Interaction of human cyclophilin hCyp-18 with short peptides suggests the existence of two functionally independent subsites

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Abstract The binding of peptides, derived from the model substrate Suc-Ala-Ala-Pro-Phe-pNA, to the human cyclophilin hCyp-18 was investigated. HCyp-18 is able to bind 2–4-mer peptides as well as shorter *para*-nitroaniline (pNA) derivatives and pNA surrogates. Although Suc-Ala-Phe-pNA binds hCyp-18, only proline-containing peptides are able to block efficiently the peptidyl-prolyl *cis/trans* isomerase activity. Competition experiments strongly suggest the existence of two independent subsites: a S1′ 'proline' subsite and a S2′–S3′ 'pNA' subsite. The interaction at S2'–S3' requires either a Phe-pNA C-terminus or a Phe-pNA surrogate bearing an H-bond acceptor able to bind Trp121 and Arg148 simultaneously. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Peptidyl-prolyl *cis/trans* isomerase; Proline; *Cis/trans* isomerization; Subsite

1. Introduction

Immunophilins are a family of ubiquitous proteins which possess a peptidyl-prolyl *cis/trans* isomerase (PPIase) activity. They catalyze the *cis/trans* interconversion of the amino acyl-prolyl amide bond in both peptides and proteins [1]. As a consequence, they accelerate one of the limiting steps of protein folding and are involved in many related biological processes such as cellular multiplication [2] and communication [3]. The human cyclophilin (hCyp)-18, a cytosolic member of the cyclophilin subfamily, is also the main receptor of the cyclic undecapeptide cyclosporine A and is hence implicated in immunosuppression [4]. Moreover, hCyp-18 has been shown to play a critical role in the infection of $CD4^+$ T-cells by HIV-1 [5,6] and is an essential constituent of the mature and infectious virus [7,8]. Therefore, hCyp-18 is an attractive target for the design of non-immunosuppressive drugs able to block HIV-1 entry and multiplication [9].

Resolution of several structures of peptide:cyclophilin complexes [10–13] as well as kinetic studies have shown that the proline residue with the (L) configuration is essential for the PPIase activity [13,14] although it may be replaced with proline isosteres [15]. The influence of other residues of the substrate on the interaction is not so critical. HCyp-18 is able to bind a wide range of substrates including cyclic peptides [16] and pseudopeptides [17]. The nature of amino acids at P1[1] and P2 does not influence much the second order rate k_{cat}/K_m [19,20] provided that P1 has the (L) configuration [13]. The cyclophilin PPIase activity is also impaired by stereochemistry at P2′; (D)-Phe-containing peptides are weak substrates of hCyp-18 and are unable to compete with the reference substrate Suc-Ala-Ala-Pro-Arg-pNA (4-nitroaniline) [13]. In turn, the N-terminal part of model substrate tetrapeptides Suc-Ala-Xaa-Pro-Phe-pNA protrudes outside the catalytic site [11] and affects neither the affinity nor the activity [13].

The effect of peptide-length and C-terminus on the biological activity of model peptides is not well documented. Resolution of structures of dipeptides Xaa-Pro (Xaa is Gly, Ala, Ser, His) complexed with hCyp-18 shows that the interactions pattern is different from those observed with larger peptides. This suggests that peptides are able to interact in different ways with the catalytic site [12].

The design of novel inhibitors of hCyp-18, in particular substrate analogues, necessitates a delineation of the most important interactions which influence the binding of peptides to the catalytic pocket. In order to determine the functional significance of the interactions revealed by resolution of different structures, we investigated the influence of the length of peptides derived from the model substrate Suc-Ala-Ala-Pro-Phe-pNA on their binding to hCyp-18. The affinity of the peptides for the enzyme was correlated to their ability to inhibit the PPIase activity. We also explored the influence of the C-terminus of peptides on both affinity and activity. For this purpose, succinyl (Suc) or acetyl (Ac) peptides bearing various substituted aniline (An) moieties were synthesized. In particular, we studied the interaction of hCyp-18 with carboxy and amino derivatives of anilide peptides (Fig. 1). We also investigated the effect of the replacement of the (L)-Phe-pNA motif with protease-resistant patterns. The influence of (D)-Phe and reduced derivatives on the PPIase activity was prospected as well.

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Abbreviations: Ac, acetyl; An, aniline; DCC, dicyclohexyl carbodiimide; DCM, dichloromethane; DFA, 2,4-difluoroaniline; DIPEA, diisopropylethylamine; DMSO, dimethylsulfoxide; HOBT, *N*-hydroxybenzotriazole; Pheψ(CH_2-NH)pNA, (2-amino-3-phenyl-propyl)-4-nitroaniline; pNA, 4-nitroaniline; Suc, succinyl; TFA, trifluoroacetic acid

[1] Derived nomenclature of peptide residues according to Schechter and Berger [18]: P2-P1-P1′-P2′-P3′ corresponds to Ala-Ala-Pro-Phe-pNA. The corresponding subsites are noted S2, S1, S1′, S2′ and S3′.

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2. Materials and methods

2.1. Materials

All the reagents employed were of analytical grade and were purchased from Aldrich Chemical Co. or Sigma. Amino acids and coupling reagents were from Novabiochem, Bachem or Sigma. Substrate peptides were obtained from Bachem. Trypsin and α-chymotrypsin were from Sigma. Recombinant hCyp-18 was prepared as described previously [9].

2.2. Synthesis of peptides and pseudopeptides

Peptide **9** was synthesized by manual solid-phase peptide synthesis on a rink-amide resin (Novabiochem, 0.47 mmol g^{-1}) using four equivalents of dicyclohexyl carbodiimide (DCC), *N*-hydroxybenzotriazole (HOBT) and diisopropylethylamine (DIPEA) in dry *N*-methylpyrolidone. The peptide was cleaved from the resin using a mixture of trifluoroacetic acid (TFA):triisopropylsilane:water 95:2.5:2.5 and purified by RP-HPLC (high performance liquid chromatography) using a C_{18} Vydac semipreparative column and a linear gradient of 0.1% TFA in water:acetonitrile 25:75 to 75:25 in 30 min (flow rate: 4 ml min^{-1}).

Other peptides were synthesized in solution using standard methods. Phenylalanine-aninilide derivatives were synthesized by the asymmetric anhydride method as previously reported [21]. Other couplings were performed in dichloromethane (DCM) using 1.5 equivalent of DCC, one equivalent of HOBT and three equivalents of DIPEA (after neutralization of residual acid used in the deprotection step). Anilide-containing peptides were deprotected with 4.0 N hydrogen chloride in dioxane at 0°C. Other peptides were deprotected with a 50:50 mixture of TFA in DCM at room temperature. Acetylation and succinylation were achieved by treatment of the deprotected peptide with respectively two equivalents of acetic anhydride or succinic anhydride, and four equivalents of DIPEA in acetonitrile. Peptide **13** was obtained by saponification of methyl ester **9** with lithium hydroxide in methanol:water 50:50. Reduction of peptide **3** yielded the para-aminoaniline derivative **14**. The corresponding aryltrimethylammonium could not be obtained in the Suc series. Reduction of the Ac tripeptide Ac-Ala-Pro-Phe-pNA **12**, followed by permethylation using a large excess of iodomethane and pentamethylpiperidine [22], gave compound **16**. Peptide **18** was prepared according to Schultz and coworkers [17]. All protected intermediates were purified by flash chromatography on 40–60 μm (230–400 mesh) Merck silica gel. Peptides were purified by RP-HPLC as described above. All compounds were characterized by ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy and ES′MS (positive or negative ionization). The purity of all compounds was checked by thin-layer chromatography and RP-HPLC.

2.3. Enzymatic assays

The PPIase uncoupled assay [23] and the PPIase α-chymotrypsin-coupled assay were carried out in a 35 mM HEPES buffer, pH 7.8, at 10 ± 0.5°C as previously described [24]. The PPIase trypsin-coupled assay was done in a 35 mM HEPES buffer, pH 8.6, at 10 ± 0.5°C [25]. Data were processed according to the literature [9].

2.4. Fluorimetric titration

2.4.1. Determination of K_d. Fluorimetric titration was carried out as previously reported [16].

2.4.2. Competition experiments. Solutions of hCyp-18 (320 nM) in a 35 mM HEPES buffer, pH 7.8, containing compounds 2–5 (concentration fixed at a value corresponding to 2 K_d) in dimethylsulfoxide (DMSO) were incubated 2 min with increasing concentrations of peptide 7 (up to 0.1 M). Fluorescence was monitored as reported above. 80–90% fluorescence quenching was obtained by using a concentration corresponding to 2 K_d of pNA-containing peptides. Maximum fluorescence was recorded by incubating hCyp-18 with 10% DMSO and without pNA compounds.

3. Results

Catalytic efficiency k_{cat}/K_m of hCyp-18 for peptide 1 was determined using the standard uncoupled PPIase assay [23], whereas k_{cat}/K_m for compounds **2** and **3** were obtained with the α-chymotrypsin-coupled PPIase assay [20,23]. These values ($k_{cat}/K_m = 11.8\times10^6$ M^{-1} s^{-1} (**1**) and 7.4×10^6 M^{-1} s^{-1} (**2**)) are similar to previously reported data [18,19]. In turn, peptide **3** was a weaker substrate ($k_{cat}/K_m = 1.0\times10^6$ M^{-1} s^{-1}) than the corresponding tetrapeptide **2**. Dipeptide Suc-Pro-

Table 1
Determination of K_d and evaluation of the IC_{50} of compounds **1–18**

Product	K_d (μM) ± S.D.[a]	IC_{50} (μM) ± S.D.[b]
Suc-Ala-Ala-Pro-Phe-pNA **2**	135 ± 20	540 ± 70
Suc-Ala-Pro-Phe-pNA **3**	170 ± 40	570 ± 60
Suc-Pro-Phe-pNA **4**	180 ± 10	1090 ± 170
Phe-pNA **5**	84 ± 4	NI[c]
pNA **6**	350 ± 70	NI[c]
Ala-Pro **7**	[d]	30 000[e]
Suc-Ala-Phe-pNA **8**	94 ± 7	NI[c]
Suc-Ala-Ala-Pro-NH_2 **9**	[d]	14 000[e]
Suc-Ala-Ala-Pro-Phe-DFA **1**	(> 500)[f]	4400 ± 500
DFA **10**	> 100 000	NI[c]
Suc-Ala-Pro-Phe-An **11**	990 ± 60	> 10 000[g]
Suc-Ala-Pro-Phe-pCMA **12**	55 ± 6	700 ± 90
Suc-Ala-Pro-Phe-pCA **13**	105 ± 10	> 10 000[g]
Suc-Ala-Pro-Phe-pAA **14**	315 ± 40	5800 ± 400
Ac-Ala-Pro-Phe-pNA **15**	140 ± 40	770 ± 200
Ac-Ala-Pro-Phe-pTMAA **16**	1450 ± 100	7000 ± 1000
Suc-Ala-Pro-(D)-Phe-pNA **17**	95 ± 10	NI[c]
Suc-Ala-Pro-Pheψ(CH_2-NH)pNA **18**	1190 ± 30	NI[c]

pAA, 4-aminoaniline; pCA, 4-carboxyaniline; pCMA, 4-carboxymethylaniline; pTMAA, 4-(trimethylammonium)aniline; S.D., standard deviation

[a]Determined by fluorimetric titration of hCyp-18 Trp121 (λ_{excit} 285 nm, λ_{emis} 322 nm) at 20°C in presence of recombinant hCyp-18 (320 nM) and DMSO (10%) in a 35 mM HEPES buffer at pH 7.8 ($n = 2$).

[b]Determined using the standard trypsin-coupled assay in the presence of recombinant hCyp-18 (16 nM), trysine (4.2 μM) in a 35 mM HEPES buffer at pH 8.6 and DMSO (2%) ($n = 2$).

[c]No inhibition was observed at inhibitor concentration up to 100 mM.

[d]Addition of increasing concentration of peptide did not modify the fluorescence of Trp121.

[e]Evaluated IC_{50}.

[f]Compound **1** produces a hCyp-18-independent rise of the fluorescence at high concentration; a plateau was reached at 2 mM.

[g]Evaluated IC_{50}, 50% inhibition of the PPIase activity was not reached at concentrations up to 10 mM.

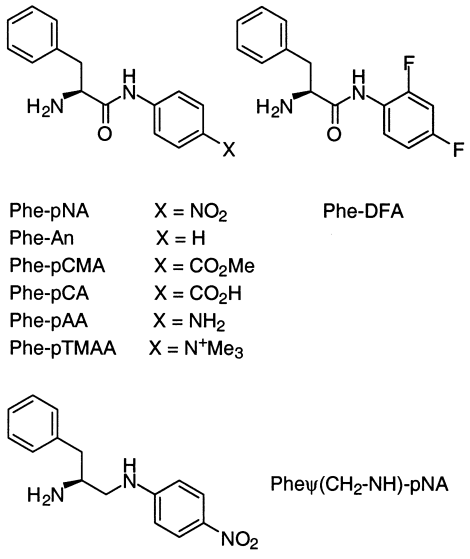

Fig. 1. Chemical structures of various phenylalanyl anilide derivatives used in the present study.

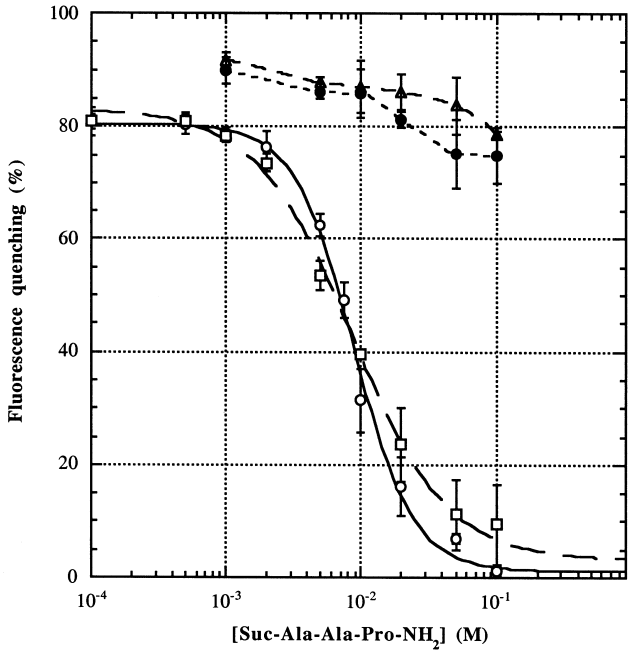

Fig. 2. Competition experiments of various pNA derivatives using Suc-Ala-Ala-Pro-NH_2 **9**. Recombinant hCyp-18 was preincubated 2 min with **2–8** (dissolved in DMSO) in a 35 mM HEPES buffer at pH 7.8 ($n=2$). Increasing concentrations of peptide **9** were added and hCyp-18 Trp121 was titrated by fluorimetry ($n=2$); ○: 340 μM Suc-Ala-Pro-Phe-pNA **3** ($IC_{50}=8.8\pm0.4$ mM); □: 360 μM Suc-Pro-Phe-pNA **4** ($IC_{50}=8.6\pm0.6$ mM); ▲: 190 μM Suc-Ala-Phe-pNA 8; ●: 170 μM Phe-pNA **5**; results obtained with tetrapeptide **2** ($IC_{50}=9.0\pm0.4$ mM) are very similar to those recorded with 270 μM of compound **3** and are not represented for clarity.

Phe-pNA **4** was resistant to α-chymotrypsin in the standard conditions. Use of a 10-fold excess of protease showed that compound **4** is a poor substrate of hCyp-18.

We investigated the effects of peptides derived from the standard substrate **2** on both the binding to hCyp-18 [16] and the PPIase activity[2][19,23–25]. As expected, ablation of the N-terminus did not modify significantly the binding to hCyp-18 (Table 1). Due to the existence of the specific α-chymotrypsin cleavage site in peptides **1–4, 8, 11–16**, the IC_{50} were evaluated using the standard trypsin-coupled PPIase assay [25] using the substrate Suc-Ala-Ala-Pro-Arg-pNA. IC_{50} values were not affected by length changes provided that peptides possessed a proline residue. Surprisingly, compounds **5**, **6**, and **8** devoid of proline were found to be ligands of cyclophilin. However, these compounds are not able to block the PPIase activity. In contrast, dipeptide Ala-Pro **7**, which is a ligand of hCyp-18 [10], and Suc-Ala-Ala-Pro-NH_2 **9** are weak inhibitors of the *cis/trans* isomerization of the substrate peptide Suc-Ala-Ala-Pro-Arg-pNA.

In order to probe the mode of interaction of the different pNA-containing compounds, we used tripeptide **9** as a competitor of peptides **2–5** and **8**. As anticipated, compound **9** was able to inhibit the PPIase activity (Table 1) without affecting the fluorescence of Trp121. Indeed, peptide **9** is truncated at the C-terminus and hence is not able to quench the fluorescence of Trp121. Therefore, hCyp-18 was incubated with concentrations of pNA-containing compounds corresponding to the value of 2 K_d which causes a 80–90% quenching of fluorescence. Recovery of the initial fluorescence of hCyp-18 Trp121 was obtained by adding increasing concentrations of tripeptide 9. As expected Suc-Ala-Ala-Pro-NH_2 **9** was able to displace proline-containing peptides **2–4**. However, concentrations up to 100 mM in tripeptide **9** did not restore the fluorescence after preincubation of hCyp-18 with Phe-pNA **5** or Suc-Ala-Phe-pNA **8** (Fig. 2).

We also investigated the functional significance of the interaction of Arg148 with the pNA nitro group as revealed by X-ray crystallography [11]. This was carried out using either tetrapeptide **1**, or tripeptides **11–16** (Table 1). Indeed, deletion of the first alanine residue has only a little effect on both the binding to hCyp-18 and the inhibition of the PPIase activity. Even though it was a good substrate, peptide **1** was a weaker ligand of cyclophilin than peptides **2** and **3**. 2,4-Difluoroaniline (DFA) did not bind hCyp-18. Replacement of pNA with An caused a significant decrease of affinity. Substitution of the nitro group with a H-bond donor such as carboxymethyl (compound **12**) and carboxylate (compound **13**) resulted in a slight improvement of the binding. Replacement with an amine (peptide **14**) had a slightly negative effect on K_d, whereas introduction of a trimethylammonium induced a 10-fold decrease of affinity relative to the corresponding nitro compound **15**[3].

We also investigated the ability of these compounds to inhibit the PPIase activity. Peptide **12**, the best ligand in the series, efficiently competed with the substrate. All other peptides were very weak inhibitors of the PPIase activity. Unexpectedly, pCA-containing peptide **13**, which binds hCyp-18

[2] Although the peptides tested herein are expected to be substrates rather than inhibitors of the PPIase activity of hCyp-18, the competitive effect relative to the catalyzed isomerization of Suc-Ala-Ala-Pro-Arg-pNA will be called 'inhibition' for conciseness and clarity.

[3] Permethylation of the aminoaniline (peptide **13**) was carried out with the Ac-peptide due to synthetic problems encountered with the Suc derivative. We checked that this N-terminal change had no effect on the interaction (peptide **12**).

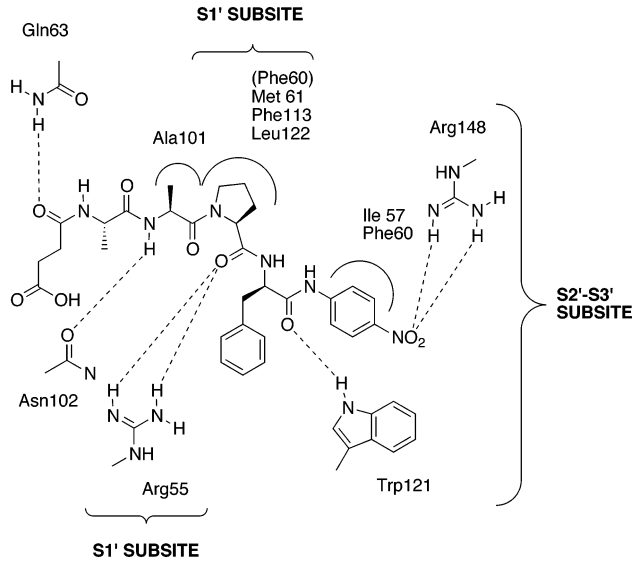

Fig. 3. Main interactions of tetrapeptide **2** with hCyp-18 as revealed by the X-ray study of the complex [11]. The functionally independent subsites S1′ and S2′ have been delineated from the data obtained in the present study.

with an affinity equivalent to the reference tripeptide **3**, was not a cyclophilin inhibitor.

Peptide **17** and pseudopeptide **18**, which possess respectively a (D)-Phe residue at P2′ and a reduced amide (2-amino-3-phenyl-propyl)-4-nitroaniline (Pheψ(CH_2-NH)pNA) (Fig. 1), were assayed as ligands and inhibitors of the hCyp (Table 1). As previously observed by Fischer and coworkers, inversion of the Cα configuration at P2′ caused a complete lack of inhibition of the PPIase activity [13]. However, this substitution did not affect the affinity as demonstrated by fluorescence titration. Reduction of the Phe-pNA carboxyl group (peptide **18**) caused a dramatic decrease of affinity and an unexpected lack of inhibition [17].

4. Discussion

Fig. 3 summarizes the interactions of tetrapeptide **2** with hCyp-18 as revealed by the X-ray resolution of the complex. Compilation of structural data of several cyclophilin:peptide complexes have led to the delineation of peptide moieties which are important for the interaction. However, only a little number of data allow to estimate the relative importances of each interaction for both the binding and the activity of hCyp-18.

Results showed that ablation of the N-terminal part of tetrapeptide **2** up to a dipeptide neither affected significantly the affinity nor its ability to inhibit hCyp-18. However, reduction of the length caused a decrease of catalytic efficiency. These results are consistent with previous studies which have shown that *cis*/*trans* isomerization [26] as well as proteolysis [27] require a minimum binding sequence. Unexpectedly, Suc-Ala-Phe-pNA **8** as well as other pNA derivatives interacted with a good affinity as well. Binding of peptides devoid of the classical sequence Xaa-Pro-Xaa to a yeast cyclophilin [28] and to the trigger factor [29], another important immunophilin, have been already reported. Interaction of hCyp-18 with peptides which do not contain proline is well-known with cyclic peptides [30,31] and linear peptidomimetics [32–34] as well. However, although peptide **8** and pNA derivatives tightly bind close to Trp121, they are unable to block the PPIase activity of hCyp-18. The (D)-Phe-containing peptide **17** cannot inhibit cyclophilin [13], whereas it binds the enzyme with a good affinity. The disconnection between K_d and IC_{50} strongly suggests the existence of two functionally distinct subsites: (i) a S1′ proline subsite delineated by Met61, Ala101, Phe113 and Leu122; (ii) a S2′–S3′ Phe-pNA subsite surrounded by Ile57, Phe60, Trp121 and Arg148 (Fig. 3). Moreover, this is also supported by our results which demonstrate that An and difluoroaniline-containing peptides interact at the pNA subsite with a relatively low affinity and are weak inhibitors of the PPIase activity although they contain a proline residue. Competition experiments confirm this hypothesis since tripeptide **9** can compete with peptides **2–4**, whereas it is unable to displace neither Suc-Ala-Phe-pNA **8** nor Phe-pNA **5**. This demonstrates the existence of two subsites and implies that subsites S1′ and S2′–S3′ are functionally independent. As a consequence, hCyp-18 can accommodate both S1′- and S2′–S3′-selective ligands simultaneously. We checked that the pNA-dependent fluorescence quenching was not the result of a non-specific interaction with the hydrophobic core delineated by Ile57, Phe60 and Trp121 (Fig. 3). Indeed, hCyp-18 is able to interact with peptides bearing a hydrophobic C-terminus such as an amidomethyl coumarin moiety [35,36]. This might be explained by the rotation of the Arg148 side-chain which enables the binding of hydrophobic molecules [13,33]. However, no binding of DFA to hCyp-18 could be detected even at a concentration up to 100 mM although peptide **1** is a ligand of cyclophilin. Since DFA possesses an electron-deficient phenyl moiety roughly similar to pNA, the lack of interaction suggests that the H-bond between Arg148 and the nitro group of pNA plays a major role in the binding.

The influence of various substituents at the *para*-position of An confirmed the important role of a H-bond acceptor at this position. Indeed, hCyp-18 binds more efficiently peptide **12** which has a carboxymethyl C-terminus. On the other hand, peptides bearing either a charged or an uncharged H-bond donor have a lower affinity and are weaker inhibitors of the PPIase activity. Hence, Arg148 seems to play an important role in the binding of An derivatives and some other compounds such as cyclophilin [37]. However, Arg148 is not implicated in the binding of the Gag polyprotein [38,39].

Structural data of all complexes [10–13] have underlined the importance of the H-bond between the P2′ carbonyl and indole moiety of Trp121. This was also observed with fragments of the Gag polyprotein [38,39] and cyclophilin [37]. On the other hand, Schultz and coworkers have demonstrated that a transition-state analogue of the PPIase activity, bearing a Pheψ(CH_2-NH)pNA C-terminus, is a good inhibitor of hCyp-18 [17]. However, we observed that reduction of the Phe carboxamide (peptide **18**) caused a significant decrease of the affinity. This indicates that the interaction made by the P2′ carboxamide with Trp121 seems to be critical in most cases [10,37–39] and cannot be suppressed.

In conclusion, our results show that the specific binding of peptides to hCyp-18 is not sufficient for inhibiting the *cis*/*trans* isomerization of the reference substrate. The N-terminal part of the peptide can be truncated up to a dipeptide without significantly affecting K_d and IC_{50} values, provided that the proline residue is maintained at P1′. Cyclophilin is able to

bind smaller ligands and non-proline-containing peptides as well although they cannot inhibit the PPIase activity. These results as well as competition experiments strongly suggest the existence of two functionally independent subsites. The S1′ subsite, encompasses the relatively open catalytic site together with the narrow proline recognition pocket. The S2′–S3′ subsite, might control the binding by interacting both with the guanidinium group of Arg148 and the indole of Trp121. The nitro group can be replaced with a carboxymethyl group which is anticipated to make a H-bond with Arg148. The H-bond between the P2′ carbonyl and Trp121 indole seems to control the interaction at both S1′ and S2′–S3′ subsites.

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