Effect of Juzentaihoto/Shi-Quan-Da-Bu-Tang on Malignant Progression and Metastasis of Tumor Cells

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Abstract

A Japanese herbal (Kampo, 汉方) medicine, 十全大补汤 (juzentaihoto/Shi-Quan-Da-Bu-Tang), is one of the nourishing agents, a so-called “补剂 (Hozai/Bu-Ji),” that is used for improving disturbance and imbalances in the homeostatic condition of the body. This formulation is orally administered to patients in various weakened states, including postsurgery patients and patients with chronic illness, where it can alleviate general symptoms such as extreme fatigue, pale complexion, loss of appetite, dry or scaly skin, night sweating, and dryness of the mouth. Recently, juzentaihoto/Shi-Quan-Da-Bu-Tang has shown to have a variety of biological activities, including activation of natural killer cells and macrophages, cytokine induction, antibody production, antitumor effects in combination with surgical excision or other drugs, and protection against the adverse effects of anticancer drugs and radiation, including immunosuppression and bone marrow toxicity. This article focuses on the antitumor and antimetastatic properties of some Kampo medicines and mainly describes the effects of juzentaihoto and its related formulations on tumor development, progression, and metastasis in vivo. We also discuss the inhibitory mechanism of action and the importance of the prescription and constituent crude drugs in determining the efficacy.

Keywords: Epithelial-to-mesenchymal transition, high-performance liquid chromatography pattern analysis, Japanese herbal (Kampo) medicine, juzentaihoto/Shi-Quan-Da-Bu-Tang, macrophages, malignant progression, natural killer cells, shimotsuto/Si-Wu-Tang, tumor metastasis, tumor vaccine

INTRODUCTION

Japanese herbal (汉方, Kampo) medicines have conventionally been used as formulations to be administered orally in the form of a decoction for treatment. Several herbal extract preparations have been developed and improved as modern pharmaceuticals in granule or powder forms by the lyophilization of decoctions to facilitate their use. Since 1976, the Ministry of Health and Welfare of Japan has approved 148 Kampo formulations (汉方方剂) as extract preparations covered by the National Health Insurance System in Japan. Herb/crude drugs in such Kampo prescriptions (汉方处方) are also covered by the insurance system. Kampo medicines have been used during their treatments at one time or another by more than 80% of practicing physicians in Japan.

The Kampo medicine, 十全大补汤 (juzentaihoto/Shi-Quan-Da-Bu-Tang), was first described in Daipinghuimin-hejijufang (A.D.1151) of the Song dynasty (A.D.960–1279) in China and introduced to Japan in the Kamakura dynasty (A.D.1192–1333). Since then, it is one of the nourishing agents, so-called “补剂 (Hozai/Bu-Ji),” for improving deficiency syndrome, “虚证 (Kyosho/Xu-Zheng),” for improving disturbances and imbalances in the homeostatic condition of the body diagnosed by Kampo medicine. It has been used as a cure for consumption, general debility, deficiency, and impairments of “阴 (In/Yin),” “阳 (Yo/Yang),” “血 (Ketsu/Xue)” (a concept referring to blood, hormones, autonomic nervous system, and other regulatory functions of the body’s internal environment), or vital energy, “气 (Ki/Qi)” in the viscera or bowels, and loss of appetite. It is currently used for patients weakened by prolonged illness, fatigue, loss of appetite, night sweats, circulatory problems, and anemia.

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Clinically, juzentaihoto/Shi-Quan-Da-Bu-Tang has been used for cancer patients to improve the general systemic condition of cancer patients and reduce the adverse effects of chemotherapy, radiation therapy, and surgical treatment, which may lead to the induction of “Kyoshō/Xu-Zheng” (deficiency of “Ki/Qi” and “Ketsu/Xue”). It has also been administered to patients with rheumatoid arthritis, atopic dermatitis, chronic fatigue syndrome, and ulcerative colitis. To evaluate the clinical efficacy of juzentaihoto/Shi-Quan-Da-Bu-Tang, pharmacological studies have been carried out to find out effects such as the enhancement of phagocytosis,[45] cytokine induction,[25,43,70] antibody production,[21] mitogenic activity of spleen cells,[80] an antitumor effect when combined with surgical excision[46] or other drugs,[25,80] and protection against immunosuppression and bone marrow toxicity caused by anticancer drugs.[81] and irradiation.[38,57]

This article shows the effect of juzentaihoto/Shi-Quan-Da-Bu-Tang on the malignant progression and metastasis of tumor cells and the inhibitory mechanism of action.

**Juzentaihoto/Shi-Quan-Da-Bu-Tang and its Constituents**

Juzentaihoto/Shi-Quan-Da-Bu-Tang is composed of 10 herbs/crude drugs, of which the quality is controlled by Japanese Pharmacopoeia XVII. 黄芪 (Astragali Radix) (3.0 g), 桂皮 (Cinnamomi Cortex) (3.0 g), 黄芩 (Rehmanniae Radix) (3.0 g), 芍药 (Paeoniae Radix) (3.0 g), 川芎 (Cnidii Rhizoma) (3.0 g), 苍术 (Atractyloides lanceae Rhizoma) (3.0 g), 当归 (Angelicae Radix) (3.0 g), 人参 (Ginseng Radix) (3.0 g), 甘草 (Glycyrrhizae Radix) (3.0 g), and 茯苓 (Poria) (3.0 g), and 黄精 (Glycyrrhiza Radix) (1.5 g) are extracted in 285 mL of water at 100°C for 1 h. The extracted solution is filtrated and spray-dried to obtain the dry extract powder (2.3 g). In Japan, juzentaihoto/Shi-Quan-Da-Bu-Tang is now available as either a decoction or extract preparation.

**Quality Control of Juzentaihoto/Shi-Quan-Da-Bu-Tang and its Constituents**

Kampo medicines have been increasingly recognized in the modern medical system. Since these formulations are generally prepared from the combination of many crude drugs, they may have effects that differ from the sum of the effects of the individual constituent crude drugs and apparently must have an acceptable efficacy and quality when used as therapeutic medicines. Formulations prepared from crude drugs with different qualities would have different biological activities and efficacies. Therefore, it is necessary to control the quality of the formulations and their constituent crude drugs and processing procedures, to ensure reproducibility of the formulation and the efficacy, because their quality varies with the origins of crude drugs and the time and place of harvest. Japanese Pharmacopoeia XVII controls the quality of crude drugs based on (1) regulation of the botanical origin, (2) crude drug test of foreign matter, (3) loss by drying, (4) total ash, (5) acid-insoluble ash, (6) extract content, (7) essential oil content, and (8) microscopic examination. However, to our knowledge, these methods for quality control have not been studied in detail in the case of juzentaihoto/Shi-Quan-Da-Bu-Tang although much information about Kampo formulations and their constituents is available in Japanese and Chinese archaic writings.

**High-performance liquid chromatography profiles of juzentaihoto/Shi-Quan-Da-Bu-Tang and its constituents**

To obtain accurate formulations with consistent quality and efficacy, high-performance liquid chromatography (HPLC) pattern analysis of juzentaihoto/Shi-Quan-Da-Bu-Tang was carried out using its chemically defined components as standard references.[67] Figure 1 shows the HPLC profiles of juzentaihoto/Shi-Quan-Da-Bu-Tang by single monitor (220 nm) and contour plot (190–400 nm) using a photo-diode array system as a detector. The contour plot of the ultraviolet (UV) absorbance intensity of the compounds indicates all of the compounds that have detectable UV absorbance in the extracts from the formulation. The origin of each peak of juzentaihoto/Shi-Quan-Da-Bu-Tang was identified by comparison with the retention time and UV spectrum of each extract of crude drug or the chemically defined standard compounds [A–L in Figure 1]. For example, the peaks of paeoniflorin from Paeoniae Radix and glycyrrhizin from Glycyrrhiza Radix were detected at the D and L positions of the contour plot in Figure 1. Thus, in addition to determine whether the standard compounds possess pharmacological efficacy to inhibit tumor metastasis, this HPLC pattern analysis, a so-called fingerprint method, could provide a useful means of identifying the crude drugs and preparing batches with a consistent formulation. Although many compounds with no UV absorbance may not be detected by this method, a reproducible fingerprint pattern of the formulation would be primarily useful for assessment of the homogeneity of the formulation and consequently lead to consistent efficacy.

As described in Section “Attempt to obtain juzentaihoto/Shi-Quan-Da-Bu-Tang preparations with constant efficacy,” to evaluate the efficacy of juzentaihoto/Shi-Quan-Da-Bu-Tang prepared in this way, we examined the antimetastatic effect by the oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang formulations with different HPLC fingerprint patterns.

**Effect of Juzentaihoto/Shi-Quan-Da-Bu-Tang on the Processes of Malignant Tumor Progression and Metastasis**

Carcinogenesis generally involves many steps, each of which governed by multiple factors. Most cancers are probably initiated by a change in the cell’s DNA sequences, but a single mutation is not enough to cause cancer. Tumor progression is the process whereby an initial population of slightly abnormal cells progressively becomes more deleterious through successive cycles of mutation and natural selection. Metastasis is one of the
major causes of cancer-related mortality and a complex cascade of events involving tumor dissemination from the primary site of growth to distant organs as shown in Figure 2. The pathogenesis of metastases following tumor development and progression can be subdivided into a variety of sequential steps: (1) release from the primary tumor and invasion of the surrounding tissues, (2) entry into the vascular or lymphatic circulation, (3) transit in the circulation, (4) arrest in the capillary bed of a distant organ, (5) extravasation from the circulation, (6) growth at possibly selected sites that are distant from the original tumor site. Only a few cells in a primary tumor can complete all these steps necessary to achieve metastasis. Specific interactions of tumor cells with host cells or components, such as lymphocytes and extracellular matrix components, are therefore fundamental events in preferential organ colonization, whereby metastases occur in specific organs and not randomly.

The cancer-associated mortality rate has been increasing steadily, despite the advances in diagnostic techniques for the early detection of various cancers and the significant improvement in surgical procedures, and metastasis is a frequent cause of cancer-related death. For instance, the liver is the most common target of hematogenous metastasis in gastrointestinal tract cancer, especially colon cancer, and the prognosis of patients with liver metastasis is extremely poor. If occult micrometastases that have already been established at the time of surgery could be inhibited, then the prognosis of patients with colon carcinoma would improve.

**Prevention of malignant tumor progression**

Malignant tumor progression is the process by which tumor cells acquire a more malignant phenotype, such as enhancement of the ability to proliferate, invade, or metastasize, and is affected by various factors. However, there have been few studies on the mechanisms, facilitating factors, and inhibitors of progression, because of the lack of a suitable experimental animal model. As shown in Figure 3, the weakly malignant QR-32 fibrosarcoma cells alone gradually grew over 15 days after subcutaneous (s.c.) inoculation and thereafter regressed spontaneously for up to 25 days. In contrast, co-implantation of QR-32 cells with a foreign body, gelatin sponge, permitted the progressive growth of the tumor at the inoculated site,
and these resultant tumor cells (QRsP) irreversibly acquired the ability to grow progressively at the inoculated sites even without gelatin sponge. Such progressive growth of tumors has also been shown to be induced by the enhancement of prostaglandin E2 (PGE2) production in the tumors or by oxygen radicals and inflammatory cytokines produced by host cells reactive with the gelatin sponge. This may be analogous to the fact that the malignant progression of tumors followed by metastasis is clinically observed to be elicited by various factors and circumstances, including stresses, anticancer drugs, and inflammation. Oral administration of bismuth subnitrate and liposomal vitamin C prevented the progressive growth after s.c. co-implantation of QR-32 cells with a gelatin sponge through the endogenous induction of antioxidative enzymes or scavengers such as manganese superoxide-dismutase or metallothionein at the tumor sites.

The oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang (Pharmaceutical preparation, TJ48 by Tsumura and Co.)
for 7 days after tumor inoculation with gelatin sponge caused the significant inhibition of the progressive growth of QR-32 regressor tumors [Figure 4] and prolonged the survival of tumor-bearing mice. This result indicates that juzentaihoto/Shi-Quan-Da-Bu-Tang may effectively prevent weakly malignant tumors from growing progressively upon co-implantation with gelatin sponge.\[54] Such progressive growth, however, may not necessarily be equivalent to malignant progression because even cells that do not acquire a more malignant phenotype can show transient proliferation depending on the host circumstances and implantation conditions. As shown in Figure 4, the resultant progressive tumor (QRsP) indicated gelatin sponge-independent growth after re-inoculation into fresh mice (6 out of 6 mice) as also demonstrated previously.\[54] This phenomenon might be regarded as malignant progression. Actually, juzentaihoto/Shi-Quan-Da-Bu-Tang-treated group did not show the progressive growth after re-inoculation into syngeneic mice without gelatin sponge [0 out of 6 mice, Figure 4].

On the other hand, the oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang for 7 days after inoculation of QRsP progressive cells led to a significant inhibition of tumor growth and enhancement of the survival rate in tumor-bearing mice as compared with the control (data not shown).\[54] Since juzentaihoto/Shi-Quan-Da-Bu-Tang has been indicated to have antitumor effects based on macrophage activation,\[45] cytokine induction,\[25,43] augmentation of natural killer (NK) cell activity,\[82] etc., the inhibitory mechanisms of the progressive growth of QR-32 regressor cells and the growth of the resultant QRsP progressor cells may be partly associated with the induction of host-mediated immune surveillance by juzentaihoto/Shi-Quan-Da-Bu-Tang. However, the oral administration of bismuth subnitrate resulted in a significant inhibition of gelatin sponge-elicited progressive growth through the induction of metallothionein as a scavenger of oxygen radicals in the tumor tissue.\[54,73] These results indicate that juzentaihoto/Shi-Quan-Da-Bu-Tang may act to induce antioxidants and reduce PGE\(_2\), production\[49] during tumor progression, in addition to enhancing the host-mediated immune responses.

**Inhibition of tumor growth and metastasis and the inhibitory mechanism**

The antitumor activity of Kampo medicines including 补剂 (Hozai/Bu-Ji) has been investigated using various experimental tumor models. Oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang (150 or 300 mg/kg) twice a day for 10 days after the intraperitoneal (i.p.) inoculation of Ehrlich ascites tumor in ICR mice significantly suppressed the growth and also prolonged the survival time of tumor-bearing mice.\[32] In addition, 猪苓汤 (choreito/Zhu-Ling-Tang) and 猪苓汤 (shosaikoto/Xiao-Chai-Hu-Tang) also showed antitumor activities against the Ehrlich ascites tumor. However, juzentaihoto/Shi-Quan-Da-Bu-Tang did not inhibit or slightly inhibited the growth of P388 leukemia, Lewis lung carcinoma (LLC), and the sarcoma 180 ascites tumor.\[1,31,41] The oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang and shosaikoto/Xiao-Chai-Hu-Tang significantly increased the relative organ weights of the spleen, thymus, and liver and also enhanced the reticuloendothelial cell function including the phagocytic index, as compared with the control.\[35] Intravenous (i.v.) injection of peritoneal macrophages stimulated by juzentaihoto/Shi-Quan-Da-Bu-Tang inhibited lung metastasis of LLC.\[35] Thus, the mechanism of antitumor activity of juzentaihoto/Shi-Quan-Da-Bu-Tang may be partly due to the stimulation of the reticuloendothelial system, C3 activation, and depression of the liver microsomal drug-metabolizing enzymatic system.

It has been reported that juzentaihoto/Shi-Quan-Da-Bu-Tang significantly suppressed the growth of human U-87MG glioma cells transplanted s.c. into BALB/c nude mice and that prolonged the survival of the mice.\[82] The administration of juzentaihoto/Shi-Quan-Da-Bu-Tang augmented endogenous tumor necrosis factor (TNF) production without a secondary stimulus. Similar results were also obtained using murine 203-glioma cells in syngeneic mice. The antitumor mechanism of juzentaihoto/Shi-Quan-Da-Bu-Tang may partly involve the ability to induce endogenous TNF production.

We have examined the effect of juzentaihoto/Shi-Quan-Da-Bu-Tang on liver metastasis resulting from the intraportal vein injection of colon 26-L5 carcinoma cells *in vivo*\[85] and the role of the immune system after administration.
Figure 5 showed that the oral administration of juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang before tumor inoculation dose dependently inhibited liver metastasis of colon 26-L5 carcinoma cells and consequently led to a significant enhancement of the survival rate compared with the untreated control.[53] An anticancer agent cis‑diammine dichloroplatinum II (CDDP) significantly inhibited liver metastasis at the dose of 80 µg/mouse, but it had severe adverse effects such as decreasing the body weight and leading to a 50% death rate of the mice. Juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang did not have any other side effects, nor did it directly affect the tumor cell growth in vitro. Thus, juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang may be effective as a biological response modifier for inhibiting micrometastasis and differing from chemotherapeutic agents. Similarly, the oral administration of juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang before the i.v. inoculation of B16‑BL6 melanoma cells resulted in the marked inhibition of lung metastasis without causing any reduction in the body weight (data not shown).[56]

Since metastasizing tumor cells interact with host cells such as lymphocytes, NK cells, and monocytes, which are important in the destruction of tumor cells,[14,22] we investigated whether juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang could stimulate such immune cells to inhibit tumor metastasis. Administration of anti‑asialo GM1 serum can selectively eliminate NK cells[20,66] and in contrast 2-chloroadenosine can eliminate macrophages.[66,69] Therefore, pretreatment of mice with anti‑asialo GM1 serum or 2-chloroadenosine, and T-cell‑deficient syngeneic nude mice resulted in the enhancement of liver metastasis produced by colon carcinoma cells as compared with untreated control, thus indicating that NK cells, macrophages, and T-cells play important roles in the prevention of the metastatic spread of tumor cells. Juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang significantly decreased the enhancement of liver metastasis of colon 26-L5 cells in mice pretreated with anti‑asialo GM1 serum [Figure 6] as well as untreated normal mice, whereas it did not affect the enhanced liver metastasis in 2-chloroadenosine-pretreated (macrophage-depleted) mice [Figure 7] or T-cell-deficient nude mice (data not shown).

Since juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang was inactive for inhibiting liver metastasis when the contributions of macrophages and T-cells were eliminated from our system, its inhibitory mechanism is likely to be related to the activation of these immune cells. We also found that peritoneal exudate immune macrophages from the mice orally administered by juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang became cytostatic against tumor cells in vitro. Although the exact mechanism responsible for the inhibition of liver metastasis by juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang is not fully understood, this inhibitory effect would be partly associated with the activation of macrophages.[53] Further investigation is needed to determine the detailed mechanisms responsible for the inhibition of tumor metastasis by juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang. However, the results of an in vitro study using this formulation, which is called “Furikake” (in Japanese) study, may be difficult to evaluate the efficacy because the expression of in vivo efficacy by the formulation is sometimes mediated or influenced by active (e.g., intestinal bacterial) metabolites after oral administration.

In conclusion, juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang inhibited liver metastasis of colon 26-L5 carcinoma cells and increased the survival rate by the oral administration, possibly through the activation of macrophages and T-cells.[53] Thus, juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang may be therapeutically effective for the prevention of cancer metastasis.

**Combination with other treatment modalities (chemotherapy, hyperthermia, radiation, etc.)**

In addition to produce new types of anticancer agents, it is also important to develop methods or agents which can enhance the therapeutic efficacy and/or reduce the side effects of anti-cancer agents. Combination treatment with juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang and other treatment modalities including anticancer agents has also been investigated using various tumor models to augment the efficacy by juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang.[30,48,50,72,86,90]

It has been reported that the daily administration of juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang alone after the s.c. inoculation of various tumor cells such as Meth A fibrosarcoma, sarcoma-180, or B16 melanoma cells resulted in almost no inhibition of tumor growth. In contrast, combination treatment with juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang (per orally) and mitomycin C (i.p.) was more effective for inhibiting the growth of Meth A fibrosarcoma or B16 melanoma cells than either treatment alone.[41] On the other hand, the combination of juzentaihoto/Shi‑Quan‑Da‑Bu‑Tang and mitomycin C showed significant prolongation of the survival rate in mice inoculated with P388 leukemia cells and also marked reduction of the side effects caused by mitomycin C.[1]
Oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang for 50 days after s.c. inoculation of ICR mice with sarcoma 180 markedly enhanced the antitumor effect of the combination of mitomycin C and hyperthermia (43°C, 30 min). Similar results using this treatment modality were obtained after the inoculation of BDF1 mice with B16 melanoma cells. In addition to the augmentation of the combined effect of mitomycin C and hyperthermia by juzentaihoto/Shi-Quan-Da-Bu-Tang, the

Figure 6: Effect of anti-asialo GM1 serum on juzentaihoto/Shi-Quan-Da-Bu-Tang-mediated inhibition of experimental liver metastasis produced by the intraportal vein injection of colon 26-L5 cells. Five BALB/c mice per group were orally administered with or without juzentaihoto/Shi-Quan-Da-Bu-Tang (40 mg/day) for 7 days before tumor inoculation. Colon 26-L5 cells (10⁴) were intraportally injected into groups of control mice or mice pretreated 24 h earlier with anti-asialo GM1 serum (20 µL/mouse). Mice were sacrificed 14 days after tumor inoculation and the number of tumor colonies in the liver and liver weight were manually calculated. *P < 0.05; **P < 0.001, as compared with an untreated control by Student’s two-tailed t-test.

Figure 7: Effect of 2-chloroadenosine on juzentaihoto/Shi-Quan-Da-Bu-Tang-mediated inhibition of experimental liver metastasis produced by the intraportal vein injection of colon 26-L5 cells. Five BALB/c mice per group were orally administered with or without juzentaihoto/Shi-Quan-Da-Bu-Tang (40 mg/day) for 7 days before tumor inoculation. Colon 26-L5 cells (10⁴) were intraportally injected into groups of control mice or mice pretreated 24 h earlier with 2-chloroadenosine (50 µg/mouse). Mice were sacrificed 14 days after tumor inoculation and the number of tumor colonies in the liver and liver weight were manually counted. **P < 0.001, NS: Not significant as compared with an untreated control by Student’s two-tailed t-test.

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imunotoxicity induced by mitomycin C and the subsequent marked growth of the tumors were reduced by the oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang.[42]

The combined treatment of juzentaihoto/Shi-Quan-Da-Bu-Tang and CDDP inhibited tumor growth of MBT-2 bladder tumor in C3H/He mice and prolonged the survival rate of mice more effectively than CDDP alone.[9] In addition, the oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang for 2 weeks significantly ameliorated the adverse effects caused by a high dose of CDDP, including lethal toxicity, renal and hepatic toxicity, and myelosuppression.[9]

Juzentaihoto/Shi-Quan-Da-Bu-Tang alone indicated no inhibition of the growth of Meth A fibrosarcoma inoculated intradermally. However, juzentaihoto/Shi-Quan-Da-Bu-Tang displayed a marked antitumor effect when combined with surgical excision.[46] Since the antitumor immunity by spleen cells from mice treated with juzentaihoto/Shi-Quan-Da-Bu-Tang was abolished by treatment of the spleen cells with anti-L3T4 monoclonal antibody + complement, but not with anti-Lyt-2 monoclonal antibody + complement, such antitumor effect is considered to be mediated by L3T4-positive helper T cells.

Combination with shosaikoto/Xiao-Chai-Hu-Tang, juzentaihoto/Shi-Quan-Da-Bu-Tang, or 桂皮 (Cinnamomi cortex) and Streptococcus pyogenes products (OK432) strongly inhibited the growth of Ehrlich ascites or Meth A fibrosarcoma cells through the increased endogenous production of TNF.[23,25] Marked lymphocytosis, hyperplasia, and hypertrophy of Kupffer cells in the liver were shown in the tumor-bearing mice receiving Kampo medicines or OK432.[23,25] These results suggest that the antitumor activities and capacity to induce TNF production of the preparations are probably due in part to stimulation of the reticuloendothelial system, including macrophage activation and TNF induction as immunopotentiators. Furthermore, antitumor activity of juzentaihoto/Shi-Quan-Da-Bu-Tang and OK432 in combination was observed in one patient with hepatocellular carcinoma.[24]

Protection against the deleterious effects of anticancer drugs and radiation-induced immunosuppression

Oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang for 2 weeks in tumor-bearing mice has been shown to significantly protect CDDP-induced adverse effects such as lethal toxicity, renal and hepatic toxicity, and myelosuppression.[9] Juzentaihoto/Shi-Quan-Da-Bu-Tang enhanced the decreased immune responses to the normal level in MBT-2 tumor-bearing mice.[10] It also protected aged mice (13–15 months old) from the decrease in immune function induced by CDDP and restored the lowered cytotoxic activity in CDDP-treated tumor-bearing mice. Sugiyama et al.[80] has reported that the oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang for 12 days prevented the increases in blood urea nitrogen, serum creatinine, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminases, and relative stomach weight and the decreases in the white blood cell count, platelet count, relative spleen and thymus weights, food intake, and body weight caused by CDDP, to nearly the control levels without reducing the antitumor activity of CDDP against sarcoma 180 cells. Juzentaihoto/Shi-Quan-Da-Bu-Tang were also observed to prevent against carboplatin-induced myelosuppression and hepatic toxicity.[181] Administration of juzentaihoto/Shi-Quan-Da-Bu-Tang for 7 days has been reported to delay deaths due to lethal doses of MMC or CDDP and markedly improve survival rate.[20] In addition, juzentaihoto/Shi-Quan-Da-Bu-Tang reduced the atrophy of the testis, thymus, and spleen caused by MMC and also had protective effects against the leukopenia, anemia, and body weight loss caused by MMC, and against the increases of BUN and creatinine caused by CDDP. These results indicate that combination with juzentaihoto/Shi-Quan-Da-Bu-Tang may be a new way to minimize the toxicity of anticancer agents, MMC or CDDP.

The administration of juzentaihoto/Shi-Quan-Da-Bu-Tang before MMC injection was not able to protect the mice from the damage to the hematopoietic function caused by MMC, but markedly accelerated the recovery of colony-forming units in the spleen (CFU-S) and granulocyte-macrophage CFU cells (CFU-GM).[183] Juzentaihoto/Shi-Quan-Da-Bu-Tang was also effective when its administration was begun after MMC injection. These results suggest that juzentaihoto/Shi-Quan-Da-Bu-Tang has the ability to accelerate hematopoietic recovery from bone marrow injury by MMC.

The continuous oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang 2–3 weeks before a dose of X-irradiation causing bone marrow death, especially enhanced the recovery of thrombocytes as well as blood-forming stem cells (CFUs) in the bone marrow, and consequently led to the increase of the 30-day survival ratios of X-irradiated mice.[28] In addition, the administration of juzentaihoto/Shi-Quan-Da-Bu-Tang 7 days after X-irradiation (20 Gy) to the intramuscularly inoculated tumors significantly prolonged the survival of X-irradiated mice, thus indicating radioprotective effects by increasing the number and size of day-14 CFU-S.[57]

Juzentaihoto/Shi-Quan-Da-Bu-Tang prevented the decrease in body weight of the mice bearing colon 26 carcinoma cells and consequently prolonged the survival rate of the mice. This suggests that juzentaihoto/Shi-Quan-Da-Bu-Tang has the ability to ameliorate the cachexia induced by transplantable colon 26 carcinoma.[68]

Attempt to obtain Juzentaihoto/Shi-Quan-Da-Bu-Tang Preparations with Constant Efficacy

To analyze the relationship between the efficacy of thus prepared juzentaihoto/Shi-Quan-Da-Bu-Tang and HPLC pattern [Figure 1], we investigated the antimetastatic effect of two juzentaihoto/Shi-Quan-Da-Bu-Tang formulations
(batches #1 and #2) which were independently prepared using the same 10 crude drugs by the same procedure. Fingerprint analysis of the two batches of juzentaihoto/Shi-Quan-Da-Bu-Tang showed HPLC profiles similar to that shown in Figure 1. The oral administration of the two juzentaihoto/Shi-Quan-Da-Bu-Tang preparations (batches #1 and #2) at the effective dose of 40 mg/day[55, 56] significantly inhibited liver metastasis of colon 26-L5 carcinoma cells, and similar results were observed with both batches of the juzentaihoto/Shi-Quan-Da-Bu-Tang formulation (data not shown).[67]

Regarding the usage and preparation of Kampo formulations, certain constituent crude drugs in the formulation are, in some cases, replaced with related crude drugs. To investigate the antimetastatic effect by the replacement of the constituents in original formulation with different crude drugs, we prepared the variant formulations of juzentaihoto/Shi-Quan-Da-Bu-Tang whereby one crude drug was substituted with a related crude drug from different sources or places of origin. As shown in Figure 8, the HPLC pattern of the extract of the root of A. mongholicus (Astragalus membranaceus) was very similar to that from A. mongholicus (Astragalus mongholicus). Variant formulation (A. mongholicus) showed HPLC profiles similar to that shown in Figure 1. In contrast, the variant formulation (A. lancéa) showed HPLC profiles similar to those of the substituted crude drug. This suggests that the reduced efficacies of the variant formulations may be associated with the remarkable differences in the fingerprint patterns between the original and substituted crude drugs. Thus, HPLC pattern analysis of Kampo medicines may provide a useful method for obtaining their optimal efficacy associated with consistent quality of the formulation.[67]

In conclusion, we demonstrated differential effects of variant formulations of juzentaihoto/Shi-Quan-Da-Bu-Tang on tumor metastasis. The decreased antimetastatic effect of the variant formulations used in this study may be related to the differences in the fingerprint patterns between the original and substituted crude drugs. Thus, HPLC pattern analysis of Kampo medicines may provide a useful method for obtaining their optimal efficacy associated with consistent quality of the formulation.[67]
Figure 8: HPLC profile and UV spectra of substitutable crude drugs of juzentaihoto/Shi-Quan-Da-Bu-Tang. I: HPLC pattern analyzed by absorbance at 220 nm, II: contour plot of HPLC pattern by UV absorbance (190–400 nm). Some significant peaks are indicated by arrowheads on the chromatograms, compared with the original standards. HPLC: High-performance liquid chromatography, UV: Ultraviolet.

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六君子湯 (rikkunshito/Liu-Jun-Zi-Tang) and ninjinyoeito/Ren-Shen-Yang-Rong-Tang, which contain shikunshito/Si-Jun-Zi-Tang constituents, did not affect the inhibition of liver metastasis, as is true of shikunshito/Si-Jun-Zi-Tang. However, because juzentaihoto/Shi-Quan-Da-Bu-Tang was more effective at inhibiting tumor metastasis than shimotsuto/
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Si-Wu-Tang, it is likely that some constituents other than shimotsuto/Si-Wu-Tang constituents are associated with the enhancement of antimetastatic effect. Thus, juzentaihoto/Shi-Quan-Da-Bu-Tang has been shown to be an efficacious formulation with beneficial effects on both “Ki/Qi” and “Ketsu/Xue”-deficiency.

補中益気湯 (hochuekkito/Bu-Zhong-Yi-Qi-Tang) as well as juzentaihoto/Shi-Quan-Da-Bu-Tang and ninjinyoeto/Ren-Shen-Yang-Rong-Tang are known to be three representative nourishing agents “Hozai/Bu-Ji,” with an ability to modulate host-mediated immune responses. As shown in Figure 10, hochuekkito/Bu-Zhong-Yi-Qi-Tang also exhibited a significant inhibition of liver metastasis produced by colon 26-L5 cells, similarly to juzentaihoto/Shi-Quan-Da-Bu-Tang. The constituents in hochuekkito/Bu-Zhong-Yi-Qi-Tang are different from those in juzentaihoto/Shi-Quan-Da-Bu-Tang as shown in Table 1 and some constituents have the ability to improve “Ki/Qi”-deficiency. Hochuekkito/Bu-Zhong-Yi-Qi-Tang has been reported to be able to stimulate NK cells, thereby enhancing the inhibition of tumor growth. Hochuekkito/Bu-Zhong-Yi-Qi-Tang stimulated splenic NK cells of WKA rats and showed enhanced cytotoxicity against tumors, including NK cell-sensitive YAC-1 targets. Therefore, the mechanism responsible for the inhibition of liver metastasis

Table 1: List of Kampo formulations and their constituent crude drugs

<table>
<thead>
<tr>
<th>Crude drug</th>
<th>Japanese name</th>
<th>Juzentaihoto</th>
<th>Shimotsuto</th>
<th>Unsein</th>
<th>Shikunshito</th>
<th>Rikkunshito</th>
<th>Ninjin-yoeto</th>
<th>Tokishakuyakusan</th>
<th>Hochuekkito</th>
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<tr>
<td>Astragali Radix</td>
<td>Ogi</td>
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<td>Cinnamomi Cortex</td>
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<td>Rehmanniae Radix</td>
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<td>Shakuyaku</td>
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Figure 9: Effect of the oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang, shimotsuto/Si-Wu-Tang, and shikunshito/Si-Jun-Zi-Tang on liver metastasis by the intraportal injection of colon 26-L5 carcinoma cells. Five BALB/c mice per group were orally administered juzentaihoto/Shi-Quan-Da-Bu-Tang, shimotsuto/Si-Wu-Tang, or shikunshito/Si-Jun-Zi-Tang at a dose of 40 mg/day/mouse for 7 days before the intraportal vein injection of colon 26-L5 carcinoma cells (2 × 10⁴). Sixteen days after tumor inoculation, mice were sacrificed and the livers were removed. The number of tumor colonies in the livers and liver weights were calculated manually. *P < 0.01, **P < 0.001 as compared with the control.
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Figure 7 shows that juzentaihoto/Shi-Quan-Da-Bu-Tang did not inhibit liver metastasis of colon 26-L5 cells when the contributions of macrophages and T-cells were removed from the model system.\[53\] In contrast, hochuekkito/Bu-Zhong-Yi-Qi-Tang significantly inhibited the liver metastasis of colon 26-L5 cells in mice pretreated with 2-chloroadenosine as well as untreated normal mice, whereas it did not inhibit the metastasis in anti-asialo GM1 antibody-pretreated (NK cell-deleted) mice [Figure 10]. These results clearly indicate that the antimetastatic mechanism of hochuekkito/Bu-Zhong-Yi-Qi-Tang is apparently different from that of juzentaihoto/Shi-Quan-Da-Bu-Tang and mainly involves the activation of NK cells.

In conclusion, the inhibitory effect of juzentaihoto/Shi-Quan-Da-Bu-Tang on tumor metastasis is primarily associated with its shimotsuto/Si-Wu-Tang-derived constituents, i.e., 地黄 (Rehmanniae Radix), 芍药 (Paeoniae Radix), 川芎 (Cnidii Rhizoma), and 当归 (Angelicae Radix). This is also supported by the evidence that some formulations containing shimotsuto/Si-Wu-Tang constituents were effective for inhibition of tumor metastasis. Because antimetastatic mechanisms by juzentaihoto/Shi-Quan-Da-Bu-Tang and hochuekkito/Bu-Zhong-Yi-Qi-Tang are primarily mediated by the stimulation of macrophages and NK cells, respectively, the contributions of these cell populations to metastasis inhibition may partly relate to the improvement of dysfunctions of “Ki/Qi” and “Ketsu/Xue,” which represent disturbances in the homeostatic condition of the body.

**Organ Selectivity of Juzentaihoto/Shi-Quan-Da-Bu-Tang and Ninjinyoeito/Ren-Shen-Yang-Rong-Tang in the Expression of Antimetastatic Efficacy**

The above results indicated that Kampo formulations containing four shimotsuto/Si-Wu-Tang constituents as well as juzentaihoto/Shi-Quan-Da-Bu-Tang were active in inhibiting liver metastasis of colon 26-L5 carcinoma cells, while ninjinyoeito/Ren-Shen-Yang-Rong-Tang, which does not include all shimotsuto/Si-Wu-Tang constituents, did not show a significant antimetastatic effect.\[56\] Although data are not shown, our preliminary study revealed that the oral administration of ninjinyoeito/Ren-Shen-Yang-Rong-Tang but not by juzentaihoto/Shi-Quan-Da-Bu-Tang after tumor implantation significantly inhibited mediastinal lymph node metastasis, following orthotopic (intrapulmonary)
implantation of murine LLC. Considering this study, we here investigated whether the difference in the antimetastatic effects of juzentaihoto/Shi-Quan-Da-Bu-Tang and ninjinyoeito/Ren-Shen-Yang-Rong-Tang is due to the existence of selective organs and tissues (e.g., liver and lung) for the expression of the efficacy.

As shown in Figure 11, juzentaihoto/Shi-Quan-Da-Bu-Tang significantly inhibited liver metastasis \( (P < 0.001) \) by the intraportal vein (i.p.v.) injection of colon 26-L5 carcinoma cells. These results are consistent with our previous report. In contrast, juzentaihoto/Shi-Quan-Da-Bu-Tang showed no inhibitory effects against lung metastasis caused by i.v. injection of the same colon 26-L5 carcinoma cells into the same syngeneic mice [Figure 11]. Although the oral administration of ninjinyoeito/Ren-Shen-Yang-Rong-Tang for 7 days before i.p.v. injection of tumor cells was not effective at inhibiting liver metastasis, it significantly inhibited metastasis of the same tumor cells as compared with the control [Figure 11]. Thus, juzentaihoto/Shi-Quan-Da-Bu-Tang and ninjinyoeito/Ren-Shen-Yang-Rong-Tang clearly exhibited the differential inhibitory effects on liver and lung metastases caused by the inoculation of the same colon 26-L5 cells in same syngeneic mice. This suggests certain relationship between the efficacy of these formulations and specific organs/circumstances for the expression of the efficacy.

Although further study is needed to examine the underlying mechanism for organ-selective expression of the antimetastatic effects of Kampo formulations, we here interpreted our findings according to the theory of “经络 (Kei-Raku/Jing-Luo),” in traditional Chinese medicine. Namely, “经 (Kei/Jing)” means route or channels and “络 (Raku/Luo)” means collateral net or branch of channels. It is thought that “Kei-Raku/Jing-Luo” connect all parts of the five viscera and six bowels to regulate their functions and keep them balanced. When dysfunction occurs in some organs, relevant pathological changes would take place. Based on the medicinal guides according to this theory, so-called “引经报使 (Inkei-hoshi/Yin-Jing-Bao-Shi)” or “气经 (Qi-Jing),” traditional Chinese medicines are classified based on their respective therapeutic effect on the disease of a special “Kei-Raku/Jing-Luo” and its pertaining organs. As shown in Table 1, 川芎 (Cnidii Rhizoma) in juzentaihoto/Shi-Quan-Da-Bu-Tang and shimotsuto/Si-Wu-Tang formulations, but not in ninjinyoeito/Ren-Shen-Yang-Rong-Tang formulation, is known to possess a selective effect for “肝胆经(Kan-Tan-Kei/Gan-Dan-Jing).” “肝经 (Kan-Kei/Gan-Jing)” runs from a point on the big toe just behind the nail to a point, “气门 (Qi-Mon/Qi-Men),” located about 8 cm below the nipple on either side. “Kan-Kei/Gan-Jing” pertains to the organ “肝/Gan,” which is considered to regulate the mind and mood, digestion, and absorption in traditional Chinese medicine, similarly to the functions of the liver in Western medicine. The indications of the “Kan-Tan-Kei/Gan-Dan-Jing” are stuffiness in the chest and pain in the costal regions and at the top of the head. The most commonly used point, “经血 (Kei-Ketsu/Jing-Xue),” is

Figure 11: Effect of the oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang and ninjinyoeito/Ren-Shen-Yang-Rong-Tang on liver or lung metastasis of colon 26-L5 carcinoma cells. Seven BALB/c mice per group were orally administered the indicated Kampo medicines at a dose of 40 mg/day/mouse for 7 days before intraportal vein (A) or intravenous (B) injection of colon 26-L5 cells. The mice were sacrificed 14 days after tumor inoculation. The number of tumor colonies in the liver (A) or lung (B) was measured manually. \(*P < 0.05, **P < 0.001\) as compared with the control.
“Ki-Mon/Qi-Men,” whose indication is pain in the chest and hypochondriac region.

On the other hand, 陈皮 (Aurantii Nobilis Pericarpium), 远志 (Polygalae Radix), and 五味子 (Schisandraceae Fructus) in the ninjinyoeto/Ren-Shen-Yang-Rong-Tang formulation, but not in juzentaihoto/Shi-Quan-Da-Bu-Tang formulation, are believed to possess a selective effect for “肺经 (Hai-Kei/Fei-Jing).” Indications of “Hai-Kei/Fei-Jing” are cough, asthma, tightness of the chest, sore throat, and pain of the forearm where this channel passes. The physiological function of “Hai/Fei” is taking control of “Ki/Qi” of respiration. Therefore, “Hai/Fei” in traditional Chinese medicine plays the same role as the respiratory system in Western medicine.

Although the concepts of “Kan/Gan” and “Hai/Fei” in traditional Chinese medicine do not correspond to those of the liver and lung in Western medicine, juzentaihoto/Shi-Quan-Da-Bu-Tang containing 陈皮 (Aurantii Nobilis Pericarpium), 远志 (Polygalae Radix), and 五味子 (Schisandraceae Fructus) without 陈皮 (Cnidii Rhizoma), predominantly inhibited tumor metastases to the liver and lung, respectively. Thus, it is of marked interest that our pharmacological data are partly due to the theory of “Kei-Raku/Jing-Luo” formed in the 13th century. Further study is needed to examine the antitumor effects using different types of tumors.

**Immunoadjuvant Effect of Juzentaihoto/Shi-Quan-Da-Bu-Tang on Cytokine Production and Tumor Vaccine Therapy**

As described above, juzentaihoto/Shi-Quan-Da-Bu-Tang was effective at inhibiting liver metastasis of murine colon 26-L5 carcinoma cells through the activation of macrophages and T-cells. Toll-like receptors (TLRs) have recently been characterized as receptors of innate immunity, and also been expressed on macrophages and dendritic cells to recognize pathogen-specific molecular patterns. Pathogen recognition by TLRs promotes the rapid activation of innate immunity by inducing the production of pro-inflammatory cytokines and upregulation of co-stimulatory molecules and subsequently leads to effective acquired immunity. Figure 12 summarizes that lipopolysaccharide (LPS)-induced production of interleukin (IL)-12 and interferon-gamma was augmented via TLR-4 signaling pathways in macrophages obtained from juzentaihoto/Shi-Quan-Da-Bu-Tang-administered mice. Using immunoblotting with phosphorylation-site specific antibodies (data not shown), enhancement of the antitumor cytokine IL-12 production is closely associated with the data that juzentaihoto/Shi-Quan-Da-Bu-Tang upregulated nuclear factor-κB (NF-κB) and p38 signaling pathways and downregulated ERK and JNK signaling pathways in macrophages stimulated by LPS. We also confirm the similar data that selective inhibitors of NF-κB and p38 decreased IL-12 production, whereas inhibitors of JNK and ERK increased IL-12 production in macrophages. Thus, juzentaihoto/Shi-Quan-Da-Bu-Tang is considered to be a medicine with potential as a biological response modifier, boosting its efficacy and activating innate immunity.

Currently, various trials of tumor vaccine therapies have been reported to be clinically carried out worldwide. However, it would be difficult to obtain an effective therapeutic effect, because tumor antigens inoculated as vaccines do not possess high-level immunogenicity. Therefore, it would be now imperative to develop novel immunoadjuvants for tumor vaccines with higher safety and efficacy. We recently found that Kampo medicines are useful as oral immunoadjuvants in viral vaccine treatment. The long-term administration of juzentaihoto/Shi-Quan-Da-Bu-Tang to elderly people who may be in a high-risk group for influenza infection was effective for increasing and prolonging antibody production after influenza vaccination in a randomized controlled trial. Similarly, we have shown that juzentaihoto/Shi-Quan-Da-Bu-Tang was able to induce a tumor vaccine-specific immune response against EL4 murine thymoma cells transfected with ovalbumin (OVA) as a tumor-model antigen. As shown in Figure 13, the oral administration of juzentaihoto/Shi-Quan-Da-Bu-Tang in combination with tumor vaccination (OVA) could significantly suppress the growth of OVA-expressing EL4 tumors inoculated subcutaneously and also prolong the survival, compared with either treatment alone. Thus, these findings confirmed that juzentaihoto/Shi-Quan-Da-Bu-Tang has immunopotentiating efficacy for tumor vaccine treatment.
This physiological phenomenon is important for resistance to apoptosis, the maintenance of cancer stem cells, and production of extracellular matrix, as well as tumor metastasis. Thus, some molecules involved in EMT would be an attractive therapeutic target to regulate tumor invasion and metastasis. Therefore, we focused on EMT to investigate how juzentaihoto/Shi-Quan-Da-Bu-Tang directly affects tumor cells during the metastatic process and the herbal crude drugs in juzentaihoto/Shi-Quan-Da-Bu-Tang that are involved in inhibiting the metastatic potential. Figure 14 shows that only water extract of桂皮 (Cinnamomi Cortex), one of 10 crude drugs of juzentaihoto/Shi-Quan-Da-Bu-Tang, inhibits transforming growth factor beta-induced EMT, such as the downregulation of E-cadherin and upregulation of N-cadherin, and that, consequently, procyanidin C1 is a major active compound in this extract for EMT inhibition. [39]

**Antitumor Effect of Constituent Crude Drugs in Juzentaihoto/Shi-Quan-Da-Bu-Tang**

Because Kampo medicines are generally prepared from the combination of many crude drugs, they may have effects that differ from the sum of the effects of the individual constituent crude drugs. However, to investigate the antitumor mechanism of juzentaihoto/Shi-Quan-Da-Bu-Tang in vivo and identify active components, some studies have also been performed using individual crude drugs of juzentaihoto/Shi-Quan-Da-Bu-Tang and their components, although it is doubtful whether the results obtained using one or some crude drugs can fully reflect the efficacy of the whole formulation, i.e., juzentaihoto/Shi-Quan-Da-Bu-Tang.

**桂皮 (Cinnamomi Cortex)**

As described in Section “Combination with other treatment modalities (chemotherapy, hyperthermia, radiation, etc.),” the combination of juzentaihoto/Shi-Quan-Da-Bu-Tang with either mitomycin C (MMP) or OK432 inhibited the growth of the Ehrlich ascites tumor in ddY mice or of Meth A fibrosarcoma in Balb/c mice and enhanced the production of endogenous TNF. Similar effects by combination therapy were also observed using the extract of桂皮 (Cinnamomi Cortex) in place of the juzentaihoto/Shi-Quan-Da-Bu-Tang.

Epithelial-to-mesenchymal transition (EMT) is a phenomenon in which cobblestone-like epithelial cells change into spindle-like mesenchymal cells with the downregulation of E-cadherin as an epithelial marker as well as upregulation of N-cadherin as a mesenchymal marker. [96] They are also involved in an early step of metastasis. [10, 35] This physiological phenomenon is important for resistance to apoptosis, the maintenance of cancer stem cells, and production of extracellular matrix, as well as tumor metastasis. Thus, some molecules involved in EMT would be an attractive therapeutic target to regulate tumor invasion and metastasis. Therefore, we focused on EMT to investigate how juzentaihoto/Shi-Quan-Da-Bu-Tang directly affects tumor cells during the metastatic process and the herbal crude drugs in juzentaihoto/Shi-Quan-Da-Bu-Tang that are involved in inhibiting the metastatic potential. Figure 14 shows that only water extract of桂皮 (Cinnamomi Cortex), one of 10 crude drugs of juzentaihoto/Shi-Quan-Da-Bu-Tang, inhibits transforming growth factor beta-induced EMT, such as the downregulation of E-cadherin and upregulation of N-cadherin, and that, consequently, procyanidin C1 is a major active compound in this extract for EMT inhibition. [39]

**人参 (Ginseng Radix)**

Ginseng (人参, the root of Panax ginseng C. A. Meyer), a constituent crude drug of juzentaihoto/Shi-Quan-Da-Bu-Tang, has been used for traditional medicine in China, Korea, Japan, and other Asian countries for the treatment of various diseases, including psychiatric and neurologic diseases as well as diabetes mellitus. So far, ginseng saponins (ginsenosides) are glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton and have been regarded as the principal components responsible for the biological activities of ginseng, including the enhancement of cholesterol biosynthesis, stimulation of serum protein synthesis, immunomodulatory effects, and anti-inflammatory activity. [71, 73, 77, 87, 95] Several studies using ginsenosides have also shown antitumor effects, particularly the inhibition of tumor-induced angiogenesis, [74] tumor invasion and metastasis, [47, 78] and control of the phenotypic expression and differentiation of tumor cells. [52, 62] Protopanaxadiol-type and protopanaxatriol-type ginsenosides have been reported to be metabolized by intestinal bacteria after oral administration to their final derivative 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (referred to as compound K) or M1 (27) or 20(S)-protopanaxatriol (referred to as M4) [27]. This indicates
that the expression of the antimitastatic effect by the oral administration of ginsenosides can be induced by their metabolites through intestinal bacteria.

Previously, we have shown that protopanaxadiol- or protopanaxatriol-type ginsenosides and their major metabolites M1 and M4 markedly inhibited lung metastasis of B16-BL6 melanoma cells when they were administered five times orally.\cite{91,92} In contrast, three consecutive i.v. administrations of the metabolite M4 after tumor inoculation resulted in a significant inhibition of lung metastasis, whereas the ginsenosides Re or Rg\textsubscript{1} did not show any inhibitory effect. These findings suggest that the expression of the in vivo antimitastatic effect by the oral administration of both types of ginsenosides was primarily attributable to their metabolites M1 and M4. This may also be supported by the evidence that metabolites were detected in serum from mice orally given ginsenosides, but ginsenosides themselves were not detected in serum by HPLC analysis. This pharmacokinetic study is well consistent with previous reports on the low absorption rate of Rb\textsubscript{1} from the intestines\cite{51,85} and high metabolic rate of Rb\textsubscript{1} to M1\cite{85} in rats and humans using HPLC and enzyme immunoassay.\cite{27,36} Moreover, it has also been noted that ginsenosides are hardly decomposed by gastric juice with the exception of slight oxygenation.\cite{37} Therefore, ginsenosides may act as a natural pro-drug that can be transformed to metabolite M1 by intestinal anaerobes after oral administration and consequently lead to the expression of an in vivo antimitastatic effect.

To examine the incidence of intestinal bacteria possessing a ginsenoside Rb\textsubscript{1}-hydrolyzing potential, the hydrolyzing potential of intestinal bacteria, expressed as the transformation rate of Rb\textsubscript{1} to M1, was performed using fecal specimens of mice. Significant correlations of the transformation rate of Rb\textsubscript{1} to M1 between the groups of litters born to dams with different rates of hydrolyzing potential were observed, thus suggesting that the intestinal microflora of a litter are primarily infected from the dam. On the other hand, the consecutive 2 week-administration of ginseng extract to mice with a moderate transformation rate from Rb\textsubscript{1} to M1 of 25\%±11\% led to a significant increase in the transformation rate, as compared with the untreated group. However, the induction of Rb\textsubscript{1}-hydrolyzing potential by the administration of ginseng extract was not effective for mice with a hydrolyzing potential of less than 10\%. In addition, the inoculation of fecal microflora from mice with a marked hydrolyzing potential was not effective for such mice with low hydrolyzing potential. Therefore, the location of the bacteria capable of hydrolyzing Rb\textsubscript{1} on intestinal epithelial cells may be associated with genetic factors of hosts.

When Rb\textsubscript{1} was orally administered to two sets of mice with low and high Rb\textsubscript{1}-hydrolyzing potential after s.c. inoculation with LLC, a positive relationship between the Rb\textsubscript{1}-hydrolyzing potential and inhibition of lung metastasis was observed as shown in Figure 15.\cite{97} This indicates that transformation rate of Rb\textsubscript{1} to its active metabolite M1 by intestinal bacteria resulted in the expression of the antimitastatic efficacy of
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Therefore, to clarify the mechanism of M1-induced apoptotic cell death, we examined the effect of M1 on the expression of the apoptosis-related proteins p21, p27G1p1, c-Myc, and cyclin D1. Treatment of B16-BL6 melanoma cells with 40 μM of M1 markedly increased the expression of p27G1p1 as compared with the untreated control. No expression of the other CDK inhibitor, p21, was detected in this experiment (data not shown). The upregulation of p27G1p1, which is known to inhibit CDK activity, has also been shown during the apoptotic process caused by anticancer agents such as etoposide and camptothecin. On the other hand, a proto-oncogene product c-Myc and cyclin D1 have been reported to be overexpressed in the proliferative phase of various types of tumor cells.[15,64,89] Treatment of tumor cells with M1 downregulated the expression of c-Myc and cyclin D1 in a time-dependent manner. This indicates that M1 might cause the cell-cycle arrest in tumor cells through the up/down-regulation of these cell-growth related molecules and consequently lead to apoptosis induction.

Some molecules including Bcl-2 (an inhibitor of apoptotic cell death), Bax (promotion of apoptosis by antagonizing the function of Bcl-2), and caspases (interleukin-1β converting enzymes to trigger the execution of cell death) have been shown to be involved in positively or negatively regulating apoptosis signaling.[79,80,90] Recent studies have proposed some signaling pathways for apoptosis mediated by different regulatory molecules.[112,79] Therefore, further study will be needed to examine the possibility that M1 inhibits or promotes these apoptosis-related molecules in detail.

When tumor cells were incubated with dansyl M1 to examine the intracellular distribution of M1, the fluorescent signal of dansyl M1 was detected in the cytosol and nuclei 15 min after incubation and thereafter was shown predominantly in the nuclei. These results suggest that the apoptotic cell death is induced by intracellular M1 through the transcriptional regulation of several cell-growth-associated molecules. Since M1 possesses a steroid-like chemical structure, it might interact with some intracellular receptors including a steroid receptor, which are known to be involved in the rapid regulation of nuclear proto-oncogene transcription.[76] The further regulatory mechanisms of M1 at the transcriptional level needs to be investigated in detail.

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Conflicts of interest
There are no conflicts of interest.
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