Activation of the PI3′K-AKT Pathway Masks the Proapoptotic Effects of Farnesyltransferase Inhibitors

Wei Du, Aixue Liu, and George C. Prendergast

The Wistar Institute, Philadelphia Pennsylvania 19104

Abstract

Farnesyltransferase inhibitors (FTIs) usually cause growth inhibition, but in certain preclinical settings they have been shown to induce apoptosis, a clinically desirable response. In this study, we show that the proapoptotic effects of FTIs in Ras-transformed cells are masked by activation of phosphatidylinositol 3′-kinase (PI3′K) or AKT, which are controlled by cytokines and integrins. The results implied that FTIs disrupt a signal that is crucial for survival of malignant cells, but not normal cells, if the PI3′K-AKT pathway is inactivated. Our findings have implications for clinical applications of FTIs where apoptotic responses would be preferred.

Introduction

The antitransforming properties of FTIs, a novel class of cancer therapeutics, have been widely investigated. FTIs were originally developed with the aim of inhibiting the posttranslational prenylation and oncogenic activity of Ras (1–4), although it has become apparent that Rho and possibly other cellular proteins are also important targets and oncogenic activity of Ras (1–4), although it has become apparent that Rho and possibly other cellular proteins are also important targets. Recent work in a variety of preclinical models has established conclusively that FTIs can effectively reverse malignant phenotypes and even cause tumor regression (8–18). A significant question has been how range is progression possible in vivo because FTIs have not generally been found to be cytotoxic to either normal or malignant cells in vitro. In a previous study, we showed that FTIs are cytotoxic to Ras-transformed cells if they are deprived of substrate attachment, implicating an adhesion signal(s) as a requisite for survival in the presence of drug (19). Here, we report that FTIs can also be rendered cytotoxic by cytokine deprivation, and that adhesion or cytokine signals, which are controlled by the PI3′K-AKT pathway, are responsible for the survival of drug-treated Ras-transformed cells. Notably, normal cells are not susceptible to FTI-induced apoptosis and do not rely on the PI3′K-AKT pathway for survival in the presence of drug, pinpointing a difference in survival mechanisms. The findings of this study provide proof-of-principle for combinatorial applications of FTIs to exploit differential survival signals and target malignant cells for cell death.

Materials and Methods

Cell Culture. The Rat1/ras cell system used in this study has been described (16, 19). Rat1/ras derivatives were obtained by modified calcium phosphate cotransfections with pcDNA3.1/hyg, a hygromycin-resistance gene vector (Intronigene) that was added at a 10:1 ratio with indicated transgene vectors, followed by selection in 100 μg/ml hygromycin B (BMB). At least three cell lines of the same type were assayed, and representative results were presented. Vectors encoding HA-tagged wild-type AKT-1 and myristoylated AKT-1 have been described (20). Where indicated, the FTI L-744,832 was added to cell culture media at a final concentration of 10 μM, and the specific PI3′K inhibitor LY 294002 (Calbiochem) was added to a final concentration of 7 μM.

Apoptosis Assays. (25) × 10^6 cells were seeded into 10-cm dishes and treated 12–14 h later with either FTI L-744,832 or methanol carrier. After incubation for 18–24 h, the cells were harvested, fixed, and stained with propidium iodide for flow cytometry analysis, as described (6, 19). The appearance of Rat1/ras cells with sub-G1 phase DNA has previously been shown to be correlated with two other markers of apoptosis-associated DNA degradation induced by FTIs, including chromatin condensation and the production of nucleosomal DNA ladders (19).

Jnk Kinase Assay. Cell extracts were prepared by lysis in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% glycerol, 1% NP40, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM sodium PPi, 1 mM sodium vanadate. Jnk kinase assay was performed with lysates essentially as described (21). Kinase assays were carried out for 30 min at room temperature in the presence of 10 μCi [γ-32P]ATP (New England Nuclear) and 4 μM unlabelled ATP in 25 μl of reaction buffer containing 20 mM Heps (pH 7.5), 10 mM MgCl2, 10 mM MnCl2, 1 mM DTT, and 2 μg GST-c-Jun (1–79) as substrate, then fractionated by SDS-PAGE and visualized by autoradiography.

Western Analyses. Cell extracts were prepared in NP40 lysis buffer, fractionated by SDS-PAGE, and analyzed by standard Western blotting methods (22) using anti-HA antibody 12C5 (BMB) at 2 μg/ml, anti-Flag antibody M2 (Kodak) at 2 μg/ml, and using the conditions recommended by the vendor for anticaspase-3 (Transduction Laboratories; catalogue no. C31720), anti-β-tubulin (ICN Biomedicals; catalogue no. 65952), and anti-Jnk1 (Santa Cruz Biotechnology; catalogue no. sc-474-G). Proteins were visualized by incubation with a 1:1000 dilution of horseradish peroxidase-conjugated anti-IgG secondary antibody (BMB) and a chemiluminescence kit (Pierce Co.).

Results and Discussion

FTIs cause Ras-transformed rodent fibroblasts (Rat1/ras cells) to revert to a benign state that supports anchorage-dependent growth (16). However, this response is converted to massive p53-independent apoptosis if cells are deprived of substrate attachment (19). Subsequent observations showed that FTIs could also induce apoptosis of attached Rat1/ras cells if they were deprived of serum (see Fig. 1, A and B). Supraphysiologic concentrations of insulin substituted efficiently for serum, converting the response back to reversion, implicating activation of the IGF1 receptor as a sufficient event to promote survival and lead to reversion (see Fig. 1, A and B). The kinetics of FTI-induced apoptosis elicited by serum deprivation were identical to those caused by adhesion deprivation in the presence of serum (data not shown), consistent with a similar mechanism. Thus, loss of either an integrin-mediated or IGF1-mediated signal led to the reliance of Ras-transformed cells on a remaining survival mechanism(s) that was disrupted by FTI treatment. In cells destined to undergo apoptosis,
Jnk-1 was activated and pro-caspase-3 was cleaved. In vitro kinase assays showed that neither Jnk-1 activity nor steady-state expression was affected by serum deprival in Rat1/ras cells, but that Jnk-1 activity was elevated within 6 h of treatment in the absence of serum (see Fig. 1C). Pro-caspase-3 was also unaffected by either FTI treatment or serum withdrawal, but a combination of these treatments led to reduction of the levels of pro-caspase-3 in a manner consistent with its cleavage and activation as a prelude to apoptosis (see Fig. 1D). Loss of pro-caspase-3 was not an artifact of gel loading or general degradation of the cell extract because β-tubulin levels were unaffected. To evaluate possible p53 involvement, we compared the response of a previously characterized Rat1/ras derivative that overexpresses a dominant inhibitory mutant of p53, termed Ras/p53mut, with the corresponding vector control line Ras/vect (19). Ras/vect and Ras/p53mut cells were both susceptible to apoptosis when deprived of serum and exposed to FTI, but the extent of death was slightly reduced in the latter (see Fig. 1E). Thus, p53 was largely dispensable, similar to the situation where apoptotic susceptibility was caused by substratum deprival (19). We concluded that FTIs induced apoptosis of Ras-transformed cells if they were deprived of a cytokine-dependent survival signal.

We investigated a role for PI3’K in suppressing the apoptotic potential of FTIs because PI3’K mediates important survival signals from both integrins and the IGF1 receptor (23, 24). We also examined and compared the response of several cell lines resistant to the growth inhibitory effects of FTI, including normal Rat1 fibroblasts, v-Raf transformed Rat1 cells (Rat1/raf cells), and Rat1/ras cells ectopically expressing the antiapoptotic Bcl-2 family protein Bcl-XL (all of which have been described previously; Refs. 10, 16, and 19). We reasoned that if a serum-regulated PI3’K-dependent survival mechanism was responsible for masking FTI-induced apoptosis, then inhibition of PI3’K in the presence of serum should unleash this property. This prediction was confirmed with a potent and specific inhibitor of PI3’K in cells, LY 294002 (referred to below simply as LY). In the presence of serum, cells treated with either FTI or LY maintained viability similar to controls, whereas cells treated with both agents detached from substratum, displayed morphological signs of apoptosis, and sustained extensive DNA degradation (see Fig. 2A and data not shown). Under low serum conditions, LY exaggerated the apop-
totic response to FTI treatment and Bcl-XL suppressed this response to control levels (see Fig. 2C). Significantly, neither Rat1 nor v-Raf-transformed Rat1 cells (Rat1/raf cells) were susceptible to apoptosis under the same conditions, which led to the demise of Rat1/ras cells (see Fig. 2C). Thus, the survival of Rat1/ras cells was unique in its requirement for activation of at least one of two mechanisms, one of which was inhibited by FTIs and the other of which was inhibited by LY. We concluded that the ability of FTIs to induce apoptosis was limited by PI3K activity.

We next examined a role for the serine-threonine kinase AKT (PKB) in mitigating FTI-dependent death, because AKT is a key effector of PI3K (25–27). AKT activation is controlled by membrane recruitment, which is mediated by interaction of the AKT pleckstrin domain with 3′-phosphate-containing phosphatidylinositols generated by PI3K activation. AKT is activated in a PI3K-independent manner by N-myristoylation, which mediates constitutive binding to membranes (28). Therefore, to determine whether AKT activation was sufficient to mediate the inhibitory effects of serum on FTI-induced apoptosis, we ectopically expressed in Rat1/ras cells an N-myristoylated version of AKT-1 (Myr-AKT) or a wild-type version of AKT-1 (AKT WT; Ref. 20) to control for nonspecific effects of overexpression. Several cell clones expressing HA epitope-tagged proteins were obtained that had similar properties (see Fig. 3A). Control and AKT WT-expressing cells remained susceptible to apoptosis following FTI+LY treatment, but Myr-AKT-expressing cells were protected from death under the same conditions (see Fig. 3B). In the presence of 10% FCS, control or AKT WT-expressed cells responded to FTI
sub-G1 phase DNA is plotted as the percentage of apoptosis. The data represent the mean processed for flow cytometry. The proportion of cells in the population that displayed with 10% FCS were left untreated or treated 24 h with FTI and LY and then harvested and expressing Rat1/ras cells to FTI-induced apoptosis. Cells cultured in media supplemented recognizes the HA epitope on each AKT polypeptide.

B activity nor does inhibition of NF-κB is not fully active, Ras-transformed cells depend on a Rho-dependent survival signal. FTIs apparently target a survival mechanism important to Ras-transformed cells, but not to normal or Raf-transformed cells. We have previously demonstrated that Rho alteration is a crucial step for the antitransforming effects of FTIs, but whether Rho participates in this survival mechanism is not yet known. Rho can activate NF-κB (31), and NF-κB is reported to mediate survival in Ras-transformed NIH3T3 cells (32). However, FTIs do not affect NF-κB activity nor does inhibition of NfκB by overexpression of an IκB “super-repressor” affect survival of Rat1/ras cells in the presence or absence of FTIs. However, consistent with some role for Rho alteration in the survival mechanism, activation of AKT activity by oncogenic Ras can be inhibited in transient cell transformation assays by FTI treatment or by coexpression of geranylgeranylated RhoB,6 which mediates the antitransforming effects of FTIs (6). Fig. 4 presents a model for how FTIs may impede Rho-dependent survival signals. How PI3K–AKT activation deactivates FTI-mediated apoptosis is still unclear. AKT may directly phosphorylate and inhibit the activity of key death machinery components (e.g., Bad or caspase-9; Refs. 33 and 34). Alternately, other PI3K-regulated events may be important, such as Bak down-regulation, which is required for Ras transformation of certain cells (35). In any case, the results of this study suggest that efforts to inhibit the PI3K–AKT pathway may unmask the proapoptotic effects of FTIs in malignantly transformed, but not normal, cells.

The findings of this study suggest insights into the basis for the cytostatic versus cytotoxic effects of FTIs in different preclinical models (11–13, 15, 17, 36). Cytostatic responses may be inherent in xenograft models where AKT is activated (e.g., by PTEN mutation), such that the apoptotic potential of FTIs is compromised. In contrast, cytotoxic responses may remain intact in transgenic mouse models, where malignancy is driven by specific genetic alterations that are more limited than those in xenograft assays. Two predictions of our findings are: (a) less advanced cancer cells where AKT is not acutely activated will retain proapoptotic responses to FTIs, thereby exhibiting more pronounced responses to monotherapeutic application, and (b) inhibition of AKT or its key effectors will improve drug efficacy in more advanced tumors in preclinical and clinical settings. In future work, it will be important to learn whether there is a correlation between FTI response and dysregulation of the PI3K–AKT pathway in cancer cells. If so, it may be useful to know the status of this pathway to interpret data from clinical trials.

Acknowledgments

We are grateful to A. Oliff and G. Hartman for providing FTI L-744,832, D. Ballard for the mutant IκB “super-repressor” vector, and M. Chou for AKT activity.

Fig. 4. Model. Activated Ras stimulates signals to PI3K and Rho that synergize to promote cell survival and proliferation. Rho promotes proliferation by suppressing p21WAF1 (37) and promotes survival by a second mechanism. FTIs interrupt proliferation by inhibiting Rho, through the production of RhoB-GG, a geranylgeranylated species of RhoB that has dominant inhibitory properties (6). RhoB-GG may interfere with survival in part by affecting AKT activity (see text), perhaps by interfering with the ability of the Rho effector PRK2 to regulate PDK1-dependent activation of AKT (38), and in part by affecting pathways that act in parallel to AKT. Ras promotes PI3K and AKT activity (B), but full activation requires signals from integrins and cytokines, such as IGF1 (A). If AKT is not fully active, Ras-transformed cells depend on a Rho-dependent survival signal.

5 W. Du, unpublished observations.
6 A. Liu, unpublished observations.
References


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