

The precise chemical-physical nature of the pharmacore in FK506 binding protein inhibition: ElteX, a new class of nanomolar FKBP12 ligands

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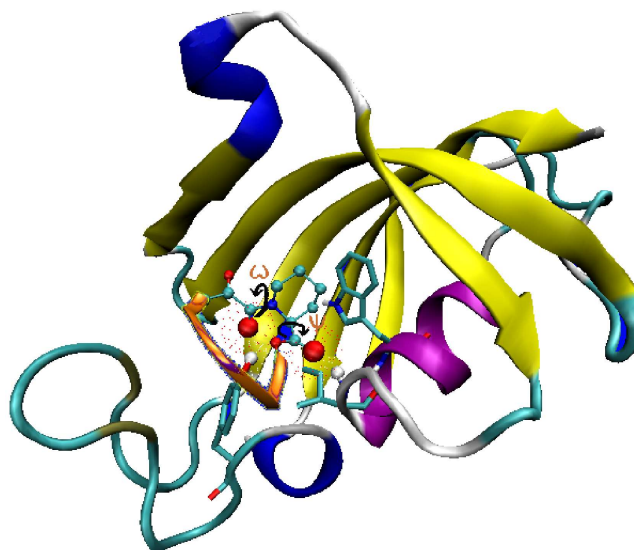
Abstract

Due to its central role in immunosuppression and cell proliferation and due to its specific peptidyl-prolyl-isomerase (PPI) function, the FKBP protein family is at the crossroad of several important metabolic pathways. Members of this family, and notably FK506 binding protein (FKBP12), are thought to be involved in neurodegenerative diseases such as Alzheimer disease, Parkinson disease, Multiple Sclerosis, Amyotrophic Lateral Sclerosis, as well as in proliferation disorders and cancer. Using an interdisciplinary approach based on computational, synthetic and experimental techniques, we show that the best potential binders for FKBP proteins optimally expose the two contiguous carbonyl oxygen in the proline-mimetic chain for FKBP docking and are characterized by the abundance of rigid *quasi*-cyclic structures stabilized in aqueous solution by intraligand hydrophobic interactions mimicking the macrolide structure of the natural FKBP binders FK506 and Rapamycin. These peculiar structural and

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chemical-physical features define at the same time an ElteX compound and the minimal pharmacore in the FKBP family, shedding new light on the isomerization mechanism of the PPI domain. Based on the above hypothesis, we have successfully designed and synthesized several nanomolar ElteX FKBP12 ligands. Among these, ElteN378 is a new low atomic weight ligand with activity comparable to that of the macrolide Rapamycin.

keywords: *m-TOR*, *Calcineurin*, *cis-trans isomerase*, *effector domain*, *configurational entropy*, *Replica Exchange*, *Elte421*



TOC figure: pharmacore for the PPI domain. The H-bonds are indicated with a dotted trait. The proline-mimetic pipecolyl α -keto amide subunit is shown in CPK representation. Tyr82, Ile56 and Trp59 of FKBP12 are shown in bond representation. The rigid through-bond (as in FK506) or through space (as in ElteX) connector is the loop in orange color.

Introduction

The FK506 binding proteins (FKBP) form an important subset of the family of the immunophilins (to date more than 20 FKBP are known¹), all sharing at least one common peptidyl-prolyl isomerase (PPI) domain. FKBP proteins are ubiquitous in the body of mammals, and are highly enriched in the central and peripheral nervous system.^{2,3} FKBP have regulatory and/or chaperone function, possibly related to PPI mediated protein folding/unfolding assistance.^{1,4,5} The archetypal FKBP12 protein, the smallest member of the family, contains just one PPI domain and is notable in humans for binding to the immunosuppressant molecules FK506 and Rapamycin. The FK506-FKBP12 complex serves as a ligand to Calcineurin, a cellular target for signal transduction in T-cells activation,^{6,7} while the Rapamycin-FKBP12 complex binds to the mammalian target of Rapamycin (mTOR), a kinase that has a regulatory function in cell growth, cell motility, and cell proliferation.⁸ The immunosuppression or cell growth regulation power of FK506 or Rapamycin natural drugs is allosteric in nature as their binding ability vs Calcineurin or mTOR requires the formation of the ligand-FKBP12 complex.

Because of its central role in immunosuppression or cell proliferation and because of its specific PPI-related function, the FKBP family is definitely at the crossroad of several important metabolic pathways.⁹ Members of this family, and notably FKBP12, are thought to be involved in neurodegenerative diseases such as Alzheimer disease^{10,11} (AD), Parkinson disease¹² (PD), Multiple Sclerosis¹³⁻¹⁵ (MS) Amyotrophic Lateral Sclerosis¹⁶ (ALS), as well as in proliferation disorders and cancer.^{8,17}

While FK506 and FK506-related ligands^{18,19} are long time known for their Calcineurin-related immunosuppressant properties and are routinely used in treating patients after organ transplant, their involvement in neurodegenerative disease such as AD and PD has been reported only recently.^{1,4} The FK506 ligand, for example, has been shown to produce neuroprotection and neuroregenerative effects in protein conformational disorders²⁰⁻²² envisaging a possible role of Calcineurin signalling pathways in AD and PD as well as in ALS. However, most reported neuroprotective or neuroregenerative effects of FKBP ligands are likely to be independent of Calcineurin ac-

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tivity as these were obtained using synthetic ligands without affinity for Calcineurin (the so-called non-immunosuppressive immunophilin ligands (NI-IL)).^{4,23} The neuroprotection/neurotrophic power of FKBP ligands could be hence due to the direct inhibition of the specific PPI function when a NI-IL binds in the active site of the PPI domain. As a matter of fact, by serendipity it was recently observed that an FKBP-type *Escherichia coli* PPIase impurity found in a recombinant α -Synuclein preparation (α -SYN is the precursor of Lewy body fibrils in PD) remarkably increased its aggregation kinetics.^{4,24,25} FKBP12 clearly accelerated aggregation of recombinant α -SYN in vitro, an effect that was inhibited by IL or NI-IL ligands.¹²

Evidences of the efficacy of the Rapamycin FKBP ligand in animal models of human Multiple Sclerosis have been also recently gathered.^{13,14,26} In particular oral or intraperitoneal treatment with Rapamycin at the peak of disease or at the end of the first clinical attack, dramatically ameliorated the clinical course of experimental autoimmune encephalomyelitis in mice.¹³ The efficacy of Rapamycin FKBP ligand in experimental autoimmune disease has been related to the mTOR signalling pathways and hence to the formation of the Rapamycin-FKBP-mTOR ternary complex.^{14,15} Moreover, in recent times, significant anecdotal clinical improvement or even complete remission of Multiple Sclerosis has been reported in human patients treated with FK506 following organ transplantation,^{27,28} pinpointing to a possible Calcineurin mediated role for MS in an ongoing immune system activation after transplant.

Interference with mTOR signalling network regulating cellular growth and survival is indeed at the center of the well documented anti-cancer activity of FKBP12 ligands (in the past decade, The FKBP ligand Rapamycin has been clinically approved for some cancer indications¹⁷). The role of FKBP12 ligands such as Rapamycin in cancer therapy is related to their ability to allosterically bind mTOR in complex with FKBP12. As for the FK506-Calcineurin interaction, the mTOR-Rapamycin interaction occurs via the so-called *effector domain*²⁹ of the ligand (opposed to the FKBP12 *binding domain* in Rapamycin) and the flexible A81-T96 loop in FKBP12. Such FKBP12-Rapamycin complex and mTOR interaction is hydrophobic in nature as it involves stacking of the trienic chain in the effector domain of Rapamycin with some planar residues in the FRB

domain of mTOR.^{30,31} A similar situation can be observed in the Calcineurin-FK506-FKBP12 complex, where again the hydrophobic effector domain of FK506 mediates the interaction between the A81-T96 loop in FKBP12 with the catalytic subunit of Calcineurin.³²

The centrality of the FKBP family, and notably of the archetypal FKBP12 PPI single domain, in the protein metabolism and its involvement in many incurable diseases provides a solid rationale for understanding the chemistry of FKBP inhibition at the PPI domain so as to effectively design new potent synthetic ligands with, or devoid of, their immunosuppressant power.^{11,19,24} Small NI-IL ligands for example, selectively inhibiting the PPI function of FKBP, may be used in the treatment of protein conformational disorders without negatively interfering with mTOR or Calcineurin mediated biological activity. On the other hand, when the target of a possible FKBP allosteric drug is the immune system and cell growth regulation, it is important to design compounds with an optimal balance and minimal interference between effector (vs mTOR or Calcineurin targets) and binding domain (vs FKBP).

In a recent computational study we identify a correlation between the activity of FKBP12 FK506-related ligands with disparate FKBP12 inhibition constants and their average structure in water solution as detected by Replica Exchange Simulations (REM).³³ In particular, we showed that the most effective of such ligands, all sharing a common pipecolyl α -keto amides moiety (the putative pharmacore for FKBP12^{1,18,34,35}) optimally expose the two binding carbonyls flanking from opposite side the proline-mimetic piperidinic ring. In all resolved complexes of FKBP proteins with FK506-related pipecolic derivatives,³³ the carbonyl in the α -keto amide region H-binds the hydroxy group of Tyr82, while that of the carboxylate moiety H-binds the NH backbone unit of Ile56. These two H-bonds are probably at the very basis of molecular recognition in the pair involving the guest NI-IL or IL ligand and the host FKBP. Not surprisingly Tyr82 and Ile56 are the two most conserved residues across the entire FKBP family, with a conservation ratio of 1 and 0.8, respectively.¹ In the proline-mimetic pipecolyl α -keto amide subunit (PKA), the optimal mutual exposure of the two binding carbonyls is regulated by the two angles ω and ψ . The distribution probability of these angles is in turn modulated in aqueous environment via the intraligand

hydrophobic interactions of the two groups on opposite side of the PKA that impart to the the lig- and a *quasi* cyclic structure mimicking the rigidity of the macrolides FK506 or Rapamycin, while maintaining solvent-exposed the binding domain of the ligand. When all these requirements, i.e. optimal mutual exposure of the CO binding units, hydrophobic interactions of the two accessory groups of the PKA and low configurational entropy, are met, the synthetic molecule has the potential to strongly bind the PPI domain of FKBP in an aqueous environment, as it occurs in the potent FKBP12 inhibitor (1R)-1,3-Diphenyl-1-propyl(2S)-1-(3,3-Dimethyl- 1,2-dioxopenty1)-2-piperidinecarboxylate (15R-sb3).¹⁸ The essential ingredients of the pharmacore for the PPI domain in FKBP family members are summarized in Figure 1. According to this new perspective,

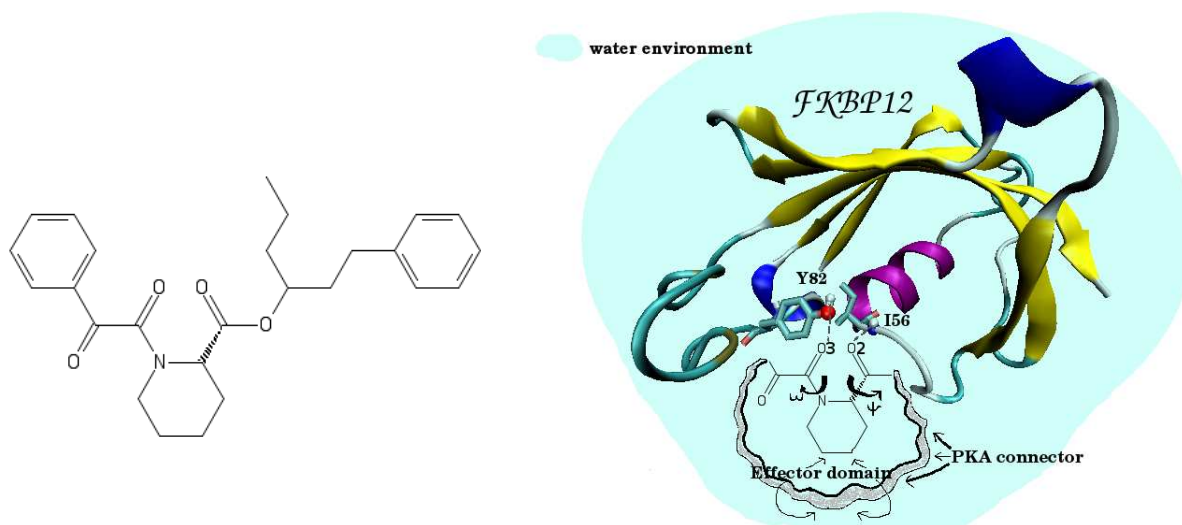


Figure 1: Left: Chemical Structure of Elte421. Right: Pharmacore elements for binding to the PPI domain in FKBP family. The binding domain involves the two carbonyls of the pipecolyl- α -keto-amide (PKA) unit. The optimal 3D exposure of the two CO units for forming the H-bonds with I56 and Y82 of FKBP12 is controlled by the two angles ω and ϕ shown in the figure. The effector domain pointing outward for the allosteric interaction with mTOR or Calcineurin can be appropriately placed either on the rigid connector or as a substituent on the piperidinic ring.

the inhibition of the PPIase activity by FKBP ligands mainly occurs via *polar*, rather hydrophobic, protein-ligand interactions, thus contradicting the common belief^{29,36} that the binding pocket of the PPI domain is hydrophobic. The hydrophobic groups at opposite sides of the PKA that characterize potent NI-IL ligands (such as in V10,³⁶⁷²⁹ or Elte421³⁷) play the indeed crucial twofold role, via hydrophobic intraligand interactions,³³ of i) stabilizing semi-rigid pseudo-cyclic confor-

mations (mimicking the natural FKBP12 macrolide binders) *in water environment* and ii) correctly exposing the two binding carbonyl units of the PKA. While the PKA moiety tightly binds to the protein, a potential effector domain for allosteric interactions should be located in the non polar portion of the ligand, opposed to the binding PKA domain (as occurs in e.g. Rapamycin where the effector domain is the trienic chain subunit of the macrolide interacting with hydrophobic residues in m-TOR).

Developing upon these ideas, in a follow-up paper,³⁷ we have set up a rational drug design based on preliminary costless simulations of potential binders in bulk solution (i.e. in absence of the protein) and on the analysis of their conformational behavior. The best candidates meeting the pharmacore criteria highlighted in Figure 1 were then assigned to organic synthesis. The structural features and resulting activity vs FKBP of the selected compounds in bulk solution were finally measured and assessed using again fluorescence spectroscopy.

Such simulation-driven multidisciplinary design turned out to be very successful leading to the synthesis of a new potent nanomolar inhibitor of FKBP protein, 1-phenylhexan-3-yl 1-(2-oxo-2-phenylacetyl) piperidine-2-carboxylate (named Elte421 and shown in Figure 1, left). Such compound was designed by optimally re-dislocating the hydrophobic groups in sb3 (phenyl and alkyl chains) so as to stabilize *at the same time* via $\pi - \pi$ stacking interactions the R stereo-isomer and, as in 15R-sb3, via phenyl-1-alkyl hydrophobic interactions the S partner. The binding affinity of the 1:1 diastereoisomeric mixture of R-Elte421 and S-Elte421 vs FKBP12 was found of the same order of magnitude of that of FK506 and significantly stronger than R-sb3.

In the present study, we have synthesized separately the two diastereoisomers 15R-Elte421 and 15S-Elte21 and measured their activity for FKBP12, finding comparable binding affinities (both of nanomolar order) fully confirming the prediction of Ref.³³ Then, by using a multidisciplinary approach for rational drug design based on advanced all atoms simulation techniques, fluorescence measurements and organic synthesis,³⁷ we further characterized the chemical-physical determinants of the peptidomimetic FKBP ligands, eventually obtaining a new non diastereoisomeric sub-nanomolar FKBP12 binder. Such compound, (2S)-1-(2-oxo-2-phenylacetyl)-N-(3-phenylpropyl)

piperidine-2-carboxamide (named ElteN378), is *de facto* a peptidomimetic for a δ -turn, with the carboxylate group replaced by an amide group. The activity of this synthetic drug is significantly higher than that of Elte421 or of other potent NI-IL inhibitors such as v10,367²⁹, higher than that of FK506 and comparable to that of natural Rapamycin, making of ElteN378, to our knowledge, the most potent synthetic inhibitor of FKBP12 to date.

The paper is organized as follows. In the section “Methods” we succinctly describe the molecular dynamics simulation methodologies, the synthesis and characterization of the ElteX compounds and the fluorescence-based techniques used to determine the binding affinity of x-Elte ligands vs FKBP12. In the section Results and Discussion we present computational and experimental results on the (R,S)-Elte421, ElteN420 and ElteN378 compounds focusing on their conformational landscape. In the concluding section we provide perspectives and future directions.

Materials and Methods

In this study we have simulated using Hamiltonian Replica Exchange³⁸ with solute tempering (ST-HREM),^{39,40} the following ligands: (R,S)-Elte421, (R,S)-ElteN420 and ElteN378. These compounds are depicted in Figure 2. R-Elte421 and S-Elte421 were separately synthesized while ElteN420 was obtained as a 1:1 diastereoisomeric mixture (the synthesis of R,S-Elte421 and of ElteN420 is described in the SI). The synthesis of ElteN378 is described in this section. FK506 (M.W. 804.02) was obtained from Sigma-Aldrich (Italy) with purity greater than 98% (obtained by HPLC). FKBP12 (expressed in *Escherichia coli*, M.W. 11600) for fluorescence determination of binding affinity of ElteX compounds was supplied by Consorzio Interuniversitario Risonanze Magnetiche di Metalloproteine Paramagnetiche, CIRMMP (Italy, Florence). The purity of FKBP12 was greater than 95% determined by SDS electrophoresis; DMSO, NaCl, K₂HPO₄ and KH₂PO₄ were obtained from Sigma-Aldrich (Italy). Water (resistivity = 18 M Ω cm, pH = 5.6 at 20 °C) was obtained from a Milli-RO coupled with a Milli-Q set up (Millipore, Italy).

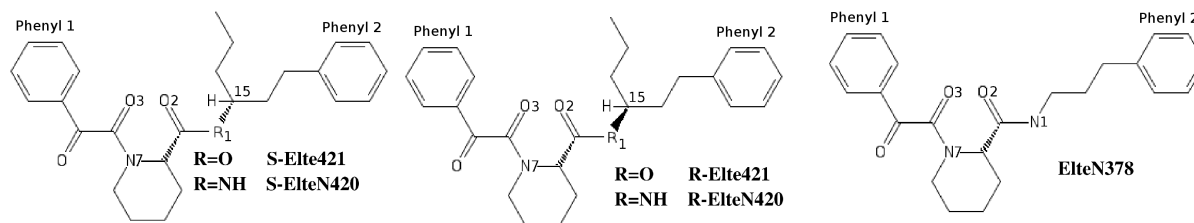


Figure 2: Chemical formula of Elte compounds. The atom numbering follows the PDB numbering of FK506 related ligands.¹⁸ Phenyl-1 and phenyl-2 (on the left and on the right, respectively of the central PKA unit) are invariant elements in ElteX ligands.

Computational techniques

The force field for all ligands is based on the AMBER03 parametrization.^{33,41} The atomic charges were determined using Density Functional Theory (DFT) with the B3LYP exchange-correlation functional⁴² and a 6-31Gd split valence basis set by evaluating on the optimized geometry a gridded electrostatic potential (ESP) according to the Merz-Singh-Kollman scheme.⁴³ Equivalent charges (e.g. charges on the hydrogen atoms of methyl groups) have been symmetrized. Parameters of force field for all simulated ligands are given in the SI. The ST-HREM simulation for each ligand in bulk water was performed using the Hamiltonian REM approach as enforced in the program ORAC.^{33,39} Simulation details are given in the SI. Structural insights on the equilibrium population of the target replica can be attained through a Quality Threshold clustering.⁴⁴ This algorithm requires a distance matrix for all pairs of structures of the population. The metrics that we chose when evaluating the “distance” between two structures was the maximum difference between corresponding pairs of heavy atoms atoms. For each structure in the ensemble the algorithm builds a candidate cluster in such a way that the distance between any two structures of the cluster do not exceed the cutoff distance (that we chose to be $d_{cut} = 3.0 \text{ \AA}$). The program then retains only the largest cluster and removes its structures from the population. The procedure is iterated until all structures of the populations are used. The distance criterion based on the above metrics produces clusters of highly related structural conformers and compares favourably with the more customary RMSD-based metrics.⁴⁵

Organic Synthesis of Elte-like compounds

The synthesis of compounds ElteX was carried out with the same procedure previously reported for the preparation of Elte421³⁷ using commercial available N-Boc(L)-pipecolic acid and phenylglyoxylic acid as common starting materials. As an example, the synthesis of ElteN378 is described in Figure 3. In particular, commercially available 3-phenylpropan-1-amine was reacted

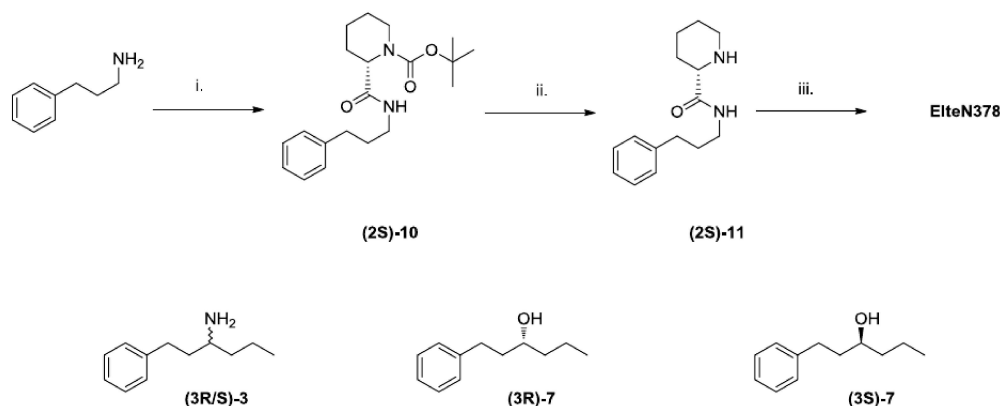


Figure 3: Synthesis of ElteN378: i. N-Boc(L)-pipecolic acid (2 equiv), DIC (2 eq.), DMAP (0.1 equiv), DCM r.t, overnight. ii. TFA (24 equiv) in DCM, r.t, 4 h. iii. phenylglyoxylic acid (1.3 equiv), DIC (1.3 equiv), DMAP (0.2 equiv), DCM, r.t, 18 h.

with N-Boc(L)-pipecolic acid in the presence of DIC and a catalytic amount of DMAP to obtain the amide (2S)-10. After deprotection of Boc group of (2S)-10 with TFA in DCM, the pipecolic nitrogen was reacted with phenylglyoxylic acid under the same amidation conditions described above allowing the isolation of ligand ElteN378 used in this study (Figure 3). Ligands ElteN420, R-Elte421 and S-Elte421 have been prepared with the same procedure, using amine (3R/S)-3, and alcohols (3R)-7 and (3S)-7 in the first coupling step. Racemic amine 3 was prepared from the corresponding racemic alcohol 7 via tosylation, nucleophilic substitution with NaN₃ and hydrogenation of azido group. The synthesis of enantiopure alcohols (3R)-7 and (3S)-7 was achieved (Figure 4) by enantioselective allylation of aldehyde 5 using, respectively, (-)- and (+)-allyldiisopinocampheylborane to obtain the secondary homoallylic alcohols (3S)-6 and (3R)-6 with yields and e.e. equivalent to those already reported in literature.⁴⁶ Eventually, catalytic hy-

drogenation of terminal double bond in 6 provided the desired alcohols (3R)-7 and (3S)-7 as shown in Figure 4.

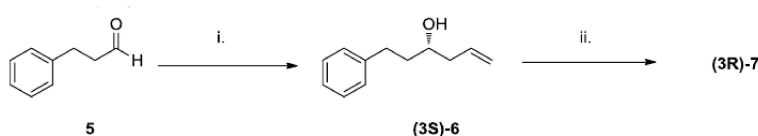


Figure 4: Synthesis of (3R)-7. i. a) (-)- β -allyldiisopinocampheylborane (1 equiv), EtO₂, -100 °C r.t. °C; b) NaOH 3M (3.5 mL), 30% H₂O₂ (1 mL), reflux, 1h. ii. H₂, Pd/C 5% (0.1 eq.) in MeOH, r.t., 2.5 h.

Fluorescence based experimental techniques

Absorption spectra were recorded on a Lambda900 spectrophotometer (Perkin Elmer, Italy) with cuvette cell holder connected to a Haacke thermostatic bath. Fluorescence spectra were recorded on a LS50B spectrofluorimeter (Perkin Elmer, Italy), excitation and emission slits were set to 3.5 or 5 nm depending on the optical path (OP) length of the cell employed. QS cell with 0.3 cm optical path length from Hellma (Hellma GmbH & Co. KG, Muellheim, Germany) were used for quenching experiments, whereas 1 cm OP cuvettes were used for absorption measurements and single component solutions. Cuvettes were cleaned with piranha solution and carefully rinsed with water and ethanol. The cuvettes were dried by nitrogen flushing prior to each measurement. All fluorescence measurements were run at 15 °C unless otherwise stated in the text, a temperature probe was inserted in the cuvette to ensure optimal temperature control. Stock FKBP12 concentration in PBS buffer (pH 7.4, 0.15 M NaCl) was determined by UV/Vis ($\epsilon_{280} = 9970 \text{ M}^{-1}\text{cm}^{-1}$). Aliquots of thrais solution were used to prepare adequate concentrations for quenching experiments by dilution with standard phosphate buffer. Emission spectra of the ElteN378 ligand were collected for DMSO, PBS/NaCl buffer and pure water (pH:5.6) solutions for different ligand concentrations in the range $7 \times 10^{-7} \text{ M}$ to $7 \times 10^{-5} \text{ M}$, the emission spectrum of the corresponding blank solution containing only the selected solvent was always recorded separately and subtracted. Emission spectra

of ElteN378 solutions were performed using different excitation wavelengths in the range 250-310 nm. Excitation spectra were run in the emission range $\lambda_{\text{em}} = 310 - 450$ nm.

Fluorescence quenching assays were performed measuring the decrease of intrinsic tryptophan fluorescence at 330 nm as a function of ligand concentration ($\lambda_{\text{exc}} = 280$ nm). The final FKBP12 concentration used for all measurements was 1 μM unless otherwise specified in the text. The desired ligand concentration in the sample was obtained by appropriate dilution of ligand stock solution. Ligand concentrations in the range 1 nM to 200 nM were used. In all cases the final DMSO concentration in the sample was kept lower than 0.3% V/V. Data points are the averaged of at least two independent measurements. Fluorescence measurements for low ligand concentrations, i.e. in a concentration range where the experimental errors are larger, were repeated three/four times and averaged. Emission spectra of the corresponding FKBP12-free samples were subtracted from the corresponding FKBP12-ligand fluorescence spectra. The apparent dissociation constant for each ligand was calculated from the decrease of fluorescence intensity values at $\lambda_{\text{em}} = 325$ nm as a function of ligand concentration. The quenching data were analyzed as previously described¹⁹ (see SI for further details). Measurements for the FK506 and Elte421 were repeated with the FKBP12 sample used in the present work and the quenching results were compared with the data obtained in a previous work.³⁷ The results, collected in the SI (SI, Table 1 and Table 2), showed the same relative quenching efficiency for Elte421 with respect to FK506 for both protein samples although the apparent dissociation constant K_d resulted to be significantly different, a result observed in the literature⁴⁷ and often overlooked.

Results and discussion.

Molecular recognition by FKBP12 ligands is invariably based in all known PDB structures of ligand-FKBP complexes^{18,32,48-50} on the formation two strong hydrogen bonds on the PPI domain involving two highly conserved residues across the FKBP family,¹ namely Tyrosine and Isoleucine (see Figures 2 and 1; when referring to protein residues, we shall use the FKBP12 [PDB id: 1fkg])

residue number throughout the paper). In all nanomolar ligands bearing a piperidyl α -keto amide moiety the formation of these H-bonds is possible if i) the stereochemistry of the C2 carbon on piperidinic ring is of the S type; ii) ω and ψ angles (see 1) are in the *trans* and *cis* configuration, respectively; iii) the 1-substituent on the piperidinic ring is in axial configuration. Such conformation yields a distance between the two binding carbonyl oxygens O2 and O3 of the ligand of approximately 4.6 Å and is found in both FKBP12 and FKBP35 proteins^{49,50} and whether the ligand is a macrolide as Rapamycin⁴⁸ or FK506^{32,49,50} or a small synthetic drugs such as R-sb3.¹⁸ As shown in Ref.³³ where the conformational features of PKA ligands in bulk solution were analyzed using molecular dynamics techniques, it appears that the most important aspect of the FKBP pharmacore for molecular recognition resides in the correct 3D orientation and in the stability of the two binding carbonyls of the ligand acting like a sort of “pipecolic clamp” in bulk water solution. In strong non macrolide ligands such as R-sb3, such feature is favoured by persistent through-space intraligand hydrophobic interactions that significantly reduce the entropy loss upon binding by imparting to the drug a pseudo-cyclic structure with solvent exposure of the highly polar pipecolic clamp. A similar situation is found also in the potent non immunosuppressant v10,367 compound,²⁹ ($K_i/K_{FK506} = 1.25$) where the hydrophobic intraligand interactions of the aromatic moieties on opposite site of the PKA, as in Elte-421, confer to the drug in solution a quasi-cyclic structure mimicking FK506. Based on these findings, in Ref.³⁷ by performing ST-HREM simulations of the drug *alone* in water bulk solution, we were able to design a new potent FKBP ligand by appropriately re-shuffling the hydrophobic groups on sb3 so as to obtain in *both* the Elte421 diastereoisomers a significant conformational population in the FKBP12 binding $\omega - \psi$ region comparable to that of the potent 15R-sb3 ligand. Such compound, named Elte421, compound was prepared as a 1:1 diastereoisomeric mixture with respect to the asymmetric carbon C15, while maintaining the S stereochemistry at C2. The resulting binding affinity relative to that of the FK506 compound was measured using fluorescence spectroscopy and found to be of the order of 3,³⁷ i.e. the Elte421 mixture has nanomolar activity vs FKBP12.

In the following we shall discuss the conformational behaviour in bulk solution of all ElteX

compounds depicted in Figure 2 form both the experimental and computational viewpoint as a rationale for their binding affinity vs FKBP proteins.

The R- and S-Elte421 diastereoisomers

When working in conditions of excess FKBP12 protein concentration ($C_p \simeq 1\mu$), the instability constant⁵¹ exhibited by the 1:1 (15S)-Elte (15R)-Elte mixture, is given by

$$K_{\text{eff}} = K_R \frac{x}{x+y} + K_S \frac{y}{x+y} \quad (1)$$

where $K_R = C_p(C/2 - x)/x$ and $K_S = C_p(C/2 - y)/y$ are the individual binding affinities for R and S diastereoisomers, x , y are the concentration at equilibrium of 15R-Elte421-FKBP12 and 15S-Elte421-FKBP12 complexes, respectively and C is the overall concentration of the diastereoisomeric mixture of the ligand. Using the definitions for K_R and K_S to eliminate x and y from Eq. 1, and exploiting the fact that $C_p \gg K_R$ and $C_p \gg K_S$, we arrive at

$$K_{\text{eff}} = \frac{K_R + K_S}{2} \quad (2)$$

In case of large disparities between K_R and K_S , the instability constant of the mixture must be dominated by the largest one, i.e. by that of the less potent binder among the two diastereoisomers. In Table 1 we report the relative values (with respect to FK506) of the binding affinities obtained for the Elte421 1:1 mixture and for the individual S-Elte421 and R-Elte421 diastereoisomers (see 2) synthesized in the present study. As reported in the SI, we found the *absolute* values of the binding affinities K_i for nanomolar ligands are very sensitive to the experimental settings, but the *relative* K_i obtained with respect to an “anchor” ligand (in our case FK506) in the same experimental conditions are not.⁴⁷ By inspection of Table 1, as correctly inferred in our previous work,³⁷ we first note that R-Elte421 and S-Elte421 have both nanomolar activity in PBS, with S-Elte having a slightly larger affinity. Such behavior can be rationalized by assessing the exposure of the two carbonyls in the PKA as derived from the Free Energy Surface (FES) in the ω , ψ space calculated

Table 1: conformational parameters for Elte-like compounds as found from the Hamiltonian REM simulation in water bulk solution; TS is the conformational entropy computed according to Eq. $TS = RT \sum_i P_i \log P_i$, where R is the gas constant, P_i is the probability of observing the conformation of the ligand in the i -th cluster and the sum goes over all clusters obtained with the QT algorithm (see method section). K_{tc} corresponds to the equilibrium constant for the ω -trans-cis rotamers. P_b is defined as the ratio between conformational states with $\omega > 130$, $\psi < 90$ and $\omega > 130$; P_{oo} is defined as the fraction of conformations with O2-O3 distance in the FKBP12 binding range 4.2-5.0 Å for O3-Tyr82 and O2-Ile56 H-bond formation (such distance in the Rapamycin-FKBP12 complex is 4.6 Å⁴⁸). The experimental binding affinity K_i vs FKBP12 is expressed as the ratio with respect to that of the FK506 compound measured in the same experimental conditions

Ligand	$TS/\text{kJ mol}^{-1}$	K_{tc}	P_b	P_{oo}	K_{inst}
R-sb3 ⁵²	9.2	2.3	0.38	0.39	25
S-sb3 ⁵²	8.9	3.6	0.29	0.30	750-1500
R-Elte421	6.5	4.8	0.32	0.42	1.6
S-Elte421	6.7	1.4	0.40	0.55	1.1
R-ElteN420	7.8	2.2	0.57	0.45	5 ^(a)
S-ElteN420	7.4	2.6	0.50	0.33	
ElteN378	5.0	4.5	0.91	0.53	0.5 ^(b)
ElteN378 ⁺	5.5	75	0.90	0.60	
v10367 ⁵³	9.9	>100	0.91	0.33	1.2
FK506 ⁵²	< 1	> 100	≈ 1	≈ 1	1
Rapamycin ⁵³	< 1	> 100	≈ 1	≈ 1	0.5

^(a) Apparent binding affinity of the 1:1 diastereoisomeric mixture.

^(b) Apparent binding affinity in PBS at pH 7.

in a REM simulation of the drug in water environment in standard conditions. Such FES has been computed in our previous work³⁷ and is reported for completeness also in Figure 22 of the SI. The S-Elte421 compound is characterized by a deep minimum in the FES close to the optimal ω , ψ values found in experimental FKBP12 complex with various ligands bearing the PKA unit.^{18,32,48–50} Such minimum is less pronounced in the R-Elte compound and somewhat shifted towards the ω , ψ trans-trans rotameric state. Correspondingly the measured binding affinity of S-Elte421 is larger than that of the R-compound. From Figure 22 of the SI we also notice that the R-Elte421 epimer has a less populated ω -cis rotamer with respect to S-Elte. As we shall see later on, the incidence of ω -cis conformation in R-Elte421 has a negative impact on the FKBP affinity since only the ω -trans rotamer is able to form the two conserved H-bonds in the PPI domain. Therefore, the low population of the cis conformer at equilibrium in bulk water solution partly compensates, in R-Elte421,

the non optimal exposure of the two binding carbonyls. As a consequence, and in contrast to the sb3¹⁸ parent compound where the S and R diastereoisomer affinities differ by two order of magnitude, in Elte421 the stereocenter C15 has a marginal role: the two Elte421 diastereoisomers have both nanomolar activity vs FKBP12, having comparable binding affinities.

As depicted in Figure 1, a third important ingredient for FKBP binding in Elte-like compounds is the reduced conformational entropy due to persistent intraligand hydrophobic interactions. In the REM simulation such entropy can be evaluated using the clustering analysis described in the section methods. The conformational entropies in bulk solution (reported in Table 1) for the R-Elte421 and S-Elte421 are similar and are about half kcal lower than those found for sb3. All other factors influencing the K_i being equal, this would translates in an increase of the FKBP binding strength by a factor 2:3.

Amide Elte derivatives

As stated above, the binding conformation of the PKA Elte421 ligands lies in the right bottom quadrant corresponding of the ω , ψ FES (see Figure 22 in the SI) to a *cis-trans* configuration of the ω , ψ dihedral angles. In order to stabilize such conformation, we may try to substitute the carboxylate group in Elte421 with an amide group, arriving at (15R/S,2S)-1-(2-oxo-2-phenylacetyl)-N-(1-phenylhexan-3-yl)piperidine-2-carboxamide (named ElteN420; see Figure 2). The N1-H moiety, by interacting via H-bond with O3 thus mimicking a tight turn of δ type, should force at the same time the *trans* conformation for ω dihedral and the *cis* conformation for ψ dihedral. In Figure 5 we report the FES with respect to the ω , ψ dihedral angles obtained from the REM simulation of the diastereoisomers R-ElteN420 and S-ElteN420 in water bulk solution. The substitution of the carboxylate unit with the amide unit indeed appears to stabilize the binding structures of the ligand (the H(N1)-O3 H-bonds are indicated in the binding clusters reported in the bottom right quadrant of 5). As a matter of fact, the optimal exposure of the PKA (as measured by the fraction P_b reported in Table 1) raises, with respect to Elte421, beyond 0.5 for both the ElteN420 diastereoisomers. Encouraged by these results and by the success in the parent Elte421 design (where the

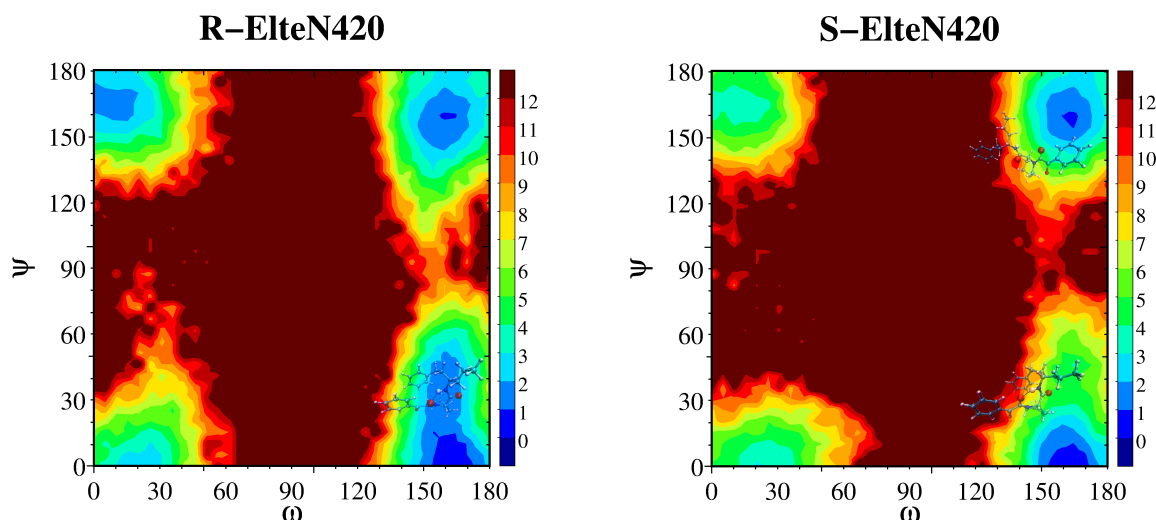


Figure 5: Free energy surface (FES) with respect to the dihedral angles ω and ψ (in degrees) of R-ElteN420 (left) and S-ElteN420 (right) calculated via solute tempering REM in bulk solution. The energy scale (labels on the color coded bar on the right of each diagram) is in kJ mol^{-1} . Perspective views of representative structures belonging to the most populated binding clusters with optimal PKA exposure for R-ElteN420 and S-ElteN420 (population, 29% and 16%, respectively) are reported in the 2D diagrams in the ω , ψ FKBP12 binding region (bottom right quadrant). The most populated *non binding* (ω and ψ both in trans) cluster for S-ElteN420 (population 21%) is also shown in the upper part of the 2D diagram. The O2 O3 and H(N1) atoms are highlighted with an enhanced sphere radius.

dislocation of the hydrophobic moieties on the molecule yielded comparable binding strength for the two epimers), following the scheme outlined in the SI we have synthesized ElteN420 as a 1:1 diastereoisomeric mixture and measured the corresponding binding affinity vs FKBP12 with respect to FK506 using fluorescence technique as described in the section methods. Disgruntledly, the potency of the mixture ElteN420 turned out to be slightly lower than that of the Elte421 mixture, yielding a binding affinity (relative to FK506) of 5. Several factors could responsible for such disappointing outcome: the ω *cis-trans* ratio of the ElteN421 epimers are not as favorable as

in R-Elte421 (see 1); the conformational entropy appears slightly larger, in the average, than that of Elte421. Also, at variance with S-Elte421, where the pseudo-cyclic conformations with strong hydrophobic intraligand interactions involving the propyl and phenyl-1 moiety were also those populating the ω, ψ binding region, in the case of the S-ElteN420 diastereoisomer, the open conformations with the three hydrophobic groups pointing away from each other (such as that depicted in the trans-trans quadrant of the corresponding 2D-FES, Figure 5, left) are comprised in the most populated cluster. Such open conformations present poor PKA exposure and are characterized by a large conformational entropy that do not conform with the semi-rigid pseudo-cyclic structure of the FKBP pharmacore (see Figure 1).

The differences in the conformational behaviour of the two ElteN420 diastereoisomers may be best appreciated in the Figure 23 of the SI where we observe the conformational details of the two ElteN420 epimers from the perspective of the 2D-FES taken as a function of the two distances O3-H(N1) and O3-O2 (plots in the bottom part of the Figure 23 of the SI). As for the FES of Figure 5, also in the diagram of Figure 23 of the SI the four minima in the R-ElteN420 diastereoisomer correspond to the four possible ω, ψ rotamers: the ω -trans and ψ -cis rotameric conformation represents the binding FKBP12 state where a weak O3-H(N1) H-bond is formed and O2-O3 is in the binding range around 4.5 Å. Surprisingly, in the S-Elte420 diastereoisomer the minimum corresponding to the ω -trans and ψ -cis rotamer is much less pronounced and partly fused with the ω -cis and ψ -cis non binding state at (low O2-O3 and O3-H(N1) distances). As further indicated by its low P_{oo} ratio reported in Table 1, S-ElteN420 conforms less satisfactorily with the FKBP pharmacore structure and should be thus significantly less active with respect to the R companion, thus degrading the overall affinity of the mixture according to Eq. 2.

From the cluster analysis on the REM configurations (see also Figure 26 of the SI), we note that population of the most populated *binding* clusters in both the diastereoisomers are characterized by a parallel displaced stacking of the two phenyl ring with one of these ring further stacked right above the planar α -keto amide moiety (see clusters structures in Figure 5, ω, ψ trans-cis region). Such rigid structures present in general optimal PKA exposure, typically exhibiting the δ -turn of

the peptidomimetic unit stabilized via the backbone H(N1)-O3 H-bond. At variance with Elte421, where the pseudo-cyclic structure of the S-Elte421 diastereoisomer was stabilized by the propyl-phenyl-1 interaction,³⁷ the propyl moiety in ElteN420 appears to have a marginal role in stabilizing transient pseudo-cyclic conformations. Actually, inspection of the highly populated binding cluster structures reported in the ω, ψ *trans-cis* region 5 reveal that the propyl moiety points in both cases *outward*, i.e. in the same direction of the pipecolic clamp, possibly interfering, at least in one epimer, with FKBP binding. Hence we decided to eliminate altogether the propyl group along with the stereocenter C15, finally landing on the compound (2S)-1-(2-oxo-2-phenylacetyl)-N-(3-phenylpropyl)piperidine-2-carboxamide (named ElteN378; see Figure 2).

ElteN378: a minimal FKBP pharmacore

In Figure 6 we show on the left the FES with respect to ω and ψ for Elte378 calculated in water bulk solution using ST-HREM. Again, as in ElteN420 (see Figure 5), the conformations of ElteN378 populate approximately the four conformational state in the ω, ψ space (*cis-cis*, *cis-trans*, *trans-cis*, *trans-trans*). However, in the case of ElteN378 we can detect only one very profound minimum (whose stabilization energy exceeds by several kJ mol^{-1} that of the other minima), indicating that most of the conformations populate the ω, ψ *trans-cis* binding region. The ball and stick representation depicted in the diagram corresponds to a representative conformation of the most populated cluster (accounting for more than 50% of sampled conformations, see Figure 27 of the SI) found in the ST-HREM simulation of Elte378 in water bulk solution with ω, ψ angles in the *trans-cis* binding region. As in ElteN470, also in ElteN378 the binding conformations are stabilized by a parallel displaced stacking of the two phenyl ring with one of these ring further stacked right above the planar α -keto amide moiety. In such rigid conformation, as shown in the ball and stick model reported in 6, the two binding oxygens, O2 and O3, points outwards with the optimal exposure of the PKA unit. The rigidity of ElteN378 in solution, due to intraligand hydrophobic interactions, is testified by the fact that all 20000 sampled configurations in the target replica can be partitioned, using the quality threshold algorithm⁴⁴ in less than 100 clusters yielding a very low entropy (see

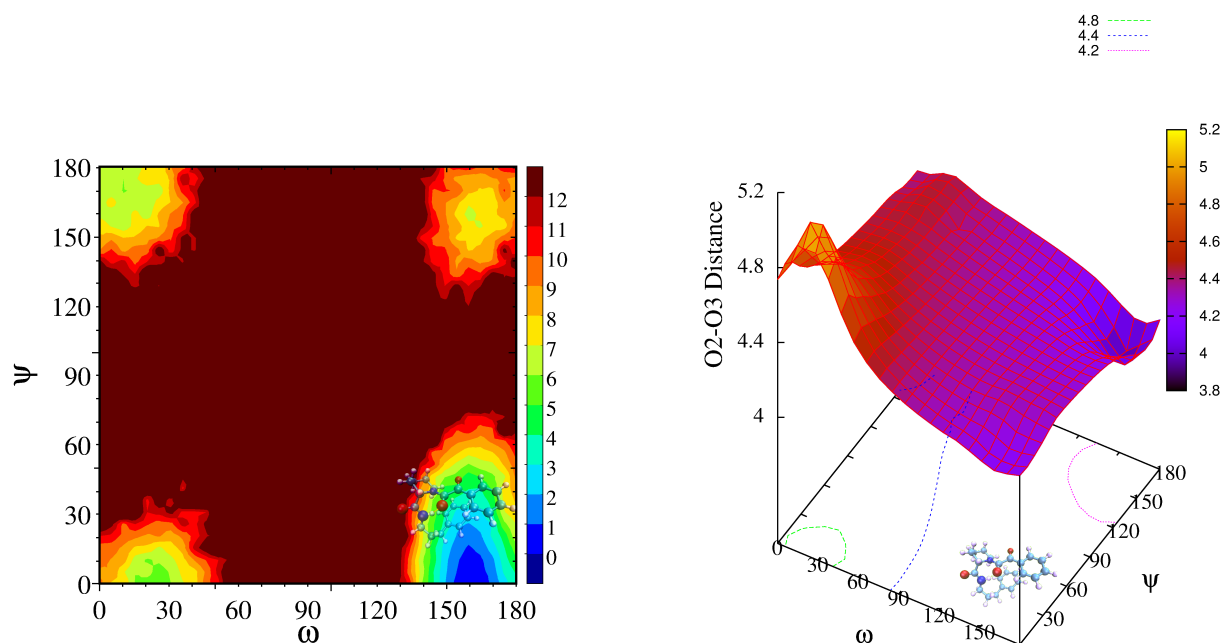


Figure 6: Left: Free energy surface (FES) with respect to the dihedral angles ω and ψ (in degrees) of ElteN378 calculated via solute tempering REM in water bulk solution. The energy scale (labels on the color coded bar on the right of each diagram) is in kJ mol^{-1} . A representative structure of the most populated cluster is reported in the ω , ψ FKBP12 binding region (bottom right quadrant). The O2 O3 and H(N1) atoms are highlighted with an enhanced sphere radius. Right: Contour map of the O2-O3 distance (in Å) as a function of ω and ψ (in degrees) in ElteN378.

Table 1). The rigidity along with the optimal PKA exposure can be best appreciated by plotting the O2-O3 distance involving the carbonyl of the binding domain as a function of the $\omega\psi$ angles for all sampled conformations. A 3D interpolation of the resulting surface using the pm3d algorithm⁵⁴ is shown in Figure 6 on the right. If one compares such function with that found in the two ElteN420 diastereoisomers (see Figure 23 top of the SI), we note that basically the entire O2-O3 distance spread in the case of Elte378 is restricted (except for some states in the ω , ψ *cis-cis* region) to the binding range 4.2-5.0 found in the experimental structures of ligand-FKBP complexes. All these peculiar features optimally satisfies the requirements of the FKBP pharmacore (see Figure 1 and makes of ElteN378 a potent binder for the FKBP PPI domain as actually shown by the measured

K_i in PBS (see Table 1).

The quasi-cyclic structure typical of ElteN378 in water solution is the resultant of the chemical topology of the molecule and of the media effect. More precisely, the extensive stacking system (see Figure 6) involving the two phenyl and the planar amide moiety is stabilized⁵⁵ by solvophobic effects due to the free energy gain (notably of the intra-solvent enthalpy⁵⁶) promoted by the minimization of the solvent-Elte378 interface upon intraligand stacking). In solvent with amphiphilic properties such as DMSO, according to our ST-HREM simulation of ElteN378 in DMSO in standard conditions, we found that stacked conformation of the phenyl moieties in Elte378 are no longer stable (see for more details Figure 27 in the SI). On the other side, in water and in DMSO as well the ω, ψ *trans-cis* structures in amide Elte derivatives are stabilized by an H-bond between the H(N1) and the O3 atom also. Therefore the stability of ω, ψ *trans-cis* conformational states should increase with the protonation of the amide nitrogen and hence with decreasing pH. As the fluorescence emission spectra in Elte compounds are sensible to the existence of transient intraligand structures for the monomeric excited states,³⁷ we can use the fluorescence profile of the ligand as a probe for its mean conformational behaviour in various solvent. In Figure 7 we

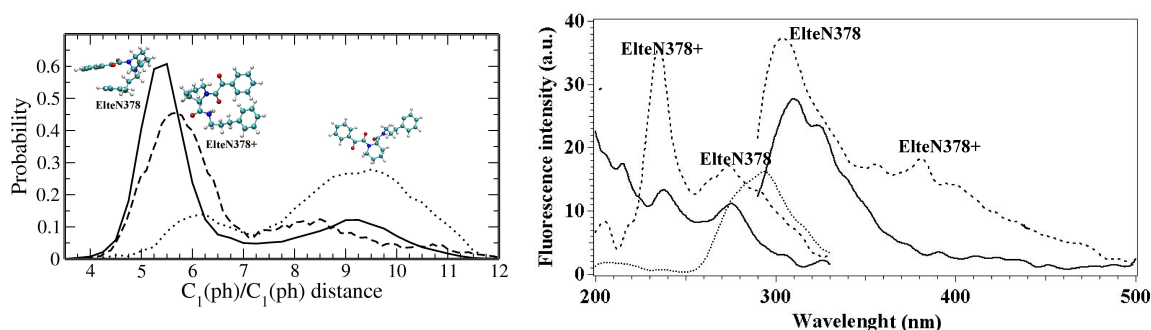


Figure 7: Left: Distribution probability of the C1(phenyl)-C1(phenyl) distance in neutral ElteN478 (solid blue) in N1 protonated ElteN478 (dashed blue) in water and in neutral ElteN478 in DMSO (solid red) calculated using the ST-HREM simulation approach. Right: Emission (right side) and excitation (left side) spectra for ElteN378 in PBS buffer (solid line), water (dashed line) and DMSO (dotted line). Emission spectra were collected at $\lambda_{\text{exc}} = 270$ nm, excitation spectra were obtained at $\lambda_{\text{em}} = 350$ nm.

show the distribution probability of the phenyl-phenyl distance ($P(R_{\text{ph-ph}})$) in neutral and N1 protonated ElteN78 in water bulk solution and in neutral ElteN378 in DMSO solution calculated using

ST-HREM simulation along with the fluorescence intensity profiles of the corresponding species. The high peak of $P(R_{\text{ph-ph}})$ seen in water at short distance ($R_{\text{ph-ph}} \simeq 4.5 \text{ \AA}$) in Figure 7 (left) reflects the abundance of the parallel displaced stacked or tilted structures reported in the binding region of 2D-FES of Figure 6 and in Figure 7. Note that for the protonated ElteN378 in water, such peak is shifted towards larger distances. This is due to the strengthening of the H-bond involving the H(N1)-O3 pair, that favours, in protonated ElteN378, edge-to-edge structures (Pop. $\simeq 32\%$) rather than parallel displaced (Pop. $\simeq 19\%$) conformations. In DMSO the ring-ring peak is much less pronounced. The broad peak at large distance reflects the prevalence in this solvent of open structures with the phenyl rings far apart from each other. Such average conformational behaviour in different media and for different protonation state of ElteN378 can be traced and monitored in the excitation and emission spectra of ElteN378. In the Figure 7 (right) we report the emission and excitation spectra for ElteN378 in PBS (pH=7.4), water (pH=5.6) at low ligand concentration (7×10^{-7}) M, i.e. safely below concentrations where intermolecular aggregation phenomena may occur (see SI, Figure 31). The spectra on the left are the excitation spectra at $\lambda_{\text{em}} = 350 \text{ nm}$. In ElteN378 in water, a sharp and intense peak at 240 nm flanks a less intense and broader peak at 270-280 nm. In ElteN378 in PBS, the absorbing species at 240 yields a much less intense peak with respect to that seen in ElteN378 in water. These bands must be evidently assigned to compact persistent structures of the neutral and protonated species like those depicted in 7 (left) as the excitation spectrum ($\lambda_{\text{em}} = 350 \text{ nm}$) of ElteN378 in DMSO (characterized mostly by open structures) has no bands in this region. Lowering the pH (in water), the ratio between protonated and neutral ElteN378 increases. In the excitation spectra reported in Figure 7 (right) we may thus reasonably assign the 240 nm signal to the species ElteN378^+ and the band at 270-280 nm to the neutral ElteN378. The emission spectra collected at $\lambda_{\text{ex}} = 270 \text{ nm}$ produces a broad and intense band at 310 nm in both water and PBS and a secondary structured band in the range 350-400 nm that is much more intense in case of ElteN378 dissolved in water. Such emission band grows in intensity as the excitation wavelength decreases (see SI Figure 33), thus confirming the connection between the emitting species in the range 350-400 nm to the absorbing species at 240 nm, a species that is

much less abundant in PBS buffer.

The fluorescence bands in the 350-400 nm range (strictly related with the 240 nm excitation band attributed to the protonated species) are hence very likely a signature of the compact conformations of the protonated species (see Figure 27 of the SI), while the intense fluorescence peak at 310 nm ($\lambda_{\text{exc}} = 270$) can be assigned mostly to neutral emitting species. In further support of such assignment, in the SI we also report (SI, Figure 32) the excitation spectra obtained at $\lambda_{\text{em}} = 310$ where only the peak at 270 nm shows up in both PBS and water due to fraction of the neutral species. Notably, the fluorescence intensity ratio I_{310}/I_{400} is almost constant for ElteN378 in water for concentrations up to 5×10^{-5} M whereas this ratio decreases in PBS (see Figure 31 in the SI) indicating that the interacting groups are of intermolecular origin; that is to say aggregation of monomeric neutral structures occurs. This results was expected since in PBS buffer the fraction of protonated species is lower and residual electrostatic repulsion is screened by the presense of the salt.

cis-trans isomerism in ElteX ligands and binding kinetics

It has been recognized that the activity of these FKBP12 ligands is related to *cis-trans* isomerism of the α -keto amide planar bond.^{18,57} In standard condition, these *cis-trans* rotamers are separated in DMSO or CDCl_3 solvents by barriers high enough to allow rotamer discrimination via ^1H NMR.^{18,33} In the Xaa-proline or piperidine bonds, the N cyclo-alkylation reduces considerably the steric advantage of the trans configuration.⁵⁷ In aqueous solution, *cis-trans* rotamers for most pipecolic based ligands exhibit at equilibrium a fraction of 2:3 in favour of the *trans* isomer.^{18,57} ElteX compounds make no exception with computed *trans/cis* ratio in water in the range 1.5:6 (see Table 1). The individual binding affinity of the *cis/trans* isoforms and the overall apparent binding affinity of the *cis/trans* rotameric mixture of ElteX compounds in presence of the FKBP12 protein are given by

$$K_c = \frac{[E_c][P]}{[PE_c]}; \quad K_t = \frac{[E_t][P]}{[PE_t]}; \quad K_m = \frac{([E_c] + [E_t])[P]}{([PE_c] + [PE_t])} \quad (3)$$

with $[E_{c/t}]$, $[P]$ being the concentrations of free *cis* and *trans* rotamers and protein in solution. The *cis/trans* isomers obey the equilibrium $K = [E_t]/[E_c]$. Combining the above equations, the apparent binding affinity of an ElteX ligand can be written as

$$K_m = \frac{K_t(1+K)}{K + K_t/K_c}. \quad (4)$$

In the assumption that $K \gg K_c$, $K \gg K_t$ and $K_t \ll K_c$, we obtain that

$$K_m = K_t \frac{1+K}{K}, \quad (5)$$

i.e. the observed K_m , in presence of a significant share ($K > 1$) of the ω -*trans* conformer at equilibrium, is essentially equal to the lowest binding affinity K_t corresponding to the most active (binding) rotamer. Equation 5 shows that if $K \geq 1$, then the difference in the activity due to the *trans/cis* ratio can account *at most* (i.e. for $K = 1$) for a factor of 2. Hence, as all ligands in Table 1 have $K > 1$, the *trans-cis* ratio should not be the essential element affecting their binding activity. On the other hand, the ω -*trans-cis* ratio, due to the high barrier, could be one of the rate-determining step in the kinetics of the FKBP binding and hence on the kinetics of Trp56 quenching by ElteX ligands. In figure 8 we report the decrease in fluorescence intensity as a function of time observed when FKBP12 ligands are added to a solution of FKBP12. Indeed, we do observe a faster decrease in fluorescence for the compounds that, according to the REM simulation, have large *trans/cis* ratio, as the potent ElteN378 and the diastereoisomer R-ElteN420 (see the inset in the Figure). In Figure 8 (right), the differences in the kinetic behaviour of R-ElteN420 ($K = 4.8$) and S-ElteN420 ($K = 1.4$) can be best appreciated at room temperature where the kinetic component due to diffusive processes is accelerated.

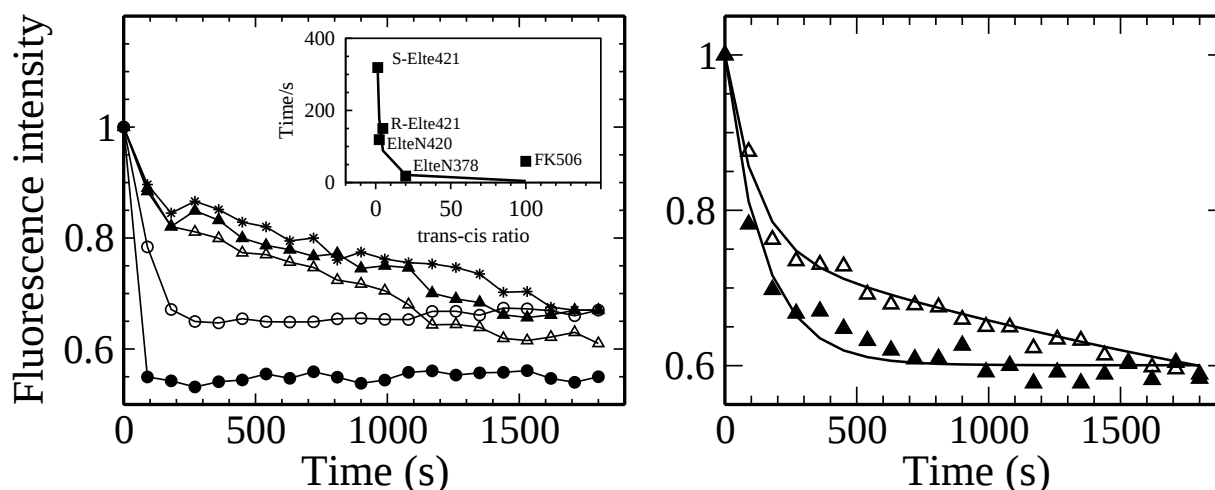


Figure 8: Left: Decrease of fluorescence intensity with time at T=15 °C. Ligand concentration 4 nM for all samples. Full circles: ElteN378; empty circles: FK506, full triangles: S-Elte421; empty triangles: R-Elte421; asterisks: ElteN420 1:1 diastereoisomeric mixture. Inset: *trans-cis* ratio as calculated from the REM simulations as a function of the the decay time determined by fitting the experimental kinetic data with a mono-exponential decay (first order reaction). The *trans/cis* ratio for the 1:1 ElteN420 diastereoisomeric mixture has been obtained by averaging the *trans/cis* ratio of the two epimers in Table 1. The *trans/cis* ratio of ElteN378 has been obtained by averaging the values of the neutral and protonated species (reported in Table 1) assuming a pKa of 7. Right: Decrease of fluorescence intensity with time at T=25 °C. Ligand concentration 4 nM for all samples. Empty triangles: S-Elte421; full triangles: R-Elte421; the curves are obtained by fitting the experimental data with a mono-exponential function

Conclusions and Perspectives

In the present study, we have demonstrated, using an interdisciplinary approach based on computational, synthetic and experimental techniques, that the best potential FKBP binders (and very likely the best PPI inhibitors) are characterized in water solution by the abundance of quasi-cyclic structures stabilized by intraligand hydrophobic interactions. The conformational entropy in such ligands, as occurring in the rigid natural macrolides FK506 and Rapamycin, is low. At the same time such ligands optimally expose, again as in FK506 and Rapamycin, the two contiguous carbonyl oxygen (O2,O3 see Figure 1) in the peptidomimetic chain for FKBP docking. These peculiar structural and chemical-physical features define at the same time an ElteX compound and the minimal pharmacore in the FKBP family. Based on the above hypothesis, we have successfully designed and synthesized ElteN378, a new low atomic weight FKBP ligand with activity comparable

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2
3 to that of the macrolide Rapamycin.
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5 The strict correlation, revealed in our study, between potency and mean conformation of the
6 FKBP ligand in bulk solution sheds new light on the functioning mechanics of the PPI machinery.
7 The PPI in the FKBP enzyme is known to catalyze the *cis-to-trans* isomerization of the peptide-
8 bond in proline.^{36,58–60} The reaction mechanism is thought to occur via an “unassisted confor-
9 mational twist mechanism with rate enhancement in due part to desolvation of the peptide bond
10 at the active site.”³⁶ Such hypothesis was based on site-directed mutagenesis by replacements of
11 several potential catalytic residues in FKBP with Ala.³⁶ However, none of these mutated residues
12 included the highly conserved Tyr82 and Ile56 (FKBP12 numbering). In the light of our findings,
13 such consensus mechanism appears unlikely. The discovery of ElteX ligands and of their binding
14 mechanism suggests the following alternative picture: the substrate reaches the concave PPI pocket
15 exposing its curved convex side and binds with the proline flanking carbonyl to Tyr82. When the
16 substrate is in place, the five membered ring of proline is stacked onto Trp59. The electron flux
17 from the O=C-N resonance system to the polar H of Tyr82 weakens the peptide bond lowering the
18 *cis-trans* energy barrier and triggering the isomerization. Such event suddenly rectifies the sub-
19 strate making it unfit for the docking in the convex binding pocket. The straightened *trans*-proline
20 substrate, in order to avoid steric clashes with nearby protein residues, cooperatively releases the
21 H-bond with Tyr82 eventually leaving the PPI convex binding pocket.
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40 The closed conformations in compact ElteX ligands can fit easily in the PPI binding pocket,
41 exposing the PKA unit with the ω angle in *trans* conformation mimicking the proline residue in
42 a *trans* conformation. The O3-Tyr82 H-bond is formed with the piperidinic ring approximately
43 stacked on Trp59 and interacting with the nearby Tyr26. The O2 oxygen of the following carbonyl
44 in the peptidomimetic chain, being the piperidinic substituent at C2/C α in axial conformation, can
45 bind the NH of Ile56 with ψ in its *circa cis* conformation, hence *locking the ligand in the PPI*
46 *binding pocket*. Further stabilization in the complex FKBP-ElteX arises from the hydrophobic
47 three-body interactions between phenyl-2 and phenyl-1 of ElteX and Phe36. Such mechanism
48 explains why only the (S)-C2 epimer in piperidine derivatives is active:¹⁸ the R piperidinic di-
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astereoisomer is in fact unable to bind Ile56 and Tyr82 *at the same*, thus preventing the locking of the ligand in the pocket. A schematic picture of the PPI working mechanism and the PPI inhibition is shown in Figures 28 and 29 of the SI.

We indeed believe that our computationally driven design of new potent NI-IL inhibitors, based on preliminary and costless simulations of the ligand in water bulk solution to monitor its conformational behaviour, may provide also a valuable mean to identify the optimal position for inserting a so-called *effector* domain²⁹ for the allosteric interactions with a third partner protein. An effective IL ligand must bind FKBP via the PKA driven mechanism as well as the partner protein with the effector domain on the opposite (convex) side of example, the PKA unit. Therefore, due care must be taken to ensure that structural modifications or group insertion in NI-IL's such as ElteN378 do not abrogate the potency of the ligand by interfering with or altering the mean conformational structure of the PKA binding domain *in water environment*, e.g by severely perturbing the ω, ψ free energy surface and/or obstructing the solvent-exposed carbonyl group in the PKA unit.

Such computational/synthetic procedure, for example, could assist the design of a sensors for FKBP12, a possible biomarker for early diagnosis in AD or PD. An FKBP sensor device can be envisaged by judiciously attaching to ElteN378 a suitable polymeric chain ending with an anchoring group for biochemical sensing in Self-Assembled Monolayers or Supported Lipid Bilayers deposited on Gold surfaces.^{61,62} Furthermore, the availability of this new class of FKBP12 ligands provides an effective mean for elucidating the role of this protein in amyloidogenesis.^{4,63} Results in these directions will be presented in forthcoming studies.

Supporting information

Details of the synthesis of R-Elte421, S-Elte421, ElteN420, ElteN378. Computational details: force field used for the ligands; accessory data for R-Elte421, S-Elte421, R-ElteN420, S-ElteN420, V10,367, ElteN378 (in water and DMSO) and ElteN378⁺ in water. Details of the experimental determination of the binding affinity via fluorescence quenching. This material is available free of

charge via the Internet at <http://pubs.acs.org>. The authors declare no competing financial interest.

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References

- (1) Blackburn, E. A.; Walkinshaw, M. D. *Curr. Op. Pharm.* **2011**, *11*, 365–371.
- (2) Chattopadhyaya, S.; Harikishore, A.; Yoon, H. S. *Curr. Med. Chem.* **2011**, *18*, 5380–5397.
- (3) Galat, A. *J. Chem. Inf. Model.* **2008**, *48*, 1118–1130.
- (4) Gerard, M.; Deleersnijder, A.; Demeulemeester, J.; Debyser, Z.; Baekelandt, V. *Mol. Neurobiology* **2011**, *44*, 13–27.
- (5) Ivery, M. *Med. Res. Rev.* **2000**, *20*, 452–484.
- (6) Rosen, M. K.; Schreiber, S. L. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 384–400.
- (7) Kissinger, C. R. et al. *Nature* **1995**, *378*, 641–644.
- (8) Waickman, A. T.; Powell, J. D. *J. Immunol.* **2012**, *188*, 4721–4729.
- (9) Kang, C.; Hong, Y.; Dhe-Paganon, S.; Yoon, H. *Neurosignals* **2008**, *16*, 318–325.
- (10) Sugata, H.; Matsuo, K.; Nakagawa, T.; Takahashi, M.; Mukai, H.; Ono, Y.; Maeda, K.; Akiyama, H.; Kawamata, T. *Neurosci. Lett.* **2006**, *350*, 472–477.
- (11) Liu, F.; Liu, P.; Shao, H.; Kung, F. *Biochem. Biophys. Res. Commun.* **2006**, *350*, 472–477.

- (12) Gerard, M.; Deleersnijder, A.; Daniels, V.; Schreurs, S.; Munck, S.; Reumers, V.; Pottel, H.; Engelborghs, Y.; Van den Haute, C.; Taymans, J.; Debyser, Z.; Baekelandt, V. *J. Neurosci.* **2010**, *30*, 2454–2463.
- (13) Esposito, M.; Ruffini, F.; Bellone, M.; Gagliani, N.; Battaglia, M.; Martino, G.; Furlan, R. *J. Neuroimmun.* **2010**, *220*, 52–63.
- (14) Lisi, L.; Navarra, P.; Cirocchi, R.; Sharp, A.; Stigliano, E.; Feinstein, D. L.; Dello Russo, C. *J. Neuroimmun.* **2012**, *243*, 43–51.
- (15) Lin, J. T.; Stein, E. A.; Wong, M. T.; Kalpathy, K. J.; Su, L. L.; Utz, P. J.; Robinson, W. H.; Fathnann, C. G. *Clin. Immunol.* **2012**, *142*, 127–138.
- (16) Manabe, Y.; Warita, H.; Murakami, T.; Shiote, M.; Hayashi, T.; Omori, N.; Nagano, I.; Shoji, M.; Abe, K. *Brain Res.* **2002**, *935*, 124–128.
- (17) Benjamin, D.; Colombi, M.; Moroni, C.; Hall, M. N. *Nature* **2011**, *10*, 868–880.
- (18) Holt, D. A.; Luengo, J. J. I.; Yamashita, D. S.; Oh, H. J.; Konialian, A. L.; Yen, H. K.; Rozamus, L. W.; Brandt, M.; Bossard, M. J.; Levy, M. A.; Eggleston, D. S.; Liang, J.; Schultz, L. W.; Stout, T. J.; Clardy, J. *J. Am. Chem. Soc.* **1993**, *115*, 9925–9938.
- (19) Roehrig, C. H.; Loch, C.; Guan, J.-Y.; Siegal, G.; Overhand, M. *Chem. Med. Chem.* **2007**, *2*, 1054–1070.
- (20) Avramut, M.; Zeevi, A.; Achim, C. *Brain Res Dev Brain* **2001**, *132*, 151–157.
- (21) Gold, B. *Drug Metab. Rev.* **1999**, *31*, 649–663.
- (22) Jost, S.; Doolabh, V.; Mackinnon, S.; Lee, M. H. *Restor. Neurol. Neurosci.* **2000**, *17*, 39–44.
- (23) Pong, K.; Zaleska, M. *Curr. Drug Targets CNS Neurol. Disord.* **2003**, *2*, 349–356.
- (24) Gerard, M.; Debyser, Z.; Desender, L.; Kahle, P. J.; Baert, J.; Baekelandt, V.; Engelborghs, Y. *FASEB J* **2006**, *20*, 524–526.

- (25) M., G.; Debyser, Z.; Desender, L.; Baert, J.; Baekelandt, V.; Engelborghs, Y. *J. Neurochem.* **2008**, *106*, 121–133.
- (26) Gold, B. G.; Voda, J.; Yu, X.; McKeon, G.; Bourdette, D. *J. Neurosci. Res.* **2004**, *77*, 367–377.
- (27) Behrbohm, J.; Neid, M.; Stolzel, U.; Wittekind, C.; Hauss, J. P.; Tillmann, H. L. *Transpl. Int.* **2007**, *20*, 1077–1079.
- (28) Bonatti, H.; Gillis, J.; Berger, N.; Mark, W.; Kofler, H.-J.; Margreiter, R.; Pfausler, B. *Transpl. Int.* **2008**, *21*, 916–918.
- (29) Armistead, D. M.; Badia, M. C.; Deininger, D. D.; Duffy, J. P.; Saunders, J. O.; Tung, R. D.; Thomson, J. A.; Decenzo, M. T.; Futer, O.; Livingston, D. J.; Murcko, M. A.; Yamashitaand, M. M.; Navia, M. A. *Acta Cryst.* **1995**, *D51*, 522–528.
- (30) Banaszynski, L.; C.W., L.; Wandless, T. *J. Am. Chem. Soc.* **2004**, *127*, 4715–4721.
- (31) Liang, J.; Cho, i. J.; Clardy, J. *Acta Crystallogr. D Biol. Crystallogr.* **1999**, *55*, 736–744.
- (32) Griffith, J.; Kim, J.; Kim, E.; Sintchak, M.; Thomson, J.; Fitzgibbon, M.; Fleming, M.; Caron, P.; Hsiao, K.; Navia, M. *Cell* **1995**, *82*, 507–522.
- (33) Bizzarri, M.; Marsili, S.; Procacci, P. *J. Phys. Chem. B* **2011**, *115*, 6193–6201.
- (34) Holt, D. A.; Konialian-Beck, A. L.; Oh, H. J.; Yen, H. K.; Rozamus, L. W.; Krog, A. J.; Erhard, K. F.; Ortiz, E.; Levy, M. A.; Brandt, M.; Bossard, M. J.; Luengo, J. I. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 315–320.
- (35) Hamilton, G. S.; Catonsville, M.; Snyder, S.; Baltimore, M. Inhibitors of rotamase enzyme activity effective at stimulating neuronal growth. US Patent 5696135, 1997.
- (36) Park, S. T.; Aldape, R. A.; OlgaFuter,; DeCenzo, M. T.; Livingston, D. J. *J. Biol. Chem.* **1992**, *267*, 3316–3324.

- (37) Bizzarri, M.; Tenori, E.; Martina, M. R.; Marsili, S.; Caminati, G.; Menichetti, S.; Procacci, P. *J. Phys. Chem. Lett.* **2011**, 2, 2834–2839.
- (38) Fukunishi, H.; Watanabe, O.; Takada, S. *J. Chem. Phys.* **2002**, 116, 9058–9067.
- (39) Marsili, S.; Signorini, G. F.; Chelli, R.; Marchi, M.; Procacci, P. *J. Comp. Chem.* **2010**, 31, 1106–11161.
- (40) Liu, P.; Kim., B.; Friesner, R. A.; Berne, B. J. *Proc. Acad. Sci.* **2005**, 102, 13749–13754.
- (41) Duan, Y.; Wu, C.; Chowdhury, S.; Lee, M. C.; Xiong, G.; Zhang, W.; Yang, R.; Cieplak, P.; Luo, R.; Lee, T.; Caldwell, J.; Wang, J.; Kollman, P. *J. Comp. Chem.* **2003**, 24, 1999–2012.
- (42) Stephens, P. J.; Devlin, F. J.; Chablowski, C. F.; Frisch, M. *J. Phys. Chem.* **1994**, 98, 11623–11627.
- (43) Singh, U. C.; Kollman, P. A. *J. Comput. Chem.* **1984**, 5, 129–145.
- (44) Heyer, L. J.; Kruglyak, S.; Yooseph, S. *Genome Res.* **1999**, 9, 1106–1115.
- (45) Guardiani, C.; Signorini, G. F.; Livi, R.; Papini, A. M.; Procacci, P. *J. Phys. Chem. B* **2012**, 116, 5458–5467.
- (46) Brown, H. C.; Jadhav, K. J. *J. Am. Chem. Soc.* **1983**, 105, 2092–2093.
- (47) Sun, F.; Li, P.; Ding, Y.; Wang, L.; Bartlam, M.; Shu, C.; Shen, B.; Jiang, H.; Li, S.; Rao, Z. *Biophys. J.* **2003**, 85, 3194–3201.
- (48) Van-Duyne, G.; R.F.Standaert; S.L.Schreiber; J.Clardy, *J. Am. Chem. Soc.* **1991**, 113, 7433–7434.
- (49) Kotaka, M.; Ye, H.; Alag, R.; Hu, G.; Bozdech, Z.; Preiser, P.; Yoon, H.; Lescar, J. *Biochemistry* **2008**, 47, 5951–5961.

- (50) Alag, R.; Qureshi, I.; Bharatham, N.; Shin, J.; Lescar, J.; Yoon, H. S. *Protein Sci.* **2010**, *19*, 1577–1586.
- (51) The conformations of sb3 diastereoisomers in water were obtained using the trajectory data of a previous work [Bizzarri *et al.* *J. Phys. Chem. B*, *115*, 6193-6201 (2011)].
- (52) The binding affinities K_i relative to FK506 was taken from [Holt. *et al.*, *J. Am. Chem. Soc.* *115*, 9925-9938 (1993)].
- (53) The binding affinities K_i relative to FK506 was taken from [Armistead *et al.*, *Acta Cryst.*, *D51*, 522-529 (1995)].
- (54) Williams, T.; Kelley, C. Gnuplot 4.4, An Interactive Plotting Program. 2011; <http://sourceforge.net/projects/gnuplot>.
- (55) Procacci, P. *Annu. Rep. Progr. Chem. Sect. C* **2011**, *107*, 242–262.
- (56) Hummer, G. *Nature Chem.* **2010**, *2*, 906–906.
- (57) Wand, X.; Etzkorn, F. *Biopolymers* **2006**, *84*, 125–146.
- (58) Harding, M.; Galat, A.; Uehling, D.; Schreiber, S. *Nature* **1989**, *341*, 758–760.
- (59) Siekierka, J.; Hung, S.; Poe, M.; Lin, C.; Sigal, N. *Nature* **1989**, *341*, 755–757.
- (60) Fischer, S.; Michnick, S.; Karplus, M. *Biochemistry* **1993**, *32*, 13830–13837.
- (61) Gambinossi, F.; Lorenzelli, L.; Baglioni, P.; Caminati, G. *Colloids Surf. A Physicochem. Eng. Asp.* **2008**, *321*, 87–93.
- (62) Morandi, S.; Puggelli, M.; Caminati, G. *Colloids Surf. A Physicochem. Eng. Asp.* **2008**, *321*, 125–130.
- (63) Al-Kayal, T.; Russo, E.; Pieri, L.; Caminati, G.; Berti, D.; Bucciantini, M.; Stefani, M.; Baglioni, P. *Soft Matter* **2012**, *8*, 9115–9126.