



Non-Ras targets of farnesyltransferase inhibitors: focus on Rho

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Farnesyltransferase inhibitors (FTIs) are a novel class of cancer therapeutics whose development was based on the discovery that the function of oncogenic Ras depends upon its posttranslational farnesylation. Significantly, experiments in animal models have shown that FTIs have promise as nontoxic cancer therapeutics. However, cell biological studies have suggested that FTIs may act at a level beyond that of suppressing Ras function, so the exact mechanism of action has emerged as a question of major interest. Here, we review evidence that proteins other than Ras are important targets for inhibition, summarize findings suggesting a role for farnesylated Rho proteins prompted by studies on RhoB, and suggest a new model for how FTIs exert their biological effects. The 'FTI-Rho hypothesis' proposes that FTIs act in part by altering Rho-dependent cell adhesion signals which are linked to pathways controlling cell cycle and cell survival and which are subverted or defective in neoplastic cells. This model offers a novel framework for addressing the questions about FTI biology, including the basis for lack of toxicity to normal cells, cytotoxic versus cytostatic effects on tumor cells, and the persistence and drug resistance of malignant cells in FTI-treated animals.

Keywords: protein prenylation; transformation; apoptosis; cell adhesion; signal transduction

Original rationale for the development of farnesyltransferase inhibitors as anti-tumor drugs

A potential strategy for combating Ras-mediated cancers was prompted by the finding that oncogenic Ras must be posttranslationally prenylated to transform cells (reviewed in Gibbs *et al.*, 1994). In general, members of the Ras superfamily of small GTPases, which includes Ras, Rho/Rac, and Rab proteins, undergo three types of C-terminal modification at their C-terminal 'CAAX box' motifs, where C is cysteine, A an aliphatic residue and X any amino acid. The first modification is prenylation, in which either farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoid moieties, each precursors in cholesterol biosynthesis, are covalently attached to the cysteine residue of the CAAX motif. The predominant prenylation reaction which occurs in cells is geranylgeranylation, but most Ras proteins and some Rho proteins are farnesylated. Prenylation reactions are carried out by one of three enzymes, namely farnesyl transferase (FT), geranylgeranyl

transferase type I (GGT-I), or geranylgeranyl transferase type II (GGT-II; Rab GGT). FT and GGT-I modify Ras superfamily proteins which have a CAAX motif, whereas GGT-II mediates geranylgeranylation of non-CAAX-containing proteins in a reaction that is mechanistically distinct from the FT and GGT-I reactions (reviewed in Casey and Seabra, 1996; Zhang and Casey, 1996). Prenylation facilitates association with cellular membranes where Ras undergoes further modifications (Cox and Der, 1992) and, in addition, may mediate protein-protein interactions (Marshall, 1993; Zhang and Casey, 1996). After prenylation, CAAX-containing proteins such as Ras undergo C-terminal cleavage, carboxymethylation, and palmitoylation. In the case of Ras, only farnesylation is crucial for oncogenicity (Kato *et al.*, 1992). Therefore, as an indirect strategy to inhibit oncogenic Ras function in tumor cells, many pharmaceutical and academic laboratories have sought chemical agents that selectively inhibit FT and leave cellular geranylgeranylation intact. From a biological standpoint, this project has been very successful, insofar as proof-of-principle experiments have definitively demonstrated the ability of FT inhibitors to block the growth of Ras-dependent tumors in a manner which is nontoxic to normal cells (Gibbs and Oliff, 1997).

Farnesyltransferase inhibitors have selective anti-transforming properties

While several structural classes of farnesyltransferase inhibitors (FTIs) have been developed, most studies reporting biological results have employed CAAX peptidomimetic compounds which exhibit excellent potency, FT specificity, cell penetration, and little normal cell toxicity (recently reviewed in Gibbs and Oliff, 1997; Leonard, 1997; Sebti and Hamilton, 1997a, b). In cell culture models, FTIs have been shown to effectively inhibit H-Ras prenylation and to selectively suppress the anchorage-independent growth of H-Ras-transformed cells (Garcia *et al.*, 1993; James *et al.*, 1993; Kohl *et al.*, 1993). Associated with the loss of anchorage-independent potential is a reversion phenomenon, characterized by cell flattening, enlargement, and acquisition of the morphological and growth regulatory characteristics of nontransformed parental cells (James *et al.*, 1994; Prendergast *et al.*, 1994). Cells transformed by oncogenes such as *src*, that utilize Ras signaling, but not those such as *raf*, whose action is Ras-independent, are also inhibited by FTIs (Kohl *et al.*, 1993; Prendergast *et al.*, 1994).

Consistent with their *in vitro* effects, FTIs block tumor formation in mouse xenograft models (Kohl *et al.*, 1994; Nagasu *et al.*, 1995; Sun *et al.*, 1995) and, more dramatically, regress tumors that arise in transgenic mice which harbor oncogenic *H-ras* genes

(‘ras oncomice’) (Kohl *et al.*, 1995). Remarkably, given that Ras is required for normal cell growth and differentiation, in all *in vitro* and *in vivo* studies to date, FTIs have been observed to be essentially nontoxic even at doses that completely block processing of H-Ras protein. In cell culture, while the anchorage-independent growth of Ras-transformed cells is inhibited, there is essentially no cytotoxicity and at most only slight effects on proliferation (James *et al.*, 1994; Prendergast *et al.*, 1994). Similarly, in mouse models, there is no apparent systemic toxicity at doses capable of causing tumor regression (Kohl *et al.*, 1995; Barrington *et al.*, 1998). Thus, FTIs apparently distinguish and target a unique aspect of transformed cell physiology.

Evidence that non-Ras proteins are important targets for FTIs

While it is clear that FTIs can both inhibit Ras farnesylation and block the transformed phenotype *in vitro* and *in vivo*, results from several studies have raised questions about whether FTIs must actually inhibit Ras function to exert their anti-transforming effects. Thus, an emerging theme in FTI research is that farnesylated targets for inhibition other than Ras are also important. Several lines of evidence illustrate this line of thought. First, the kinetics of phenotypic reversion induced in transformed cells by FTI treatment do not fit with a simple loss-of-function of Ras. Detailed investigation of the reversion process in H-Ras transformed cells indicates that it is essentially complete within 24 h of drug treatment (Prendergast *et al.*, 1994). However, fully processed Ras has a half-life of ~24 (Shih *et al.*, 1982) and its steady-state levels are reduced only ~50% by the time that reversion is complete (due to the inability of the cell to prenylate newly synthesized Ras) (Prendergast *et al.*, 1994). Strikingly, once it is initiated, the reverted phenotype is also maintained without the continued need to depress Ras levels (Prendergast *et al.*, 1994). Thus, FTIs do not have to reduce processed H-Ras levels significantly to either initiate or maintain the reverted phenotype. Second, while it has been suggested that soluble species of oncogenic Ras may exert a dominant inhibitory activity in drug-treated cells, it is unclear whether the generation of such species has much biological impact. Overexpression of unfarnesylated mutant but not normal H-Ras has been reported to partially inhibit MEK activation by fully modified Ras (Miyake *et al.*, 1996). However, the significance of this effect is unclear since unfarnesylated H-Ras does not accumulate to significant steady-state levels in Ras-transformed cells treated with FTI, presumably due to rapid turnover (Prendergast *et al.*, 1994). In addition, while soluble forms of the Ras L61 mutant allele can exert biologically significant effects (Gibbs *et al.*, 1989), soluble forms of the Ras V12 mutant allele used in all published experimental models do not (GCP unpublished results). Thus, whether soluble Ras exerts any biologically significant effect in drug-treated cells is uncertain. Third, cells transformed with oncogenic Ras proteins that are engineered to function independently of farnesylation, due to N-myristylation or C-terminal geranylgeranylation, are still susceptible to anchorage-independent growth inhibition by FTIs (Cox *et al.*, 1994;

Lebowitz *et al.*, 1995; GCP, unpublished results). Fourth, cells transformed by oncogenic K-Ras are still susceptible to anchorage-independent growth inhibition by FTIs, even though K-Ras remains prenylated in the presence of FTI; this phenomenon reflects the fact that K-Ras is geranylgeranylated by GGT-I in drug-treated cells, maintaining its oncogenic activity (James *et al.*, 1995; Lerner *et al.*, 1997; Rowell *et al.*, 1997; Whyte *et al.*, 1997). Lastly, FTIs can inhibit the anchorage-independent growth of many human tumor cell lines in soft agar culture, but there is no correlation between biological susceptibility with the presence of Ras mutations (Nagasu *et al.*, 1995; Sepp-Lorenzino *et al.*, 1995). Since the biological effects of FTIs can be separated to a significant degree from their ability to block Ras function, inhibiting the farnesylation of proteins other than Ras is likely to be important.

Farnesylated Rho proteins may be important targets for FTIs

The list of farnesylated proteins that constitute biologically relevant non-Ras targets of FTIs continues to grow (Cox and Der, 1997). At the current time, one class of appealing targets that have emerged are farnesylated Rho proteins, in particular RhoB, a member of the Rho/Rac family of small GTPases that regulate cytoskeletal actin, focal adhesion formation, cell adhesion signaling, and transcription (reviewed in Symons, 1996; Tapon and Hall, 1997; Van Aelst and D’Souza-Schorey, 1997; Hall, 1998). An initial clue that Rho alteration may be part of the drug mechanism was prompted by the observation that FTIs stimulate stress fiber formation and cell enlargement in normal as well as many transformed cells (Prendergast *et al.*, 1994). RhoB is a member of the Rho family with two features making it a logical target. First, unlike most Rho/Rac proteins, which are geranylgeranylated in cells, RhoB exists in one population that is farnesylated and a second that is geranylgeranylated (Adamson *et al.*, 1992). Second, there is evidence that RhoB is involved in the control of cell cycle and proliferation (Jahner and Hunter, 1991a,b; Zalcman *et al.*, 1995).

The ‘FTI-Rho hypothesis’ proposes that the anti-transforming effects of FTIs are based in part on alteration of the function of RhoB or a related farnesylated Rho protein (Prendergast *et al.*, 1994). Several predictions of this hypothesis were corroborated in later studies. First, FTIs specifically inhibited the farnesylation of RhoB in cells and this effect was correlated with loss of its cell growth-stimulating activity (Lebowitz *et al.*, 1997a). In support of its status as a non-Ras target, RhoB is relocalized in cells by FTI treatment (Lebowitz *et al.*, 1995). Second, fully processed RhoB is short-lived in cells, with a half-life of 2–4 h (Lebowitz *et al.*, 1995; Zalcman *et al.*, 1995), so farnesylated RhoB levels are rapidly reduced by drug treatment. This feature addresses the rapid kinetics of FTI biology mentioned above and supports a model for FTI action based on simple loss-of-function of a farnesylated protein. As a relatively weak FT substrate, one even weaker than H-Ras (Lebowitz *et al.*, 1997a), RhoB offers an ideal target in the sense that its farnesylation is comparatively easy to block.

Third, inhibition of Rho function by expression of a dominant inhibitory mutant genetically mimics pharmacological inhibition, blocking Ras transformation (Prendergast *et al.*, 1995). Thus, even though Ras transformation is associated with stress fiber dissolution (Bar-Sagi and Feramisco, 1986; Ridley and Hall, 1992), certain Rho functions that may be affected by FTI treatment are apparently required for Ras transformation (Prendergast *et al.*, 1994, 1995; Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). Consistent with the possibility that RhoB itself is important for some feature of the transformed phenotype, but dispensable for normal cell function, mice with homozygous deletion of the RhoB gene are viable (S Liu and T Jessell, pers. comm.). Lastly, Ras-transformed cells could be rendered drug resistant by ectopic expression of an N-myristylated RhoB species whose membrane localization is prenylation-independent (Lebowitz *et al.*, 1995). While change-of-function of myr-Rho is a caveat to this result, the simplest interpretation of the data was that the biological effects of FTIs are mediated at least in part by altering RhoB prenylation patterns. Although a role must be considered for other related farnesylated Rho proteins, such as RhoD and RhoE (Foster *et al.*, 1996; Murphy *et al.*, 1996), and other farnesylated proteins generally (Cox and Der, 1997), RhoB represents the first non-Ras target for which there is biochemical and biological evidence that altering its prenylation may be important to the mechanism by which FTIs reverse the transformed phenotype.

Utility of the FTI-Rho hypothesis: identification of cell adhesion as a critical parameter for distinguishing cytotoxic and cytostatic properties of FTIs

To date, perhaps the most dramatic demonstration of the potential of FTIs as anti-cancer agents involve experiments using a transgenic *v-H-Ras* mouse model. In these animals, which harbor an oncogenic *v-H-ras* gene and develop spontaneous carcinomas, FTI treatment led to a dramatic and nearly complete tumor regression (Kohl *et al.*, 1995). However, at the time of this study, the basis for regression was unclear, because FTIs had not displayed cytotoxicity *in vitro* or *in vivo*, even at concentrations significantly beyond the minimal inhibitory concentration (MIC) required to inhibit Ras transformation. Three other issues were also raised by animal experiments. First, resistance to tumor regression was observed in some animals, a phenomenon that could be selected for by repeated cycles of drug exposure and withdrawal (Kohl *et al.*, 1995). Second, even among tumors that appeared to regress completely, cessation of FTI treatment led to a rapid return of the tumor (Kohl *et al.*, 1995), indicating that some malignant cells could persist even while the bulk of the tumor disappeared. Tumor persistence, if also seen with the treatment of human cancer, would require continuous, long-term FTI treatment that could increase side effects and the development of resistance. Finally, while FTIs induced regression in the *v-H-Ras* oncomice model, in xenograph models (Kohl *et al.*, 1994; Nagasu *et al.*, 1995) and against human tumor cell lines (Sepp-Lorenzino *et al.*, 1995; Nagasu *et al.*, 1995, 1996), FTIs appeared to be cytostatic. Therefore,

to maximize the clinical effectiveness of FTIs, it was desirable to determine the basis for cytotoxic *versus* cytostatic effects, how regression was possible, and how tumor persistence and resistance might occur in drug-treated animals.

The FTI-Rho hypothesis offers some explanative power for these issues as well as a new viewpoint to frame questions. Since Rho proteins are implicated in cell adhesion (reviewed in Hotchin and Hall, 1996), Ras can subvert the requirement of normal cells for substratum (Rak *et al.*, 1995b), and Rho is required for Ras transformation (Khosravi-Far *et al.*, 1995; Prendergast *et al.*, 1995; Qiu *et al.*, 1995), a logical experiment was to compare the effects of FTIs on H-Ras-transformed cells under conditions where cell-cell or cell-matrix attachment were favored. In this manner, cell-substratum attachment potential was found to be a crucial parameter in dictating the physiological response to FTIs (Lebowitz *et al.*, 1997c). H-Ras-transformed cells cultured in suspension, where cell-cell but not cell-substratum attachment is possible, respond to FTI treatment by apoptosis instead of reversion. The cell death mechanism was deemed Rho-dependent, because the kinetics and dose response were similar to those for reversion, and because myristylated RhoB blocked apoptosis similar to reversion. Death was p53-independent but inhibited by Bcl-X_L (Lebowitz *et al.*, 1997c), a member of the Bcl-2 family of apoptosis regulators which broadly influence cell death responses. Consistent with these findings, p53-independent apoptosis has been confirmed recently as the predominant mode of regression of *v-H-ras*-induced tumors in transgenic animals (Barrington *et al.*, 1998). Since cell attachment dramatically influences the phenotypic response of Ras-transformed cells *in vitro*, Rho-dependent integrin signaling pathways may prove to be an important realm for understanding the drugs' anti-transforming and anti-tumor properties. It will be important in future work to investigate the effects of FTIs on Rho-dependent integrin signal transduction pathways.

Some recent studies support the likelihood that there are Rho signaling pathways which specifically regulate cell viability. In one study, where recombinant *C. botulinum* C3 exoenzyme was expressed in cells as a method to inactivate Rho function, the results suggested that viability and cell adhesion pathways overlap partly, and that some Rho-sensitive signal or feedback continues to operate in cells even after integrin-matrix interaction and focal adhesion assembly are complete (Bobak *et al.*, 1997). While the relevant signals are undefined, one attractive candidate downstream effector is the transcription factor nuclear factor- κ B (NF- κ B), because it is activated by Rho/Rac family proteins (Perona *et al.*, 1997) and has been implicated in maintaining cell viability (Baichwal and Baeuerle, 1997). It is important to note that in some cell contexts certain Rho proteins can also induce apoptosis (Jimenez *et al.*, 1995). Therefore, the exact mechanism by which Rho induces survival may be complicated, perhaps involving differential functions of Rho family members or a balance between signals which control proliferative and apoptotic responses (Lacal, 1997).

The identification of a possible link between the biological action of FTIs and their effects on Rho-

dependent, cell adhesion-linked cell viability pathways suggests mechanisms for tumor cell persistence and drug resistance. First, results from the *in vitro* model suggest that cells which lack appropriate substratum attachment *in vivo* might respond to FTI treatment by apoptosis, thereby causing tumor regression. Tumor cells at privileged locations, perhaps in the periphery of the tumor, where normal anchorage cues exist and could be accessed by drug-treated cells, might instead achieve appropriate attachment status, revert to a benign phenotype, and survive. A second explanation for persistence and resistance is suggested by the Bcl-X_L experiment. Genetic alterations that block the apoptotic response, such as Bcl-X_L overexpression, might arise in a percentage of tumor cells thereby allowing them to survive drug treatment and regrow upon FTI removal. The recurrent tumor might then be resistant to tumor regression when FTI treatment is reimplemented. The ability of Bcl-X_L to defeat FTI-induced death raises clinical concerns, because Bcl-X_L is overexpressed in a substantial fraction of human carcinomas (Krajewska *et al.*, 1996). It will be important in future work to determine whether there is any *in vivo* correlation between drug resistance and aberrant overexpression of Bcl-2 family genes, defects in Rho targets of FTIs, or dysfunction of Rho-dependent signals that control cell viability.

FTI-induced suppression of VEGF expression in tumor cells: another cytotoxic mechanism linked to Rho?

In addition to variant cell adhesion properties, another unique aspect of tumors is their dependence on angiogenesis. Since FTIs caused nontoxic tumor regression but did not completely eliminate the tumor (Kohl *et al.*, 1995), two logical questions were whether Ras could affect the expression of any angiogenic factors which are deregulated in palpable tumors, and whether FTIs might reverse such effects. In two rodent epithelial models, it has been demonstrated that oncogenic H-Ras can upregulate secretion of vascular endothelial growth factor (VEGF) (Rak *et al.*, 1995a; Larcher *et al.*, 1996), a crucial angiogenic factor in cancer, and that FTI treatment can suppress this effect (Rak *et al.*, 1995a). Although it is not yet clear this mechanism operates *in vivo*, there are additional *in vitro* observations which would support such a role and which are consistent with a role for Rho inhibition. First, VEGF message levels that are elevated in H-Ras-transformed Rat1 cells are subject to suppression by FTI treatment, with kinetics consistent with RhoB inhibition/alteration (GCP, unpublished observations). Second, H-Ras-transformed cells which are selected for biological resistance to FTIs (Prendergast *et al.*, 1996), or which are rendered FTI resistant by expression of myristylated RhoB (Lebowitz *et al.*, 1995), each exhibit resistance to FTI-induced suppression of VEGF overexpression (GCP, unpublished results). Thus, in addition to possible effects on cell adhesion-dependent viability, FTIs might also mediate cell killing by indirectly inhibiting a pathway that leads to VEGF overexpression and therefore maintenance of tumor vasculature.

Recently, phosphatidylinositol 3-kinase (PI3'K) and the Akt kinase have been implicated in a transcrip-

tional mechanism by which hypoxia activates VEGF expression in Ras-transformed cells (Mazure *et al.*, 1997). Experiments using dominant inhibitory mutants suggest that Rho is not necessary for Ras to upregulate VEGF expression at the transcriptional level (W Du and GCP, unpublished results). However, a role for Rho in the regulation of VEGF at some other level by FTIs in transformed cells can not be ruled out, because Ras can also upregulate VEGF by a second mechanism that is independent of PI3'K (Arbiser *et al.*, 1997). In future work, it will be important to explore the linkage between the biological and genetic response to FTIs, and to determine whether their ability to suppress VEGF overexpression is a cause or effect of either tumor cell reversion and/or cytostatic effects induced by FTI treatment.

Why don't FTIs affect normal cells?

Given that FT is a housekeeping enzyme and that there are numerous farnesylated cellular proteins which are important to cell function (e.g. lamins (Farnsworth *et al.*, 1989)), it is puzzling that FT can be inhibited without detrimental effects to normal cells. Consistent with the notion that some non-Ras aspects of the transformed phenotype are an important target of drug treatment, FTIs do not inhibit the proliferation of normal cells which are known to be Ras-dependent, nor do they block the ability of Ras to drive, for example, the differentiation of PC12 cells (Dalton *et al.*, 1995). The basis for how this distinction is possible is unclear, although the response of transformed and tumor cells has been correlated with an inhibition of MAPK activation (James *et al.*, 1994; Sepp-Lorenzino *et al.*, 1995; Nagase *et al.*, 1996). However, considerations of Rho function and 'cross-prenylation' effects which occur in cells treated with FTIs offer a new vantage point to consider how FTIs might selectively target the transformed state.

Rho regulates certain cell adhesion signaling pathways, so the different responses of normal and transformed cells may relate to differences in their requirements for these pathways. Normal cells exist in a state where their survival and proliferation is wedded to physiological adhesion, but this is not the case for transformed and tumor cells. Indeed, to become malignant, tumor cells must evolve resistance to apoptotic triggers normally engaged by loss of physiological attachment. If FTIs force cells to make a physiological attachment response, perhaps by altering an integrin activation pathway (Schwartz *et al.*, 1995), normal cells might not be expected to be affected, since they already exist in a state where they are physiologically attached. This would not be the case for a transformed or tumor cell, where an attempt at physiological attachment may lead to reversion, due to successful matrix engagement, focal adhesion formation, and cytoskeletal organization, or to apoptosis, due to the generation of an anoikis ('homelessness') signal characteristic of a normal cell (Frisch and Ruoslahti, 1997). A prediction of this model is that one might not expect 'real' human tumor cells to efficiently engage a death program in response to FTIs, since such cells must have necessarily evolved some resistance to anoikis to progress to malignant (i.e.

invasive) status. Consistent with this possibility, although FTIs can inhibit the anchorage-independent growth of human tumor cells (Sepp-Lorenzino *et al.*, 1995), they do not undergo apoptosis similar to H-Ras-transformed cells when completely deprived of anchorage (i.e. when cultured on polyHEMA-coated substrate; PFL, unpublished observations). Thus, the utility of FTIs against clinical cancer may rely solely upon cytostatic effects, which predominate in xenograph and non-*v-H-Ras* transgenic models as well in human tumor cell lines (Kohl *et al.*, 1993; Nagasu *et al.*, 1994; Sepp-Lorenzino *et al.*, 1995; Miquel *et al.*, 1997; Barrington *et al.*, 1998).

Although cell adhesion properties might help understand how normal cells could escape the cytotoxic effects of FTIs, it is unclear why such cells should escape the drugs' cytostatic effects. This issue may be partly due to cross-prenylation of certain important proteins, such that in the absence of FT their function is maintained by their ability to be geranylgeranylated by GGT-I (a sort of prenylation shunt pathway). Two examples of proteins that are crossprenylated in this way are RhoB and K-Ras, which become preferentially geranylgeranylated in cells following FTI treatment (Lebowitz *et al.*, 1997a; Rowell *et al.*, 1997; Whyte *et al.*, 1997). In yeast, there is strong evidence that cell survival and proliferation can be supported by cross-prenylation: growth inhibition caused by deletion of the FT β subunit can be suppressed by overexpression of the related GGT-I β subunit (Trueblood *et al.*, 1993). If Ras-related proteins that are normally geranylgeranylated can compensate for the loss of farnesylated Ras in growth regulation, then cell proliferation may proceed relatively normally. At least one such protein with features of such a 'toxicity stopgap' molecule has been identified, TC21/R-Ras-2 (Graham *et al.*, 1996), and there is evidence that protein geranylgeranylation but not farnesylation is crucial for cell cycle transit in animal cells (Vogt *et al.*, 1996). Why normal and transformed or tumor cells should respond differently to cross-prenylation patterns, given what is currently known, however, is unclear.

Cytostatic effects of FTIs: role for accumulation of geranylgeranylated RhoB (a gain of function mechanism?)

As mentioned above, it is clear that FTIs can block the proliferation of tumor cells even if they can not kill them (Kohl *et al.*, 1994; Nagasu *et al.*, 1995; Sepp-Lorenzino *et al.*, 1995; Sun *et al.*, 1995; Miquel *et al.*, 1997; Barrington *et al.*, 1998). How are cytostatic effects mediated? While cell cycle arrest or retardation presumably underlies this phenomenon, the mechanism awaits definition. Some linkage to cell adhesion signaling may be germane, because varying the culturing conditions for H-Ras-transformed cells not only reveals apoptotic properties of FTIs, but also distinguishes whether growth inhibition occurs or not. Thus, when cells are cultured under conditions where only cell-cell adhesion is permitted, FTIs induce apoptosis (Lebowitz *et al.*, 1997c); if they are cultured in soft agar, where cells can deposit matrix but can not generate actin tension or spread, FTIs arrest cell proliferation but do not induce cell death (Kohl *et al.*, 1993); lastly, when cells are cultured on plastic, where

cells can deposit matrix and spread, FTIs induce reversion (flattening) but have little effect on cell proliferation (Prendergast *et al.*, 1994). While it must be emphasized that not all FTI responsive transformed cells nor human tumor cell lines display this range of phenotypes, on the basis of the extensive studies of H-Ras-transformed cells it seems reasonable to suggest that subtleties of integrin-dependent attachment and/or spreading signals, which are probably universally subverted in cancer, may contribute to FTI-induced growth arrest as well as apoptosis. In future work, it will be important to learn whether cells regain some feature of matrix-dependent cell cycle-apoptosis regulation following FTI treatment.

The simplest model for how FTIs act is that they indirectly cause loss-of-function of farnesylated target proteins. However, because FTI treatment increases the levels of geranylgeranylated RhoB (RhoB-GG), as well as reduces the levels of farnesylated RhoB (RhoB-F), it is possible that accumulation of RhoB-GG species with an altered cell localization may be a part of the drug mechanism (Lebowitz *et al.*, 1997a). In this manner, FTI action in tumor cells may be comprised of gain-of-function as well as loss-of-function effects. There is evidence that the growth-regulatory properties of RhoB-F and RhoB-GG are different: FTIs inhibit the growth promoting activity of RhoB even though RhoB-GG persists in drug-treated cells where RhoB-F has been depleted (Lebowitz *et al.*, 1997a). A precedent for the notion that differential prenylation alters the growth regulatory functions of small GTPases can be found in studies on H-Ras, in which H-Ras-GG has been shown to inhibit and H-Ras-F to potentiate cell proliferation (Cox *et al.*, 1992). Consistent with the possibility that RhoB-GG is growth-inhibitory, ectopic expression of a solely geranylgeranylated form of RhoB in Ras-transformed cells causes reversion and loss of anchorage-independent growth potential in soft agar, but not in polyHEMA culture, and this effect is associated with induction of the CKI p21 WAF1 (W Du, PFL and GCP, manuscript in preparation). These results directly support the possibility that there may be two arms to the FTI mechanism, one which involves loss of farnesylated functions such as RhoB-F, perhaps related to apoptosis, and a second involving gain of geranylgeranylated functions such as RhoB-GG, perhaps related to cell cycle and/or cell growth inhibition. The latter mechanism may prove to be more clinically important in applications for advanced cancers, where apoptotic responses are usually defective or suppressed.

If FTIs target Rho for inhibition, why do they induce actin stress fibers in normal and transformed cells?

The induction of increased levels of RhoB-GG by FTIs may also explain why the drugs induce stress fibers in normal cells (Prendergast *et al.*, 1994; Manne *et al.*, 1995), as well as how cytostatic effects may be mediated in tumor cells. Given that Rho proteins cause stress fiber formation, loss of RhoB function in Ras-transformed cells might have been expected to lead to the disappearance rather than the appearance of actin stress fibers. However, since FTIs increase levels of RhoB-GG, which has a more RhoA-like character, one aspect of FTI treatment is to increase levels of a

RhoA-like function that may promote stress fiber formation. As noted above, RhoB-GG overexpression partially reverts the morphology of Ras-transformed cells and inhibits their anchorage-independent growth capacity. Therefore, accumulation of RhoB-GG may provide a mechanism to explain the cytostatic effects of FTIs observed in xenograph models and human tumor cell lines (Kohl *et al.*, 1994; Nagasu *et al.*, 1995; Sepp-Lorenzino *et al.*, 1995; Miquel *et al.*, 1997), where apoptotic responses are lacking. It is unclear why gain of RhoB-GG associated with stress fiber formation would be compatible with anchorage-dependent but not anchorage-independent growth. Nevertheless some insights into cytostatic effects might be offered by comparing the functions of RhoB-GG and RhoB-F, or other proteins whose prenylation patterns are similarly altered by FTI treatment (e.g. K-ras) in normal and transformed cells. While differences in localization of these proteins may be sufficient to dictate different functions, biochemical binding specificities might also contribute, since two RhoB-binding proteins have been identified, RhoGDI-3 (Zalcman *et al.*, 1996) and DB1 (Lebowitz and Prendergast, 1998), which are sensitive to prenylation status and which can discriminate RhoA and RhoB from each other, despite the exceptionally high structural similarity between these proteins (~90%). In future work, it will be important to investigate the biological consequences of different types of prenylation on various candidate targets of FTIs, and whether these properties are affected by neoplastic transformation.

Why is FTI-induced phenotypic reversion so long-lived?

An intriguing feature of FTI biology is that drug treatment is required to initiate but not maintain the phenotypic reversion of H-Ras-transformed cells which occurs in normal culture. For example, a single application of FTI is sufficient to revert H-Ras-transformed cells to a normal morphology and anchorage-dependent proliferative status that persists for up to 7–10 days (Prendergast *et al.*, 1994), even though the drug is not maintained in culture. Strikingly, cells remain reverted even though FT activity and farnesylated H-Ras return to their initial steady-state levels within 2–4 days. Experiments using protein synthesis inhibitor cycloheximide have shown that inhibition of new protein production significantly

delays FTI-induced reversion, and, consistent with effects on gene expression, a set of FTI-regulated genes has been identified (PFL and GCP, unpublished results). If gene induction is part of reversion, as this finding suggests, then long-lived products required for reversion may lengthen its persistence such that drug maintenance is unnecessary. Identification of FTI-regulated genes may provide insights into this phenomenon, as well as offer a starting point for bottom-up investigation of target-to-gene regulatory pathways altered by FTI treatment. A role for Rho proteins in such pathways can be considered, based on the ability of RhoA and RhoB to activate serum response factor (Hill *et al.*, 1995; Lebowitz *et al.*, 1997b) (although in the case of RhoB this effect does not require protein prenylation (Lebowitz *et al.*, 1997b), and on the potential ability of RhoB to associate with and inhibit the activity of certain transcription factors (Lebowitz and Prendergast, 1998).

Future prospects

While it is impossible to predict the fate of FTIs as they enter clinical trials, it must be admitted that their biological properties are fascinating and potentially informative. If FTIs prove to be effective for treatment of human cancer, further study of their cellular effects will be instrumental for optimizing their application and combating or avoiding drug resistance which has been observed to arise *in vitro* and in animal models (Kohl *et al.*, 1995; Sepp-Lorenzino *et al.*, 1995; Prendergast *et al.*, 1996). Even if they prove to be clinically problematic, they offer an excellent probe of biological mechanisms that are selective for the transformed or malignant phenotype. Thus, continued investigation of their mechanism of action may help identify selective prevention strategies. By targeting a transformation-specific mechanism(s), a 'holy grail' of cancer biology research, FTIs will continue to provide important tools for cancer researchers.

Acknowledgements

We thank Ulrich Rodeck for discussion and Kapaettu Satyamoorthy for criticizing the manuscript. GP is the recipient of an American Cancer Society Junior Faculty Award and is a Pew Scholar in the Biomedical Sciences. PL is the recipient of a fellowship award from Merck and Co, Inc.

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