Communications to the Editor

Rapamycin and FK506 Binding Proteins (Immunophilins)

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The structurally related macrolides FK506 and rapamycin have recently been shown to inhibit distinct signaling pathways in T lymphocytes.[1,2] Whereas FK506 and the structurally unrelated undecapeptide cyclosporin A (CsA) potently inhibit a T cell receptor-mediated signal transduction pathway, rapamycin appears to act at a later stage of T cell activation by interfering with a signaling pathway that is mediated by a lymphokine receptor. In addition, FK506 and rapamycin, but not CsA, inhibit each other's actions in a variety of functional assays, indicating that FK506 and rapamycin are competitive antagonists and may have a common biological receptor site.[2,3] However, concentrations 10–100 times greater than the effective drug concentration of either agent ($IC_{50} \sim 0.5$ nM) are required in order to observe this mutual antagonism. The requirement for a large excess of the antagonizing agent may be accounted for by either the existence of separate immunophilins (immunosuppressant binding proteins) with different affinities for the two agents or by an excess of a common immunophilin. In the latter case, the abundant, uncomplexed immunophilin would sequester the antagonist. Only after the excess binding sites were saturated would the concentration of the antagonizing agent rise to sufficient levels to displace the drug effectively from its biological receptor, i.e., the inhibition would be subject to a buffering action by the excess immunophilin.

The predominant receptor for FK506 has been shown to be the immunophilin FKBP (FK506 binding protein),[4,5] which binds to FK506 and rapamycin with similar affinities [K_d(rapamycin) = 0.2 nM, K_d(FK506) = 0.4 nM].[2] The concentration of FKBP in Jurkat T cells is approximately 5 nM,[6] i.e., approximately 10-fold greater than the IC_{50} of either drug. This suggests that FKBP may be responsible for the aforementioned concentration dependence of the mutually inhibitory actions of rapamycin and FK506. In order to further probe the role of immunophilins in mediating both the mutually inhibitory and biological actions of these agents, the cellular receptor(s) for rapamycin must be characterized. In this communication, we demonstrate that the predominant receptor for rapamycin in T lymphocytes is identical with FKBP by several analytical techniques, suggesting that FKBP is the common immunophilin responsible for buffering the actions of both drugs, and we report the initial characterization of several lower abundance immunophilins that have affinity for both drugs.

Since affinity chromatography proved successful in our initial isolation of FKBP,[4] a rapamycin affinity matrix was synthesized. As the chemistry used to prepare this reagent was superior to our earlier chemistry,[4] a new FK506 affinity matrix was also prepared. Both drugs were attached to the matrix through the secondary alcohol of the common cyclohexyl moiety. The esterification of the C32-hydroxyl of FK506 (**1a**) (Scheme I) and C42-hydroxyl of rapamycin (**2a**) with the allyloxycarbonyl (Aloc) protected amino acid **3** was achieved with phenylphosphorodichloridate–dimethylformamide complex **4** as the coupling reagent.[7] The Aloc protecting group of **1b** and **2b** was removed by using $Pd(PPh_3)_4$ as catalyst (10 mol %) and dimedone as an allyl acceptor.[8] The crude reaction mixture, containing the amino derivative **1c** or **2c**, was added to Affigel-10 that had been equilibrated with THF.[9]

To test the matrices, affinity chromatography of an extract from the human T cell line Jurkat using **1d** and **2d** was performed.[10] Briefly, three aliquots of Jurkat cell lysate (5×10^7 cells/aliquot) were first passed over ethanolamine-capped Affigel-10 and then twice over either FK506- or rapamycin-linked Affigel-10. After repeated washings, the retained proteins were batch-eluted with the corresponding drug. Subsequent SDS/PAGE analysis and silver staining[11] revealed the immunophilins as shown in Figure 1. In a control experiment, the same process was performed with the ethanolamine-capped Affigel-10. Only bovine serum albumin, which was present at high concentration in the lysis buffer, was detected by silver staining.

The profiles of eluted proteins from the FK506 and rapamycin affinity matrices are similar (Figure 1A). This similarity was also observed in extracts prepared from calf thymus (Figure 1B) and bakers' yeast (data not shown). The major human immunophilin from the rapamycin affinity matrix [densitometric analysis indicates a ratio of 42 (12 kDa):<1 (13 kDa):4.2 (30 kDa):9.2 (60 kDa):1.0 (80 kDa)] has a mobility on SDS/PAGE (M_r 12000) identical with that of recombinant human FKBP; moreover, the N-terminal sequence (10 amino acids, determined by Edman degradation), isoelectric point (pI = 8.8–8.9), amino acid composition, and SDS/PAGE mobility of the major protein retained from calf thymus tissue extract are identical with those of FKBP.[4] The minor proteins from human T cells and calf thymus, which are present for both drugs, may bind immobilized drug, the drug/FKBP complex, or both. Analyses of purified proteins from calf thymus indicate that the immunophilins of M_r 30 000 and 13 000, which are more prominent in the eluates from the rapamycin matrix, bind directly to FK506[12] and, as inferred from their partial sequence determination, are members of a previously unknown family of immunophilins that includes FKBP.[13] Affinity chromatography as described above with an extract prepared from Jurkat cells labeled with [^{32}P]orthophosphate and subsequent

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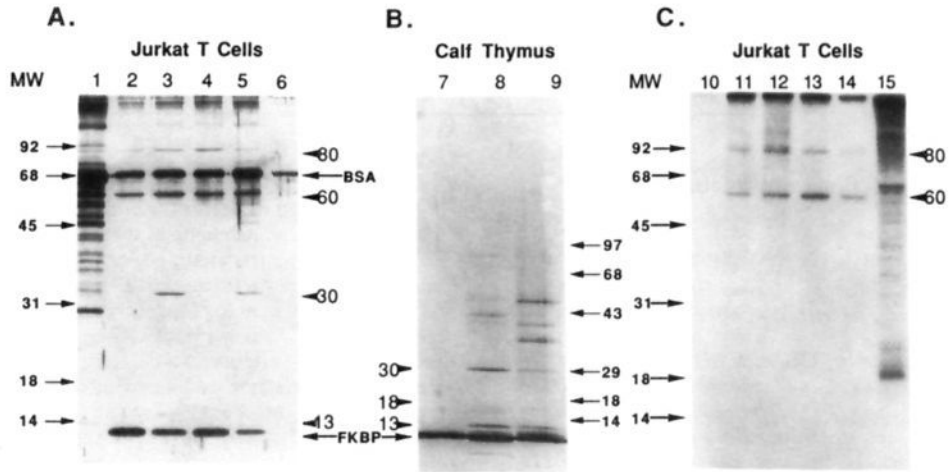
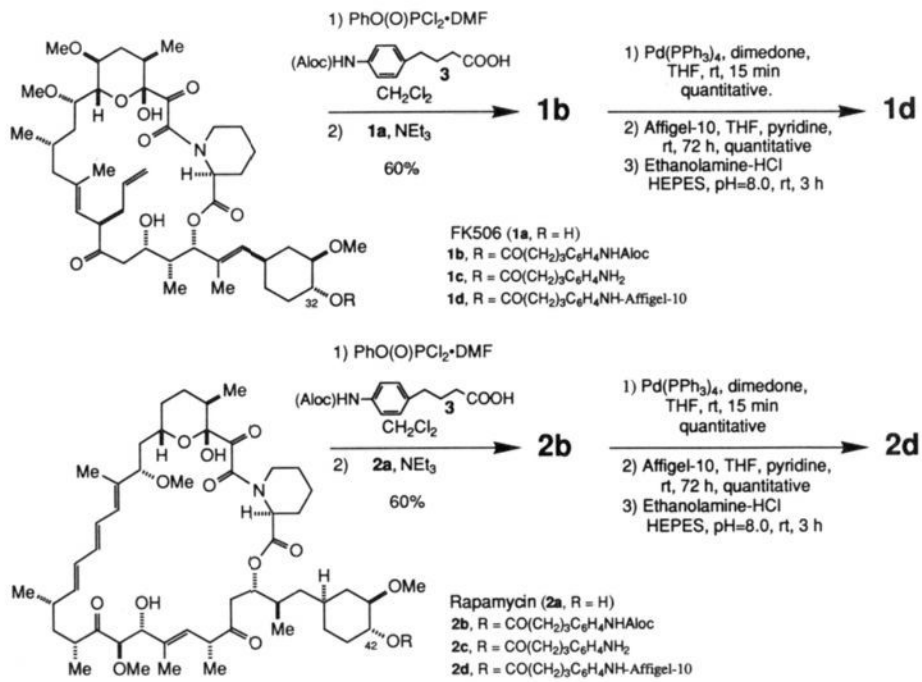


Figure 1. SDS/PAGE analysis of the eluates from (A) Jurkat T cells and (B) calf thymus and (C) autoradiogram of Jurkat T cells. Lanes: (A) (1) Jurkat lysate flow through. (2) Unstimulated cells: FK506 matrix. (3) Unstimulated cells: rapamycin matrix. (4) Stimulated cells: FK506 matrix. (5) Stimulated cells: rapamycin matrix. (6) Unstimulated cells: control matrix. (B) (7) Recombinant human FKBP. (8) Calf thymus: rapamycin matrix. (9) Calf thymus: FK506 matrix. (C) (10) Unstimulated cells: control matrix. (11) Stimulated cells: rapamycin matrix. (12) Stimulated cells: FK506 matrix. (13) Unstimulated cells: rapamycin. (14) Stimulated cells: FK506 matrix. (15) Jurkat lysate flow through. The major band for both tissue sources and both matrices is FKBP. The band at 68 000 in the Jurkat experiment (A) is BSA (bovine serum albumin), which is present at high concentrations in the lysis buffer. The minor bands from Jurkat cells (M_r ca. 13 000, 30 000, 60 000 and 80 000) and calf thymus (M_r ca. 13 000, 18 000, 30 000, >40 000) are present in eluates from both drug matrices, and the M_r ca. 60 000 and 80 000 proteins are phosphorylated (see text).

Scheme I



analysis by autoradiography revealed that the bands at M_r 60 000 and M_r 80 000 are phosphorylated proteins (Figure 1C). It will be of considerable interest to elucidate any role these proteins may play in cell signaling pathways.

The results reported herein indicate that the predominant rapamycin binding protein in Jurkat T lymphocytes is FKBP, and thus implicate FKBP as the major "buffer" for FK506's inhibition of the actions of rapamycin and vice versa. In addition, several previously unidentified rapamycin- and FK506-binding immunophilins have been detected that may also be relevant to the biological actions of these drugs.[14] Current research is focused on the complete characterization of immunophilins and immunophilin (or immunophilin/drug complex) binding proteins in hopes of unraveling the mysteries of signal transduction through the cytoplasm.[15,16]

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(14) Recently it has been suggested that immunophilin/drug complexes are the species responsible for interfering with normal signal transmission pathways in T lymphocytes.[2,15] The direct binding of these immunophilins to drug makes them candidates, as is FKBP, for the protein component of these inhibitory complexes.

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Hughes Medical Institute for a predoctoral fellowship (awarded to M.W.A.).

Supplementary Material Available: Experimental details for preparation of the FK506 and the rapamycin matrix, isolation of the FK506 and rapamycin binding proteins, and ^{32}P labeling of Jurkat cells and autoradiography (3 pages). Ordering information is given on any current masthead page.

Enantiomeric Resolution of $Ru(phen)_3^{2+}$ and $Ru(bpy)_2ppz^{2+}$ on a DNA-Hydroxylapatite Column

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It has been demonstrated[1,2] that various six-coordinate metal complexes in which the ligands are bidentate diimines with fused aromatic ring systems are capable of enantiomerically selective interaction with double-stranded DNAs. The basis of this enantioselectivity is reported[2] to be the more favorable steric fit of the Δ isomer within the major groove of DNA induced by an intercalative interaction of one of the metal-bonded aromatic ligands. At the same time, a lesser enantioselectivity has been observed for the Λ isomer via interaction with the minor groove. Such complexes have been demonstrated[2-4] to be useful as selective reagents for cleaving both DNA and RNA. We report here a novel application of these interactions in separating enantiomers of such complexes by immobilizing double-stranded DNA on a column of hydroxylapatite and passing a racemic solution of a complex through the column. In one case described here, the first resolution of a complex is achieved by using such a column. A racemic mixture of the complex separated into two bands, which are demonstrated to contain mainly (~95% or higher purity) the separated Λ and Δ isomers.

We have been interested[5a] in the photophysical and photochemical properties of mixed-ligand diimine complexes of ruthenium(II) and have synthesized and characterized mononuclear and dinuclear complexes containing the ligands 4′,7′-phenanthrolino-5′,6′:5,6-pyrazine (ppz) (**1**)[5b] and 2,3-di-2-pyridylpyrazine (dpp) (**2**). The complexes $Ru(bpy)_2\cdot\mathbf{1}^{2+}$ and $Ru(bpy)_2\cdot\mathbf{2}^{2+}$ (bpy = 2,2′-bipyridine) were tested for evidence of intercalative interaction with DNA. The visible-region metal to ligand charge transfer (MLCT) band of $Ru(bpy)_2\cdot\mathbf{1}^{2+}$, which is associated with the ppz ligand (Figure 1), shows hypochromicity in the presence of calf thymus DNA, while the MLCT bands of $Ru(bpy)_2\cdot\mathbf{2}^{2+}$ show no hypochromicity under the same conditions. While this is not definitive proof of an intercalative interaction, it is strongly suggestive. At the least, it provides evidence of strong association of $Ru(bpy)_2\cdot\mathbf{1}^{2+}$ with DNA. During the course of our investigations we attempted to resolve each into Δ and Λ isomers by fractional crystallization with potassium antimony tartarate without success. Because of limited amounts of complex, we have devised a novel means of separation for $Ru(bpy)_2\cdot\mathbf{1}^{2+}$.

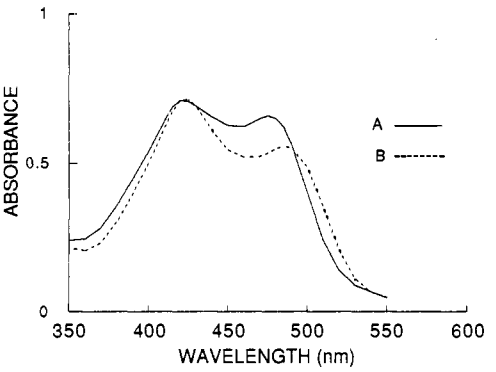
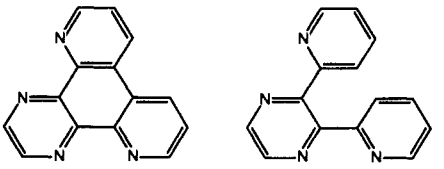


Figure 1. Absorption spectra of $Ru(bpy)_2\cdot\mathbf{1}^{2+}$ alone (A) and in the presence of calf thymus DNA, mole ratio of DNA phosphate/Ru of 20 (B). Complex is 65 μM in both spectra.



1 **2**

A column of hydroxylapatite (BIO-GEL HTP, Biorad Laboratories), 1.6 cm × 21 cm, was first poured and washed with several column volumes of buffer (0.01 M sodium phosphate, pH 6.8; 100 mM NaCl). Calf thymus DNA (Sigma) was dissolved at a concentration of 1 mg/mL in the same buffer, and 40 mL of this solution was washed onto the column, followed by several hundred milliliters of buffer. Double-stranded DNA (and some single-stranded, if present) adsorbs[6,7] to the column under these conditions. The absorbance of the column wash at 260 nm was checked, and no DNA was detected.

As a test of the resolving capabilities of the column, we passed a 0.100-mL sample of racemic $Ru(phen)_3^{2+}$ (1.2 mM) through the column, eluting with the column buffer. Peak ratios for ultraviolet absorption bands of the complex were routinely checked to exclude the presence of DNA in the eluted samples. Circular dichroism spectra of the fractions eluted showed that the fractions eluting first were enriched in Λ-$Ru(phen)_3^{2+}$ and that the tailing fractions were enriched in Δ-$Ru(phen)_3^{2+}$. This was not unexpected since Δ-$Ru(phen)_3^{2+}$ has been shown,[2] primarily via

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