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Design, Synthesis and Biological Activity of Novel Non-Peptidyl Endothelin Converting Enzyme Inhibitors, 1-Phenyl-tetrazole-formazan Analogues

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Abstract—A novel non-peptidyl endothelin converting enzyme inhibitor was obtained through a pharmacophore analysis of known inhibitors and three-dimensional structure database search. Analogues of the new inhibitor were designed using the structure–activity relationship of known inhibitors and synthesized. In anesthetized rats, intraperitoneal administration of the analogues suppressed the pressor responses induced by big endothelin-1.

Introduction

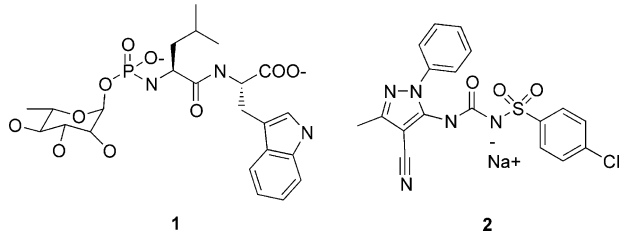
Since endothelin-1 (ET-1) was discovered in 1988 as a 21 amino acid peptide causing a potent and continuous blood vessel contraction, many studies have been conducted to uncover its mode of action.[1] ET-1 is biosynthesized by cleavage of the latent precursor big ET-1 at Trp21-Val22 bond by endothelin converting enzyme (ECE), a zinc metalloprotease.[2] ET-1 receptor antagonists or ECE inhibitors are expected to be effective against such diseases as hypertension, cardiovascular diseases and renal failure.[3]

Phosphoramidon (**1**, Fig. 1), a potent inhibitor of neutral endopeptidase (NEP), inhibits ECE with IC_{50} of 690 nM.[4] Some peptide-mimetic analogues of **1** are also reported as ECE inhibitors.[5] SM-19712 (**2**, Fig. 1) inhibits ECE with IC_{50} of 42 nM, 16-fold more potent than **1**.[6] SM-19712 was expected to be an orally available drug for chronic diseases since it is a non-peptidyl small molecule. In fact orally administered **2** suppressed the pressor response induced by big ET-1 in anesthetized rats.[6] However, the in vivo potency of **2** was not consistent with that anticipated from in vitro activity, probably because of strong binding to the plasma proteins.[6]

To develop an oral drug for chronic diseases, we set out to find a new non-peptidyl small molecule lead. We have identified as new leads novel 1-phenyl-tetrazole-formazan analogues through pharmacophore analysis by superposition of the binding features of **1** and **2** followed by a combination of 3-dimensional structure database search using the pharmacophore and incorporation of some substituents consisted of **2**. Intraperitoneal administration of these leads in anesthetized rats suppressed the pressor responses induced by big ET-1, and the potency was consistent with that anticipated from in vitro activity.

Pharmacophore Analysis

To superpose molecular structures for pharmacophore analysis, conformational coordinates of the compounds in solution were determined. Conformational analysis



1 2

Figure 1. Chemical structures of phosphoramidon (**1**) and SM-19712 (**2**).

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of **2** was performed with the Macromodel, version 4.5.[7] The Monte Carlo search was employed to identify the local energy minimum conformations. At each Monte Carlo step, the geometry was optimized using AMBER* force field[7] with a distance-dependent dielectric constant in aqueous solution of GB/SA model,[7] and with a 0.001 kcal/mol energy gradient convergence criterion. In 3000 Monte Carlo steps, two lowest energy conformations and 22 independent low-energy ones within 10 kcal/mol were obtained (Fig. 2).

On the other hand, **1** had a large number of low-energy conformations. Fortunately, some crystal structures of the derivatives of **1** complexed with thermolysin (TL), one of the zinc protease family members, were reported.[8] C terminal domain of ECE, consisted of 304 amino acids and containing the active site, has about 20% sequence identity to TL.[9] Therefore we assumed that active conformation of **1** on ECE was similar to that on TL. To simplify the superposition analysis, a model molecule **3** (Fig. 3) was actually used in place of **1**. In **3** the sugar moiety of **1** was replaced with a benzyl group and the leucyl residue with a phenylalanyl, replacements justified by the structure–activity relationship information of **1** against ECE.[10] The crystal structure coordinates in TL were used for the main chain and the beta carbons of **3**. Coordinates of other atoms were optimized locally under the same conditions as that used for conformational analysis of **2**.

Three enzyme binding features, hydrogen bond acceptor, hydrogen bond donor and hydrophobic center, were assigned manually to both **2** and **3** using the DISCO module of the SYBYL, version 6.3.[11] The hydrogen bond features were defined as projection points at the position of 300 nm to the lone pair direction, in order to simulate the actual environment better. The phosphorous and carboxyl groups of **3** and the sulfonamide group of **2** were treated as deprotonated. Twelve features were assigned to **2** and nine to **3** (Fig. 3).

The best superposition between the features of **2** and **3** was searched exhaustively using all the conformations with the DISCO module of the SYBYL, version 6.3.[11] Consequently two independent superpositions were obtained. In one of the two, seven features were superposed within 0.11 nm r.m.s. (Fig. 4), and in the other eight features were within 0.14 nm r.m.s. (not shown). Since the molecular shapes in the former superposition were more similar, the pharmacophore model was adopted for the molecular design of new lead.

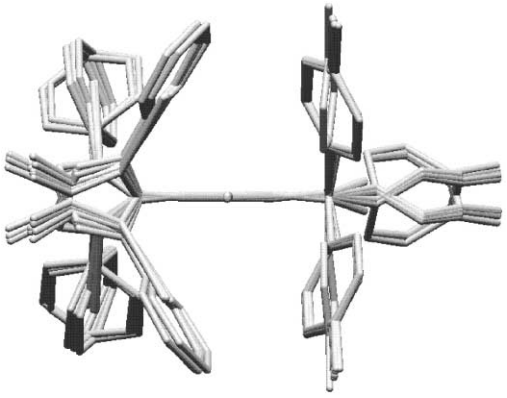


Figure 2. 24 independent low-energy conformations of **2**.

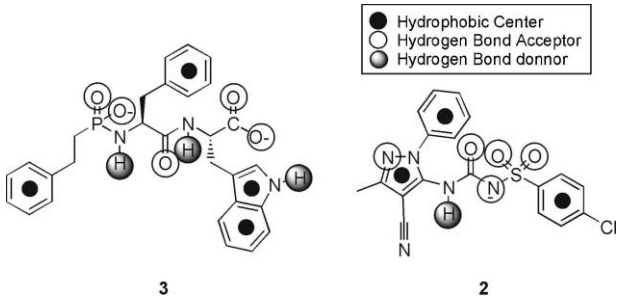


Figure 3. Enzyme binding features assigned to compounds **2** and **3**.

Molecular Design

The superposition model suggested that the nitrogen atom of pyrazole of **2** would interact with the zinc atom of ECE. It also suggested that the phenyl rings adjacent to pyrazole and sulfonyl were at P1′ and P2′ sites respectively in the active site of ECE.

To design a novel ECE inhibitor, a three-dimensional structure database was searched with the pharmacophore information using the ISIS, version 2.2.[12] The three substructures mentioned above in the relative arrangement shown in Figure 5 was used as a query, and the ACD database containing commercially available compounds[12] was searched. The tolerance level in the search was set at 0.03 nm. Seventeen compounds were found as hits by the search. Only S13946-7 (**10a**, Salor Inc.)

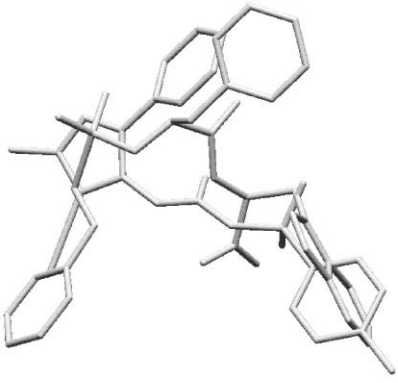


Figure 4. The superposition model of compounds **2** and **3**.

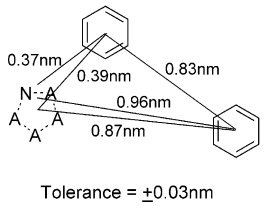


Figure 5. The query for the three-dimensional structure database search.

among the hits was consistent with the structure–activity relationship of **2** (in-house data).

It was suggested that the phenyl ring attached to the diazo group of **10a** was equivalent to the phenyl ring attached to the sulfonyl of **2**. The phenyl ring of **2** was substituted with a chlorine at the *p*-position. Thus, compounds **10b–i** were designed by substituting the *p*-position of the phenyl ring of **10a**.

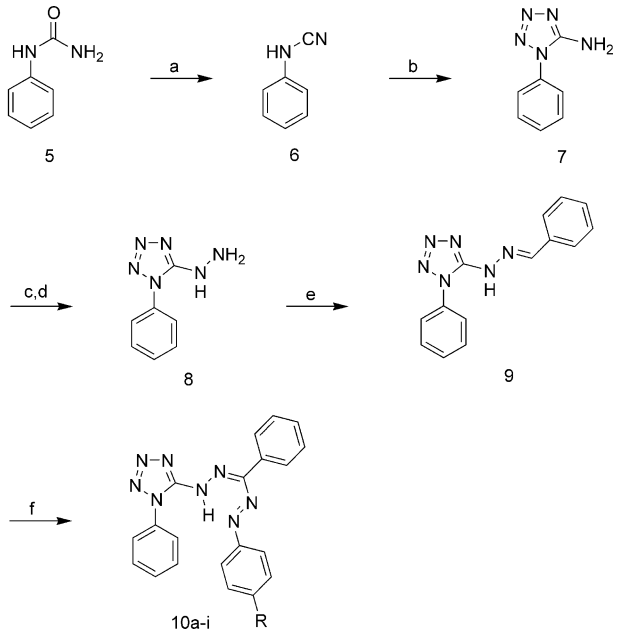
Chemistry

The compound **10a** was obtained from Salor Inc. (Catalog Code. S13946-7). Derivatives of **10a** (**10b–i**) were synthesized as outlined in Scheme 1. The common intermediate, **9**, was prepared by known methods.[13] The starting material **5** was commercially available. In the last process, the objective compounds were obtained by the azo-coupling of **9** with the corresponding *p*-substituted phenyldiazonium chloride.

The structures of **10b–i** were verified by LC–MS and NMR spectroscopy. The geometrical isomerism of these compounds was not determined. However, it was known that the main product is *cis–trans* form because of making intramolecular hydrogen bonding.[13]

Results and Discussion

The inhibitory activities of **10a–i** against ECE in the solubilized fraction from rat lung membrane were shown in Table 1.[6]



Scheme 1. Synthesis of compounds **10a–10i**. Reagents, conditions and yields: (a) $Pb(OAc)_2$, KOH, H_2O, 100 °C, 5 min, 80%; (b) NaN_3, NH_4Cl, DMF, 80 °C, 2.5 h, 66%; (c) $NaNO_2$, HCl, H_2O, 0 °C, 1 h; (d) Zn, AcOH, H_2O, 10 °C, 30 min, 28% (two steps); (e) PhCHO, EtOH, rt, 10 min, 70%; (f) *p*-R-PhN_2Cl, pyridine, EtOH, rt, 5 min, 50–96%.

The compound identified by the database search, **10a**, showed an ECE inhibitory activity ($IC_{50} = 10$ μM). The *p*-chloro substituted compound **10b** showed less potency, but the alkyl substituted analogues **10c–i** showed more potent activities than **10a**. Among them **10e** showed the highest activity ($IC_{50} = 2.6$ μM). Although **10e** is 2 orders of magnitude less potent than **2**, it is about 1/3 as potent as **1**, thus we consider it a good lead for optimization.[6]

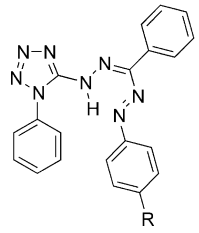
Intraperitoneal administration (10 mg/kg ip) of **10e** in anesthetized rats suppressed the pressor responses induced by big ET-1.[6] Actually, the plasma level of ET-1 was lowered to 42.9 ± 4.1% of that observed by intraperitoneal administration of saline ($n = 3$).

Protein binding rate of **10e** in rat serum was determined as 99%, identical to that of **2**, though protein binding at this high potency level could not be determined precisely. A minute difference of the plasma protein binding could have a large effect on ECE inhibitory activity.

The proposed pharmacophore model suggests that **2** binds at P1′ and P2′ in the active site of ECE and the methyl group at 3-position of pyrazole is directed toward P1 and P2. It is known that the replacement of this methyl group with a phenylalkoxylcarbonylmethyl group leads to more potent activity.[14] On the other hand, it was reported that the benzyloxycarbamate (Z) group at P2 site of **1** was suitable for ECE inhibitory activity.[15] This correspondence at P2 site verifies further the pharmacophore model. We will report the structure–activity relationship of **2** in more detail in another article.

Conformational profile of **10a** was analyzed with the same conditions as **2**, giving six independent conformations

Table 1. In vitro assay results for compounds **10a–10i**



Compd	R	In vitro assay[a] IC_{50} (μM)
1		0.69
2		0.042
10a	H	10.0
10b	Cl	37%[b]
10c	Me	8.4
10d	Et	3.8
10e	*n*Pr	2.6
10f	*i*Pr	3.4
10g	*s*Bu	2.7
10h	*t*Bu	3.8
10i	Cyclohexyl	3.6

[a] $n = 2$.
[b] % inhibition at 10 μM.

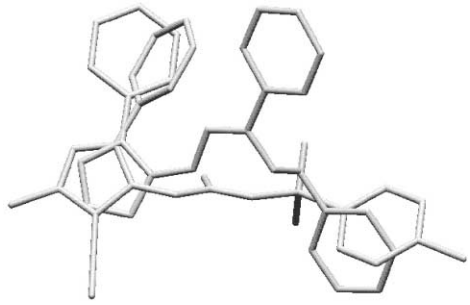


Figure 6. Superposition model of **2** and **10a**.

with minimum energy. One of the minimum energy conformations with a potential energy 3.9 kcal/mol higher than the lowest was similar to **2** in the topology of three rings used as the query in the three-dimensional structure database search (Fig. 6). None of the formazane analogues has a negative charge fragment that corresponds to the sulfonamide in **2**. On the other hand, the phenyl ring next to hydrazone of **10a** corresponds to no fragment in **2** but to the side chain of phenylalanine in **3**. These results suggest that the absence of the negative charged fragment in **10a** is compensated with this phenyl ring.

Conclusions

New non-peptidyl ECE inhibitors were found through a pharmacophore analysis of two known ECE inhibitors and a rational design based on the analysis. Intraperitoneal administration to anesthetized rats of one of these inhibitors suppressed the pressor responses induced by big ET-1. The inhibitors found in this study apparently lacked a negative ionizable fragment that can be a zinc chelater. Only a few other ECE inhibitors without a negative ionizable fragment are known to date.[16] This study demonstrated that rational drug design of metalloprotease inhibitors without a chelater is relevant for lead discovery.

Acknowledgements

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