Synergy between Anions and Farnesyl Diposphate Competitive Inhibitors of Farnesyl:Protein Transferase*

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Investigation of the comparative activities of various inhibitors of farnesyl:protein transferase (FPTase) has led to the observation that the presence of phosphate or pyrophosphate ions in the assay buffer increases the potency of farnesyl diposphate (FPP) competitive inhibitors. In addition to exploring the phenomenon of phosphate synergy, we report here the effects of various other ions including sulfate, bicarbonate, and chloride on the inhibitory ability of three FPP competitive compounds: Cbz-His-Tyr-Ser(OBn)TrpNH₂ (2), Cbz-HisTyr-(OPO₄²⁻)-Ser(OBn)TrpNH₂ (3), and α-hydroxyfarnesyl phosphonic acid (4). Detailed kinetic analysis of FPTase inhibition revealed a high degree of synergy for compound 2 and each of these ions. Phosphorylation of 2 to give 3 completely eliminated any ionic synergistic effect. Moreover, these ions have an antagonistic effect on the inhibitory potency of compound 4. The anions in the absence of inhibitor exhibit non-competitive inhibition with respect to FPP. These results suggest that phosphate, pyrophosphate, bicarbonate, sulfate, and chloride ions may be binding at the active site of both free enzyme and product-bound enzyme with normal substrates. These bound complexes increase the potency of FPP competitive inhibitors and mimic an enzyme product-structure of the enzyme. None of the anions studied here proved to be synergistic with respect to inhibition of geranylgeranyl transferase I. These findings provide insight into the mechanism of action of FPP competitive inhibitors for FPTase and point to enzymatic differences between FPTase and geranylgeranyl transferase I that may facilitate the design of more potent and specific inhibitors for these therapeutically relevant target enzymes.

Since mutations rendering the Ras protein (p21) oncogene are prevalent in many human cancers (1) and farnesylation of the C-terminal region of the Ras protein is essential for activation of Ras function in vivo (2, 3), a potential therapeutic approach to tumor regression would be to inhibit the farnesyltransferase reaction. Farnesyl:protein transferase (FPTase) catalyzes the transfer of a 15-carbon group to several cellular proteins containing the requisite C-terminal CAAX recognition sequence. The specificity for transfer of farnesyl relies on the CAAX motif comprised of a cysteine followed by two aliphatic amino acids and ending mainly with a methionine or serine. FPTase isolated from rat brain is a 97-kDa αβ heterodimeric protein requiring both zinc and magnesium metal ions (4, 5). The reaction mechanism for this enzyme is shown in Fig. 1 where a thioether bond is formed upon transfer of the farnesyl moiety of farnesyl diposphate (FPP) to the thiol of the cysteine residue. The kinetic mechanism for the two-substrate reaction is functionally ordered where FPP binds first onto FPTase (6, 7).

Several classes of compounds have been identified as potent inhibitors of FPTase. These include peptide mimetic structures based on the tetrapeptide CVFM (5, 8–11), bisubstrate analog structures (12), and farnesyl pyrophosphate competitive compounds (13–15). These inhibitors show a wide range of specificity with respect to other prenylating enzymes including geranylgeranyl transferase. Geranylgeranyl transferase 1 transfers a 20-carbon geranylgeranyl group to proteins characterized by a CAAX motif, generally with a terminal leucine. The α subunit is identical for both rat FPTase and rat geranylgeranyl transferase I while the β subunit has 30% sequence identity (16).

Recently, the pentapeptide (1) was identified as a potent inhibitor of FPTase (17, 18). This compound was shown to be competitive with respect to FPP and was also demonstrated to be more potent in phosphate buffer than in a Hepes buffered system under otherwise identical conditions (19). In this study, the mechanism by which phosphate enhances inhibition of the tetrapeptide (2) was studied kinetically. We also wished to determine whether other anions (in particular product pyrophosphate) would cause this same enhancement, and whether other known FPP analog inhibitors share this anion requirement. Furthermore, we wanted to determine whether the phosphate enhancement of binding could be accomplished by covalently linking the phosphate to the inhibitor on an available tyrosine hydroxyl, and whether this abolished the enhancement by exogenous phosphate ion. Finally, we wanted to determine whether the phosphate enhancement of inhibitor binding was specific for FPTase compared with geranylgeranyl transferase I since distinguishing inhibition characteristics for these two similar enzymes may be critical in generating selective agents with distinctive cellular activities and further delineating the specificity by which these enzymes operate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tritiated farnesyl pyrophosphate and geranylgeranyl pyrophosphate were obtained from American Radiolabeled Chemicals (St. Louis, MO). Thr-Lys-Cys-Val-Ile-Met and biotin-Aha-Thr-Lys-Cys-Val-Ile-Met were synthesized according to solid phase peptide chemistry techniques (21, 22). α-Hydroxyfarnesyl phosphonic acid was synthesized as described (15). Potassium phosphate and sodium chloride were obtained from Sigma. Potassium bicarbonate was obtained from Mallinkrodt and Hepes buffer was obtained from Life Technologies, Inc. Geranylgeranyl transferase I was obtained from the Departments of ‡Biochemistry and §Chemistry, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105.

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§ The abbreviations used are: FPTase, farnesyl:protein transferase; FPP, farnesyl diposphate; DTT, dithiothreitol; HPLC, high performance liquid chromatography.
a gift from Dr. Michael Gelb, University of Washington, Seattle, WA. FPTase was expressed by baculovirus in SF9 cells and purified according to the following procedure adapted from Reiss et al. (4).

Purification of Farnesyl:Protein Transferase—SP9 cell pellets from 1.5 liters of suspension growth were resuspended in buffer containing 20 mM Tris chloride (pH 7.5), 50 mM NaCl, 20 mM ZnCl₂, and 1 mM DTT (Buffer A) and homogenized by French Press at 700 kpsi. Homogenates were centrifuged at 100,000 × g for 45 min and supernatants adjusted to 55% saturation with ammonium sulfate. Precipitated material was filtered (0.4 μm) and loaded onto a Q-Sepharose column (HiLoad 26/10) using a fast protein liquid chromatography system (Pharmacia Biotech Inc.). The column was washed with 220 mL of Buffer A followed by 60 mL of Buffer B (20 mM Tris chloride (pH 7.5), 150 mM NaCl, 20 μM ZnCl₂, and 1 mM DTT). The enzyme was eluted with a linear gradient (440 μL of buffer containing 150 mM to 1 mM NaCl at a flow rate of 4 mL/min. Fractions containing FPTase activity were pooled, brought to 10% glycerol, and stored at −80 °C for later affinity purification.

The affinity column for FPTase purification was prepared by mixing 12.8 mg of CNBr-activated CH-Sepharose with 26 mg of the peptide Thr-Lys-Cys-Val-Ile-Met in 43 ml of coupling buffer (100 mM NaHCO₃, 500 mM NaCl, pH 8.2) for 4 h with constant stirring at room temperature. The resin was then washed with 300 ml of Buffer C (50 mM Tris chloride, 100 mM NaCl, and 1 mM DTT) and loaded onto the column. The column was stored in 200 mM Tris chloride (pH 7.5), 0.02% sodium azide at 4 °C.

Prior to FPTase purification, the affinity column (2.5 × 12 cm) was washed with 300 ml of cold Buffer C. Thawed, active fast protein liquid chromatography fractions (80–100 mg) were then loaded onto the column and eluted by 3 times. The column was washed with 300 mL of Buffer D (50 mM Tris chloride, 100 mM NaCl, 1 mM DTT, and 0.2% PEG 8000) and the enzyme eluted with 300 ml of elution buffer (50 mM Tris succinate, 1 mM DTT, 500 mM NaCl, 0.2% PEG 8000, and 10% glycerol (pH 5)). The eluent was concentrated in an Amicon concentrator with a YM10 membrane to 5–10 ml, washed twice with 30 ml of Buffer E (50 mM Tris, 100 mM NaCl, 1 mM DTT, 0.2% PEG 8000, and 10% glycerol), and quick frozen in a dry ice-ethanol bath. The enzyme was a single band for each subunit by Coomassie Blue stain on a Novex 4–20% Tris glycline SDS-polyacrylamide electrophoresis gel.

Peptide Synthesis, Purification, and Characterization—The peptide analogs were synthesized by solid phase peptide synthetic methodologies (21, 22). The peptide analogs were prepared using an N-Fmoc protecting group strategy on a Rink-amide resin (4,2’,4’-dimethoxyphenoxy-Fmoc-aminoethyl-phenoxy resin) (23).

The N-Fmoc group was removed with 20% piperidine in N-methylpyrrolidone prior to coupling with the next protected amino acid. All amino acids were double coupled as their t-Boc derivatives, except for dimethylamino acid, which was coupled as its t-Boc derivative. The peptide was then deprotected and cleaved from the resin by treatment with 25–70% trifluoroacetic acid in methylene chloride, dependent on the side chain protecting groups, at room temperature for 2–3 h.

Crude peptides were then purified to homogeneity by preparative reversed-phase high performance liquid chromatography (RP-HPLC) eluting with linear gradients of 0% to 100% aqueous trifluoroacetic acid with increasing concentrations of 0.1% trifluoroacetic acid in acetonitrile (CH₃CN). Peptide fractions found homogeneous by analytical reversed-phase HPLC were combined, concentrated, and lyophilized. All the peptides were analyzed for homogeneity by analytical HPLC, and characterized by amino acid analysis, element analysis, fast atom bombardment or electrospray mass spectrometry, and proton nuclear magnetic resonance (¹H NMR) spectroscopy.

Phosphorylation of the tyrosine residue was carried out while the peptide was still linked to the resin using an excess of di-t-butyly-N,N-diethylphosphoramidite and tetrazole, followed by oxidation with 70% t-butyliodide in methylene chloride. The t-butyliodide groups were removed simultaneously under the cleavage conditions (60% trifluoroacetic acid in methylene chloride).

In Vitro FPTase Enzyme Assay—Enzyme activity was monitored using scintillation proximity assay technology from Amersham. Standard reactions were carried out in a 100-μL volume containing 50 mM Hepes (pH 7.4), 5 mM MgCl₂, 20 μM ZnCl₂, 1 mM DTT, 0.1% PEG 8000, 200 mM peptide (biotin-Aha-Thr-Lys-Cys-Val-Ile-Met), 134 nM tritiated farnesylphosphorylated, and 0.3–0.5 mM affinity purified farnesyl:protein transferase. Inhibitors were assayed at a final concentration of 5% dimethyl sulfoxide. When the effect of various ions were studied, buffered potassium phosphate, Na₂HPO₄, Na₂SO₄, KCl, or KHCO₃ were added to the assay buffer at the indicated concentrations. All reactions were initiated with the addition of enzyme followed by incubation at 37 °C for 30 min. The reactions were terminated with the addition of 150 μL of stop reagent (prepared by diluting 20 mg/mL scintillation proximity assay beads (resuspended in phosphate-buffered saline + 0.05% NaN₃) 1:10 with buffer containing 1.5 M magnesium acetate, 200 mM H₃PO₄, and 0.5% bovine serum albumin). Radioactive product was then counted on a Wallac Microbeta 1450 scintillation counter.

Data Analysis—Initial velocity data were obtained from the counts obtained from radiolabeled product using the scintillation proximity assay technology and analyzed using the kinetics software package KinetAsyst II (IntelleKinetics, Princeton, NJ) on a Macintosh computer. The data for the double inhibition experiments were fitted to Equation 1 where one inhibitor is noncompetitive for FPP, and the other inhibitor is competitive for FPP, and the inhibitors are not mutually exclusive (25).

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_c(1 + [I]/K_i + [I][X]/K_{ii} + [I][X][S]/K_{ii}[K_c] + [S][1 + [X]/aK_i) - I} \quad (\text{Eq. 1})
\]

In Equation 1, β is the interaction factor for the two inhibitors and α is the factor by which K_i changes when inhibitor (I) occupies the enzyme active site. Individual K_i constants were derived by nonlinear least squares fit to Equation 2 in the case of competitive kinetics or to Equation 3 in the case of noncompetitive kinetics.

\[
v = V_{\text{max}}[S]/K_{c_i}(1 + [I]/K_i) + [S] \quad (\text{Eq. 2})
\]

\[
v = V_{\text{max}}[S]/K_{c_i}(1 + [I]/K_i) + [S](1 + [I]/K_i) \quad (\text{Eq. 3})
\]

RESULTS

Peptide Inhibitors of Farnesyl:Protein Transferase Are Competitive with Respect to Farnesyl Diphosphate—We have previously seen an effect of phosphate anion on the inhibitory potency of the FPP competitive pentapeptide (1). Truncation of this compound yielded Cbz-His-Tyr-Ser(OBz)-Trp-NH₂ (compound 2, Fig. 2) which is equally potent and also competitive for FPP. As shown in Table 1, compound 2 has a K_i of 984 nm when assayed in Hepes buffer in the absence of phosphate. However, when 5 mM potassium phosphate is added to this buffer system, the inhibition becomes 53-fold more potent showing a K_i of 18 nm. Because of these large differences in inhibitory potency against the enzyme, we wished to analyze kinetically this phenomenon using compound 2 and several different anions.

Several Anions Inhibit FPTase and Are Synergistic with Cbz-
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Inhibitors of farnesyl:protein transferase by anions

Table I

| Compound | IC<sub>50</sub><sup>a</sup> | K<sub>i</sub> | IC<sub>50</sub><sup>b</sup> | K<sub>i</sub> |
|----------|-----------------|-------------|-----------------|-------------|
| 2        | 6.100 μM        | 984 ± 311 μM| 160             | 18.6 ± 2.3  |
| 3        | 6 μM            | 2.6 ± 0.6 μM| 3               | 3.6 ± 0.5   |
| 4        | 49 ± 10 μM      |             | 488 ± 32        |             |

<sup>a</sup> Hepes buffer system.

<sup>b</sup> Hepes plus 5 mM phosphate buffer system.

Fig. 2. Inhibitors of farnesyl:protein transferase.

His-Tyr-Ser(OBn)-Trp-NH₂ (Compound 2)—Table II shows the inhibition constants of several anions against FPTase. Each ion shows noncompetitive kinetics with respect to FPP and an inhibition constant in the mM range. Pyrophosphate is the most potent inhibitor shown with a K<sub>i</sub> of 1.4 mM due to the fact that pyrophosphate is also a product inhibitor (13). When inhibitor 2 is tested in the presence of these anions, a significant enhancement of inhibition is observed. By varying anion concentration and compound 2, a kinetic analysis can be made based on possible synergy of inhibition. The degree of synergy can be analyzed using Equation 1 in a system where a competitive inhibitor and a noncompetitive inhibitor may bind to the active site in combination favorable to binding and resulting in an enhancement of inhibition (25). A β value is derived that is a measure of cooperativity and can also be described as an interaction factor between the two molecules. If β is greater than 1, the binding of one inhibitor is hindering the binding of the other. If β is equal to 1, the binding of each species has no effect on the other, and if β is less than 1, then the binding of the two species are synergetic. In Fig. 3, a Dixon plot of compound 2 versus varying concentrations of phosphate anion show lines that intersect above the x axis indicative of a synergy of inhibition and gives a β value = 0.012. The β value actually represents the reciprocal of the maximal enhancement that can be observed, for the case of phosphate and compound 2, this increase is over 80-fold. The β values for several anions tested and their possible fold enhancement of inhibition with compound 2 are listed in Table III and all show synergy of inhibition.

Covalent Attachment of a Phosphate onto Compound 2 Disrupts the Synergism of Inhibition—Because of the large increase in inhibition by phosphate anion on compound 2, a synthetic strategy was devised to covalently link the phosphate group onto the tyrosine hydroxyl group of 2. This yielded a much more potent inhibitor (compound 3, Fig. 2) while retaining the characteristic of being competitive for FPP as shown in Fig. 4. In addition to being a low nanomolar inhibitor, this compound is unaffected by the presence of phosphate in the buffer. As shown in Table I, the K<sub>i</sub> values of this compound are 2.6 and 3.6 nM in a Hepes buffer system with and without added phosphate. Comparing compound 2 and 3 where the only difference is the added phosphate group, their K<sub>i</sub> values in a Hepes buffer system are 984 and 2.6 nM, respectively. However, in the presence of 5 mM phosphate, the K<sub>i</sub> for compound 2 is 53-fold lower while the K<sub>i</sub> for compound 3 remains relatively unchanged.

Inhibition of Farnesyl:Protein Transferase by (α-Hydroxyfarnesyl)phosphonic acid (4)—(α-Hydroxyfarnesyl)phosphonic acid (4) is shown in Fig. 2 and is competitive with respect to FPP exhibiting a K<sub>i</sub> = 49 mM in a Hepes buffer system. This value is consistent with that reported in the literature (13). However, when 5 mM phosphate is added to the assay buffer, the K<sub>i</sub> of this compound increases 10-fold to 488 mM (see Table I) yet remains competitive with respect to FPP. This result seems to imply that there is now mutual competition between compound 4 and phosphate anion.

Inhibition of Geranylgeranyl Transferase I—Compound 2 was also assayed against geranylgeranyl transferase I in the presence of the anions shown in this study. However, in this case, there was no increase in inhibition observed when any of the anions were included in the assay buffer.

**DISCUSSION**

As the ongoing search for potent inhibitors of FPTase continues, a better understanding of the enzyme mechanism of FPTase is necessary to provide insight into the design and synthesis of potent FPTase inhibitors. Employing a phosphate buffer system, we identified several compounds as very potent inhibitors of the farnesylation reaction. Subsequent examination of their inhibitory potency in a nonphosphate buffering system revealed significantly lower enzyme inhibition in vitro. Further investigation showed that the phosphate anion was...
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Inhibitor synergy of compound 2 with various anions

| Anion   | β      | Fold increase in inhibition (1/β) |
|---------|--------|----------------------------------|
| HPO₄²⁻  | 0.012  | 83                               |
| H₂PO₄⁻  | 0.92   | 1.2                              |
| HSO₄⁻   | 0.31   | 3.2                              |
| HCO₃⁻   | 0.043  | 23                               |
| Cl⁻     | 0.007  | 146                              |

Kinetic Analysis of Synergy of Inhibition of FPTase—In a Heps buffer system compound 2 is competitive with respect to FPP having a $K_i$ of 984 nM. In the same buffer system plus 5 mM potassium phosphate, compound 2 is still competitive with respect to FPP but the $K_i$ is reduced 53-fold to 18 nM. This effect could be indicative of synergy of inhibition between two molecules operating together at the active site. Similar inhibitory effects have been observed with phosphoenolpyruvate mutase (26) and with phosphoenolpyruvate carboxylase from Zea mays (27). Phosphoenolpyruvate carboxylase proceeds through a random sequential mechanism where a high level of synergy of binding of substrates is observed. Very high levels of synergistic inhibition was found between oxalate and carboxylyl phosphate ($β = 0.0013$). In the case of phosphoenolpyruvate mutase, synergy of inhibition between oxalate and anions that alone were noncompetitive inhibitors suggested a “combined presence” in the enzyme active site as a bimolecular transition state analog. In both of these cases, the inhibitor oxalate was a very close mimic of the original substrate phosphoenolpyruvate. In our case, the peptidic inhibitors bear little resemblance to the original substrates.

Using affinity purified FPTase from rat brain, phosphate was kinetically analyzed as an inhibitor. We found noncompetitive inhibition with respect to FPP in the high millimolar concentration range. In cases where one inhibitor is noncompetitive and the other is competitive, the amount of synergism between two compounds can be measured using a Dixon plot to arrive at an interaction $β$ value. $β$ is a measure of the amount of synergistic cooperativity between the two inhibitors. A Dixon plot is shown in Fig. 3 for the case of compound 2 and phosphate anion. Here, $β$ is 0.012 showing that an increase of over 80-fold (1/β) in inhibition is possible.

Because an improvement of inhibition with phosphate anion was observed with a tetrapeptide based molecule, other anions were used to test whether synergism would also be found. The anions sulfate, carbonate, chloride, and pyrophosphate were found to retain a synergistic effect with compound 2, whereas nitrate and acetate showed little or no effect. When the anions were tested for direct inhibition of FPTase, all showed noncompetitive inhibition with respect to farnesyl diphosphate. This kinetic pattern is consistent with these anions binding to two different forms of the enzyme. One logical binding site for phosphate, for example, could be at the portion of the active site of FPTase that actively binds the pyrophosphate group of farnesyl diphosphate known as the pyrophosphate binding pocket.

Evidence for Synergy of Inhibition at the Active Site of FPTase—There are four pieces of supportive evidence that the anion contributing to synergy of inhibition is acting at the enzyme active site. First, noncompetitive kinetics for phosphate was observed with respect to FPP against farnesyl:protein transferase. Second, based on the effect of phosphate anion on inhibition of FPTase, a second inhibitor, compound 3, was synthesized which contained a covalently linked phosphate group. For this compound, the effect of phosphate anion to enhance inhibition was no longer found. The inhibition of compound 3 itself against FPTase was 3 nM making this compound one of the most potent inhibitors reported to date against FPTase (see Fig. 4). Third, when α-hydroxyfarnesyl phosphate was studied, there appeared to be a direct competition for this binding pocket and antisynergy was observed. The $K_i$ in Heps buffer was 10-fold lower than that observed in the presence of 5 mM phosphate. Finally, when inhibitors that are competitive with respect to the peptide substrate were studied (CVF for example), no phosphate effect was observed.

Kinetic analysis reveals that the anions are noncompetitive with respect to FPP. Therefore, the phosphate must bind to two different forms of the enzyme. Phosphate can bind to the free form of the enzyme because the pyrophosphate binding pocket is still unoccupied. In considering other forms of the enzyme to which phosphate could bind, the E-FPP form of the enzyme where the pyrophosphate pocket is already occupied seems unlikely. Furthermore, once the bulky peptide substrate binds at the active site, catalysis happens extremely fast and product release is thought to be rate-limiting (7). If there is an ordered mechanism of product release, the pyrophosphate would leave prior to...
Fig. 5. Possible mechanism of action for farnesyl:protein transferase.

the farnesylated peptide providing a second form of the enzyme which would then be accessible to phosphate binding at the putative pyrophosphate binding pocket of the active site. Fig. 5 shows how phosphate anion could exhibit noncompetitive kinetics of inhibition with respect to FPP and bind to two different forms of the enzyme. In this mechanistic scheme, phosphate binds to the free form of the enzyme (I) because the pyrophosphate pocket is still unoccupied. In addition, after release of pyrophosphate from the E:farnesylated peptide:PP form of the enzyme, there would exist an E:farnesylated peptide form of the enzyme (V) that would have a pyrophosphate binding pocket now accessible to phosphate anion. If the inhibitor 2 was acting like a farnesylated product (F-CAAX), then phosphate could bind to the enzyme:inhibitor form of the enzyme. The consequence would presumably be a closely mimicked step of the enzyme catalytic mechanism. Since product release is rate-limiting, the E>F-CAAX species would be considered a kinetically long lasting species. This may account for the synergy of inhibition observed.

There are several examples in the literature of such synergism like phosphoenolpyruvate mutase (26) where many anions were used as a close substrate analog and synergy of inhibition was observed. In addition, synergy of inhibition with anions and a substrate analog was observed for phosphoenolpyruvate carboxylase (27). In both cases, the data could be analyzed in terms of a transition state analog. But in this case, the inhibitor is not a structural analog of either substrate in the enzyme catalyzed reaction. Nevertheless, anions have a dramatic impact on the degree of inhibition with certain FPP competitive compounds and may be used to design more effective inhibitors against the enzyme possibly as transition state analogs.

Comparison of Synergy of Inhibition for FPTase and Geranylgeranyl Transferase—Since FPTase and geranylgeranyl transferase I share a common subunit and undergo very similar reactions (the length of the prenyl chain differing by five carbon units) it was of interest to examine if there still existed the same synergy of inhibition. The IC50 for compound 2 against geranylgeranyl transferase I is 12 μM and no effect of phosphate or other anions was found. The lack of an anion effect may point out differences between these two enzymes that could be used to increase specificity of inhibition for each respective protein. Since the two enzymes share an identical α subunit, the differences in synergy of inhibition may lie elsewhere. One possibility may be that kinetically, geranylgeranyl transferase I does not release PPi in an ordered fashion leaving no room for any anions to combine with a partial product form of an inhibitor. It would also be plausible that the specific inhibitor 2 does not mimic a product complex for geranylgeranyl transferase I.

It is interesting that phosphate has such an effect on FPTase-I as opposed to geranylgeranyl transferase I. The amount of phosphate found in vivo at the cellular level is thought to be in the low millimolar range (28–30). This anion would then be present in high enough concentrations to see an observable effect in cellular assays and could be used advantageously to increase the specificity of inhibition between the two prenyl transferases. Finally, enzymatic differences between these two catalytic enzymes may become more apparent based on the phosphate synergy phenomenon.

Conclusion—We have shown that in the presence of various anions there is a large increase in potency of inhibition for compound (2). This effect has been analyzed kinetically to reveal a synergy of inhibition in the case of FPTase that does not exist for geranylgeranyl transferase I. Furthermore, an inhibitor (3) has been designed to use this effect to increase potency against FPTase.

REFERENCES

1. Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827
2. Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., and Der, C. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6403–6407
3. Meadey, P., Kuroda, S., Tomizawa, K., Takai, Y., and Gelb, M. H. (1995) J. Biol. Chem. 270, 26347–26351
4. Reiss, Y., Goldstein, J. L., Seabara, M. C., Casey, P. J., and Brown, M. S. (1999) Cell 82, 81–88
5. Reiss, Y., Brown, M. S. and Goldstein, J. L. (1999) J. Biol. Chem. 274, 6403–6408
6. Pompian, D. L., Schaber, M. D., Mosser, S. D., Omer, C. A., Shafer, J. A., and Gibbs, J. B. (1993) Biochemistry 32, 8541–8547
7. Furfine, E. S., Leban, J. J., Landavazo, A., Mooshaw, J. F., and Casey, P. J. (1995) Biochemistry 34, 6857–6862
8. Leftheris, K., Kline, T., Natarajan, S., DeVirgilio, M. K., Cho, Y. H., Placese, J., Ricca, C., Robinson, S., Seizinger, B. R., Manne, V., and Meyers, C. A. (1994) Bioorg. & Med. Chem. Lett. 4, 887–892
9. Wai, J. S., Bamberger, D. L., Fisher, T. E., Graham, S. L., Smith, R. L., Gibbs, J. B., Mosser, S. D., Oliff, A. I., Pompian, D. L., Rands, E., and Kohl, N. E. (1994) Bioorg. & Med. Chem. 2, 939–947
10. Garcia, A. M., Rowell, C., Ackermann, K., Kowalczyk, J., and Lewis, M. D. (1993) J. Biol. Chem. 268, 18414–18418
11. Vogt, A., Qian, Y., Blaskovich, M. A., Fassum, J. D., Hamilton, A. D., and Sebti, S. M. (1995) J. Biol. Chem. 270, 660–664
12. Patel, D. V., Gordon, E. M., Schmidt, R. J., Weller, H. N., Young, M. G., Zahler, R., Barbacid, M., Carboni, J. M., Gullo-Brown, J. L., Hunihan, L., Ricca, C., Robinson, S., Seizinger, B. R., and Tsunumi, A. Y. (1995) J. Med. Chem. 38, 435–442
13. Pompian, D. L., Rands, E., Schaber, M. D., Mosser, N. J., Anthony, N. J., and Gibbs, J. B. (1992) Biochemistry 31, 3890–3897
14. Singh, S. P., Zink, D. L., LeClerc, J. M., Goetz, M. A., Jenkins, R. G., Nallin-Omstead, M., Silverman, K. C., Bills, G. F., Mosley, R. T., Gibbs, J. B., Albers-Schonberg, G., and Lingham, R. B. (1993) Tetrahedron 49, 5917–5926
15. Dolence, J. M., and Poulter, C. D. (1995) Proc. Natl. Acad. Sci. U. S. A 92, 5008–5011
16. Armstrong, S. A., Seabara, M. G., Sudhoff, T. C., Golsteyn, J. L., and Brown, M. S. (1995) J. Biol. Chem. 268, 12221–12229
17. Sebolt-Leopold, J., Gowen, R., Su, T., and Leonard, D. (1994) 85th Annual Meeting of AACR, Toronto, Canada, April 10, 1994
18. Leonard, D., Eaton, S., Sawyer, T., and Bolton, G. (1994) 107th ACS National Meeting, San Diego, CA, March 13, 1994
19. Scholten, J. D., Zimmerman, K., Oxender, G. M., Sebol-Leopold, J., Gowen, R., Leonard, D., and Hupe, D. J. (1996) Bioorganic and Medicinal Chemistry 4, 1537–1543
20. Leonard, D. M., Shuler, K. R., Poulter, C. J., Eaton, S. R., Sawyer, T. K., Hodges, J. C., Scholten, J. D., Gowen, R. C., Scholten, J. F., and Doherty, A. M. (1997) J. Med. Chem. 40, 192–200
21. Stewart, J. M., and Young, J. D. (1984) Solid Phase Peptide Synthesis, Pierce Chemical Co.
22. Bodansky, M., and Bodansky, A. (eds) (1984) in The Practice of Peptide Synthesis, Springer-Verlag, Berlin, Heidelberg
23. Rink, H. (1987) Tetrahedron Lett. 28, 3777–3780
24. Kaiser, E., Colecott, R. S., Bussing, C. D., and Cook, P. (1970) Anal. Biochem. 34, 595–598
25. Sepal, I. (1975) Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, pp. 493–495 John Wiley & Sons, Inc., New York
26. Seidel, H. M., and Knowles, J. R. (1994) Biochemistry 33, 5641–5646
27. James W. J., O'Leary M. H., and Cleland, W. W. (1992) Biochemistry 31, 6421–6426
28. Amatrudra, T. T., Hollingsworth, D. R., D'Esepo, N. D., Upton, G. V., and Bondy, P. K. (1996) J. Clin. Endocrinol. Metab. 239, 339–379
29. Buchli, R., Martin, E., Boesiger, P., and Bunzel, V. (1994) Pediatr. Res. 35, 431–435
30. Siess, E. A., Kienitz-Engel, R. I., Gahimi, F. M., and Wieland, O. H. (1984) Eur. J. Biochem. 141, 543–548