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α-Cyanocinnamide Derivatives: A New Family of Non-Peptide, Non-Sulfhydryl Inhibitors of Ras Farnesylation

Enrique Poradosu,[a] Aviv Gazit,[b] Hadas Reuveni[a] and Alexander Levitzki[a,*]

[a]*Department of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*
[b]*Department of Organic Chemistry, The Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

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Abstract—Farnesylation of Ras and other proteins is required for their membrane attachment and normal function. Here we report on the synthesis of α-cyanocinnamide derivatives, a new family of farnesyltransferase inhibitors. These compounds are nonpeptidic and do not contain sulfhydryl groups. The most potent compound is a pure competitive inhibitor with respect to the Ras protein and mixed competitive with respect to farnesyl diphosphate. Selectivity studies against geranylgeranyltransferase and biological activities of selected compounds are described.

Introduction

The Ras proteins are a class of plasma membrane associated G-proteins that act as a molecular switch in normal and pathogenic mitogenic signalling pathways across the cell membrane. Point mutations in the ras oncogenes which lock the Ras switch in its active GTP-bound state are found in 40% of all cancers.[1,2] The Ras protein is localized to the inner leaflet of the plasma membrane. Anchoring to the membrane is achieved through a series of post-translational modifications directed by its carboxy terminal CAAX motif (C-cysteine, A-aliphatic, X-methionine or serine), which is farnesylated at the cysteine residue by the protein Ras farnesyl transferase (FT). After a subsequent proteolytic removal of the three C-terminal amino acids the farnesylated cysteine residue is methyl esterified.[3,4] The initial farnesylation is a prerequisite for all subsequent covalent modifications.[5]

The biological role of Ras prenylation has been extensively studied; the modification of Ras with a specific isoprenoid is required for hSOS promoted guanine nucleotide exchange,[6] and the activation of Raf-1,[7] B-Raf,[8] and ERK.[9] Moreover, membrane localization of Ras is essential for its normal function and the transforming activity of its oncogenic version.[10] Thus, interfering with the Ras pathway by inhibiting Ras farnesylation and membrane localization was hypothesized to be a potentially specific chemotherapeutic strategy.

Although biological effects exerted by FT inhibitors were correlated with their ability to abolish Ras membrane anchorage, several lines of evidence suggest that the biological effects of FT inhibitors are mediated by the inhibition of farnesylation of proteins other than Ras, like Rho B.[11–13]

Despite the open questions concerning the mechanism of action of FT inhibitors, experimental data obtained in the past four years has clearly demonstated their biological activity. FT inhibitors cause reversal of Ras induced transformation in intact cells,[14–18] inhibition of Ras tumor growth in nude mice[19–22] and tumor regression in Ha-Ras transgenic animals,[23] with minimal toxic effects. These findings strongly support the use of FT inhibitors as potential anticancer drugs.

Analysis of the crystal structure of FT shows that it possesses a bound zinc ion within an open coordination sphere that includes a water molecule, suggesting a catalytic function as opposed to the closed spheres characteristic of structural metal ions. The crystallographic data also indicates that the sulfhydryl group in the CAAX motif is localized in close proximity to the zinc atom and adjacent to the farnesylpyrophosphate α phosphate.[24] Accordingly, in CAAX motif peptidic analogues the cysteine residue confers improved potency to the inhibitor.[25–28] However, the sulfhydryl moiety has been replaced with various degrees of success in various

* Corresponding author.

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peptidic derivatives[16,17,29–34] and is not present in some non-peptide inhibitors.[35–37]

In this study we report on the synthesis and evaluation of a new class of non-peptide, non-sulfhydryl FT inhibitors based on the α-cyano-cinnamide structure. These compounds exhibit FT inhibition activity in vitro and in intact cells. The structure–activity relationship of α-cyano-cinnamide derivatives, selectivity studies of FT versus geranyl–geranyl transferase type I (GGT I) inhibition,[38,39] and their biological activities are described.

Results and Discussion

α-Cyano-cinnamide derivatives design

With the aim to design non-peptide FT inhibitors lacking a sulfhydryl group we first synthesized a series of CAAX peptides in which the cysteine residue was replaced by various groups with different affinities towards Zn^{++} (data not shown). A direct correlation was observed between the chelating capability to the inhibitory activity towards FT. The best inhibitor of this series was HVFM with an IC_{50} of 12 μM, in agreement with published results in which the cysteine residue in CAAX peptidic analogues was replaced by histidine.[29]

Since tyrosine kinase inhibitors from the tyrphostin family which are hydroxy derivatives of a-cyanocinnamide were shown to be tyrosine mimics,[40] we chose as a non-peptidic scaffold the α-cyanocinnamide structure with the assumption that it will potentially mimic the phenylalanine residue in the potent CVFM peptide inhibitor.

α-Cyanocinnamide derivatives were prepared by a straight-forward synthesis (Fig. 1); phenolic aldehydes were reacted with excess α,ω-dibromoalkanes and the monobromo product purified by chromatography. The various thio-analogues were prepared from these bromo compounds and the corresponding mercapto-heterocycles, which were then condensed in the Knoevenagel reaction with aryl cyano-acetamides[41] or benzyl cyano-acetamides (prepared analogueously). Yields were not optimized.

The inhibitory activity of a series of compounds based on the α-cyanocinnamide structure substituted with

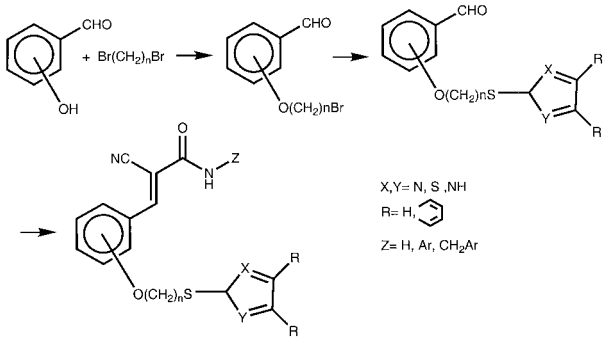


Figure 1. A general scheme for the synthesis of α-cyanocinnamide derivatives.

imidazole and related heterocyclic groups is shown in Table 1. The most effective compound was the imidazole substituted α-cyanocinnamide (**3**) with an IC_{50} of 11.5 μM.

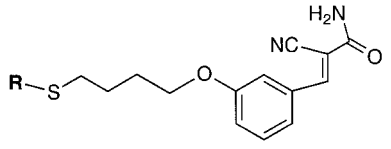
Next we examined the optimal length of the alkyl linker between the pharmacophore and the cinnamide ring and the optimal position on that ring (Table 2). The most effective analogues had linkers in position *ortho* and *meta* with an optimal length of 3 and 4 methylenes. These inhibitors showed an IC_{50} of 17.5 and 11.5 μM for the imidazole derivatives (compounds **10** and **3**, respectively) and 36.6 and 33.3 μM for the benzimidazole analogues (compounds **5** and **2**, respectively).

In order to further explore the binding pocket of the inhibitors a phenyl (**14**) and benzyl (**29**) derivatives of compound **10** were prepared. While the benzyl ring improved the inhibitory activity twofold (IC_{50} of 10.3 μM) the phenyl ring has a deleterious effect compared to the parental compound (IC_{50} of 162 μM). Further examination of phenyl and benzyl amide derivatives yielded two different families in which the rings were systematically substituted with chemically different groups (Table 3). The most potent compound from this series was **26**, which inhibited FT with an IC_{50} of 1.8 μM.

Interestingly, substitutions of different chemical nature in the phenyl ring at position R2 like the polar carboxyl in compound **13** (IC_{50} 12.1 μM) or Cl in compound **12** (IC_{50} 7 μM) gave analogues with similar inhibitory activities (Table 3). Free rotation of the phenyl ring in space suggests the possibility that these substituents are oriented to opposite sites in the same pocket. A similar result was obtained in CAAX non-peptidic analogues.[42,43]

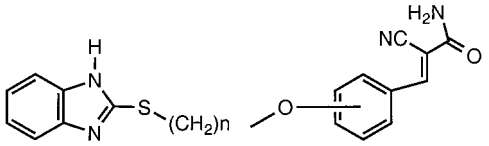
As a preliminary test of this hypothesis we prepared the phenyl imidazole derivatives with the double substitution

Table 1. Imidazole and related substitutions of α-cyanocinnamide. In vitro IC_{50}s against FT are reported for each compound. Each result represents results of two to four tests. Assays were conducted as described in Experimental

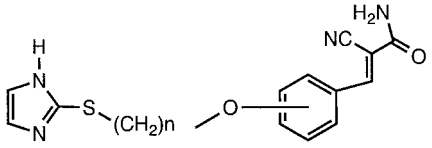


Compound no.	AGR	R	FT IC_{50} (μM)
1	109		500
2	71		33.3
3	124		11.5

Table 2. Optimization of linker positioning and length in benzimidazole and imidazole derivatives of α-cyanocinnamide. In vitro IC_{50}s against FT are reported for each compound. Each result represents results of two to four tests. Assays were conducted as described in Experimental



Compound no.	AGR	n (*ortho*)	FT IC_{50} (μM)
4	93	2	153.1
5	85	3	36.6
6	94	4	115.1
Compound no.	AGR	n (*meta*)	FT IC_{50} (μM)
7	87	2	112.3
8	79	3	81.6
2	71	4	33.3
9	74	5	100



Compound no.	AGR	n (*ortho*)	FT IC_{50} (μM)
10	128	3	17.5
Compound no.	AGR	n (*meta*)	FT IC_{50} (μM)
3	124	4	11.5

3,4 and 3,5-chloro,carboxy. These compounds inhibited the FT reaction with an IC_{50} of 50.6 and 40 μM (**18** and **25**, respectively) (Table 3). This negative result implies that the geometry of the hydrophobic and carboxylic subpockets within this cleft requires a much more flexible structure than the phenyl ring to reach both sites simultaneously. Further work is in progress to examine this hypothesis.

Although compound **10**, which is the core of the inhibitors described, was designed to mimic the HVF portion of the HVFM peptide, the molecular structure of the FT inhibitors developed differ markedly from the CAAX motif. Therefore we studied the kinetics of the FT inhibition by the most potent in vitro inhibitor, **26**. Inhibition modalities other than Ras competition would indicate a binding site different from the catalytic pocket to which the inhibitors were targeted based on theoretical cosiderations. Compound **26** is a pure competitive inhibitor of FT with respect to the Ras protein and mixed competitive with respect to farnesyl pyrophosphate, as shown by the double reciprocal plots (1/v versus 1/[S]) and the slope replots (slope versus [I]) (Fig. 2). The intersection of the reciprocal plots below the 1/FPP axis means that the constant affecting K_i and KFPP resulting from the mutual influence between FPP and the inhibitor is <1. The inhibition pattern of CAAX alternative substrates was found to be pure competitive with respect to Ras and non-competitive with respect to FPP.[44] Although the mechanism of inhibition of compound **26** implies a different space orientation within the active site than CAAX alternative substrates it indicates that the inhibitor binds to the catalytic pocket as expected.

Inhibition of FT versus GGT I

Early studies showed that the closely FT related prenyl transferase GGT I transfers a geranyl–geranyl group to CAAX containing sequences where X is preferably leucine or phenylalanine.[45–47] Since geranyl-geranylation of normal proteins is 5–10 times more common than farnesylation,[25,48,49] the common assumption was that a compound displaying a strong selectivity towards FT will have the advantage of reduced side effects than a more general prenylation inhibitor. However, the discovery that in cells resistant to FT inhibitors the K- and N-Ras isoforms become geranyl-geranylated by GGT I,[50,51] and the ability of geranyl-geranylation inhibitors to block platelet-derived and epidermal growth factor dependent tyrosine phosphorylation,[52] and to block cells at G0/G1[53,54] has aroused new interest in this more ubiquitous prenyl transferase reaction.

A selectivity study of some FT inhibitors described here was performed by testing their inhibitory activity towards GGT I (Table 4). Most of the compounds tested inhibit both enzymes in vitro at comparable concentrations, (0.2–5 selectivity factor), with the highest factor of selectivity towards FT (selectivity factor = 33) observed for compound **26**. Similar to other series of FT inhibitors[35,55,56] selectivity was achieved despite the lack of a structural element clearly corresponding to the specificity determining X residue in the CAAX motif.

Biological results

Compounds **13, 26**, and **29** were tested for their ability to inhibit protein prenylation in intact cells. Compounds **13** and **26** completely inhibit Ras farnesylation at a concentration of 120 μM, Rap-1 geranyl-geranylation was not affected at the same concentrations (Fig. 3). The effect of these compounds on cell growth was assessed on a LIM1899 colon carcinoma cell line expressing a mutant K-Ras($Gly^{12}\rightarrow Cys^{12}$), NIH3T3 and v-H-Ras transformed NIH3T3 cell lines (Table 5). The IC_{50}s obtained (70–180 μM) are in correlation with the concentrations required for FT inhibition in whole cells. Previously it was reported that in K-Ras transformed cells resistance to FT inhibitors could be attributed to K-Ras geranyl-geranylation,[50] however, LIM1899 cells which express a mutated K-Ras were inhibited to the same extent as NIH3T3 cells transformed with v-H-Ras and normal NIH3T3 cells (Table 5). Compound **26** (AGR129) also inhibited colony formation in soft agar of v-H-Ras transformed NIH3T3 cells (Fig. 4). At 100 μM, the concentration required for complete inhibition of FT in whole cells, a 75% inhibition of colony formation was observed.

Conclusions

Imidazole derivatives of α-cyano-cinnamide constitute a novel class of non-peptidic, non-sulfhydryl FT inhibitors. The most potent inhibitors of this class inhibit FT with IC_{50} values at the low micromolar range and are targeted to the catalytic site of the FT enzyme. These compounds inhibit both the farnesylation of Ras in intact cells and the cell growth of cells harboring mutated v-H-Ras and K-Ras($Gly^{12}\rightarrow Cys^{12}$).

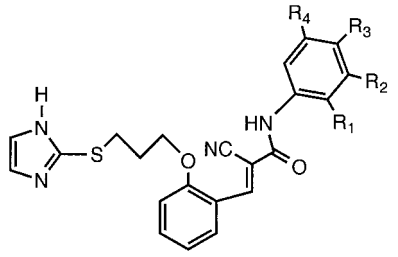
Experimental

General chemical procedures

All starting materials were purchased from Aldrich. NMR spectra were recorded on a Bruker 300 pulsed FT spectrometer. Chemical shifts are in ppm relative to TMS internal standard. Mass spectra were recorded with a MAT311 instrument. Combustion analyses for all new compounds were within 0.4% of the theoretical value. Work up means adding to water, extracting with

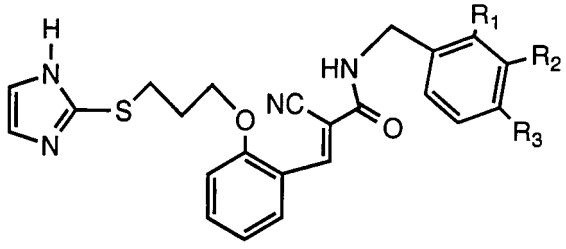
Table 3. In vitro FT inhibition by phenyl (A) and benzyl (B) derivatives of AGR128 (compound **10**). In vitro IC_{50}s against FT are reported for each compound. Each result represents results of two to four tests. Assays were conducted as described in Experimental

(A)



Compound no.	AGR	R1	R2	R3	R4	FT IC_{50} (μM)
11	139	H	CH_3	H	H	72.6
12	141	H	Cl	H	H	7
13	142	H	COOH	H	H	12.1
14	144	H	H	H	H	162
15	154	H	H	$COOCH_2CH_3$	H	> 300
16	158	H	H	COOH	H	16.3
17	168	H	Cl	$COOCH_3$	H	58.4
18	169	H	Cl	COOH	H	50.5
19	178	H	$COOCH_3$	H	$COOCH_3$	> 64
20	180			H	H	13.6
21	182	pH	H	H	H	26
22	189	H	NH_2	H	$COOCH_3$	> 128
23	190	H	$COOCH_3$	OH	H	> 128
24	196	H	Cl	H	$COOCH_3$	> 64
25	197	H	Cl	H	COOH	40

(B)



Compound no.	AGR	R1	R2	R3	FT IC_{50} (μM)
26	129	H	Cl	H	1.8
27	130	H	H	Cl	12.8
28	137	Cl	H	H	4.3
29	143	H	H	H	10.6
30	146	H	OCH_3	OCH_3	21.5
31	147	H	Cl	Cl	23.6
32	148	OCH_3	H	OCH_3	20.5

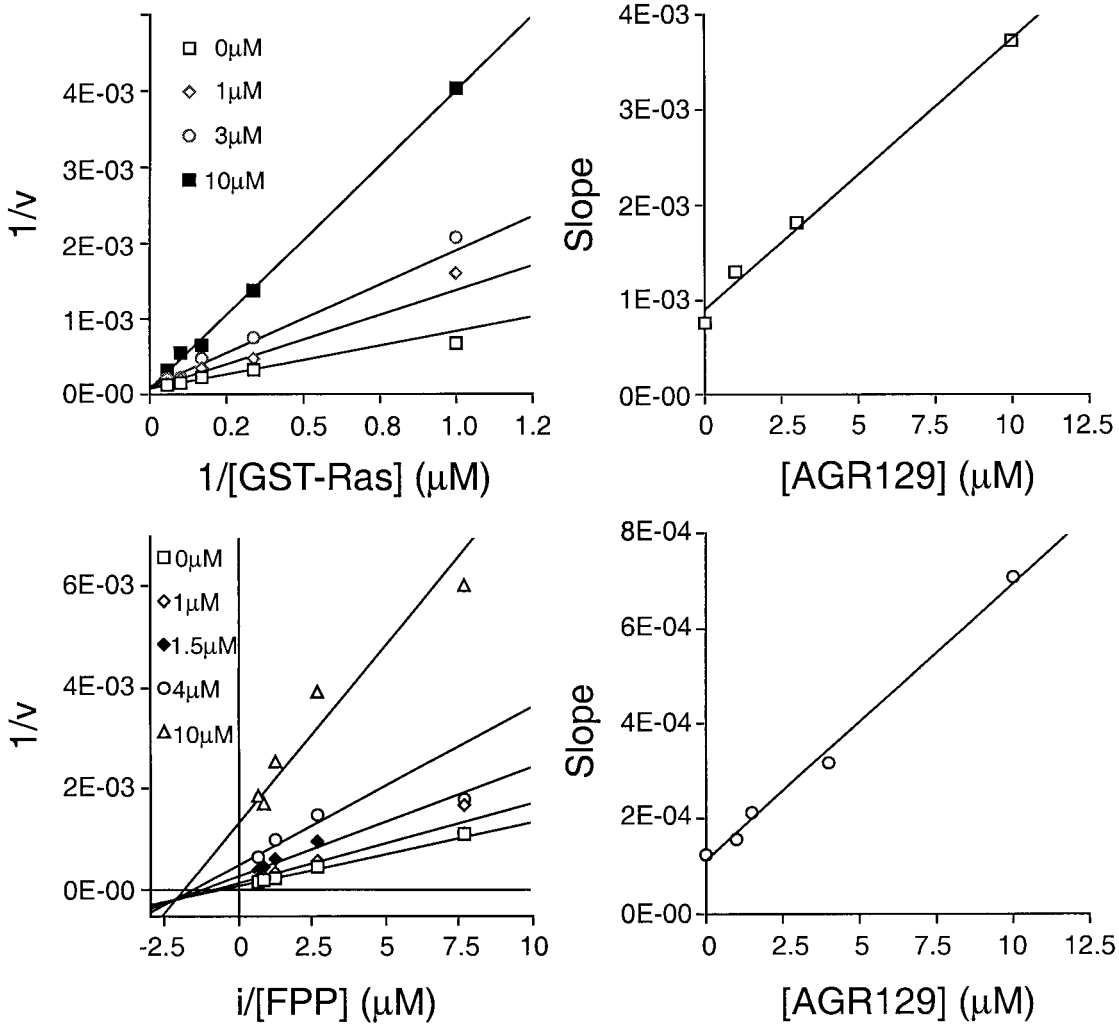


Figure 2. Kinetics of inhibition of the FT reaction by AGR129. The activity of FT was determined as described in Experimental except that the reaction was performed in the presence of 1, 3, 6, 10, and 18 μM of GST-Ras, and a fixed concentration of 0.5 μM FPP; or in the presence of 0.1, 0.3, 0.5, 1, and 1.8 μM of FPP and a fixed concentration of 5 μM GST-Ras. Initial reaction velocity (v) is expressed as pmol of farnesylated GST-Ras/h/mg enzyme. Families of double reciprocal plots and their respective slope replots are shown. AGR129 concentrations were as indicated in the figure.

dichloromethane (or with ethyl acetate when indicated), drying the organic phase and evaporating to dryness.

Compound 1. (a). Bromo aldehyde. 25 g, 0.2 M, 3-hydroxy benzaldehyde, 70 g, 0.32 M, 1,4-dibromo butane, and 17 g KOH in 150 mL ethanol were refluxed 18 h. Work up and chromatography (silica gel, 70–230 mesh, elution with CH_2Cl_2) gave 10.3 g, 20% yield, white oil. NMR ($CDCl_3$) δ 9.97 (1H, s, CHO), 7.40 (3H, m), 7.18 (1H, m), 4.06 (2H, t, $J=6.0$ Hz), 3.50 (2H, t, $J=6.0$ Hz), 2.0 (4H, m). (**b**) Benzothiazole aldehyde. 0.5 g, 2.2 mM, of the above bromo aldehyde, 0.38 g, 2.3 mM, 2-mercapto benzothiazole, and 0.2 g KOH in 30 mL ethanol were stirred 19 h at room temperature. Work up (HCl and EtAc) and chromatography gave after trituration with benzene–hexane 0.11 g, 37% yield, white solid, mp 104°C. NMR ($CDCl_3$) δ 9.95 (1H, s, CHO), 7.60 (4H, m), 7.40 (3H, m), 7.22 (1H, m), 4.03 (2H, t, $J=6.0$ Hz), 3.53 (2H, t, $J=6.0$ Hz), 2.10 (4H, m). (**c**) 170 mg, 0.5 mM, **1b**, 50 mg, 0.6 mM, cyano acetamide, and 15 mg β-alanine in 15 mL ethanol were refluxed 4 h. Evaporation and recrystalization from benzene gave 125 mg, 61% yield, white solid, mp 175°C. NMR (DMSO-d_6) δ 8.22 (1H, s, vinyl), 7.65 (4H, m), 7.30 (4H, m), 4.10 (2H, t, $J=5.8$ Hz), 3.68 (2H, t, $J=5.8$ Hz), 2.12 (4H, m).

Table 4. A comparison of FT and GGT I in vitro inhibitory activity by selected inhibitors. Selectivity factor is expressed as the ratio of the GGT I to FT inhibitory activites

Compound no.	FT IC_{50} (μM)	GGT I IC_{50} (μM)	Selectivity factor
3	11.5	53.6	4.7
12	7	9.2	1.3
13	12.1	42.6	3.5
16	16.3	7.2	0.4
21	26	6.7	0.3
25	40	7.5	0.2
26	1.8	59.3	33
27	12.8	19.5	1.5
28	4.3	15.8	3.7
29	10.6	19.3	1.8

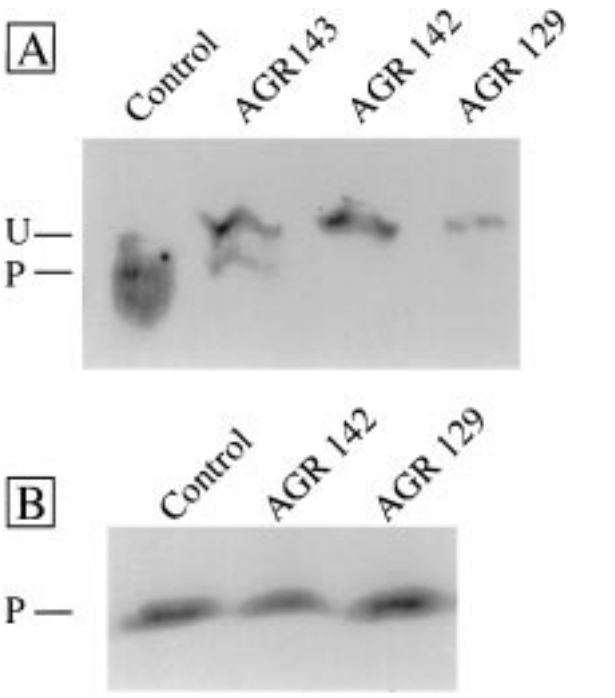


Figure 3. Effect of α-cyanocinnamide derivatives on post-translational processing of Ras and Rap1A/K-rev. v-H-Ras transformed NIH3T3 cells were treated with the indicated compounds at a concentration of 120 μM for 48 h or vehicle alone (control). Cell extracts were separated by SDS–Page (40 μg of protein/lane) and visualized by western blot. (A) v-H-Ras; (B) Rap1A. P, prenylated proteins; U, unprenylated protein. Compound **13** = AGR142, compound **29** = AGR142, compound **26** = AGR129.

Table 5. Cell growth inhibition. Inhibitions are expressed as IC_{50}s in μM. (3T3 = NIH3T3, vRas = v-H-Ras transformed NIH3T3, LIM = LIM1899)

Compound no.	3T3	vRas	LIM
13	98	80	73
26	186	152	100

Compound 2. (a). 1.6 g, 6.2 mM, bromo aldehyde **1a**, 1 g, 6.5 mM, 2-mercapto benzimidazole, and 1 g, 10 mM, Et_3N in 30 mL ethanol were stirred 26 h at room temperature. Work up and recrystalization from benzene gave 0.6 g, 30% yield, viscous solid. NMR (acetone-d_6) δ 10.0 (1H, s, CHO), 7.50 (4H, m), 7.25 (4H, m), 4.18 (2H, t, J = 6.0 Hz), 3.86 (2H, t, J = 6.0 Hz), 2.2 (4H, m). **(b)** 310 mg, 0.95 mM, **2a**, 84 mg, 1 mM cyano acetamide, and 15 mg β-alanine in 20 mL ethanol were refluxed 8 h. Evaporation and trituration in acetone–hexane gave 200 mg light-yellow solid, 54% yield, mp 154°C. NMR (acetone-d_6) δ 8.28 (1H, s, vinyl), 7.63 (4H, m), 7.30 (4H, m), 4.15 (2H, t, J = 6.0 Hz), 3.72 (2H, t, J = 6.0 Hz), 2.10 (4H, m).

Compound 3. (a). 1.6 g, 6.2 mM, **1a**, 0.67 g, 6.7 mM, 2-mercapto imidazole, and 0.5 KOH in 30 mL ethanol were stirred 20 h at ambient temperature. Work up and chromatography gave after trituration from acetone–hexane 540 mg, 32% yield, white solid, mp 165°C. NMR ($CDCl_3$) δ 11.17 (1H, br.s, NH), 9.93 (1H, s, CHO), 7.4 (3H, m), 7.15 (2H, s, imidazole), 7.10 (1H, m), 3.93 (2H, t, J = 5.7 Hz), 3.05 (2H, t, J = 5.7 Hz), 1.60 (4H, m). **(b)** 260 mg, 0.93 mM, **3a**, 84 mg, 1 mM, cyano acetamide, and 18 mg β-alanine in 20 mL ethanol were refluxed 3 h. Work up and trituration with acetone–hexane gave 150 mg, 47% yield, white solid, mp 162°C. NMR (acetone-d_6) δ 8.18 (1H, s, vinyl), 7.45 (3H, m), 7.12 (2H, s), 7.08 (1H, m), 3.90 (2H, t, J = 5.8 Hz), 3.10 (2H, t, J = 5.8 Hz), 1.70 (4H, m).

Compound 4. (a). Bromo aldehyde. 7 g, 57 mM, salicyl aldehyde, 15 g, 80 mM, dibromo ethane, and 7 g KOH in 30 mL water and 50 mL ethanol were refluxed 20 h. Work up and chromatography gave 0.83 g, 6% yield, white oil. NMR ($CDCl_3$) δ 9.95 (1H, s, CHO), 7.40 (4H, m), 4.33 (2H, t, J = 6.0 Hz), 3.62 (2H, t, J = 6.0 Hz). (**b**) 0.8 g, 3.5 mM, **4a**, 0.5 g, 3.3 mM, 2-mercapto benzimidazole, and 0.2 g KOH in 30 mL ethanol were stirred at room temperature 14 h. Work up and trituration in CH_2Cl_2–hexane gave 0.45 g, 44% yield, oily solid. NMR (acetone-d_6) δ 9.93 (1H, s, CHO), 7.53 (4H, m), 7.30 (4H, m), 4.45 (2H, t, J = 6.0 Hz), 3.72 (2H, t, J = 6.0 Hz). (**c**) 400 mg, 1.3 mM, **4b**, 130 mg, 1.5 mM, cyano acetamide, and 18 mg β-alanine in 20 mL ethanol were refluxed 3 h. Work up and trituration with acetone–hexane gave 120 mg, 25% yield, white solid, mp 212°C. NMR (acetone-d_6) δ 8.26 (1H, s, vinyl), 7.45 (4H, m), 7.30 (4H, m), 4.40 (2H, t, J = 5.8 Hz), 3.60 (2H, t, J = 5.8 Hz).

Compound 5. (a). Bromo aldehyde, 7 g, 57 mM, salicyl aldehyde, 16 g, 79 mM, 1,3-dibromo propane, and 7 g KOH in 30 mL water and 50 mL ethanol were refluxed 24 h. Work up and chromatography gave 1.3 g, 9% yield, white oil. NMR ($CDCl_3$) δ 9.95 (1H, s, CHO), 7.83 (1H, m), 7.55 (1H, m), 7.06 (2H, m), 4.24 (2H, t, J = 6.0 Hz), 3.62 (2H, t, J = 6.0 Hz), 2.40 (2H, quint, J = 6.0 Hz). (**b**) 0.45 g, 1.8 mM, **5a**, 0.2 g, 1.5 mM, 2-mercapto benzimidazole, and 0.2 g KOH in 30 mL ethanol were stirred at room temperature 30 h. Work up and trituration in CH_2Cl_2–hexane gave 0.29g, 61% yield, white solid, mp 168°C. NMR (acetone-d_6) δ 9.95 (1H, s, CHO), 7.53 (4H, m), 7.30 (4H, m), 4.45 (2H, t, J = 6.0 Hz), 3.72 (2H, t, J = 6.0 Hz), 2.30 (2H, quint., J = 6.0 Hz). (**c**) 160 mg, 0.5 mM, **5b**, 45 mg, 0.5 mM, cyano acetamide and 5 mg β-alanine in 20 mL ethanol were refluxed 3 h. Work up and chromatography gave 40 mg, 21% yield, white solid, mp 77°C. NMR (acetone-d_6) δ 8.24 (1H, s, vinyl), 7.48 (4H, m), 7.36 (4H, m), 4.40 (2H, t, J = 5.8 Hz), 3.60 (2H, t, J = 5.8 Hz), 2.30 (2H, quint., J = 6.0 Hz).

Compound 6. (a). Bromo aldehyde. 7 g, 57 mM, salicyl aldehyde, 15 g, 70 mM, 1,4-dibromo butane, and 7 g KOH in 30 mL water and 40 mL ethanol were refluxed 24 h. Work up and chromatography gave 0.76 g, 5% yield, white oil. NMR ($CDCl_3$) δ 9.95 (1H, s, CHO), 7.42 (4H, m), 4.06 (2H, t, J = 6.0 Hz), 3.52 (2H, t, J = 6.0 Hz), 2.0 (4H, m). (**b**) 0.73 g, 2.8 mM, **6a**, 0.4 g, 2.7 mM, 2-mercapto benzimidazole, and 0.2 g KOH in 30 mL ethanol were stirred at room temperature 14 h. Work up and trituration in CH_2Cl_2–hexane gave 0.6 g, 65% yield, white solid, mp 68°C. NMR (acetone-d_6) δ 9.95 (1H, s, CHO), 7.53 (4H, m), 7.31 (4H, m), 4.44 (2H, t, J = 6.0 Hz), 3.70 (2H, t, J = 6.0 Hz), 2.30 (2H, m).

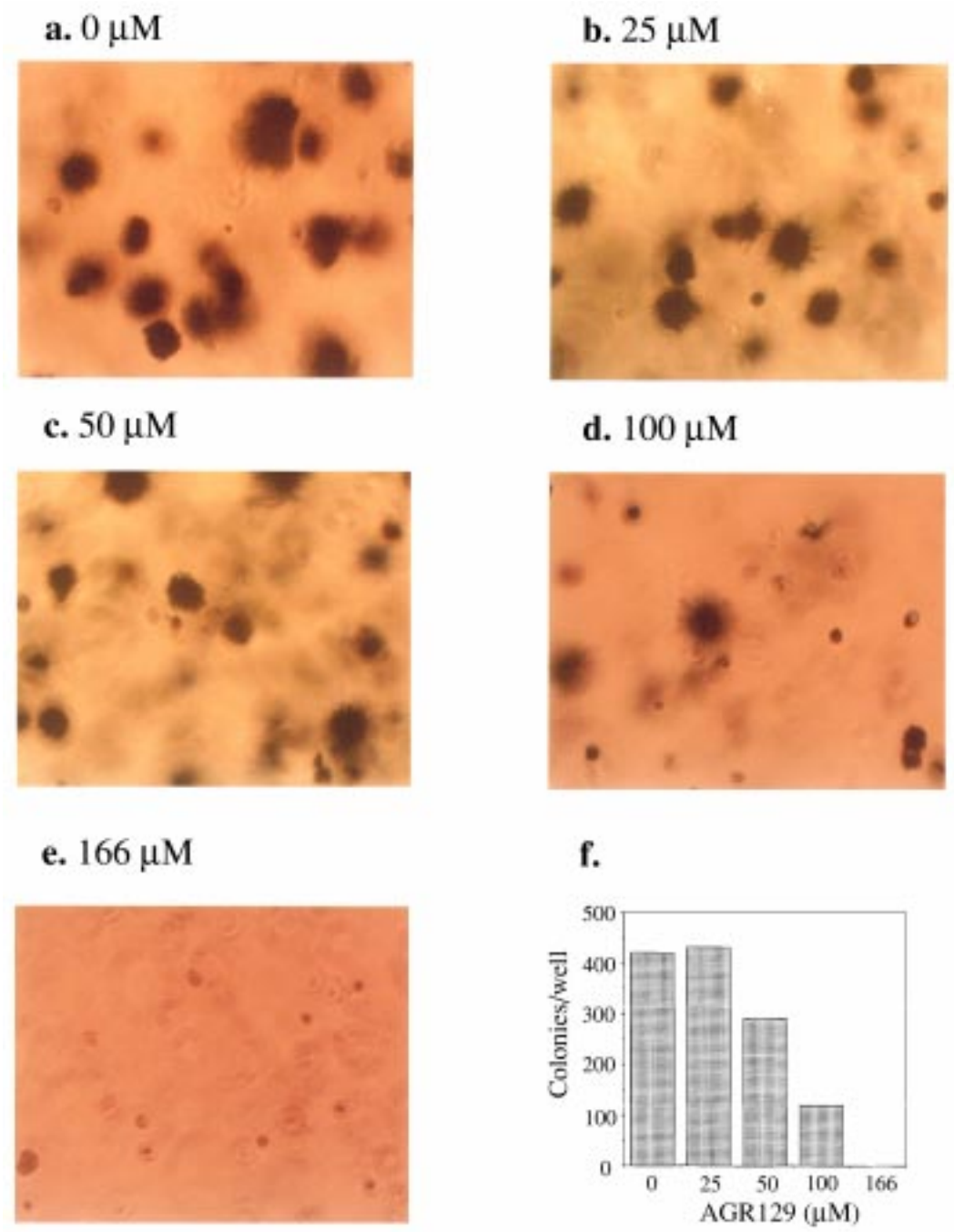


Figure 4. Inhibition of colony formation in soft agar by AGR129 (compound **26**). v-H-Ras transformed NIH3T3 cells were seeded on a 96-well plate (3500 cells/well) in soft agar (see Materials and Methods). Serial dilutions of the inhibitor were applied in the growth medium from the top. After 12 days the colonies were stained with MTT, counted in the microscope and photographed. (a) 0 μM, (b) 25 μM, (c) 50 μM, (d) 100 μM, (e) 166 μM, (f) number of colonies per well plotted against the concentration of the inhibitor.

(**c**) 400 mg, 1.2 mM, **6b**, 130 mg, 1.5 mM, cyano acetamide, and 10 mg β-alanine in 20 mL ethanol were refluxed 3 h. Work up and chromatography gave 120 mg, 25% yield, white solid, mp 138°C. NMR (acetone-d_6) δ 8.28 (1H, s, vinyl), 7.48 (4H, m), 7.36 (4H, m), 4.40 (2H, t, $J = 5.8$ Hz), 3.60 (2H, t, $J = 5.8$ Hz), 2.34 (2H, m)

Compound 7. (a). Bromo aldehyde, 4.9 g, 40 mM, 3-hydroxy benzaldehyde, 12.2 g, 65 mM, 1,2-dibromo ethane, and 5 g KOH in 40 mL water and 40 mL ethanol were refluxed 24 h. Work up and chromatography gave 1.05 g, 11% yield, white oil. NMR ($CDCl_3$) δ 9.97 (1H, s, CHO), 7.42 (3H, m), 7.20 (1H, m), 4.35 (2H, t, $J = 6.0$ Hz), 3.66 (2H, t, $J = 6.0$ Hz). (**b**) 0.43 g, 2.0 mM, **7a**, 0.25 g, 1.6 mM, 2-mercapto benzimidazole, and 0.2 g KOH in 30 mL ethanol were stirred at room temperature 30 h. Work up and trituration in CH_2Cl_2–hexane gave 0.115 g, 24% yield, white solid, mp 164°C. NMR (acetone-d_6) δ 9.95 (1H, s, CHO), 7.53 (3H, m), 7.31 (4H, m), 7.15 (1H, m), 4.44 (2H, t, $J = 6.0$ Hz), 3.70 (2H, t, $J = 6.0$ Hz). (**c**) 105 mg, 0.35 mM, **7b**, 30 mg, 1.5 mM, cyano acetamide, and 5 mg β-alanine in 20 mL ethanol were refluxed 6 h. Workup and chromatography gave 40 mg, 31% yield, white solid, mp 121°C. NMR

(acetone-d_6) δ 8.28 (1H, s, vinyl), 7.50 (3H, m), 7.38 (4H, m), 7.20 (1H, m), 4.40 (2H, t, J = 5.9 Hz), 3.61 (2H, t, J = 5.9 Hz).

Compound 8. (a). Bromo aldehyde, 5.1 g, 42 mM, 3-hydroxy benzaldehyde, 14 g, 69 mM, 1,3-dibromo propane, and 5 g KOH in 60 mL water and 50 mL ethanol were refluxed 18 h. Work up and chromatography gave 4.3 g, 33% yield, white oil. NMR ($CDCl_3$) δ 9.97 (1H, s, CHO), 7.45 (3H, m), 7.18 (1H, m), 4.16 (2H, t, J = 6.0 Hz), 3.62 (2H, t, J = 6.0 Hz), 2.34 (2H, quint., J = 6.0 Hz). (**b**) 1.5 g, 6.2 mM, **8a**, 0.9 g, 6.0 mM, 2-mercapto benzimidazole, and 0.2 g KOH in 30 mL ethanol were stirred at room temperature 26 h. Work up and chromatography gave 0.29 g, 24% yield, viscous oil. NMR (acetone-d_6) δ 9.95 (1H, s, CHO), 7.53 (3H, m), 7.30 (4H, m), 7.25 (1H, m), 4.43 (2H, t, J = 6.0 Hz), 3.74 (2H, t, J = 6.0 Hz), 2.30 (2H, quint., J = 6.0 Hz). (**c**) 300 mg, 0.96 mM, **8b**, 90 mg, 1.07 mM, cyano acetamide, and 5 mg β-alanine in 20 mL ethanol were refluxed 3 h. Workup and trituration in CH_2Cl_2–hexane gave 160 mg, 44% yield, white solid, mp 132°C. NMR (acetone-d_6) δ 8.20 (1H, s, vinyl), 7.48 (3H, m), 7.36 (4H, m), 7.19 (1H, m), 4.40 (2H, t, J = 5.8 Hz), 3.60 (2H, t, J = 5.8 Hz), 2.30 (2H, quint., J = 5.8 Hz).

Compound 9. (a). Bromo aldehyde, 4.9 g, 40 mM, 3-hydroxy benzaldehyde, 12.6 g, 55 mM, 1,5-dibromo pentane, and 5 g KOH in 40 mL water and 40 mL ethanol were refluxed 20 h. Workup and chromatography gave 2.7 g, 25% yield, white oil. NMR ($CDCl_3$) δ 9.97 (1H, s, CHO), 7.40 (3H, m), 7.18 (1H, m), 4.03 (2H, t, J = 6.0 Hz), 3.44 (2H, t, J = 6.0 Hz), 2.0 (4H, m), 1.7 (2H, m). (**b**) 1.3 g, 5.0 mM, **9a**, 0.6 g, 4.0 mM, 2-mercapto benzimidazole, and 0.3 g KOH in 30 mL ethanol were stirred at room temperature 23 h. Workup and chromatography gave 0.5 g, 29% yield, viscous oil. NMR (acetone-d_6) δ 9.95 (1H, s, CHO), 7.47 (3H, m), 7.30 (4H, m), 7.20 (1H, m), 4.43 (2H, t, J = 6.0 Hz), 3.54 (2H, t, J = 6.0 Hz), 2.20 (4H, m), 1.85 (2H, m). (**c**) 250 mg, 0.73 mM, **9b**, 67 mg, 0.8 mM, cyano acetamide, and 5 mg β-alanine in 20 mL ethanol were refluxed 8 h. Work up and trituration in CH_2Cl_2–hexane gave 195 mg, 65% yield, white solid, mp 82°C. NMR (acetone-d_6) δ 8.25 (1H, s, vinyl), 7.48 (3H, m), 7.36 (4H, m), 7.19 (1H, m), 4.40 (2H, t, J = 5.8 Hz), 3.60 (2H, t, J = 5.8 Hz), 2.20 (4H, m), 1.90 (2H, m).

Compound 10. (a). 0.8 g, 3.3 mM, **5a**, 0.32 g, 3.2 mM, 2-mercapto imidazole, and 0.2 KOH in 30 mL ethanol were stirred 20 h at ambient temperature. Work up and chromatography gave after trituration from acetone hexane 356 mg, 43% yield, white solid, mp 88°C. NMR ($CDCl_3$) δ 10.4 (1H, s, CHO), 7.80 (1H, d, J = 7.8 Hz), 7.54 (1H, t), 7.10 (2H, s, imidazole), 7.04 (1H, t, J = 7.5 Hz), 6.96 (1H, d, J = 8.4 Hz), 4.22 (2H, t, J = 6.0 Hz) 3.24 (2H, t, J = 6.0 Hz), 2.22 (2H, quint., J = 6.0 Hz). (**b**) 45 mg, 0.17 mM, **3a**, 20 mg, 0.24 mM, cyano acetamide, and 4 mg β-alanine in 20 mL ethanol were refluxed 4 h. Workup and trituration with acetone–hexane gave 44 mg, 80% yield, white solid, mp 164°C. NMR (acetone-d_6) δ 9.0 (1H, s, vinyl), 8.4 (1H, d, J = 8.2 Hz), 7.50 (1H, t, J = 7.8 Hz), 7.10 (1H, m), 7.07 (2H, s, imidazole), 6.93 (1H, m), 4.16 (2H, t, J = 6.0 Hz) 3.28 (2H, t, J = 6.0 Hz), 2.19 (2H, quint., J = 6.0 Hz).

Compounds 11–32. These compounds were prepared from the imidazole aldehyde **5a** and the appropriate aryl or benzyl cyano acetamide. One illustrating example is given to each followed by data to the analogues of its group.

Aryl analogues 11–25

Compound 12. (a). 4 g, 31 mM, 3-Cl aniline, and 4.7 g, 47 mM, methyl cyanoacetate were heated at 120°C without solvent at open flask, for 15 h, the cooled reaction was chromatographed directly (silica gel, 70–230 mesh, elution with dichloromethane) to give 0.92 g, 15% yield, white solid, mp 132°C. NMR (acetone-d_6) δ 7.84 (1H, m), 7.40 (2H, m), 7.16 (1H, m), 3.85 (2H, s). (**b**) 41 mg, 0.156 mM, **5a**, 31 mg, 0.16 mM, **12a**, and 6 mg β-alanine in 20 mL ethanol were refluxed 4 h. Evaporation and trituration in CH_2Cl_2–hexane gave 60 mg, 87% yield, light-yellow solid, mp 152°C. NMR (acetone-d_6) δ 9.06 (1H, s, vinyl), 8.25 (1H, m), 7.82 (1H, m), 7.5–7.0 (7H, m), 7.10 (2H, s, imidazole), 4.19 (2H, t, J = 6.0 Hz), 3.30 (2H, t, J = 6.0 Hz), 2.22 (2H, quint., J = 6.0 Hz). MS m/e 438,440 (M^+, 12, 4%), 312 (M-NHAr, 14), 193 (20), 127 (100).

***N*-Aryl cyano acetamides.** Compound, yield (%), mp (°C). **11**, 19, 127. **13**, 25, 256. **14**, 17, 193. **15**, 17, 138. **16**, 30, 254. **17**, 25, 178. **18**, 30,222. **19**, 47, 132. **20**, 6, 138. **21**, 7, 158. **22**, 7, 112. **23**, 5, 127. **24**, 30, 187. **25**, 13, 236.

Compounds 11–25. Compound, yield (%), mp (°C). **11**, 44, 148. **13**, 60, 214. **14**, 90, 182. **15**, 92, 117. **16**, 55, 215. **17**, 66, 158. **18**, 44, 148. **19**, 97, 173. **20**, 95, 197. **21**. 84, 159. **22**, 43, 133. **23**, 50, 145. **24**, 38, 170. **25**, 42, 192.

Benzyl analogues 26–32

Compound 29. (a). 15 mL, 0.14 M, benzyl amine, and 13 mL, 0.14 M, methyl cyanoacetate were heated at 120°C without solvent at open flask, for 16 h, the cooled reaction was chromatographed directly (silica gel, 70–230 mesh, elution with dichloromethane) to give 9.4 g, 38 yield, white solid, mp 132°C. NMR ($CDCl_3$) δ 7.4 (5H, m), 4.47 (2H, d, J = 6.0 Hz), 3.38 (2H, s). (**b**) 63 mg, 0.24 mM, **5a**, 46 mg, 0.26 mM, **29a**, and 6 mg β-alanine in 20 mL ethanol were refluxed 4 h. Evaporation and trituration in CH_2Cl_2–hexane gave 36 mg, 37% yield, light-yellow solid, mp 132°C. NMR (acetone-d_6) δ 9.00 (1H, s, vinyl), 8.15 (1H, m), 7.82 (1H, m), 7.5–7.0 (7H, m), 7.12 (2H, s, imidazole), 4.52 (2H, d, J = 5.8 Hz), 4.24 (2H, t, J = 6.0 Hz), 3.32 (2H, t, J = 6.0 Hz), 2.26 (2H, quint., J = 6.0 Hz).

N-Benzyl cyano acetamides. Compound, yield (%), mp (°C). **26**, 80, 96. **27**, 90, 115. **28**, 51, 98. **30**, 96, 133. **31**, 85, 126. **32**, 81, 134.

Compounds 26–32. Compound, yield (%), mp (°C). **26**, 43, 172. **27**, 57, 147. **28**, 53, oil. **30**, 47, 141. **31**, 41, 89. **32**, 58, 111.

Biological assays

FT and GGT I inhibition assays and protein processing assay in intact cells were conducted as described.[57] Kinetic assays were performed in the same way as the inhibition assays except for adding serial dilutions of the substrates as indicated in the figure legend.

Cell growth inhibition assay. NIH3T3, v-H-Ras transformed NIH3T3 and LIM1899[58] were seeded in 96-well plates (2000 cells/well), after incubation overnight serial dilutions of the inhibitors were added. Medium and inhibitor solution were replaced every 24 h. After growing the cells for 3 days they were fixed by adding glutaraldehyde to a final concentration of 0.5%. Plates were washed with DDW, 200 L/well*3 and borate buffer, pH 8.5, 0.1 M, 200 L/well. Plates were incubated for 60 min at room temperatures with methylene blue 1%, 100 mL/well, in borate buffer (pH 8.5, 0.1 M) and washed in DDW until background was cleared. After drying the dye was dissolved with 200 mL HCl 0.1 M and the OD was measured in a plate reader at 620 nm.

Soft agar assay. Each well of a 96-well tissue culture plate was coated with 100 μL of bottom agar mixture (DMEM, 10% FCS and 1% agarose). v-H-Ras transformed NIH3T3 cells were suspended in top agar mixture (DMEM, 10% FCS, 0.3% agarose, 70,000 cells/mL), and seeded in 96-well plates (3500 cells/well) on top of the base layer. Serial dilutions of the inhibitors were added in 50 μL medium (DMEM, 10% FCS) on top. Plates were incubated for 12 days in a humidified 37°C incubator. At the end point 25 μL MTT (5 mg/mL in PBS) was added and the plates were incubated at 37°C for an additional 4 h. The upper solution was washed with PBS and colored colonies were counted under a light microscope, magnification ×100. Colonies from four fields of 2.5×2 mm from each duplicate well were counted and the average number of colonies per well was calculated. Subsequently 100 μL of solubilization solution (20% SDS, 2% acetic acid, 25 mM HCl, 50% dimethylformamide) was added and the plate was incubated in a sealed container overnight, absorbance was read at 570 nm with a reference wavelength of 630 nm using a ELISA plate reader to account for possible effects on colony size.

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