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Inhibitors of Farnesyl:Protein Transferase—A Possible Cancer Chemotherapeutic

Jeffrey D. Scholten,[a,*] Karen Zimmerman,[a] Maritza Oxender,[a] Judith Sebolt-Leopold,[a] Richard Gowan,[a] Daniele Leonard[b] and Donald J. Hupe[a]

Departments of [a]Biochemistry and [b]Chemistry, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, MI 48105, U.S.A.

Abstract—The recent interest in inhibitors of farnesyl:protein transferase (FPTase) has resulted in a better understanding of the enzymology of this protein. Rationally designed inhibitors of prenyl transfer have emerged as potential new drug candidates because of the insight gained over how a prenyl group is enzymatically transferred onto a peptide thiol. This paper will explore how advances in our understanding of FPTase mediated catalysis has affected the design of FPTase inhibitors as possible cancer therapeutic agents. Without structural information of the enzyme, substrate analogues comprise the first area of drug design: these include peptidomimetics of the four *C*-terminal amino acids of rasP21 as well as farnesyl diphosphate analogs. In addition, phosphate anion was found to enhance the inhibitory potency of certain compounds known to be competitive with respect to farnesyl diphosphate and therefore incorporation of the phosphate anion may also provide a basis for improved inhibitor design.

Introduction

Post-translational modifications such as farnesylation, geranylgeranylation, and palmitoylation play critical roles in controlling activity of several proteins important in cellular signaling. Many cellular proteins require post-translational modification for biological activity. Prenylation is one such modification that is needed for the functional activity of a wide variety of proteins. Such proteins include: p21ras, rho, rab, and arf proteins.[1] The ras protein is a GTP binding protein that functions as a key molecular switch acting upstream of a cascade of signal transduction pathways responsible for cell proliferation. Mutations of p21ras that compromise the function of ras and the GTPase function of ras have cell transforming activity and several mutations have been found at a high frequency in many pancreatic and colon tumors.[2] Farnesylation of p21ras stimulates kinase activity by either acting to anchor the protein to the cell membrane[3] or in a direct but nonspecific manner;[4] therefore, numerous pharmaceutical companies have targeted the prenylation enzymatic step as a way to inhibit growth of tumors expressing the p21 oncogene.

This article will review the many approaches that have been taken to design inhibitors of farnesyl:protein transferase (FPTase) highlighting how knowledge of enzyme mechanism and substrate specificity has contributed to the design of potentially therapeutic compounds. Without prior knowledge of the three-dimensional structure of the target protein, several methods for designing FPTase inhibitors were employed. After outlining these methods, we will then review our current understanding of enzyme mechanism. This will be followed by a description of the discovery and use of peptide competitive inhibitors, farnesyl diphosphate (FPP) competitive inhibitors, and transition state analogue inhibitors. Finally, we will focus on the anionic synergism of inhibition observed for certain FPP competitive inhibitors focusing on how this effect could be exploited to design more potent inhibitors for farnesyl:protein transferase.

Key words: farnesyl transferase, inhibitors, phosphate synergism, and drug design.

Background on the Enzymology of FPTase

Enzyme mechanism

FPTase is a 97 kDa $\alpha\beta$ dimeric protein that catalyses the post-translational attack of a cysteine thiol from the *C*-terminal region of various proteins to the substrate farnesyl diphosphate at the carbon α to a pyrophosphate leaving group (see Fig. 1). The products are pyrophosphate and a 15-carbon lipophilic tail covalently attached to the protein that, after further modification, acts to stimulate the signal transduction pathway. FPTase will accept several carboxyl terminal protein sequences as substrates in which a cysteine is followed by two aliphatic amino acids and another amino acid, usually methionine or serine. This has become known as the CaaX motif. In this two substrate reaction, the kinetic mechanism for human and bovine protein farnesyl transferase was determined by steady state initial velocity and dead-end inhibitor studies and found to be formally random but functionally ordered sequential (see Scheme 1).[5]

On the basis of single turnover kinetics, fluorescent binding studies show a clear preferential binding of

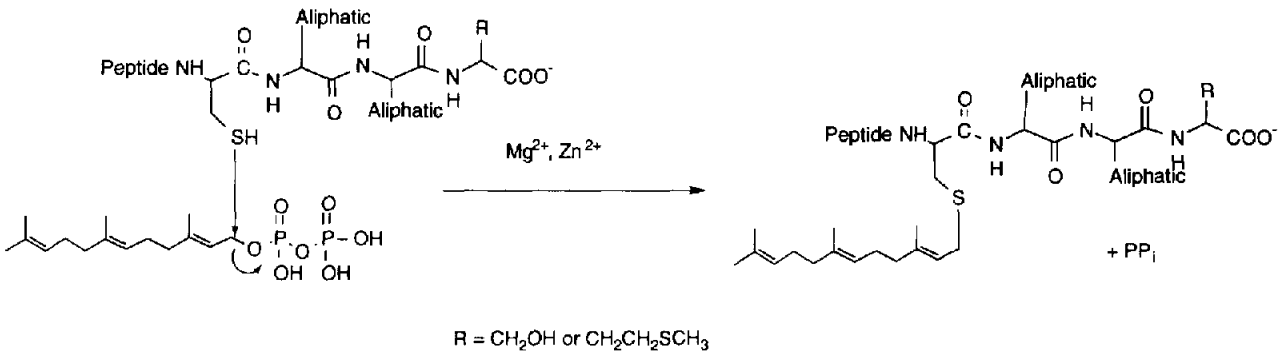


Figure 1. Reaction catalysed by FPTase.

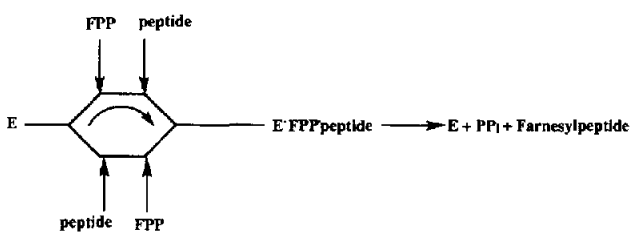
FPP onto the enzyme as the first step.[6] Farnesyl diphosphate is known to be bound to FPTase very tightly (K_d = 15 nM).[6] This tight binding of farnesyl substrate for FPTase may eventually be shown to play a role in substrate specificity between FPTase and geranylgeranyl:protein transferase in vivo.

Role of the metals in catalysis

Both zinc and magnesium have been shown to be essential for the enzyme catalysed farnesylation reaction.[7,8] The exact role of zinc remains unknown except for its requirement in the protein substrate binding step.[7] Several reasonable possibilities for the necessity of the zinc ion are (a) catalytic: the zinc directly liganded to the cysteine thiol to facilitate thioether formation producing inorganic pyrophosphate, (b) structural: playing a role in the stabilization of the αβ subunits, (c) substrate binding: coordinated with the free carboxyl of the peptide substrate, and (d) substrate binding: coordinated with the pyrophosphate group. The role of magnesium is also unknown, except that it is absolutely essential for transfer of the bound farnesyl group to the peptide thiol.[7] The magnesium ion may be needed to bind/activate the pyrophosphate group of FPP.

FPTase inhibitors overview

In the past several years an extensive array of compounds have been produced using rational drug design that have proven to be potent FPTase inhibitors, some with reported in vivo efficacy. Table 1 gives a summary of some of the more potent inhibitors reported to date. The key compounds highlighted here have provided important insight into the enzyme mechanism. Early compounds contained limitations which made them poor drug candidates.



Scheme 1.

Peptide mimetics of the Caax motif

The most successful compounds for inhibiting FPTase have been peptide mimetic inhibitors derived from the Cys-aliphatic-aliphatic-Met/Ser (CaaX) C-terminal region of the natural farnesylated proteins. Many potent inhibitors have originated from the unfarnesylated tetrapeptide CVFM (**1**), which is itself a 57 nM inhibitor of rat FPTase.[8] Leftheris and colleagues[9] have published an extensive structure–activity relationship around the CaaX motif peptides, as have Wai et al.[10] Scientists at Eisai have reported on two potent FPTase inhibitors, B515 and B581 (**2**), with IC_{50}'s in the 30 nM range.[11] The pseudopeptide L-731,735 (**3**) is very potent against human FPTase with an IC_{50} of 18 nM and the prodrug L-744,832 (**4**) has shown remarkable in vivo activity where tumor regression was observed in MMTV-v-Ha-ras mice.[12] The drawback of the CaaX inhibitors is their inherent peptidic character, which make them poor pharmacological agents.[13] Thus, one strategy taken to improve pharmacological properties

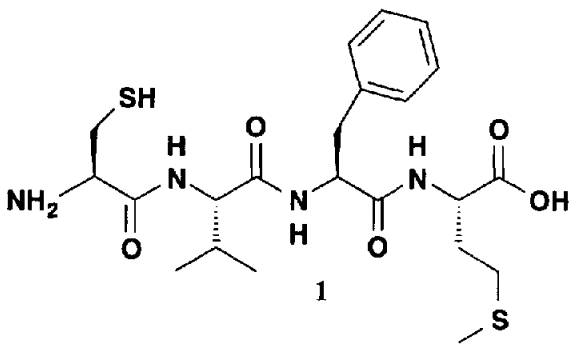
Table 1. FPTase inhibitors

Compd	FPTase inhibition (nM)	FPTase source
1	57	Bovine
2	21	Bovine
3	1.8	Human recombinant
4	240	Human recombinant
5	1800	Rat
6	0.3–1	Rat
7	150	Human Burkitt cells
8	6	Porcine
9	5[a]	Bovine
10	830[a]	Bovine
11	55	Recombinant human
12a	140[a]	Yeast
12b	36[a]	
12c	11[a]	
13a	1000	Rat
13b	300	
13c	6	
14	600	Rat

[a] K_i determination.

has been to establish potency in vitro with peptidic compounds and then to modify the inhibitor where a few modifications could make them viable as drug candidates. For example, as seen with compound **3** or **4**, the amide bonds of the peptide backbone have been replaced with a secondary amine decreasing possible proteolysis or cellular metabolism instability.

The peptide mimetic inhibitors have revealed a great deal about the enzyme's mode of action. For example, we have observed uncompetitive patterns of inhibition with respect to FPP for the peptide mimetic compound



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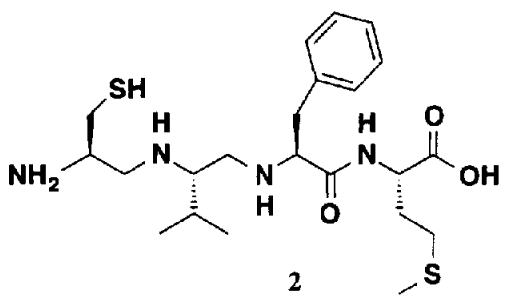

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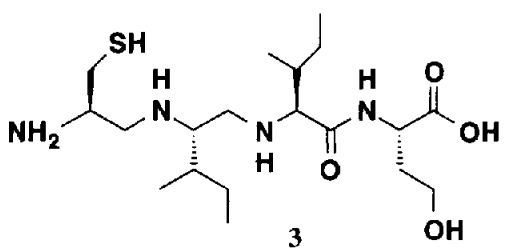

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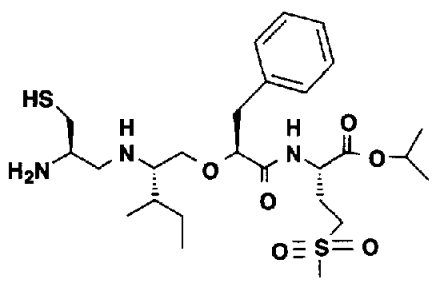

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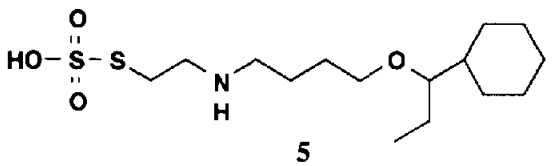

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B581 (**2**) and with PD 061938 (**5**), indicating that inhibitor binds preferentially to E:FPP instead of E.

The inhibitor B581 is competitive for the peptide *C*-terminal substrate yet is uncompetitive for FPP (see Fig. 2). In addition, PD 061938, which was found by random screening, also shows this uncompetitive pattern against FPP (see Fig. 3). These inhibition patterns imply FPP is bound first to the enzyme followed by peptide substrate.

Key inhibitors have also been used to elucidate the nature of zinc binding to FPTase. Specifically, zinc has been proposed to be bidentate with the peptide substrate CVFM (**1**).[14] trNOE NMR experiments have

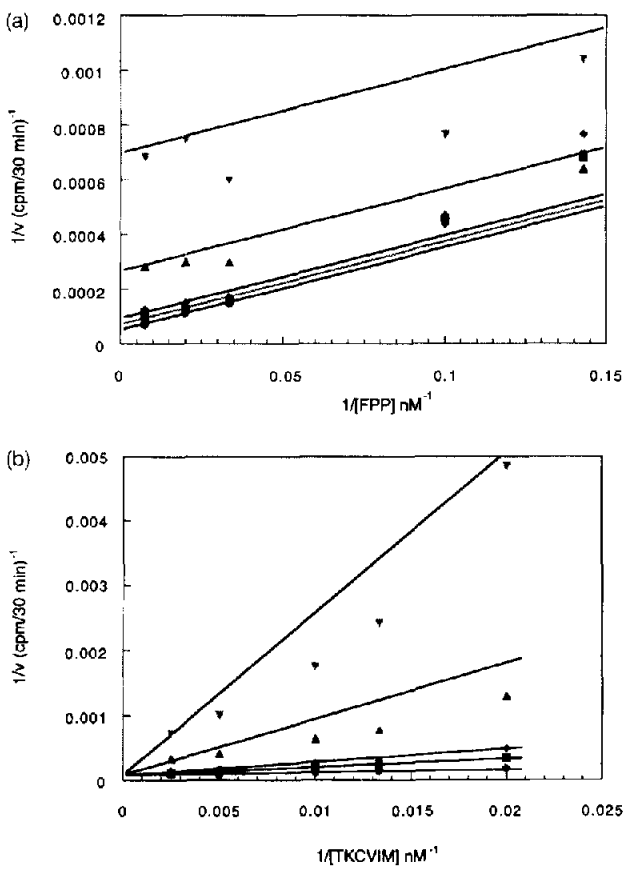


Figure 2. (**a**) Double-reciprocal plot of initial velocity versus varying farnesyl diphosphate concentration. Concentrations of inhibitor, B581, are as follows: (▼) [I] = 1.5 μM; (▲) [I] = 500 nM; (◆) [I] = 100 nM; (■) [I] = 50 nM; (●) [I] = 0 nM. $K_m = 63 \pm 7$ nM; $V_{max} = 20{,}860 \pm 1240$ cpm/30 min; $K_i = 110 \pm 12$ nM. (**b**) Double-reciprocal plot of initial velocity versus varying TKCVIM concentration. Concentrations of inhibitor, B581, are as follows: (▼) [I] = 1.5 μM; (▲) [I] = 500 nM; (◆) [I] = 100 nM; (■) [I] = 50 nM; (●) [I] = 0 nM. $K_m = 56 + 8$ nM; $V_{max} = 13{,}740 \pm 600$ cpm/30 min; $K_i = 25 \pm 3$ nM.

suggested that the cysteine thiol of the substrate acts as a direct ligand to the zinc metal and that in the peptide backbone of CVFM there is an indication of a β-turn.[14] Subsequently, inhibitor modifications were made to take advantage of a proposed β-turn in the peptide structure.[15] Compound **6** was found to be a potent inhibitor for FPTase with an IC_{50} between 0.3 and 1 nM. The idea that a β-turn being necessary for coordinate binding and increased potency against FPTase has also been looked at with the tetrapeptide CVWM. In this case, 2-DtrNMR experiments have also revealed a peptide backbone in a nonideal reverse turn conformation.[16] On the other hand, an unconventional β-turn in the peptide inhibitor was not observed by the inhibitor design and computer modeling experiments of Hamilton et al., who evaluated the conformationally constrained peptidomimetic compound **7**.[17] Compound **7** is not capable of forming the necessary spatial orientation to form a bidentate ligand with the putative zinc metal nor can it be in a nonideal β-turn conformation, yet it is a potent inhibitor with an IC_{50} of 150 nM. It remains to be seen, however, whether binding to the peptide active site is the same for compounds **6** and **7**.

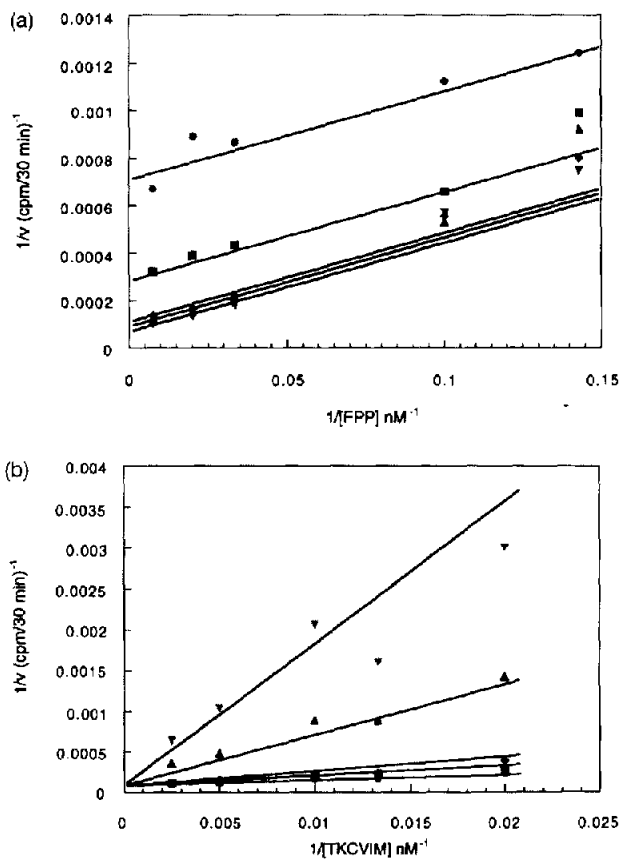
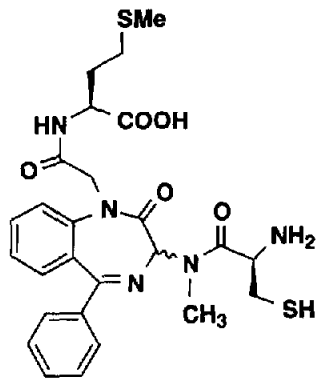


Figure 3. (**a**) Double-reciprocal plot of initial velocity versus varying farnesyl diphosphate concentration. Concentrations of inhibitor, PD 061938, are as follows: (●) [I] = 15 μM; (■) [I] = 5 μM; (♦) [I] = 1 μM; (▲) [I] = 500 nM; (▼) [I] = 0 nM. $K_m = 54.01 \pm 5.35$ nM; $V_{max} = 14{,}430 \pm 703$ cpm/30 min; $K_i = 1.627 \pm 0.164$ μM. (**b**) Double-reciprocal plot of initial velocity versus varying TKCVIM concentration. Concentrations of inhibitor, PD 061938, are as follows: (▼) [I] = 15 μM; (▲) [I] = 5 μM; (♦) [I] = 1 μM; (■) [I] = 500 nM; (●) [I] = 0 nM. $K_m = 83.06 \pm 13.05$ nM; $V_{max} = 12{,}550 \pm 677$ cpm/30 min; $K_i = 0.591 \pm 0.092$ μM.



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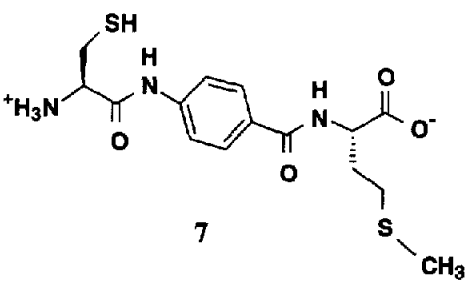
Transition state analogues

Patel et al. took a unique approach to inhibitor design by constructing a hypothetical active site and synthesizing a putative transition state analogue.[18] The ras *C*-terminal tripeptide and the farnesyl group were attached by a phosphonic or phosphinic linker to produce several very potent compounds (e.g., compound **8**). Compound **8** exhibited an IC_{50} of 6 nM against porcine FPTase. This is one of the first examples of a peptide-like substrate showing extremely potent inhibition against the FPTase, but lacking a sulfhydryl group and showing cellular activity.[19] The mode of action against the enzyme has not been reported.

Farnesyl diphosphate mimetics

Two of the first inhibitors discovered to be competitive for farnesyl diphosphate were structures **9** and **10**



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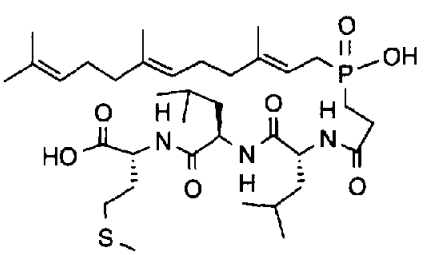

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characterized by K_is against bovine FPTase of 5 nM and 830 nM, respectively.[20] These compounds are structurally similar to FPP, but are not substrates for the enzyme. Other classes of compounds found to be competitive inhibitors against FPP are the chaetomellic acids (e.g., compound **11**,[21] as well as compounds **12a–c**[22]) that are competitive with respect to FPP in the yeast FPTase system.

Although these compounds are slowly active as substrates, their inhibitory potency increases with the electron-withdrawing ability of the R substituent. These inhibitors were shown to be competitive with respect to FPP having K_is of 140 nM, 36 nM, and 11 nM, respectively using yeast FPTase.[22]

Results and Discussion

Phosphate synergism of PD 083176

PD 083176, a pentapeptide (**13a**) identified through random screening of the Parke–Davis sample collection, was found to be a competitive inhibitor with respect to FPP.[23,24] Inhibition of FPTase by compound **13a** was found to be strongly dependent on the concentration of phosphate anion exhibiting an IC_{50} of 24 nM in 30 mM potassium phosphate buffer and is 60-fold less potent in Hepes buffer containing no phosphate (IC_{50} = 1600 nM, see Fig. 4). This significant change in inhibition is clearly dependent on the concentration of phosphate anion in the buffer as shown in Figure 5. The effect of phosphate anion on enhancing the inhibition of PD 083176 essentially leveled off at concentrations greater than 10 mM phosphate. This observation suggested that phosphate from the buffer solution has a synergistic effect with the inhibitor to increase inhibitory activity against FPTase. Compounds derived from the original template of PD 083176 that remained competitive for FPP also continued to show an enhanced inhibition with phosphate anion. One example of this type of compound is PD 152440 (**14**). Due to the dramatic increase in inhibition observed for PD 083176 a synthetic strategy was devised to increase the effective concentration of the phosphate moiety. Compounds **13b** and **c** were synthesized in an effort to capitalize on the phosphate anion phenomenon. The inhibition data for these compounds (**13a–c** and **14**) are shown in Table 2. The effect of adding 5 mM phosphate anion to a Hepes buffer assay system improved potency greater than 10-fold, this effect was still seen when the R group of the parent compound **13** was changed from a benzyl group to a hydrogen (**13b**). However, when phosphate is covalently attached at this same position, the effect of the phosphate anion collapses yielding an IC_{50} of around 7 nM in both buffer systems. In addition, the covalent attachment of

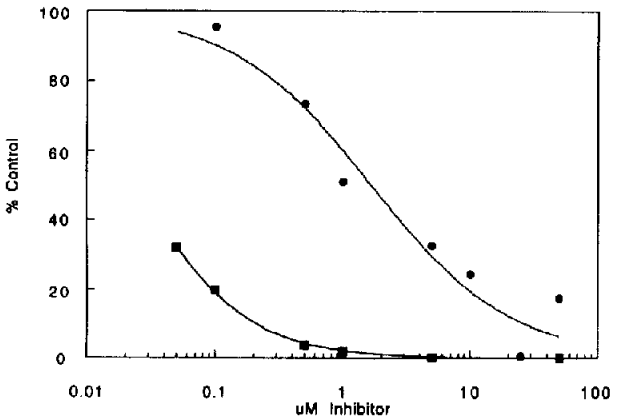
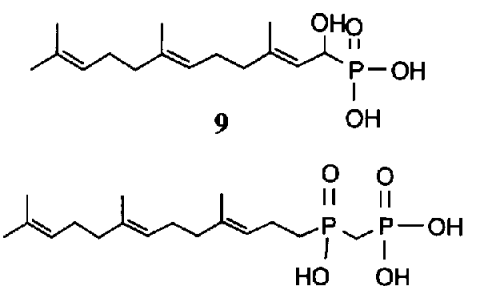


Figure 4. IC_{50} curves of PD 083176 against rat FPTase. Assay buffers are as follows: (●) 50 mM Hepes + no phosphate; (■) 50 mM Hepes + 30 mM potassium phosphate.



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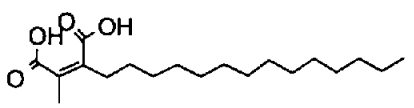

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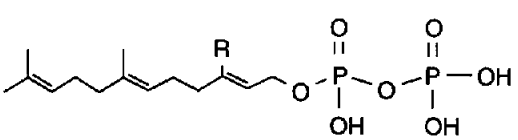


12a = CH_2F
12b = H
12c = CF_3

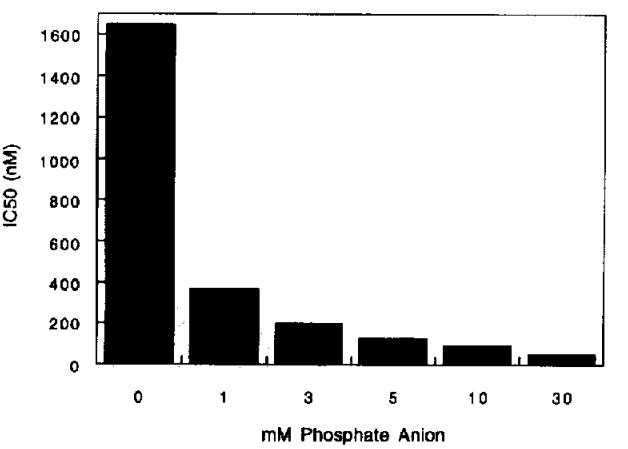


Figure 5. IC_{50} against rat FPTase of PD 083176 as a function of phosphate concentration in a 50 mM Hepes assay buffer.

Table 2. Effect of phosphate ion on inhibition of FPTase

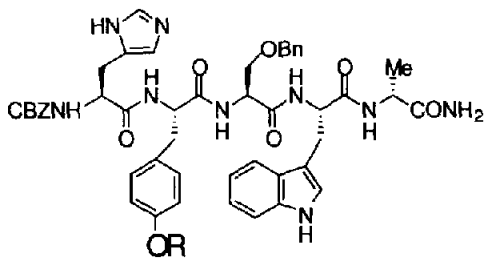
Concn of phosphate (mM)	IC_{50} determination of compd (nM)			
	13a	**13b**	**13c**	**14**
0	1950	510	7.5	3400
5	165	100	6.8	415
30	20	40	nd[a]	145

[a]nd = not determined.

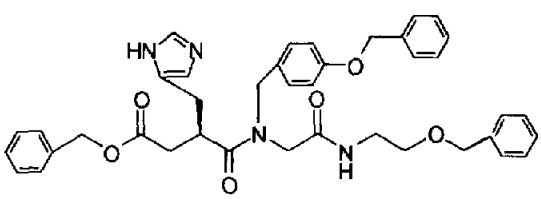
phosphate, which would eliminate any reliance on solution phosphate, improved the inhibition two- to threefold over the parent compound. This result suggests that the phosphate ion that is enhancing inhibition is not tightly bound at the enzyme active site. Compound **13c** remains competitive with respect to FPP ($K_i = 3$ nM) using rat FPTase and is noncompetitive with respect to peptide (data not shown).

This ion effect was only observed with compounds that were found to be competitive inhibitors with respect to FPP. Compound **9**, on the other hand, which is also a competitive inhibitor with respect to FPP, did not show an increased inhibition with greater concentrations of phosphate anion. The IC_{50} in Hepes buffer assay with no additional phosphate was 28 nM but when 5 mM phosphate was added to the assay buffer the IC_{50} was actually higher at 200 nM. In this case, the phosphate may be competing with the inhibitor. One way this effect could be explained kinetically is shown in Scheme 2. Phosphate or another anion could be interacting in an E:anion:inhibitor complex leading to better inhibition than a normal E:inhibitor complex. In the case of compound **9** the putative pyrophosphate binding site is already bound and no enhancement of inhibition should be observed. Buffer-dependent inhibition has also been observed with another prenyldiphosphate processing enzyme squalene synthetase which was attributed to an inhibitor:pyrophosphate ion-pair mechanism.[25] Poulter et al. showed that pyrophosphate 'promoted' binding of an inhibitor. In our case, the effect is seen with phosphate anion.

Unfortunately, in cellular assays compounds **13a–c** were found to be inactive, presumably due to inadequate cell permeability. However, based on the above observations it can be envisioned that truncated analogues of PD 083176 (**13a**) may be able to break the cell permeability barrier. Cellular potency is observed, for example, with compound **14**. The inhibition of processed H-ras was found to be as low as 1 μM upon Western analysis of NIH-3T3 cells (see Fig. 6). Having cellular activity at 1 μM when the IC_{50} is 3 μM in enzyme assay with no phosphate may be an indication that cellular phosphate could work in tandem with the inhibitor for improved inhibition of FPTase. Further experimental work is ongoing elucidating this phosphate-dependent inhibition. Finally, it is conceivable that the future design of FPP competitive inhibitors could benefit from the incorporation of a phosphate moiety and provide another rational way to increase enzyme inhibitory potency.



13a = benzyl
13b = hydrogen
13c = phosphate



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Experimental

In vitro FPTase enzyme assay

Enzyme activity was monitored using SPA (Scintillation Proximity Assay) technology. Reactions were carried

Scheme 2.

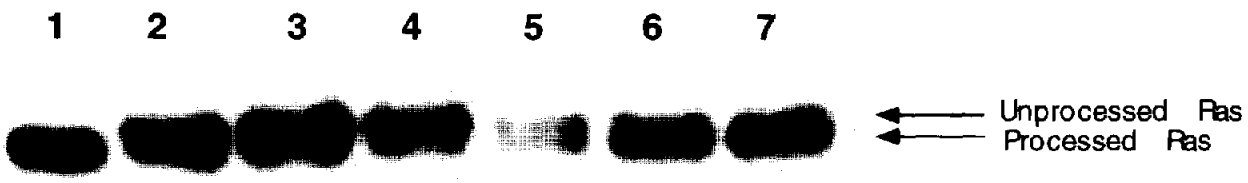

Figure 6. Effect of PD 152440 on ras-transformed Rat1 cells. Cells were treated with inhibitor, lysed, and lysates subjected to Western blotting. Lane 1, vehicle control (DMSO); lanes 2–5, PD 152440 at concentrations of 25, 10, 5, and 2.5 μM, respectively.

out in buffer containing 50 mM Hepes (pH 7.4) with and without buffered potassium phosphate at varying concentrations. In addition the assay buffer contained 5 mM DTT, 20 μM $ZnCl_2$, 5 mM $MgCl_2$, and 0.1% PEG. Upon the addition of rat affinity purified FPTase, tritiated farnesyldiphosphate (134 nM) and peptide (biotinyl-Aha-Thr-Lys-Cys-Val-Ile-Met, 200 nM) assay mixtures were incubated at 37 °C for 30 min. Addition of a stop reagent and SPA beads was added and radioactive product was counted on a Wallac Microbeta 1450 scintillation counter. The kinetic fits were derived from a nonlinear least squares computer fit of the data and were a result of one experiment.

Gel shift assay

Twenty-four hours after planting 2×10^6 ras-transformed cells per treatment condition, the farnesylation inhibitor was added at varying concentrations. Following an 18 h incubation period, cells were lysed in phosphate-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.4 in the presence of several protease inhibitors (PMSF, antipain, leupeptin, pepstatin A, and aprotinin all at 1 μg/mL). Ras protein was immunoprecipitated from the supernatants by the addition of 3 μg v-H-ras Ab-2 (Y13–259 antibody from Oncogene Science). After overnight immunoprecipitation, 30 μL of a 50% protein G-Sepharose slurry (Pharmacia) was added followed by 45 min incubation. Pellets were resuspended in 2X tris-glycine loading buffer (Novex) containing 5% B-mercaptoethanol and then denatured by 5 min boiling prior to electrophoresis on 14% Tris–glycine SDS gels. Using Western transfer techniques, proteins were transferred to nitrocellulose membranes followed by blotting in blocking buffer. Upon overnight incubation with primary antibody (pan-ras Ab-2 from Oncogene Science), an anti-mouse HRP conjugate secondary antibody (Amersham) was employed for detection of the ras protein. Blots were developed using ECL techniques (Amersham).

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