Probing Angiotensin Converting Enzyme (ACE) Domain-Dependent Inhibition of Onopordia, Isolated from Onopordon acanthium L., Using a Continuous Fluorescent Assay

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ABSTRACT

Background: Somatic ACE is a two-domain protein, C and N which are resulted from gene duplication. Presence of two active sites with particular properties, demonstrates functional significance of each domain. Increased levels of circulating N-acetyl-seryl-aspartyl-lysly-proline (Ac-SDKP), could be the result of ACE N-domain selective inhibition. Moreover, ACE C-domain specific inhibitors are able to inactivate bradykinin and inhibit the conversion of angiotensin I to angiotensin II in order to regulate blood pressure as well as reduced side effect profiles.

Methods: The present study was designed to determine ACE domain specificity of the novel ACE inhibitor, onopordia which was recently isolated from Onopordon acanthium L. The ACE inhibition activity was determined using Abz-SDK (Dnp)P-OH and Abz-LFK(Dnp)-OH as ACE domain selective substrates. IC₅₀ values of onopordia determined and compared with those of captopril as the standard.

Results: IC₅₀ values of onopordia for ACE N and C- domains were 180 μM and 244 μM respectively which demonstrated approximately similar affinity of the mentioned compound to ACE C and N-domains. A pharmacophore model was further generated based on onopordia interactions with the relevant ACE domain active sites.

Conclusion: According to onopordia interactions in the ACE C and N-domain active sits, it is a potential to generate more potent and also specific inhibitor based on this new scaffold by doing accurate adjustments. Therefore, this study provides the molecular basis for further designing ACE inhibitors, which are new therapeutics in combating tissue fibrosis diseases.

Introduction

Angiotensin-I converting enzyme (ACE; EC 3.4.15.1) is a zinc metallopeptidase of the M2 family which removes dipeptides from the C-terminal of polypeptides.¹ One of the most important functions of ACE is regulation of blood pressure via converting angiotensin I (Ang I) into a potent vasoconstrictor angiotensin II (Ang II) and also inactivation of bradykinin (a hypotensive peptide) by hydrolyzing it into inactive components.² In this regard, ACE inhibitors are widely used for treating hypertension and heart failure.² ACE is capable of cleaving some other substrates including AcSDKP, β-amyloid, angiotensin (1–7), etc.³ On the other hand, ACE inhibitors possessed some side effects including skin rash, hyperkalemia, renal insufficiency, dry cough and angioedema.⁴

ACE exists in two forms: somatic ACE and testicular ACE, representing unique physiological roles. While testicular ACE plays a conclusive role in reproduction,⁵ somatic ACE which is composed of two catalytic domains (N and C),⁶ is responsible for blood pressure regulation and electrolyte homeostasis.⁷ Despite the 60% sequence homology between the two domains, there are functional and structural differences between actives sites, which leads to substrate specificity and eventually inhibitor specificity.⁸,⁹

N-domain specific inhibitors including RXP407⁰ and 33RE¹¹ (Figure 1), resulted in increased levels of N-domain specific substrate, AcSDKP. The mentioned substrate is subjected to hydrolysis by the N-domain 50 times faster than C-domain¹² without side effects.

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associated with complete ACE inhibition. AcSDKP prevents the fibroblasts proliferation and eventually fibrosis in hypertensive patients. In addition, the high levels of AcSDKP contributes to the prevention of stem cell proliferation by preventing their entry into S-phase. On the other hand, C-domain specific inhibitors such as RXPA380, lisW-S, kAF and kAW (Figure 1), contributes in avoiding some of the common undesirable non-selective ACE inhibitors side effects, which include cough and angioedema. These data suggest contribution of the two domains in different biological processes. Therefore, searching for specific substrates and inhibitors, is an ongoing process. Present approved ACE inhibitors inhibit both domains with approximately similar affinities. The interests in finding domain selective inhibitors, led to design a fluorescent ACE assay using selective Fluorescence Energy Resonance Transfer (FRET) peptides as domain selective substrates. Medicinal plants are potential sources for drug discovery. In our recent reports, we have found six Iranian medicinal plants with promising ACE inhibition activity, and also isolated the active compound of one of them, called onopordia and introduced it as a new scaffold for ACE inhibition (Figure 2).

Materials and Methods

Chemical reagents

FRET substrates, Abz-SDK (Dnp)-OH and Abz-LFK(Dnp)-OH, were synthesized according to the literature. Onopordia was isolated from O. acanthium. Angiotensin converting enzyme (ACE) from rabbit lung
was purchased from Sigma-Aldrich Co. (England). Tris buffer (Tris(hydroxymethyl)-aminomethan), captopril, zinc chloride, dimethyl sulfoxide (DMSO) and hydrochloric acid, were purchased from Merck Co. (Germany).

**Apparatus**
A fluorescence spectrophotometer with thermostat and stirrer (Synergy H1, Hybrid Reader) (Winooski, VT, USA), and a black flat bottom polystyrene 96-well micro plate (SPL), were used to perform enzymatic incubation.

**ACE inhibition assay**
ACE C and N-domain inhibition activity was determined according to the strategy developed by Sturrock et al. The assay is based on hydrolysis of FRET substrates by ACE and measuring the amount of liberating fluorescence. The assay buffer was prepared using 0.1 M Tris, 50 mM NaCl, 10 µM ZnCl₂ and then adjusting the pH to 7. According to $K_m$ values for specific FRET substrates, Abz-SDK(Dnp)P-OH (50.6 µM ± 1.47) for N-domain and Abz-LFK(Dnp)-OH (3.43 µM ± 0.5) for C-domain, the stock substrate solutions were prepared by dissolving 1 mg of each of them in 1 ml DMSO. The final concentration of ND specific substrate was 151.8 µM and that of CD specific substrate was 10.29 µM. The inhibitor onopordia (1 mg) was dissolved in 1 ml of solvent containing buffer/DMSO (90:10, v/v). 30 µl substrate solution was added to the inhibitor solution (or buffer as positive control). The reaction was started by adding 50 µl ACE solution (25 mU/ml), followed by incubating the mixture at 37 °C for 60 min. Blank samples were prepared in the absence of enzyme and substrate, and the results were subtracted from samples. Cleavage of the FRET substrates induced an increased fluorescence that was measured continuously at $\lambda_{ex}/\lambda_{em} = 320/420$ nm.

**ACE inhibition measurement**
ACE inhibition activity was calculated based on the measured fluorescence at $\lambda_{ex}/\lambda_{em} = 320/420$ nm in an inhibitor sample to that of a control sample (without inhibitor) ratio as it is expressed by Eq. (1):

$$\text{ACE inhibition} \% = \frac{1 - \left(\frac{\text{fluorescence inhibitor}}{\text{fluorescence blank}}\right)}{\left(\frac{\text{fluorescence control}}{\text{fluorescence blank}}\right)} \times 100$$  

**Pharmacophore modeling**
LigandScout 3.0.5 was employed to create a 3D pharmacophore model of onopordia. Our previous study involved docking analysis of onopordia on ACE C and N-domains which predicted that the mentioned compound should represent approximately similar affinity for ACE domains. The pharmacophore model of essential interactions like hydrogen bond donors, hydrogen bond acceptors, hydrophobic interactions, etc, was developed using the best pose of onopordia at ACE-N catalytic site as a filter for screening databases (Figure 3). Hydrogen bonds between hydroxyl groups of onopordia and polar residues, Arg381 and Tyr369, are the prominent interactions in S₂ subsite of the ACE N-domain which was considered in pharmacophore model creation. Other interactions were detected including hydrogen bond interactions of His361 and His365 in S₁ subsite, hydrophobic interaction of Thr358 in S₁ subsite and hydrogen bond of Tyr501 with oxygen atom in onopordia.

**Result and Discussion**

**Determination of ACE inhibition activity**
Most of the clinical approved ACE inhibitors inhibit both ACE domains with similar affinity. While this permits efficient reduction in blood pressure, bradykinin accumulation caused by dual domain inhibition resulted in adverse drug effects.

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**Figure 3.** Chemical features that represent binding mode of onopordia to the ACE N-domain active site. Pharmacophore model was generated by LigandScout 3.0.5.
Therefore, selective inhibition of ACE C or N-domain leads to effective treatment with reduced adverse drug occurrence. IC\textsubscript{50} values for captopril as the standard ACE inhibitor and also those of the onopordia were determined using Excel software 2013 and demonstrated in Table 1. Comparison the IC\textsubscript{50} value of captopril in ACE N-domain with that of C-domain demonstrated similar but almost higher affinity of captopril to N-domain compare to C-domain. Inhibitors that selectively inhibit the N-domain (N-selective) could be useful in treating fibrosis and inflammatory due to build-up of N-domain specific substrate AcSDKP. Indeed, selective inhibition of N-domain, which is the primary site for the clearance of tetrapeptide AcSDKP, resulted in vivo increasing plasma levels of AcSDKP which play a prominent anti-inflammatory and anti-fibrotic role in heart, liver, kidney and lung tissues.\textsuperscript{13} 

According to our previous study, in silico experiments suggested a binding mode for onopordia with approximately similar affinities for ACE C and N-domains. Although, the current study confirmed the previous results, it demonstrated that the inhibition of the N-domain was slightly higher than that of C-domain at the same concentration of the inhibitor (Table 1). IC\textsubscript{50} values were determined from dose-response curve, using Excel software 2013 (Figure 4). Therefore, there should be a potential for onopordia in order to reach a specific N-domain inhibitor and subsequently be useful in the treatment of fibrosis and tissue injury by performing precise modifications. 

**Onopordia interactions with ACE C and N-domains** 
The major differences observed for the binding modes of the inhibitor in the two domains are due to the presence of the acrylate double bond, the isochroman-1-one ring and the hydroxyl groups on the phenyl ring. In our previous study interaction modes and also similar lowest binding energies of onopordia in ACE C and N-domains (resulted from docking studies), led to predict approximately similar affinities of onopordia to ACE C and N-domains.\textsuperscript{21} 

Docking studies of onopordia in ACE C and N-domains, hydrophobic interaction of acrylate double bond with Val518 in S\textsubscript{1} subsite and also that of isochroman-1-one ring with Val379 and Val380 in S\textsubscript{1} subsite resulted in moderate affinity of the ligand to ACE C-domain.\textsuperscript{21} Lower affinity of onopordia to ACE- S\textsubscript{1} and -S\textsubscript{1} subsites in N-domain in comparison with C-domain could be explained by the V518/T496, V379/T358 and V380/S357 alterations in ACE C/ACE N domains. The favorable hydrophobic interactions with hydrophobic residues in ACE -S\textsubscript{1} and -S\textsubscript{1} subsites have been lost because of replacing those hydrophobic residues with the polar ones in ACE N-domain. 

On the other hand, the hydroxyl groups on the phenyl ring were found to be in a right direction and enough close to polar residues, Tyr369 and Arg381, in S\textsubscript{2} subsite to develop favorable hydrogen bond interactions with ACE N-domain active site. These interactions have been lost due to replacing the mentioned residues with Phe391 and Glu403 in C-domain, which results in a reduced affinity of the inhibitor to ACE C-domain (Figure 5). In compound 33RE, which is recently reported as a selective ACE N-domain inhibitor, liability of the tetrazole ring permitted aromatic stacking binding of 33RE with His388 and also hydrogen bond with the hydroxyl group of Tyr369. Therefore, it has been proved that hydrogen bond interactions of S\textsubscript{2} subsite with the inhibitor plays the major role in N-domain selectivity.\textsuperscript{11} Moreover, N-domain selectivity in another selective ACE N-domain inhibitor, RXP407 is related to hydrogen bond interactions of S\textsubscript{2} subsite with the carboxylate moiety in the inhibitor.\textsuperscript{11} 

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
Inhibitor & IC\textsubscript{50} value for C-domain & IC\textsubscript{50} value for N-domain \\
\hline
Captopril & 2.81 ± 0.07 nM & 0.9 ± 0.06 nM \\
Onopordia & 244 ± 9.0 µM & 180 ± 1.8 µM \\
\hline
\end{tabular}
\caption{IC\textsubscript{50} values of captopril and onopordia for the C and N-domains of ACE.}
\end{table}

![Figure 4](image)

**Figure 4.** The IC\textsubscript{50} values of onopordia for (A, B) ACE C and N-domain.
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According to these results, ACE N-domain inhibition would be due to the presence of hydroxyl groups on phenyl ring in *onopordia*. However, presence of the acrylate double bond and the isochroman-1-one ring is responsible for ACE C-domain inhibition.

**Pharmacophore and ligand based design**

Pharmacophore model generation and virtual screening are established strategies in the rational development of small molecule hits. In this research, the best pose of *onopordia* at ACE-N domain, resulted from docking the inhibitor into the N-domain active site was used to generate a pharmacophore model. To better understanding the interactions and pharmacophore requirements of the inhibitor, binding site analysis was carried out prior to creating the pharmacophore model. Two hydrogen bond interactions which were pointed toward Tyr369 and Arg381, play the major role in ACE N-domain inhibition activity (Figure 5). Therefore, generated pharmacophore model could be the starting point in screening databases and discovering new scaffolds as ACE N-domain specific inhibitors.

**Figure 5.** Schematic diagram of hydrogen bonds (dashed lines, distances in 3.0 Å) and hydrophobic interactions (red symbols) between ACE N-domain (brown) and *onopordia* (blue) obtained from Ligplot.
Conclusion
According to the in vitro ACE assay, onopordia, a moderate ACE inhibitor, reveals almost similar affinities to ACE C and N-domains which approves docking results in our previous study. Based on IC₅₀ values of onopordia (244 µM for C and 180 µM for N-domain), it seems that there is slightly more affinity of onopordia to ACE N-domain than C-domain. One possible explanation for this aspect, is that despite the presence of hydrophobic interactions between acrylate double bond and the isochroman-1-one ring of the inhibitor with hydrophobic residues in ACE- S₁ and - S₂ subsites, the presence of hydroxyl groups on the other side of the inhibitor is the motive for a little increased affinity of onopordia to ACE N-domain. Therefore, this mild ACE inhibitor could be used as a suppressive medication over hypertension. Moreover, due to the onopordia structure, reaching a specific ACE N-domain inhibitor may be achievable by doing precise optimization.

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Conflict of interests
The authors claim that there is no conflict of interest.

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