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DIFFERENT ANALOGUES OF FARNESYL PYROPHOSPHATE INHIBIT SQUALENE SYNTHASE AND PROTEIN:FARNESYLTRANSFERASE TO DIFFERENT EXTENTS

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Abstract—The inhibitory potency of farnesyl pyrophosphate analogues was investigated on two farnesyl pyrophosphate-consuming enzymes: squalene synthase, a secondary regulation site in the cholesterol synthesis pathway, and protein:farnesyl transferase, which plays a role in the function of Ras-proteins. For the transferase determination a rapid *in vitro* assay, using Sepharose-bound Ras-peptides, was developed. The distinct farnesyl pyrophosphate analogues showed a different order of potency in the inhibition of these two enzymes. Using the farnesyl transferase assay with pre-$p21^{Ha\text{-}ras}$ as substrate the same result was obtained. The difference observed in the *in vitro* assays was also reflected in the inhibition of cholesterol synthesis, protein prenylation in general and Ha-ras farnesylation in Rat-1.H-ras13 cells, a rat fibroblast cell line that overproduces human $p21^{Ha\text{-}ras}$. This work shows that farnesyl pyrophosphate analogues can be developed for specific inhibition of different processes such as cholesterol synthesis and protein prenylation.

Key words: farnesyl pyrophosphate analogues; cholesterol synthesis inhibitors; ras farnesylation; protein prenylation inhibitors; protein:farnesyltransferase assay

Inhibitors of cholesterol biosynthesis are currently in use as cholesterol lowering drugs. The vastatins, potent and specific inhibitors of the rate-limiting enzyme of this pathway, HMG-CoA¶ reductase (EC 1.1.1.34), are well-known examples [1]. However, it has been shown that these compounds can influence other processes which are dependent on the synthesis of isoprene intermediates of the cholesterol biosynthesis pathway as well. Of these, the post-translational modification of members of certain protein families by covalent linkage of a farnesyl or geranylgeranyl group to a cysteine residue by the enzymes protein:farnesyl transferase [2] and protein:geranylgeranyl transferase [3], respectively, addressed as protein (iso)prenylation, has attracted much attention [for reviews see Refs. 4–6]. For instance, the farnesylation of $p21^{ras}$ was shown to play a role in the interaction with its site of action in the plasma membrane [7] and possibly in protein–protein recognition [8]. Furthermore, prevention of farnesylation (by inhibiting HMG-CoA reductase) abolished the transforming potency of mutated $p21^{ras}$ [9]. It is preferable to develop inhibitors of cholesterol synthesis as cholesterol lowering drugs, which do not interfere with protein isoprenylation, as well as to develop specific inhibitors of the latter process [10, 11], without influencing cholesterol biosynthesis. Squalene synthase (EC 2.5.1.21) is thought to be a good target for such cholesterol synthesis inhibitors. Besides the very recent discovery of the squalestatins/zaragozic acids [12, 13], analogues of FPP, the substrate of this enzyme, have been developed as inhibitors of squalene synthase [14, 15]. However, FPP is also the substrate of protein:farnesyltransferase and indirectly, via the enzyme geranylgeranyl pyrophosphate synthase, of protein geranylgeranylation.

Indeed, a few FPP analogues have been shown to inhibit protein farnesylation [16, 17]. In this report we have looked for differences in the inhibitory potency of several FPP analogues in both pathways by studying their influence on squalene synthase and protein:farnesyltransferase activity *in vitro* and have shown that analogues of FPP that are different in their inhibitory action towards these processes can be developed.

MATERIALS AND METHODS

Synthesis of farnesyl pyrophosphate analogues. Six different farnesyl pyrophosphate analogues (FPPA 1–6; see Table 1 for chemical structures) were

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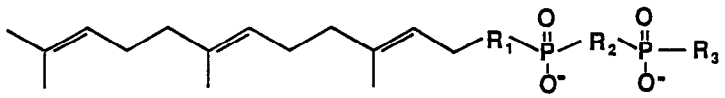
¶ Abbreviations: FPP, farnesyl pyrophosphate; FPPA, FPP analogue; DTT, dithiothreitol; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; DMEM, Dulbecco's modified Eagles medium.

Table 1. Chemical structure of the FFPAs



Compound	R_1	R_2	R_3
FPP	—O—	—O—	—O^-
FPPA 1	—	—O—	—O^-
FPPA 2	—	—O—	—CH_3
FPPA 3	—CH_2—	—O—	—O^-
FPPA 4	—CH_2—	—O—	—CH_3
FPPA 5	—OCH_2—	—CH_2—	—O^-
FPPA 6	—OCH_2—	—O—	—O^-

synthesized according to the procedures described by Kolodyazhnyi *et al.* for FPPA 1 [18], Valentijn *et al.* for FPPA 2–4 [19] and by Biller *et al.* for FPPA 5 [15]. FPPA 6 was synthesized according to a modification of the procedure of Valentijn *et al.* (to be published elsewhere).

Squalene synthase assay in rat liver microsomal preparations. Squalene synthase activity was determined in rat liver microsomal preparations according to a modification of a previously described procedure [20], which shortened assay time considerably. Although incubation conditions remained unchanged, the extraction and TLC separation of squalene was replaced by the following procedure: after the reaction was stopped by addition of 150 μL 5 M NaOH, [^{3}H]squalene (80,000 dpm) as a recovery standard, unlabelled squalene (5 μg) as carrier and subsequently 1 mL of chloroform/methanol (1:2) were added. Vigorous mixing resulted in a homogeneous organic/unorganic phase containing the lipids in a soluble form. Following centrifugation to remove precipitated proteins the supernatant was loaded onto a prepacked Amprep octadecyl C18 column (Amersham, U.K.). After the column was washed with 4 mL of methanol/20 mM NaOH (1:1), ^{3}H/^{14}C-labelled squalene was eluted with 2 mL of hexane into a scintillation vial and radioactivity determined in a Tricarb liquid scintillation counter (Packard). This column procedure had been developed in such a way that the radioactivity in the hexane eluate is only from squalene. The ^{14}C-cpm of the squalene formed was corrected for the recovery of [^{3}H]squalene, which was between 70 and 90%.

Assay of protein: farnesyltransferase activity using a C-terminal peptide of pre-p21$^{N\text{-}ras}$ coupled to sepharose beads as substrate. Two peptides, i.e. peptide A containing the carboxyl-terminal amino acid sequence of human pre-p21$^{N\text{-}ras}$

(NH_2-Aca-Met-Gly-Leu-Pro-Cys-Val-Val-Met-COOH)
|
S-*tert*-butyl

(Aca = ε-aminocaproic acid), and peptide B (control peptide; peptide A with Cys replaced by Ala) with sequence NH_2-Aca-Met-Gly-Leu-Pro-Ala-Val-Val-Met-COOH were coupled to CH-Sepharose 4B (Pharmacia, Uppsala, Sweden) through the sole amino group according to the manufacturer's instructions. The thiol of the cysteine residue of peptide A was protected by a *tert*-butylthio group, which was removed before use in the assay by reduction with DTT. The coupled peptides are designated pepAsep and pepBsep, respectively. Using pepAsep as substrate and pepBsep as control the assay of protein: farnesyltransferase activity was performed in the presence of various concentrations of FPPAs as indicated in Fig. 2A. Twenty-five microlitres of a mixture containing 5 μL of pepAsep or pepBsep (containing 80 pmol of peptides), 13 μL rabbit reticulocyte lysate (Promega) used as enzyme source [21], 0.5 mM $MgCl_2$, 1 mM DTT, 50 mM Tris–HCl (pH 7.4) and 0.7 μM [^{3}H]FPP (sp. radioact. 15 Ci/mmol; American Radiolabeled Chemicals, U.S.A.) were incubated at 37° for 30 min under continuous shaking. The reaction was terminated by addition of 1 mL of 2% SDS and the beads were washed three times with 2% SDS under shaking for 45 min at 50°. The radioactivity bound to the sepharose, as determined in a Packard Tricarb liquid scintillation counter, was strongly dependent on the presence of the cysteine residue in peptide A (compare values in the legend of Fig. 2A). For the calculation of protein: farnesyltransferase activity the ^{3}H-counts bound to pepBsep are subtracted from the counts bound to pepAsep.

Assay of protein: farnesyltransferase activity using pre-p21$^{Ha\text{-}ras}$ as substrate. Protein: farnesyltransferase assay was performed essentially according to Reiss *et al.* [22] with a slight modification: the 25 μL-incubation mixture contained 0.5 μg recombinant pre-p21$^{Ha\text{-}ras}$, 0.5 mM $MgCl_2$, 1 mM DTT, 50 mM Tris–HCl (pH 7.4), 0.7 μM [^{3}H]FPP (American Radiolabeled Chemicals), 13 μL rabbit reticulocyte lysate (Promega) and various concentrations of FPPAs as indicated in Fig. 2B and was incubated for 30 min at 37°.

Culture and determination of protein isoprenylation in Rat-1.H-ras13 cells. Rat-1.H-ras13 cells [23] were cultured in 10 cm^2 wells in DMEM, supplemented with 10% foetal calf serum. For determination of protein prenylation and the influence of FPPAs on that process the cells were preincubated in DMEM containing 1% human serum albumin, 2.5 μM of

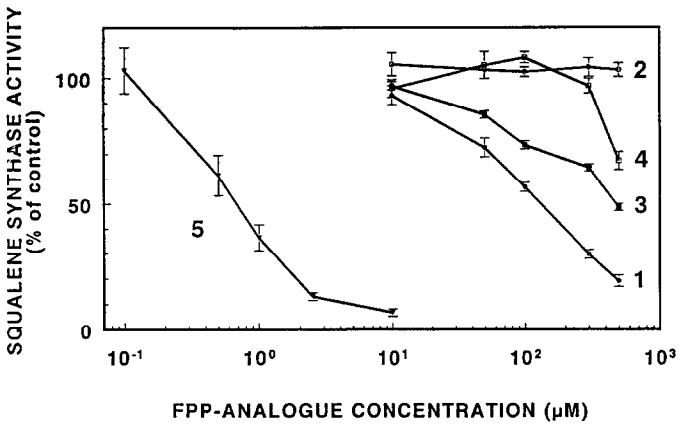


Fig. 1. Inhibition of squalene synthase by FFPAs. Squalene synthase activity was determined in rat liver microsomal preparations in the presence of indicated concentrations of FPPA 1 (●), FPPA 2 (○), FPPA 3 (■), FPPA 4 (□) and FPPA 5 (▼). The enzyme assay was performed in duplicate determinations in three separate experiments (bars indicate SEM) and the activity is expressed as percentage of control values, which were in the range of 1.7–2.9 nmol/min/mg of microsomal protein.

simvastatin, and 0–200 μM of FPPA 1 or FPPA 5 for 2 hr at 37° in a 5% CO_2 atmosphere. Incubation for 18 hr was started by addition of 20 μL (20 μCi) of [^{3}H]mevalonolactone (American Radiolabeled Chemicals, U.S.A.; sp. radioact. 50 Ci/mmol). After incubation the cells were lysed in 300 μL PBS supplemented with 1% Triton X-100, 0.5% sodium deoxycholate, and the protease inhibitors phenylmethylsulfonylfluoride (1 mM), leupeptin (50 μg/mL), pepstatin A (50 μg/mL) and trasylol 300 K.I.U./mL), collected and stored at −20°. For analysis of the ^{3}H-labelled polypeptides, the lysates were thawed, centrifuged (13,000 rpm for 15 min at 4°) and the polypeptides precipitated from the supernatant by adding acetone/NH_4OH (18:1). The precipitate was dissolved in 75 μL of SDS-sample buffer [2.5% SDS, 5% β-mercaptoethanol, 10 mM Tris (pH 8.0), 1 mM EDTA, 10% glycerol, 0.005% bromophenol blue] and electrophoresis was performed on a 14% polyacrylamide slab gel according to Laemmli [24]. Labelled bands were visualized by fluorography.

Immunodetection of p21^{ras}-farnesylation in Rat-1.H-ras13 cells. For assaying the influence of FPPAs on the farnesylation of p21$^{Ha\text{-}ras}$ in H-ras13 cells, p21$^{Ha\text{-}ras}$ was collected from the cell lysates using the monoclonal antibody Y13-259 coupled to protein-G-Sepharose (Pharmacia) as described by Osterop *et al.* [25]. The immuneprecipitated polypeptides were analysed by SDS–PAGE followed by fluorography.

Determination of cholesterol synthesis in Rat-1.H-ras13 cells. One day prior to measurement the culture medium was replaced by 1 mL of DMEM, supplemented with 10% of human lipoprotein deficient serum. Determination of cholesterol synthesis in H-ras13 cells in the absence or presence of 50 μM or 200 μM of FPPA 1 or FPPA 5 was performed according to a modification of a previously described method [26]. After the cells had been incubated for 2 hr with the same medium additionally containing the FPPAs, [^{14}C]acetate (Amersham; sp. radioact. 55 mCi/mmol) was added (0.4 μCi/well containing 1 mL of medium). The incubation was continued for 18 hr and the medium was then removed, the cells lysed in 300 μL of 0.2 M NaOH and subsequently neutralized with 30 μL 2 M HCl. Media and cell lysates were store at −20°. After thawing samples were taken for protein determination, lipids extracted from cell lysate and medium together, saponified, and cholesterol purified in TLC system I as described previously [26]. ^{14}C-radioactivity incorporated into cholesterol was counted in a Tri-carb liquid scintillation analyzer (Packard), corrected for the recovery of [^{3}H]-cholesterol and expressed as ^{14}C-dpm/mg of cellular protein. Values are the average of duplicate cell incubations. The data presented are expressed as percentages of the control values, as means ± SEM, obtained from three separately performed experiments.

RESULTS AND DISCUSSION

Inhibition of squalene synthase activity by farnesyl pyrophosphate analogues

Five FPP analogues shown in Table 1 (FPPA 1–5) were tested for inhibition of squalene synthase activity in rat liver microsomal preparations. The results as depicted in Fig. 1 are generally in agreement with earlier reports by Biller and co-workers [14, 15] and show that an ether-oxygen at R_1 and methylene group at R_2 in FPPA 5 strongly potentiated the inhibitory capacity as compared to the phosphonate analogue FPPA 3 (an increase of 3 orders of magnitude). FPPA 1, which is one carbon atom shorter than FPPA 3, is only approximately five times more potent. Replacement of the charged oxygen on the terminal phosphate with a methyl group (FPPA 2 and 4) strongly diminished inhibitory potential. Therefore, as reflected by the IC_{50} values (Table 2), the order of potency is: FPPA 5 ⩾ FPPA 1 > FPPA 3 > FPPA 4 ⩾ FPPA 2.

Table 2. IC_{50} values for inhibition of squalene synthase and *N*-ras-peptide: farnesyltransferase by FFPAs

FPPA	IC_{50} values (μM) (mean ± SEM; N = 3) Squalene synthase	*N*-ras-peptide farnesylation
1	129 ± 11	0.34 ± 0.05
2	NI*	8.9 ± 2.2
3	518 ± 12	0.93 ± 0.23
4	>500	>200
5	0.68 ± 0.14	7.15 ± 2.32

* NI, non-inhibitory.

In separately performed experiments IC_{50} values were calculated from inhibitor curves composed of at least five different FPPA concentrations (compare e.g. the concentrations indicated in Figs 1 and 2A). The 50% inhibition value was determined using a mathematical function derived by curve fitting. Mean values ± SEM obtained from three experiments have been given.

Inhibition of protein: farnesyltransferase activity by farnesyl pyrophosphate analogues

The most potent squalene synthase inhibitors—compounds 1, 3 and 5—were tested for their ability to inhibit protein: farnesyltransferase activity in rabbit reticulocyte lysates. The assay was performed using a sepharose-coupled peptide substrate as described in Materials and Methods in the presence of various concentrations of the FPPAs. As depicted in Fig. 2A all three compounds are inhibitors of the farnesylation reaction. However the order of potency (FPPA 1 > FPPA 3 > FPPA 5—IC_{50} values are given in Table 2), was different from that in the squalene synthase reaction. The relative diminished potency of FPPA 5 in this case may suggest an important role for the oxygen atom in position R_2, present in FPPA 1 and 3 but not in 5, in the inhibition of the enzyme. Therefore we tested FPPA 6, which was the same as FPPA 5 except that the CH_2-group, denoted as R_2 in Table 1, was replaced by an oxygen atom, in the peptide-farnesylation reaction. Indeed FPPA 6 was a stronger inhibitor (IC_{50} = 0.5 μM) than FPPA 5 and almost as strong as FPPA 1 (data not shown).

The results of experiments with FPPA 2 and 4, which were not or hardly active in inhibiting squalene synthase, further support the notion that the order of inhibitory potency can be different for both enzymes tested. By having one negative charged oxygen atom less than FPPA 1 and 3, respectively, FPPA 2 and FPPA 4 were weaker inhibitors of farnesyltransferase (Table 2). However, FPPA 2 was much more potent than FPPA 4 in inhibiting the transferase activity, with an IC_{50} comparable to that of FPPA 5 (Table 2).

In order to verify the results obtained using the sepharose-coupled peptides, the farnesylation reaction was also performed using a biological substrate, in this case pre-$p21^{Ha\text{-}ras}$, and the inhibitors FPPA 1, 3 and 5. As shown in Fig. 2B the order of inhibitory potency in this reaction was the same as that in the assay with the bead-bound peptide. This

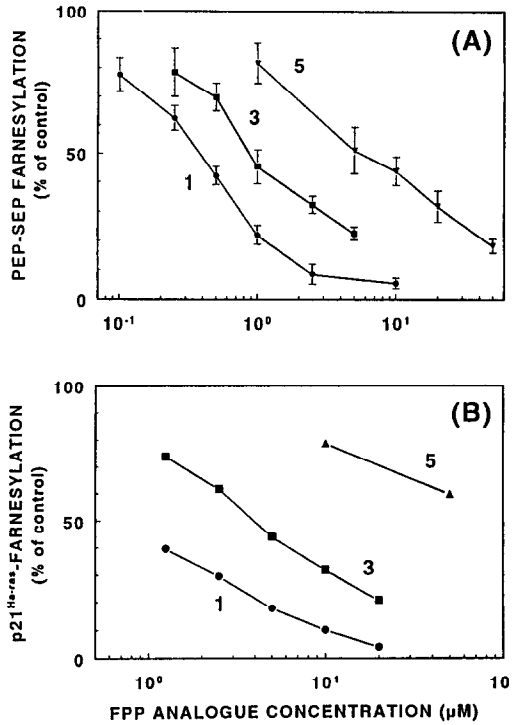


Fig. 2. Inhibition of protein: farnesyltransferase by FFPAs, using (A) sepharose-bound *N*-ras peptides or (B) pre-$p21^{Ha\text{-}ras}$ as substrate. (A) Peptide substrates, pepAsep and pepBsep, or (B) pre-$p21^{Ha\text{-}ras}$ were incubated with reticulocyte lysate in the presence of 0.7 μM of [^{3}H]-FPP and the indicated concentrations of the farnesyl pyrophosphate analogues FPPA 1 (●), 3 (■) and 5 (▼). As described in Materials and Methods peptide-farnesylation activity (A) was calculated as ^{3}H-dpm bound to pepAsep minus the pepBsep-bound dpm and expressed as percentage of control (control values pepAsep 12740 ± 2060 dpm, pepBsep 670 ± 290 dpm). The data shown are mean values ± SEM from three separately performed experiments. Using pre-$p21^{Ha\text{-}ras}$ as substrate (B) the ^{3}H-radioactivity bound to the protein precipitate is expressed as percentage of control (16,110 ± 880 dpm).

demonstrates that the latter assay will be useful for rapid screening of inhibitors of protein: farnesyltransferase.

Inhibition of protein-isoprenylation by FPPAs in Rat-1.H-ras13 cells in culture

In order to show that these compounds can also act in cultured cells, Rat-1.H-ras13 cells, which overexpress human $p21^{Ha\text{-}ras}$ [23], were incubated with 200 μM of either FPPA 1 or 5 in the presence of [^{3}H]mevalonate as source for the label in protein-isoprenyl groups and 2.5 μM of simvastatin to inhibit endogenous mevalonate production. After incubation, the labelled isoprenylated proteins were analysed by SDS–PAGE followed by autoradiography. The result is shown in Fig. 3A. Compared to control incubations (Fig. 3A, lanes 1, 2) FPPA 1 prevented the incorporation of [^{3}H]mevalonate into a number of proteins (Fig. 3A, lanes 3, 4), showing its inhibitory action in cultured cells as well. Since

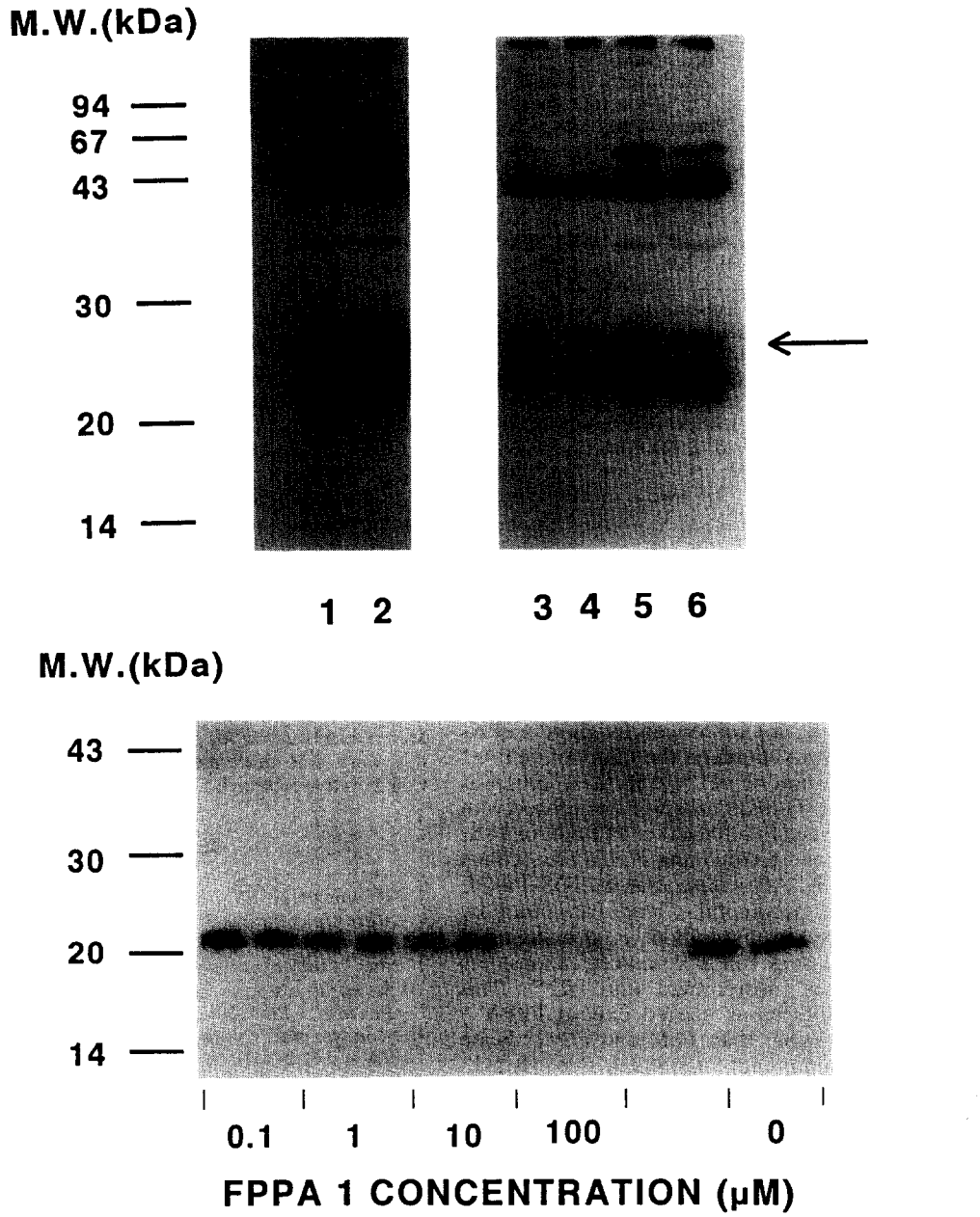


Fig. 3. Inhibition of protein prenylation in Rat-1.H-ras13 cells by FFPAs. (A) H-ras13 cells were incubated for 20 hr in the absence (lanes 1 and 2) or presence of 200 μM of FPPA 1 (lanes 3 and 4) or FPPA 5 (lanes 5 and 6). After the first 2 hr, 20 μCi of [^{3}H]mevalonate were added (see Materials and Methods for incubation conditions). ^{3}H-Labelled proteins were precipitated with acetone/ammonia, analysed by SDS–PAGE (14%) and visualized by fluorography. Molecular weight markers indicate the size of the polypeptides shown. The arrow indicates a 26-kDa band, which contained relatively less radioactivity in lanes 5 and 6. (B) H-ras13 cells were incubated with the indicated concentrations of FPPA 1 for 20 hr and with 20 μCi of [^{3}H]mevalonate for the last 18 hr. Cells were lysed and [^{3}H]p21$^{Ha\text{-}ras}$ was collected on sepharose-coupled antibody Y13-259. The precipitate was analysed by electrophoresis on a 14% polyacrylamide slab gel and the radiolabelled bands were visualized by fluorography.

the majority of mammalian prenylated proteins bear the geranylgeranyl group [27, 28], it seems likely that FPPA 1 also inhibits the geranylgeranylation process. On the other hand, FPPA 5 hardly inhibited protein isoprenylation, except that a 26-kDa band (arrow in Fig. 3A) seems to be reduced as compared to the control pattern. These results are in concordance with the differences in inhibitory

Table 3. Inhibition of cholesterol synthesis by FFPAs in Rat-1.H-ras13 cells

FPPA concentration (μM)	Cholesterol synthesis (% of control values) (mean ± SEM; N = 3)	
	FPPA 1	FPPA 5
—	100	100
50	92.4 ± 4.5	71.7 ± 17.4
200	93.0 ± 2.9	55.5 ± 10.2

H-ras13 cells were preincubated for 24 hr in DMEM/10% lipoprotein deficient serum and incubated with 50 or 200 μM of FPPA 1 or FPPA 5 for 20 hr. Two hours after the addition of the analogues, 0.4 μCi of [^{14}C]acetate/mL of culture medium was added and at the end of the incubation the incorporation of ^{14}C-counts into cholesterol was determined as described in Materials and Methods. The values (mean ± SEM; N = 3) are expressed as percentage of controls (238,840 ± 72,190 dpm/mg of cellular protein).

potency of the compounds in the *in vitro* protein farnesylation assays. Protein synthesis in these cells (measured as the incorporation of [^{35}S]methionine into protein) was not influenced by these FPPAs (results not shown). Further, the effect of FPPA 1 on the farnesylation of p21$^{Ha\text{-}ras}$ in these cells was also determined. H-ras13 cells were incubated with [^{3}H]mevalonate and 0.1–100 μM of FPPA 1 for 24 hr and p21^{ras} was collected on immobilized monoclonal antibody Y13-259. After separation by SDS–PAGE the ^{3}H-labelled polypeptides were visualized by autoradiography. In Fig. 3B it is demonstrated that with increasing FPPA 1 concentrations less radioactivity was incorporated into p21^{ras}. This decrease in farnesylation started only at FPPA 1 concentrations higher than 1 μM and may indicate that this compound is not easily taken up by H-ras13 cells.

The effect of FPPA 1 and FPPA 5 on cholesterol synthesis in H.ras13 cells was examined as well. As shown in Table 3, FPPA 1 has no significant inhibitory effect on [^{14}C]acetate incorporation into cholesterol. On the other hand FPPA 5 inhibited cholesterol synthesis to approximately 45% at the highest concentration used. This is in agreement with the *in vitro* potencies of both compounds to inhibit squalene synthase activity (Fig. 1, Table 2).

Our results show that FPPAs can have different relative potencies in their inhibitory action towards different FPP-consuming enzymes as we herewith have shown for squalene synthase and protein: farnesyltransferase. While the compounds described here are all negatively charged and therefore may have limited cellular uptake, modifications, such as the addition of masking groups which are removed within the cell, can be considered.

Recently it has been published that a very potent inhibitor of squalene synthase, zaragozic acid [13] is, to a lesser extent, also an inhibitor of protein: farnesyltransferase *in vitro*, although not in cell culture [29]. Further, α-hydroxyfarnesylphosphonic acid, another FPPA, has been reported to be a strong inhibitor of farnesyltransferase activity [17] as well as of squalene synthase [29]. The potency of yet another FPPA, [[(farnesylmethyl)-hydroxyphosphinyl]methyl] phosphonic acid, to inhibit protein: farnesyltransferase was much lower than that of α-hydroxyfarnesylphosphonic acid [17]. These and our observations show that FPPAs can be developed as specific and potent inhibitors for different processes, such as cholesterol synthesis and protein-farnesylation.

As shown in Fig. 3A, FPPA 1 and maybe more specifically FPPA 5 as well may influence protein geranylgeranylation. Several enzymes are involved in this process, such as geranylgeranyl pyrophosphate synthase [30], protein: geranylgeranyltransferase-I [31, 32] and protein: geranylgeranyltransferase-II [31, 33]. It is therefore possible that these and other FPPAs or analogues of geranylgeranyl pyrophosphate will possess different specificities in the inhibition of these enzymes. This is currently under investigation.

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