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CAAX PEPTIDOMIMETIC FTI-244 DECREASES PLATELET-DERIVED GROWTH FACTOR RECEPTOR TYROSINE PHOSPHORYLATION LEVELS AND INHIBITS STIMULATION OF PHOSPHATIDYLINOSITOL 3-KINASE BUT NOT MITOGEN-ACTIVATED PROTEIN KINASE[1]

Terence F. McGuire,[◇] Yimin Qian,[$] Michelle A. Blaskovich,[◇] Renae D. Fossum,[$] Jiazhi Sun,[◇] Tara Marlowe,[◇] Seth J. Corey[‖], Steven P. Wathen,[$] Andreas Vogt,[◇] Andrew D. Hamilton[$] and Saïd M. Sebti[◇2]

University of Pittsburgh, ◇School of Medicine, Department of Pharmacology, $School of Arts and Sciences, Department of Chemistry and ‖Children's Hospital of Pittsburgh, Department of Pediatrics, Pittsburgh, Pennsylvania 15261

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Cysteine farnesylation of the Ras carboxyl terminal tetrapeptide CAAX motif (where C=cysteine, A=leucine, isoleucine, or valine, and X=methionine or serine) is required for Ras biological activity. In this report, we describe the effects of inhibitors of farnesyltransferase (FTase), the enzyme responsible for this lipid modification, on platelet-derived growth factor (PDGF) signaling in NIH-3T3 cells. *In vitro*, the CAAX peptidomimetic FTI-232 exhibits potent inhibition of FTase activity (IC_{50}=150 nM) and its carboxyl-methylated counterpart, FTI-244, inhibits Ras processing *in vivo*. Treatment of NIH-3T3 cells with FTI-244 inhibits PDGF-induced DNA synthesis but not stimulation of mitogen-activated protein kinase (MAPK). However, FTI-244 significantly reduces PDGF-induced tyrosine phosphorylation levels of PDGF receptor (PDGFR) as well as its association with, and activation of, phosphatidylinositol-3-kinase (PI-3-K), a key enzyme in PDGF-induced mitogenesis.

Ras is a small guanine nucleotide-binding protein that plays a critical role in transducing growth signals from receptor tyrosine kinases to the nucleus. Ras farnesylation is required for the plasma membrane association as well as the biological activity of both normal and oncogenic

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[2]Requests for reprints should be addressed to Dr. Saïd M. Sebti and/or Dr. Andrew D. Hamilton.

Abbreviations: FTI-232, Cys-4-aminobenzoic acid-Met; FTI-244, carboxyl methylated form of FTI-232; PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; PI-3-K, phosphatidylinositol-3-kinase; FTase, farnesyltransferase; GGTase, geranylgeranyltransferase; ABA, 4-aminobenzoic acid; mSOS, mammalian Son of Sevenless.

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Ras. Farnesyltransferase (FTase), the enzyme responsible for this lipid modification, transfers farnesyl to cysteine on the Ras carboxyl terminal tetrapeptide CAAX (1). FTase is a heterodimer which shares its α subunit with the closely-related enzyme geranylgeranyltransferase I (GGTase I) (2), an enzyme that recognizes and geranylgeranylates proteins possessing a C-terminal CAAX motif where leucine or isoleucine is typically in the X position (3). FTase is known to recognize and farnesylate peptides as short as four residues in length provided that the CAAX sequence required for farnesylation is respected (4). We (5-7) and others (8-10) have designed peptidomimetics based on the CAAX sequence of K_B-Ras (CVIM) that competitively inhibit FTase.

Recently, it has been demonstrated that Ras CAAX peptidomimetics antagonize signaling events downstream of Ras (i.e. mitogen-activated protein kinase, MAPK) in cells transformed by the *ras* oncogene (8,9). However, in non-transformed rat-1 cells, epidermal growth factor (EGF)-induced MAPK activation was not affected by these peptidomimetics (9), suggesting that in these cells the EGF receptor utilizes Ras-independent pathways to activate MAPK. Whether CAAX peptidomimetics disrupt other normal signaling pathways originating from tyrosine kinase receptors has not been investigated.

Platelet-derived growth factor (PDGF) is a potent mitogen which carries out its biological effects on cells through binding to its tyrosine kinase receptor located on the plasma membrane (11). Binding of PDGF induces receptor dimerization, autophosphorylation at multiple tyrosine residues, and subsequent recruitment of signaling proteins to specific phosphotyrosine sites on the receptor (11,12). In fibroblasts, one signaling protein that is recruited and activated by the PDGF receptor (PDGFR) and that has been demonstrated to be required for PDGF-induced mitogenesis is phosphatidylinositol-3-kinase (PI-3-K). Both deletion and point mutation analyses of the PDGFR leading to selective elimination of PI-3-K association demonstrate a critical role for PI-3-K in the transduction of the signal leading to DNA synthesis (12-14).

In this report, we demonstrate that the CAAX peptidomimetic FTI-244 blocks PDGF-induced DNA synthesis but not MAPK activation in NIH-3T3 cells. However, FTI-244 was found to significantly reduce levels of tyrosine phosphorylated PDGFR as well as its association with and activation of PI-3-K.

MATERIALS AND METHODS

FTase and GGTase I Activity Assays. NIH-3T3 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Pen-Strep in a humidified 10% CO_2 incubator at 37°C. FTase was partially purified from NIH-3T3 cells as described previously (7). FTase and GGTase I were assayed by measuring the transfer of [^{3}H]farnesyl and [^{3}H] geranylgeranyl from their pyrophosphate forms to recombinant H-Ras-CVLS and H-Ras-CVLL, respectively, in the exact fashion as previously described (7). The

bacteria expressing H-Ras-CVLL was a gift from Dr. Channing J. Der and Dr. Adrienne D. Cox, Departments of Pharmacology and Radiation Oncology, University of North Carolina.

Ras and Rap1A Processing Assays. The ability of CAAX peptidomimetics to inhibit Ras and Rap1A processing in whole cells was analyzed essentially as described previously (7).

DNA Synthesis. NIH-3T3 cells were starved for 24 h, treated with peptidomimetic or vehicle overnight and subsequently stimulated with 30 ng/ml PDGF in 2% FBS. 19 h after PDGF stimulation, 2 μCi/ml [methyl-^{3}H]thymidine (ICN, Irvine, CA) was added for 2 h. The cells were then released by trypsinization, harvested from the wells, and placed on glass fiber filters. The cells were disrupted with ice-cold 0.1 M NaOH and the DNA precipitate was washed once with ice-cold ethanol. Filters were dried and counted in a liquid scintillation counter.

MAP Kinase Activity. Starved NIH-3T3 cells were treated with or without FTI-244 and then stimulated with 30 ng/ml PDGF at 37°C for 10 min. Lysate proteins were chromatographed by FPLC using a Mono-Q column and a linear salt gradient from 0.15 to 0.30 M NaCl in 50 Mm Hepes, pH7.5, containing 25 mM NaF, 1 mM EDTA, 1 mM EGTA and 2 mM vanadate. MAPK activity was assayed across the collected fractions using myelin basic protein (MBP) and [γ-^{32}P]ATP as the substrates essentially as previously described (15).

Phosphatidylinositol-3-kinase Activity. Starved NIH-3T3 cells were treated overnight with either peptidomimetic or vehicle and then stimulated with 30 ng/ml PDGF at 37°C for 10 minutes. Cells were lysed and PI-3-K activity was measured in the lysates as described previously (16).

Immunoprecipitation and Western Blotting. NIH-3T3 cells were treated with inhibitor, stimulated with PDGF, and lysates prepared. Immunoprecipitation of tyrosine phosphorylated proteins or the p85 subunit of PI-3-K and immunoblotting were carried out essentially as described previously (16). In some experiments, whole cell lysates or aliquots of Mono-Q fractions were electrophoresed and subsequently blotted with either anti-phosphotyrosine or anti-MAPK (*erk2*) (Upstate Biotechnology Inc., Lake Placid, NY). Western blotting with anti-MAPK detected both the inactive (nonphosphorylated) as well as the slower-migrating, active (phosphorylated) forms of MAPK.

RESULTS AND DISCUSSION

CAAX peptidomimetics inhibit FTase and Ras processing. The design and biochemical properties of the FTase inhibitors FTI-232/-244 have been described (6,7). In the present study, FTI-232 inhibited FTase from murine NIH-3T3 cells with an IC_{50} value of 150 nM (Fig. 1A). FTI-232 was also found to be modestly more selective (10-fold) towards inhibiting FTase over the closely-related enzyme GGTase I (Fig. 1A). Furthermore, the corresponding methyl ester of FTI-232, FTI-244, inhibited processing of Ras, but not Rap1A, a geranylgeranylated protein, at 400 and 200 μM in NIH-3T3 cells. FTI-232 treatment did not result in detectable levels of unprocessed Ras or Rap1A (Fig. 1B), while lovastatin, an inhibitor of HMG-CoA reductase (which inhibits the biosynthesis of both farnesyl- and geranylgeranyl-pyrophosphates), is a positive control that inhibits the processing of both Ras and Rap1A (Fig. 1B).

FTI-244 inhibits PDGF-induced DNA synthesis. Since FTI-244 inhibited the processing of Ras and, most likely, that of other farnesylated proteins that are potentially involved in mitogenic signaling, we determined the effect of this CAAX peptidomimetic on PDGF-induced DNA synthesis in NIH-3T3 cells. In the absence of peptidomimetic, PDGF elicited a 5-6-fold

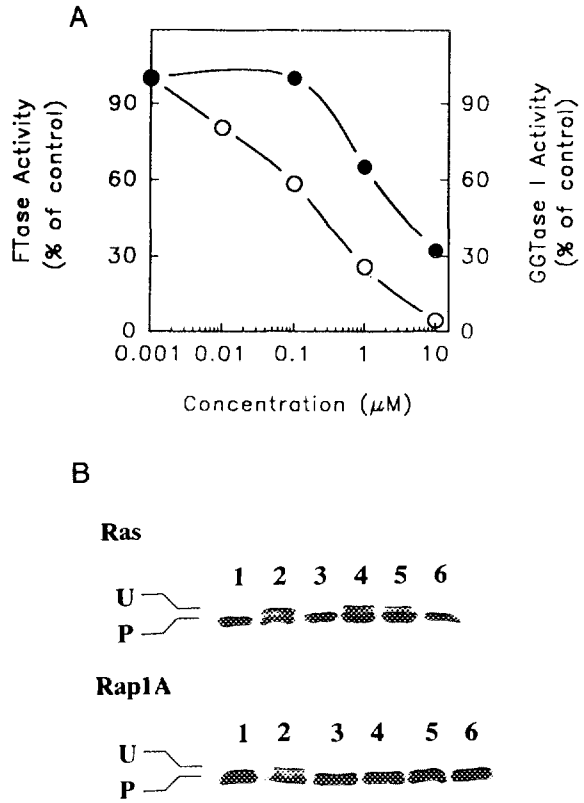

FIGURE 1. Inhibition of FTase and GGTase I by FTI-232. *A*, inhibition of FTase (○) and GGTase I (●) activities by FTI-232. *B*, inhibition of Ras and Rap1A posttranslational processing. NIH-3T3 cells were treated with vehicle (1), 60 μM lovastatin (2), 400 μM FTI-232 (3), and FTI-244 at 400 μM (4), 200 μM (5), and 100 μM (6). *Upper panel*, whole cell lysates were immunoprecipitated and then immunoblotted with an anti-Ras antibody that recognizes both processed (P) and unprocessed (U) forms of Ras. *Lower panel*, whole cell lysates were immunoblotted with anti-Rap1A antibody. Data are representative of two (*A*), three (*B, upper panel*) and two (*B, lower panel*) independent experiments.

increase in DNA synthesis (Fig. 2). FTI-244 was effective at blocking PDGF-induced DNA synthesis at concentrations between 200 and 400 μM (Fig. 2). FTI-232, as expected, was much less effective.

Divergent effects of FTI-244 on PDGF-induced MAPK activation and PDGFR tyrosine phosphorylation levels. Since Ras activation is known to lead to MAPK activation, we investigated the effects of FTI-244 on PDGF-induced MAPK activation. NIH-3T3 cells were treated and lysed and the lysate proteins were separated by ion exchange chromatography on a Mono Q column as described under "Materials and Methods". The first of two peaks (Fig. 3A, fractions 15 - 18) represented true MAPK activity since this demonstrated significant PDGF dependence and contained the immunoreactive material when immunoblotted with anti-MAPK antibodies (data not shown). PDGF stimulation of control cells increased MAPK activity in

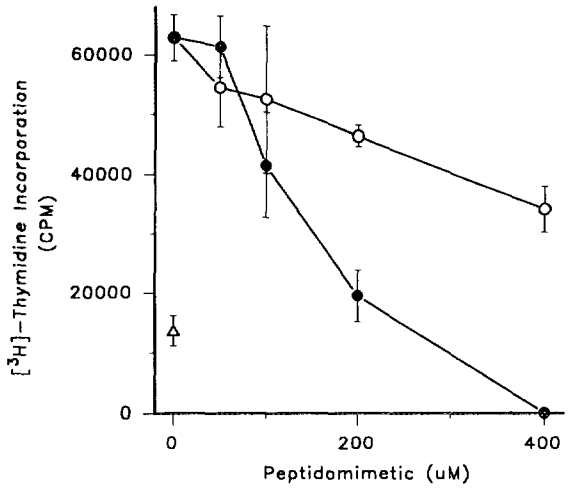

FIGURE 2. Inhibition of DNA synthesis. Starved NIH-3T3 cells were treated with FTI-232 (○) or FTI-244 (●), stimulated with PDGF, and after 19h pulsed with [^{3}H]thymidine and the amount of tritium incorporated into DNA was determined. Amount of [^{3}H]thymidine incorporated into DNA of cells that were not treated with peptidomimetic or PDGF (▵) is also shown. Data are representative of three independent experiments.

these fractions from basal levels of 99,000 cpm/mg protein to 454,000 cpm/mg protein, a 4.6-fold increase (Fig. 3A). Pretreatment of cells with FTI-244 did not prevent PDGF stimulation of MAPK (442,000 cpm/mg protein) (Fig. 3A). The lack of effect of FTI-244 on MAPK stimulation by PDGF was confirmed by immunoblotting the *identical* whole cell lysates with anti-MAPK antibody to assess the extent of formation of the active (phosphorylated) form of MAPK. Fig. 3B shows that unstimulated control cells contain only the inactive form of MAPK (lane 1) whereas PDGF-stimulated control and FTI-244-treated cells contain not only the inactive form but also the same level of the active form (lanes 2 and 3). In an effort to assess the effects of FTI-244 on one of the earliest steps in PDGF signaling, PDGFR tyrosine phosphorylation, the *identical* lysates were reprobed with anti-phosphotyrosine antibodies. PDGF-treated control cells exhibited one major tyrosine phosphorylated band corresponding to the PDGFR (Fig. 3C, lane 2). However, levels of PDGFR tyrosine phosphorylation from cells pretreated with FTI-244 were significantly reduced (Fig. 3C, lane 3). Thus, while PDGF-induced DNA synthesis and PDGFR tyrosine phosphorylation levels were nearly completely blocked by 400 μM FTI-244, MAPK activation remained unaffected. Although inhibition of Ras processing by FTI-244 is incomplete and enough Ras may remain in the plasma membrane that can be used to activate MAPK, the activation of this Ras via signaling molecules that are recruited and activated by tyrosine phosphorylated PDGFR (14) (i.e., PI-3-K and Grb-2/mSOS) would be expected to be inhibited. Hence, the activation of MAPK in this system may not only be Ras-independent but tyrosine phosphorylation-independent as well. Similar observations have been made with EGF

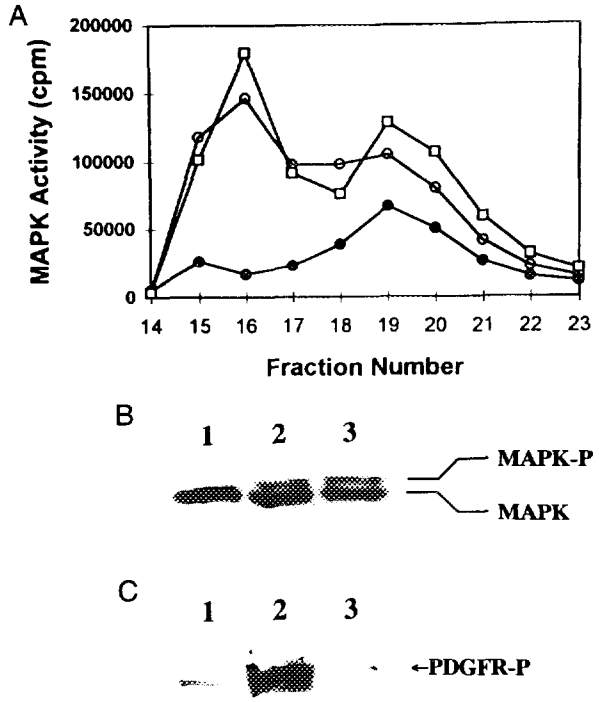

FIGURE 3. Effect of FTI-244 on PDGF-stimulated MAPK and levels of PDGFR tyrosine phosphorylation. Starved NIH-3T3 cells were treated with or without FTI-244 and stimulated with or without PDGF. *A*, cell lysates were chromotographed on a Mono Q column and eluted fractions analyzed for MAPK activity. (●), control lysates, (○), lysates from cells stimulated with PDGF, and (□), lysates from cells treated with FTI-244 and stimulated with PDGF. *B* and *C*, the identical cell lysates used to measure MAPK activity were blotted with antibodies against either MAPK (*B*) or phosphotyrosine (*C*). *Lane 1*, lysates from control cells; *lane 2*, lysates from PDGF-stimulated cells; and *lane 3*, lysates from cells treated with FTI-244 and stimulated with PDGF. MAPK-P and PDGFR-P represent the phosphorylated forms of MAPK and PDGFR, respectively. Data are representative of two independent experiments.

receptor (EGFR) signaling in fibroblasts where not only has MAPK activation been demonstrated to occur via Ras-dependent and Ras-independent pathways (17), but also by a mechanism that is not dependent upon a tyrosine phosphorylated EGFR (18).

Association and activation of PI-3-K are blocked by FTI-244. In order to further characterize FTI-244 inhibition of PDGFR early signaling events, PI-3-K, previously shown to play a key role in PDGF-induced mitogenesis in fibroblasts (12-14), was assessed as to whether its association with PDGFR and subsequent activation is inhibited by FTI-244. Fig. 4A (lanes 1 and 2) shows that when cells were stimulated with PDGF, in the absence of peptidomimetic, a major tyrosine-phosphorylated, 180 kDa protein co-immunoprecipitated with p85, the regulatory subunit of PI-3-K. This 180 kDa protein corresponds to the PDGFR since its association with p85 was PDGF-dependent, and previous investigations had demonstrated its association with PI-3-K upon PDGF stimulation (12,13). FTI-244 inhibited this association in

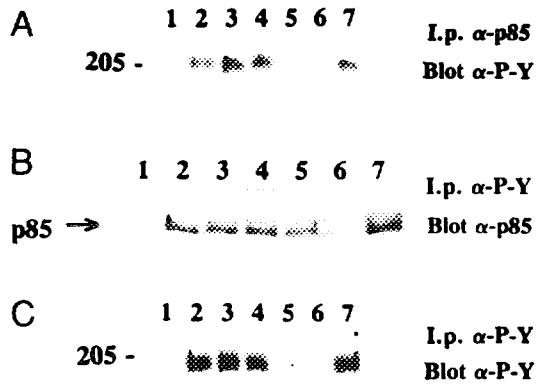

FIGURE 4. Inhibition of PDGF-induced PDGFR/PI-3-K association. Starved NIH-3T3 cells were treated with peptidomimetics and stimulated with or without PDGF. Cell lysates were immunoprecipitated with antibodies against either the PI-3-K p85 subunit (*A*) or phosphotyrosine (*B* and *C*). Immunoprecipitated proteins were immunoblotted with either antiphosphotyrosine (*A* and *C*) or anti-p85 (*B*). Cells were treated with vehicle (1,2), FTI-244, 50 μM (3), 100 μM (4), 200 μM (5), and 400 μM (6); and with 200 μM FTI-232 (7). Cells from all samples were treated with PDGF except those loaded onto lane 1 which were treated with vehicle. Data are representative of three independent experiments.

a concentration-dependent manner (Fig. 4A, lanes 4-6). The ability of FTI-244 to inhibit this association was confirmed by immunoprecipitating the same set of cell lysates with anti-phosphotyrosine antibody and detecting PI-3-K with anti-p85 antibody (Fig. 4B). When levels of tyrosine phosphorylated PDGFR were assessed, the extent of receptor phosphorylation was found to reflect the extent of PDGFR/p85 complex formation (Fig. 4C, lanes 4-6). Since PDGF stimulation of PI-3-K activity depends upon its ability to associate with the PDGFR (19), we determined whether this stimulation is blocked by FTI-244. Our results, shown in Fig. 5, demonstrate that PDGF activation of PI-3-K is blocked by FTI-244 in a concentration-dependent manner.

The above results clearly demonstrate that the CAAX mimic FTI-244 disrupts a major PDGF signaling pathway and inhibits DNA synthesis by a mechanism that does not appear to be due to inhibition of MAPK but rather involves inhibition of receptor tyrosine phosphorylation. Though it is not yet known as to how this might occur, the fact that activation of MAPK remains intact suggests that the inhibitory effect of FTI-244 is not due to general cytotoxicity. The possibility that FTI-244 directly inhibits the kinase activity of the PDGFR is unlikely since *in vitro* PDGFR kinase experiments performed as previously described (20) on partially purified NIH-3T3 plasma membranes (21) demonstrated that neither FTI-232 nor FTI-244 (at concentrations as high as 400 μM) inhibited the PDGF-dependent tyrosine autophosphorylation of the receptor (data not shown). Moreover, time course studies which showed that at least 5h of FTI-244 treatment of NIH-3T3 cells is required before observing any inhibition of receptor

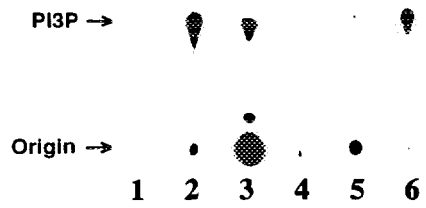

FIGURE 5. Inhibition of PDGF activation of PI-3-K. Starved NIH-3T3 cells were treated with vehicle (1,2), FTI-232, 400 μM (3), FTI-244, 400 μM (4), 200 μM (5), and 100 μM (6). The cells were then stimulated with (2-6) or without (1) PDGF, lysed and the lysates were assayed for PI-3-K activity as described previously (29). Phosphatidylinositol-3-phosphate (PI3P), the product of the reaction, was analyzed by thin layer chromatography and autoradiography. Data are representative of three independent experiments.

tyrosine phosphorylation and Ras processing (data not shown) also suggest that direct inhibition of the receptor tyrosine kinase activity by the inhibitors is unlikely.

Therefore, a reasonable mechanism to propose is that FTI-244 inhibits the processing of one or more farnesylated proteins which potentially function as regulators of growth factor-induced PDGFR tyrosine phosphorylation levels. In fact, not only is FTI-244 likely to inhibit the processing of farnesylated proteins, but some geranylgeranylated proteins displaying a lower affinity towards GGTase I than Rap1A may also be inhibited. We are presently using farnesylation-specific and geranylgeranylation-specific inhibitors to further investigate this issue.

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