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Review

Farnesyltransferase inhibitors define a role for RhoB in controlling neoplastic pathophysiology

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Summary. A long-standing goal in cancer research is to identify cellular functions that have selective roles in regulating neoplastic pathophysiology. Farnesyltransferase inhibitors (FTIs) are a novel class of cancer chemotherapeutics which have little effect on normal cell physiology but which inhibit or reverse malignant cell phenotypes. FTIs were originally developed as a strategy to inhibit oncogenic Ras, the activity of which depends upon posttranslational farnesylation. However, recent work indicates the antineoplastic effects of FTIs are not linked to Ras inhibition but instead to alteration of RhoB, a small GTPase of the Rho family of cytoskeletal regulators that controls trafficking of cell surface receptors. Rho proteins integrate signals from integrins and cytokine receptors with cell shape via the actin cytoskeleton. A connection between FTIs and Rho alteration is interesting given that histological differences have long been used to define clinical cancer. RhoB is dispensable for normal cell growth and differentiation in mice. Thus, research into the antineoplastic effects of FTIs has led to the identification of a function(s) that is unnecessary for normal cell physiology but crucial for controlling malignant phenotypes.

Key words: Cancer, Malignant transformation, Experimental therapeutics, Signal transduction, Ras

Introduction

Pharmacological inhibitors of the housekeeping enzyme farnesyltransferase (FTIs) inhibit malignant growth in a wide variety of murine models and human tumor cell lines. In contrast, FTIs have little effect on normal cell proliferation, viability, or differentiation. The different responses of normal and malignant cells are interesting because they suggest that FTIs identify a unique feature of neoplastic pathophysiology. Notably, while it has become apparent that FTIs can interfere specifically with malignant growth, it also has become apparent that they do not have to inhibit isoprenylation of Ras to do so. The evidence that the antineoplastic effects of FTIs can be unlinked from Ras and instead linked to Rho is surveyed here. A Rho-based model can explain why FTIs produce such diverse biological responses in different cell and animal models and suggests new ways to apply and interpret the effects of FTIs in the oncology clinic. Possible connections that are emerging between Rho function and the actions and histopathology of cancer cells are also covered in this review. On the basis of recent advances we propose that Rho effector signaling molecules may be useful targets for cancer drug development.

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Farnesyltransferase inhibitors selectively target cancer cell phenotypes

The development of FTIs was based on the need of oncogenic Ras for posttranslational farnesylation (reviewed in Gibbs et al., 1994; Oliff, 1999). This founding discovery initiated the area of protein isoprenylation research and led to the identification of three protein-isoprenyl transferases in cells, farnesyl transferase (FT), geranylgeranyl transferase type I (GGT-I), and geranylgeranyl transferase type II (GGT-II). Each of these housekeeping enzymes catalyze the transfer of farnesyl (C15) or geranylgeranyl (C20) isoprenoids from farnesyl or geranylgeranyl pyrophosphate to cysteine thiol groups in the C-termini of protein substrates. FT and GGT-I share a subunit and similar mechanism of action whereas the action of GGT-II is mechanistically different (Casey and Seabra, 1996; Zhang and Casey, 1996). Small GTPases of the Ras superfamily represent a major fraction of isoprenylated proteins in the cell (~0.5% of the proteome). In general, FT and GGT-I modify Ras and Rho proteins whereas GGT-II modifies Rab proteins. Since all mutant Ras proteins involved in human cancer are modified exclusively by FT, compounds that specifically inhibit FT were sought as a strategy to block the activity of oncogenic Ras in cancer

cells (Gibbs and Oliff, 1997; Oliff, 1999). Most isoprenylated proteins are geranylgeranylated. Also, while there is some overlap in the protein substrate specificities between FT and GGT-I, in cells FT is solely responsible for the transfer of farnesyl isoprenoid and GGT-I is solely responsible for the transfer of geranylgeranyl isoprenoid. Thus, 'pure' FTIs affect only the relatively fewer number of farnesylated proteins in cells. Interestingly, some FT substrates are geranylgeranylated by GGT-I if FT activity is abolished (see below). This effect of FTI treatment, which leads to an increase in overall protein geranylgeranylation in cells, turns out to be important to the mechanism through which FTIs inhibit malignant cell growth, as described below.

In H-Ras transformation models, FTIs inhibit modification of newly synthesized H-Ras protein and suppress anchorage-independent cell growth. Loss of anchorage-independence correlates with reversion to a flat cell phenotype which is permissive for proliferation under anchorage-dependent conditions, with division rates similar to normal cells (James et al., 1993; Prendergast et al., 1994). Notably, little if any toxicity is associated with phenotypic reversion and loss of anchorage-independence under standard *in vitro* culture conditions. Cells transformed by K-Ras and N-Ras are also susceptible to growth suppression by FTIs (although less so than H-Ras-transformed cells), as are many human tumor cell lines that harbor activated K-Ras (Sepp-Lorenzino et al., 1995). Similarly, FTIs block malignancy in a variety of xenograft and transgenic mouse models, where FTIs either impede tumor growth or elicit tumor regression, respectively (Kohl et al., 1994, 1995; Nagasu et al., 1995; Barrington et al., 1998; Liu et al., 1998; Mangues et al., 1998; Norgaard et al., 1999). Significantly, regressions in transgenic animals are not confined to H-Ras models but can also be seen in models where tumorigenesis is driven by N-Ras or K-Ras (Mangues et al., 1998; C. Omer, pers. comm.). Regressions are associated with cell cycle inhibition but also with increased apoptosis (Barrington et al., 1998; Norgaard et al., 1999). This is notable because most FTIs do not induce apoptosis of Ras-transformed cells in vitro unless cells are deprived of either substratum or cytokines (Lebowitz et al., 1997b; Suzuki et al., 1998), events which lead to loss of PI3'K-AKT signals that are crucial for survival of FTI-treated cells (Du et al., 1999c). Significantly, normal cells are not susceptible to FTI-induced apoptosis under the same conditions which lead to killing of Ras-transformed cells, highlighting a difference in survival requirements between normal and Ras-transformed cells (Du et al., 1999c). Based on the fact that AKT status can influence FTI response in certain setting, I have suggested that differences in AKT activity might explain the differences in FTI response seen in xenograft and transgenic models, where cytokine and adhesive environments likely differ (see below). In any case, in support of clinical utility the survival mechanism disrupted by FTIs appears to be largely p53-independent (Lebowitz et al., 1997b; Barrington et al., 1998; Du et al., 1999c). In summary, FTIs exhibit antiproliferative and proapoptotic effects that are quite selective for malignant cells.

Ras alteration is not crucial to the antineoplastic effects of FTIs

Although not widely appreciated, a close examination of the FTI literature reveals that the antineoplastic effects of FTIs can in many instances be separated from their effects on Ras farnesylation (Cox and Der, 1997; Lebowitz and Prendergast, 1998b). The first indication was that the kinetics of FTI-induced phenotypic reversion were simply too rapid to be accounted for by depletion of processed H-Ras from cells (Prendergast et al., 1994). Fully modified H-Ras is long-lived, with a half-life of 24 hours (Ulsh and Shih, 1984). FTIs block modification of modified Ras, not its steady-state level or activity once modified, so 2-3 days of drug treatment is needed to deplete it from cells. However, reversion of H-Ras transformation is achieved within 18 to 24 hours of drug treatment, when modified Ras levels persist at 50% their pretreatment level. Furthermore, once initiated by a single dose of drug, the reverted phenotype persists for days after FT activity and fully modified Ras returns to pretreatment levels (Prendergast et al., 1994). This interesting long-lived phenotype, which resembles a pseudo-differentiation state in its stability, has been traced to the ability of FTIs to upregulate collagen Iα2 period. This stable growth inhibitory collagen isoform must be suppressed by Ras for it transform cells (Travers et al., 1996; Andreu et al., 1998) and that FTIs must derepress to elicit and maintain the reverted phenotype (Du et al., 1999b). Consistent with a Ras-independent effect, the kinetics of this gene induction parallels phenotypic reversion, occurring by 16 hr after drug treatment. The ability of FTIs to induce a long-lived reversion through upregulation of long-lived growth inhibitory protein(s) may have clinical benefit. In any case, neither initiation nor maintenance of reversion to a benign phenotype is correlated with significant steady-state depletion of farnesylated Ras protein.

There is other evidence that Ras alteration can be uncoupled from biological response. First, rodent fibroblasts transformed with N-myristoylated Ras remain susceptible to FTI-induced reversion and suppression of anchorage-independent growth, even though the activity of N-myristoylated Ras is independent of farnesylation (Cox et al., 1994; Lebowitz et al., 1995). Second, while approximately 70% of human tumor cell lines are susceptible to growth inhibition by FTIs, there is no correlation with Ras mutation (Nagasu et al., 1995; Sepp-Lorenzino et al., 1995). Lastly, FTIs can inhibit malignant growth induced by activated K-Ras or N-Ras, but they do not inhibit isoprenylation of these proteins. This unexpected result, which have raised concerns among some investigators whether FTIs will be useful anti-cancer drugs, is explained by the observation that

GGT-I can geranylgeranylate K-Ras and N-Ras in the absence of FT activity (Sepp-Lorenzino et al., 1995; Lerner et al., 1997; Whyte et al., 1997; Mangues et al., 1998; Servais et al., 1998). This "GGT-I shunt pathway" allows oncogenic K-Ras and N-Ras to remain membrane bound and active in FTI-treated cells. Notably, as mentioned above, FTIs still inhibit malignant growth induced by K-Ras or N-Ras. This fact suggests that FTIs may exhibit anti-cancer activity through Ras-independent mechanisms.

The antineoplastic effects of FTIs can be explained by alteration of RhoB

I first noticed that FTIs affected actin stress fibers in normal cells in 1992. Since the kinetics of biological response and H-Ras depletion in the fibroblast model under study did not fit, I hypothesized that alteration of a farnesylated Rho protein may be crucial to the mechanism through which FTIs inhibit malignant cell growth (Prendergast et al., 1994). Subsequent studies focused on RhoB as a paradigm for testing this hypothesis, which was corroborated by several lines of evidence. RhoB is a member of the Rho family that is ~90% similar to RhoA, but which unlike RhoA is located on early endosomes and to a lesser extent on the nuclear membrane and periphery (Adamson et al., 1992; Lebowitz et al., 1995; Zalcman et al., 1995; Lebowitz and Prendergast, 1998a). Consistent with an endosomal location, RhoB functions in trafficking of the EGF receptor through a mechanism that requires recruitment of the Rho effector kinase PRK/PKN to endosomes (Mellor et al., 1998; Gample et al., 1999). The role of RhoB in recruiting the PRK/PKN kinase(s) to endosomal membranes is analogous to the role of Ras in recruiting the Raf kinase to plasma membrane. Unlike most small GTPases proteins, RhoB is short-lived (Lebowitz et al., 1995). Therefore, farnesylated RhoB is depleted rapidly from cells by FTI treatment. One unusual feature of RhoB is that it exists in both farnesylated or geranylgeranylated forms in the cell, in approximately equal populations, for reasons that are unknown.

RhoB has several features arguing it is a crucial FTI target (Lebowitz and Prendergast, 1998b). First, FTI treatment inhibits farnesylation of RhoB and leads to an elevation of geranylgeranylated RhoB, due to the 'shunt pathway' for isoprenylation provided by GGT-I. These events correlate with a change in intracellular localization and loss of the growth-stimulatory activity of RhoB (Lebowitz et al., 1995, 1997a). Thus, although RhoB remains isoprenylated, it is depleted from its normal localization in the cell and likely suffers loss-of-function as a result. Second, Rho is crucial for Ras transformation: dominant inhibitory mutants of RhoB suppress transformation by H-Ras (Prendergast et al. 1995). Third, ectopic expression of N-myristoylated RhoB makes Ras-transformed cells resistant to FTIs (Lebowitz et al., 1995; Prendergast et al., 1995). This observation argues that loss of farnesylated RhoB is part of the mechanism at some level. Lastly, the elevation of geranylgeranylated RhoB (RhoB-GG) elicited by FTI treatment is sufficient on its own to cause phenotypic reversion and growth inhibition (Du et al., 1999a). Since RhoB-GG appears to be mislocalized in cells, for reasons that are unclear, we have hypothesized that RhoB-GG in FTI-treated cells may sequester effectors such as PRK/PKN away from sites of action on endosomal membranes. Recent work has established that RhoB-GG is necessary, as well as sufficient, for apoptotic and antineoplastics effects of FTIs (Liu et al., 2000).

It should be emphasized that the growth inhibitory effects of RhoB-GG are not confined to 'morphological' contexts and are not specific to rodent fibroblast models: RhoB-GG also selectively inhibits the growth of FTI-susceptible human carcinoma cells (Du and Prendergast, 1999). Thus, there is a good correlation between the susceptibility to growth inhibition by FTIs and RhoB-GG. Moreover, RhoB alteration is germane to human epithelial cancer cells, where phenotypic effects may or may not be apparent. Taken together, these studies establish that the antineoplastic properties of FTIs can be traced to alteration of RhoB, and they identify a gain of function aspect of the mechanism involving production of a potentially dominant inhibitory RhoB-GG.

Implications of a Rho-based mechanism for FTI action

One old question is why FTIs should not affect the proliferation and differentiation of normal cells in vitro and in vivo, since Ras is necessary for these processes. This issue may be partly addressed by the action of a GGT-I-mediated 'shunt pathway', which could lead to alternate isoprenylation and maintenance of the function of K-Ras and possibly other FT substrates. However, it is notable that mice that have homozygous deletions of rhoB develop without any apparent defect and are fertile (S. Liu and T. Jessell, unpublished observations). Thus, loss of RhoB does not have consequences for normal cell growth or differentiation, mirroring the lack of any discernable effect of FTI treatment in normal mice. It is also notable that while elevated levels of RhoB-GG can inhibit the growth of FTI-susceptible malignant cells, this same event does not affect the growth of normal cells (Du et al., 1999a; Du and Prendergast, 1999). In summary, a RhoB-based mechanism for FTI action is consistent with the lack of FTI effects on normal cells.

A second question concerns why malignantly transformed cells respond so differently to drug treatment in various preclinical assays. For example, cytostatic responses predominate in xenograft models but cytotoxic responses can be seen transgenic models. The known connections between Rho function and integrin-dependent adhesion signaling offers a new vantage point to address this question. Environmental influences mediated by adhesion might be expected to

determine how cells respond to FTIs if the drugs work by altering a Rho signal(s). In support of this view, our laboratory has shown that the response of H-Ras-transformed Rat1 cells to FTI treatment in vitro can be dramatically altered simply by varying substratum adhesion parameters. In tissue culture dishes, cells revert but continue to proliferate in an anchorage-dependent manner. In soft agar, where neither focal adhesions nor a tensile actin cytoskeleton can be organized, but where integrin-ligand interactions can persist (due to the deposition of ECM by cells in the agar media), cells slow or arrest division but remain viable. Lastly, when placed in frank suspension, which completely deprives integrin-ligand interactions, cells undergo apoptosis (Kohl et al., 1993, 1994; Prendergast et al., 1994; Lebowitz et al., 1995, 1997b). Importantly, all these responses to FTIs are abolished by ectopic expression of N-myristoylated RhoB (Lebowitz et al., 1995, 1997b). Thus, one can establish a link between RhoB alteration and the effects of FTIs in different adhesive environments.

We have suggested that the role of adhesive cues in determining response may also address why malignant cells can not totally destroyed in FTI-treated H-Ras transgenic mice. In this model, tumors can be completely regressed from a histological standpoint. However, latent cells apparently persist in a 'reverted' state, because even after long periods of drug treatment removal from the treatment protocol leads to rapid recurrence of the tumor (Kohl et al., 1995). We suggest that in the periphery of the tumor, where normal adhesion cues may be available (e.g. basement membrane), cells may revert to a benign phenotype upon drug exposure such that they could survive and persist. The flat phenotype seen *in vitro* may occur also *in vivo* such that the 'reverted' but malignant cell could not be identified histologically at this point. In contrast, cells without access to normal adhesion cues would engage an anoikis program and die. Upon drug removal, malignant cells in the 'reverted' and benign state would re-revert, as seen in vitro, allowing the tumor to rapidly re-emerge as observed.

A role for RhoB-GG in the FTI mechanism addresses an apparent conundrum of the model: if FTIs act by blocking Rho function why should they stimulate stress fiber formation, since this would be expected to require Rho activity? In its structure and localization in FTI-treated cells, RhoB-GG partly mimics RhoA. Thus, in FTI-treated cells RhoB-GG may mimic some functions of RhoA, including its ability to induce stress fibers. One can not rule out the possibility that the stress fiber-inducing activity of FTIs is due to loss of function in a different farnesylated Rho protein that opposes the activity of RhoA. However, in support of the first interpretation, ectopic expression of RhoB-GG increases actin stress fiber formation in a manner similar to FTI treatment. In summary, Rho-GG may have both dominant positive and dominant inhibitory functions in cells.

Cancer cell histopathology and Rho function: correlation or causation?

For many years, cancer has been defined by histological criteria and by the propensity of malignant cells to move around the body inappropriately. Given these links, one might expect that Rho proteins would be important in cancer, because of their roles in controlling cell shape and adhesion and because altered adhesive properties are a hallmark of the malignant cell. Evidence and argument can be drawn in support of a causal connection between Rho function and cancer. Rho proteins seem likely to mediate environmental cues that determine where tumor cells go and what they can do, including responses to chemotherapeutics which are in many cases controlled by the particular tissue site of tumor deposition (Killion et al., 1999). Notably, Rho proteins are elevated strongly in cancer cells, including RhoA (Fritz et al., 1999). Commercially available RhoB antibodies known to us do not discriminate very well between the closely-related RhoA and RhoB proteins, so immunohistological experiments using RhoB antibodies need to be controlled carefully. Perhaps the most direct causal link is suggested by evidence that the Rho effector ROCK kinase is important for invasion (Itoh et al., 1999). We hypothesize that RhoB-GG may dominantly inhibit RhoA and other Rho functions based on elevations and mislocalization of RhoB-GG in FTI-treated cells. Lastly, the RhoB effector kinase PRK/PKN has been reported to bind to and alter the substrate specificity of the AKT regulatory kinase PDK1 (Balendran et al., 1999). This observation suggests there may be crosstalk between RhoB-dependent and AKT-dependent survival signals that may be important in malignantly transformed cells. *rhoB*-/- cells under study in our laboratory exhibit alterations in motility, integrin modification, and focal adhesion formation, supporting a role for RhoB in these processes (A. Liu and G.C.P., unpublished observations). In summary, we anticipate that continued investigations of Rho functions in cancer cells will provide additional support for the notion that there is a causative rather than correlative relationship between Rho activity and the histology and pathophysiology of cancer.

FTIs in the clinic

Data from human trials presented at recent oncology meetings by several pharmaceutical companies suggest that systemic exposure produces only limited toxicities (e.g. neutropenia), even at high doses, and that it is quite possible to achieve serum concentrations which are sufficient to effectively inhibit FT activity. The former results are consistent with the lack of toxicity to normal cells in preclinical models. However, while cancer preclinical models do a fair job of predicting toxicity they do poorly at predicting efficacy. Phase II trials will ultimately address this question. It may require patience and persistence to learn how to obtain efficacy in

humans, since mechanistic studies from preclinical models suggest that the cytotoxic activity of FTIs can be inhibited in many ways. Resistance to FTIs can be selected readily *in vitro* by a variety of MDR-independent mechanisms (Kohl et al., 1995; Prendergast et al., 1996), including by mutation of FT in ways that lead to reduced FTI affinity (Del Villar et al., 1999). Drug responses may be shifted also by overexpression of FT itself, an event which has been shown to be sufficient on its own to transform murine 3T3 fibroblasts (Nagase et al., 1999). In addition, Bcl-XL overexpression can block FTI-induced apoptosis, offering another possible mechanism to achieve cytotoxic resistance (Prendergast et al., 1996; Lebowitz et al., 1997b). Other mechanisms of resistance are involved because Bcl-XL-overexpressing cells remain susceptible to growth inhibition by FTIs (Lebowitz et al., 1997b). Lastly, AKT activation can block both the proapoptotic and antitransforming effects of FTIs in cells (Du et al., 1999c). The proapoptotic Bcl-2 family member Bad can be phosphorylated by AKT and then proceed to bind to and inhibit Bcl-XL activity. Therefore, there may exist a common linear pathway involving PI3'K-AKT-BAD-BCL-XL signals which can elicit FTI resistance. While further investigations are needed in this area, the preclinical data predict that FTIs may have lesser efficacy against tumors that have activated AKT (e.g. due to PTEN mutation) or that overexpress antiapoptotic Bcl-2 family proteins. Therefore, knowing the status of these genes in patients under study may help one interpret clinical data.

Efficacy may be improved by combinatorial application with other modalities that promote apoptosis. For example, since PI3'K-AKT activation can mask the proapoptotic effects of FTIs (Du et al., 1999c), inhibiting the PI3'K-AKT pathway may be valuable. One way this might be achieved is to combine FTIs plus PI3'K inhibitors, which exist currently. As noted above, this combination does not interfere with the viability of normal cells even though it leads to a loss of viability of Ras-transformed cells (Du et al., 1999c). Similarly, combinations with classical chemotherapeutics or radiotherapy may be useful. FTIs can sensitize the response of human tumor cell lines when added in combination with cisplatin, taxanes, or gemcitabine (Moasser et al., 1998; Sun et al., 1999). Similarly, FTIs can sensitize transformed rodent cells and human tumor cell lines to gamma irradiation (Bernhard et al., 1996, 1998; Cohen-Jonathan et al., 1999). Ras has been suggested to be a key target in these settings but RhoB may actually be germane. RhoB expression is elevated by DNA damage and other stresses (Fritz et al., 1995; Fritz and Kaina, 1997). Moreover, when transformed by oncogenes, *rhoB-/-* cells exhibit differences in the cytotoxic response to gamma irradiation or DNA damaging drugs (A. Liu and G.C.P., unpublished observations). Thus, the ability of FTIs to sensitize cells to radiotherapy or traditional chemo-therapeutics may also be linked to altering Rho-dependent processes. In closing, preclinical data argue that FTIs may be valuable as chemosensitizers or radiosensitizers even if they have limited efficacy as primary therapeutics.

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