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Research Article

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-lysyl-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. IC_{50} values of *onopordia* determined and compared with those of captopril as the standard.
Results: IC_{50} 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 metalopeptidase 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 RXP407[10] and 33RE[11] (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-domain[12] without side effects

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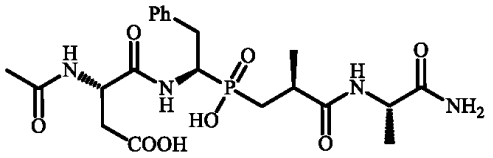
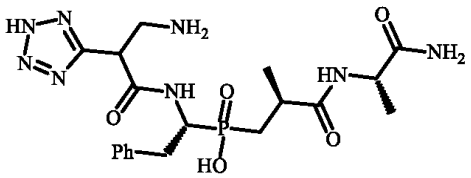


RXP407



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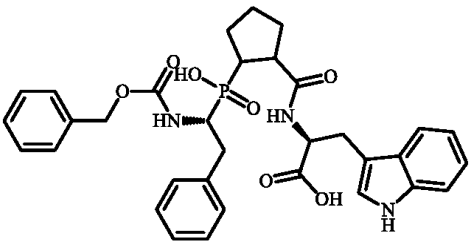
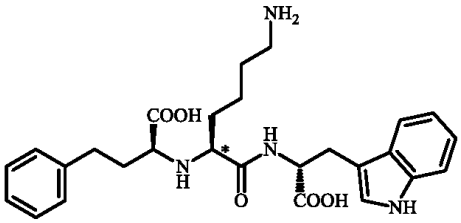
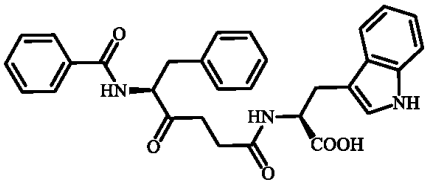


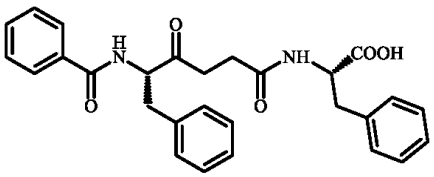
RXPA380



lisW-S



kAW



kAF

Figure 1. Chemical structure of ACE N-domain and ACE C-domain selective inhibitors.

associated with complete ACE inhibition. AcSDKP prevents the fibroblasts proliferation and eventually fibrosis in hypertensive patients.[13] In addition, the high levels of AcSDKP, contributes to the prevention of stem cell proliferation by preventing their entry into S-phase.[12] On the other hand, C-domain specific inhibitors such as RXPA380,[14] lisW-S,[15] kAF and kAW[16] (Figure 1), contributes in avoiding some of the common undesirable non-selective ACE inhibitors side effects, which include cough and angioedema.[17] These data suggest contribution of the two domains in different biological processes. Therefore, searching for specific substrates and inhibitors, is an ongoing process.[12]

Present approved ACE inhibitors inhibit both domains with approximately similar affinities.[18] 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.[19]

Medicinal plants are potential sources for drug discovery. In our recent reports, we have found six Iranian medicinal plants with promising ACE inhibition activity,[20] and also isolated the active compound of one of them, called *onopordia* and introduced it as a new scaffold for ACE inhibition (Figure 2).[21]

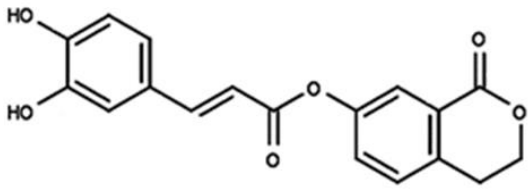


Figure 2. Structure of *onopordia*.

Since developing 3D-pharmacophore models are utilized in virtual screening of available databases and designing new hits, we have used this strategy to generate such a pharmacophore model in this study.

The aim was using an *in vitro* fluorescent assay to determine ACE domain specific inhibition activity of *onopordia* and then creating a pharmacophore model of *onopordia* interactions in ACE N-domain to perform virtual screening in the future in order to discover novel hits rationally as ACE inhibitors.

Materials and Methods

Chemical reagents

FRET substrates, Abz-SDK (Dnp)P-OH and Abz-LFK(Dnp)-OH, were synthesized according to the literature.[19] *Onopordia* was isolated from *O. acanthium*.[21] 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 H_1, 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.[22]

The assay buffer was prepared using 0.1 M Tris, 50 mM NaCl, 10 µM $ZnCl_2$ 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):

$$ACE\ inhibition\ \% = [1 - ((fluorescence\ inhibitor - fluorescence\ blank) / (fluorescence\ control - fluorescence\ blank))] \times 100 \quad \text{Eq. (1)}$$

Fluorescence $_{inhibitor:}$ fluorescence in the presence of inhibitor

Fluorescence $_{control:}$ fluorescence in the absence of inhibitor

Fluorescence $_{blank:}$ fluorescence in the absence of enzyme and substrate

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.[21] 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[21] 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 S_2 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 S_1 subsite, hydrophobic interaction of Thr358 in $S_{1'}$ 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.[18] While this permits efficient reduction in blood pressure, bradykinin accumulation caused by dual domain inhibition resulted in adverse drug effects.[3]

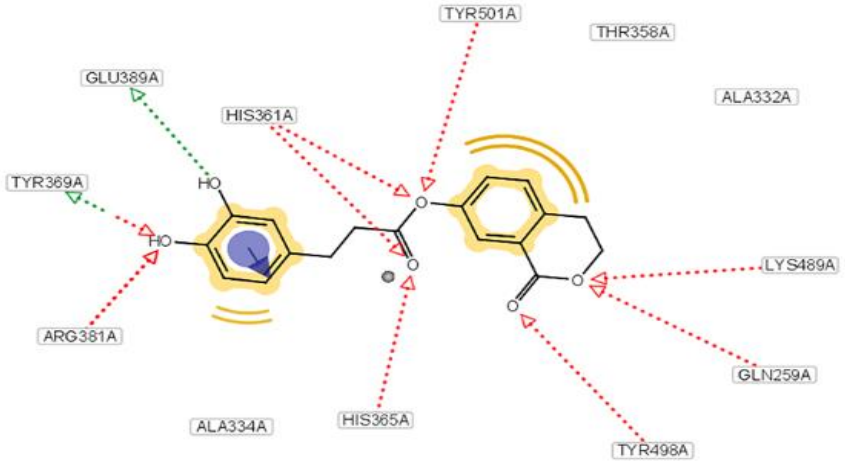


Figure 3. Chemical features that represent binding mode of *onopordia* to the ACE N-domain active site. Pharmacophore model was generated by LigandScout 3.0.5.

Table 1. IC_{50} values of captopril and *onopordia* for the C and N-domains of ACE.

Inhibitor	IC_{50} value for C-domain	IC_{50} value for N-domain
Captopril	2.81 ± 0.07 nM	0.9 ± 0.06 nM
Onopordia	244 ± 9.0 µM	180 ± 1.8 µM

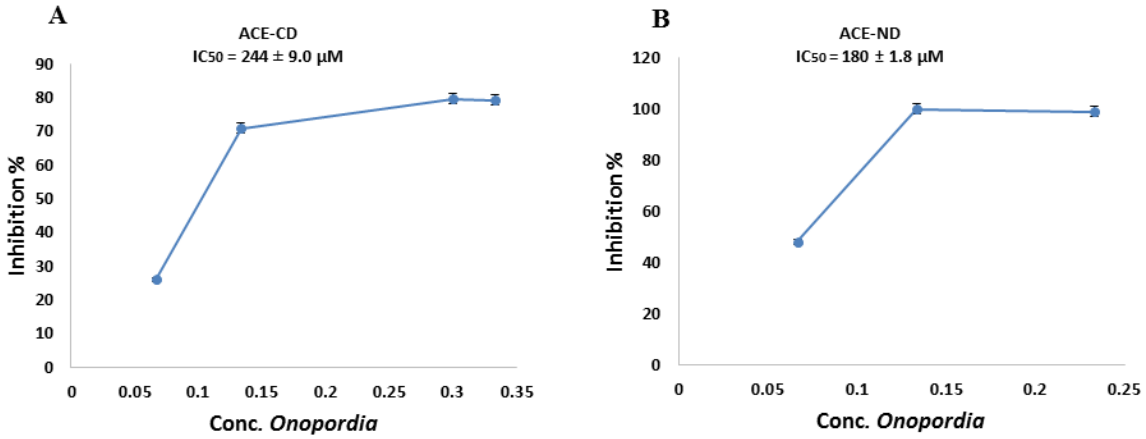


Figure 4. The IC_{50} values of *onopordia* for (A, B) ACE C and N- domain.

Therefore, selective inhibition of ACE C or N-domain leads to effective treatment with reduced adverse drug occurrence.

IC_{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_{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 Ac-SDKP. Indeed, selective inhibition of N-domain, which is the primary site for the clearance of tetrapeptide Ac-SDKP, 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.[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_{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 to 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.[21]

Docking studies of *onopordia* in ACE C and N-domains, hydrophobic interaction of acrylate double bond with Val518 in S_1 subsite and also that of isochroman-1-one ring with Val379 and Val380 in $S_{1'}$ subsite resulted in moderate affinity of the ligand to ACE C-domain.[21] Lower affinity of *onopordia* to ACE- S_1 and -$S_{1'}$ 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_1 and -$S_{1'}$ 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_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_2 subsite with the inhibitor plays the major role in N-domain selectivity.[11] Moreover, N-domain selectivity in another selective ACE N-domain inhibitor, RXP407 is related to hydrogen bond interactions of S_2 subsite with the carboxylate moiety in the inhibitor.[11]

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.

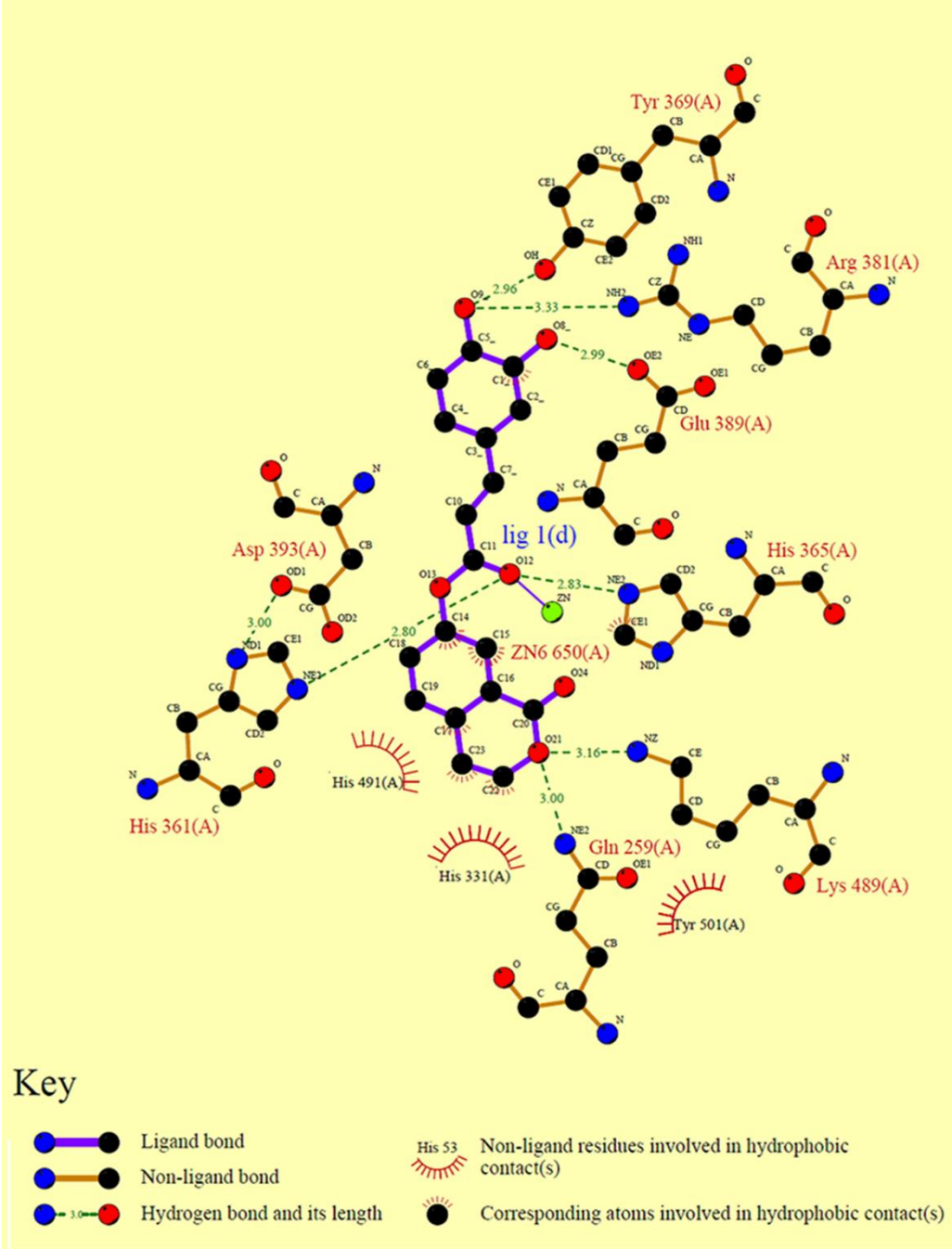


Figure 5. Schematic diagram of hydrogen bonds (dashed lines, distances in 3.0 A°) and hydrophobic interactions (red symbols) between ACE N-domain (brown) and *onopordia* (blue) obtained from Ligplot.

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 pervious study.[21] Based on IC_{50} 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- S_1 and - $S_{1'}$ 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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