Cyclophilin Inhibition by a (*Z*)-Alkene *cis*-Proline Mimic

Scott A. Hart and Felicia A. Etzkorn*,†

Department of Chemistry, University of Virginia, McCormick Road, Charlottesville, Virginia 22901

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The peptidyl-prolyl isomerases (PPIases) cyclophilin (CyP) and FK506 binding protein (FKBP) catalyze the cis−trans isomerization of Xaa−Pro amide bonds. Several compounds that bind these enzymes, including cyclosporin and FK506, are potent immunosuppressants,[1,2] although the immunosuppressant activity is independent of PPIase activity.[3−7] Understanding the mechanism by which these enzymes catalyze cis−trans amide isomerization will help elucidate the native roles of these ubiquitous enzymes in cellular processes.[8] We present here a *(Z)*-alkene-based substrate mimic that inhibits the PPIase activity of human cyclophilin A (hCyPA).

Proline is unique among the 20 natural amino acids due to its dialkylated amine, which causes amide bonds preceding proline to adopt a significantly higher percentage of cis configuration than other amino acids. Xaa-*cis*-Pro amides appear in 10−30% of short prolyl peptides[9] (Figure 1) and approximately 6% of Xaa-Pro amides in proteins of known structure.[10] This relatively high occurrence of cis amides complicates protein folding when a particular prolyl amide must isomerize before a protein can reach its native folded structure. On the time scale of protein folding, thermal cis−trans amide isomerization is slow, leading to slow steps in the folding of proline-containing proteins.[9,11−13] PPIases facilitate this process in nature by catalyzing prolyl cis−trans amide isomerization. A twisted amide mechanism was proposed on the basis of both the observation that the keto-carbonyl of the FK506 α-keto amide was orthogonal to the amide plane in the bound conformation[14−16] and secondary kinetic isotope effects.[17−19] More recently, it has been suggested that these enzymes bind the Xaa-Pro substrate and distort the amide bond by pyramidalizing the prolyl nitrogen through hydrogen bonding.[20−22] It was proposed that the hydrogen bond in FKBP is donated intramolecularly from the prolyl C-terminal amide in the substrate,[20−22] while the active site Arg-55 is proposed to act as the hydrogen donor in hCyPA, since the orientation of the substrate in the hCyPA active site does not allow a similar intramolecular hydrogen bond.[8] Indeed, the Arg55Ala mutant was catalytically inactive yet retained the ability to bind cyclosporin.[3,8]

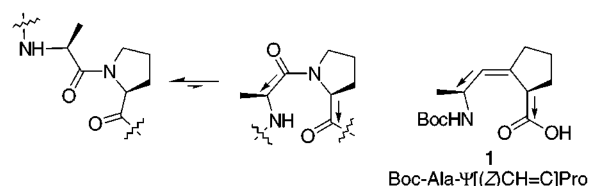

Figure 1. *cis/trans*-Pro equilibrium and (*Z*)-alkene Ala-*cis*-Pro mimic **1**.

While the immunosuppression exhibited by cyclosporin and FK506 is independent of PPIase activity, it has been shown these drugs do bind in the isomerase active site[23,24] and are competitive inhibitors of PPIase activity.[25] A better mechanistic understanding may lead to other drugs divorced of immunosuppression activity, as well as insights into other PPIase dependent processes such as protein folding[13] and related chaperone activity,[26] voltage gating in ion channels,[27] maturation and infectivity of HIV-1,[28−30] selectivity of Xaa-Pro amides by HIV-1 protease,[31] molecular switching in the HIV-1 capsid protein,[32] and regulation of mitosis by the newly discovered PPIase Pin1.[33−35]

Structural studies have demonstrated that CyP is highly selective for cis substrates. The tetrapeptide Ac-Ala-Ala-Pro-Ala-amidomethylcoumarin was shown bound in the hCyPA active site with a cis conformation about the central Ala-Pro amide.[36] Crystallography has also demonstrated that the Ala-Pro dipeptide binds to hCyPA in the cis conformation.[37] Additionally, the tripeptide succinyl-Ala-Pro-Ala-*p*-nitro-

† Tel: (804)-924-3135. Fax: (804)-924-3567. email: etzkorn@virginia.edu.

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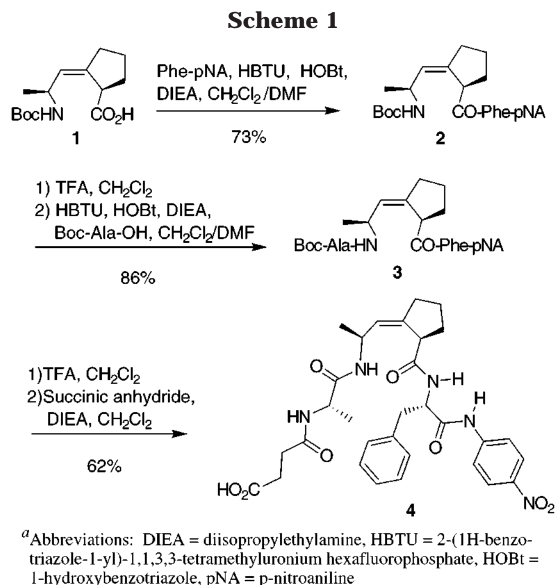
Scheme 1



[a]Abbreviations: DIEA = diisopropylethylamine, HBTU = 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, pNA = p-nitroaniline

anilide crystallized in the active site of *Escherichia coli* CyP was found in the cis conformation.[38] These observations take on greater significance considering that 70−90% of small prolyl peptides in solution are trans.[9]

We previously reported the stereoselective synthesis of **1**, a (*Z*)-alkene *cis*-Pro mimic of the Ala-Pro dipeptide (Figure 1),[39] by a route similar to the stereoselective synthesis of (*E*)-alkene non-proline dipeptide isosteres.[40] The (*Z*)-alkene *cis*-Pro mimic overlays the analogous dipeptide on the two vectors shown in Figure 1 with an RMS deviation of 0.17 Å, indicating that it is an ideal conformationally locked mimic of the bound substrate.[39] Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (succ-AAPF-pNA) is an excellent short peptide substrate for hCyPA, with a $K_{m(cis)}$ of 870 μM.[25] We designed mimic **4** based directly on this substrate by replacing the central Ala-Pro core with (*Z*)-alkene *cis*-Pro mimic **1**.

The peptide mimic was synthesized as shown in Scheme 1. Amides were coupled in solution using HBTU, and the succinyl N-terminus was installed with succinic anhydride. The sterically demanding coupling of acid **1** proceeded in good yield (73%). Concerns about β,γ-unsaturated acid or amide isomerization to the α,β-unsaturated compound proved unfounded, as no migration of the alkene was observed in any of the reactions.[39]

The chymotrypsin coupled assay was used to evaluate inhibition.[3,41,42] Final substrate (succ-AAPF-pNA) concentration was 100 μM (10 μM cis),[25] hCyPA concentration was 20 nM, and the concentration of inhibitor **4** was varied from 0.5 to 240 μM. Progress of the assays was monitored by release of *p*-nitroaniline (pNA), since it is known that chymotrypsin hydrolyzes only trans substrates.[41] However, we observed that chymotrypsin recognized peptide mimic **4** and slowly released pNA in the absence of hCyPA, indicating that chymotrypsin may recognize cis substrates, although poorly. The amount of pNA released from **4** during a typical assay was approximately 0.6% of the total inhibitor concentration. In control experiments containing only inhibitor **4** and chymotrypsin, over the course of approximately 1 h at room temperature, the amount of pNA released corresponded to the total amount of inhibitor present, confirming that chymotrypsin was not simply cleaving a small amount of (*E*)-alkene impurity. The presence of hCyPA had no affect on the rate of pNA release from **4**, eliminating the mechanistically intriguing possibility of alkene isomerization by hCyPA.

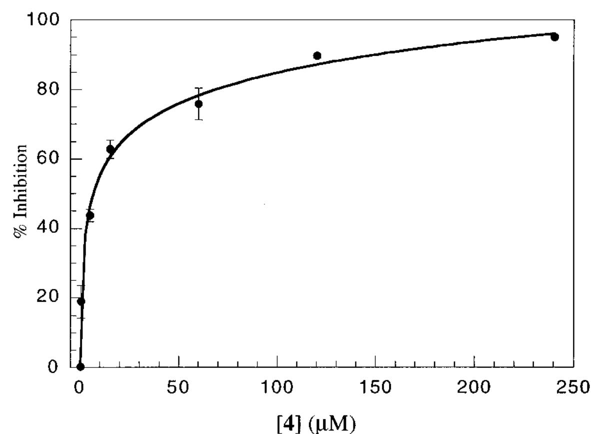


Figure 2. % inhibition vs concentration of **4** (μM). Calculated value of IC_{50} = 6.5 ± 0.5 μM was from a logarithmic fit by Kaleidagraph.

Due to *p*-nitroaniline insolubility, steady-state data to determine competitive inhibition could not be obtained by this assay.[25] The IC_{50} was determined to be 6.5 ± 0.5 μM from a plot of percent inhibition vs inhibitor concentration (Figure 2). A bicyclic lactam *cis*-Pro mimic has been found to bind hCyPA with a K_d of 5 μM by fluorescence saturation.[43−45] An (*E*)-alkene *trans*-Pro mimic included in an FKBP substrate sequence was shown to inhibit the PPIase activity of FKBP with a K_i of 8.6 μM.[46] Since cis substrates have been found several times in the active site of CyP, it is interesting that a trans substrate mimic inhibited FKBP PPIase activity with a similar magnitude. FKBP is known to bind α-keto amides with the ketone orthogonal to a cis amide,[15] which led to the hypothesis of a twisted amide transition state.[14,16] Unlike CyP, no structures of FKBPs have been reported with peptide substrates bound, so the preference for the cis or trans conformation is unknown, and these two inhibitor/PPIase complexes cannot be compared directly.

We have designed and synthesized an inhibitor of cyclophilin based on a (*Z*)-alkene amide bond isostere. The central Ala-*cis*-Pro core of the substrate succ-AAPF-pNA was replaced by a (*Z*)-alkene isostere. Substrate mimic **4** inhibits the PPIase activity of hCyPA with an IC_{50} of 6.5 ± 0.5 μM.

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Supporting Information Available: Experimental procedures for assays, synthesis, and characterization of compounds **2**, **3**, and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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