Disruption of Oncogenic K-Ras4B Processing and Signaling by a Potent Geranylgeranyltransferase I Inhibitor*

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Prenylation of the carboxyl-terminal CAAX (C, cysteine; A, aliphatic acid; and X, any amino acid) of Ras is required for its biological activity. We have designed a CAAX peptidomimetic, GGTI-287, which is 10 times more potent toward inhibiting geranylgeranyltransferase I (GGTase I) in vitro (IC50 = 5 nM) than our previously reported farnesyltransferase inhibitor, FTI-276. In whole cells, the methyl ester derivative of GGTI-287, GGTI-286, was 25-fold more potent (IC50 = 2 μM) than the corresponding methyl ester of FTI-276, FTI-277, toward inhibiting the processing of the geranylgeranylated protein Rap1A. Furthermore, GGTI-286 is highly selective for geranylgeranylation over farnesylation since it inhibited the processing of farnesylated H-Ras only at much higher concentrations (IC50 > 30 μM). While the processing of H-Ras was very sensitive to inhibition by FTI-277 (IC50 = 100 nm), that of K-Ras4B was highly resistant (IC50 = 10 μM). In contrast, we found the processing of K-Ras4B to be much more sensitive to GGTI-286 (IC50 = 2 μM). Furthermore, oncogenic K-Ras4B stimulation of mitogen-activated protein (MAP) kinase was inhibited potently by GGTI-286 (IC50 = 1 μM) but weakly by FTI-277 (IC50 = 30 μM). Significant inhibition of oncogenic K-Ras4B stimulation of MAP kinase by GGTI-286 occurred at concentrations (1-3 μM) that did not inhibit oncogenic H-Ras stimulation of MAP kinase. The data presented in this study provide the first demonstration of selective disruption of oncogenic K-Ras4B processing and signaling by a CAAX peptidomimetic. The higher sensitivity of K-Ras4B toward a GGTase I inhibitor has a tremendous impact on future research directions targeting Ras in anticancer therapy.

Ras is a small guanine nucleotide binding protein that cycles between its active (GTP-bound) and inactive (GDP-bound) forms to transduce growth and differentiation signals from receptor tyrosine kinases to the nucleus (1, 2). Binding of epidermal and platelet-derived growth factors to their receptor tyrosine kinases results in autophosphorylation and recruitment of key signaling proteins to the receptor. Among these proteins are the Ras exchange factors that activate Ras by catalyzing the exchange of GDP for GTP. GTP-bound Ras activates a cascade of mitogen-activated protein (MAP) kinases by recruiting Raf to the plasma membrane. Raf, a serine/threonine kinase, phosphorylates MAP kinase, which in turn activates MAP kinase by phosphorylating it on threonine and tyrosine. Hyperphosphorylated MAP kinase translocates to the nucleus where it phosphorylates transcription factors that are involved in the regulation of growth-related genes. The growth signal is terminated when Ras hydrolyzes GTP to GDP (1-3). However, mutations that lock Ras in its GTP-bound form result in an uninterrupted growth signal and are believed to contribute to the development of more than one-third of human cancers (4, 5).

In order for Ras to transduce its normal and oncogenic signal it must be anchored to the plasma membrane, which is accomplished by post-translational modifications that increase its hydrophobicity (6-8). A key step in this process is catalyzed by farnesyltransferase (FTase), an enzyme that transfers farnesyl from farnesylpyrophosphate, a cholesterol biosynthesis intermediate, to the cysteine of the carboxyl-terminal CAAX of Ras (C, cysteine, A, aliphatic amino acid; X, serine or threonine) (9, 10). A closely related enzyme, geranylgeranyltransferase I (GGTase I), attaches the lipid geranylgeranyl to the cysteine of the CAAX box of proteins, where X is leucine (11, 12). FTase and GGTase I are αβ heterodimers that share the α subunit (13, 14). Cross-linking experiments suggested that both substrates (farnesylpyrophosphate and Ras CAAX) interact with the β subunit of FTase (15, 16). Although GGTase I prefers leucine at the X position, its substrate specificity was shown to overlap with that of FTase in vitro (17). Furthermore, GGTase I was also able to transfer farnesyl to a leucine terminating peptide (18).

Because farnesylation of Ras is required for its oncogenic activity, we (19-22) and others (23-27) have designed potent inhibitors of FTase as potential anticancer drugs. These inhibitors are CAAX peptidomimetics, which show great selectivity for FTase over GGTase I in vitro and selectively block the processing of farnesylated but not geranylgeranylated proteins in whole cells (22). Furthermore, FTase inhibitors can selectively block oncogenic Ras signaling and reverse malignant phenotype at concentrations that do not affect normal cells (24, 25). However, mammalian cells express four types of Ras proteins (H-, N-, KA-, and KB-Ras) among which K-Ras4B is the most frequently mutated form of Ras in human cancers (4, 5). Although several laboratories have demonstrated potent inhibition of oncogenic H-Ras processing and signaling (26, 28), this disruption has not been shown with K-Ras4B. Hence, a drawback of the previous studies is the use of H-Ras and not

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† The abbreviations used are: MAP, mitogen-activated protein; FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; PAGE, polyacrylamide gel electrophoresis; CAAX, tetrapeptide where C is cysteine, A is aliphatic amino acid, and X is serine or methionine; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase inhibitor.
K-Ras4B as a target for the development of these inhibitors. Recently, we have shown that a potent inhibitor of FTase disrupts K-Ras4B processing but only at very high concentrations that also inhibited the processing of geranylgeranylated proteins (29). This suggested that K-Ras4B may be geranylgeranylated, particularly in cells where FTase is inhibited. Consistent with this possibility is the recent observation that K-Ras4B can be geranylgeranylated in vitro, but its $K_m$ for GGTase I is 7 times higher than its $K_m$ for FTase (30). GGTase I CAAX-based inhibitors that can block geranylgeranylation processing have not been reported. In the present study, we have designed a CAAX peptidomimetic that selectively inhibits GGTase I and demonstrate that oncogenic K-Ras4B processing and signaling are disrupted at concentrations that affect geranylgeranylation but not farnesylation processing.

**EXPERIMENTAL PROCEDURES**

Synthesis of FTase and GGTase I Inhibitors—Peptidomimetics FTI-276 and FTI-277 were prepared as described previously (29). The GGTase I inhibitors GGTI-286 and -287 were prepared from 2-phenyl-4-nitrobenzoic acid (29) by reaction with l-leucine methyl ester followed by reduction with stannous chloride. The resulting 4-aminophenylbenzoyl leucine methyl ester was reacted with N-t-butyloxycarbonyl-S-trityl-cysteinyl and deprotected by procedures similar to those described for the FTase inhibitors (29) to give GGTI-286 and -287 as their hydrochloride salts.

FTase and GGTase Activity Assays—FTase and GGTase activities from 60,000 g supernatants of human Burkitt lymphoma (Daudi) cells (ATCC, Rockville, MD) were assayed exactly as described previously for FTase (22). Inhibition studies were performed by determining the ability of Ras CAAX peptidomimetics to inhibit the transfer of [3H]farnesyl and [3H]geranylgeranyl from [3H]farnesylpyrophosphate and [3H]geranylgeranylpyrophosphate to H-Ras-CVLS and H-Ras-CVLL, respectively (22).

Ras and Rap1A Processing Assays—Ras cells (31) and K-Ras4B cells (32) were kind gifts from Dr. Channing Der and Dr. Adrienne Cox (University of North Carolina, Chapel Hill). Cells were seeded on day 0 in 100-mm dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and 1% penicillin/streptomycin. On days 1 and 2, cells were refed with medium containing various concentrations of FTI-277, GGTI-286, or vehicle (10 mM dithiothreitol in dimethyl sulfoxide). On day 3, cells were washed and lysed in lysis buffer containing 50 mM HEPES, pH 7.5, 10 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM MgCl$_2$, 1 mM EDTA, 25 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, 1 mg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, 6.4 μg/ml Sigma-1044 phosphatase substrate. Lysates were cleared (14,000 rpm, 4°C, 15 min), and equal amounts of protein were separated on 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-Ras antibody as described under “Experimental Procedures.”

**RESULTS AND DISCUSSION**

The carboxyl-terminal CAAX tetrapeptide of Ras is a substrate for FTase and serves as a target for designing inhibitors of this enzyme with potential anticancer activity (23). We have recently made a highly potent (IC$_{50}$ = 500 pm) inhibitor of FTase, FTI-276 (Fig. 1) (29). Its cell-permeable methyl ester FTI-277 inhibited H-Ras processing in whole cells with an IC$_{50}$ of 100 nm (29). Furthermore, FTI-276 is highly selective (100-fold) for FTase over GGTase I (Table 1). Although oncogenic H-Ras processing and signaling were essentially sensitive to FTI-277, those of K-Ras4B were highly resistant. However, at high concentrations of FTI-277, when the processing of the geranylgeranylated Rap1A protein was inhibited, K-Ras4B processing was also inhibited (29). We, therefore, set out to determine whether a GGTase I-selective inhibitor would disrupt K-Ras4B processing and signaling. Our approach involved replacing the central “AA” of CAAX tetrapeptides by a hydrophobic spacer and incorporating a leucine residue in the carboxyl-terminal position to optimize recognition by GGTase I. We herein report a CAAL peptidomimetic, GGTI-287, where reduced cysteine is linked to leucine by 2-phenyl-4-aminobenzoic acid. Figure 1 shows that GGTI-287 is more than 15-fold selective for inhibition of geranylgeranylated Rap1A protein (IC$_{50}$ = 100 nM) and 50 μM, respectively (Fig. 2). Thus, GGTI-286 is...
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FIG. 2. Disruption of H-Ras and Rap1A processing. NIH 3T3 cells that overexpress oncogenic H-Ras were treated with various concentrations of FTI-277 (0–50 μM) or GGTI-286 (0–30 μM). The cells were lysed, and the lysates were electrophoresed on SDS-PAGE and immunoblotted with either anti-Ras or anti-Rap1A antibodies as described under "Experimental Procedures." U and P designate unprocessed and processed forms of the proteins. Data are representative of three independent experiments.

FIG. 3. Disruption of K-Ras4B processing. NIH 3T3 cells that overexpress oncogenic K-Ras4B were treated with FTI-277 or GGTI-286 (0–30 μM). The cells were lysed and the lysates were electrophoresed on SDS-PAGE and immunoblotted with anti-Ras antibodies as described under "Experimental Procedures." U and P designate unprocessed and processed forms of Ras. The data are representative of three independent experiments.

FIG. 4. Inhibition of oncogenic activation of MAP kinase. NIH 3T3 cells that overexpress oncogenic H-Ras or K-Ras4B were treated with FTI-277 or GGTI-286 (0–30 μM). The cells were lysed, and the lysates were electrophoresed on SDS-PAGE and immunoblotted with an anti-MAP kinase antibody. P-MAPK designates hyperphosphorylated MAP kinase. The data are representative of three independent experiments.

25-fold more potent than FTI-277 at inhibiting geranylgeranylation in whole cells (Table I).

We next evaluated the ability of GGTI-286 to inhibit the processing and signaling of oncogenic K-Ras4B. NIH 3T3 cells, which overexpress oncogenic K-Ras4B (32), were treated with either GGTI-286 (0–30 μM) or FTI-277 (0–30 μM), and the lysates were immunoblotted with an anti-Ras antibody as described under "Experimental Procedures." Fig. 3 shows that GGTI-286 inhibited potently the processing of K-Ras4B with an IC50 of 2 μM. The ability of GGTI-286 to inhibit the processing of K-Ras4B was much closer to its ability to inhibit the processing of geranylgeranylated Rap1A (IC50 = 2 μM) than that of farnesylated H-Ras (IC50 > 30 μM) (Fig. 2 and Table I). This suggested that K-Ras4B might be geranylgeranylated. Consistent with this is the fact that K-Ras4B processing was very resistant to the FTase-specific inhibitor FTI-277 (IC50 = 10 μM) (Fig. 3). Furthermore, GGTI-286 inhibited K-Ras4B processing at concentrations (1–3 μM) (Fig. 3) that had no effect on the processing of farnesylated H-Ras (Fig. 2). These results are not consistent with the work of Casey et al. (7), who used [3H]mevalonate acid to label cellular proteins and provided evidence for a farnesylated K-Ras4B based on high pressure liquid chromatography of the radiolabeled prenyl group. However, the mass of the prenyl group in these studies was not determined.

To determine whether inhibition of K-Ras4B processing by GGTI-286 results in disruption of oncogenic signaling, we evaluated the ability of GGTI-286 to antagonize oncogenic K-Ras4B constitutive activation of MAP kinase. Activated MAP kinase is hyperphosphorylated and migrates slower than hypophosphorylated (inactive) MAP kinase on SDS-PAGE (26, 29). Fig. 4 shows that K-Ras4B-transformed cells contained mainly activated MAP kinase. Treatment of these cells with the FTase-specific inhibitor FTI-277 (0–30 μM) did not inhibit MAP kinase activation until 30 μM (Fig. 4). In contrast, GGTI-286 inhibited MAP kinase activation with an IC50 of 1 μM, and the block was complete at 10 μM. Thus, GGTI-286 blocked oncogenic K-Ras4B MAP kinase activation at a concentration (10 μM) where FTI-277 had no effect. In contrast, oncogenic H-Ras activation of MAP kinase was inhibited only slightly by GGTI-286 whereas FTI-277 completely blocked this activation at 3 μM (Fig. 4). Furthermore, GGTI-286 blocked K-Ras4B activation of MAP kinase at a concentration (10 μM) that had little effect on H-Ras activation of MAP kinase (Fig. 4). It should be noted that GGTI-286 was not toxic to cells at concentrations as high as 10 μM. However, at higher concentrations (30 μM), GGTI-286 did show some signs of toxicity as reflected by a rounded morphology of the cells. Thus, GGTI-286 was not toxic at concentrations (10 μM) that resulted in complete inhibition of MAP kinase activation.

Recently, we have demonstrated that the FTase-specific inhibitor FTI-277 inhibits oncogenic H-Ras processing and signaling (29) and blocks in vivo tumor growth of H-Ras-transformed NIH 3T3 cells and a human lung carcinoma that expresses a K-Ras mutation (33). However, processing of K-Ras4B was inhibited by FTI-277 only at high concentrations similar to those needed to inhibit the processing of the geranylgeranylated protein Rap1A (29). In the present study, we have described the design of a geranylgeranylation-specific inhibitor and its effects on oncogenic K-Ras4B processing and signaling. Our results demonstrate that oncogenic K-Ras4B processing and constitutive activation of MAP kinase are potently inhibited by a GGTase I-selective inhibitor (GGTI-286) but are resistant to one selective for FTase (FTI-277). This is in direct contrast to the processing and signaling of oncogenic H-Ras, which was very sensitive to FTI-277 and highly resistant to GGTI-286. The resistance of K-Ras4B to disruption by FTase inhibitors could be explained by the 50-fold higher affinity of K-Ras4B for FTase compared with H-Ras (30). Our current data strongly suggest, however, that K-Ras4B may be resistant to FTase inhibition because it is post-translationally processed by a geranylgeranyl rather than a farnesyl group. This is consistent with the recent observation that in vitro K-Ras4B can be geranylgeranylated by GGTagase I (30). Al-
though this previous work shows that K-Ras4B is a 7 times better substrate in vitro for FTase (Km = 0.2 μM) than GGTase I (Km = 1.5 μM) (30), our data suggest that, in cultured cells, K-Ras4B is geranylgeranylated. This is supported by the fact that GGTI-286 inhibited oncogenic K-Ras4B processing and MAP kinase activation at concentrations (1 and 3 μM) that did not affect farnesylation-dependent processing.

The results presented in this study are critical to the further design and development of inhibitors of Ras prenylation as potential anticancer agents. The results identify the GGTase I-specific inhibitor GGTI-286 as a small molecule capable of antagonizing selectively oncogenic K-Ras4B (not H-Ras) signaling. This is a key finding since K-Ras4B is the most frequently identified mutated Ras in human cancers, and its function has been resistant to FTase inhibitors. Furthermore, we have recently shown that a GGTase I inhibitor selectively suppressed activated DRas1 in Drosophila without side effects demonstrating the utility of these Ras CAAX peptidomimetics in whole animals (34). Finally, the availability of K-Ras4B-selective inhibitors (i.e. GGTI-286) in addition to H-Ras-selective inhibitors (i.e. FTI-277) will enhance our understanding of the distinctive roles of these two forms of Ras in normal and oncogenic signaling.

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