Mechanistic Studies on Human Platelet Isoprenylated Protein Methyltransferase: Farnesylcysteine Analogs Block Platelet Aggregation without Inhibiting the Methyltransferase†

Yu-Ting Ma,‡ Yi-Qun Shi,‡ Young Hee Lim,‡ Sonja H. McGrail,§ J. Anthony Ware,‡,§ and Robert R. Rando*,‡

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Avenue, Boston, Massachusetts 02115, and the Cardiovascular Division, Department of Medicine, Beth Israel Hospital, Boston, Massachusetts 02215

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ABSTRACT: The kinetic mechanism of the human platelet *S*-adenosyl-L-methionine (AdoMet)-linked isoprenylated protein methyltransferase was studied and determined to be ordered bibi. AdoMet binds first, and *S*-adenosyl-L-homocysteine (AdoHcy) departs last. Simple N-acetylated farnesylated cysteine analogs, such as *N*-acetyl-*S*-farnesyl-L-cysteine (AFC), are excellent substrates for the enzyme. Although many N-acetylated farnesylated cysteine analogs are excellent substrates for the enzyme, analogs with bulky moieties adjacent to the farnesylcysteine are neither substrates nor inhibitors of the enzyme. Two molecules of this class, *N*-benzoyl-*S*-farnesyl-L-cysteine (BzFC) and *N*-pivaloyl-*S*-farnesyl-L-cysteine (PFC) are useful in sorting out the putative physiological role of the methyltransferase in mediating human platelet aggregation because their pharmacological activities are unlinked to methyltransferase inhibition. When studied as inhibitors of platelet aggregation, the analogs are as active, or more active, than bona fide methyltransferase inhibitors of similar structure. Therefore, although it is possible that methyltransferase inhibitors, such as AFC, inhibit the enzyme when applied to cells, the observed pharmacological effects appear to be unrelated to this blockade. The new FC analogs described here have revealed a new signal transduction target which will be of some interest to explore.

Protein carboxymethyltransferase enzymes have been implicated in signal transduction pathways associated with bacterial chemotaxis (Kort et al., 1975). The situation in eucaryotes is complicated. Methyltransferases which are involved in the carboxymethylation of aspartate and glutamate residues appear not to be involved in signal transduction (Clarke, 1985). Recently, a novel eucaryotic methyltransferase has been discovered which methylates carboxyl terminal isoprenylated cysteine residues (Gutierrez et al., 1989). This modification is part of the biochemical sequence of events which occurs during protein isoprenylation (Hancock et al., 1989; Farnsworth et al., 1990; Reiss et al., 1990; Lai et al., 1990; Clarke et al., 1988; Maltese, 1990; Sinensky & Lutz, 1992; Gibbs, 1991). As this modification occurs with all known heterotrimeric G proteins and many "small" G proteins, it is possible that methylation here has a role to play in signal transduction. The fact that this methylation reaction is reversible and the fact that a methylesterase exists which is specific for isoprenylated cysteine-containing methyl esters support the view that methylation may be of regulatory significance (Tan & Rando, 1992).

Although the membrane-bound isoprenylated protein methyltransferase has not been purified, aspects of its chemical biology have been explored. For example, the bovine enzyme processes very simple substrates, such as *N*-acetyl-*S*-farnesyl-L-cysteine (AFC)[1] and *S*-farnesylthiopropionic acid (FTP), with K_M values in the micromolar range (Pérez-Sala et al., 1991). Moreover, the enzyme is potently inhibited by *S*-farnesylthioacetic acid (FTA) (Tan et al., 1991). The kinetics of the bovine enzyme have also been studied, and an ordered mechanism has been demonstrated, with *S*-adenosylmethionine (AdoMet) binding first and *S*-adenosylhomocysteine departing last (Shi & Rando, 1992).

Recently, AFC has been reported to have remarkable effects on a variety of human cells. It has been reported that AFC inhibits stimulated platelet aggregation (Akbar et al., 1993), superoxide formation induced by FMLP in human neutrophils (Philips et al., 1993), chemotaxis of macrophages (Volker et al., 1991), and insulin release from pancreatic cells (Metz et al., 1993), implicating carboxymethylation in these processes. Although it was stated that the mechanistic basis for these effects is to be found in the inhibition of the isoprenylated protein methyltransferase by AFC, no evidence was provided to support this view. Quantitative issues arise as well. For example, without knowing what the K_M values for molecules like AFC are for the human methyltransferase enzyme(s), it is impossible to know if the observed dose–response curves for farnesyl-L-cysteine analogs on target tissues are consistent with expectations. Finally, a recent report has shown that AFC can also inhibit receptor-mediated activation of G-

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* To whom correspondence should be addressed.
‡ Harvard Medical School.
§ Beth Israel Hospital.

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[1] Abbreviations: AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; AFCMe, *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester; FC, *S*-farnesyl-L-cysteine; FTA, *S*-farnesylthioacetic acid; FTP, *S*-farnesylthiopropionic acid; iBFC, *N*-isobutyryl-*S*-farnesyl-L-cysteine; iVFC, *N*-isovaleryl-*S*-farnesyl-L-cysteine; PFC, *N*-pivaloyl-*S*-farnesyl-L-cysteine; BzFC, *N*-benzoyl-*S*-farnesyl-L-cysteine; BzGFC, *N*-(benzoylglycyl)-*S*-farnesyl-L-cysteine; BzGGFC, *N*-((benzoylglycyl)glycyl)-*S*-farnesyl-L-cysteine; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; DMSO, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ROS, rod outer segment.

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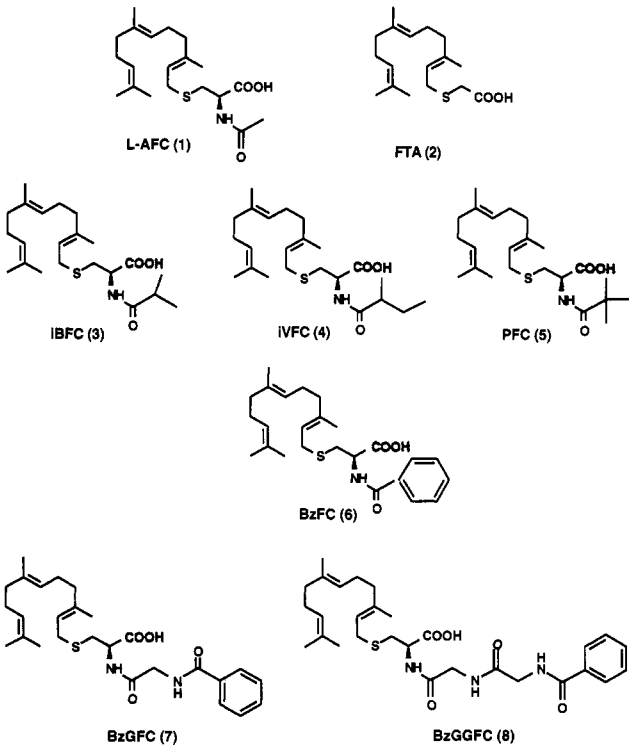
Chart 1



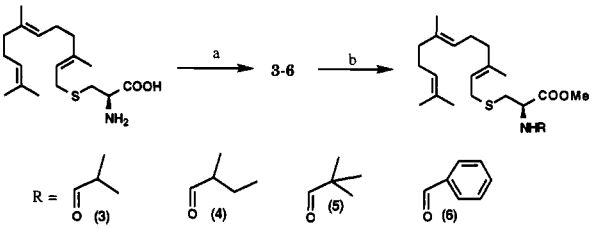
proteins in isolated membranes by a mechanism independent of protein carboxymethylation (Scheer & Gierschik, 1993). Thus it is of some interest to determine quantitative aspects of the mechanism of action of the human enzyme and to elucidate the modes of interaction of substrates and inhibitors with it to inform pharmacological studies. In this article, we begin to explore the kinetics and substrate profile from the human enzyme. The kinetic mechanism of the human platelet enzyme is determined to be ordered bibi, just as it is in the case of the bovine enzyme (Shi & Rando, 1992). New farnesyl-L-cysteine (FC) analogs are reported which are either good substrates for the enzyme or do not interact with it at all. These analogs are shown in Chart 1, along with AFC and FTA. The farnesyl-L-cysteine analogs, which are neither substrates nor inhibitors of the methyltransferase, have sterically bulky moieties attached to the amino group of FC, suggesting a lack of bulk tolerance here. These inert FC analogs allow for a direct test of whether the methyltransferase is the actual target for AFC and similar analogs.

MATERIALS AND METHODS

Materials

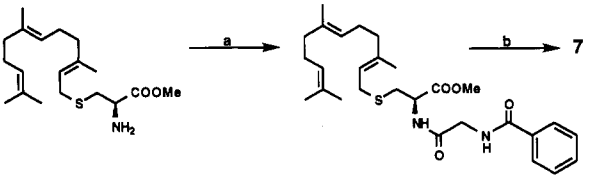
Frozen bovine retinas were obtained from J. W. Lawson Co. (Lincoln, NE). *S*-Adenosyl-L-[methyl-^{3}H]methionine (15 Ci/mmol) was purchased from Amersham Inc. GlyGlyCys was purchased from Bachem Bioscience Inc. L-Cysteine, benzoic acid *N*-hydroxylsuccinimide ester, 1-hydroxybenzotriazole, and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride were purchased from Sigma Chemical Co. *all-trans*-Farnesyl bromide, *N*-methylmorpholine, propionic anhydride, butyric anhydride, isobutyric anhydride, valeric anhydride, (*S*)-(+)-2-methylbutyric anhydride, trimethylacetic anhydride, hexanoic anhydride, and hippuric acid were acquired from Aldrich Chemical Co. Benzoic anhydride and triethylamine were obtained from Fluka. AFC, FTA, and AFCMe were synthesized as previously reported

Scheme 1: Synthesis of Simple Farnesylcysteine Derivatives[a]



[a] Reagents: (a) $(RCO)_2O$, Et_3N, DMAP, CH_2Cl_2; (b) HCl, MeOH.

Scheme 2: Synthesis of *N*-(Benzoylglycyl)-*S*-farnesyl-L-cysteine[a]



[a] Reagents: (a) hippuric acid, EDC, HOBT, DMF; (b) 10% Na_2CO_3.

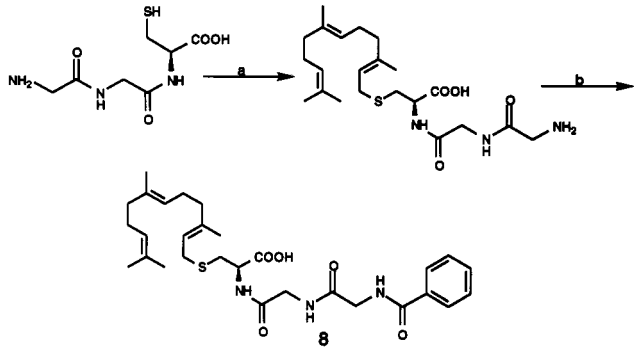
(Tan et al., 1991). Platelets were obtained from human blood drawn from healthy volunteers who had not taken aspirin for the preceding 8 days.

Methods

Syntheses. Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy was recorded on a Varian VRX 500S spectrometer operating at a proton frequency of 499.843 MHz. Dimethyl sulfoxide (DMSO-d_6) or chloroform ($CDCl_3$) was used as the ^{1}H NMR solvent. The residual proton absorption of the deuterated solvent was used as the internal standard. All ^{1}H NMR chemical shifts are reported as δ values in parts per million (ppm), and the coupling constants (J) are given in Hertz. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet, br, broad; m, multiplet; dd, doublet of doublets; dt, doublet of triplets. FAB (fast atom bombardment) mass spectra were performed in a JEOL Mass Spectrometer JMS-SX102. HPLC solvents were from J. T. Baker, Inc. All chemicals and solvents purchased were of the highest purity commercially available. All synthetic samples were ≥98% pure as judged by both TLC and 500-MHz NMR and HPLC analysis. The HPLC retention times are given for the methyl esters of the analogs. The HPLC conditions used were hexane/isopropanol (85:15) on a 250 × 4.6 mm^2 Rainin silica column run at a flow rate of 1.5 mL/min. Detection was at 210 nm.

Compounds **4**–**11** were prepared by acylation of *S-all-trans*-farnesyl-L-cysteine with the appropriate acid anhydride as shown in Scheme 1. The corresponding methyl esters were obtained from their parent carboxylic acids by treatment with methanolic HCl. *trans,trans*-Farnesyl-L-cysteine was prepared according to the published procedure (Brown et al., 1991). The synthesis of *N*-(benzoylglycyl)-*S-all-trans*-farnesylcysteine was performed beginning with *S-all-trans*-farnesylcysteine methyl ester. Coupling of *S-all-trans*-farnesylcysteine methyl ester with hippuric acid under standard conditions (Ma et al., 1993), followed by mild alkaline hydrolysis of the methyl ester, provided *N*-(benzoylglycyl)-*S-all-trans*-farnesylcysteine (**7**) (Scheme 2). Farnesylation of (glycylglycyl)cysteine and benzoylation of the amino group gave *N*-((benzoylglycyl)glycyl)-*S-all-trans*-farnesylcysteine (**8**) (Scheme 3).

Scheme 3: Synthesis of *N*-((Benzoylglycyl)glycyl)-*S*-farnesyl-L-cysteine[a]



[a] Reagents: (a) farnesyl bromide, diisopropylamine, MeOH, *n*-BuOH, H_2O; (b) benzoic acid *N*-hydroxysuccinimide ester, Et_3N, CH_2Cl_2.

Synthesis of N-Benzoyl-S-all-farnesyl-L-cysteine (6) (BzFC). A mixture of *S-all-trans*-farnesylcysteine (813 mg, 2.49 mmol), benzoic anhydride (564 mg, 2.49 mmol), triethylamine (694 μL, 504 mg, 4.98 mmol), and a catalytic amount of DMAP in methylene chloride (40 mL) was stirred at 25 °C for 24 h. Water (30 mL) was added, and the organic solvent was evaporated. The aqueous layer was extracted with ethyl acetate (3 × 100 mL), and the combined organic layer was washed with 10% HCl and with water and then dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was separated by silica gel chromatography (hexane/acetone 90:10, 80:20, 70:30) to give the title compound in a yield of 71% (760 mg). ^{1}H NMR (DMSO-d_6, 500 MHz): 8.50 (1H, br s), 7.84 (2H, d, J = 8 Hz), 7.51 (1H, t, J = 7.5 Hz), 7.42 (2H, t, J = 8 Hz), 5.14 (1H, t, J = 8 Hz), 5.04 (2H, br d, J = 6 Hz), 4.45 (1H, br d, J = 3.5 Hz), 3.18 (1H, dd, J = 8, 12.5 Hz), 3.11 (1H, dd, J = 8, 13 Hz), 2.98 (1H, dd, J = 4, 13.5 Hz), 2.82 (1H, dd, J = 9, 14 Hz), 2.04–1.88 (8H, m), 1.60 (3H, s), 1.59 (3H, s), 1.52 (6H, s). FAB (glycerol) m/e $(M - H^+)^-$ = 428, calcd for $C_{25}H_{35}NO_3S$ MW = 429.

Synthesis of Methyl N-Benzoyl-S-all-trans-farnesylcysteine. A solution of *N*-benzoyl-*S-all-trans*-farnesylcysteine (50 mg) and 3 drops of concentrated HCl in 20 mL of methanol was stirred at 25 °C for 12 h. The methanol was evaporated, and the residue was extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with saturated sodium bicarbonate and with water and then dried over anhydrous magnesium sulfate, filtered, evaporated, and purified by silica gel chromatography (hexane/acetone 90:10, 80:20, 70:30) to give the title compound (45 mg). ^{1}H NMR ($CDCl_3$, 500 MHz): 7.81 (2H, d, J = 8 Hz), 7.51 (1H, t, J = 7.5 Hz), 7.43 (2H, t, J = 8 Hz), 6.98 (1H, d, J = 8 Hz), 5.19 (1H, t, J = 8 Hz), 5.07 (2H, br s), 4.99 (1H, dd, J = 4.5, 12.5 Hz), 3.79 (3H, s), 3.21 (1H, dd, J = 7.5, 12.5 Hz), 3.14 (1H, dd, J = 8, 13.5 Hz), 3.08 (1H, dd, J = 4.5, 13.5 Hz), 2.99 (1H, dd, J = 5.5, 13.5 Hz), 2.10–1.93 (8H, m), 1.66 (3H, s), 1.63 (3H, s), 1.58 (3H, s), 1.57 (3H, s). The HPLC retention time under the conditions described above was 3.18 min.

Synthesis of Methyl N-(Benzoylglycyl)-S-all-trans-farnesylcysteine. 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (610 mg, 3.19 mmol) in one portion was added to a mixture of methyl *S-all-trans*-farnesyl-L-cysteine (990 mg, 2.9 mmol), hippuric acid (520 mg, 2.9 mmol), and 1-hydroxybenzotriazole monohydrate (470 mg, 3.48 mmol) in DMF (20 mL) at 0 °C. The mixture was stirred at 0 °C for 2 h and then at room temperature for 21 h. The reaction was quenched with water (40 mL), and the mixture was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with aqueous 10% HCl, saturated $NaHCO_3$, and brine and then dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by silica gel chromatography (hexane/acetone 80:20, 70:30) to afford the product with a yield of 54% (830 mg). ^{1}H NMR (DMSO-d_6, 500 MHz): 8.70 (1H, t, J = 6 Hz), 8.42 (1H, d, J = 8 Hz), 7.85 (2H, d, J = 7.5 Hz), 7.52 (1H, t, J = 6.5 Hz), 7.45 (2H, t, J = 8 Hz), 5.13 (1H, t, J = 7.5 Hz), 5.04 (2H, br d, J = 7 Hz), 4.47 (1H, dd, J = 8, 14 Hz), 3.92 (2H, d, J = 6 Hz), 3.62 (3H, s), 3.18 (1H, dd, J = 8.5, 13.5 Hz), 3.10 (1H, dd, J = 8, 13.5 Hz), 2.80 (1H, dd, J = 6, 13.5 Hz), 2.67 (1H, dd, J = 9, 13.5 Hz), 2.06–1.80 (8H, m), 1.61 (3H, s), 1.60 (3H, s), 1.53 (6H, s). The HPLC retention time under the conditions described above was 16.06 min.

Synthesis of N-(Benzoylglycyl)-S-all-trans-farnesylcysteine (7) (BzGFC). A mixture of methyl *N*-(benzoylglycyl)-*S-all-trans*-farnesylcysteine (700 mg) and 10 mL of 10% sodium carbonate in 20 mL of methanol/acetonitrile (v/v 1:1) was stirred at 25 °C for 20 h. 10% HCl was added until the pH = 2. The solvent was evaporated, and the residue was extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with water, dried, and evaporated. The residue was purified by silica gel chromatography (hexane/acetone 70:30, 50:50 and then hexane/acetone/methanol 40:40:20) to give the title compound (503 mg). ^{1}H NMR (DMSO-d_6, 500 MHz): 8.93 (1H, br s), 7.88 (2H, t, J = 7 Hz), 7.80 (1H, d, J = 7 Hz), (1H, t, J = 6.5 Hz), 7.43 (2H, t, J = 8 Hz), 5.12 (1H, t, J = 7.5 Hz), 5.03 (2H, br, d, J = 7 Hz), 4.20 (1H, br d, J = 4 Hz), 3.97 (1H, dd, J = 5.5, 16.5 Hz), 3.85 (1H, dd, J = 4.5, 15.5 Hz), 3.10 (2H, m), 2.89 (1H, dd, J = 5, 13.5 Hz), 2.72 (1H, dd, J = 7, 13.5 Hz), 2.03–1.88 (8H, m), 1.60 (3H, s), 1.57 (3H, s), 1.53 (6H, s).

Synthesis of (Glycylglyl)-S-all-trans-farnesylcysteine. A mixture of glycylglycylcysteine (101 mg, 0.43 mmol), *S-all-trans*-farnesyl bromide (117 μL, 123 mg, 0.43 mmol), and *N,N*-diisopropylethylamine (0.6 mL, 445 mg, 3.44 mmol) in *n*-BuOH/MeOH/H_2O (1:1:1, 10 mL) was stirred at 0 °C for 3 h. Water (30 mL) was added, and the mixture was extracted with *n*-BuOH (3 × 60 mL). The organic layer was washed with water and dried over anhydrous magnesium sulfate, filtered, and evaporated to give the title compound (150 mg).

Synthesis of N-((Benzoylglycyl)glycyl)-S-all-trans-farnesylcysteine (8) (BzGGFC). A mixture of (glycylglycyl)-*S-all-trans*-farnesylcysteine (100 mg, 0.228 mmol), benzoic acid *N*-hydroxysuccinimide ester (100 mg), triethylamine (0.1 mL), and a catalytic amount of DMAP in methylene chloride (15 mL) was stirred at 25 °C for 13 h. Water (20 mL) was added, and the methylene chloride was evaporated. The aqueous layer was extracted with ethyl acetate (3 × 50 mL), and the combined organic layer was washed with water, dried over anhydrous magnesium sulfate, filtered, and evaporated. The residue was separated by silica gel chromatography (hexane/acetone 70:30 and then hexane/acetone/methanol 40:40:20) to give the title compound with a yield of 85% (105 mg). ^{1}H NMR (DMSO-d_6, 500 MHz): 8.82 (1H, t, J = 6 Hz), 8.23 (1H, br s), 7.88 (2H, d, J = 7 Hz), 7.80 (1H, br s), 7.52 (1H, t, J = 7.45 (2H, t, J = 8 Hz), 5.13 (1H, t, J = 7.5 Hz), 5.04 (2H, d, J = 6 Hz), 4.16 (1H, br d, J = 4 Hz), 3.90 (2H, d, J = 5.5 Hz), 3.78 (1H, dd, J = 6, 16.5 Hz), 3.70 (1H, dd, J = 4.5, 16.5 Hz), 3.10 (2H, m), 2.86 (1H, dd, J = 4, 13 Hz), 2.65 (1H, dd, J = 7, 13 Hz), 2.04–1.88 (8H, m), 1.61 (3H, s), 1.58 (3H, s), 1.53 (6H, s). FAB (glycerol) m/e $(M - H^+)^-$ = 542, calcd for $C_{25}H_{35}NO_3S$ MW = 543.

Synthesis of Methyl N-((Benzoylglycyl)glycyl)-S-all-trans-farnesylcysteine. A solution of *N*-((benzoylglycyl)glycyl)-*S-all-trans*-farnesylcysteine (20 mg) and 3 drops of concentrated HCl in 10 mL of methanol was stirred at 25 °C for 18 h. The methanol was evaporated, and the residue was extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with water, dried over anhydrous magnesium sulfate, filtered, and evaporated to give the title compound (19 mg). ^{1}H NMR (DMSO-d_6, 500 MHz): 8.80 (1H, t, J = 6 Hz), 8.32 (1H, d, J = 7.5 Hz), 8.17 (1H, t, J = 5 Hz), 7.87 (2H, d, J = 8 Hz), 7.52 (1H, t, J = 7 Hz), 7.46 (2H, d, J = 8 Hz), 5.13 (1H, t, J = 7.5 Hz), 5.04 (2H, d, J = 5 Hz), 4.44 (1H, dd, J = 7.5, 13.5 Hz), 3.87 (2H, d, J = 6 Hz), 3.76 (2H, d, J = 6 Hz), 3.61 (3H, s), 3.17 (1H, dd, J = 8.5, 13.5 Hz), 3.09 (1H, dd, J = 7.5, 13.5 Hz), 2.80 (1H, dd, J = 6, 14.5 Hz), 2.66 (1H, dd, J = 8.5, 13.5 Hz), 2.05–1.87 (8H, m), 1.61 (3H, s), 1.60 (3H, s), 1.53 (6H, s). The HPLC retention time under the conditions described above was 31.12 min.

The compounds listed below were prepared in the same way as in the synthesis of *N*-benzoyl-*S-all-trans*-farnesylcysteine and its methyl ester. Analytical data for these compounds are compiled below.

Methyl N-Isobutyl-S-all-trans-farnesyl-cysteine. ^{1}H NMR ($CDCl_3$, 500 MHz): 6.24 (1H, d, J = 6.5 Hz), 5.19 (1H, t, J = 8 Hz), 5.08 (2H, t, J = 6.5 Hz), 4.78 (1H, dd, J = 6, 13.5 Hz), 3.75 (3H, s), 3.18 (1H, dd, J = 8, 12.5 Hz), 3.11 (1H, dd, J = 7, 13 Hz), 2.96 (1H, dd, J = 4, 13.5 Hz), 2.86 (1H, dd, J = 5.5, 13.5 Hz), 2.44 (1H, m), 2.10–1.94 (8H, m), 1.67 (3H, s), 1.65 (3H, s), 1.59 (6H, s), 1.18 (3H, d, J = 6.5 Hz), 1.17 (3H, d, J = 7 Hz). The HPLC retention time under the conditions described above was 2.54 min.

Methyl N-(2-Methylbutyl)-S-all-trans-farnesylcysteine. ^{1}H NMR ($CDCl_3$, 500 MHz): 6.20 (1H, d, J = 8 Hz), 5.19 (1H, t, J = 8 Hz), 5.09 (2H, t, J = 5.5 Hz), 4.81 (1H, dd, J = 8, 13 Hz), 3.12 (1H, dd, J = 6.5, 12.5 Hz), 2.96 (1H, dd, J = 5, 13.5 Hz), 2.87 (1H, dt, J = 4, 13.5 Hz), 2.20 (1H, m), 2.10–1.94 (8H, m), 1.70 (1H, m), 1.67 (3H, s), 1.66 (3H, s), 1.59 (6H, s), 1.45 (1H, m), 1.15 (3H, dd, J = 23, 7 Hz), 0.93 (3H, t, J = 7.5 Hz). The HPLC retention time under the conditions described above was 2.42 min.

Methyl N-((Trimethylacetyl)pivaloyl)-S-all-trans-farnesylcysteine. ^{1}H NMR ($CDCl_3$, 500 MHz): 6.42 (1H, d, J = 7 Hz), 5.18 (1H, t, J = 7 Hz), 5.08 (2H, t, J = 6 Hz), 4.74 (1H, dd, J = 6, 12.5 Hz), 3.75 (3H, s), 3.17 (1H, dd, J = 8.5, 13.5 Hz), 3.11 (1H, dd, J = 8, 14 Hz), 2.96 (1H, dd, J = 5, 13.5 Hz), 2.87 (1H, dd, J = 5.5, 13.5 Hz), 2.10–1.94 (8H, m), 1.66 (3H, s), 1.65 (3H, s), 1.58 (6H, s), 1.22 (9H, s). The HPLC retention time under the conditions described above was 3.54 min.

N-Isobutyl-S-all-trans-farnesylcysteine (3) (iBFC). ^{1}H NMR (DMSO-d_6, 500 MHz): 7.36 (1H, d, J = 7 Hz), 5.13 (1H, t, J = 7.5 Hz), 5.05 (2H, dd, J = 6.5, 11.5 Hz), 4.07 (1H, d, J = 4 Hz), 3.08 (2H, d, J = 8 Hz), 2.89 (1H, dd, J = 5, 13.5 Hz), 2.66 (1H, dd, J = 7.5, 13.5 Hz), 2.42 (1H, m), 2.03–1.89 (8H, m), 1.61 (3H, s), 1.59 (3H, s), 1.53 (6H, s), 0.99 (3H, d, J = 7 Hz), 0.97 (3H, d, J = 7 Hz). FAB (glycerol) m/e (M – H$^+$)$^-$ = 394, calcd for $C_{22}H_{37}NO_3S$ MW = 395.

N-Isovaleryl-S-all-trans-farnesylcysteine (4) (iVFC). ^{1}H NMR (DMSO-d_6, 500 MHz): 7.35 (1H, d, J = 6.5 Hz), 5.14 (1H, t, J = 7.5 Hz), 5.04 (2H, dd, J = 6.5, 12.5 Hz), 4.10 (1H, br s), 3.09 (2H, d, J = 7.5 Hz), 2.90 (1H, dt, J = 4, 13 Hz), 2.65 (1H, dd, J = 7.5, 14.5 Hz), 2.20 (1H, m), 2.03–1.89 (8H, m), 1.61 (3H, s), 1.59 (3H, s), 1.53 (6H, s), 1.48 (1H, m), 1.25 (1H, m), 0.96 (3H, dd, J = 6.5, 14.5 Hz), 0.81 (3H, dt, J = 8, 10.5 H). FAB (glycerol) m/e (M = H$^+$)$^-$ = 408, calcd for $C_{22}H_{39}NO_3S$ MW = 409.

N-((Trimethylacetyl)pivaloyl)-S-all-trans-farnesylcysteine (5) (PFC). ^{1}H NMR (DMSO-d_6, 500 MHz): 7.10 (1H, d, J = 7 Hz), 5.13 (1H, t, J = 7.5 Hz), 5.04 (2H, dd, J = 7, 11 Hz), 3.99 (1H, br s), 3.07 (2H, d, J = 8 Hz), 2.92 (1H, dd, J = 3.5, 12.5 Hz), 2.79 (1H, dd, J = 5.5, 13.5 Hz), 2.03–1.88 (8H, m), 1.61 (3H, s), 1.58 (3H, s), 1.53 (6H, s), 1.09 (9H, s). FAB (glycerol) m/e (M – H$^+$)$^-$ = 408, calcd for $C_{22}H_{39}NO_3S$ MW = 409.

Preparation of Human Platelet Membranes. Outdated human platelets were obtained from the Beth Israel Hospital, Boston, MA. The platelets were centrifuged at 5000*g* for 20 min, and the supernatant was discarded. The platelets were then suspended in a 10-fold excess (with respect to initial platelet volume) of low ionic strength buffer (10 mM Tris pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA) and GTP, resuspended in 50 mM Hepes–Na (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM (phenylmethyl)sulfonyl fluoride, and 0.1 mM dithiothreitol. The platelets were homogenized by passing them through a fine-needle syringe. The homogenate was sonicated for 5-min bursts on ice with a Kontes microsonicator. This procedure was repeated three times. The homogenate was centrifuged at 600*g* and the pellet was discarded. The supernatant was centrifuged at 100 000*g* for 50 min, and the pellet was suspended in the buffer described above at a concentration of 8.7 mg/mL. This membrane preparation served as the source of methyltransferase. The methyltransferase enzyme is membrane-bound and has thus far not been detergent-solubilized in a stable form; this precludes its purification.

Kinetic Measurements. Kinetic measurements were performed as previously described using AFC dissolved in DMSO as the substrate and with *S*-adenosyl[methyl-^{3}H]methionine (15 Ci/mmol) in the presence of buffer containing 200 mM Hepes (pH = 7.4), 100 mM NaCl, and 5 mM $MgCl_2$ (Shi & Rando, 1992). The substrates were pre-equilibrated at 37 °C for 5 min, and then washed platelet membranes (0.22 mg of protein per mL final concentration) were added to the buffer. The final concentration of DMSO in the assay was 4%–8% (v/v), and the incubation time for the assay was 45 min at 37 °C. At an AFC concentration of 20 μM and an AdoMet concentration of 10.7 μM, product formation was linear for at least 90 min. The reaction was quenched with a mixed solution of chloroform and methanol (1:1), followed by the addition of water. After vortexing and centrifuging, the lower layer was removed and the solvent was gently evaporated with nitrogen. The amount of the corresponding methyl-^{3}H ester produced was determined by HPLC analysis of the chloroform extracts obtained from the incubation mixtures as described in Pérez-Sala et al. (1991). The residues were dissolved in isopropanol/hexane (15:85), and the samples were analyzed on a normal phase HPLC column (Dynamax 60) connected to an on-line Berthold LB 506-C HPLC radioactivity monitor and eluted with isopropanol/hexane (15:85) at a flow rate of 1.5 mL/min. In all cases, the methyl-^{3}H esters were readily separated from the corresponding parent acids.

Platelet Aggregation Studies. Human blood was collected into 0.1 volume of 3.8% sodium citrate and then centrifuged at 200*g* for 20 min at room temperature. Platelet-rich plasma was removed and centrifuged in the presence of 1 μM prostaglandin E1 at 800*g* for 20 min to form a platelet pellet. The platelets were suspended in a modified HEPES–Tyrode's buffer, pH = 7.4, with 2 units/mL apyrase added (Johnson et al., 1989). The platelet suspension was then layered onto

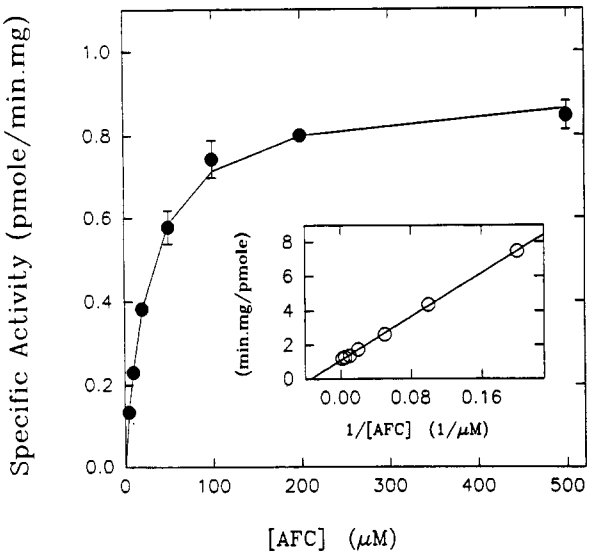


FIGURE 1: Plot of specific activity vs concentration of AFC. The concentration of AdoMet was set at 10.7 μM, and the concentrations of AFC were varied. The incubation time was 45 min. The symbols represent the average of three determinations. AFC was added in DMSO at a final concentration of 4%.

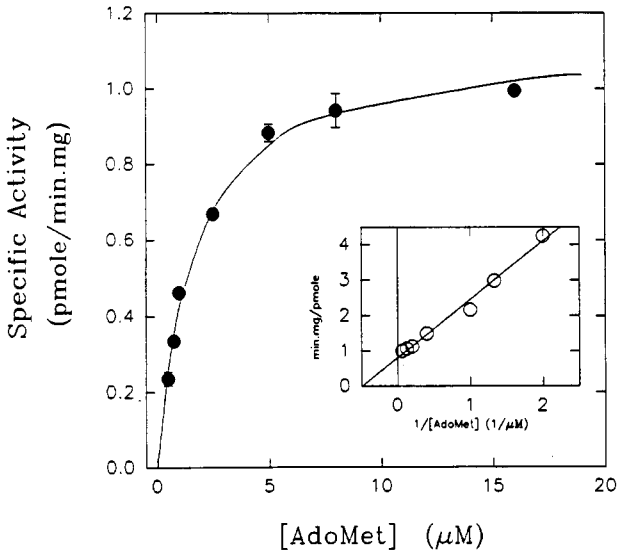


FIGURE 2: Plot of specific activity vs concentration of AdoMet. The concentration of AFC was set at 20 μM, and the concentrations of AdoMet were varied. The incubation time was 45 min. The symbols represent the average of three determinations. AFC was added in a final concentration of 4%.

a Sepharose 2B column pre-equilibrated with this same buffer. Platelets (2×10^8 per mL), suspended in the modified HEPES–Tyrode's buffer containing 1 mM Ca^{2+}, were caused to aggregate by adding either thrombin (0.01–0.025 units/mL) or the endoperoxide analog U46619 (100 nM). Farnesyl-L-cysteine analogs were added 60 s before the addition of either aggregating agent. Aggregation was recorded on a Chronolog Ionized Calcium Lumi-Aggregometer.

RESULTS

Kinetic Experiments. Initial experiments were performed to determine the kinetic parameters for AFC and AdoMet using the human platelet methyltransferase. In Figures 1 and 2, curves were generated either by varying AFC at saturating AdoMet or by varying AdoMet at saturating concentrations of AFC. The K_M values for AFC and AdoMet were measured to be 27.8 ± 1.7 μM and 1.6 ± 0.14 μM, respectively. The V_{max} values were measured to be 0.91 ± 0.01 pmol/(min mg of protein) when varying AFC and 1.12 ± 0.03 pmol/(min mg of protein) when varying AdoMet.

Table 1: Modes of Inhibition and the Measured K_I Values

varied substrate	FTA (K_I (μM))	AdoHcy (K_I (μM))	AFCMe (K_I (mM))
AFC	competitive (13.7)	mixed-type (4.40)	mixed-type (2.07)
AdoMet	uncompetitive	competitive (4.13)	mixed-type (5.02)

Table 2: Interactions of Farnesylcysteine Analogs with the Platelet Enzyme[a]

analog studied	K_M (μM)	V_{max} (pmol/min mg)
iBFC	2.95 ± 0.40	0.3 ± 0.025
iVFC	2.55 ± 0.78	0.27 ± 0.037
PFC[b]		
BzFC[b]		
BzGFC	2.55 ± 0.10	1.23 ± 0.20
BzGGFC	10.50 ± 0.58	2.84 ± 0.55

[a] The kinetic constants were determined as described in the Methods section and in a previous publication (Shi & Rando, 1992). [b] No substrate activity was measured with either PFC or BzFC at concentrations up to 100 μM. The sensitivity of the assay used allows the detection of methyltransferase activities above 1% typically seen with AFC as substrate. At 40–50 μM analogs, no inhibition of the processing of AFC at 10 μM was observed.

Previous studies in our laboratory demonstrated that the kinetic mechanism of the bovine rod outer segment methyltransferase was ordered bibi, with AdoMet binding first, followed by AFC (Shi & Rando, 1992). On the product side, AFC methyl ester departs, followed finally by AdoHcy (Shi & Rando, 1992). Identical studies were carried out here on the platelet enzyme and are described in the supplementary material. The various constants are compiled in Table 1. The data make it very clear that the kinetic mechanism of the human enzyme is ordered bibi and is thus the same as that found in the case of the bovine counterpart.

Inhibition of Human Platelet Methyltransferase. A series of new FC analogs was prepared and studied kinetically with the human platelet enzyme. The particular analogs prepared are shown in Chart 1, along with AFC and FTA. The K_M for AFC for the human platelet enzyme is 27.8 μM, and the K_I for FTA is 13.7 μM. Studies on the aliphatic series iBFC, iVFC, and PFC turned out to be quite interesting. While iBFC and iVFC had K_M values of 2.95 and 2.55 μM, respectively (Table 2), PFC proved to be inert both as a substrate and as an inhibitor of the enzyme. When the benzoyl series BzFC, BzGFC, and BzGGFC was studied, BzGFC and BzGGFC were found to be excellent substrates (Table 2), with K_M values of 2.55 and 10.5 μM, respectively, but BzFC proved to be inert both as a substrate and as an inhibitor of the methyltransferase. Similar results were obtained using the bovine ROS enzyme. Neither BzFC nor PFC bound to the enzyme, whereas the remaining molecules described here were all excellent substrates. These series of compounds provide a useful method to reveal the physiological roles of the isoprenylated methyltransferase, because BzFC and PFC should be inert with respect to methyltransferase function.

Effects of Analogs on Human Platelet Aggregation. It had previously been reported that AFC inhibits human platelet aggregation induced by either thrombin or the endoperoxide mimetic U46619 (Akbar et al., 1993). This activity of AFC was ascribed to its putative ability to inhibit the platelet methyltransferase. This notion could be directly tested by observing the abilities of the analogs described here to block

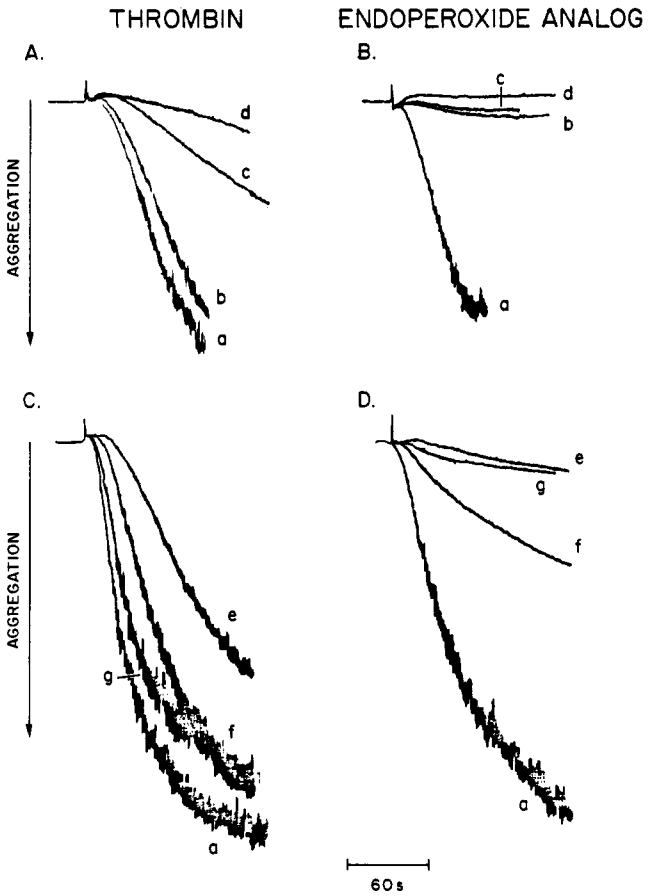


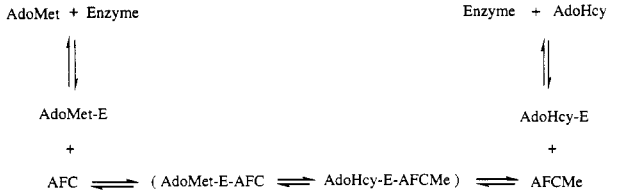
FIGURE 3: Representative aggregation tracings from platelets stimulated with thrombin or the endoperoxide analog. Platelets were treated with thrombin (panel A) or the endoperoxide analog U46619 (panel B) after incubation for 60 s with BzFC (b), BzGGFC (c), BzGFC (d), or control (a). In panels C and D, platelets were treated with thrombin (panel C) or U46619 (panel D) after 60-s incubation with PFC (e), iVFC (f), or iBFC (g). Agonists were added at the point of positive deflection in each set of tracings. These results are representative of three similar experiments.

platelet function. Platelet aggregation was induced either with thrombin or U46619, and the abilities of the analogs described in Chart 1 were studied as possible inhibitors of aggregation (Figure 3). In the aliphatic amide series, the three compounds studied (**3**–**5**) were all potent antagonistis (Figure 3) of aggregation, even though PFC (**5**) is not a measurable inhibitor of the platelet methyltransferase. In fact, PFC is the most potent analog in the group. Similar results were obtained using the aromatic analogs (**6**–**8**, Figure 3). All three analogs were active antagonists, but compound **6** (BzFC) is neither a substrate nor an antagonist of the methyltransferase. These results make it unlikely that the methyltransferase is the functional target for FC derivatives.

DISCUSSION

The isoprenylated protein methyltransferase is the terminal step in the protein isoprenylation pathway. As all known G-proteins, as well as a substantial number of membrane-associated enzymes and proteins, are modified by isoprenylation (Maltese, 1990); antagonists of the methyltransferase are potentially interesting as probes to reveal the physiological function of methylation. The methylation reaction is potentially interesting in a regulatory sense because it is the only reaction in the pathway which is reversible. In fact, G-proteins are reversibly methylated in rod outer segment membranes (Pérez-Sala et al., 1991), and a specific methylesterase that

Scheme 4: Ordered BiBi Mechanism for the Methyltransferase



can hydrolyze isoprenylated methyl ester substrates has been identified (Tan & Rando, 1992). Inhibitors of this enzyme also inhibit the hydrolysis of methylated G-proteins (Tan & Rando, 1992).

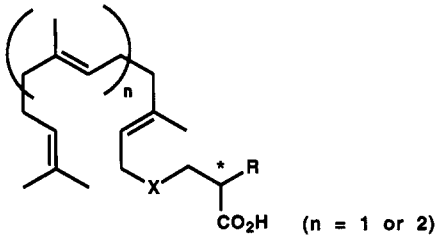
Although the isoprenylation reaction itself appears to be essential for G-protein function (Gutierrez et al., 1989), what role the methylation reaction might play is unclear. However, the design and synthesis of small molecule substrates and inhibitors of the methyltransferase is straightforward, and a large number of analogs have been synthesized and studied with respect to their abilities to be substrates or to be inhibitors of the bovine enzyme (Tan et al., 1991; Gilbert et al., 1992). Some of these molecules could be useful with respect to revealing the physiological role(s) of the methyltransferase. In fact, one of the inhibitors of AFC has been reported to inhibit the aggregation of human platelets via inhibition of the methyltransferase (Akbar et al., 1993). It was of some interest to study the kinetic mechanism of action of the human platelet enzyme and to determine the mode of interaction of L-farnesylcysteine analogs with this enzyme; these studies could serve as a starting point for pharmacological and physiological investigations on the role(s) of the methyltransferase.

Initial work centered on determining the kinetic mechanism of the human platelet enzyme and characterizing the interactions of the farnesylcysteine analogs with it. The kinetic mechanism of the enzyme proved to be ordered bibi as shown in Scheme 4. This overall mechanism is identical to that determined for the bovine rod outer segment enzyme (Shi & Rando, 1992). Moreover, the K_M values for AFC and AdoMet are virtually identical for the two enzymes. The V_{max} of the platelet enzyme is 15–20-fold lower than is the corresponding V_{max} of the bovine rod outer segment enzyme (Tan et al., 1991). However, this may simply reflect the tissue type from which the enzymes originated and the fact that we were using outdated human platelets. Also, with both enzymes, FTA is not a substrate but is a pure competitive inhibitor, although its K_I is somewhat lower in the case of bovine enzyme.

It is interesting to note that the K_M for AFC on the human platelet enzyme is approximately 28 μM. Published studies on the effects of AFC on human platelet aggregation show it to be highly active in the 1–10 μM range (Akbar et al., 1993), concentrations which are considerably below the measured K_M for the methyltransferase. Without even being concerned with expected barriers to AFC uptake, this result would suggest that the target for AFC is not the methyltransferase. Further experiments which bear on this issue come from structure–activity studies on farnesyl-L-cysteine analogs.

A considerable amount is known about structure–activity preferences of the isoprenylated protein methyltransferases (Tan et al., 1991; Gilbert et al., 1992). The composite minimal structure shown in Chart 2 shows where permissible structural modifications can be made in the basic FTP (**2**) structures so that substrate activity is retained. As shown in Chart 2, methyltransferase substrates are only obtained under a limited set of circumstances. As implied in Chart 2, an *all-trans-*

Chart 2: Active Methyltransferase Substrates Are Obtained Only When X = S or Se, When R Is an H, an Alkyl, or an *N*-Acyl Group, and When the Absolute Stereochemistry at * Is L



geranylgeranyl or *all-trans*-farnesyl substitution is permitted. Modifications at the isoprenoid side chain led to clear alterations in substrate activity. The reduction of the isoprenoid moiety led to inactive molecules, and shortening the isoprenoid chain to geranyl (C10) led to a marked diminution in activity (Tan et al., 1991).

It is important to extend current structure–activity profiles to generate analogs of very similar structures which either do or do not bind to the enzyme. Analogs of these types would be of great use for defining the physiological roles of the methyltransferase in cells. It is the purpose of the work reported here to describe such molecules. As is clear from Chart 2, alterations at the R group are an obvious place to begin these studies. Various similar acyl derivatives were studied with respect to their abilities to interact with the methyltransferase. In the farnesyl cysteine series, analogs iBFC (**3**) and iVFC (**4**) are active substrates for the enzyme, whereas PFC (**5**), an isomer of iVFC, is neither a substrate nor an inhibitor of the enzyme. In the benzoyl farnesyl series, BzFC (**6**) is also inactive, while BzGFC (**7**) and BzGGFC (**8**) are excellent substrates for the methyltransferase. From these limited studies it would appear that the enzyme does not tolerate bulky groups adjacent to the L-farnesylcysteine moiety.

The finding that there are farnesylcysteine analogs structurally similar to active methyltransferase substrates should make it possible to test whether or not the use of putative methyltransferase inhibitors is a cogent way of determining what the physiological role(s) of methyltransferase might be. As previously mentioned, AFC has already been reported to have significant pharmacological effects on a variety of cell types, including human platelets (Akbar et al., 1993). However, a recent report has shown that AFC can also inhibit receptor-mediated activation of G-proteins in isolated membranes by a mechanism independent of protein carboxymethylation (Scheer & Gierschik, 1993).

One difficulty in working with AFC is that appropriate control molecules have been unavailable. For example, although D-AFC is not a substrate for the methyltransferase, it is a mixed-type inhibitor of it, with a K_I of approximately 3–4-fold less than the K_M of AFC as a substrate (Gilbert et al., 1992). Interestingly, we have found that D-AFC is at least as active as L-AFC in blocking platelet aggregation (Ware & Rando, unpublished experiments) suggesting a nonstereospecific component to the inhibition. As demonstrated here, both PFC and BzFC were as active or more active than AFC at inhibiting platelet aggregation, although neither analog can bind to the enzyme. Therefore, although AFC may partially inhibit the isoprenylated methyltransferase under the conditions of the experiments, the observed pharmacological effects cannot be ascribed to the inhibition of this enzyme. Similar results have also been obtained with human neutrophils (Badwey & Rando, unpublished experiments) where the abilities of farnsylcysteine derivatives to block stimulated superoxide release were completely uncorrelated with the abilities of the analogs to serve as inhibitors of the methyltransferase. None of this is to say that the isoprenylated protein methyltransferase does not play a significant role in signal transduction mechanisms but rather that this putative role cannot be revealed by AFC. Finally, it will be of some interest to determine what additional targets AFC and similar molecules are interacting with. It is likely that these targets wil involve the isoprenylated and methylated G-proteins, in which case these analogs will have revealed a new pharmacological target essential in signal transduction. It is possible that some of the recently revealed activities of isoprenylated and methylated G-protein β_γ subunits may be of interest here (Lefkowitz, 1993).

SUPPLEMENTARY MATERIAL AVAILABLE

Details of the enzyme kinetic studies (10 pages). Ordering information is given on any current masthead page.

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