Cyclosporins: Structure−Activity Relationships for the Inhibition of the Human *FPR1* Formylpeptide Receptor

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The human formylpeptide receptor (FPR) is a seven-transmembranous G-protein-coupled receptor (7TM-GPCR) for chemotactic peptides of bacterial origins, possibly involved in the recruitment and activation of neutrophils in various inflammatory diseases of mucosal epithelia. Mutational analyses suggest that interactions of formylated peptides with FPR occur on the outer exoplasmic leaflet/domains of the plasma membrane. The immunosuppressive and antifungal antibiotic cyclic undecapeptide cyclosporin A (CsA; cyclo-[MeBmt^1-Abu^2-MeGly^3-MeLeu^4-Val^5-MeLeu^6-Ala^7-D-Ala^8-MeLeu^9-MeLeu^{10}-MeVal^{11}]) and some tested analogues such as [Ala^2]-CsA, [Thr^2]-CsA, [Val^2]-CsA, and [Nva^2]-CsA were able of inhibiting the binding of formylpeptides to the FPR, with [D-MeVal^{11}]-CsA (CsH) being much more active than the other analogues. CsH is devoid of immunosuppressive and antifungal activities, and its large potency for human FPR inhibition is of inverse agonism origin. Formylpeptide binding to FPR-expressing cells does not only induce chemotaxis; it also causes a rapid release of granule enzymes in the extracellular medium, allowing the easy monitoring of any inhibition of FPR function "in vivo" (with intact live cells). With such an assay, CsH was confirmed to be the most potent FPR inhibitory cyclosporin, although a far related immunosuppressive cyclosporin analogue, FR901459 ([Thr^2, Leu^5, Leu^{10}]-CsA), was found to display a high FPR inhibitory activity (FPR-InhA). To establish structure−activity relationships (SAR) for FPR function inhibition, 59 cyclosporins were now studied by this standardized assay (with differentiated human leukemic cell line HL-60 as FPR-expressing cells and with *N*-acetyl-β-D-glucosaminidase release as read-out). These SAR confirmed the low FPR-InhA of classical cyclosporins, where such activity was only seldom found: the most active ones ([Thr^2, Ile^5]-CsA, [aMeIle^{11}]-CsA, and [MeAla^{11}]-CsA) remained 3−10-fold less potent than CsH. In contrast, the SAR disclosed that N^{10}-desmethylated cyclosporins were particularly prone to display a large FPR-InhA: their most potent one was a [Thr^2, Gly^3, Leu^5, D-Hiv^8, Leu^{10}]-CsA, found to be only 2−4-fold less active than [D-MeVal^{11}]-CsA (CsH), with which it shows six differences out of 11 residues. Because the free conformations of both CsH and N^{10}-desmethylated cyclosporins differ from those of "classical" (N^{10}-methylated, [L-MeVal^{11}]-using) cyclosporins, these potent FPR inhibitory cyclosporins probably bind to FPR pharmacophores for which classical cyclosporins show little affinity. Moreover, because the conformations of the N^{10}-desmethylated cyclosporins widely differ from the CsH one, they probably bind to different pharmacophores on the FPR molecules.

Introduction

The well-known IM activity of cyclo-[MeBmt^1-Abu^2-MeGly^3-MeLeu^4-Val^5-MeLeu^6-Ala^7-D-Ala^8-MeLeu^9-MeLeu^{10}-MeVal^{11}] (**1**, cyclosporin A [CsA]) depends on its binding to its intracellular receptor CyP and the further binding of the cyclosporin−CyP complex to calcineurin. The early in vitro assays used to study IM activity led to numerous data that were not prone for an easy establishment of SAR.[1,2] Indeed, such SAR were intrinsically complex, as the eventual IM activity depended on the cyclosporin ability to insert into the cell PM exoplasmic leaflet, to move to its cytosolic leaflet, to partition back to the cytosol, and then to refold and bind to CyP molecules, and eventually to form a complex suitable enough to sequester calcineurin.[3] Immunosuppression is probably not the reason why various fungi produce cyclosporins, and it remains unknown whether fungi find some advantage to interfere with some other functions that imply CyP or calcineurin binding. Nevertheless, by interacting with a variety of TM glycoproteins, cyclosporin molecules display other biological activities that show no obvious correlation with the expression of IM activity.[1] Possibly, one such membranous activity of cyclosporin might be related to the normal, yet elusive function of the cyclosporin for the variety of fungi producing them.

In all naturally occurring cyclosporins, the four amide-NH groups of residues 2, 5, 7, and 8 are always free (nonmethylated), while the other residues are most often *N*-Me. Several major forms of natural cyclosporins share with CsA a methylation of residues 1, 3, 4, 6, and 9−11, on the amide group (MeBmt^1, MeGly^3 (= Sar^3), MeLeu^4, MeLeu^6, MeLeu^9, MeLeu^{10}, and MeVal^{11}). In

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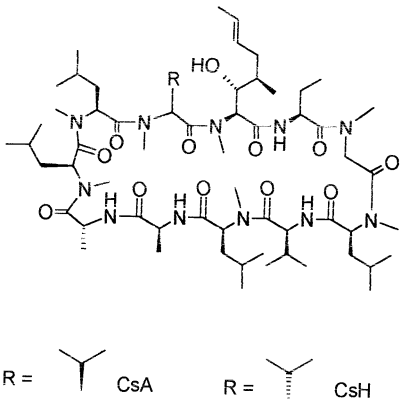
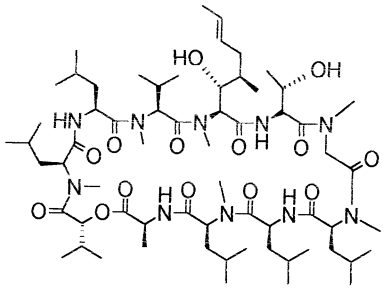
Scheme 1




SDZ 214-103

these cyclosporins, the free amides at positions 2, 5, 7, and 8 are involved in forming four intramolecular H-bonds. This imprints the "classical" cyclosporin conformation, made of a compact β-sheet that involves the antiparallel $^{[5-6-7]}$tripeptide and $^{[11-1-2]}$tripeptide and a loop contributed by the $^{[7-8-9-10-11]}$pentapeptide, two domains that those natural cyclosporins share with CsA. The naturally occurring analogues show only single or double side chain differences concerning residues 1, 2, 4, 5, and 7, with none available for residues 3, 6, and 8−11. Yet, there is an intriguing preference for [Thr^2] analogues, which are the sole cyclosporin forms produced by most cyclosporin-producing fungi.[4−7] Other side chain variants can be obtained by precursor-directed biosynthesis or microbial transformation[6,7] or by chemical synthesis.[4,5]

Moreover, a large number of naturally occurring cyclosporins show one or two additional free amide groups concerning residues 1, 3, 4, 6, and 9−11,[4,6,7] which may introduce new H-bonding opportunities within the cyclosporin molecule itself and with other molecules. In aqueous solution, the latter amide group variants might thus display much distorted framework shapes in comparison to CsA, which may dramatically change their initial interaction with the cell PM. Most of these simple *N*-desMe analogues are closely related to classical cyclosporin analogues by a single or double *N*-methyl difference.

Although most *N*-desMe cyclosporins show largely decreased IM activity, some fungi-produced [Thr^2]-cyclosporins combine an N^{10}-desmethylation with other residue side chain or *N*-methyl differences concerning residues 1, 3−5, 8, and 10 and display a large IM activity.[6−8] The frequent occurrence of selective *N*-desMe cyclosporins among producing fungi is intriguing as it might indicate their potential, yet unknown functions for the fungus interest.

More than a decade ago, another biological activity of cyclosporins was discovered; their interference with the function of the human *MDR1* Pgp involved in multidrug resistance (MDR) of cancer cells, a model 12TM adenosine 5′-triphosphate (ATP) binding cassette (ABC) transporter. The SAR for this Pgp-InhA are the topic of the companion paper.[9] This promiscuous "hydrophobic vacuum cleaner" or flippase for selective membrane phospholipids can also restrict anticancer drug accumulation by the cells causing their MDR phenotype.[10] For this Pgp inhibition, several cyclosporins devoid of IM activity were potent Pgp inhibitors, but SDZ 214-103 (**3**) that shows a large IM activity is a more potent Pgp inhibitor than CsA (**1**).[9] Because SDZ 214-103 (**3**) is a cyclo-[MeBmt^1-Thr^2-MeGly^3-MeLeu^4-Leu^5-MeLeu^6-Ala^7-D-Hiv^8-MeLeu^9-Leu^{10}-MeVal^{11}] or [Thr^2, Leu^5, D-Hiv^8, Leu^{10}]-CsA,[8] cyclosporins **1** and **3** are two far related analogues. They show largely different shapes in the free form, although they acquire very similar ones when bound to CyP molecules.[11,12]

Yet another CsA activity is the inhibition of signaling through 7TM-GPCR.[1] This is particularly the case for a model 7TM-GPCR usually described as a chemoattractant receptor with specificity for formylated peptides of bacterial and mitochondrial origins.[13−15] For this formylpeptide receptor (FPR), previous comparisons of a few analogues underlined the exceptional property of CsH (**2**) as it is a highly potent and selective inhibitor, being much more potent than CsA and lacking activity on several other tested chemoattractant 7TM-GPCR.[16−20]

While the immunosuppressive and cancer MDR reversing properties of cyclosporins have obvious clinical applications, the medicinal interest of their FPR inhibitory potential remains elusive. Indeed, both the actual function of FPR on leukocytes and the physiological relevance of an involvement of formylpeptides in human health and disease are still being debated.[13−15,21]

Although less active than CsH (**2**), FR901459 (**54**), a distant analogue of CsA (**1**) but a close analogue of SDZ 214-103 (**3**) as it is a [D-Ala^8]-SDZ 214-103, was recently shown to display a large FPR-InhA.[21] While CsH (**2**) is devoid of IM activity, both FR901459 (**54**) and SDZ 214-103 (**3**) show a large IM activity.[2,8,22] The structures of the key reference compounds **1**−**3** are shown as Scheme 1.

Because the substantial Pgp-InhA and the weak FPR-InhA shown by CsA (**1**)[9,10,16−20] occur together with its potent IM activity,[1] CsA must obviously display all suitable structural features. Earlier SAR studies of cyclosporins showed that these cyclic peptides were large enough to display various effector regions. A priori, either common, or overlapping, or totally different regions could be involved in interactions with different cyclosporin activities such as IM activity (by gluing calcineurin to CyP), MDR reversion (by binding to the Pgp transporter), and chemotaxis inhibition (by binding to FPR chemoattractant receptors).

Thus, with regards to the sole expression of IM activity, one cyclosporin side binds to CyP through an

adequate conformation of [MeLeu[9]-MeLeu[10]-MeVal[11]-MeBmt[1]-Abu[2]-MeGly[3]] hexapeptide; opposite to this "CyP binding domain" of the cyclosporin, some residues (residues 4 and 6 in the [MeLeu[4]-Val[5]-MeLeu[6]] tripeptide, together with the extremity of the MeBmt[1] side chain) are essential for the binding of the cyclosporin−CyP complex to calcineurin and constitute a "calcineurin binding domain".[1,3,23]

With regard to Pgp inhibition by CsA (**1**) at least, the CyP binding domain would not bind the Pgp molecules but would be exposed to the cytosol; the "contact residues" with Pgp molecules (or "Pgp binding domain") would not only include the calcineurin binding domain ([MeLeu[4]-Val[5]-MeLeu[6]] and MeBmt[1] side chain) but also include the [Ala[7]-D-Ala[8]-MeLeu[9]] tripeptide.[24] Nevertheless, our recent studies disclosed that not every residue side chain in the Pgp binding domain of cyclosporin showed obvious requirement of size or hydrophobicity, while other residue side chains outside the Pgp binding domain were not inert.[9] This suggests either that the Pgp binding domain concept was too restricted or that other features of cyclosporin interactions with the membrane may have a major impact on their eventual interaction with Pgp molecules. The SAR for Pgp-InhA further suggested additional requirements for: (i) the occurrence of suitable conformers for insertion in the cell membrane, (ii) a sufficient conformational plasticity for gaining access to Pgp binding sites, (iii) an adequate conformer structure there to achieve such binding with a high enough affinity, and (iv) possibly an escape from sequestration on CyP molecules.[9]

If however the inhibitory interactions between cyclosporins and FPR do occur at the cell surface level (directly from the extracellular medium), their SAR might be simpler than those for IM activity or inhibition of Pgp. The cyclosporin capacity to undergo conformational shifts needed to partition into the inner leaflet of the lipid bilayer, as required for Pgp-InhA, or to cross the cell PM to bind to the cytosolic CyP, as required for IM activity, might not dominate or at least have less impact on their ability to inhibit FPR function. Thus, the most representative cyclosporin conformers for the interaction of cyclosporins with FPR molecules should belong to those found in solution in the extracellular medium. The shapes of the different cyclosporin conformers in solution may indeed be directly relevant to their binding to the extracellular moieties of FPR molecules, and their conformational plasticity may modulate their capacity to gain access to their intramembranous domain, thus would feature their binding to pharmacophores within the FPR molecules.

Most cyclosporins do actually belong to two major structural conformations: the classical (β-sheet and loop structure) backbone,[25] whose prototype is CsA (**1**), and the "twisted" one,[11,12] whose prototype is SDZ 214-103 (**3**), itself similar to the FR901459 conformation (**54**);[22] the "right angle" conformation of CsH (**2**)[25,26] is so far unique for that analogue. These three major "consensus" structures of CsA and SDZ 214-103, as well as the CsH one, obtained from crystallographic or NMR studies were recalled and discussed in the companion paper.[9] To summarize the differences of conformation, the backbone conformations (derived from unpublished X-ray data of A. Widmer and H. P. Weber) of three cyclosporins are shown superposed for CsA (**1**) and CsH (**2**) in the first case (Figure 1) and for CsA (**1**) and SDZ 214-103 (**3**) in the second case (Figure 2).

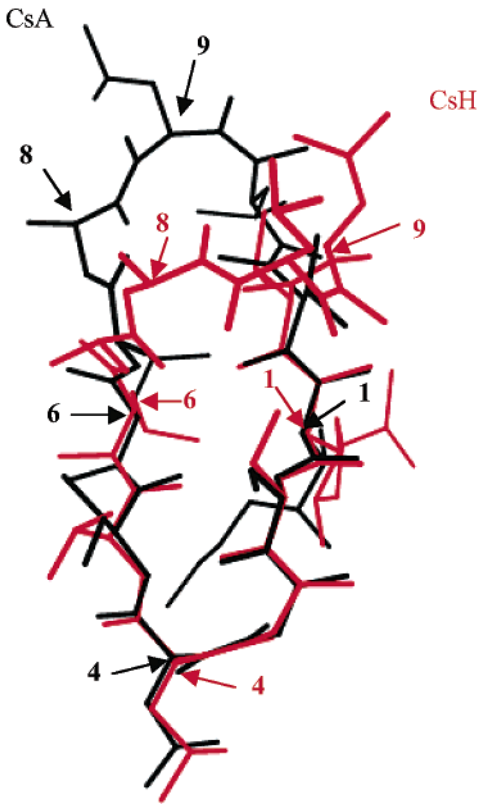


Figure 1. Comparison of the 3D structures of CsA (**1**) and CsH (**2**). These backbone conformations of CsA (**1**, black) and CsH (**2**, red) were derived from X-ray data. The Cα atoms of residues 1, 4, 6, 8, and 9 of each cyclic peptide are labeled by arrows. In sum (see details in companion paper),[9] the β-pleated sheet that involves antiparallel tripeptides ([MeVal[11]-MeBmt[1]-Abu[2]] and [Val[5]-MeLeu[6]-Ala[7]]) is conserved when **1** and **2** are superposed as done here (superposition is on Cα atoms of residues 1−6 (β-turn region)). The D-MeVal[11] residue causes a 90° shift of the backbone conformation of **2** in comparison to **1**. In fact, both structural features (loop and β-pleated sheet) could be superposed, even though the relative orientation of the loop (formed by the pentapeptide [Ala[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-MeVal[11]]) to the β-pleated sheet was radically changed. Computer modeling optimization of **2** suggested that the conformation of the loop backbone part of the peptidic ring in **2** (Ala[7] to D-MeVal[11]) was identical to the loop backbone in **1**, although being at a right angle with regards to the structure of **1**. However, the side group conformation of native MeBmt[1] in **2** is not fully established, because the X-ray data have been measured with a cyclic iodo derivative of MeBmt[1] (H. P. Weber, unpublished results).

In the present study, 59 representative cyclosporins were available to establish cyclosporin SAR for FPR inhibition in vivo in intact live cells. Our earlier comparisons of the feasibility of various functional assays led us to select an enzyme release assay as readout for comparisons of numerous cyclosporins.[20] Indeed, formylpeptide binding to FPR-expressing cells causes a rapid release of granule enzymes in the extracellular medium, and any inhibition of such an activation can be conveniently monitored as recently described for *N*-acetyl-β-D-glucosaminidase release from FPR-expressing differentiated human leukemic HL-60 cells.[20]

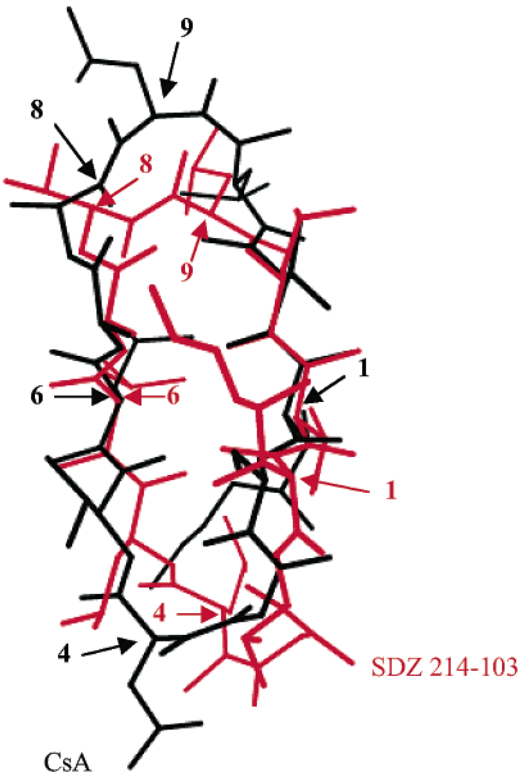


Figure 2. Comparison of the 3D structures of CsA (**1**) and SDZ 214-103 (**3**). These backbone conformations of CsA (**1**, black) and SDZ 214-103 (**3**, red) were derived from X-ray data. In sum (see details in companion paper),[9] the two conformations show substantial differences with no conserved domain between the two, so that the superposition performed here is somewhat arbitrary on Cα atoms of residues 1, 6, 7, and 11. The Cα atoms of residues 1, 4, 6, 8, and 9 of each cyclic peptide are labeled by arrows. Both **1** and **3** show the intramolecular [O^2- - -N^5] H-bond between the, respectively, Abu^2 and Thr^2 carbonyl and Val^5 and Leu^5 amide, but in **3**, there are no [N^2- - -O^5] and [O^{11}- - -N^7] H-bonds. One peculiarity of the crystal conformation of **3** is the cis amide bond between residues 3 and 4, which allows the formation of a type Via β-turn stabilized by the [O^2- - -N^5] H-bond between the Thr^2 and Leu^5 residues. Therefore, **3** does not have the peculiar β-sheet of **1** but a short extended antiparallel sheet involving residues 7−10, with two [O^{10}- - -N^7] and [O^7- - -N^{10}] H-bonds between Ala^7 and Leu^{10} stabilizing a type II′ β-turn.

Chemistry

All 59 cyclosporins tested here for their activity on the FPR function are described in the companion paper on the SAR of cyclosporins for Pgp inhibition.[9] The same compound numbering is used throughout both papers. The reader is thus referred to the corresponding section of the companion paper for all data and references concerning the sources of these naturally occurring or biosynthetically produced analogues or to major reviews describing most of the analogues used here.[4−7]

Biological Tests

Cells. The promyelocytic human leukemia HL-60 cells were differentiated along the neutrophilic lineage by culture in the presence of 0.2 mM dibutyryl cAMP (N^6,2′-O-dibutyryl adenosine 3:5′-monophosphate; Sigma) for 48 h to acquire receptors for and responsiveness to ligands of 7TM-GPCR such as f-MLF or C5a as described.[20]

Cyclosporins. All cyclosporins were dissolved as 10 mg/mL stock solutions and diluted to obtain a range of three and 10 stepwise concentrations in dimethyl sulfoxide (DMSO). They were further diluted in the adequate buffer just prior to the FPR inhibition assay, which contains a final 0.125% DMSO for all tested cyclosporin concentrations. At variance with other cellular assays that can support much higher DMSO concentrations, such as those used recently for the SAR on the Pgp-InhA of cyclosporins (1% DMSO), the low final 0.125% DMSO concentration was used here to avoid any interference of DMSO with the performance of the degranulation assay.[20] As a consequence, most cyclosporin analogues remained obviously stable in solution only up to a final 10 μM concentration, with some analogues stable only up to 3 μM.

FPR-InhA. The FPR activity and its inhibition were assayed by a ligand-induced granule enzyme release using human leukemia HL-60 cells.[20] The FPR ligand f-MLF (Sigma) was used at 30 nM. Briefly, the release of *N*-acetyl-β-D-glucosaminidase was measured upon stimulation of HL-60 cells with f-MLF in the presence of ranges of concentrations of potential antagonists (CsA or analogues). The released enzyme was collected by the use of filtration, using the MultiScreen system, which consists of one filtration unit and 96 well microplates with porous membranes (MAVM 096 01, MAFC microplates, Millipore). Before the assay, the cells were washed twice by centrifugation (200 g for 10 min, in a centrifuge operating at 20 °C) and by resuspension in an isotonic "release buffer" at room temperature. For assays monitoring *N*-acetyl-β-D-glucosaminidase release, the specific release buffer consisted of 136 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 5 mM D-glucose, 1 mg/mL bovine serum albumin, and 20 mM Hepes buffer (pH 7.4). Before use, the porous microplates were washed once by filling and filtration of release buffer. Just prior to their distribution in the wells, the cell suspensions at 2.5×10^6/mL were exposed to 5 μM cytochalasin B (Calbiochem) for 5−10 min at 37 °C in a water bath. These assays included a step of cell pretreatment with the potential antagonists: 50 μL of a range of compound (three and 10 stepwise) concentrations in release buffer (or release buffer only as control) was first distributed in the wells, and 100 μL of the cell suspensions was added (thus, 2.5×10^5/well). The plates were incubated for 10 min at 37 °C (incubator). Then, 50 μL of f-MLF (30 or 100 nM final) in release buffer (or release buffer alone as control) was added to each well. The plates were reincubated for 10 min at 37 °C, and the extracellular medium was immediately collected by filtration into classical 96 well flat bottom microplates (NUNC Maxisorp) containing 50 μL per well of the enzyme substrate, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma), which was prepared just before use at 10 mM in 125 mM citrate buffer, pH 4.6. After 3−4 h at 37 °C (incubator), the glucosaminidase activities were measured by the released paranitrophenol, revealed by adding 50 μL of glycine buffer (0.3 M, pH 10.4) and reading the absorbency at 410 nm at the Titertek Multiskan MCC/340 MkII (Flow Labs). For each assay, duplicated microculture wells were used and the mean was recorded. For all compounds here described, spontaneous release was not affected by the

highest antagonist concentrations; to calculate the percentage of f-MLF response in the presence of cyclosporin, the spontaneous release (absolute absorbency in the absence of f-MLF) was subtracted from all values. Dose−response curves were built with the antagonist concentrations on the *X*-axes, and absorbency was expressed as percentage of the normal response obtained with 30 nM f-MLF on the *Y*-axes. The IC_{50} and IC_{20} values were measured on independent experiments (three for most analogues) and expressed as a mean ± standard deviation.

SAR Analyses by IC_{20} Value Comparisons. A number of cyclosporins were obviously inhibiting FPR function although nearly two-thirds of the analogues did not provide an IC_{20} value, only a dozen did not provide an IC_{50} value below 10 μM. Because the IC_{20} level of specific FPR inhibition was largely above the experimental fluctuations due to spontaneous degranulation, the cyclosporin IC_{20} values, rather than the IC_{50} ones, were used to make cross-comparisons of the cyclosporins to establish the SAR for FPR inhibition. At variance with the assay conditions for the study of Pgp inhibition in the companion paper,[9] FPR inhibition is assayed in the presence of 0.125% DMSO only (higher ones impairing the read-out of specific degranulation), which limits to 3−10 μM the maximally achievable cyclosporin concentrations in the assay. For some compounds, the IC_{50} values even showed a larger than proportional increase (>2.5-fold) in comparison to the IC_{20} values. Indeed, because of the low final 0.125% DMSO solvent concentration, such analogues were unstable in solution at higher concentrations, so that the IC_{50} values might underevaluate their FPR-InhA potentials and are only given for information.

Internal Control and Data Normalization. Throughout the whole experimental program, single concentrations of reference compounds (most often, 50 or 100 nM of compounds **1**−**3**) were included to control the normal drug sensitivity of the f-MLF response of the differentiated HL-60 cells. In a series of experiments on nine analogues (**6**, **22**, **31**, **33**, **37**, **40**, **43**, **53**, and **60**), which were performed in a different laboratory and with a different source of HL-60 cells, we observed an apparently larger sensitivity to cyclosporin inhibition, which was then shown to be roughly 1.5-fold (e.g., IC_{20} of 20 ± 10 nM and IC_{50} of 90 ± 80 nM (n = 6) instead of the 30 nM IC_{20} and 150 nM IC_{50}, respectively, usually found for the CsH FPR-InhA; a similar 1.5-fold factor was found with other reference cyclosporins). To allow fair comparisons with the other 50 cyclosporins, the independently generated data of the nine compounds were normalized; their actual measured IC_{20} and IC_{50} values were 1.5-fold lower than those reported here.

Results

The SAR of cyclosporins for FPR inhibition (FPR-InhA) will be shown like those for Pgp inhibition in the companion paper.[9] Like in the latter, the cyclic peptide sequence variations of all cyclosporins will principally refer to CsA (**1**) and in some cases to SDZ 214-103 (**3**). In some sections, two other trivial names may also be used, CsH (**2**) and FR901459 (**54**).

While CsA only shows a modest FPR-InhA, both CsH (**2**) and FR901459 (**54**) share a large capacity to reverse the FPR-mediated cell response.[21] This property is also shared by SDZ 214-103, as shown (Figure 3) by the concentration-dependent inhibition of FPR function by CsA (**1**) and SDZ 214-103 (**3**) in comparison with CsH (**2**).

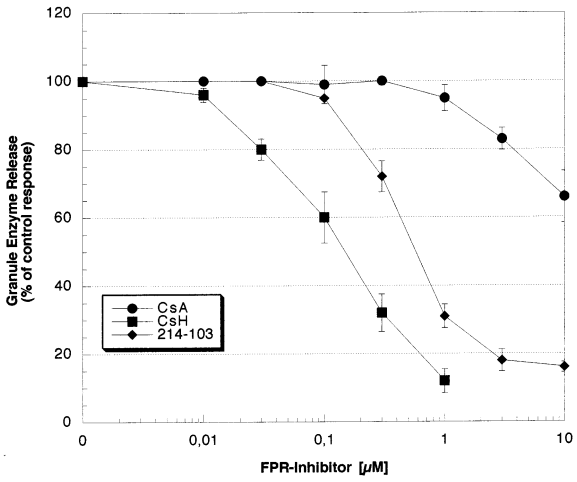


Figure 3. Comparison of CsA (**1**), CsH (**2**), and SDZ 214-103 (**3**) for their capacity to inhibit the FPR−f-MLF interaction-induced response. The diagram shows the cyclosporin dose-dependent inhibition of the 30 nM f-MLF-induced release of *N*-acetyl-β-D-glucosaminidase by neutrophilic HL-60 cells. The data are expressed as percentages (*Y*-axis) of the enzyme release responses as a function of the CsA (circles), CsH (squares), or SDZ 214-103 (lozenges) cyclosporin concentrations (μM, *X*-axis). The data are shown as means and SD obtained from three independent experiments.

Thus, to inhibit a typical cellular response induced by f-MLF ligand binding to the human FPR, CsA is much less potent than analogues CsH and SDZ 214-103, with free conformations different from the CsA one.[9] Because the free conformations of CsH and SDZ 214-103 are also widely different from each other,[11,25] the SAR of cyclosporins for FPR inhibition are very complex. While most studied side chain alterations may leave the overall cyclosporin structure rather unaffected, desmethylation of *N*-Me residues may induce considerable alterations of the overall cyclosporin conformation. A first SAR section will only concern CsA (**1**) and all classical cyclosporins, numbered **4**−**33**, that show single or double side chain difference of residues 11 through 8 (none was available for residues 9 and 10); CsH (**2**), which does not have the classical conformation, is also included. Most of these cyclosporins either were naturally occurring analogues or were prepared by precursor-directed biosynthesis or microbial transformation.[6] A second SAR section concerns SDZ 214-103 (**3**) and *N*-desMe cyclosporins, numbered **34**−**60**, all of which are naturally occurring analogues.[6]

1. Impact of Residue Side Chains of Classical Cyclosporin on FPR Inhibition. 1.1. Single Amino Side Chain Variations on Classical Cyclosporins. All cyclosporin analogues can be directly compared with CsA as reference (Table 1).

L-MeVal[11]. In comparison with CsA (**1**), the FPR-InhA was largely (roughly 12-fold) increased by a smaller side chain (**6**, [MeAla[11]]-CsA), whereas residues with larger side chains conferred either an unchanged FPR-InhA (**5**, [MeIle[11]]-CsA) or a markedly (12-fold) increased one (**4**, [aMeIle[11]]-CsA). These best [L-resi-

Table 1. FPR-InhA of CsA Analogues with Single Residue Differences[a]

cyclosporin analogues			FPR-InhA		
compd no.	residue alterations	trivial name	fold diff	IC_{20} (μM) mean ± SD (n)	IC_{50} (μM) mean ± SD
1	**CsA**	CsA	= **1**	3.6 ± 0.8 (3)	>10
2	D-MeVal[11]	CsH	× 120	0.03 ± 0.007 (3)	0.15 ± 0.035
4	aMeIle[11]		× 12.4	0.29 ± 0.18 (6)	1.57 ± 0.63
5	MeIle[11]		× 1.2	2.9 ± 1.4 (9)	>10
6	MeAla[11]		× 11.6	0.31 ± 0.15 (3)	2.52 ± 0.49
7	deoxy-MeBmt[1]	CsF	× 1.5	2.4 ± 0.4 (3)	>10
8	MeAoa[1]	CsZ	× 2.1	1.7 ± 0.4 (3)	≥10
9	MeLeu[1]	Cs28	/ >2.8	>10 (3)	
10	Me-cyclohexyl-Ala[1]		× 2.1	1.69 ± 1.18 (6)	6.33 ± 2.13
11	Me-cyclized-Bmt[1]		/ >2.8	>10 (5)	
12	8′-OH-MeBmt[1]	AM1	/ >2.8	>10 (3)	
13	Ala[2]	CsB	× 2.4	1.51 ± 0.04 (3)	≥10
14	Val[2]	CsD	/ 1.1	3.9 ± 0.4 (3)	>10
15	Nva[2]	CsG	× 1.4	2.6 ± 0.2 (3)	>10
16	Thr[2]	CsC	× 1.6	2.2 ± 1.1 (3)	≥10
17	D-MePhe[3]		/ >2.8	>10 (2)	
18	L-Pro[3]		/ >2.8	>10 (2)	
20	MeIle[4]	Cs29	× 3.6	1.0 ± 0.37 (3)	7 ± 2.4
21	γ-OH-MeLeu[4]		× 2.1	1.7 ± 0.0 (3)	>10
22	MeVal[4]		× 2.5	1.44 ± 0.47 (3)	>5
23	MePhe[4]		/ 1.1	4.0 ± 1.9 (8)	>10
24	MeAla[6]		/ >2.8	>10 (4)	
25	Abu[7]	CsV	/ ≥2.8	≥10 (5)	
26	D-Lys[8]		× 7.2	0.5 ± 0.1 (3)	6.3 ± 1.9
27	D-Ser[8]		/ 1.8	6.3 ± 1.5 (3)	>10

[a] Concerned residues of CsA (**1**) are MeVal[11], MeBmt[1], Abu[2], MeGly[3], MeLeu[4], MeLeu[6], Ala[7], and D-Ala[8]. Fold differences are based on IC_{20} values.

due[11]] variants nonetheless remained about 10-fold less active than the CsH analogue (**2**), whose FPR-InhA was consistently 120-fold larger than the one of CsA (**1**).

L-MeBmt[1]. The 8′-hydroxylation of MeBmt[1] resulted in a marked decrease of FPR-InhA (**12**). In [MeLeu[1]]-CsA (**9**), the substitution of MeBmt[1] by the nonpolar and shorter MeLeu[1] abrogated the FPR-InhA of CsA, as was also observed for another CsA analogue lacking the 3′-hydroxyl group (**11**, Me-cyclized-Bmt[1]]-CsA). This effect should not depend on the loss of the polar 3′-hydroxyl group, since its absence in [deoxy-MeBmt[1]]-CsA (**7**) slightly improved (1.5-fold) the FPR-InhA and since the substitution of MeBmt[1] by a Me-aminooctanoic acid in [MeAoa[1]]-CsA (**8**) or a Me-cyclohexylalanine in [Me-cyclohexyl-Ala[1]]-CsA (**10**) led in both cases to a 2-fold increase in FPR-InhA.

L-Abu[2]. Larger second residue side chains either did not change the FPR-InhA (**14**, Val[2]) or slightly increased it (**15**, 1.4-fold; Nva[2]), as was the case for a polar side chain (**16**, Thr[2]; 1.6-fold increase). Only the smaller side chain (**13**, Ala[2]) conferred a substantially increased (2.4-fold) FPR-InhA.

MeGly[3]. Both available analogues with substitutions of the MeGly[3] residue by larger hydrophobic ones, i.e., [L-Pro[3]]-CsA (**18**) and [D-MePhe[3]]-CsA (**17**), were devoid of detectable FPR-InhA.

L-MeLeu[4]. Replacement of the MeLeu[4] residue of CsA (**1**) by a bulkier [MePhe[4]] residue (**23**) led to about the same FPR-InhA, while its replacement by a smaller one in [MeVal[4]]-CsA (**22**) resulted in a 2.5-fold increase in FPR-InhA. The MeLeu[4] residue replacement by the similar-sized one in [MeIle[4]]-CsA (**20**) or its γ-hydroxylation in [γ-OH-MeLeu[4]]-CsA (**21**) led to obviously more potent FPR inhibitors (3.6- and 2.1-fold, respectively).

L-MeLeu[6]. The small side chain [MeAla[6]]-CsA (**24**) lacked detectable FPR-InhA.

L-Ala[7]. The larger side chain in [Abu[7]]-CsA (**25**) markedly decreased (≥2.8-fold) FPR-InhA.

D-Ala[8]. The capacity to inhibit FPR function ranked as Lys ≫ Ala > Ser, and the substitutions of D-Ala[8] (**1**) for D-Ser[8] (**27**) or D-Lys[8] (**26**) in CsA led to, respectively, decreased (1.8-fold) and largely increased (7.2-fold) FPR inhibition.

1.2. Combined Variations of Second and First or Fifth Residues. The impact of Abu[2] and/or Val[5] substitution on the FPR-InhA is studied in reference to their closest analogues (Table 2).

The lack of the 3′ hydroxyl on MeBmt[1] on the deoxy-MeBmt[1]-analogues (**7** and **28**) led to a slight increase (1.5-fold) of FPR-InhA for CsA but a clear decrease (>2.6-fold) for [Val[2]]-CsA. The substitution of the large MeBmt[1] by the nonpolar and smaller MeLeu[1] residue (**9**, **29**, and **30**) did not change the [Val[2]]-CsA FPR-InhA but clearly decreased the ones of CsA and of [Nva[2]]-CsA. The MeLeu[1] (**29**) and deoxy-MeBmt[1] (**28**) analogues of [Val[2]]-CsA thus behaved differently from the corresponding [Abu[2]]- and [Nva[2]]-using analogues (**9**, **30**, and **7**).

The impact of the fifth residue in [Nva[2]]-CsA (**15**, with Val[5]) on FPR-InhA was shown by the 13-fold larger activity of [Nva[2], Nva[5]]-CsA (**31**) but the 3.4-fold lower activity of [Nva[2], Leu[5]]-CsA (**32**). However, the substitution of the Val[5] in [Thr[2]]-CsA (**16**) by another large residue in [Thr[2], Ile[5]]-CsA (**33**) led to a surprisingly 24-fold larger FPR-InhA.

2. Impact of Cyclosporin *N*-Methylation on FPR Inhibition. The occurrence of either free amides or methylated amides should obviously impact the overall

Table 2. FPR-InhA of CsA Analogues with Combined Second and First or Fifth Residue Differences[a]

cyclosporin analogues					FPR-InhA		
compd no.	residue no. alteration			trivial name	fold diff	IC_{20} (μM) mean ± SD (n)	IC_{50} (μM) mean ± SD
1	**Abu^2**	**MeBmt^1**	**Val^5**	CsA	= **1**	3.6 ± 0.8 (3)	>10
7	:	deoxy-MeBmt^1	:	CsF	× 1.5	2.4 ± 0.4 (3)	>10
9	:	MeLeu^1	:	Cs28	/ >2.8	>10 (3)	
14	**Val^2**	**MeBmt^1**	**Val^5**	CsD	= **1**	3.9 ± 0.4 (3)	>10
28	:	deoxy-MeBmt^1	:	CsK	/ >2.6	>10 (3)	
29	:	MeLeu^1	:	Cs30	/ 1.1	4.2 ± 1.7 (3)	>10
15	**Nva^2**	**MeBmt^1**	**Val^5**	CsG	= **1**	2.6 ± 0.2 (3)	>10
30	:	MeLeu^1	:	CsO	/ 2.9	7.6 ± 1.5 (3)	>10
31	:	MeBmt^1	Nva^5	CsM	× 13	0.20 ± 0.13 (3)	3.4 ± 0.7
32	:	MeBmt^1	Leu^5	Cs26	/ 3.4	8.9 ± 1.9 (3)	>10
16	**Thr^2**	**MeBmt^1**	**Val^5**	CsC	= **1**	2.2 ± 1.1 (3)	>10
33	:	MeBmt^1	Ile^5		× 24	0.09 ± 0.04 (3)	2.3 ± 0.8

[a] Concerned residues of CsA (**1**) are MeBmt^1, Abu^2, and Val^5. Fold differences are based on IC_{20} values.

Table 3. FPR-InhA of Simple *N*-DesMe Cyclosporin Analogues[a]

cyclosporin analogues			FPR-InhA		
compd no.	residue no. alterations	trivial name	fold diff[b]	IC_{20} (μM) mean ± SD (n)	IC_{50} (μM) mean ± SD
1	**CsA-analogues (Abu^2)**	CsA	= **1**	3.6 ± 0.8 (3)	>10
34	Val^{11}	CsE	/ 1.1	3.8 ± 1.3 (3)	>10
35	Bmt^1	CsL	× 1.9	1.9 ± 0.4 (3)	6.45 ± 0.5
36	Leu^4	AM4N	/ ≥2.8	≥10 (4)	
37	Ile^4	Cs31	(× 1.3)[b]	2.8 ± 1.1 (3)	>5
38	Val^4	CsQ	(/ >2.8)[b]	>10 (3)	
39	Leu^6	CsU	/ >2.8	>10 (2)	
40	Leu^9		× 6.8	0.53 ± 0.20 (4)	3.0 ± 0.7
41	Leu^{10}	CsT	× 5.5	0.65 ± 0.1 (3)	9.2 ± 1.4
42	Leu^6 Leu^{10}	CsR	× 9.7	0.37 ± 0.07 (3)	1.80 ± 0.67
13	**[Ala^2]-CsA analogue**	CsB	= **1**	1.51 ± 0.04 (3)	≥10
43	Leu^{10}		× 7.5	0.20 ± 0.09 (3)	2.2 ± 0.7
16	**[Thr^2]-CsA analogues**	CsC	= **1**	2.2 ± 1.1 (3)	≥10
44	Val^{11}	CsW	/ 2.9	6.4 ± 1.2 (3)	>10
45	Bmt^1	CsP	× 1.5	1.5 ± 0.4 (3)	5.5 ± 0.6
46	Val^4	CsS	(/ ≥4.5)[b]	≥10 (2)	
47	Leu^{10}		× 1.1	2.05 ± 0.86 (3)	>3
14	**[Val^2]-CsA analogues**	CsD	= **1**	3.9 ± 0.4 (3)	>10
48	Bmt^1	Cs27	× 1.9	2.0 ± 0.4 (3)	>10
49	Leu^{10}	CsI	× 2.8	1.40 ± 0.14 (3)	>3
15	**[Nva^2]-CsA analogues**	CsG	= **1**	2.6 ± 0.2 (3)	>10
50	Leu^6	CsY	/ 3.1	8.1 ± 2.8 (2)	>10
51	Leu^9	CsX	× 1.2	2.1 ± 0.3 (3)	>10
52	Leu^{10}	CsN	× 3.9	0.66 ± 0.38 (3)	>3
27	**[D-Ser^8]-CsA analogue**		= **1**	6.3 ± 1.5 (3)	>10
53	Gly^3	Cs32	× 3.3	1.9 ± 1.2 (4)	>5

[a] Reference residues of CsA (**1**) are MeBmt^1, MeGly^3, MeLeu^4, MeLeu^6, D-Ala^8, MeLeu^9, MeLeu^{10}, and MeVal^{11}. Fold differences are based on IC_{20} values. [b] For compounds **37**, **38**, and **46**, the comparisons shown in the table are made with the [MeLeu^4]-using cyclosporins. For compounds **37** and **38**, more relevant comparisons with the [MeIle^4]- and [MeVal^4]-using analogues were also possible and described in the text.

shape of cyclosporin and/or its surface for interaction with the FPR.

2.1. FPR Inhibition by Simple *N*-DesMe Cyclosporin Analogues. With analogues of classical cyclosporins, there was a variable impact of selective residue *N*-desmethylation (Table 3).

For the available cyclosporins, *N*-desMe^4, *N*-desMe^6, or *N*-desMe^{11} analogues showed decreased FPR-InhA, whereas *N*-desMe^1, *N*-desMe^3, *N*-desMe^9, or *N*-desMe^{10} analogues showed increased FPR-InhA, two exceptions to this "rule" depending on the second residue used.

***N*-DesMe^1.** [Bmt^1]-CsA (**35**), [Bmt^1, Thr^2]-CsA (**45**), and [Bmt^1, Val^2]-CsA (**48**) showed a moderate but clear increase of FPR-InhA, being 1.5−1.9-fold more active than their parent [MeBmt^1]-cyclosporins.

***N*-DesMe^3.** The [Gly^3, D-Ser^8]-CsA (**53**) had a 3.3-fold larger FPR-InhA than [D-Ser^8]-CsA (**27**).

***N*-DesMe^4**. The *N*^{4}-desmethylation caused obvious decreases of FPR-InhA, [Leu^4]-CsA (**36**), and [Ile^4]-CsA (**37**) about 2.8-fold weaker than, respectively, CsA (**1**, with MeLeu^4), [MeIle^4]-CsA (**20**), and [Val^4]-CsA (**38**) lacking detectable activity and being at least 7-fold

Table 4. FPR-InhA of Complex N^{10}-Desmethylated [Thr^2]-Cyclosporin Analogues[a]

compd no.	ref: analogue or trivial name	key residues: residue diff or alternative name	FPR-InhA: fold diff	FPR-InhA: IC_{20} (μM) mean ± SD (n)	FPR-InhA: IC_{50} (μM) mean ± SD
47	[Thr^2 Leu^{10}]-CsA	Val^5 D-Ala^8 Leu^{10}	= **1**	2.05 ± 0.86 (3)	>3
54	(FR901459)	Leu^5	× 10	0.20 ± 0.10 (8)	0.59 ± 0.12
55	analogue	D-Hiv^8	× 2.2	0.93 ± 0.1 (3)	2.50 ± 0.35
16	analogue	MeLeu^{10}	/ 1.1	2.2 ± 1.1 (3)	10.1 ± 3.5
54	FR901459	Leu^5 D-Ala^8 Leu^{10}	= **1**	0.20 ± 0.10 (8)	0.59 ± 0.12
47	analogue	[Val^5]-FR901459	/ 10	2.05 ± 0.86 (3)	>3
3	(SDZ 214-103)	[D-Hiv^8]-FR901459	=	0.20 ± 0.03 (3)	0.57 ± 0.06
56	analogue	[Ala^{10}]-FR901459	/ 4	0.8 ± 0.3 (3)	3.25 ± 0.20
3	SDZ 214-103	MeBmt^1 MeGly^3 Leu^5 D-Hiv^8	= **1**	0.20 ± 0.03 (3)	0.57 ± 0.06
57	analogue	[8′-OH-MeBmt^1]-SDZ 214-103	/ 2.3	0.47 ± 0.07 (3)	1.48 ± 0.12
58	analogue	[Gly^3]-SDZ 214-103	× 1.6	0.13 ± 0.02 (3)	0.3 ± 0.1
55	analogue	[Val^5]-SDZ 214-103	/ 4.7	0.93 ± 0.1 (3)	2.50 ± 0.35
59	analogue	[Ile^5]-SDZ 214-103	/ 1.5	0.29 ± 0.01 (3)	0.75 ± 0.08
60	analogue	[Leu^4]-SDZ 214-103	× 1.4	0.14 ± 0.05 (4)	0.56 ± 0.02
54	(FR901459)	[D-Ala^8]-SDZ 214-103	=	0.20 ± 0.10 (8)	0.59 ± 0.12

[a] Reference residues of CsA (**1**) are MeVal^{11}, MeBmt^1, Abu^2, MeGly^3, MeLeu^4, MeLeu^6, Ala^7, D-Ala^8, MeLeu^9, and MeLeu^{10}. FR901459 (**54**) is [Thr^2, Leu^5, Leu^{10}]-CsA, and SDZ 214-103 (**3**) is [Thr^2, Leu^5, D-Hiv^8, Leu^{10}]-CsA. Fold differences are based on IC_{20} values.

weaker than [MeVal^4]-CsA (**22**). In the case of [Thr^2, Val^4]-CsA (**46**), the replacement of MeLeu^4 by a non-methylated Val^4 residue also resulted in an inactive analogue, but the possible impact of the smaller side chain is unknown.

***N*-DesMe^6**. Both N^6-desmethylated cyclosporins ([Leu^6]-CsA [**39**] and [Nva^2, Leu^6]-CsA [**50**]) showed definitely weaker FPR-InhA than their parent compounds, from a loss of detectable activity for the CsA analogue to a 3.1-fold decreased one for the [Nva^2]-CsA one.

***N*-DesMe^9**. While [Leu^9]-CsA (**40**) showed a near 7-fold larger FPR-InhA than compound **1**, [Nva^2, Leu^9]-CsA (**51**) showed about the same FPR-InhA as its analogue **15**.

***N*-DesMe^{10}**. There was most often a much increased FPR-InhA: 7.5-fold for [Ala^2, Leu^{10}]-CsA (**43**), 5.5-fold for [Leu^{10}]-CsA (**41**), 3.9-fold for [Nva^2, Leu^{10}]-CsA (**52**), and 2.8-fold for [Val^2, Leu^{10}]-CsA (**49**). This trend also appears from the clear FPR-InhA shown by the doubly *N*-desMe analogue [Leu^6, Leu^{10}]-CsA (**42**), while the [Leu^6]-CsA (**39**) lacks detectable activity. Curiously, no enhancing effect of N^{10}-desmethylation on the FPR-InhA was observed with [Thr^2, Leu^{10}]-CsA (**47**).

***N*-DesMe^{11}**. In comparison with their parent compounds, [Val^{11}]-CsA (**34**) showed unchanged features, while [Thr^2, Val^{11}]-CsA (**44**) showed a clearly decreased FPR-InhA (2.9-fold). This difference might be linked to the apolar Abu^2 vs polar Thr^2 difference.

2.2. FPR Inhibition by Complex *N*-DesMe^{10}, [Thr^2]-Cyclosporins. Most of these natural cyclosporins showed a large capacity to inhibit FPR function (Table 4).

These analogues have the consensus sequence cyclo-[R^1- Thr^2- R^3- R^4- R^5- MeLeu^6-Ala^7- R^8- MeLeu^9- R^{10}-MeVal^{11}]. For the broadly occurring CsC (**16**, [Thr^2]-CsA), R^1 = MeBmt^1, R^3 = MeGly^3, R^4 = MeLeu^4, R^5 = Val^5, R^8 = D-Ala^8, and R^{10} = MeLeu^{10}, but all other analogues showed an N^{10}-desmethylation. They are best compared in reference to three different cyclosporins, [Thr^2, Leu^{10}]-CsA (**47**), FR901459 (**54**), and SDZ 214-103 (**3**), as groups where cyclosporins show single residue differences.

In reference to [Thr^2, Leu^{10}]-CsA (**47**), single residue differences showed different impacts on FPR-InhA: unchanged by an *N*-methyl on the Leu^{10} residue (**16**), increased over 2-fold by a D-Hiv^8 residue (**55**) instead of D-Ala^8, but largely (10-fold) increased by a larger fifth residue side chain, Leu^5 in FR901459 (**54**) instead of Val^5.

In reference to FR901459 (**54**), single residue differences had a markedly different impact on the FPR-InhA. Thus, besides its large decrease caused by the smaller fifth residue side chain Val^5 (**47**), a large (4-fold) decrease resulted from the smaller tenth residue side chain Ala^{10} (**56**) instead of Leu^{10}. In contrast, replacement of the D-Ala^8 residue (**54**) by D-Hiv^8 (**3**) did not affect FPR-InhA.

In reference to SDZ 214-103 (**3**), a few analogues showed single residue alterations: 8′-OH-MeBmt^1, Gly^3, Leu^4, Val^5, Ile^5, or D-Ala^8. While D-Ala^8 (**54**) and D-Hiv^8 (**3**) conferred the same large FPR-InhA, both the N^3- and N^4-desmethylations favored FPR-InhA expression, as shown for [Gly^3]-SDZ 214-103 (**58**) and for [Leu^4]-SDZ 214-103 (**60**). The [Val^5]-SDZ 214-103 (**55**) and [Ile^5]-SDZ 214-103 (**59**) were, respectively, 4.7- and 1.5-fold less active for FPR inhibition than the reference Leu^5-using compound **3**. Finally, a 2.5-fold FPR-InhA decrease resulted from 8′-hydroxylation of MeBmt^1 (**57**).

Discussion

The most potent FPR inhibitory cyclosporin, CsH (**2**), lacks IM activity and shows only modest Pgp-InhA. CsA (**1**), SDZ 214-103 (**3**), and FR901459 (**54**) display both very large IM activity,[1,8,22] and Pgp-InhA,[9] but while CsA (**1**) shows little FPR-InhA, both SDZ 214-103 (**3**) and FR901459 (**54**) are potent FPR inhibitors. In the free form, SDZ 214-103 and FR901459 display very similar conformations,[12,22] which are largely different from the CsA one, while CsH shows a unique conformation, which is largely different from all others. Therefore, cyclosporins that show largely different shapes in the free form may nonetheless achieve large FPR-InhA levels.

1. Relevance of Free Cyclosporin Conformation to FPR Inhibition. As recalled in the companion

paper,[9] cyclosporins can adopt a variety of conformations: a hydrophobic environment will favor a conformation maximizing the intramolecular hydrogen bonds, whereas a polar environment will favor a conformation shielding the *N*-methyl groups from the solvent. Moreover, the cyclosporin SAR for IM activity and for Pgp-InhA do not only reflect their binding to, respectively, CyP and Pgp but also depend on other structural features, which may control their distribution within the cells.

In principle, an interaction between a cyclosporin and an FPR molecule might be visualized as the simple binding of the cyclosporin molecule diffusing from the medium to the FPR molecule on the PM cell surface; in this case, the shape of the cyclosporin molecule when it binds might be the one achieved in free solution, with its intramolecular H-bonding-determined structural features. Therefore, for what concerns inhibition of FPR function, cyclosporin features such as hydrophobicity and plasticity (capacity to undergo conformational changes) should have less impact than for other activities, which require the deep penetration of the cyclosporin within the cell PM such as for Pgp inhibition[9] or its PM crossing such as for IM activity.[1]

Yet, features of the cyclosporins already discussed in the companion paper[9] may obviously influence their FPR-InhA on intact cells. The overall FPR-InhA of any cyclosporin may depend not only on its intrinsic affinity for some pharmacophore in or on the FPR molecules but also on the microbioavailability of cyclosporins in the adequate FPR-containing PM microdomain. Moreover, the free amide N−H and free carbonyl C═O known to pair within the cyclosporin molecule in aqueous solution might not do so when the cyclic compound gets in touch with the cell PM. These free groups may rather find other binding partners among various cell PM components, and the switch from intramolecular H-bonds to intermolecular ones may seriously impair any attempt to extrapolate cyclosporin structure in any solvent to an FPR bound structure. This may be particularly striking in the case of *N*-desMe analogues. Many cyclosporins may thus be "unspecifically" prevented to reach the current target (FPR) or specifically favored for binding to it, although this may occur through molecular interactions that could not be predicted from their conformation in any solvent. The discovery of a unique cyclosporin analogue that shows in solution the CyP binding conformation was not a finding leading to the final mechanism of IM activity but some kind of a posteriori search for such a conformer after the resolution of the CyP-bound cyclosporin conformation.[27]

Thus far, studies on the inhibition of FPR function by cyclosporins only show that a variety of alterations that affect either the backbone structure or flexibility or the cyclosporin surface, or both, may nevertheless influence the occurrence of the cyclosporin−FPR encounters in the PM and the more-or-less good fit of cyclosporin−FPR binding. Only when knowledge of the three-dimensional (3D) structure and dynamics of the FPR molecule within its physiological membranous microenvironment will be known, will it be possible to integrate the present cyclosporin−FPR-InhA SAR into cyclosporin−FPR binding data.

2. CsH Case. Early studies on the inhibitory effects of cyclosporins on various responses of FPR-expressing cells to formylpeptides consistently showed the much larger potency of CsH (**2**), which shows the substitution of L-MeVal[11] by a D-MeVal[11], in comparison to other cyclosporins (CsA, [Ala[2]]-CsA, [Thr[2]]-CsA, and [Val[2]]-CsA),[16−18] as well as their selectivity for the FPR in relation to other chemoattractant receptors.[17,18,20] Their inhibition of agonist binding was at the time interpreted as antagonism, and the most active cyclosporin (CsH) was logically taken as the most potent and selective competitive antagonist for formylpeptide binding by the human FPR.[17,18] With the newly emerging concepts of inverse agonism (i.e., the capacity of some inhibitors to recruit and stabilize 7TM-GPCR in an inactive conformation, therefore to inhibit even the fraction of constitutively active receptors),[28] a reevaluation of data and further experiments led to the demonstration that CsH at least was an inverse agonist rather than a neutral antagonist or a partial agonist.[19]

While devoid of IM activity,[1−3] and displaying only a low Pgp-InhA,[9] CsH (**2**) actually shows the largest FPR-InhA.[16−21] In early assays with an 100 nM f-MLF concentration to induce the FPR-mediated response (not shown), CsH consistently showed a more than 90-fold larger inhibitory capacity than CsA (**1**). In the present assays with a 30 nM f-MLF concentration, the CsH was a 120-fold more potent FPR inhibitor than CsA. This larger potency could reach up to 180-fold, depending on experimental conditions, among which the neutrophilic HL-60 cell differentiation (not shown).

This large FPR-InhA of CsH (**2**) must be due to favored interactions with the FPR but of which kind? Although early studies of CsH actually showed complicated NMR spectra indicating possibly up to seven peptide ring conformers,[5] one hypothesis could privilege the very special shape of CsH. More specifically, the L- to D-epimerization of MeVal[11] has two structural consequences (Figure 1), the first one being a drastic distortion of the ring conformation[25,26] and the second one being a modification of the orientation of the MeVal[11] side chain,[5,26] either of which could account for its lack of IM activity and its lack of antifungal activity.[1,4,25,26] For instance, in comparison with CsA (**1**) and other natural [L-MeVal[11]]-using analogues, the markedly different conformation of CsH (**2**) might change its capacity to cross the cell PM and/or to glue calcineurin to CyP, resulting in a lack of IM activity.[1−3] As shown in the companion paper, CsH could inhibit, although moderately, Pgp function.[9] Yet, CsH should be able to bind to and to partition in the cell PM to interact with the FPR as it would be less hydrophobic but also less folded than CsA. Another hypothesis for the large FPR-InhA of CsH would be that the D-MeVal[11] itself might be directly involved as a contact residue conferring better fit of CsH into a cyclosporin binding pocket of the FPR. Unfortunately, no other [D-MeVal[11]] analogues were available to approach SAR for the CsH−FPR interaction. Thus, despite its highest FPR-InhA and its very peculiar 3D conformation, the inverse agonist CsH does not help to understand what structural features bring its very large FPR-InhA.

Besides the unique CsH analogue, the available data for SAR analyses essentially belong to three catego-

ries: (i) alterations of residue side chains of classical cyclosporins, most of which may share a common, CsA-like backbone conformation; (ii) *N*-desmethylation of various residues, which may lead to many different cyclosporin conformations; and (iii) alterations of residue side chains of N^{10}-desMe cyclosporins, most of which may share another common conformation.

3. SAR for Cyclosporins with the Classical Conformation. The classical cyclosporins may be defined as using L-MeVal as the eleventh residue and showing *N*-methylation of all residues 1, 3, 4, 6, and 9−11. Their gross 3D conformation is like the one of CsA (**1**), with differences essentially due to the size, shape, and orientation of the residue side chains. That classical cyclosporin 3D conformation differs from both the CsH one and the N^{10}-desMe cyclosporin one. For classical cyclosporin conformers, a rather large panel of residue side chain variations could be explored for FPR-InhA. There was no simple rule such as the larger or the more hydrophobic side chain, the better inhibition, as was found for Pgp inhibition.[9] Besides the low to very low FPR-InhA of most classical cyclosporins, a substantial FPR-InhA was seldom found for some of them, the most active ones remaining 3−10-fold less potent than CsH (**2**). Some FPR-InhA differences might depend more on alterations of some cyclosporin side chain exposure than on a structurally altered backbone, as discussed in the following impact analysis for FPR-InhA of side chain variants of residues 11 through 8, none being available for residues 9 and 10.

Eleventh Residue. Ala (**6**) ≥ aIle (**4**) ≫ Ile (**5**) ≥ Val (**1**). The [aMeIle^{11}]-CsA (**4**) and [MeAla^{11}]-CsA (**6**) belong to the most active among classical cyclosporins, being about 10-fold more active than [MeIle^{11}]-CsA (**5**) and CsA (**1**, with MeVal^{11}), and approaching levels of FPR-InhA more commonly found with N^{10}-desMe cyclosporins. Because a large FPR-InhA might depend on the orientation of a large eleventh residue side chain (aMeIle^{11}), although being achieved as well by a small side chain (MeAla^{11}), this might suggest steric hindrance of MeIle^{11} for FPR binding.

First Residue. Cyclohexyl-Ala (**10**) = Aoa (**8**) > deoxy-Bmt (**7** and **28**) > Bmt (**1**) ≫ Leu (**9**, **29**, and **30**) = cyclized-Bmt (**11**) = 8′-OH-Bmt (**12**). Special attention was given to MeBmt^1 because of its highly restrictive occurrence in cyclosporins, its 3′-OH function, and its very large side chain. Its 8′-hydroxylation (**12**) was deleterious for the FPR-InhA, as was also its replacement by a MeLeu^1 residue (**9**, **29**, and **30**), but the loss of the free 3′-hydroxyl group was not responsible for the activity loss since some deoxy-MeBmt^1 and MeAoa^1 analogues of CsA (**7** and **8**) showed increased FPR-InhA. With the exception of **12**, which is a side chain tail alteration, all other MeBmt^1 cyclosporin analogues (**7**−**11**, **29**, and **30**) do not provide the crucial 3′-OH function required for CyP binding. A consequence of their lack of sequestration on CyP could be their larger cellular bioavailability for interaction with FPR. However, this is obviously not sufficient to confer them a larger FPR-InhA, some analogues being more inhibitory than the MeBmt^1 cyclosporins and some others being less inhibitory! The [8′-OH-MeBmt^1]-CsA (**12**) was of special interest as it occurs in vivo in CsA-treated human beings, as an early and major CYP3A metabolite (AM1) devoid of IM activity and of nephrotoxicity.[1] The presence of a free hydroxyl moiety at the tail of the long MeBmt^1 side chain (like in AM1) may impair its binding to the FPR molecule. Although this could also apply to the 3′-hydroxyl moiety of MeBmt^1, the latter might not play a direct role in FPR binding. In fact, MeAoa (**8**) confers a much larger (over 6-fold) FPR-InhA than MeLeu (**9**), and since two out of three MeLeu^1 analogues (**9**, **29**, and **30**) were less potent than their MeBmt^1 analogues, the most obvious finding is that the presence of a large first residue side chain favors the inhibitory interaction with the FPR molecule. This is also in line with the larger potency of [Me-cyclohexyl-Ala^1]-CsA (**10**) in comparison to CsA (**1**, MeBmt^1). Together, the data discard the direct involvement of the 3′-hydroxyl function of the Bmt^1 residue in FPR binding, and they suggest that the presence of a large first residue side chain may favor the interaction.

Second Residue. Ala (**13**) > Thr (**16**) > Nva (**15**) > Abu (**1**) = Val (**14**). FPR-InhA was thus influenced by the occurrence of polar side chains as well as by the size of nonpolar side chains. Nevertheless, with the exception of an obviously larger FPR-InhA for [Ala^2]-CsA (**13**), the range of FPR-InhA remains too narrow to draw firm conclusions. Moreover, the aforementioned ranking is only valid for unmodified [MeBmt^1]-using classical cyclosporins: for those using deoxy-MeBmt^1, the FPR-InhA ranking is now Abu (**7**) ≫ Val (**28**), while for those using MeLeu^1, it is Val (**29**) > Nva (**30**) > Abu (**9**). Thus, each residue impact may depend on the cyclosporin context, i.e., on the nature of some other residues.

Third Residue. Gly (**1**) ≫ Pro (**18**) = Phe (**17**). There was an obviously larger FPR-InhA of the naturally occurring, side chain-lacking residue, since the other residues with large side chains lacked any detectable activity. However, such substitutions do more than change the residue side chain. The replacement of the flexible MeGly^3 at one edge of the cyclosporin β-sheet by such residues such as L-Pro^3 or D-MePhe^3 should restrict the flexibility of the cyclic peptide and change its conformer equilibrium. An alteration of the cyclosporin backbone may be evoked in the MeGly^3 to L-Pro^3 substitution case, as it would both prevent the formation of the type II′ β-turn and reduce cyclosporin flexibility.[5] The lack of inhibitory activity by substituting MeGly^3 by D-MePhe^3 may come both from conformational alterations of the backbone, larger hydrophobicity of that cyclosporin domain, and steric hindrance to a good binding fit in the FPR. Thus, inadequate PM localization or impaired binding to FPR might account for the lack of detectable FPR inhibition.

Fourth Residue. Ile (**20**) > Val (**22**) > γ-OH-Leu (**21**) > Leu (**1**) ≥ Phe (**23**). This ranking suggested some involvement of the fourth side chain in FPR binding, possibly with restrictions of side chain size and/or orientation. Because all of these classical cyclosporin conformers can bind CyP but only one (CsA, with a MeLeu^4) can then sequester calcineurin, there is an obvious lack of relation between the latter binding activities and the FPR inhibition.

Fifth Residue. The rankings Nva (**31**) ≫ Val (**15**) > Leu (**32**) for [Nva^2] analogues, and Ile (**33**) ≫> Val (**16**) for [Thr^2] analogues suggested that not only the size but also the shape of the fifth residue side chain was

important for the interaction with FPR. It is intriguing that these FPR-InhA differences come from combined differences at the levels of second and fifth cyclosporin residues, which are known to be involved in intramolecular H-bonding in the free state. The *Hypoderma eucalyptii*-produced [Thr^2, Ile^5]-CsA (**33**) shows the highest FPR-InhA potential among classical cyclosporins, larger than the best residue 11 analogues (**4**, [aMeIle^{11}]-CsA, and **6**, [MeAla^{11}]-CsA), and at least as potent as the best N^{10}-desMe cyclosporins, although remaining less potent than CsH (**2**).

Sixth Residue. Leu (**1**) ≫ Ala (**24**). Loss of detectable FPR-InhA by use of a smaller side chain suggests the need for a large hydrophobic side chain for FPR binding.

Seventh Residue. Ala (**1**) ≫ Abu (**25**). A larger side chain markedly decreased (≥2.8-fold) FPR-InhA, possibly indicating steric hindrance in binding to FPR. If not, this minor variation in [Abu^7]-CsA either disorders or reinforces the γ-turn structure formed by the H-bond between the free amide of D-Ala^8 and the carbonyl of MeLeu^6.

Eighth Residue. Lys (**26**) ≫ Ala (**1**) > Ser (**27**). In CsA (**1**), the D-Ala^8 is at the center of at least one H-bond involving the amide proton of D-Ala^8 and the carbonyl of the MeLeu^6. Replacement of D-Ala^8 or D-Ser^8 by the longer and more polar D-Lys^8 leads to an obviously larger FPR-InhA, which does not necessarilly indicate a more efficient fit to the FPR. With the obviously less hydrophobic [D-Lys^8]-CsA (**26**), the long and positively charged Lys^8 side chain might rather unspecifically increase FPR-InhA, by favoring the cyclosporin adsorption onto the extracellular cell surface and impairing its cell PM crossing, while allowing its insertion into the exoplasmic PM leaflet and favoring encounters with FPR extracellular moieties.

In conclusion, whether the combination of each best residue in a [MeAoa^1, Ala^2, MeIle^4, Ile^5, D-Lys^8, MeAla^{11}]-CsA would give the largest FPR-InhA is speculation only. Indeed, each residue impact on the FPR-InhA may depend on the cyclosporin context, i.e., on the nature of some other residues. Nevertheless, the observed changes of FPR-InhA caused by polar vs nonpolar and by shorter vs longer side chains essentially showed a distribution of the important residues all around the cyclosporin molecule. This suggests that a large fraction of the classical cyclosporin conformer surface must show a particular shape to achieve a best fitting interaction with the FPR molecule in order to inhibit its function. Thus, most of the classical cyclosporin residue side chains might be involved as contact residues with the human FPR. The cyclosporin may not be "adsorbed" onto the surface of the FPR but deeply inserted within its TM−extracellular interface. The L- to D-epimerization of residue 11 in CsH (**2**) might have more impact on the backbone conformation and plasticity for the cyclosporin insertion in the intramembranous portions of the FPR molecules than on the cyclosporin surface for interaction with their extracellular portions. However, the large conformational differences between CsH and classical cyclosporins may also suggest that they would not inhibit FPR function through the same pharmacophore or even through the same mechanism. Thus, they might bind to different conformers of the FPR, CsH working as inverse agonist through a large affinity for inactive FPR conformers, whereas the other classical cyclosporins with a CsA-like conformation might simply work as neutral agonists (competitive antagonists) for formylpeptide binding.

4. General Impact of the *N*-Desmethylation on FPR-InhA. Single or combined residue *N*-desmethylation is known to occur naturally at residues 1, 3, 4, 6, or 9−11 for cyclosporins produced by various fungi.[6,7] Such selective lack of residue *N*-methylation during cyclosporin biosynthesis[4,6] may also occur during in vivo cyclosporin metabolism in man.[1] Although the impact of any selective *N*-desmethylation for the intramolecular H-bonding potential and folding of such cyclosporins in their free form is either known or can be modeled, its impact on the intermolecular H-bonding potential with FPR molecules and other PM proteins can only be speculative. Their unclassical conformations may account for the low or undetectable IM activity shown by most *N*-desMe cyclosporins.[1,2] Conformational alterations caused by *N*-desmethylation may also account for the generally marked decrease of Pgp-InhA reported in the companion paper[9] and for the more variable effects of selective residue *N*-desmethylation on the FPR-InhA. Indeed, *N*-desmethylation may largely affect the cyclosporin FPR-InhA, the SAR disclosing that selective *N*-desmethylation of MeLeu^4, MeLeu^6, or MeVal^{11} generally decreased FPR-InhA, while selective *N*-desmethylation of MeBmt^1, MeGly^3, MeLeu^9, or MeLeu^{10} generally increased FPR-InhA. A simple hypothesis would be that the N^1-, N^3-, N^9-, and N^{10}-methyl groups of cyclosporins may impair binding to FPR because of steric hindrance, whereas the N^4-, N^6-, and N^{11}-methyl groups would improve those cyclosporin fits into the FPR pharmacophores.

However, the residue selective *N*-desmethylation may also introduce new constraints within the cyclosporin molecule with novel intramolecular H-bonding opportunities participating in various alternative twists of the backbone, which are presumably reflected by favorable or deleterious conformational changes in the cyclic peptides. It may also allow novel intermolecular H-bonding opportunities, allowing alternative interactions with other molecules (solvent, receptors, and transporters) with unpredictable consequences for the cyclosporin activity on the single human *FPR1*-encoded 7TM-GPCR tested. Nevertheless, because all studied *N*-desMe cyclosporins are naturally occurring fungal products, such *N*-desMe analogues may be actually relevant to the yet unknown normal biological functions of cyclosporins for fungi. These effects of *N*-desmethylation for FPR-InhA are now further discussed.

4.1. FPR-InhA-Decreasing *N*-Desmethylations. The N^4-, N^6-, and N^{11}-methyl groups of cyclosporins may be directly involved in interactions with FPR. Loss of FPR-InhA by *N*-desmethylation at residues 4, 6, or 11 suggests that these *N*-methyl moieties are either involved in the FPR binding as contact residues with the FPR molecule or prevent the occurrence of cyclosporin conformations inadequate for FPR binding.

Fourth Residue. The N^4-desmethylation of classical cyclosporins, already known to abrogate both IM activity and Pgp-InhA,[2,9] led to a decreased FPR-InhA (**36**−**38** and **46**). Analogues with a free amide on residue 4 were of special interest, since the naturally occurring cy-

closporin [Leu[4]]-CsA (**36**, AM4N) is also an early and major metabolite of CsA in transplant patients.[1,6,7] The metabolization of CsA (**1**) into AM4N (**36**) in man is fast,[1] suggesting that the *N*-methyl moiety of the fourth residue of the cyclosporin must be exposed in the cytosol or on the endoplasmic reticulum for cleavage by the cytochrome P450 enzyme (CYP3A). This exposure of the N^4-methyl in the physiological conditions of the cytosol suggests that it may also be similarly exposed in other aqueous environments such as might occur on the outer cell surface at the time of potential interactions with the FPR. Because this N^4-desmethylation decreases FPR-InhA, the N^4-methyl may confer substantial binding energy of cyclosporin to the FPR. Nevertheless, this latter speculation cannot be extended to the N^{10}-desMe cyclosporin conformers, whose single known *N*-desMe[4] analogue, [Leu[4]]-SDZ 214-103 (**60**), did not show a decreased FPR-InhA.

Sixth Residue. The N^6-desmethylation of classical cyclosporins, reported to largely decrease both IM activity and Pgp-InhA,[2,9] also led to a largely decreased capacity to inhibit FPR function (**39** and **50**). This suggests either a direct involvement of the cyclosporin N^6-methyl in binding to the FPR or a function for a free amide on residue 6 in establishment of novel intramolecular constraints. However, this would not apply to the N^6-desmethylation of a N^{10}-desMe cyclosporin, as it increased FPR-InhA (**42**).

Eleventh Residue. The N^{11}-desmethylation of classical cyclosporins leads to analogues with little IM activity,[2] presumably because of considerable conformational changes in the loop fragment due to an additional H-bond between the L-Val[11] amide proton and the D-Ala[8] carbonyl group.[4,5] Yet, when tested for their capacity to inhibit Pgp function, two tested [*N*-desMe[11]] analogues showed unchanged to clearly decreased Pgp-InhA depending on the nature of the second residue.[9] Similarly, a largely decreased capacity to inhibit FPR function was found here only in the polar Thr[2] context (**44**) and not in the apolar Abu[2] one (**34**).

4.2. FPR-InhA-Enhancing *N*-Desmethylations. Enhanced FPR-InhA resulted from lack of N^1-, N^3-, N^9-, or N^{10}-methyl, the N^{10}-desMe cyclosporins being particularly prone to display a large FPR-InhA. Generally, either these *N*-methyl moieties sterically interfere with binding to FPR molecule or they prevent the occurrence of cyclosporin conformations adequate for FPR binding.

First Residue. The MeBmt[1] residue is the most typical cyclosporin residue. Its *N*-desmethylation led to a moderate but clear increase of FPR-InhA in all three tested [Bmt[1]] analogues. [Bmt[1]]-cyclosporins also retained their IM activity in vitro,[2] but N^1-desmethylation caused from a slight increase to a clear decrease of Pgp-InhA, depending on the second residue.[9] The free amide on Bmt[1] might be involved in intermolecular interactions with FPR or allow novel intramolecular H-bonding patterns and conformations favoring FPR binding.

Third Residue. The MeGly[3] residue is most conserved in cyclosporins; the FPR-InhA was substantially increased by N^3-desmethylation, although this naturally occurred only in combination with the substitution of D-Ala[8] either by D-Ser[8] (**53**) or by D-Hiv[8] plus an N^{10}-desmethylation (**58**). Because the free Gly[3] amide is located at one "edge" of the cyclosporin molecule opposite to the D-Ser[8], D-Hiv[8], and/or Leu[10] residue side, the obvious 3D conformations of CsA and SDZ 214-103 might remain unchanged in that domain, although with altered flexibility. As the C═O and the N−H of β-turns are often implicated in intermolecular interactions, an alteration of the local peptidic surface that provides the contact residues for binding with the FPR may be suggested: the available free Gly[3] amide would provide a better fit of the cyclosporin into an FPR pharmacophore (moreover, as earlier seen, third residues with bulky side chains abrogated FPR-InhA).

Ninth Residue. The N^9-desmethylation largely increased FPR-InhA of one analogue (**40**) but did not change the FPR-InhA of another (**51**). This enlarged or unchanged FPR-InhA is in contrast with the complete loss of Pgp-InhA[9] and the marked decrease of IM activity,[2] brought by N^9-desmethylation. The MeLeu[9] residue is localized at the most extreme end of the loop formed by the amino acids 7−11, and the N^9-desmethylation might provide a free proton available for interaction with another molecule.

Tenth Residue. Numerous naturally occurring N^{10}-desMe cyclosporins are produced by *Stachybotris chartratum* (**54**), *Acremonium luzulae* (**56**), *Cylindrotrichum oligospermum* (**3**, **55**, and **57**−**59**), and *Tolypocladium inflatum* (**41**−**43**, **47**, **49**, and **52**). With regards to other biological activities, the N^{10}-desmethylation of classical cyclosporins leads to analogues with clearly decreased Pgp-InhA[9] but to a variable decrease of IM activity ranging from moderate (**41**) to medium (**52**) to marked (**49**), depending on the nature of the second residue.[2] Where *N*-Me analogues were known, a largely enhancing effect of N^{10}-desmethylation on the FPR-InhA was obvious (**41**−**43**, **49**, and **52**), with one exception (**47**). Therefore, sharing a potential to form a [O^7- - -N^{10}] bond leading to the occurrence of a type II′ β-turn in the cyclic peptide (see below) may actually represent a crucial element for its interaction with the FPR molecule. In view of the generally large impact of the N^{10}-desmethylation on the cyclosporin conformation, the effects of the few other residue side chain variations on the FPR-InhA of N^{10}-desMe cyclosporins will be analyzed specifically.

5. Focus on N^{10}-DesMe Cyclosporins, Particularly SDZ 214-103 Analogues. The reference compound SDZ 214-103 (**3**) is cyclo-[MeBmt[1]-Thr[2]-MeGly[3]-MeLeu[4]-Leu[5]-MeLeu[6]-Ala[7]-D-Hiv[8]-MeLeu[9]-Leu[10]-MeVal[11]], which shows a high IM activity like CsA (**1**) and [Thr[2]]-CsA (**16**).[1,2,8] It was found to be very efficient at inhibiting the FPR-mediated signaling, being much more potent than CsA and [Thr[2]]-CsA (18- and 11-fold, respectively [by IC_{20} comparisons]) although remaining less active than CsH (8- to 4-fold by, respectively, IC_{20} or IC_{50} comparisons). Yet, it differs from CsH (**2**) by five residues:[8] Thr[2] instead of Abu[2], Leu[5] instead of Val[5], D-Hiv[8] instead of D-Ala[8], Leu[10] instead of MeLeu[10], and L-MeVal[11] instead of D-MeVal[11]. While the Thr[2] and Leu[5] variations and an N^{10}-desmethylation also occur, either individually or in combination, in natural cyclosporins, particularly in the *Stachybotris*-produced FR901459 (**54**) and the *Acremonium*-produced [Thr[2], Leu[5], Ala[10]]-CsA (**56**),[6,22] the D-2-hydroxy-isovaleric acid D-Hiv[8] residue, which introduces an ester bond, is in a

cyclosporin context the major chemical peculiarity of all the *Cylindrotrichum*-produced SDZ 214-103 (**3**) analogues.

As recalled in the Introduction (Figure 2) and in the companion paper,[9] not only the overall shape of SDZ 214-103 may differ from the CsA one but also the cyclopeptolide conformation may be more flexible. The natural [D-Ala^8]-using analogue, which is closest to SDZ 214-103 (**3**), is FR901459 (**54**).[22] Its reported conformation in solution looks similar to the SDZ 214-103 one,[22] despite the lack of repulsive interaction between the ester oxygen of the D-Hiv^8 and the carbonyl of the MeLeu^6 found in the latter. This potential dominance of the N^{10}-desmethylation on the conformation might be extended to other N^{10}-desMe analogues of cyclosporins, which might also show a [O^7- - -N^{10}] H-bond-driven type II′ β-turn. However, other conformational features of SDZ 214-103 (**3**) and FR901459 (**54**) might also be influenced by the occurrence of a Leu^5 residue instead of a Val^5 one. Although the overall shape of FR901459 (free conformation) is similar to the SDZ 214-103 one, the cyclopeptolide conformation may be more flexible than FR901459. Indeed, the potential formation of an [N^8- - -O^6] H-bond might account for some FR901459 conformers with the restricted flexibility of classical cyclosporins (but whether the [N^8- - -O^6] H-bond actually occurs in a conformation disturbed by the N^{10}-desmethylation is unclear).

As N^{10}-desmethylation results in a large conformational change of the whole molecule, the impact of the different residue substitutions in this conformational context could not be correlated with the same residue alterations in cyclosporins with the classical, CsA-like conformation. Here, however, replacement of the typical MeBmt^1 by a MeLeu^1 as found in classical cyclosporins[4] does not occur among natural N^{10}-desMe cyclosporins, neither are deoxy- nor *N*-desMe-Bmt^1 analogues found.[6] Also not available among N^{10}-desMe cyclosporins were other first, third, fourth, sixth, seventh, or eleventh residue side chain variants. Particularly lacking are variants, such as with D-MeVal^{11}, L-aMeIle^{11}, or L-MeAla^{11}, which had high impact on classical cyclosporin FPR-InhA. However, N^{10}-desMe cyclosporins provided fifth residue variants (Ile^5 or Leu^5 instead of Val^5) and an [8′-hydroxy-MeBmt^1] analogue like the classical N^{10}-Me analogues, while in contrast to the latter, they provided a tenth residue side chain variant (Ala^{10} instead of Leu^{10}) and a peculiar eighth residue variant (D-Hiv^8 instead of D-Ala^8). Although on the basis of few analogues only, comparisons of the SAR of N^{10}-desMe cyclosporins and of N^{10}-Me cyclosporins suggest possibly different impacts of residue side chain alterations on FPR-InhA.

First Residue. For the typical MeBmt^1, hydroxylation naturally occurs at the terminal methyl group (**57**, [8′-hydroxy-MeBmt^1]-SDZ 214-103),[6] as can be found in in vivo-generated analogous metabolites of cyclosporins.[1] Even though the 8′-OH-Bmt (**57**) maintained a large FPR-InhA, it was 2.3-fold lower than the one conferred by Bmt (**3**), in line with the loss of activity shown by [8′-OH-MeBmt^1]-CsA (**12**) metabolite found in transplant patients.[1] Thus, the insertion of a polar radical by hydroxylation at the tip of the MeBmt^1 side chain impairs the capacity of the cyclic peptide to interact with FPR conformers. No N^{10}-desMe analogues were available to assay other first residue features critical for N^{10}-Me cyclosporins FPR-InhA (such as lack of free 3′-hydroxyl group or the occurrence of a large side chain).

Second Residue. In contrast to N^{10}-Me cyclosporins where the ranking order of FPR-InhA was Ala (**13**) > Thr (**16**) > Nva (**15**) > Abu (**1**) = Val (**14**), here it was Ala (**43**) > Abu (**41**) = Nva (**52**) ≫ Val (**49**) > Thr (**47**) for N^{10}-desMe analogues. This different requirement for the second residue side chain strongly suggests largely different modes of interaction of FPR with classical cyclosporins or with N^{10}-desMe analogues, either binding different pharmacophores on a unique FPR conformer or recognizing different FPR conformers.

Fifth Residue. At variance with N^{10}-Me cyclosporins, where the fifth residue variants ranked for FPR-InhA as Nva (**31**) ≫ Val (**15**) > Leu (**32**) for [Nva^2] analogues, and as Ile (**33**) ≫ Val (**16**) for [Thr^2] analogues, their ranking order was Leu (**3**) > Ile (**59**) ≫ Val (**55**) for the N^{10}-desMe analogues. Thus, like for residue 2, this suggests the probable involvement of different FPR pharmacophores or conformers for these analogues and for classical cyclosporins. In the free state of both cyclosporin types, whose leads are compounds **3** and **1** (with a similar H-bonding in **15**), the second and fifth residues are involved in intramolecular H-bonding, yet differently in the different conformations achieved (see Figure 2); one [N^2- - -O^5] H-bond present in **1** or **15** is absent in **3**.[9] Therefore, such different conformers require different residue 2 and 5 side chains for a large FPR-InhA. Nevertheless, the similar ranking Ile ≫ Val for both N^{10}-Me (**33** ≫ **16**) and N^{10}-desMe (**59** ≫ **55**) forms of [Thr^2] cyclosporins (although different ones) might also suggest that the Thr^2 side chain rather than the difference of H-bonding has a high impact on the suitability of the cyclosporin shape for its interaction with the FPR.

Eighth Residue. In the N^{10}-desMe analogues, the larger size of the Hiv side chain than the Ala one does not change the FPR-InhA: Ala (**54**) = Hiv (**3**), possibly suggesting their lack of direct involvement in FPR binding. The D-Hiv^8-using SDZ 214-103 (**3**) and D-Ala^8-using FR901459 (**54**) displayed large FPR-InhA similar enough to suggest that their capacity to interact with FPR might essentially come either from a large occurrence of the right conformers or from a similar peculiar display of contact radicals with the FPR molecules (none of which would be contributed by the eighth side chain). Unfortunately, other residue 8 variants of classical cyclosporins (such as D-Lys, which had high impact on FPR-InhA) were not available as N^{10}-desMe cyclosporins. Similarly, the replacement of D-Ala^8 by the larger D-Hiv^8 residue (as found in SDZ 214-103 analogues) or by a D-Val^8 or larger residue did not occur in classical (N^{10}-Me) cyclosporin conformers to evaluate its impact on their FPR-InhA.

Tenth Residue. Comparisons of all available pairs of cyclosporin analogues with MeLeu^{10}, Leu^{10}, or Ala^{10} residues suggest that the occurrence of the Leu^{10} residue may be the most critical element for a large FPR inhibition. This might be due both to its *N*-desmethylation and to its large side chain: Leu^{10} generally brought a larger FPR inhibition than MeLeu^{10}, while

Ala[10] brought a lower FPR inhibition than Leu[10] in the single case studied (**56** vs **54**).

Additional N^3- or N^4-Desmethylation. Both most potent N^{10}-desMe analogues (**58** and **60**) were *C. oligospermum*-produced compounds that showed an additional free amide on residues 3 and 4, respectively. The [Gly[3]]-SDZ 214-103 (**58**) and [Leu[4]]-SDZ 214-103 (**60**) were about 1.5-fold more active than the parent compound **3**.

6. On the Mechanism(s) of FPR Inhibition by Cyclosporins. How cyclosporins do inhibit FPR function is complex as it must account for potent FPR-InhA by widely different structures. Large FPR-InhA levels were indeed shown by a few classical cyclosporins (**4**, **6**, **20**, **26**, **31**, and **33**), by a few analogues with simple *N*-desmethylation—one N^9-desMe one (**40**) and four N^{10}-desMe ones (**41**−**43** and **52**)—and by most complex N^{10}-desMe [Thr[2]] cyclosporins (**3** and **55**−**60**). Particularly, compounds **33**, **58**, and **60** are approaching the large FPR-InhA shown by CsH (**2**), although remaining 2−10-fold less potent (depending on whether comparisons are made on bases of IC_{20} or IC_{50} values). Because CsH may not be naturally produced by the fungi but may result from an artifactual epimerization of CsA during the extraction procedure,[6] compounds **33**, **58**, and **60** are thus possibly the most potent naturally occurring cyclosporins known.

The present SAR are obviously limited to establishment of a repertoire of potential interpretations and cannot exclude the possibility that different cyclosporins might act at different levels of the multicomponent FPR-mediated signaling (e.g., ligand to receptor binding, receptor to G-protein coupling, G-protein function itself), which leads to the fully blown response (degranulation). Although data showing that cyclosporins inhibit the binding of formylpeptides to the FPR[16−18] were reported for only few analogues and might not be extrapolated to all, there are no data against the FPR itself being the common target of every inhibitory cyclosporin.

While CsH (**2**) differs by three residues only from the classical cyclosporin (**33**) ([Thr[2], Ile[5]]-CsA), the N^{10}-desMe compounds **58** and **60** ([Thr[2], Gly[3], or Leu[4], Leu[5], D-Hiv[8], and Leu[10]]-CsA) differ by six residues out of 11 from CsH! It is impressive how such structurally different cyclosporins may achieve about the same levels of FPR-InhA, recalling the earlier known case of shared high IM activity for classical cyclosporins and N^{10}-desMe [Thr[2]] cyclosporins. In the IM activity case, the mechanism could be interpreted as an induced fit of the different cyclosporins to the CyP and calcineurin, and it would be tempting to extrapolate the conclusion to the FPR inhibition, i.e., that all inhibitory cyclosporins, whichever their sequence and conformation, would bind to a single pharmacophore on the FPR and inhibit its function.

For such a hypothesis, the general shapes of classical cyclosporins and N^{10}-desmethylated cyclosporins may be looking closer to each other (Figure 2) than those of classical cyclosporins and CsH (Figure 1). Yet, in CsA and CsH, both structural domains of the cyclic peptide, i.e., the β-pleated sheet and the loop, can be superposed and it is only the angle between the two that is widely different in their free forms (Figure 1). However, this is possibly not so in their FPR-bound form, and one "induced fit" hypothesis could be the easier shape adaptation of CsH than CsA to the FPR pharmacophore. If not, the higher activity of CsH might correspond to a large occurrence of conformer with the right FPR binding fit in the free form, while for CsA it would be much less frequent or require an induced fit. Among classical cyclosporins, [Thr[2], Ile[5]]-CsA (**33**) would be the most prone to acquire the right FPR binding conformation.

In contrast, in the case of a comparison of classical and N^{10}-desMe analogues (Figure 2), the intramolecular H-bonding between the second and the fifth residue is not the same in the two cyclosporin classes. Although the MeBmt[1] residue 8′-hydroxylation decreased and the N^3-desmethylation increased FPR-InhA of both cyclosporin classes, different ranking for FPR-InhA was shown for the second and fifth residue side chains and opposite effects on FPR-InhA were caused by the N^4- and N^6-desmethylations in classical vs N^{10}-desMe cyclosporins. The probable reason for such differences is that these residue alterations occur on cyclopeptides with two widely different 3D conformations and that classical and N^{10}-desMe cyclosporins bind to different pharmacophores either on a common conformer or on distinct ones. If however, the binding of a cyclosporin to an FPR implies a refolding—or induced fit—of one or both molecular partners, the residue side chain preferences for classical and N^{10}-desMe cyclosporins might also represent different permissivity for such refolding of the two cyclosporin classes.

Until recently, the FPR was taken as rather selective and restricted to formylpeptides and a few unformylated ones, the origins of formylpeptides being both exogenous (microbial) and endogenous (mitochondrial).[14,15] Whether the formylpeptides are the most physiologically relevant agonists or simply surrogate agonists of this model 7TM-GPCR is currently challenged by the discovery of various other ligands, which bind and trigger cytosolic calcium transients in human FPR-bearing cells.[29−33] Therefore, the human FPR might be more promiscuous than initially thought, and this may imply the need for different domains on a single FPR conformer or for different FPR conformers to achieve specific recognition of a variety of different ligands.

According to one hypothesis, the FPRs would be maintained in an inactive conformation by an ion pair between a Lys[85] residue in the second TM α-helix and an Asp[284] residue localized in the seventh TM α-helix, near the TM−extracellular interface.[34−36] The binding of agonistic ligands (formylpeptides) might thus activate the receptor by disrupting such pairing (if this is an active process), or it would recruite FPR conformers with a disrupted ion pair (if the whole FPR population on the cell surface occurs as an equilibrium of different active and inactive FPR conformers). It could be suggested that CsH acts as an inverse agonist of FPR, i.e., by recruiting inactive FPR conformers, and that other cyclosporins with a large FPR-InhA might do so as well. However, as the primary sequences and conformations of the N^{10}-desMe cyclosporins widely differ from those of CsH and of classical cyclosporins, they might recruit different inactive FPR conformers or they might bind to different pharmacophores on the inactive FPR molecules. So long as no structural information on the

FPR−cyclosporin complexes in situ in the normal cell membrane will not be available, the molecular mechanisms of FPR inhibition by different cyclosporins will remain speculative.

Abbreviations Used

Amino acid abbreviations: Abu, α-aminobutyric acid; aIle, allo-isoleucine; aMeIle, *N*-methyl allo-isoleucine; Hiv, 2-hydroxy-isovaleric acid; MeAla, *N*-methyl-alanine; MeAoa, *N*-methyl-amino-octanoic acid; MeBmt, *N*-methyl-4-butenyl-4-methyl-threonine; MeGly, *N*-methyl-glycine (sarcosine, Sar); MeIle, *N*-methyl-isoleucine; MeLeu, *N*-methyl-leucine; MePhe, *N*-methyl-phenylalanine; MeVal, *N*-methyl-valine; N^1- − N^{11}, N^1-amide through N^{11}-amide; *N*-Me, *N*-methylated; *N*-desMe, *N*-desmethylated. Other abbreviations: 7TM-GPCR, seven TM GPCR; CsH, [D-MeVal^{11}]-CsA; CyP, cyclophilin; DMSO, dimethyl sulfoxide; f-MLF, formyl-Met-Leu-Phe; FPR, formylpeptide receptor; FPR-InhA, FPR inhibitory activity; FR901459, [Thr^2, Leu^5, Leu^{10}]-CsA; GPCR, G-protein-coupled receptor; IC_{20}, concentration giving 20% inhibition; IM, immunomodulatory; Pgp, P-glycoprotein; Pgp-InhA, Pgp inhibitory activity; PM, plasma membrane; TM, transmembrane; SAR, structure−activity relationships; SDZ 214-103, [Thr^2, Leu^5, D-Hiv^8, Leu^{10}]-CsA.

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