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Inhibition of Human Smooth Muscle Cell Proliferation in Culture by Farnesyl Pyrophosphate Analogues, Inhibitors of *In Vitro* Protein:Farnesyl Transferase

Louis H. Cohen,† Elsbet Pieterman,* Rick E. W. van Leeuwen,* Junrong Du,*#*
Pascale Negre-Aminou, A. Rob P. M. Valentijn,‡ Mark Overhand,‡*
Gijs A. van der Marel‡ and Jacques H. van Boom‡

*Gaubius Laboratory, TNO Prevention and Health and ‡Department of Organic Chemistry, University of Leiden, Leiden, The Netherlands

ABSTRACT. In this study, it was investigated whether and how inhibitors of protein:farnesyl transferase (PFT) can inhibit the proliferation of human smooth muscle cells (HSMC) in culture. Several farnesyl pyrophosphate (FPP) analogues were synthesized and tested *in vitro* for their specificity in inhibiting squalene synthase (SS), PFT, or protein:geranylgeranyl transferase-1 (PGGT-1) activities (the latter was determined using a newly designed assay). One of these compounds appeared to be a strong PFT inhibitor (IC_{50} value: 340 nM) and a weak inhibitor in the other two enzyme assays. This compound (designated as TR006) inhibited the farnesylation of *Ras* in a Ha-*ras* transfected cell line (Cohen *et al.*, *Biochem Pharmacol* **49**: 839–845, 1995) and concomitantly slowed down the growth of these cells. Twenty-five μM of TR006 inhibited the proliferation of HSMC isolated from left internal mammary artery, as measured by counting the cells over a period of three cell cycles (10 days). A structurally related compound (TR007), a specific SS inhibitor, did not influence HSMC proliferation under the same conditions. The inhibition by TR006 was concentration-dependent. In HSMC, synchronized by serum depletion, platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF)-induced DNA synthesis was decreased by a 29-hr pretreatment with 100 μM of TR006, indicating that this inhibitor acted in an early phase of the cell cycle, probably by preventing protein isoprenylation. Some other FPP analogues with comparable IC_{50} values in the *in vitro* PFT assay were also able to decrease bFGF-induced DNA synthesis without affecting cell viability. A more negatively charged member of this group, TR018, did not influence the growth factor-induced DNA synthesis, probably due to an impaired uptake into the cells. However, the pivaloyloxomethyl derivative of this compound, which is uncharged, and is thought to be converted into TR018 within the cells, showed a strong decrease in bFGF-induced DNA synthesis in HSMC. These data suggest that the compounds investigated may be developed further for treatment of conditions in which undesirable proliferation of smooth muscle cells plays an important role. BIOCHEM PHARMACOL **57**;4:365–373, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. *Ras*; signal transduction; PDGF; bFGF; DNA synthesis; protein:geranylgeranyl transferase-1; squalene synthase

Proliferation of vascular smooth muscle cells plays an important role in several pathological conditions. In the process of atherosclerosis, smooth muscle cell proliferation is often observed [1, 2], and even more frequently in patients with diabetes [3] and homocysteinemia [4]. It is the major event in restenosis of the coronary artery after percutaneous transluminal coronary angioplasty [5, 6]. Furthermore, it plays a role in the pathogenesis of chronic hypertension [7–9]. Inhibition of the growth of vascular smooth muscle cells is thought to be beneficial under these conditions. Recently, we have shown that depletion of cellular mevalonate by HMG-CoA reductase inhibitors (statins, currently in use as serum cholesterol-lowering drugs) inhibits the proliferation of cultured HSMC§ isolated from left internal mammary artery [10]. Depletion of the mevalonate-derived isoprenyl pyrophosphates FPP and/or GGPP, which are essential for the formation and activity of (iso)prenylated G-proteins, is thought to be the cause of the

† Corresponding author: Louis H. Cohen, Ph.D., TNO Prevention and Health, Gaubius Laboratory, P.O. Box 2215, 2301 CE Leiden, The Netherlands. FAX +31-71-5181904; E-mail: LH.Cohen@pg.tno.nl

Present address: Dept. of Pharmacology, School of Pharmacy, West China University of Med Sci., Chengdu, China.

§ *Abbreviations:* HSMC, human smooth muscle cells; PFT, protein: farnesyl transferase; PGGT-1, protein:geranylgeranyl transferase-1; SS, squalene synthase; FPP, farnesyl pyrophosphate; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; GGPP, geranylgeranyl pyrophosphate; MTT, 3,-[4,5-dimethyl-thiazo-2-yl]-2,5-diphenyltetrazolium bromide; PepAsep, sepharose-coupled peptide for farnesylation by PFT; PepBsep, non-isoprenylatable sepharose-coupled peptide; PepCsep, sepharose-coupled peptide for geranylgeranylation by PGGT-1; and POM, pivaloyloxomethyl.

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inhibition, since several of these proteins (e.g. *Ras*, Cdc42, Rho) play a role in growth factor-induced signal transduction and cell cycle progression [11, 12]. Enzymes involved in this prenylation process are PFT, which recognizes the C-terminal amino acid sequence CaaX (C = cys, a = aliphatic amino acid, and X = Met or Ser), as e.g. in *Ras*, for the covalent linkage of the farnesyl group from FPP to the cysteine residue [13] and PGGT-1, which transfers the GG group from GGPP to cysteine in the C-terminal CaaX box of proteins, containing a leucine at the X-place [14, 15].

According to the reasoning above, specific inhibition of PFT and/or PGGT-1 should result in inhibition of HSMC proliferation. Peptidomimetic compounds, as mimics of the CaaX motif, have been developed as specific inhibitors of PFT [16]. Since these compounds inhibited the proliferation of *Ras*-transformed cells and because of the involvement of mutated *Ras* in certain human cancers, the development of these inhibitors as anticancer drugs is currently in progress [17]. Remarkably, these peptidomimetics did not inhibit the proliferation of nontransformed cells, possibly due to differences in the *Ras*-proteins in transformed and nontransformed cells [18]. We have synthesized several analogues of FPP, the other substrate of PFT, and have shown that some were specific inhibitors of either PFT or SS, another enzyme which uses FPP as substrate [19]. In addition, a strong inhibitor of PFT (FFPA1), now designated as TR006, inhibited the farnesylation of *Ras* in Rat-1.H*ras*13 cells, a transformed rat fibroblast cell line overexpressing H-*Ras* [20]. It is important to know whether this and other FPP analogues are inhibitors of the proliferation of nontransformed cells like HSMC. The present study describes a series of synthetic FPP analogues tested for their inhibition of SS, PFT, and PGGT-1 (the latter in a newly designed *in vitro* assay). The potencies of these compounds to inhibit the proliferation of H*ras*13 cells and HSMC in culture are described.

MATERIALS AND METHODS

Materials

FPP analogues have been synthesized and characterized as described previously ([21–26]; see refs. in Table 1). Simvastatin sodium salt was a kind gift from Sankyo Co. (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

Cell Culture

H-*ras*-transformed Rat-1.H*ras*13 cells [20] were cultured as described previously [19]. HSMC were isolated from left internal mammary artery and cultured as described previously [10].

Cell Proliferation Assay

Rat-1.H*ras*13 cells were plated at 5×10^3 cells per 10 cm^2 well and cultured for 10 days in DMEM supplemented with 10% FBS and the indicated concentrations of TR006 (see Results). HSMC were cultured for 10 days in DMEM supplemented with 10% human serum in the presence of 0, 25, or 100 μM of TR006 or TR007. At day 0, cells were plated and, on day 3 and 7, were re-fed with the same media. On the same days and day 10, cells were harvested by trypsinization and counted using a standard haemocytometer chamber. Values given are the means of triplicate counting of duplicate incubations.

Growth Factor-induced DNA Synthesis in HSMC

About 2.5×10^4 cells were plated in 2.5 cm^2 wells, and incubated in serum-poor medium (containing 0.4% FBS) for 48 hr to obtain quiescent, synchronized cells. After this growth arrest period, cells were incubated with or without 10 or 100 μM of a particular FPP analogue for 29 hr before addition of PDGF (0.8 nM) or bFGF (1.1 nM). After further incubation for 24 hr, DNA synthesis was determined by measuring the incorporation of [^{3}H]-thymidine into cellular DNA during the next 2 hr, as described previously [10].

Determination of Cellular Mitochondrial Dehydrogenase Activity (MTT Test)

The viability of the HSMC after incubation with the FPP analogues was investigated using the MTT test as described previously [10].

Assay of Squalene Synthase

The activity of SS in rat liver microsomal preparations was determined in the absence or presence of the different FPP analogues, as described previously [19].

Assay of Protein:Farnesyl Transferase

PFT activity was determined using a C-terminal peptide of pre-p21$^{N\text{-}ras}$ coupled to sepharose beads as substrate (pep-Asep) as described previously [19], except that a crude preparation of protein:farnesyl transferase from rat brain [13] was used as the source for the enzyme. Figure 1 shows the dependencies of substrate and enzyme concentrations and the time-course of this reaction. On the basis of these data, the experimental conditions (25 μL reaction mixture) were as follows: 80 pmol/25 μL sepharose-coupled peptide A or B, 0.7 μM of [^{3}H]-FPP (American Radiolabeled Chemicals Inc.; specific radioactivity 15 Ci/mmol), and 13 μL of rat brain enzyme preparation. Incubation was performed at 37° for 30 min. For the determination of IC_{50} values of the FPP analogues, the assay was performed three times in the presence of different concentrations of the compounds in duplicate as described previously [19].

Assay of Protein:geranylgeranyl Transferase-1

Determination of PGGT-1 activity was performed by using a sepharose-coupled peptide as substrate. The amino acid

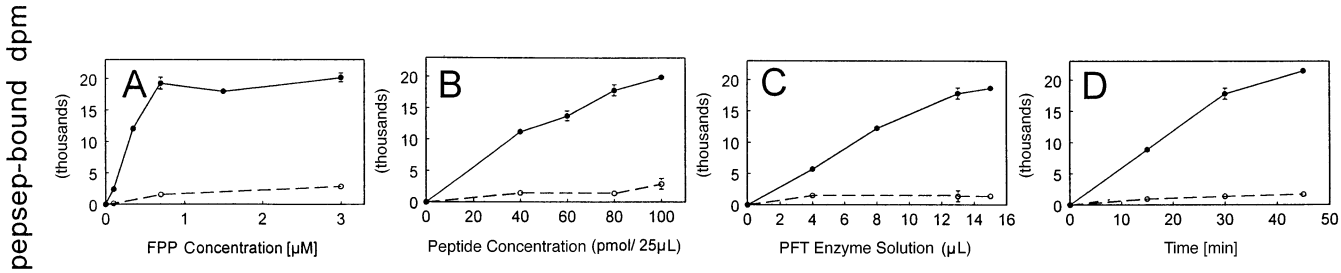


FIG. 1. Parameters of the PFT assay. The assay of the binding of radioactivity from [^{3}H]-FPP to pepAsep (–●–) and pepBsep (–○–) catalyzed by rat brain PFT was performed as described under Materials and Methods. The dependencies of the reactions on FPP concentration (A), concentration of peptide bound to sepharose (B), enzyme concentration (C), and incubation time (D) were investigated. The additional reaction conditions which are not shown in the figure were: (A) 80 pmol/25 μL of sepharose-coupled peptides, 13 μL (/25 μL) PFT solution, 30-min incubation time; (B) 0.7 μM FPP, 13 μL (/25 μL) PFT solution, 30-min incubation time; (C) 80 pmol/25 μL of sepharose-coupled peptides; 0.7 μM FPP, 30-min incubation time; and (D) 80 pmol/25 μL of sepharose-coupled peptides, 13 μL (/25 μL) PFT solution, 0.7 μM FPP.

sequence of the peptide was the same as that described for the PFT assay (pepAsep), except that the C-terminal methionine was replaced by leucine, thereby introducing the CaaL box which is the consensus sequence for geranylgeranylation by PGGT-1 [14, 15, 27]. This substrate has been designated as pepCsep. PepBsep, the non-isoprenylatable sepharose-coupled peptide (see PFT assay in [19]), was used as control to measure nonspecific association of radiolabeled GGPP. A crude PGGT-1 preparation was isolated from bovine brain according to Yokoyama *et al.* [14]. Time, substrate, and enzyme dependencies of the geranylgeranylation of pepCsep and pepBsep are shown in Fig. 2. The radioactivity bound to the sepharose was strongly dependent on the presence of the cysteine residue in pepCsep, and the reaction was linear up to 40 min and up to 5 μL of the enzyme preparation. On the basis of these data, the conditions for the determination of PGGT-1 activity were as follows: The incubation mixture (25 μL) contained 2.5 μL pepBsep or pepCsep (1 nmol peptides), 3 μL bovine brain enzyme, 1 μM [^{3}H]-GGPP (specific radioactivity 15 Ci/mmol, American Radiolabeled Chemicals), 50 μM $ZnCl_2$, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.004% Triton X-100, 50 mM Tris–HCl (pH 7.4). For the determination of the inhibitory potencies of the various FPP analogues, at least 5 different concentrations of these compounds were added to the mixture. The incubation was performed at 37° for 40 min with continuous shaking. The reaction was terminated by addition of 1 mL of 2% (w/v) SDS, and the beads were spun down and washed successively 3 times with 2% (w/v) SDS under shaking for 45 min at 50°. For the calculation of PGGT-1 activity, the ^{3}H-counts bound to pepBsep were subtracted from the counts bound to pepCsep. For the determination of the IC_{50} values of the FPP analogues, the assay was repeated at least three times in the presence of the various concentrations of the compounds, and the concentration at 50% inhibition was determined using a mathematical function fitting to the concentration/inhibition curve.

RESULTS AND DISCUSSION

In Vitro *Inhibitory Potency of FPP analogues on SS, PFT, and PGGT-1 Activity*

Several FPP analogues have been synthesized (see Table 1 for chemical structures and references) and tested for their inhibitory potency *in vitro* for two FPP-consuming enzymes, SS and PFT. In order to gain insight into their specificities in inhibiting protein isoprenylation in a broader sense, these compounds were tested as well in the *in vitro* assay of PGGT-1. The IC_{50} values of the different compounds in these three *in vitro* assays are shown in Table 1.

In the subgroup of linear diphosphonate analogues (Ta-

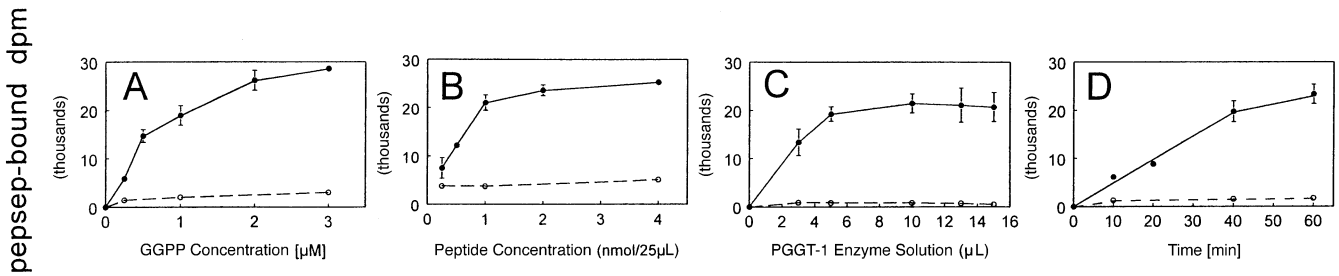


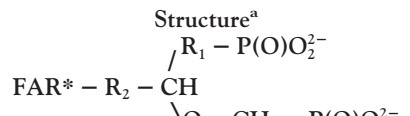
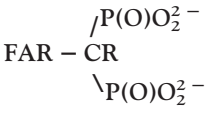
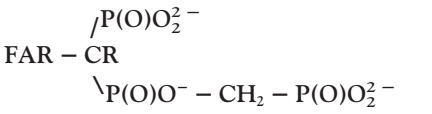
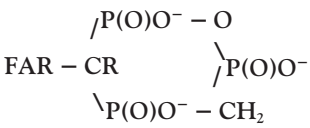
FIG. 2. Parameters of the PGGT-1 Assay. The assay of the binding of radioactivity from [^{3}H]-GGPP to pepCsep (–●–) and pepBsep (–○–) catalyzed by bovine brain PGGT-1 was performed as described under Materials and Methods. The dependencies of the reaction on GGPP concentration (A), concentration of peptide bound to sepharose (B), enzyme concentration (C), and incubation time (D) were tested. The additional reaction conditions were: (A) 1 nmol/25 μL of sepharose-coupled peptides, 2.5 μL (/25 μL) PGGT-1 solution, 40-min incubation time; (B) 1 μM GGPP, 2.5 μL (/25 μL) PGGT-1 solution, 40-min incubation time; (C) 1 nmol/25 μL of sepharose-coupled peptides; 1 μM GGPP, 40-min incubation time; and (D) 1 nmol/25 μL of sepharose-coupled peptides, 2.5 μL (/25 μL) PGGT-1 solution, 1 μM GGPP.

TABLE 1. Effect of FPP analogues on squalene synthase (SS), protein:farnesyl transferase (PFT) and protein:geranylgeranyl transferase-1 (PGGT-1) *in vitro*

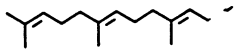
1A. Linear diphosphonates

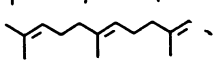
	Structure[a] FAR-R_1-P(O)O^--R_2-P(O)O^--R_3				IC_{50} Values (μM)		
Code	R_1	R_2	R_3	Refs.	SS	PFT	PGGT-1
TR006	—	O	O^-	30	129 ± 11	0.34 ± 0.05	19.1 ± 6.0
TR005	—	O	CH_3	21	>500	8.90 ± 2.15	>100
TR003	CH_2	O	O^-	21	518 ± 12	0.93 ± 0.23	6.5 ± 0.8
TR004	CH_2	O	CH_3	21	>500	>200	>100
TR007	OCH_2	CH_2	O^-	28	0.68 ± 0.14	7.15 ± 2.32	>100
TR008	OCH_2	O	O^-	22	1.29 ± 0.22	0.52 ± 0.07	41.0 ± 2.7
TR012	OCH_2	O	CH_3	22	655 ± 42	6.67 ± 0.70	>100

1B. Branched and cyclic di- and triphosphonates

	Structure[a] 			IC_{50} Values (μM)		
Code	R_1	R_2	Refs.	SS	PFT	PGGT-1
TR010	CH_2	—	24	357 ± 22	6.57 ± 0.86	55.4 ± 18.8
TR013	—	—	24	18.3 ± 1.0	1.08 ± 0.23	>100
TR015	—	CH_2	24	16.6 ± 3.4	0.58 ± 0.45	1.15 ± 0.13
						
TR011	R = H		24,29	0.018 ± 0.002	13.5 ± 1.1	39.7 ± 0.9
TR014	R = FAR		24	25.8 ± 5.1	147 ± 37	>100
						
TR018	R = H		26	1.10 ± 0.24	1.81 ± 0.38	3.60 ± 0.35
TR021	R = FAR		26	23.3 ± 6.7	3.99 ± 0.84	3.87 ± 1.24
						
TR019	R = H		26	46.4 ± 7.5	34.2 ± 3.7	>100
TR022	R = FAR		26	>50	56.2 ± 5.0	33.3 ± 3.2

[a]Values are means ± SEM, N = 3.

FAR = 

FAR* = 

ble 1A), TR006 and TR007 were the strongest inhibitors of PFT and SS, respectively, and TR003 was a rather moderate inhibitor of PGGT-1. The first two compounds seemed to be specific inhibitors of PFT and SS, respectively. Increasing the length of the farnesyl chain by one carbon atom (compare TR006 and TR003) decreased the inhibition of PFT and increased that of PGGT-1. Biller *et al.* [28] showed previously that the ether oxygen is important for inhibition of SS (TR007 and TR008), whereas the oxygen between the two phosphates seems to play a role in the inhibition of PGGT-1 and PFT (cf. TR007/TR008). Decreasing the negative charge from 3^- to 2^- generally resulted in a decrease in the inhibitory capacity in all three assays (cf. TR006/5, TR003/4, and TR008/12). Therefore, compounds with 4^- and 5^- charges, as in the branched di- and triphosphonate analogues shown in Table 1B, were also tested in the same enzyme assays. The diphosphonate TR011 is a strong and specific inhibitor of SS (see also Ciosek *et al.* [29]). The other 4^--charged compounds, TR010, TR013, and TR015, are more potent inhibitors of PFT, and additionally, TR015, which is one carbon longer than TR013, is an inhibitor of PGGT-1. Increasing the

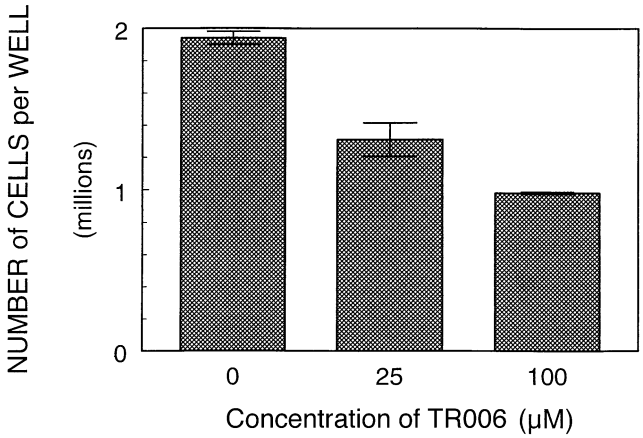


FIG. 3. TR006 inhibits proliferation of Rat-1.H*ras*13 cells. Rat-1.H*ras*13 cells were plated in monolayer culture at 5 × 10^3 cells per well and cultured further for 10 days in DMEM supplemented with 10% FBS and 25 or 100 μM of TR006. On days 3 and 7, the medium was refreshed and cells were harvested and counted at day 10. Values are the means (±SD) of triplicate counting of duplicate incubations.

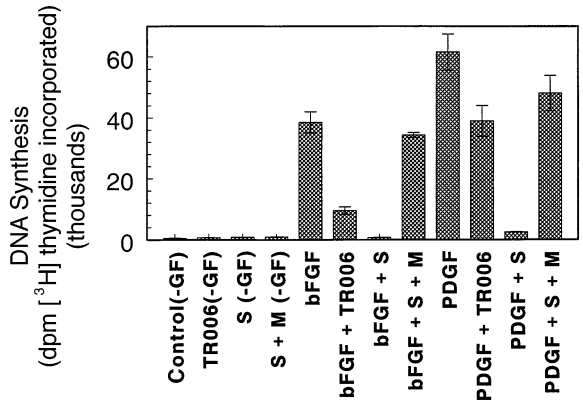


FIG. 5. Effect of TR006 on growth factor (GF)-stimulated DNA synthesis in HSMC. Quiescent HSMC (see Materials and Methods) were incubated with or without 100 μM TR006, 1 μM simvastatin (S), or 1 μM simvastatin + 100 μM mevalonate (S + M). After 29 hr, human PDGF (0.8 nM) or human bFGF (1.1 nM) were added to induce cell cycle progression. DNA synthesis was determined, 24 hr later, as incorporation of [^{3}H]-thymidine into cellular DNA. Shown are the average (±SEM) values of triplicate measurements.

charge to 5^- in TR018 by introducing a third phosphate group (compare with TR011 and TR015) turned the compound into an inhibitor in all three assays.

The structure of TR018 made it possible to introduce an oxygen atom between two phosphate atoms (which was favorable for inhibition of PFT and PGGT-1) by cyclization to TR019. However, TR019 proved to be a weak or no inhibitor of the enzymes studied.

During the organic chemical synthesis of the latter di- and triphosphonates, bisfarnesyl analogues were obtained as well (see refs. in Table 1B). The addition of a second farnesyl group had an unfavorable effect on the inhibitory capacity, except for the fact that the inhibition of PFT and PGGT-1 by the 5^--charged triphosphonate (cf. TR018/TR021) hardly changed.

Inhibition of Proliferation of Rat1.H-ras13 Cells by TR006

As can be seen in Table 1, TR006 was the strongest and rather specific inhibitor of PFT. It was shown previously [19] that a 20-hr incubation with TR006 inhibited the farnesylation of *Ras* in H-*ras*13 cells. The same compound was able to inhibit the proliferation of these cells for 10 days in a concentration-dependent fashion (Fig. 3). Starting with 5000 cells per well, the control cells proliferated exponentially to 1.94 × 10^6 during the incubation time. Incubation with TR006 (25 and 100 μM) led to a reduction in cell numbers of 32.5 and 49.5% of the control value, respectively. Nonviable cells, as determined by the trypan blue exclusion method, were not observed.

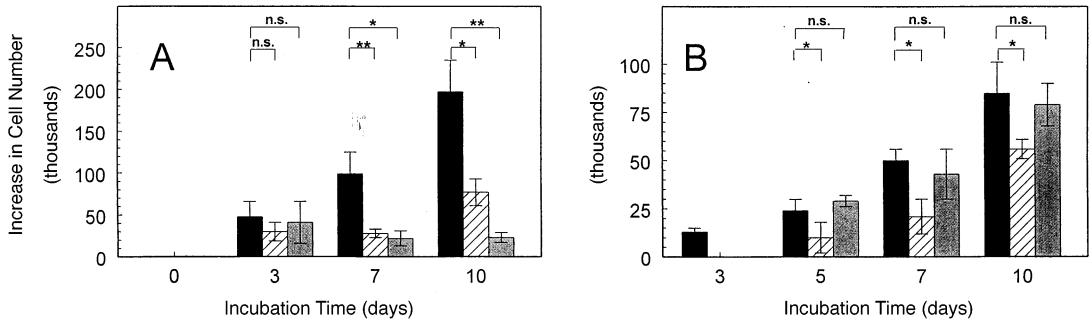


FIG. 4. Effect of TR006 and TR007 on the proliferation of HSMC. In two separately performed experiments (A and B), HSMC were cultured for 10 days in DMEM supplemented with 10% FBS and 10% human serum in the presence of (A) 0 (black bars), 25 μM (hatched bars), and 100 μM (gray bars) of TR006, or (B) 0 (black bars), 25 μM of TR006 (hatched bars), and 25 μM of TR007 (gray bars). At day 0, cells were plated in monolayer culture in: (A) 9.10^4 and (B) 10^4 cells per 10 cm^2 well. On the indicated days, half of the cells were re-fed with the same medium and the other half were harvested and counted using a standard haemocytometer chamber. The increase in cell number from day 0 has been depicted. Values are the means (±SD) of triplicate counting of duplicate incubations. Significance of the difference of each time point was tested using a Student *t*-test: *$P < 0.05$; **$P < 0.01$; n.s., not significant.

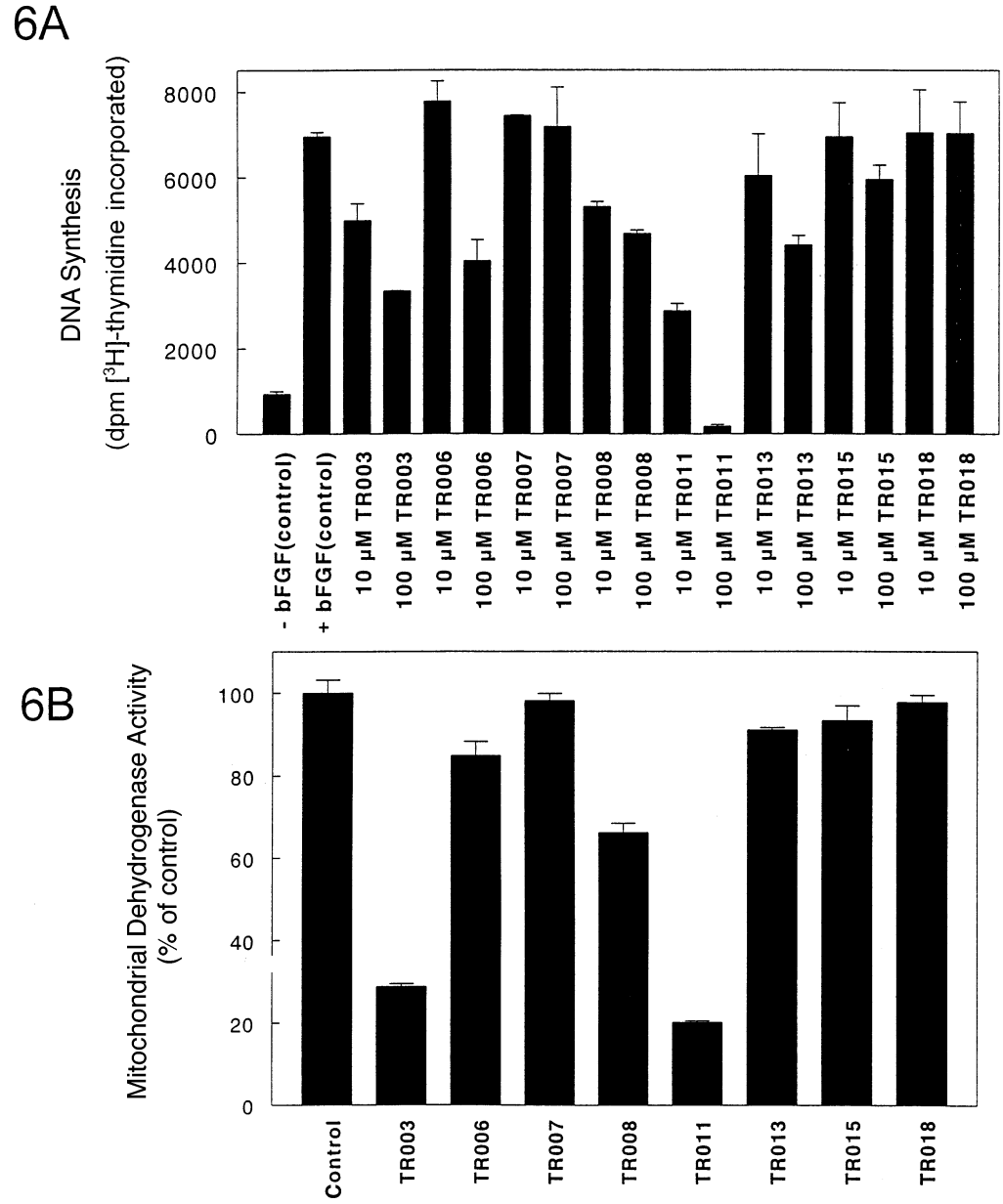


FIG. 6. Effect of FPP analogues on bFGF-stimulated DNA synthesis (A) and on cell viability (B) in HSMC. Quiescent HSMC (see Materials and Methods) were preincubated with or without 10 or 100 µM of the indicated FPP analogue. After 29 hr, human bFGF (1.1 nM) was added and 24 hr later, DNA synthesis (A) and mitochondrial dehydrogenase activity using the MTT test (B) were determined as described under Materials and Methods. Shown are the average (±SEM) values of triplicate measurements.

Inhibition of Proliferation of HSMC by TR006

In the same proliferation assay, using HSMC, TR006 slowed down the increase in cell number during 10 days of incubation in a concentration-dependent way (Fig. 4A). In this period, control cells proliferated to 2.9 ($\pm$0.4) $\times$ 10^5 cells per well. Incubation with 25 and 100 µM of TR006 severely retarded the growth in a concentration-dependent fashion and led at day 10 to 39% and 12% of the increase in the control cells, respectively. Again, nonviable cells were absent after the incubation period. This antiproliferative effect must be related to specific characteristics of TR006, because in an experiment comparing TR006 with TR007, a specific inhibitor of SS, TR007 did not inhibit the proliferation of HSMC (Fig. 4B). TR011, a stronger SS inhibitor than TR007, exhibited cell toxicity under these experimental conditions (results not shown; cf. results in Fig. 6).

Inhibition of bFGF- and PDGF-induced DNA Synthesis in HSMC by TR006

The growth factors bFGF and PDGF are thought to stimulate cell growth by activating the *Ras*-MAP kinase signal transduction pathway in the G1-phase of the cell cycle. In the event that inhibition of the farnesylation of *Ras*-protein played a role in the inhibition of HSMC proliferation, TR006 should block the cell cycle in the

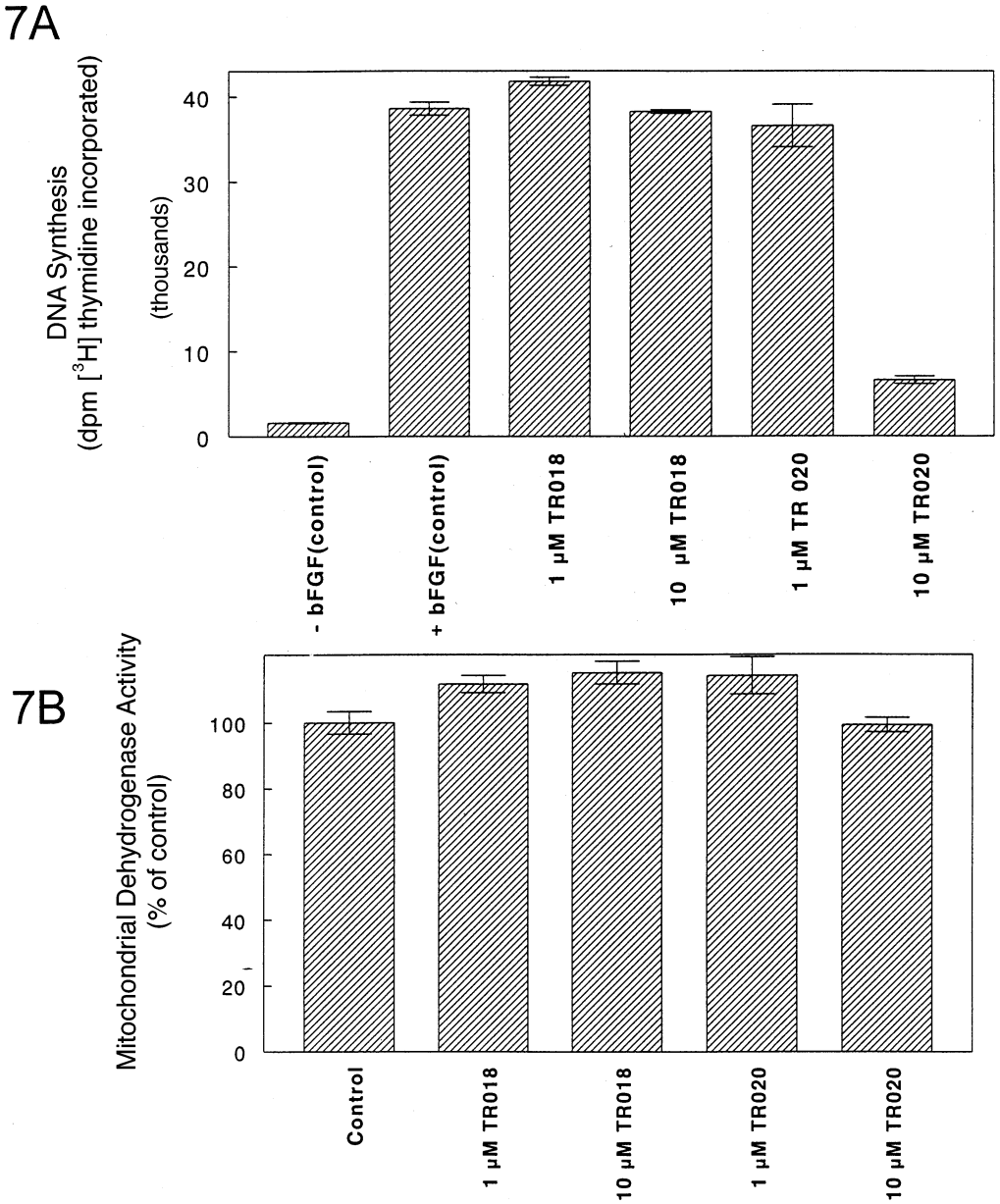


FIG. 7. Effect of TR018 and TR020 on bFGF-stimulated DNA synthesis (A) and on cell viability (B) in HSMC. Quiescent HSMC (see Materials and Methods) were preincubated with or without 1 or 10 µM of TR018 and TR020. After 29 hr, human bFGF (1.1 nM) was added and 24 hr later, DNA synthesis (A) and mitochondrial dehydrogenase activity using the MTT test (B) were determined as described under Materials and Methods. Shown are the average (±SEM) values of triplicate measurements.

G1-phase and, consequently, decrease the growth factor-induced DNA synthesis. In order to investigate this hypothesis, quiescent HSMC were activated by PDGF or bFGF and the entering of the S-phase, was determined 24 hr later by measuring DNA synthesis. In a time-course study of the incorporation of [^{3}H]-thymidine into DNA in HSMC, it was observed that the maximum of DNA synthesis took place around 27 hr after PDGF addition (results not shown). The effect of TR006 on growth factor-induced DNA synthesis was investigated in three separately performed experiments using HSMC from different donors. A typical example of the results is shown in Fig. 5. PDGF and bFGF markedly stimulated DNA synthesis (compared to control without growth factors). TR006, added 29 hr previous to GF addition, was able to inhibit DNA synthesis, showing its inhibitory effect in the G1-phase. Simvastatin, which reduced the farnesylation of *Ras* by FPP depletion through inhibition of mevalonate formation, and greatly decreased DNA synthesis in HSMC [10], was used as control (Fig. 5). As proof that the latter effect was indeed through mevalonate depletion, concomitant addition of 100 µM mevalonate together with simvastatin largely abolished the inhibition of GF-induced DNA synthesis by simvastatin (Fig. 5). These observations support the notion that TR006 acts in the G1-phase, probably by preventing protein isoprenylation, such as the farnesylation of

TABLE 2. Summary of inhibitory effects of FPP analogues on SS, PFT, and PGGT-1 activities *in vitro* and on growth factor-stimulated DNA synthesis and viability of synchronized HSMC in culture

		Extent of inhibition				
		Enzyme activity *in vitro*			Synchronized HSMC	
Code	Charge	SS	PFT	PGGT-1	DNA synthesis	Cell viability
TR003	3^-	−	+	+	+	++
TR006	3^-	−	+	±	+	−
TR007	3^-	+	±	−	−	−
TR008	3^-	+	+	±	+	+
TR011	4^-	++	±	±	++	++
TR013	4^-	±	+	−	+	−
TR015	4^-	±	+	+	±	−
TR018	5^-	+	+	+	−	−
TR020	0	−	−	−	++	−

−, none; ±, moderate; +, strong; ++, very strong.

Ras. However, the involvement of other signaling G-proteins, such as Cdc42 or Rho, cannot be excluded at the moment.

Inhibition of bFGF-induced DNA Synthesis in HSMC by FPP Analogues

Analogous to the previous experiment with TR006, we tested the possible inhibition of the bFGF-induced DNA synthesis in HSMC by those FPP analogues which exhibited comparable low IC_{50} values in the *in vitro* PFT assay or the PGGT-1 assay. Two strong inhibitors of SS, TR007 and TR011, were tested as well. Each compound was tested in two to three separately performed experiments, and the results of a typical experiment are shown in Fig. 6A. In order to exclude the possibility that a decrease in DNA synthesis may be the result of a decrease in cell viability, the MTT test (see Materials and Methods) was performed after incubation of the cells with the highest concentration (100 μM) of the FPP analogues (Fig. 6B). Besides the previously observed inhibition by TR006 (the increase at 10 μM TR006 is not significantly different from control), the PFT inhibitors TR003, TR008, and TR013 also decreased the bFGF-induced DNA synthesis. However, the effect of TR003 can be attributed to a strong decrease in viability. One hundred μM of TR008 also caused a small decrease in MD activity. TR015, which structurally differs only slightly from TR013, had only a small effect under these conditions. It is not clear why TR015 inhibited less than TR013 while being a comparable inhibitor of PFT and a strong inhibitor of PGGT-1 in the *in vitro* assays. TR018 had no effect. This may be explained, however, by a decreased uptake of this compound into the cells due to the increase in the charge of this compound (5^-). As expected from the results shown in Fig. 4B, the SS inhibitor TR007 had no effect, whereas the much stronger SS inhibitor TR011 caused a strong decrease in DNA synthesis. However, as shown in Fig. 6B, this is probably the result of its negative effect on cell viability.

To improve the uptake of TR018 in the cells, we synthesized TR020, the POM derivative of TR018. This compound is not charged because the POM groups are covalently bound to the free oxygen atoms of the phosphates. TR020 proved not to be active as an inhibitor in the *in vitro* assays (results not shown). It is supposed that after entering the cells, the POM groups are removed, releasing the active TR018 [31]. A typical example of the results of incubations of HSMC with TR020 is shown in Fig. 7A. TR020 markedly decreased the bFGF-induced DNA synthesis in HSMC, with an IC_{50} value between 1 and 10 μM. At the same concentrations, TR018 had no effect and neither of the compounds influenced cell viability as measured by the MTT test (Fig. 7B). These results suggest that TR018 can act as a PFT and/or PGGT-1 inhibitor within the cell. However, we cannot exclude the possibility that TR020, as such, decreased the DNA synthesis by a yet unclear mechanism. TR020 is a much stronger inhibitor of bFGF-induced DNA synthesis than TR006 (cf. Figs. 6A and 7A), but in the *in vitro* farnesylation assay (Table 1), TR006 was a somewhat stronger inhibitor compared to TR018, a putative TR020 derivative within the cells. We explain this discrepancy by assuming that the uptake of the uncharged TR020 is much higher than the negatively charged TR006. Furthermore, TR018 is a somewhat stronger inhibitor of *in vitro* geranylgeranylation than TR006, which may influence growth factor-stimulated DNA synthesis in HSMC as well.

The effects of the different FPP analogues on *in vitro* enzyme activities and the cellular effects in synchronized HSMC are summarized in Table 2. Those charged compounds which did not affect cell viability and inhibited DNA synthesis, i.e. TR006, TR013, and TR015, have in common that they are strong inhibitors of PFT activity *in vitro*. TR018, which has the latter property as well, did not influence DNA synthesis, but we suggest that the increase in charge to 5^- impaired the uptake of this compound into the cells. This is supported by the effect of the noncharged POM-TR018, TR020, which strongly inhibited DNA synthesis. However, we cannot exclude at the moment that the

inhibition of DNA synthesis by the different compounds is additionally caused by (an)other mechanism(s).

In conclusion, we have developed certain FPP analogues which are both inhibitors of PFT *in vitro* and inhibitors of HSMC proliferation in culture. The mechanism of action of these compounds in these cells is currently under investigation. Our data support the notion that the investigated compounds may be developed further for treatment of pathological conditions in which proliferation of smooth muscle cells plays an important role, such as in atherosclerosis and restenosis.

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References

1. Ross R, The pathogenesis of atherosclerosis: A perspective for the 1990's. *Nature* **362:** 801–806, 1993.
2. Schwartz SM, de Blois D and O'Brien ER, The intima. Soil for atherosclerosis and restenosis. *Circ Res* **77:** 445–465, 1995.
3. Oikawa S, Human arterial smooth muscle cell proliferation in diabetes. *Diabetes* **45:** S114–S116, 1996.
4. McCully KS, Homocysteine and vascular disease. *Nat Med* **2:** 386–389, 1996.
5. Libby P, Schwartz D, Brogi E, Tanaka H and Clinton SK, A cascade model for restenosis. A special case of atherosclerosis progression. *Circulation* **86:** 47–52, 1992.
6. Mattsson E and Clowes AW, Current concepts in restenosis following balloon angioplasty. *Trends Cardiovas Med* **5:** 200–204, 1995.
7. Lever AF, Slow pressor mechanisms in hypertension: a role for hypertrophy of resistance vessels? *J Hypertens* **4:** 515–524, 1986.
8. Mulvany MJ, Role of vascular structure in blood pressure development of the spontaneously hypertensive rat. *J Hypertens* **4:** 61–63, 1986.
9. Jackson CL and Schwartz SM, Pharmacology of smooth muscle cell replication. *Hypertension* **20:** 713–736, 1992.
10. Nègre-Aminou P, van Vliet AK, Van Erck M, van Thiel GCF, van Leeuwen REW and Cohen LH, Inhibition of proliferation of human smooth muscle cells by various HMG-CoA reductase inhibitors: Comparison with other human cell types. *Biochim Biophys Acta* **1345:** 259–268, 1997.
11. Mulcahy LS, Smith MR and Stacey DW, Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. *Nature* **313:** 241–243, 1985.
12. Olson MF, Ashworth A and Hall A, An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* **269:** 1270–1272, 1995.
13. Reiss Y, Goldstein JL, Seabra MC, Casey PJ and Brown MS, Inhibition of purified $p21^{Ras}$ farnesyl-protein transferase by Cys-AAX tetrapeptides. *Cell* **62:** 81–88, 1990.
14. Yokoyama K, Goodwin GW, Ghomashchi F, Glomset JA and Gelb MH, A protein geranylgeranyltransferase from bovine brain-Implications for protein prenylation specificity. *Proc Natl Acad Sci* USA **88:** 5302–5306, 1991.
15. Moores SL, Shaber MD, Mosser SD, Rands E, O'Hara MB, Garsky VM, Marshall MS, Pompliano DL and Gibbs JB, Sequence dependence of protein isoprenylation. *J Biol Chem* **266:** 14603–14610, 1991.
16. James GL, Goldstein JL, Brown MS, Rawson TE, Somers TC, McDowell RS, Crowley CW, Lucas BK, Levinson AD and Marters JC Jr., Benzodiazepine peptidomimetics: potent inhibitors of *Ras* farnesylation in animal cells. *Science* **260:** 1937–1942, 1993.
17. Gibbs JB, Oliff A and Kohl NE, Farnesyltransferase inhibitors-Ras research yields a potential cancer therapeutic. *Cell* **77:** 175–178, 1994.
18. James GL, Goldstein, JL and Brown MS, Polylysine and CVIM sequences of k-*Ras*b dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic *in vitro*. *J Biol Chem* **270:** 6221–6226, 1995.
19. Cohen LH, Valentijn ARPM, Roodenburg L, van Leeuwen REW, Huisman RH, Lutz RJ, van der Marel GA and van Boom JH, Different analogues of farnesyl pyrophosphate inhibit squalene synthase and protein:farnesyltransferase to different extents. *Biochem Pharmacol* **49:** 839–845, 1995.
20. Downward J, De Gunzburg J, Riehl R and Weinberg RA, p21*ras*-induced responsiveness of phosphatidylinositol turnover to bradykinin is a receptor number effect. *Proc Natl Acad Sci* USA **85:** 5774–5778, 1988.
21. Valentijn ARPM, van der Marel GA, Cohen LH and van Boom JH, An expeditious synthesis of pyrophosphate analogues of farnesyl pyrophosphate using the phosphonylating agent methyl methylphosphonomorpholidate. *Synlett* 663–664, 1991.
22. Valentijn ARPM, van den Berg O, van der Marel GA, Cohen LH and van Boom JH, A novel approach towards the synthesis of pyrophosphate analogues of farnesyl pyrophosphate. *Recueil des Travaux Chimiques des Pays-Bas* **113:** 563–566, 1994.
23. Valentijn ARPM, de Haan R, Hagens S, de Kant E, van der Marel GA, Cohen LH and van Boom JH, Synthesis of a potential enzyme-specific inhibitor of squalene synthase. *Recueil des Travaux Chimiques des Pays-Bas* **115:** 332–336, 1995.
24. Valentijn ARPM, van den Berg O, van der Marel GA, Cohen LH and van Boom JH, Synthesis of pyrophosphonic acid analogues of farnesyl pyrophosphate. *Tetrahedron* **51:** 2099–2108, 1995.
25. Valentijn ARPM, Broxterman HJG, van der Marel GA, Cohen LH and van Boom JH, Synthesis of 1,6-anhydro-2,3-di-O-farnesyl-5-O-([{phosphonomethyl}phosphinyl]methyl)-α-D-galactofuranose: A zaragozic acid–presqualene pyrophosphate hybrid. *J Carbohyd Chem* **14:** 737–749, 1995.
26. Overhand M, Pieterman E, Cohen LH, Valentijn ARPM, van der Marel GA and van Boom JH, Synthesis of triphosphonate analogues of farnesyl pyrophosphate, inhibitors of squalene synthase and protein:farnesyl transferase. *Bioorg Med Chem Lett* **7:** 2435–2440, 1997.
27. Kinsella BT, Erdman RA and Maltese WA, Posttranslational modification of Ha-*ras* p21 by farnesyl versus geranylgeranyl isoprenoids is determined by the COOH-terminal amino acid. *Proc Natl Acad Sci* USA **88:** 8934–8938, 1991.
28. Biller SA, Sofia MJ, Delange B, Forster C, Gordon EM, Harrity T, Rich LC and Ciosek CP, The 1st potent inhibitor of squalene synthase-a profound contribution of an ether oxygen to inhibitor enzyme interaction. *J Am Chem Soc* **113:** 8522–8524, 1991.
29. Ciosek CP, Magnin DR, Harrity TW, Logan JVH, Dickson JK, Gordon EM, Hamilton KA, Jolibois KG, Kunselman LK, Lawrence RM, Mookhtiar KA, Rich LC, Slusarchyk DA, Sulsky RB and Biller SA, Lipophilic 1,1-bisphosphonates are potent squalene synthase inhibitors and orally active cholesterol-lowering agents *in vivo*. *J Biol Chem* **268:** 24832–24837, 1993.
30. Kolodyazhnyi OI, Yakovlev VI, Grishkun EV, Kovalenko AB and Kukhar VP, Carbon-phosphorous analogues of natural prenyl pyrophosphates. *Dopov Akad Nauk Ukr RSR Ser B: Geol Khim Biol Nauki*, 51–53, 1987 *Chem Abstr* **108:** 38140g, 1988.
31. Dickson JK, Biller SA, Magnin DR, Petrillo EW, Hillyer JW, Hsieh DC, Lan SJ, Rinehart JK, Gregg RE, Harrity TW, Jolibois KG, Kalinowski SS, Kunselman LK, Mookhtiar KA and Ciosek CP, Orally active squalene synthase inhibitors: Bis((acyloxy)alkyl)prodrugs of the alpha-phosphonosulfonic acid moiety. *J Med Chem* **39:** 661–664, 1996.