Identification of Arctigenin as an Antitumor Agent Having the Ability to Eliminate the Tolerance of Cancer Cells to Nutrient Starvation

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Abstract
Tumor cells generally proliferate rapidly and the demand for essential nutrients as well as oxygen always exceeds the supply due to the unregulated growth and the insufficient and inappropriate vascular supply. However, cancer cells show an inherent ability to tolerate extreme conditions, such as that characterized by low nutrient and oxygen supply, by modulating their energy metabolism. Thus, targeting nutrient-deprived cancer cells may be a novel strategy in anticancer drug development. Based on that, we established a novel screening method to discover anticancer agents that preferentially inhibit cancer cell viability under the nutrient-deprived condition. After screening 500 medicinal plant extracts used in Japanese Kampo medicine, we found that a CH2Cl2-soluble extract of Arctium lappa exhibited 100% preferential cytotoxicity under the nutrient-deprived condition at a concentration of 50 μg/mL with virtually no cytotoxicity under nutrient-rich condition. Further bioassay-guided fractionation and isolation led to the isolation of arctigenin as the primary compound responsible for such preferential cytotoxicity; the compound exhibited 100% preferential cytotoxicity against nutrient-deprived cells at a concentration of 0.01 μg/mL. Furthermore, arctigenin was also found to strongly suppress the PANC-1 tumor growth in nude mice, as well as the growth of several of the tested pancreatic cancer cell lines, suggesting the feasibility of this novel antiausterity approach in cancer therapy. Further investigation of the mechanism of action of arctigenin revealed that the compound blocked the activation of Akt induced by glucose starvation, which is a key process in the tolerance exhibited by cancer cells to glucose starvation.

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Introduction
A wealth of accumulated clinical experience and indigenous knowledge of traditional medicine is still waiting to be tapped as an important source for the modern drug discovery process. In traditional medicine systems, medicinal plants and their extracts have formed the main basis for the treatment of diseases for thousands of years. The unique and very broad chemical diversity of medicinal plant extracts constitutes immense potential in the search for novel medicaments.

Pancreatic cancer is one of the most serious forms of cancer that shows resistance to almost all known chemotherapeutic agents because of resistance of the cells to apoptosis (1). Almost all patients of pancreatic cancer rapidly develop metastases and die within a short period of time after the diagnosis; this cancer is associated with the lowest 5-year survival rates known for cancers (2, 3) and continues to be one of the major health problems that remain unresolved at the start of the 21st century. Currently, surgery is the only treatment modality that offers any prospect of potential cure. 5-Fluorouracil and gemcitabine are the most commonly used chemotherapeutic agents for palliative therapy of advanced pancreatic cancer. However, recent advances in the molecular biology of pancreatic cancer and the identification of molecular signals that mediate the resistance of the cancer cells to apoptosis have prompted the development application of a novel screening strategy for drugs with activity against this cancer from natural sources (4).

Earlier, we reported that certain pancreatic cancer cell lines, such as PANC-1, AsPC-1, BxPC-1, and KP-3, exhibited marked tolerance and survive for prolonged periods of time even under extremely nutrient-starved conditions, and that a high expression level of protein kinase B (PKB)/Akt was associated with this tolerance of the cells to nutrient starvation (5). Tumor cells, in general, are well known to show high glycolytic activity and, as the tumor progresses through the multiple steps of carcinogenesis and increases in size, it is faced with an insufficient supply of oxygen and other nutrients, including glucose, and growth factors. Thus, development of drugs aimed at countering this resistance of the cells to nutrient deprivation may serve as a novel biochemical approach to cancer therapy (5–7).

In this regard, we recently screened 500 medicinal plants used in Kampo medicine for their preferential cytotoxicity against PANC-1 cancer cell lines under nutrient-deprived conditions and found that a dichloromethane (CH2Cl2)-soluble extract of Arctium lappa exhibited 100% preferential cytotoxicity against nutrient-deprived cells at the concentration 50 μg/mL. Further activity-guided fractionation and separation led to the isolation of arctigenin as the active principle of A. lappa. In this article, we report the isolation of arctigenin as a natural product with potent inhibitory activity against PANC-1 tumor cell growth both in vitro and in vivo.

Materials and Methods

Materials. The dried seeds of A. lappa were commercially purchased from Tochimoto Tenkaido Co. (Osaka, Japan). The method of extraction and purification is described in detail in Results.

Cell lines and nutrient starvation. The human pancreatic cancer cell lines PANC-1, AsPC-1, PSN-1, BxPC-3, and the hepatoma cell line Alexander, obtained from the American Type Culture Collection, were maintained in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FCS.
Statistical comparisons were conducted using Student's t test after ANOVA. The results were considered to be significant when P < 0.05.

Results

Purification and structure determination of the active principle extracted from the seeds of *A. lappa*. The dried seeds of *A. lappa* (10 kg) were chopped into small pieces and percolated overnight with CH₂Cl₂ (30 L × 3). The extracts were concentrated under reduced pressure to yield the CH₂Cl₂ extract (1.2 kg), which was chromatographed on silica gel (8 × 70 cm) eluted with hexane, and then with CHCl₃ to obtain the hexane-eluted fraction (900 g) and the CHCl₃-eluted fraction (800 g), respectively. The CHCl₃-eluted fraction was further subjected to silica gel chromatography (8 × 70 cm) using methanol-CHCl₃ (0-10%) to obtain three fractions (fraction 1, 275 g; fraction 2, 430 g; and fraction 3, 185 g). The preferential cytotoxic activity of each fraction against the PANC-1 cells was then evaluated as described in Materials and Methods, and fraction 2 (430 g) was found to be the most active with 100% preferential cytotoxicity under the nutrient-deprived condition at a concentration of 10 μg/mL. Fraction 2 was viscous oily and its TLC analysis indicated that it contained one major compound. To separate the major compound from the oily fraction, it was subjected to crystallization at room temperature for 2 days, which led to the formation of colorless prism (60.0 g). The crystals showed [x]D₂⁰ 28.4° (c = 0.077, CHCl₃). Its molecular formula was determined by HR-FABMS to be C₂₁H₂₄O₆ [M + H]⁺. Its IR spectrum indicated the presence of hydroxyl (3,550 cm⁻¹), lactone carbonyl (1,760 cm⁻¹), and phenyl (1,610 and 1,460 cm⁻¹) functional groups. Analysis of [¹H]NMR (NMR), [¹³C]NMR, correlation spectroscopy, heteronuclear multiple- bond quantum coherence, heteronuclear multiple-bond correlation, and circular dichroism spectral data confirm its chemical structure to be arctigenin (Fig. 1).

Arctigenin showed preferential cytotoxicity against nutrient-deprived cells in a concentration- and time-dependent manner. As shown in Fig. 2A, PANC-1 is highly resistant to even extreme nutrient deprivation and can survive under this condition for >48 hours (5). In this study, this tolerance to nutrient deprivation was remarkably eliminated by arctigenin in a concentration-dependent as well as time-dependent manner. Cells exposed to arctigenin at a concentration of 0.01 μg/mL showed 100% cell death within 24 hours of starvation and this sensitivity to starvation was even more pronounced when arctigenin was added at a concentration of 1 μg/mL, at which 100% cell death was observed within 12 hours. To investigate the precise mechanism of the cell death induced by arctigenin under nutrient-deprived conditions, we examined the mode of cell death using the Hoechst 33342/propidium iodide staining method, as described previously (6, 7). As shown in Fig. 2B, arctigenin at a concentration of 0.01 μg/mL induced nuclear condensation in the PANC-1 cells within 12 hours of incubation under nutrient-deprived conditions. Because propidium iodide staining of the nuclei in the absence of nuclear damage has been characterized as representing necrotic cell death, our results suggest that the nutrient-deprived PANC-1 cells exposed to arctigenin underwent necrotic cell death.

Arctigenin sensitizes the PANC-1 cells to death only under glucose-deprived conditions. We then examined the conditions under which arctigenin induced cell death in the PANC-1 cells by depriving the cell culture of glucose, amino acids, serum, or none of these. As shown in Fig. 3, arctigenin (0.01 μg/mL) caused cell death within 24 hours preferentially under the glucose-deprived condition, irrespective of the presence or absence of amino acids and/or serum.
whether arctigenin also exerted antitumor activity in vivo indicated that arctigenin was effective against many cancer cell lines. These results showed that arctigenin was the most sensitive, among all the cancer cell lines tested, to deprivation in all the tested cell lines, although to only a relatively small extent in KP-3 cells. Also, the AsPC-1 cell line was found to be responsible for the cell death.

**Effect of arctigenin on the survival of various cancer cell lines under nutrient-deprived conditions.** To investigate whether the preferential cytotoxicity of arctigenin was limited to PANC-1 cells or was observed against various other cancer cells, we examined the effect of arctigenin on the survival of various cancer cell lines under nutrient-deprived conditions. As shown in Fig. 5, arctigenin diminished the tolerance of the cells to nutrient deprivation in all the tested cell lines, although to only a relatively small extent in KP-3 cells. Also, the AsPC-1 cell line was found to be the most sensitive, among all the cancer cell lines tested, to arctigenin under nutrient-deprived conditions. These results indicated that arctigenin was effective against many cancer cell lines.

**Antitumor activity of arctigenin in vivo.** To determine whether arctigenin also exerted antitumor activity in vivo, mice were inoculated with $5 \times 10^6$ PANC-1 cells s.c. on the back and then administered arctigenin or vehicle, as described in Materials and Methods. The body weight of the animals was monitored weekly (Fig. 6A) and no significant body weight loss was recognized in the treated group versus the vehicle control group at any time during the experimental period. The treatment was initiated from the 15th day by i.p. injection of the drug at the dose of 50 μg/mouse/d on 6 days of the week (or vehicle in the control group) until the 64th day. The tumor size was measured weekly. As is evident from the tumor growth curve shown in Fig. 6B, the tumor volume increased steadily in the control group, whereas the increase was significantly less prominent in the arctigenin-treated group. There was a significant difference in the tumor size between the arctigenin-treated group and the control group ($P < 0.05$), and the mean wet weight of the tumor was significantly higher in the control group than in the arctigenin-treated group (Fig. 6C). These data indicate that arctigenin also exerted antitumor activity in vivo. The mean tumor doubling time was 49 days in the arctigenin-treated group compared with 23 days in the control group. More importantly, no toxicity was observed in any of the animals at the dose used.

**In vitro activity of arctigenin-related compounds.** To compare the anti-austerity capability of arctigenin with that of other plant-derived lignans, we further tested the preferential cytotoxicity of arctiin, nordihydroguaiaretic acid, secoisolarirecinol, and sesamin. Interestingly, only arctigenin showed potent preferential cytotoxicity against PANC-1 cells under conditions of nutrient deprivation (Fig. 7), suggesting its unique anti-austerity capability among the lignans. The glycoside arctiin is converted into arctigenin by the human intestinal flora (10, 11) after consumption, and thus the indirect role of the intestinal bacteria should also be considered in cancer chemoprevention activity of traditional medicines like A. lappa.

**Discussion**

Regulation of cell survival is crucial to the normal physiology of multicellular organisms and oxygen and nutrient supply to the cells and tissues is pivotal to the maintenance of their functions and integrity. However, the physiology of solid tumors differs from that of normal tissues in a number of important respects, the majority of which stem from the differences of the vasculature between...
tumors and normal tissues. As compared with normal cells, tumor cells proliferate more rapidly and the oxygen as well as essential nutrition demand often exceeds the supply due to the unregulated growth of the cells caused by genetic and epigenetic alterations (12–14). Thus, tumor growth and progression largely depend on angiogenesis for functional blood supply, and antiangiogenic therapy was considered to be an attractive approach for cancer therapy. However, despite marked angiogenesis, large numbers of cells in a tumor are still in a state of oxygen and nutrient deprivation, partly because they proliferate more rapidly than normal endothelial cells and partly because the newly formed vascular networks are disorganized and, therefore, not optimally functional (12–14). Nevertheless, cancer cells have an inbuilt capability of surviving even under extreme conditions, such as that characterized by low nutrient and oxygen supply. This is particularly valid for highly aggressive tumors, such as pancreatic cancer, which are relatively hypovascular. Angiographic investigations have shown that most pancreatic cancers are hypovascular and that tumor vessels are noted only in a small number of cases (15–18). Ultrasonographic and computed tomographic studies have also shown that most pancreatic cancers receive poor blood supply despite considerable angiogenesis being noted histopathologically (19–21). The question, therefore, now, is how do pancreatic cancer cells survive and proliferate under conditions of severe hypoxia and nutrient deprivation? There must be some mechanism that facilitates the survival and proliferation of cancer cells under such extreme adverse environments.

In our earlier works, we hypothesized that tolerance to nutrient starvation might be a part of the biological response to insufficient blood supply and that targeting cancer cells under conditions of nutrition deprivation may be a novel approach in anticancer drug development. In this regard, we developed a novel screening methodology for anticancer agents based on this antiausterity strategy (6) and screened 500 medicinal plant extracts used in Japanese Kampo medicine. We found from this screening that a strategy (6) and screened 500 medicinal plant extracts used in methodology for anticancer agents based on this antiausterity nutrition deprivation may be a novel approach in anticancer blood supply and that targeting cancer cells under conditions of extreme adverse environments.

The disparity is possibly due to either the difference of the culture condition (the normal medium was used in that study whereas the nutrient-deprived medium was used in our study), or the difference of the concentration of arctigenin used (the concentration of arctigenin used in the previous study was hundreds of times higher than that used in our study). As nutrition deprivation seldom occurs in normal tissues, it may be considered that the antiausterity capability of arctigenin is specific to cancer cells. Interestingly, in our present study, arctigenin markedly inhibited the Panc-1 tumor formation in nude mice. Conventional screening strategies for agents exhibiting cytotoxicity use normal medium containing all the essential elements, such as glucose, amino acids, and serum, required for normal cell growth, and in this study, only natural product extracts having cytotoxicity were considered for further isolation, identification, and drug development. However, many compounds that exhibit cytotoxicity in vitro fail to show any significant cytotoxic activity in vivo in animal models. This is partly because the in vitro culture conditions of the cells fail to precisely mimic the actual in vivo environment of tumors. In this regard, nutrient-deprived conditions might closely resemble the natural austere environment of tumors. In the present study, whereas arctigenin did not exhibit any cytotoxicity against the Panc-1 cancer cell line under normal conditions (IC50 > 100 μg/mL), it exhibited preferential cytotoxicity under nutrient-deprived conditions; such anticancer activity in vivo indicates that our antiausterity strategy could be a feasible and novel approach for cancer therapy. Because the aim of anticancer treatment is to kill only the cancer cells, the therapeutic efficacy of anticancer agents under nutrient-deprived conditions may serve as a highly feasible approach for cancer prevention and treatment. It is noteworthy that the conventional chemotherapeutic drugs, such as 5-fluorouracil, Taxol, doxorubicin, cisplatin, and camptothecin, show only weak

**Figure 3.** Nutrient deprivation and toxicity of arctigenin. Arctigenin (0.01 μg/mL) was added to the medium and the cell survival rate was examined 24 hours after the start of nutrient deprivation. Cell survival was examined by the WST-8 cell counting kit method. Columns, mean from triplicate experiments.

50 μg/mL. We thus carried out further bioassay-guided fractionation and isolation, which led to the isolation of arctigenin as the primary compound exhibiting preferential cytotoxicity against nutrient-deprived cells at a concentration of 0.01 μg/mL. Furthermore, the activity of arctigenin was found to be both concentration-dependent and time-dependent, and arctigenin was found to induce the necrosis of cancer cells under the nutrient-deprived condition in our study. We noticed that arctigenin was reported to induce apoptosis in colorectal adenoma and carcinoma cells (22). The disparity is possibly due to either the difference of the culture condition (the normal medium was used in that study whereas the nutrient-deprived medium was used in our study), or the difference of the concentration of arctigenin used (the concentration of arctigenin used in the previous study was hundreds of times higher than that used in our study). As nutrition deprivation seldom occurs in normal tissues, it may be considered that the antiausterity capability of arctigenin is specific to cancer cells. Interestingly, in our present study, arctigenin markedly inhibited the Panc-1 tumor formation in nude mice. Conventional screening strategies for agents exhibiting cytotoxicity use normal medium containing all the essential elements, such as glucose, amino acids, and serum, required for normal cell growth, and in this study, only natural product extracts having cytotoxicity were considered for further isolation, identification, and drug development. However, many compounds that exhibit cytotoxicity in vitro fail to show any significant cytotoxic activity in vivo in animal models. This is partly because the in vitro culture conditions of the cells fail to precisely mimic the actual in vivo environment of tumors. In this regard, nutrient-deprived conditions might closely resemble the natural austere environment of tumors. In the present study, whereas arctigenin did not exhibit any cytotoxicity against the Panc-1 cancer cell line under normal conditions (IC50 > 100 μg/mL), it exhibited preferential cytotoxicity under nutrient-deprived conditions; such anticancer activity in vivo indicates that our antiausterity strategy could be a feasible and novel approach for cancer therapy. Because the aim of anticancer treatment is to kill only the cancer cells, the therapeutic efficacy of anticancer agents under nutrient-deprived conditions may serve as a highly feasible approach for cancer prevention and treatment. It is noteworthy that the conventional chemotherapeutic drugs, such as 5-fluorouracil, Taxol, doxorubicin, cisplatin, and camptothecin, show only weak
cytotoxicity against cancer cells cultured in nutrient-deprived media (6), which indicates that a different mechanism may underlie the antitumor activity of arctigenin against tumor cells growing in a low-nutrient, low-oxygen environment.

Arctigenin and arctiin have been reported to inhibit heterocyclic amine-induced colon, pancreatic, and hepatic carcinogenesis in rats, whereas 7,12-dimethylbenz(a)anthracene and 12-O-tetradecanoyl phorbol-13-acetate induced mouse skin carcinogenesis (23, 24). Arctigenin has also been reported to modulate immunologic responses (25) and to induce apoptosis in colon cancer cell lines (22). Therefore, the mechanism of antitumor activity of this agent may not be simple and the results of the present study suggest the involvement of the Akt pathway. Carcinogenesis is a complex process that includes both progressive acquisition of genetic

Figure 5. Effect of arctigenin on the survival of various cancer cell lines in normal DMEM (●) and nutrient-deprived medium, NDM (○). A, ASPC-1; B, BXPC-3; C, Alexander; D, PSN-1.

Figure 6. Effect of arctigenin on the growth of PANC-1 cells in nude mice. A, body weight of mice. ○, control group; ●, treated group. Y axis, body weight (g); X axis, time (day). B, the tumor volume in the mice. ○, control group; ●, treated group. Y axis, tumor volume (mm³); X axis, time (day). C, wet weight of the tumor in the mice on the last day of the experiment. Filled column, control group; clear column, treated group. (n = 6). Y axis, wet weight of the tumor (g). D, photographs of the tumor after sacrifice on the last day.
alterations by cancer cells and reorganization of cancer tissues. During these complex processes, adaptation of the cancer cells to their microenvironiments is pivotal to tumor growth and progression. Therefore, it would be reasonable to consider agents that inhibit this adaptation of the cells to their microenvironment as chemopreventive agents. In this sense, inhibition of carcinogenesis by arctigenin might be related to the activity presently identified, namely, antiausterity.

Many lines of evidence show that Akt plays a critical role in the development, growth, and acquisition of therapeutic resistance of cancer cells. Inhibition of the Akt pathway might have therapeutic value in patients with cancer and this has formed the basis for widespread efforts to develop agents that inhibit Akt (26–28). Although the molecular and biochemical mechanism of austerity of cancer cells and the pharmacologic mechanisms of action of arctigenin are yet to be understood, inhibition of Akt phosphorylation by arctigenin under nutrient-deprived conditions found in this study might have relevance to its preferential cytotoxicity.

Glucose deprivation occurs in many solid tumors as a consequence of the local decrease in blood supply and is an intrinsic variable of ischemia (12, 16, 19, 29). The severity of glucose deprivation may even be aggravated by a higher nutrient consumption in neoplastic tissues (30, 31). Under various stress conditions, including nutrient deprivation, PKB/Akt is involved in cell survival reactions. In our earlier study, we found that a high level of expression of PKB/Akt was associated with the tolerance of pancreatic cancer cells against nutrient deprivation (5). One mechanism by which Akt may promote cell survival is through the inhibition of a component of the cell death machinery. We have also found that PKB/Akt was strongly phosphorylated during glucose starvation, under both normoxic and hypoxic conditions. Therefore, inhibition of Akt phosphorylation by arctigenin may, at least in part, be responsible for the preferential cytotoxicity of arctigenin against nutrient-deprived cells. Actually, in our previous study, transfection of antisense RNA expression vector for Akt significantly diminished the tolerance of pancreatic cancer cell to nutrient deprivation, and LY294002, a representative phosphoinositide 3 kinase inhibitor, remarkably decreased cell survival of PANC-1 cell under nutrient-deprived condition (5). All evidences suggest that the inhibition of Akt activation by arctigenin have some relations with its preferential cytotoxicity to cancer cells, although the Akt pathway only might not be sufficient to explain the intricate mechanism for preferential cytotoxicity.

In conclusion, our findings provide a novel mechanistic insight into the actions of arctigenin in cancer therapy, based on antiausterity strategy, indicating again the feasibility of antiausterity strategy for cancer therapy.

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Figure 7. Effect of different lignans on the survival of PANC-1 cells in nutrient-deprived medium. ◆, arctigenin; ☐, nordihydroguaiaretic acid; A, sesamin; ◇, secoisolaricrinol; ○, arctin. Cells were seeded at a density of $1 \times 10^4$ per well in 96-well plates and incubated in completely fresh medium for 24 hours. The cells were then washed with PBS and the medium was changed to nutrient-deprived medium containing graded concentrations of arctigenin. The cell number at the start of the starvation was considered to be 100%.

References


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