European Journal of Medicinal Chemistry 46 (2011) 5556–5561

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Oligopeptide cyclophilin inhibitors: A reassessment

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ARTICLE INFO

Article history:
Received 1 September 2011
Received in revised form
12 September 2011
Accepted 14 September 2011
Available online 21 September 2011

Keywords:
Cyclophilin
Cyclosporin A
Prolyl bond *cis*/*trans* isomerization
Inhibition
cis/*trans* ratio
Isothermal titration calorimetry

ABSTRACT

Potent cyclophilin A (CypA) inhibitors such as non-immunosuppressive cyclosporin A (CsA) derivatives have been already used in clinical trials in patients with viral infections. CypA is a peptidyl prolyl *cis*/*trans* isomerase (PPIase) that catalyzes slow prolyl bond *cis*/*trans* interconversions of the backbone of substrate peptides and proteins. In this study we investigate whether the notoriously low affinity inhibitory interaction of linear proline-containing peptides with the active site of CypA can be increased through a combination of a high *cis*/*trans* ratio and a negatively charged C-terminus as has been recently reported for Trp-Gly-Pro. Surprisingly, isothermal titration calorimetry did not reveal formation of an inhibitory CypA/Trp-Gly-Pro complex previously described within a complex stability range similar to CsA, a nanomolar CypA inhibitor. Moreover, despite of *cis* content of 41% at pH 7.5 Trp-Gly-Pro cannot inhibit CypA-catalyzed standard substrate isomerization up to high micromolar concentrations. However, in the context of the CsA framework a net charge of −7 clustered at the amino acid side chain of position 1 resulted in slightly improved CypA inhibition.

1. Introduction

Cyclophilins represent a subfamily of peptidyl prolyl *cis*/*trans* isomerases (PPIases, EC. 5.2.1.8) that can bind and catalytically interconvert prolyl *cis*/*trans* isomers in all folding states of a proline-containing polypeptide chain. The functional importance of the prototypic enzyme cyclophilin A (CypA) for a variety of biological processes such as viral infections [1–4], chronic inflammation [5–8] and malignancies [9–13] has stimulated a considerable interest in the development of CypA inhibitors as mechanistic tools and potential drugs for various diseases.

The immunosuppressive cyclic undecapeptide cyclosporin A (CsA) and non-immunosuppressive CsA derivatives represent low-nanomolar inhibitors of the PPIase activity of CypA [14,15]. Beside inhibition of CypA catalytic activity, CypA–CsA complex formation also precedes calcineurin inhibition and subsequent suppression of IL-2 expression which may explain how CsA mediated immunosuppression can arise [16]. Non-peptidic small molecule inhibitors have been designed but require considerable improvement to exhibit CsA-like inhibitory potencies [17]. While the active cleft of CypA orients CsA and oligopeptide substrates in the opposite direction of the contacting sites in their respective complexes topologies are defined by the superposition of the proline ring of the substrate and the MeVal-11 side chain of CsA [18]. The marked increase of about $>10^4$ fold in the CypA affinity for CsA when compared to a linear oligopeptide substrate can be rationalized by the transition state-like structural constraints imposed by the macrocycle [19]. In an extensive search of heptapeptide sequences derived from the HIV-1 Gag polyprotein capsid domain containing -Xaa-Gly-Pro- moieties only very weak CypA binders could be identified [20]. On the other hand, the internally positioned -Gly-Pro- sequence motif was found as an essential feature for CypA binders identified by phage display screening and by planar peptide arrays [21]. Consequently, the question arises whether linear peptide inhibitors of low molecular masses, whose particular amino acid sequences and internal charge distributions allow for a transition state complementary geometry, might exist. Recently, based on the identification of critical residues of CypA for the CypA–CsA interaction by calculating the electronic structure of both molecules [22], a virtual oligopeptide library database has been ranked for CypA affinity by using a molecular docking algorithm [23]. Interestingly, the calculations have resulted in a few charged oligopeptides that exhibited interaction energies superior to those of the CypA–CsA interaction. Based on these calculations the authors have carried out an experimental approach with PPIase assays, surface plasmon resonance analyses and viral replication data. The inhibitory tripeptide Trp-Gly-Pro eventually emerged, exhibiting a CypA binding and inhibiting affinity nearly as strong as

Abbreviations: CypA, cyclophilin A; CsA, cyclosporin A; PPIase, peptidyl prolyl *cis*/*trans* isomerase; ITC, isothermal titration calorimetry.

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doi:10.1016/j.ejmech.2011.09.019

those found for CsA. Even more, marked antiviral effects in a HIV-1 replication assay have been noted [23]. From the cellular viewpoint, these findings would be particularly intriguing since they implicate protein degradation products, especially those resulting from catalysis by proline-specific proteases, in the regulation of endogenous CypA activity.

Structurally, the major differences found between previously tested peptides and Trp-Gly-Pro were found in the charge distribution next to the critical proline and the presence of a Trp residue. To explain the possible origin for the said CsA-like biochemical effects, the interplay between a negative charge at the backbone and a potentially Trp-mediated increase of the *cis*/*trans* ratio was considered to play a dominating role.

Here we investigate anionic peptides in conjunction with an NMR-based analysis of prolyl *cis*/*trans* isomers to evaluate their CypA inhibitory potency. We show below that various PPIase assays and isothermal titration calorimetry revealed a drastic loss of CypA-peptide complex stability for peptides bearing a negative charge next to proline on the backbone. Taken together, our results do not only contradict CypA inhibition but furthermore indicate a lack of affinity of short linear oligopeptides, such as Trp-Gly-Pro, for CypA.

2. Results and discussion

2.1. Isothermal titration calorimetry

Direct comparison of surface plasmon resonance, which has been used as an activity independent binding assay by Pang et al. [23], and isothermal titration calorimetry (ITC) has been already utilized to analyze CypA inhibitor interactions [24]. Our ITC experiments indicated an exothermally formed CypA–CsA complex (ΔH = 10.6 kcal mol^{-1}) with a K_d value of 7.6 nM together with a complex stoichiometry of 1.02 in good agreement with published data [24,25]. However, under the same conditions, titration of Trp-Gly-Pro with CypA did not indicate any binding up to 25 μM peptide in the titration cell (Fig. 1).

It can be hypothesized that the reportedly high affinity of Trp-Gly-Pro resulted from the combination of a high *cis* prolyl isomer content and a negatively charged proline. Indeed, ^{1}H NMR investigations on linear oligopeptide substrates revealed that the K_M value of a *cis* isomer is significantly lower than that of the respective *trans* isomer [26,27]. It is also known that proline-containing linear peptides undergo relatively slow *cis*/*trans* equilibration after dissolution in aqueous buffer and that this might lead to a lag and latency issue for the *cis* isomer content. It follows that very slow isomerization kinetics might be able to disturb our ITC experiment [28]. Thus, we characterized the prolyl isomer composition of Trp-Gly-Pro in buffer solution at pH 7.5 by NMR spectroscopy.

2.2. Isomer-specific NMR signals

To identify isomer-specific ^{1}H and ^{13}C resonances of Trp-Gly-Pro the signals were completely assigned by means of homo- and heteronuclear 2D NMR spectra (Supplementary Tables S1 and S2). The *trans* and *cis* prolyl bond isomers were identified based on the Cβ and Cγ chemical shift values [29], and additionally confirmed by sequential ROEs between Gly2 Hα and either Pro3 Hα or Hδ. Due to strong resonance overlap, the *cis*/*trans* ratio was obtained from the

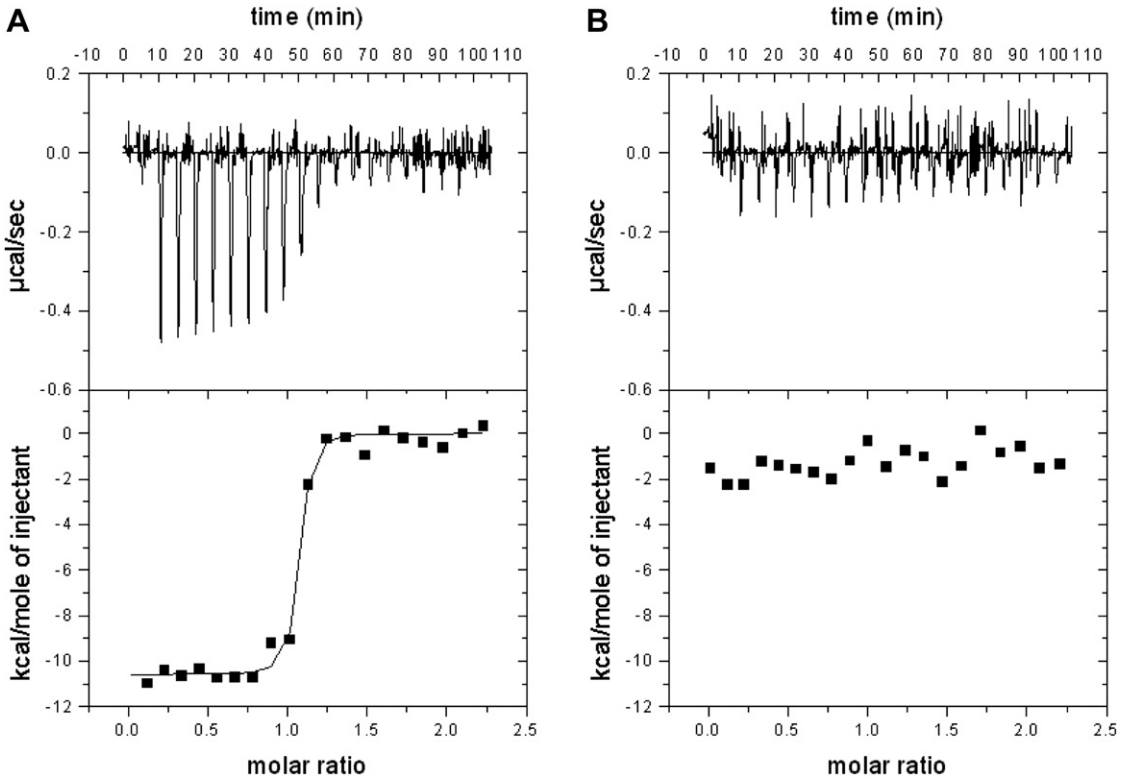

Fig. 1. Representative isothermal titration calorimetry of CsA (A) and Trp-Gly-Pro (B) with CypA in 10 mM HEPES buffer pH 7.5, 100 mM NaCl at 20 °C. For the CsA experiment each peak corresponds to the injection of 15 μl of 100 μM cyclophilin into the titration cell containing 10 μM CsA. For the tripeptide the titration cell contained 10 μM CypA which was titrated with 100 μM peptide solution. Capacity of the titration cell was 1.4 ml. Reference titration of buffer versus buffer, CypA versus buffer and peptide versus buffer were separately obtained and subtracted from the thermogram of the sample titrations. Cumulative heat of reaction is displayed as a function of the mole ratio of inhibitor/CypA (lower part). The solid line of the CsA titration represents the least square fit of the compiled data points. (A) CsA binding to CypA gave an association constant $K_a = (1.3 \pm 0.6) \times 10^8$ M, a complex stoichiometry $N = 1.023 \pm 0.08$ and a reaction enthalpy $\Delta H = 10.6 \pm 0.1$ kcal mol^{-1} (B) CypA did not show any affinity for the tripeptide in the isothermal titration calorimetry.