

# New Perspective on How and Why Immunophilin FK506-Related Ligands Work

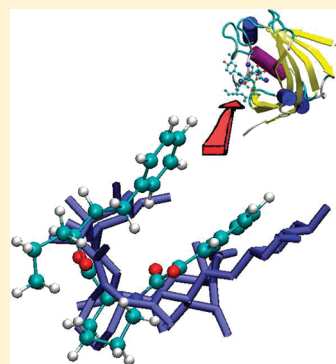
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 Supporting Information

**ABSTRACT:** We report on theoretical and experimental data that help elucidate the intimate structural and thermodynamical cooperative mechanisms that are responsible for the inhibitory activity of FK506-related ligands of the FKBP12 protein, a cytosolic enzyme that catalyzes the cis–trans isomerization of prolyl amide bonds and that is involved in immunosuppression and neuronal functioning. Effective FKBP12 ligands are those that mimic in bulk solution the structure of the Tacrolimus natural drug with respect to (i) the orientation of the two carbonyl groups units of the ligand that will form, upon binding, H-bonds with specific residues in the protein binding pocket and (ii) the reduced conformational entropy as in the rigid macrolide Tacrolimus. On this basis, we have rationally designed in silico and synthesized a new compound, Elte421, as a simple variant of an existing FK506-related ligand. The experimental characterization of the synthesized compound via fluorescence quenching shows that Elte421 has a binding affinity for human FKBP12 comparable to that of FK506 natural drug.

**SECTION:** Statistical Mechanics, Thermodynamics, Medium Effects



We report experimental and computational results that help clarify the structural properties that determine the affinity of FK506-related ligands for peptidyl–prolyl cis–trans isomerase protein (FKBP12). FKBP12 is notable in humans for binding the immunosuppressant molecule Tacrolimus (originally designated FK506), forming a very stable complex that in turn serves as a ligand to calcineurin, a cellular target for signal transduction in T-cell activation and proliferation.<sup>1,2</sup> FK506 and FK506-related ligands<sup>3,4</sup> are routinely used in treating patients after organ transplant and in patients suffering from autoimmune disorders.<sup>5,6</sup> In recent times, significant clinical improvement or even complete remission of multiple sclerosis has been reported in patients treated with FK506 following organ transplantation.<sup>7,8</sup> As to the specific function of FKBP12, it has been recently reported that this protein plays a role in neuronal survival. Nonimmunosuppressive ligands of FKBP12 have been shown to interfere in the interaction of FKBP12 with amyloidogenic proteins with neuroprotective and neuroregenerative activity both in vitro and in vivo,<sup>9</sup> thus stimulating the interest in the design and/or identification of novel FKBP12 ligands.<sup>10–12</sup>

The greatest number of designed FKBP ligands arose from the so-called dual-domain concept<sup>13</sup> whereby ligands bind FKBP12 through the  $\alpha$ -keto amide binding domain and act, in the resulting complex, as an immunosuppressant agent via the complementary effector domain. These designs of FKBP inhibitors were based on the binding domain of the common structural elements of FK506 and Rapamycin that dock the FKBP protein in the primary site<sup>4</sup> through two H-bonds between the donors OH of Tyr82 and NH of Ile56 and the acceptor carbonyl groups

(C8–O3 and C1–O2) in the pipecolyl  $\alpha$ -keto amide region<sup>14,15</sup> and through two more H-bonds between hydroxy groups in the macrocycle and Asp37 and Glu54 (1FKG numbering) in the secondary binding site.<sup>4</sup>

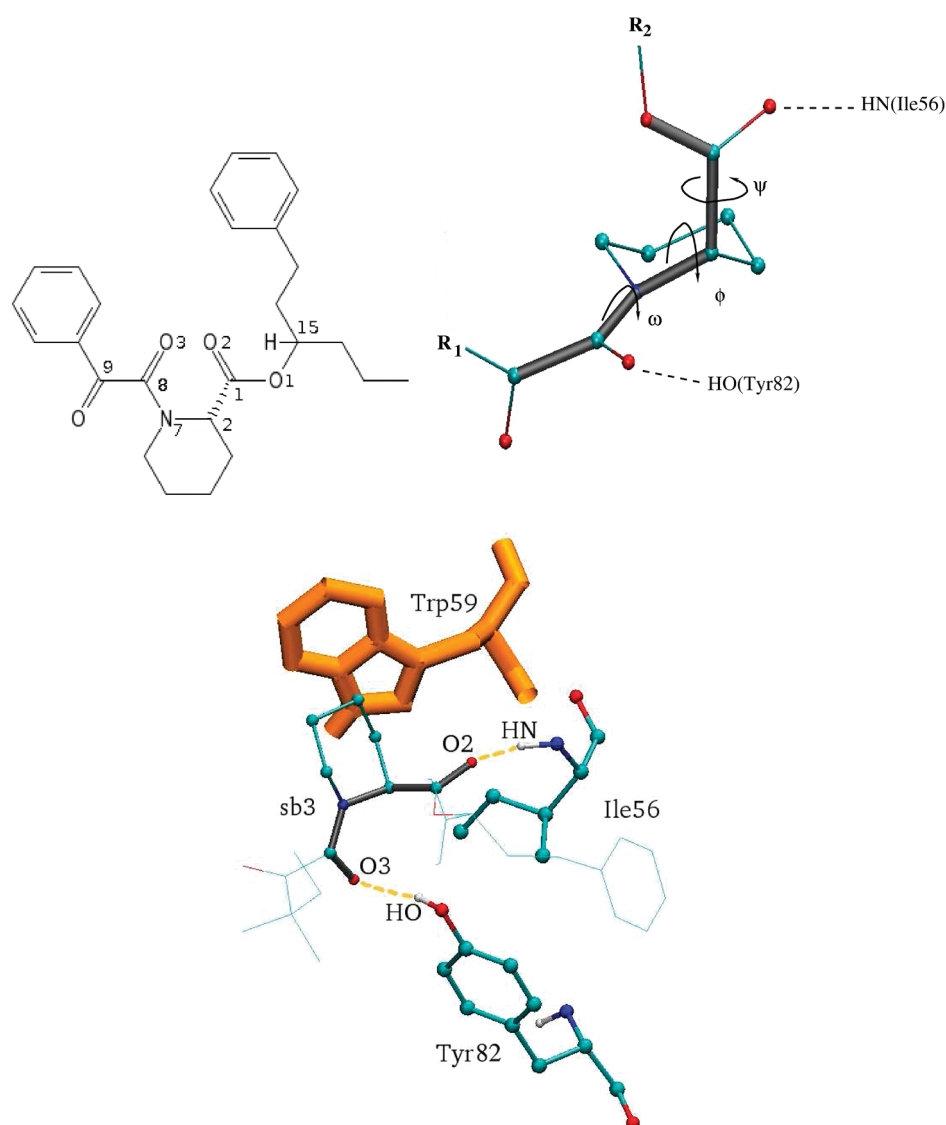
Holt et al.<sup>3</sup> and, more recently, Roehring et al.<sup>4</sup> analyzed the minimal binding domain of FK506-related drugs by evaluating individually the effect of each part on the potency of the FKBP inhibition. Pipecolyl-containing compounds (i.e., primary site binders) presented better inhibition than any acyclic amide or prolyl-containing compound, while efforts to synthesize non-macrolide compounds that bind at both the primary and secondary site of FKBP12 went frustrated,<sup>4</sup> indicating that site 2 is likely to play a minor role in the FKBP12–ligand complexes.

In a recent work,<sup>16</sup> using solute tempering replica exchange (REM) molecular dynamics simulations,<sup>17,18</sup> we have shown that the activity of known FKBP12 proline-mimetic drugs such as (1R)-1,3-diphenyl-1-propyl-(2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-piperidinecarboxylate (sb3)<sup>3</sup> is related to the mean mutual orientation of the two carbonyl units C1–O2 and C8–O3 in bulk solution that is in turn controlled by the mutual arrangement of the dihedral involving the C2 and N7 substituents, that is, C9C8N7C2 and O1C1C2N7 (see Figure 1). When the former torsional angle is in the trans and the latter in the cis configuration, the orientation of the carbonyl units and the O2–O3 distance (around 4.5 Å) is optimal for binding.

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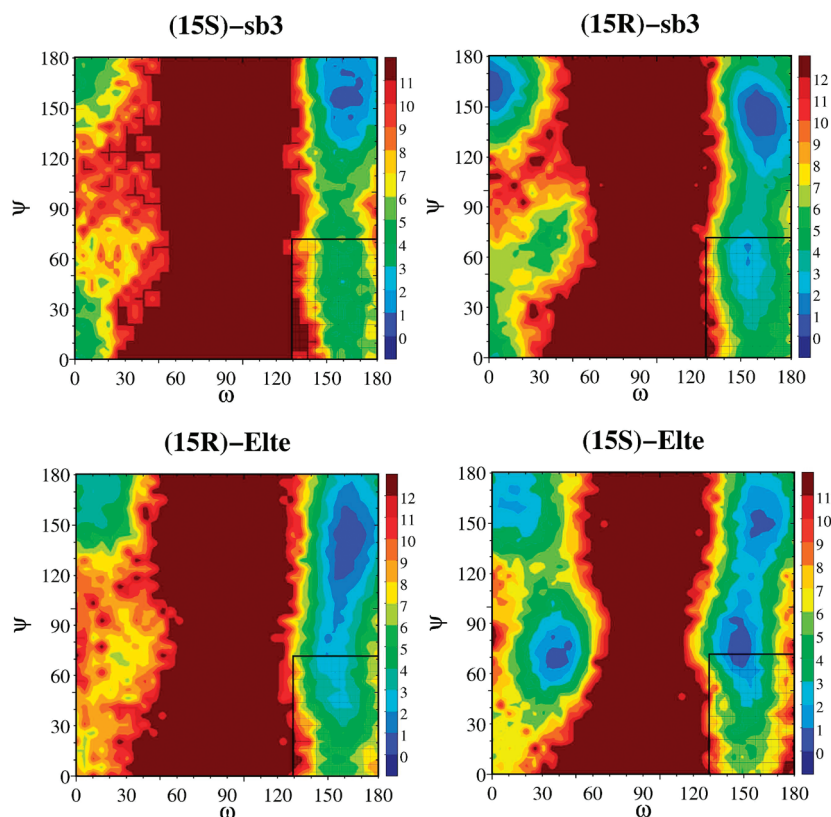


**Figure 1.** (Left) Elte421. The binding carbonyl groups in the FKBP12 complex are C8—O3 and C1—O2. C15 is the stereogenic center. (Right) Conformational distribution of the  $\omega$ ,  $\phi$ , and  $\psi$  angles of the  $\alpha$ -keto amide region of FK506-related ligands at the binding primary site<sup>4</sup> in the sb3—FKBP12 complex (substructure taken from the 1FKG pdb file). Values of these dihedral angles in the sb3—FKBP12 experimental structure 1FKG<sup>3</sup> are  $\omega = 173^\circ$ ,  $\phi = 92^\circ$ , and  $\psi = 17^\circ$ . The gray bonds follow the backbone of the proline mimetic compound. The dashed lines indicate the H-bonds with the protein residues in the primary binding site. (Bottom) Binding pocket of the (15R)-sb3—FKBP12 complex obtained from the pdb file 1FKG.<sup>3</sup> In the figure are shown (i) the ligand (15R)-sb3 [blue lines with the binding moiety (see upper right) evidenced in CPK/bonds representation], (ii) the binding Ile56 and Tyr82 residues (CPK representation), and (iii) the tryptophan fluorescent residue (bonds representation, orange color). The H-bonds between HN(Ile56)—O3(sb3) and HO(Tyr82)—O2(sb3) are indicated with yellow dashed lines.

In ref 16, it was further shown that in the right-handed stereoisomer (15R)-sb3, this optimal trans—cis arrangement of the C9C8N7C2 ( $\omega$ ) and O1C1C2N7 ( $\psi$ ) dihedral angles (with the C2 substituent in the axial position) for FKBP binding is stabilized in bulk solution by a stereospecific intraligand hydrophobic interaction of the 1,1-dimethyl propyl group of the N2 substituent with one phenyl ring of the C2 substituent. These hydrophobic interactions are not observed in the (15S)-sb3 epimer, consequently reducing the probability of potential binding conformation in bulk solution, with a remarkable impact on the observed potency of the compound (30–60 times less effective than the (15R) counterpart).

On the basis of these observations, starting from sb3, we can envisage a possible route to rationally design new potent inhibitors of FKBP protein by optimally redislocating the hydrophobic

groups in sb3 (phenyl and alkyl chains) so as to stabilize at the same time via  $\pi$ — $\pi$  stacking interactions the R stereoisomer and, as in (15R)-sb3, via phenyl—alkyl hydrophobic interactions the 15S partner. Using this guidance, starting from sb3 (see Figure 1 of the Supporting Information (SI)), we replaced (i) the isopropyl moiety at carbon 9 with a phenyl ring and (ii) the phenyl ring at the stereocenter C15 with a propyl group, ending up with the new compound 1-phenylhexan-3-yl-1-(2-oxo-2-phenylacetyl)piperidine-2-carboxylate (shown in Figure 1). These minimal changes with respect to the sb3 topology are expected to substantially preserve the conformational landscape of the  $\omega$ ,  $\phi$ , and  $\psi$  angles of the potent (15R)-sb3 and to impart to the proposed compound, via optimal intraligand hydrophobic interactions, a rigidity (in both stereoisomers) that significantly reduces the configurational



**Figure 2.** Free-energy surface with respect to the dihedral angles  $\omega$  and  $\psi$  (see Figure 1 for definitions) of Elte421 and sb3 in water at  $T = 300$  K and  $P = 1$  atm calculated via solute tempering REM. The gridded box frame in the right–bottom quadrant corresponds to the region of the  $\omega$ – $\psi$  coordinates sampled when Elte421 or sb3 is bound to FKBP12 in standard conditions<sup>3</sup> (see also results on the  $\omega$ – $\psi$  FES obtained from REM simulations of (15R)-Elte–FKBP12 and (15R)-sb3–FKBP12 in standard conditions, described in the SI). The probability distribution of the  $\phi$  angle in both (15R)-Elte and (15S)-Elte is peaked at about  $90^\circ$ , corresponding to the C2 substituent of the piperidinic ring in the axial configuration (data not shown). Such a probability distribution of the  $\phi$  angle is found in all strong  $\alpha$ -keto amide FKBP12 ligands.<sup>16</sup>

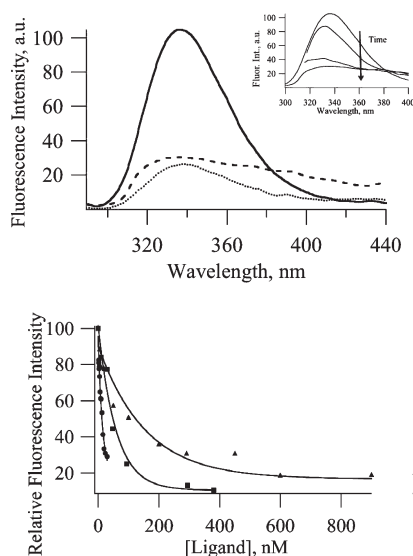
entropy in bulk with respect to (15R)-sb3 and (15S)-sb3, at the same time mimicking the macrocycle in natural macrolide drugs such as Tacrolimus or Rapamycin. Such a rationally designed compound, named Elte421, is shown in Figure 1.

The relevant conformational landscape of FK506-related ligands in bulk water can be obtained by evaluating via molecular dynamics (MD) at the atomistic level the free-energy surface (FES) with respect to  $\omega$ ,  $\phi$ , and  $\psi$  angles characterizing the conformation of the binding carbonyl groups. Due to the high free-energy barriers between ( $\omega$ ) cis–trans rotamers and for the axial/equatorial interconversion of the C2 substituent of the piperidinic ring in FK506-related ligands, standard MD is inadequate for a complete sampling along the dihedral coordinates.<sup>16</sup> We have thus performed 18 ns REM simulations with solute tempering<sup>17</sup> of (15R)-Elte and (15S)-Elte in water solution at  $T = 300$  K and  $P = 1$  atm using the ORAC program.<sup>18</sup> Further details of the simulation methodologies are given in the SI. In Figure 2, we report the  $\omega$ – $\psi$  2D FES obtained at the target (unscaled) replica for (15R)-Elte and (15S)-Elte in water solution. For comparison, we also report in Figure 2 the FES calculated for (15R)-sb3 and (15S)-sb3 in the same thermodynamic conditions using the trajectory data of a previous work.<sup>19</sup> Inspection of Figure 2 reveals that the exchange between the alkyl and phenyl moieties in sb3 to arrive at Elte421 produced the expected result, that is, both the Elte421 diastereoisomers exhibit a significant conformational population in the FKBP12 binding  $\omega$ – $\psi$

region (gridded quadrant), comparable to that of the potent (15R)-sb3 ligand. (15S)-Elte, in particular, has a free-energy minimum ( $\omega \cong 150^\circ$ ,  $\psi \cong 70$ – $80^\circ$ ) close to the binding conformational region.

(15S)-Elte and (15R)-sb3 are characterized by a similar pattern of the  $\omega$ ,  $\psi$  FES that in both cases bears two important minima in the ( $\omega$ ) trans and ( $\omega$ ) cis non-binding region and a less significant conformational population in the bottom-left binding quadrant. Note that the less active<sup>3</sup> (15S)-sb3 ( $K_d = 300$ – $600$  nM) in solution has all free-energy basins laying far away from the binding region with a very limited population in the binding region. The synthesis of Elte421 (1:1 (C15) diastereoisomers mixture) is described in the SI. The activity of the Elte421 is measured with respect to that of the Tacrolimus FKBP12 natural drug by fluorescence quenching, as described in the SI.

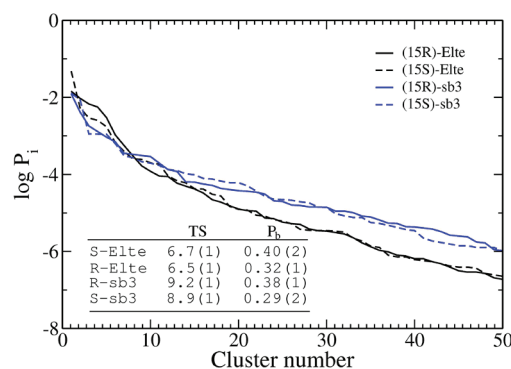
In Figure 1 (bottom), the details of the FKBP12 binding pocket taken from the experimental structure of the complex (15R)-sb3–FKBP12 (pdb file 1FKG) are shown. The tryptophan fluorescent residue (Trp59) lies just above the piperidinic ring of the ligand in a quasi-stacked configuration with interatomic Trp–piperidine distances as low as 3.7 Å, thus making FKBP12 amenable for intrinsic fluorescence quenching determination of binding affinities. A decrease in the intrinsic tryptophan fluorescence of FKBP12 protein is readily observed upon addition of Elte421, indicating binding of the Elte421 ligand in the region of the Trp59 in FKBP12, as shown in Figure 3 (top). The decrease in protein fluorescence for a similar concentration of



**Figure 3.** (Top) Fluorescence spectra of FKBP12 in PBS (solid line), FKBP12 with FK506 in PBS (dotted line), and FKBP12 with Elte421 in PBS (dashed line) at 288 K. The FKBP12 concentration was  $1.09 \mu\text{M}$  in all samples,  $[\text{FK506}] = 284 \text{ nM}$  and  $[\text{Elte421}] = 297 \text{ nM}$ . The excitation wavelength was  $\lambda = 280 \text{ nm}$ . (Inset) Fluorescence emission spectra of the FKBP12–Elte421 system as a function of time after ligand addition: 5 min, 20 min, 1 h, and 2 h. (Bottom) Relative fluorescence intensity decrease of FKBP12 fluorescence with FK506 (squares), Elte421 (triangles), and Rapamycin (circles, taken from ref 20).

FK506 was found to be of comparable extent. Closer inspection of the concentration dependence of the quenching data for Elte421, FK506, and Rapamycin (reported in Figure 3, bottom) shows that all three ligands exhibit a similar binding behavior. The data were fitted as described in the SI to extract the dissociation constant  $K_d$  for the three ligands. Due to the well-known dependence of  $K_d$  on experimental conditions and systematic concentration errors,<sup>4</sup> the binding activity of the Elte421 is reported with respect to that of the Tacrolimus natural drug. The experimentally determined dissociation constant of the Elte421–FKBP12 complex is found to be only 3 times larger than that of Tacrolimus and 5 times larger than the  $K_d$  for Rapamycin calculated from data extracted from the literature.<sup>20</sup>

Confirming the prediction based on the above free-energy conformational analysis, the (15R)-Elte and (15S)-Elte 1:1 mixture therefore is three times as potent as (15R)-sb3 ( $K_d[(15R)\text{-sb3}]/K_d(\text{FK506}) = 10$ ).<sup>3</sup> Simple considerations on independent chemical equilibria of the 1:1 diastereoisomeric mixture reported in section 4 of the SI imply that the two diastereoisomers exhibit dissociation constants of the same order of magnitude. The ideal behavior of the 1:1 mixture in the examined concentration range is supported by the photophysical fingerprint of the 1:1 mixture as a function of concentration, that is, the invariance of the fluorescence intensity ratio at 308 and 380 nm excludes significant intermolecular aggregation processes (see Figure 9, bottom of the SI). We also point out that only the trans rotamers bind FKBP12. Consequently, the cis–trans equilibrium of non-macrolide FK506-related ligands progressively shifts toward the trans form upon binding to the protein FKBP12 (see section 4 of the SI), with a kinetics that is typical of the cis–trans isomerization in proline. Such behavior is confirmed by the decrease of fluorescence of the FKBP12 as a function of time after Elte421 is added to the protein in solution (see the inset of Figure 3). In the

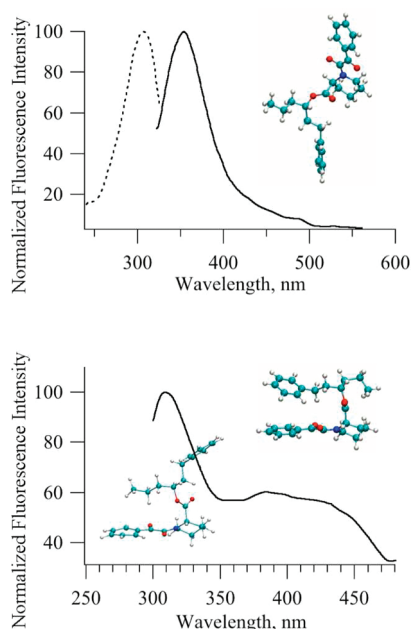


**Figure 4.** Cluster analysis of Elte421 and sb3 C15-diastereoisomers in water bulk solution at  $T = 300 \text{ K}$  and  $P = 1 \text{ atm}$  obtained from REM simulations. Clusters are ordered according to decreasing populations. In the inset table, we report the molar conformational entropy TS at  $T = 300 \text{ K}$  (in  $\text{kJ mol}^{-1}$ ) and the binding conformation probability of the ligands in bulk solution as obtained by the REM simulations. The molar conformational entropy contribution of the solute is evaluated according to the equation  $\text{TS} = RT \sum_i P_i \log P_i$ , 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.  $P_b$  is defined as the ratio between conformational states with  $\omega > 130$  and  $\psi > 90$  and  $\omega > 130$ .

natural drug Tacrolimus, which exhibits only the ( $\omega$ ) trans form, such kinetic behavior is not observed (data not shown).

The observed enhanced affinity of Elte421 for the FKBP12 protein with respect to that of (15R)-sb3 is due to a combination of two factors; (i) in the 1:1 diastereoisomeric mixture, potentially binding conformations of the  $\alpha$ -keto amide region (typical of macrolide ligands such as Tacrolimus) are stabilized by the phenyl–propyl interactions in (15S)-Elte and by the  $\pi$ – $\pi$  stacking interaction between the two phenyl rings in (15R)-Elte, and (ii) both Elte421 diastereoisomers are characterized in water by low-entropy structures, whereby hydrophobic moieties tend to aggregate in intraligand interactions in order to minimize the surface of the solute–solvent interface, thus minimizing the loss of water–water enthalpy.<sup>21–24</sup> This restrained conformational mobility, a key factor in the activity of the natural FKBP12 drugs such as the macrolide FK506 or Rapamycin, is an important ingredient for the efficacy of Elte421 as well.

In order to elucidate the origin of the peculiar FES pattern in Elte421 in water with respect to intraligand interactions, we have analyzed the REM trajectory data of Elte421 in water and DMSO and done fluorescence measurements on Elte421 dissolved in water and in DMSO. From the computational standpoint, we have performed a conformational analysis by clustering the solute structures sampled in the 18 ns REM simulations according to a quality threshold criterion. Full methodological explanations of the clustering analysis are given in the SI. In Figure 4, we show the behavior of the log of the cluster conformational probabilities in water as a function of the cluster number (ordered by decreasing cluster population) of (15R)-Elte and (15S)-Elte compared to those of the parent compounds R-sb3 and (15S)-sb3.<sup>19</sup> We can see that the populations of (15R)-Elte and (15S)-Elte follow a similar trend, consistently below that of the sb3 diastereoisomers for large cluster numbers. This fact translates in a molar conformational entropy content of Elte421 diastereoisomers in water and in standard thermodynamic conditions that are more than  $0.5 \text{ kcal mol}^{-1}$  lower than that of (15R)-sb3 and (15S)-sb3 (see data



**Figure 5.** (Top) Normalized fluorescence emission (solid line) and excitation (dashed line) spectra of Elte421 in DMSO; concentration = 1.35 mM,  $\lambda_{\text{exc}} = 300$  nm, and  $\lambda_{\text{em}} = 350$  nm. The chemical structure represents the central element of cluster n.1 of (15S)-Elte in DMSO (see Table 2 of the SI). (Bottom) Fluorescence emission (solid line) spectrum of Elte421 in PBS; concentration = 8  $\mu$ M and  $\lambda_{\text{exc}} = 270$  nm. The chemical structure on the left represents the central element of cluster n.1 of (15S)-Elte in water, whereas the compact structure on the right corresponds to the central element of cluster n.1 of (15R)-Elte in water (see Table 2 of the SI).

reported Figure 4). Such peculiar conformational behavior of Elte421 in water solution that emerges from REM simulations has been fully confirmed by the comparison of the fluorescence spectra of 1.35 mM Elte421 in water and in DMSO (see Figure 5 top). In DMSO, the clustering analysis done using the data from REM simulations shows that Elte421 overwhelmingly adopts open configurations, with the three hydrophobic groups pointing away from each other, as in the inset structure reported in Figure 5 top. Such behavior is due to the amphiphilic character of the DMSO molecule<sup>25,26</sup> that can interact favorably with both hydrophobic and hydrophilic groups in Elte421. Consequently, in the fluorescence spectrum of Elte421 in DMSO, only one broad peak is observed at  $\lambda = 360$ . In water solution, the Elte421 major fluorescence peak undergoes a blue shift, while a broad secondary peak at larger wavelengths ( $\lambda = 380\text{--}430$ ) appears. This is reminiscent of formation of aggregates, a common behavior found in molecules containing aromatic moieties in water solution or confined environment.<sup>27</sup> The intramolecular character of this secondary peak is demonstrated by the constancy of the intensity ratio  $I_{380}/I_{308}$  as a function of the concentration of Elte421 (see section 5 of the SI). Moreover, we found an emission-dependent behavior for the excitation spectra of Elte421 in water, suggesting the presence of a significant population of long-lived intraligand ground-state structures stabilized by  $\pi\text{--}\pi$  stacking as those depicted in Figure 5 bottom (see Figure 8 of section 3 of the SI). These experimental indications are in full agreement with the results of the REM simulations that assign to Elte421 in water, a much more compact structure than that in DMSO (see the cluster analysis in the SI for further details).

Theoretical as well as experimental data collected in the present study have elucidated, in our view, the intimate structural and thermodynamical cooperative mechanisms that are responsible for the inhibitory activity of the FK506-related ligand of the FKBP12 protein. Effective FKBP12 ligands are those that mimic in bulk solution the structure of the Tacrolimus natural drug with respect to (i) the orientation of the carbonyl groups units and (ii) the reduced conformational entropy as in the rigid macrolide Tacrolimus. On this basis, we have rationally designed and synthesized Elte421 as a simple variant of the (15R)-sb3 ligand, producing a new compound with an instability constant of the nanomolar order. However, following the proposed route, one can further optimize the nature and dislocation of the hydrophobic groups in the molecule so as to increase the fraction of rigid and potentially binding  $\alpha$ -keto amide conformations in bulk solution, an effect that can be verified, prior to undertaking the synthesis, by performing a simple REM simulation of the drug alone in bulk solution. Within the dual domain concept of FK506-related ligands, and in the quest for immunosuppressive drugs, the proposed rational design methodology can be straightforwardly applied to assess whether a candidate effector domain, added to the  $\alpha$ -keto amide ligand for targeting calcineurin, may or may not interfere with the conformation of the FKBP12 binding  $\alpha$ -keto amide domain, thus reducing its FKBP12 binding affinity and, consequently, its overall immunosuppressant potency.

## ■ ASSOCIATED CONTENT

**S Supporting Information.** Compounds, synthesis of Elte421, computational details of REM simulations, chemical equilibria, fluorescence data and experimental details, NMR spectra, and references for Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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