CHAPTER **20**

Peptidyl Prolyl Isomerase Inhibitors

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Contents

ABBREVIATIONS

AD	Alzheimer's disease
CNS	central nervous system
CsA	cyclosporine A
FKPB	FK506 binding protein
HCV	hepatitis C virus
HIV	human immunodeficiency virus
Hsp	heat-shock protein
HTS	high-throughput screen
ITC	isothermal calorimetry
LSF	late SV40 factor

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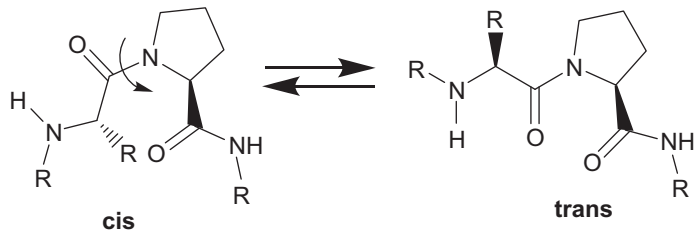
NFT	neurofibrillary tangle
PPI	peptidyl prolyl isomerase
pSer	phosphoserine
pThr	phosphothreonine
siRNA	small interfering RNA
TOSCY	total correlation spectroscopy
TRPC1	transient receptor potential cation channel 1
TS	thymidylate synthase
Vpr	multifunctional viral protein R

1. INTRODUCTION

Posttranslational modifications of newly synthesized proteins include proper folding of proteins and covalent modification of side-chain residues with auxiliary groups. Properly folded proteins possess unique physical and biological properties and exhibit unique capacities to undergo subsequent covalent posttranslational modification. c*is–trans* peptidyl prolyl isomerases (PPIs) catalyze the conversion of nascent protein strands containing *cis*-proline residues to the more stable *trans*-proline conformation. The less common interconversion from *trans* to the thermodynamically unfavored *cis*-proline form is catalyzed by *trans–cis* PPIs. Although PPIs [EC 5.2.1.8] all catalyze the *cis–trans* interconversion of proline residues, they can be divided into three enzyme families on the basis of structure and mechanism. Each PPI family exhibits selective binding of both ligands and inhibitors.



2. CATEGORIES OF PEPTIDYL PROLYL ISOMERASES

Review of the biochemistry of the PPIs is well represented in the literature with an emphasis on overall biological relevance [1–4], foundational biochemistry [5–12], mechanism [7,8,10,13], inhibitors [14–19], and possible therapeutic utility [5,13,17].

2.1. Cyclophilins

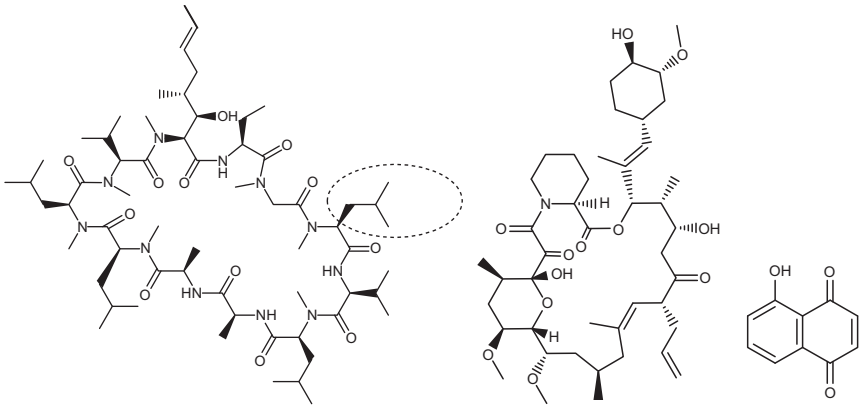
Cyclophilins were the first PPIs discovered and currently are represented by at least 60 enzymes [20]. There have been 17 human isoforms of cyclophilin identified [21]. Of the three families, cyclophilins display the greatest sequence homology within their PPI family. The current nomenclature is to assign a letter suffix. Older nomenclature employs a numeric suffix derived from the enzyme weight in kilodaltons. As a result, a discrete cyclophilin may have multiple names: for example, cyclophilin A is identical to cyclophilin 18. Cyclophilins all contain a *cis–trans* PPI domain with an eight-strand β-bundle and two associated α-helices; this site binds a portion of the macrocyclic immunosuppressive drugs represented by cyclosporine A (CsA) **1**. This dimeric complex then binds a third partner, such as calcineurin. The identity of this third binding partner and the ensuing formation of an active or inactive ternary complex are dependent on the identity of bound ligand; this has been referred to as ligand specific protein binding and activation [22].

Pathological states associated with cyclophilin activity include viral infection [23,24], chemotaxis during inflammation, cancer [25,26], and mitochondrial stress, during which cyclophilin inhibitors are protective [27]. The most widely studied cyclophilins are A, B, and D.

The ternary complex of cyclophilin A/CsA/calcineurin generates active pSer (pThr) 2B protein phosphatase which, in part, mediates the immunosuppressive properties of CsA.

Cyclophilin A also catalyzes a *cis/trans* interconversion at a requisite Pro35 residue of multifunctional viral protein R (Vpr), an essential component for HIV virion assembly [28]. This is consistent with a chaperonin role for cyclophilin A facilitating virion assembly and HIV virulence. Cyclophilin A has also been identified as essential for the assembly of NS5B into the virion replication complex of HCV [23,28–31]. The role of cyclophilin B in HIV and HCV continues to be examined [11,32,33], and although siRNA strategies [24] offer better isoform selective analysis, the exact role of cyclophilin B is not clear. Additional interactions of cyclophilin A, B, and **1** with the membrane bound immunological glycoprotein CD147 have been reviewed [34] with an emphasis on neutrophil chemotaxis and migration.

X-ray crystal structures have recently been obtained with cyclophilin B and calnexin, calreticulin, or calmegin [35], providing a structural basis for the folding of *N*-glycosylated proteins in the calnexin cycle. Possible roles for cyclophilin B in the prolyl-3-hydroxylase complex have also been examined with an emphasis on osteogenesis imperfecta [36,37].



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Cyclophilin D has also been shown to activate the mitochondrial permeability transition pore complex in the inner mitochondrial membrane [38]. This pore regulates mitochondrial Ca^{2+} stores, and inhibition of pore activation has been postulated to provide a significant protective effect in multiple models of neurodegeneration [3,39]. Cyclophilin D also associates with the ATP synthase complex in the inner mitochondrial membrane, although the specific nature of this interaction requires further characterization [40]. Cyclophilin D knockout mice, however, display cardiac hypertrophy and less tolerance to physical stress consistent with less metabolic flexibility in the myocardium and a shift from fatty acid to glucose utilization [41]. The conversion of cyclophilin D from a PPI to a pore-activating complex has been proposed as arising from oxidative formation of a disulfide bond between Cys203 and Cys157 [27] and a resultant change in its biological function.

Cyclophilins contain additional protein binding domains and have been demonstrated to form complex multi-protein assemblies [21].

2.2. FK506 binding proteins

PPIs that display preferential binding to the immunosuppressive drug FK506 (Tacrolimus), **2**, [ARMC 29, p. 347] are categorized as FK506 binding proteins (FKBPs). The ternary complex of FKBP, FK506, and calcineurin negatively regulates calcineurin activity in a manner analogous to the ternary complex of cyclophilin A, CsA, and calcineurin. At least 50 FKBPs have been identified and exhibit potent biological activities including signal transduction and cellular adaption to increased metabolic demand and oxidative stress. Both cyclophilins and FKBPs are commonly referred to as immunophilins as they bind

immunosuppressive drugs [22]. There are 15 principle members of the human FKPB family. FKPBs have six antiparallel β-strands, one α-helix, several loop regions near the α-helix, and a deep hydrophobic pocket facilitating proline binding. FKBPs are 10–50 times elevated in the CNS (depending on isoform) compared to the periphery. Four of these isoforms exist primarily in the brain: FKBP12, FKBP38, FKBP52, and FKBP65. The role of FKBPs in the brain has been difficult to study due to limited CsA partitioning into the CNS. Nonimmunological pathological disease states associated with FKBPs include neurodegeneration and depression. Emphasis has been placed on FKBP/tau/microtubule interactions [42], neurite outgrowth [43], protein fibrilization [44], and FKBP/glucocorticoid receptor [45,46] interactions [18].

FK506 reduced α-synuclein fibrilization in SHSY5Y neuroblastoma cells in a dose-dependent manner and reduced apoptotic cell death. siRNA or stable knockdown of FKBP12 or FKBP52 (to a lesser extent) produced similar effects [47]. Subsequently, extensive tertiary structure analysis with NMR and modeling has been conducted on FKBP12 [44,48]. Modification of α-synuclein aggregation may find relevance in understanding the pathology of Parkinson's disease or Lewy body dementia; both of these neurodegenerative disorders present Lewy bodies composed of fibrilized α-synuclein.

FKBP51 and 52 assist in stabilizing the microtubule-associated protein tau and stabilize microtubules. The proposed mechanism is salvage of hyperphosphorylated tau from ubquitination through stepwise sequestration by heat-shock protein (Hsp) 90, tau dephosphorylation, and finally FKBP-mediated refolding of tau to its original microtubule stabilizing form [42,49]. Additionally, FKBP52 may interact with the TRPC1 channel assisting with neuron growth cone steering and direction of neurite outgrowth [46]. These processes may underlie synaptic remodeling events relevant to memory and deposition of hyperphosphorylated tau protein in Alzheimer's disease (AD).

Both FKBP51 and 52 interact with the glucocorticoid receptor. FKPB51 effects selective downregulation of receptor activity, although the nature of these interactions is not currently well characterized [45]. Interaction of FKBP5 with the glucocorticoid receptor has been postulated as mediating unipolar depression driven by elevated cortisol levels [46,50].

2.3. Parvulins

Parvulins are PPIs that preferentially bind 5-hydroxy-1,4-naphthoquinone (juglone) **3** rather than immunosuppressive drugs **1** and **2**. Consequently, parvulins are not classified as immunophilins. Parvulins are present in many species, but there are only three human isoforms of the parvulins. Pin1 is the most widely studied isoform. Parvulins effect a

phosphoserine (pSer) or a phosphothreonine (pThr) directed *cis–trans* isomerization of the prolyl bond. The enzyme has two distinct domains: a phosphate-recognizing domain that associates with the pSer or pThr and the catalytic PPI domain. The PPI domain of parvulins consists of four antiparallel β-sheets, an α-helix, and convergent loops [51]. Pin1 inhibitors have been the focus of extensive research [15,52] and only recent developments will be discussed here. Pathological states associated with parvulins include AD, cancer, and immunological response.

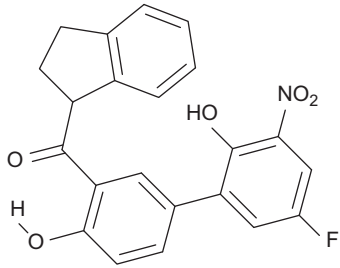
Many studies have examined the role of Pin1 in tau hyperphosphorylation as a contributing factor in Alzheimer's and other neurodegenerative diseases [51,53,54]. Pin1 has also been colocalized in NFT deposits. Its current role in AD requires additional characterization. Overexpression of Pin1 in hepatic carcinomas suggests Pin1 inhibition as a plausible therapeutic strategy. Pin1 binds the thymidylate synthase (TS) regulating transcription factor late SV40 factor (LSF) at pThr329Pro330, resulting in the *trans*Pro330 product. This resultant pThr329 *trans*Pro330 form of LSF is then dephosphorylated at Ser291 and Ser301 permitting TS expression and successful navigation of the G1/S cell cycle transition [55]. Additionally, Pin1 phosphorylates and activates Notch, which subsequently undergoes γ-secretase-mediated proteolysis. Notch upregulation was correlated with increased growth of breast tumor cells; Pin1 levels were also increased [56].

Pin1 activity has been proposed as essential for eosinophil survival. Pin1 generates pThr167 *trans*Pro168 Bax that is refractory to calpain hydrolysis and the subsequent apoptosis-inducing caspase cascade. As a consequence, Pin1 inhibition has been proposed as a drug target for asthma and other eosinophilic diseases [57]. However, Pin1 knockout mice display cell proliferation deficiency phenotypes [58] and display deficient telomere maintenance [59].

3. SMALL-MOLECULE INHIBITORS

Although small-molecule inhibitors of PPIs were identified over 15 years ago, initially in the context of FK506 work, it has only recently been shown that small molecules can differentiate between isoforms of cyclophilins, specifically cyclophilins A and B. These results have emanated primarily from the laboratories of Fischer [14,60,61] and Li [62–64]. Recent structural analysis of the 17 known human cyclophilins utilizing both X-ray crystal structures and homology modeling assuming an invariant Arg (Arg55 in the case of cyclophilin A) has identified two specific locations near the catalytic site likely to confer isoform selectivity: the S2 pocket and the S1′ pocket. Unique residues on the S2 pocket exist for each isoform suggesting the potential for rationally developing selective inhibitors. Analysis of

p-nitrophenyl-tagged tetra-peptide-based ligands with isothermal calorimetry (ITC) and $^{1}H/^{1}H$ TOSCY experiments was consistent with the hypothesis of isoform variation in the S2 pocket contributing to ligand selectivity for a given cyclophilin [21].



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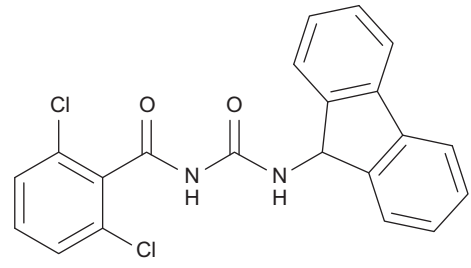

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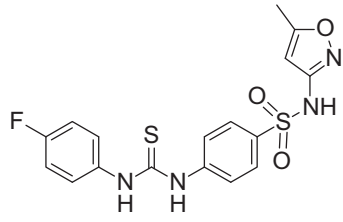
Compound **4** from the Fisher lab represents a parvulin series compound modified for selective cyclophilin A inhibition [14,65]. The requisite *ortho*-hydroxyl and the dihydroindane ring has been proposed to mimic the prolyl acyl group, and the 3′-nitro biphenyl system increases potency. As a result, **4** displays 520 nM inhibition of cyclophilin A as determined with a fluorescence quench assay. This compound shows no inhibition at the B or C isoforms and is four times less active against cyclophilin D. ITC studies are consistent with the biphenyl group making an entropic contribution that surpasses hydrogen bonding contributions from the acyl group. This is consistent with Dugave's model of a hydrophobic pocket in cyclophilin A comprising residues from Ile57 to Phe60 [7]; this would correspond to the S1′ pocket [21]. Reduction of the 3′-*meta*-nitro group of **4** to the corresponding amine and subsequent testing gave a compound with a K_i of 300 nM for cyclophilin A and a reduction of 40-fold for cyclophilin A versus cyclophilin B selectivity. The differential activity of the 3′-*meta*-nitro and 3′-*meta*-amino, the symmetry of inhibition curves presented, and the concentration-dependent displacement of CsA in the ITC experiments are consistent with a competitive binding in the prolyl isomerase site rather than artifacts arising from bulk hydrophobic properties of aggregates [66,67]. Compound **5** is reported to have near equipotent activity in cyclophilin A inhibition compared to CsA [64]. Selectivity for cyclophilin A over other PPIs was not presented. These observations build upon prior observations from a series of thiourea and related derivatives by the same research group [68,69]. These have been proposed to bind the catalytic site of cyclophilin A. Docking simulations indicate a 90° angle between the plane of the 2,6-dichloro phenyl ring and the amide carbonyl of **5**. Additionally, the aromatic ring may exhibit hydrophobic interactions with the aryl ring of Phe60 of the S1′ pocket of cyclophilin A [70]. Also, the amide acyl is proposed to interact with the

catalytic Arg55 residue. A similar series of thiourea derivatives, represented by **6**, have also been explored as dual inhibitors of cyclophilin A and viral capsid assembly in the design of anti-HIV therapeutic agents [71]. The cyclophilin A portion of the design strategy utilized an ionic interaction of the sulfonamide isoxazole with the catalytic Arg55 and extension of the thiourea portion into the lipophilic portion of the S2 pocket [71].



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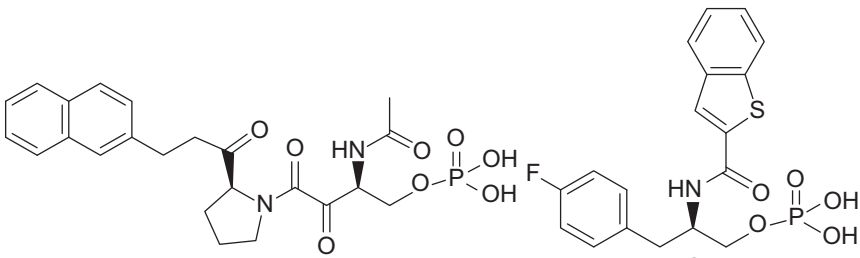


7 **8**

Recent small-molecule inhibitors of Pin1 have also been reported. Starting with a known core for FKBP inhibition, the 2-amido phosphate moiety, structural variations were examined to optimize inhibition at Pin1 in terms of both potency and selectivity.

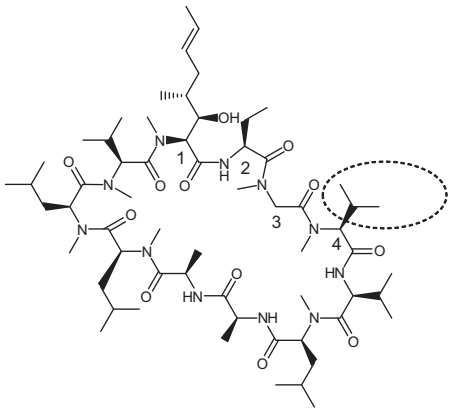
Mechanistic probes including **7** were synthesized to evaluate competing enzymatic proposals for Pin1 prolyl bond catalysis [72]. Two broad categories of catalysis by PPIs are frequently invoked: a twisted amide versus a Cys-mediated tetrahedral intermediate. Compound **7** used the α-keto amide functional group, a motif that has led to potent FKPB inhibitors and is consistent with a twisted amide transition state. Although this compound was subsequently prepared in diastereomerically pure form from enantiopure *S*-proline, both compounds were modestly active in the high millimolar range. These results indicate that either the enzymatic mechanism(s) employed by parvulins differ from FKBP or that the structural characteristics of **7** are not yet sufficiently optimized.

A series of single and double-digit nanomolar amido phosphate inhibitors of Pin1 represented by **8** were identified using a structure-based design strategy [73]. A phosphate group was identified (PDB ID: 3IK8) as interacting with an anionic binding domain (Arg68 and Arg69) of Pin1

originally identified in the X-ray crystal structure of 3IK8. Several prolyl acyl bioisosteres were examined with the amide group presented as optimal. Additional exploration of the proline binding catalytic binding pocket identified hydrophobic regions that were explored with aryl and heterocyclic aryl groups resulting in the 6 nM Pin1 inhibitor **8**. Hydrophobic interactions of **8** with Pin1 not accessed by traditional proline mimics include Phe134 and Leu122. Data for cross-inhibition with other parvulins were not presented, and inactivity in cellular assays was attributed to poor transport into the cell due to the phosphate group.

4. MACROCYCLIC INHIBITORS

Despite the imposing structural components presented by **1** and **2**, both are used clinically and have been the subject of multiple total syntheses [65]. Another related compound DEBIO-025, **9**, is in clinical trials as a potential treatment for HCV [74].



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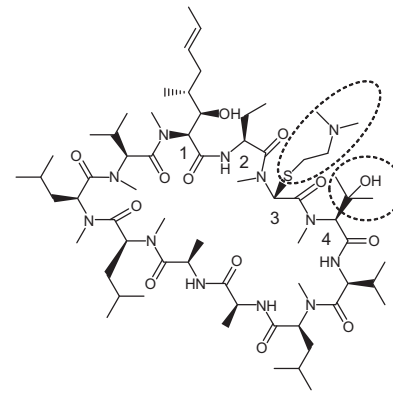

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DEBIO-025, a semisynthetic derivative differing from **1** by a single amino acid side chain, is predominantly a cyclophilin inhibitor [75,76] and is under examination for treatment of HCV particularly in individuals coinfected with both HCV and HIV. DEBIO-025 has been shown to reduce HIV-1 infection and replication *in vitro* and has also been shown to reduce infection and replication against the more challenging HIV-2 genotype, but only when used in combination with existing HIV therapeutic agents. Unlike CsA, **9** does not activate calcineurin and as a result has reduced immunosuppressive effects. SCY-635, **10**, exhibits equipotent binding to CsA and like DEBIO-025 does not activate calcineurin [77]. DEBIO-025 and SCY-635 deviate from the structure of CsA by variation on the three and four amino acid residues; this portion of the macrocycle

is directed away from the prolyl isomerase active site toward calcineurin. Three significant points should be emphasized: (1) immunosuppressive and antiviral effects of CsA are possibly independent, (2) inhibition of cyclophilin is a potentially useful strategy for reducing HIV infectivity and replication *in vivo*, and (3) this antiviral effect may be synergistic with currently used therapies.

5. CONCLUSION

The understanding of PPIs on a biological level continues to advance with a current emphasis on mechanism and relevance to disease states. Drug design strategies from one family of PPI are finding application in other PPIs as evidenced by compounds **4**, **8**, DEBIO-025, and SCY-635. Several structural classes of molecules have demonstrated the ability to inhibit cyclophilin with the possibility of isoform selectivity. Additionally, small molecules have been demonstrated to inhibit the active catalytic site without a direct proline mimic. The presentation of a comprehensive structure-based analysis of all cyclophilins suggests that the development of rationally designed selective inhibitors of PPIs is a reasonable goal. The development of HTS assays should facilitate efficient identification of PPI inhibitors [44,78,79]. However, the complexity of mechanism of activity evidenced by Pin1 and the possibility of significant modification of enzymatic activity in response to cellular metabolism evidenced by cyclophilin D suggest that *in vitro* analysis of PPI activity should be supported with relevant whole animal models of relevant human disease states.

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