ABSTRACT

Ras proteins play fundamental roles in cell signal transduction pathways that regulate cell growth, differentiation, proliferation, and survival. Ras mutations are among the most frequently encountered genetic abnormalities in human cancers and play a key role in tumorigenesis. The enzymatic attachment of a 15- or 20-carbon moiety to the Ras protein through farnesylation or geranylgeranylation, respectively, is a required step in the proper localization and activation of Ras. Inhibition of the catalytic enzymes, farnesyl transferase and geranylgeranyl transferase, is a novel, mechanism-based, targeted approach to cancer therapy development. Geranylgeranyl transferase inhibitors suppress tumor growth by accumulating cells in the G1/S cell cycle phase. One mechanism by which farnesyl transferase inhibitors suppress tumor growth is by inhibiting bipolar spindle formation, thereby blocking progression from prophase to metaphase. Although the exact molecular target responsible for the antitumor activity of farnesyl transferase inhibitors is unclear, at least in some tumor cells, inhibition of phosphoinositide-3-OH kinase/Akt-mediated cell survival pathways may play a critical role. Identifying the farnesylated proteins that are targeted by farnesyl transferase inhibitors and the tumor molecular signatures that dictate which set of patients will respond to farnesyl transferase inhibitors are critical end points for future mechanistic studies.

INTEGRATED IMAGE

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Learning Objectives
After completing this course, the reader will be able to:

1. Describe how mutations in ras oncogenes affect cell signal transduction pathways and disrupt cell growth, differentiation, proliferation, and survival.

2. Identify the mechanisms of action of two classes of prenylation inhibitors, the farnesyl transferase inhibitors (FTIs) and the geranylgeranyl transferase-I inhibitors (GGTIs).

3. Explain the in vitro and in vivo antitumor activity of FTIs and GGTIs in multiple myeloma and myelodysplastic syndrome.
cancer, Ras has become an important target for antineoplastic therapy [2]. Selective blockade of Ras farnesylation, which essentially blocks subsequent membrane attachment and interrupts Ras effector pathways, has emerged as a strategic approach to the development of novel cancer therapies [3, 4]. Ras farnesylation inhibitors have shown promising preclinical and clinical antitumor activities, so their benefits as cancer therapy continue to be explored [4, 5]. In addition to farnesylation, a closely related posttranslational modification called geranylgeranylation is also a target for cancer therapy because other Ras family members, such as RhôA and Rac1, require geranylgeranylation for their ability to mediate oncogenesis and metastasis.

Here we discuss the proposed mechanisms of action of two classes of prenylation inhibitors, the farnesyl transferase inhibitors (FTIs) and the geranylgeranyl transferase inhibitors (GGTIs). Preliminary data are presented, demonstrating the clinical activity of the FTI tipifarnib (formerly R115777; ZARNESTRA™; Ortho Biotech Products, L.P.; Bridgewater, NJ) in multiple myeloma and myelodysplastic syndrome.

**RAS PROCESSING AND MEMBRANE ASSOCIATION**

A mutation in the ras oncogene is one of the most frequently detected genetic mutations in human cancers [1]. K-, H-, and N-ras mutations are known to be involved in approximately 25%-30% of all human cancers, with as many as 90% of pancreatic cancers expressing a mutated form of ras [1, 2]. Ras is a low-molecular-weight, membrane-bound, guanine-nucleotide-binding GTPase that plays a key role in the signal transduction pathways that control cellular growth, differentiation, and proliferation [2]. Ras cycles between an inactive, GDP-bound state and an active, GTP-bound state [1, 2]. Mutations in the ras gene result in a constitutively active, GTP-bound Ras. This leads to unregulated signaling through a cascade of downstream effectors and results in an unrestricted stimulation of cellular proliferation and tumor growth [2, 6].

Posttranslational Ras lipid modification and subsequent localization of Ras to the inner surface of the plasma membrane are required for Ras activity. These processes are also essential for the oncogenic activity of ras mutations [1, 3, 4]. Ras modification at the C-terminus end, specifically farnesylation, is essential for membrane association (Fig. 1) [3]. All farnesylated proteins have a specific amino acid sequence at the C-terminus, termed the CAAX recognition sequence or motif [1, 3]. CAAX refers to the tetrapeptide consisting of a cysteine at the fourth position from the C-terminus end, then any two aliphatic amino acids (AA), followed by a specific amino acid (X), usually serine (Ser), methionine (Met), glutamine, or cysteine (Cys) [1, 3, 4]. Farnesylation is the process whereby a 15-carbon farnesyl moiety is enzymatically attached to the C-terminus cysteine residue of the target protein via the formation of a covalent thio-ether bond [1]. Farnesyl transferase (FT) is the enzyme that catalyzes this prenylation reaction [4].

FT is structurally related to another CAAX prenyltransferase, geranylgeranyl transferase (GGT)-1 [1, 3]. GGT-2 is a third enzyme that catalyzes isoprenoid attachment to proteins but it uses a mechanistically distinct process [1, 3]. GGT-2 is not discussed in this paper. GGT-1 modifies the target Ras family protein by attaching a 20-carbon geranylgeranyl isoprenoid to proteins with CAAX, where X is often either leucine (Leu) or isoleucine (Ile) [1].

Whether through farnesylation or geranylgeranylation, the introduction of an isoprenoid lipid “tail” results in a more lipophilic protein, which allows Ras and other proteins to anchor to cell membranes [1]. In both FT- and GGT-1-catalyzed pathways, following prenylation, the terminal AAX tripeptide is cleaved from the cysteine residue by specific endoproteases, and the resulting C-terminal prenylcysteine is carboxymethylated [2, 3]. In some cases, a palmitate lipid moiety also is added to cysteines upstream of the CAAX box [1-3]. Each of these reactions (prenylation, proteolysis, methylation, palmitoylation) contributes to neutralizing the negative charge and increasing the lipid solubility of proteins so that stable membrane association can occur.

The selectivity of FT and GGT-1 for their substrates is determined by the specific sequence of the CAAX tetrapeptide, specifically the carboxy-terminal residue (X) [1, 3, 4]. FT generally catalyzes the transfer of a farnesyl moiety to the cysteine residue when X is Met, Ser, glutamine, or Cys. Alternatively, if X is Leu or Ile, attachment of the geranylgeranyl moiety occurs via GGT-1. While FT and GGT-1 have distinct preferences for substrates, these are not absolute, and cross-prenylation can occur [1, 3].

FT and GGT-1 substrates also include many non-Ras proteins containing the CAAX motif, including: lamin A and B; retinal proteins; centromere-associated proteins; and Ras-related small G-proteins such as Rac, Rho, and Rap [1-3]. The existence of multiple substrates for FT and GGT-1 has important implications in the development of inhibitors of these enzymes as antitumor agents, as discussed in later sections.

**DEVELOPMENT OF FT AND GGT-I INHIBITORS AS CANCER THERAPY**

Interrupting the Ras-signaling pathway through inhibition of Ras processing and localization to the membrane, specifically through blockade of FT, is a mechanistic approach to the development of novel cancer therapies that has been pursued throughout the last decade [4]. Specific inhibitors of FT were
among the first classes of targeted anti-Ras agents tested in preclinical and clinical studies for antitumor properties. Although CAAX tetrapeptides, such as those from K_{B}-Ras (Cys-Valine (Val)-Ile-Met), are potent inhibitors of FTase in vitro, their low cellular uptake and protease sensitivity make them poor anticancer drugs in vivo. The development of CAAX peptidomimetics that are potent and selective inhibitors of FT has led to improved stability of the tetrapeptides toward proteolytic degradation, thus increasing their cellular uptake [7, 8]. Molecular refinement focused on designing CAAX peptidomimetics (Fig. 2) such as FTI-276, where the dipeptide Val-Ile was replaced by 2-phenyl aminobenzoic acid, and FTI-2148, where the reactive cysteine of FTI-276 was replaced with an imidazole derivative and the phenyl group was substituted with a tolyl group (Fig. 2) [4]. Farnesyl pyrophosphate (FPP) mimetics and bisubstrate transition state analogs (e.g., BMS184878) have also been made, in which structural elements of both the CAAX tetrapeptide (or mimetics) and FPP substrate are incorporated into the inhibitors. Identification of novel FTIs has also come from screening synthetic combinatorial libraries. For example, unrelated heterocyclic derivatives, such as SCH66336, R115777, and RPR130401, were identified as potent FTase inhibitors from the screening of chemical archives at pharmaceutical companies. Many of these inhibitors have been described thoroughly elsewhere [9]. In addition to FTI-276 and FTI-2148, CAAX peptidomimetics specific to GGT-1 inhibition (e.g., GGTI-298 and GGTI-2154) also have been developed by incorporating a leucine at the terminal tetrapeptide position instead of methionine (Fig. 2) [4]. The chemical structures of two FTIs used in the clinic to treat hematologic malignancies, tipifarnib and lonafarnib (SCH66336, Sarasar™; Schering-Plough; Kenilworth, NJ), are presented in Figure 2.

**IN VITRO AND IN VIVO ANTITUMOR ACTIVITY**

In vitro studies demonstrate that FTIs and GGTIs selectively inhibit intrinsic enzyme activity at nanomolar to low-
FTI-276 showed a 50% inhibitory concentration (IC₅₀) value for FT of 0.5 nM (in vitro), with a 100-fold difference in activity for GGT (IC₅₀ = 50 nM) [4]. The selectivity of FTI-276 for FT over GGT also was demonstrated in cultured cells using a gel shift assay [4]. GGTI-2154 showed similar preferential inhibition of GGT over FT in vitro [4].

In vivo, FTIs have been shown to improve survival of nude mice that were implanted intracranially with the human glioblastoma U87 cell line [10]. Both FTIs and GGTIs also induced tumor regression in animal models [4]. In a transgenic mouse model with oncogenes under the control of mammary tumor virus promoter (MMTV), FTIs induced dramatic cytoreduction [11]. These compounds also were tested in the nude mouse xenograft model using the A-549 human lung carcinoma cell line. Reductions in tumor volume following FTI administration were further confirmed in that model [8]. Apoptosis was suggested as the mechanism by which FTIs induced tumor regression in those studies, with the extent of apoptosis dependent on the genetic background of the tumors [4, 11].

**MECHANISM OF ACTION: INVESTIGATIONAL STUDIES**

GGTIs inhibit tumor growth by inducing G₁ arrest, thereby blocking the G₁-to-S transition and accumulating cells in the G₁/S cell cycle phase. In the normal physiologic state, one possible mechanism could involve the geranylgeranylated protein, RhoA, which is known to suppress expression of the cyclin-dependent kinase (cdk) inhibitor p21WAF (Fig. 3A). This results in an increase in cdk, subsequent retinoblastoma (Rb) protein hyperphosphorylation, and G₁-to-S transition. In tumor cells exposed to GGTI-298, RhoA is not geranylgeranylated and therefore nonfunctional, so that p21WAF expression is upregulated, cdk decreases, Rb is hypophosphorylated, and the cell is arrested in G₁ [4, 12, 13].

The intent of FTI development was to target farnesylation-dependent Ras signaling and block the oncogenic activity of ras mutations. This approach led to the identification of FTIs as novel agents with demonstrated antitumor effects [4]. It is clear that FTIs block the farnesylation of proteins essential for tumor growth and induce apoptosis of tumor cells, but the precise intracellular protein or the complex interplay of proteins that are the targets of FTIs remains to be established [4, 14]. Several mechanistic studies suggest that inhibition of Ras (particularly K-Ras) farnesylation may not sufficiently explain the antitumor activity of FTIs. For example, FTIs appear to inhibit tumor cell growth independently of the mutation status of Ras in human cancer cell lines [15, 16]. Further, the kinetics of FTI-induced reversal of the transformed phenotype do not correlate with the kinetics of FTI-mediated inhibition of Ras farnesylation in H-Ras-transformed rodent fibroblasts [17]. Finally, despite cross-prenylation (geranylgeranylation) of K-Ras and possi-
bly N-Ras in human cancer cells treated with FTIs, the growth of these cells continues to be inhibited [8, 18-21]. However, several arguments can be made in support of a role for Ras in the antitumor activity of FTIs. For example, in cross-prenylation studies, tumor cell growth may have been mediated by H-Ras, which does not become geranyl-geranylated when human cancer cells are treated with FTIs [19, 20]. Furthermore, there are differences among the various Ras proteins in their effector pathways; for example, H-Ras preferentially activates the phosphoinositide-3-OH kinase (PI3K)/Akt pathway, whereas K-Ras preferentially activates the Raf/mitogen-activated protein kinase/extracellular-signal-regulated kinase (MEK)/Erk pathway [22, 23]. This complex network of pathways may influence the effects of FTIs on tumor cells. Finally, inhibition of wild-type Ras prenylation might also contribute to the antitumor activity of FTIs, particularly in tumors that arise due to aberrant pathways upstream of Ras [4].

Identifying the intracellular pathway or pathways targeted by FTIs is currently the subject of active research. Three lines of investigations are discussed here. The first involves elucidating the cellular mechanism by which FTIs induce arrest of tumor cell cycle progression. Another line of experimentation involves investigating specific intracellular proteins, for example, RhoB, as the targets for FTIs. Finally, the cellular mechanism by which FTIs lead to apoptosis of tumor cells is discussed.

Cell Cycle Progression

FTIs have been shown to interfere with cell cycle progression in human cancer cells [4]. In most cells, FTIs induce accumulation of human cancer cells in the G2/M phase of the cell cycle, but in some cells, FTIs could either induce a G0/G1 block or have no effect on cell cycle progression. Furthermore, it has been shown that the G2/M accumulation is due to inhibition of the prophase/metaphase transition during mitosis [24-27]. For example, after a 24-hour exposure to FTI-2153, cancer cells attempted to cycle from prophase to metaphase in preparation for chromosomal alignment, but were unable to form bipolar spindles, and their chromosomes failed to align to form a metaphase plate (Fig. 3B) [24]. Instead, a rosette chromosome morphology could be visualized with monopolar spindles surrounded by chromosomes. This inhibition of bipolar spindle formation correlated with an accumulation of cells in prometaphase [24, 25].

Further investigation into the mechanism of action of FTIs, therefore, involved the analysis of farnesylated proteins required for prophase/metaphase transition and bipolar spindle formation. Centromere-associated proteins (CENP-E and CENP-F) were the first targets to be investigated, as they are critical to the processes of chromosomal alignment and bipolar spindle formation [25-27]. However, localization of farnesylated CENP-E and CENP-F to the kinetochore was unaffected by FTI-2153 [24], suggesting that these centromeric proteins may not be molecular targets for the inhibition of bipolar spindle formation by FTIs [25]. However, other investigators have argued that these proteins may be involved in the mechanism by which
FTIs inhibit cell cycle progression [26, 27]. Further investigations are needed in order to elucidate the mechanism by which FTIs induce cell cycle arrest in human tumor cells.

**RhoB Is Not the Target for FTIs**

Cellular RhoB is both farnesylated (RhoB-F) and geranylgeranylated (RhoB-GG). Initial studies showed that GGTase I geranylgeranylates as well as farnesylates RhoB, which would eliminate RhoB as a direct target for FTIs [28]. However, a recent study showed that RhoB can be farnesylated by FTase, and its farnesylation is inhibited by FTIs [29]. Nevertheless, the fact that RhoB is farnesylated and geranylgeranylated makes its candidacy as a target for FTI somewhat complex. Although there is some evidence for, there is overwhelming evidence against inhibition of RhoB farnesylation as a mechanism by which FTIs inhibit tumor growth. The fact that, in one study, treatment of cells with FTIs resulted in lower levels of farnesylated RhoB (RhoB-F) and higher levels of geranylgeranylated RhoB (RhoB-GG) [29], coupled with the observation that an RhoB/RhoA chimeric protein that is exclusively geranylgeranylated is growth inhibitory [30], suggest that RhoB-GG may be a mediator of some of the effects of FTIs. Further support for this was provided by experiments using murine fibroblasts that were deleted for the RhoB gene [31]. In those experiments, the ability of FTIs to induce apoptosis was shown to be dependent on RhoB, but the ability of FTIs to inhibit tumor growth in soft agar was completely independent of RhoB. Furthermore, the growth in nude mice of Ras-transformed RhoB(-/-) fibroblasts was less sensitive but not resistant to FTIs [31]. These observations support the suggestion that the FTI-stimulated increase in RhoB-GG protein levels may be important for FTI-induced apoptosis, but not anchorage-independent growth.

There are several key observations that argue against the involvement of RhoB in mediating FTI antitumor activity. First, ectopic overexpression of wild-type RhoB suppressed human tumor growth in soft agar and nude mice. Thus, whether FTI treatment converts RhoB into a growth-inhibitory protein is unclear [32]. Second, RhoB-deficient mouse embryo fibroblasts transformed by H-Ras retained sensitivity to FTI-induced inhibition of growth in soft agar and in nude mice in vivo. This result indicates that RhoB is not an important target for FTI inhibition of anchorage-independent growth, yet FTIs do block this growth property in a wide variety of human tumor cells [16, 22]. Third, in a variety of untreated cell types, it has been established that the proportion of RhoB-GG is greater than that of RhoB-F [29], questioning whether the FTI-induced changes in this ratio could explain the potent antitumor activity of FTIs. Fourth, the most direct evidence against RhoB-F as a target comes from cloning and biologically characterizing forms of RhoB that are either exclusively farnesylated or geranylgeranylated [32]. Those studies showed that RhoB-F is just as potent as RhoB-GG at inhibiting human tumor cell growth. RhoB-F is also more potent than RhoB-GG in inducing apoptosis and inhibiting human tumor growth in soft agar and nude mice [32]. In support of the tumor suppressive role of RhoB, results of a recent study in 19 head and neck cancer patients have shown that RhoB protein levels are high in nonneoplastic adjacent tissue and very low to undetectable in highly invasive carcinoma [33].

Taken together, these observations argue against a mechanism where inhibition of RhoB farnesylation mediates FTI antitumor activity.

**H-Ras/PI3K/Akt/BAD Pathway as a Potential Target for FTIs**

H-Ras has been proposed as a potentially critical target for FTI-induced apoptosis through inhibition of the PI3K/Akt-mediated survival pathways. PI3K is required for Ras transformation [34]. Thus, inhibition of H-Ras farnesylation could be a potential mechanism by which FTIs inhibit human tumor growth in cells that overexpress Akt. To investigate this hypothesis, the ability of FTI-277 to induce apoptosis was examined in the human ovarian carcinoma cell line, OVCAR-3, as well as in other ovarian and pancreatic carcinoma cell lines that overexpress Akt2 (Fig. 4) [34]. In this Akt2-overexpressing human cancer cell line, FTI-277 induced apoptosis [34], and this was rescued by constitutively activated Akt2 [34]. These data suggest that the apoptotic activity of FTIs requires inhibition of the PI3K/Akt cell survival pathways.

**Clinical Trials**

Phase I and II clinical trials have been conducted with several different FTIs [4, 35] as single agents and in combination regimens in patients with a variety of cancers, including hematologic malignancies [36-39] and solid tumors [40, 41]. The activity of one of these, tipifarnib (Fig. 2), was investigated in a phase II trial in 42 heavily pretreated patients with advanced multiple myeloma [42]. Tipifarnib, 300 mg, was administered orally twice daily in 4-week cycles (3 weeks on, 1 week off). Clinical laboratory studies for biochemical correlates (FT, GGT, farnesylation, oncogenic and tumor survival pathways, ras mutation status) were performed at baseline and during the third week of the first cycle of treatment in blood and bone marrow. Of the 42 evaluable patients, 66% showed disease stabilization. Mild-to-moderate fatigue was a common toxicity, occurring in 64% of patients. An evaluation of the activity of tipifarnib and its correlation with clinical response revealed that FT in bone marrow was inhibited in every patient, at
a range of approximately 60%-90%. However, bone marrow GGT activity was not suppressed, supporting the specificity for FT seen in preclinical studies. When protein farnesylation (HDJ-2/DNAJ) in the bone marrow and peripheral blood mononuclear cells (PBMNs) was investigated, tipifarnib clearly inhibited its farnesylation. There was no correlation between inhibition of protein farnesylation and clinical activity of the drug.

In a phase I study in myelodysplastic syndrome, 21 patients were treated with tipifarnib at doses ranging from 600-900 mg/day. There was a 30% objective response rate (three patients with hematologic improvement, two patients with partial response, one patient with complete response); however, there was no correlation among clinical response, dose used, and ras mutation status. This result supports preclinical findings that showed no correlation between ras mutation status and sensitivity of human cancer cells to FTIs. As in the previous study in advanced multiple myeloma, FT activity but not GGT activity was inhibited by tipifarnib and this inhibition was not correlated with clinical response (complete or partial response) [43].

**SUMMARY**

Inhibition of FT and GGT represents a novel mechanism-based approach to cancer therapy. FTIs and GGTIs have shown promising antitumor activity in vitro and in human tumor animal models. In phase I/II clinical studies, response rates on the order of 30% have been seen in hematologic malignancies, such as acute myeloid leukemia and myelodysplastic syndrome, as well as in solid tumors, such as breast cancer [4]. Yet, studies to date have failed to demonstrate a correlation between enzyme inhibitory activity and clinical activity. The fact that no correlation was found between the ability of FTIs to inhibit FTase and clinical activity suggests that only some tumors depend on protein farnesylation for oncogenicity and tumor survival. A key issue to be resolved is the identification of a set of patients that would respond to FTIs. This will be aided by gene-expression profiling and proteomics studies that are currently under way.

The extent of the correlation between clinical response and FTI activity may also be influenced by the marker used for measuring the biochemical activity of FTIs [4, 44]. In some of the earlier phase I trials, FTase inhibition in PBMNs was used as the surrogate marker [4]. In the clinical trials discussed here, prenylation of HDJ-2 was the biochemical marker. Typically, proteins that are farnesylated but not geranylgeranylated, for example, prelamin A, lamin B, and HDJ-2, are used as markers for investigating the effects of FTIs. However, the role of these proteins, if any, in growth, survival, and tumorigenesis is not yet known [4]. A more suitable biochemical marker for correlation studies will likely emerge once the intracellular pathway targeted by FTIs is established.

In conclusion, identifying the tumor molecular signatures that predict response to FTIs and GGTIs is a critical issue to address in future hypothesis-driven mechanistic...
studies. Microarray gene-expression profiling, proteomics, and the elucidation of oncogenic and survival pathways are potential avenues by which these questions could be answered. Additional studies should be developed to determine signaling pathways critical to FTI activities and to identify appropriate clinical end points for future trials [4].

**References**

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