Abstract—This paper explores the use of computational methods to direct engineered biosynthesis based on the desired properties of the target compounds. The immunosuppressive properties of rapamycin are a result of the formation of the complex FKBP12–rapamycin–FRAP. Neuroregenerative properties are exhibited by the complex or complexes of rapamycin with FKBP proteins. Our objective has been to design biosynthetically available analogues of rapamycin that bind tightly to FKBP12 but not to FRAP. This has been carried out by successive single ketide deletions from the effector domain of rapamycin. The approach described here has yielded modified rapamycin analogues (RP2 and RP3) as targets for biosynthesis by modified polyketide synthases. RP2 and RP3 have an identical binding affinity (linear interaction energy calculation) to FKBP12 as rapamycin but little or no affinity for binding to FRAP. © 2000 Published by Elsevier Science Ltd. All rights reserved.

Introduction

The immunosuppressive drugs FK506 and rapamycin (Chart 1) form complexes with the immunophilin FKBP12. The FKBP12–FK506 complex subsequently binds to the protein calcineurin, and the FKBP12-rapamycin complex binds to a protein known as FRAP. The binding of the second protein to the FKBP12-drug complexes is responsible for the immunosuppressive activities of the drugs.1–5 Modifications to FK506 or rapamycin that preclude binding to calcineurin or FRAP, respectively, lead to loss of immunosuppressive activity.

Binding of rapamycin and FK506 to FKBP12 occurs at structurally similar regions of the drugs known as the binding domain (Chart 2). The binding domains of rapamycin and FK506 have been proposed to act as proline-leucine mimics.4,5 Structural features resembling the binding domains of rapamycin and FK506 are found in a large number of known FKBP12 binders.

The binding of the FKBP12–rapamycin and FKBP12–FK506 complexes to FRAP and calcineurin, respectively, occur at structural regions of the drugs known as the effector domains (Chart 2). The effector domains of the drugs differ greatly, as is required by their different target proteins. The effector domain of rapamycin, which is the target of this study, has been proposed to act as a peptide mimic in binding to FRAP.6 It has been reported that the interactions between rapamycin and FRAP are mainly hydrophobic interactions with the three adjacent double bonds in the effector domain of rapamycin (Fig. 1).7

Rapamycin and FK506 have been reported to promote neurite outgrowth as secondary activities.8 Furthermore, the immunosuppressive and neurite outgrowth activities of these drugs can be separated: the toxicity of these compounds is related to their immunosuppressive abilities while neuroregenerative activity is retained by non-immunosuppressive agents which bind to FKBP12 but not to the effector proteins Calcineurin or FRAP.8 Synthetic, non-immunosuppressive FKBP12 ligands have been shown to promote nerve regrowth in vitro and in vivo without the addition of exogenous growth factors.9–12 Several synthetic FKBP binders that resemble the binding domain of FK506 and rapamycin have also been shown to have neuroregenerative abilities, as has been reviewed by Hamilton and Steiner.12 Recent studies indicate that the neuroregenerative properties of these compounds are due to their ability to bind to the protein FKBP52.13 Far less is known about the structure of FKBP52 than the structure of FKBP12, but it has been shown that the structure of the FK506 binding site of FKBP52 differs by less than 1Å from the binding site of FKBP12.14 A strong, albeit not fully linear,
correlation is also seen between FKBP12 binding affinity and neuroregenerative ability.\textsuperscript{12}

Rapamycin and FK506 are biosynthesized by enzyme systems known as modular polyketide synthases (PKS). The chemistry of PKSs was reviewed extensively in the 10 November 1997 issue of Chemical Reviews and will not be outlined in detail here. Modular PKSs, e.g. the PKS responsible for the biosynthesis of erythromycin, have been modified to produce non-natural analogues of the naturally occurring polyketide.\textsuperscript{15–18} The biosynthetic gene cluster responsible for synthesizing rapamycin has been sequenced, \textsuperscript{19} making engineered modifications to rapamycin feasible. The goal of this study has been to propose modifications to rapamycin that give rise to non-natural analogues which: (i) bind to FKBP12 in an identical manner as rapamycin; (ii) do not form the ternary FKBP12–ligand–FRAP complex of rapamycin; and (iii) are accessible by engineered biosynthesis through modifications of the PKS responsible for generating rapamycin.

Methods

The crystal structures of the FKBP12–rapamycin complex\textsuperscript{20} and the FKBP12–rapamycin–FRAP complex\textsuperscript{21} were used as the basis for the molecular dynamics (MD) studies in this work.
hydrogens were constrained using the SHAKE algorithm and interactions involving hydrogens were omitted. During the production portion of the dynamics runs, all interactions were calculated and the SHAKE algorithm was, therefore, not used. Subsequent runs consisted of a geometry optimization (200 steps steepest descent, 9800 steps conjugate gradient), a 10 ps constant pressure equilibration run in which only waters were allowed to move, a 10 ps constant volume equilibration run using SHAKE, and a 100 ps constant volume production MD run (without geometry constraints or SHAKE) for each system examined. Each ligand was studied unbound (in water) and bound to FKBP12. Additionally, the dynamics of some of the ligands were studied in ternary FKBP12–ligand–FRAP complexes.

Charges for the ligands were calculated using averaged PM3 Mulliken charges for eight to ten distinct conformations of each ligand, using Ampac 6.0.23 This was done after the calculation of standard RESP charges had proven prohibitively expensive (due to the large size and flexibility of the ligands, multiple conformations are required to get a reasonable ESP fit). The β-keto amide in the binding domain was assigned zero torsional parameters (i.e. the torsional energy profile was purely determined by electrostatic and van der Waals interactions) resulting in the energy profile shown in Figure 2. The single bonds separating the adjacent double bonds in the binding domain were assigned parameters calculated by Hermone and Kuczera for retinal,24 which reproduced the HF/6-31G(d) energy profile for rotation around the C3–C4 bond in 2,4-hexadiene at torsional angles close to 180° (Fig. 3). The fit to the energy profile at torsional angles close to 0° was poor. This is, however, not a problem, since the adjacent double bonds cannot assume a 0° torsion angle due to constraints placed by the macrocycle.

The binding of rapamycin and the modified ligands was assessed based on the following criteria: (i) The mobilities of the protein and ligands during the MD simulations. (ii) The differences in the average solvent accessible surface area of the binding site of the protein bound to the different ligands. (iii) The formation of cavities between the protein and ligands during the MD simulations. (iv) The difference in the interaction energies of the ligands with the protein relative to the free ligand and protein, scaled using the linear interaction energy (LIE) method of Aqvist et al.25,26 Each of the analysis methods used is described in more detail below.

(i) The mobilities of the protein and ligands during the MD simulation were determined by estimating the average deviation of each atom from the average structure during the MD simulation. These can be compared to the crystal structure Debye–Weller B-factor (assuming that the B-factors are free from contributions from lattice effects and that the motions of atoms are isotropic) using the relationship \( \Delta r_i^{1/2} = (3B/8\pi)^{1/2} \).27 The comparison of the atom mobilities in the FKBP12–rapamycin and FKBP12–rapamycin–FRAP complexes with the Debye–Werrer B-factors was used to verify that the secondary structure of the crystal structure remained intact during the MD simulation. The comparison of the mobilities of the protein bound to different ligands was used to indicate how the protein–ligand binding was affected by the modifications to the ligand.

(ii) The differences in the average solvent accessible surface area of the binding site of the protein bound to the different ligands was used as a gauge of whether the modifications to the ligands affected the surface of FKBP12 as presented to FRAP. This was only relevant for the binary FKBP12–ligand complexes. Solvent accessible surface areas were calculated at each point during the simulation using the program NACCESS.28 All residues that had at least one atom within 5 Å of rapamycin in the binary complex of rapamycin with FKBP12 (Tyr26, Gly28, Phe36, Asp37, Phe46, Gln53, Glu54, Val55, Ile56, Arg57, Trp59, Tyr82, His87, Ile90, Ile91, Leu97 and Phe99) were used in the SASA calculation.
(iii) The formation of cavities between the protein and ligands during the MD simulations was used to gauge how closely the ligand filled the binding sites on FKBP12 and FRAP. This measurement was mainly relevant for the ternary FKBP12–ligand–FRAP complexes as a test of whether the binding to FRAP had been sufficiently affected by the modifications to rapamycin to let water into the binding site. The volumes of gaps formed between the ligand and protein were calculated at each step of the simulation using the program SURFNET. The gaps were calculated using a 6 × 6 × 6 Å boundary around the atom range, a grid separation of 0.8 Å, an initial sphere size of 1 Å, a maximum sphere size of 4 Å, and a scaling factor of 1.0.

(iv) The difference in the interaction energies of the ligands with the proteins relative to the free ligand and protein, scaled using the LIE method of Åqvist et al., was used to estimate the relative free energy of binding for rapamycin and the modified ligands to FKBP12. The LIE binding free energy was also estimated for the ternary complexes, but it should mainly be taken as a qualitative measurement for the relative binding free energy in those cases. The LIE method is based on the assumption that, using molecular dynamics or Monte Carlo conformational simulations, the free energy of ligand binding to a protein target can be expressed using the equation:

\[
\Delta G_{\text{bind}} = \left( \alpha_{\text{prot}} \langle V_{\text{vdW}} \rangle_{\text{prot}} - \alpha_{\text{wat}} \langle V_{\text{vdW}} \rangle_{\text{wat}} \right) + \left( \beta_{\text{prot}} \langle V_{\text{el}} \rangle_{\text{prot}} - \beta_{\text{wat}} \langle V_{\text{el}} \rangle_{\text{wat}} \right) + \gamma
\]

where \( \alpha_{\text{prot}} \) and \( \alpha_{\text{wat}} \) are scaling factors for the average potential van der Waals energies \( \langle V_{\text{vdW}} \rangle \), \( \beta_{\text{prot}} \) and \( \beta_{\text{wat}} \) are scaling factors for the average potential electrostatic energies \( \langle V_{\text{el}} \rangle \), \( \gamma \) is an empirical constant to reproduce experimental free energies of binding, and the subscripts prot and wat refer to the FKBP12–ligand

Chart 3. The structures of the modified rapamycin analogues evaluated in this study.
complex in water and the free ligand in water, respectively. Åqvist and co-workers fit eq (1) to a large number of experimental binding constants and arrived at several possible combinations for the scaling factors $\alpha$ and $\beta$. In this study, we used parameters which gave a good general fit in Åqvist's studies, which are $\alpha_{\text{prot}} = 0.163$, $\beta_{\text{prot}} = 0.348$, $\beta_{\text{wat}} = 0.340$, and $\gamma = -1.89$. Snapshots were taken at 1 ps intervals from the MD studies. The potential energies (van der Waals and electrostatic) were calculated for the ligand at each snapshot conformation using the ‘anal’ program in the Amber suite of programs, and the average potential energies were used in calculating the free energies of binding from the LIE equation.

Results and Discussion

The rapamycin analogues studied here are shown in Chart 3. All the analogues studied here share the same binding domain but have effector domains that differ by the number of deletions from the effector domain of rapamycin (Chart 3). It is reasonable to expect larger differences in the interaction energies of the ligands with FRAP than with FKBP12, since all the differences focus on the part of rapamycin associated with binding to FRAP. The changes in interactions between the ligands and the target proteins can be evaluated both from the interaction energies between the ligands and proteins and from the different dynamics of the ligand–protein complexes.

Binary ligand–FKBP12 complexes

The ability of the FKBP12–rapamycin complex to bind to FRAP is due, in large part, to the rigid nature of rapamycin bound to FKBP12. When the binding domain of rapamycin is bound to FKBP12, the three adjacent double bonds in the rapamycin effector domain protrude away from the protein surface, forming a rigid arm suitable for affixing to FRAP. Figure 4 provides a sense of how the different ligands fit into the binding site on FKBP12. The largest ligands protrude significantly from the protein surface, whereas the shortest ones are nearly completely engulfed by the protein. Figure 5 is a tube representation of the ligand RP7, with the remaining portion of the effector domain shown in magenta. When ketides were removed from the effector domain of rapamycin, the solvent accessible surface area (SASA)
of the residues near the binding site of FKBP12 stayed nearly constant during the first three deletions, decreased during the next three deletions, and then rose sharply (Table 1). These changes in SASA reflect that the shorter effector domains of RP4, and RP6 do not protrude from the surface of FKBP12 as much as the effector domain of rapamycin does, but are instead in rather close contact with the protein surface. These close contacts between protein and ligand are reflected in less favorable free energies of binding than for rapamycin, RP2, and RP3. For the shortest ligands, RP7 and RP8, the effector domain is too short to protrude from the protein surface, and the ligands no longer favor the conformation required to bind to FKBP12. The poor fit of RP8 in the binding site of FKBP12 is reflected by the mobilities of the binding domains of the two ligands bound to FKBP12 (Fig. 6), which indicates that RP8 is making little or no favorable contacts with the binding site.

These findings are also reflected in the LIE free energies of binding, although RP1 does not fit the overall energy profile (Fig. 7). The free energy of binding for RP2 and RP3 is nearly identical to the free energy of binding calculated for rapamycin, but the free energies of binding start getting less favorable as the effector domain is truncated further. The results of this study, therefore, indicate that up to three ketide units can be removed from the effector domain of rapamycin without adversely affecting the ability of the drug to bind to FKBP12.

Table 1. The effects of sequentially deleting ketides from the effector domain of rapamycin on the formation of FKBP12–ligand complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>SASA (Å²)</th>
<th>RMS dev.</th>
<th>LIE (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapamycin</td>
<td>645</td>
<td>42</td>
<td>−7.2</td>
</tr>
<tr>
<td>RP1</td>
<td>612</td>
<td>33</td>
<td>−6.5</td>
</tr>
<tr>
<td>RP2</td>
<td>616</td>
<td>32</td>
<td>−7.7</td>
</tr>
<tr>
<td>RP3</td>
<td>621</td>
<td>30</td>
<td>−7.4</td>
</tr>
<tr>
<td>RP4</td>
<td>595</td>
<td>38</td>
<td>−6.5</td>
</tr>
<tr>
<td>RP6</td>
<td>571</td>
<td>44</td>
<td>−5.6</td>
</tr>
<tr>
<td>RP7</td>
<td>630</td>
<td>45</td>
<td>−4.6</td>
</tr>
<tr>
<td>RP8</td>
<td>694</td>
<td>43</td>
<td>−4.3</td>
</tr>
</tbody>
</table>

*The ligands are shown in Chart 3.

The average solvent accessible surface area (SASA) is given along with its standard deviation for each dynamics run.
**Ternary ligand–FKBP12 complexes**

The interactions of rapamycin and the modified ligands RP1, RP2, and RP3 bound in ternary complexes with the proteins FKBP12 and FRAP were evaluated using the same methods as were used for the binary FKBP12–ligand complexes. The way that the modified ligands interact with FRAP was likely to be significantly different from the interactions between rapamycin and FRAP. The formation of cavities between the ligand and protein were, therefore, used as a qualitative measure of the effect that the changes in ligand structure had on the FRAP binding. The LIE free energies of binding were also calculated. The LIE interaction energies might differ significantly from the experimental free energies of binding due to the long equilibration periods required to fully accommodate the nesting of the novel ligand in the binding site of FRAP, which are outside the scope of this project.

As ketide units were removed from the effector domain of rapamycin, increased cavities were formed between the ligand and proteins. In the case of the smaller ligands, RP2 and RP3, water molecules from the simulation environment found their way into these cavities as the simulation progressed, indicating the loss of favorable interactions between FRAP and the modified effector domain of the ligands (Table 2). The same trend is reflected in the LIE free energies of binding, which grow steeply less favorable as the first double bonded ketide unit is removed and are identical for the modified ligands RP2 and RP3.

**Table 2.** The effects of sequentially deleting ketides from the effector domain of rapamycin on the formation of FKBP12–ligand–FRAP complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Gap (Å³)</th>
<th>RMS dev.</th>
<th>LIE (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapamycin</td>
<td>904</td>
<td>147</td>
<td>−10.7</td>
</tr>
<tr>
<td>RP1</td>
<td>997</td>
<td>144</td>
<td>−7.1</td>
</tr>
<tr>
<td>RP2</td>
<td>1027</td>
<td>127</td>
<td>−5.8</td>
</tr>
<tr>
<td>RP3</td>
<td>1098</td>
<td>108</td>
<td>−5.8</td>
</tr>
</tbody>
</table>

*The ligands are shown in Chart 3.*

*The average gap volume between the ligand and proteins is given along with its standard deviation for each dynamics run.

**Conclusion**

The binding energies of rapamycin and the modified ligands RP1 through RP8 are summarized in Figure 7. These results indicate that several deletions can be performed from the effector domain of rapamycin without adversely affecting the ability of the ligands to bind to FKBP12. Indeed, the binding free energies calculated using the LIE method are predicted to become more favorable when one or more of the adjacent double bonded ketide units in the rapamycin binding domain are deleted. This indicates that the role of the adjacent double bonds in binding to FRAP might be important enough for nature to accept the unfavorable effect this part of the ligand has on the binding to FKBP12. The affinities of the modified ligands for binding to FKBP12 are predicted to drop off sharply when the deletions from the effector domain are numerous enough to bring substituents on the effector domain close to the surface of the protein (ligands RP6 and higher). The drop in binding affinities becomes even sharper when the deletions become severe, as is seen for the ligands RP7 and RP8.

The binding energies for the rapamycin and the modified ligands RP1, RP2, and RP3 in the ternary FKBP12–ligand–FRAP complexes are summarized in Figure 8. As can be seen from these results, the prediction of the MD simulations is that the favorable interactions between rapamycin and FRAP are severely affected by the removal of one of the adjacent double bonded ketide units in the effector domain of rapamycin. The interactions between the ligand and FRAP are nearly eliminated with the removal of the second of the adjacent double bonds. An examination of Figure 4 shows that the effector domains of the smaller ligands (notably RP6 through RP8) barely protrude from the protein surface, such that these ligands would not be able to associate with FRAP.

The best ligands found in the current study, RP2 and RP3, show that the ease of formation of the ternary FKBP12–rapamycin–FRAP complex can be selectively varied through modifications to the effector domain of rapamycin. It is also clear that the modifications performed to achieve these changes can be kept within the range of changes accessible by modifications to polyketide synthases.

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References and Notes

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