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Review

Protein farnesylation in mammalian cells: effects of farnesyltransferase inhibitors on cancer cells

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Abstract. Protein farnesylation, catalyzed by protein farnesyltransferase, plays important roles in the membrane association and protein-protein interaction of a number of eukaryotic proteins. Recent development of farnesyltransferase inhibitors (FTIs) has led to further insight into the biological significance of farnesylation in cancer cells. A number of reports point to the dramatic effects FTIs exert on cancer cells. In addition to inhibiting anchorage-independent growth, FTIs cause changes in the cell cycle either at the G1/S or at the G2/M phase. Furthermore, induction of apoptosis by FTIs has been reported. FTIs also affects the actin cytoskeleton and cell morphology. This review summarizes these reports and discusses implications for farnesylated proteins responsible for these FTI effects.

Key words. Protein farnesylation; farnesyltransferase inhibitor; cell cycle; apoptosis.

Introduction

Posttranslational modification of proteins by the addition of a farnesyl group has emerged as an important event for the function of a variety of proteins, including Ras, the γ-subunit of some heterotrimeric G proteins and nuclear lamins [1–5]. After farnesylation, these proteins undergo further C-terminal modification events that consist of the removal of three C-terminal amino acids and carboxyl methylation (fig. 1). These C-terminal modifications facilitate membrane association of these proteins. In addition, farnesylation has been shown to be important for protein-protein interaction [5, 6]. Farnesylation is important for the function of these proteins. For example, an oncogenic Ras is incapable of transforming cells without farnesylation [7, 8].

Farnesylation is catalyzed by protein farnesyltransferase (FTase), an enzyme which recognizes the CaaX motif (C is cysteine, a is aliphatic amino acid and X is usually serine, glutamine or methionine) present at the C-termini of substrate proteins [3]. FTase transfers a farnesyl group from farnesyl pyrophosphate to the cysteine in the CaaX motif forming a thioether linkage (fig. 1). FTase is a heterodimer consisting of α and β subunits. The crystal structure of FTase revealed that the enzyme consists mainly of α helices with the β subunit forming a barrel structure [9]. Residues involved in the recognition of substrates have been defined by mutational analyses [9, 10]. FTase contains one zinc ion per active site that is directly involved in catalysis [11]. A closely related enzyme, protein geranylgeranyltransferase type I (GGTase-I), recognizes the C-terminal CaaL motif (similar to the CaaX motif except that the C-terminal residue is leucine or phenylalanine) present in proteins such as RhoA, Rac and Cdc42 [3].

Recently, small molecule inhibitors of FTase have been developed as anticancer drugs [1, 12–14]. Preclinical

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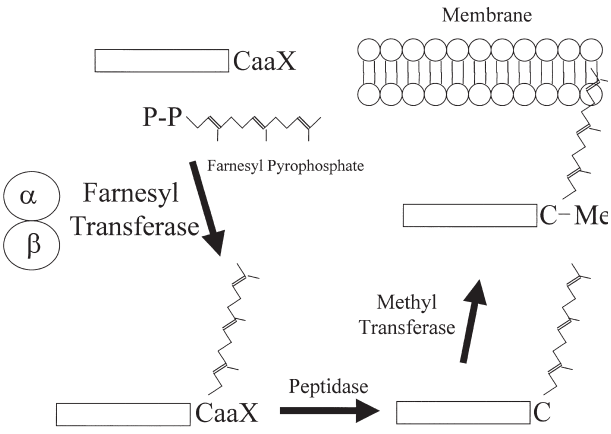


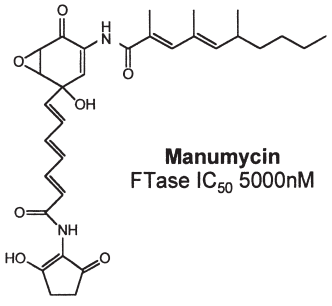
Figure 1. C-terminal modification of CaaX ending proteins. Proteins ending with the C-terminal CaaX motif will be farnesylated by protein farnesyltransferase. The modified protein will undergo further C-terminal processing events that include removal of the three C-terminal amino acids, carboxyl methylation. The mature form has increased affinity to the membrane.

studies utilizing xenografts and transgenic mice showed that these farnesyltransferase inhibitors (FTIs) inhibit the growth of tumors or even regress tumors without causing major side effects. Currently, FTIs are being evaluated by clinical trials [15–17]. The development of FTIs was also an important event in the study of protein farnesylation, since FTIs provided valuable tools to investigate the biological significance of protein farnesylation in mammalian cells. The compounds currently used in clinical trials are highly specific to FTase, as much higher concentrations are needed to inhibit a closely related enzyme, GGTase-I. Use of these compounds enabled investigation into the effects of specifically inhibiting farnesylation in mammalian cells.

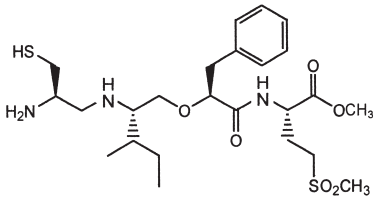
In the past several years, a variety of reports have been published that described the effects of FTIs on cancer cells. It has been reported that FTIs cause dramatic effects including morphological changes, cell cycle changes and induction of apoptosis. This review summarizes these developments and discusses farnesylated proteins involved in the FTI effects.

Farnesyltransferase inhibitors

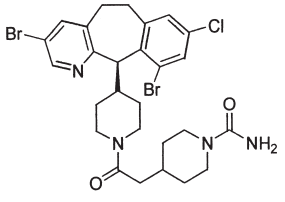
Some representative FTI compounds are shown in fig. 2. The two compounds at the top are examples of the first-generation drugs. We identified manumycin from a screen of culture media from a large variety of microbial organism [18]. The screen utilized a microbial assay based on a yeast mutant [19]. The mutant cells are growth arrested due to the activation of a farnesylated protein, Ste18p. Inhibition of farnesylation allows the growth of the yeast cells. Another approach taken to identify FTI was to look for substrate analogues. A particularly suc-



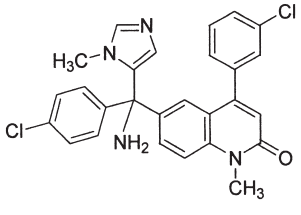
Manumycin
FTase IC_{50} 5000nM



L-739,749
FTase IC_{50} 240nM



SCH 66336
FTase IC_{50} 2nM



R115777
FTase IC_{50} 2nM

Figure 2. Structure of FTI compounds. Some representative FTI compounds are shown. Manumycin and L739,749 represent first-generation FTI compounds. L-739,749, shown here, is a prodrug, and it is an acid form (L-739,750) that is active in inhibiting FTase. SCH66336 and R115777 are used in clinical trials. The IC_{50} values for FTase inhibition are shown.

cessful strategy was to develop CaaX peptidomimetics. This approach began with the realization that a tetrapeptide with the CaaX sequence inhibits FTase activity [20]. This observation was quickly followed by the finding that a tetrapeptide CVFM serves as a nonsubstrate inhibitor [21]. Modifications to make the peptides resistant to protease degradation and to make them cell permeable led to the development of CaaX peptidomimetics such as L-739,749 [22–25]. Finally, the third approach was to screen chemical libraries using an in vitro FTase assay. This resulted in the identification of powerful FTIs such as tricyclic inhibitors [26].

Two compounds shown at the lower part of this figure, SCH66336 [27] and R115777 [28], represent more recent compounds. They inhibit FTase with an IC_{50} (concentration needed to achieve 50% inhibition) of approximately 2 nM. On the other hand, SCH66336 does not inhibit GGTase-I at concentrations as high as 50 μM. Likewise, R115777 shows only 40% inhibition of GGTase-I at 50 μM. These compounds are currently used in clinical trials. In addition, there are other compounds such as BMS-214662 [29] and FTI-2148 [30] that exhibit excellent potency. BMS-214662 is also evaluated in clinical trials.

Effects of FTIs on cancer cells

FTIs cause dramatic effects on cancer cells. As schematized in Fig. 3, these effects can be classified into mainly four different types. First, FTIs inhibit anchorage-independent growth of a wide variety of cancer cells. Second, FTIs cause changes in cell cycle progression. The effect could be either at the G1/S phase or at the G2/M phase depending on the cell line examined. Third, FTIs induce apoptosis of cancer cells when the cells are exposed to low serum conditions or are inhibited in their attachment to the extracellular substratum. Finally, FTIs are reported to cause morphological changes and induce formation of actin stress fibers.

FTIs inhibit anchorage-independent growth

One of the transformed phenotypes of cancer cells is their ability to grow in soft agar [31]. FTIs are capable of inhibiting this anchorage-independent growth. This was demonstrated first with H-*ras*-transformed cells [22–25]. On the other hand, FTIs were much less efficient in inhibiting the soft agar growth of *raf*- or *mos*-transformed cells [22,23,32]. Also, FTIs were much less efficient in inhibiting soft agar growth of NIH3T3 cells transformed with oncogenic Ras that is modified by alternative lipids such as a geranylgeranyl or the fatty acid myristate [32]. These results demonstrate the ability of

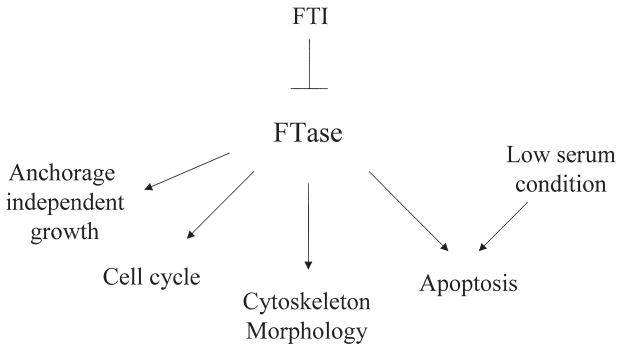


Figure 3. Overall effects of FTI on transformed cells.

FTIs to inhibit H-*ras*-activated phenotypes. On the other hand, FTIs were much less efficient in inhibiting soft agar growth of K-*ras*-transformed cells; these cells required at least one order of magnitude higher concentration of FTI than H-*ras*-transformed cells [33–35]. Another interesting example is ST88-14, a cell line established from malignant schwannoma of a neurofibromatosis type I (NF1) patient [36]. FTIs were effective in inhibiting soft agar growth of this cell line [36]. Upregulation of Ras activity due to a deficiency in neurofibromin, which functions as a GTPase activating protein for Ras appears to be responsible for this sensitivity.

FTI's ability to inhibit anchorage-independent growth is not limited to *ras*-transformed cells. Sepp-Lorenzino et al. [37] examined a variety of human cancer cell lines for their sensitivity to L-744,832. The soft agar growth of over 70% of all tumor cell lines tested (42 cell lines) was inhibited by 2–20 μM FTI. FTI also inhibited anchorage-dependent growth of a variety of human cancer cell lines. No correlation was observed between response to FTI and the origin of the tumor cell. In addition, no correlation was observed between the *ras* mutation status and the response to the FTI. These results show that FTI has activity against many types of human cancer cell lines, including those with wild-type *ras*. A correlation between the presence of wild type p53 and an increased FTI sensitivity was reported [35].

FTIs cause accumulation of G0/G1 phase cells in some cell lines

One of the major effects of FTIs consistently observed in a number of cancer cell lines is alteration of the cell cycle. In contrast to GGTase-I inhibitors, which block most human tumor cells in G0/G1, the cell cycle effects of FTIs depend on the cell lines used [35, 38, 39]. Some cells respond to FTIs by inducing accumulation of G0/G1 phase cells with a concomitant decrease in S-phase cells. Other cell lines show accumulation of G2/M phase cells by the treatment with FTIs. The cell cycle profile of some

Table 1. Cell cycle effects of FTI.

	% age of cells in G0/G1		% age of cells in G2/M		% age of cells in S	
		+ FTI		+ FTI		+ FTI
Exp. 1 (SCH56582)						
LNCaP	57	91	10	3	33	6
MDA-MB468	50	70	14	20	36	10
PA1	54	74	15	9	31	17
HeLa	62	72	11	15	27	13
HL60	34	41	51	50	15	9
Exp. 2 (SCH66336)						
NIH3T3/H-ras	60	80	5	5	35	15
T24	75	90	7	2	18	8
HCT116	49	29	12	36	39	35
NCI-H460	49	43	11	25	40	32
MCF-7	36	32	26	36	38	32
MIA Pa Ca-2	52	34	13	34	35	32
T47-D	47	49	16	17	37	34
Exp. 3 (FTI-277)						
T24	37	63	26	20	37	17
HT1080	32	78	24	18	44	4
Calu-1	53	34	16	42	31	24
Colo-357	51	43	15	28	34	29
SKBr3	59	52	13	22	28	26
A-253	59	52	13	22	28	26
MDA-MB231	55	60	17	16	28	24
Panc-1	55	46	21	24	24	30

Percentage of cells in G0/G1, G2/M or S phase are shown for FTI treated (+FTI) and untreated cells. Exp. 1 is from Tamanoi, unpublished results. Exp. 2 is from [35]. Exp. 3 is from [39].

cell lines is unaffected by FTIs. Table 1 summarizes these results.

We have examined the effects of SCH56582 on a variety of human cancer cell lines (Exp. 1). Significant enrichment of G0/G1 phase cells was observed with a prostate cancer cell line, LNCaP; a breast cancer cell line MDA-MB468; an ovarian cancer cell line PA1; and HeLa cells. A concomitant decrease in S-phase cells was observed in these cases. The percentage of G2/M phase cells, on the other hand, was not significantly affected. We have also shown that SCH56582 causes significant increase of G0/G1 phase cells and a slight increase of G2/M phase cells with K-*ras*-transformed NRK cells [40]. Ashar et al. [35] reported that SCH66336 caused accumulation of G0/G1 phase cells in H-*ras*-transformed NIH3T3 and the human bladder carcinoma cell line T24 (Exp. 2). Vogt et al. [38, 39] reported that treatment with FTI-277 resulted in the accumulation of G0/G1 phase cells with T24 and the fibrosarcoma cell line HT1080 (Exp. 3). Another farnesyl transferase inhibitor, L-744,832, was also found to interfere with cell cycle distribution. It could induce cell cycle arrest at both the G1/S and G2/M checkpoints in astrocytoma cell lines [41]. Cell cycle arrest was accompanied by the induction of $p21^{WAF1/CIP1}$ [41].

Sepp-Lorenzino and Rosen [42] showed that FTI L-744,832 inhibited proliferation of MCF7 cells and caused an increase in the proportion of G1-phase cells at the expense of cells in S phase. The G1 arrest was accompanied by hypophosphorylation of retinoblastoma protein (pRb) and inhibition of cyclin E- and A-associated kinase activities. On the other hand, Cdk4 kinase activity remained unchanged. FTI induced a decline in the steady-state level of cyclin A and an increase of cyclin D1 level, whereas the level of cyclin E and D3 remained largely unchanged. Induction of $p21^{Waf1/Cip1}$ was observed following the FTI treatment that was accompanied by an increase in the steady-state levels of $p21^{Waf1/Cip1}$ messenger RNA (mRNA). This transciptional activation of $p21^{Waf1/Cip1}$ appears to depend on p53, as revealed from studies characterizing the $p21^{Waf1/Cip1}$ promoter. Experiments to decrease the p53 level by expression of the E6 gene of the human papilloma virus 16 also supported this idea. Knockout of $p21^{Waf1/Cip1}$ alleles in HCT116 rendered these cells resistant to FTI-induced decrease in Cdk activity and pRb hypophosphorylation. These observations suggest that the inhibition of cyclin E-associated kinase activity and induction of pRb hypophosphorylation by FTI require the p53-dependent induction of $p21^{Waf1/Cip1}$. It is, however, important to point out that FTI still caused inhibition of anchorage-dependent and -independent growth of HCT116 cells expressing HPV16 E6 or lacking the $p21^{Waf1/Cip1}$ gene [42]. Thus, the $p21^{Waf1/Cip1}$ induction constitutes only one of the pathways through which FTI inhibits cell growth.

FTIs were also observed to inhibit cell cycle progression at the G1 phase in studies using transgenic mice. Administration of L744,832 to mammary tumor virus (MMTV)-v-H-*ras* transgenic mice resulted in an increase in G1-phase cells with a corresponding decrease in the S-phase fraction [43]. With mouse mammary tumor virus-transforming growth factor-α (MMTV-TGFα) and MMTV-TGFα/neu transgenic mice, L744,832 induced regression of mammary tumors [44]. Analyses of tumors showed that FTI caused an increase in G1 phase and a decrease in S-phase cells [44]. In addition, the tumor regression was associated with $p70^{S6K}$ inhibition. Rapid inhibition of $p70^{s6k}$ by FTI was observed with mouse keratinocytes, prostate cancer cell line LNCaP cells and breast cancer cell line MDA-MB468 cells [45, 46].

FTIs cause accumulation of G2/M-phase cells in some cell lines

Accumulation of G2/M-phase cells by SCH66336 was observed with the colon cancer line HCT116, lung carcinoma cell line NCI-H-460, breast cancer cell line MCF7 and pancreatic tumor line MIA $PaCa^{-2}$ (exp. 2) [35]. Treatment with FTI-277 caused accumulation of G2/M phase cells with lung carcinoma cell lines Calu-1 and A-549 as well as with the pancreatic carcinoma cell line Colo357 and breast cancer cell line SKBr3 (Exp. 3) [38, 39].

The effects of FTIs at the G2/M phase of the cell cycle have been further investigated using lung cancer cell lines. Ashar et al. [47] reported that SCH66336 induced a G2/M-pause in the lung carcinoma cell line A549. Immunohistochemistry showed that prophase and metaphase cells accumulated upon FTI treatment. Crespo et al. [48] carried out detailed analysis of the effects of FTI-2153 on G2/M phase progression with two lung cancer cell lines, A-549 and Calu-1. Normally, progression of the cell cycle during the M phase begins with prophase when the nuclear membrane is disassembled, and DNA condensation and separation of mitotic bipolar spindle assembly begin. This is followed by metaphase when the chromosomes are aligned at the metaphase plate and the mitotic bipolar spindles are formed. At anaphase, depolymerization of spindles begins and the chromosomes are pulled toward each pole. FTI-treated cells, in contrast, showed a very different appearance. They accumulated in mitosis, with chromosomes forming a rosette configuration in which microtubules radiate from the center [48].

G2/M accumulation by FTIs is particularly interesting in light of the observed synergy of FTI with microtubule-disrupting agents. FTI compound L-744,823 and taxol could act synergistically to inhibit tumor cell growth and arrest cells in metaphase [49]. Although treatment with up to 300 nM taxol alone caused moderate growth inhibition of MCF-7 cells in a 4-h treatment, addition of FTI caused a dramatic increase in growth inhibition. A similar synergy between FTI and taxol was observed with MDA-MB468 breast cancer, T47D breast cancer and DU145 prostate cancer cells [49]. Another microtubule-stabilizing agent with little structural similarity to taxol, epothilone, also synergized with FTI, suggesting that there is a mechanistic relationship between FTI and microtubule-stabilizing agents [49]. Immunohistochemistry with anti-tubulin and anti-centromere antibodies revealed the presence of abnormal chromosome alignment and disordered spindle apparatus that is consistent with metaphase arrest. A similar synergistic effect of FTI and microtubule drugs was reported by Suzuki et al. [40], who showed that vincristine synergized with SCH56582 to enrich G2/M phase cells in v-K-*ras*-transformed NRK cells. Shi et al. [50] examined the synergistic effects of taxanes and SCH66336 using a variety of tumor cell lines as well as xenografts and transgenic mice. This study showed that SCH66336 synergized with paclitaxel to inhibit the proliferation of 10 out of 11 tumor cell lines. These sensitive cell lines are MDA-MB-468 (breast), DLD-1 (colorectal), NCI-H460 (lung), PA-1 (ovarian), DU-145 (prostate), LNCaP (prostate), AsPC-1 (pancreatic), BxPC-3 (pancreatic), MIA PaCa2 (pancreatic) and PANC-1 (pancreatic). In the NCI-H460 lung cancer xenograft model, combination of FTI and paclitaxel resulted in 86% inhibition of tumor growth and was more effective than therapy with either single agent [50]. Mammary tumors developed in the wap-*ras* transgenic mice are known to be resistant to paclitaxel therapy [51]. Oral treatment with SCH66336 sensitized the tumor to paclitaxel chemotherapy, and tumors in mice treated with FTI and paclitaxel underwent regression [50].

FTIs induce apoptosis of cancer cells

Another major effect of FTIs observed with a number of cancer cell lines is induction of apoptosis. This cell death is characterized by the appearance of apoptotic morphology, subG1 peak in FACS analysis, chromosomal DNA fragmentation and chromatin condensation [52–54]. FTIs induce apoptosis preferentially in oncogene-transformed cells and in tumor cells. We have shown that SCH56582 induces apoptosis of v-K-*ras*-transformed normal rat kidney (NRK) cells but not of nontransformed NRK cells [52]. Similarly, L-744,832 induces apoptosis of H-*ras*-transformed rat embryonic fibroblast Rat-1 cells but does not induce apoptosis of wild-type Rat-1 cells [53]. We have examined 30 different human cancer cell lines for their sensitivity to FTI-induced apoptosis [unpublished observations]. This analysis showed that more than 50% of cell lines tested underwent apoptosis by the treatment with FTI. Cell lines that showed a sig-

nificant degree of apoptosis include leukemic, colon cancer, prostate cancer, stomach cancer and pancreatic cancer cell lines. We observed that cancer cell lines with mutant p53 are also sensitive to FTI, suggesting that the FTI-induced apoptosis is p53 independent. In addition, cell lines with wild-type *ras* were also sensitive to FTI-induced apoptosis. Thus, FTIs are capable of inducing apoptosis of a wide variety of human cancer cell lines.

FTI-induced apoptosis may explain regression of tumors observed with xenograft and transgenic mice studies. Barrington et al. [43] showed that L-744,832 was capable of inducing regression of tumors in the nude mouse xenograft models. This regression was due to apoptosis, as detected by the increase of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL)-positive cells. The increase of apoptotic cells by FTI was detected in *ras*/*p53*+/+, *ras*/*p53*–/–, and *ras*/*myc* model mice [43]. These results suggest that apoptosis of tumors in these model mice is not dependent on p53. Induction of apoptosis by L-744,832 was also reported with MMTV-TGFα and MMTV-TGFα/*neu* transgenic mice [44]. Induction of apoptosis by SCH66336 was detected with wap-*ras* transgenic mice harboring activated H-*ras* [55]. Significant levels of apoptosis were detected that were associated with the regression of mammary and salivary gland tumors.

FTI-induced apoptosis is tightly regulated by the caspase cascade which consists of a series of cysteine proteases. Apoptosis of HL-60 cells by SCH56582 was inhibited by caspase inhibitor z-VAD-fmk [56]. Similarly, apoptosis of ovarian cancer PA-1 cells by FPT inhibitor III was inhibited by caspase inhibitor z-VAD-fmk [57]. Furthermore, apoptosis of KNRK cells induced by SCH56582 was inhibited by z-DEVD-fmk, a specific inhibitor of caspase-3 [52]. Caspase-3 is a key regulator to trigger a variety of apoptotic changes including nuclear condensation and chromosomal DNA fragmentation, and this enzyme is critical for FTI-induced apoptosis. We showed that human breast cancer MCF-7 cells, which lack caspase-3 activity due to a deletion in exon3 of the caspase-3 gene, were resistant to SCH56582-induced apoptosis [52]. However, expression of exogenous caspase-3 sensitized MCF7 cells to SCH56582-induced apoptosis [52]. During apoptosis induced by FTI, caspase-9 is activated in addition to caspase-3, but caspase-8 is not activated, indicating that FTI-induced apoptosis is dependent on caspase-9 rather than caspase-8 (unpublished). Also, FTI did not activate caspase-1 [52].

The activation of caspase-9 is initiated by cytochrome *c* released from mitochondria into the cytosol followed by the formation of a protein complex, apoptosome, that consists of pro-caspase-9, Apaf1 and cytochrome *c*. This complex activates caspase-9. We have shown that treatment with SCH56582 induces cytochrome *c* release in v-K-*ras*-transformed KNRK cells and in human leukemic HL-60 cells [52,56]. FTI also induces changes in mitochondrial membrane potential [56]. This was demonstrated by using $DiOC_6$, a dye that accumulates in mitochondria by the membrane potential. Treatment of HL-60 cells with SCH56582 resulted in the appearance of cells with decreased accumulation of $DiOC_6$ [56]. The cytochrome *c* release and mitochondrial membrane potential change appear to occur prior to the caspase activation, since the addition of a pan-caspase inhibitor, z-VAD-fmk, did not inhibit these events [56]. These results demonstrate that FTI stimulates the cytochrome *c*/caspase-9/caspase-3 pathway to induce apoptosis (fig. 4). Effects of FTI on Bcl2 family proteins have been examined in some cases, but no systematic analysis has been reported. Increased expression of Bax and Bak in astrocytoma cells treated with L-744,832 was reported [41]. Upregulation of Bax and Bcl-xs has been reported with human ovarian cancer cells treated with FPT inhibitor III [54].

Enhancement of FTI-induced apoptosis

Although FTIs are competent in inducing apoptosis of cancer cells, this is masked under normal growth conditions. FTIs induce apoptosis efficiently when cells are exposed to the media with low concentration of serum or are prevented from attaching to the extracellular substratum [52, 53]. These results suggest that a survival pathway prevents cells from undergoing apoptosis by FTIs. One of the survival pathways activated in a variety of cancers is the phosphatidylinositol 3-kinases (PI3 kinase)/Akt pathway, which phosphorylates Bad and procaspase-9 [58]. Inhibition of this pathway is expected to enhance FTI-induced apoptosis. In agreement with this idea, Du et al. [59] showed that FTI in combination with a PI3 kinase inhibitor, LY294002, induced apoptosis of H-*ras* transfected Rat1 cells under normal growth conditions. We have shown that PI3 kinase inhibitors such as LY294002 and wortmannin are efficient in enhancing FTI-induced apoptosis with a number of cell lines [56]. Several-fold enhancement of FTI-induced apoptosis by PI3 kinase inhibitor, LY294002 was observed with a leukemic cell line, CEM; prostate cancer cell line LNCaP, ovarian cancer cell line, OV2008; and colon cancer cell line, HT29. Furthermore, introduction of a myristoylated version of constitutively active Akt (PKB) into H-*ras*-transformed Rat-1 cells inhibited FTI-induced apoptosis [59].

Jiang et al. [60] proposed that FTI directly inhibits the PI3 kinase/Akt pathway. Their argument is based on the observation that FTI-277 inhibited the IGF-1- or serum-stimulated PI3 kinase/Akt2 activation in COS7, OVCAR-3 and A2780 cells, and that ovarian cancer cells overexpressing Akt2 are highly sensitive to FTI-induced apoptosis. Liu et al. [61] found that Akt1 activation by

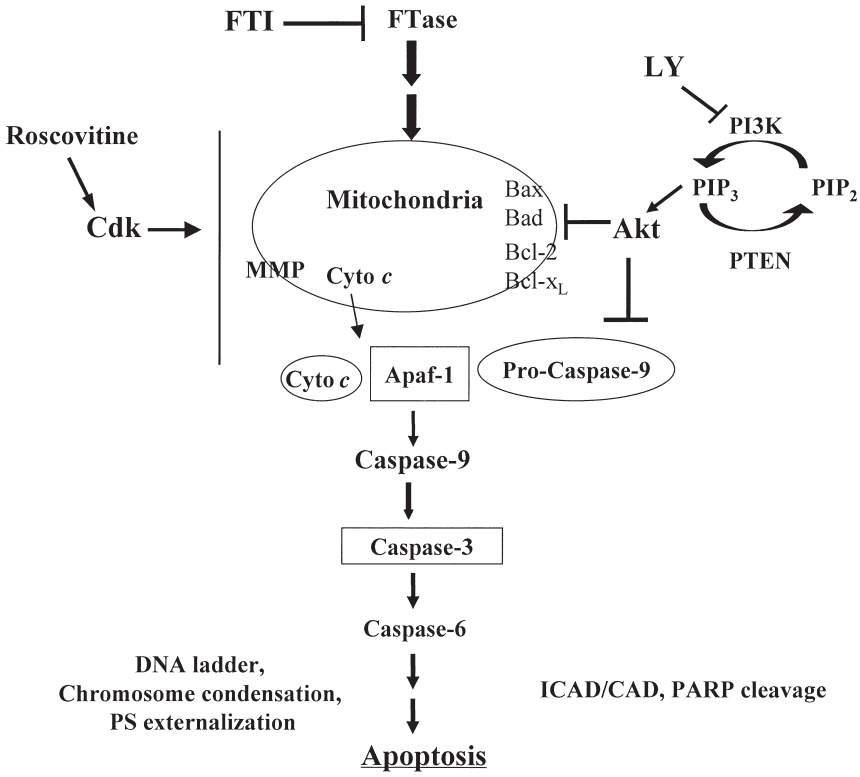


Figure 4. A scheme for the induction of apoptosis by FTI. FTI-induced apoptosis involves a decrease in mitochondrial membrane potential (MMP) and release of cytochrome *c* into the cytosol. Cytochrome *c* forms a complex with Apaf-1 and pro-caspase-9 that results in the activation of caspase-9 and caspase-3. Caspase-3 activates caspase-6 as well as other effectors involved in apoptotic changes. This FTI-induced apoptosis can be enhanced by LY294002, which inhibits PI3 kinase. Roscovitine, a Cdk inhibitor, is also capable of enhancing the FTI-induced apoptosis with some cell lines.

oncogenic Ras or EGF could be disrupted by FTI treatment in COS and MCF7 cells, repectively. However, in other systems, FTI does not affect the PI3 kinase/Akt pathway. Liu et al. [62] reported that L-744,832 could not suppress basal activity of endogenous Akt1 in either RhoB(+/–) or RhoB(–/–) mouse embryonic fibroblast (MEF) cells. In addition, SCH56582 did not affect the Akt phosphorylation status in LNCaP and PC3 cells [56]. Furthermore, SCH56582 can induce apoptosis of these cells without affecting the Akt phosphorylation status [56]. These results suggest that even if FTI suppresses the PI3 kinase/Akt pathway in some cell lines, the suppression of Akt activity is not the only mechanism for the induction of apoptosis by FTI.

We have identified another reagent that enhances FTI-induced apoptosis with some cancer cell lines. By screening a variety of kinase inhibitors for their ability to enhance FTI-induced apoptosis of HL-60 cells, we found that a Cdk inhibitor, roscovitine or olomoucine, synergizes with FTI to induce apoptosis [56]. FTI and roscovitine synergized to release cytochrome *c* from mitochondria. Synergistic effects of roscovitine on FTI-induced apoptosis were observed with another leukemic cell line, CEM, as well as with a prostate cancer cell line, LNCaP [56]. Roscovitine and olomoucine inhibit activities of Cdk 1, 2 and 5; however, they also inhibit other enzymes. Further study is needed to explore how these compounds enhance FTI-induced apoptosis.

An interesting effect of nitric oxide on FTI-induced apoptosis was detected using a breast cancer cell line, MDA-MB468 [63]. High concentrations of DETA-NONOate, a donor for nitric oxide, caused inhibition of proliferation and apoptosis of MDA-MB468 cells. Activation of caspases 3 and 9 and cytochrome *c* release accompanied this apoptosis. Addition of SCH56582 potentiated this effect and enabled apoptosis to occur even at lower concentrations of DETA-NONOate. Importantly, apoptosis induced by the combination of FTI and DETA-NONOate was preferential with transformed cells.

FTI effects on actin stress fibers and cell morphology

Whereas the growth inhibitory effects of FTIs are well documented, their effects on cytoskeleton, i.e. microtubules, intermediate filaments and actin filaments, are less clear. The use of different cell lines and different FTI compounds makes the comparison difficult. Here, we

aim to document the observations concerning effects of FTIs on cytoskeleton.

There have been a number of reports that point to the effect of FTIs on actin stress fibers. Treatment of H-*ras*-transformed fibroblasts with FTI resulted in morphological reversion to an enlarged or flattened untransformed phenotype [26, 32, 33, 64] and induction of actin stress fibers [33, 64]. On the other hand, FTI showed no effect on actin filaments in *raf*-transformed Rat-1 cells [64]. Furthermore, in the case of v-K-*ras*-transformed cells, FTI did not induce actin stress fiber formation [40]. Recently, Reuveni et al. [65] showed that a constitutively active MEK mimicked the effect of *ras*-transformation and prevented FTI HR12-induced actin filaments in H-*ras*-transformed Rat1 cells. Taken together, these observations suggest that the effects of FTI on actin cytoskeleton are due to the inhibition of oncogenic H-Ras. A likely scenario is that the inhibition of the Raf/MEK/Erk pathway interferes with the expression of Rnd3/Rho8/RhoE, an endogenous inhibitor of Rho function [66]. Effects of FTIs on actin stress fibers in untransformed NIH3T3 cells are controversial. Treatment with FTI-277 or BMS-186511 did not affect actin stress fibers [33, 38]. On the other hand, a slight increase of actin filaments in Rat1 cells upon treatment with FTI, L-739,749 or L-744,832 was reported [62, 64].

Microtubules, extending throughout the cytoplasm, are formed from tubulin, and regulate the location of organelles and other cell components. We previously showed that tricyclic FTI, SCH56582, induces morphological change with significant induction of microtubule assembly in v-K-*ras* transformed NRK (KNRK) cells [40]. Furthermore, the morphological change induced by SCH56582 was inhibited by the addition of vincristine at a concentration that did not affect cell cycle progression [40]. Tubulin has no CaaX motif, and further work is needed to investigate the molecular mechanism by which FTI induces microtubule assembly.

Intermediate filaments, including keratins, vimentin, neurofilaments and nuclear lamins, are polymers of fibrous molecules, providing cells with mechanical strength. Among a number of intermediate filaments, nuclear lamins are known to be farnesylated [67,68]. Treatment of CHO-K1 cells with the FTase inhibitor BZA-5B inhibited farnesylation of prelamin A and lamin B, which results in the accumulation of prelamin A in the nucleus [69]. However, BZA-5B did not have significant effect on the cell growth or viability of CHO-K1 cells. No effects on the formation of the nuclear lamina were observed in HeLa cells that had been treated with BZA-5B for up to 23 days. Upon immunofluorescent visualization, it was observed that lamin A and B were still being assembled normally [69].

Multiple farnesylated proteins may be involved in FTI effects

FTIs induce multiple effects on cancer cells. What farnesylated proteins could be involved in these effects? Table 2 lists farnesylated proteins found in mammalian cells. This is still a partial list, and there are many proteins end-

Table 2. Farnesylated proteins in mammalian cells.

H-Ras		C L V S	proliferation, differentiation
N-Ras		C V V M	proliferation, differentiation
K-Ras		C V I M	proliferation, differentiation
Rheb		C S V M	cell cycle (G1/S)?
Rap2		C N I Q	
RhoB		C K V L	endocytosis, transcriptional regulation and apoptosis
RhoD		C V V T	cytoskeleton and endocytosis
Rho6/Rnd1		C S I M	cytoskeleton and adhesion
Rho7/Rnd2		C N L M	cytoskeleton
RhoE/Rho8/Rnd3		C T V M	cytoskeleton and adhesion
TC10		C L I T	cytoskeleton and transformation
CENP-E		C K T Q	cell cycle (G2/M), centromere binding
CENP-F		C K V Q	
PTPCAAX1/hPRL1		C C I Q	tyrosine phosphatase
PTPCAAX2/hPRL2/OV-1		C C V Q	
hPRL3		C C V M	
$InsP_3$ 5-phos.	I	C V V Q	inositol signaling
	IV	C S V S	
Hdj2		C Q T S	molecular chaperone
Nuclear lamin	A	C S I M	nuclear lamins
	B	C A I M	nuclear lamins

Farnesylated proteins in mammalian cells that have been characterized are summarized in this table. Their C-terminal CaaX sequence as well as their function are described.

ing with the CaaX motif that have not been characterized. Effects of FTI on some farnesylated proteins have been reported. Here, we will summarize these studies.

Ras proteins

H-Ras, K-Ras and N-Ras play critical roles in the growth factor signal transduction that involves activation of a number of downstream signaling pathways, including the Raf/MAP kinase and the PI3 kinase/Akt pathways [7,70]. Farnesylation of these Ras proteins have been established [71]. Because Ras proteins are capable of transforming cells and farnesylation is critical for this activity, effects of FTIs on the prenylation of Ras proteins have been extensively investigated [22–26, 72, 73].

FTIs clearly inhibit farnesylation of H-Ras. This was shown by the appearance of a slow migrating unmodified form in A SDS polyacrylamide gel as well as by the increase of Ras in the cytosolic fraction [22–26]. Some of the earlier observations on the ability of FTIs to inhibit anchorage-independent growth of H-*ras*-activated cancer cell lines may be attributed to the FTI effects on H-Ras. In addition, morphological changes and actin stress fiber formation induced by FTIs appear to be largely due to the inhibition of H-Ras, as described before.

The situation with K-Ras and N-Ras, on the other hand, is different. Whereas these proteins are modified by farnesylation, they become geranylgeranylated when their farnesylation is inhibited [72, 73]. This modification, catalyzed by GGTase-I, is termed alternative modification. Thus, FTI is not effective in inhibiting signaling events induced by the activation of K-ras. Similarly, K-*ras* transformed cells are less sensitive to FTIs [33–35].

Rheb and Rap2 proteins

There are two other members of Ras family proteins that are farnesylated. Rap2A is demonstrated to be farnesylated [74], but its function is unknown. A novel subfamily of Ras-superfamily G proteins, Rheb, is also farnesylated [75–78]. It has been reported that FTI affects cellular localization of this protein; after FTI treatment, the amount of Rheb in the cytosol increases [76]. FTI also inhibits farnesylation of Rheb, as detected by the conversion to a slow migrating species in an SDS polyacrylamide gel [unpublished observation]. Rheb does not undergo alternative prenylation. Rheb is conserved in a variety of organisms, including budding and fission yeasts [78]. Studies with fission yeast showed that the inhibition of Rheb expression results in G0/G1 arrest [79]. It would be interesting to examine whether Rheb plays a role in the FTI-induced G1 pause.

Rho proteins

RhoB has been proposed as a key target of FTI. RhoB possesses a unique CaaX sequence, CKVL, and has been shown to be both farnesylated and geranylgeranylated [80,81]. Prendergast and his co-workers have developed a hypothesis called the RhoB hypothesis [82]. They suggest that FTI shifts the prenylation status of RhoB to an exclusively geranylgeranylated form which causes dominant effects to induce apoptosis and the formation of stress fibers. In addition, it has been reported that cellular localization of RhoB is altered by the addition of FTI [83]. Cells transformed with myristoylated RhoB exhibit increased resistance to FTI [83]. Expression of geranylgeranylated RhoB (RhoB-GG) causes inhibition of soft agar growth in cell lines that are sensitive to FTI [84, 85]. Finally, FTI does not exert its apoptotic and antineoplastic effects when RhoB is absent [62]. This was demonstrated by the use of RhoB (–/–) MEF cells transformed with E1A and activated H-*ras*. These cells are incapable of undergoing apoptosis upon treatment with L-744,832. RhoB (–/–) cells are also defective in inducing actin stress fibers, but the stress fiber formation can be induced by the expression of RhoB-GG. RhoB-GG expression in epithelial cells can mimic the effects of FTI on H-ras or EGF activated Akt1 [61]. Thus, many results point to the idea that RhoB is responsible for FTI effects on apoptosis and stress fiber formation.

The RhoB hypothesis, however, needs further investigation. It has been reported that overexpression of either the farnesylated or geranylgeranylated version of RhoB induces apoptosis of Panc-1 cells, suggesting that both RhoB-F and Rhob-GG function similarly [86]. This argues against the idea that RhoB-GG has a function different from that of RhoB-F. Another issue concerns the observation that RhoB(–/–) MEF cells harboring E1A and activated H-Ras are resistant to FTI-induced apoptosis. Although this was interpreted to mean that RhoB is required for FTI-induced apoptosis, this result may simply reflect the fact that RhoB knockout cells acquired general resistance to apoptosis rather than exhibiting specific effects on FTI-induced apoptosis. In fact, these cells are also insensitive to other apoptosis inducers, such as irradiation and doxorubicin [62]. Recently, it was reported that RhoB represses nuclear factor kappa B(NF-κB) activity [87]. Thus, the resistance to apoptosis induction in the RhoB knockout cells may be due to the activation of NF-κB.

Besides RhoB, there are other Rho family small G proteins that are farnesylated. RhoD, which regulates actin dynamics and endocytosis, is farnesylated [88]. Rnd3/Rho8/RhoE is exclusively farnesylated and lacks intrinsic GTPase activity [89]. In contrast to RhoA, which induces stress fibers and cell spreading, Rnd3/Rho8/ RhoE disrupts stress fibers and induces cell rounding [90]. Other members of Rnd proteins Rnd1/Rho6 and Rnd2/

Rho7 have striking sequence similarity to Rnd3/Rho8/RhoE [90]. Finally, TC10 also ends with the CaaX motif [91]. This protein is interesting, because it is implicated in transformation; TC10 synergizes with Raf to transform NIH3T3 cells [91]. Thus, possible effects of FTI on the prenylation of these Rho proteins need to be investigated.

CENP proteins

CENP-E and CENP-F proteins contain a rather unusual CaaX motif (CKTQ for CENP-E and CKVQ for CENP-F), but were shown to be farnesylated [47]. These proteins function as a centromere-associated kinesin motor and play critical roles during mitosis [92]. Ashar et al. [47] proposed that CENP-E and F are involved in the FTI-induced G2/M enrichment. Farnesylation of these proteins is inhibited by FTI treatment and FTI inhibits association of these proteins with microtubules. There are two microtubule binding domains on CENP-E and -F, one at the N-terminus and the other at the C-terminus, and farnesylation is critical for the binding to microtubules [47]. FTI does not appear to alter the association of CENP-E and F proteins with the centromeres. Crespo et al. [48], on the other hand, suggested that CENP-E and F proteins are not involved in the G2/M enrichment. Like the study by Ashar et al. [47], FTI did not affect CENP-E and -F localization. But they point out that the rosette configuration observed after the FTI treatment is different from the pattern observed with cells that are deficient in CENP-E function, suggesting that a farnesylated protein(s) other than CENP-E/F is responsible for the effect of FTI on M-phase cells [48].

PTPCAAX

Human proteins PTPCAAX1/hPRL1 and PTPCAAX2/hPRL2/OV-1 were identified as farnesylated proteins through an in vitro prenylation screen [93]. These proteins belong to the protein tyrosine phosphatase superfamily. Three homologues, PRL-1, -2 and -3 were identified in rat [94]. Overexpression of PTPCAAX1 and PTPCAAX, in D27 hamster pancreatic ductal epithelial cells caused loss of contact inhibition. Nude mice injected with these cell lines exhibited tumors [93]. Similarly, overexpression of PRL-1 has been shown to increase growth rate and alter morphology of NIH3T3 cells [94]. Overexpression led to the transformation of NIH3T3 cells, as they formed colonies in soft agar at a rate comparable to that of v-Src transfected cells [94].

Farnesylated tyrosine phosphatase is a potential target of FTI, because FTI is reported to alter its cellular localization [95]. PRL-1, -2, and -3 are localized in the early endosomes and the plasma membrane in NIH3T3 cells stably expressing myc-tagged proteins. After the cells were treated with FTI, these proteins were found in the nucleus. Similar nuclear localization was observed when the CaaX motif was deleted. Zeng et al. [95] suggest that the inhibition of farnesylation leads to translocation of PRL. In agreement with this idea, a putative bipartite nuclear localization sequence is present within the C-terminal region just upstream of the farnesylation site [95]. The idea that FTI causes translocation of this protein to the nucleus is intriguing. However, further characterization is needed to establish the cellular localization of PTPCAAX and the effect of FTI on their localization.

Other farnesylated proteins

Another interesting class of enzymes that undergo farnesylation is inositol polyphosphate 5-phosphatase ($InsP_3$ 5-phosphatase) type I and IV [96-100]. The type I enzyme is a 43-kDa protein ending with CVVQ sequence. This enzyme is membrane associated ,and farnesylation is critical for its membrane association. The enzyme possesses the activity to hydrolyze inositol 1,4,5 triphosphate [Ins(1,4,5)P_3] and inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] [96, 97]. The type IV enzyme ends with the CSVS sequence and has a molecular weight of 70 kDa [98,100]. This enzyme hydrolyzes only lipid substrates, phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) [98]. PtdIns 4,5-P_2 directly binds to a variety of actin-binding proteins and regulates depolymerization and polymerization of actin [101]. Overexpression of rat IP5-phosphatase type IV induces dendritic structures in fibroblasts [99]. These proteins, however, are unlikely to be responsible for the FTI effects on cancer cells. Decreased expression of the type I phosphatase in NIH3T3 cells leads to transformed phenotypes including growth in soft agar and tumor formation in nude mice [102]. Intracellular localization of $InsP_3$ 5-phosphatase type IV is independent of farnesylation [100].

Nuclear lamins A and B belong to the family of lamin proteins. They are important for the formation of the nuclear lamina, which plays a role in anchoring the chromatin to the nuclear envelope, as well as DNA replication [103]. The lamins are also important for nuclear growth and DNA replication [103]. For example, lamin B-depleted nuclei are half the size of control nuclei, and are unable to replicate DNA [67,103]. Proper disassembly and assembly of the nuclear lamina are important for progression through the cell cycle. Prelamin A and lamin B have been shown to be farnesylated and this prenylation is important for the proper localization of lamin A and B to the nuclear membrane [67, 68]. However, as discussed before, FTI does not affect the formation of the nuclear lamina [69].

Heat shock protein, Hdj2, is farnesylated [104]. Inhibition of farnesylation by FTIs causes a change in the mo-

bility of this protein in an SDS polyacrylamide gel. This simple detection of the inhibition of farnesylation has been valuable in assessing inhibition of farnesylation during clinical trials of FTIs [105].

Conclusions and future directions

It is clear that FTIs cause multiple effects on cancer cells. First, FTIs inhibit anchorage-independent and dependent growth of cancer cells. Second, FTIs affect cell cycle progression of a wide range of cells. FTIs are also capable of inducing apoptosis. These changes are accompanied by changes in gene expression, such as induction of p21 [42] and α2(I) collagen [106]. Further studies utilizing complementary DNA (cDNA) microarrays may provide information on gene expression changes caused by FTIs. These studies should provide a clearer description of the effects of FTIs on cancer cells.

A major challenge at the moment is to identify the farnesylated proteins responsible for these FTI-induced events. Knowledge obtained so far on this subject points to the idea that multiple farnesylated proteins are involved. For example, H-Ras may be responsible for some events, such as induction of actin stress fibers. However, farnesylated proteins other than Ras are clearly responsible for other effects of FTIs, such as the induction of $p21^{Waf1/Cip1}$. This is unlikely to be mediated by H-ras, since inhibition of Ras is expected to inhibit $p21^{Waf1/Cip1}$ expression rather than increase it. Rho proteins may be important for this effect, since RhoA is known to suppress $p21^{Waf1/Cip1}$ expression. CENP-E/F proteins might play a role in the FTI-induced G2/M accumulation in some cell lines. Ultimate understanding of how FTI exerts its effects on cancer cells requires elucidation of the effect of FTI on each farnesylated protein and the sum of effects of FTI on multiple farnesylated proteins. We should also take into account that the expression of farnesylated proteins may differ among different cancer cells. Depending on which cancer cell is examined, different outcomes of FTI effect may be observed.

It is also important to point out that there are potential consequences of the inhibition of FTase by FTIs other than those involving farnesylated proteins. For example, it is possible that FTase inhibition leads to the accumulation of farnesyl pyrophosphate, which could alter the steady state level of isoprenoids within the cell. Although this possibility is believed to be unlikely because farnesyl pyrophosphate is mainly used for the synthesis of squalene, further investigation is needed to address this point. Another possible consequence of the inhibition of FTase that needs to be considered concerns cellular responses to the accumulation of the FTase/FTI complex. Formation of the enzyme/inhibitor complex may trigger some kind of stress response.

Significance of farnesylation in the physiology of mammalian cells may also be gleaned from a different line of study to inhibit farnesylation. For example, antisense strategy may be employed to examine the consequences of the inhibition of farnesylation. Furthermore, studies on mice with the FTase β-subunit gene knocked out may yield valuable information on biological processes which depend on protein farnesylation in mammalian cells. These lines of research, together with further studies using FTI, should provide us with a deeper understanding of the significance of farnesylation in mammalian cells.

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