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Sanglifehrin A, a Novel Cyclophilin-Binding Compound Showing Immunosuppressive Activity with a New Mechanism of Action

Gerhard Zenke,1* Ulrike Strittmatter,* Serge Fuchs,* Valerie F. J. Quesniaux,* Volker Brinkmann,* Walter Schuler,* Mauro Zurini,† Albert Enz,‡ Andreas Billich,§ Jean-Jacques Sanglier,¶ and Theo Fehr†

We report here on the characterization of the novel immunosuppressant Sanglifehrin A (SFA). SFA is a representative of a class of macrodiles produced by actinomycetes that bind to cyclophilin A (CypA), the binding protein of the fungal cyclic peptide cyclosporin A (CsA). SFA interacts with high affinity with the CsA binding side of CypA and inhibits its peptidyl-prolyl isomerase activity. The mode of action of SFA is different from known immunosuppressive drugs. It has no effect on the phosphatase activity of calcineurin, the target of the immunosuppressants CsA and FK506 when complexed to their binding proteins CypA and FK binding protein, respectively. Moreover, its effects are independent of binding of cyclophilin. SFA inhibits alloantigen-stimulated T cell proliferation but acts at a later stage than CsA and FK506. In contrast to these drugs, SFA does not affect IL-2 transcription or secretion. However, it blocks IL-2-dependent proliferation and cytokine production of T cells, in this respect resembling rapamycin. SFA inhibits the proliferation of mitogen-activated B cells, but, unlike rapamycin, it has no effect on CD154/IL-4-induced Ab synthesis. The activity of SFA is also different from that of other known late-acting immunosuppressants, e.g., mycophenolate mofetil or brequinar, as it does not affect de novo purine and pyrimidine biosynthesis. In summary, we have identified a novel immunosuppressant, which represents, in addition to CsA, FK506 and rapamycin, a fourth class of immunophilin-binding metabolites with a new, yet undefined mechanism of action. The Journal of Immunology, 2001, 166: 7165–7171.

The immunosuppressive drugs cyclosporin A (CsA)2, FK506, and rapamycin exert their effects by forming complexes with intracellular binding proteins (immunophilins) (1), which inhibit effector molecules involved in intracellular signal transduction (2–4). FK506 and rapamycin, despite binding to the same immunophilin, i.e., the FK506 binding protein (FKBP) (5, 6), inhibit two different effector molecules. The FK506/FKBP complex blocks the serine-threonine phosphatase calcineurin (7–9), whereas the rapamycin/FKBP complex inhibits the FKBP-rapamycin-associated kinase called mammalian target of rapamycin (mTOR) (10–14). As a consequence, FK506 and rapamycin interfere with T cell activation at different stages of the cell cycle (15, 16), and thus have different activity profiles. FK506 prevents T cell activation at the $G_0$-$G_1$ phase transition by selectively blocking transcriptional activation of early T cell-specific genes (17), resulting in the inhibition of the production of T cell growth factors like IL-2. In contrast, rapamycin acts at a later stage of the cell cycle, namely at the transition from $G_1$ to $S$ phase, thus inhibiting the proliferation of cells in response to growth factors (18–21). CsA forms a complex with the immunophilin cyclophilin (22), which like the FK506/FKBP complex, inhibits calcineurin (7, 23) and thus also selectively prevents transcription of early T cell activation genes (24).

Taking into account the different ligands for FKBP, which have different biological effects, we asked whether ligands for cyclophilin other than the fungal secondary metabolite CsA might exist. Those ligands might have different biological activities as well. Screening of microbial broth extracts for metabolites blocking the CsA-cyclophilin A (CypA) molecular interaction led to the discovery of the actinomycetes strain Streptomyces A92-308110 (25), which produces a class of novel macrocyclic compounds, named sanglifehrins (26). Sanglifehrins exhibit a high affinity for cyclophilins and show activity in the MLR (25). Here, we report a detailed analysis of the biological activity of sanglifehrin A (SFA), a representative of this novel class of immunosuppressants (see Fig. 1), and present studies to elucidate its mechanism of action.

Materials and Methods

Compounds

CsA, N-methyl-Val-4 cyclosporin (4-Cs), SFA, FK506, rapamycin, mycophenolic acid (MPA), mycophenolate mofetil (MMF), leflunomide, and brequinar were produced at Novartis Pharma (Basel, Switzerland). Dexamethasone and azathioprine were obtained from Sigma (St. Louis, MO). Diseasemodifier and azathioprine were obtained from Sigma (St. Louis, MO). The compounds were dissolved in DMSO at 10–2 to 10–3 M and stored at −20°C. Samples were diluted on the day of the experiment in assay buffer or medium. $IC_{50}$ values were determined from seven 2- to 3-fold dilution steps in duplicate.

Cyclophilin and FKBP binding assays

Binding of compounds to CypA and FKBP12 was assessed in competitive ELISA formats, which are based on the interaction of solid-phase coupled CsA-BSA, SFA-BSA, or FK506-BSA conjugates and biotinylated CypA or biotinylated FKBP12, respectively. The CsA-CypA ELISA and the...
FKS06-FKBPI2 ELISA have been described previously (27, 28). For the SFA-CypA ELISA, the SFA-BSA conjugate was prepared as follows: A SFA derivative bearing in position 53 (see Fig. 1) a 18-membered linker terminated by an activated succinimide ester was prepared in two steps. First, the C5 ketone of SFA was reductively aminated with adipic acid dihydrazide in the presence of sodium cyanoborohydride. The product of this reaction was then coupled with disuccinimidyl suberate. The resulting SFA-derived coupling agent was dissolved in dimethylformamide (3 mg/ml) and 300 µl were added to 6 mg of BSA dissolved in phosphate buffer (50 mM, 2.7 mM) at pH 7.5, incubated for 24 h at 4°C, and used without further purification. The SFA-BSA conjugate (0.5 µg/ml in PBS) was coated to ELISA plates (100 µl per well) overnight at 4°C. All subsequent steps were performed as described (27). In competition experiments appropriate serial dilutions of test compounds were added together with CypA-biotin.

CypA isomerase assay

SFA was tested for its effects on the peptidyl prolyl cis-trans isomerase activity of CypA according to Kofron et al. (29). SFA was added to a 25-nM solution of CypA in 50 mM HEPES, 100 mM NaCl, pH 8.0 (875 µL), in a photometer cuvette (held at 10°C) followed by 50 µl of α-chymotrypsin (Serva, Heidelberg, Germany) (10 mg/ml in 1 mM HCl) and 25 µl 4 M N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (Bachem, Bubendorf, Switzerland) in trifluoroethanol/470 mM lithium chloride. The increase in absorbance at 390 nm was recorded with a Cary 1E spectrophotometer (Varian, Mulgrave, Australia). Data points were fitted to a first-order rate law to obtain the reaction rate.

Inosine monophosphate dehydrogenase (IMPDH) assay

IMPDH was purified from bovine thymus essentially as described by Jackson et al. (30). The enzyme activity was measured spectrophotometrically at 290 nm by following NADH-dependent formation of xanthosine monophosphate from inosine monophosphate at 37°C according to the procedure described by Magasanik (31).

Dihydro-rotate dehydrogenase (DDHODH) assay

A truncated form of human DHODH (32) was expressed in Escherichia coli and partially purified. Enzyme activity was measured spectrophotometrically at room temperature by following the reduction of 2,6-dichlorophenolindophenol essentially as described (33).

Calcineurin phosphatase assay

The calcineurin phosphatase activity was determined by HPLC assay previously described in detail by monitoring the dephosphorylation of a 19-aa phosphopeptide, a partial sequence of the regulatory subunit of cyclic adenosine 3′,5′-monophosphate-dependent protein kinase (34).

Cyclophilin binding in whole cells

Cellular uptake and binding to cytosolic binding proteins was assessed by competition with [3H]CsA as described (35).

IL-2 reporter gene assay

The assay was performed as described (35) with the exception that the β-galactosidase reporter gene was replaced by the luciferase gene (36). Briefly, Jurkat transfected with a human IL-2 promoter/luciferase reporter gene construct were stimulated with 20 ng/ml PMA and 1 µg/ml PHA in the presence of either SFA and CsA alone or a combination of both compounds and incubated in RPMI 1640/10% FCS for 5 h at 37°C in 5% CO2.

IL-2 production by Jurkat cells

Jurkat cells were stimulated with PMA and PHA as described for the IL-2 reporter gene assay. After 18 h, the IL-2 content in the supernatant was determined with the IL-2-dependent murine T cell line CTLL (40).

Cytokine production by human effector T cells

Human CD4+45RO+ T cells were separated by FACS from PBMC stained with FITC-labeled anti-CD4 mAb plus PE-labeled anti-CD45RO mAb (Becton Dickinson, Basel, Switzerland) to a purity of >98% as described (41). CD4+45RO+ cells were primed (105 cells/ml) for 7 days, washed, and restimulated (2 × 107/ml) for 3 days by plate-bound anti-CD3 mAb OKT3 (25 µg/ml coated) in the presence of IL-2 (100 U/ml). Supernatants of restimulated cells were harvested and analyzed for IL-4 and IFN-γ by ELISA as described (41).

LPS-splined spleen cell proliferation

Spleen cells of CBA mice (2 × 106 cells per well) in 200 µl RPMI 1640/10% FCS medium were stimulated with 50 µg/ml LPS (LPS from E. coli, serotype 011:B4; Difco, Detroit, MI) for 2 days (42). Proliferation was determined by incubation with 1 µCi [3H]thymidine (15 Ci/mmol; Amer sham, Little Chalfont, U.K.) for 5 h.

Ab production by human B cells

Human CD19+ B cells were isolated to a purity of >95% by magnetic cell sorting from PBMC as described previously (43). B cells were stimulated in triplicate for 10 days by soluble CD154 (20% supernatant) and IL-4 (100 U/ml) (43). Culture supernatants were analyzed for IgG by ELISA (44).

TNF-α release from human PBMC

Mononuclear cells were isolated from human blood by Ficoll-Hypaque density separation and incubated with serial dilutions of compounds for 30 min before addition of IFN-γ (100 U/ml; Boehringer Mannheim, Mannheim, Germany) and LPS (5 µg/ml; LPS from E. coli, serotype 026:B6; Sigma). After a 3-h incubation in RPMI 1640/10% FCS, TNF-α present in the cell culture supernatant was determined with a commercially available ELISA kit (Innogenetics N.V., Zwijndrecht, Belgium).

Proliferation of mouse bone marrow cells

Bone marrow cells from CBA mice (2.5 × 107 cells per well) were incubated for 4 days in 100 µl RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Basel, Switzerland), 50 µM 2-ME (Fukusa, Buchs, Switzerland), WEHI-3-conditioned medium (7.5% v/v), and L929-conditioned medium (3% v/v) as a source of growth factors. Proliferation was assessed by [3H]thymidine incorporation as described above.

Nucleoside reversal experiments

A murine MLR was performed as described above with the exception that proliferation was assessed by MTT (45). Serial dilutions of brequinar and SFA were performed either in the absence of the presence of 50 µM uridine.

Human T cells were purified from buffy coats of normal donors by density centrifugation and negative selection with anti-HELA-DR-, anti-CD14-, anti-CD16-, and anti-CD19-coated magnetic beads (Miltenyi Biotech Gmbh, Bergisch-Gladbach, Germany) according to instructions of the manufacturer. Purified T cells (5 × 104) were stimulated with 1.25 µg/ml PHA for 3 days. Serial dilutions of MMF and SFA were performed in the presence or absence of 50 µM guanosine. Proliferation was assessed by [3H]thymidine incorporation.

Results

SFA binds to CypA and inhibits its isomerase activity

Binding of SFA to CypA was assessed in two different competitive ELISA formats in which the interaction between solid-phase
coated CsA-BSA or SFA-BSA conjugates and biotinylated CypA is inhibited by SFA free in solution. In both assays, free SFA inhibited these interactions at significantly lower concentrations than CsA (Table I), indicating a higher affinity of SFA for CypA. CsA has been shown to inhibit the peptidyl-prolyl cis-trans isomerase activity of CypA (46, 47). We thus tested SFA for inhibition of the isomerase activity of CypA in a cell-free assay; again SFA was more potent than CsA (Table I). In contrast, when the binding to cyclophilin was assessed in whole cells, the concentration of SFA needed to displace cell-associated radioactive CsA was ~3-fold higher than that of CsA (Table II). This suggests that the permeability of SFA into cells is lower compared with that of CsA.

SFA inhibits proliferation of activated T cells

We next assessed the effect of SFA on T cells. SFA inhibited the proliferation of alloantigen-stimulated murine and human T cells in MLR cultures with IC50 values of 95–170 nM (Table III). CsA was 15- to 35-fold more potent in these assays. SFA also suppressed the proliferation of a human T cell clone stimulated with IL-2 (Table I). In contrast, CsA had no effect on the IL-2-dependent proliferation. Rapamycin was active in this assay with an IC50 value comparable to that reported for the Ag-specific proliferation of this T cell clone (data not shown; Ref. 28).

Different effects of SFA on cytokine production of T cells

SFA did not affect IL-2 secretion by PMA/PHA-stimulated Jurkat cells, whereas CsA was active at low nM concentrations (Table IV). In line with these results, SFA did not inhibit PMA/PHA-induced IL-2 transcription as assessed with an IL-2 reporter gene assay with Jurkat cells (Fig. 2). In contrast, low nM concentrations of CsA completely prevented IL-2 transcription. SFA dose dependently abrogated this inhibitory effect of CsA (Fig. 2), indicating that both compounds compete for the same binding side on cyclophilin but have different effector mechanisms. To assess the effects on cytokine production by nontransformed human T cells, CD4+45RO+ T cells were primed and restimulated in vitro with anti-CD3 and IL-2. The production of both Th1 and Th2 cytokines (IFN-γ and IL-4, respectively) by these effector T cells was inhibited by SFA and CsA with similar potency (IC50: 55–310 nM; Table IV). Taken together, these results show that SFA does not affect IL-2 production but rather inhibited IL-2-dependent proliferation and IL-2-dependent cytokine production of human T cells.

SFA has no effects on targets of known immunosuppressants

To get further insight into the mechanism of action, the effects of SFA on targets of known immunosuppressants were studied. FKBP12 is the common intracellular binding protein of FK506 and rapamycin (5). SFA did not bind to FKBP12 as assessed with a FK506-FKBP12 binding assay (Table V). Furthermore, SFA did not affect the phosphatase activity of calcineurin (Table V), the effector molecule of CsA and FK506 (7–9, 23). The immunosuppressants MPA/MMF (49) and brequinar/leflunomide (50, 51) affect de novo purine and pyrimidine biosynthesis by inhibition of their respective targets IMPDH (52) and DHODH (53, 54). SFA had no effect on the enzymatic activities of these enzymes (Table V). Furthermore, although the inhibitory effects of brequinar on the proliferation of murine MLR cultures and of MMF on PHA-stimulated human T cells were fully abrogated by addition of uridine and guanosine, respectively, these nucleosides had no effect on the activity of SFA (Fig. 4). Also, all other nucleosides did not affect the activity of SFA (data not shown). Taken together, these data indicate that SFA has a different mechanism of action than the well

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**Table I.** Cell-free binding to CypA and inhibition of the CypA isomerase activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>CypA-Cyp ELISA</th>
<th>SFA-Cyp ELISA</th>
<th>CypA Isomerase Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>3.6 ± 1.2</td>
<td>6.9 ± 0.9</td>
<td>12.8</td>
</tr>
<tr>
<td>CsA</td>
<td>82 ± 16</td>
<td>420 ± 56</td>
<td>54.9</td>
</tr>
<tr>
<td>4-Cs</td>
<td>13 ± 1.5</td>
<td>42 ± 2.4</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

*The binding of SFA, CsA, and the nonimmunosuppressive 4-Cs to CypA was determined in two competitive ELISA formats (CypA-CypA and SFA-CypA) as described in Materials and Methods. The inhibition of the CypA isomerase activity was determined according to Kofron et al. (29).

The results are expressed as mean IC50 values of ELISA from three to four experiments. Isomerase results of one experiment are shown.

**Table II.** Binding to cyclophilin in whole cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean IC50 ± SD (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>14.9 ± 1.6</td>
</tr>
<tr>
<td>CsA</td>
<td>5.9 ± 1.4</td>
</tr>
</tbody>
</table>

*Jurkat cells were incubated with [3H]CsA in the presence of increasing molar excess of unlabelled SFA or CsA.

*Mean values of four experiments.

**Table III.** Inhibition of T cell proliferation

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nM)</th>
<th>Mean IC50 ± SD (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>170 ± 30</td>
<td>95 ± 40</td>
</tr>
<tr>
<td>CsA</td>
<td>11 ± 1.6</td>
<td>2.7 ± 1.4</td>
</tr>
</tbody>
</table>

*The results are expressed as mean IC50 values of ELISA from three to four experiments. Isomerase results of one experiment are shown.

**Table IV.** Different effects of SFA on cytokine production by T cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IL-2</th>
<th>IFN-γ</th>
<th>IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>&gt;10,000</td>
<td>310 ± 62</td>
<td>55 ± 11</td>
</tr>
<tr>
<td>CsA</td>
<td>7</td>
<td>230 ± 46</td>
<td>120 ± 24</td>
</tr>
</tbody>
</table>

*Jurkat cells were stimulated with PMA/PHA for 18 h, and the IL-2 content of the supernatant was determined using the IL-2-dependent murine T cell line CTLL.

*Human CD4+45RO+ cells were primed for 7 days and restimulated with anti-CD3 and IL-2. IL-4 and IFN-γ in the supernatant was determined by ELISA. Mean ± SD values of three experiments are shown.
characterized immunosuppressants CsA, FK506, MMF/MPA, brequinar, and leflunomide.

**SFA acts late in T cell activation**

Presently known immunosuppressants act at different time points during the process of T cell activation and proliferation. CsA and FK506 block early, TCR-mediated gene activation, thereby preventing cell cycle progression from G0 to G1 phase (17, 24). Rapamycin inhibits later stages, namely, the G1/S transition by blocking cell proliferation in response to growth factors (18–21). MMF and brequinar, inhibiting purine and pyrimidine synthesis, respectively, interfere with DNA synthesis and thus block the S phase of the cell cycle (55, 56). Effects on individual cell cycle phases can be analyzed by delayed addition of the compounds to MLR cultures, in which T cells pass through all the above mentioned phases within 96 h. CsA was no longer active in the MLR when added 48 h after initiation of the culture (Fig. 5). Its IC50 value was 110-fold higher at this time point compared with the IC50 value obtained when added immediately at the beginning of the culture (i.e., “normalized IC50” = 110). The effect of rapamycin decreased when added after 48 h (normalized IC50 = 50). Brequinar was fully active when added after 48 h and essentially inactive when added after 72 h. MMF showed full activity at all time points and was still active when added after 91 h just before assessment of proliferation by [3H]thymidine incorporation. SFA showed a profile different from all these compounds; it was still active when added 72 h after MLR initiation, but essentially inactive when added after 91 h (normalized IC50 = 130).

**Effects of SFA on B cells, monocytes, and bone marrow cells**

To further profile the in vitro activities, SFA was analyzed for its effects on B cells, monocytes, and bone marrow cells. SFA inhibited the proliferation of murine B cells stimulated with LPS with a potency comparable to that of MMF (Table VI). SFA affected the IgG production by purified human B cells stimulated with CD154 and IL-4 only at μM concentrations, which is very potently blocked by rapamycin (Table VI). In comparison to dexamethasone, SFA weakly affected TNF-α secretion by IFN-γ/LPS-stimulated PBMC. The proliferation of murine bone marrow cells in response to growth factors was only inhibited by SFA at μM concentrations, whereas the nucleoside analog azathioprine, whose main metabolite 6-mercaptopurine interferes with DNA synthesis (57), prevents proliferation of these cells at nM concentrations.

**Discussion**

Aside from their clinical application as immunosuppressants to prevent transplant rejection, CsA, FK506, and rapamycin have also been proven to be powerful experimental tools. These drugs have significantly contributed to our understanding of T cell signal transduction pathways and molecular events involved in T cell activation (58). A prerequisite for their activity is the binding to intracellular binding proteins (immunophilins). Although this binding is required it is not sufficient for immunosuppression: only further, tertiary complex formation with effector molecules leads to the biological effect. FK506 and rapamycin, although binding to the same immunophilin FKBP, exert their activities through binding to two different effector molecules, i.e., calcineurin and mTOR.

**Table V. No effect of SFA on targets of known immunosuppressants**

<table>
<thead>
<tr>
<th>Assay</th>
<th>IC50 (nM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKBP12-binding</td>
<td>&gt;10,000</td>
<td>Rapamycin: 0.6</td>
</tr>
<tr>
<td>Calcineurin phosphatase</td>
<td>&gt;3,000</td>
<td>CsA: 200</td>
</tr>
<tr>
<td>IMPDH</td>
<td>&gt;10,000</td>
<td>MPA: 25</td>
</tr>
<tr>
<td>DHODH</td>
<td>&gt;50,000</td>
<td>Leflunomide: 190</td>
</tr>
</tbody>
</table>

*FKBP12-binding was measured in a competitive ELISA format that is based on the interaction between solid-phase coupled FK506-BSA and biotinylated FKBP12. The calcineurin phosphatase, IMPDH, and DHODH enzyme assays are described in Materials and Methods. SFA and reference compounds were tested in the same experiment. IC50 values of a representative experiment are shown.*
For cyclophilins, so far only the fungal metabolite cyclosporin has been identified as a ligand. This encouraged us to search for novel immunosuppressive cyclophilin-binding structures that might act on new targets and reveal novel T cell-regulatory pathways.

By screening microbial fermentation extracts in a CypA-binding assay we identified an actinomycetes strain producing several closely related metabolites with novel structures (25, 26). The so-called sanglifehrins bind with high affinity to several cyclophilin isofoms (25). Several lines of evidence suggest that SFA and CsA share the same binding site on cyclophilin. Both SFA and CsA inhibit CsA-CypA as well as SFA-CypA interactions and both compounds inhibit the isomerase activity of CypA (Table I). Furthermore, SFA antagonizes the activity of CsA in an IL-2 reporter gene assay by displacing CsA from cyclophilin (Fig. 2) similar to a cyclophilin-binding, nonimmunosuppressive cyclosporin derivative with an altered effector domain (48). These data suggest that, despite their overall distinct structure, SFA and CsA may display a common three-dimensional cyclophilin-binding domain as do FK506 and rapamycin with respect to their FKBP-binding domain (59). Indeed, this has been recently confirmed by the x-ray structure of the CypA/SFA complex (J. Kallen, R. Sedrani, J.-J. Sangl, and T. Fehr, manuscript in preparation).

Despite the higher affinity of SFA over CsA for CypA under cell-free conditions, SFA showed a significantly lower activity than CsA in displacing radiolabeled CsA in a cellular binding assay (Table II). We believe that the binding of CsA to the cells in this assay is mainly due to its interaction with intracellular cyclophilins. A possible explanation for the difference in SFA binding to cyclophilin in the molecular vs the cellular assay could be a lower permeability into cells. This might also explain the relatively low activities of SFA in MLR when compared with CsA.

In contrast to CsA, SFA did not affect IL-2 production of activated T cells either at the transcriptional level (IL-2 reporter gene assay; Fig. 2) or at the level of IL-2 secretion (Table IV). SFA did not inhibit IL-2 production by mitogen-activated T cells (Table IV) or by T cells activated by a combination of anti-TCR and anti-CD28 Abs (data not shown). In contrast, SFA blocked the IL-2-dependent proliferation (Table III) and cytokine production (Table IV) of T cells. SFA still showed activity when added 72 h after initiation of MLR cultures (Fig. 5). The latter results indicate that SFA acts at a later stage of the cell cycle, most likely at the G1/S transition. Taken together, these results suggest that SFA binds to the CsA-binding side of cyclophilin but displays a different effector domain than CsA (like FK506 vs rapamycin), resulting in a different mechanism of action and activity profile.

When SFA was added to MLR cultures in combination with a cyclophilin-binding, nonimmunosuppressive cyclosporin derivative, its activity was not abrogated even at a 10-fold molar excess (Fig. 3). At this molar excess the cyclosporin derivative reversed >50% of the activity of CsA in the IL-2 reporter gene assay (48). These observations indicate that SFA exerts its effect independent of cyclophilin.

To characterize the mechanism of action of SFA in more detail we first analyzed its effects on targets of well characterized immunosuppressants. SFA did not bind to FKBP nor did it inhibit the enzymatic activities of calcineurin, IMPDH, and DHODH (Table V), the targets of FK506, MMF/MPA, and brequinar/leflunomide, respectively. Furthermore, the findings that the inhibition of the proliferation of activated T cells by SFA could not be reversed by addition of exogenous nucleosides (Fig. 4) indicates that SFA has no effects on targets involved in the de novo nucleoside synthesis.

SFA also showed effects on other cell types. It inhibited the proliferation of mitogen-activated B cells (Table VI). However, it had no effect on Ab production induced by CD154 and IL-4, which, in contrast, is blocked by rapamycin. The differential effects of SFA and rapamycin in this assay indicate that mTOR might not be involved in the mechanism of action of SFA. Unlike dexamethasone, SFA only weakly inhibited TNF-α production by IFN-γ/ LPS-stimulated PBMC. SFA exerts no general cytotoxic activity because it did not affect the proliferation of bone marrow cells (Table VI).

In conclusion, the data presented here indicate that SFA represents a new immunosuppressive compound whose mode of action is different from that of known immunosuppressants. However, the

![FIGURE 4. No effects of exogenous nucleosides on SFA activity. A. Brequinar or SFA (1 μM) were added to murine MLR cultures either in the absence or presence of 50 μM uridine. B. PHA-stimulated human T cells were incubated with MMF or SFA (1 μM) either in the absence or presence of 50 μM guanosine. In both cases the nucleosides completely reversed the inhibitory effect of brequinar and MMF, respectively, whereas they had no effect of the activity of SFA. Mean values of duplicate determinations are shown.](http://www.jimmunol.org/)

![FIGURE 5. Delayed addition of immunosuppressive compounds to murine MLR. The indicated compounds were added to MLR either at the initiation of the cultures (time of addition 0 h) or 24, 48, 72, and 91 h later. [3H]thymidine incorporation was performed for an additional 5 h as indicated by the horizontal bar until harvesting of the cells. Maximal proliferation in the absence of compound was 2–4 × 10⁶ cpm. At all time points, IC₅₀ values were determined from a complete titration of each compound. Results are expressed as “normalized IC₅₀” values where the IC₅₀ value obtained after addition at the initiation of the MLR was set to 1. When added at the initiation of the cultures, the compounds showed the following mean IC₅₀ values: CsA 10 nM, SFA 70 nM, rapamycin 1.3 nM, brequinar 550 nM, and MMF 20 nM. Mean normalized IC₅₀ ± SD values of three experiments are shown.](http://www.jimmunol.org/)
precise mechanism of action and in particular the effector protein of SFA remains to be identified.

Note added in proof. L.-H. Zhang and I. O. Liu recently showed that SFA inhibits IL-2-dependent T cell proliferation at the G1 phase of the cell cycle (60).

Acknowledgments
We thank R. Sedrani and J. Wagner for preparing the SFA-BSA conjugate.

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