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Role of Immunophilins in Therapeutic Drug Monitoring of Immunosuppressive Drugs

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Introduction

Immunophilins are proteins that bind one or more of the three immunosuppressive drugs cyclosporine (CsA), tacrolimus (FK-506), and sirolimus (RAPA). Studies have shown that quantitatively immunophilins can be divided into two categories, major and minor. The major immunophilins are cyclophilin (17 kDa) which binds CsA and FK-Binding Protein (FKBP, 12 kDa) which binds FK-506 and RAPA. The major immunophilins are ubiquitous and account for about 0.1% of total cytosolic protein. They are present in μmolar concentrations. Both the major immunophilins have cis-trans-peptidylprolyl isomerase (PPI-ase) also called rotamase activity and both have isoelectric points close to 9.0 (1–14). The dissociation constants (Kds) of the drugs for the immunophilins are shown in Table 1. The minor immunophilins can be divided into two groups. Those with PPI-ase activity (12, 25 and 56–50 kDa) and those without PPI-ase activity (14, 37, and 52 kDa) (7,8,15). The 14, 37, and 52 kDa immunophilins are not ubiquitous and have been found in the cytosol of T cells, spleen, and thymus (Table 2). Unlike cyclophilin and FKBP-12 they have not been isolated from other tissues or cells. The PIs of the 14, 37, and 52 kDa immunophilins are in the range 6.5 to 7.5 (7,9,15). It should be emphasized that the minor immunophilins are present in nmolar concentrations and drugs which bind to these proteins are pharmacologically active in the nmolar range. Although CsA binds to both the 37 and 52 kDa proteins this binding is weak. Whether these three minor immunophilins play a central role in the mechanism of action of these drugs is unknown. However, the 37 kDa immunophilin has been shown to possess glyceraldehyde-3-phosphate dehydrogenase activity (EC:1.2.1.12) as well as uracil DNA glycosylase activity (15,16).

TABLE 1
Dissociation Constants of Immunosuppressive Drugs for the Binding Proteins

Protein	Drug	Kd (nM)
Cyclophilin	CsA	50–150
FK BP-12	FK-506	0.4–1.3
14 kDa	FK-506	1.8
37 kDa	FK-506	4.5
	RAPA	0.8
52 kDa	CsA	64
	FK-506	2.0
	RAPA	1.1

The rationale for receptor assays

Theoretically, receptor assays possess many advantages over current immunoassays. For example, whenever a drug is metabolized to both active and inactive metabolites, there will be great difficulty quantifying only the parent drug or, better still, the parent drug and the pharmacologically active and inactive metabolites relative to their pharmacological potency using antibodies (monoclonal or polyclonal). One of the potential advantages of receptor assays over immunoassays is that the cross-reactivity of metabolites in receptor assays may well parallel their pharmacological activity. Consider the situation with several drugs that fall into this category.

CsA

The clinical relevance of CsA metabolites, of which there are around 30, is still controversial. Immunosuppressive activity has been found *in vitro* for AM1, AM9, and AM4N (3,14,17,18). Only about one-third of CsA in the blood is in the form of the

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TABLE 2
Binding Proteins to
Immunosuppressive Drugs
(CsA, FK-506, RAPA)

Cyclophilin	CsA
FKBP-12	FK-506, RAPA
14 kDa	FK-506, RAPA
37 kDa	CsA, FK-506, RAPA
52 kDa	CsA, FK-506, RAPA

parent drug. Approximately two-thirds is in the form of active and inactive metabolites, although this ratio varies from patient to patient and, also, within patients. Measurement of only the parent drug by specific immunoassay or high-performance liquid chromatography does not take into account the immunosuppressive activity of the major metabolites, which may well be present at concentrations greater than the parent drug. Current immunoassays cross-react to a variable extent with CsA metabolites. Table 3 shows the cross reactivity of seven CsA metabolites with two receptors assays, two immunoassays and the mixed lymphocyte culture assay (MLC) (19).

From this data, it is clear that the current RRAs for CsA using the 52 kDa binding protein or Cyclophilin leave much to be desired because of the degree of metabolite cross-reactivity in this case not paralleling the immunosuppressive activity as measured in the MLC.

Additionally, other investigators (20) have compared the RRA for cyclophilin with a standard monoclonal radioimmunoassay and found the RRA to give higher values suggesting greater cross-reactivity from CsA metabolites. While their report suggests the cross-reactivity of metabolites in the RRA for CsA, metabolite interference experiments were not included in their study. The crossreactivity of the seven CsA metabolites using the Abbott monoclonal assay closely matched the metabolites for their pharmacological potency as measured in the MLC indicating that this may currently be the preferred method.

The metabolite immunosuppressive activity, when combined, could account for about 25 to 30% of the immunosuppressive activity of the parent CsA present. All CsA assays (immunoassays, HPLC, and receptor assays) predict CsA toxicity fairly well. Prediction of organ rejection is far more difficult.

FK-506

FK-506 is metabolized into at least 9 metabolites (21). The metabolites, M-III and M-V, have no pharmacological activity *in vitro*. Both commercially available immunoassays (which use the same antibody) cross-react significantly with these inactive metabolites. One of the metabolites is pharmacologically active (M-II) and also cross-reacts significantly in both the Abbott IMx® and the Incstar ELISA® procedures. An ideal immunophilin-binding protein still needs to be found that will bind only to the parent drug and the active metabolites relative to their pharmacological potency. The binding of tacrolimus metabolites to FK BP-12 is not proportional to the pharmacologic potency of the metabolites (22).

Table 4 (23) shows the crossreactivity of three FK-506 metabolites in the RRAs, two immunoassays, and the MLC. From this data, it is clear that the receptor assays compare favorably with the current state of the art immunoassay procedures.

RAPA

RAPA has been found to have up to 10 metabolites (24,25). The four major metabolites were isolated from the urine of renal transplant patients and their binding to the 14 and 52 kDa immunophilins was compared to their immunosuppressive activity relative to parent RAPA. The correlation between binding to these immunophilins and pharmacological potency was found to be excellent (26; Table 5), indicating that either immunophilin would provide an excellent receptor assay for RAPA. Commercially available immunoassays are currently unavailable for this drug, so that investigators are limited to HPLC or HPLC/MS. The results of the receptor assays for RAPA compare well with those obtained by HPLC (26,27), indicating that receptor assays

TABLE 3
% Cross-Reactivity of CsA Metabolites with RRA and Immunoassay as Compared to Mixed Lymphocyte Culture Assay

Drug Metabolite	Relative Concentration[a]	MLC	RRA		Immunoassay	
			52 kDa	Cyclophilin	Incstar RIA	TDx
CsA	100	100	100	100	100	100
AM19	69	2	271	490	0.5	2
AM1c9	5	1	1	51	0.8	0.7
AM4n9	10	2	1	61	0.2	<0.1
AM1	150	16	268	492	1	11
AM9	75	14	246	421	5	12
AM1c	40	3	33	12	0.3	7
AM4n	25	4	67	26	4	7

[a]Kidney transplant patients (18).

TABLE 4
% Cross-Reactivity of FK-506 Metabolites with RRA and Immunoassay as Compared to Mixed Lymphocyte Culture Assay

Metabolite	MLC	Relative Concentration[a]	RRA			Immunoassay	
			52 kDa	37 kDa	14 kDa	IncStar	Abbott
MI	0	6	2.2	0.6	9.1	0.2	0.4
MII	100	15	8.1	1.9	11.1	22.3	27.9
MIII	0	6	9.2	10.0	2.7	7.5	5.8
FK-506	100	100	100	100	100	100	100
Final Result Obtained[b]			119.5	112.5	122.9	130.0	134.1
Target[c]			115	115	115	115	115

[a]Yatscoff personal communication. Refers to steady-state trough concentrations in renal transplant recipients.
[b]The Final Result Obtained is the sum of the parent drug plus metabolites in each column.
[c]The Target is the sum of the parent drug and the only active metabolite (MII).

TABLE 5
Binding of RAPA Metabolites to the 14 and 52 kDa Immunophilins and Comparison to Immunosuppressive Activity as Measured by the Mixed Lymphocyte Culture (MLC) Assay

RAPA metabolites[a]	Ratio of metabolite to drug RRA-14 kDa	Ratio of metabolite to drug RRA-52 kDa	Ratio of metabolite to drug MLC
Parent Drug	1.00	1.00	1.00
RM-1	0.21	0.25	0.02
RM-II	0.02	0.05	0.09
RM-III	<0.01	<0.01	0.08
RM-IV	<0.01	0.03	0.04

[a]Assayed at 40 μg/L.

provide a viable alternative to HPLC. The advantages of the former over the latter are many and include smaller sample requirement (200 μL *vs.* 1–2 mL), simplicity, and speed. For example, only 10 to 12 assays/day can be performed using current HPLC procedures for RAPA and 50–70 can be run/day by receptor assay. Also the receptor assay for RAPA is amenable to automation. At the present time, receptor assays for immunosuppressive drugs are fairly time-consuming. Automating these assays to enable them to be used on commercial platforms is clearly urgently needed.

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