Farnesyl Transferase Inhibitor (R115777)–Induced Inhibition of STAT3(Tyr705) Phosphorylation in Human Pancreatic Cancer Cell Lines Require Extracellular Signal-Regulated Kinases

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Abstract

In this study, we report that R115777, a nonpeptidomimetic farnesyl transferase inhibitor, suppresses the growth of human pancreatic adenocarcinoma cell lines and that this growth inhibition is associated with modulation in the phosphorylation levels of signal transducers and activators of transcription 3 (STAT3) and extracellular signal-regulated kinases (ERK). Treatment of cells with R115777 inhibited the tyrosine phosphorylation of STAT3(Tyr705), while increasing the serine phosphorylation of STAT3(Ser727). We found the differential phosphorylation of STAT3 was due to an increase and prolonged activation of ERKs. The biological significance of ERK-mediated inhibition of STAT3(Tyr705) phosphorylation was further assessed by treating the cells with an inhibitor (PD98059) of mitogen-activated protein kinase kinase (MEK) or by transfecting the cells with a vector that expresses constitutively active MEK-1. Expression of constitutively active MEK-1 caused an increase of ERK activity and inhibited STAT3(Tyr705) phosphorylation. Conversely, inhibition of ERK activity by PD98059 reversed the R115777-induced inhibition of STAT3(Tyr705) phosphorylation. R115777 also caused the inhibition of the binding of STAT3 to its consensus binding element. An increase in the activation of ERKs either by overexpressing MEK-1 or treatment of cells with R115777 caused an up-regulation in the levels of a cyclin-dependent kinase (cdk) inhibitor, p21cip1/waf1. These observations suggest that R115777-induced growth inhibition is partly due to the prolonged activation of ERKs that mediates an inhibition of STAT3(Tyr705) phosphorylation and an increase in the levels of p21cip1/waf1 in human pancreatic adenocarcinoma cell lines. (Cancer Res 2005; 65(7): 2861-71)

Introduction

Carcinoma of the human exocrine pancreas represents the fourth most common cause of cancer deaths in the United States. There has been little improvement in patient survival because the 5-year survival rate for these patients is <5%, with a median survival of <6 months (1). A number of genetic mutations occur in pancreatic cancer cells (2). The most predominant (>90%) genetic alterations are mutations in codon 12 of the K-RAS gene and the cell cycle regulator p16(INK4a) gene. More than 50% of pancreatic tumors show inactivation of DPC4 (deleted in pancreas cancer locus 4/Smad4) due to homozygous deletions and intragenic mutations and mutations of p53 (3). In addition, overexpression of several tyrosine kinase receptors and their respective ligands leads to enhanced mitogenesis (4). Together, these genetic modifications contribute to a selective growth advantage over normal cells, suppression of apoptotic pathways, and facilitation of invasion and metastasis of pancreatic cancer.

Farnesyl transferase inhibitors represent a new class of agents that were originally developed to specifically inhibit the activity of oncogenic RAS, which is mutated in many cancer types and is associated with poor prognoses (5–8). RAS proteins are guanine nucleotide binding proteins that play a major role in cancer development. RAS activates several downstream effectors after stimulation by various growth factors and cytokines (9). RAS undergoes several post-translational modifications that facilitate its attachment to the inner surface of the plasma membrane. The most crucial modification is the covalent attachment of a farnesyl isoprenoid lipid to a cysteine residue in the COOH-terminal of RAS proteins that is catalyzed by the enzyme farnesyl transferase and has become an important target for anticancer drug discovery. However, subsequent studies suggest that another closely related enzyme, geranyl geranyl transferase is also involved in RAS prenylation when farnesylation is inhibited by farnesyl transferase inhibitors (10). A large number of preclinical studies using various farnesyl transferase inhibitors have examined their spectrum of efficacy in an effort to understand the biological mechanism of their antitumor activity. Although a large number of preclinical studies show that farnesyl transferase inhibitors have examined their spectrum of efficacy in an effort to understand the biological mechanism of their antitumor activity, the mechanisms of action of these drugs seem complex and still remain largely unknown (11). Other studies show that farnesyl transferase inhibitors have antitumor activity regardless of RAS mutation suggesting that these compounds may target other potential prenylated proteins involved in growth regulation (11).

Signal transducers and activator of transcription (STAT) were originally discovered as latent cytoplasmic transcription factors that mediate pivotal cellular responses to diverse cytokines and growth factors (12–14). STATs are Src homology 2 (SH2) domain-containing transcription factors that exist as latent cytoplasmic transcription factors until stimulated by growth factors or cytokines. Apart from the cytokine receptor gp130, initial studies led to the identification of receptor-associated Janus kinase family of proteins as mediators for the activation of STATs (12–14). Further studies revealed that growth factor receptor tyrosine kinases or cytoplasmic tyrosine kinases such as Src also mediate activation of STATs. The STAT family consists of seven members designated as STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Following ligand binding and activation of cytokines or growth factor receptors, STATs are phosphorylated at their tyrosine residues. The tyrosine phosphorylated STAT monomers dimerize and subsequently translocate to the nucleus and bind to STAT-specific

References

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DNA-response elements of target genes and induce gene expression. Among the STAT family, only STAT3 ablation leads to embryonic lethality. The tyrosine phosphorylation of STAT3 occurs at residue 705. In addition to the phosphorylation at tyrosine residues, STAT3 also undergoes phosphorylation at serine residue 727. The role of STAT3 serine phosphorylation in transcriptional regulation is not well defined because it may enhance or inhibit the ability of tyrosine phosphorylated STAT3 to bind to DNA.

Besides its normal functions, emerging evidence strongly suggests that aberrant constitutive activation of STAT3 can lead to cellular transformation and tumorigenesis. Many cancer cell lines and primary tumors are reported to possess constitutively activated STAT3. Other investigations have shown that inactivation of STAT3 leads to an inhibition of cell proliferation. A recent study from our laboratory shows that STAT3 phosphorylation depends on ErbB2 tyrosine kinase activity and is partially responsible for the mediation of autocrine growth factor–independent phenotype in human pancreatic cancer cells. We further showed in that study that functional inhibition of STAT3 signaling by expression of dominant-negative STAT3 vector reduced the growth of human pancreatic cancer cells (26). Thus, due to its intrinsic oncogenic potential, STAT3 may be an attractive target for therapeutic intervention.

This led us to investigate the mechanism of farnesyl transferase inhibitor–induced growth inhibition in pancreatic cancer cells and whether any tumor-promoting signaling pathways apart from RAS signaling are affected by this compound. In this study, we used the nonpeptidomimetic farnesyl transferase inhibitor, R115777 that was shown to have antitumor effects both in vivo and in vitro. Treatment of pancreatic cancer cells with R115777 caused a dose-dependent growth inhibition due to G1 arrest. While analyzing various oncogenic signaling pathways, we found that R115777 caused a differential modulation of STAT3(Tyr705) and STAT3(Ser 727) phosphorylation. Although previous studies have been attempted to analyze the effects of R115777 on STAT3 phosphorylation, the underlying mechanism has not been established. Whereas a recent phase I clinical study (31) suggests no correlation between clinical response and variations in STAT3(Tyr705) phosphorylation in myelodysplastic syndrome, an in vitro study showed that higher concentrations of R115777 inhibited the ability of interleukin 6 (IL-6) to induce STAT3(Tyr705) phosphorylation in human myeloma cells. The present study shows that R115777 causes a differential modulation of STAT3 phosphorylation in pancreatic cancer cell lines. Treatment of cells with R115777 inhibited STAT3(Tyr705) phosphorylation but caused an increase in the phosphorylation of STAT3(Ser 727). We further show that this differential modulation of STAT3(Tyr705) and STAT3(Ser 727) phosphorylation is mediated by prolonged activation of ERKs. The role of ERKs in this phenomenon was further verified by either blocking or increasing ERK activity in these cells. Inhibition of ERK activity reverses the R115777-induced inhibition of STAT3(Tyr705) activation. On the other hand, increasing the activation of ERKs inhibited the phosphorylation of STAT3(Tyr 705). Treatment of cells with R115777 also caused an increase in expression of the cyclin-dependent kinase (cdk) inhibitor p21(Cip1/waf1). Thus, the growth inhibitory effects of R115777 were associated with the inhibition of STAT3(Tyr705)-mediated tumorigenic signaling pathway and an increase in the levels of p21(Cip1/waf1) mediated by prolonged activation of ERKs. This study suggests that although farnesyl transferase inhibitors are developed to target RAS signaling pathways, these compounds may lead to inhibition of other important tumorigenic pathway(s) in pancreatic cancer.

Materials and Methods

Cell culture. The human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 were purchased from American Type Culture Collection (Manassas, VA) and the cell line UK Pan-1 was established in our laboratory (33). Cells were grown in DMEM (Cell Grow-Mediatech, Herndon, VA) containing 10% fetal bovine serum. The medium was supplemented with 2 mmol/L glutamine and 100 units/mL penicillin/streptomycin. Cell cultures were maintained under 5% CO2.

R115777 treatment and cell culture stimulation. The farnesyl transferase inhibitor used in the study is R115777 from Janssen Pharmaceuticals (Titusville, NJ). The compound was solubilized in DMSO and working dilutions were made as described previously (29). Unless otherwise mentioned, for all R115777 treatments conducted in this study, exponentially growing cells were plated in medium supplemented with 10% serum. Forty-eight hours after plating the cells, serum-containing media was replaced with serum-free medium. Cells were maintained under serum-free conditions for 48 hours. R115777 dissolved in DMSO was added at indicated concentrations during the last 24 hours of serum starvation. For the untreated control set of cells, only vehicle (DMSO) was added. The cells were maintained under low serum conditions to arrest cell growth and for partial synchronization. At the end of serum starvation, cells were stimulated to reenter the cell cycle with medium containing transferrin (20 μg/mL), insulin (4 μg/mL), and epidermal growth factor (5 ng/mL), referred to as growth factor medium (34) with or without freshly supplemented R115777. Cells were harvested after 24 hours of growth factor medium stimulation and used for various analyses. When mitogen-activated protein kinase (MAPK) inhibitors were used, cells were plated, serum starved as mentioned above, and the specific MAPK inhibitors were added at indicated concentrations 6 hours before the beginning of pretreatment with R115777. At the end of serum starvation, cells were stimulated with fresh growth factor medium with or without freshly added R115777 and/or MAPK inhibitors. To analyze the effects of IL-6 on STAT3 phosphorylation, cells were plated, serum starved as mentioned above, treated with R115777, and IL-6 (10 ng/mL) was added 6 hours before harvesting cells for Western blot analysis.

Cell growth assays. The growth rate of cells was determined by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as described previously (35). Briefly, exponentially growing cells were plated in 96-well dishes. After 48 hours of plating, cells were treated with indicated concentrations of R115777. MTT assays were done after 1, 3, and 5 days of R115777 treatment. The time of assay, cells were stained with 0.5 mg/mL MTT (Sigma Chemical Co., St. Louis, MO) at 37°C for 2 hours and cells were solubilized in 200 μL of DMSO. Colorimetric determination was done with a FLUostar Optima plate reader (Durham, NC). The data are represented as the mean value of eight wells per treatment group and the experiments were repeated a minimum of three times.

Mitogenesis assays. [3H]-Thymidine incorporation assay that determines the rate of DNA synthesis, which is reflective of proliferation of cells, was done as described previously (36). Briefly, cells were plated and treated with R115777 as described for MTT assays. After 2 days of treatment with R115777, cells were pulsed with [3H]-thymidine (1 μCi; 46 Ci/mmol; Amersham Pharmacia Biotech, Inc., Piscataway, NJ) for 2 hours. DNA was then precipitated with 10% trichloroacetic acid and the amount of [3H]-thymidine incorporated was analyzed by liquid scintillation counting in a Beckman LS8301 Scintillation Counter. The data represented as the mean value of quadruplicate wells per treatment group and the experiments were repeated a minimum of two times.

Cell cycle analysis by flow cytometry. To determine the percentage of cells at various phases of cell cycle, serum-starved cells were stimulated with growth factor medium as described in cell culture stimulation section. Untreated...
control or R115777-treated cells (1 × 10⁶) cells were fixed in 70% ethanol for 30 minutes at room temperature. Fixed cells were stained with PBS with 50 μg/mL propidium iodide and 300 μg/mL RNase A at 37°C. Flow cytometric analysis was done in FACSCalibur (Becton Dickinson Immunocytometry Systems, Inc., San Jose, CA). Data collected from 20,000 cells for each experiment was analyzed using ModFit software (Verity Software House, Topsham, ME).

Western immunoblots. Western immunoblots were prepared to analyze the protein levels of phosphorylated or total STAT3, ERKs, ErbB2, human β-actin, MEK-1, c-Myc, and p21cip/waf1. Cells were harvested at indicated time points after treatment with R115777, and total cellular proteins were extracted using radiolabeled precipitation assay buffer (1 × PBS containing 1% NP40, 0.5% sodium deoxycholate and EDTA-free protease inhibitor cocktail, 2 mMol/L phenylmethylsulfonyl fluoride and 2 mMol/L sodium orthovanadate). Fifty micrograms of protein lysates were electrophoresed on an 8% SDS-polyacrylamide gel and then transferred to Hybond-P; polyvinilidiene difluoride membrane (Amersham Pharmacia Biotech, Inc.). The blots were probed with specific antibodies for the above mentioned proteins and detected by enhanced chemiluminescence methods (Klenow Life Science Products, Boston, MA). Wherever total cellular levels of STAT3, ERKs, MEK-1, and β-actin were to be detected, the exposure time was minimized to avoid saturation effects of these proteins due to their abundant amount present in the cells.

STAT3-DNA binding studies. Preparation of nuclear extracts, EMSA, and competitive analyses were carried out as described previously (37, 38). The wild-type and mutant EMSA oligonucleotide probes for the STAT3 consensus element were the same as reported previously (37, 38). The wild-type probe sequence is 5'-GATCTCCTTCCCCGGAGAGCA-3'. The bold face letters represent the STAT3 binding sequence. The sequence of mutant STAT3 SIE is as follows: 5'-GATCTCCAGGCTGAAGCA-3'. The underlined sequence represents the mutated STAT3 binding sequence. Densitometric analysis was done for the autoradiograms to determine the variations in the binding of STAT3 to its consensus element using Alphalmager software.

Transient transfection of mitogen-activated protein kinase kinase-1 vector. To determine the biological role of increased expression of ERKs, on the modulation of STAT3 phosphorylation, PANC-1 and UK Pan-1 cells were transiently transfected with a vector that expresses constitutively active MEK-1. Exponentially growing cells were transfected with 1 and 2 μg of the MEK-1 vector per mL of medium in a 6-cm dish using commercially available transfection reagent Fugene (Roche, Indianapolis, IN). As a control, 2 μg of an empty vector was transfected under identical conditions. Forty-eight hours after transfection, whole cellular lysates were extracted and subjected to Western blot analysis.

Results

R115777 causes a dose-dependent decrease of growth of pancreatic cancer cell lines. Exponentially growing PANC-1, UK Pan-1, and MIA PaCa-2 cells were treated with different concentrations of R115777. The growth was measured by MTT assays after 1, 3, and 5 days of treatment. As shown in Fig. 1A, R115777 caused a dose-dependent growth inhibition of these cells. The highest concentration used in this study (5 μmol/L) inhibited growth by about 50% in the cell lines tested. Furthermore, [³H]-thymidine assays, which measure DNA synthesis, that reflect the rate of proliferation of cells was determined in PANC-1 cells (Fig. 1B). Cells were treated either with DMSO alone or with different concentrations of R115777, followed by determination of [³H]-thymidine incorporation. The data shows that [³H]-thymidine incorporation was inhibited in cells treated with R115777 (Fig. 1B) in a dose-dependent manner. We further examined the effect of R115777 on cell cycle progression in these cells. PANC-1 cells were grown arrested by serum deprivation and stimulated with a growth factor enriched medium (34) to reenter the cell cycle. Serum-deprived, control and R115777-treated cells were mostly in G₁ phase, 79.6% and 84.9%, respectively. After 24 hours of stimulation of cells with growth factor medium, control cells showed reentry into the cell cycle as indicated by a decrease of cells in G₁-phase cells (from 79.6 to 57.9) and an increase in S-phase cells (from 11.8 to 35.3; Fig. 1C). However, cells treated with R115777 and stimulated with growth factor medium failed to show reentry into the cell cycle and were mostly maintained in G₁ phase (86.1; Fig. 1C). Taken together, these observations indicate that R115777 causes a dose-dependent suppression of pancreatic cancer cell growth.

R115777 causes a differential modulation of STAT3-Tyr705 and STAT3Ser727 phosphorylation that is associated with an increase in the activation of extracellular signal-regulated kinases in pancreatic cancer cells. It has become increasingly evident that activated STAT3-Tyr705 plays a role in tumorigenesis in many types of cancer. Recent studies from our lab (26) and others (24, 25, 27, 28) implicate a growth-promoting role of activated STAT3-Tyr705 in human pancreatic cancer cells. While analyzing various oncogenic pathways in R115777-treated cells, we also determined whether treatment of pancreatic cancer cells with R115777 could interfere with the activation of STAT3-Tyr705. To determine the levels of various proteins analyzed in this study, pancreatic cancer cells were serum starved and stimulated with growth factor medium in the presence or absence of R115777. Western immunoblot analysis revealed that treatment of pancreatic cancer cells (MIA PaCa-2, PANC-1, and UK Pan-1) with R115777 inhibited STAT3-Tyr705 phosphorylation (Fig. 2A). It has been reported that STAT3 also undergoes phosphorylation of its serine residues, especially STAT3Ser727, and it was thought to be involved in the transcriptional regulation of target genes (39). We ascertained whether R115777 treatment causes alterations to STAT3Ser727 phosphorylation. Western immunoblots revealed that R115777 treatment caused an increase of STAT3Ser727 phosphorylation (Fig. 2A). This suggests that R115777 treatment may lead to differential phosphorylation of STAT3. While inhibiting STAT3-Tyr705 phosphorylation, R115777 resulted in an increase of STAT3Ser727 phosphorylation.

ERKs are phosphorylated via several signal transduction pathways, including signaling through protein-tyrosine kinases or by activated RAS through RAF/MEK-1 kinases. Because farnesyl transferase inhibitors affect functionality of the RAS signaling pathway, we sought to determine whether this pathway was inhibited by R115777 in pancreatic cancer cells, and analyzed the levels of ERK activation. Surprisingly, we found that the levels of activated ERKs were increased in the pancreatic cancer cells treated with R115777 (Fig. 2A). These findings suggest that R115777-mediated effects are not restricted to one specific cell line. Similar observations were also seen in experiments conducted with exponentially growing cells (data not shown). The total cellular levels of STAT3 and ERKs, however, were not altered by R115777 treatment. Cells treated with R115777, however, showed accumulation of unfarnesylated RAS in the cell lines studied (data not shown).

Next, we determined the dose-dependent effects of R115777 in the activation of ERKs and STAT3-Tyr705. Cells were serum starved and treated with indicated concentrations of R115777. Total cellular lysates were subjected to Western immunoblot analysis. A representative Western blot analysis of PANC-1 cells treated with R115777 is shown in Fig. 2B. Treatment with R115777 caused an increase of the phosphorylation of ERKs (Fig. 2B). As shown in Fig. 2B, the levels of phosphorylated STAT3-Tyr705 in R115777-treated cells were reduced in a dose-dependent manner. On the other hand, the phosphorylated levels of STAT3Ser727 increased in R115777-treated cells. Total cellular

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levels of STAT3 and ERKs, however, remain unchanged (Fig. 2B). Treatment of cells with R115777 did not cause any changes in the activated levels of a separate MAPK member, p38 (data not shown). Thus, treatment with R115777 specifically caused a prolonged and sustained activation of ERKs in pancreatic cancer cells.

To determine the time course of phosphorylation of ERKs and STAT3(Tyr705), serum-deprived PANC-1 cells were stimulated with growth factor medium and harvested at various time points. In control cells, accumulation of phosphorylated STAT3(Tyr705) occurred from 12 hours after stimulation with growth factor medium and these levels were maintained through 24 hours. However, in R115777-treated cells, the levels of phosphorylated STAT3(Tyr705) were low when compared with untreated control cells (data not shown).

**R115777 prevents the ability of interleukin-6 to induce phosphorylation of STAT3(Tyr705).** IL-6 activates the Janus kinase/STAT3 pathway (14, 40). To determine whether IL-6 can abrogate R115777-mediated inhibition of STAT3(Tyr705) phosphorylation, cells were treated with R115777 and stimulated with growth factor medium and then assayed for STAT3 phosphorylation. Figure 1 shows that treatment with R115777 markedly inhibited IL-6-induced phosphorylation of STAT3(Tyr705).

**Figure 1.** Farnesyl transferase inhibitor R115777 causes a dose-dependent decrease of pancreatic cancer cell growth by causing a G1 arrest. A, exponentially growing PANC-1, UK Pan-1, and MIA PaCa-2 cells were exposed to indicated concentrations of R115777 and MTT assays were performed as explained in Materials and Methods to determine the growth of cells. Growth was measured 1, 3, and 5 days after exposure to R115777. Points, mean of three separate experiments; bars, ±SE. B, rate of DNA synthesis was measured in PANC-1 cells untreated control or treated with R115777 by [3H]-thymidine incorporation as explained in Materials and Methods. Columns, mean % [3H]-thymidine incorporated in untreated control cells of two separate experiments; bars, ±SE. The increasing concentration of R115777 caused a dose-dependent decrease of DNA synthesis. C, flow cytometric analysis of control and R115777-treated PANC-1 cells to assess the various phases of the cell cycle. DNA histograms (top) and % of each phase of cell cycle (table).
factor medium supplemented with IL-6 (10 ng/mL) as explained under cell culture stimulation section. As expected, IL-6 stimulation caused an increase in the phosphorylation of STAT3(Tyr705) (Fig. 3A, lane 3). However, in R115777-treated cells, IL-6 did not cause appreciable increase in the phosphorylation of STAT3(Tyr705) (Fig. 3A, lane 4). This suggests that the treatment of cells with IL-6 did not completely abrogate the R115777-induced inhibition of STAT3(Tyr705). Thus, R115777 can partially inhibit the phosphorylation of STAT3(Tyr705), even in the presence of a strong activator of STAT3(Tyr705) phosphorylation.

R115777-induced STAT3(Tyr705) inhibition and extracellular signal-regulated kinase activation are not transient. To determine whether R115777-induced effects are transient and reversible, cells were serum starved and treated with R115777. At the end of treatment, cells were allowed to grow in the growth factor medium without R115777 for an additional 72 hours. Western immunoblots revealed that R115777-induced inhibition of STAT3(Tyr705) phosphorylation is not reversed at 72 hours after withdrawal of treatment (Fig. 3B). The sustained activation of ERKs also was not reversed to basal levels due to treatment withdrawal suggesting that the effects caused by R115777 are not transient and not reversible up to 72 hours after treatment withdrawal.

Functional inhibition of extracellular signal-regulated kinases by PD98059 reverses the R115777-induced inhibition of STAT3(Tyr705) phosphorylation. Because we observed a correlation between ERK activation and inhibition of STAT3(Tyr705) phosphorylation in R115777-treated cells, next we determined the biological significance of ERKs in this process. To assess this phenomenon, we blocked the activation of ERKs by using the chemical inhibitor of MEK, PD98059. Serum-starved cells were treated with R115777 and supplemented with growth factor medium. Western immunoblots were analyzed for phosphorylated STAT3(Tyr705) and ERKs (Fig. 4A). As loading controls, total cellular levels of both STAT3 and ERKs remain unchanged.

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**Figure 2.** Differential modulation of STAT3 phosphorylation by R115777 is associated with prolonged activation of ERKs in pancreatic cancer cells. PANC-1, UK Pan-1, and MIA PaCa-2 cells were serum starved and stimulated with growth factor medium in the presence or absence of R115777 as explained in Materials and Methods. Total cellular lysates were isolated from control and R115777-treated cells and subjected to Western immunoblot analysis to determine the levels of phosphorylated STAT3 and ERKs. A. Western immunoblot showing that R115777 caused an inhibition of STAT3(Tyr705) phosphorylation and an increase of STAT3(Ser727) phosphorylation associated with an increase of ERK activation of pancreatic cancer cell lines, MIA PaCa-2, PANC-1 and UK Pan-1. As a loading control, total cellular levels of STAT and ERKs were measured. B, representative Western immunoblot of phosphorylated and total levels of STAT3 and ERKs. PANC-1 cells treated with indicated concentrations of R115777. Treatment with R115777 caused a dose-dependent inhibition of STAT3(Tyr705) phosphorylation, while increasing the phosphorylation of STAT3(Ser727) associated with an increase of ERK activation. The total cellular levels of both STAT3 and ERKs remain unchanged.
Functional activation of extracellular signal-regulated kinases by mitogen-activated protein kinase kinase-1 overexpression inhibits STAT3(Tyr705) phosphorylation. We further determined whether increasing ERK signaling pathway would prevent STAT3(Tyr705) phosphorylation. PANC-1 and UK Pan-1 cells were transiently transfected with an empty vector, Neo, or with a vector that express constitutively activated MAPK kinase (MEK-1). The protein lysates obtained from these transiently transfected cells were analyzed by Western immunoblotting for STAT3(Tyr705) phosphorylation levels and activated ERKs and shown in Fig. 4B and C for PANC-1 and UK Pan-1 cells, respectively. As loading controls, total cellular levels of STAT3 and ERKs were measured. Transient transfection of cells with MEK-1 expression vector, as expected, showed an increase in the total cellular levels of MEK-1 and also caused an increase of ERK activation (Fig. 4B and C) in comparison with cells transfected with an empty vector, Neo (Fig. 4B and C). With increasing amounts of MEK-1 vector, a dose-dependent decrease of STAT3(Tyr705) was also observed (Fig. 4B and C, lanes 2-3). This data further supports the notion that ERK activation negatively regulates STAT3(Tyr705) phosphorylation. Similar to R115777-treated cells, MEK-1 transfection also increased the STAT3(Ser727) activity. These observations suggest that ERK activation induced by either MEK-1 expression or R115777 results in the differential modulation of STAT3(Tyr705) versus STAT3(Ser727) phosphorylation levels.

Binding of STAT3 to its consensus element is inhibited by R115777 treatment in pancreatic cancer cells. Enhanced DNA binding activity of activated STAT3 has been reported in several cell systems (18, 41). Hence, we determined whether R115777 causes functional inhibition of STAT3-DNA binding. Nuclear extracts were prepared from control and R115777-treated cells and then analyzed by electrophoretic mobility shift assay. The STAT3-DNA protein complex is indicated by an arrow (Fig. 5). The specificity of STAT3 was verified by competition analysis using either a nonspecific oligonucleotide probe (lane 2) or with an oligonucleotide probe with a mutation in STAT3 binding site (lane 3). These probes did not affect the STAT3 binding to its consensus element. However, competition with 25 and 50 mol/L excess of unlabeled wild-type oligonucleotide probes caused a reduction in the intensity of the bands (lanes 4 and 5, respectively), suggesting the specificity of STAT3 binding. Treatment of cells with R115777 caused a dose-dependent decrease of STAT3 binding to its consensus element (lanes 7-10) compared with nuclear extracts from untreated control cells (lane 6). Treatment of cells with the ERK inhibitor PD98059 did not affect the STAT3-DNA binding ability (lane 11). However, inhibition of ERK activity by PD98059 partially reversed R115777-induced inhibition of STAT3-DNA binding (lane 12). Similar to PANC-1 cells, UK Pan, and MIA PaCa-2 cells showed a diminished binding of STAT3 in R115777-treated cells (lanes 13-16).

Activation of extracellular signal-regulated kinases by either expression of mitogen-activated protein kinase kinase-1 or treatment of cells with R115777 up-regulated the cyclin-dependent kinase inhibitor p21<sup>cip1/waf1</sup>. The cdk inhibitor p21<sup>cip1/waf1</sup> plays a crucial role in several physiologic functions including controlling G<sub>1</sub>-S progression, differentiation, apoptosis, senescence, and affects DNA replication (42). ERK activation was previously reported to be involved in the up-regulation of p21<sup>cip1/waf1</sup> (43, 44). In this context we sought to determine the levels of p21<sup>cip1/waf1</sup> since R115777 caused a prolonged activation of ERKs. Western immunoblots were analyzed for p21<sup>cip1/waf1</sup> levels (Fig. 6A and B). Transient transfection of cells with MEK-1 also caused a similar increase of p21<sup>cip1/waf1</sup> suggesting that prolonged ERK activation may lead to an increase of the cdk inhibitor p21<sup>cip1/waf1</sup> (Fig. 6A).
Similarly, treatment of cells with R115777 caused a dose-dependent increase of p21cip1/waf1 levels. The induction of p21cip1/waf1 may also be responsible for G1 arrest of cells, suggesting that R115777 treatment may lead to alterations of multiple signaling pathways to suppress the growth of cancer cells.

R115777 causes inhibition of c-Myc, a transcriptional target of STAT3. It has been shown earlier that c-Myc is a transcriptional target of STAT3. We sought to determine whether treatment of pancreatic cancer cells with R115777 leads to an inhibition of c-Myc expression. Western blot analysis revealed that treatment of cells with R115777 causes a decrease of c-Myc expression (Fig. 7).

Discussion

Farnesyl transferase inhibitors were originally developed to target tumors with mutated RAS genes. However, a majority of tumor cell lines were found sensitive to farnesyl transferase inhibitor–induced growth inhibition irrespective of the presence of RAS mutation. Hence, it is not clear, whether farnesyl transferase inhibitor–induced growth inhibition was only due to inhibition of RAS (11). It is possible that the mechanism underlying the farnesyl transferase inhibitor–induced growth inhibition may be due to the inhibition of prenylated proteins, besides RAS family of proteins, which may also be involved in tumor promoting pathways. In this study, we show that a farnesyl transferase inhibitor, R115777 caused inhibition of pancreatic cancer cell growth apparently due to a cell cycle block in G1. Furthermore, we show this growth inhibition is associated with a decrease in phosphorylation of STAT3Y705. R115777 treatment also led to a reduction of DNA binding of STAT3 to its consensus element. Emerging evidence suggests that activated STAT3 signaling is involved in tumorigenesis of several cancer types.

**Figure 4.** Down-modulation of STAT3Y705 phosphorylation requires ERK activation. A, selective inhibition of ERK activity in PANC-1, UK Pan-1 and MIA PaCa-2 cells reverses the R115777-induced inhibition of STAT3Y705 phosphorylation. Cells were serum-starved and pretreated with R115777 as described in Materials and Methods. For cell receiving combination treatments, the MAPK inhibitors were added to the medium 6 hours before the beginning of R115777 pretreatment. At the end of serum starvation and pretreatment, cells were stimulated with growth factor medium with or without R115777 and freshly supplemented MAPK inhibitors for 24 hours. Total cellular lysates isolated were subjected to Western immunoblot analysis. Treatment of cells with MEK inhibitor PD98059 reversed the R115777-induced inhibition of STAT3Y705 phosphorylation. However, treatment with p38 MAPK inhibitor did not cause any alteration of R115777-induced down modulation of STAT3Y705 phosphorylation. Treatment of cells with either of MAPK inhibitors alone did not alter the levels of STAT3Y705 phosphorylation. As loading controls, total cellular levels of both STAT3 and ERKs were used. Transient expression of constitutively active MAPK kinase (MEK-1) vector caused differential phosphorylation of STAT3Y705 and STAT3S727 in PANC-1 (B) and UK Pan-1 (C) cells. Exponentially growing PANC-1 and UK Pan-1 cells were transiently transfected with indicated amounts of an empty vector, Neo, or a vector that expresses constitutively active MEK-1. Total cellular lysates were extracted after 48 hours of transfection and subjected to Western immunoblot analysis. As expected, MEK-1 caused a dose-dependent increase of ERK activation associated with an inhibition of STAT3Y705 phosphorylation. Constitutive MEK-1 expression, however, caused an increase of STAT3S727 phosphorylation. Expression of the vectors, Neo or MEK-1, did not cause any alteration in the total cellular levels of STAT3 or ERKs. Expression of MEK-1 vector, however, caused an increase of the total cellular levels of MEK-1 as expected.
and inhibition of STAT3 signaling reverses tumor-promoting ability (20–23). Activated STAT3 is also reported to play a role in pancreatic carcinogenesis (24–28).

We found that the inhibition of STAT3\(^{(Ty705)}\) is causally linked to a prolonged activation of ERKs. Initially, we speculated that R115777 treatment would lead to a reduction of ERK activation, because many reports show that treatment with farnesyl transferase inhibitor causes a decrease in ERK signaling (45–47). Contrary to our expectation, we observed an increase of ERK activation upon treatment of pancreatic cancer cells with R115777. IL-6 activates STAT3\(^{(Ty705)}\) in several cell systems (13, 14), and we tested whether IL-6 stimulation causes abrogation of R115777-induced hypophosphorylation of STAT3\(^{(Ty705)}\). Our observations show that IL-6 induced STAT3\(^{(Ty705)}\) phosphorylation was diminished in the presence of R115777. This is in agreement with an earlier study that shows higher concentrations of R115777 caused a reduction of STAT3\(^{(Ty705)}\) phosphorylation in IL-6 induced human myeloma cells (32). Furthermore, the R115777-induced modulations of ERKs and STAT3 phosphorylation were not transient as these effects were still persistent after 72 hours of treatment withdrawal. It is not clear at this time how the ERKs are activated in response to R115777 treatment. Further studies are under way to determine the specific pathways leading to the activation of ERKs mediated by R115777. To our knowledge, this study is the first to show that an inhibitor of farnesylation leads to activation of ERKs. The findings of this study contradict the classic view that activated ERK signaling is involved in growth promotion. It is probable that prolonged activation of ERK, caused by R115777 treatment as seen in this study, may have a detrimental effect on growth. This suggestion is in agreement with a recent study (48) that showed high intensity ERK signaling leads to growth arrest by maintaining the hypophosphorylated forms of Rb protein in human hepatocellular carcinoma cell lines. Prolonged activation of ERKs is also reported to induce cell death mediated by synthetic vitamin K analogues in pancreatic cancer cells (49, 50). Activation of ERKs by treatment of cells with chemotherapeutic drugs induced apoptosis of several cancer cells (51–53). Sustained activation of ERKs was also shown to play a role in oxidative stress-induced apoptosis of hydrogen peroxide–treated mouse fibroblast cells (54). Thus, it is probable that sustained activation of ERKs can result in very different cellular effects than the effects caused by transient induction upon growth factor stimulation.

Figure 5. DNA binding ability of STAT3 is inhibited in R115777-treated pancreatic cancer cells. Nuclear extracts isolated from cells either untreated or treated with R115777. EMSA performed to determine the DNA binding ability of STAT3 to its consensus element as explained in Materials and Methods. Representative electrophoretic mobility shift assay which indicates a dose-dependent decrease of STAT3 binding to its consensus element in R115777 treated cells (lanes 6–12). STAT3 nuclear protein complex (arrow). Incubation of nuclear extracts with radio-labeled STAT3 consensus element probe in the presence of 25 and 50 mol/L excess of unlabeled wild-type (W) probe diminished the intensity of STAT3 protein-DNA complex (lanes 4 and 5). However, competition with a mutant STAT3 probe (M, lane 3) or a nonspecific probe (N, lane 2) did not affect the STAT3 protein-DNA complex, indicating the specificity of the STAT3-DNA complex. Nuclear extracts from cells treated with MEK inhibitor PD98059 alone did not cause any alterations in the DNA binding ability of STAT3 protein (lane 1). However, treatment of cells with both PD98059 and R115777 showed an increase in the binding of STAT3 to its consensus element (lane 12) suggesting a partial reversal of R115777-induced inhibition of STAT3-DNA binding. R115777-treated cells also showed a decreased binding of STAT3 to its consensus element in UK Pan-1 (lane 13 versus lane 14) and MIA PaCa-2 cells (lane 15 versus lane 16). Inset, densitometric values of autoradiogram showing variations in the binding of STAT3 to its consensus element (lanes 6–12).
STAT3(Tyr705) phosphorylation was further verified in this study by western blot analysis. The biological significance of ERK-mediated modulation of STAT3 in human pancreatic cancer cells was explained in Materials and Methods and cellular lysates were isolated and subjected to Western blot analysis. Human pancreatic cancer cells were transfected with a vector that expresses constitutively active MEK-1 (Vector: µg / ml) and treated or left untreated as explained in Materials and Methods and cellular lysates were subjected to Western blot analysis. Human c-Myc, a downstream transcriptional target of STAT3, was also reported to inhibit the phosphorylation of STAT3(Tyr705) that was mediated by ERKs in gastric cancer cells (59). Collectively, these studies show a direct role of ERKs in the down-modulation of STAT3(Tyr705) phosphorylation.

Furthermore, we show that R115777 treatment caused an increase of the cdk inhibitor p21cip1/waf1 in a dose-dependent manner. p21cip1/waf1 plays a crucial role in controlling G1-S progression and also affects DNA replication (42). The induction of p21cip1/waf1 occurs at the transcriptional and post-translational levels in a p53-dependent (42, 63), or p53-independent (64), or transforming growth factor-β/Smad–dependent mechanisms (65, 66). ERK activation was previously reported to be involved in the up-regulation of the cdk inhibitor p21cip1/waf1 (43, 44, 67, 68). Several studies have suggested that the up-regulation of p21cip1/waf1 in response to farnesyl transferase inhibitors occurred in a p53-dependent manner in cells with wild-type p53 (69, 70).
However, the cell lines used in our study harbors a mutant p53 gene. Thus, R115777-induced p21cip1/waf1 levels are independent of p53 in these cells. It has been shown that oncogenic STAT3 activation negatively regulates p21cip1/waf1 in cancer cells as opposed to normal cells (71). ERKs are also known to activate p21cip1/waf1 in some cell types (43, 44). It is of interest to note that inhibition of STAT3 upstream activator Janus kinase 2 by tyrphostin also resulted in growth arrest by a delayed G1-S phase progression and an increase in the expression of p21cip1/waf1 in pancreatic tumor cells (25). Taken together, our study suggests that induction of p21cip1/waf1 that occurred in concert with inhibition of STAT3 (72) and activation of ERKs, play a role in mediating the R115777-induced growth inhibition. Further studies are under way to determine the mechanism of up-regulation of p21cip1/waf1 and whether STAT3 (Ser727) plays a role in transcriptional activation of p21cip1/waf1 in pancreatic cancer cells. Furthermore, we show that the levels of c-Myc, a well-known transcriptional downstream target of STAT3 were found to be decreased as a consequence of the inhibition of STAT3 (72). phosphorylation in R115777-treated pancreatic cancer cells. It is of interest in this context to note that a recent report suggests that STAT3 can negatively regulate p21cip1/waf1 expression whereas increasing the levels of myc in cancer cells (71). It is probable that treatment of pancreatic cancer cells with R115777 caused a reversal of this phenomenon by increasing the levels of p21cip1/waf1, while reducing the levels of c-Myc in pancreatic cancer cells.

In summary, this study suggests that inhibiting farnesylation activities blocks phosphorylation of STAT3 (72), an important tumor promoting pathway in pancreatic cancer cells. This inhibition of STAT3 (72) phosphorylation by R115777 is mediated by prolonged ERK activation. The prolonged ERK activity also resulted in an increase in the levels of cdk inhibitor p21cip1/waf1 that play a role in G1-S cell cycle progression. Surmounting evidence indicate that targeting STAT3 is of therapeutic importance (72, 73).

Thus, this study identifies a novel target for farnesyl transferase inhibitor–induced growth inhibition of pancreatic cancer cells. Understanding the biochemical basis for the growth inhibitory actions of farnesyl transferase inhibitors will lead to a rational clinical design for efficient therapy of several types of cancers. Also it may be possible that inhibition of oncogenic STAT3 signaling may also increase the tumoral response to conventional chemotherapy and radiotherapy.

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Farnesyl Transferase Inhibitor (R115777)–Induced Inhibition of STAT3 (Tyr705) Phosphorylation in Human Pancreatic Cancer Cell Lines Require Extracellular Signal-Regulated Kinases

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