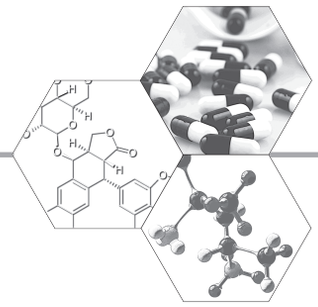

Hsp90-binding immunophilins as a potential new platform for drug treatment

Immunophilins are proteins that contain a PPIase domain as a family signature. Low-molecular-weight immunophilins were first described associated to immunosuppressive action and protein folding. Recent studies of other members of the family have led to the identification of their participation in basic processes such as protein-protein interactions, signal transduction cascades, cell differentiation, cell cycle progression, metabolic activity, apoptosis mechanisms, microorganisms infection, cancer, neurotrophism and neuroprotection, among several other physiological and pathophysiological processes. Due to all these emerging features, the developing of specific ligands for immunophilins appears to have promising perspectives in the coming years, in particular in the cancer biology and neuroregeneration fields. In this article, the emerging role of immunophilins in protein transport, transcription regulation, malignancies development and neurotrophic action, a number of biological properties that transforms these proteins in potential targets for novel therapeutic strategies, are discussed.

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The immunuphilin family

Immunophilins (IMMs) comprise a family of intracellular proteins with **PPIase** activity, that is, the reversible *cis*↔*trans* interconvertion of Xaa-Pro bonds **(Figure 1A)**. PPIases (EC 5.2.1.8) are abundant foldases that are classified into two large subfamilies according to their ability to bind immunosuppressant drugs [1]. They are named cyclophilins (or CyPs) when they bind cyclosporine A (CsA), or FKBPs when they bind the macrolide **FK506** (or **tacrolimus**). Many members of this subfamily also bind rapamycin **(Figure 1B)**. A third subfamily of IMMs comprises parvulins. Although these IMMs conserve the homology in the PPIase domain, they do not bind immunosuppressive drugs.

The signature domain of the IMM family is the PPIase domain, which is, in turn, the drug-binding domain for most of the CyPs and FKBPs family members. **Table 1** represents an overview of the known IMMs for each subfamily, where the gene name and molecular weights are also included. Only the low-molecular-weight IMMs FKBP12 (gene name *FKBP1A*,) and CyPA (gene name *PP1A*) are related to the immunosuppressive effect when the drug–IMM complex inhibits the Ser/Thr-phosphatase activity of PP2B/calcineurin. This prevents the activation by dephosphorylation of the transcription factor NFAT (nuclear factor of activated T cells) and its subsequent nuclear translocation, which avoids the production of interleukins and interferon-γ (see [2] for a recent review).

High-molecular-weight IMMs have a more complex architecture and are not related to the immunosupression process. The archetype of this subfamily is the 52-kDa FK506-binding protein, FKBP52 **(Figure 2A)** [3]. In addition to the active PPIase domain (also called FKBD1 or FK1 domain in FKBP proteins), there are other additional domains. The best studied is the TPR domain formed by sequences of 34 amino acids repeated in tandem through which they bind to Hsp90 [4]. The TPR-domain IMMs such as FKBP51 and FKBP52 are abundant and ubiquitous proteins that were first discovered associated to steroid receptors. The four more classical TPR-domain IMMs that have been relatively well characterized, due to their association with these receptors are FKBP52 (gene name *FKBP4*), FKBP51 (gene name *FKBP5*), the cyclophilin CyP40 (gene name *PPID*), and the FKBP-like protein phosphatase PP5 (gene name *PPP5C*). All of them have their counter-part in plants [5], are highly ubiquitous and are also able to form complexes (many of them still to be characterized) with several factors, although their biological functions and their molecular mechanism of action are still poorly understood.

Another important TPR-domain IMM is FKBP37 (gene name *AIP*), also known as XAP2/AIP. It was first discovered associated to aryl-hydrocarbon receptor, or 'dioxane' receptor, where the IMM favors the biological actions of the receptor. FKBP37 is also able to interact and repress the biological activity of other member of the nuclear receptor superfamily,

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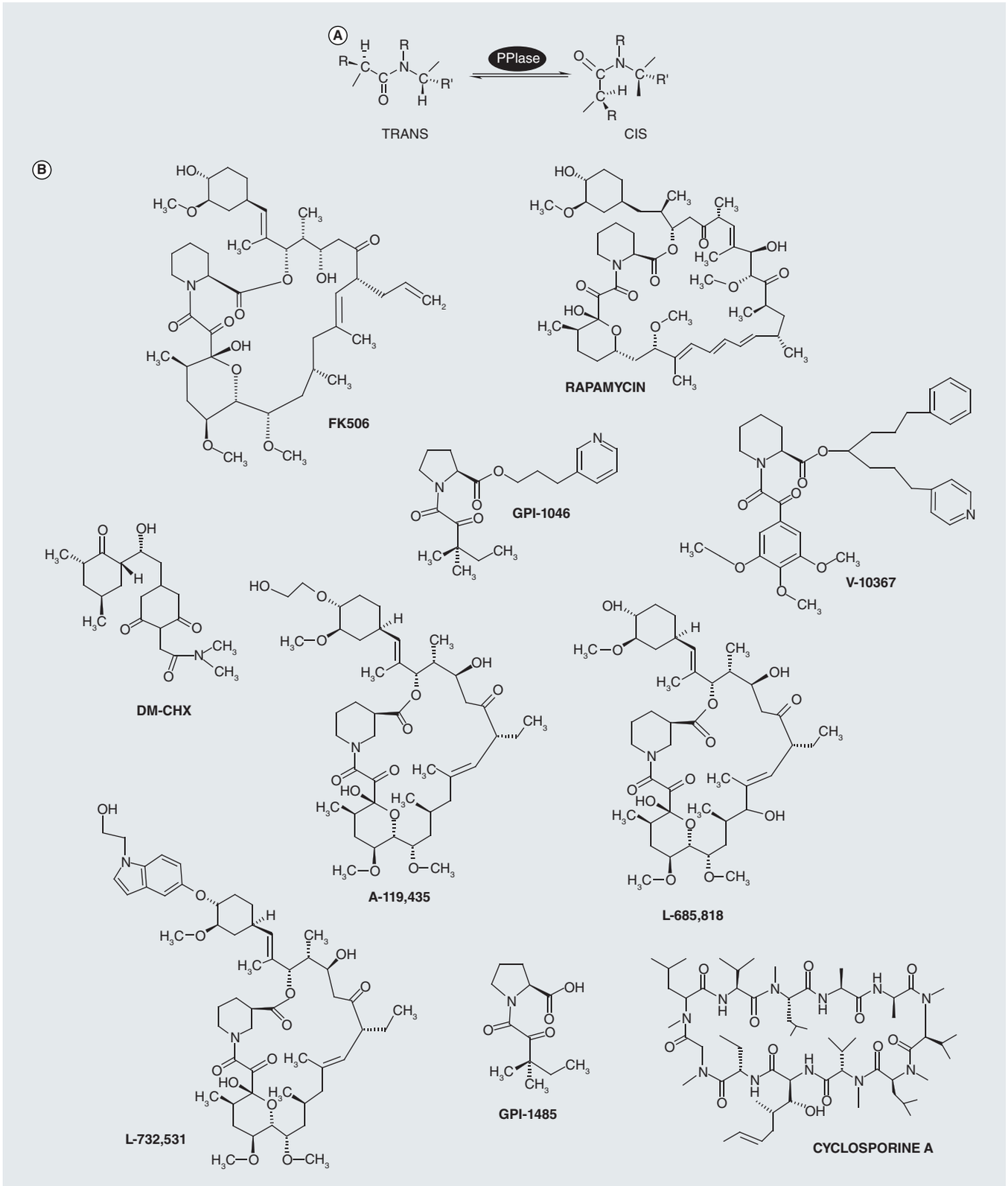

Figure 1. *Cis*/*trans* isomerization and structures of some inhibitors of this activity. (A) The PPIase-catalyzed peptide bond *cis*/*trans* isomerization. **(B)** Macrolide FK506 (tacrolimus) and some derivatives with inhibitory activity on the PPIase activity. Rapamicyn (sirolimus) is also a PPIase-binding drug with no inhibitory action on PP2B/calcineurin. Cyclosporine A is the typical cyclic oligopeptide with inhibitory action on cyclophilins.

Table 1. Human immunophilins.

Cyclophilin	Gene	MW (Da)	FK506-binding protein	Gene	MW (Da)
CyP18 / CyPA	*PPIA*	18,012	FKBP12 / FKBP1A	*FKBP1A*	11,951
CyP18.1 / CyPJ	*PPIL3*	18,155	FKBP12.6 / FKBP1B	*FKBP1B*	11,782
CyP18.2 / COAS2 / PPIase A-like 4B	*PPIAL4B,C,D*	18,182	FKBP15.6 / FKBP2 / FKBP13	*FKBP2*	15,649
CyP18.2a / CyPL1	*PPIL1*	18,237	FKBP22 / FKBP11 / FKBP19	*FKBP11*	22,180
CyP19.2 / Snu-Cyp20 / PPIase H	*PPIH*	19,208	FKBP24 / FKBP22 / FKBP14	*FKBP14*	24,172
CyP22 / CyPF / CyP3 / CyP D	*PPIF*	22,040	FKBP25 / FKBP3	*FKBP3*	25,177
CyP22a / CyPC	*PPIC*	22,763	FKBP30 / FKBP23 / FKBP7	*FKBP7*	30,009
CyP23 / CyPB / SCYLP	*PPIB*	23,742	FKBP36 / FKBP6	*FKBP6*	37,214
CyP33 / CyPE	*PPIE*	33,431	FKBP37 / AIP / ARA9 / XAP2	*AIP*	37,636
CyP35	*PPIL6*	35,228	FKBP38 / FKBP8 / FKBPR8	*FKBP8*	44,562
CyP40	*PPID*	40,764	FKBP44 / AIPL1	*AIPL1*	43,903
CyP54 / SDCCAG10 / NY-CO-10	*SDCCAG10*	53,847	FKBP51 / FKBP54 / FKBP5	*FKBP5*	51,212
CyP57 / PPIL4	*PPIL4*	57,225	FKBP52 / FKBP59 / FKBP4 /p59	*FKBP4*	51,804
CyP58 / CyP60 / PPIL2	*PPIL2*	58,823	FKBP63 / FKBP9	*FKBP9*	63,804
CyP73 / KIAA0073	*PPWD1*	73,575	FKBP65 / FKBP10	*FKBP10*	64,245
CyP88 / CARS-CyP / CyPG / Srcyp	*PPIG*	88,617	FKBP135 / WAFL / FKBP15	*FKBP15*	133,630
CyP165 / NKTR	*NKTR*	165,677	n/a	n/a	n/a
CyP358 / Nup358 / RanBP2	*RANBP2*	358,218	n/a	n/a	n/a

The names for each immunophilin belonging to the cyclophilin subfamily (left) or FK506-binding protein subfamily (right) are in the first column following a recently suggested nomenclature [105]. *The middle column is the gene name, and the right column the predicted molecular weight according to amino acid sequence.*

PPARα [6], which modulates lipid metabolism and inflammation.

There are two more relevant TPR-domain IMMs whose biological roles have been elucidated more recently. One is FKBP38, which demonstrate a mitochondrial localization signal and has been related to apoptosis (see [7] for a recent review). In spite of its almost identical 3D structure of the PPIase domain with the immunosuppressive IMM FKBP12, FKBP38 lacks enzymatic activity and does not bind immunosuppressive drugs. The other relevant TPR-domain IMM is FKBP36 because it is crucial to spermatogenesis since it is able to interact with components of the synaptonemal complex [8], and is also a natural inhibitor of GADPH activity [9]. GADPH is involved in the mechanism of vesicle transport from the endoplasmic reticulum to the Golgi and is also recruited by Rab2 to the vesicular–tubular clusters of the reticulum where it helps to form vesicles. Consequently, FKBP36 demonstrate the additional potential to affect vesicle **trafficking** and the secretory pathways [9]. To date, there are no compounds able to specifically recognize this IMM.

In the mid-1990s, a 92-residue member of the parvulins was identified in *Escherichia coli,* forming the prototype of the third family of PPIases. Soon after, human isoenzymes were described and the small subfamily of parvulins was born. It has only three members in humans: Pin1, Par14 and Par17. Pin1 is the best studied and its name is often used as synonym of parvulin itself. They are also able to accelerate protein folding *in vitro*, but they demonstrate unique specificity for prolines preceded by phosphorylated Ser or Thr residues. Thus, Pin1 possesses the potential to regulate several phosphorylation signaling cascades by modifying the conformation of the target protein around its phosphorylation site, making Ser or Thr residues less or more accessible for dephosphorylation (see a recent reviewed in [10]). In this sense, Pin1 may act as a molecular timer to make the first move or bring to an end signaling cascades at certain time points of the cell cycle. Pin1 is prevalently overexpressed in human cancers and its expression levels correlate with poor clinical outcome. Pin1 inhibitors may simultaneously block multiple oncogenic signaling pathways and thereby overcome cancer cell resistance to inhibition of specific kinases or phosphatases [10]. The best-known Pin1 inhibitors include the natural product juglone, the small molecule PiB and others of a peptidic nature (see [11] for a recent review), but their specificity and potency remain a major concern, and further design and optimization of novel small-molecule Pin1 inhibitors are required. Even though recent efforts have been made to obtain better compounds with higher cell membrane permeability and better affinity for this

Key Terms

FK506: Immunosuppressive macrolide that binds FKBP proteins.

PPIase: Enzyme that catalyzes the reversible cis/trans interconversion of X-Pro bonds.

Tacrolimus: Pharmacological name of the drug FK506.

Trafficking: Transport of factors between the compartments of the cell.

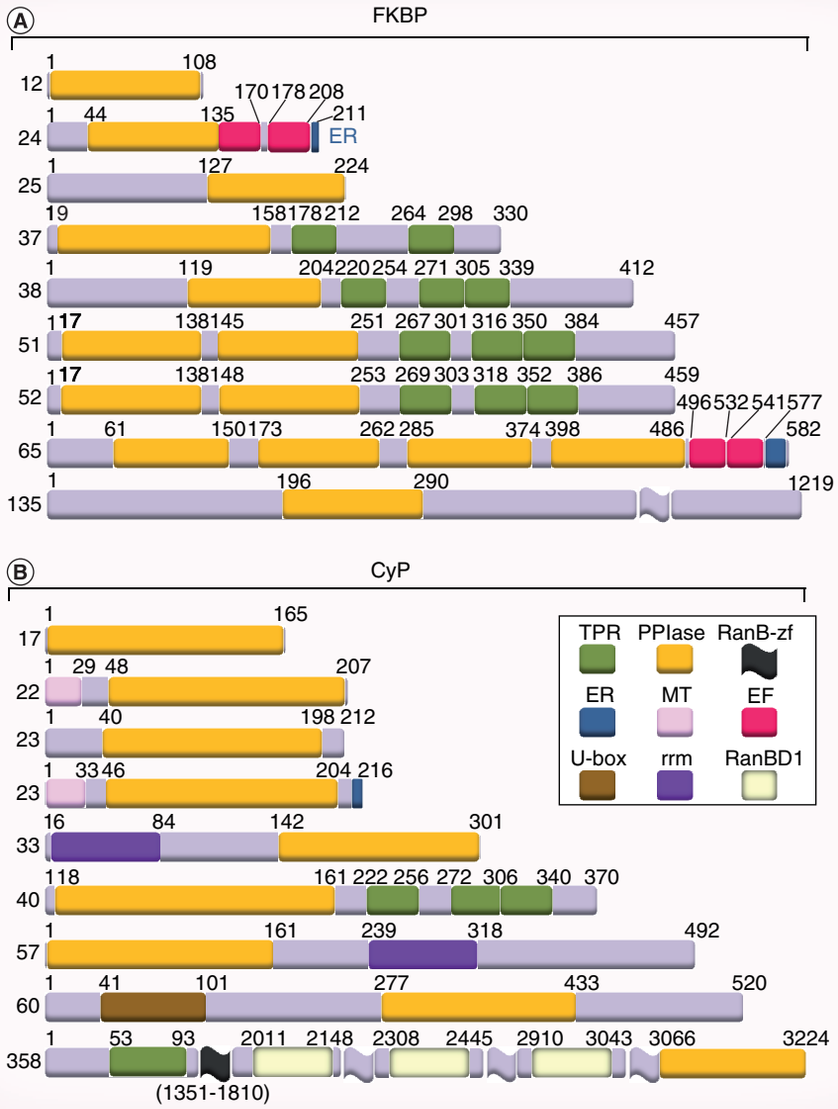

Figure 2. Domains observed by some human PPIases. (A) Human FKBPs. **(B)** Human CyPs. The insert represents the color code. Numbers on the left-hand side are the molecular weights of the immunophilins.
ER: Endoplasmic reticulum; MT: Mitochondrial localization signal; rrm: RNA-recognition motif.

IMM [12,13], the specificity of these novel small molecules still remains to be proved.

Protein folding

Most of the peptide bonds of nascent polypeptides emerge from the ribosome in the *trans*-conformation and the majority retains that energetically favored state. Nonetheless, there is a significant amount ranging from 5–7% of the proteins with structures of peptidyl–prolyl bonds (i.e., Xaa-Pro bonds) that switch to the *cis*-conformation during folding, transport and assembly [14]. The *cis–trans* isomerization of Xaa-Pro bonds **(Figure 1A)** is one of the rate-limiting steps of protein folding. The obvious influence of CyPs and FKBPs on the conformations, oligomeric states and consequent biological activities of these proteins cannot be explained on the sole bases of the PPIase activity alone. Actually, IMMs also act as molecular **chaperones** in an analogous manner to certain members of stress protein families. CyPs and FKBPs have been observed to influence the folding of a number of synthetic peptides

Key Term

Chaperones: Proteins that assist the folding of other factors and permit their biological actions.

and natural proteins, such as collagen, chymotrypsin inhibitor, carbonic anhydrase and ribonuclease T1; the latter considered the standard protein substrate of PPIases (see [15] for a recent review). As such, ribonuclease T1 was analyzed structurally during the refolding process using NMR techniques and the results suggest that the presence of a non-native *trans*-prolyl bond, at Pro39 in a folding intermediate, may represent the targeted species for enzyme catalysis. Structural modifications of this region, owing to the catalytic action of PPIases, propagate the effect of prolyl isomerization, not only to regions adjacent to the proline, but also to remote parts of the polypeptide chain. This implies that an apparent minor structural modification generates an overall larger modification of the topological structure of the substrate protein. Even so, this is simply a particular case and the exact catalytic mechanism of the intrinsic PPIase activity on several substrate proteins still remains hazy.

An interesting feature of IMMs is that although both CyP and FKBP families possess PPIase activity, the sequence and structure of the two families are dissimilar, though in both proteins the substrate and the inhibitory immunosuppressants compete for binding to the PPIase active site. Thus, the PPIase domain has become synonymous with drug-binding domain. The rest of the IMM sequence often plays a family-specific role, such as interacting with specific subsets of other proteins.

FK506 is a natural macrolide isolated from the bacterium *Streptomyces tsukubaensis* that demonstrates profound immunosuppressive activities both *in vitro* and *in vivo*, and is effective in a wide variety of models of experimental transplantation and autoimmunity. In addition to its obvious clinical importance, the discovery of FK506 yielded new insights into the mechanisms underlying the activation of T cells and its use is likely to impart even more important scientific information. Interestingly, FK506 also exhibits neuroprotective and neurotrophic effects [16,17], such that several derivatives have been developed to avoid the immunosuppressive side effect **(FIGURE 1B)**. The other drug also able to exert immunosupressive action via FKBPs is rapamycin, a macrolide discovered as a product of the bacterium *Streptomyces hygroscopicus* in a soil sample from Easter Island. Even though it binds to FKBP12 in a similar manner as FK506, rapamycin is not a PP2B/calcineurin inhibitor, but a mTOR pathway inhibitor by direct binding to the mTORC1. Thus, rapamycin inhibits the response to IL-2-blocking T- and B-cells activation (for a recent review, see [18]).

The mechanistic properties are not exclusive of PPIase domain proteins, but are somehow reminiscent of the strategies adopted by other families of proteins, such as Ser/Thr phosphatases. All members of this family catalyze the same basic reaction of dephosphorylating P-Ser and/or P-Thr residues [19]. However, the phosphatases of the PPP class share a highly conserved sequence and structure in their catalytic core, whereas the metal-dependent PPM phosphatases have a very different sequence and a novel protein folds [20]. Finally, both high-molecular-weight IMMs and the IMM-like phosphatase contain protein–protein interaction domains outside the catalytic core, such as the TPR domains. Perhaps the best example is the IMM-like Ser/Thr phosphatase PP5, also a common Hsp90-binding factor associated to steroid receptor complexes.

IMMs are more abundant in the nervous system than in other tissues and have participation in several neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, prion protein diseases, polyglutamine repeat diseases (e.g., huntingtin in Huntington's disease) and amyotrophic lateral sclerosis (for a recent reviews see [21]). All these diseases have been classified under the concept of 'protein conformational disorders' and IMMs are potential therapeutic targets for them.

Transport of soluble factors

Among all members of the TPR-domain subfamily of IMMs, FKBP51 and FKBP52 are the best characterized due to their presence in steroid receptor complexes. Both proteins are highly homologous as it is demonstrated by their amino acid sequences, domain organization and 3D structures [22] . They share 60% identity and 75% similarity in their amino acid sequences. Both proteins contain an N-terminal FKD1 domain that is responsible for the PPIase activity and a PPIase-like FKD2 domain, which shares 32% sequence homology with FKD1 and exhibits no PPIase activity **(FIGURE 2A)**. The C-terminal TPR domain, which is made up of three units of a consensus 34-amino acid motif, is the responsible for their association with the heat-shock protein of 90 kDa, Hsp90, and through this chaperone, with the client protein. Nonetheless, direct interactions between FKBP52 and the glucocorticoid receptor also take place [23].

The first report related to a biological function

Key Term

Retrotransport: Transport of a factor from the cell periphery to the nucleus.

of FKBP52 in the steroid receptor–Hsp90 heterocomplex was the demonstration that the hormone-dependent **retrotransport** of the glucocorticoid receptor is favored by the association of PPIase domain of FKBP52 with the dynein/dynactin motor complex **(Figure 3)** [24]. Both FKBP52 and dynein are recruited by the steroid receptor–Hsp90 heterocomplex upon steroid binding and also in a hormone-dependent manner [25]. On the other hand, FKBP51, an IMM that binds inefficiently to dynein and also impairs ligand binding and receptor-dependent transcriptional activity, is released [25–27]. Further, complexes of IMMs and motor proteins were related to trafficking of other soluble factors such as mineralocorticoid receptor [27], androgen receptor (AR) [28], p53 [29], the AIF/Rac3 complex [30], adeno-associated virus 2 [31], HERG [32], polyQ-aggregated proteins in Kennedy disease cells [28], the brain-specific protein PAHX-AP1 [33], insect ecdysteroid receptor [34] and so forth.

Therefore, it is possible that factors able to interact with the Hsp90–FKBP52 complex could share a similar molecular machinery of movement via motor proteins. Interestingly, while FKBP12 does not interact with dynein, CyP A, the other low-molecular-weight IMM involved in immunosupression, associates to dyenin via its PPIase domain [35]. While viral infection requires the presence of CyP A, it is still uncertain whether both processes are related. Interestingly, CyP A is also required for the replication of the RNA of the hepatitis C virus and, probably, it is also required for its assembly and transport. Accordingly, CyP A binds to the non-structural viral protein NS5A and facilitates replication through an unknown mechanism. Based on these observations, CsA **(Figure 1B)** was first successfully used to inhibit hepatitis C virus replication (see [36] for a recent review). Subsequent CsA derivatives without immunosuppressive properties were developed as potential antivirals, among them, alisporivir (formerly known as Debio-025), which is currently in a Phase III trial for

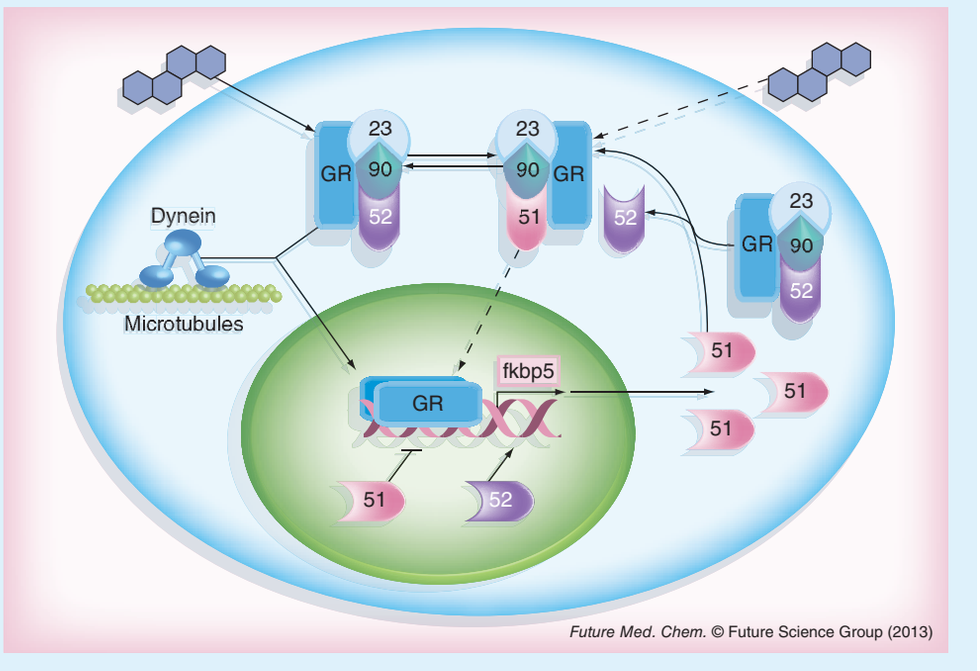

Figure 3. Glucocorticoid receptor regulation by steroid binding via FKBP51 and FKBP52 signaling. Steroid hormones bind with higher affinity to the GR–Hsp90 heterocomplex that contains FKBP52 associated to the TPR acceptor site of Hsp90. Ligand binding favors the recruitment of FKBP52 and dynein motors to facilitate GR retrotransport on cytoskeletal tracks, whereas FKBP51 is released from the complex. Upon receptor transformation (i.e., Hsp90 dissociation) in the nuclear compartment, GR dimers interact with specific promoter sites. While FKBP52 favors GR action, FKBP51 impairs steroid-binding affinity, GR retrotransport and transcriptional activity. One of the genes activated by GR is *fkbp5*, whose product, FKBP51, competes with FKBP52 for the acceptor binding site on Hsp90. Therefore, the circular activation–inhibition cycle of glucocorticoid action is closed. Dashed lines represent weaker activity.
GR: Glucocorticoid receptor.

the treatment of treatment-naive hepatitis C virus genotype 1 patients [37].

Importantly, the biological function of IMMs associated to **heat-shock proteins** on protein trafficking is not limited to the cytoplasm. Once the cargo has reached the nuclear envelope using microtubules as molecular tracks, it must traverse the nuclear pore. The Hsp90-based heterocomplex (which includes FKBP52, but not the highly homologous partner FKBP51) is also able to interact with factors of the nuclear import machinery such as nucleoporins and importins. This facilitates the nuclear import of cargoes [38]. In addition to FKBP52, Cyp40 and PP5 are also able to bind dynein [39], suggesting some type of redundancy between IMMs for this biological property in the retrotransport of soluble cargoes. Importantly, all these IMMs bind to the same TPR acceptor site in Hsp90 [23,27], indicating that there is a functional competition among them for the same acceptor site of Hsp90 dimers. Nonetheless, the biological consequences may differ drastically. Whereas FKBP52 is regarded as a positive regulator of the activity of corticosteroid receptors, FKBP51 is a negative regulator. Accordingly, the overexpression of FKBP51 usually prevents the positive regulation by FKBP52, most likely due to the competition of FKBP51 for the same TPR-binding site on Hsp90. Importantly, glucocorticoids self-regulate their own effects by inducing the expression of the negative regulator FKBP51 [40]. On the other hand, while FKBP52 itself is able to interact with cytoskeletal structures [41,42] and links steroid receptor with microtubules, FKBP51 does not demonstrate these properties. Perhaps related to these properties, it has recently been shown that the macrolide FK506 favors neuronal differentiation via FKBP52, whereas FKBP51 antagonizes these effects [17,43]. Thus, binding of either **TPR protein** to Hsp90 complexes could show inhibitory or stimulatory action. In turn, these antagonistic properties between both IMMs and their relative expression (i.e., FKBP52 to FKBP51 ratio) in each particular tissue may regulate various aspects of the cell physiology [25,27]. For example, it can be predicted that higher expression levels of FKBP51 may turn a particular tissue resistant to glucocorticoid action, whereas greater amounts of FKBP52 should favor glucocorticoid receptor (GR) action.

Transcriptional activity

It was first observed that in yeast, FKBP12 reduces the transcriptional action of the ubiquitous transcription factor YY1, and that FK506 and rapamycin disrupt the interaction between both factors by releasing the IMM from YY1. This causes YY1 to become an active repressor. Such binding of the immunosuppressant drugs to FKBP12, allowing the release of the IMM from the promoter site, has recently been proposed as an underlying mechanism for switching YY1 from an activator to a repressor [44].

Subsequently, it was also demonstrated that FKBP25 is able to interact with YY1 [45], such that while YY1 interacts with FKBP12 at the conserved PPIase domain, YY1 interacts with the N-terminal end of FKBP25. Interestingly, rapamycin or FK506 has no effect on the interaction between YY1 and FKBP25. In turn, FKBP25 interacts with HDAC1 and HDAC2, suggesting that FKBP25 functions as a scaffold protein to assemble histone deacetylase complex around YY1 affecting the chromatin structure. These findings further demonstrate the functional diversity of an FKBP protein in the regulation of a single transcription factor. FKBP25 also interacts with MDM2 via its PPIase domain, leading to enhanced MDM2 ubiquitination and proteosomal degradation [46]. Accordingly, the expression of p53 and its downstream effector p21 are significantly reduced, affecting the cell cycle regulation.

A similar case of an IMM acting as a coregulator of transcription factors is FKBP38. This IMM associates to the oxygen sensor PHD2 promoting its degradation, which, in turn, favors the expression of HIF1α target genes [47]. Taken together, these two findings regarding FKBP25 and FKBP38 demonstrate yet another mechanism of how FKBPs regulate transcription through modulations of regulatory proteins of transcription factors.

Recently, it was demonstrated that Nanog, a transcription factor crucial for unlimited self-renewal of embryonic stem cells, undergoes Ser/Thr phosphorylation that favors its interaction with the IMM Pin1 [48]. This association leads to Nanog stabilization by suppressing its ubiquitination. Inhibition of either Pin1 activity or Pin1–Nanog interaction suppresses the capability of this complex to favor self-renewal of stem-cells and to form teratomas in immunodeficient mice. Therefore, in addition to the rigorous transcriptional regulation of Nanog, its expression level is also modulated by post-translational modifications that involve a key regulatory association with the IMM.

Among the high-molecular-weight IMMs, it was first demonstrated that FKBP52 acts as a

Key Terms

Heat-shock proteins: Molecular chaperones inducible by heat-shock and other stressing stimuli.

TPR protein: Protein with tetratricopeptide repeat domains.

Key Term

Shuttling: Backwards and forwards transport of molecules.

transcriptional coregulator of the transcription factor IRF-4 in a manner that is reversed by PPIase inhibitors. Furthermore, FKBP52 was regarded as a positive regulator for several steroid receptors (see [49] for a recent review). It was postulated that one of the possible reasons for such potentiation of steroid hormone signaling could be related to a proved increase in receptor affinity for hormone. Nonetheless, there may also be additional mechanisms by which FKBP52 enhances receptor activity, such as direct effects on chromatin remodeling (Erlejman AG, Galigniana MD, Unpublished Data). On the other hand, the FKBP52's highly homologous partner, FKBP51, has the overall effect of reducing receptor transcriptional activity for most steroid receptors, except for the case of the AR where the IMM favors its biological action [50]. Interestingly, experiments using FKBP52 knockout cells demonstrated that FKBP52 is required for the transactivation of nuclear AR [51]. All these results agree with the notion that FKBP51 and FKBP52 could directly regulate transcription at the site of gene promoters. Nonetheless, there is no direct experimental evidence published to date supporting this postulate, as there is for other members of the family. Importantly, the potential development of specific inhibitors for each IMM in the near future may be of extreme interest for the elucidation of their respective biological actions and for therapeutic purposes (as discussed further below).

The cyclophilin CyP40 is the only TPR-domain IMM belonging to the CyP subfamily that is found associated to steroid receptors. CyP40 has not been recovered with mineralocorticoid receptor, but it is present in complexes with the estrogen receptor (ER), the progesterone receptor and GR. It has also been described as a positive regulator of AR, such that an effective inhibition of androgen-induced growth of tumor prostatic cells was possible through the action of the immunosuppressive drug CsA [52]. Currently, there are no developments for specific CyP40-targeting drugs.

The effects of IMMs on transcriptional activity could be indirect, due to the regulatory action of IMMs on other transcription factors, but we also postulate that IMMs influence chromatin structure at the promoter site of the genes. An interesting evidence for a putative direct involvement of FKBPs in the regulation of chromatin structure comes with the discovery that FKBP25 associates with histone deacetylases [45]. FKBP25 interacts with HDAC1 and HDAC2 via its distinctive N-terminal domain, whereas the PPIase domain does not demonstrate a relevant role. Interestingly, FKBP25 is also able to interact with other chromatin-related proteins, such as casein kinase II, nucleolin [53] and high-mobility group II protein [54]. Whereas high-mobility group II influences DNA-binding capacity, casein kinase II and nucleolin are involved in the regulation of rDNA transcription. In short, it is possible that the role of FKBP25 is to be a scaffold protein related to structural modifications of chromatin and, consequently, to the regulation of transcription. In line with these studies, the yeast FKBP Fpr4 binds to the N-terminal tails of H3 and H4; its PPIase activity being required for the methylation of H3 affecting the regulation of chromatin architecture and transcriptional activity by localizing in actively transcribed regions by RNA-pol II [45]. Additionally, RNA-pol II shares the same nuclear speckles with FKBP52 [Gallo L, Galigniana M, Unpublished Data].

Role of IMMs in malignancies

Several studies have shown that FKBPs can have a major impact on the initiation, progression and therapy of cancer through perturbation of steroid receptor signaling (see [44,50,55] for recent reviews). While FKBP51 expression is induced through the activation of steroid receptors, it can also modulate its own expression in a mechanism of negative feedback loop. In a recent study, the authors demonstrated that FKBP51 is not only a soluble factor, but also a mitochondrial protein that undergoes nuclear-mitochondrial **shuttling**, according to the cell status and external stimuli [56]. Thus, FKBP51 concentrates in the mitochondria of resting interphase cells, but moves towards the nucleus upon the onset of several stresses, or during the early steps of cell differentiation. While FKBP51 overexpression protects cells against oxidative stress, its knockdown makes cells more sensitive to injury and/or cell death. These observations correlate with an enhanced antiapoptotic mechanism, mediated by FKBP51.

In line with those properties, FKBP51 has the tendency to be overexpressed in most cancer cell lines and tumors, although there are some reports demonstrating that the expression of FKBP51 could be low in some particular cases. In other cases, the results were variable such that high expression of FKBP51 was related to either suppression or promotion of tumor growth, depending on the specific tumor type

and its relative microenvironment (see [57] for a recent review in this field). While some studies have reported that FKBP51 is downregulated in pancreatic cancer cells [58], it has also been demonstrated that its expression is increased in neoplastic melanocytes of non-invasive cutaneous melanomas, whereas an even stronger signal was found in tumor cells of the invasive (vertical) growth phase [59].

Importantly, the highest immunoreactivity for FKBP51 was observed in all the metastatic melanoma cases [59]. Interference studies by siRNA have demonstrated that FKBP51 suppresses the proliferation of colorectal adenocarcinoma and that GR inhibitors abrogate the effect of siRNA for FKBP51 on the proliferation of colorectal adenocarcinoma. Thereby, the suppression of the proliferation via FKBP51 may be due to the suppression of function of the glucocorticoid [60].

Interestingly, the FKBP ligand FK506 promotes the inhibition of androgen-dependent activity of ARs in prostate cancer cells by a mechanism that appears to reduce the intrinsic steroid-binding capacity of the receptor [52]. When AR-positive human prostate carcinoma LNCaP cell line was compared with PC-3 and DU145 AR-negative prostate cancer cell lines, the inhibitory action of FK506 on cell growth was only demonstrated for the AR-positive cells when they were treated with steroid [61]. Therefore, it is possible that the drug may function on the AR-dependent mechanism of cell growth, via their associated IMMs, in particular FKBP51. Thus, the knockdown of FKBP51 impaired cellular events where this IMM promotes AR-dependent transcriptional activity. Interestingly, similar results to those reported for FKBP51 were also observed for the IMM CyP40 [52].

High expression of FKBP51 has been observed in metastatic melanomas, and the knockdown of this IMM sensitized cells to ionizing radiation [59]. The effect was assigned to the potential decreased anti-apoptotic signaling through NF-κB in response to low levels of FKBP51. This effect could be cell or tissue specific since a reduced expression of FKBP51 resulted in a low sensitivity to chemotherapeutic agents in breast, lung and pancreatic cancer cell lines.

On the other hand, FKBP52 is overexpressed in breast tumors and is upregulated transcriptionally and post-transcriptionally by estrogens (recently reviewed in [49]). Interestingly, it has been demonstrated that the FKBP52 gene is methylated in the estrogen receptor-negative MDA-MB-231 cell line, but not in MCF7 cells (ER-positive cells), suggesting that the repression of FKBP52 may itself affect the expression of the ER [62]. Even more importantly, this observation implies that this IMM may be a potential target for breast cancer.

The tyrosine kinase activity of the Hsp90-binding oncoprotein NPM-ALK favors the expression of the Cyp40 and FKPB52, but not FKBP51 [63] in cases of the aggressive non-Hodgkin lymphoma of T/null cell called ALK+/ALCL. However, it seems that CyP40 is the critical IMM since its knockdown, but not FKBP51 or FKBP52, reduced the viability of ALK+ ALCL cell lines.

As it was advanced before, CyP40 and FKBP52 are universally expressed in breast carcinomas and breast cancer cell lines in an estrogen-induced manner. Both IMMs are associated to the ER in a mutually exclusive manner and its expression and recruitment to the receptor are increased in breast cancer cells. Although both isoforms ERα and ERβ are the mediators of the effects of estrogen, both receptors have distinct effects. Actually, the biological action of estrogen ligands as risk factors for developing tumors depends primarily on the balance between these two receptors [64], as well as on the high levels of exposure to estrogens during the life-time period. While the tumorigenic actions of estrogens are primarily mediated by the ERα isoform, the role of ERβ in breast cancer growth and development is not as clear as that of ERα, although several lines of evidence argue in favor of a negative dominance of ERβ versus ERα (see [65] for a recent review). Thus, ERβ opposes ERα on cyclin D1 gene expression and represses c-myc, cyclins D1 and A gene transcription whereas increases the expression of p21Cip1 and p27Kip1 leading to a G2 cell cycle arrest. Accordingly, ERβ levels are high in normal mammary tissue and decrease as tumor progresses from pre-invasive to invasive tumor [66]. While IMM-binding immunosupressants weakly decreased the E2-induced expression, this inhibitory activity is more pronounced in case of ERβ-expressing cells. This suggests that ERβ is more sensitive to immunophilin inhibitors than ERα, a property that was also observed when Hsp90 inhibitors were assayed [66]. Nonetheless, the immunosuppressive primary action of a conventional IMM-binding drug, such as FK506 or CsA, make them unlikely to be used for other types of therapeutic uses different from immunosuppression (for example, to inhibit ER

response) if novel derivatives devoid of that primary action are not designed and clinically studied in trials. Good examples of these attempts are some of the compounds represented in **Figure 1B**.

FKBP37 (also known XAP2/AIP) was originally identified as a negative regulator of the hepatitis B virus X-associated protein and a member of the aryl hydrocarbon receptor–Hsp90 complex [67]. Other studies have expanded the range of FKBP37 client proteins to include the nuclear receptor family of transcription factors such as the PPARα and, more recently, ERα [68]. In breast cancer cells, FKBP37 downregulates the estradiol-dependent transcriptional activation of ERα, but not ERβ-mediated transcription. Thus, knockdown of intracellular FKBP37 leads to increased ERα activity, whereas FKBP37 mutations that affect the interaction of the IMM with ERα can no longer regulate ER target gene transcription.

Taken all these antecedents together, it may be concluded that IMMs perform important functions in a number of cancers and represent attractive novel therapeutic targets in malignancies. In this regard, a recent study by M.Cox's group [69] showed that small molecules can be used to inhibit AR function by preventing hormone-dependent dissociation of the AR–Hsp90–FKBP52 complex, which results in a lower amount of steroid-bound receptor in the nucleus. Interestingly, assays in early- and late-stage human prostate cancer cells demonstrated that these compounds inhibit AR-dependent gene expression and androgen-stimulated prostate cancer cell proliferation [69].

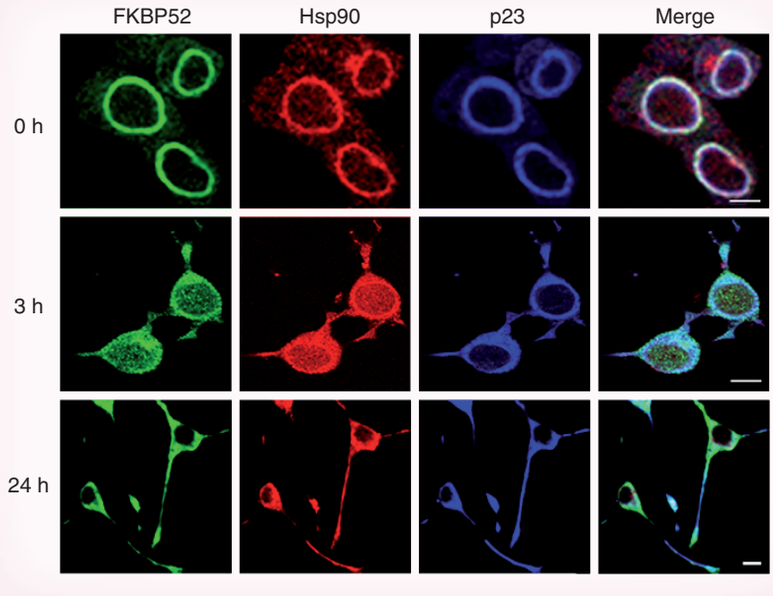

Figure 4. Distribution of FKBP52 and associated proteins during neuronal differentiation. Undifferentiated neurons show the three components of the FKBP52–Hsp90–p23 complex in a perinuclear ring associated to the inner face of the nuclear envelope. Upon stimulation with FK506, all three proteins redistribute rapidly throughout the cytoplasm. Bars = 10 μM.

Neuronal differentiation

IMMs also play relevant functions in the brain. As it was mentioned above, their expression levels are 10–50-fold higher in the nervous system than in the immune system. Both neuroregenerative and neuroprotective properties have been ascribed to FK506 in animal models. At first, it was thought that these effects could be mediated by the inhibitory action of the drug bound to FKBP12 on calcineurin activity. However, FKBP12-knockout mice still respond to FK506 treatment and the development of synthetic ligands lacking immunosupressive action, such as GPI-1046, GPI-1485 and V10,367 (see structures in **Figure 1B**) demonstrated that calcineurin should not be involved. In design of these compounds, the functional groups of FK506 that interact with both FKBP12 and calcineurin were omitted [70–75] with the purpose of avoiding the unwanted side effects. However, none of these compounds were explored intensively with pharmacologic purposes in clinical trials and most of the studies performed to date were only exploratory using animal models.

More recently, the authors reported that in both undifferentiated neuroblastoma cells and embryonic hippocampal neurons, the FKBP52–Hsp90–p23 heterocomplex concentrates in perinuclear structures **(Figure 4)** [76]. Upon cell stimulation with FK506 (in the absence of any other trophic factor, including serum in the culture medium), these structures disassemble and the perinuclear area becomes transcriptionally active. In turn, the acquisition of a neuronal phenotype is accompanied by increased expression of neuronal markers and also Hsp90, Hsp70, p23, and FKBP52, but not FKBP51. The authors hypothesize is that the heterocomplex may be repressing the expression of key genes of the neuron required for differentiation, and the activation of the process releases the chaperones from those areas allowing the early expression of essential factors.

During the early differentiation steps triggered by FK506, the perinuclear heterocomplex redistributes along the cytoplasm and nascent neurites, p23 binds to intermediate filaments, and microtubules acquire higher filamentary

organization, whereas FKBP52 moves towards neurites and concentrates in arborization bodies and terminal axons. In contrast, FKBP51 replaces FKBP52 in the perinuclear structure [76]. Importantly, neurite outgrowth is favored by FKBP52 overexpression or FKBP51 knockdown, and it is impaired by FKBP52 knockdown or FKBP51 overexpression, indicating that the expression balance between both FK506-binding proteins plays a key role during the early mechanism of neuronal differentiation.

Importantly, it was also demonstrated that when the axons of differentiated neurons are damaged or cut with a laser beam, the chaperones reorganize back to similar perinuclear structures like those originally observed in undifferentiated cells [17]. This suggests that this type of nuclear arrangement of the IMM complex must precede the neuroregeneration process after the injury. In other words, the authors hypothesize that both **neurodifferentiation** and neuroregeneration, appear to be processes mechanistically linked to the FKBP52–Hsp90–p23 heterocomplex and are essentially alike.

Another interesting hint to a role of FKBP52 in neurons was the demonstration of its interaction with the copper-binding metallochaperone Atox1 [77]. The overexpression of FKBP52 favors copper efflux, suggesting that FKBP52 protects neurons against copper toxicity. This could be of therapeutic interest since alterations in metal homeostasis have been related to neurodegenerative diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and prion diseases [78,79]. Nonetheless, this cannot explain the neuroprotective and neuroregenerative properties of FK506.

Recently, it was discovered that FKBP52 binds directly to tau, in particular to the phosphorylated isoforms [80]. Tau has received great attention because mounting evidence indicates that hyperphosphorylation of Tau is the origin of Alzheimer's disease [81]. Tau proteins interact with tubulins to promote their assembly into microtubules, and FKBP52 inhibits the promotion of microtubule assembly by tau. This intriguing property of FKBP52 has not been tested to date for Alzheimer's disease in *in vivo* models.

FKBP51 was also recently implicated in Alzheimer's disease [82]. As a consequence of age-related changes in cellular chaperones, FKBP51 expression was shown to be increased, preventing tau clearance. Also, FKBP51 negatively regulates tau phosphorylation status. The latter was dependent on its PPIase activity and resulted in an increased stability of microtubules. In this context, these relevant findings indicate that FKBP51 would have a beneficial role in Alzheimer's disease, making it a desirable novel therapeutic target.

One of the known roles of FKBPs is to interact with proteins belonging to the transient receptor potential channel superfamily (TRPC). These ion channels are relatively non-selectively permeable to cations, including sodium, calcium and magnesium [83]. The association of FKBP12 and FKBP52 with TRPCs in rat brain lysates is displaced by FK506. Inasmuch as FK506 disrupts both the binding of the IMM to TRPCs and also inhibits the PPIase activity and calcineurin activity, it is unlikely that such inhibitory action of FK506 may be responsible for neuron differentiation, neurite outgrowth, and nerve regeneration since all these effects are also obtained in FKBP12-knockout mice or with FK506 derivatives lacking the ability to activate calcineurin. Our recent results involving FKBPs in the neurotrophic action [23,53] suggest a more complex network of biological processes, in addition to the possible (and tempting) speculation that activity of FKBP52 action may be related to some gating mechanism of those channels. TRPCs are not exclusive of neurons, but they are widely expressed in several cell types [84], whereas the effects of FK506 on cell differentiation via Hsp90-binding immunophilins are not observed in other tissues and seem to be exclusive for neurons. Nonetheless, the use of FK506 or FK506 derivatives may have therapeutic relevance.

There are reports linking IMMs from the CyP subfamily with both neuroprotective and neuroregenerative actions [75,85–87]. For example, CyPs have been associated to prion diseases [88]. Inhibition of CyPs with CsA results in the accumulation of aggresomes containing the abnormal conformer of prion protein. Interestingly, proline substitutions in the prion protein is a familial form of the disease and mutations of two prolines in prion protein mimicked some of the effects of the drug, suggesting that CyPs should have a beneficial role in the maintenance of prion protein in its proper conformation, whereas PPIase inhibition of CyPs activity is clearly detrimental for the cell.

CyPA was also related to Alzheimer's disease via the apolipoprotein E pathway that controls cerebrovascular integrity [87]. Thus, the expression of ApoE4 (a major factor risk in Alzheimer's disease) activates the proinflammatory CypA–NFκB–MMP-9 pathway in pericytes leading to

Key Term

Neurodifferentiation: Differentiation of neuronal cells.

neuronal uptake of multiple blood-derived neurotoxic proteins, as well as microvascular and cerebral blood flow reductions. This, ultimately, leads to neuronal dysfunction and neurodegeneration.

Parvulins have also been linked to neurodegeneration. In Alzheimer's disease, the Pin1-regulated phosphorylation of tau and the amyloid precursor protein affects respectively tangle and Aβ formation, which influences the production of aggregates [89]. Therefore, Pin1 is certainly a protective factor, as it is also shown by the fact that in Alzheimer's disease Pin1 is downregulated or inhibited [90]. However, Pin1 was also observed to facilitate α-synuclein aggregation in Parkinson's disease models [91].

Future perspective

The developing of IMM ligands appears to have promising perspectives during the forthcoming years. The ability to regulate the functions of a specific protein using cell-permeable small molecules, such as those that bind IMMs, is an unquestionable powerful method not only to study biological systems, but also a desired alternative to be used in therapeutic treatments. IMMs are rising as novel targets that could offer new therapeutic opportunities in many fields, most likely in cancer therapy, neurodegenerative diseases and other neurological disorders, such as depression. However, after several years of interest in the study of IMMs, there is still a strong requirement for novel drug scaffolds. Despite some advances in the development of fluorescent probes to examine ligand specificity and few drugs that are still in the experimental step, concrete advances in the therapeutic role of IMM ligands is still a pending assignment. This would be particularly important for Hsp90-binding IMMs, due to their involvement in such a great number of basic biological processes. Simply, as a sort of example to reinforce this concept, the Hsp90–IMM-regulated transport of soluble proteins discussed in the previous sections deals with a process where protein mistargeting demonstrates obvious dire cellular consequences. Ideally, the biological function of certain nuclear factors could be regulated if we can influence the mechanisms by which they reach their sites of action. For example, it is well known that NFκB is active in many cancer cells and its persistent localization in the nucleus relates to tumor development, as well as p53 does, due to its mislocalization in the cytoplasm that prevents the defensive mechanisms leading to cell cycle arrest and apoptosis. Therefore, targeting p53 to the nucleus and/or retaining NFκB in the cytoplasm could be desirable for the control of cell survival. This effect could be potentially achieved by targeting the associated IMMs related to the regulation of their respective subcellular localizations.

Following the isolation of rapamycin and CsA, FK506 was the most-used drug for the prevention of liver transplant rejection and, since then, its use has expanded rapidly into the transplantation of other organs. Importantly, treatments with CsA have also been associated with increased idiopathic susceptibility to atherosclerosis and the development of hyperlipidemia and cardiovascular side effects [92]. Other natural compounds were also assayed for inhibiting CyPA, such as sanglifehrin. The immunosuppressive action of this drug is still poorly understood and not related to calcineurin. Transplant experiments indicated that addition of sanglifehrin to CsA efficiently suppresses graft arteriosclerosis in comparison to CsA monotherapy [93], suggesting that sanglifehrin may represent a novel class of IMM-binding agent. In turn, this compound has recently been proved in dendritic cells as an efficient chemokine suppressor and cell migration inhibitor [94]. Nevertheless, studies in lymphocytes are necessary to be complete the potential pharmacological use of this compound.

Even though there is some evidence in the literature indicating that CsA could also have some neurotrophic action, CsA demonstrates low permeability through the blood–brain barrier (in contrast to FK506). One possible alternative to deliver the drug could be the use of the recently developed technology of drug-containing biodegradable microspheres [60], or alternative methodologies such as ultrasonic atomization to microencapsulate drugs within a polymer construct [95] so to feasibly the use CsA in neurological disorders.

Regarding non-immunosupressive derivatives of FK506, in addition to their obvious clinical importance, the relatively recent discovery that FK506 also demonstrates neurotrophic action makes it even a more experimental attractive base compound to develop specific compounds. The fact that the neurotrophic mechanism seems to respond to the expression balance of the neurotrophic IMM FKBP52 and its closely related and antagonistic partner FKBP51, could also prompt the development of specific small molecules able to activate the positive IMM (FKBP52), or prevent the opposite effects of FKBP51 for the efficient progression of differentiation [43]. Great efforts are invested in this

objective, although the results obtained to date are still of relative efficacy.

The fact that FKBP51 is also an antiapoptotic factor in many types of cancer represents another stimulating challenge for the pharmaceutical industry. Additionally, it should be also considered its relevance as a regulator of the steroid receptor action. In this sense, functional domain mapping in yeast suggested that the FKBP52 FK1 domain is critical for regulating steroid hormone receptor function through interaction with the receptor ligand-binding domain. This was recently used to identify a surface region on the AR ligand binding domain named BF3 that allosterically regulates coactivator binding [96]. This region also displays increased functional dependence on FKBP52 when it is mutated, suggesting that FKBP52 can indirectly influence receptor function through this surface. It was recently demonstrated [69] that FKBP52 regulation of receptor function can be blocked by small molecules such as the cyclohexanecarboxamide MJC13, which is predicted to bind to the BF3 surface of the AR. In agreement with the previously discussed role of FKBP52 in receptor retrotransport, this experimental drug prevented AR nuclear accumulation and consequently, AR activity. This is a relevant and encouraging observation in view of the fact that when the other main chaperone of the complex, Hsp90, was targeted with geldanamycin derivatives, many complications were detected in animal models and also in clinical trials because the doses of the Hsp90-disrupting agents that were required for inducing apoptosis also resulted in severe adverse effects [97].

Human genetic studies have demonstrated intronic single nucleotide polymorphisms in the gene encoding FKBP51 to be associated with FKBP51 expression and with various stress-related psychiatric disorders (see [98] for a recent review). Recent characterization of FKBP51-knockout mice has provided a wonderful model to analyze these actions [99,100], suggesting that, therefore, the selective inhibition of FKBP51 might also be of therapeutic benefit for psychiatric disorders. Nonetheless, to further dissect the role of larger FKBPs and to better understand the underlying biology, selective inhibitors targeting FKBP51 and FKBP52 are required. Attempts are currently being focused on the design of these class of drugs [101,102], but the biological effectiveness and pharmacological specificity of these novel compounds are still to be validated.

In contrast to FKBP51 and FKBP52, the developing of specific inhibitors for the apoptosis-related IMM FKBP38 is less prolific to date. Interestingly, a well-known inhibitor of eukaryotic protein synthesis, such as cycloheximide, also inhibits the PPIase activity of FKBP12. This prompted the development of derivative named *N*-(*N*′,*N*′-dimethylcarboxamidomethyl) cycloheximide that functions as a relatively specific inhibitor FKBP38 [70]. Nonetheless, its use is quite limited.

The reversible inhibition of parvulins by small molecules has not yet been properly established. Since inhibition of the pSer(pThr)-Pro specific parvulin Pin1 can lead to a new strategy for cancer therapy, to date there is no efficient and specific inhibitor of this activity and great effort should be invested in the search for it. One of the few attempts to inhibit parvulin was the use of juglone. However, although juglone differentiates the parvulin subfamily of PPIases from CyPs and FKBPs subfamilies, its use has been hampered by lack of specificity [103]. Impressively, screening of more than 1 million compounds with a Pin1 fluorescence polarization binding assay and a Pin1 PPIase activity assay did not yield any inhibitory compounds that could be confirmed by gold standard binding assays, such as isothermal titration calorimetry or NMR-based ligand-binding analysis [104].

In summary, probing physiological pathways in response to specific inhibitors of PPIases will become increasingly important in the following years. Characterizing and understanding of their molecular pathways is an emerging strategy for the design of novel pharmacological targets. This is particularly true as the molecular role of IMMs in nerve regeneration and cancer development will become more clearly defined in the near future.

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Executive summary

Immunophilins

- Family of proteins with peptidyl prolyl-(*cis/trans*)-isomerase activity. Most of them are able to bind immunosuppressive drugs.

Cyclophilins

- Immunophilins that selectively bind the immunosupressive fungal decapeptide cyclosporine A.
- The human genome has 16 genes encoding for cyclophilins, but the smallest member of the subfamily (CyPA or CyP17) is the only intracellular receptor of the drug able to form an immunosuppressive complex.

FKBPs

- Immunophilins that selectively bind the immunosuppressive macrolide FK506. The human genome has 17 genes encoding for FKBPs, but the smallest member of the subfamily (FKBP12) is the only intracellular receptor of the drug able to form an immunosuppressive complex.

TPR domains

- Tetratricopeptide repeats consisting in degenerated sequences of 34 amino acids that form tandem domains, usually antiparallel alpha-helices. They function as a protein-protein interacting domain.

Macrolides

- Group of drugs whose activity (typically antibiotics) stems from the presence of a large macrocyclic lactone ring.

Heat-shock proteins

- Family of proteins that were first characterized by a great induction of expression due to the exposure of cells to heat-stress. They were subsequently identified as molecular chaperones.

Nanog

- Transcription factor critically involved with self-renewal and pluripotency of undifferentiated embryonic stem cells and blastocyst inner cell mass along with Sox2 and Oct4, the other two members of a key functional triad.

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