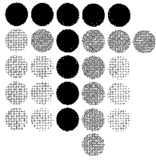

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# Anti-proliferation Effect of Damina 909 on Pancreatic Cancer Cells in Tumor-Xenografted Nude Mice Model

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## Abstract

**In this study, we investigated the anti-proliferative effect of Damina 909 in human cancer cell lines and tumor-xenografted nude mice to elucidate its potential in treating many cancers. Damina 909 treatment resulted in inhibition of cell proliferation of human pancreatic cancer cells. Our *in vivo* study showed that the weight of pancreatic tumors in Damina 909-treated group were the lighter than control group. Consequently, the intake of food and water in Damina 909-treated group did not change, while those in control group were steadily decreased over a period of treatment. Moreover, Damina 909 treatment elevated the protein expression of p53 and p21 in pancreatic tumor of xenografted nude mice. In summary, compare to other human cancer cells such as prostate and hepatocyte, Damina 909 is most effectively inhibited proliferation of pancreatic cancer cells by increasing the expression of tumor suppressor genes. This led us to speculate that a candidate substance for effective cancer therapy of pancreatic cancer might be contained in Damina 909.**

**Keywords:** Damina 909, Herb, Pancreatic cancer, Tumor-xenografted model, p21

Pancreatic cancer is one of the most fetal types of cancer, and represents the fifth leading cause of cancer death in the United State[1,2]. Since this pancreatic adenocarcinoma has 1-3% of a 5-year survival rate, many patients with pancreatic cancer die within few months after diagnosis[3,4]. There are a few palliative therapies for pancreatic cancer including surgery, chemotherapy and radiation therapy; however these therapies are known to be ineffective against the cancer[5,6]. Therefore, to increase the anti-cancer effect of these therapies, a new combination therapy for treating pancreatic cancer is needed.

One example of Damina 909 is made from many herbs such as *Rhodiola sachalinensis*, *Torilis japonica*, *Cuscutae Semen* and *Alnus japonica*. herb materials of Damina 909 have been used in the traditional medicine in Koreans to cure several diseases and many studies reported that the active substances of these herbs also have an anti-cancer effect. For example, high pressure extract of *R. sachalinensis* inhibited growth of many types of human cancer cells including lung, stomach, breast and liver[7,24]. Oral treatment of *R. rosea* root extract, also, suppressed growth of ascites tumors and lung carcinomas in tumor afflicted mice cancer models[25]. Acupuncture of *C. Semen* solution is effective in anti-cancer and anti-metastasis *in vivo* and *in vitro*[26]. In addition, diarylheptanoids from *A. japonica* has cytotoxicity activities on various cancer cell lines[8]. Moreover, previous *in vivo* studies demonstrated that Damina 909 promoted stamina and prevented reduction of spermatozoa stemming from environmental hormones. However, the anti-cancer effect of Damina 909 and its underlying mechanism (s) have not been established yet.

$p21^{cip/waf1}$ is well known as tumor suppressor gene and is considered as target for anti-cancer drug because it plays an important role in cell proliferation, differentiation, and apoptosis. p21 transcript is up-regulated by p53 in response to cellular stress such as chemical or radiation-induced DNA damage[6,9]. p21 inhibits the activity of cyclin/cdk2 complexes, thereby blocking the cell cycle progression[7]. p21 also inhibits DNA replication by binding to the proliferating cell nuclear antigen (PCNA)[10,11]. Recently, p21 overexpression resulted in growth inhibition and apoptosis in many human carcinoma cell lines including pancreatic can-

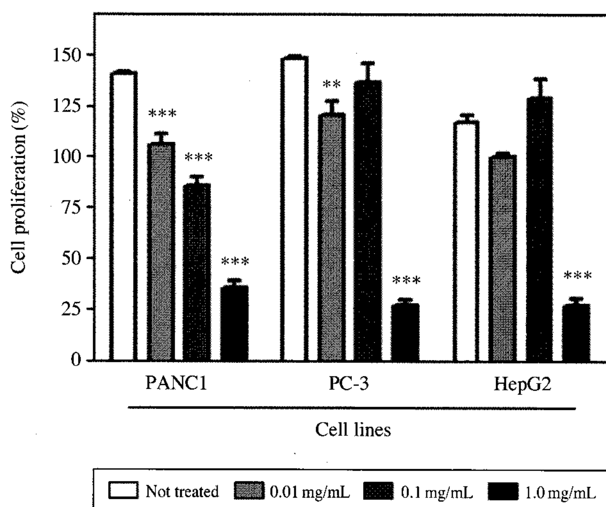



**Figure 1.** Inhibitory effect of Damina 909 on the growth of human cancer cells. Cell viability was determined by the MTT assay. Cells were treated with 0.01 mg/mL, 0.1 mg/mL, and 1.0 mg/mL Damina 909 for 24 hr. The results are expressed as percentage viability, with nontreated cells being set at 100% viability. **, *** indicates statistically significant differences ($P<0.01$ and $P<0.001$) vs untreated cells (ANOVA).

**Table 1.** Quantitative and Qualitative Analysis of 4 Reagents of Damina 909.

| | Reagents of Damina 909 | | | | Units |
|---|---|---|---|---|---|
| | No.1 | No.2 | No.3 | No.4 | |
| Water | 85.6 | 84.8 | 84.4 | 83.2 | g/100g |
| Lipid | 0.1 | 0.1 | 0.1 | 0.1 | g/100g |
| Protein | 0.1 | 0.2 | 0.2 | 0.3 | g/100g |
| Ash | 0.2 | 0.3 | 0.3 | 0.4 | g/100g |
| Carbohydrate | 14.0 | 14.6 | 15.0 | 16.0 | g/100g |
| Calory | 57 | 60 | 62 | 66 | kcal/100g |

cer cell line[6,12,13].

In this study, we investigated the anti-proliferative effect of Damina 909 in human cancer cell lines and tumor-xenografted nude mice to elucidate its potential in treating many cancers.

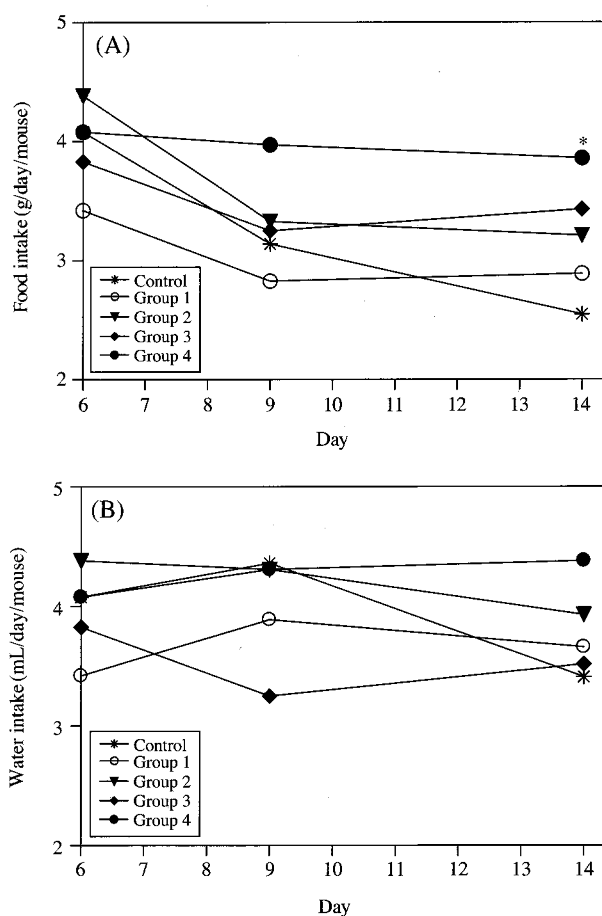



**Figure 2.** The effect of Damina 909 reagents on intake of food (A) and water (B) in tumor-xenografted nude mice. Two weeks after inoculated later, nude mice were treated 1.0 mg/mL Damina 909 reagent for 2 weeks. Animals were divided into 5 groups containing 4 mice each: Control, oral administration of autoclaved distilled water, Group 1, oral administration of No.1 reagent of Damina 909, Group 2, oral administration of No.2 reagent of Damina 909, Group 3, oral administration of No.3 reagent of Damina 909, and Group 4, oral administration of No.4 reagent of Damina 909. Food and drink intake were measured at 6, 9 and 14 days after oral administration. *indicates statistically significant differences ($P<0.05$) vs untreated nude mice (ANOVA).

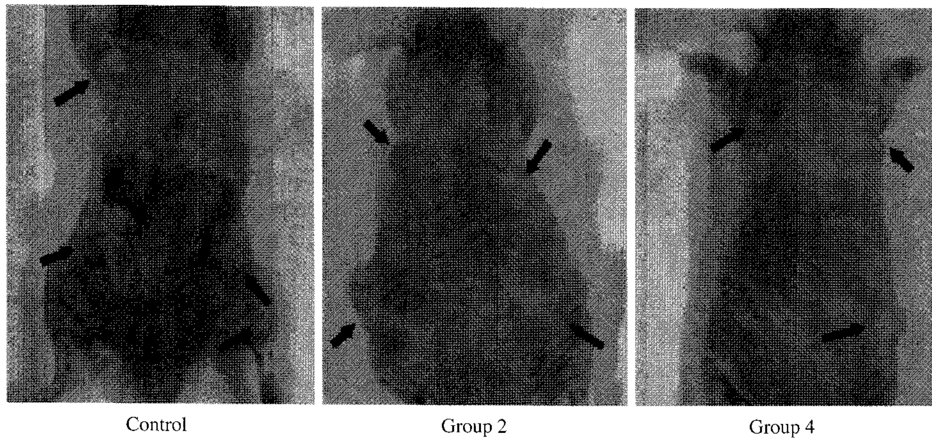
## Anti-proliferative Effect of Damina 909 in Human Cancer Cell Lines

To determine the optimal concentration of Damina 909 for *in vivo* and *in vitro* experiments, various concentrations of Damina 909 were treated to PANC-1, PC-3 and HepG2 cells for 24 hours and cell viability was assessed by using the MTT assay. 0.01 mg/mL or 0.1 mg/mL of Damina 909 inhibited the growth of PANC-1 cells, but did not affect the growth of PC-3 and HepG2 cells. Only 1.0 mg/mL of Damina 909 inhibited the growth of 3 types of cells (Figure 1). Especially, the growth of PANC-1 cells was decreased by Damina 909 in a dose-dependent manner. Thus, compared to untreated PANC1 cells, 0.01 mg/mL, 0.1 mg/mL or 1.0 mg/mL of Damina 909 treatment decreased the cell growth by 76.2%, 61.0% and 25.1%, respectively (Figure 1).

## Quantitative and Qualitative Analysis of 4 Reagents of Damina 909

To measure the amount of different classes of nutrients such as carbohydrates, lipids, proteins, ashes and

(A)




(B)

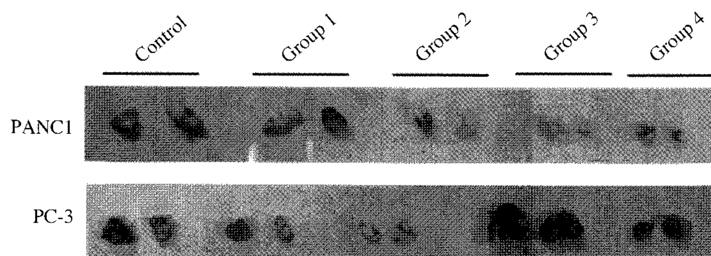



**Figure 3.** Representative photograph of tumor-xenografted nude mice (A). Two weeks after inoculated later, nude mice were treated 1.0 mg/mL Damina 909 reagent for 2 weeks. Animals were divided into 5 groups containing 4 mice each: Control, oral administration of autoclaved distilled water, Group 1, oral administration of No.1 reagent of Damina 909, Group 2, oral administration of No.2 reagent of Damina 909, Group 3, oral administration of No.3 reagent of Damina 909 and Group 4, oral administration of No.4 reagent of Damina 909. Mice were sacrificed and collected the tumors (B) including human prostate cancer cell lines PC-3 and human pancreatic adenocarcinoma cell line PANC-1.

**Table 2.** The body weight of Damina 909-treated tumor-xenografted nude mice.

| Group | Control | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|---|
| Body weight (g) | 20.585±4.872 | 24.505±1.927 | 24.920±3.712 | 19.890±3.279 | 20.855±4.958 |

The data shown are means±SEM of 4 controls, Group 1, Group 2 and Group 4, and 3 Group 3, respectively. Animals were divided into 5 groups containing 4 mice each : Control, oral administration of autoclaved distilled water, Group 1, oral administration of No.1 reagent of Damina 909, Group 2, oral administration of No.2 reagent of Damina 909, Group 3, oral administration of No.3 reagent of Damina 909 and Group 4, oral administration of No.4 reagent of Damina 909.

water in Damina 909, we perform the quantitative and qualitative analysis according to testing methods of Food Code (2007) (Table 1). Our results showed that the largest amount of proteins, ashes and carbohydrates was found in No.4 reagent, while the amount of lipid did not differ. For example, the amount of nutrients in No.4 reagents were 0.3 g/100 g for protein, 16 g/100 g for carbohydrate, and its calorie was 66 kcal/100 g (Table 1). These results suggest that the active substances, which have anti-cancer effect, may contain proteins and carbohydrates.

### Effects of 4 Reagents of Damina 909 on Food and Drink Intake in Tumor-xenografted Nude Mice Model

Food and water intake in 4 reagents of Damina 909 treated groups over a period of 14 days during Damina 909 treatment are shown in Figure 2, which are presented as mean g/day/mouse and mL/day/mouse, respectively. The food intake of control decreased progressively, but that of No.4 reagent-treated group did not change over a period of treatment. In addition, food intake of No.1-, No.2- and No.3 reagent-treated groups decreased at day 9, and then recovered to basal level (Figure 2A).

The pattern for water intake was similar to that for food intake. Overall, the water intake in the No.4-reagent treated group steadily maintained the basal level, but that of the control decreased 9 days later during Damina 909 treatment (Figure 2B). Especially, food and water intake in the reagent No.4-treated group was significantly higher than those of the control at day 14 (Figure 2).

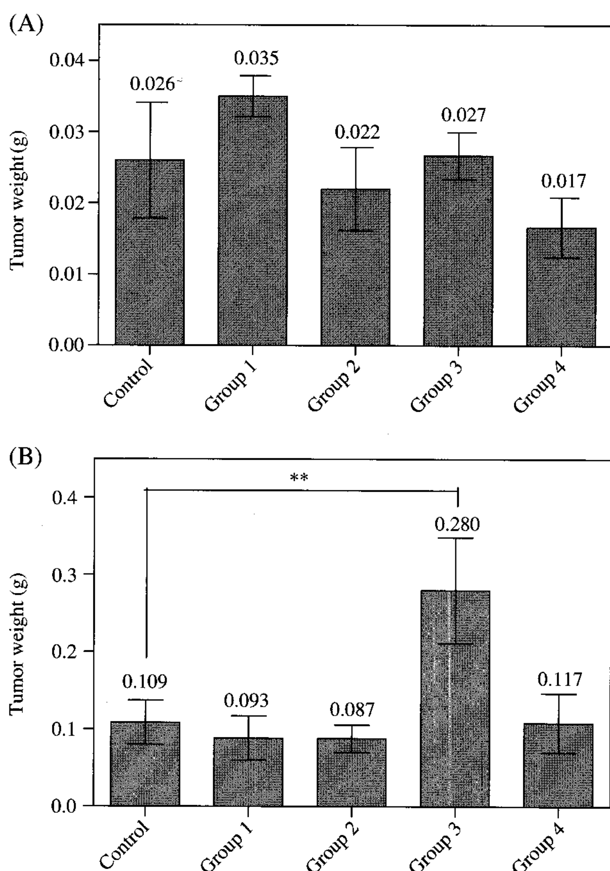



**Figure 4.** Anti-proliferative effect of Damina 909 reagent in tumor xenografted nude mice. Two weeks after inoculated later, and nude mice were divided into 5 groups containing 4 mice each : Control, oral administration of autoclaved distilled water, Group 1, oral administration of No.1 reagent of Damina 909, Group 2, oral administration of No.2 reagent of Damina 909, Group 3, oral administration of No.3 reagent of Damina 909 and Group 4, oral administration of No.4 reagent of Damina 909. Mice were sacrificed 2 weeks after oral administration to harvest the pancreatic tumor (A) and prostatic tumor (B) for measuring its weight. ** indicates statistically significant differences ($P<0.01$) vs untreated nude mice (ANOVA).

### Anti-proliferative Effects of Damina 909 on Pancreatic Cancer in Tumor-xenografted Nude Mice Model

Effect of 4 reagents of Damina 909 on the growth of human pancreatic and prostatic cancers in tumor-xenografted nude mice was shown at Figure 3. The average body weight of the mice did not differ significantly between experimental groups (Table 2). Compared to the control, oral administration of the No.4 reagent suppressed tumor weight of the pancreas, but other Damina 909 reagents were not inhibited (Figure 4A). For example, the final tumor weights of pancreatic cancers were $0.026\pm0.018$ g for control and $0.017\pm0.006$ g for the No.4 reagent-administrated group. In contrast, there were not significant differences in tumor weights of the prostate between the control and experimental groups (Figure 4B). Our results of *in vitro* and *in vivo* experiments showed that anti-proliferative effect of Damina 909 was more effective for pancreatic cancer than prostatic and hepatic cancers (Figure 1 and 4A). Moreover, the No.4 reagent produced the greatest anti-cancer effect in reagents of Damina 909 and this correlated with our quantitative and qualitative analysis of Damina 909 (Table 1).

### Effects of Damina 909 on Expression of Tumor Suppressor Genes in Pancreatic Cancer in Tumor-xenografted Nude Mice

To determine the effect of Damina 909 treatment of expression of tumor suppressor genes such as p21 and p53, we performed the Western blot analysis using pancreatic cancers collected from tumor-xenografted nude mice. After 2 weeks of treatment, a higher protein expression of p21 and p53 was observed in Damina 909-treated groups than in control group (Figure 5). As shown Figure 5B, No. 3 and No.4 reagent treatment increased expression of p53 by ~2.0 ($P<0.01$) and ~3.0 fold ($P<0.01$), respectively. Correspondingly, the expression of p21, which is critical downstream effector of p53, was induced by No.4 reagent treatment by ~7.2 fold ($P<0.01$) compared to p21 expression in control (Figure 5C). Moreover, this data is consistent with our observation that the No.4 reagent suppressed the tumor weight of the pancreatic cancer (Figure 4B).

## Discussion

Pancreatic adenocarcinoma is one of the most common causes of cancer-related death in the world, and least responds to palliative therapies[14]. Although chemotherapies for pancreatic cancer patients have been advanced, these have a number of unwanted side effects. Many patients tend to use herbal therapies as new combination therapies because natural products are well known to contain various chemopreventive or chemotherapeutic compounds[15,16].

In this study, we used *in vitro* and *in vivo* experiments to examine the anti-cancer effect of Damina 909 which mixed extract of many plants including *R. sachalinensis*, *T. japonica*, *C. Semen* and *A. japonica*. Our results showed that low concentration (0.01 mg/mL and 0.1 mg/mL) of Damina 909 significantly inhibited the cell proliferation of PANC-1 cells, but it did

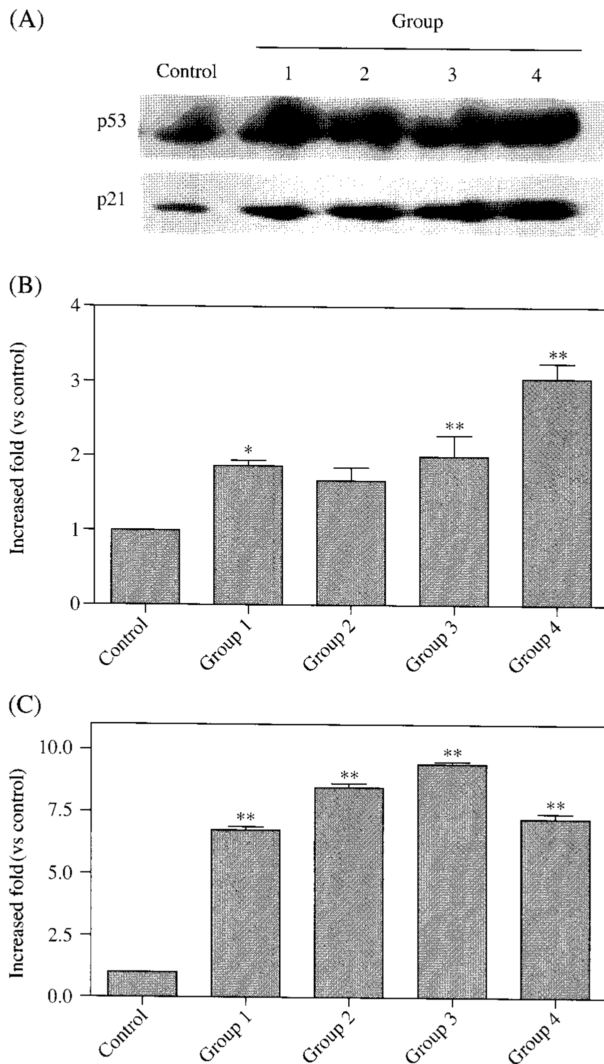



**Figure 5.** Western blotting analysis of expression of p21 and p53 in the pancreatic cancers after Damina 909 treatment. The pancreatic tumors were collected 2 weeks after oral treatment with 4 reagnet of Damina 909 (1.0 mg/mL) and equal amounts of protein extract (30 μg) were size-separated on a 12% SDS-PAGE gel, transferred to an NC membrane (0.45 μm) and incubated with antibodies of p53 and p21. (A) Representative Western blots showing protein levels of p53 and p21 in the Damina 909-treated groups. (B), (C) Quantitative analysis of the protein levels of p53 and p21 in the gel shown in (A). *, **indicates significant differences, *$P<0.05$, **$P<0.01$ (ANOVA).

not affect the cell growth of PC-3 and HepG2 cells. Damina 909 also inhibited the cell proliferation of PANC-1 cells as dose-dependent manner (Figure 1). Similarly, our *in vitro* data demonstrated that oral treatment of No.4 reagents of Damina 909 suppressed tumor weight of pancreas; however it did not inhibit tumor weight of the prostate in tumor-xenografted nude mice when compared to tumor weight in untreated group (Figure 4). From these observations, we speculated that anti-proliferative effect of Damina 909 was more effective for pancreatic cancer than prostate and liver cancers.

In addition the intake of water and food, a criterion of health condition, did not change in No.4 reagent-treated group over a period of treatment, progressively decreased in the untreated groups (Figure 2). Moreover, the food intake of the No.4 reagent-treated group was significantly higher than that of untreated groups ($P<0.01$) (Figure 2A). In relation to these results, quantitative and qualitative analysis showed that the No.4 reagent contained the largest amount of protein and carbohydrate in 4 reagents of Damina 909 (Table 1). Thus, the No.4 reagent produced the greatest anti-cancer effect in 4 reagents of Damina 909, and anti-cancer effect of Damina 909 might be affected by the amount of protein carbohydrate. Recently, many studies investigated that phytochemicals isolated from plant extracts play important role in anti-cancer process[14,17,18]. For example, plant lectin, which is one of the glycoprotein components of mistletoe, inhibited the cell proliferation and induced apoptosis in cancer cells such as melanoma and T-lymphocyte[17,19].

Our observations reveal that Damina 909 contained large amounts of proteins and carbohydrates that clearly inhibited the cell proliferation of pancreatic cancer (Figure 1 and Figure 4). However, the cellular mechanism(s) of Damina 909 by which Damina 909 treatment regulate expression of tumor suppressor genes such as p53 and p21, which are target of anti-cancer drugs. p53 is well known to play important role in apoptosis and cell cycle control over a period of cancer treatment[20]. In addition, p21, which is induced by p53 protein, is critical mediator of growth inhibition and DNA replication[21,22]. In this study, Damina 909 treatment elevated protein expression of p53 and p21 of pancreatic cancers in tumor-xenografted nude mice (Figure 5). Similarly, pancreatic tumor cells infected with p21 recombinant adenovirus showed significant growth inhibition and accumulation in G0/G1 phase compared to non-infected cells[6]. In addition, the opioid growth factor (OGF), which is a critical regulator of the progression of pancreatic cancer, inhibited cell growth of pancreatic cancer cells by up-regulating p21 expression[23].

In conclusion, we observed that Damina 909 treatment inhibited the cell proliferation of human cancer cells such as pancreas, prostate and liver. The anti-cancer effect of Damina 909, also, was more effective for pancreatic cancer than for prostatic cancer in tumor-xenografted nude mice. Moreover, Damina 909 treatment increased the expression of p21 and p53 by pan-

creatic cancer cells and this inhibited their proliferation. Thus, these observations supports that up-regulation of p21 may participate in the Damina 909-induced cell death of pancreatic cancer cells. Therefore, the anti-proliferative effect of Damina 909 may be useful for treating pancreatic cancer.

## Materials & Methods

### Cell Culture

The androgen-independent human prostate cancer cell lines PC-3 (ATTCC CRL 1435), human pancreatic adenocarcinoma cell line PANC-1 (ATCC CRL-1469) and human liver adenocarcinoma cell lines HepG2 (ATCC HB 8065) were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

PC-3 cells were cultured in RPMI 1640 (WelGENE Inc.) supplemented with 10% fetal bovine serum (FBS) (WelGENE Inc.), 100 units/mL penicillin G sodium, 100 μg/mL streptolycin sulfate and 0.25 μg/mL amphotericine B.

PANC-1 and HepG2 cells PC-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (WelGENE Inc.) supplemented with 10% FBS (WelGENE Inc.), 100 units/mL penicillin G sodium, 100 μg/mL streptolycin sulfate and 0.25 μg/mL amphotericine B. All cell lines were maintained at 37°C and 5% $CO_2$ in a humid environment.

### Cell Viability Assay

After treatment with Damina 909, cell viability was determined by the MTT assay. Briefly, the cells were plated in 96-well tissue culture plates, treated with 0.01, 0.1 or 1.0 mg/mL of Damina 909 for 24 hr, and incubated for 4 hr at 37°C with 20 μL per well of 5 mg/mL MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution (Sigma Aldrich, USA). To dissolve the insoluble purple formazan crystal that was then formed, the medium was replaced with dimethyl sulfoxide (DMSO). The absorbance in each well was then recorded at 540 nm by using an ELISA reader, and the percentage of cell viability was calculated relative to the viability of untreated cells.

### Animal Experiments

Male BALB/cS1c-nu mice, 5 weeks old, were used in this study. All mice were housed in plastic cages with stainless-steel grid tops in an air-conditioned room (25°C, 12 hr light : 12 hr dark cycle) and fed with autoclaved standard laboratory chow pellets and water. Animal experiments were started after 1 weeks of acclimatization.

### Tumor-xenografted Nude Mouse Model

PANC-1 cells $5 \times 10^5$ were suspended in 200 μL PBS (pH 7.4), then inoculated subcutaneously into the upper region of front legs of nude mice. PC-3 cells, also, $5 \times 10^5$ were suspended in 200 μL PBS (pH 7.4), then inoculated subcutaneously into the upper region of hind legs of nude mice. Two weeks after inoculated later, the diameter of tumor became 0.15 cm, and nude mice were divided into 5 groups containing 4 mice each : Control, oral administration of autoclaved distilled water, Group 1, oral administration of No.1 reagent of Damina 909, Group 2, oral administration of No.2 reagent of Damina 909, Group 3, oral administration of No.3 reagent of Damina 909 and Group 4, oral administration of No.4 reagent of Damina 909. Food and drink intake were measured at 6, 9 and 14 days after oral administration. Mice were sacrificed 2 weeks after oral administration to harvest the tumors for measuring its weight.

### Western Blot Analysis

After 2 weeks of Damina 909 treatment, the pancreatic tumors were collected from tumor-xenografted nude mice. Tumors were rinsed in 1X PBS, homogenized with lysis buffer (iNtRON BIOTECHNOLOGY, Inc.) and centrifuged at 13,000 rpm for 30 min at 4°C. The protein concentrations in the supernatants were determined using a BCA Protein Assay Kit (Sigma Aldrich, USA) and equal amounts of extracted protein samples were separated by SDS-PAGE and transferred to 0.45 μm nitrocellulose (NC) membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween20 (TBS-T) for 2 hr at room temperature and then incubated overnight at at 4°C with specific antibodies against p53 and p21 diluted 1 : 200. Detection was performed by using a horseradish peroxidase-conjugated goat-anti-mouse secondary antibody or a horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody diluted 1 : 1,000, and then enhanced by using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.).

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