Articles

Cyclosporins: Structure−Activity Relationships for the Inhibition of the Human *MDR1* P-Glycoprotein ABC Transporter

Francis Loor,*,† Françoise Tiberghien,†,‡ Tom Wenandy,† Agnès Didier,† and René Traber§

Strasbourg 1 University, F-67083 Strasbourg, France, and Novartis Pharma Inc., CH-4002 Basel, Switzerland

Cyclic undecapeptide cyclo-[MeBmt[1]-Abu[2]-MeGly[3]-MeLeu[4]-Val[5]-MeLeu[6]-Ala[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-MeVal[11]], the immunosuppressive and antifungal antibiotic cyclosporin A (CsA), was reported to interfere with the *MDR1* P-glycoprotein (Pgp), a transmembranous adenosine 5′-triphosphate binding cassette (ABC) transporter with phospholipid flippase or "hydrophobic vacuum cleaner" properties that mediate multidrug resistance (MDR) of cancer cells. By use of photoaffinity-labeled cyclosporins and membranes from Pgp-expressing cells, it was recently shown that in vitro, Pgp molecules could bind a large cyclosporin domain involving residues 4−9 as well as the side chain of residue 1. Tumor cell MDR can also be reversed by a product more distantly related to cyclosporin with the structure [Thr[2], Leu[5], D-Hiv[8], Leu[10]]-CsA (SDZ 214-103). In a standardized assay that measures Pgp function in vivo (on intact live cells) by the Pgp-mediated efflux of the calcein-AM Pgp substrate and uses human lymphoblastoid MDR-CEM (VBL[100]) cells as highly resistant Pgp-expressing cells, SDZ 214-103 was found to be one of the most active Pgp inhibitors among naturally occurring cyclosporins, with an IC_{50} of 1.6 μM in an assay where CsA gives an IC_{50} of 3.4 μM. Using the in vivo assay, 60, mostly natural, cyclosporin analogues were analyzed to establish structure−activity relationships (SAR). Our SAR are compatible with the in vitro-defined Pgp binding domain model and further disclose that in vivo Pgp inhibition is favored by larger hydrophobic side chains on cyclosporin residues 1, 4, 6, and 8 and a smaller one on residue 7, although with no effect on the residue 5 side chain; moreover, larger hydrophobic side chains on other residues 2, 3, 10, and 11 (outside the in vitro-defined Pgp binding domain) also favor the eventual inhibition of Pgp function. The *N*-desmethylation of any of the seven *N*-methylated amides, as naturally occurring in numerous cyclosporins, regularly leads to a decreased Pgp inhibitory activity (Pgp-InhA), up to its abrogation if it occurs at residues 4 and 9. Nevertheless, despite unfavorable use of [Thr[2]] and [Leu[10]] residues, all [D-Hiv[8]] analogues whose lead is SDZ 214-103 show a large Pgp-InhA. The SAR for Pgp inhibition by cyclosporins are thus very complex. Because CsA and SDZ 214-103 show largely different conformations when free in solution, but remarkably similar ones when bound to the cytosolic cyclophilins, SAR for Pgp inhibition must similarly include requirements for occurrence of suitable conformers for insertion in the cell membrane, sufficient conformational plasticity for gaining access to Pgp binding sites, and an adequate conformer structure there to achieve such binding with a high enough affinity and possibly escape from sequestration on cyclophilins.

Introduction

Expression of immunomodulatory (IM) activity may not be the most fundamental activity of the cyclosporins, cyclic undecapeptides with a pronounced hydrophobic character that are produced by several fungal taxa and whose number now exceeds the 17 different ones known in 1994.[1] The remarkable success of cyclosporin A (CsA, Sandimmun, Neoral) in transplantation actually focused attention on its IM activity.[2] Yet, the large heterogeneity of assays used to study it[2,3] made it difficult to define consensus SAR for IM activity. It is now well-known that the latter depends on its binding to its intracellular receptor cyclophilin A (CyP-A) and the further sequestration of calcineurin to the [cyclosporin-CyP-A] complex.[2,4] From then on, other biological activities of CsA were often linked to the ubiquitous presence of T cells or to that peculiar mechanism of calcineurin inhibition. However, essentially before the mechanism of its action as a T cell-directed IM agent was found to lie in the induction of such an illegitimate [CyP−calcineurin] interaction,[2,4] various other activities were reported for cyclosporins that do not obviously involve calcineurin and are most difficult if not nearly impossible to reconcile with [CyP−calcineurin]-based mechanisms.[2,5−10] Among these are the various antifungal and antiparasitic activities of CsA, its enhancing effects on vasocon-

* To whom correspondence should be addressed. Tel: +33 390 24 18 67. Fax: +33 390 24 18 67. E-mail: francis.loor@bota-ulp.u-strasbg.fr.
† Strasbourg 1 University.
‡ Present address: EntoMed, Pôle API, Bd Sébastien Brandt, F-67400 Illkirch, France.
§ Novartis Pharma Inc.

striction, its inhibitory effects on antigen presentation, its interference with prolactin binding, its inhibition of ligand-induced degranulation of basophils, its modulation of membrane component mobility, and so on.[2]

Most cyclosporin activities involve an essential step at the PM level, including IM activity (i.e., PM crossing), but some cyclosporin activities actually take place at the cell PM level by interactions with a variety of transmembrane (TM) glycoproteins. Thus, CsA is a broad and relatively unselective inhibitor of seven TM-G-protein-coupled receptors and/or of 12 TM channels and transporters, whose functions could often be more efficiently modulated by various cyclosporin analogues devoid of IM properties.[2,5–10] One of these transporters is the *MDR1*-encoded Pgp, a 12 TM ABC transporter. The cell specific expression of the *MDR1* Pgp sustains house-keeping functions (e.g., at the blood–brain barrier), toxin exclusion (e.g., in the gut), and toxic metabolites clearance (e.g., in the liver), but the *MDR1* Pgp is also a flippase for selective membrane phospholipids.[11,12] This Pgp activity also restricts anticancer drug accumulation by the cells, causing the MDR phenotype of some tumor cells. Several cyclosporin derivatives were found to behave as highly potent and selective inhibitors of drug transport by the *MDR1* Pgp. For such a Pgp-InhA, as well as for the reversion of the MDR phenotype itself, the [3′-keto-MeBmt^1, Val^2]-CsA derivative (research name, SDZ PSC 833; development name, Valspodar; trade name, Amdray) is more potent and more selective than CsA.[2,13–15]

Early studies on the MDR reversal potential of cyclosporin analogues suggested that nearly every amino acid residue in the cyclic undecapeptide could modulate that activity.[11,13,14] However, precise SAR were actually lacking as they could not be established from screening studies that were only designed toward the selection of a most suitable candidate for cancer MDR chemosensitization: most cyclosporin analogues provided little if any SAR for the functional inhibition of the Pgp molecules, as they were a priori disregarded because they showed IM activity, were less active than CsA, or inhibited the growth of cells lacking Pgp.[13,14] Finally, inhibition of Pgp function was not the only factor involved in the study of cyclosporin analogues, since chemosensitization assays required several days of culture with mostly epithelial or epitheloid cells (Chinese hamster ovary (CHO), KB, and LoVo).[14] Such cells display intrinsic resistance to anticancer drugs, which may result from synergistic interactions of their metabolism and of their intracellular retention deficiency.[15] Thus, the chemosensitization potency of different cyclosporins did not simply correlate with *MDR1* Pgp inhibition but could also involve the capacity of different cyclosporins to inhibit the CYP3A-dependent metabolization of the tested anticancer drug, as well as the resistance of the cyclosporin themselves to the CYP3A-mediated metabolization.[15–17]

Therefore, a reliable establishment of SAR for the inhibition of Pgp molecule function by cyclosporins required the development of a more specifically targeted read-out. As reviewed elsewhere,[11,15] the cyclosporins are also slow (e.g., CsA) to very slow (e.g., SDZ PSC 833) substrates of Pgp molecules, and this provides a possible mechanism for their inhibition of Pgp function. How-

ever, besides this current hypothesis, it must be considered that cyclosporins may also alter diverse normal cell physiology processes by interacting with various intracellular isoforms of the CyP family.[7,18]

The *MDR1* Pgp activity and its inhibition can be conveniently measured by use of Pgp-overexpressing leukemic cells and a substrate of Pgp (calcein-AM), which, if not "effluxed" by the Pgp, is quickly converted in the cytosol into fluorescent calcein, which is then retained there as it is no longer a Pgp substrate.[19] In the present SAR study, 60 representative cyclosporins[20–28] that essentially included all naturally occurring and some biosynthetically produced analogues were thus compared in vivo in intact live cells in such an assay that measures their capacity to inhibit the efflux of the Pgp substrates from the cell PM.

Natural cyclosporins are cyclic undecapeptides that all share four free (nonmethylated) amide-NH groups of residues 2, 5, 7, and 8. The other seven residues 1, 3, 4, 6, and 9–11 are methylated on the amide group in several members of the family, the "classical" cyclosporins. Besides the latter, there is, among producing fungi, a frequent occurrence of selective *N*-desMe cyclosporins lacking methylation of one or two of the seven *N*-Me residues,[23,28] which may imply potential yet unknown functions for such molecules.

All natural cyclosporins thus belong to those two classes of structural variations: side chain variants and *N*-desMe variants. These two classes of structural variations in cyclosporins are qualitatively different enough to separately describe and analyze their effects on Pgp-InhA. Indeed, while most of the side chain alterations studied (Section 1) should leave the overall cyclosporin structure rather unaffected (i.e., the CsA or classical cyclosporin conformation), desmethylation of *N*-Me residues (Section 2) might induce substantial alterations of the overall cyclosporin conformation in aqueous solution. Special attention was also given to N^{10}-desMe and/or [Thr^2] analogues (Section 2.2), in view of a rather large occurrence in nature of this residue specific *N*-desmethylation variation and of the intriguing fact that most cyclosporin-producing fungi use Thr as a second residue.[21,22,24,25]

Chemistry

All cyclosporins were from Novartis Pharma Ltd., Basel, Switzerland. The major lead CsA was cyclo-[MeBmt^1-Abu^2-MeGly^3-MeLeu^4-Val^5-MeLeu^6-Ala^7-D-Ala^8-MeLeu^9-MeLeu^{10}-MeVal^{11}]. A minor lead, SDZ 214-103, cyclo-[MeBmt^1-Thr^2-MeGly^3-MeLeu^4-Leu^5-MeLeu^6-Ala^7-D-Hiv^8-MeLeu^9-Leu^{10}-MeVal^{11}], differs from CsA by Thr^2 instead of Abu^2, Leu^5 instead of Val^5, a D-2-hydroxyisovaleric acid (D-Hiv^8) instead of D-Ala^8, and Leu^{10} instead of MeLeu^{10}. All other cyclosporins were named after the CsA or SDZ 214-103 leads.

Most naturally occurring cyclosporins studied, including most of *N*-desMe analogues, can be produced by the common strain of *Tolypocladium inflatum* Gams (NRRL 8044),[20] but several other fungi could provide either limited panels of cyclosporin analogues, such as *Cylindrotrichum oligospermum* (SDZ 214-103 and all of its [Thr^2, D-Hiv^8, Leu^{10}]-CsA congeners), or unique cyclosporins such as *Acremonium luzulae* (Fuckel) W. Gams ([Thr^2, Leu^5, Ala^{10}]-CsA), *Tolypocladium terricola*

([Leu[4]]-CsA), a mutant strain of *Tolypocladium inflatum* NRRL 8044 ([Thr[2], Leu[10]]-CsA), and a *Leptostroma* anamorph of *Hypoderma eucalyptii* Cooke and Harkn ([Thr[2], Ile[5]]-CsA).[1,20−24] As a matter of fact, [Thr[2]]-CsA (CsC) should, rather than CsA, become the reference compound in SAR studies since most cyclosporin-producing microorganisms invariably use Thr as a second residue. FR901459, produced by *Stachybotris chartarum*,[22] was not studied here, but the results from an independent study performed in identical conditions with CsA as the reference will be used for comparison. Although classically taken as a naturally occurring cyclosporin, CsH, which shows the substitution of L-MeVal[11] by a D-MeVal[11], might not be so; during the extraction of CsA with an acidic alcoholic solution, a small fraction isomerizes to form iso-CsA, which can then convert into either CsH or CsA.[25]

Most other analogues were obtained by precursor-directed biosynthesis,[25−28] and a few analogues were obtained by chemical modification.[29] The study also included two CsA metabolites: AM1, which was named here as [8′-OH-MeBmt[1]]-CsA, and AM4N, which was named here as [Leu[4]]-CsA. These two CsA analogues were early and major metabolites devoid of IM activity and produced in vivo in CsA-treated human beings as a result of metabolization by the CYP3A enzyme.[17] Although not reported as a naturally occurring fungal product, AM1 (for CsA metabolite on residue 1, formerly named M17),[2,17] which showed an 8′-hydroxylation of MeBmt,[1] was used here for comparison with a similar modification on a natural cyclosporin. The [Leu[4]]-CsA analogue was usually referred as AM4N, according to the nomenclature used for fully synthetic CsA metabolites,[17] but CsA was also produced in such a N[4]-desmethylated form either by biosynthetic manipulations[28] or naturally by mycelium of surface-cultivated fungus *T. terricola*.[23]

Biological Tests

Cell Lines. A pair of human T cell leukemia CEM cells, the highly resistant, *MDR1* Pgp-overexpressing MDR CEM cells (VBL[100] CEM), and their Pgp-lacking parental (Par-)CEM cells (CEM 1.3) for controls were maintained at 37 °C in RPMI medium supplemented with 10% fetal calf serum (FCS), penicillin, and strep-tomycin, as described earlier.[19]

Cyclosporins. All cyclosporins were dissolved as 10 mg/mL stock solutions and diluted to obtain a range of (three and 10 stepwise) molarities in DMSO. They were further diluted in the assay buffer just prior to the test to achieve final molarities ranging from 100 to 0.01 μM in medium with a final 1% DMSO content. Some analogues were not available in sufficient amounts to be assayed at 100 μM, and a few were tested at a larger molarity.

Pgp Inhibitory Activity. All assays for Pgp activity were performed by use of a standard calcein-AM efflux method with the human leukemia CEM cells, which measured calcein retention.[19] This assay is robust and can support high DMSO concentrations, with no effect on calcein-AM uptake or calcein retention by the control cells. Whatever the concentration of cyclosporin ana-logue, the final DMSO content in the assay was adjusted to 1% (10 μL DMSO/mL medium).[19] Briefly, Pgp-expressing MDR CEM cells were first exposed to the Pgp modulator for 15 min at 37 °C; calcein-AM (0.25 μM final; Molecular Probes Europe BV, Leiden, The Netherlands) was then added, and the cells were kept at 37 °C for a further 15 min. The microplates were centrifuged, medium was flicked out, and the cells were resuspended in medium. After three cell washes, inhibi-tion of Pgp function in Pgp-expressing MDR CEM cells was measured as calcein specific fluorescence and expressed as the percentage of the calcein retention in Pgp-lacking Par-CEM cells exposed to the same range of Pgp inhibitor concentrations. Dose−response correla-tions were built with the antagonist concentrations on the *X*-axes (log scales) and the percentage of calcein retention in MDR cells on the *Y*-axes (arithmetic scales). The Pgp modulator IC$_{50}$ and IC$_{20}$ were routinely mea-sured as the cyclosporin molarities, which, in MDR CEM cells, restored 50 and 20% of the calcein retention shown by similarly treated Par-CEM cells. All data are means [± standard deviations (SD)] of at least three indepen-dent experiments (each in duplicates). All IC$_{50}$ molari-ties shown did not detectably alter calcein retention by control Pgp-lacking Par-CEM cells. In this experimental series, the calcein retention in untreated MDR CEM cells ranged from 3 to 7% of the calcein retention by the Par-CEM cells, and the mean IC$_{50}$ of 0.49 ± 0.02 μM for the restoration of calcein retention in MDR CEM cells by the reference Pgp inhibitor SDZ PSC-833 showed that the sensitivity of their Pgp function to cyclosporin-mediated inhibition was similar to the one reported earlier.[19] As most cyclosporins provided IC$_{50}$ values, the SAR for the Pgp inhibitory potencies were studied by comparing the IC$_{50}$ of analogues with those of reference cyclosporins.

Internal Control and Data Normalization. Throughout the whole experimental program, single near-IC$_{50}$ concentrations of reference cyclosporins (most often **1**, **3**, or SDZ PSC 833) were regularly included to control the drug resistance level of the MDR CEM cells. In a series of experiments on four analogues (**37**, **43**, **53**, and **60**), which had to be performed in a different laboratory and with another batch of MDR CEM cells, we obtained an apparently larger resistance to cy-closporin inhibition that was calculated to be roughly 1.5-fold (IC$_{50}$ of 4.6 ± 0.2 μM instead of 3.4 μM for CsA and IC$_{50}$ of 0.87 ± 0.04 μM instead of 0.49 μM for SDZ PSC 833). To allow fair comparisons with the other 56 cyclosporins, the independently generated data of the four compounds were normalized (their actual measured IC$_{50}$ values were 1.5-fold larger than those reported here).

Results

The cyclic peptide sequence variations of cyclosporins are shown in reference to CsA (**1**) principally, and in some sections in reference to SDZ 214-103 (**3**), whose structures are shown in Scheme 1.

Most cyclosporins studied here belong to two major structural conformations (see Discussion): the "classical one" (β-sheet and loop structure) backbone,[30] whose prototype is CsA (**1**), and the "twisted one",[32] whose prototype is SDZ 214-103 (**3**). The conformation of CsH (**2**; Scheme 1) is so far unique for that analogue.[31]

The first SAR section concerns CsA (**1**) and all classical cyclosporins, numbered **4−33**, that show single

Scheme 1

R = CsA   R = CsH   SDZ 214-103
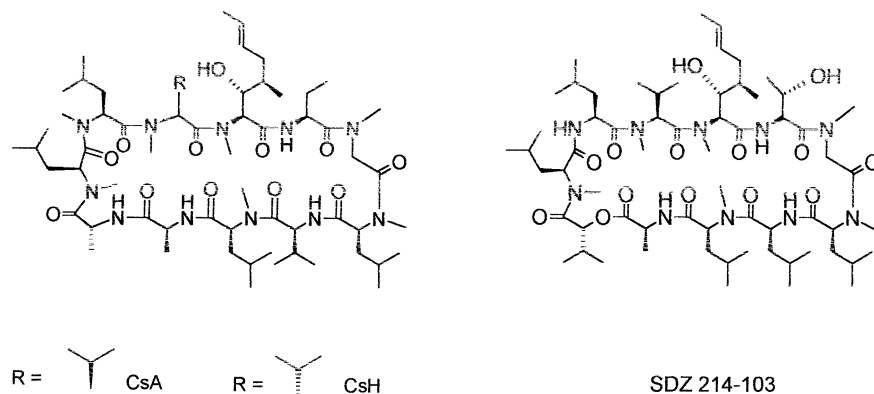
or double side chain differences of residues 11 through 8 (none are available for residues 9 and 10); CsH (**2**), which does not have the classical conformation, is also included. Most of these cyclosporins were either naturally occurring analogues or were prepared by precursor-directed biosynthesis or microbial transformation.[1,20,25] The second SAR section concerns SDZ 214-103 (**3**) and *N*-desMe cyclosporins, numbered **34−60**, all of which are naturally occurring analogues.[20,21] A comparison of the concentration-dependent inhibition of Pgp function by CsA (**1**), CsH (**2**), and SDZ 214-103 (**3**) is shown (Figure 1).
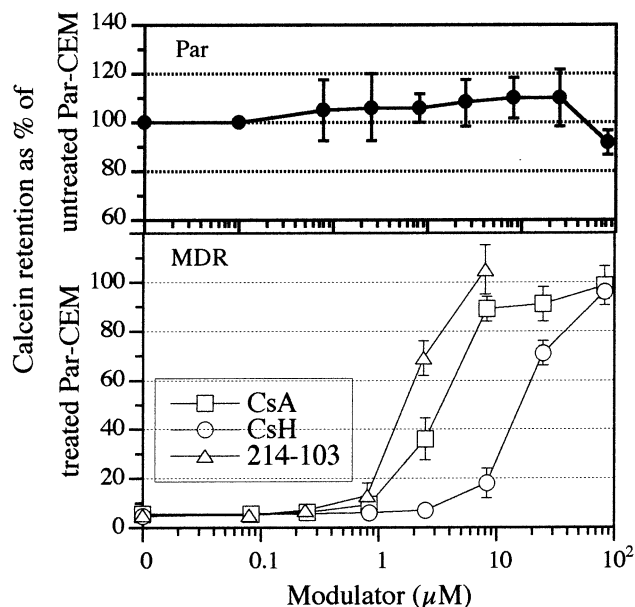

Figure 1. Dose dependence of human *MDR1* Pgp inhibition by CsA, CsH, and SDZ 214-103. The diagram shows the dose-dependent inhibition of Pgp function as an increasing calcein retention (*Y* axes) at increasing concentrations of Pgp inhibitor. For the Pgp-expressing MDR-CEM cells (bottom part, open symbols), the restoration of calcein retention is expressed as percentages of the calcein retention shown by similarly treated Par-CEM cells (exposed to the same Pgp inhibitor concentration). CsA (squares) is used as reference Pgp inhibitor for cyclosporin H (CsH, circles) and the SDZ 214-103 analogue (= [Thr^2, Leu^5, D-Hiv^8, Leu^{10}]-CsA, triangles). The dose−response curves are means and standard deviations obtained from three independent experiments. For the control Pgp-lacking Par-CEM cells (top part), the levels of calcein retention are expressed as percentages of the levels in Par-CEM cells exposed to 0 μM Pgp inhibitor (full symbols, squares, circles, and triangles as for bottom part).

1. SAR of Amino Acid Side Chain Variations on Classical Cyclosporins. 1.1. Single Amino Acid Side Chain Variations. All cyclosporin analogues studied here are directly compared with CsA (**1**) as a reference (Table 1).

L-MeVal^{11}. In comparison to CsA (**1**), the Pgp-InhA was largely decreased (nearly 5-fold) for one analogue with a smaller side chain (**6**, [MeAla^{11}]-CsA) and either unchanged or substantially increased (about 2-fold) for two analogues with a larger side chain, respectively (**4**, [aMeIle^{11}]-CsA, and **5**, [MeIle^{11}]-CsA). For CsH (**2**), the L-MeVal^{11} to D-MeVal^{11} isomerization caused a large (4.6-fold) decrease of Pgp-InhA.

L-MeBmt^{1}. The 8′-hydroxylation of the highly cyclosporin specific residue MeBmt^1 (**12**) resulted in a marked (15-fold) decrease of Pgp-InhA, and its substitution by a nonpolar and shorter side chain residue (**9**,

Table 1. Pgp Inhibitory Activity (Pgp-InhA) of CsA Analogues with Single Residue Differences[a]

	cyclosporin analogues		Pgp-InhA	
compd no.	residue no. alterations	trivial name	fold diff	IC$_{50}$ (μM) mean ± SD (n)
1	**CsA**	CsA	**= 1**	**3.4 ± 0.5** (5)
2	D-MeVal^{11}	CsH	/ 4.6	15.5 ± 1.4 (4)
4	aMeIle^{11}		=	3.4 ± 1.2 (3)
5	MeIle^{11}		× 2.3	1.5 ± 0.1 (3)
6	MeAla^{11}		/ 4.7	16.1 ± 0.9 (4)
7	deoxy-MeBmt^{1}	CsF	× 1.5	2.2 ± 0.1 (3)
8	MeAoa^{1}	CsZ	/ 1.2	4.0 ± 0.6 (3)
9	MeLeu^{1}	Cs28	/ 2.4	8.0 ± 2.1 (3)
10	Me-cyclohexyl-Ala^{1}		/ 2.4	8.1 ± 6.2 (3)
11	Me-cyclized-Bmt^{1}		/ 3.8	13.1 ± 1.3 (3)
12	8′-OH-MeBmt^{1}	AM1	/ 15	50 ± 6 (3)
13	Ala^{2}	CsB	/ 1.4	4.7 ± 0.3 (3)
14	Val^{2}	CsD	× 1.7	2.0 ± 0.2 (3)
15	Nva^{2}	CsG	× 1.8	1.9 ± 0.4 (6)
16	Thr^{2}	CsC	/ 1.5	5.1 ± 1.5 (9)
17	D-MePhe^{3}		× 2.3	1.5 ± 0.1 (3)
18	L-Pro^{3}		/ 1.5	5.2 ± 0.4 (3)
19	D-Pro^{3}		=	3.5 ± 0.3 (3)
20	MeIle^{4}	Cs29	/ 1.9	6.4 ± 0.7 (n)
21	γ-OH-MeLeu^{4}		/ >9	>30 (3)
22	MeVal^{4}		/ 3.3	11.1 ± 2.1 (3)
23	MePhe^{4}		× 1.4	2.4 ± 0.3 (3)
24	MeAla^{6}		/ 1.4	4.6 ± 0.6 (3)
25	Abu^{7}	CsV	/ 15	50 ± 3.8 (3)
26	D-Lys^{8}		/ 38	130 ± 28 (3)
27	D-Ser^{8}		/ 12	42 ± 5 (3)

[a] Reference CsA residues are MeVal^{11}, MeBmt^1, Abu^2, MeGly^3, MeLeu^4, MeLeu^6, Ala^7, and D-Ala^8.

Table 2. Pgp Inhibitory Activity (Pgp-InhA) of CsA Analogues with Combined Second and First or Fifth Residue Differences[a]

	cyclosporin analogues			Pgp-InhA	
compd no.	residue no. alterations		trivial name	fold diff	IC$_{50}$ (μM) mean ± SD (n)
1	**MeBmt^1**	**Abu^2** **Val^5**	CsA	= 1	**3.4 ± 0.5** (5)
7	deoxy-MeBmt^1	: :	CsF	× 1.5	2.2 ± 0.1 (3)
9	MeLeu^1	: :	Cs28	/ 2.4	8.0 ± 2.1 (4)
14	**MeBmt^1**	**Val^2** **Val^5**	CsD	= 1	**2.0 ± 0.2** (3)
28	deoxy-MeBmt^1	: :	CsK	/ 1.3	2.7 ± 0.5 (4)
29	MeLeu^1	: :	Cs30	/ 3.5	7.0 ± 0.3 (4)
15	**MeBmt^1**	**Nva^2** **Val^5**	CsG	= 1	**1.9 ± 0.4** (6)
30	MeLeu^1	: Val^5	CsO	/ 2.9	5.6 ± 0.6 (3)
31	MeBmt^1	: Nva^5	CsM	× 1.5	1.3 ± 0.3 (5)
32	MeBmt^1	: Leu^5	Cs26	=	2.0 ± 0.3 (3)
16	**MeBmt^1**	**Thr^2** **Val^5**	CsC	= 1	**5.1 ± 1.5** (9)
33	MeBmt^1	: Ile^5		/ 1.6	8.4 ± 2.1 (3)

[a] Reference CsA residues are MeBmt^1, Abu^2, and Val^5.

Table 3. Pgp Inhibitory Activity (Pgp-InhA) of Simple *N*-desMe Cyclosporin Analogues[a]

	cyclosporin analogues		Pgp-InhA	
compd no.	residue no. alterations	trivial name	fold diff	IC$_{50}$ (μM) mean ± SD (n)
1	**CsA analogues (Abu^2)**	CsA	= 1	**3.4 ± 0.5** (5)
34	Val^{11}	CsE	=	3.4 ± 0.6 (3)
35	Bmt^1	CsL	× 1.4	2.3 ± 0.1 (3)
36	Leu^4	AM4N	/ >29	>100 (3)
37	Ile^4	Cs31	(/ 4.4)[b]	15 ± 7 (3)
38	Val^4	CsQ	(/ >16)[b]	>54 (4)
39	Leu^6	CsU	/ 6.6	23 ± 6 (4)
40	Leu^9		/ >29	>100 (3)
41	Leu^{10}	CsT	/ 1.7	5.7 ± 1.2 (7)
42	Leu^6 Leu^{10}	CsR	/ >29	>100 (4)
13	**[Ala^2]-CsA analogues**	CsB	= 1	**4.7 ± 0.3** (3)
43	Leu^{10}		× 1.6	3.0 ± 0.3 (3)
16	**[Thr^2]-CsA analogues**	CsC	= 1	**5.1 ± 1.5** (9)
44	Val^{11}	CsW	/ 3.8	19 ± 3 (4)
45	Bmt^1	CsP	/ 3.3	17 ± 4 (4)
46	Val^4	CsS	(/ >19)[b]	>100 (3)
47	Leu^{10}		/ 2.7	14.1 ± 3.0 (5)
14	**[Val^2]-CsA analogues**	CsD	= 1	**2.0 ± 0.2** (3)
48	Bmt^1	Cs27	=	2.1 ± 0.2 (3)
49	Leu^{10}	CsI	/ 4.4	8.8 ± 2.5 (4)
15	**[Nva^2]-CsA analogues**	CsG	= 1	**1.9 ± 0.4** (6)
50	Leu^6	CsY	(/ 4.2)[c]	NA (4)[c]
51	Leu^9	CsX	/ >53	>100 (4)
52	Leu^{10}	CsN	/ 4.6	8.8 ± 1.8 (3)
27	**[D-Ser^8]-CsA analogues**		= 1	**42 ± 5** (3)
53	Gly^3	Cs32	=	≫30 (3)[d]

[a] Reference residues of CsA (**1**) are MeBmt^1, MeGly^3, MeLeu^4, MeLeu^6, MeLeu^9, MeLeu^{10}, and MeVal^{11}. [b] Comparisons made with the [MeLeu^4]-using analogues. For compounds **37** and **38**, more relevant comparisons with the [MeIle^4]- and [MeVal^4]-using analogues are described in the text. [c] No IC$_{50}$ value achievable; fold difference is based on IC$_{20}$ values of 0.96 ± 0.09 μM for compond **15** and 4.0 ± 1.2 μM for compound **50**. [d] IC$_{20}$ > 30 μM for **53**.

[MeLeu^1]-CsA) clearly decreased (2.4-fold) Pgp-InhA. This was also observed for analogues lacking the polar 3′-hydroxyl group, [Me-cyclohexyl-Ala^1]-CsA (**10**, 2.4-fold decrease), and [Me-cyclized-Bmt^1]-CsA (**11**, 3.8-fold decrease). However, the absence of a polar 3′-hydroxyl group does not per se lead to low Pgp-InhA: its absence in [deoxy-MeBmt^1]-CsA (**7**) slightly improved the Pgp-InhA (1.5-fold), and the substitution of MeBmt^1 by a Me-aminooctanoic acid in [MeAoa^1]-CsA (**8**) did not change Pgp-InhA. Moreover, chemically produced [3′-O-acetyl-MeBmt^1] or [3′-keto-MeBmt^1] cyclosporin derivatives are also known to display enlarged Pgp-InhA (see Discussion).[13,14]

L-Abu^2. A larger hydrophobic side chain of the second residue favors Pgp inhibition, since increased Pgp-InhA was present with the larger Val^2 (**14**, 1.7-fold) and Nva^2 (**15**, 1.8-fold) side chains, whereas slightly decreased Pgp-InhA was observed with both the shorter Ala^2 (**13**, 1.4-fold) side chain and the polar Thr^2 (**16**, 1.5-fold) side chain.

L-MeGly^3. Residues with bulky side chains led to cyclosporins with larger (**17**, [D-MePhe^3]-CsA), slightly weaker (**18**, [L-Pro^3]-CsA), or unchanged (**19**, [D-Pro^3]-CsA) Pgp-InhA.

L-MeLeu^4. Its γ-hydroxylation (**21**) largely decreased Pgp-InhA (over 9-folds); its replacement by an as large (**20**, MeIle^4) or smaller residue (**22**, MeVal^4) also decreased it (1.9- and 3.3-fold, respectively), while its replacement by a bulkier residue (**23**, MePhe^4) slightly increased Pgp-InhA.

L-MeLeu^6. A shorter side chain (**24**, [MeAla^6]-CsA) marginally decreased Pgp-InhA.

L-Ala^7. A larger side chain (**25**, [L-Abu^7]-CsA) largely (15-fold) decreased Pgp-InhA.

D-Ala^8. The polar D-Ser^8 (**27**) largely (12-fold) decreased Pgp-InhA, and the longer, more polar and positively charged D-Lys^8 (**26**) decreased it more (38-fold).

1.2. Combined Variations of Second and First or Fifth Residues. Their impacts on the Pgp-InhA are studied in comparison to their closest analogues (Table 2). The substitution of MeBmt^1 by the nonpolar and smaller MeLeu^1 clearly decreased the Pgp-InhA of all cyclosporins whether their second residue was Abu^2 (**9**,

2.4-fold), Val^2 (**29**, 3.5-fold), or Nva^2 (**30**, 2.9-fold). This impact on Pgp-InhA should result from the smaller side chain, rather than from the loss of the polar 3′-hydroxyl group, since the Pgp-InhA of deoxy-MeBmt^1 cyclosporins **7** and **28** were rather similar to those of their parent compounds.

Comparisons of fifth residue variants of [Nva^2]-cyclosporins (**15**, **31**, and **32**) and of [Thr^2]-cyclosporins (**16** and **33**) showed that differences of the side chain of the fifth residue (Nva, Leu, or Ile, instead of Val) did not have a major impact on the cyclosporin Pgp-InhA.

2. SAR for *N*-DesMe Cyclosporin Analogues. 2.1. Simple *N*-DesMe Cyclosporin Analogues. Most are closely related to the CsA (**1**, with Abu^2), [Ala^2]-CsA (**13**), [Thr^2]-CsA (**16**), [Val^2]-CsA (**14**), and [Nva^2]-CsA (**15**) by a simple *N*-methyl difference (Table 3). The impact of *N*-desmethylation of a cyclosporin was not equivalent for all residues, although, relative to the parent compounds, it slightly increased or left unchanged Pgp-InhA only in rare cases: the vast majority of the *N*-desMe analogues showed substantially to largely decreased Pgp-InhA in comparison to their *N*-Me analogues.

Table 4. Pgp Inhibitory Activity (Pgp-InhA) of Complex N^{10}-Desmethylated [Thr^2]-Cyclosporin Analogues[a]

	ref		key residues		Pgp-InhA	
compd no.	analogue or trivial name		residue diff or alternative name		fold diff	IC$_{50}$ (μM) mean $\pm$ SD (n)
47	**[Thr^2 Leu^{10}]-CsA**		Val^5 D-Ala^8 Leu^{10}		= **1**	**14.1 $\pm$ 3.0** (5)
54	(FR901459)		Leu^5		$\times$ 2.4	6.0 $\pm$ 0.8 (4)
55	analogue		D-Hiv^8		$\times$ 6.7	2.1 $\pm$ 0.4 (3)
16	[Thr^2]-CsA (CsC)		MeLeu^{10}		$\times$ 2.8	5.1 $\pm$ 1.5 (9)
54	**FR901459**		Leu^5 D-Ala^8 Leu^{10}		= **1**	**6.0 $\pm$ 0.8** (4)
47	analogue		[Val^5]-FR901459		/ 2.4	14.1 $\pm$ 3.0 (5)
3	(SDZ 214-103)		[D-Hiv^8]-FR901459		$\times$ 3.8	1.6 $\pm$ 0.2 (5)
56	analogue		[Ala^{10}]-FR901459		/ 9.3	56 $\pm$ 5 (3)
3	**SDZ 214-103**		MeBmt^1 MeGly^3 Leu^5 D-Hiv^8		= **1**	**1.6 $\pm$ 0.2** (5)
57	analogue		[8'-OH-MeBmt^1]-SDZ 214-103		/ 9.9	15.8 $\pm$ 4.4 (3)
58	analogue		[Gly^3]-SDZ 214-103		/ 2.5	4.0 $\pm$ 0.5 (3)
55	analogue		[Val^5]-SDZ 214-103		/ 1.3	2.1 $\pm$ 0.4 (3)
59	analogue		[Ile^5]-SDZ 214-103		=	1.6 $\pm$ 0.1 (3)
60	analogue		[Leu^4]-SDZ 214-103		/ 2.7	4.3 $\pm$ 0.3 (3)
54	(FR901459)		[D-Ala^8]-SDZ 214-103		/ 3.8	6.0 $\pm$ 0.8 (4)

[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.

Although the Pgp-InhA may be modulated by the nature of the second residue, the impact of *N*-desmethylation is now analyzed for each of the seven concerned residue specific *N*-desmethylations.

N-DesMe^{11}. The [Val^{11}]-CsA (**34**) and [Thr^2, Val^{11}]-CsA (**44**) showed, respectively, unchanged and much decreased (3.8-fold) Pgp-InhA in comparison to their N^{11}-methylated forms (**1** and **16**).

N-DesMe^1. Depending again on the nature of the second residue, the N^1-desmethylation slightly increased (**35**, [Bmt^1]-CsA), did not change (**48**, [Bmt^1, Val^2]-CsA), or clearly (3.3-fold) decreased (**45**, [Bmt^1, Thr^2]-CsA) the cyclosporin Pgp-InhA.

N-DesMe^3. The low Pgp-InhA of **27** ([D-Ser^8]-CsA) is removed by N^3-desmethylation in **53** ([Gly^3, D-Ser^8]-CsA).

N-DesMe^4. Analogues lacking N^4-methylation showed decreased Pgp-InhA, as most obviously shown by its loss for **36** ([Leu^4]-CsA) vs **1**, but less obviously by the 2.5-fold decreased Pgp-InhA for **37** ([Ile^4]-CsA) vs **20**. For the two other compounds **38** ([Val^4]-CsA) and **46** ([Thr^2, Val^4]-CsA), the N^4-desmethylation was paired with a shorter side chain (Leu^4 to Val^4) substitution that already causes a Pgp-InhA loss (3.3-fold; see Section 1). With reference to **22**, N^4-desmethylation leads to a major loss (>5-fold) of compound **38** Pgp-InhA. The lack of detectable Pgp-InhA shown by **46** probably results from both the N^4-desmethylation and the shorter side chain, although this cannot be accurately determined, since no [Thr^2, MeVal^4]-CsA was available as reference.

N-DesMe^6. The N^6-methylation is clearly required for a large Pgp-InhA, as shown for **39** ([Leu^6]-CsA) and **50** ([Nva^2, Leu^6]-CsA), which were definitely weaker than their N^6-methylated analogues. The doubly *N*-desMe [Leu^6, Leu^{10}]-CsA (**42**) lacks Pgp-InhA.

N-DesMe^9. The N^9-desmethylation dramatically abrogated the Pgp-InhA (**40**, [Leu^9]-CsA, and **51**, [Nva^2, Leu^9]-CsA), suggesting that MeLeu^9 might directly interact with Pgp.

N-DesMe^{10}. While [Ala^2, Leu^{10}]-CsA (**43**) showed a slightly increased Pgp-InhA in comparison to **13**, the sole *N*-desmethylation of MeLeu^{10} clearly decreased Pgp-InhA from nearly 2–3-fold for CsA (**41** vs **1**) and

[Thr^2]-CsA (**47** vs **16**), to over 4-fold for [Val^2]-CsA (**49** vs **14**), [Nva^2]-CsA (**52** vs **15**), and [Leu^6]-CsA (**42** vs **39**).

2.2. Special Focus on Complex N^{10}-DesMe [Thr^2]-Cyclosporin Analogues. Several variants of [Thr^2]-cyclosporins accumulate a N^{10}-desmethylation with several residue side chain differences. Previous sections have shown that the occurrence of a nonpolar residue at position 2 enhanced the Pgp-InhA of classical cyclosporins, whereas a decreased Pgp-InhA resulted from the N^{10}-desmethylation. Despite that, most N^{10}-desmethylated [Thr^2]-cyclosporin analogues are not devoid of Pgp-InhA (Table 4).

These natural analogues are best compared in reference to compounds [Thr^2, Leu^{10}]-CsA (**47**), FR901459 (**54**), and SDZ 214-103 (**3**), as groups where each cyclosporin differs by a single residue from the reference. In reference to [Thr^2, Leu^{10}]-CsA (**47**), three compounds differ by a single residue leading to an obviously larger Pgp-InhA. The presence of a larger fifth residue side chain, Leu^5 (**54**) instead of Val^5 (**47**), increases Pgp-InhA more than 2-fold, and the presence of the *N*-methyl on the Leu^{10} residue (**16**) potentiates Pgp-InhA nearly 3-fold. However, a major near 7-fold increase of Pgp-InhA is obtained by the replacement of the D-Ala^8 residue (**47**) by the D-Hiv^8 residue (**55**).

In reference to FR901459 (**54**), single residue differences show markedly different impacts on the Pgp-InhA. Besides the decreased Pgp-InhA due to the Val^5 (**47**) to Leu^5 (**54**) difference just seen, the much smaller tenth residue side chain, Ala^{10} (**56**) instead of Leu^{10} (**54**), results in a near loss of Pgp-InhA. Again, the replacement of the D-Ala^8 (**54**) by D-Hiv^8 (**3**) results in a major (nearly 4-fold) increase of Pgp-InhA.

In reference to SDZ 214-103 (**3**), fifth residue side chain alterations did not substantially change the Pgp-InhA, the smaller Val^5 side chain (**55**) affording nearly the same level of inhibition as the larger Ile^5 (**59**) or Leu^5 (**3**) ones. The *N*-desmethylation of the third or fourth residue was deleterious since the [Gly^3]-SDZ 214-103 (**58**) and [Leu^4]-SDZ 214-103 (**60**) congeners showed decreased Pgp-InhA as compared to **3**. The 8'-hydroxylation of the MeBmt^1 residue (**57**) led to a markedly decreased (10-fold) Pgp-InhA. As already seen above,

the presence of the D-Ala[8] (**54**) also led to a large decrease of Pgp-InhA.

Discussion

1. General Considerations on Cyclosporins SAR. Although MDR cancer cell chemosensitization was clearly the best conceivable selection procedure to screen for potential drug candidates,[11,13−15] such earlier studies with cyclosporins were not suitable to establish SAR for the inhibition of the Pgp molecules, since other resistance factors could impact on the overall MDR reversal potential. In the present study, the cyclosporins were more directly compared for their Pgp-InhA by their capacity to inhibit the efflux of Pgp substrates from the cell PM.[19] The same method and *MDR1* Pgp-expressing human leukemic cell lines were recently used to establish the SAR for the Pgp-InhA of aureobasidins,[33] and our current studies of *Septoria*-derived cyclic peptolides are also providing more refined SAR for Pgp inhibition (unpublished) than those earlier concluded from their MDR reversal capacity.[34]

1.1. Lack of Straightforward SAR of Cyclosporins on Cellular Activities. As for all drugs that act via intracellular mechanisms, the cellular activity of cyclosporin analogues depends not only on their action on their target receptor but also on their ability to cross the cell membrane and reach their target. Interpretation of their SAR is further complicated in the case of flexible cyclic peptides by the fact that in nonpolar media they will tend to cluster polar groups on the inside and expose hydrophobic residues on the surface but in aqueous or polar solvents they will cluster the hydrophobic residues in the interior and expose their hydrophilic residues to the outside. Cyclosporin derivatives have been shown to adopt several distinct conformations depending on the solvent or environment.[30−32,35−44] Presumably, this property is important for membrane passage as cyclosporins do not fulfill the "rule of 5",[45] i.e., the normal requirements for oral absorption and membrane passage, and must be able to adopt different conformations with appropriate surface properties for various stages of the transport process and finally for receptor recognition. A second complication is that the Pgp receptor has evolved to bind many hydrophobic substrates, and different cyclosporin analogues may have different binding modes.

The SAR of cyclosporins for Pgp inhibition are thus extremely complex, as were their SAR for IM activity, which are not yet completely solved at the whole cell level. Previous SAR studies for IM activity showed that cyclosporins were large enough to display various effector regions, with a "calcineurin binding domain" that included the [MeLeu[4]-Val[5]-MeLeu[6]] tripeptide and the extremity of its MeBmt[1] side chain, and a "CyP binding domain" that corresponded to an adequate conformation of [MeLeu[9]-MeLeu[10]-MeVal[11]-MeBmt[1]-Abu[2]-MeGly[3]] hexapeptide.[2,4,46] Yet, no such SAR could be defined from the immunosuppressive potencies studied in cellular in vitro assays, even in rather direct ones such as IL-2 reporter gene assays,[2−4] simply because their read-out did not only reflect the cyclosporin ability to glue calcineurin to CyP but also primarily depended on its eventual cellular bioavailability. Indeed, the inhibitory activity of any given cyclosporin on lymphocytes depends on its ability to cross the cell PM, to adopt in the cytosol a conformation that can bind to CyP molecules, and finally to form a complex capable of interacting with calcineurin.[2−4,35]

1.2. Relevance of Free Cyclosporin Conformation to Pgp Inhibition. Similarly to their IM activity, the overall Pgp inhibitory potential of any cyclosporin does not only depend on its intrinsic affinity for some pharmacophore on the Pgp molecules but also depends on the microbioavailability of cyclosporins in the Pgp-containing PM microdomain. Therefore, a variety of structural features of the cyclosporins may obviously influence their Pgp-InhA on intact cells. As several different conformations may be acquired by the cyclosporins in the extracellular medium depending on the solvent (but also within a given solvent), the shapes of the different cyclosporin conformers in solution and their conformational plasticity may be major features that control their access to their pharmacophores on the Pgp molecules. They may be more or less favorable for the cyclosporin capacity to penetrate the outer leaflet of the bilayer, to move to the inner leaflet, to show translational mobility in the cell PM plane, to partition into the cytosol, and for their capacity to escape capture and sequestration on the ubiquitous and abundant CyPs. Therefore, both intramembranous solubility properties and CyP binding features may impact the eventual microavailability of the different cyclosporins for interaction with the Pgp molecules in the cell PM.

To set the limits of relevance of solution conformation of cyclic peptides to their activity on intramembranous or intracellular targets, it is recalled that SDZ 214-103 (**3**) and FR901459 (**54**, [Thr[2], Leu[5], D-Ala[8], Leu[10]]-CsA) share with CsA not only a large capacity to reverse the Pgp-mediated MDR[11,47,48] that was shown to correlate with a large Pgp-InhA but also its high IM-activity.[2,21,22] Despite showing largely different conformations in crystalline state, CsA (**1**) and SDZ 214-103 (**3**) acquire very similar ones when bound to CyP,[32,35] explaining how such distantly related analogues may share a high IM activity. While one cyclosporin analogue was shown to acquire in water a conformation that was nearly identical to the CyP-bound one,[41] this interesting finding primarily underlines that only one of a large number of solution conformations might mimic the receptor binding conformation for each of the 60 analogues that are studied here for Pgp-InhA.

Therefore, it is not known whether any of the structures known from crystallographic or NMR studies of free cyclosporins in solution are relevant for the structures that they acquire within the cell PM domains involved in Pgp function. Moreover, there is also no reason to believe that the free amide N−H and free carbonyl C=O known to pair within the cyclosporin molecule still do so when the cyclic compound is dissolved in the PM. Rather, it is probable that these free groups may find other binding partners among various cell PM components. In each PM microenvironment with distinct solvent properties, each cyclosporin analogue may acquire a panel of conformations and establish different intermolecular interactions. Nevertheless, even though there cannot be simple correlations between the cyclosporin conformations in solution in the extracellular medium and those they may acquire

within the cell PM, the repertoire and distribution of the cyclosporin conformers in solution should impact their membranous activities, such as Pgp inhibition (as they do on their intracellular activities, such as calcineurin sequestration). Thus, there is some general relevance of the solution conformation of cyclosporins to their inhibitory potential on membranous Pgp molecules.

Most of the 60 cyclosporins studied in the present report belong to two major structural classes of cyclosporins that show widely different shapes in solution: the classical one (with a β-sheet and loop backbone),[30] whose prototype is CsA (**1**), and the twisted one,[32] whose prototype is SDZ 214-103 (**3**), a feature that may modulate their capacity to gain access to the intramembranous and/or cytosolic domains of Pgp molecules, as well as their interactions with CyP molecules. These two major "consensus" structures of CsA and SDZ 214-103 are first recalled, as most other analogues may be compared in reference to one or the other of them. Few or no conformational data are available for some analogues, but peculiar attention is given to the "CsH" (**2**) conformation,[31] which is so far unique for that analogue, as it can be chemically altered to become a most efficient inhibitor of Pgp activity (unpublished data) and as intact CsH seems to be the most favorable structure for interaction with a 7 TM GPCR (see companion paper).

2. Analogues with the Classical Cyclosporin Conformation. 2.1. CsA Conformation and Pgp Interaction.

CsA (**1**) is cyclo-[MeBmt[1]-Abu[2]-MeGly[3]-MeLeu[4]-Val[5]-MeLeu[6]-Ala[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-Me-Val[11]]; it shows the typical backbone β-sheet and loop structure (Figure 2).[32] As a consequence of the rather rigid framework conformation, the amino acid side chains, which are nearly oriented at a right angle with the plane of the peptide ring, form two hydrophobic clusters: [Abu[2], Val[5], Ala[7], and MeVal[11]] projecting on one side and [MeBmt[1], MeLeu[4], MeLeu[6], and MeLeu[10]] projecting on the opposite side. Most side chain alterations do not change the overall cyclosporin structure, i.e., its backbone β-sheet and loop structure.[32]

Pilot studies suggested at an early stage that cyclosporins might directly affect Pgp function by actually binding to the Pgp molecules themselves,[49,50] and later studies showed indeed that cyclosporins could be substrates of the Pgp molecules.[11,15] Pgp photolabeling was then reported to be inhibited with various cyclosporins with potencies that correlated with their potency known from in vitro studies with cell membranes from Pgp-expressing cells.[51] A priori, either the CyP binding and calcineurin binding domains or the overlapping ones, or totally different ones, could be involved in interactions with the Pgp molecule in the cell PM. By using different photoactivable CsA analogues with a diazirine group, either on position 8 (within the calcineurin binding domain) or on position 3 (at the edge of the CyP binding domain), and specific anticyclosporin monoclonal antibodies capable of recognizing these two domains, the calcineurin domain appeared to play a role in the physical interaction of CsA (**1**) with Pgp molecules, whereas the "CyP domain" on the other hand appeared to be exposed on the surface of the inner (cytosolic) leaflet of the PM of Pgp-expressing cells.[51]
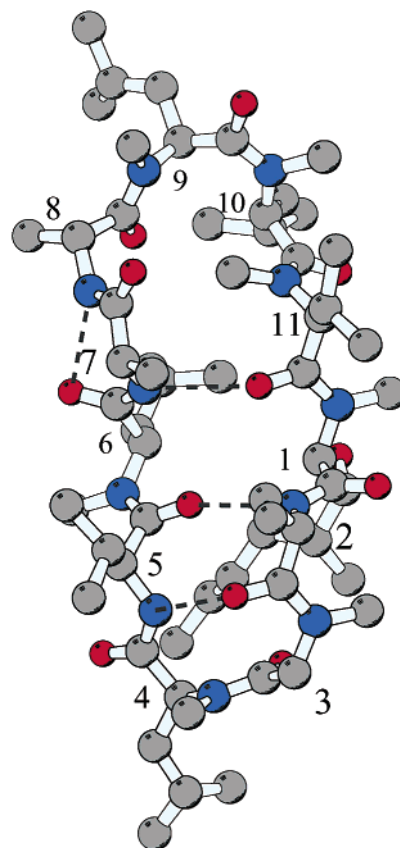

Figure 2. 3D structure of CsA (**1**). The picture shown corresponds to X-ray data. The NMR structure in chloroform and the X-ray crystal structure are virtually identical, showing that in both environments the backbone forms a twisted β-sheet that involves antiparallel tripeptides [MeVal[11]-MeBmt[1]-Abu[2]] and [Val[5]-MeLeu[6]-Ala[7]]. The MeGly[3] and MeLeu[4] residues participate in a type II' β-turn. A loop formed by the pentapeptide [Ala[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-MeVal[11]] contains the only cis amide linkage in the molecule between the two adjacent MeLeu[9] and MeLeu[10] residues. This structure is held by four intramolecular H-bonds involving the four nonmethylated NH amides of the residues 2, 5, 7, and 8: one H-bond defines the type II' β-turn [N[5]- - -O[2]], two other H-bonds extend the β-sheet ([N[2]- - -O[5]] and [N[7]- - -O[11]]), and one additional and unusual H-bond between D-Ala[8] and MeLeu[6] ([N[8]- - -O[6]]) defines a γ-turn centered around Ala[7].

For CsA at least, the CyP binding domain would not bind the Pgp molecules but would be exposed to the cytosol, whereas 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.[51] While providing very valuable information on the potential molecular interactions between cyclosporins and Pgp molecules, such data cannot completely reflect the SAR for the functional inhibition of Pgp molecules expressed by intact live cells by cyclosporins added in the extracellular space. Restrictions to this intramembranous localization of cyclosporins, because either of structural (e.g., polar side chains) or of dynamic (e.g., rigidity) constraints, may impair the occurrence of cyclosporin–Pgp interactions.

2.2. Side Chain Impact on the Pgp Inhibitory Potential of Classical Cyclosporins.
For such cyclosporins that show the same general framework conformation as CsA in solution, our in vivo assays (with

live cells) provide further SAR data. The occurrence of polar groups on residues 1, 2, 4, and 8 side chains tends to impair Pgp-InhA: 8′-OH-Bmt[1] ≪ Bmt[1] (compare **12** and **1**); Thr[2] < Abu[2] (**16** and **1**); γ-OH-Leu[4] < Leu[4] (**21** and **1**); and Lys[8] ≪ Ser[8] ≪ Ala[8] (**26**, **27**, and **1**). On the contrary, bulkier and more hydrophobic side chains in the domain constituted by residues 11 and 1−4 tend to increase Pgp-InhA: Ile[11] > Val[11] > Ala[11] (**5**, **1**, and **6**); deoxy-Bmt[1] ≥ Aoa[1] > Leu[1] (**7**, **8**, and **9**; **28** and **29**); Nva[2] ≥ Val[2] ≥ Abu[2] > Ala[2] (**15**, **14**, **1**, and **13**; **30**, **29**, and **9**; **28** and **7**); Phe[3] > Gly[3] (**17** and **1**); Phe[4] > Leu[4] > Ile[4] > Val[4] (**23**, **1**, **20**, and **22**), the exception being Ala[7] > Abu[7] (**1** and **25**). No analogue was available to probe the importance of the residue 9 and 10 side chains.

Therefore, the Pgp binding domain of cyclosporin (residues 4−9 plus the Bmt[1] side chain), as defined from other studies,[51] may be involved but not each one shows an obvious requirement of size or hydrophobicity. However, other residue side chains are not inert, although they are not a part of the Pgp binding domain,[51] since the size and/or hydrophobicity of the side chains of residues 11, 2, and 3 show a marked impact on Pgp-InhA. This suggests that either the Pgp binding concept is too restricted or the other features of cyclosporin interactions with the membrane or cytosolic molecules (e.g., CyP) may have a major impact on their eventual interaction with Pgp molecules.

2.2.1. Residues That Would Not Belong to the Pgp Binding Domain. For what concerns those residues (11, 2, and 3; no data on 10), the residue side chain may actually be involved in Pgp binding or indirectly impact the Pgp-InhA through controlling availability for Pgp molecules. Thus, for residue 11, a lower absorption of a cyclosporin analogue onto the cytosolic CyP, with a larger availability for interacting with Pgp, cannot simply account for a stronger inhibition of Pgp function, since out of two low CyP binding analogues,[46] one (**5**) was a more potent Pgp inhibitor and another (**6**) was a fairly weak Pgp inhibitor. Because the loss of Pgp-InhA of **6** vs **1** cannot be attributed to a larger deviation by the CyP, efficient inhibitory interactions with Pgp might depend on the eleventh residue side chain being favored by a larger size (**5**) although not of any shape (**4**). In the case of residue 2, larger and more hydrophobic side chains also favor Pgp-InhA, but this could be interpreted as due to a larger membrane solubility. This idea might be extended to the lower Pgp-InhA shown by [Thr[2]]-CsA (**16**), but this idea is difficult to reconcile with the fact that this compound shows a large IM activity,[2,3] thus can cross the cell PM. Besides its polar features, the Thr[2] hydroxyl group might have a specific impact in potential intermolecular H-bonding features of [Thr[2]]-CsA, which would reduce the intramembranous bioavailability for interaction with Pgp molecules. However, these speculations cannot discard a direct involvement of the second residue side chains in Pgp binding. For residue 3 variants, finally, the Pro[3] substitutes only showed slightly weaker (**18**) or unchanged (**19**) Pgp-InhA. The replacement of the flexible MeGly[3] at one edge of the cyclosporin β-sheet by Pro residues not only provides an increased steric bulk but also reduces the cyclic peptide flexibility[29] and changes its conformer distribution: the substitution of MeGly[3] by L-Pro[3] (**18**) prevents the formation of the type II′ β-turn, while its

substitution by D-Pro[3] (**19**) does not alter the CsA backbone.[29] The increased Pgp-InhA shown by the [D-MePhe[3]] CsA analogue (**17**) might come from the bulky side chain and suggests that this residue may also be involved in binding the cyclosporin to Pgp at some step of its efflux.

2.2.2. Residues That Would Belong to the Pgp Binding Domain. For this large part of the cyclosporin molecule ([MeLeu[4]-Val[5]-MeLeu[6] Ala[7]-D-Ala[8]-MeLeu[9]] plus the MeBmt[1] side chain), some residue side chain variations, such as Ala or Leu for residue 6, and Val, Nva, Leu, or Ile for residue 5, have little or no impact on the Pgp-InhA, although for unclear reasons. Other residue side chains had more impact on Pgp-InhA. Thus, a larger hydrophobic side chain of residue 4 (Phe, Leu, or Ile, instead of Val) may favor Pgp-InhA through an increased binding fit to Pgp. Conversely, the much decreased Pgp-InhA found with Abu (**25**) instead of Ala (**1**) as residue 7 might show that this residue side chain sterically hinders binding to Pgp if not small enough. Alternatively, this minor variation might either disorder or reinforce the γ-turn structure formed by the H-bond between the free amide of D-Ala[8] and the carbonyl of MeLeu[6] in CsA (**1**) and this would impair the Pgp-InhA either at the Pgp binding level or at another one. As part of the Pgp binding domain, this D-Ala[8] residue may be critical. As shown for several compounds in Section 2.2, its replacement by D-Hiv[8], which both introduces a larger side chain and excludes formation of an [NH[8]- - - CO[6]] H-bond, largely increases Pgp-InhA, which could possibly be through increased binding to Pgp. The lack of Pgp-InhA shown by [D-Lys[8]]-CsA (**26**) might be related to the fact that cationic hydrophobic compounds usually are faster substrates of Pgp.[11] However, the very low Pgp-InhA of [D-Ser[8]]-CsA (**27**) cannot be due to a reduced membranous availability, as its large IM activity[2,3] shows that it does penetrate (and move through) the cell PM.

In any cyclosporin SAR, the importance of their most characteristic residue (MeBmt[1]) is always intriguing, and potent Pgp-InhA might primarily depend on a very long side chain such as for Bmt[1] (**1**) or Aoa[1] (**8**), a large Leu[1] (**9**, **29**, and **30**) side chain being already less effective. The 8′-hydroxylation of MeBmt[1], which naturally occurs at the terminal methyl group, leads to a marked Pgp-InhA decrease. Together with the good Pgp-InhA of [MeAoa[1]]-CsA, this suggests that hydrophobicity of the long side chain of the first cyclosporin residue favors interaction with Pgp molecules. Most likely, the orientation of this long side chain may be a major element that determines the cyclosporin affinity for Pgp. This [8′-OH-MeBmt[1]]-CsA (**12**) is of special interest as it occurs in vivo in CsA-treated human beings, as an early and major CYP3A metabolite (named AM1) devoid of IM activity and with low nephrotoxicity,[52] like analogous in vivo-generated metabolites of cyclosporins.[17] At variance, the polar 3′-hydroxyl group of MeBmt[1], whose involvement in CyP binding is critical for the cyclosporin IM activity, is not involved in Pgp binding, as its absence in both [deoxy-MeBmt[1]] and [MeAoa[1]] analogues (**7**, **8**, and **28**) does not decrease Pgp-InhA. Moreover, blockade of the 3′-hydroxyl group on Bmt[1] does not impair but may largely increase Pgp-InhA of various cyclosporins (unpublished data), such

as reported for [3′-O-acetyl-MeBmt[1]]-CsA and for [3′-keto-MeBmt[1], Val[2]]-CsA (SDZ PSC 833, Valspodar, Amdray).[11,13−15] The free crystalline shape of Amdray shows similar intramolecular H-bonds, which hold the structure together and are responsible for the rigid backbone. In apolar solvents, NMR reveals an almost identical backbone conformation to the crystal structure one, but like for classical cyclosporins, many different Amdray conformers are present in solvents of higher polarity, presumably because of the breaking of intramolecular H-bonds and the formation of H-bonds to the solvent molecules.[39,40,53] Therefore, for both Amdray and classical cyclosporins, the [1−6]hexapeptide forms an antiparallel β-pleated sheet, the [7−11]pentapeptide forms an open loop with the unusual H-bond between the amide of D-Ala[8] and the carbonyl of MeLeu[6], and the residue side chains are similarly staggered.[53] Although the MeBmt[1] side chain is folded over the peptide backbone in both Amdray and classical cyclosporins, there are some differences in its conformation, because a carbonyl group (Amdray) replaces an hydroxyl group in the 3′ position of MeBmt[1]. Thus, blockade of the 3′-OH group on Bmt[1] might relieve the long side chain from interactive constraints with other cyclosporin residues, increase its interactions with Pgp molecules, and contribute to the large increases of Pgp-InhA seen.[13,14,53]

3. CsH. Although low, the Pgp-InhA of CsH (**2**) is substantial, as it is even 2−3-fold larger than the Pgp-InhA shown in the present assay by early MDR reversing agents such as verapamil or quinidine.[11,13,14] The lack of IM activity of CsH was thought to come from a reduced partition to the cytosol and/or a reduced capacity to contribute to calcineurin deviation.[2,3] Similarly, Pgp inhibition in live cells does not solely depend on the affinity of cyclosporins for Pgp molecules but also on their access to the right cell compartment, as illustrated by an earlier difficulty in interpreting SAR for Pgp inhibition from apparently conflicting data on CsH. CsH was much less potent than CsA in restoring the sensitivity of Pgp-overexpressing MDR cells to anticancer drugs.[13] Yet, when PM vesicles prepared from Pgp-overexpressing MDR CHO cells were used,[49,50] thus in conditions providing direct access to the Pgp domains expressed on the inner, cytosolic leaflet of the bilayered cell PM, CsH could interfere as efficiently as CsA with radiolabeled CsA binding to Pgp molecules. This does not show a lack of involvement of the eleventh residue side chain of classical cyclosporins for binding to Pgp molecules, as the overall CsH conformation is so different from a classical cyclosporin one. Indeed, the L- to D-epimerization of MeVal[11] has actually two structural consequences, the first one being a modification of the orientation of the MeVal[11] side chain; the second one is a drastic distortion of the ring conformation, actually conferring CsH a markedly different shape from classical cyclosporins (Figure 3).[31]

Because CsH can bind to Pgp molecules in PM vesicles, its low Pgp-InhA in intact cells would come from its distorted cyclosporin backbone conformation. In fact, early studies of CsH had shown complicated NMR spectra indicating possibly up to seven peptide ring conformers.[29] The L-MeVal[11] to D-MeVal[11] epimerization also changes the cyclosporin backbone flexibility
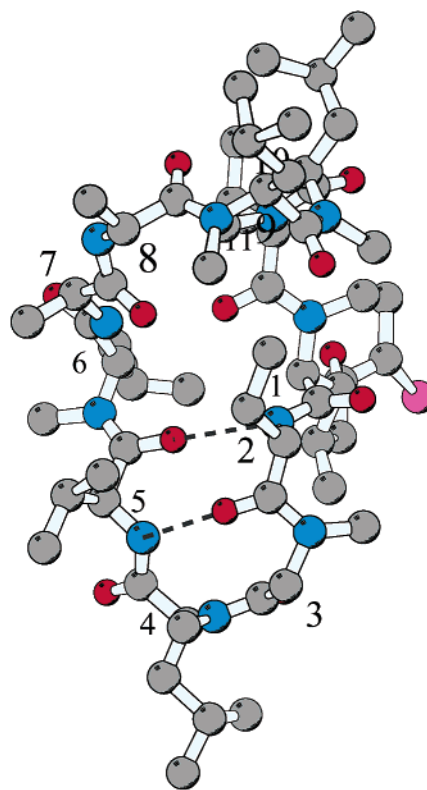

Figure 3. 3D structure of CsH (**2**). The picture shown corresponds to X-ray data. These X-ray data as well as NMR studies show the occurrence in CsH of a similar β-pleated sheet as in CsA, involving the two nonmethylated NH amides of residues 2 and 5, with the [N[2]- - -O[5]] and [N[5]- - -O[2]] H-bonds. Comparisons of infrared spectra of CsA and CsH in several organic solvents might be interpreted as a weakening of the [N[7]- - -O[11]] H-bond together with a strengthening of the γ-turn ([N[8]- - -O[6]] H-bond in CsH.[40] Computer modeling optimization of CsH also suggested that the conformation of the loop backbone part of the peptidic ring in CsH (Ala[7] to D-MeVal[11]) was identical to the loop backbone in CsA, although being at right angle with regards to the CsA structure (H. P. Weber, personal communication).

since the CsH three-dimensional (3D) conformation would be less folded than the CsA one, with an unstable [O[11]- - -N[7]] H-bond.[31] Comparisons of the infrared spectra of CsH (**2**), CsA (**1**), [Thr[2]]-CsA (**16**), and [Val[2]]-CsA (**14**) in several organic solvents showed that[40] in the completely apolar solvent CCl₄, the CsH spectrum differs from the others and can be interpreted as a weakening of the [N[7]- - -O[11]] H-bond together with a strengthening of the γ-turn ([N[8]- - -O[6]] H-bond). However, the CsA, [Thr[2]]-CsA, or [Val[2]]-CsA conformation does also markedly differ from the CsH one in its susceptibility to solvent disruption:[40] with the former cyclosporins, the strong H-bonds that are evident in apolar solvents remained relatively strong in acetonitrile, whereas the analogous bonds were broken in the CsH case. Thus, although CsH would be less folded, it would also be less hydrophobic than classical cyclosporins. CsH should thus be able to partition into the cell PM, but its fairly twisted backbone would reduce its capacity to move and distribute within the cell PM and its availability to interact with Pgp in the inner PM leaflet (as well as to cross the PM to interact with CyP). Because efficient inhibitory interactions of classical cyclosporins with Pgp were shown to depend on the size

and shape of the eleventh residue side chain (see Table 1 or previous section), the different orientation of the MeVal[11] side chain in CsH (**2**) and CsA (**1**) might also control Pgp-InhA, the L- to D-epimerization of MeVal[11] obviously causing a lower Pgp-InhA.

4. N-DesMe Cyclosporins. The *N*-desmethylation of nearly any of the seven residues that are *N*-Me in classical cyclosporins (1, 3, 4, 6, and 9–11) decreased Pgp-InhA. Most tested *N*-desMe cyclosporins were known to show a marked loss of IM activity;[2,3,20,29] these alterations of their immunosuppressive potential might be accounted for by a variety of mechanisms, from a loss of interaction with calcineurin to an inability to cross the cell PM. Similarly, their general impaired Pgp-InhA may be interpreted in various ways. One could suggest that lower Pgp-InhA might simply come from the decreased hydrophobicity of *N*-desMe derivatives reducing their PM retention. Although possibly playing a role for some derivatives, the latter interpretation is not generally satisfactory, as at least some others showed an obvious capacity to cross the PM as needed to display IM activity or to be released as a CYP3A-metabolite from the cytosol. Within the cyclosporin molecule itself, residue selective *N*-desmethylation may confer new H-bonding opportunities participating in various alternative twists of the backbone. In aqueous solution, the *N*-desMe analogues may indeed display much distorted conformations in comparison to cyclosporins with the consensus CsA shape and show alterations of conformational plasticity.[22,32] Such new intramolecular constraints on the cyclosporin molecules may lead in the extracellular medium to a lower occurrence of conformers suitable for insertion in the cell PM, or within the PM, to a reduced plasticity of the cyclosporin framework and a lower capacity to move to the inner PM leaflet. However, selective *N*-desmethylation of cyclosporins may also allow the formation of novel intermolecular H-bonds or other alternative interactions with other molecules (solvent, receptors, and transporters). While the impact of any selective *N*-desmethylation for the intramolecular H-bonding potential and cyclosporin folding is either known or can be modeled for the cyclosporins in their free form, its impact on the intermolecular H-bonding potential (the C=O and the N–H of β-turns are often implicated in intermolecular interactions) with Pgp molecules and other PM proteins would be speculative. However, a lower Pgp-InhA might also come from the more specific loss of structural features required for an efficient binding to Pgp molecules as might be the case for the MeLeu[9], which would be part of the Pgp binding domain.[51]

Depending on the *N*-desMe residue, the losses of Pgp-InhA temptatively ranked, with few exceptions, from complete for the ninth residue (**40** and **51**), to very large for the fourth residue (**36**, **38**, and **46**), and large for the sixth residue (**39** and **50**), obvious for the tenth residue (**41**, **47**, **49**, and **52**), not obvious or variable for the eleventh (**34** and **44**) and first (**35**, **45**, and **48**) residues, and not really known for the third residues (the *N*-desmethylation of MeGly[3] in [D-Ser[8]]-CsA (**53**) did not detectably change its already weak Pgp-InhA).

Thus, the deleterious effects of single *N*-desmethylation on Pgp-InhA of tested cyclosporins were most dramatic for what concerned residues 4 and 9, at the two edges of the molecule, whose *N*-desmethylation of either tended to suppress Pgp-InhA. The total loss of Pgp-InhA by *N*[9]-desmethylation (**40** and **51**) may suggest that MeLeu[9] might directly interact with Pgp, but the *N*[9]-desmethylation might be prone to offer a free proton that would interact with another molecule, as the Leu[9] residue is localized at the most extreme end of the loop formed by residues 7–11. As **51** only displays a marginal IM activity,[3] its capacity to partition into the cell PM might also be impaired.

The *N*[4]-desmethylation also impacts very much on Pgp-InhA although with an exception. Besides [Leu[4]]-CsA (**36**) that completely lost Pgp-InhA, *N*[4]-desmethylation of [MeIle[4]]-CsA (**20**) only leads to a 2.5-fold decreased Pgp-InhA for **37** ([Ile[4]]-CsA), suggesting that the impact of the *N*[4]-methylation on the overall Pgp-InhA depends on the size and shape of the fourth residue side chain. This interpretation is compatible with the lack of Pgp-InhA shown by the other natural *N*[4]-desmethylated analogues that used a smaller (Val) fourth residue (**38** and **46**). The [Leu[4]]-CsA (**36**) is of peculiar interest as it is also an early, natural, and major metabolite of CsA in man (named AM4N).[17] Because [Leu[4]]-CsA (**36**) was claimed to be the most nephrotoxic cyclosporin,[52] its lack of Pgp-InhA shows that Pgp inhibition is not a major factor of cyclosporin treatment-associated nephrotoxicity.

The large impairment of Pgp-InhA, as well as of IM activity,[2,3] by *N*[6]-desmethylation (**39** and **50**) is interesting since the *N*[6]-methyl points out on the surface of all intact cyclosporins, both classical ones such as CsA (**1**) and others such as CsH (**2**) and SDZ 214-103 (**3**). Because residue 6 is part of the Pgp binding domain,[51] the lack of *N*[6]-methyl might impair binding to Pgp because of suboptimal binding fit of the cyclosporin. If not, *N*[6]-desmethylation might cause a large conformational alteration (as happens for *N*[10]-desmethylation) or the release of the free NH[6] amide might provide various intermolecular interactions that would prevent [Leu[6]] analogues from interacting with Pgp.

The combined *N*-desmethylation of residues 6 and 10 completely abrogated Pgp-InhA (**42**), the structural reasons of which are unknown but should include large conformational alterations. The sole *N*[10]-desmethylation decreases Pgp-InhA for all classical cyclosporins, with the exception of the *N*[10]-desMe-[Ala[2]]-CsA (**43**). The *N*-desmethylation of MeLeu[10] might disrupt the conformation of the peptide ring by favoring an H-bond [N[10]- - -O[7]], similar to the one found in the SDZ 214-103 (**3**) analogues, although for the latter this structure is compatible with a large Pgp-InhA.

The impact of the *N*-desmethylation of the MeBmt[1] residue on Pgp-InhA (**35**, **45**, and **48**) largely depends on the nature of the second residue side chain: decreased for [Bmt[1], Thr[2]]-CsA (**45**) and unchanged for [Bmt[1]]-CsA (**35**) and [Bmt[1], Val[2]]-CsA (**48**). Because both **45** and **35** retain their IM activity in vitro (thus showing that they are able to cross the cell PM to gain access to cytosolic CyP),[2,3] it is obvious that cyclosporin domains involved in Pgp molecule interaction and in the CyP–calcineurin interactions are not similarly touched by the lack of *N*-methyl group on the first residue. Yet, at variance with the *N*-desMe CsA analogues with a nonpolar second residue side chain, the Thr[2]-hydroxyl

group might have a specific impact in allowing novel H-bonding features of the *N*-desMe [Thr[2]]-CsA analogues, with more complex conformational alterations.

Similarly, the N^{11}-desmethylation showed substantial deleterious effects for [Thr[2]]-CsA (**44**) but only marginal ones for CsA (**34**), and it is difficult to structurally relate the Pgp-InhA difference with the short apolar Abu[2] vs polar Thr[2] difference. In both cases, an additional H-bond between the amide proton of L-Val[11] and the carbonyl group of D-Ala[8] is formed leading to considerable conformational changes in the loop fragment.[20] Both [Val[11]]-CsA (**34**) and [Thr[2], Val[11]]-CsA (**44**) displayed only marginal IM activity,[3,20] which was speculated as possibly reflecting a low capacity to enter the cell PM. Because [Val[11]]-CsA (**34**) is an as good Pgp inhibitor as CsA (**1**), it is probable that its low IM activity must rather be related to its capacity to glue calcineurin to CyP.

5. Twisted Cyclosporin (SDZ 214-103) Conformation. While *Tolypocladium*-derived cyclosporins may use various nonpolar residues as second residue, most cyclosporin-producing fungi only produce [Thr[2]] analogues, several of which also show an *N*-desmethylation of the tenth residue.[21,22,24,25] The latter occurs in some natural cyclosporins from both *Tolypocladium* and some other fungi.[22,24,27] Six of the eight naturally occurring N^{10}-desMe-[Thr[2]]-analogues are cyclopeptolides produced by the fungus *C. oligospermum*.[21,25] While a D-Ala[8] residue most commonly occurs in cyclosporins, a D-Hiv[8] residue is found in all cyclosporins produced by *Cylindrotrichum* but only these.[21] The major one, SDZ 214-103 (**3**), differs from CsA (**1**) by Thr[2] instead of Abu[2], Leu[5] instead of Val[5], Leu[10] instead of MeLeu[10], and D-Hiv[8] instead of D-Ala[8] residue.[21] While Thr[2], Leu[5], and Leu[10] variations also occur in other natural cyclosporins,[25] the D-Hiv[8] residue, which introduces an ester bond, is in a cyclosporin context the major chemical peculiarity of the SDZ 214-103 (**3**).[21] Besides a large capacity to reverse the Pgp-mediated MDR,[47] this naturally occurring cyclosporin-related cyclopeptolide shares with CsA (**1**) a high IM activity.[21] In the CyP-bound state, the 3D structure of SDZ 214-103 is very similar to the CsA one.[32] However, in the free crystalline state, SDZ 214-103 does not show the classical cyclosporin 3D conformation despite its high sequence homology to CsA and several other classical cyclosporins (Figure 4). Thus, not only the overall shape of SDZ 214-103 may differ from the CsA one but also the cyclopeptolide conformation may be less rigid and more flexible.

The closest natural [D-Ala[8]] using cyclosporin to SDZ 214-103 (**3**) is FR901459 (**54**, [Thr[2], Leu[5], Leu[10]]-CsA), which also shows a potent IM activity and a large Pgp-InhA.[22,48] The reported conformation of **54** in solution looks similar to the SDZ 214-103 one (**3**), 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 (**3**).[22] This suggests a dominance of the desmethylation at the tenth residue on the cyclosporin conformation, which might be extended to some other N^{10}-desMe analogues of cyclosporins. Therefore, the impact of some residue substitutions in this N^{10}-desMe-cyclosporin conformational context might not be correlated with the same residue alterations in cyclosporins with the classical conformation.
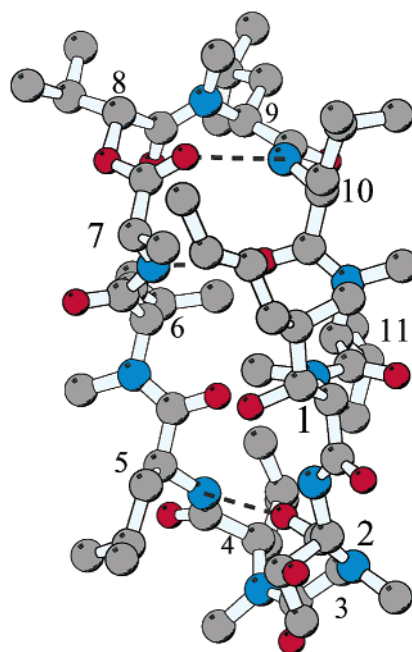

Figure 4. 3D structure of SDZ 214-103 (**3**, [Thr[2], Leu[5], D-Hiv[8], Leu[10]]-CsA). The picture shown corresponds to X-ray data. In the crystal, the cis amide bond between residues 3 and 4 allows the formation of a type Via β-turn stabilized by an intramolecular [O[2]- - -N[5]] H-bond between the carbonyl of Thr[2] and the amide of the Leu[5] residue. However, besides this intramolecular [O[2]- - -N[5]] H-bond also found in **1**, there are no [N[2]- - -O[5]] and [O[11]- - -N[7]] H-bonds, so that **3** does not have the peculiar β-sheet of **1** (also found in **2**).[32] Instead, **3** shows a short extended antiparallel sheet involving residues 7−10, with two H-bonds between Ala[7] and Leu[10] ([O[10]- - -N[7]] and [O[7]- - -N[10]]) stabilizing a type II′ β-turn. The main difference with **1** is the obviously less-folded conformation of **3**. The replacement of D-Ala[8] by D-Hiv[8] also results in substituting the unusual attractive H-bond between the amide of D-Ala[8] and the carbonyl of MeLeu[6] [N[8]- - -O[6]] in **1** (defining a γ-turn centered around Ala[7])[30] by a repulsive interaction between the ester oxygen of the D-Hiv[8] and the carbonyl of the MeLeu[6] in **3**.[32]

Replacement of the cyclosporin typical MeBmt[1] by a MeLeu[1] or other residues does not occur among natural SDZ 214-103 analogues, neither are deoxy- nor *N*-desmethyl-Bmt[1] analogues found.[21,25] However, 8′-hydroxylation of the MeBmt[1] residue naturally occurs at the terminal methyl group (**57**).[21,25] It leads to a markedly (10-fold) decreased Pgp-InhA, although it remains substantial and 3-fold larger than the one shown by the equivalent [8′-hydroxy-MeBmt[1]]-CsA (**12**). This suggests that hydrophobicity of the long side chain of the first cyclosporin residue obviously favors interaction with Pgp molecules, even in the compound **3** conformation. Similarly, for classical cyclosporins, the substitution of the Leu[5] of SDZ 214-103 (**3**) by the smaller Val[5] (**55**) typical of CsA or by a similar-sized Ile[5] (**59**) did not significantly change the Pgp-InhA. As also observed with classical cyclosporins, it appears that the *N*-desmethylation of SDZ-214-103 is also deleterious, since the MeGly[3] to Gly[3] mutation (**58**) and MeLeu[4] to Leu[4] mutation (**60**) gave obvious 2.5−2.7-fold Pgp-InhA decreases. Yet, these naturally occurring analogues remain in the class of potent inhibitors of Pgp function, and although little could be concluded from the N^3-desmethylation of classical cyclosporins, the N^4-desm-

ethylation was much more dramatic for them than for compound **3**.

In the [D-Ala[8]]-cyclosporins from *Tolypocladium*, the N^{10}-desMe analogues showed substantially less Pgp-InhA than the related N^{10}-Me analogues. In contrast, SDZ 214-103 analogues showed a large Pgp-InhA despite their N^{10}-desmethylation. In comparison to classical cyclosporins with the consensus shape and despite its unfavorable N^{10}-desmethylation, SDZ 214-103 (**3**) was shown to display a surprisingly large Pgp-InhA, which was 2.1-, 3.2, and 8.8-fold larger than those of CsA (**1**), [Thr[2]]-CsA (**16**), and [Thr[2], Leu[10]]-CsA (**47**), respectively. The presence of the D-Hiv[8] residue might be principally responsible for a better interaction with Pgp. Indeed, the sole choice of a D-Hiv[8] residue instead of a D-Ala[8] residue conferred large increases of Pgp-InhA, which were 4-fold when comparing [Thr[2], Leu[5], Leu[10]]-cyclosporins (**3** and **54**) and nearly 7-fold when comparing [Thr[2], Val[5], Leu[10]]-cyclosporins (**47** and **55**). The use of a D-Hiv[8] residue in the SDZ 214-103 congeners, instead of the D-Ala[8] one in classical cyclosporins, might thus be a critical chemical pecularity for a large Pgp-InhA. This replacement of D-Ala[8] by D-Hiv[8] replaces an amide by an ester bound in the cyclic peptide between the seventh and the eighth residues, excludes formation of an [NH[8]- - -CO[6]] H-bond, and also introduces a larger residue side chain on the eighth residue.[25] The larger Pgp-InhA may result from the latter feature inherent to the D-Ala[8] to D-Hiv[8] substitution, but unfortunately, this could not be confirmed as no classical cyclosporin analogue with a D-Val[8] or even larger D-Leu[8] or D-Ile[8] residues was available for comparison.[25] Nevertheless, the impact of the eighth cyclosporin residue for Pgp inhibition is compatible with the in situ photoaffinity studies showing that the contact residues with Pgp molecules would not only include the calcineurin binding domain ([MeLeu[4]-Val[5]-MeLeu[6]] and the MeBmt[1] side chain) but also include the [Ala[7]-D-Ala[8]-MeLeu[9]] tripeptide.[51]

Alternatively, other structural features of SDZ 214-103 (**3**) and its analogues may also play a role in its Pgp-InhA. Thus, the occurrence of a β-sheet involving residues 7−10 might confer a specific conformation for interaction with the Pgp molecules. Moreover, the replacement of an amide (peptidic) bond by an ester (peptolidic) one between the seventh and the eighth residues might confer a larger plasticity to the already flexible molecules of the cyclosporin class and facilitate the acquisition by SDZ 214-103 of a peculiar 3D conformation favorable for inhibitory interaction with Pgp molecules. Whether this presence of a peptolidic bond in a cyclic peptide is generally favorable for their eventual inhibitory interactions with Pgp molecules can only be speculative, but it is intriguing to note that two other classes of cyclic peptides with a peptolidic bond were found to be very potent inhibitors of Pgp function: the depsipeptidic aureobasidins, which use either a 2-hydroxy-3-methyl-butanoic acid [= Hiv] or a 2-hydroxy-3-methyl-pentanoic acid,[33] and the decapeptidic "septorins", which use a D-lactic acid.[34] Therefore, together with a higher plasticity allowing the peptolidic cyclosporin SDZ 214-103 to enter the cell PM from the extracellular milieu and its passive flipping from the outer phospholipid leaflet to the inner one, the occur-

rence of a larger eighth residue side chain and the aforementioned antiparallel β-sheet may thus represent key structures for the interaction with the Pgp molecules.

6. Pgp-InhA Relation to PM Structure and Cyclosporin Interactions. How do cyclosporins actually inhibit function(s) of Pgp molecules shown to be a phospholipid flippase or pictured as an hydrophobic vacuum cleaner-like pump? An interaction between a cyclosporin and a Pgp molecule implies a penetration of the molecule into the cell PM, its move from the outer leaflet of the lipid bilayer to the inner one, and its diffusion to the Pgp molecule at the inner PM leaflet−cytosol interface. Thus, the cyclosporin conformers that interact with the Pgp molecule might be very different from those in free solution. Because some cyclosporins at least were shown to be slow to very slow substrates of the Pgp molecules, saturation of the Pgp flippase or hydrophobic pump by slow substrates may account for the reduced retention of anticancer drugs that are Pgp substrates in MDR cells. Yet, CsA itself cannot be seen as made of a hydrophobic tail and a polar head, and whether it may be handled by the phospholipid binding, the flippase active site of the Pgp molecule is unlikely. Rather, other mechanisms may be postulated, such as cyclosporin-induced allosteric modifications in the Pgp molecules causing a reduced availability of drug binding sites or cyclosporin-mediated recruitment of inactive Pgp conformers with displacement of an equilibrium between active and inactive forms.[15] Indeed, the Pgp molecules may occur in a variety of oligomeric forms though principally as dimers;[54] they show selective association with some PM phospholipids;[55] and they are localized in discrete PM domains, such as caveolae.[56,57] This supports our earlier suggestion,[15] that like several other multi-TM glycoproteins, the Pgp molecules might occur on the cell surface as selective lipidic rafts and/or within specific microdomains that would be defined by the 12 TM moiety of the molecule. The lipidic composition and organization of such Pgp-rich rafts will impact on the cyclosporin retention and activity on the Pgp molecules.

All together, our SAR data suggest that structural features of cyclosporins that modulate their interaction with Pgp molecules deal both with modifications that would principally change the surface of the cyclic peptide and others that may have major effects on the backbone conformation. The cyclosporin backbone was known to take various conformations depending on their solvent environment,[36−44] and this obvious molecular plasticity may increase Pgp-InhA, by facilitating the passive move of the cyclic compound from the exoplasmic PM leaflet to the cytoplasmic one. Efficient inhibition of Pgp function by a cyclosporin may first require its good access to the inner PM leaflet, which seems to be the preferred side for action of cyclosporins on Pgp function. Thus, any interpretation of the SAR for Pgp-InhA of cyclosporin analogues should take into account not only the contribution of cyclosporin surface for the efficacy of interaction with Pgp molecules but also the capacity of the cyclosporin analogues to undergo conformational changes. Besides the epimerization of the eleventh residue or the introduction of some amino acid side chains, which are too bulky, the major alterations

that change the cyclosporin backbone were the *N*-desmethylation of some amide functions (as found to occur in natural cyclosporins): they may be more or less favorable for occurrence of one or another conformation of the cyclosporin backbone.

Concluding Remarks

Our analyses of a large number of cyclosporin analogues with a variety of structural variations provide SAR that constitute a framework for modeling the interaction of cyclosporin with Pgp molecules. They confirm the expected involvement of both unspecific, e.g., hydrophobicity, and specific features. The Pgp-InhA of cyclosporin may depend both on its biodistribution in the cell and on its interactions with the Pgp molecules. Whether this depends on restricted cyclosporin domains made of a few residues only, the whole framework conformation itself, or both remains unclear. Structural alterations such as those affecting the level of hydrophobicity or the bulkiness of the analogues may be crucial for the binding of cyclosporin molecules to the Pgp molecules, but it might also change their microdistribution within the various lipidic domains of the cell PM. While most naturally occurring *N*-desMe cyclosporins that are produced by various fungi show regularly decreased potential to inhibit Pgp function, those produced by *C. oligospermum* (SDZ 214-103 and congeners) showed a larger potential. Besides its relevance to the present SAR, what this difference may represent as an advantage for this fungus is intriguing, particularly as it was recently found to be paired with a very large potential to interact with some seven TM G-protein-coupled receptors.[58]

Abbreviations Used

Amino acid abbreviations: Abu, α-aminobutyric acid; aIle, allo-isoleucine; Hiv, 2-hydroxy-isovaleric acid; MeaIle, *N*-methyl allo-isoleucine; MeAoa, *N*-methyl-amino-octanoic acid; MeBmt, *N*-methyl-4-butenyl-4-methyl-threonine; MeGly, *N*-methylglycine (sarcosine); MeLeu, *N*-methylleucine; MePhe, *N*-methylphenylalanine; MeVal, *N*-methylvaline; N^1- – N^{11}-, N^1-amide through N^{11}-amide; *N*-Me, *N*-methylated; *N*-desMe, *N*-desmethylated. Other abbreviations: ABC, ATP binding cassette; CsH, [D-MeVal^{11}]-CsA; CyP, cyclophilin; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; FR901459, [Thr^2, Leu^5, Leu^{10}]-CsA; MDR, multiple drug resistance; MDR, multidrug resistant; Par-, parental; Pgp, P-glycoprotein; PM, plasma membrane; Pgp-InhA, Pgp inhibitory activity; SAR, structure–activity relationships; SDZ 214-103, [Thr^2, Leu^5, D-Hiv^8, Leu^{10}]-CsA.

References

(1) Dreyfuss, M. M.; Chapela, I. H. Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. In *The Discovery of Natural Products with Therapeutic Potential*; Gullo, V. P., Ed.; Butterworth-Heinemann, Boston, 1994; pp 49–80.

(2) Borel, J. F.; Baumann, G.; Chapman, I.; Donatsch, P.; Fähr, A.; Mueller, E. A.; Vigouret, J. M. In vivo pharmacological effects of ciclosporin and some analogues. *Adv. Pharmacol.* **1996**, *33*, 115–246.

(3) Hiestand, P.; Gubler, H. U. Cyclosporins: Immunopharmacologic properties of natural cyclosporins. *Handb. Exptl. Pharmacol.* **1988**, *85*, 487–502.

(4) Schreiber, S. L.; Crabtree, G. R. The mechanism of action of cyclosporin A and FK506. *Immunol. Today* **1992**, *13*, 136–142.

(5) Sherry, B.; Yarlett, N.; Strupp, A.; Cerami, A. Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3511–3515.

(6) Billich, A.; Hammerschmid, F.; Peichl, P.; Wenger, R.; Zenke, G.; Quesniaux, V.; Rosenwirth, B. Mode of action of SDZ NIM 811, a nonimmunosuppressive Cyclosporin A analogue with activity against human immunodeficiency virus (HIV) type 1: interference with HIV protein-cyclophilin A interactions. *J. Virol.* **1995**, *69*, 2451–2461.

(7) Crompton, M. The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* **1999**, *341*, 233–249.

(8) Lo Russo, A. L.; Passaquin, A. C.; André, P.; Skutella, M.; Rüegg, U. T. Effect of cyclosporin A and analogues on cytosolic calcium and vasoconstriction: possible lack of relationship to immunosuppressive activity. *Br. J. Pharmacol.* **1996**, *118*, 885–892.

(9) Gitter, B. D.; Waters, D. C.; Threlkeld, P. G.; Lovelace, A. M.; Matsumoto, K.; Bruns, R. F. Cyclosporin A is a substance P (tachykinin NK$_1$) receptor antagonist. *Eur. J. Pharmacol.* **1995**, *289*, 439–446.

(10) Wenzel-Seifert, K.; Seifert, R. Cyclosporin H is a potent and selective formyl peptide receptor antagonist. Comparison with *N-tert*-butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L-leucyl-L-phenylalanine and cyclosporins A, B, C, D, and E. *J. Immunol.* **1993**, *150*, 4591–4599.

(11) Loor, F. Cyclosporins and related fungal products in the reversal of P-glycoprotein-mediated multidrug resistance. In *Multidrug Resistance in Cancer Cells*; Gupta, S., Tsuruo, T., Eds.; John Wiley & Sons Ltd.: Chichester, 1996; pp 387–412.

(12) Van Helvoort, A.; Smith, A. J.; Sprong, H.; Fritzsche, I.; Schinkel, A. H.; Borst, P.; Van Meer, G. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* **1996**, *87*, 507–517.

(13) Gavériaux, C.; Boesch, D.; Boelsterli, J. J.; Bollinger, P.; Eberle, M. K.; Hiestand, P.; Payne, T.; Traber, R.; Wenger, R.; Loor, F. Overcoming multidrug resistance in Chinese hamster ovary cells in vitro by cyclosporin A (Sandimmune) and nonimmunosuppressive derivatives. *Br. J. Cancer* **1989**, *60*, 867–871.

(14) Gavériaux, C.; Boesch, D.; Jachez, B.; Bollinger, P.; Payne, T.; Loor, F. SDZ PSC 833, a nonimmunosuppressive cyclosporin analogue, is a very potent multidrug-resistance modifier. *J. Cell. Pharmacol.* **1991**, *2*, 225–234.

(15) Loor, F. Valspodar: current status and perspectives. *Exp. Opin. Invest. Drugs* **1999**, *8*, 807–835.

(16) Vickers, A. E. M.; Alegret, M.; Jiménez, R. M.; Pflimlin, V.; Fisher, R.; Spaans, C.; Brendel, K. Changes in human liver and kidney slice function related to potential side-effects in the presence of biotransformation of four cyclosporin derivatives – CsA, IMM, OG, and PSC. *In Vitro Mol. Toxicol.* **1998**, *11*, 119–132.

(17) Fähr, A. Cyclosporin clinical pharmacokinetics. *Clin. Pharmacokinet.* **1993**, *24*, 472–495.

(18) Hamilton, G. S.; Steiner, J. P. Immunophilins: Beyond immunosuppression. *J. Med. Chem.* **1998**, *41*, 5119–5143.

(19) Tiberghien, F.; Loor, F. Ranking of P-glycoprotein substrates and inhibitors by a calcein-AM fluorometry screening assay. *Anti-Cancer Drugs* **1996**, *7*, 568–578.

(20) Von Wartburg, A.; Traber, R. Chemistry of the natural cyclosporin metabolites. *Prog. Allergy* **1986**, *38*, 28–45.

(21) Dreyfuss, M. M.; Schreier, M. H.; Tscherter, H.; Wenger, R. Cyclosporin peptolides, their preparation by fermentation or chemical synthesis and their use as pharmaceuticals. European Patent Application 0296 123 A2 (15.06.1988); *Chem. Abstr.* **1989**, *111*, 152146a.

(22) Sakamoto, K.; Tsujii, E.; Miyauchi, M.; Nakanishi, T.; Ya-mashita, M.; Shigematsu, N.; Tada, T.; Izumi, S.; Okuhara, M. FR901459, a novel immunosuppressant isolated from *Stachybotrys chartarum* no. 19392. Taxonomy of the producing organism, fermentation, isolation, physicochemical properties and biological activities. *J. Antibiot.* **1993**, *46*, 1788−1798.

(23) Jegorov, A.; Matha, V.; Sedmera, P.; Havlicek, V.; Stuchlik, J.; Seidel, P.; Simek, P. Cyclosporins from *Tolypocladium terricola*. *Phytochemistry* **1995**, *38*, 403−407.

(24) Traber, R.; Dreyfuss, M. M. Occurrence of cyclosporins and cyclosporin-like peptolides in fungi. *J. Ind. Microbiol.* **1996**, *17*, 397−401.

(25) Traber, R. Biosynthesis of cyclosporins. *Biotechnology of Antibiotics*, 2nd ed.; Strohl, W.R., Ed.; Dekker: New York, 1997; pp 279−313.

(26) Traber, R.; Hofmann, H.; Kobel, H. Cyclosporins − New analogues by precursor directed biosynthesis. *J. Antibiot.* **1989**, *42*, 591−597.

(27) Lawen, A.; Traber, R.; Geyl, D. In vitro biosynthesis of [Thr², Leu⁵, D-Hiv⁸, Leu¹⁰]-cyclosporin, a cyclosporin related peptolide, with immunosuppressive activity by a multienzyme polypeptide. *J. Biol. Chem.* **1991**, *265*, 11355−11360.

(28) Kuhnt, M.; Bitsch, F.; France, J.; Hofmann, H.; Sanglier, J. J.; Traber, R. Microbial biotransformation Products of Cyclosporin A. *J. Antibiot.* **1996**, *49*, 781−787.

(29) Wenger, R. M. Synthesis of ciclosporin and analogues: structural and conformational requirements for immunosuppressive activity. *Prog. Allergy* **1986**, *38*, 46−64.

(30) Wenger, R. M. Cyclosporine: Conformation and analogues as tools for studying its mechanism of action. *Transplant. Proc.* **1988**, *XX*, 313−318.

(31) Walkinshaw, M. D.; Weber, H. P.; Widmer, A. Molecular recognition in biological systems: models for rational drug design. *Triangle* **1986**, *25*, 131−142.

(32) Taylor, P.; Mikol, V.; Kallen, J.; Burkhard, P.; Walkinshaw, M. D. Conformational polymorphism in peptidic and nonpeptidic drug molecules. *Biopolymers* **1997**, *40*, 585−592.

(33) Tiberghien, F.; Kurome, T.; Takesako, K.; Didier, A.; Wenandy, T.; Loor, F. Aureobasidins: Structure−Activity Relationships for the inhibition of the human MDR1 P-glycoprotein ABC-transporter. *J. Med. Chem.* **2000**, *43*, 2547−2556.

(34) Emmer, G.; Grassberger, M. A.; Schulz, G.; Boesch, D.; Gavériaux, C.; Loor, F. Derivatives of a novel cyclopeptolide. 2. Synthesis, activity against multidrug resistance in CHO and KB cells in vitro, and structure−activity relationships. *J. Med. Chem.* **1994**, *37*, 1918−1928.

(35) Mikol, V.; Taylor, P.; Kallen, J.; Walkinshaw, M. D. Conformational differences of an immunosuppressant peptolide in a single crystal and in a crystal complex with human cyclophilin A. *J. Mol. Biol.* **1998**, *283*, 451−461.

(36) Kessler, H.; Köck, M.; Wein, T.; Gehrke, M. 164. Reinvestigation of the conformation of cyclosporin A in chloroform. *Helv. Chim. Acta* **1990**, *73*, 1818−1832.

(37) Kessler, H.; Gehrke, M.; Lautz, J.; Köck, M.; Seebach, D.; Thaler, A. Complexation and medium effects on the conformation of cyclosporin A studied by NMR spectroscopy and molecular dynamics calculations. *Biochem. Pharmacol.* **1990**, *40*, 169−173.

(38) Ko, S. Y.; Dalvit, C. Conformation of cyclosporin A in polar solvents. *Int. J. Pept. Protein Res.* **1992**, *40*, 380−382.

(39) El Tayar, N.; Mark, A. E.; Vallat, P.; Brunne, R. M.; Testa, B.; van Gunsteren, W. F. Solvent-dependent conformation and hydrogen-bonding capacity of cyclosporin A: Evidence from partition coefficients and molecular dynamics simulations. *J. Med. Chem.* **1993**, *36*, 3757−3764.

(40) Shaw, R. A.; Mantsch, H. H.; Chowdhry, B. Z. Solvent influence on the conformation of cyclosporin. An FT-IR study. *Can. J. Chem.* **1993**, *71*, 1334−1339.

(41) Wenger, R. M.; France, J.; Bovermann, G.; Walliser, L.; Widmer, A.; Widmer, H. The 3D structure of a cyclosporin analogue in water is nearly identical to the cyclophilin-bound cyclosporin conformation. *FEBS Lett.* **1994**, *340*, 255−259.

(42) O'Donohue, M. F.; Burgess, A. W.; Walkinshaw, M. D.; Treutlein, H. R. Modeling conformational changes in cyclosporin A. *Protein Sci.* **1995**, *4*, 2191−2202.

(43) Janowski, B.; Fischer, G. Modulation of Cyclosporin A/Cyclophilin interactions by drug vehicles. *Bioorg. Med. Chem.* **1997**, *5*, 179−186.

(44) Kallen, J.; Mikol, V.; Quesniaux, V. F. J.; Walkinshaw, M. D.; Schneider-Scherzer, E.; Schörgendorfer, K.; Weber, G.; Fliri, H. G. Cyclosporins: Recent developments in biosynthesis, pharmacology and biology, and clinical applications. In *Biotechnology*, 2nd edition, Vol. 7; Rehm, H. J., Reed, G., Eds.; VCH: Weinheim, 1997; Chapter 12, pp 535−591.

(45) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23*, 3−25.

(46) Quesniaux, V.; Schreier, M. H.; Wenger, R. M.; Hiestand, P. C.; Harding, M. W.; van Regenmortel, M. H. V. Cyclophilins binds to the region of cyclosporine involved in its immunosuppressive activity. *Eur. J. Immunol.* **1987**, *17*, 1359−1365.

(47) Schwabe, K.; Steinheider, G.; Lawen, A.; Traber, R.; Hildebrandt, A. Reversal of multidrug resistance by novel cyclosporin A analogues and the cyclopeptolide SDZ 214−103 biosynthesized in vitro. *J. Cancer Res. Clin. Oncol.* **1995**, *121*, 407−412.

(48) Tiberghien, F.; Wenandy, T.; Loor, F. The potent immunosuppressive cyclosporin FR901459 inhibits the human P-glycoprotein and formyl peptide receptor functions. *J. Antibiot.* **2000**, *53*, 509−515.

(49) Foxwell, B. M. J.; Mackie, A.; Ling, V.; Ryffel, B. Identification of the multidrug resistance-related P-glycoprotein as a cyclosporine binding protein. *Mol. Pharmacol.* **1989**, *36*, 543−546.

(50) Ryffel, B.; Woerly, G.; Rodriguez, C.; Foxwell, B. M. J. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for cyclosporine. *J. Recept. Res.* **1991**, *11*, 675−686.

(51) Demeule, M.; Wenger, R. M.; Beliveau, R. Molecular interactions of cyclosporin A with P-glycoprotein. Photolabeling with cyclosporin derivatives. *J. Biol. Chem.* **1997**, *272*, 6647−6652.

(52) Copeland, K. R.; Thliveris, J. A.; Yatscoff, R. W. Toxicity of cyclosporine metabolites. *Ther. Drug Monit.* **1990**, *12*, 525−532.

(53) Pohl, E.; Herbst-Irmer, R.; Sheldrick, G. M.; Dauter, Z.; Wilson, K. S.; Bölsterli, J. J.; Bollinger, P.; Kallen, J.; Walkinshaw, M. D. Crystal structures of two modifications of [3,O-didehydro-MeBmt¹, Val²]-cyclosporin and comparison of three different X-ray data sets. *Helv. Chim. Acta* **1995**, *78*, 355−366.

(54) Jette, L.; Potier, M.; Béliveau, R. P-glycoprotein is a dimer in the kidney and brain capillary membranes: effect of cyclosporin A and SDZ PSC 833. *Biochemistry* **1997**, *36*, 13929−13937.

(55) Sharom, F. J.; Yu, X.; Chu, J. W. K.; Doige, C. A. Characterization of the ATPase activity of P-glycoprotein from multidrug-resistant chinese hamster ovary cells. *Biochem. J.* **1995**, *308*, 381−390.

(56) Demeule, M.; Jodoin, J.; Gingras, D.; Beliveau, R. P-glycoprotein is localized in caveolae in resistant cells and in brain capillaries. *FEBS Lett.* **2000**, *466*, 219−224.

(57) Lucker, G. M.; Pica, C. M.; Kumar, A. S.; Covey, D. F.; Piwnica-Worms, D. Effects of cholesterol and enantiomeric cholesterol on P-glycoprotein localization and function in low-density membrane domains. *Biochemistry* **2000**, *39*, 7651−7661.

(58) Loor, F.; Tiberghien, F.; Wenandy, T.; Didier, A.; Traber, R. Cyclosporins: Structure−Actitivy Relationship for the inhibition of the human FPR1 formylpeptide receptor. *J. Med. Chem.* **2002**, *45*, 4613−4628.