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Suppression of Human Pancreatic Cancer Growth in BALB/c Nude Mice by Manumycin, a Farnesyl:protein Transferase Inhibitor

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Activating mutations of Ki-*ras* have been detected in most human pancreatic adenocarcinomas. Since Ras protein requires farnesylation to function, we investigated the effects of manumycin, a potent farnesyl:protein transferase inhibitor, on the growth in nude mice of a human pancreatic cancer cell line, MIA PaCa-2, with a point mutation in the Ki-*ras* gene. Tumor-bearing mice received intraperitoneal injection of 1 or 5 mg/kg manumycin daily for 5 days, or 2 mg/kg manumycin daily for 2 weeks. Growth of inoculated tumors was significantly inhibited by the treatment. The treatment significantly ($P<0.05$) lowered the numbers of bromodeoxyuridine-incorporating tumor cells. Manumycin did not have apparent hepatotoxicity *in vivo*. Farnesyl:protein transferase inhibitors could offer a new approach for cancer chemotherapy.

Key words: Farnesyl:protein transferase inhibitor — Manumycin — Pancreatic cancer — Nude mouse — Ras

The Ki-*ras* gene is the oncogene that is most frequently mutated in human tumors. More than 90% of human pancreatic cancers and 50% of colon cancers are reported to have mutationally activated forms of the *ras* oncogene.[1] Mutationally activated *ras* oncogenes have been suggested to have significant roles in the development and progression of various human malignancies, including pancreatic cancer.[1]

Ras gene product, Ras, is synthesized as a cytosolic precursor, but Ras does not function unless it is attached to the inside of the cell membrane. Post-translational modification of Ras is requisite for localization in the plasma membrane.[2] The first and obligatory step in this post-translational modification is the addition of a farnesyl moiety to the cysteine residue located at the Ras COOH-terminal CAAX motif (C, cysteine; A, any aliphatic amino acid; X, any other amino acid). This reaction is catalyzed by farnesyl:protein transferase (FPTase).[3,4] Inhibition of Ras function by inhibiting the farnesylation reaction could be a critical target for cancer therapy.[5–7] Monoterpenes and inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase were considered as candidates to reduce the farnesylation of Ras protein. However, monoterpenes were reported not to affect the localization of Ras protein.[8] Recently, a number of selective and potent inhibitors of FPTase have been reported,[9–13] which could inhibit *ras*-dependent transformation in *ras*-transformed cells *in vitro*. UCF1-C, which is identical to manumycin, is a potent and specific FPTase inhibitor, which was isolated from a strain of *Streptomyces* by Hara *et al.*[11] Manumycin is an analog of farnesyl diphosphate (FPP) and acts as a competitive inhibitor of FPTase with respect to FPP and non-competitively with respect to Ras protein.[11]

In this study, to clarify whether FTPase inhibitors could have potential as anticancer agents, we investigated the effects of manumycin on the growth of human pancreatic cancer cells inoculated into nude mice.

Cells of the human pancreatic carcinoma cell line MIA PaCa-2[14] were seeded at a density of 3.0×10^3/well on 96-well microplates. We confirmed that the cell line has a point mutation in codon 12 of the Ki-*ras* gene by sequence analysis. After 24 h, the medium was replaced with fresh media containing various concentrations of manumycin (provided by Kyowa Hakko Kogyo Co., Tokyo). After 42 h, 1 mCi/well of ^{3}H-thymidine was added to the culture media , and the radioactivity of each cell sample was determined by using a liquid scintillation counter.

MIA PaCa-2 cells (4×10^6 cells/mouse) were injected subcutaneously into the intrascapular region of nude mice (BALB/c congenitally athymic male mice, 5 weeks old, weighing 18–22 g). Twenty days after inoculation, tumor-bearing animals were randomly assigned to 3 groups ($n=5$ for each group). We confirmed that there were no significant differences in body weight and tumor volume between groups. Two groups received manumycin dissolved in 0.2 ml of a vehicle (0.3% carboxymethyl cellulose) at a dose of 1 or 5 mg/kg, daily by intraperitoneal injection for five days, and the other

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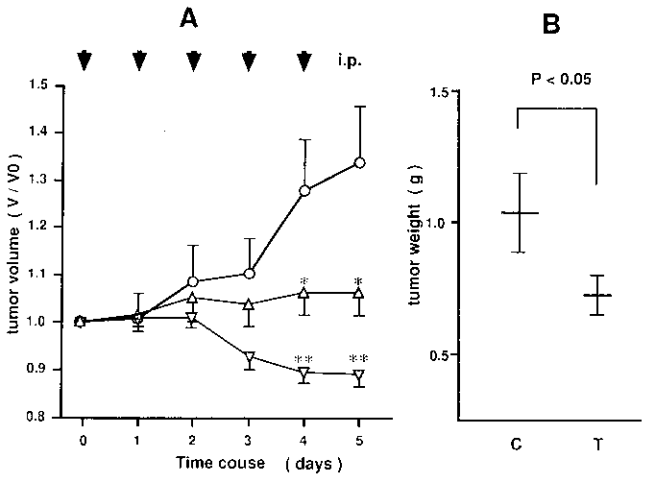

Fig. 1. Effects of manumycin on tumor growth of MIA PaCa-2 in nude mice. (A) Tumor-bearing mice received manumycin at a dose of 1 (△) or 5 mg/kg daily (▽) by intraperitoneal injection for 5 days, and control mice (○) received vehicle alone. V represents the tumor volume on the day of evaluation and V_0 represents that on the day of the initial treatment with the drug (day 0). Values expressed are mean ±SEM of five animals. Statistical analysis was done using the nonparametric Mann-Whitney test. * $P<0.05$, ** $P<0.01$ (vs. control). (B) Tumor-bearing mice received manumycin at a dose of 2 mg/kg daily for 2 weeks. Tumors were removed and weighed after the animals had been killed. Values expressed are mean±SEM of 5 animals. Statistical analysis was done using Student's t test.

group (control) received the vehicle alone. The greatest two perpendicular diameters of the tumors were measured daily, and the tumor volumes were calculated as length × (width)^2 × 0.5. Tumor regression rate (T/C) was calculated as the ratio of the mean V/V_0 value of each treated group (T) to that of the control group (C), where V is the tumor volume on the day of evaluation and V_0 is the tumor volume on the day of the initial treatment with drug (day 0). Body weights were also measured daily. On day 5 (day after the final injection), mice were killed and the tumor, heart, liver, and kidney were removed for histopathological examinations. Sera were also obtained and serum aspartate aminotransferase (AST) and total cholesterol levels were assayed using GOT-UV Test Kowa and Free Cholesterol E Test (Wako Co., Osaka), respectively.

To investigate the effect of manumycin on DNA synthesis of tumor cells, tumor-bearing mice prepared as described above were administered 0 or 2 mg/kg of manumycin daily for 2 weeks (5 mice for each groups). Two hours before the animals were killed, 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St. Louis, MO) was administered intraperitoneally (3 mice per group). The tumors were fixed in 10% neutral formalin. For immunostaining of BrdU incorporated into tumor DNA, we used a cell proliferation kit (Amersham, Backinghamshire, England) according to the manufacturer's instructions. The number of stained nuclei in 3,000 total nuclei per section was counted and the labeling index was calculated as number of stained nuclei per 1,000 total nuclei.

Manumycin dose-dependently inhibited DNA synthesis of MIA PaCa-2 cells *in vitro*. The values of the ratio of DNA synthesis to that of untreated cells were 0.72±0.11 (mean±SD, n=3), 0.42±0.04, and 0.21±0.13 in cells treated with 5, 10, and 15 m*M* manumycin, respectively. No distinct morphological change was observed after the treatment with manumycin *in vitro*.

Manumycin inhibited the growth of MIA PaCa-2 inoculated into nude mice in a dose-dependent manner. As shown in Fig. 1A, V/V_0 of the mice treated with 1 or 5 mg/kg of manumycin was significantly lower than that of untreated mice on day 4 and day 5. The T/C values of the mice treated with 1 and 5 mg/kg of manumycin on day 5 were 0.79 and 0.67, respectively. We also studied the anti-tumor effect of manumycin by administration for a longer period. In mice treated with 2 mg/kg manumycin daily for 2 weeks, V/V_0 became 1.60±0.09 (mean± SEM, n=5), which was significantly ($P<0.05$) lower than that in controls (2.35±0.21, n=5). Tumors of mice treated with 2 mg/kg manumycin daily for 2 weeks weighed significantly less than those of controls at the termination (Fig. 1B).

Histopathological examination of hematoxylin and eosin-stained samples revealed that manumycin did not induce obvious necrosis or degeneration of tumor cells. Numbers of BrdU-positive tumor cells in the treated mice (2 mg/kg manumycin daily for 2 weeks) were significantly ($P<0.05$) smaller than those in controls (Fig. 2). These data suggested that manumycin had a cytostatic effect on tumor cells.

Histological examination revealed no apparent pathological change in liver, kidney and heart after the treatment. Serum AST levels of the mice treated with 1 and 5 mg/kg manumycin daily for 5 days were 57.2±10.6 (mean±SD, n=5) and 49.7±27.6 IU/liter (n=5), respectively. These values are not significantly different from the control (64.0±17.5, n=5). Serum total cholesterol concentrations were 47.8±2.2 (mean±SD, n=5) and 48.1±3.8 mg/dl (n=5) in mice treated with 1 and 5 mg/kg manumycin daily for 5 days, respectively, and were not significantly different from the control (44.5± 2.9, n=5). The mice treated with 1 mg/kg manumycin gained weight to almost the same extent as untreated mice, but those treated with 5 mg/kg manumycin lost weight. On day 5, the body weight of the mice treated

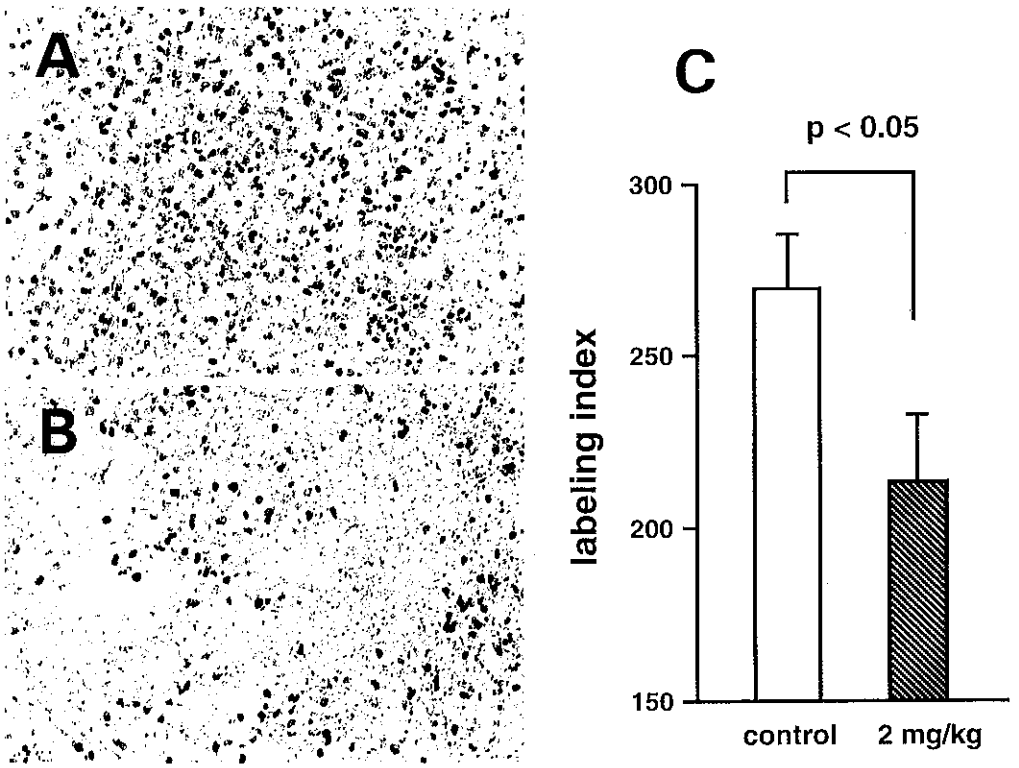

Fig. 2. Immunohistochemical staining of bromodeoxyuridine in tumors. (A) Tumor of mice treated with 2 mg/kg/day of manumycin for 2 weeks (original magnification ×412.5). (B) Tumor of control mice (original magnification ×412.5). (C) Numbers of bromodeoxyuridine-positive cells per 1,000 cells in tumors. Values represent mean±SEM of three animals. Statistical analysis was done using Student's *t* test.

with 5 mg/kg manumycin was approximately 85% of that of the controls.

Recent studies have defined several components in the signaling pathway for cell growth. These components of signal transduction are potential targets for cancer therapy. Ras is one of the key components of the signal pathway. Membrane-bound Ras exists in two states: the active form is bound to GTP, while the inactive molecule is bound to GDP. Mutationally activated Ras is locked into the GTP-bound "on" state. However, Ras does not function unless it is post-translationally modified. Farnesyl:protein transferase, which catalyzes the post-translational modification of Ras, presents a tempting chemotherapeutic target to block the *ras*-mediated signaling.[5]

Kohl *et al.*[9] and James *et al.*[10] reported that a prodrug of a tetrapeptide analog of the CAAX motif and benzodiazepine peptidomimetics restored a normal growth pattern to Ras-transformed cells *in vitro*. Hara *et al.* have demonstrated that manumycin inhibits the growth of K-*ras*-transformed fibrosarcoma cells inoculated into syngeneic mice.[11] However, the effect of FPTase inhibitors on human carcinoma-derived cells *in vivo* has not been clarified. In this paper, we have demonstrated that the FPTase inhibitor, manumycin, has potential to inhibit the growth in nude mice of human pancreatic cancer cells which have a Ki-*ras* mutation at codon 12. Pancreatic cancer is among the most difficult human malignancies to treat. It is resistant to chemotherapy and radiation therapy. More than 90% of human pancreatic cancer is reported to have an activating mutation of the Ki-*ras* gene, which may play an important role in the development and progression of pancreatic cancer.[1, 15] Our study has indicated that blockage of *ras*-dependent signaling has potential as a novel approach to chemotherapy for pancreatic cancer. Manumycin also inhibited the growth of human hepatoma cells with or without *ras* mutation *in vitro* (unpublished data). Further studies are needed to clarify the effect of FPTase inhibitors on the *in vivo* growth of tumors which do not have a mutation in the *ras* gene.

Our results indicate that manumycin does not have any marked, acute hepatotoxic effect *in vivo*. Akinaga *et al.* reported that administration of 6.25 mg/kg manumycin

daily for 5 days did not affect the levels of leukocytes and platelets in peripheral blood during and up to 3 weeks after the administration (unpublished data), which implies that manumycin does not have toxicity to bone marrow. However, further efforts to develop more potent FPTase inhibitors with low toxicity are needed.

In summary, we have shown that a farnesyl:protein transferase inhibitor, manumycin, inhibited the *in vivo* growth of a human pancreatic cancer cell line, MIA PaCa-2. These results indicate that farnesyl:protein transferase inhibitors could offer a novel approach for cancer therapy.

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