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Short communication

Phenol-derived CVFM analog inhibitors of Ras Farnesyltransferase possessing cellular in vitro activity[1]

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Abstract – A study was performed on structure–activity relationships of a series of phenol-derived, CVFM analogs, inhibitors of Ras Farnesyltransferase (FTase). The effect of various substituents on the phenol ring was examined, while the VFM moiety of the potent inhibitor CVFM was kept constant. The FTase inhibitory activity, reported as IC_{50} in *table I*, was influenced by both the chemical properties and the relative position of the substituents on the phenolic ring. The most active compounds in this series contained a chloro or bromine substituent on the phenolic ring. Subsequently we have tested the effects of these FTase inhibitors on the anchorage-dependent growth of two rat epithelial cell lines, FRTL-5 and the same line v-Ha-ras transformed. While most of the compounds were inactive, two showed a growth inhibitory effect: compound **4** was active against normal as well against transformed cells while derivative **13** was active only against transformed cells.

Ras Farnesyltransferase inhibitor / phenol derivative / cellular in vitro activity

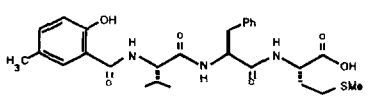
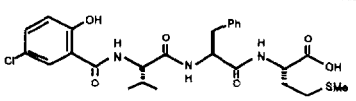
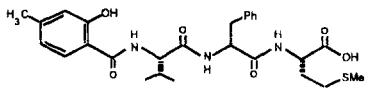
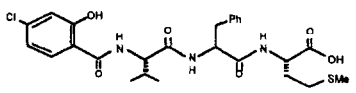
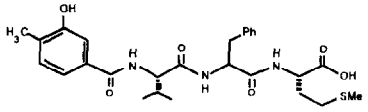
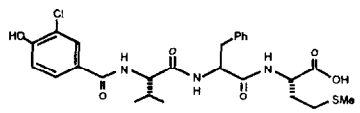
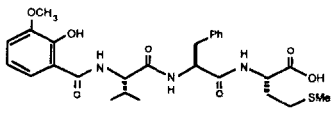
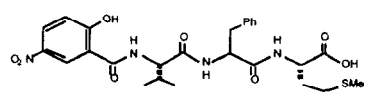
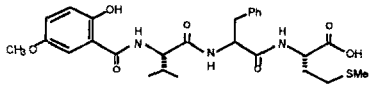
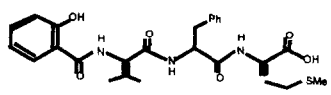
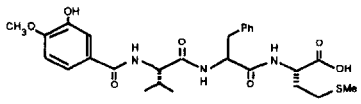
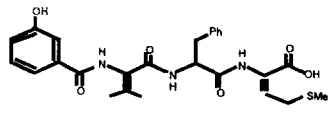
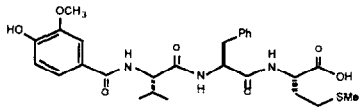
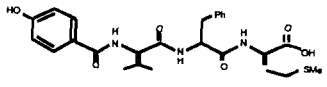
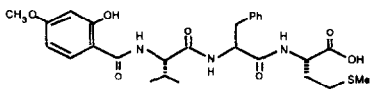
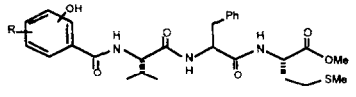
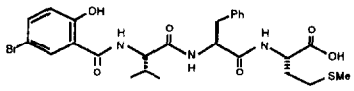
1. Introduction

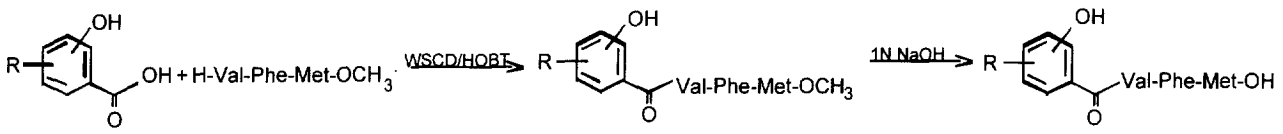
Current cancer chemotherapy is highly effective in only approximately 15% of human malignancies. In fact the most common adult solid tumors are rather refractory to the existing therapeutic drugs. Therefore it would be desirable to develop new agents with improved efficacy and broader clinical application. It is likely that these new drugs will exploit the informations that have recently been accumulated on the molecular basis of cell transformation by the action of oncogenes. Some of the most common oncogenes involved in human pathology are the Ras genes that are found activated in about 30% of all neoplasms [2, 3].

An essential step required for Ras proteins activity is the localization to the inner membrane of cells that depends on three posttranslational modifications: farnesylation, proteolysis and methyl esterification [4]. Once localized into plasma membrane the processed Ras proteins participate in the signal transduction pathways that control growth and differentiation [5]. The critical biochemical modification that is common to all Ras proteins, both wild-type and mutated, is the farnesylation step [6]. This step is catalyzed by the farnesyltransferase enzyme (FTase) that uses farnesyl pyrophosphate as a donor and attaches a 15-carbon isoprenoid moiety to the cysteine residue at the fourth position from the COOH-terminus. This cysteine belongs to the terminal tetrapeptide sequence CAAX, where A stands for an aliphatic residue and X stands for methionine or serine [7]. This motif is shared by nuclear lamins A and B, skeletal muscle phosphorylase kinase, and three retinal proteins that are all substrates for FTase [8].

*Correspondence and reprints
[1]Symbols and abbreviations are in accord with recommendations from [1].

Table I. Farnesyltransferase inhibitory activity of phenolic CVFM analogs.

Compounds		IC_{50} [μM] FTase	Compounds		IC_{50} [μM] FTase
1		10	**10**		58
2		> 100	**11**		1
3		> 100	**12**		> 100
4		42	**13**		46
5		> 100	**14**		> 100
6		> 100	**15**		> 100
7		> 100	**16**		> 100
8		> 100	**17**		> 100
9		7	**18**	**CVFM**	0.1



R = H, CH_3, OCH_3, Cl, Br, NO_2

Figure 1. Synthetic procedure of compounds.

Inhibitors of this enzyme may block the proliferation of cells that are dependent on oncogenic Ras proteins for their growth. Such inhibitors, of course, could also inhibit the farnesylation of wild-type Ras protein, but there exists the possibility that the dependence on oncogenic Ras might render a transformed cell population more sensitive than the normal counterpart, by lack of compensatory mechanisms. Since the peptide sequence requirement for the substrates of farnesyl transferase have been elucidated, in the past few years intense efforts of several research groups have been dedicated to the development of numerous highly potent peptidic and nonpeptidic inhibitors of this enzyme [9–15]. As an example the tetrapeptide CVFM is a potent FTase inhibitor (IC_{50} = 0.06 μM), not itself farnesylated by the enzyme [16–18]. Peptide drugs, however, may have limitations such as poor permeability/bioavability and degradation by proteases. Moreover, peptides incorporating cysteine suffer from complications specifically due the thiol moiety, which can be involved in oxidation reactions, thus rendering the compound inactive.

To circumvent this problem we prepared a series of CVFM analogs in which the thiol group was replaced by several hydroxybenzoic acids opportunely substituted. A limited number of substituents in ortho, meta and para position of the hydroxybenzoic acid were selected with various hydrophobic, electronic and steric properties. Phenol based peptide inhibitors of FTase have been prepared by others [19, 20], however no evaluation of the cellular activity of these compounds has been reported. In this paper we describe the synthesis and report the inhibitory activity against FTase, as well as the cellular activity, of a first series of pseudopeptides that we have synthesized (*table I*).

2. Chemistry

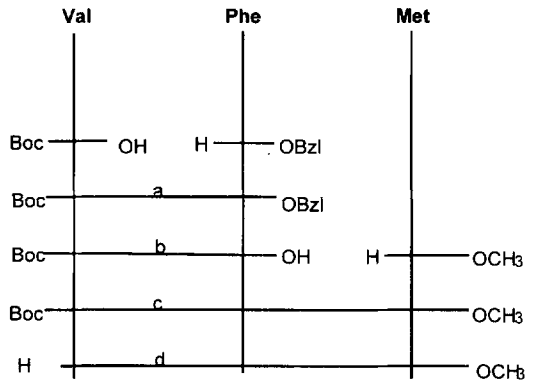
Compounds used in this study were prepared by condensation of a hydroxybenzoic acid derivative with H-VFM-OCH_3 as reported in *figure 1*.

The target tripeptide was synthesized by standard solution-phase peptide synthesis using the general procedure outlined in *figure 2*.

Figure 2 comprises a cycle of deprotection of the Boc group on the tripeptide H-VFM-OCH_3 with hydrochloric acid in dioxane and the subsequent coupling process with an appropriate salicylic acid derivative. The 1-ethyl-3-[3-(N,Ndimethylamino)-propyl]carbodiimide (WSCD) and 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate (TBTU) 1-hydroxybenzotriazole (HOBT) methods were applied to the coupling reactions [20–22]. The starting dipeptide Boc-Val-Phe-OBzl was produced from Boc-Val-OH and HCl•H-Phe-OBzl by the WSCD method. Successively the protecting group of the C-terminal was removed by catalytic hydrogenation (H_2, 10% C/Pd) and coupled with methionine protected as methylester by TBTU method. The Boc protecting group of the protected tripeptide was removed with hydrochloric acid in dioxane, purified by crystallization and characterized by FAB-mass spectroscopy. The final tripeptide was then coupled with an appropriate salicylic acid derivative by WSCD-HOBT method. The resulted compounds were characterized (*table II*) and converted to their carboxylic form by saponification with 1 N NaOH, successively purified by reversed-phase HPLC (RP-HPLC) and characterized by FAB-mass spectroscopy (*table II*).

3. Pharmacology

All the compounds were tested in vitro for their FTase inhibitory activity as well as the cellular activity [9, 23]. The biological data are reported, respectively, in *table I* and *figures 3–6*. The synthesized compounds were compared for their activity with CVFM, taken as reference drug.



(a) WSCD, HOBT, DMF; (b) H_2 C/Pd 5%; (c) TBTU, DIPEA, HOBT, DCM; (d) HCl, dioxane 4N

Figure 2. Synthetic routes of VFM tripeptide.

4. Results and discussion

Initially, to validate the assay, we tested three farnesyl diphosphate analog inhibitors of FTase: I, II and III [24] (commercially available by Calbiochem, San Diego, CA, USA), and the synthetic tetrapeptide CVFM. The IC_{50} values obtained under our assay conditions were somewhat higher compared to those reported in the literature for these compounds (as an example the IC_{50} of CVFM was 0.1 μM), but the respective rank of inhibitory activity was in agreement with the values reported. The inhibitory values of the new compounds that we have tested together with the reference compound **18** (CVFM) are shown in *table I*. The range of the IC_{50} values was wide: from 1 μM of the most active compound **11** to > 100 μM of a series of compounds that must be considered inactive. The most active compounds, **11** and **9** contained a chloro or bromo substituents on the phenolic ring. In contrast the parent ortho-, meta- and para-unsubstituted phenols were inactive (*table I*, **14–16**). Therefore the higher potency of this series of compounds, compared to those described by Patel [19] is not due solely to the V-F-M tripeptide used by us versus the V-L-S tripeptide. This activity may be partially due, instead, to the electronic-withdrawing properties of the halogens, since another electron-withdrawing group as NO_2 also confers inhibitory activity to the compound **13**. However this is not a general rule, in fact as an example, compound **1**, which contains a methyl group (electron-donating), is more active than compound **10** containing an electron withdrawing residue (Cl). It is possible, therefore, that the increased activity of compounds containing chloro or bromo substituents may be due to some direct interactions of the halogens with the enzyme. Moreover the relative position of the substituent on the phenolic ring seems to be important as shown by the striking difference

Table II. Physical constants of the investigated peptides.

Compounds	FAB-MS *m/z*				HPLC [a,b] t_R (min)		Yield (%) [c]	
	Calc. [d]	Calc. [e]	Found [d]	Found [e]	d	e	d	e
1	529.68	543.69	529.0	543.0	9.3	18.5	20.5	23.2
2	529.68	543.69	529.0	543.0	9.4	18.9	22.2	25.2
3	529.68	543.69	529.0	543.0	9.2	18.2	21.5	22.0
4	545.70	559.71	545.5	559.5	8.7	17.5	18.2	19.8
5	545.70	559.71	545.5	559.5	8.9	17.8	19.1	20.3
6	545.70	559.71	545.5	559.5	8.6	18.0	17.2	19.5
7	545.70	559.71	545.5	559.5	8.2	17.2	20.7	23.5
8	545.70	559.71	545.5	559.5	8.2	17.4	21.8	23.6
9	594.60	608.61	594.0	608.0	9.8	19.5	23.4	24.6
10	550.10	564.11	550.2	564.0	9.6	19.2	24.5	27.8
11	550.10	564.11	550.2	564.0	9.7	19.5	22.6	23.8
12	550.10	564.11	550.2	564.0	9.5	19.3	23.4	25.6
13	561.11	575.12	561.0	575.0	8.4	17.2	18.6	20.1
14	515.68	529.69	515.5	529.5	7.8	16.5	21.5	23.6
15	515.68	529.69	515.5	529.5	7.9	16.8	23.6	24.8
16	515.68	529.69	515.5	529.5	8.0	17.6	24.3	26.5

[a] Eluents: A (0.1% TFA in CH_3CN) and B (0.1% TFA in H_2O); analytical HPLC on a μ-Bondapak C_{18} silica column (125 Å, 15–20 mm, 30 × 300 mm); linear gradient from 5% A – 95% B to 80% A – 20% B over 25 min, UV detection at 220 nm, flow rate 1 mL/min. [b] The final HPLC purity of the peptides was always > 98%. [c] Yields of purified peptides were calculated as percentage of the theoretical yield, based on the starting material. [d] Carboxylic derivatives. [e] Methylester derivatives.

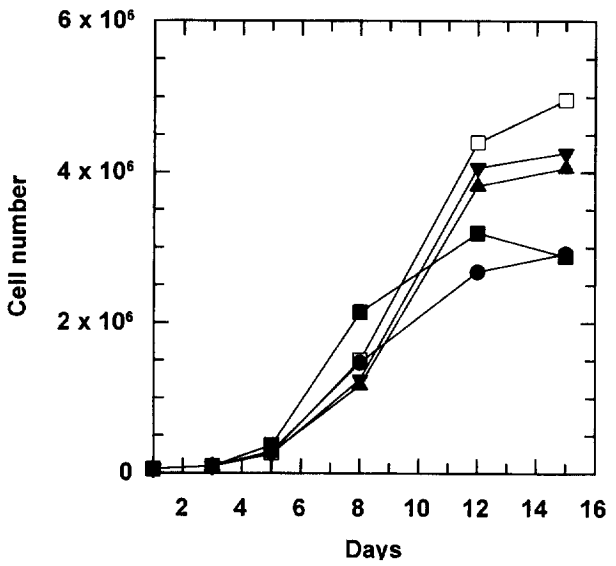


Figure 3. 40000 cells were seeded on day 0. At day 1 and twice weekly after, 100 μM inhibitor was added to the cultures. Mean cell counts from triplicate samples harvested on the days indicated are shown. FRTL-5 v-Ha-ras (open squares); **1** (triangles up); **4** (closed circles); **9** (triangles down); **13** (closed squares).

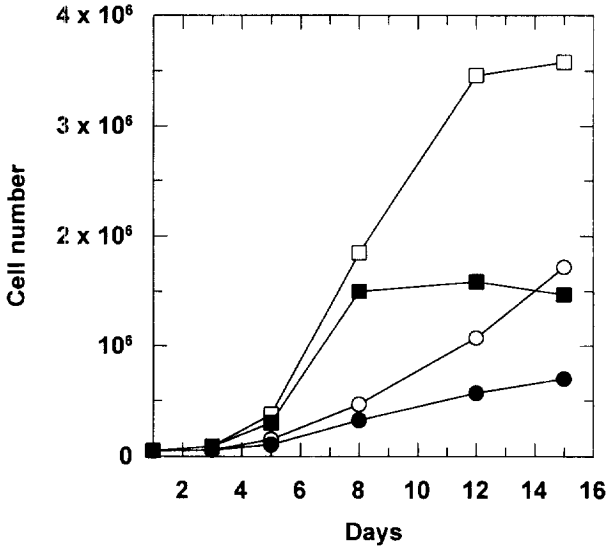


Figure 4. 40000 cells were seeded on day 0. At day 1 and twice weekly after, 100 μM compound **4** was added to the cultures. Mean cell counts from triplicate samples harvested on the days indicated are shown. FRTL-5 v-Ha-ras (open squares); FRTL-5 v-Ha-ras + compound **4** (closed squares); FRTL-5 (open circles); FRTL-5 + compound **4** (closed circles).

in activity among compounds **10**, **11** and **12**. As reported by others, also in our set of compounds the methyl ester analogs were inactive in inhibiting FTase, as shown in *table I* (**17,** general structure of the considered compounds). However, when tested in the cellular assay, the esterified derivatives showed a cytostatic activity similar to the parent compounds against transformed cells. The growth inhibition curves for the methyl ester derivatives of compounds **9** and **11**, characterized by the lower values of IC_{50} among our series of compounds, are shown in *figure 6*. In conclusion FTase inhibitory activity seems to be influenced by both the chemical properties and the relative position of the substituents on the phenolic ring. We next evaluated the pharmacological activity of these compounds in an in vitro assay on an epithelial thyroid cell line transformed by v-Ha-ras oncogene. We tested all the compounds with an $IC_{50} < 100$ μM on the normal rat thyroid cell line FRTL-5, as well as on the v-Ha-ras transformed counterparts. Only two, among all the compounds tested, **4** and **13**, had a growth inhibitory activity as shown in *figure 3*. We then tested these two latter compounds on FRTL-5 and FRTL-5 v-Ha-ras cells. Compounds **4** inhibited normal as well as transformed cells, as shown in *figure 4*. On the contrary, compound **13** had a growth inhibitory activity only against transformed cells and was not toxic to normal FRTL-5 cells, as shown in *figure 5*.

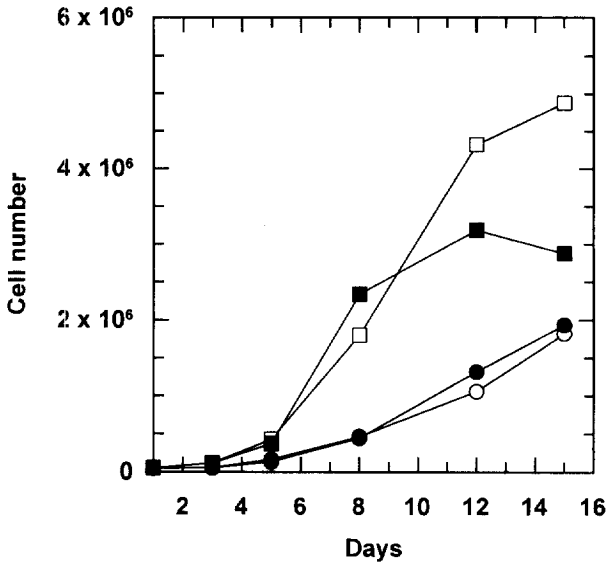


Figure 5. 40000 cells were seeded on day 0. At day 1 and twice weekly after, 100 μM compound **13** was added to the cultures. Mean cell counts from triplicate samples harvested on the days indicated are shown. FRTL-5 v-Ha-ras (open squares); FRTL-5 v-Ha-ras + compound **13** (closed squares); FRTL-5 (open circles); FRTL-5 + compound **13** (closed circles).

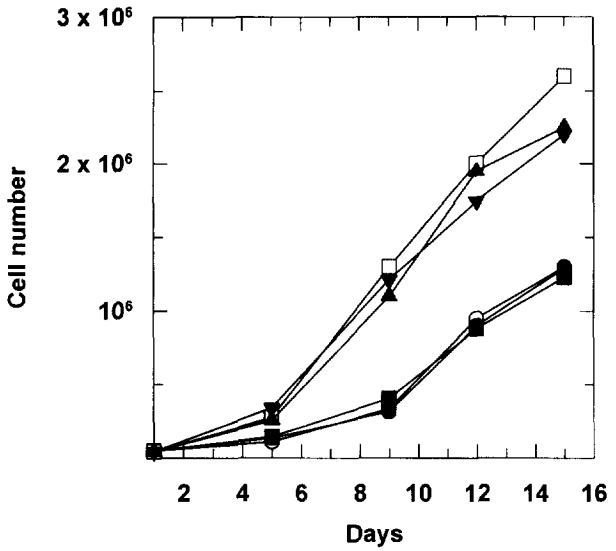


Figure 6. 40000 cells were seeded on day 0. At day 1 and twice weekly after, 100 μM inhibitor was added to the cultures. Mean cell counts from triplicate samples harvested on the days indicated are shown. FRTL-5 (open circles); FRTL-5 + methyl ester derivative of compound **9** (closed circles); FRTL-5 + methyl ester derivative of compound **11** (closed squares); FRTL-5 v-Ha-ras (open squares); FRTL-5 v-Ha-ras + methyl ester derivative of compound **9** (triangles up); FRTL-5 v-Ha-ras + methyl ester derivative of compound **11** (triangles down).

5. Conclusion

Although general conclusions cannot be drawn from this small series of compounds, it appears that the IC_{50}, as evaluated in our assay system, has a limited value in predicting the pharmacological activity of these drugs against cell lines transformed by Ras oncogenes. Compound **11** that had the lower IC_{50} was totally ineffective in the cellular assay (results not shown) and, moreover, the attempt to improve its membrane permeability, masking the carboxyl group did not result in a higher activity in cells (*figure 6*). However, the avaibility in our laboratory of rat thyroid epithelial cell lines transformed by Ras oncogenes as well as of their normal counterpart should allow us to select compounds active against malignant cells and non toxic against normal cells, as shown by the analysis of the growth inhibitory effects of compound **13**.

6. Experimental protocols

6.1. Chemistry

Melting points were measured on a Buchi SPM-20 apparatus and are reported uncorrected. Optical rotations were recorded on a Atago Polax-D polarimeter. Elemental analysis were performed on a Perkin-Elmer 2400 CHN analyzer. Analytical results were within ± 0.4% of the theoretical values unless otherwise noted. Thin layer chromatography was performed on precoated silica gel plate Kieselgel 60F254 (E. Merck, A.G., Darmstadt, Germany) with the following solvent systems: A, $CHCl_3$–MeOH (10:1); B, $CHCl_3$–MeOH (20:1); C, $CHCl_3$–MeOH–AcOH (8:1:1). The products on thin layer chromatography plates were detected by UV light and either chlorination followed by a solution of 1% starch-15 KI (1:1 v/v) or ninhydrin. Silica gel column chromatography was performed on Kieselgel 60 (230–400 mesh). Extraction solvents were dried over magnesium sulfate. Solvents used for reactions were dried over 3 Å molecular sieves. Reversed-phase was routinely performed on a Waters Delta-Prep 4000 system equipped with a Waters 484 multiwavelength detector on a m-Bondapak C-18 silica (125 Å, 15–20 mm, 30 x 300 mm) high performance liquid chromatography (HPLC) column. The gradient used was identical to that of analytical determinations. The operational flow rate was 30 mL/min. Homogeneity and retention times (t_R) of the purified products were assessed by analytical reverse phase (RP) HPLC with μ-Bondapak C18-125 Å column 10 mm, 3.9 x 300 mm, spherical, with the following solvent system: A 0.1% trifluoroacetic acid (TFA) in CH_3CN, B 0.1% TFA in H_2O, linear gradient from 5% A – 95% B to 80% A – 20% B over 25 min, UV detection at 220 nm, flow rate 1 mL/min. The final HPLC purity of the peptides was always > 98%. The yields of purified peptides were calculated as percentage of the theoretical yield, based on the starting material. All solvents were filtered and degassed prior to use. Reagent grade material were purchased from Novabiochem and from Aldrich Chemical Co. and were used whitout further purification. Molecular weights of pseudopeptides were determined by fast atom bombardement (FAB) mass spectrometry on a ZAB 2 SE-FISONS. Dimethylformamide (DMF) was distilled immediately before use over CaH_2.

6.1.1. General procedures for compounds 1–16

The main procedures used for the preparation of the protected peptides reported in *figures 1* and *2* and *table 1* varied little in the individual steps and are therefore summarized in the following general form.

6.1.1.1. WSCD/HOBT method. A solution of Boc-amino acid (1.0 mmol), amine component (1.0 mmol), and HOBT (1.1 mmol) in CH_2Cl_2 (10 mL) was ice cooled. To this solution was added WSCD•HCl (1.1 mmol). The resulting solution was stirred at this temperature for 2 h and overnight at room temperature and then concentrated, diluted with water, and extracted with EtOAc. The organic layer was washed successively with $NaHCO_3$ solution, water, 0.5 N HCl, and brine and evaporated under reduced pressure. The obtained residue was chromatographed on silica gel eluting with $CHCl_3$/MeOH (9:1, v/v) and then crystallized from EtOAc–diisopropylether.

6.1.1.2. TBTU/HOBT method. To a DMF mixture of 1 mmol of Boc-dipeptide, 1.1 mmol of HOBT, 1.1 mmol of TBTU, and 1 mmol of H-Met-OCH_3, 2 mmol of diisopropylethylamine were added under cooling to 0 °C. After 16 h at room temperature the reaction mixture was separated from the solvent, the crude residue was taken up in ethyl acetate and washed successively three times with citric acid (5%), sodium bicarbonate (5%), and a saturated

solution of sodium chloride. The oily residue was then triturated in ether and pentane to obtain a crude solid compound which was further purified by silica gel column chromatography using $CHCl_3$/MeOH (9:1, v/v) as eluent.

6.1.1.3. Deprotection procedures. The procedures used for the removal of peptide protecting groups are summarized in the following general form.

6.1.1.4. HCl/Dioxane deprotection. To a solution of 1 mmol of peptide and anisole (4 mL) in CH_2Cl_2 (15 mL) was added 4 N HCl (15 mL) in dioxane under ice cooling. The resulting mixture was stirred at this temperature for 15 min and at room temperature for 1 h. The solvent was evaporated under reduced pressure and the residue was crystallized from diethylether. The crystalline product was filtered and dried.

6.1.1.5. Catalytic hydrogenation. The –OBzl protective group (0.01 mol) was removed by hydrogenation (10% palladized charcoal, H_2, 10 mL of MeOH) under an atmospheric pressure of hydrogen. The catalyst was removed by filtration after the uptake of hydrogen ceased, and the filtrate was concentrated to produce the corresponding deprotected crude peptides.

6.1.1.6. Saponification. Methyl ester groups were removed by treating 1 mmol of peptide in MeOH (8 mL) with 1.2 equiv. 1 N NaOH for 6–8 h at room temperature. The solution was then diluted with water, concentrated in vacuo to remove the methanol, and washed with AcOEt. After cooling at 0 °C, the aqueous solution was acidified with 1 N HCl and the product extracted with ethyl acetate. The organic layer was washed with water, dried and evaporated; the residue was purified by preparative RP-HPLC.

6.2. Biological evaluation

6.2.1. Farnesyltransferase protein

The enzyme was a partially purified fraction from a bovine brain homogenate, prepared essentially as described by Reiss et al. [9].

6.2.2. Farnesyltransferase inhibition assay

The assay that we have used, based on the scintillation proximity principle, is the Amersham's Farnesyl Transferase kit. A human lamin-B carboxy terminus sequence peptide (biotin-YRASNRSCAIM) is ^{3}H-farnesylated at the cysteine residue when processed by farnesyl transferase. The resultant complex is captured by a streptavidin-linked SPA beads. In a 100 µL final reaction volume, the test compound was added in 10 µL of DMSO to a 70 µL reaction mixture containing 20 µL of 1:20 diluted [^{3}H]farnesyl pyrophosphate (0.2 µCi), 46 µL of assay buffer (50 mM HEPES, 30 mM $MgCl_2$, 20 mM KCl, 5 mM DTT and 0.01% Triton X-100) and 20 µL of 0.5 mM Biotin-Lamin B peptide in a buffer at pH 7.5 (50 mM HEPES, 25 mM Na_2HPO4, 20 mM KCl, 5 mM DTT and 0.01% Triton X-100). This mixture was allowed to stand at 37°C for 5 minutes. The reaction was started by adding 4 µL of diluted enzyme (~ 2 µg of protein) to the mixture that was incubated at 37 °C for 1 h. To stop the reaction 150 µL of the stop/bead reagent were added. The samples were counted in a Beckman LS 1801 scintillation counter. Every test compound was assayed at least twice. FTase inhibitors I, II and III were obtained from Calbiochem Corporation, La Jolla, CA, USA.

6.2.3. Cell lines

FRTL-5 is a continuous line of differentiated epithelial cells derived from normal Fisher rat thyroids and represents one of the few available models to study the mechanism of thyroid cell transformation by cellular oncogenes in vitro. These cells have retained the typical markers of thyroid differentiation: thyroglobulin (TG), thyroperoxidase (TPO) and thyrotropin receptor (TSH-R) and depend for growth upon the continuous presence of thyroid stimulating hormone (TSH) in the medium. In vitro experiments on FRTL-5 rat thyroid cells have shown that this oncogene is able, in a single step, of malignantly transforming this cell line [23]. v-Ha-ras transformation results in changes in cellular morphology, an increase in anchorage-dependent growth, a modification of the specific differentiated cell properties and the ability to form tumors when transplanted into syngenic rats.

The FRTL-5 cells were grown in Coon's modified Ham F12 medium supplemented with 5% calf serum and a six-hormone mixture: TSH 1×10^{-10} M, insulin 10 mg/mL, somatostatin 10 ng/mL, glycyl-L-Histidyl-L-Lysine acetate 10 ng/mL, hydrocortisone 1×10^{-8} M and transferrin 5 mg/mL. FRTL-5 cells fully transformed by the v-Ha-ras oncogene were grown in Coon's modified Ham F12 medium supplemented with 5% calf serum.

6.2.4 Growth experiments

On day 0, 40000 cells were plated on 60 mm tissue culture dishes. One day following plating, cells were treated with a 100 µM concentration of the drug. Cultures were refed and treated twice per week. Cell counts were taken on triplicates by hemocytometer. All growth curves experiments were repeated at least twice.

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