ([Thr^2]-CsA) was also weak (IC_{50}>10 μM). The potency of CsA for FPR inhibition was similar to the potency of *N-t*-BOC-Nle-Leu-Phe (the classically used fMLF antagonist) using the present[19] or other assays[13~15]. Both CsH and FR901459 are thus exceptionally potent FPR inhibitors.

Discussion

The capacity of cyclosporins to bind to cyclophilins and to sequester calcineurin[2] is unrelated to their capacity to give a functional inhibition of either the *MDR1* Pgp efflux

pump (a twelve transmembranous ABC-transporter/flippase responsible for a multiple drug resistance phenotype of some cancer cells) or the FPR function (a seven transmembranous G-protein coupled receptor for formylated peptides of bacterial and mitochondrial origins). Indeed, considering two reference cyclosporin analogs devoid of IM-activity, the most potent Pgp inhibitor SDZ PSC 833 (a chemical CsD-derivative with an IC_{50} of about 0.5 μM)[18] shows no detectable FPR inhibition; conversely, the most potent FPR inhibitor CsH (a natural analog with an IC_{50} of 0.15 μM)[19] is a weak Pgp function inhibitor.

The naturally occurring cyclosporin FR901459 displays a large IM-activity, reported to be about a third the one shown by CsA both *in vitro* (principally, mixed lymphocyte reaction assays) and *in vivo* (principally, delayed type hypersensitivity and skin allograft survival assays)[20]. The 3-fold lower IM-activity of FR901459 could simply come from a lower efficacy of its binding to cyclophilin and/or of the sequestration of calcineurin by the complex, but such data were not reported. However, these immune responses involve complex reaction mechanisms which may be eventually dependent on the level of calcineurin sequestration by cyclophilin-cyclosporin complexes in the cytosol of selected T-cell subsets[1,2], but which are obviously dependent on other features of the cyclosporin molecule: its pharmacokinetics (*i.e.* its bioavailability in the plasma which is controled by Pgp activity at the absorbtion and clearance borders)[1,3~6,12], its intracellular uptake (*i.e.* its capacity to reach the cytosolic cyclophilin which is controled by Pgp activity at the cell membrane level)[3,4,6~11], and its interference with the recruitment of leukocytes and lymphocytes to the sites of these immune response assays (*i.e.* its capacity to inhibit cell chemotaxis involving chemotactic receptors of the FPR type)[1,13~17].

In comparison with CsA, FR901459 was now shown to display a much larger capacity to inhibit FPR function while maintaining a substantial capacity to inhibit Pgp function. How this might be related to its roughly 3-fold lower IM-activity will be discussed below.

The expression of functional Pgp molecules on cells at the drug absorption and clearance borders is known to control the pharmacokinetics of a variety of drugs including many immunosuppressants. In particular, cyclosporins are slow substrates and competitive inhibitors of the Pgp molecules[3,4,5,8~12]. In view of the distribution of Pgp molecules and of their polarization on the concerned epithelial borders, the 2-fold lower Pgp inhibitory potency of FR901459 may lead to its lower absorption at the gut level and its larger clearance at the liver level in comparison to CsA, thus to a lower plasma bioavailability. Yet, the latter cannot show a straightforward correlation with Pgp function, since additional factors, principally the susceptibility of different cyclosporins to CYP3A-mediated metabolism[21], play a role in their pharmacokinetics. A lower exposure to FR901459 and its easier clearance may account for its better tolerability (using dose-dependent renal calcification as toxicity index)[20].

The expression of functional Pgp molecules on some subsets of leukocytes and lymphocytes may also alter cyclosporin pharmacodynamics: indeed, there is a differential Pgp expression by different subsets of immunocompetent cells, and the Pgp molecules can restrict their uptake of cyclosporin[3,6~11]. Therefore, uptake of FR901459 by Pgp-expressing immunocompetent cells is lower or slower than their uptake of CsA, and this lower intracellular bioavailability may result in a less efficient cyclophilin-mediated sequestration of calcineurin.

The expression of functional FPR molecules on some subsets of leukocytes and lymphocytes may also alter cyclosporin pharmacodynamics: ligand binding to the FPR triggers chemotactic responses by a variety of cells[22], and mice with a disrupted FPR receptor gene display an impaired antibacterial immunity[17]. The FPR function may not be restricted to the recruitment of leukocytes to sites of bacterial infections and it could also be involved in cell chemotaxis towards other chemoattractants (*e.g.* mitochondrial peptides[22]). No immunological, anti-inflammatory or other biological activity of CsH, which would be obviously related to its capacity to inhibit FPR function, was observed in rodent models *in vivo*[1]. However, the species-selectivity displayed by low molecular weight antagonists of 7TM G protein-coupled receptors[23] can account for the observed lack of effects since cells expressing the rodent homolog of the human FPR were little responsive to the formylpeptides used for human cells studies[22] and since no inhibition by CsH could be shown with murine cells (unpublished data).

For the functional inhibition of the human FPR receptor, FR901459 was nearly 20-fold more potent than CsA, and only 4-fold less potent than reported for CsH[19]. Since CsH and FR901459 differ from each other by four out of eleven residues (Abu^2/Thr^2, Val^5/Leu^5, MeLeu^{10}/Leu^{10}, and D-MeVal^{11}/L-MeVal^{11}), whether they act through the same mechanism is a challenging question. It is obvious that three widely different conformations are achieved in solution by CsH[24], by FR901459[20], and by the classical cyclosporins such as CsA and CsC[25], suggesting that the first two may act through different FPR pharmacophores whereas the others cannot bind efficiently to any of them.

FR901459 and CsC (=[Thr^2]-CsA) showed the same Pgp

inhibition IC_{50} of about 6.0 μM, but largely different FPR inhibition IC_{50} (0.6 μM for FR901459 *versus* >10 μM for CsC). Thus with regards to pharmacodynamics, FR901459 and CsC must show a similar Pgp-dependence (same uptake by Pgp-expressing immunocompetent cells) but may express widely different anti-inflammatory effects (chemotaxis of FPR-expressing immunocompetent cells). With regards to pharmacokinetics, the bioavailability of these two cyclosporins should show the same Pgp-mediated control, but FR901459 (=[Val^5 Leu^{10}]-CsC) and CsC (=[Val^5 $MeLeu^{10}$]-FR901459) may actually display different pharmacokinetics since their two residue difference may confer them different susceptibility to CYP3A-mediated metabolism[1,21].

In conclusion, until the present disclosure of FR901459 activity for FPR inhibition, CsH was the only obviously active cyclosporin reported, other cyclosporins such as CsA or CsC giving little or no inhibition of the chemotactic receptor function[13~15,19]. The consequences of the FPR inhibitory and Pgp-inhibitory capacities of these different cyclosporins on their *in vivo* pharmacokinetics and pharmacodynamics have been discussed. These differences may confer FR901459 an IM-activity which would be not only quantitatively, but rather qualitatively different from the one displayed by CsA, for the modulation of some immune responses.

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