INHIBITORS OF PROTEIN GERANYLGERANYLTRANSFERASE-I AND RAB GERANYLGERANYLTRANSFERASE IDENTIFIED FROM A LIBRARY OF ALLENOATE DERIVED COMPOUNDS

Masaru Watanabe1, Hannah D. G. Fiji2, Lea Guo1, Lai Chan3, Sape S. Kinderman2, Dennis J. Slamon4, Ohyun Kwon2 and Fuyuhiko Tamanoi1

From Dep. of Microbiology, Immunology & Molecular Genetics1, Dept. of Chemistry & Biochemistry2, Molecular Biology Institute3, Department of Medicine, Jonsson Comprehensive Cancer Center4, University of California, Los Angeles, Los Angeles, CA 90095

Running Title: GGTIs and RabGGTIs

Address correspondence to: Fuyuhiko Tamanoi, Dep. of Microbio., Immunol. & Molec. Genet., UCLA, Los Angeles, CA 90095. Fax: 310-206-7318 ; E-mail: fuyut@microbio.ucla.edu

Protein geranylgeranylation is critical for the function of a number of proteins such as RhoA, Rac and Rab. Protein geranylgeranyltransferase type I (GGTase-I) and Rab geranylgeranytransferase (RabGGTase) catalyze these modifications. In this paper, we first describe identification and characterization of small molecule inhibitors of GGTase-I with two novel scaffolds from a library consisting of allenolate derived compounds. These compounds exhibit specific inhibition of GGTase-I and act by competing with a substrate protein. Derivatizing a carboxylic acid emanating from the core ring of one of the GGTI compounds dramatically improved their cellular activity. The improved GGTI compounds inhibit proliferation of a variety of human cancer cell lines and cause G1 cell cycle arrest and induction of p21 CIP1/WAF1. We also report identification of novel small molecule inhibitors of RabGGTase. These compounds were identified first by screening our GGTI compounds for ones that also exhibited RabGGTase inhibition. This led to the discovery of a common structural feature for RabGGTase inhibitors; presence of a characteristic 6-atom aliphatic tail attached to the penta-substituted pyrrolidine core. Further screen led to the identification of compounds with preferential inhibition of RabGGTase. These compounds inhibit RabGGTase activity by competing with the protein substrate. These novel compounds may provide valuable reagents to study protein geranylgeranylation.

Protein geranylgeranylation is critical for the function of a number of proteins such as RhoA, Rac and Rab. Protein geranylgeranyltransferase type I (GGTase-I) and Rab geranylgeranytransferase (RabGGTase) catalyze these modifications. In this paper, we first describe identification and characterization of small molecule inhibitors of GGTase-I with two novel scaffolds from a library consisting of allenolate derived compounds. These compounds exhibit specific inhibition of GGTase-I and act by competing with a substrate protein. Derivatizing a carboxylic acid emanating from the core ring of one of the GGTI compounds dramatically improved their cellular activity. The improved GGTI compounds inhibit proliferation of a variety of human cancer cell lines and cause G1 cell cycle arrest and induction of p21 CIP1/WAF1. We also report identification of novel small molecule inhibitors of RabGGTase. These compounds were identified first by screening our GGTI compounds for ones that also exhibited RabGGTase inhibition. This led to the discovery of a common structural feature for RabGGTase inhibitors; presence of a characteristic 6-atom aliphatic tail attached to the penta-substituted pyrrolidine core. Further screen led to the identification of compounds with preferential inhibition of RabGGTase. These compounds inhibit RabGGTase activity by competing with the protein substrate. These novel compounds may provide valuable reagents to study protein geranylgeranylation.

Recent studies highlight physiological significance of protein geranylgeranylation. Knockout mice specific for the β-subunit of GGTase-I have been established (6). Characterization of GGTase-I-deficient cells showed proliferation inhibition and accumulation of p21(CIP1/WAF1), pointing to the significance of GGTase-I in cell proliferation and cell cycle progression (6). GGTase-I deficiency reduced oncogenic K-ras-induced lung tumor formation in mice, pointing to the significance of inhibiting GGTase-I to block tumor formation (6). Recent
studies also showed that a number of geranylgeranylated proteins play important roles in tumorigenesis and metastasis. In addition to RhoA and Cdc42 proteins, RalA protein was recently found to be activated downstream of Ras in most pancreatic cancer cells harboring oncogenic K-ras mutation (7). RalB plays critical roles in the survival pathway (8). RhoC is overexpressed in metastatic cancer and RhoC knockout mice exhibit defect in metastasis (9,10). Overexpression of Rab25 in breast and ovarian cancer cells has been reported, and this mutation is a determinant for aggressiveness of these cancers (11,12). Rab25 is also upregulated in prostate cancer and transitional-cell bladder cancer (11). Overexpression of other Rab proteins such as Rab5a and Rab7 in cancer has been reported (13,14).

Protein geranylgeranylation is catalyzed by two types of enzymes. Geranylgeranyl-transferase type I (GGTase-I) catalyzes mono geranylgeranylation of proteins such as Rho, Rac and Cdc42. This enzyme is a heterodimer consisting of α- and β-subunits (15). RabGGTase (or GGTase-II) catalyzes digeranylgeranylation of Rab proteins (16,17). This enzyme also contains α- and β-subunits, but contains an additional subunit Rab Escort Protein (REP) (16,18). The REP subunit binds to the substrate Rab protein (19). The α- and β-subunits share homology with corresponding subunits of GGTase-I.

Small molecule inhibitors of GGTases provide novel reagents to study geranylgeranylation. Development of peptidomimetic GGTI compounds derived from the CAAL peptide was pioneered by Sebti and Hamilton groups. These compounds (GGTI-298, GGTI-2154, etc.) exhibit cellular effects including cell cycle arrest and apoptosis induction and inhibit tumor growth in mice (20-24). However, development of non-peptidomimetic inhibitors lagged behind. Recently, the first non-peptidomimetic compound, GGTI-DU40, was reported (25). As for RabGGTase inhibitors, no effective inhibitors have been identified so far (26). 2-Hydroxy-2-phosphono-3-(3-pyridinyl) propanoic acid has been shown to inhibit geranylgeranylation of Rab proteins, however a high concentration (mM) of compound is used to achieve inhibition (27). Thus, further development of compounds that inhibit GGTase-I or RabGGTase is of importance, as they provide novel reagents to deepen our understanding of protein geranylgeranylation.

In this study, we first report identification and characterization of novel inhibitors of GGTase-I which were identified from a chemical compound library that we constructed through diversity oriented synthesis using allenoic acid derived starting materials (28). A specific modification that improves their cellular activity has been identified. Second, we report identification and characterization of novel compounds with preferential inhibition of RabGGTase. These compounds have a scaffold that is shared with GGTI but possess an extra structural feature.

**Experimental Procedures**

**Cell Lines and Cell Culture-** NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Cellgro, Herndon, VA) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT), 2% L-glutamine, 1% penicillin, and 1% streptomycin stock solutions (Life Technologies, Gaithersburg, MD). K562 cells were maintained in RPMI-1640 medium (Cellgro) supplemented with 10% (v/v) FBS and penicillin/streptomycin. PANC-1 cells were maintained in DMEM/F12 medium (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) FBS and penicillin/streptomycin. MCF-7 cells were maintained in Eagle’s Minimum Essential Medium (EMEM; Cellgro) supplemented with 10% (v/v) FBS and penicillin/streptomycin.

**Materials-** [3H]-farnesyl diphosphate (FPP) (21.5 Ci/mmol) and [3H]-geranylgeranyl diphosphate (GGPP) (23.0 Ci/mmol) were purchased from PerkinElmer Life Sciences. BMS-225975 was kindly provided by Dr. Veeraswamy Manne (Bristol-Myers Squibb). GGTI-298 was purchased from Calbiochem (La Jolla, CA). Prenyltransferases used are recombinant enzymes. GGTase-I, FTase, RabGGTase, REP-1 and Rab7 were purchased from JENA BIOSCIENCE (Jena, Germany). Other chemicals were obtained from Sigma. The allenoate derived compounds library
including P3-E5 and P5-H6 were synthesized as described (28). Detailed methods for the synthesis of P63-F10, P63-C7, P63-E11, P62-A5, P62-C11, P62-E4 and modified P5-H6 compounds (P61-A2, P61-A5, P61-A6, P61-A7 and P61-B4) will be described elsewhere.

**In vitro Enzyme Assays-** GGTase-I and FTase activities were determined by following the incorporation of radiolabeled isoprenoid [3H]-geranylglyceranyl or [3H]-farnesyl into substrate proteins. FTase or GGTase-I (50 nM) were used to initiate reactions containing 0.4 µM of [3H]-FPP or 0.5 µM of [3H]-GGPP and 2 µM of MBP-tagged substrates (K-Ras4B for FTase; RhoA for GGTase-I) in 20 µl of buffer {50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 50 mM ZnCl2 and 5 mM DTT}. Inhibitors were added at the indicated concentrations. Final DMSO concentration was 2.5% for all samples. Reactions were carried out for 10 min at 30 °C. The reaction mixture was spotted onto a filter paper, treated with 10% trichloroacetic acid (TCA) followed by ethanol and acetone washing. The filter was counted using a scintillation counter. Kinetic assays in which the GGPP concentration was varied employed fixed concentrations of GGTase-I and RhoA, and reactions were carried out for 5 min. Similarly, fixed concentrations of GGTase-I and GGPP were used when the amount of RhoA was varied. For RabGGTase assays, the reaction contained the following components in 20 µl; 0.625 µl of [3H]-GGPP (0.7 µM), 25 nM RabGGTase, 0.6 µM REP-1, 0.6 µM purified Rab7 or Ypt1 protein, 40 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM DTT, 3 mM MgCl2 and 0.3% CHAPS. Reactions were carried out for 20 min at 37 °C and the products were analyzed as described above for the GGTase-I reaction. Graphing and Michaelis-Menten analysis were performed using Prism ver.5 (GraphPad, San Diego CA).

**Inhibition of geranylglyceranyl in cells-** Inhibition of GGTase-I catalyzed protein geranylglyceranyl was assessed by examining the accumulation of unprenylated Rap1. To measure the level of unprenylated Rap1, cells were cultured in DMEM plus 10% (v/v) FBS overnight, and then DMSO or appropriate inhibitors were added. Incubation was continued for 48 hours. The cells were harvested and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 1x Protease Inhibitor Cocktail). Whole cell lysates of NIH3T3 cells were electrophoresed on a 12% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with the antibody against unprenylated form of Rap1 (Santa Cruz Biotechnology catalog number sc-1482, goat), total-Rap1 (Santa Cruz: sc-65) or actin (Sigma: A4700). Actin was used as a loading control. Inhibition Rab geranylglyceranyl in cells (Fig. S2) was examined according to (29). Briefly, whole cell lysates were subjected to 15% SDS-PAGE containing 4M urea followed by immunoblotting with the antibody against Rab5b (Santa Cruz: sc-598) or actin. Subcellular fractionations (Fig. S3) were performed as described by Gomes et al. (30). Briefly, cells were treated with P49-F6 for 48 hours. After osmotic lysis, cell debris were removed by centrifugation at 500 x g for 10 min, and the supernatant was subjected to ultracentrifugation at 100,000 x g for 60 min. The supernatant of ultracentrifugation was collected as a soluble fraction. The pellet was collected as a membrane fraction. These fractions were subjected to electrophoresis on 10% SDS-PAGE gels followed by immunoblotting with the antibody against Rab5b. RhoGDI (Santa Cruz: sc-360) and Na+/K+ ATPase (Sigma: A276) were used as markers for soluble and membrane fractions, respectively.

**Cell Viability, Cell Cycle Analysis -** Cell viability was determined by Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) as described previously (31). Briefly, cells (5 x 10^4) were plated onto 96-well plates and treated with the appropriate inhibitor as indicated in figure legends. Cell viability was calculated relative to the DMSO control. Cell cycle profile was analyzed by flow cytometry as described previously (32).

**Transcriptional Reporter Assays-** For the p21^WAF1/CIP1 promoter luciferase assay, NIH3T3 cells were transfected with p21^WAF1/CIP1 promoter-Luc or vector plasmids (33) (both plasmids are provided by Dr. Genhong Cheng). Cells were treated with GGTI compounds. Promega luciferase assay kit was used according to the manufacturer's protocol.

**Statistical analysis-** Statistical significance of
difference was determined using the unpaired Student's t-test. *P* value < 0.05 was considered statistically significant.

**RESULTS**

*Allenoic acid based chemical compound library and identification of GGTase-I inhibitors.*

Previously, we have reported construction of a library of allenoate derived compounds and identification of novel GGTI compounds (28). The library construction involved the use of allenoates as multireactive core molecules. Using the second set of building blocks (imines, aldehydes and maleimides) that react with allenoates under similar reaction conditions (phosphine catalysis), we have produced diverse compounds including dihydropyrrroles (34), tetrahydropyridines (35), bicyclic succinimides (unpublished results), dioxanylidenes (36) and α-pyrones (37). The identification of GGTI compounds was carried out first by screening a 171-compound pilot library using an *in vitro* assay with RhoA protein as a substrate. Scaffolds that initially showed activity were optimized by solid-phase split-and-pool combinatorial synthesis. This enabled us to identify two types of novel compounds; one group containing a tetrahydropyridine ring as its core scaffold and the other group having a dihydropyrole ring as its core scaffold. Fig. 1 shows structure and potency of four representative compounds from each group together with a general structure of each group.

Two compounds with highest potency in each group, P3-E5 and P5-H6, were further characterized. As described, P3-E5 and P5-H6 inhibit GGTase-I with IC$_{50}$ values of 313 and 466 nM, respectively (Fig. 1). Specificity of GGTase-I inhibition by P3-E5 and P5-H6 was examined by assaying their ability to inhibit two closely related enzymes, FTase and RabGGTase. As can be seen, no significant inhibition of FTase activity was observed by these compounds even when the concentration was increased to 50,000 nM (Fig. 2). Similarly, P3-E5 showed little inhibition against RabGGTase even at 50,000 nM. P5-H6 showed little inhibition against RabGGTase up to 10,000 nM.

GGTIs compete with substrate protein. Michaelis-Menten analysis of the inhibition of GGTase-I is shown in Fig. 3. The upper panels show the data derived from the results obtained using varying concentrations of geranylgeranyl pyrophosphate (GGPP), while the lower panels show data derived from the results obtained using varying concentrations of the substrate protein, RhoA. These results revealed that P3-E5 and P5-H6 are competitive inhibitors with respect to the protein substrate and uncompetitive inhibitors with respect to GGPP. P3-E5 and P5-H6 compete for binding of the protein substrate but not the isoprenoid substrate of GGTase-I. $K_i$ values of 187 ± 13 nM and 408 ± 32 nM, respectively, were calculated for P3-E5 and P5-H6.

*Improvement of cellular activity of GGTI by the modification of P5-H6.*

We found that replacing a carboxyl group of the dihydropyrrrole ring of P5-H6 significantly improves its cellular activity. This is shown in Fig. 4A where we synthesized a series of compounds with different moieties replacing the carboxyl group. Potency of these compounds to inhibit proliferation of K562 leukemic cells was examined and their IC$_{50}$ values are shown. As can be seen, original P5-H6 compound containing a carboxylic acid moiety exhibited inhibition of proliferation of K562 cells with IC$_{50}$ of 20 µM. Converting the free acid to an ethyl ester did not improve its cellular potency. However, amidation of this moiety led to improvement in its potency. Moreover, coupling of P5-H6 with an L-phenylalanine group resulted in significant improvement of cellular potency. Two compounds, P61-A6 and P61-B6, showed good potency with IC$_{50}$ of 2.2 µM and 5.0 µM to inhibit proliferation of K562 cells, respectively. Comparison of inhibitory activity of P5-H6 and P61-A6 on a pancreatic cancer cell line PANC-1 and Jurkat cells is shown in Fig. 4B.

Improved potency of these compounds to inhibit cell proliferation correlates with their increased ability to inhibit protein geranylgeranylation inside the cell. Results on this point using P5-H6 and P61-A6 are shown in Fig. 4C. In this experiment, inhibition of protein geranylgeranylation is evaluated using an antibody that specifically detects unprenylated Rap1. As can be seen, treatment with P5-H6 or P61-A6 led to the appearance of the unprocessed Rap1 band in a dose dependent manner. Note that
the appearance of the Rap1 band is observed at 2.5 µM concentration with P61-A6, while this is not seen with P5-H6, reflecting significant improvement in the potency of P61-A6 to inhibit protein geranylgeranylation.

In contrast to its effects on GGTase-I, P61-A6 did not inhibit protein farnesylation. This was examined by using a farnesylated protein H-Ras. While farnesyltransferase inhibitor (FTI) (BMS-225975) slowed the mobility of H-Ras protein on a SDS polyacrylamide gel, no such mobility shift was observed with P61-A6 or another GGTI compound, GGTI-298. Similarly, P61-A6 did not inhibit geranylgeranylation of Rab5b, as a slow migrating band representing that of unmodified Rab5b was detected only after the treatment with RabGGTase inhibitors (P49-F6, see below) and not with P61-A6 (Fig. 4E).

Although P61-A6 exhibits improved ability to inhibit geranylgeranylation in cells, its ability to inhibit GGTase-I enzyme was less than that of P5-H6, as the IC₅₀ value for the enzyme inhibition was 1 µM. No significant inhibition of FTase or RabGGTase activity was observed by P61-A6 compound even when the concentration was increased to 100,000 nM (data not shown).

GGTI compounds inhibit proliferation of various human cancer cell lines and cause G₁ cell cycle arrest. As can be seen in Table S1, significant inhibition of proliferation of a variety of human cancer cell lines was observed. Thus, a broad range of human cancer cell lines is inhibited by these GGTI compounds.

The inhibition of proliferation by GGTI appears to be due to the inhibition of cell cycle progression. As shown in Fig. 5A, treatment of a breast cancer cell line MCF-7 with P61-A6 or P61-B6 caused dose-dependent inhibition of proliferation. This is associated with a significant dose-dependent enrichment of G₁ phase cells, while the percentage of S-phase cells decreased (Fig. 5B, Fig. S1). Similar G₁ enrichment was observed with leukemic cell line, Jurkat, two pancreatic cancer cell lines, PANC-1 and MiaPaCa2, as well as with another breast cancer cell line MDA-MB-231 (Table S2).

One of the proposed mechanisms of GGTI effects on cell cycle progression is to inhibit RhoA that negatively regulates expression of a Cdk inhibitor p21CIP1/WAF1. To investigate whether our GGTI compound induces p21CIP1/WAF1 expression, luciferase transcriptional activation from the p21CIP1/WAF1 promoter (33) was measured. Transient expression systems with NIH3T3 cells were used to examine the ability of P61-A6 to induce p21CIP1/WAF1-luciferase expression. P61-A6 induced significant (4-fold) inductions of luciferase activity versus DMSO in a dose dependence manner (Fig. 5C).

Identification of dual specificity inhibitors of GGTase-I and RabGGTase. Characterization of our library of allenoate derived compounds yielded novel compounds that have the ability to inhibit RabGGTase. Identification of these novel compounds was initially prompted by the observation that P5-H6 exhibits a slight inhibition of RabGGTase at concentrations higher than 10,000 nM (Fig. 2; lower panel). This suggested to us that these GGTIs have the ability to act weakly on RabGGTase. If this were the case, we speculated that it might be possible to identify a subset of GGTI compounds that inhibit RabGGTase. With this reasoning, we examined our library of 3601 compounds related to P5-H6 and listed all the compounds that exhibited GGTase-I inhibition and screened them to see whether we can identify compounds that also inhibited RabGGTase. The assay was carried out using RabGGTase as described in “Experimental Procedures”. Out of 428 compounds that exhibited GGTase-I inhibition (more than 50% inhibition at 50 µM concentration), we found that 60 compounds also exhibited the ability to inhibit RabGGTase (more than 50% inhibition at 25 µM concentration). Fig. 6 shows four most potent compounds (P8-G7, P8-H6, P8-H7 and P49-F5) that exhibit inhibition of both GGTase-I and RabGGTase at a single µM range. On the other hand, these compounds do not inhibit FTase even with more than 100 µM concentration (Fig. 6B and C). Examination of their structures led us to notice a feature that is common to these dual specificity compounds; they all have a characteristic 6-atom aliphatic tail attached to the penta-substituted pyrrolidine core via thioether linkage (putative RabGGTI feature).

Identification of RabGGTase inhibitors. The finding of the putative RabGGTI feature is important, as this feature may be used to predict RabGGTI activity. We first examined our 3601
compound library and identified 524 compounds that have this feature. We then screened these compounds by carrying out RabGGTase assay. This resulted in the identification of 23 compounds that exhibited preferential inhibition of RabGGTase (less than 50% inhibition of GGTase-I at 50 µM and more than 50% inhibition of RabGGTase at 25 µM concentration). Fig. 7 shows the structure and IC₅₀ values for the five most potent RabGGTase preferential inhibitors, P23-D6, P47-D11, P49-A6, P49-F6 and P50-E11. Three of these compounds contain n-hexylmercapto substituent at C4 of the pyrrolidine ring. Additionally, we find n-pentyl thioether (P47-D11) and para-methoxyphenyl thioether (P23-D6) appendages in the group of RabGGTase preferential inhibitors.

As can be seen in Fig. 7B and 7C, all these compounds inhibit RabGGTase with the IC₅₀ value of 2-5 µM, while the inhibition of GGTase-I required more than 50 µM. No inhibition of FTase was observed with 100 µM of these compounds. Inhibition of Rab geranylgeranylation in cells was examined using Rab5b protein. In this experiment, we examined the mobility of Rab5b proteins (Fig. S2A). P49-F6, one of the RabGGTase preferential inhibitors caused the appearance of a slow migrating Rab5b protein which represents an unprenylated form (29). On the other hand, P49-F6 did not cause mobility shift of H-Ras and did not induce appearance of unprenylated Rap1, suggesting that it does not inhibit FTase or GGTase-I (Fig. S2B). We also examined intracellular localization of Rab5b proteins (Fig. S3). Treatment with P49-F6 resulted in the increase of Rab5b protein in the soluble fraction.

RabGGTI competes with the substrate protein. Kinetic analysis was carried out to examine whether our RabGGTI compounds compete with substrate protein. Rab geranylgeranyltransferase consists of a tightly bound core complex, the α- and β-subunits, and the third subunit REP protein. Therefore, these subunits were first mixed together in the presence of a low concentration of GGPP and then the concentration of each substrate was altered and the effect of inhibition by RabGGTI was examined. As can be seen in Fig. 8, a RabGGTI compound P49-F6 inhibits RabGGTase activity with respect to the substrate protein Rab7 (Fig. 8B). On the other hand, increasing concentration of GGPP did not influence the inhibition by RabGGTI (Fig. 8A), suggesting that they are uncompetitive inhibitors with respect to GGPP. Ki value of 1.36 ± 0.38 µM was calculated from these experiments.

**DISCUSSION**

In this paper, we first report identification of novel small molecule inhibitors of GGTase-I from the diversity library of allenoate-derived compounds. Two novel scaffolds were identified. Our compounds are non-peptidomimetic inhibitors that compete with the substrate protein. Specific inhibition of protein geranylgeranylation but not protein farnesylation was established with the purified enzymes as well as with treated cells. Another non-peptidomimetic GGTI compounds have been reported (25). However, 2-20 µM concentration was used to exert cellular activity with tissue culture cells (25). Our compounds represent an important addition to the growing list of GGTI compounds.

We found that derivatizing the carboxyl group of the dihydropyrrole ring of our initial compound P5-H6 results in a dramatic increase in their cellular potency. In particular, changing the carboxyl group to the corresponding amide by coupling it with L-phenylalanine is effective. On the other hand, converting the free carboxylic acid into the ethyl ester did not lead to any improvement. Similarly, converting to a methyl ester did not result in improvement either (unpublished results). These results suggest that the improvement is not simply due to the removal of the charge but that the phenylalanine moiety exerted additional effect. The improvement of the cellular activity of our GGTI compound is correlated with the increase in the ability to inhibit protein geranylgeranylation, as detected by the appearance of unprenylated Rap1 protein. On the other hand, the modification did not improve potency of these compounds to inhibit GGTase-I enzyme. Therefore, the improvement of cellular activity may reflect increased cellular uptake or stability of the compound.

Our GGTI compounds exhibit inhibition of proliferation of human cancer cell lines including leukemic, pancreatic cancer and breast cancer cell
lines. One of the hallmarks of GGTI is that this class of inhibitors causes cell cycle arrest at the G1 phase (38,39). Our inhibitors exhibit significant G1 arrest with the human cancer cell lines examined (Fig. 5B, Table S2). In particular, dramatic G1 arrest is observed with a breast cancer cell line MCF-7. In addition, our GGTI compounds induce p21\textsuperscript{CIP1/WAF1} expression, as observed by using a luciferase reporter assay. These results are in line with the idea that our GGTI inhibits RhoA that acts as a negative regulator of p21\textsuperscript{CIP1/WAF1} expression (39,40,41). These results also agree with the recent observation of the accumulation of p21\textsuperscript{CIP1/WAF1} in GGTase-I-deficient cells from knockout mice (6).

In this paper, we also report identification of a novel type of RabGGTase inhibitors. These compounds share the scaffold with one of the GGTI compounds but possess an extra hydrophobic tail emanating from the core ring. Only a handful of RabGGTase inhibitors have been identified in the past (26). Commonly used inhibitors are bisphosphonate type compounds, however the inhibition of RabGGTase requires about mM concentration of the compounds. In contrast, our compounds inhibit RabGGTase with a single \mu M concentration. While we did observe inhibition of geranylgeranylation of Rab protein in cells, further modification of the compounds is needed to improve their cellular activity. Recent studies suggest that RabGGTI may be valuable as anticancer drugs. A study using siRNA showed that the inhibition of RabGGTase leads to apoptosis induction in human cancer cells (42). Elevated levels of RabGGTase are detected in a number of human cancers (42). Furthermore FTI compounds which inhibit RabGGTase induce mislocalization of Rab protein and apoptosis (42).

We have shown that our RabGGTI compounds inhibit the enzyme by competing with the substrate protein. Because our RabGGTI compounds are derived from GGTI compounds, RabGGTI and GGTI share the same scaffold. This may be the reason that both our GGTI and RabGGTI work by competing with the protein substrate. GGTase-I and RabGGTase share similar active site structures (16,26); both enzymes have a core structure that consists of \(\alpha\)- and \(\beta\)-subunits. In addition, the corresponding subunits in these enzymes share significant homology. It will be interesting to examine whether these inhibitors bind to similar pockets in these enzymes. On the other hand, we found that RabGGTI compounds possess a structural feature that is unique to this group of inhibitors. They contain a characteristic long aliphatic tail attached to the penta-substituted pyrrolidine core. It will be interesting to understand how this feature contributes to the inhibition of RabGGTase. One possibility is that the aliphatic tail fits into a pocket or interferes with an enzymatic process that is specific to RabGGTase but not to GGTase-I. Significance of lipid binding pockets of REP for RabGGTase reaction has been suggested (18,43). Further characterization of our GGTI and RabGGTI should lead to increased understanding about possible differences and similarities in active sites of the two enzymes.

In summary, we report three different types of inhibitors of protein geranylgeranylation. First, we report novel GGTase-I inhibitors. They inhibit proliferation of a variety of human cancer cell lines and cause G1 cell cycle arrest. Second, we report novel RabGGTase inhibitors. Interestingly, those compounds possess structural features that define this class of inhibitors. In the course of the identification of RabGGTI compounds, we have also identified dual specificity inhibitors that inhibit both GGTase-I and RabGGTase. Use of these novel compounds may lead to further understanding of the roles of protein geranylgeranylation. In addition, they may provide lead compounds for the development of anticancer drugs based on the inhibition of protein geranylgeranylation.

REFERENCES

4. Gelb, M. H., Brunsveld, L., Hrycyna, C. A., Michaelis, S., Tamanoi, F., Van Voorhis, W. C., and

**FOOTNOTES**

This work is supported by NIH grant CA32737 (to F. T.) and GM071779 (to O. K.) as well as by a grant from the Susan E. Riley Foundation. We would like to thank Alan Ikeda and Kathleen Sakamoto for discussion on the effect of GGTI on leukemic cells. We also thank Richard Finn for discussion on breast cancer cells. The flow cytometric analysis was done in the University of California at Los Angeles Flow Cytometry Core Facilities that are supported in part by grants from the NIH (CA16042 and AI28697).

**FIGURE LEGENDS**

**Fig. 1.** Novel core structures of GGTI and molecular structures of potent inhibitors. IC\textsubscript{50} values of GGTase-I inhibition *in vitro* by compounds were measured as described in “Experimental Procedures”.

**Fig. 2.** Effect of P3-E5 (A) and P5-H6 (B) on the enzymatic activity of GGTase-I (left), RabGGTase (middle) and FTase (right). Varying concentrations of compounds were added to each enzyme reaction. Data represent the mean +/- S.D. of two measurements from two independent experiments.
Fig. 3. Kinetic analysis of GGTase-I inhibition. Double reciprocal plots obtained from substrate velocity curves for the inhibition of GGTase-I by P3-E5 (left) and P5-H6 (right). (A) Varying GGPP concentrations with a fixed RhoA protein concentration were used. (B) Varying RhoA protein concentrations with a fixed GGPP concentration were used. The amount of GGTI used is indicated.

Fig. 4. Improved cellular activity of modified GGTI compounds. (A) Molecular structure of P5-H6 and modified P5-H6 compounds. K562 cells were treated with modified compounds for 72 hours and then cell number was counted as described in “Experimental Procedures”. IC50 values of cell viability relative to the DMSO are measured. (B) Inhibitory effect of 12.5 µM P5-H6 or P61-A6 on PANC-1 and Jurkat cell viability. Data represent the mean +/- S.D. of two measurements from two independent experiments. *, P < 0.05 compared with the value for DMSO. (C-E) P5-H6 or P61-A6 treatment inhibits Rap1 geranylgeranylation in NIH3T3 cells. Whole cell lysates from cells treated with DMSO, P5-H6, P61-A6, GGTI (GGTI-298), FTI (BMS-225975) or RabGGTI (P49-F6) for 48 hours were prepared and processed for immunoblot analysis using antibody against unprenylated form of Rap1 (C: upper panel), Total-Rap1 (C: middle panel), H-Ras (D: upper panel), Rab5b (E: upper panel) or actin (C, D, and E: lower panel). The immunoblots shown here are representative of two independent experiments for each treatment.

Fig. 5. Effects of P61-A6 or P61-B6 on cell proliferation and cell cycle in MCF-7 cells. (A) Inhibition of proliferation of MCF-7 by P61-A6 and P61-B6. MCF-7 cells were treated with P61-A6, P61-B6 or DMSO for 72 hours. Cell number was counted as described in “Experimental Procedures”. Cell viability relative to the DMSO control (100% value) is plotted. (B) MCF-7 cells were treated with indicated concentrations (µM) of P61-A6, P61-B6 or DMSO for 48 hours. Cell cycle profiles were monitored by flow cytometry. Percentages of cells in each phase of the cell cycle are indicated by different shades. (C) NIH3T3 cells were transfected with p21CIP1/WAF1-luciferase or empty vector. Cells were treated with P61-A6 or P61-B6 compound at indicated concentrations or with DMSO for 48 hours and luciferase assay was performed. Data represent the mean +/- S.D. of two measurements from two independent experiments. *, P < 0.05; **, P < 0.005 compared with the value for DMSO.

Fig. 6. Dual specificity inhibitors of GGTase-I and RabGGTase. (A) Molecular structure of dual specificity inhibitors. (B) IC50 values of dual specificity inhibitors against GGTase-I, RabGGTase and FTase. (C) Inhibitory effect of 25 µM P49-F5 compound on in vitro activities of GGTase-I, RabGGTase and FTase.

Fig. 7. RabGGTase preferential inhibitors. (A) Molecular structure of RabGGTase inhibitors. (B) IC50 values of RabGGTase preferential inhibitors against GGTase-I, RabGGTase and FTase. (C) Inhibitory effect of 25 µM P49-F6 compound on in vitro activities of GGTase-I, RabGGTase and FTase.

Fig. 8. Characterization of P49-F6 inhibition of RabGGTase. in vitro RabGGTase assay was carried out with varying concentration of GGPP (A) or Rab7 (B). Each reaction mixture contained recombinant RabGGTase and recombinant REP-1 protein. They were mixed with a low concentration of GGPP (0.125µM) to form an active enzyme first. Duplicate reactions were carried out as described under "Experimental Procedures". The amount of RabGGTI used is indicated.
Supplemental data

**Fig. S1.** Primary FACS data. MCF-7 cells were treated with 10 µM of P61-A6, P61-B6 or DMSO for 48 hours. Data shown here are representative of two independent experiments for each treatment.

**Fig. S2.** Inhibition of RabGGTase activity in cells. (A) P49-F6 treatment inhibits Rab5b geranylgeranylation in NIH3T3 cells. Whole cell lysates from cells treated with DMSO or P49-F6 for 48 hours were prepared and processed for immunoblot analysis using antibody against Rab5b (upper panel) or actin (lower panel). (B) Whole cell lysates from NIH3T3 cells treated with DMSO or P49-F6 for 48 hours were prepared and processed for immunoblot analysis using antibody against unprenylated form of Rap1 (upper panel), total-Rap1 (upper middle panel), H-Ras (lower middle panel) or actin (lower panel).

**Fig. S3.** Effects of RabGGTI on membrane association of Rab5b protein. Western blots of Rab5b in the soluble fractions or membrane fractions prepared from NIH3T3 cells treated for 48 hours as indicated in "Experimental Procedures". Rho GDI and Na⁺/K⁺ ATPase were used as marker proteins for the soluble and membrane fractions, respectively. Bars indicate intensity of protein bands after normalization using loading control.
Fig. 1

Tetrahydropyridine ring
IC_{50}:
- P3-E5: 313 nM
- P63-F10: 841 nM
- P63-C7: 1020 nM
- P63-E11: 576 nM

Dihydropyrrole ring
IC_{50}:
- P5-H6: 466 nM
- P62-A5: 1050 nM
- P62-C11: 980 nM
- P62-E4: 1050 nM
Fig. 3

A: GGPP

B: RhoA
Fig. 4

A

<table>
<thead>
<tr>
<th>IC₅₀ values of proliferation of K562 cells (μM)</th>
<th>20.0</th>
<th>&gt;25.0</th>
<th>11.0</th>
<th>10.5</th>
<th>2.2</th>
<th>5.8</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5-H6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P61-A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P61-A7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P61-A5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P61-A6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P61-B4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P61-B6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Cell viability (% control)

DMSO  P5-H6  P61-A6

PANC-1  Jurkat

C

Unproenylated Rap1
Total Rap1

Actin

D

H-Ras

Actin

E

Rab5b

Actin
Fig. 5

A. Cell viability (% control) vs. Concentration, μM

B. % Cell Cycle Phases

C. p21-Luciferase activity (x1000 Units)
Fig. 6

A

B

<table>
<thead>
<tr>
<th>IC50 values of dual specificity inhibitors (μM)</th>
<th>GGTase-I</th>
<th>RabGGTase</th>
<th>FTase</th>
</tr>
</thead>
<tbody>
<tr>
<td>P8-G7</td>
<td>8.7</td>
<td>4.7</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>P8-H6</td>
<td>8.9</td>
<td>3.1</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>P8-H7</td>
<td>2.4</td>
<td>7.0</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>P49-F5</td>
<td>5.1</td>
<td>7.0</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

C
**A**

Chemical structures of inhibitors:

- P23-D6
- P47-D11
- P49-A6
- P49-F6
- P50-E11

**B**

IC₅₀ values of preferential inhibitors (µM)

<table>
<thead>
<tr>
<th></th>
<th>GGTase-I</th>
<th>RabGGTase</th>
<th>FTase</th>
</tr>
</thead>
<tbody>
<tr>
<td>P23-D6</td>
<td>&gt; 50</td>
<td>4.5</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>P47-D11</td>
<td>&gt; 50</td>
<td>3.6</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>P49-A6</td>
<td>&gt; 50</td>
<td>4.8</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>P49-F6</td>
<td>&gt; 50</td>
<td>2.1</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>P50-E11</td>
<td>&gt; 50</td>
<td>2.2</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

**C**

Graph showing enzyme activity (% control) for P49-F6 with error bars.
Fig. S1

DMSO

P61-A6

P61-B6
Fig. S2

**A**

<table>
<thead>
<tr>
<th></th>
<th>P49-F6</th>
<th>GGTI-298</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rab5b

Actin

**B**

<table>
<thead>
<tr>
<th></th>
<th>P49-F6</th>
<th>GGTI-298</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unprenylated-Rap1

Total-Rap1

H-Ras

Actin
Fig. S3
Table S1. Potencies of GGTIs toward human cancer cell lines

<table>
<thead>
<tr>
<th>Cancer Cell Type</th>
<th>Tissue Type</th>
<th>P61-A6</th>
<th>P61-B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>Blood</td>
<td>2.9</td>
<td>1.3</td>
</tr>
<tr>
<td>SE-Mk2</td>
<td>Blood</td>
<td>6.4</td>
<td>14.1</td>
</tr>
<tr>
<td>PANC-1</td>
<td>Pancreas</td>
<td>5.2</td>
<td>12.6</td>
</tr>
<tr>
<td>MiaPaCa2</td>
<td>Pancreas</td>
<td>4.7</td>
<td>13.9</td>
</tr>
<tr>
<td>AsPc-1</td>
<td>Pancreas</td>
<td>11.7</td>
<td>&gt;20.0</td>
</tr>
<tr>
<td>Capan-2</td>
<td>Pancreas</td>
<td>11.5</td>
<td>11.1</td>
</tr>
<tr>
<td>CFpac-1</td>
<td>Pancreas</td>
<td>6.3</td>
<td>8.1</td>
</tr>
<tr>
<td>HPAC</td>
<td>Pancreas</td>
<td>3.9</td>
<td>11.7</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>8.7</td>
<td>12.5</td>
</tr>
<tr>
<td>BT474</td>
<td>Breast</td>
<td>8.5</td>
<td>11.6</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>4.5</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Cancer cells were treated with the indicated GGTIs for 3 days (Jurkat, PANC-1, MiaPaCa2, Capan-2, CFpac-1, HPAC, MDA-MB-231 and BT474) or 6 days (SE-MK2, AsPc-1 and MCF-7), and cell number was counted using CCK-8 and compared with vehicle (DMSO) treated cells. Values are the IC50 (μM) for at least two separate experiments.
Table S2. Effects of GGTIs on cell cycle phase distribution in cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue</th>
<th>Percent G0/G1</th>
<th>P value</th>
<th>Percent G2/M</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DMSO</td>
<td>P61-A6</td>
<td></td>
<td>DMSO</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Blood</td>
<td>46.92</td>
<td>57.87</td>
<td>&lt; 0.05</td>
<td>11.80</td>
</tr>
<tr>
<td>PANC-1</td>
<td>Pancreas</td>
<td>42.08</td>
<td>54.13</td>
<td>&lt; 0.005</td>
<td>24.21</td>
</tr>
<tr>
<td>MiaPaCa2</td>
<td>Pancreas</td>
<td>77.85</td>
<td>87.98</td>
<td>&lt; 0.05</td>
<td>14.12</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>73.78</td>
<td>85.16</td>
<td>&lt; 0.05</td>
<td>7.74</td>
</tr>
</tbody>
</table>

NOTE: Cancer cells were treated with the P61-A6 (10 μM) for 48 hours, and cell cycle distribution was determined by flow cytometry as described under "Experimental Procedures". Data are representative of at least two independent experiments.
Inhibitors of protein geranylgeranyltransferase-I and rab geranylgeranyltransferase identified from a library of allenoate derived compounds
Masaru Watanabe, Hannah D.G. Fiji, Lea Guo, Lai Chan, Sape S Kinderman, Dennis J Slamon, Ohyun Kwon and Fuyuhiko Tamanoi

J. Biol. Chem. published online January 28, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M706229200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2008/01/29/M706229200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2008/01/28/jbc.M706229200.citation.full.html#ref-list-1