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STRUCTURE-ACTIVITY STUDIES OF NONMACROCYCLIC RAPAMYCIN DERIVATIVES

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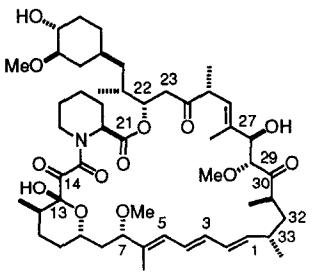
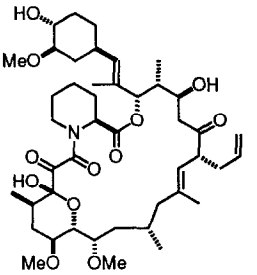
Abstract: X-ray crystallography suggests the C23-C28 segment of rapamycin may act more as an element of the FKBP binding domain than as part of the immunosuppressant effector domain. Selective excision of this region from the natural product followed by minor reconstruction of the binding domain resulted in compounds with high affinity for FKBP but no immunosuppressive activity. This, along with data from other secorapamycin analogs, suggest the importance of C23-C28 in the orientation of the effector domain.

Rapamycin (**1**) and FK506 (**2**), two structurally related macrocyclic natural products, have received remarkable attention due to their extraordinary immunosuppressant properties as well as to their fascinating biomolecular mechanisms of action.[1-4] Both compounds interrupt specific yet different signal transduction pathways in T-cells through the common intermediacy of a highly abundant intracellular protein receptor, FKBP.[5,6] FKBP catalyzes the *cis-trans* peptide bond isomerization of proline (PPIase or rotamase), however, the inhibition of this enzyme activity by rapamycin or FK506 has been demonstrated to be insufficient for immunosuppression.[7] Instead, the FKBP and rapamycin or FK506 complexes interact with "downstream" targets associated with distinct cell-signaling pathways. The FK506-FKBP complex has been shown to inhibit the calmodulin-dependent protein phosphatase PP2B (calcineurin).[8] The downstream target for the rapamycin-FKBP complex has yet to be determined.[9]



Rapamycin (**1**)



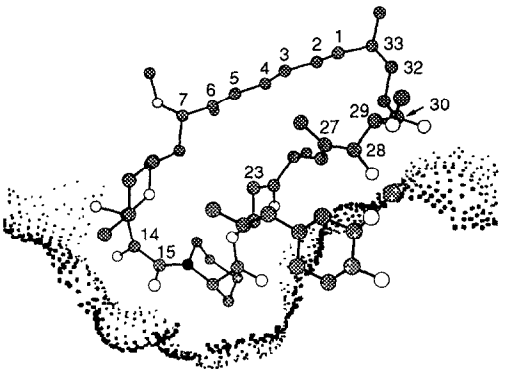
FK506 (**2**)

The structural relationship between FK506 and rapamycin, coupled with X-ray crystal structures of their complexes with FKBP[10,11] and biochemical studies with modified ligands,[12-14] has led to a view of these drugs as dual domain entities.[2] The common structural motif, centered on the pipecolyl ester and extending on either side to the pyranose ring and cyclohexylethyl macrocyclic appendage, constitutes the "binding domain" which is essential for high affinity to FKBP.[3] The remaining halves of each macrocycle, which bear little structural

resemblance to each other, constitute the "effector domains" and are presumably essential for immunosuppressive activity.

The X-ray crystal structures of these two drug-FKBP complexes show the binding domain of each drug to be deeply buried in a hydrophobic cavity of the protein while the respective effector domains protrude and contribute to distinct composite protein-ligand surfaces which form the immunosuppressant pharmacophores. In the case of the rapamycin-FKBP complex,[11] the most exposed section of the putative effector domain is the triene region (C1-C7) along with one face of the adjacent chain of atoms C33-C29 (Figure 1). Other than the methyl attached to C27, the stretch of macrocycle extending from C28 through C23 is essentially buried beneath the triene -- making contact with FKBP but having minimal exposure to solvent. Thus, C23-C28 is perhaps more appropriately viewed as an element of the binding domain, unique to rapamycin, rather than part of the effector domain.

Figure 1. *Crystal sructure of rapamycin bound to FKBP*[11] *with cross section of protein surface.*

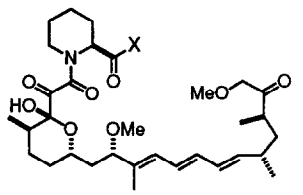


We recently described[15] synthetic manipulations of rapamycin which allow for the clean excision of the C22-C28 region of the macrocycle along with the cyclohexylethyl appendage (via retroaldol cleavage of the C28-C29 bond followed by base-catalyzed fragmentation of the C21-C22 lactone). Thus, ready access to acid **3** (Table 1) has allowed for the examination of several rapamycin derivatives lacking this molecular region. Although **3** itself possessed little affinity for FKBP,[16] our experience with simple pipecolate derivatives[17] suggested that refunctionalization of acid **3** as an ester or amide might restore sufficient FKBP binding while retaining enough elements of the effector domain (as C29-C7) for immunosuppressive activity.

In the event, coupling of acid **3** to amines using 2-chloro-1-methylpyridinium iodide proved to be straightforward. However, ester formation through alcohol coupling with a variety of reagents (2-chloro-1-methylpyridinium iodide, DCC, trichlorobenzoyl chloride, phenyl dichlorophosphate) was unsuccessful.[19] Esterification was only accomplished by alkylation of the carboxylate with alkyl bromides or iodides and DBU in DMF. As indicated in Table 1, esters and amides alike proved to have high affinity for FKBP, although about 100-fold lower than rapamycin ($K_{i,app}$ = 0.5 nM). Most significantly, none of the compounds exhibited any suppressive activity in a splenocyte mitogenesis assay, an *in vitro* model for immunosuppression,[20] at non-toxic drug concentrations in the range of 1 pM to 10 μM (IC_{50} for rapamycin = 1 nM). In addition to the C22-C28 excision products (Table 1), a number of secorapamycins have been examined which retain all of the parent carbons (Figure 2). The methyl and benzyl esters **12** and **13**, products of lactone opening and esterification, are most closely related to esters **4** and **6** and exhibited comparable binding to FKBP. However, these compounds

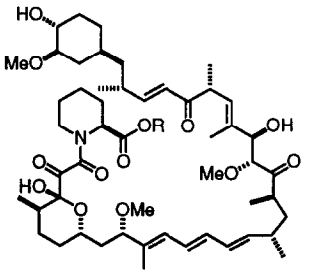
(**12** and **13**) were also devoid of *in vitro* immunosuppressive activity. The retroaldol product, 28,29-secorapamycin (**14**), showed 4 nM FKBP affinity, but it too lacked activity in the splenocyte assay. Finally, cleavage of the C13-C14 bond in **1** with lead tetraacetate provided the methoxalyl pipecolyl derivative, which selectively reacted with t-pentylmagnesium bromide to provide compound **15**.[21] Again, seco-derivative **15** exhibited good FKBP affinity but no measurable immunosuppressive activity.

Table 1.

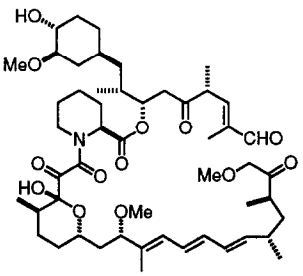


compd no.	X	$K_{I,app}$ (nM)
3	OH	500
4	OMe	12
5	OiPr	51
6	OBn	15
7	OCHMePh	41
8	$OCH_2CHCHPh$	31
9	$OCH_2CH_2CH_2(3,4\text{-}OMe_2)Ph$	43
10	NHBn	110
11	$NHCH_2CH_2CH_2Ph$	32

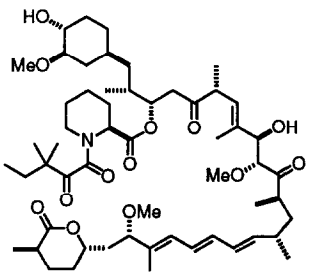
Figure 2.



12: R = Me $K_{i,app}$ = 29 nM
13: R = Bn $K_{i,app}$ = 2-6 nM



14 $K_{i,app}$ = 4 nM



15: $K_{i,app}$ = 20 nM

While the C23-C28 section of rapamycin may actually contribute to the immunosuppressant pharmacophore directly through a bound conformation which is not observed in the crystal state, it seems more likely that this transition zone between the binding and effector domains is critically involved in orienting the C29-C7 effector motif. Attachment of the binding domain to C30 in the form of a macrocycle may be essential and could, in principle, be accomplished using a simplified replacement for the C23-C28 chain. Toward this end we have prepared a 29-nor-21,30-dicarboxylic acid analog of **3**[22] which may be ideally suited for insertion of simple cassettes. Alternatively, proper orientation of the effector domain may still be achievable with non-macrocyclic relatives of those in Table 1 which possess esters that optimally fill the spatial volume left unoccupied by the removal of the C23-C28 segment. Studies to further elucidate the functional relationship between the binding and effector domains of **1** and **2** are ongoing.

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20. Spleen cells from BDF1 female mice were established in RPMI with 10% fetal calf serum at $5x10^6$/mL. One hundred μL aliquots of this suspension ($5x10^5$ cells) were dispensed into 96-well round-bottomed microtiter plates (Linbro, Flow Laboratories). Concanavalin A (5 μg/mL) was added as the mitogenic stimulus and the final volume in the microtiter wells was adjusted to 200 μL with RPMI. Cell cultures were incubated for 72 h at 37°C in a 5% CO_2 atmosphere and pulsed with 0.5 μCi ^{3}H-thymidine (specific activity 2.00 Ci/mmol) for the last 18 h of the 72 h culture. The cells were harvested on an automated multiple sample harvester and cell-associated radioactivity counted in a Beckman liquid scintillation counter. The results are expressed as the mean values derived from quadruplicate measurements. Cell viability was determined by trypan blue exclusion after 72 h of incubation. Compounds to be tested were added to the microtiter plates at the appropriate dilutions prior to the addition of cells.
21. The utilization of the pipecolyl 3,3-dimethyl-2-oxo-valeramide as a high-affinity binding motif has been described.[17]
22. Luengo, J. I., unpublished results.