

Prolyl Isomerases as New Therapeutic Targets

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Prolyl isomerase inhibitors are an emerging class of drugs, acting on an unexploited class of therapeutic targets and representing a novel category of drugs acting by novel mechanisms.

Abstract

Prolyl isomerases comprise three main protein families totalling over thirty mammalian genes, and several hundred orthologues across the biological domains, with a very broad spectrum of physiological functions and disease implications. Potent small molecule inhibitors exist for members of the three main mammalian families (cyclophilins, FKBP and parvulins) but, until recently, these proteins have not received the attention of the pharmaceutical industry that they merit. Perceptions and experimental difficulties of lead finding and functional screens made the whole class unattractive for drug discovery. Over the last ten years however, interest in prolyl isomerase-directed drug discovery has started to increase. This article discusses the history of prolyl isomerases, their physiological functions in health and disease, the therapeutic potential of inhibitors and drug discovery challenges.

Keywords: Prolyl isomerase; protein-protein interaction; medicinal chemistry; natural products; capillary electrophoresis.

History

The discovery of peptidyl-prolyl *cis-trans* isomerases ("PPIases") began in the 1980s and was driven by two independent research activities. Fischer, at the University of Halle (then in East Germany) was working on the enzymology of protein folding. Independently and unknown to each other, Handschumacher at Yale was investigating the molecular basis of the immunosuppressive activity of cyclosporin, a cyclic peptide isolated from cultures of the fungus *Tolypocladium inflatum*. This latter compound had been launched in 1983 by Sandoz (now Novartis) to suppress organ transplant rejection and truly revolutionised this field. Whilst its activity was relatively well understood at the cellular level, details of a molecular understanding were lacking, prompting the search for a

receptor mediating the activity. In 1984, Handschumacher isolated a cyclosporin-binding protein and named it cyclophilin [1]. Soon thereafter, the existence of many isoforms of cyclophilin with ubiquitous distribution across all living organisms and tissues was reported [2]. This ubiquitous distribution was in apparent conflict with the high specificity of inhibitory activity of cyclosporin against T cells. Further doubts over the relevance of cyclophilin for immunosuppression were raised by the existence of extremely close structural analogues of cyclosporin, such as NIM-811, which had equal affinity for cyclophilin yet were not only completely devoid of immunosuppressive activity but also failed to show functional antagonism of cyclosporin in the assays available at the time [3].

At approximately the same time as cyclophilin was isolated at Yale, Fischer characterised a protein from pig kidney capable of catalysing the *cis-trans* isomerisation of peptide bonds to the amino acid proline, one of the rate-determining steps of protein folding [4]. The co-identity of this newly discovered protein with the cyclosporin-binding protein cyclophilin was reported in 1989 [5,6].

In 1984, researchers at Fujisawa isolated a macrolide from cultures of *Streptomyces tsukubaensis*, that had a profile of immunosuppressive activity very similar to cyclosporin, but was up to a thousand fold more potent *in vitro*. This compound was named FK506, published in 1987 [7] and approved in 1994 for liver transplantation (Tacrolimus®). Soon after the publication of FK506, a binding protein for FK506 was reported by the Merck research laboratories and shown to be a PPIase similar to cyclophilin. However, not only was its protein sequence different from cyclophilin, so too was its substrate specificity and susceptibility to inhibition [8]: FKBP is not inhibited by cyclosporin nor is cyclophilin inhibited by FK506. In 1990, Schreiber et al. showed that the long-known macrolide rapamycin also strongly bound and inhibited FKBP but that its profile of immunosuppression was distinctly different from that of FK506 [9].

Thus, a picture emerged that saw cyclosporin, FK506, and rapamycin and their binding proteins under the common umbrella of immunosuppression, prompting the creation of the term “immunophilin” for the two families of cyclophilins and FKBP [10]. This term has since become synonymous with PPIases, suggesting, somewhat unfortunately, main roles of these proteins in the immune system and not doing justice to the full scope of their physiological functions or their many and diverse roles in disease, which go far beyond the immune system.

In 1994, a third family of PPIases, the parvulins, was added, again by the Fischer group [11]. The number of known parvulins is remarkably small, only two genes are known in humans, in contrast to the high number of human cyclophilins (17) and FKBP (16) [12]. In 1996 the mitotic regulator Pin1 was discovered and shown to be a member of the parvulin family with specificity for phosphoserine or phosphothreonine preceding proline [13]. Pin1 was immediately recognised as a promising antitumour target and many pharmaceutical and biotechnology companies embarked on a search for small

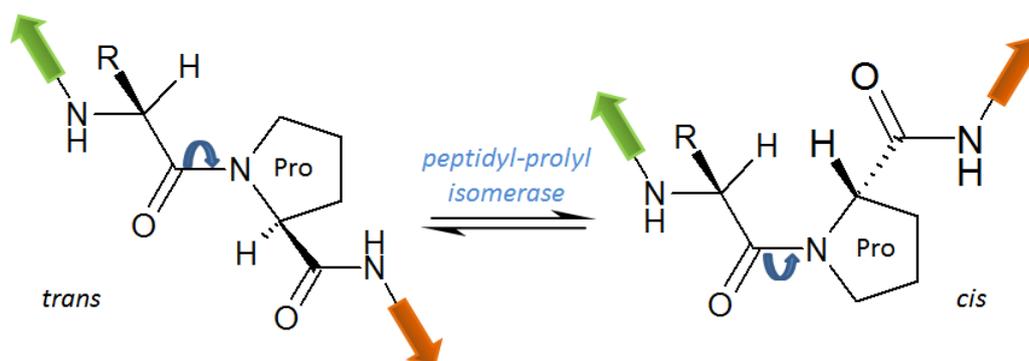
molecule Pin1 inhibitors. To date, none of these efforts is known to have resulted in a drug candidate [14].

It is important to note that the chemical families, to which cyclosporin, FK506, and rapamycin belong, were discovered not as PPIase inhibitors but rather due to their antifungal activity. All three compounds, which are the best known inhibitors of PPIases (all are marketed drugs), have gained prominence due to the medical breakthroughs they have enabled. Moreover, it was their first discovery that enabled the subsequent discovery of cyclophilins and FKBP. It is doubtful that a screen for protein folding inhibitory activity would ever have taken place in an industrial environment and, given the abandonment by the pharmaceutical industry of natural products research, even more unlikely that these compounds would have been found as a result of a modern day drug discovery strategy.

Physiological functions

To gain an insight into possible physiological roles of PPIases, it is useful to look first at the basics of prolyl isomerisation. Peptide bonds adopt a planar structure, the plane being defined by the C, O, and N atoms of the peptide bond (figure 1). From this plane, the C- and N-terminal residues of the peptide chain can point either to the same (*cis*) or opposite (*trans*) sides, resulting in two different shapes of the peptide/protein. Because shape defines the properties of peptides and proteins, *cis* and *trans* isomers differ in their biological properties. Most peptide bonds are not very rigid and under physiological conditions *cis* and *trans* isomers can easily convert into each other by rotation around the peptide bond. The ease of this rotation is at the core of the flexibility of peptides and proteins, which allows proteins to acquire their fully folded form ("native state") after being made but also to change this shape for interactions with other proteins. In fact, flexibility has been recognised as an essential factor for biological activity [15].

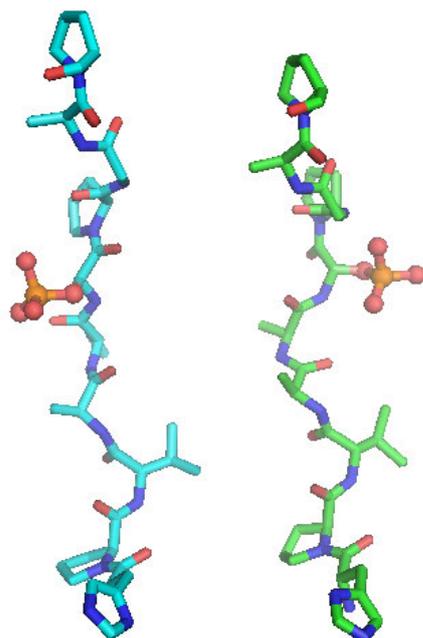
Figure 1: *cis-trans* isomerisation of peptide bond to proline



In the case of the amino acid proline, the rotation that converts *cis* and *trans* isomers into each other is more difficult due to there being a higher energy barrier to inter-conversion. The increased energy requirement means that peptide bonds to proline are

conformationally more restrained and consequently can act as points of rigidity (“stiffness”) in an otherwise very flexible peptide chain. This rigidity not only affects protein secondary structure but also influences important physiological functions. On the other hand, changes in the environmental conditions of the organism may require that this rigidity is removed, a role that is fulfilled by PPIases. It should be pointed out, that in a polypeptide chain, such aspects of *cis/trans* conformation often have very localised manifestations as illustrated in Figure 2.

Figure 2: how the *cis-trans* switch can have clear local effects on peptide shape



Models of two 10-mer peptides with the sequence 1-HPVAAS*PGAP-11 are shown. In the left hand (blue) strand, Pro-7 is in the *trans* conformation and in the right hand (green) strand, Pro-7 is in the *cis* conformation. Ser-6 has been modelled in the phosphorylated state. The figure shows how the *cis-trans* switch can have clear local effects on peptide shape (by presenting or hiding the side chain on the preceding residue) but that residues two or three residues distant in sequence adopt very similar positions. The models were based on experimental X-ray structures (pdb code 1M9Y) in which both *cis* and *trans* peptides were solved as complexes with cyclophilin A. (The actual sequence in the X-ray structures which was used as a template for the two models was HPVAAAPIAP).

Thus, prolyl isomerisation can be viewed as a mechanism that governs the activity of proteins *via* control of their flexibility and is complementary to covalent posttranslational modification such as e.g. phosphorylation, glycosylation, prenylation and others. The ubiquitous and important nature of this control mechanism is underlined by the large number of PPIases that have evolved. The existence of drugs that inhibit PPIases opens up, therefore, the possibility of a whole new category of pharmacological agents acting by unprecedented mechanisms and providing new ways of biomedical innovation.

Why have prolyl isomerases been neglected as drug discovery targets?

Perceptions

The evolutionary conservation as well as the ubiquitous distribution of cyclophilins and FKBP's across many tissues in all living organisms suggests important physiological functions. However, these have remained remarkably elusive over the years. Whilst by the end of 2012 the total number of publications on cyclophilins since 1990 had surpassed 2,000, a publication with the title “Cyclophilins: Proteins in Search of a Function” provides a good illustration of the still scarce understanding of their biological roles [16].

The early pioneering work by Fischer concerned the enzymology of protein folding, a biochemical event unlikely to show up in the massive “target identification and validation” efforts that have taken place in pharmaceutical and biotechnology companies over the last 20 years. Moreover, a publication by Dolinski in 1997 reporting that all cyclophilins (8) and FKBP (4) in yeast could collectively be deleted without resulting in a discernible phenotype seemed to emphasise the non-essential role of these proteins and all but ruled out their relevance as therapeutic targets [17]. Thus, the perception of “immunophilins” as something concerning the immune system and their “invisibility” in target discovery and validation approaches provide a first explanation for industrial lack of interest.

Mode of action complexity

The action of PPIases on their substrate proteins is essentially an interaction between two proteins. Hence, the activity of PPIase inhibitors is by nature the **inhibition of protein-protein interactions**. The PPIase inhibitors currently on the market have all been developed and launched not as inhibitors of protein-protein interactions but because of their immunosuppressive activities. These, however, are only indirectly linked to inhibition of PPIase enzymatic activity. The natural PPIase inhibitors all have in common the rather unusual property of **enabling protein-protein interactions**: Both the FK506-FKBP12 complex as well as the complex between some cyclosporins (e.g. cyclosporins A, C, D, G) and some cyclophilins interact with the phosphatase calcineurin and inhibit its activity, resulting in immunosuppression. Neither the drugs alone nor the proteins without the drugs have this property. On the other hand, the complex formed between FKBP12 and rapamycin does not inhibit calcineurin, the complex rather interacts and inhibits another protein best known as mTOR (mammalian target of rapamycin), resulting in a different mechanism of immunosuppression. Sanglifehrin, a natural inhibitor of cyclophilins, acts *via* an unknown mechanism on dendritic cells, which form the bridge between the innate and adaptive immune system. Chemical degradation yields compounds that still have high affinity for cyclophilin but lack the activity on dendritic cells, implying that it is again the complex between the whole natural molecule and cyclophilin that has this property, which smaller, cyclophilin-binding, fragments of sanglifehrin lack [18].

Based on what is outlined above, it thus becomes clear that these drugs have two levels of bioactivity: (1) activities due to the **inhibition of interaction** between PPIases and their physiological substrate proteins, (2) activities resulting from **enabling protein interactions** by inducing the formation of a ternary complex with other proteins affecting the function of these partner proteins. It is therefore possible to have drugs that inhibit PPIases without ternary complex formation, i.e. more selective drugs that lack the pharmacology resulting from ternary complex formation. It does not, however, appear to be possible to have drugs that have only the effects caused by formation of a ternary complex without the pharmacology associated with PPIase inhibition.

Medicinal chemistry

The medicinal chemistry optimisation of PPIase inhibition is largely a “classical” problem of enzyme inhibition which has, in some cases, been greatly aided by protein X-ray crystallography and NMR studies. There are a number of synthetic small molecule inhibitors of cyclophilin and FKBP reported in the literature. However, no such compound is known to have entered clinical development and no reports on their pharmacological activities *in vivo* appear to have been published.

In contrast, the natural PPIase inhibitors all have a rich and medically useful pharmacological profile. The main (but by no means prohibitive) difficulty is due to the somewhat higher structural complexity of the natural PPIase inhibitors as compared to “classical” synthetic molecules typically made by medicinal chemists. Unfortunately, there exists nowadays a certain reluctance amongst medicinal chemists to work with natural products. This reluctance was adopted by decision makers and medicinal chemistry programmes based on natural products have, unfortunately, almost disappeared from the industry. More importantly, optimisation attempts at the level of the ternary complex (i.e. their protein-protein interaction enabling properties) are a chemistry challenge of a different dimension. Structural biology on such “supra-molecules” is in principle feasible [19] but experimentally much more demanding, since guidance of chemistry by structural information is not available in a useful timeframe. The interface between the three interacting partners often requires the participation of all three of them and chemistry on one partner alone (the small molecule) is unlikely to achieve the desired outcome. Moreover, ultimately, the economic constraints of synthesis cost are likely to define the limits of such endeavours.

Difficulty of screens

The biochemical reaction catalysed by PPIases interconverts *cis* and *trans* isomers of a peptide bond to proline. Not only have substrate and product of this reaction the same chemical constitution, it also occurs spontaneously in the absence of catalysis within a few minutes, at least when using short synthetic peptide substrates. Inhibitor screens therefore have to distinguish between the catalysed and spontaneous reaction, which are separated by a time window of a few minutes. Such screens have been developed, but they require low temperatures and elaborate experimental protocols, are invariably of low throughput and are unsuitable as industrial screens for hit finding. However, these screens are useful tools for verifying the functional biochemical activity of compounds found by other screens.

For hit finding purposes, the best screening formats are binding assays. At Novartis, competitive binding assays for cyclophilin and FKBP12, using cyclosporin and FK506, were developed and used to screen synthetic compound collections as well as microbial extracts [20]. Whilst the synthetic compound collections gave no useful results whatsoever, microbial extracts proved a rich source of FKBP ligands, re-discovered cyclosporin many times, and also found new natural products such as cymbimicins [21] or sanglifehrins [22].

A particularly suitable screening technology not dependent on existing ligands is affinity capillary electrophoresis (ACE), based on a detectable change in the electrophoretic mobility of a protein upon ligand binding. Applications of ACE for drug screening were initially developed by Cetek and shown to be particularly useful for screening microbial extracts [23]. Applying ACE to cyclophilin and screening a library of several thousand microbial extracts, Cetek Corp. found many new cyclosporin producing organisms, strains producing sanglifehrin, as well as several known natural products with weaker, hitherto unknown cyclophilin-binding activity [24].

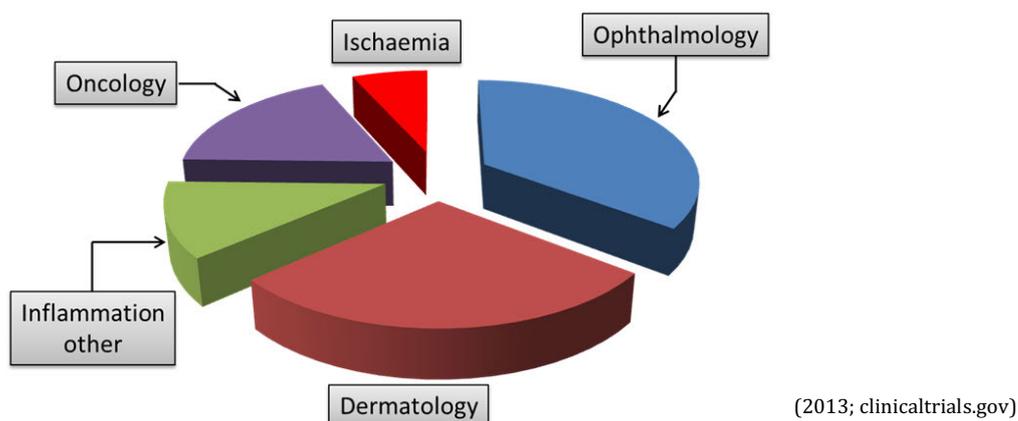
Sample libraries

The catalytic site of prolyl isomerases has evolved to bind relatively large substrates spanning several amino acid residues. Screening libraries assembled using current concepts of drug-likeness have intrinsically low probabilities of containing compounds binding to a PPIase catalytic site, especially with an affinity typically targeted by HTS approaches. Therefore, it is perhaps not surprising that all pharmacologically active PPIase inhibitors are natural products, derivatives of natural products, or synthetic molecules whose structures have been inspired by a natural product. There are a number of reports of synthetic small molecule inhibitors of cyclophilins, but most of them seem to be artefacts of the complex test systems described above. The well-documented neurotrophic activities of cyclosporin and FK506 have triggered an intensive search for small molecule ligands of cyclophilin and FKBP that would cross the blood-brain barrier [25, 26, 27]. None of these efforts seems to have resulted in a clinical development compound. Fragment screening approaches have been published for Pin1 but appear not to have succeeded in generating a drug candidate [28].

Therapeutic potential

The diverse therapeutic potential of PPIase inhibition is best demonstrated using cyclosporin as an example. A search of the clinical trials database ClinicalTrials.gov using the search term “cyclosporin” excluding “transplantation” returns over 300 clinical trials in the areas shown in figure 3. For none of these indications does immunosuppressive activity appear to be necessary.

Figure 3: clinical trials with cyclosporin other than transplantation



Cyclophilin inhibitors have shown promise as replication inhibitors of several RNA viruses including HIV, HCV, SARS corona virus and influenza virus. The non-immunosuppressive cyclosporin derivatives Alisporivir (Debio-025; Debiopharm, Novartis) [29] and SCY-635 (Scynexis Inc.) [30] have reached clinical development for the treatment of hepatitis C infection. Formulations of cyclosporin itself have also shown some promise in new therapeutic applications, for example in traumatic brain injury [31]. Preclinical research has suggested the utility of these or other cyclophilin inhibitors in diseases as diverse as muscular dystrophy [32], respiratory disease [33], cardiovascular disease [34] and Alzheimer's disease [35], to cite but a few. Furthermore, the FKBP and parvulin families of PPIases show similarly diverse potential.

PPIases are found across both prokaryotes and eukaryotes, including plants, and bacteria, but the functions of the majority of these proteins are mostly unknown. Some proteins, e.g. the macrophage infectivity protein (Mip) of *Legionella* and *Chlamydia spp.*, seem to act as virulence factors [36]. It is highly likely, therefore, that a pharmacological understanding of PPIases could give rise to products for both infectious and non-infectious diseases and also agricultural uses.

Summary and conclusions

Prolyl isomerases represent a large class of biological targets, including cyclophilins, FK506-binding proteins and parvulins, with broad physiological functions and have been shown to be valid targets for therapeutic intervention by small molecules. Drug discovery approaches based on the traditional paradigm of HTS of synthetic libraries have met with limited success; all pharmacologically useful inhibitors are of natural origin. However, the application of new screening technologies paired with natural product samples and underpinned by strong skills and experience in medicinal chemistry of natural products will open up the whole class of PPIases as a rich source of new pharmacological targets. PPIase inhibitors are predicted to act via unprecedented mechanisms and offer new

modes of therapeutic intervention in many different diseases, representing a new chapter in biomedical innovation.

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