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Analogs of farnesylcysteine induce apoptosis in HL-60 cells

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Abstract ***S*-Farnesyl-thioacetic acid (FTA), a competitive inhibitor of isoprenylated protein methyltransferase, potently suppressed the growth of HL-60 cells and induced apoptosis, as evidenced by the development of increased annexin-V binding, decreased binding of DNA dyes and internucleosomal DNA degradation. FTA did not impair the membrane association of ras proteins, conversely, it brought about a decrease in the proportion of ras present in the cytosolic fraction. Farnesylated molecules which are weak inhibitors of the methyltransferase also induced DNA laddering and reduced the proportion of cytosolic ras. These findings suggest that neither inhibition of isoprenylated protein methylation nor impairment of ras membrane association are essential for apoptosis induced by farnesylcysteine analogs.**
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Key words: Farnesylcysteine; Apoptosis; ras protein; HL-60 cell

1. Introduction

G proteins (Gp) are posttranslationally modified by isoprenylation and methylation. While isoprenylation is essential for Gp membrane association and function, methylation is required for full activity (see [1] and [2] for review). Gp methylation is carried out by specific methyltransferases [3,4], which recognize the isoprenylated carboxyl terminal cysteine [5,6]. Extensive structure-activity studies on the partially purified isoprenylated protein methyltransferase (PPMTase) from bovine retinal rod outer segments have allowed to obtain a battery of small molecule analogs of the isoprenylated Gp C-terminus, which behave as substrates and/or inhibitors of the enzyme [7]. Among these molecules, *N*-acetyl-*S*-farnesyl-L-cysteine (L-AFC) is a good substrate for retinal PPMTase, with a K_m of approximately 20 μM [6], while the steroisomer D-AFC is a poorer substrate, with a K_m of 70 μM [7]. Another compound, farnesyl-thioacetic acid (FTA), is a potent competitive inhibitor both of L-AFC methylation, with a K_i of 5 μM, and of the methylation of the endogenous retinal isoprenylated proteins in in vitro assays [6]. L-AFC has been widely used as a competitive inhibitor of PPMTase to explore the importance of isoprenylated protein carboxyl methylation in signal transduction processes in numerous experimental systems. The effects encountered evidence an impairment of various cellular functions elicited by the activation of G protein-coupled receptors, including the release of superoxide anion induced by formyl-peptide in human neutrophils [8,9], agonist-receptor-mediated platelet aggregation [10] and the chemotactic responses of mouse peritoneal macrophages [11], while other signal transduction pathways, like those elicited by calcium ionophores or by direct activation of protein kinase C with phorbol esters, seem unaffected [8–10]. In spite of the selectivity that analogs of AFC exhibit for Gp-dependent processes, some of their biological effects appear to be independent from their behavior as methylation inhibitors. On one hand, L-AFC has been reported to interfere with receptor-mediated Gp activation by a mechanism independent of the inhibition of Gp methylation [12]. On the other hand, farnesylcysteine analogs have been shown to exert either positive or negative effects on neutrophil activation, measured as superoxide release, in a way not related to the blockade of PPMTase activity [9]. In addition, it has been shown that farnesylated molecules which are not inhibitors of the methyltransferase are able to block thrombin-induced platelet aggregation [13]. Accumulating evidence suggests that the isoprenoid moiety of certain proteins could play an important role in protein–protein interactions [14–17].

Interestingly, an isoprenoid acceptor structure has been recently identified in the guanosine diphosphate (GDP)-dissociation inhibitor of rho proteins, rho-GDI [18]. These observations raise the possibility that farnesylated compounds may exert their diverse biological effects through the interaction with intracellular targets not yet identified. It has been recently reported that certain farnesyl-based inhibitors of PPMTase can inhibit ras-dependent cell growth [19]. In this study we have explored the effect of several farnesylated molecules, with widely divergent activities towards PPMTase, on the growth and viability of human leukemic HL-60 cells.

2. Materials and methods

2.1. Materials

N-Acetyl-*S*-farnesyl-L-cysteine (L-AFC), *N*-acetyl-*S*-farnesyl-D-cysteine (D-AFC) and farnesyl-*S*-thioacetic acid (FTA) were synthesized as previously described [6,7]. The synthesis of farnesoic acid (FA), farnesyl-phosphate methyl ester (FPMe) and farnesyl-phosphate dimethyl ester ($FPMe_2$) will be reported in detail elsewhere. Pan-Ras-10 (Ab-3) monoclonal antibody was from Oncogene Science (Uniondale, NY). Mouse monoclonal anti-calmodulin antibody was from Zymed Laboratories, Inc. (San Francisco, CA), and peroxidase-conjugated anti-mouse immunoglobulins were from Dako (Glostrup, Denmark). Annexin V- FITC and RNase A were from Boehringer Mannheim. *S*-Adenosyl-methyl-[^{3}H]methionine (80 Ci/mmol) and Amplify were from Amersham (Buckinghamshire, UK). All other reagents were of the highest purity available from Sigma Co. (St. Louis, MO).

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Abbreviations: FTA, *S*-farnesyl-thioacetic acid; L-AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; D-AFC, *N*-acetyl-*S*-farnesyl-D-cysteine; FA, farnesoic acid; FPMe, farnesylphosphate methyl ester; $FPMe_2$, farnesyl-phosphate dimethyl ester; PPMTase, isoprenylated protein methyltransferase; Gp, GTP binding protein; GDS, guanine nucleotide dissociation stimulator; GDI, guanine nucleotide dissociation inhibitor; SAM, *S*-adenosylmethionine

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2.2. Cell culture

Human promyelocytic HL-60 leukemic cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 U/ml) and gentamycin (24 μg/ml), at 37°C under 5% CO_2 in a humidified atmosphere. For experiments, cells were incubated in serum-free medium supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml) [20], at a density of 2.5×10^5 cells/ml. Farnesylcysteine analogs were added in 0.1% (v/v) dimethyl sulfoxide (DMSO), final concentration. Control cells received an equivalent amount of DMSO. This DMSO concentration did not induce cell differentiation or apoptosis. Cell viability was evaluated by trypan blue dye exclusion.

2.3. Cell cycle analysis

Approximately 10^6 cells per experimental condition were harvested, washed with phosphate buffered saline (PBS) and resuspended in 250 μl of the same buffer. Cell suspensions were incubated for 30 min at r.t. in the presence of 0.1% Nonidet P-40, 50 μg/ml propidium iodide and 50 μg/ml RNase final concentrations, and the DNA content per cell was estimated by flow cytometry as previously described [21].

2.4. Analysis of annexin V-FITC binding

Approximately 5×10^5 cells per experimental condition were harvested, washed with phosphate buffered saline (PBS) and resuspended in 150 μl of HEPES buffer (10 mM HEPES-Na, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$). Cells were incubated for 15 min at 37°C in the presence of 2 μg/ml of annexin V-FITC, washed twice with HEPES buffer and resuspended in 400 μl of the same buffer. To distinguish cells which had lost membrane integrity, propidium iodide was added to a final concentration of 50 μg/ml before flow cytometry analysis [22].

2.5. DNA fragmentation

Fragmented DNA was obtained basically as described [23]. Briefly, approximately 4×10^6 cells from each experimental condition were lysed by incubation in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100, for 30 min at 4°C. Cell organelles were removed by centrifugation at $12\,000\times g$ for 20 min and the fragmented DNA present in the supernatant was precipitated as described [23], and resuspended in 50 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Aliquots (5 μl) were analyzed by electrophoresis on 1% agarose gels.

2.6. SDS-PAGE and immunoblotting

For SDS-PAGE, HL-60 cells were homogenized by forced passes through a $26^{1/2}$ G needle in 10 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM β-mercaptoethanol, containing 2 μg/ml of each of the protease inhibitors leupeptin, aprotinin and trypsin inhibitor. S100 (cytosol) and P100 (membrane) fractions were obtained by centrifugation at $200\,000\times g$ for 30 min in a Beckman TLA100 rotor. Aliquots from each fraction containing 20 μg of protein were electrophoresed on 15% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Even loading and transfer were ensured by Ponceau staining. Blots were incubated with pan-Ras-10 antibody (1:500 dilution) and the proteins of interest were visualized using an ECL detection system from Amersham. In some experiments, blots were re-probed with anti-calmodulin antibody (1:10 000 dilution).

2.7. Methylation of HL-60 cell proteins in vitro

The activity of PPMTase towards endogenous substrates was monitored in vitro essentially as described [9]. Briefly, cell extracts (2 mg of total protein/ml) were incubated with 14 μCi of [^{3}H]-*S*-adenosylmethionine ([^{3}H]SAM) (1.75 μM; 80 Ci/mmol) in 100 mM HEPES, pH 7.4, 100 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT at 37°C for 30 min, in the absence or presence of analogs at 50 μM. For electrophoresis, samples were precipitated by the addition of 0.1% (w/v) deoxycholate and 10% (w/v) trichloroacetic acid and centrifuged at $14\,000\times g$ for 10 min. The protein pellets were solubilized in sample buffer containing 5% SDS, boiled for 5 min and run on 12.5% polyacrylamide gels. Gels were treated with Amplify (Amersham), dried and exposed to film at −80°C for 3 days.

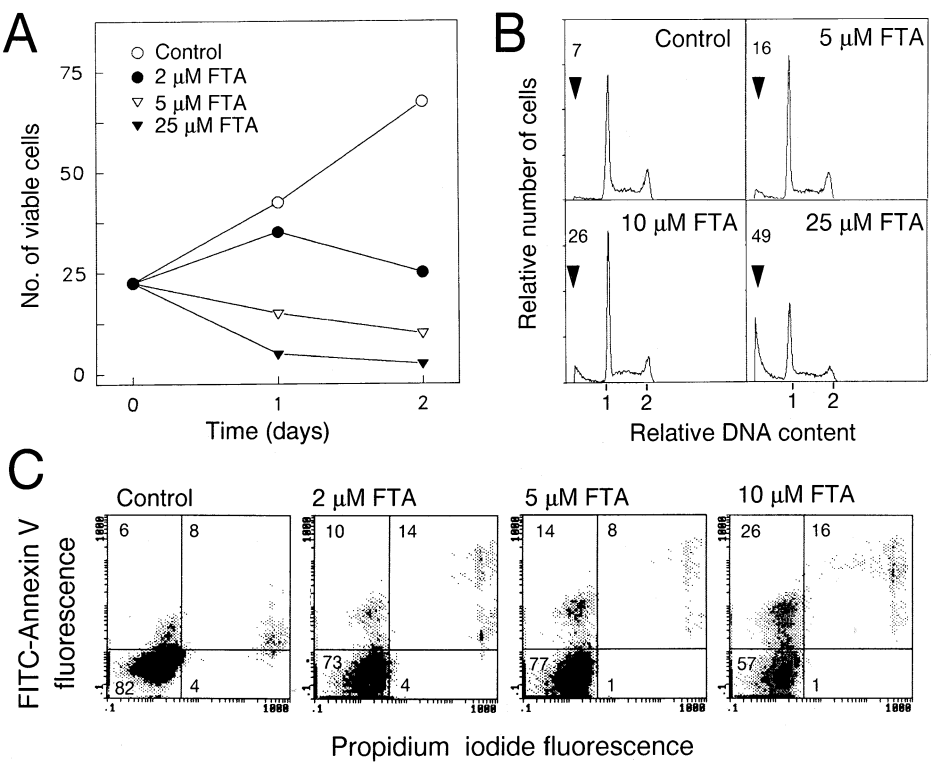


Fig. 1. Effect of FTA on the growth and cell cycle distribution of HL-60 cells. A: HL-60 cells were grown in the presence of the indicated concentrations of FTA. At the indicated time points, viable cells were counted on a hemocytometer chamber. B: Cell cycle analysis of HL-60 cells treated with the indicated concentrations of FTA for 8 h. The proportion of cells in the sub-G_1 peak (arrowheads) is given. C: Binding of annexin V-FITC to FTA-treated cells, vs. loss of plasma membrane integrity, as assessed by propidium iodide uptake. The proportion of cells within each quadrant is given in inserts. Results shown are representative of three independent experiments.

3. Results

3.1. Effect of FTA on the growth and cell cycle distribution of HL-60 cells

Incubation of HL-60 cells in the presence of FTA inhibited cell growth in a dose-dependent manner (Fig. 1A). Inhibition was complete at 5 μM FTA. This effect was accompanied by a loss of cell viability, as well as by morphological alterations including cell shrinking (results not shown). The study of the cell cycle distribution of HL-60 cells treated with FTA revealed the dose-dependent appearance of a distinct population of cells with a decreased binding of the DNA-binding dye, a characteristic of early apoptotic cells [24], which integrate the so-called sub-G1 peak (Fig. 1B). The induction of apoptosis by FTA was also suggested by the appearance of a population of cells with increased annexin-V binding (Fig. 1C), an indication of the exposure of negatively charged phospholipids to the outer surface of the plasma membrane [22]. This cell population maintained a low (close to background) staining with propidium iodide, which is indicative of membrane integrity, and therefore represents early apoptotic cells. In addition, analysis of DNA from FTA-treated cells showed an internucleosomal degradation which gave rise to the DNA laddering pattern characteristic of some apoptotic processes (see Fig. 4A).

3.2. Effect of FTA on methyltransferase activity in HL-60 cell extracts

The activity of protein methyltransferase towards the endogenous substrates was assessed by incubating HL-60 cell extracts in the presence of [^{3}H]SAM. This resulted in the incorporation of radioactivity in several polypeptides with apparent molecular masses of 14–17, 20–23, 32, 40 and 46 kDa (Fig. 2A). The most prominently methylated polypeptides were in the 20–23 kDa range, where small Gp are known to occur. FTA inhibited protein methylation in HL-60 extracts. This inhibition was more evident for the 20–23 kDa group of polypeptides (Fig. 2A). Half maximal inhibition of the methylation of this group of proteins was achieved with 30 μM

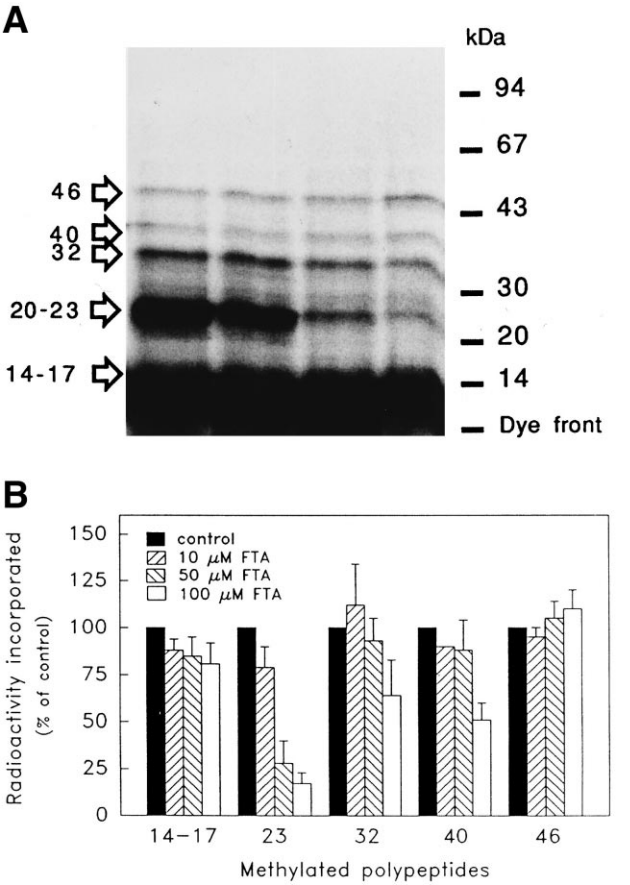
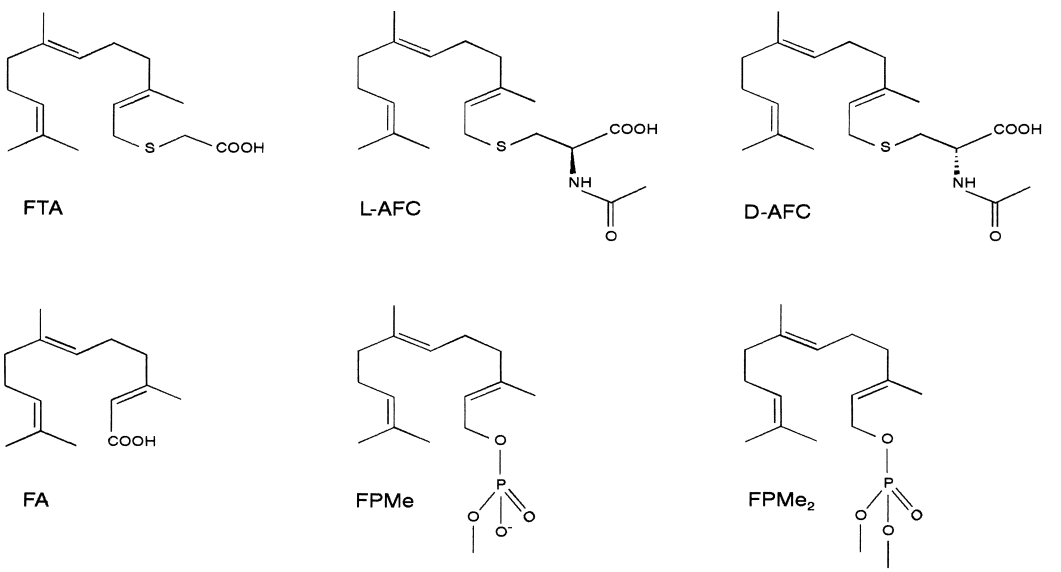


Fig. 2. Effect of FTA on the methylation of endogenous substrates in extracts of HL-60 cells. Protein methylation in HL-60 cell extracts was performed by incubation with [^{3}H]SAM in the absence or presence of FTA at the indicated concentrations. The experiment was repeated three times with different preparations of cells. A representative autoradiograph is shown in A and the densitometric quantitation of the radioactivity incorporated in the groups of polypeptides marked in A is shown in B. Results are expressed as percentage of the values obtained in the absence of FTA and are given as mean ± S.D. of the three experiments.



Scheme 1. Farnesylcysteine analogs employed in these studies.

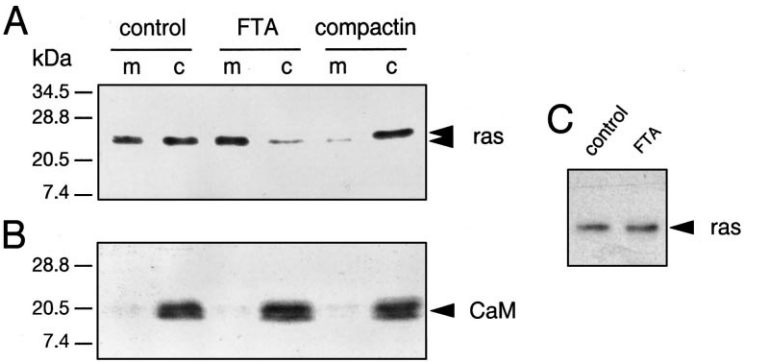


Fig. 3. Effect of FTA on the membrane association of ras proteins. HL-60 cells were incubated in the absence or presence of 10 μM FTA or 10 μM compactin for 8 h. A: The subcellular distribution of ras proteins between membrane (m) and cytosol (c) compartments was analyzed by immunoblot using a pan-ras antibody. Arrowheads mark the position of the putative unprocessed (upper) and processed (lower) ras proteins. B: The blot shown in A was probed with an anti-calmodulin antibody (CaM, calmodulin). C: The amount of ras present in cells incubated for 8 h in the absence or presence of 10 μM FTA was estimated by Western blot of total cell lysates.

FTA. The densitometric quantitation of these results appears in Fig. 2B.

3.3. *Effect of FTA on the subcellular localization of ras proteins*

It has been reported that certain farnesylated molecules can interfere with cell growth and impair membrane association of ras proteins, an important process for ras function [25]. Therefore, we explored whether FTA induction of apoptosis in HL-60 cells might be associated with alterations in ras subcellular localization. Since HL-60 cells contain the oncogene N-ras [26], we estimated the levels of ras proteins present in S100 or P100 fractions of HL-60 cell extracts by Western blot using the pan-ras 10 antibody. This antibody recognizes normal ras, as well as K-, H-, and N-ras [27]. As is shown in Fig. 3A, in control HL-60 cells, pan-ras-10 immunoreactive proteins could be detected both in the cytosolic and membrane fractions. Treatment with 10 μM FTA did not reduce the levels of membrane-associated ras proteins. Interestingly, it brought about an increase in the proportion of ras proteins present in the membrane fraction. In contrast to these results, treatment of HL-60 cells with the isoprenoid biosynthesis inhibitor

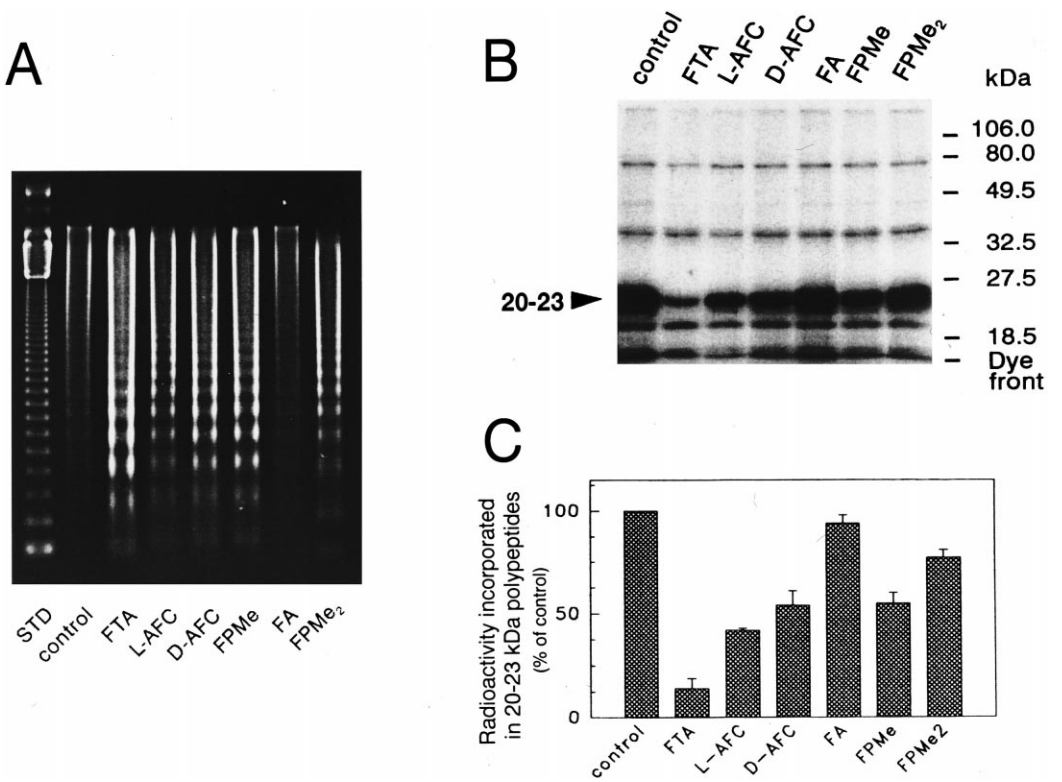


Fig. 4. Effect of farnesylcysteine analogs on DNA degradation and protein methyltransferase activity. A: HL-60 cells were cultured in the presence of several farnesylated compounds at 10 μM as indicated. After 12 h cells were harvested and the fragmented DNA was analyzed on agarose gels. STD, 123-bp ladder standard. B: Protein methylation in HL-60 cell extracts was performed by incubation with [^{3}H]SAM in the absence or presence of the various farnesylcysteine analogs at 50 μM. The autoradiograph shown is representative of three experiments. The densitometric quantitation of the radioactivity incorporated in the 20–23-kDa group of polypeptides marked in B is shown in C. Results are expressed as percentage of the values obtained in the absence of analogs and are given as mean ± S.D. of the three experiments.

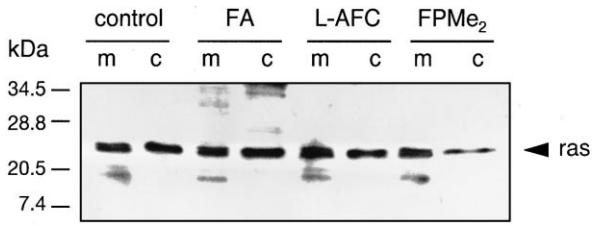


Fig. 5. Effect of farnesylcysteine analogs on ras proteins membrane association. HL-60 cells were incubated in the absence or presence of the various farnesylated compounds at 10 μM for 8 h. The subcellular distribution of ras proteins between membrane (m) and cytosol (c) compartments was analyzed by immunoblot using the pan-ras 10 antibody. The position of ras proteins is marked by an arrowhead.

compactin clearly impaired ras proteins membrane association, giving rise to the accumulation of ras in the cytosolic fraction. This effect probably occurred as a consequence of impaired isoprenylation, as suggested by the slightly slower electrophoretic mobility of the immunodetected protein (Fig. 3A). As a control of this experiment blots were re-probed with an antibody against calmodulin, a predominantly cytosolic protein. As can be observed in Fig. 3B, neither FTA nor compactin altered calmodulin subcellular distribution. Finally, we observed that in total cell lysates from FTA-treated cells, the levels of ras proteins were not significantly different from those of control cells (Fig. 3C).

3.4. *Effect of various farnesylcysteine analogs on DNA degradation and in vitro protein methylation in HL-60 cells*

In order to assess the possible importance of PPMTase inhibition in the induction of apoptosis we studied the effects of a series of farnesylcysteine analogs (see Scheme 1) of different potencies as inhibitors of the retinal rod outer segment PPMTase. Besides FTA, L-AFC and D-AFC [7], we used farnesoic acid (FA), which is a weak inhibitor of L-AFC methylation by retinal PPMTase, and the farnesyl phosphate mono- and dimethyl esters (FPMe and $FPMe_2$, respectively). These latter analogs are virtually inert both as substrates and inhibitors of this enzyme (Gilbert et al., unpublished observations). Incubation of HL-60 cells with the various compounds at 10 μM for 12 h resulted in loss of cell viability, which ranged from 20–30% of the cells in all cases, except for FA, which had no activity. Analysis of cellular DNA showed that all compounds, except FA, were effective inducers of internucleosomal DNA degradation (Fig. 4A). FTA was the most potent inducer of DNA laddering. Of the two isomers of AFC, the D-isomer was slightly more potent. Next, we compared the potency of the various analogs as inhibitors of PPMTase in HL-60 cell extracts assaying each compound at 50 μM. As shown in Fig. 4B, FTA was the most potent inhibitor of in vitro protein methylation. The extent of inhibition attained by the remaining compounds correlated well with the kinetic constants previously obtained in studies with the retinal rod outer segment methyltransferase, using AFC as a substrate [6,7]. FA was ineffective as inhibitor of the methylation of endogenous protein substrates in HL-60 cells and $FPMe_2$ was a poor inhibitor, while L-AFC was more potent than the D-isomer. The densitometric quantitation of the radioactivity incorporated into the 20–23-kDa group of polypeptides is given in Fig. 4C.

3.5. *Effect of farnesylcysteine analogs on ras proteins subcellular localization*

Finally, we explored whether some of these farnesylcysteine analogs could interfere with ras protein membrane association. As is shown in Fig. 5, none of the analogs used impaired ras membrane association. FA did not alter the partitioning of ras proteins between the membrane and cytosolic compartments compared to control conditions. However, L-AFC and, to a somewhat greater extent $FPMe_2$, reduced the proportion of ras-10 immunoreactive proteins in the soluble fraction. However, neither analog was as potent as FTA proved to be in this regard.

4. Discussion

The results presented here show that the farnesylcysteine analog FTA is a powerful inhibitor of HL-60 cell growth and induces apoptosis. A number of farnesylated molecules share these characteristics of FTA. However, these properties appear not to correlate with the ability of these molecules to inhibit the processing of isoprenylated proteins. First, the concentrations required to exert the inhibitory activities on cell growth and survival are lower than those needed to inhibit protein methylation. Moreover, analogs which are very weak inhibitors of PPMTase, are effective inducers of apoptosis. Second, interference of these molecules with the isoprenylation step is also unlikely, since none of the compounds tested diminished the electrophoretic mobility or impaired the membrane association of ras proteins. Both effects were observed when the inhibitor of isoprenoid biosynthesis compactin was used. These results suggest that isoprenylated molecules can disrupt signalling pathways through mechanisms not related to the inhibition of Gp isoprenylation or methylation. It is likely that the effects of farnesylcysteine analogs arise from their interaction with intracellular targets capable of recognizing the isoprenoid moiety.

Recent studies suggest that the modification of proteins by isoprenylation and methylation may have important consequences for protein–protein interactions (see [28] and [2] for reviews). For example, it has been shown that farnesylation of yeast Ras2 increases its apparent affinity for its effector, adenylyl cyclase, even in the absence of membranes [14]. In addition, it has recently been reported that isoprenylation of ras proteins is required for the action of hSOS1, a mammalian guanine nucleotide exchange factor responsible for activating ras in response to growth factor stimulation [29]. In the case of several members of the ras superfamily of small Gp, the isoprenoid modification is essential for their interaction with their respective regulators of guanine nucleotide dissociation, the GDP dissociation inhibitors rab and rho-GDIs, and the GDP dissociation stimulator, Smg GDS [17]. The isoprenylation of heterotrimeric Gp γ-subunits seems important for the association of α and βγ subunits [15]. In the case of the photoreceptor Gp, transducin, not only the isoprenylation, but also the methylation of T_γ, contributes to the subunit interaction between T_α and $T_{\beta\gamma}$, thus facilitating the receptor–Gp coupling [30]. The possibility exists that, as has been shown for rho-GDI [18], some of these proteins may contain domains which can recognize isoprenyl moieties. This would provide potential sites of interaction with farnesylated molecules. In fact, a specific interference of farnesylcysteine analogs with the

interaction between activated receptors and Gp has been previously suggested [31].

It has been previously shown that farnesylcysteine derivatives can inhibit the growth of ras-dependent cell lines [19]. In the case of the analog farnesylthiosalisylic acid, this effect was associated with an impairment of ras membrane association [25]. However, the results reported here clearly show that isoprenylated molecules can also inhibit cell growth in conditions under which the proportion of membrane-associated ras proteins is not affected, or is even increased, as in the case of FTA. Several possibilities could be envisaged that would explain this observation. Firstly, cytosolic ras or ras-like proteins could be subjected to increased degradation in cells treated with FTA. However, when total ras levels were monitored by Western blots, no differences could be found between control and FTA-treated cells. Secondly, farnesylcysteine analogs could interfere with the interaction of ras or ras-like proteins with a cytosolic acceptor(s). Interestingly, GDIs and smg-GDS are able to release their cognate small Gp from membranes, presumably by burying the isoprenylated moiety in a hydrophobic pocket [32,33], which may be similar to the structure recently identified in rho-GDI [18]. It has been shown previously that various fatty acids and lipids can disrupt the complex of rac protein and rho-GDI, present in the cytosol of resting human neutrophils [34]. Moreover, it has been reported that synthetic geranylgeranylated decapeptides, designed from the carboxyl-terminal region of rap proteins, can inhibit the interaction of rap proteins with smg-GDS [35]. Finally, a cytosolic acceptor(s) for ras or ras-like proteins could be subjected to increased degradation in the course of FTA-induced apoptosis, as has been shown for D4-GDI during Fas-induced apoptosis in Jurkat cells [36]. Several of these possibilities are currently under study.

In conclusion, our results show that several farnesylcysteine analogs can induce apoptosis in HL-60 cells, and suggest that the inhibition of PPMTase is not essential for this process. In the light of recent evidence, our findings suggest that farnesylcysteine analogs could provide a novel approach to explore the participation of the isoprenyl moieties of signalling proteins in protein–protein interactions.

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References

[1] Rando, R.R. (1996) Biochim. Biophys. Acta 1300, 5–16.
[2] Zhang, F.L. and Casey, P.J. (1996) Annu. Rev. Biochem. 65, 241–269.
[3] Clarke, S., Vogel, J.P., Deschenes, R.J. and Stock, J. (1988) Proc. Natl. Acad. Sci. USA 85, 4643–4647.
[4] Clarke, S. (1992) Annu. Rev. Biochem. 61, 355–386.
[5] Pérez-Sala, D., Tan, E.W., Cañada, F.J. and Rando, R.R. (1991) Proc. Natl. Acad. Sci. USA 88, 3043–3046.
[6] Tan, E.W., Pérez-Sala, D., Cañada, F.J. and Rando, R.R. (1991) J. Biol. Chem. 266, 10719–10722.
[7] Gilbert, B.A., Tan, E.W., Pérez-Sala, D. and Rando, R.R. (1992) J. Am. Chem. Soc. 114, 3969–3973.
[8] Philips, M.R., Pillinger, M.H., Staud, R., Volker, C., Rosenfeld, M.G., Weissmann, G. and Stock, J.B. (1993) Science 259, 977–980.
[9] Ding, J., Lu, D.J., Pérez-Sala, D., Ma, Y.T., Maddox, J.F., Gilbert, B.A., Badwey, J.A. and Rando, R.R. (1994) J. Biol. Chem. 269, 16837–16844.
[10] Huzoor-Akbar, Wang, W., Kornhauser, R., Volker, C. and Stock, J. (1993) Proc. Natl. Acad. Sci. USA 90, 868–872.
[11] Volker, C., Miller, R.A., McCleary, W.R., Rao, A., Poenie, M., Backer, J.M. and Stock, J.B. (1991) J. Biol. Chem. 266, 21515–21522.
[12] Scheer, A. and Gierschik, P. (1993) FEBS Lett. 319, 110–114.
[13] Ma, Y.-T., Shi, Y.-Q., Lim, Y.H., McGrail, S.H., Ware, J.A. and Rando, R.R. (1994) Biochemistry 33, 5414–5420.
[14] Kuroda, Y., Suzuki, N. and Kataoka, T. (1993) Science 259, 683–686.
[15] Iniguez-Lluhi, J.A., Simon, M.I., Robishaw, J.D. and Gilman, A.G. (1992) J. Biol. Chem. 267, 23409–23417.
[16] Fukada, Y., Takao, T., Ohguro, H., Yoshizawa, T., Akino, T. and Shimonishi, Y. (1990) Nature 346, 658–660.
[17] Takai, Y., Kaibuchi, K., Kikuchi, A. and Sasaki, T. (1995) Methods Enzymol. 250, 122–133.
[18] Gosser, Y.Q., Nomanbhoy, T.K., Aghazadeh, B., Manor, D., Combs, C., Cerione, R.A. and Rosen, M.K. (1997) Nature 387, 814–819.
[19] Marciano, D., Ben-Baruch, G., Marom, M., Egozi, Y., Haklai, R. and Kloog, Y. (1995) J. Med. Chem. 38, 1267–1272.
[20] Breitman, T., Collins, S. and Keene, B. (1980) Exp. Cell Res. 126, 494–498.
[21] Pérez-Sala, D., Collado-Escobar, D. and Mollinedo, F. (1995) J. Biol. Chem. 270, 6235–6242.
[22] Martin, S.J., Reutelingsperger, C.P.M., McGahon, A.J., Rader, J.A., Schie, R.C.A.A.v., LaFace, D.M. and Green, D.R. (1995) J. Exp. Med. 182, 1545–1556.
[23] Pérez-Sala, D. and Mollinedo, F. (1994) Biochem. Biophys. Res. Commun. 199, 1209–1215.
[24] Ormerod, M.G., Collins, M.K.L., Rodríguez-Tarduchy, G. and Robertson, D. (1992) J. Immunol. Methods 153, 57–65.
[25] Marom, M., Haklai, R., Ben-Baruch, G., Marciano, D., Egozi, Y. and Kloog, Y. (1995) J. Biol. Chem. 270, 22263–22270.
[26] Murray, M.J., Cunningham, J.M., Parada, L.F., Dautry, F., Lebowitz, P. and Weinberg, R.A. (1983) Cell 33, 749–757.
[27] Carney, W.P., Hamer, P.J., LaVecchio, J., Ng, S., Petit, D., Pullano, T.G. and Trimpe, K.L. (1989) in: Human Tumor Antigens and Specific Tumor Therapy, pp. 53–62, Alan R. Liss, New York, NY.
[28] Marshall, C.J. (1993) Science 259, 1865–1866.
[29] Porfiri, E., Evans, T., Chardin, P. and Hancock, J.F. (1994) J. Biol. Chem. 269, 22672–22677.
[30] Fukada, Y., Matsuda, T., Kokame, K., Takao, T., Shimonishi, Y., Akino, T. and Yoshizawa, T. (1994) J. Biol. Chem. 269, 5163–5170.
[31] Scheer, A. and Gierschik, P. (1995) Biochemistry 34, 4952–4961.
[32] Orita, S., Kaibuchi, K., Kuroda, S., Shimizu, K., Nakanishi, H. and Takai, Y. (1993) J. Biol. Chem. 268, 25542–25546.
[33] Ullrich, O., Stenmark, H., Alexandrov, K., Huber, L.A., Kaibuchi, K., Sasaki, T., Takai, Y. and Zerial, M. (1993) J. Biol. Chem. 268, 18143–18150.
[34] Chuang, T.-H., Bohl, B.P. and Bokoch, G.M. (1993) J. Biol. Chem. 268, 26206–26211.
[35] Shirataki, H., Kaibuchi, K., Hiroyoshi, M., Isomura, M., Araki, S., Sasaki, T. and Takai, Y. (1991) J. Biol. Chem. 266, 20672–20677.
[36] Na, S., Chuang, T.-H., Cunningham, A., Turi, T.G., Hanke, J.H., Bokoch, G.M. and Danley, D.E. (1996) J. Biol. Chem. 271, 11209–11213.