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Synthesis and Biological Activity of Semipeptoid Farnesyltransferase Inhibitors

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Abstract—Semipeptoids derived from the Ras farnesyl transferase inhibitor, CVFM, were synthesized by the Simultaneous Multiple Analogue Peptide Synthesis methodology. The semipeptoids were screened for their in vitro inhibition potency towards farnesyl transferase and geranylgeranyl transferase. Structure–activity relationship studies led to a potent and selective inhibitor, **HR-11**, which blocks Ras farnesylation in vitro with an IC_{50} of 1.2 nM. The cell permeable methyl ester derivative of **HR-11**, **HR-12**, inhibits Ras farnesylation in intact cells with an IC_{50} of 10 μM and with no detectable inhibition of Rap1A/K-rev geranylgeranylation. Copyright © 1997 Elsevier Science Ltd

Introduction

Oncogenic Ras is found in 40% of all cancers and is associated with over 90% of pancreatic tumors and over 50% of colon carcinomas.[1] Thus, inhibition of the Ras function is believed to be a crucial target for cancer chemotherapy.[2] Membrane localization of Ras is essential for its normal function and the transforming activity of its oncogenic version.[3] Membrane anchoring is achieved through a series of post-translational modifications directed by its carboxy-terminal CAAX motif (where C is cysteine, A is an aliphatic residue and X is preferably serine or methionine). The first and most critical modification is farnesylation of the conserved cysteine, catalysed by farnesyl protein transferase (FT). Subsequent modifications are dependent on its previous occurrence.[4] Inhibition of the farnesylation reaction either by site directed mutagenesis,[5] or by synthetic FT inhibitors nullifies Ras membrane anchorage and reverses transformation by oncogenic Ras.[1] Recent findings show that inhibition of FT by CAAX peptidomimetics causes reversal of ras-induced transformation in whole cells,[6–10] inhibition of ras-dependent tumor growth in nude mice[11–13] and tumor regression in Ha-Ras transgenic animals[14] with minimal toxic effects, strongly suggesting FT inhibitors as potential anti-cancer agents.

A new and promising class of peptidomimetics was formed by shifting the amino acid side chains from the Cα to the backbone nitrogen atom to yield Nα-alkylated oligoglycine derivatives, called peptoids.[15] The main biological advantage of peptoids as compared to peptides is their resistance to proteases.[16,17] In addition this method enables detailed structure–function relationship studies using both natural and unnatural side chains by simple chemistry. A few peptoids have already been found to be biologically active.[15,18,19]

In this study we describe the application of the peptoid approach to the peptide Cys-Val-Phe-Met (CVFM), known as a potent, selective and non-substrate FT inhibitor.[20,21] We describe structure–activity relationship studies of a series of semipeptoids, in which methionine and cysteine are retained, and valine and/or phenylalanine are replaced by N-substituted glycines.

Biological Effects

An effective inhibitor must exhibit high affinity and high selectivity towards the target enzyme, but must not serve as an alternative substrate if its product is less potent. These properties were tested in vitro for the semipeptoid FT inhibitors. The first assay determined the concentration needed for 50% inhibition (IC_{50}). FT activity was quantified by measuring the incorporation of [^{3}H]farnesyl from all-*trans*-[^{3}H]farnesylpyrophosphate into purified Gst-Ha-Ras. The second assay determined the selectivity of the inhibitor towards FT over a related enzyme, the geranylgeranyl-protein-transferase-I (GGT), which transfers a C_{20}-isoprenoid to the thiol of the cysteine of a C-terminal CAAL peptide sequence from geranylgeranylpyrophosphate. Geranylgeranylation of normal cellular proteins is 5–10 times more common than farnesylation.[1,2,22] A selective FT-inhibitor is considered

Abbreviations: Abbreviations of amino acids are according to the IUPAC-IUB Commission on Biochemical Nomenclature (1975); FT, farnesyltransferase; GGT, geranylgeranyltransferase; NSG, N-substituted glycine; MAPS, multiple analogue peptide synthesis; Fmoc, fluoren-9-ylmethoxycarbonyl; Boc, *tert*-Butyloxycarbonyl; Trt, Trityl (triphenylmethyl); DIC, *N,N'*-diisopropyl carbodiimide; PyBroP, bromo-tris-pyrrolidino-phosphonium hexafluorophosphate; BOP, benzotriazolyl-*N*-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, diisopropylethylamine; DMSO, dimethylsulfoxide; DMF, dimethylformamide; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; DTT, 1,4-dithiothreitol; SDS, sodium dodecyl sulfate; Gst, glutathione-S-transferase; RP, reverse phase; FABMS, fast atom bombardment mass spectroscopy; PAGE, polyacrylamide gel electrophoresis.

to cause fewer cytotoxic effects than a nonselective inhibitor of both FT and GGT. The GGT inhibition assay was performed as described for the FT, using purified Gst-HRas-CVLL and [^{3}H]geranylgeranylpyrophosphate as substrates for the partially purified GGT enzyme.[23] The third assay determined whether the inhibitor itself undergoes farnesylation. For this purpose, the inhibitor was incubated with FT and [^{3}H]farnesylpyrophosphate in the absence of Ras. It has been previously shown that farnesylated-CAAX-peptides were about 13-fold (for CVLS) or 16-fold (for CVIM) less potent as inhibitors than their corresponding tetrapeptides.[20,24]

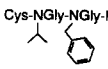
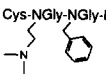
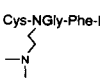
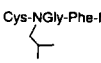
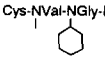
The most effective inhibitor derived from the in vitro screen was methyl esterified and subjected to intact cells, to examine its effect on Ras processing. Its selectivity towards FT as compared to GGT in whole cells, was determined by its relative inhibitory effects on Ras farnesylation and on Rap1A/Krev geranylgeranylation,[25–28] respectively. v-Ha-ras transformed NIH3T3 cells were incubated with lovastatin or increasing concentrations of the inhibitor. Cells were lysed and the processed and unprocessed forms of Ras or Rap1A were separated by SDS-PAGE and immunoblotted with anti-Ras or anti-Rap1A antibodies, respectively. Lovastatin is a compound which blocks the processing of isoprenylated proteins in cells by inhibiting an early step of the isoprenoid biosynthetic pathway, and therefore serves as a positive control for the inhibition of both geranylgeranylation and farnesylation.[6,27,28]

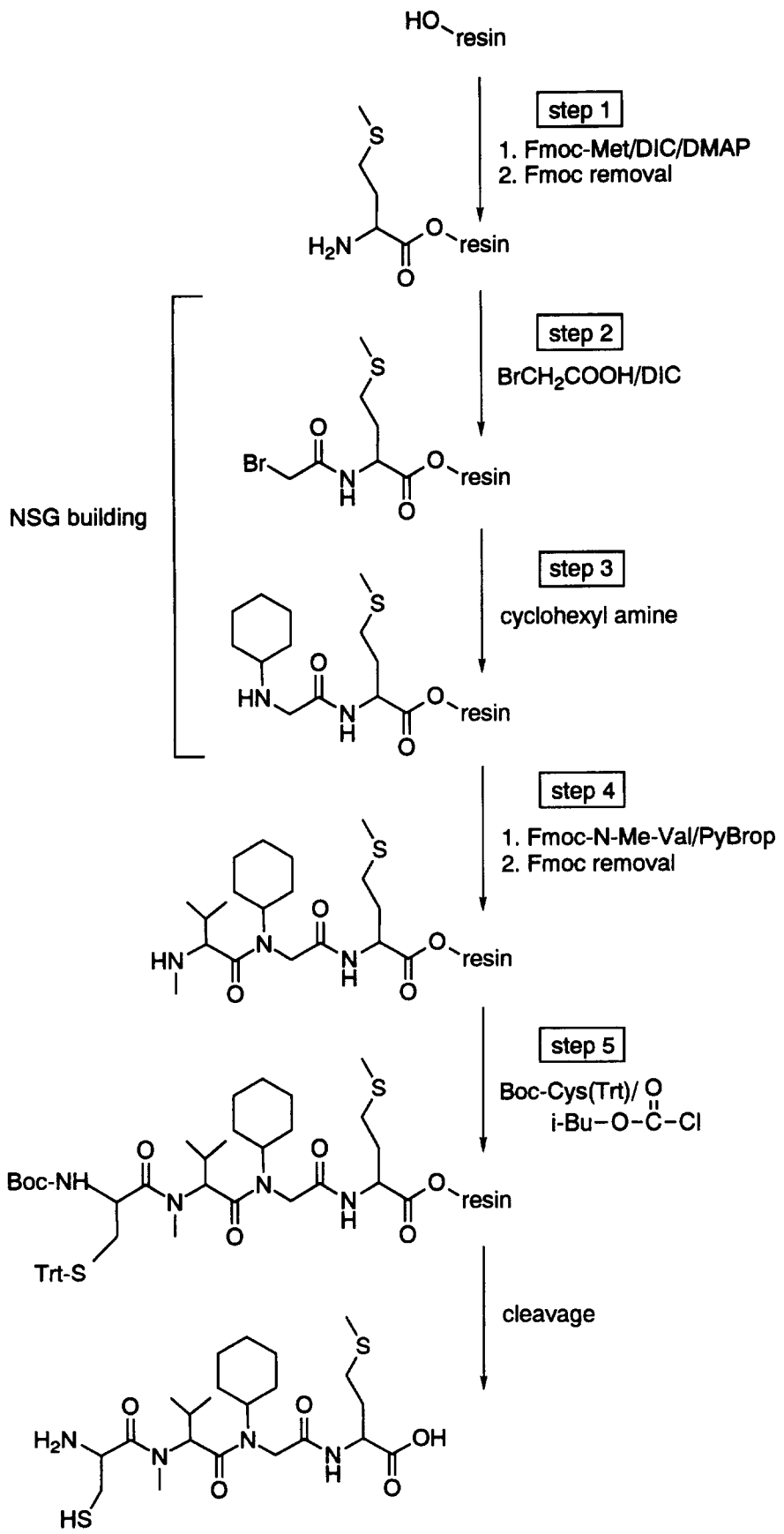
Chemistry

It has been previously shown that for FT inhibitors based on the CA_1A_2X structure, the cysteine is essential for improved potency of the inhibitor[1,20,21,29] and the methionine at the X position is the preferred residue for high potency and selectivity,[7,21,23,27] although recently it was replaced successfully by aromatic substituents,[12,28,30] methionine sulfone[11,14] or glutamine.[13] The A_1A_2-spacer may tolerate certain modifications.[13,31–33] Thus, for initial screening we decided to conserve the N-terminal cysteine and the C-terminal methionine and to build N-substituted-glycine (NSG) units at positions A_1 and/or A_2. The goals of the modifications were (i) to increase the metabolic stability of the parent peptide, (ii) to examine whether shifting the side chains of the A_1/A_2 residues would influence the potency and selectivity towards FT by performing structure–activity relationship (SAR) studies on these side chains. For this purpose a library of 10 semipeptoids was constructed by the 'tea bags' multiple analogue peptide synthesis (MAPS) method.[34,35] The library was divided into three classes (Table 1). In class I (semipeptoids **1–4,** Table 1) valine was conserved at position A_1 while phenylalanine at the A_2 position was replaced by NSG with different substituents on the amine. In class II (semipeptoids **5–7,** Table 1) both A_1 and A_2 residues were replaced by NSG units. While the substituents at the A_2 position were mainly aromatic, those at the A_1 position were aliphatic, as in the original CVFM peptide inhibitor. In class III (semipeptoids **8–10**, Table 1), phenylalanine was retained and only the A_1 residue was replaced by NSGs.

Scheme 1 shows the sequential synthetic steps for building **HR-11**, the most potent and selective semipeptoid FT-inhibitor. **HR-11** synthesis includes all the coupling types used for preparation of semipeptoids **1–10**. The NSG units of the semipeptoids were built on the resin via the 'submonomer' synthesis reported by Zuckermann.[36] Each cycle of monomer addition consisted of two steps: an acylation step followed by a nucleophilic displacement step. Coupling of bromoacetic acid to the resin-bound amine was performed by *N,N'*-diisopropyl carbodiimide (DIC) (step 2, scheme 1). In the second step the side chain was introduced by nucleophilic substitution of the bromide with an excess of alkyl or aryl amine (step 3, Scheme 1). N-protected amino acids were coupled using various coupling reagents, depending on the steric hindrance of the amine attached to the peptide-resin. Bromo-tris-pyrro-

Table 1. Semipeptoid inhibitors of farnesyltransferase. The following structures have been synthesized by MAPS on solid phase, purified (>95%) by HPLC and their masses were verified by MS. In vitro IC_{50}s against FT and GGT are reported for each compound. Each value represents results of two to four tests. Assays were conducted as described in the Experimental section.

Semipeptoid no. (class)	Structure	IC_{50} (FT) μM	IC_{50}(GGT) μM	Alternative substrate
1 (I)	Cys-Val-NGly-Met 	0.10	40	no
2 (I)	Cys-Val-NGly-Met 	0.32	30	no
3 (I)	Cys-Val-NGly-Met 	0.12	23	no
4 (I)	Cys-Val-NGly-Met 	0.07	5	no
5 (II)	Cys-NGly-NGly-Met 	13	190	no
6 (II)	Cys-NGly-NGly-Met 	4	80	no
7 (II)	Cys-NGly-NGly-Met 	3	40	no
8 (III)	Cys-NGly-Phe-Met 	50	155	no
9 (III)	Cys-NGly-Phe-Met 	4	>400	no
10 (III)	Cys-NGly-Phe-Met 	75	240	no
HR-11	Cys-NVal-NGly-Met 	0.0012	0.91	no



HR-11

Scheme 1. Synthesis of **HR-11**.

lidino-phosphonium hexafluorophosphate (PyBroP) was used for coupling of N-protected amino acids to the secondary amine of the NSG units (step 4, Scheme 1). In the problematic attachment of N-protected amino acid to the secondary amine of the N-Me-Val, PyBroP was replaced by the mixed anhydride activation method with isobutyl chloroformate (step 5, Scheme 1).[37]

Results and Discussion

The results of the structure–activity relationship studies of the FT-semipeptoid-inhibitors are shown in Table 1. Comparing semipeptoids **1**, **5** and **8**, which have the same side chains, enables us to examine the importance of the location of the isopropyl and the benzyl side chains for the inhibitory activity. Each one of these semipeptoids represents a different class. In semipeptoid **1** (class I) the side chain of Phe is shifted to the nitrogen, in semipeptoid **8** (class III) the side chain of Val is shifted to the nitrogen, and in semipeptoid **5** (class II) both side chains are shifted. Semipeptoid **1** was found to be 130- and 500-fold more potent than compounds **5** and **8** respectively. Accordingly, **3** is 33 times more potent than **6**. Comparison of the IC_{50} ranges of class I (**1–4**), class II (**5–7**) and class III (**8–10**) generalizes the conclusion that when the Cys and Met are unmodified in the peptoid frame the position of the side chain of the valine is critical for the inhibitory activity. This is not the case, however, for the Phe side chain. CVFM ($IC_{50}=50$ nM) and **1** ($IC_{50}=100$ nM) exhibit similar inhibitory activities towards FT. Thus, the benzyl side chain of these inhibitors might occupy the same binding pocket in FT, although its position in the peptide backbone is shifted. This is possible due to the increased flexibility of the peptoid chain compared to its parent peptide. This explanation is corroborated by recent findings showing that replacing Phe with Tic, in which the benzyl side chain is attached to both Cα and Nα, improves the inhibitory activity.[13,33,38]

Among the FT inhibitors of class I, semipeptoid **4** was found to be the most potent one. It was suggested that introduction of NSG units replacing amino acid residues renders the peptide backbone achiral and flexible.[19] The proximity of the bulky cyclohexyl side chain to the backbone in semipeptoid **4** imposes a local conformational constraint which might compensate for the relatively flexible character of peptoids **1–3**. In addition, the length and/or aliphatic character of this specific side chain might better fit the pocket in the active site of the enzyme. Extension by one methylene of the spacer connecting the phenyl and the peptide backbone reduced the relative potency by 3.2-fold both in the peptoidic structure (**2** vs. **1**) and in the peptidic structure (CV-HomoPhe-M versus CVFM[39]). Moreover, it was found that in CA_1A_2X FT-peptide-inhibitors, free terminal amine and an aromatic residue at A_2 are essential to prevent farnesylation of the inhibitor.[29] Interestingly, upon shifting the side chain at A_2 to the backbone nitrogen, aromaticity was no longer required to preclude the inhibitor from becoming an alternative substrate.

One of the aims of the peptoid approach is the introduction of N-alkylated peptide bonds that increase metabolic stability. Semipeptoid **4** has one N-alkylated peptide bond at the A_1-A_2 position. Almost any modification of the C-terminal Phe–Met peptidic bond that has been attempted, e.g. N-methylation or reduction to methyleneamine,[39] led to a decrease of inhibitory potency, suggesting that either the Phe-Met amide bond is involved in a critical hydrogen bond or a certain geometry is required at this position.[39] The most effective FT inhibitors reported previously possess the original Phe-Met peptide bond.[6,7,14,27] On the other hand the N-terminal Cys-Val bond showed wide tolerance towards structural modifications. A number of studies have already shown increased potency due to reduction of this amide to methyleneamine.[6,13,27,39] N-methylation of this peptide bond led to a decreased potency of the CVFM peptide[39] but improved the inhibitory activity of CAAX peptidomimetics bearing a local conformational constraint[7,33]. N-methylation of **4** at that position (**HR-11**) improved its potency 58-fold ($IC_{50}=1.2$ nM), increased its selectivity 11-fold $\{IC_{50}(GGT)/IC_{50}(FT)=756\}$ and preserved its property of not being a substrate itself. This modification was aimed to improve both the metabolic stability and its hydrophobicity, to make it permeable to cells and produce a long acting drug.

In order for FT inhibitors to become potential anticancer drugs they must exhibit metabolic stability, cellular permeability and retention of their selectivity towards FT, thus minimizing their toxic effects. Towards this end, v-ras transformed NIH3T3 cells were treated with **HR-12**, a methyl ester derivative of **HR-11**. This prodrug strategy[6,7,11,28] masks the negative charge of the free carboxylate, thereby enables it to penetrate the cell membrane. After hydrolysis by cellular esterases the activated inhibitor is generated and trapped within the cell. After 48 h of incubation, cell extracts were separated on SDS-PAGE and immunoblotted with anti-Ras antibody. Unprocessed Ras migrates slower than its processed form on SDS-PAGE.[6,27,28,40] Figure 1A shows increased amount of unprocessed Ras vs. processed Ras in cells treated with lovastatin (lane 2) in comparison to cells treated with vehicle (lane 1). A dramatic effect is observed in the presence of **HR-12** (lanes 3–9), which causes a dose dependent shift of Ras towards its unprocessed form at a concentration range of 1–160 μM, with an in situ IC_{50} of 10 μM.

To test the selectivity of **HR-12** towards FT in whole cells, we analyzed the post-translational processing of an endogenous geranylgeranylated protein, Rap1A/K-rev. Figure 1B shows that Rap1A/K-rev processing was not affected by **HR-12** over the same concentration range (lanes 3–5). In contrast, lovastatin did inhibit Rap1A/K-rev processing, as indicated by a slight shifting of the Rap1A band upward (lane 2). These results clearly demonstrate that **HR-12** selectively

inhibits farnesylation but not geranylgeranylation. The lack of toxic effects of **HR-12** up to 250 μM (data not shown) may be at least partially attributed to its high selectivity towards FT as compared to GGT. Moreover, the lack of inhibition of Rap1A/K-rev by **HR-12** is advantageous, since Rap1 is a suppressor of Ras signaling.[41]

In summary, a series of semipeptoids derived from CVFM were synthesized and screened in vitro for their inhibitory activity and selectivity towards FT. The methyl ester form of the most potent semipeptoid, was found to selectively inhibit Ras processing in intact cells. These results are consistent with the approach claiming that a specific FT inhibitor, like **HR-11**, could be used in the future as a potential anti-cancer drug.

Experimental

Chemicals

Protected amino acids and resins were purchased from Bachem (U.S.A.) and Sigma. Chemicals were purchased from Aldrich and were of AR grade. Ultra-dry dimethyl formamide (DMF) was from Labscan. Other solvents were purchased from Aldrich or Merck and were used without further purification.

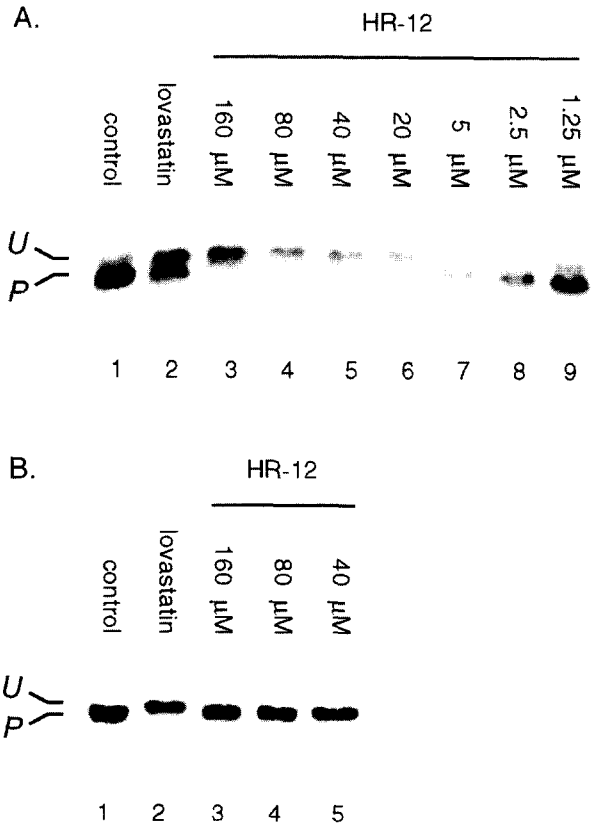


Figure 1. Effect of HR-12 on post-translational processing of Ras and Rap1A/K-rev. v-Ha-ras transformed NIH3T3 cells were treated with lovastatin (15 μM) or the indicated concentrations of HR-12, for 48 h. Cell extracts were separated by SDS-PAGE (40 μg of protein/lane) and visualized by Western blot as described in the Experimental section. A, v-Ha-Ras; B, Rap1A. P, processed protein; U, unprocessed protein.

Synthesis of semipeptoids 1–10

Solid-phase peptide synthesis was performed according to the general Merrifield synthetic protocols.[42] After each step the peptide resin was washed with DMF (3 times), CH_2Cl_2 (2 times) and DMF (3 times). After deprotection of Met, Phe and Val and coupling to their primary amines, the Kaiser test was performed to ensure deprotection and full coupling.[43] The library was synthesized by the simultaneous multiple solid-phase analogue peptide synthesis (MAPS) method,[34] using the 'tea bags' approach (see Scheme 2). The solid support (Wang resin, 0.93 meq/g) was contained in 10 polypropylene mesh bags (**I–X**, 100 mg resin per bag). After washing the resin, a mixture of Fmoc-Met (4 eq), DIC (4 eq) and dimethyl-amino-pyridine (0.16 eq) dissolved in DMF (80 mL) was added and shaken for 2 h to give 0.5 meq coupled Fmoc-Met per g resin, determined by the quantitative Fmoc-piperidine test.[44] The remaining OH-groups on the resin were capped [acetic acid anhydride (6 eq)/DMF, 30 min]. After Fmoc removal (30% piperidine/DMF, 20 min), bags **VIII–X** were shaken with preactivated 30 mL DMF containing Fmoc-Phe (6 eq), BOP (6 eq) and diisopropyl ethylamine (DIEA, 12 eq) for 2 h, followed by Fmoc deprotection.

The N-alkylated-glycine units in bags **I–VII** were built in two steps, an acylation step and a nucleophilic displacement step. The acylation reaction was performed by addition of a mixture of bromoacetic acid (30 eq) and DIC (33 eq) in DMF (70 mL), to a vessel containing bags **I–VII**. The reaction mixture was shaken for 30 min. The acylation procedure was repeated once. After washings, the displacement reactions were performed by addition of the following primary amines as 2.5 M solutions in dimethylsulfoxide (DMSO), and agitation for 2 h. Bags **I**, **V**, **VII** were reacted with benzylamine, bags **III**, **VI** with 3,4-dimethoxyphenethyl amine, bag **II** with phenethyl amine and bag **IV** with cyclohexyl amine.

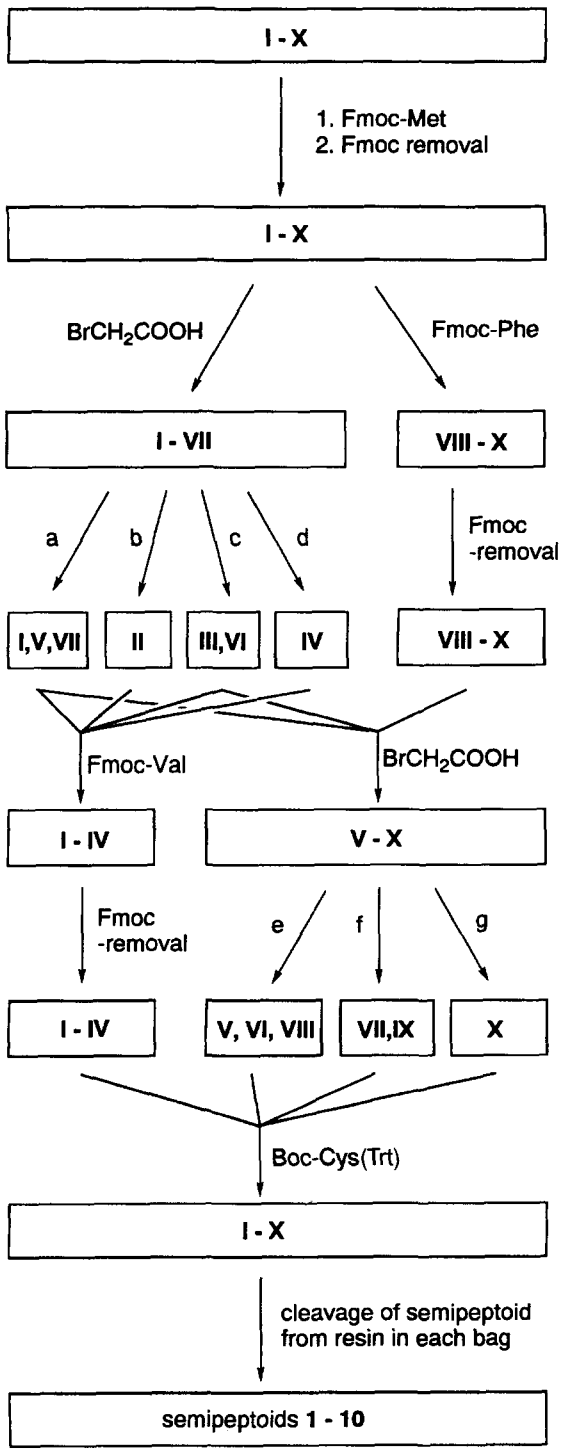
Fmoc-Val was coupled to the secondary amines of **I–IV**, by shaking them with a preactivated mixture of Fmoc-Val (6 eq), PyBroP (6 eq) and DIEA (12 eq) in DMF (35 mL) for 2 h, followed by Fmoc- removal.

The N-alkyl-glycine units in bags **V–X** were built by the same procedure described above with the following amines. Bags **V**, **VI**, **VIII** were reacted with isopropyl amine, bags **VII**, **IX** with *N*,*N*-dimethylethylenediamine, and bag **X** with isobutyl amine.

Bags **I–X** were then agitated with a preactivated mixture of 80 ml of DMF containing Boc-Cys(Trt) (6 eq), PyBroP (6 eq) and DIEA (12 eq) overnight.

Protecting groups (Boc, Trt) were removed and semipeptoids **1–10** were cleaved from the resin by treatment of each peptoid-resin with 3 mL of a

precooled mixture of thioanisole (5% v/v)/ethanedithiol (2.5% v/v)/trifluoro acetic acid (TFA, 92.5% v/v) for 2 h at room temperature, followed by filtration and cold ether precipitation. The precipitate was washed 3 times with 40 mL of cold ether. After vortex and centrifugation the ether was decanted and pooled. The final washed precipitate was dried in vacuum, dissolved in 1 mM DTT/30% acetic acid and lyophilized. The pooled ether washings were evaporated to dryness and washed 3 times with 10 mL of cold ether. The precipitate was treated as above.



Scheme 2. Synthesis of semipeptoids **1–10** library, by the simultaneous multiple analogue peptide synthesis using the 'tea bags' approach. Semipeptoids **1–10** were synthesized in bags I–X, respectively. The amines used in the nucleophilic displacement step were: a, benzylamine; b, phenethylamine; c, 3,4-dimethoxyphenethylamine; d, cyclohexylamine; e, isopropylamine; f, *N*,*N*-dimethylethylenediamine; g, isobutylamine.

The semipeptoids were purified to >95% purity level by semipreparative RPHPLC (C_{18} Vydac column, type 218TP510, column size: 1.0 × 25 cm, particle size: 5 μ, gradient: A = acetonitrile (0.1% TFA); B = water (0.1% TFA) detection 210 nm; 0–20 min 20% A, 80% B 20–40 min from 20% A, 80% B to 80% A, 20% B, flow 3 mL/min). The purified peptides were characterized by analytical RPHPLC (C_{18} Vydac column, type 218TP54, column size: 0.46 × 25 cm, particle size: 5 μ, gradient: 0–5 min 20% A, 80% B 5–35 min from 20% A, 80% B to 80% A, 20% B, flow 1 mL/min). The pure peptoids were subjected to molecular weight determination by FABMS (TSQ-70, Finnigan). k′ Values and MS determinations of the semipeptoids are given in Table 2.

Synthesis of HR-11

The preparation of **HR-11** followed the procedure of semipeptoid **4**, with two modifications. Fmoc-*N*-methyl-Val was used instead of Fmoc-Val and the mixed anhydride activation method[37] was used for the coupling of Boc-Cys(Trt) to the secondary amine of the N-Me-Val residue. Boc-Cys(Trt) (3 eq) and *N*-methylmorpholine (3 eq) were dissolved in DMF (15 mL) and cooled to −15 °C. Isobutyl chloroformate (3 eq) was added slowly under stirring at −15 °C. After 1 min the mixture was added to the resin-peptide and shaken for 8 h. The coupling was repeated for 4 h with freshly activated Boc-Cys(Trt).

Synthesis of HR-12

HR-12, the methyl ester derivative of **HR-11**, was synthesized on 2-methoxy-4-alkoxybenzyl alcohol resin (1 g, 0.96 meq/g). The assembly of the semipeptoid is the same as for **HR-11**. The compound was cleaved from the resin, retaining the Nα Boc-Cys protecting group, in 5% of TFA in DCM. The crude product was dissolved in MeOH (5 mL), cooled on an ice bath, and a satd soln of CH_2N_2 in Et_2O was added dropwise until the pale yellow color of the diazomethane soln appeared. After evapn of the solvent, the Boc protecting group was removed in a mixture of thioanisole:ethanedithiol:TFA as described for deprotection and cleavage of semipeptoids **1–10**. **HR-12** was purified to >90% purity level by semipreparative RPHPLC (gradient: 0–5 min 10% A, 90% B, 5–65 min from 10% A, 90% B to 75% A, 25% B, flow 3 mL/min). The purified **HR-12** was characterized as mentioned above (Table 2).

Farnesyl protein transferase (FT) inhibition assay

Partial purification of FT from bovine brain was performed essentially as described by Reiss et al.,[45]

Table 2. HPLC and MS characterization of compounds **1–12**.

Semipeptoid no.	*k'* HPLC[a]	MS	
		Calculated	Found
1	3.2	498	498
2	3.2	512	512
3	3.1	572	572
4	3.5	489	490
5	1.3	498	498
6	3.3	572	572
7	1.6	527	527
8	3.1	498	498
9	1.7	527	527
10	4.6	512	512
HR-11	4.7	505	505
HR-12	8	518	518

[a]The HPLC conditions are described in the Experimental section.

except that a fractogel DEAE column (150 × 16 mm, Merck) was used for the ion exchange chromatography step. GST-Ha-Ras expression vector was constructed by cloning the Ha-ras gene into a pGEX-1 vector, and expressed in the *E. coli* strain TG1. The protein was purified using glutathione agarose (Sigma, USA). FT inhibition assays were conducted in 96-well plates in a reaction vol. of 30 μL. The final reaction mixture contained 5 μM Gst-Ha-Ras, 0.9 μM [^{3}H]farnesylpyrophosphate 20,000 dpm/pmol (NEN-Dupont) 1 μg of partially purified FT, 5 mM $MgCl_2$, 3 μM $ZnCl_2$, 40 mM Tris·HCl pH 7.5, 60 mM NaCl, 1mM DTT, 0.2 mM glutathione, 0.03% octyl-β-D-glucopyranoside, and serial dilutions of the inhibitor. Following incubation at 37 °C for 30 min the reactions were stopped by adding 100 μL of 4% sodium dodecyl sulfate (SDS) followed by 100 μL 30% trichloroacetic acid (TCA). Plates were incubated for 100 min on ice, and the precipitates were filtered using a Millipore milliblot™-system (Millipore Corp., Bedford MA) with GF/C membranes. The wells were washed twice with 200 μL of 2% SDS/6% TCA and 7 times with 200 μL of 6% TCA. The filters were punched into 4 mL vials and dried at 70 °C for 10 min. Scintillation fluid (3 mL) was added and radioactivity monitored by scintillation counting. IC_{50} values were resolved from dose–response curves of percent control vs. log(drug concentration), using the Regression program (Blackwell, U.K.). A complete dose response for each compound was performed two to four times.

Geranylgeranyl transferase-I (GGT) inhibition assay

GGT was partially purified from bovine brain. Cytosol was prepared and GGT was isolated using a fractogel DEAE column (150 × 16 mm, Merck) with NaCl gradient, as for FT purification (see above). Fractions containing the GGT activity were pooled and stored at −70 °C. Ha-ras gene was point mutated using an oligonucleotide directed in vitro mutagenesis system (Amersham version 2.1). The mutated Ha-ras was cloned into the GST frame of a pGEX-1 expression vector, expressed in *E. coli* strain TG1, and a fusion protein Gst-Ha-Ras-CVLL was purified on glutathione agarose. In order to determine the IC_{50} values for GGT inhibition, the same assay as for the FT inhibition was conducted, except for using 0.9 μM [^{3}H]geranylgeranylpyrophosphate 10,000 dpm/pmol (NEN-Dupont) and 5 μM Gst-Ha-Ras-CVLL as substrates and 6.8 μg of partially purified GGT for the enzyme of the reaction.

Inhibitor farnesylation assay

For examination whether the inhibitors undergo farnesylation we used a previously described procedure.[20] Each reaction mixture contained the following components in a final volume of 15 μL: 40 mM Tris·HCl pH 7.5, 67 mM NaCl, 5 mM $MgCl_2$, 7 μM $ZnCl_2$, 1 mM DTT, 0.07% (v/v) octyl-β-D-glucopyranoside, 20 pmol of [^{3}H]farnesylpyrophosphate 45,000 dpm/pmol, 4 μM peptide or peptoid, and 1 μg of partially purified FT. After incubation at 37 °C for 30 min, the entire reaction mixture was spotted onto an aluminium backed silica gel thin layer sheet (20 × 20 cm, Merck), and placed in a tank containing n-propyl-alcohol/ammonium hydroxide/water (6:3:1, v/v/v). The chromatogram was run for 4 h, sprayed with EN^{3}HANCE (Dupont) and exposed to RX film (Fuji) for 4 days at −70 °C.

Protein processing assay in intact cells

v-Ha-ras transformed NIH3T3 fibroblasts were treated with 15 μM lovastatin or the indicated concentrations of **HR-12** or vehicle (0.25mM DTT, 0.25% DMSO), for 48 h. Cells were lysed[27] in 1% Nonidet P-40, 5 mM Tris·HCl pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotonin, 10 μg/mL soybean trypsin inhibitor and 313 μg/mL benzamidine. The lysate was separated by centrifugation and the supernatant used as cell extract. Total protein was separated by SDS-PAGE (40 μg/lane) in 15% acrylamide (0.4% bis-acrylamide) gels, transferred onto nitrocellulose (Schleicher & Schuell, optitran BA-S 85) and probed with anti-Ras antibody Y13–259, or monoclonal anti-Rap1A/K-rev antibody (transduction laboratories). The Western blots were visualized using an enhanced chemiluminescence (ECL) detection system.

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