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TAN-1813, a Novel Ras-Farnesyltransferase Inhibitor Produced by *Phoma* sp.

Taxonomy, Fermentation, Isolation and Biological Activities *In Vitro* and *In Vivo*

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A novel Ras-farnesyltransferase inhibitor designated TAN-1813 was isolated from the culture broth of a fungus strain, FL-41510, isolated as a plant endophyte. The producer was taxonomically characterized as *Phoma* sp. FL-41510. TAN-1813 inhibited rat brain farnesyltransferase and geranylgeranyltransferase I activity with IC_{50} values of 23 μg/ml and 47 μg/ml, respectively. TAN-1813 showed mixed-type inhibition with respect to farnesylpyrophosphate and noncompetitive inhibition with respect to a K-Ras *C*-terminal peptide. It also inhibited the *in situ* farnesylation of cellular Ras proteins in a K-ras transformant (NIH3T3/K-ras) of mouse embryonic fibroblast cell line NIH3T3.

TAN-1813 inhibited the proliferation of various human cancer cells, some of which harbor activated ras alleles, with IC_{50} values of 15~110 ng/ml as well as that of NIH3T3 and NIH3T3/K-ras cells with IC_{50}s of 540 and 310 ng/ml, respectively. Flow cytometric analysis indicated that TAN-1813 arrests NIH3T3/K-ras cells at both G1 and G2/M phases of the cell cycle. In addition, TAN-1813 was found to induce morphological reversion of NIH3T3/K-ras cells from the transformed phenotype. Antitumor activity of TAN-1813 against human fibrosarcoma HT-1080 and NIH3T3/K-ras tumors in nude mice was also verified.

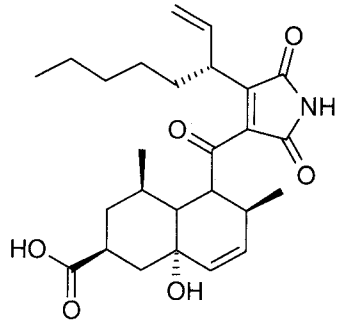
Previous studies have clearly established that small GTP-binding proteins such as Ras, Rho and Rab proteins play crucial roles in regulating cell proliferation and differentiation, organizing actin microfilaments and controlling secretory vesicle transport systems, respectively[1~3], and that they exert these distinct functions in specific cellular compartments. Ras proteins, which are representatives of the small GTP-binding protein family, are considered to function as a "molecular switch," which cycle between an active GTP-bound form and an inactive GDP-bound form, receiving signals from receptor-type tyrosine kinases *via* an adaptor protein (*e.g.* Grb2) and a GTP/GDP exchange protein (*e.g.* mSos) complex and transmitting them to downstream effectors such as c-Raf-1, phosphatidyl-inositol-3-OH kinase and GTPase activating protein (GAP)[1,4,5]. Thus, Ras proteins are regarded as one of the key regulators of cell proliferation and differentiation.

Among the oncogenes discovered so far, ras genes have been reported to be most frequently activated in human cancer, especially in colon or pancreas cancer[6]. Such oncogenic activation has been proven to be caused by some point mutations in ras genes, most of which were detected in the domain responsible for the interaction with the guanine nucleotides[5]. The resultant mutant forms of Ras proteins become refractory to negative regulation such as stimulation of their intrinsic GTPase activity by GAP[5], continuously producing active mitogenic signals in such deregulated cells. In other words, the "molecular switch"

is always "on," giving tumor cells a growth advantage. Actually, the contribution of the oncogenic Ras proteins to malignant phenotypes of tumor cells has been substantiated in a number of experiments. For instance, selective disruption of an oncogenic allele of K-ras gene using the homologous recombination technique resulted in the loss of anchorage-independent growth and tumorigenicity in nude mice[7]. In addition, anti-ras ribozymes and antisense oligonucleotides targeted against mutated ras genes have been shown to suppress tumor growth in mice[8,9]. It is therefore feasible that the blockade of oncogenic ras function leads to loss of malignant phenotypes and will be a successful approach to cancer chemotherapy.

Ras gene products are well conserved evolutionarily from yeasts to mammals, reflecting their pivotal roles in fundamental cellular processes in eukaryotic cells. They are located on the inner surface of plasma membrane in eukaryotic cells, and this is a prerequisite for Ras proteins to be biologically active[10]. The membrane localization of Ras proteins is achieved by three posttranslational enzymatic modifications[11]: addition of farnesyl isoprenoid to the cysteine residue in the Ras *C*-terminal "CAAX box", removal of the tripeptide AAX and carboxymethylation of the newly formed *C*-terminal cysteine residue. Among these three steps, the first was shown to be essential for the transforming activity of Ras proteins[11]. Therefore, inhibition of Ras farnesylation is believed to be a promising approach to block Ras function, and selective inhibitors of farnesyltransferase (FTase) have been developed as anti-cancer agents highly specific for oncogenic Ras-harboring tumors[12], because unmodified oncogenic Ras but not c-Ras was thought to exert dominant negative effects against the cell growth[13,14]. Although this hypothesis has proved to be controversial because highly specific FTase inhibitors showed antitumor activity against tumors with wild-type ras gene[15], it is still noteworthy that some FTase inhibitors have entered clinical trials[15]. With this prospect in mind, we have developed a unique screening method taking advantage of a genetically mutated strain of *Saccharomyces cerevisiae* in which *RAS1* gene has been disrupted and the cysteine residue in the CAAX box of wild type *RAS2* protein has been replaced by serine, rendering this mutant protein unsusceptible to the modification, but whose growth is supported by overproduction of the mutated protein[16]. Using those two yeast strains harboring either wild-type Ras2 or Ras2^{Ser319} protein, a microbial culture broth was selected which showed stronger antimicrobial activity against the wild-type strain than the mutant strain, and the culture filtrate of a plant endophyte fungus, FL-41510, was found to inhibit the growth of yeast strain harboring wild-type RAS2 protein preferentially. The active principle designated TAN-1813 was purified to the homogeneity and shown to be a novel compound (Fig. 1). Evidences have now been presented that TAN-1813 can inhibit FTase in the cell-free assay and in mammalian cells and reduce Ras-dependent tumor growth in some nude mouse models. In this report, we describe the taxonomy of the producing organism, and fermentation, isolation and biological activities of TAN-1813.

Fig. 1. Structure of TAN-1813 with the relative stereochemistry.



Materials and Methods

Isolation of Nystatin-resistant Mutant

S. cerevisiae FSY1-26C harboring YCp50-*RAS2* plasmid was kindly provided by Dr. JAMES R. BROACH (Princeton University, U.S.A.). Cells cultivated in 400 ml of YPD medium to a mid-logarithmic phase were harvested, washed once with 400 ml of saline and suspended in 15 ml of 0.1 M potassium phosphate buffer (pH 8.0). Ethyl methanesulfonate was added to the cell suspension at a final concentration of 20 mg/ml. After incubation at 28°C for 1 hour, cells were washed three times with 40 ml of saline, resuspended in 10 ml of saline and plated (0.1 ml/plate) on YPD plates (10 cm in diameter) containing 10 μg/ml nystatin. Colonies were isolated after incubation for 2 days.

Construction of a Mutant Strain Harboring Non-farnesylated Ras2^{Ser319} Protein

YEp51-*ras2^{Ser319}* plasmid[16] containing *LEU2* gene as a selectable marker was also generously donated by Dr. JAMES R. BROACH. The nystatin-resistant mutant, TYC4, isolated as described above was further transformed with the above galactose-inducible plasmid according to the

lithium acetate method[17] and Leu^+ clones were selected on SD plates containing 20 μg/ml of L-tryptophan. The Ura^- Leu^+ clone, TYC8, was obtained by spreading Ura^+Leu^+ clones twice on nonselective YPGal plates to remove YCp50-*RAS2* plasmid containing *URA3* gene as a selectable marker and by selecting resultant colonies on SGal plates containing 30 μg/ml of uracil and 20 μg/ml of L-tryptophan.

Taxonomic Studies

Cultural and morphological characterizations were carried out by the methods described by MALLOCH[18] or described in "Kinrui Kenkyu Ho"[19].

Fermentation

A loopful of mycelia of strain FL-41510 grown on an agar slant was inoculated into a 2-liter flask containing 500 ml of a seed medium consisting of glucose 2%, maltose 3%, soy-bean flour 1.5%, corn steep liquor 1%, Polypepton (Wako Pure Chemical Ind., Japan) 0.5%, yeast extract 0.3% and NaCl 0.3% (pH 6.0) and cultivated at 28°C for 48 hours on a reciprocal shaker. The entire seed culture thus obtained from two 2-liter flasks was transferred to a 50-liter fermenter containing 30 liters of the same medium supplemented with 0.05% Actocol (Takeda Chemical Ind., Japan). Cultivation was carried out at 28°C for 48 hours under aeration of 30 liters/minute and agitation of 280 rpm. Six liters of the seed culture thus obtained was transferred to a 200-liter fermenter containing 120 liters of a production medium consisting of glucose 1%, dextrin 4%, soy-bean flour 0.5%, malt extract 0.5%, Polypepton 0.5%, yeast extract 0.2%, $FeSO_4 \cdot 7H_2O$ 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $MnSO_4 \cdot 4H_2O$ 0.05%, KH_2PO_4 0.1%, $CaCO_3$ 0.5% and Actocol 0.05% (pH 7.5). Cultivation was carried out at 24°C under aeration of 120 liters/minute and agitation of 150 rpm. Small scale cultivation was also performed in 40 ml of the same seed or production media in a 200-ml Erlenmeyer flask. An aliquot (2 ml) of the seed culture cultivated at 28°C for 48 hours on a rotary shaker was transferred to 40 ml of the production medium, and cultivation was continued at 24°C.

Isolation

Culture broth (105 liters) obtained after cultivating for 114 hours was extracted with EtOAc (100 liters) at pH 2.6. The organic layer was washed with water (30 liters) and concentrated to give an oily residue (78 g). The residue was subjected to silica gel (1.3 liters) column chromatography. The column was washed with hexane (2 liters), hexane-acetone (9 : 1, 2 liters) and hexane-acetone (8 : 2, 5 liters), successively and then eluted with hexane-acetone (6 : 4, 2 liters). The eluate was concentrated to give a crude powder (8.9 g). The powder was subjected to preparative HPLC (ODS, YMC-Pack S-363. I-15) with a mobile phase of 58% acetonitrile-10 mM phosphate buffer (pH 3.0). The pure fraction was concentrated and extracted with EtOAc at pH 3.0. The EtOAc layer was washed with water and concentrated to give TAN-1813 as a yellow powder (2.1 g).

Preparation and Assay of FTase and Geranylgeranyltransferase I

FTase and geranylgeranyltransferase I (GGTase I) were prepared from rat brain homogenate by the method described previously[20] with slight modification. Briefly, 10 whole brains isolated from 5-week-old male Wistar rats were minced with scissors, suspended in 30 ml of 50 mM Tris/HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 20 μM leupeptin, 0.1 μM pepstatin A, 10 μg/ml aprotinin and 10 μM (*p*-amidinophenyl) methanesulfonyl fluoride (APMSF) and homogenized with a Potter-Elvehjem homogenizer. Insoluble material was removed by centrifugation at 60,000×*g* for 70 minutes at 4°C, and the supernatant was subjected to ammonium sulfate fractionation. Proteins precipitated between 30% and 50% saturation were dissolved in 2 ml of 20 mM Tris/HCl buffer (pH 7.5) supplemented with 1 mM dithiothreitol, 20 mM $ZnCl_2$, 20 μM leupeptin, 0.1 μM pepstatin A, 10 μg/ml aprotinin and 10 μM APMSF and then dialyzed overnight against 5 liters of the same buffer. The supernatant (2.5 ml) obtained by centrifugation at 15,000×*g* for 10 minutes at 4°C was further subjected to Mono Q column (Amersham-Pharmacia) chromatography with a linear gradient from 150 mM to 500 mM NaCl. The active fractions containing FTase or GGTase I activity were combined separately and stored at −80°C. FTase activity was assayed using an FTase assay kit following the manufacturer's instruction, except that biotin-K-Ras peptide was substituted for biotin-lamin B peptide. GGTase I activity was assayed by the same method as that used for FTase, using 500 nM biotin-Krev-1 peptide and 240 nM ^{3}H-geranylgeranylpyrophosphate (GGPP) instead of 100 nM biotin-K-Ras peptide and 120 nM ^{3}H-farnesylpyrophosphate (FPP).

Cell Lines and Animals

Mouse embryonic fibroblast cell line NIH3T3 and its K-ras transformant (NIH3T3/K-ras) were generously provided by Dr. L. LIOTTA (National Institutes of Health, U.S.A.). Human fibrosarcoma HT-1080, human lung fibroblast MRC-9, human melanoma G361, human lung carcinoma A549 and human colon adenocarcinoma WiDr were

purchased from Institute for Fermentation, Osaka (IFO). The following human carcinoma cell lines were obtained from the American Type Culture Collection (ATCC): human breast adenocarcinomas MCF-7 and MDA-MB-231, human hepatocellular carcinoma HepG2, human lung small cell carcinoma H69, human pancreatic carcinoma MIAPaCa-2 and human colon adenocarcinomas SW48 and SW480. PCMR-5, a variant of PC12 pheochromocytoma, was kindly supplied by Dr. Y. SUGIMOTO[21] (Shirakawa Institute of Animal Genetics, Fukushima, Japan). All cell lines except for NIH3T3, NIH3T3/K-ras, PCMR-5 and HepG2 were cultured with MEM containing 10% FCS and 30 μg/ml gentamicin. Cell lines NIH3T3, NIH3T3/K-ras and HepG2 were cultured with DMEM containing 10% FCS and 30 μg/ml gentamicin (DMEM/F10). Cell line PCMR-5 was cultured with RPMI 1640 containing 10% FCS, 5% horse serum and 30 μg/ml gentamicin. Wistar rats and BALB/c *nu/nu* mice were obtained from Charles River, Japan.

Metabolic Labeling of Ras Proteins *In Situ*

^{3}H-Mevalonolactone labeling of NIH3T3/K-ras cells was performed essentially as described previously[22]. NIH3T3/K-ras cells suspended in DMEM/F10 were seeded with 1×10^6 cells per 100-mm dish which was precoated at 37°C for 3 hours with 50 μg/ml bovine plasma fibronectin. Cells were grown at 37°C for 2 days in a humid atmosphere under 5% CO_2, and then 3 ml of DMEM supplemented with 1% FCS (dialyzed against 0.15 M NaCl), 50 μM lovastatin and varying concentrations of TAN-1813 was added. After incubating for 2 hours, 300 μCi of ^{3}H-mevalonolactone (33 Ci/mmole) was added to each monolayer culture followed by incubation for an additional 20 hours. The cells in each dish were rinsed three times with 10 ml of 50 mM Tris/HCl buffer (pH 7.5) containing 0.15 M NaCl and lysed in 700 μl of 2-fold diluted DULBECCO's phosphate-buffered saline (DPBS) supplemented with 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 μg/ml aprotinin and 10 μM APMSF (Lysis Buffer). After standing on ice for 5 minutes, the lysates were centrifuged at 12,000×*g* for 30 seconds, and supernatant containing 700 μg of protein was immunoprecipitated with 100 μl of anti-Ras immune complex at 4°C overnight with gentle shaking. The anti-Ras immune complex was prepared by incubating a mixture (10 ml) containing 50 μg of anti-Ras monoclonal antibody (Y13-259), 100 μg of rabbit anti-rat IgG and 100 μl (wet volume) of Protein A-Sepharose CL-4B at 4°C overnight, followed by washing with DPBS (10 ml×3) and resuspending in a total volume of 0.5 ml. The immunoprecipitate was washed three times with 1 ml of Lysis Buffer, suspended with 100 μl of SDS-PAGE buffer, boiled for 5 minutes and subjected to SDS-PAGE[23] (15% acrylamide). Supernatant containing 56 μg of protein, obtained after removing the immunoprecipitate, was also subjected to SDS-PAGE in the same way. After electrophoresis, the gel was fixed for 45 minutes with 50% methanol/10% acetic acid, soaked in Enlightning™ for 30 minutes, dried and exposed to X-ray film for one week at −40°C.

Assay of Growth-inhibitory Activity

Growth of various cell lines was measured colorimetrically by the tetrazolium salt (MTT) method[24]. Briefly, 2,000~5,000 cells suspended in the appropriate culture medium were seeded in the wells of a 96-well plate (0.1 ml/well) to which varying amounts of TAN-1813 dissolved in 10 μl of DPBS had previously been added. After culturing for 3 days at 37°C in a humid atmosphere under 5% CO_2, 25 μl of MTT solution (5 mg/ml) was added, and the mixture was incubated for an additional 5 hours under the same conditions. The reaction was then stopped by adding 0.1 ml of 10% SDS/0.01 N HCl solution. After incubating overnight, the absorbance at 620 nm was measured, and the growth-inhibitory activity was calculated.

Flow Cytometric Analysis

The effect of TAN-1813 on the cell cycle progression of NIH3T3/K-ras cells was evaluated. NIH3T3/K-ras cells suspended in DMEM/F10 were plated at a density of 3×10^5 cells per 100-mm dish and synchronized in S phase by 24-hour incubation in the presence of 1 μM aphidicolin. Cells were then washed three times with 10 ml of prewarmed DMEM/F10 to release them from the S phase block and were further cultured in 10 ml of DMEM/F10 to allow them to progress into G2 and M phases. Immediately or several hours after the release, TAN-1813 was added to the culture medium at a final concentration of 5 μg/ml to determine the growth-inhibitory effect in different phases of the cell cycle. Twenty-nine hours after the release, cells were harvested, fixed, stained with 50 μg/ml of propidium iodide and analyzed using a FACScan Flow Cytometer (Becton Dickinson, U.S.A.).

Assay of Antitumor Activity *In Vivo*

A human fibrosarcoma HT-1080 xenograft model was established by subcutaneous inoculation of the cells (3×10^6 cells/mouse) into adult male BALB/c *nu/nu* mice. Eleven days after tumor inoculation, TAN-1813 suspended in 0.5% methylcellulose in saline was administered daily

Table 1. Drug sensitivity of nystatin-resistant mutant TYC4.

Compound	MIC(μg/ml)	
	FSY1-26C	TYC4
Nystatin	3.1	12.5
Cycloheximide	0.31	<0.02
Adriamycin	>100	3.1
Actinomycin D	>100	1.6
5-Fluorouracil	100	6.3
Mitomycin C	>100	25

intraperitoneally or subcutaneously. Tumor size was measured nineteen days after tumor inoculation. An NIH3T3/K-ras isograft model was also established by subcutaneous inoculation of the cells (5×10^5 cells/mouse) into 5-week-old male BALB/c *nu/nu* mice. Subcutaneous administration of TAN-1813 suspended in 0.5% methylcellulose in saline was started 5 days later. Tumor size was measured 3 days after the final treatment. In both tumor models, tumor size was estimated by the formula, length$\times$width$^2\times$1/2.

Assay of Antimicrobial Activity

Antimicrobial activity against two yeast strains, TYC4 and TYC8, was determined by the conventional paper disk assay using YPGal medium as a growth medium. MICs of various compounds against FSY1-26C and TYC4 were determined by the conventional 2-fold agar dilution method using YPD plate. Yeast growth was examined after incubation at 28°C for 2 days.

Media

Liquid media used for yeast strains in this study were as follows: YPD; 1% yeast extract, 2% peptone and 2% glucose. YPGal; 1% yeast extract, 2% peptone and 2% galactose. SD; yeast nitrogen base (amino acid-free) and 2% glucose. SGal; yeast nitrogen base (amino acid-free) and 2% galactose. Plate media contained 2% agar.

Chemicals

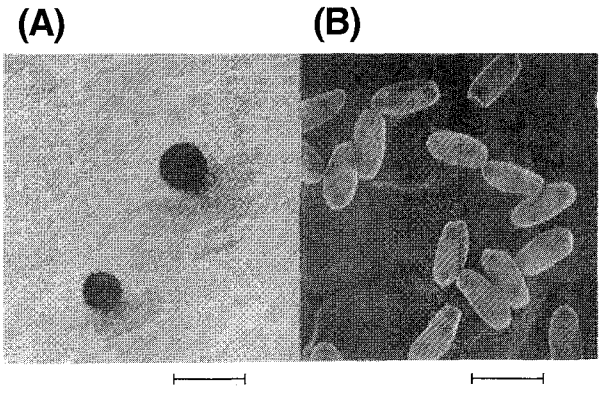
^{3}H-geranylgeranyl pyrophosphate (15 Ci/mmole), RS-[5-^{3}H]-mevalonolactone (33 Ci/mmole) and Enlightning™ were purchased from Du Pont. Biotinylated *C*-terminal undecapeptides of K-Ras (biotin-KKKSKTKCVIM) and Krev-1 (Rap1A) proteins (biotin-KKPKKKSCLLL) were synthesized by Peptide Institute, Inc., Japan. FTase assay kits were obtained from Amersham-Pharmacia. DULBECCO's modified minimum essential medium (DMEM), EAGLE's minimum essential medium (MEM) and fetal calf serum (FCS) were purchased from Whittaker Bioproducts, U.S.A. Rat anti-Ras monoclonal antibody (clone Y13-259) and rabbit anti-rat IgG (Cappel) were obtained from Oncogene Science, U.S.A. and Organon Teknika N.V., Belgium, respectively. Protein A-Sepharose CL-4B was purchased from Amersham-Pharmacia. All other chemicals used were obtained from commercial sources and of reagent grade.

Results

Drug Sensitivity of Nystatin Resistant Mutant

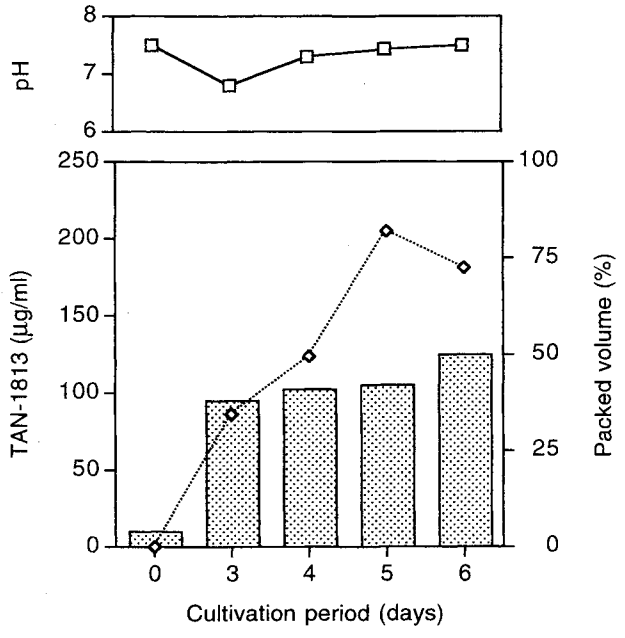
It is often found that growth-inhibitory activity against *S. cerevisiae* and mammalian cells do not always correlate to each other. Table 1 shows that *S. cerevisiae* is tolerant of some cytotoxic compounds which are known to have potent growth-inhibitory activity against mammalian cells. This could be because the yeast cell membrane constitutes a more rigid permeability barrier to toxic compounds than that of the mammalian cell. Membrane component responsible for the barrier might be ergosterol, because antifungal polyene antibiotics such as amphotericin B and nystatin, which are believed to bind to sterols and form hydrophilic pores in the plasma membrane, potentiate the activity of cytotoxic drugs[25]. Furthermore, mutants

Fig. 2. Morphology of strain FL-41510.



(A) Pycnidia formed in potato dextrose agar after cultivation for 3 weeks at room temperature. Bar represents 300 μm. (B) Scanning electron micrograph of conidia. Bar represents 4 μm.

Fig. 3. Time course of TAN-1813 production.



Strain FL-41510 was cultivated as described in Materials and Methods.

TAN-1813 production (◇), pH (□) and packed cell volume (stippled bar).

defective in ergosterol biosynthesis showed nystatin-resistant phenotype but were sensitive to a wide variety of drugs other than polyene antibiotics[26]. Based on these experimental evidences, a nystatin-resistant mutant of *S. cerevisiae* was isolated and, as expected, the mutant strain TYC4 showed marked sensitivity to various cytotoxic compounds (Table 1). Furthermore, high permeability of plasma membrane of the nystatin-resistant strain was also supported by the observation that phloxine B pigment was taken up by the strain TYC4 but not by the wild-type strain FSY1-26C (data not shown). As the next step, another nystatin-resistant strain TYC8 which grows depending on non-farnesylated $Ras2^{Ser319}$ proteins[16] was constructed as described in Materials and Methods, and, on the course of the screening program, we found a plant endophyte fungus, FL-41510, to produce an active principle which inhibited the growth of TYC4 preferentially.

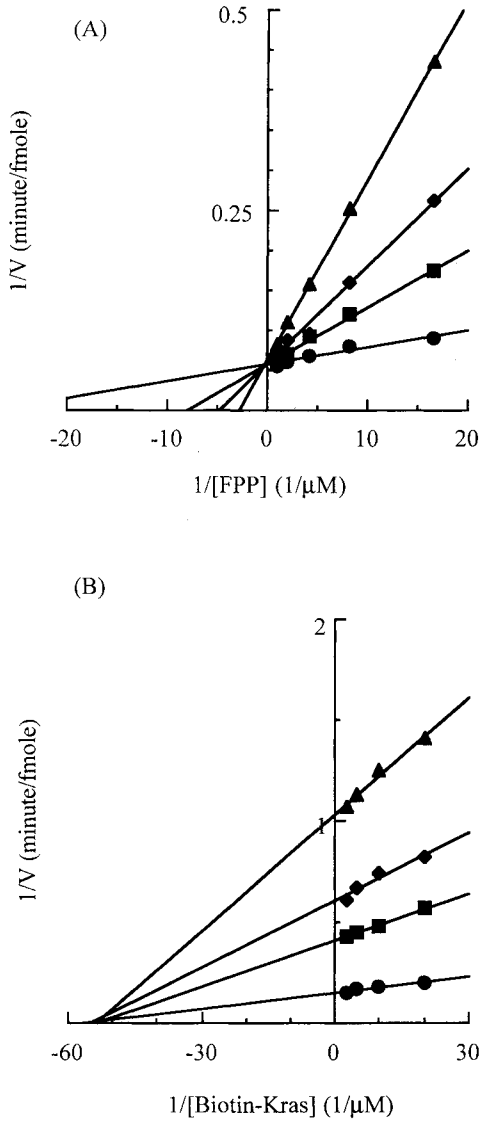
Taxonomy of the Producing Organism

Strain FL-41510 was isolated as an endophyte from a root of *Erigeron annuus* collected in Fukushima Prefecture, Japan. Cultural and morphological characteristics of strain FL-41510 were as follows: colonies were fast-growing, reaching 33~35 mm in diameter in 2 weeks at 24°C on potato-dextrose agar, producing raised wooly aerial mycelium with pale gray to whitish gray color with a slightly irregular periphery. Soluble pigment was not produced. After 3 weeks or more, many dark brownish pycnidia were observed in the agar medium. Aerial hyphae were septate, smooth to rough surface. Pycnidia were mostly globose to ellipsoidal shape, 150~200 μm in diameter, having one ostiole (Fig. 2A). Abundant conidia were produced in a pycnidium. Conidia were single-celled, smooth, hyaline, ellipsoidal to ovoid, 3~5×1.5~2 μm (Fig. 2B). The gross taxonomical characteristics of strain FL-41510 described above clearly indicate that it belongs to the genus *Phoma*[18,27]. The strain has been deposited as *Phoma* sp. FL-41510 in the Institute for Fermentation, Osaka, and the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession numbers IFO-32613 and FERM BP-4632, respectively.

Fermentation

A typical time course of the production of TAN-1813 on the 40-ml cultivation scale is shown in Fig. 3. While the growth of strain FL-41510 reached almost the maximum level as early as after 3 days of cultivation, the production

Fig. 4. Kinetics of the FTase inhibition by TAN-1813 as a function of substrate concentration.



The kinetics of the inhibition by TAN-1813 was analyzed using a Lineweaver-Burk plot as a function of FPP concentration (A) or as a function of K-Ras peptide concentration (B). Rat brain FTase preparation (19 fmole/minute) was incubated at 37°C for 1 hour with various concentrations of [^{3}H]FPP at 100 nM K-Ras peptide or with various concentrations of the K-Ras peptide at 60 nM [^{3}H]FPP.

TAN-1813; none (●), 25 μg/ml (■), 50 μg/ml (◆) and 100 μg/ml (▲).

of TAN-1813 increased time-dependently and reached the maximum (205 μg/ml) 5 days after inoculation. The large scale cultivation using a 200-liter fermenter showed a similar time course and the maximum production of TAN-1813 (approximately 80 μg/ml) was obtained 5 days after inoculation (data not shown). Therefore, the culture broth obtained after 114 hours of cultivation was used for the isolation of TAN-1813.

Isolation

Since TAN-1813 is a fat-soluble and weakly acidic compound, the culture broth was extracted with EtOAc under acidic conditions. The extract was chromatographed on a silica gel column to give a crude powder. The powder was purified by preparative HPLC using a column of ODS to give TAN-1813 as a yellow powder.

Inhibition of FTase and GGTase I *In Vitro*

Using FTase and GGTase I partially purified from rat brains, the inhibitory activity (IC_{50} value) of TAN-1813 against these enzymes was determined to be 23 μg/ml and 47 μg/ml, respectively. The kinetics of the FTase inhibition by TAN-1813 was examined in reaction mixtures containing 100 nM biotin-K-Ras peptide and varying concentrations of ^{3}H-FPP or 60 nM ^{3}H-FPP and varying concentrations of biotin-K-Ras peptide. Lineweaver-Burk plots[28] revealed a mixed type inhibition (Fig. 4A) with respect to FPP (Ki=29 μM) and a noncompetitive type inhibition (Fig. 4B) with respect to biotin-K-Ras peptide (Ki=41 μM). The kinetics of the GGTase I inhibition by TAN-1813 was tested in reaction mixtures containing 500 nM biotin-Krev-1 peptide and varying concentrations of ^{3}H-GGPP. Lineweaver-Burk plots indicated that, in contrast to the FTase inhibition, TAN-1813 was noncompetitive (Ki=166 μM) with respect to GGPP (Fig. 5).

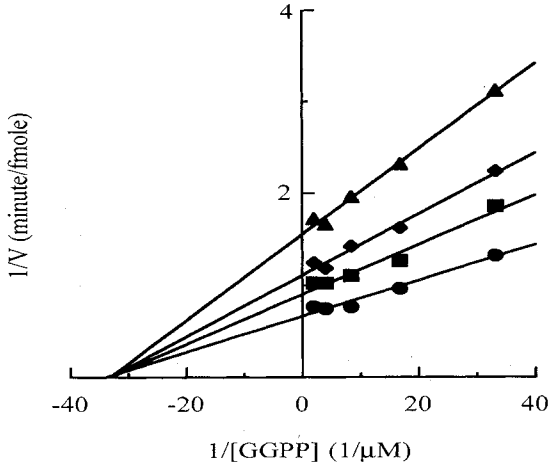
Inhibition of Cell Proliferation

TAN-1813 showed growth-inhibitory activity against cultured cells including a wide variety of human carcinoma cells (Table 2). Its inhibitory activity was comparable against NIH3T3 and its K-ras transformant cells but was about 10-fold more potent against all human tumor cells than against the normal human fibroblast MRC-9 (Table 2). However, there was no difference in the sensitivity to TAN-1813 between the cells with the normal ras allele and those with the oncogenic ras allele such as A549, MDA-MB-231, MIAPaCa-2, SW480, HepG2 and HT-1080 in a monolayer culture (Table 2).

Inhibition of Ras Protein-farnesylation in NIH3T3/K-ras Cells

To clarify whether TAN-1813 could block Ras protein farnesylation in cultured cells as well, NIH3T3/K-ras cells were metabolically labeled with ^{3}H-mevalonolactone, and Ras proteins were immunoprecipitated as described in Materials and Methods. As shown in Fig. 6B, the radiolabeled (*i.e.* farnesylated) Ras proteins could not be detected at all following treatment with TAN-1813 at more than 3 μg/ml. In contrast, geranylgeranylation of other small G-proteins was refractory to the inhibition by TAN-1813; a substantial amount of geranylgeranylated proteins could be detected even at 10 μg/ml, the highest concentration tested (Fig. 6A).

Fig. 5. Kinetics of the GGTase I inhibition by TAN-1813 as a function of GGPP concentration.



Rat brain GGTase I preparation (1.3 fmole/minute) was incubated at 37°C for 1 hour with various concentrations of [^{3}H]GGPP at 500 nM Krev-1 peptide. TAN-1813; none (●), 25 μg/ml (■), 50 μg/ml (◆) and 100 μg/ml (▲).

Morphological Reversion of NIH3T3/K-ras Cells

Consistent with the observation that TAN-1813 could reduce the amount of farnesylated Ras proteins in NIH3T3/K-ras cells, morphology of NIH3T3/K-ras cells reverted from the transformed phenotype to the normal one, when they were cultured for 2 days in the presence of 1 μg/ml of TAN-1813 (Fig. 7). Furthermore, TAN-1813 restrained H-ras-induced neurite outgrowth of PCMR-5 cells, a variant of PC-12 pheochromocytoma cells transfected with activated H-ras gene which is fused to the MMTV promotor and whose expression is inducible by addition of dexamethasone (data not shown)[21].

Table 2. Growth-inhibitory activity of TAN-1813 against murine and human fibroblasts and human tumor cells.

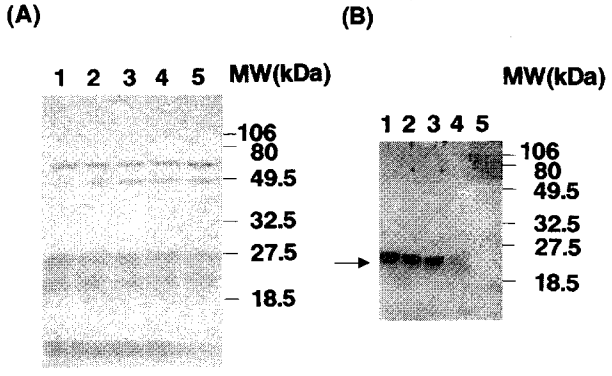
Cell line	IC_{50} (ng/ml)
NIH3T3 murine fibroblast	540
NIH3T3 K-ras transformant*	310
MRC-9 human lung fibroblast	800
G361 human melanoma	50
A549 human lung carcinoma*	110
H69 human lung small cell carcinoma	36
MCF-7 human breast adenocarcinoma	43
MDA-MB-231 human breast adenocarcinoma*	39
HepG2 human hepatocellular carcinoma*	72
HT-1080 human fibrosarcoma*	15
MIAPaCa-2 human pancreatic carcinoma*	20
WiDr human colon carcinoma	35
SW48 human colon carcinoma	68
SW480 human colon carcinoma*	43

Assays of growth-inhibitory activity of TAN-1813 were carried out as described in Materials and Methods. The cell lines with an asterisk (*) have been reported to harbor oncogenic ras mutations.

Effect on the Cell Cycle Progression of NIH3T3/K-ras Cells

To examine the point in the cell cycle where TAN-1813 acts, we carried out flow cytometric analyses using NIH3T3/K-ras cells. NIH3T3/K-ras cells synchronized in early S phase by aphidicolin treatment (Fig. 8A-2) were washed with drug-free medium. After release from the S phase block, the cells progressed into G2/M and G1 phases 5 and 10 hours later, respectively (Fig. 8A-3 and -4). Under these experimental conditions, TAN-1813 was added to the medium immediately and 5 and 10 hours after the release in order to examine the effect against cells in S, G2/M and G1 phases of the cell cycle, respectively. Irrespective of the cell cycle state at the beginning of TAN-1813 treatment, NIH3T3/K-ras cells were arrested at both G1 and G2/M phases (Fig. 8B-1, -2 and -3).

Fig. 6. Inhibition of Ras farnesylation in NIH3T3/K-ras cells.



Cells were labeled with [^{3}H]mevalonolactone in the absence or presence of varying concentrations of TAN-1813 for 20 hours. The cell-free extract was immunoprecipitated with anti-Ras immune complex at 4°C overnight and spun down briefly. Both the supernatant (A) and pellet containing Ras proteins (B) were processed and subjected to fluorography as described in Materials and Methods. The positions of the molecular weight markers are indicated on the right. Arrow in (B) indicates the position of Ras proteins. TAN-1813; none (lane 1), 0.3 μg/ml (lane 2), 1 μg/ml (lane 3), 3 μg/ml (lane 4) and 10 μg/ml (lane 5).

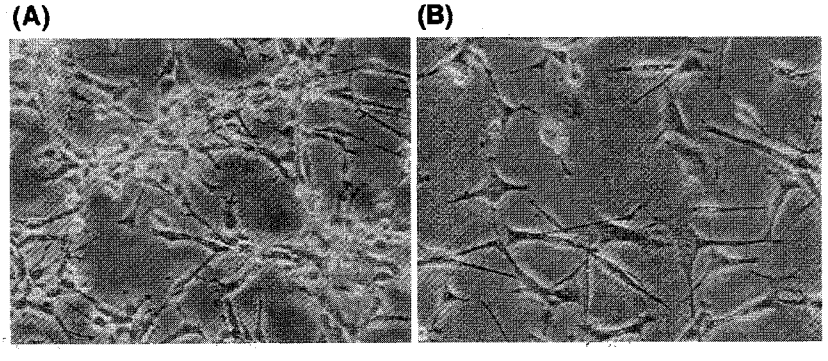
Antitumor Activity *In Vivo*

Antitumor activity of TAN-1813 was evaluated in mice implanted with two types of oncogenic ras-harboring cells. Intraperitoneal administration at doses of 6.3 and 25 mg/kg reduced the growth of HT-1080 tumor cells, which have been reported to harbor an oncogenic mutation (Q61K) in the N-ras gene[29], without causing marked body weight loss (Table 3). Subcutaneous administration at higher doses (30~100 mg/kg) was also effective against HT-1080 and NIH3T3/K-ras tumor growth (Table 3 and 4, respectively). In addition, antitumor activity was also observed in nude mice bearing AsPC-1 human pancreas carcinoma (data not shown). Minimum lethal doses of TAN-1813 were 200~400 mg/kg or 50 mg/kg when administered subcutaneously or intraperitoneally, respectively (ICR male mice, 6-week-old).

Discussion

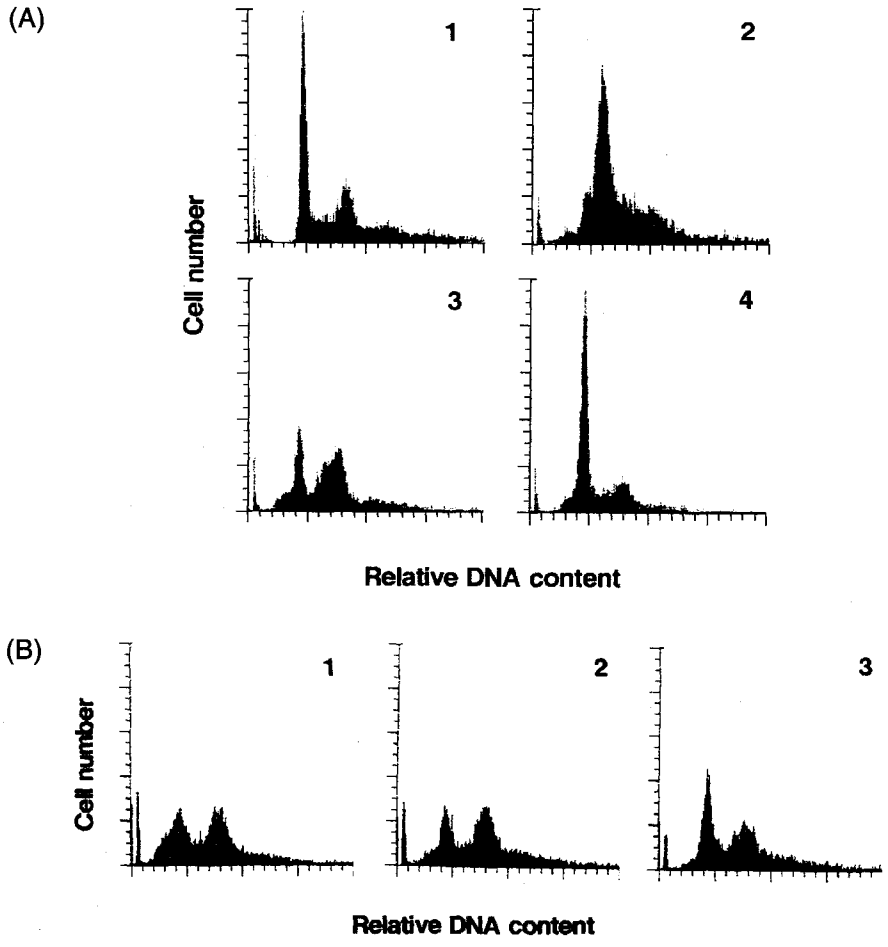
Since the frequent incidence of the gain-of-function

Fig. 7. Morphological reversion of NIH3T3/K-ras cells by TAN-1813.



NIH3T3/K-ras cells (1×10^4 cells) suspended in DMEM/F10 were plated in the wells of a 48-well plate. Cells were cultured at 37°C under 5% CO_2 for 2 days in the absence (A) or presence (B) of 1 μg/ml of TAN-1813.

Fig. 8. Effect of TAN-1813 on the cell cycle progression of NIH3T3/K-ras cells.



(A) Cells were synchronized in the early S phase by aphidicolin and released in drug-free medium as described in Materials and Methods. At appropriate time points, cells were harvested, stained and analyzed by flow cytometry. (1), asynchronous culture; (2), aphidicolin synchronized culture; (3), 5-hour after release; (4), 10-hour after release.

(B) Cells were synchronized as in (A) and released in drug-free medium. Immediately (1), 5 hours (2) or 10 hours later (3), TAN-1813 (final 5 μg/ml) was added to the medium, and 29 hours after release from the S phase block, cells were analyzed as in (A).

mutations in ras genes was reported with human tumor specimens[6], many researchers have strived for "anti-Ras drugs" based on the activity to revert the morphologically transformed phenotype to the normal one, aiming at pharmacological manipulations which suppress the activity of oncogenic Ras proteins. Such efforts, however, have not been fruitful probably because such a screening method was not directly targeted against Ras proteins. Indeed, most, if not all, of the compounds discovered through this approach don't seem to be specific against Ras proteins. However, the recent finding that the farnesylation of oncogenic Ras is essential for its cell transforming activity makes FTase a promising target, and more direct approaches, *i.e.* "target-directed screening" have been taken to discover Ras-specific inhibitors. One such approach is the design of FTase inhibitors based on the FTase substrates, Ras *C*-terminal peptides and FPP. Another is random screening for inhibitors of natural origin through the enzyme inhibition assay. During the last decade, various types of compounds have been reported including synthetic compounds such as farnesyl isoprenoid analogues[30,31], Ras *C*-terminal CAAX-based peptido-mimetics[22,32,33] or heterocyclic compounds[34,35] as well as microbial products such as manumycin[36,37], pepticinnamins[36], chaetomellic acids[30,36] and zaragozic acid A[30,36]. Among synthetic compounds, SCH66336 and R115777, which are

Table 3. Antitumor activity of TAN-1813 in human fibrosarcoma HT-1080-bearing mice.

Compound	Dose (mg/kg)	No. of mice tested	T/C	Body weight*** change(g)	No. of dead mice
Experiment 1*					
Vehicle	-	3	100	0.90	0
TAN-1813	6.3	3	44	-0.60	0
	25.2	3	42	-0.15	1
Experiment 2**					
Vehicle	-	3	100	-0.57	0
TAN-1813	10	3	94	-0.13	0
	30	3	65	-1.37	0

* TAN-1813 was intraperitoneally administered daily for 5 days (Day11-15) at 6.3 mg/kg or 3 days (Day11-13) at 25.2 mg/kg.
** TAN-1813 was subcutaneously administered daily for 4 days(Day11-14) at the two doses.
*** Body weight change was measured from Day 11 to Day 19 (Exp. 1) and Day 11 to Day 18 (Exp. 2).

Table 4. Antitumor activity of TAN-1813 in NIH3T3/K-ras tumor-bearing mice.

Compound	Dose (mg/kg)	No. of mice tested	T/C	Body weight* change(g)	No. of dead mice
Vehicle	-	5	100	2.3	0
TAN-1813	25	5	85	0.8	0
	50	5	67	0.9	0
	100	5	48	-0.9	0

TAN-1813 was subcutaneously administered daily for 5 days(Day 5-9) at all doses.
*Body weight change was measured from Day 5 to Day 12.

heterocyclic compounds mentioned above, showed orally active antitumor activity *in vivo*[35,38] and have entered clinical trials[15]. However, no microbial products except manumycin have been shown to inhibit Ras-dependent tumor growth *in vivo*[37]. We have now presented evidence that a novel fungal metabolite, TAN-1813, can inhibit FTase in the cell-free assay and in mammalian cells and reduce Ras-dependent tumor growth in some nude mouse models.

FTase catalyzes the transfer of a 15-carbon farnesyl group from FPP to the cysteine residue of the carboxyl terminal CAAX motif of peptide and protein substrates. The crystal structure of FTase revealed the zinc-binding site, as well as the overall structure of the enzyme[39]. The zinc ion in FTase is essential for catalytic activity and is required for binding of the peptide substrate but not the isoprenoid substrate[40,41]. Furthermore, mutations of amino acid residues of the β-subunit, which are responsible for zinc binding, dramatically decrease the ability of the enzyme to bind zinc and to catalyze product formation but don't affect FPP binding[42]. While a variety of evidence indicates that the bound zinc ion coordinates the sulfur of the peptide substrate, the actual role of the zinc ion in the catalytic mechanism has remainded speculative. Several

mechanisms have been proposed for the reaction catalyzed by FTase: electrophilic, with formation of a positive charge on C1 of FPP[43~45]; nucleophilic, with attack by a thiolate of the CAAX cysteine residue[43,46]; or a combination of the two[47]. Recent work using metal-substituted rat FTase and fluorinated FPP analogs, however, may provide evidence for a multiple-step catalytic mechanism, perhaps suggesting a dissociative mechanism under certain conditions[48].

TAN-1813 possesses a C_8-alkenyl moiety in its totally unique structure (Fig. 1), and FTase inhibition is supposed to be due to this moiety because this inhibitor was competitive (Ki=29 μM) with FPP and noncompetitive with Ras *C*-terminal undecapeptide (Fig. 4). This kinetic data also suggested that it might also be competitive with GGPP, but the detailed kinetic study revealed that it was noncompetitive (Ki=166 μM) with GGPP (Fig. 5). It is therefore suggested that TAN-1813 inhibits FTase in a distinct manner from that in which it inhibits GGTase I, and its FTase-inhibitory activity could fluctuate in response to the changes of the cellular FPP concentration. Furthermore, the ^{3}H-mevalonolactone labeling experiment demonstrated that *in situ* prenylation of the small G proteins other than Ras proteins (*e.g.* Rho and Rab proteins) appeared less affected by TAN-1813 treatment compared to the Ras-farnesylation (Fig. 6A and B). We have not yet explored the reason for the apparent difference in the effect of TAN-1813 between the cell-free assay and the cellular assay, but it is possible that TAN-1813 might not inhibit GGTase II activity responsible for geranylgeranylation of Rab proteins and the residual bands appearing between 18.5 kDa and 27.5 kDa in Fig. 6A might correspond to this enzyme activity. Another straightforward possibility is that FTase might be more sensitive to this compound than is GGTase in the normal cellular compartments. In any case, some small G proteins other than Ras proteins seem to be insensitive to the inhibitory effect of TAN-1813, but whether the residual activity is sufficient to support the functions of Rho and Rab proteins is not clear.

TAN-1813 showed equipotent inhibitory activity against the proliferation of NIH3T3 cells and its K-ras transformed cells (Table 2), although the recently developed FTase-specific CAAX-based inhibitors[22,49] have been reported to specifically inhibit the growth of ras-transformed cells. In spite of the nonselectivity observed in the growth-inhibition experiment, TAN-1813 was able to induce the reversion of NIH3T3/K-ras cells from the transformed morphology to the normal one (Fig. 7). This activity of TAN-1813 seems to be manifested through the inhibition of Ras-farnesylation, leading to the abrogation of Ras function, since the FTase-specific inhibitors have been reported to have this activity[22,49,50]. However, the involvement of inhibition of Rho function cannot be ruled out because Rho proteins play important roles in regulation of actin filament organization[1] which is critical for cell shape determination. It is quite difficult to determine the primary contributing factor for this activity as the functions of Rho proteins have been reported to be controlled by Ras proteins[1]. However, taking into account the fact that this activity was observed at TAN-1813 concentrations of 1 μg/ml, the concentration range which abolished the Ras-farnesylation completely but the geranylgeranylation of other small G proteins incompletely in the cells, it is likely that inactivation of Ras proteins was primarily responsible for this morphological reversion.

Addition of TAN-1813 caused NIH3T3/K-ras cells to cease their cell cycle progression at both G1 and G2/M phases, which excludes the possibility that TAN-1813 directly inhibits DNA synthesis and arrests cells at S phase. This is partially inconsistent with the previous observation that lovastatin and simvastatin, which are HMG-CoA reductase inhibitors and were reported to inhibit Ras-farnesylation by reducing the FPP content in the cells, pre-dominantly inhibited G1 to S phase transition in T24 bladder carcinoma[51] and MIAPaCa-2 pancreas carcinoma[52], respectively. It is also reported that the growth-inhibitory activity of lovastatin is not due to the inactivation of Ras[53] and that Ras function is also required for G2 to M transition in addition to G1 to S transition[54]. Therefore, the exact roles of Ras proteins in each step of the cell cycle have not been elucidated yet, and clarification of the molecular target(s) of TAN-1813 may lead to a better understanding of Ras functions in cell cycle regulation.

Although the potency of TAN-1813 to inhibit FTase in the cell-free enzyme assay is rather low (IC_{50}=23 μg/ml), inhibition of cellular Ras-farnesylation in and growth of NIH3T3/K-ras cells could be detected at about 10- and 100-fold lower concentrations, respectively (Fig. 6 and Table 2). This discrepancy cannot be explained clearly as yet, but, taking into consideration that TAN-1813 treatment time was an hour for the cell-free FTase assay, 22 hours for ^{3}H-mevalonolactone labeling experiment and 3 days for growth inhibition experiment, it is possible that the inhibitory activity becomes stronger as the treatment period becomes longer. Indeed, the CAAX-based FTase inhibitors have been shown to require a long incubation time to elicit an apparent inhibitory effect[22,49]. Alternatively, since TAN-1813 competes with FPP (Fig. 4) and its inhibitory activity is abrogated with an increase in FPP concentration (data not shown), our assay conditions for FTase may be too rigorous to reflect the intrinsic activity of TAN-1813 in cells. In any

case, we revealed in this study that TAN-1813 inhibits Ras protein farnesylation in NIH3T3/K-ras cells and it is highly likely that this is the most important contributing factor for the growth inhibition of NIH3T3/K-ras cells.

TAN-1813 exhibited *in vivo* antitumor activity against HT-1080 and NIH3T3/K-ras tumors harboring oncogenic ras genes (Table 3 and 4) but didn't against experimental murine tumor models such as P388 lymphoma and Lewis lung carcinoma harboring no oncogenic ras alleles (data not shown). This seems to be consistent with the assumption that TAN-1813 showed antitumor activity *in vivo via* malfunction of Ras protein. Experiments to clarify its precise mode of action are currently in progress.

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