In vivo antitumor effect of a novel inhibitor of protein geranylgeranyltransferase-I

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Abstract

Protein geranylgeranyltransferase-I (GGTase-I) catalyzes protein geranylgeranylation, which is critical for the function of proteins such as Rho, Rac, and Ral. We previously identified several small-molecule inhibitors of GGTase-I from an allenoate-derived compound library and showed that these compounds exhibit specific inhibition of GGTase-I resulting in the inhibition of proliferation associated with the induction of G1 cell cycle arrest of a variety of cancer cell lines. Because inhibition of GGTase-I is expected to suppress tumor growth, we investigated in vivo effects of one of these GGTase-I inhibitors (GGTI), P61A6, by using a human pancreatic cancer xenograft model in mice. The new compound GGTI P61A6 showed an excellent antitumor effect. I.p. administration of P61A6 significantly suppressed tumor growth of the Panc-1 xenograft. Even once per week administration of GGTI was enough to suppress tumor growth. Immunohistochemical examination indicated the inhibition of cell proliferation in the tumors by P61A6 treatment, but neither apoptosis nor antiangiogenesis was observed. Increased cytosolic localization of proteins such as Rap1 and RhoA in tumors was observed. In addition, the enzyme activity of GGTase-I in tumors was inhibited. Pharmacokinetic analysis showed that the plasma half-life of GGTI is 6 h, suggesting its prolonged effect. These data suggest that the novel GGTI compound P61A6 is an excellent chemotherapeutic drug candidate for human pancreatic cancer. They also provide evidence that protein GGTase-I may be a valid target for cancer therapy. [Mol Cancer Ther 2009;8(5):1218–26]

Introduction

Recent studies on protein geranylgeranylation point to the significance of this post-translational modification in oncogenesis. Proteins such as RhoA, RhoC, Rap1, and Ral are geranylgeranylated (1). Discovery of Dlc1, Rho-GAP, as a major tumor suppressor suggests that activation of the Rho type proteins is widespread in cancer (2, 3). RhoC is a key protein that is required for tumor metastasis (4, 5). Ral proteins are activated in >90% of pancreatic duct adenocarcinoma cases (6). Protein geranylgeranylation is catalyzed by protein geranylgeranyltransferase-I (GGTase-I), an enzyme that adds a C20 geranylgeranyl group to the cysteine of proteins with the COOH-terminal tetrapeptide consensus sequence CAAL (C is cysteine, A is any aliphatic amino acid, and the COOH-terminal residue is leucine or phenylalanine; refs. 7-11). Characterization of mice with conditional knockout of GGTase-I showed that the GGTase-I deficiency results in reduced oncogenic K-ras-induced lung tumor formation and dramatically increased survival (12). GGTase-I-deficient cells showed proliferation inhibition and accumulation of p21cip1/waf1, pointing to the importance of GGTase-I in cell proliferation and cell cycle progression (12). These observations prompted us and others to design GGTase-I inhibitors (GGTI) as potential anticancer drugs (13-19). To date, several GGTI compounds have been developed, such as GGTI-298 (20), GGTI-2154 (15), GGTI-2166 (21), and GGTI-286 (22). Although these are derived from CAAL peptide, there are also nonpeptidomimetic inhibitors. GGTI-DU45 was identified via high-throughput screening of a compound library (16). We have recently established a new chemical compound library of >4,000 allenoate-derived compounds, screened them for inhibitors of human GGTase-I, and identified several GGTIs that can be divided into two groups: one group containing a tetrahydropyridine ring as its core scaffold and the other group having a dihydropyrrole ring as its core scaffold (18, 23). These GGTI compounds inhibit the protein modification and block membrane association and function of Ral, Rho, and Rap subfamilies, cause cell cycle arrest at the G1 phase, and suppress the growth of several human cancer cell lines including leukemia, pancreatic cancer, and breast cancer (18).

Antitumor efficacy of GGTI compounds has been reported. Demonstration of the antitumor efficacy of GGTI-2154 was made using the human lung adenocarcinoma A549 xenograft model (24). By using the MMTV-\(v\)-Ha-Ras model, Sun et al. (15) showed that GGTI-2154 induced apoptosis, tumor regression, and differentiation as well as...
inhibited oncogenic and tumor survival pathways in nude mice. GGTI-2 was used by Lobell et al. to inhibit growth of tumor xenografts in mice in combination with FTI (25). In this experiment, administration of 3 mg/kg/d GGTI-2 alone was not deleterious; however, administration of 30 to 100 mg/kg/d caused cellular depletion in the bone marrow and spleen. These observations suggest that low concentrations of GGTIs are well tolerated. The challenge will be to achieve significant inhibition of geranylgeranylation at low concentrations of GGTI so that any deleterious effects will be minimal.

In this article, we have reassessed the issue of using GGTIs to inhibit tumor growth in mice by using our novel GGTI compounds. We show that GGTI P61A6 has an excellent antitumor effect in the human pancreatic cancer xenograft model in severe combined immunodeficient (SCID) mice. i.p. administration of GGTI P61A6 at 1.16 mg/kg/d caused a significant suppression of the growth of the human pancreatic cancer xenograft in mice. Even once per week administration of GGTI was enough to cause a similar level of suppression, and dose-dependent efficacy was observed. Immunohistochemical examinations indicated the inhibition of cell proliferation in the tumors by P61A6 treatment, but neither apoptosis nor antiangiogenesis was observed. In addition, inhibition of geranylgeranylation in tumors was observed. These data suggest that the novel GGTI compound P61A6 is an excellent chemotherapeutic drug candidate for human pancreatic cancers.

**Materials and Methods**

**GGTI Compounds**

The allenoate-derived compound library was synthesized as described in our previous publication (18, 23). A 20 mmol/L stock solution of GGTI P61A6 (Fig. 1A) in DMSO was kept at −20°C until use.

**Cells and Culture**

The human pancreatic cancer cell line, PANC-1, obtained from the American Type Culture Collection, was maintained in DMEM supplemented with 10% FCS (Sigma), 2% L-glutamine, 1% penicillin, and 1% streptomycin. The medium was routinely changed every 3 days, and the cells were separated by trypsinization before reaching confluency.

**Tumorigenesis in SCID Mice**

The stock solution of GGTI P61A6 was diluted in saline to give a final concentration of 160 μmol/L immediately before administration. Female 5-week-old C.B-17 SCID mice were obtained from Charles River Laboratories. PANC-1 cells (5 × 10^6) in 0.2 mL DMEM were s.c. injected into the right lateral abdominal wall of the mice. Two weeks later, mice bearing tumors ∼3 mm in diameter were randomly divided into treatment or vehicle groups in each experiment. Different concentrations (0.25 mL) of GGTI P61A6 solution [160 μmol/L or 1.16 mg/kg body weight, 80 μmol/L or 0.58 mg/kg body weight, 40 μmol/L or 0.29 mg/kg body weight, or vehicle (DMSO in saline, DMSO final concentration = 0.8%)] was injected i.p. from the right lower corner of the abdomen into the mice at different injection schedules until the end of the experiment. Tumor volume and body weight were measured every other day, and tumor volumes are shown. *, P < 0.05.

**In vivo Pharmacokinetic Evaluation**

For pharmacokinetic evaluation, 3 female mice, 5 weeks old, were i.p. injected with GGTI P61A6 (1.16 mg/kg body weight). Blood samples (60 μL) were drawn from retro-orbit...
at 15 min, 30 min, 60 min, 2 h, 4 h, and 24 h from each mouse, collected in EDTA-treated tubes, mixed well, and frozen immediately. Plasma was harvested and kept at −20°C until assayed.

Concentrations of P61A6 in plasma were determined using a validated analytical procedure based on high-performance liquid chromatography. Liquid chromatography-tandem mass spectrometry analyses were carried out using a SCIEX API3000 triple-quadrupole mass spectrometer (PE Sciex Instruments) operating in electrospray ionization mode. Chromatography was carried out using gradient elution (water-acetonitrile) on a Kromasil C18 reverse-phase column at a flow rate of 1 mL/min. Plasma compound concentrations were determined using a 7-point calibration curve derived from peak areas obtained from serially diluted solutions of P61A6. Pharmacokinetic analysis was done by noncompartmental analysis using PK Solutions (version 2.0) software (Summit Research Services).

**Effects of GGTI P61A6 on Prenylation of RhoA and Rap1 in Mouse Tumor Samples**

In their prenylated forms, proteins such as RhoA and Rap1 associate with cellular membranes (26). Inhibition of geranylgeranylation dissociates these proteins from the membrane. Therefore, to test for geranylgeranylation inhibition, membrane and cytosolic fractions were prepared by homogenizing tumor biopsies in ice-cold hypotonic buffer [10 mmol/L Tris (pH 7.5), 5 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and 25 μg/mL leupeptin] following the published protocol (15). The homogenates were then filtered through one layer of nylon mesh and centrifuged at 100,000 × g for 30 min at 4°C to separate membrane and cytosolic fractions. The membrane fractions were lysed by HEPES lysis buffer [30 mmol/L HEPES (pH 7.5), 1% Triton X-100, 10% glycerol, 10 mmol/L NaCl, 5 mmol/L MgCl₂, 25 mmol/L NaF, 1 mmol/L EGTA, 2 mmol/L Na₂VO₄, 10 μg/mL soybean trypsin inhibitor, 25 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mmol/L phenylmethylsulfonyl fluoride, and 6.4 mg/mL 2-nitrophenylphosphate]. The lysates were then separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies against Rap1 or RhoA (Santa Cruz Biotechnology).

**Table 1. Determining the maximum tolerance dose of GGTI P61A6**

<table>
<thead>
<tr>
<th>Dose of P61A6 (mg/kg/d)</th>
<th>Body weight change (%: 10th/1st day)</th>
<th>Dermal infection</th>
<th>Ascites</th>
<th>Mobility</th>
<th>Body condition scoring*</th>
<th>Hematologic tests*</th>
<th>Serologic tests*</th>
<th>Histopathologic alteration</th>
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<tr>
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<td>111.8</td>
<td>None</td>
<td>None</td>
<td>Good</td>
<td>3</td>
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<tr>
<td>0.29</td>
<td>114.0</td>
<td>None</td>
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<tr>
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<tr>
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<td>None</td>
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<tr>
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<td>112.0</td>
<td>None</td>
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<td>None</td>
<td>Good</td>
<td>3</td>
<td>Normal</td>
<td>Normal</td>
<td>None</td>
</tr>
</tbody>
</table>

*Body condition scoring; the body condition was scored on a scale of 1 to 5 according to the guideline of University of California-Los Angeles Department of Laboratory Animal Medicine: 1: mouse is emaciated, skeletal structure extremely prominent, vertebrate distinctly segmented; 2: mouse is underconditioned, segmentation of vertebral column being evident, dorsal pelvic bones being readily palpable; 3: mouse is well conditioned, vertebrae and dorsal pelvic being not prominent; 4: mouse is overconditioned, spine being a continuous column, vertebrae being palpable only with firm pressure; 5: mouse is obese, smooth and bulky, bone structure being disappearing under flesh and s.c. fat.

*Detailed hematologic and serologic data are provided in Supplementary Data.*
Immunohistochemistry

The effects of GGTI P61A6 on angiogenesis in the tumor xenograft experiments were investigated by immunohistochemistry. The purified rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Biosciences) was used to detect tumor vasculature. Briefly, the s.c. tumors were removed at the end of the in vivo experiments, embedded in OCT compound (Tissue-Tek), frozen, and microdissected into 4-μm-thick serial sections with a Tissue-Tek Cryo. The tissue sections were fixed with 100% acetone and Carnoy's fixative (60% absolute ethanol, 30% chloroform, and 10% glacial acetic acid) for 15 min and immersed in methanol containing 0.3% H₂O₂ to block endogenous peroxide activity. After incubation with a blocking buffer (2% bovine serum albumin in PBS buffer), the sections were exposed to the first antibody (1% dilution) for 1 h at room temperature. Biotinylated polyclonal anti-rat immunoglobulin (BD Biosciences) was used as the secondary antibody. Peroxide staining was done for 2 to 5 min with a solution of 3,3′-diaminobenzidine tetrahydrochloride in 50 mmol/L Tris-HCl (pH 7.5) containing 0.001% H₂O₂ and the sections were counterstained with 0.1% hematoxylin. Intratumoral microvessel density (MVD) was analyzed with a KS300 imaging system (Carl Zeiss Vision). Photographs of the entire area of slides were taken after staining, and all of the vessels were counted on each slide. MVD was calculated as the total number of vessels on each slide/the gross area of the slide.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used to detect apoptosis in the xenograft. Briefly, deparaffinized sections were pretreated with proteinase K and rinsed in PBS-Tween buffer. After blocking in 3% H₂O₂ in PBS for 10 min and rinsing, the sections were incubated in TdT Reaction Buffer for 10 min and then in TdT Reaction Mixture for 2 h at 37°C. After stopping and rinsing, streptavidin-horseradish peroxidase in PBS was added and the sections were incubated for 10 min and then incubated with 3,3′-diaminobenzidine tetrahydrochloride until the brown staining was obvious. Sections were counterstained with Gill’s hematoxylin and dehydrated with ethanol. The images were analyzed with an Olympus microscope/Metamorph and Ariol systems.

DNA synthesis was detected by 5-bromo-2′-deoxyuridine (BrdUrd) incorporation. BrdUrd (100 μg/g bodyweight; Sigma-Aldrich) was injected into the mice 1 h before sacrifice. The tumor sections were stained by immunohistochemistry as described above. For quantitation, the tumor slides were scanned and the number of BrdUrd-positive nuclei was counted and compared with the total number of nuclei in the same section, determined by hematoxylin staining.
Statistical Analysis
All results are expressed as mean ± SD. Statistical comparisons were made using Student’s t test after ANOVA. The results were considered to be significantly different at P < 0.05.

Results
Antitumor Effect of GGTI P61A6
The compound P61A6 (Fig. 1A) was shown to inhibit proliferation of various human cancer cells in our previous study (18). In this study, the in vivo antitumor effect of the compound was assessed using mouse xenografts of human cancer cells. To determine the maximum tolerated dose in mice, 6 female mice, 5 weeks old, were injected with various doses of P61A6 (dose range, 0.46 mg/kg/d) by i.p. administration once per day for 10 days. Body weight change, visible and/or palpable dermal infection, presence of ascites, and grooming or impaired mobility were closely monitored every day. Also, the body condition scoring system was used to evaluate the nutrition status. On the 2nd and 10th days, ∼60 μL blood was collected from retro-orbit for hematologic and serologic examination. On the 10th day, all mice were sacrificed and all organs were collected for histopathologic examination. The results show that 10-day treatment with different dosages of P61A6 did not cause any body weight loss, mobility impairment, or histopathologic alteration in any mouse but two (Table 1). The 2 mice showing slight effect were those treated with the two highest doses (4.64 and 2.32 mg/kg). These mice showed a slight increase of serum alanine aminotransferase (540 and 546 units/L on the 2nd and 10th days, respectively, for mouse treated with 4.64 mg/kg; normal range, 7-227 units/L) and aspartate aminotransferase (345 and 356 units/L on the 2nd and 10th days, respectively, for mouse treated with 2.32 mg/kg and 272 and 267 units/L on the 2nd and 10th days, respectively, for mouse treated with 2.32 mg/kg; normal range, 7-227 units/L). Therefore, we decided that 1.16 mg/kg (0.25 mL of 160 μmol/L GGTI in 0.9% NaCl) would be the highest concentration (=20 μmol/L in plasma) to be used for the following xenograft experiments.

In the first in vivo experiment, 14 female SCID mice were used. PANCl-1 cells (5 × 10⁶) were s.c. implanted into the back of each mouse. After 2 weeks, the mice with tumors ~3 mm in diameter were randomly divided into treatment and control groups (7 mice for each group), and administration of 1.16 mg/kg P61A6 (0.25 mL of 160 μmol/L GGTI in 0.9% NaCl three times per week, every other day) was initiated as described in Materials and Methods. As shown in Fig. 1B and C, after 33 days of i.p. administration of P61A6, both tumor volumes and tumor wet weight in the treated group was significantly suppressed to ~35% and 39% of that in the control mice, respectively. Examinations of hematology, serology, and tissue pathologic alteration did not show any significant changes (data not shown).

The second animal experiment was carried out with 30 female mice (Fig. 2). Similar to the first experiment, the s.c. tumors were established on the back of each mouse. Two weeks later, the mice were randomly divided into five groups (one group for control and four groups for treated mice, 6 mice for each group). The 6 mice in group 1 were treated with 0.25 mL of 0.9% NaCl every day by i.p. administration, whereas group 2 mice were treated with 1.16 mg/kg GGTI P61A6 (0.25 mL of 160 μmol/L GGTI in 0.9% NaCl) six times per week, group 3 with 1.16 mg/kg P61A6 (0.25 mL of 160 μmol/L GGTI in 0.9% NaCl), once per week, group 4 with 0.58 mg/kg P61A6 (0.25 mL of 80 μmol/L GGTI in 0.9% NaCl) three times per week (every other day), and group 5 with 0.29 mg/kg P61A6 (0.25 mL of 40 μmol/L GGTI in 0.9% NaCl) three times per week for 44 days. At the end of the experiment, the tumors in control mice grew up to 776 mm³ in average volume. The results on tumor volume are presented in Fig. 2A. In the treated groups, inhibition of tumor growth was evident. Administration of GGTI P61A6 in group 2 (1.16 mg/kg P61A6 six times per week) significantly suppressed the tumors to ~32% of control (Fig. 2A, a). Even treatment with P61A6 once per week resulted in the inhibition of tumor growth to 47% of control (Fig. 2A, b). In Fig. 2A, b, results of using lower dosages of P61A6 are shown. Treatment with 0.58 mg/kg P61A6 three times per week (group 4) effectively suppressed the tumor growth to 62% of the control. However, decreasing the dosage of P61A6 further (0.29 mg/kg three times per week; group 5) resulted in a diminished tumor-suppressing effect, although transient suppression in the first 2 weeks was observed (Fig. 2A, c). These results clearly showed the dose-dependent tumor-suppressing effect of GGTI P61A6 in animals (Fig. 2A, c).

![Figure 3. Plasma concentrations of GGTI P61A6 in mice after i.p. administration. Mice were i.p. injected with GGTI P61A6 (1.16 mg/kg body weight). Blood (60 μL) was drawn from suborbital at 15 min, 30 min, 60 min, 2 h, 4 h, and 24 h from each mouse. Concentrations of P61A6 in plasma were determined using a validated analytical procedure based on high-performance liquid chromatography.](http://mct.aacrjournals.org/)[6]

Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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We did not observe significant body weight loss in treated mice (Fig. 2A, d). To investigate possible toxicity of P61A6, the organs, such as esophagus, stomach, lung, liver, kidney, and spleen, were taken when the mice were sacrificed and subjected to H&E staining. The blood was also collected from the heart for hematologic and serologic examination. No significant abnormalities were observed (data not shown).

**Pharmacokinetics of P61A6**

Pharmacokinetic parameters of GGTI P61A6 were determined in mice dosed i.p. at 1.16 mg/kg. As can be seen in Fig. 3 and Supplementary Table S2, P61A6 showed good pharmacokinetic parameters that are amenable to the efficacious dosage amounts. P61A6 had low plasma clearance (22.7 mL/min/kg) and a relatively small volume of distribution (183 mL/kg) in mice, resulting in a terminal half-life of 5.6 h. The time of maximum concentration in plasma occurred at 30 min after dosing, with the mean maximal concentration in plasma via i.p. administration being 7.1 μg/mL (Fig. 3; Supplementary Table S2).

**P61A6 Suppresses Cell Proliferation in Tumors**

The growth of transplanted tumors depends significantly on the angiogenic ability of the tumor (27), and inhibition of protein geranylgeranylation and RhoA/Rho kinase pathway was reported to interfere with angiogenesis (28, 29). Therefore, we first examined the effect of GGTI P61A6 on tumor angiogenesis by immunohistochemical staining using the anti-CD31 antibody in tumors. MVD was counted as described in Materials and Methods. No differences in MVD were observed between the tumors in the treatment and control groups (MVD: control 192 ± 34.5 vessels/mm² and treated 200.4 ± 39.4 vessels/mm²; Fig. 4).

To assess P61A6-mediated apoptosis in xenografts, we examined cells with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay that detects DNA fragmentation characteristic of apoptotic cells in the tumors from treated and control groups. The tumor sections were analyzed with an Olympus microscope/Metamorph and Ariol systems. We did not observe statistically significant differences of the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells (apoptotic cells) between the mice from the control and treated groups, although larger tumors in control mice seemed to have more apoptotic cells (Fig. 4).

In our previous *in vitro* study, we found that GGTI P61A6 inhibited the proliferation of various human cancer cell lines and caused G1 cell cycle arrest (18). Therefore, the rate of proliferating cells in the xenograft tumors was also examined by measuring incorporation of BrdUrd in newly synthesized DNA of replicating cells. Mice were pulsed with a single BrdUrd injection, killed 1 h later, and analyzed for BrdUrd incorporation. These results are shown in Fig. 4. Interestingly, the average percentage of BrdUrd-positive cells (S-phase cells) in the P61A6-treated mice tumors is 13.6 ± 4.7% compared to 20.4 ± 3.4% in the control mice (Fig. 4), suggesting significant inhibition of DNA synthesis of tumor cells by GGTI P61A6. These results suggest that the main effect of GGTI is to inhibit proliferation of tumor cells.

**GGTI P61A6 Inhibits GGTase-I Activity in Pancreatic Cancer Xenografts in Mice**

Because proteins such as RhoA and Rap1 are modified by GGTase-I and the prenylation is required for these proteins to be associated with cellular membranes, we assessed whether GGTI treatment inhibits membrane association of these proteins. Membrane and cytosolic fractions were prepared from these tumors by ultracentrifugation and processed for SDS-PAGE Western immunoblotting. The membrane
marker, lactate dehydrogenase, was used to confirm the separation of cytosol fractions. As shown in Fig. 5A, in tumors from control mice, RhoA and Rap1 proteins were predominantly present in the membrane fractions. Treatment with GGTI P61A6 resulted in a dramatic increase of Rap1 and RhoA in the cytosolic fractions, whereas their association with the membrane fractions decreased.

Next, we measured GGTase-I enzymatic activities in tumors from the control and treated groups. GGTase-I activity was examined using the cytosolic fraction of the tumor cells. As shown in Fig. 5B, incorporation of radiolabeled isoprenoid \[^3H\]geranylgeranyl into substrate protein RhoA was significantly inhibited in the extracts of tumors from the treated mice compared with that of the control mice, suggesting significantly reduced GGTase-I enzyme activity. These results suggest that GGTI P61A6 reached the tumors and inhibited GGTase-I in animals.

Taken together, these results show that the treatment with GGTI P61A6 exhibits a clear and significant inhibition of protein geranylgeranylation in animal xenografts.

**Discussion**

In the present study, we tested in vivo anticancer effects of a novel GGTI P61A6 using human pancreatic cancer xenograft in SCID mice. GGTI P61A6 markedly inhibited tumor formation to \(\sim 35\)% of the control mice when administered three times per week at a 1.16 mg/kg concentration. Even treatment with P61A6 once per week inhibited the tumor growth to 47% of control. Moreover, half of the dosage also showed good tumor inhibition in mice. Further decreased dosage failed to inhibit tumors, although a transient suppression after 4 weeks was observed. Thus, P61A6 exhibits excellent antitumor activity. The inhibition of tumor growth by treatment once per week is encouraging. Although half-life of P61A6 is 5.6 h, the high mean maximal concentration in plasma (7.1 \(\mu\)g/mL or 12.2 \(\mu\)mol/L) and the relatively high plasma concentration at 24 h (6 \(\mu\)mol/L), considering the in vitro IC\(_{50}\) value for P61A6 is \(\sim 2.2\) \(\mu\)mol/L (18), might explain this significant inhibition, although further detailed pharmacologic study is required.

One concern in developing GGTI is that inhibiting GGTase-I might lead to severe toxicity. Remarkably, we did not observe any severe toxic effects in mice. In addition, P61A6 has desirable in vivo pharmacokinetic properties, specifically clearance and distribution parameters that are suited to the small dosages required for efficacy. The pharmacokinetic profile of GGTI P61A6 obtained from this mouse experiment is possibly different from that in human being; this should be addressed in the future clinical trial. One of the reasons for minimum toxicity observed with our GGTI compound is that we could achieve geranylgeranylation inhibition with low concentration of the compound compared with the study using other GGTI compounds, such as GGTI-2 (25). The difference may be due to the relatively long plasma half-life of our compound P61A6, which has a half-life of 5.6 h. GGTI-2418 was reported to have a half-life of 0.57 h (30). It is shown that inactivating the gene coding the \(\beta\)-subunit of GGTase-I reduces lung tumor formation, eliminates myeloproliferative phenotypes, and increases survival rates of mice (12). Interestingly, in those studies, several cell types remained viable in the absence of GGTase-I, and myelopoiesis appeared to function normally (12). These observations are consistent with our results that the inhibition of GGTase-I can significantly inhibit tumor growth without inducing severe toxicity to mice.

In our previous in vitro study, we found that the treatment with GGTI P61A6 induced the inhibition of cell proliferation and cell cycle arrest at G1 phase accompanied by decreased S phase, but not apoptosis, in various cancer cell lines, such as leukemia, breast cancer, and pancreatic cancer (18). This cell proliferation inhibition was confirmed in the present in vivo study by the observation that treatment with P61A6 resulted in decreased BrdUrd-positive cells in the tumors, whereas apoptotic cells (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining) were not affected by P61A6. We also checked the effect of P61A6 on tumor angiogenesis in the xenograft model but did not find any obvious differences in the MVD in treated mice versus control. Taken together, these results suggest that the novel GGTI P61A6 inhibited tumor growth by inhibiting proliferation leading to the suppression of tumor growth. Theoretically,
it is probable that combinations of apoptosis-inducing cytotoxic agents and this newly identified GGTI compound will have a powerful anticancer effect on human cancers, and this possibility is being pursued now.

We have confirmed GGTase-I inhibition in tumors. We found that GGTI P61A6 impeded membrane association of geranylgeranylated proteins such as RhoA and Rap1. In addition, we have recently found that our GGTI inhibits geranylgeranylation of RalA in human cancer cell lines. 7 However, how this inhibition of protein geranylgeranylation by P61A6 contributed to the tumor growth inhibition remains to be determined, requiring future investigation. One possibility is that inhibition of RhoA function by blocking its membrane association, which negatively regulates expression of the Cdk inhibitor p21<sup>CIP1/WAF1</sup>, might partly be responsible for the tumor suppression of P61A6. Although other GGTI compounds, such as GGTI-298 and GGTI2166, were reported to inhibit phosphatidylinositol 3-kinase/Akt2 pathway, leading to programmed cell death in human ovarian cancer cells (31), we observed neither apoptosis nor effect on phospho-Akt by our compound P61A6 (Supplementary Fig. S1). 6 This difference of activation between our GGTI P61A6 and GGTI-298 and GGTI-2166 is interesting and requires further investigation. In addition, treatment with GGTI P61A6 markedly inhibited the enzymatic activity of GGTase-I in tumor cells. Inhibition of GGTase-I activity in tumors was also observed with GGTI-2154 (15). Despite significant inhibition of membrane association of RhoA, we observed that tumors in treated groups kept growing, although much slower and smaller than that in the control group, even with the highest dosage. It was reported that dominant-negative forms of RhoA, Rac1, and Cdc42 only partially reverse Ras-induced malignant transformation (32, 33). This might partially explain the continuing growth of the treated tumors, although further investigations are certainly required to elucidate this phenomenon.

The i.p. administration of GGTI P61A6 in the present study showed positive tumor-suppressing effects in SCID mice at doses that did not show adverse effects; thus, we believe that i.v. administration should provide a better outcome for cancer therapy. Also, because this compound showed very good mucosa absorbance rates and long blood circulation, it is probable that combinations of apoptosis-inducing cytotoxic agents and this newly identified GGTI compound will have a powerful anticancer effect on human cancers, and this possibility is being pursued now.

We have confirmed GGTase-I inhibition in tumors. We found that GGTI P61A6 inhibited membrane association of geranylgeranylated proteins such as RhoA and Rap1. In addition, we have recently found that our GGTI inhibits geranylgeranylation of RalA in human cancer cell lines. 7 However, how this inhibition of protein geranylgeranylation by P61A6 contributed to the tumor growth inhibition remains to be determined, requiring future investigation. One possibility is that inhibition of RhoA function by blocking its membrane association, which negatively regulates expression of the Cdk inhibitor p21<sup>CIP1/WAF1</sup>, might partly be responsible for the tumor suppression of P61A6. Although other GGTI compounds, such as GGTI-298 and GGTI2166, were reported to inhibit phosphatidylinositol 3-kinase/Akt2 pathway, leading to programmed cell death in human ovarian cancer cells (31), we observed neither apoptosis nor effect on phospho-Akt by our compound P61A6 (Supplementary Fig. S1). 6 This difference of activation between our GGTI P61A6 and GGTI-298 and GGTI-2166 is interesting and requires further investigation. In addition, treatment with GGTI P61A6 markedly inhibited the enzymatic activity of GGTase-I in tumor cells. Inhibition of GGTase-I activity in tumors was also observed with GGTI-2154 (15). Despite significant inhibition of membrane association of RhoA, we observed that tumors in treated groups kept growing, although much slower and smaller than that in the control group, even with the highest dosage. It was reported that dominant-negative forms of RhoA, Rac1, and Cdc42 only partially reverse Ras-induced malignant transformation (32, 33). This might partially explain the continuing growth of the treated tumors, although further investigations are certainly required to elucidate this phenomenon.

The i.p. administration of GGTI P61A6 in the present study showed positive tumor-suppressing effects in SCID mice at doses that did not show adverse effects; thus, we believe that i.v. administration should provide a better outcome for cancer therapy. Also, because this compound showed very good mucosa absorbance rates and long blood circulation, oral administration will be worth pursuing and good tumor-suppressing effects may be expected. Because GGTI P61A6 showed cell proliferation inhibition on human cancer xenografts in addition to pancreatic cancer in our previous in vitro studies, it will be interesting to test various human cancer xenografts. Taken together, the present study clearly shows that our novel GGTI P61A6 inhibits GGTase-I activity, blocks protein geranylgeranylation, and induces tumor inhibition in human pancreatic cancer xenograft models at low doses without inducing any obvious adverse effects. These findings prove that geranylgeranylation of proteins plays a critical role in Ras-driven tumorigenesis and provide strong evidence that GGTase-I is a feasible and promising molecular target for anticancer drug discovery.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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7 Unpublished data.
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