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Bioorganic & Medicinal Chemistry Letters 15 (2005) 1075–1078
Bioorganic & Medicinal Chemistry Letters

Design, synthesis, and evaluation of oxazole transthyretin amyloidogenesis inhibitors

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Received 10 November 2004; revised 6 December 2004; accepted 9 December 2004
Available online 13 January 2005

Abstract—Ten oxazoles bearing a C(4) carboxyl group were synthesized and evaluated as transthyretin (TTR) amyloid fibril inhibitors. Substituting aryls at the C(2) position of the oxazole ring reveals that a 3,5-dichlorophenyl substituent significantly reduced amyloidogenesis. The efficacy of these inhibitors was enhanced further by installing an ethyl, a propyl, or a CF_3 group at the C(5) position. The CF_3 substitution at C(5) also improves the TTR binding selectivity over all the other proteins in human blood.

Transthyretin (TTR) is a homotetrameric protein composed of 127-amino acid subunits that functions as the primary transporter of L-thyroxine (T4) in cerebral spinal fluid, as well as a carrier of T4 and holo retinol binding protein in plasma.[1,2] Although TTR is a stable protein under physiological conditions (pH 7.2), it dissociates to monomers under partial denaturing conditions, which is typically rate-limiting for amyloidogenesis. However, dissociation is not sufficient for amyloidogenesis; the folded monomer has to partially denature in order to misassemble into aggregates including amyloid fibrils.[3] Soluble aggregates of TTR and/or amyloid fibrils have been implicated as the causative agents in diseases such as senile systemic amyloidosis,[4] familial amyloid cardiomyopathy,[5] and familial amyloid polyneuropathy.[6]

Since it is not yet clear whether the soluble aggregates or the amyloid fibrils themselves lead to neuropathology, the most conservative strategy is to stabilize the native tetramer utilizing small organic molecules.[7] Molecules that bind TTR and selectively stabilize the tetramer over the dissociative transition state raise the kinetic barrier dramatically slowing or preventing tetramer dissociation.[8]

To date, hundreds of structurally different compounds in several distinct classes have been identified as inhibitors of TTR fibril formation. Most inhibitors have two aromatic substructures connected by a variety of linkers. Generally, one aromatic ring bears hydrophobic substituents (e.g., halogens), while the other ring is substituted with hydrophilic moieties (e.g., carboxylic acid or hydroxyl groups). Examples of efficacious TTR amyloidogenesis inhibitors include substituted biaryl amines,[9] biaryl ethers,[10] anthranilic acids,[11] bivalent inhibitors,[12] *N*-phenyl phenoxazines,[13] and analogs of the non-steroidal anti-inflammatory drugs flufenamic acid,[14] diclofenac,[15] and diflunisal.[16] In our attempt to identify novel classes of TTR inhibitors with enhanced potency and improved binding selectivity to TTR over other plasma proteins, we have examined aryl oxazoles bearing a carboxyl group at the C(4) position.

The oxazole core can be accessed via the oxidation of oxazolines. For the construction of oxazolines, we relied on the method reported by Wipf and Miller which utilizes [(methoxycarbonyl)sulfamoyl]triethylammonium hydroxide inner salt (Burgess Reagent) as an agent for cyclodehydrating peptides and *N*-acyl amino esters.[17]

Starting materials **1a-1d** (Scheme 1; Supplementary data), prepared by benzoylation of L-threonine benzyl ester, were treated with Burgess Reagent in THF at reflux for 2 h. This method provides oxazolines via S_N2 intramolecular cyclization with inversion of

Keywords: Amyloidogenesis inhibitors; Aryl oxazole; Inhibitor binding selectivity; Transthyretin.
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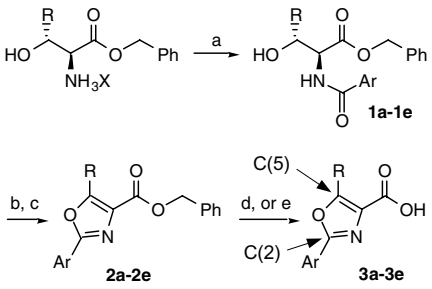
doi:10.1016/j.bmcl.2004.12.022



Scheme 1. Reagents and conditions: (a) ArCOCl, CH_2Cl_2, DIEA [X = Cl, $C_2H_2O_4$ (hemioxalate)] (70–84%); (b) Burgess Reagent, THF, reflux; (c) DBU, $BrCCl_3$, CH_2Cl_2 (34–70%, over two steps); (d) H_2, Pd–C, MeOH (96–99%); (e) LiOH, THF, MeOH, H_2O (98–99%). See Supplementary data for a detailed description of all experimental procedures. [R = Me, Ar = 3,5-difluorophenyl (**3a**); R = Me, Ar = 3-(trifluoromethyl)phenyl (**3b**); R = Me, Ar = 3,5-dichlorophenyl (**3c**); R = Me, Ar = phenyl (**3d**); R = H, Ar = 3,5-dichlorophenyl (**3e**)].

configuration at the amino acid β-carbon. However, the stereochemical consequences of these reactions were unimportant to this study, since the products were immediately converted into oxazoles by mild oxidation with $BrCCl_3$/DBU.[18] Subsequently, esters **2a-2d** were hydrogenated or hydrolyzed to their corresponding acids **3a-3d**. Compound **3e** was synthesized from *N*-benzoylated L-serine benzyl ester **1e**, using the approach outlined in Scheme 1 (see Fig. 1 for line drawings of the products).

C(5)-substituted analogs were synthesized according to a method described previously.[19] This method employs a glycine-derived thioimidate, a nitrile ylide synthon used in the synthesis of a variety of heterocyclic compounds such as oxazolines, oxazoles, and thiazoles. We envisaged that the latter method could be applied to synthesize a panel of C(5)-substituted oxazoles with a glycine-derived thioimidate serving as an advanced intermediate.

Therefore, glycine methyl ester hydrochloride was converted to methyl azidoacetate by means of a diazo transfer reaction (Scheme 2).[20] The resulting azide was treated with diphenyl methylphosphine yielding the phosphazene derivative, which was transformed in situ to its corresponding thioimidate using a method described previously.[21] The reaction of the glycine-derived thioimidate with various acyl chlorides or anhydrides in the presence of diisopropyl ethyl amine, followed by the hydrolysis of the resulting products, generated the corresponding oxazoles **4-8** in low to moderate overall yields (Scheme 2, and see Fig. 1 for line drawings of the structures prepared; Supplementary data).

Our strategy for identifying oxazole-based inhibitors is based on the synthesis and evaluation of a limited number of suitably substituted analogs depicted in Figure 1. Putative suitable substructures are based on those found in other potent TTR amyloidogenesis inhibitors including aromatic carboxylic acids and halogenated aromatics.[9–16] We envisioned that the carboxylic acid moiety that interacts with the ammonium group of Lys 15 and Lys 15′ within TTR could be directly displayed on the C(4) of the oxazole ring while the halogenated

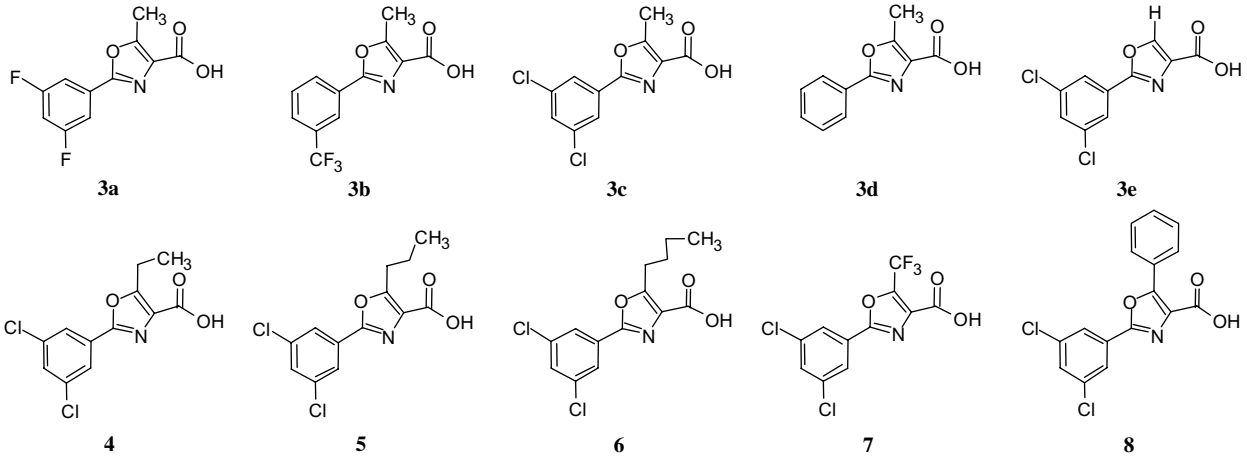
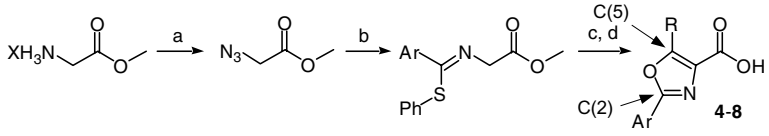


Figure 1. Oxazoles utilized in this study. Synthesis pathways, and procedures are described in Schemes 1 and 2, and in the Supplementary data section, respectively.



Scheme 2. Reagents and conditions: (a) $CF_3SO_2N_3$, CH_2Cl_2, DIEA (44%); (b) $MePh_2P$, ArCOCl (Ar = 3,5-dichlorophenyl), PhSH, DIEA (16%); (c) RCOX, DIEA, CH_2Cl_2 [R = Et (**4**), *n*-Pr (**5**), *n*-Bu (**6**), CF_3 (**7**), Ph (**8**); X = Cl, CF_3CO_2]; (d) LiOH, THF, MeOH, H_2O (10–79% over two steps). See Supplementary data for a detailed description of all experimental procedures.

aromatic ring could be linked to the oxazole core at the C(2) position.

The activity of the synthesized oxazoles was evaluated in duplicate using the TTR amyloid fibril formation assay,[10] and the human plasma binding-selectivity assay.[22] The amyloid fibril formation assay revealed that simple oxazole **3d** was a poor inhibitor of TTR fibril formation (Figs. 1 and 2A). However, the display of halogens on the phenyl substituent attached at C(2) improves the activity of the parent compound (Figs. 1 and 2A, cf. **3d** vs **3b** or **3c**). The *meta*-CF_3 and 3,5-dichloro aryl substituents appeared optimal for anti-amyloidogenesis activity (Fig. 2A). This improvement in activity is likely to be attributable to the favorable hydrophobic interaction(s) between the halogenated aryl and the so-called halogen binding pockets of TTR. In this panel of compounds, the 3,5-dichloro substituted pattern that imparts the highest level of activity serves as a lead compound for further optimization.

The second group of compounds was synthesized to probe the role of the C(5) substituent on activity relative to the lead compound **3c**. The activity of the inhibitors increased when the alkyl chain at the C(5) position was lengthened. Optimal activity is observed for the ethyl and propyl analogs (Fig. 2B, cf. **4** or **5** vs **3c** or **6**, respectively). Replacement of the methyl substituent

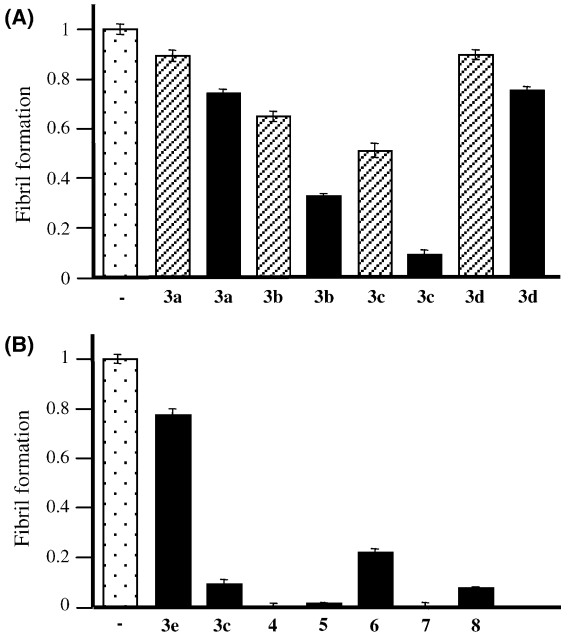


Figure 2. The influence of oxazole-based inhibitors on transthyretin amyloid fibril formation. The relative fibril yield was determined after a 72 h incubation period under partial denaturing conditions (pH 4.4, 37 °C) in the presence or absence of inhibitor. Yields were expressed relative to that obtained for TTR (3.6 μM) in the absence of inhibitor (speckled bars). (A) The influence of substituents at the C(2) position of the oxazole ring on TTR amyloidogenesis. The respective inhibitors were applied at a concentration of 3.6 μM (hatched bars) or 7.2 μM (solid bars). (B) The influence of the substituents at the C(5) position of the oxazole ring on TTR amyloidogenesis. All inhibitors were applied at a concentration of 7.2 μM (solid bars). Compounds **3a**–**3e** and **4**–**8** are substituted oxazole-4-carboxylic acids as depicted in Figure 1.

Table 1. In vitro transthyretin binding selectivity assay with human blood plasma

Entry	Compound[a]	Molar equivalence bound[b]
1	**4**	0.49 ± 0.07
2	**5**	0
3	**7**	0.68 ± 0.04
4	**8**	0

[a] Compounds identified as good inhibitors in the fibril formation assay (Fig. 2).

[b] Binding selectivity to transthyretin in human blood plasma (transthyretin concentration ≈5.4 μM based on ELISA assay) after a 24 h incubation period (37 °C) in the presence of inhibitor (10.8 μM), as determined by reverse phase HPLC.[22]

with hydrogen severely diminished the activity of the parent compound (Fig. 2B, cf. **3e** vs **3c**). The activity of the lead compound is also improved upon introduction of a CF_3 moiety (Fig. 2B, compound **7**). These results suggested that a hydrophobic group of the appropriate size is required at C(5) for optimal activity of an oxazole-based TTR amyloid inhibitor.

The more potent amyloid inhibitors (<10% fibril formation at a concentration of 7.2 μM) were further subjected to the immunoprecipitation, HPLC-based assay to evaluate compound binding stoichiometry to TTR in human blood plasma. These data reveal that compounds **4** and **7** were moderately selective for TTR in plasma (Table 1). The results suggest that the affinity of oxazole-based inhibitors for TTR over other plasma proteins is sensitive to the size of the C(5) substituent (Table 1, cf. **4** vs **5**). The information gathered from both assays indicates that the C(5) substituent is not only important for activity, but also for binding selectively to TTR over all the other plasma proteins.

In conclusion, oxazoles containing a C(4) carboxylic acid, a C(5) alkyl group and a C(2) halogenated aromatic pharmacophore were synthesized. Evaluation of these compounds reveals that several compounds in this class inhibit TTR amyloidogenesis in vitro and two bind TTR in human plasma with modest selectivity. In particular, compounds **4** and **7** completely inhibit fibril formation at 7.2 μM (TTR 3.6 μM) and bind to TTR in the presence of other plasma proteins with a stoichiometry of at least 0.5 (2 equiv being maximal).

Acknowledgements

We gratefully acknowledge financial support by the NIH (DK046335), the Skaggs Institute for Chemical Biology, and the Lita Annenberg Hazen Foundation. H. R. acknowledges the Skaggs Institute for Chemical Biology for a postdoctoral fellowship.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.12.022.

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