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NOVEL REVERSE-TURN MIMICS INHIBIT FARNESYL TRANSFERASE

Ruiping Liu, Dennis L.-Y. Dong, Rosemarie Sherlock, H. Peter Nestler*

Cold Spring Harbor Laboratory, One Bungtown Road, Cold Spring Harbor, NY 11724, USA

Cesare Gennari*, Antonia Mielgo, Carlo Scolastico*

Dipartimento di Chimica Organica e Industriale, Università di Milano. via Venezian 21, 20133 Milano, Italy

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Abstract: Reverse-turn inducing bicyclic lactams were incorporated into the substrate sequence recognized by farnesyl transferase to create inhibitors of RAS farnesylation. While the free peptides did not show any effect on the farnesylation, their Fmoc-protected counterparts impede the transformation of RAS with IC_{50}'s in the low micromolar range.

RAS proteins play a central role in normal cellular physiology and pathophysiology and are commonly found in human tumors.[1,2] RAS proteins are initially synthesized in the cytoplasm where they undergo a series of post-translational modifications at their carboxy terminal sequence, the CaaX-box (where *C* is cysteine, *a* are aliphatic amino acids and *X* is methionine or serine), resulting in their farnesylation with subsequent cleavage and carboxy methylation.[3,4] The processed proteins become localized to the cell membrane, a step which is essential to their functioning. The farnesylation cascade has been considered to be a logical target for the development of cancer therapeutics.[5,6,7,8] In fact, inhibitors of the farnesyl transferase (FTase) can induce morphological reversion of cells transformed by RAS at concentrations that do not arrest normal cell growth.[5,6,9] Moreover, non-farnesylated oncogenic ^{12}Val-RAS has shown to sequester RAF to the cytosol, thus leading to a cytostatic effect in transformed cells while untransformed cells only show a slower growing and proliferating phenotype.[10] Therefore, FTase inhibitors may be generally useful as anti-cancer agents.

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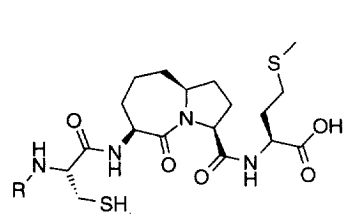
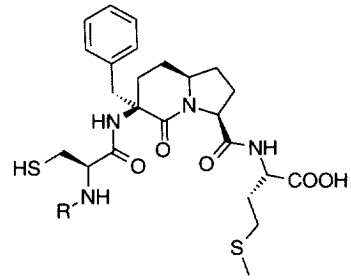
Structural studies on the FTase[11] revealed that the enzyme has two substrate recognition pockets in its active site to bring together the two partners of the farnesylation reaction, farnesyl pyrophosphate (FPP) and the CaaX-containing proteins. While compounds that compete with FPP have been used to inhibit FTase,[12] peptides and peptide mimics that compete with the protein in the CaaX-box binding site may show more potential for inhibitory activity and tuning of selectivity.[13] Initial structural investigations of substrates and inhibitors suggested an active conformation similar to a β-turn.[7,14,15,16,17] However, several other papers have postulated extended conformations for potent peptidomimetic inhibitors.[18,19,20] These findings seem to go along with a lately reported structure of the ternary complex of FTase with a CaaX-peptide and a FPP analogue.[21] In this structure the CaaX-peptide shows an extended conformation, but is not in the exact location which would allow for the farnesylation. A final and conclusive clue about the active conformation is still missing, as molecules incorporating turn-mimics as well as extended peptide mimics have yielded equally strong inhibitors.[6,20]

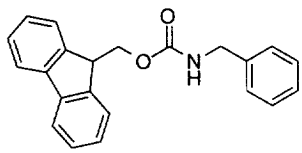
In the course of our studies on factors determining the conformation of peptides, we were investigating reverse-turn mimetic bicyclic lactams: The (5,7)-bicyclic skeleton (see compounds **1**, **2**) is able to constrain the peptide sequence in an inverse γ-turn geometry, while the (5,6)-system (see compounds **3**, **4**) is able to induce either a type II' β-turn or an inverse γ-turn conformation.[22,23] It was our expectation that the incorporation of these reverse-turn mimics into a FTase substrate sequence could lead to potent FTase inhibitors.



1 R = H
2 R = Fmoc



3 R = H
4 R = Fmoc



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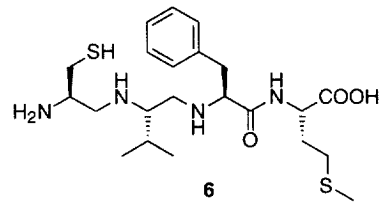

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Figure 1. Structures of the compounds tested for inhibition of FTase

Compounds **1** through **4** were prepared by standard peptide synthesis on solid support (Wang resin) using Fmoc-protected amino acids and Fmoc-protected bicyclic lactams[24,25] as building blocks.[22] The peptides were cleaved from the resin (with simultaneous deprotection of the Cys *t*-Bu group) with 10% trifluoromethane sulfonic acid in TFA and were purified by HPLC. Compound **5** was obtained by reacting benzylamine with Fmoc-Cl, the previously described FTase inhibitor **6**[26] was purchased from *CALBIOCHEM.*

In brief, the FTase inhibition assays were carried out as follows: yeast FTase[27] (expressed as a GST-fusion protein in *E.coli*) and the test compounds were preincubated on ice in transferase buffer (final buffer concentration: 50 mM Tris·HCl, pH 7.7, 20 mM KCl, 5 mM $MgCl_2$, 5 μM $ZnCl_2$, 2 mM DTT and 0.5 mM Zwittergent 3-14) for 10 min. 5 μg of GST-^{12}Val-RAS and 0.1 μCi [^{3}H]-FPP were added and incubated at 37 °C for 30 min. The reaction was stopped by addition of 250 μl of 4% SDS and the degree of farnesylation was determined by precipitation of the protein, filtration through a glass fiber membrane and subsequent scintillation counting of the membranes.

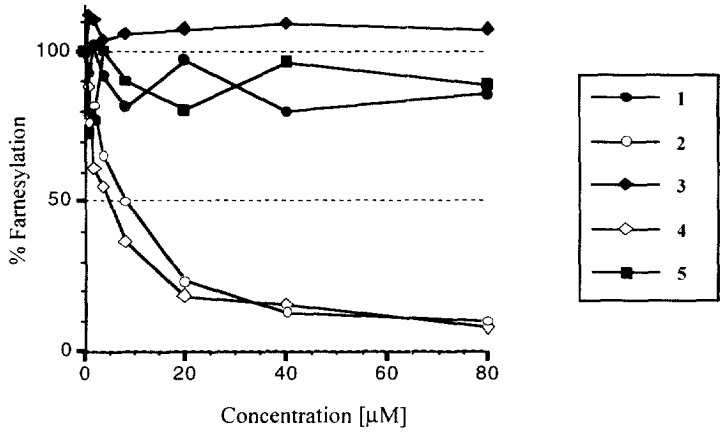

Figure 2. Inhibition of farnesylation of RAS by compounds **1** through **5**

Compound **6** was previously reported to have an IC_{50} of 21 nM against bovine FTase.[26] We used it as a positive control and to calibrate our assay system; we found for **6** an IC_{50} of about 300 nM against yeast FTase. Surprisingly, compounds **1** and **3** do not show any significant inhibitory effect on yeast FTase (IC_{50} > 200 μM). However, we found that the peptides carrying the Fmoc-protecting group at their N-terminus inhibit yeast FTase with IC_{50}'s of 5 μM (**4**) and 7 μM (**2**). To rule out the possibility that the inhibition may be merely caused by binding of the Fmoc-group in the lipophilic FPP binding site, we tested Fmoc-benzylamide **5** for its

inhibitory potential. We did not observe any effect on the farnesylation of RAS by **5** at concentrations up to 400 μM.

Two aspects of our results are noteworthy. First, replacement of the two aliphatic residues of the FTase peptide substrate with a reverse-turn inducing unit does not lead to effective inhibitors of the FTase - in contrast with our expectations. Secondly, the attachment of a Fmoc-group onto the amino terminus of the peptidomimetic inhibitor is clearly beneficial for the inhibition.

Early studies of substrates and inhibitors of FTase suggested that the active conformation of these compounds involves a β-turn conformation. In our case, the (5,7)-lactam in **1** and **2** dominantly induces a γ-turn, while the (5,6)-lactam in **3** and **4** can promote either a γ-turn or a type II' β-turn conformation with the β-turn usually preferred. Yet, this change in conformation does not seem to affect the inhibitory properties significantly [cf. IC_{50} = 5 μM (**4**) and 7 μM (**2**)]. Although the n.m.r.-studies may imply on the first glance that it is important to have a type I β-turn[16] (instead of a type II' β-turn or a γ-turn), the exact turn structure does not seem to be so relevant, considering that the most potent inhibitor so far was derived by substituting the aliphatic core of the CaaX-motif with a benzodiazepine turn-mimic (IC_{50} < 1 nM).[6] The benzodiazepine mimics a turn which is not a type I β-turn and the n.m.r.-studies revealed indeed that the bound peptide adopts "nonideal" reverse-turn conformations in the active site.[17] The poor/moderate activity of our compounds may therefore mainly be caused by a mismatch of the side-chains displayed on the turn scaffold with elements in the active site, and in particular by the lack of an aromatic residue in a position corresponding to the third amino acid of the tetrapeptide sequence (cf. the benzodiazepine peptidomimetic where the fused phenyl ring mimics the presence of Phe).[6]

The Fmoc-protected compounds (**2** and **4**) showed significantly enhanced inhibition of FTase compared to the unprotected compounds (**1** and **3**). This is in contrast to earlier studies where attachment of a hydrophobic group (Cbz) onto the amino terminus of the peptidomimetic inhibitor had a negative impact on the in vitro activity of these compounds.[18] Currently, we cannot offer a convincing rationale for this observation. It is possible that the Fmoc-group (but not the smaller Cbz-group)[18] is lipophilic enough to mimic FPP and compete with the FPP binding to FTase. Thus the Fmoc-protected peptides would offer two anchors for the two binding pockets in the FTase active site.

In light of the ongoing discussions about the active conformations of CaaX-peptide substrates and inhibitors, we cannot make a final decision about the actual inhibitory mode of action for our compounds. Yet,

our results support the validity of turn-mimics as a design tool for the generation of FTase inhibitors. It is conceivable that peptides with extended conformation exert their activity in the CaaX-binding pocket of FTase, while molecules based on turn-mimics occupy the FPP binding site. This view would be supported by the two available crystal structures of FTase which show an extended peptide in the CaaX-binding site[21] and a peptide with β-turn conformation in the FPP pocket.[11]

Further studies on better designed reverse-turn inducing peptide mimics and on the role of Fmoc amino protection are currently under way to clarify the mode of inhibition and to generate more potent inhibitors.

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