New approaches to anticancer drug design based on the inhibition of farnesyltransferase

Saïd M. Sebti and Andrew D. Hamilton

Mutated forms of the GTP-binding protein Ras are found in 30% of human cancers, with particularly high prevalence in colon and pancreatic carcinomas. Ras function in growth factor signaling requires post-translational farnesylation of a cysteine residue present as part of the CA_1A_2X carboxyl terminal tetrapeptide. The enzyme farnesyltransferase has become an important target for the design of potential new antitumor agents. The authors outline the major new approaches to inhibition of farnesyltransferase and describe how certain peptidomimetics have been shown to block oncogenic signaling and tumor growth in various animal models.

Recent investigations into signal transduction pathways stimulated by growth factors have shown that a critical role is played by the small G-protein, Ras[1]. Binding of growth factors, such as epidermal growth factor (EGF), to a membrane bound receptor tyrosine kinase results in dimerization and autophosphorylation of tyrosine residues on the protein surface. One of these is recognized by a *src*-homology 2 (SH2) domain on the growth factor receptor binding protein Grb2, which itself is complexed to a guanine nucleotide exchange protein, m-SOS-1 (Ref. 2). m-SOS-1 activates the membrane-bound Ras by catalyzing the exchange of GDP for GTP (Ref. 3). The GTP-bound form of Ras undergoes a conformational change on its surface, enabling it to bind to several effector molecules, among which c-Raf, a serine/threonine kinase, is the most thoroughly characterized[4]. Translocation of Raf to the membrane results in its activation of mitogen activated protein kinase kinase (MAPKK or MEK) and initiation of a series of steps through mitogen activated protein kinase (MAPK) leading to the activation of transcription factors involved in DNA synthesis (Figure 1)[5].

Four structurally related Ras proteins are produced in the cell (H-, K_A-, K_B- and N-Ras) with 188 or 189 amino acids and a molecular weight of 21 kDa (Ref. 6). A common feature of all Ras proteins is that the last four amino acids at the carboxyl terminus are composed of a CAAX motif, where C is cysteine, A is valine, isoleucine or leucine and X is methionine or serine. The importance currently attached to these proteins derives from the observation that mutated ras oncogenes are found in approximately 30% of all human tumors, with over 90% in human pancreatic carcinomas and 50% in human colon cancers[7].

In order to perform its on–off switching function in cell signaling, Ras must be located at the inner surface of the plasma membrane. Significant affinity for the membrane requires an increase in hydrophobicity of the protein by post-translational modification. This involves farnesylation on the cysteine residue of the carboxyl terminal

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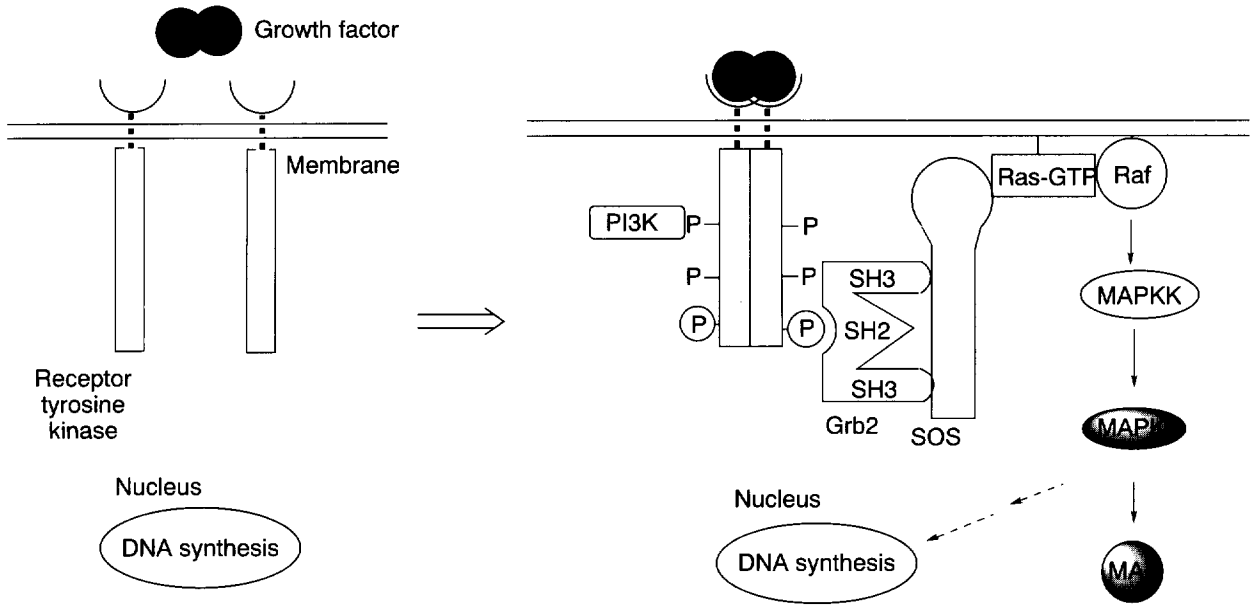


Figure 1. *Signal transduction pathways through receptor tyrosine kinases. MAP (MAPK and MAPKK), mitogen activated protein (and kinases); P, phosphate; P13K, phosphoinositide 3-kinase; SH2;* sre*-homology 2 domain.*

CAAX, cleavage of the tripeptide AAX, methylation of cysteine carboxylic acid and, in some cases, attachment of an additional palmitoyl group (Figure 2)[8]. The key post-translational modification step is farnesylation, which is catalyzed by the enzyme farnesyltransferase (FTase). This is both sufficient and required for mutated Ras to lead to cancer[9]. An important new target for anticancer research has therefore become the design of inhibitors of FTase that can potentially block the translocation of mutated Ras to the membrane and so prevent its oncogenic signaling function[10].

FTase is a heterodimer containing α and β subunits with molecular weights of 48 kDa and 46 kDa (Ref. 9). The transfer of the farnesyl group to H-Ras protein within the enzyme has been shown to require Mg^{2+}, while the association of H-Ras needs the presence of Zn^{2+} (Ref. 11). A recent X-ray structure of the protein shows a Zn^{2+} in the active site, suggesting that the metal ion coordinates to the cysteine to facilitate deprotonation of the thiol group[12,13]. It has recently been shown that farnesylation involves a randomly ordered sequential mechanism[14], although the enzyme–farnesylpyrophosphate binary complex appears to provide the faster catalytic pathway[15]. Simple CAAX tetrapeptides have been shown to act as alternative substrates and competitive inhibitors of FTase catalyzed Ras farnesylation[16].

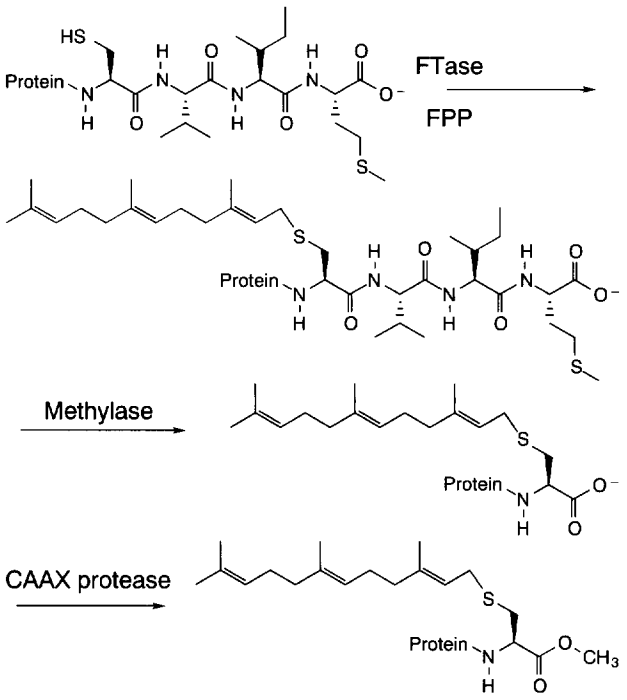


Figure 2. *Carboxyl terminal processing of Ras proteins. CAAX tetrapeptide: C, cysteine; A, valine, isoleucine or leucine; X, methionine or serine. FTase, farnesylpyrophosphate; FTase, farneslytransferase.*

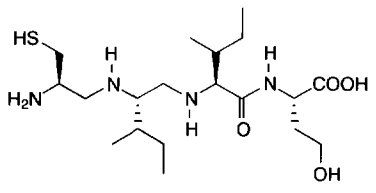
Design of Ras farnesyltransferase inhibitors

Several recent reviews have detailed progress in this area[17–20]. The primary strategies have involved the development of peptidomimetics to mimic the Ras substrate recognition region, the design of mimetics of the farnesylpyrophosphate substrate, the preparation of bisubstrate transition state analogs and the screening of synthetic or natural product libraries for potent inhibitors.

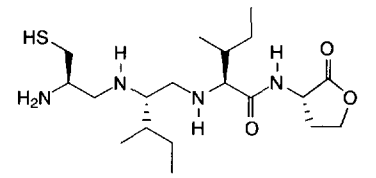
Peptidomimetics

The finding that CAAX tetrapeptides can function as potent and competitive inhibitors of FTase has led to an intensive search for peptidomimetic structures with improved potency, cellular uptake and stability to peptidase degradation.

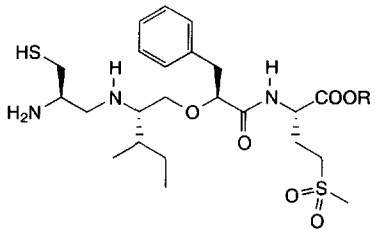
Several groups have taken a pseudopeptide strategy in which peptide bonds in CAAX are reduced to their methyleneamino forms (Figure 3)[21]. For example, **1** (L731735) is competitive to H-Ras protein and has an inhibition constant K_i = 20 nM. In the corresponding lactone **2** (L731734) the negative charge of the carboxylate was masked and cellular uptake significantly improved. This compound inhibited H-Ras farnesylation in cell culture with an IC_{50} value of 100 μM[22]. In a further development of this approach the methyleneoxy isostere **3** (L739750) was prepared and shown to be a very potent inhibitor of FTase (IC_{50} = 1.8 nM). A prodrug derivative **4** (L739749T) inhibits H-Ras processing at concentrations of 0.1–1.0 μM and has also been shown to suppress the growth of mutated H-Ras transfected tumors in nude mice[23]. A similar prodrug **5** (L744832) inhibited the growth in culture of more than 70% of all tumor cell lines at concentrations 2–20 μM[24], and was further shown to cause regression of mammary and salivary carcinomas in H-Ras transgenic mice without showing systemic toxicity[25]. A related pseudopeptide strategy has been effectively developed by a group at Eisai[26].



1, L731735



2, L731734



3, L739750 R = H

4, L739749T R = CH_3

5, L744832 R = $CH(CH_3)_2$

Figure 3. *Some compounds based on a pseudopeptide strategy whereby peptide bonds in CAAX are reduced to their methyleneamino forms.*

The approach taken to designing peptidomimetic inhibitors of FTase in our laboratories has focused on the replacement of peptidic features in the CAAX tetrapeptide by stable components. At the outset, we reasoned that the A_1A_2 region of the tetrapeptide would bind to a hydrophobic pocket in the enzyme active site and so might be replaced by a simple hydrophobic spacer based on substituted 4-aminobenzoic acid derivatives (Figure 4). Peptidomimetic **6** (FTI276) inhibited FTase *in vitro* with an IC_{50} value of 0.5 nM, and its prodrug form **7** (FTI277) inhibited H-Ras processing *in vivo* with an IC_{50} value of 100 nM[27]. Importantly, inhibition of FTase by **7** (FTI277) also resulted in the accumulation of non-farnesylated Ras which could complex Raf protein to form inactive Ras–Raf complexes in the cytoplasm. Consequently **7** (FTI277) selectively blocked constitutive activation of MAPK by oncogenic Ras but not Raf[27]. Antitumor studies in nude mouse models showed that **6** (FTI276) could selectively inhibit the growth of human lung carcinoma and H-Ras transformed NIH 3T3 cells in nude mice without apparent toxicity[28].

We used the same hydrophobic spacer strategy in the study of the related enzyme geranylgeranyltransferase-I (GGTase-I), which recognizes a leucine residue in the C-terminal CAAX sequence. Incorporation of leucine instead of methionine into our design led to a new class of inhibitors such as **8** (GGTI287), which selectively inhibits GGTase-I over FTase, IC_{50} = 5 vs 25 nM. The methyl ester of **8** (GGTI287) showed potent inhibition of Rap1A and K-Ras4B processing (IC_{50} = 2 μM), but a weak inhibition of H-Ras processing (IC_{50} > 30 μM)[29].

In order to prepare completely nonpeptide and potentially more stable inhibitors, we extended our hydrophobic strategy to replace the methionine residue. Inhibitor **9** is as potent (IC_{50} = 114 nM) as CVIM, despite the large structural differences caused by the replacement of the VIM tripeptide by a 4-amino-3′-carboxybiphenyl group[30]. A more potent

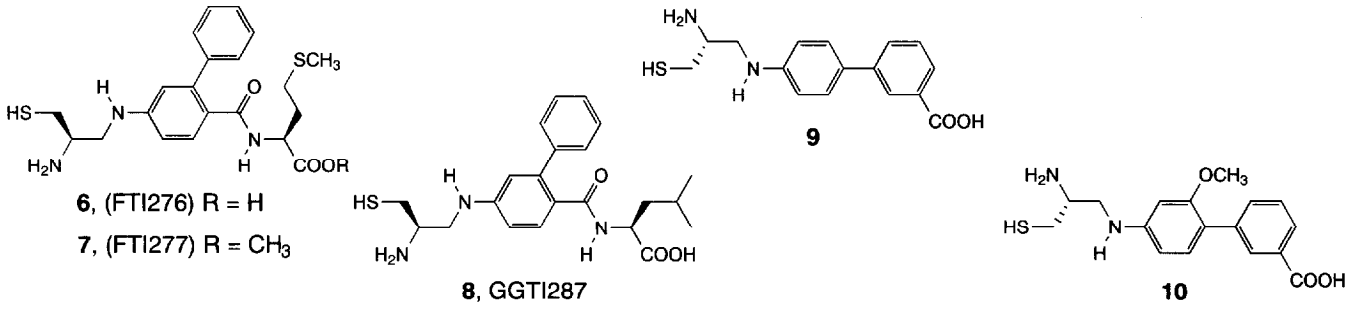


Figure 4. *Peptidomimetic inhibitors of FTase designed on the basis of replacement of peptidic features in the CAAX tetrapeptide by stable components.*

inhibitor **10** (IC_{50} = 30 nM) could be formed by substitution of the biphenyl spacer with a hydrophobic group such as methoxyl[31]. Although **9** and **10** lack the methionine residue, the compounds have a 1,000-fold selectivity for FTase over GGTase-I. Furthermore, **9** inhibited H-Ras processing at 50 μM as its free carboxylate[30].

A group at Eisai used a related hydrophobic spacer strategy in which they replaced the central two amino acid residues in CAAX tetrapeptides by alkane spacers (Figure 5). Substitution of a benzyl group at the 2-position of 5-aminopentanoic acid (**11** and **12**) gave highly potent inhibitors (IC_{50} = 20 and 18 nM, respectively)[32]. The absolute stereochemistry of the 2-position was not important in FTase binding but did affect the selectivity with respect to GGTase-I. The structure of peptidomimetic **13** (B956) was designed to mimic tetrapeptide CVFM where the cysteine amide is replaced by a trans alkene isostere and the central amide is replaced by a cis alkene isostere. **13** (B956) inhibits H- and K-Ras processing with IC_{50} values of 0.5 and 25 μM, respectively[33]. Furthermore, **13** (B956) was also shown to disrupt the growth in soft agar assays of transformed cell lines without Ras mutation at concentrations between 16 and 80 μM and also to block tumor growth in nude mice[33].

In a different strategy, a group at Genentech used a benzodiazepine subunit to enforce a turn conformation in their peptidomimetic with the potential of both thiol and terminal carboxylate group coordinating to the Zn^{2+} ion (Figure 6). In particular, a 3-amino-1-carboxymethyl-5-phenyl-benzodiazepine-2-one group replaced the two central amino acid residues in the CAAX to form **14** (BZA2B), a highly potent inhibitor of FTase (IC_{50} = 0.85 nM)[34,35]. The potency of these benzodiazepine peptidomimetics was related to the methylation state of the cysteine amide bond and the stereochemistry at the C_3 position of the diazepine ring[36]. In this series the selectivity for FTase over GGTase-I was not influenced by the C-terminal amino acid residue. However, the methyl ester **15** (BZA5B) was shown both to inhibit Ras processing at a concentration of 10 μM and to interrupt MAP kinase activation in H-Ras transformed Rat-1 cells but not in untransformed cells[34,35].

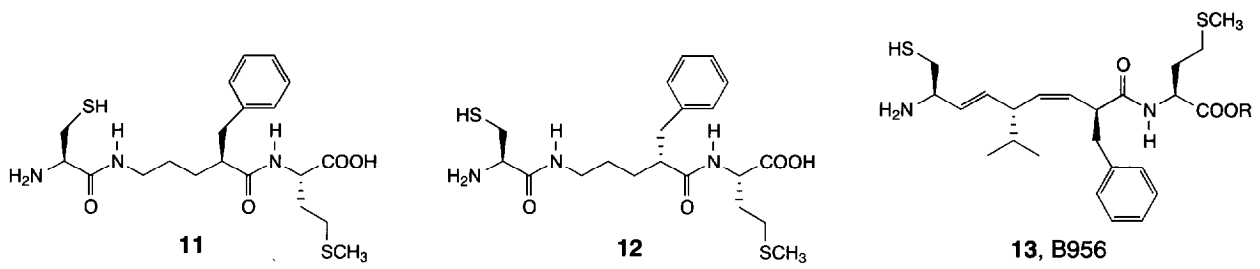
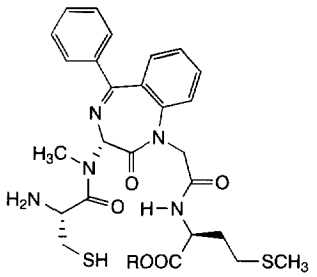


Figure 5. *FTase inhibitors designed by a group at Eisai by replacing the central two amino acid residues in CAAX tetrapeptides by alkane spacers.*

An investigation of the active conformation of the FTase-bound CAAX terminal sequence has been carried out using transferred nuclear Overhauser effect (TRNOE) NMR spectroscopy[37]. The weak heptapeptide inhibitor KTKCVFM (K_i = 4.5 μM: corresponding to the C-terminal sequence of K-Ras4B with Ile replaced by Phe) was studied and proposed to adopt a type-I β-turn conformation where the carbonyl group of cysteine forms a hydrogen bond with the amino group of methionine. Investigations at Merck showed that flexible peptide or synthetic inhibitors adopt non-ideal reverse-turn conformations similar to a type-III β-turn but without an internal transannular hydrogen bond between cysteine and methionine[38].

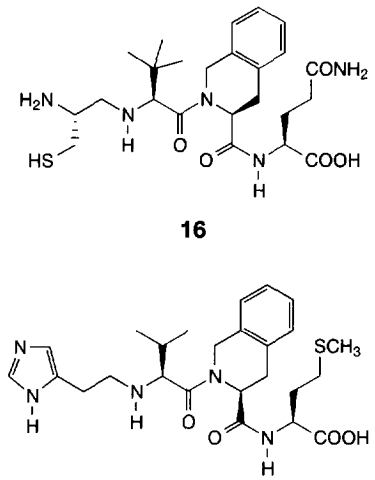
In a study at Rhône-Poulenc™, a conformationally constrained amino acid, (L)-1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid (Tic), was used to replace the A_2 residue in KCA_1A_2X. Molecular modeling suggested that potent inhibition of FTase requires an extended rather than β-turn conformation of KCVTicM[39]. A related strategy employing constrained amino acids to control the conformation of tetrapeptide analogs was also reported by Genentech and Bristol-Myers Squibb[36,40]. The success of this approach was shown by the ability of **16** (Figure 7) to inhibit tumor growth in mice[40].

In a recent key result the Bristol-Myers Squibb group has successfully replaced the cysteine residue to make a thiol-free FTase inhibitor[41]. Their strategy was based on the likelihood that the thiol group is bound to the zinc ion in the FTase active site. An imidazole ring was incorporated in inhibitor **17** (BMS193269: Figure 7) and the compound was shown to inhibit H-Ras processing with an IC_{50} of 5 μM[41]. In a related development, a group at Parke-Davis identified pentapeptide



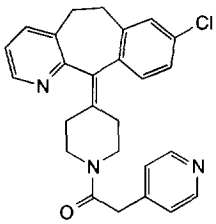
14, (BZA2B) R = H
15, (BZA5B) R = CH_3

Figure 6. *FTase inhibitors designed at Genentech.*



16

17, BMS193269



18, SCH44342

Figure 7. *Other FTase inhibitors designed using peptidomimetic approaches.*

Cbz-His-Tyr(OBz)-Ser(OBz)-Trp-(D)-Ala-NH_2 as a potent, non-thiol inhibitor of FTase (IC_{50} = 57 nM)[42,43]. The potency of the lead could be enhanced by truncation and (D)-amino acid replacement to form a tripeptide Cbz-(D)-His-Tyr-Ser (PD15169) with an IC_{50} of 11 nM for FTase inhibition and of 50 μM for Ras processing.

In a random screening exercise, scientists at Schering-Plough have identified an entirely non-peptidic inhibitor of FTase **18** (SCH44342; Figure 7) that is competitive with the Ras protein in inhibiting FTase and also inhibiting Ras processing with an IC_{50} value of 3.0 μM[44].

Other peptidomimetic strategies that have been applied to FTase inhibition include peptoid and retro–inverso approaches[45].

In the past two years, a question has arisen concerning the relative effect of these peptidomimetic FTase inhibitors on the different H-, N- and K-Ras proteins. Most of the early whole cell studies had used readily available H-Ras transformed cell lines. However, it has now become clear that the cellular processing of K-Ras is much more difficult to disrupt with FTase inhibitors. For example, **7** (FTI277) can inhibit the processing of H-Ras with an IC_{50} of 0.1 μM but requires a 100-fold higher concentration (IC_{50} = 10 μM) to block the processing of K-Ras[27,29]. This observation is particularly important because most Ras-transformed tumors in human cancers contain mutations in K-Ras rather than H- or N-Ras. This 100-fold resistance cannot be explained simply by the fact that K_B-Ras is bound eightfold more tightly to FTase than H-Ras. An alternative explanation is that when FTase is inhibited, K_B-Ras can alone be efficiently processed by the related prenyltransferase, GGTase-I. Indeed, recent results have shown that

K_B-Ras can be a substrate for GGTase-I *in vitro*[46] and that the processing of Ras in whole cells can be disrupted by specifically designed GGTase-I inhibitors[29]. Furthermore, when cells are treated with FTase inhibitors K-Ras becomes geranylgeranylated[47,48]. This is consistent with our recent observation that inhibition of K-Ras prenylation in human tumors requires cotreatment with both FTase and GGTase-I inhibitors[49].

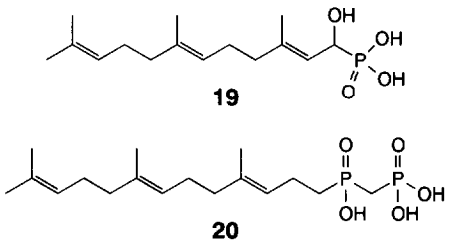
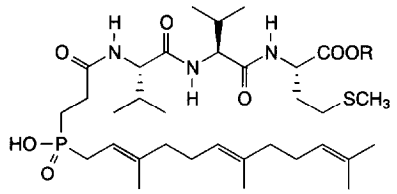


Figure 8. *FTase inhibitors that might bind into the farnesylpyrophosphate recognition site.*



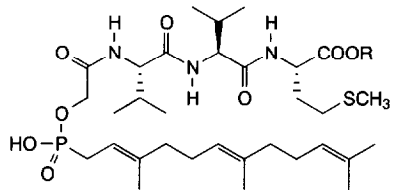


Figure 9. *FTase inhibitors incorporating features of both the farnesylpyrophosphate and peptide substrates.*

Farnesylpyrophosphate and bisubstrate analogs

The above approaches attempt to mimic the Ras protein substrate structure. However, since the FTase catalyzed reaction also involves farnesylpyrophosphate (FPP) there has been interest in the design of inhibitors that might bind into the FPP recognition site. An inherent disadvantage in this approach is that FPP is a widely used metabolite in the cell in such enzymes as squalene synthase and so FPP mimics may show toxicity. In α-hydroxyfarnesylphosphonic acid **19** (Figure 8), the pyrophosphate group in FPP is replaced by a monophosphonate. A related analog is farnesylmethylhydroxyphosphinyl methyl phosphonic acid **20** in which the diphosphate oxygen atom is replaced with a methylene group. Both **19** and **20** are competitive to FPP in inhibiting FTase with K_i values of 5.2 nM and 830 nM, respectively[14]. Compound **19** was also able to inhibit H-Ras processing in whole cells at a concentration of 1 μM[50]. The issue of selectivity appears not to be a problem with **19**, which inhibited squalene synthase *in vitro* with an IC_{50} of 630 nM.

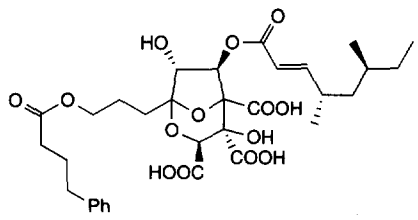
A group at Bristol-Myers Squibb has followed a bisubstrate transition state analog strategy by incorporating features of both the farnesylpyrophosphate and peptide substrates into their inhibitor design (Figure 9)[51]. Compounds **21** (BMS185878) or **22** (BMS184467) were highly potent inhibitors for FTase (both, IC_{50} = 6 nM) and show strong selectivity with respect to GGTase-I. The corresponding prodrugs **23** (BMS186511) and **24** (BMS184382) inhibited 75–80% growth in transformed foci cells at concentrations of 100 μM without showing significant effects on untransformed cells[52]. Both anchorage dependent and anchorage independent growth of Ras transformed cells were inhibited by **23** (BMS186511) at micromolar concentrations.

Natural product inhibitors

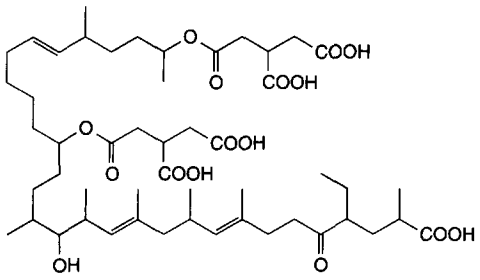
The screening of natural products has led to the discovery of several novel and potent farnesyltransferase inhibitors (Figure 10). These compounds inhibit FTase with a variety of inhibition mechanisms: competitive to FPP, competitive to Ras protein or noncompetitive to either FPP or Ras protein. In zaragozic acid A analog **25**, actinoplanic acid B **26** and chaetomellic acid A **27**, multiple carboxylate groups appear to mimic the diphosphate fragment in FPP and are competitive with its binding to FTase (IC_{50} values = 50, 55 and 12 nM, respectively)[50,53–55]. Zaragozic acid also inhibits the key cholesterol biosynthesis enzyme squalene synthase (which also utilizes FPP) with an IC_{50} of 78 pM, while both chaetomellic acid and actinoplanic acid are inactive at high concentrations.

Conclusions

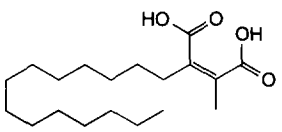
Already outstanding progress has been made in demonstrating the efficacy of FTase inhibitors to block Ras processing selectively in a range of Ras transformed tumor cell lines at concentrations as low as 100 nM. Several reports have also confirmed that FTase inhibitors will block tumor growth



25, zaragozic acid A analog



26, actinoplanic acid B



27, chaetomellic acid A

Figure 10. *Novel and potent farnesyltransferase inhibitors discovered through screening of natural products.*

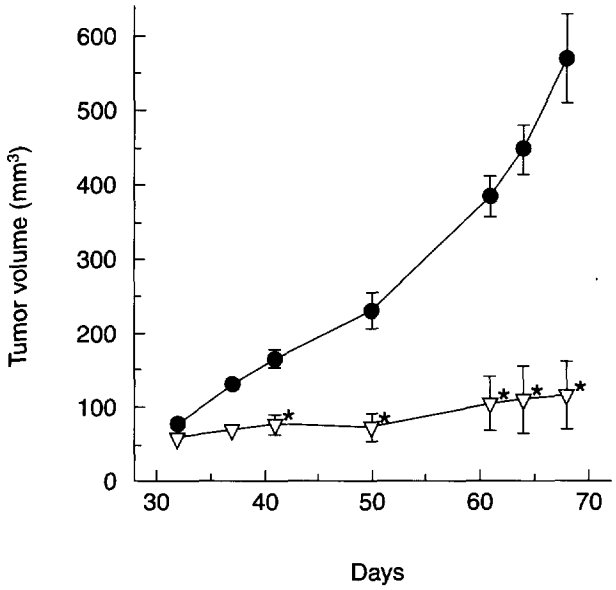


Figure 11. *Antitumor efficacy in nude mouse xenografts of* ***6*** *(FTI276) against human lung adenocarcinoma Calu-1. Mice were implanted subcutaneously with 10^7 Calu-1 cells per flank. When the tumors had reached 50–100 mm^3, the mice were treated intraperitoneally with 50 mg Kg^{-1} day^{-1} of* ***6*** *(FTI276) (▽) or saline (●) (*, P<0.05). (Reproduced, with permission, from Ref. 28.)*

in various mouse models. For example, we have evaluated the antitumor efficacy of **6** (FTI276) in nude mouse xenograft models using human lung carcinoma cell line Calu-1 with a K-Ras oncogenic mutation. Animals treated with 50 mg kg^{-1} day^{-1} of **6** (FTI276) showed a dramatic decrease in the growth of Calu-1 tumors (Figure 11)[28]. Although animals were treated once daily for 36 days, no weight loss was observed, and animals appeared to be normal without any evidence of gross toxicity.

While underlining the exciting potential for FTase inhibitors as anticancer agents, these results are nonetheless puzzling. We had previously shown that K_B-Ras processing in whole cell experiments is resistant to treatment with **6** (FTI276; see above)[29,49], yet this compound has good antitumor activity against human tumors that express an oncogenically activated K-Ras[28]. These results suggest that FTase inhibitors may be disrupting the farnesylation of proteins, other than K-Ras, that are involved in cell transformation. In surveying the *in vivo* potency of peptidomimetic FTase inhibitors, other groups have noted a lack of correlation between antitumor activity and the Ras mutation status of the tumor cell lines[24,33].

These observations all point to a more complex role for FTase inhibitors in blocking tumor growth than simply the disruption of K-Ras translocation to the membrane. In spite of this, the prospects appear good for the eventual application of FTase inhibitors in anticancer therapy, however there is much work still to be done to improve the efficiency of the inhibitors and confirm their mechanism of action.

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