Effects of active bufadienolide compounds on human cancer cells and CD4+CD25+Foxp3+ regulatory T cells in mitogen-activated human peripheral blood mononuclear cells

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Abstract. The growth inhibitory effects of bufadienolide compounds were investigated in two intractable cancer cells, a human glioblastoma cell line U-87 and a pancreatic cancer cell line SW1990. Among four bufadienolide compounds, a dose-dependent cytotoxicity was observed in these cancer cells after treatment with gamabufotalin and arenobufagin. The IC_{50} values of the two compounds were 3-5 times higher in normal peripheral blood mononuclear cells (PBMCs) than these values for both cancer cell lines. However, similar phenomena were not observed for two other bufadienolide compounds, telocinobufagin and bufalin. These results thus suggest that gamabufotalin and arenobufagin possess selective cytotoxic activity against tumor cells rather than normal cells. Moreover, a clear dose-dependent lactate dehydrogenase (LDH) release, a well-known hallmark of necrosis, was observed in both cancer cells treated with gamabufotalin, suggesting that gamabufotalin-mediated cell death is predominantly associated with a necrosis-like phenotype. Of most importance, treatment with as little as 8 ng/ml of gamabufotalin, even an almost non-toxic concentration to PBMCs, efficiently downregulated the percentages of $CD4^+CD25^+Foxp3^+$ regulator T (Treg) cells in mitogen-activated PBMCs. Given that Treg cells play a critical role in tumor immunotolerance by suppressing antitumor immunity, these results suggest that gamabufotalin may serve as a promising candidate, as an adjuvant therapeutic agent by manipulating Treg cells to enhance the efficacy of conventional anticancer drugs and lessen their side-effects. These findings provide insights into the clinical application of gamabufotalin for cancer patients with glioblastoma/pancreatic cancer based on its cytocidal effect against tumor cells as well as its depletion of Treg cells.

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Introduction

Bufadienolides are the major effective constituents of cinobufacini (Huachansu), a well-known traditional Chinese medicine (TCM) that comes from dried toad venom (Chan Su) from the skin glands of *Bufo gargarizans* or *Bufo melanostictus*, and cinobufacini has been used to treat patients with various types of cancers such as hepatoma, gallbladder carcinoma and lung cancer in China (1-3). We previously identified 10 bufadienolide compounds in cinobufacini and further suggested that some of them may be used as the quality control markers for cinobufacini (4,5). Among these bufadienolide compounds, bufalin seems to be one of the well-studied active compounds as to its cytocidal effects against a wide spectrum of cancer cell lines and the mechanisms underlying its cytotoxicity (6-9). Despite being key active bufadienolide compounds, the *in vitro* activities of gamabufotalin and arenobufagin in human cancer cells, particularly in intractable cancer cells such as glioblastoma and pancreatic cancer have not yet been well evaluated.

Immunosuppression has been widely recognized in cancer patients due to immune tolerance induced by malignant tumor cells and/or undesirable side-effects of many types of chemotherapeutic drugs (10-14). In this regard, $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells have received considerable attention due to their immunosuppressive properties *in vitro* and *in vivo* (13-16). Furthermore, accumulating evidence has shown an increased number and function of Treg cells in patients with solid tumors and hematologic malignancies, suggesting its critical role in limiting antitumor immune response and promoting immunological ignorance of cancer cells (10,13,16).

Therefore, immune enhancement therapy by attenuating the number and function of Treg cells has become a new attractive field that could be exploited to yield clinical benefit; indeed, depletion or attenuation of Treg cells has been attempted in tumor therapy (13,15). Notably, the positive immunomodulatory effects of medicinal botanicals including TCM have been recognized in both Western countries and China (17-20).

Cinobufacini has been an important TCM in China and other Asian countries for centuries, and is currently widely used as an injection in China. Concerning its antitumor activities, an *in vitro*/*in vivo* study using C3H/HeN mice has demonstrated that a water-soluble and non-dialyzable fraction of Chan Su possesses immunomodulatory effects (21). Furthermore, Deng *et al* reported that cinobufagin and telocinobufagin, two important constituents of Chan Su, significantly stimulate the activation of immunocytes prepared from mice, suggesting that these compounds can boost the host immune system (22,23). Despite this, the effects of active bufadienolide compounds on the alterations of the $CD4^+$ T and Treg cell populations in peripheral blood mononuclear cells (PBMCs) prepared from healthy volunteers, has not yet been investigated.

In the present study, the effects of four active bufadienolide compounds, gamabufotalin, arenobufagin, telocinobufagin and bufalin, were investigated by focusing on the growth inhibition in two intractable cancer cells, a human glioblastoma cell line U-87 and a pancreatic cancer cell line SW1990. Furthermore, lactate dehydrogenase (LDH) assay was conducted to explore whether cell membrane damage occurred in these tumor cells after treatment with gamabufotalin. Human PBMCs were also used to clarify whether these bufadienolide compounds possess a selective cytotoxic activity against tumor cells. Of most importance, the influence of gamabufotalin, endowed with a relatively high cytotoxic effect against cancer cells and a relatively low cytotoxic effect on PBMCs among the four active compounds tested in the present study, on the number of $CD4^+$ T and Treg cells in mitogen-activated PBMCs were further evaluated.

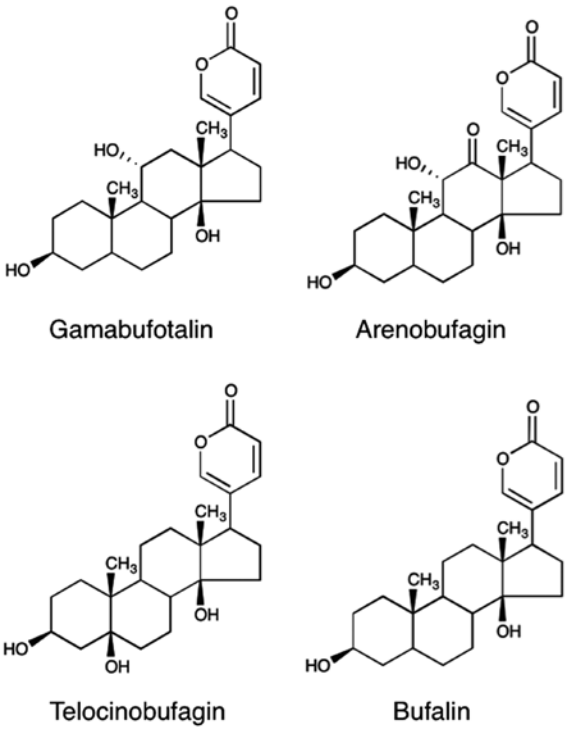

Figure 1. Chemical structures of gamabufotalin, arenobufagin, telocinobufagin and bufalin.

Materials and methods

Materials. Four active bufadienolide compounds (Fig. 1), gamabufotalin, arenobufagin, telocinobufagin and bufalin, were purchased from Baoji Herbest Bio-Tech Co., Ltd. (Baoji, Shanxi, China). RPMI-1640 medium and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbony]-2H-tetrazolium hydroxide (XTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Concanavalin A (ConA), a conventionally known T-cell mitogen, was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and phenazine methosulfate (PMS) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Nichirei Biosciences (Tokyo, Japan).

Cell lines and culture conditions. U-87, a human glioblastoma cell line; and SW1990, a human pancreatic cancer cell line, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). PBMCs were isolated from three healthy volunteers (32±9 years of age) using Mono-Poly resolving medium (DS Pharma Biomedical Co., Ltd., Osaka, Japan) according to a method previously described with slight modifications (24,25). Briefly, 3.5 ml of heparinized blood was loaded on 3 ml of Mono-Poly resolving medium. After centrifugation at 400 x g for 20 min at room temperature, the opaque interface containing PBMCs was transferred to a clean centrifuge tube and washed once with phosphate-buffered saline (PBS). The two types of cancer cells and PBMCs were cultured in DMEM and RPMI-1640 medium, respectively, supplemented with 10% heat-inactivated FBS and antibiotics [100 U/ml of penicillin and 100 μg/ml of streptomycin (Wako Pure Chemical Industries)] at 37˚C in a humidified atmosphere (5% CO_2 in air). The present study was approved by the IRB Committee of Tokyo University of Pharmacy and Life Sciences. An informed consent was obtained from all healthy volunteers.

Cell viability assay. For cell viability assay, U-87 and SW1990 cells were seeded into 96-well plates at a density of $1x10^4$ cells/well in 0.1 ml cell culture medium and allowed to cultivate for one night, followed by the treatment with various concentrations of each drug. In contrast, the cell density of PBMCs was adjusted to $1x10^6$ cells/ml, and 186 μl of the cell suspension was inoculated into 96-well plates, followed by the addition of 10 μl of ConA (0.1 mg/ml in PBS) at final concentrations of 5.0 μg/ml. Subsequently, 4 μl of each drug solution was added to give final indicated concentrations. After treatment with each drug for 48 h, cell viability was determined by the XTT dye-reduction assay according to the method previously described with slight modifications (26). The relative cell viability was expressed as the ratio of the absorbance at 450 nm of each treatment group against those of the corresponding untreated control group. Data are shown

as means ± SD from three independent experiments. The IC_{50} values of each drug for all three cell types were calculated using GraphPad Prism 5 software.

LDH assay. After treatment with the indicated concentrations of gamabufotalin for 24 h as described in the previous section of the cell viability assay, LDH leakage from both the U-87 and SW1990 cells was measured using a LDH cytotoxicity detection kit (Wako Pure Chemical Industry, Osaka, Japan) according to the method previously described with slight modifications (26). Culture medium served as the negative control (NC). Culture supernatants (S) were collected by centrifugation at 450 x g for 5 min at 4˚C and stored at -80˚C until use. Cultured cells without treatment were lysed in the culture medium containing 0.2% Tween-20, and the cell lysate after centrifugation at 12,000 x g for 5 min at 4˚C was used as the non-damaged positive control (PC). Furthermore, in order to avoid an influence of Tween-20, culture medium containing 0.2% Tween-20 served as the NC for PC and was referred to as NCT. Samples were diluted 16-fold with PBS, then 50 μl was loaded into wells of a 96-well plate. LDH activities were determined by adding 50 μl of 'reaction reagent' from the kit, followed by incubation at room temperature for 30 min. The reaction was stopped by the addition of 100 μl of 'stopping solution' provided with the kit, and the absorbance at 560 nm was measured with a microplate reader (EMax® Plus, Molecular Devices, CA, USA). Cell damage was calculated as a percentage of LDH leakage from damaged cells using the following formula: LDH leakage (%) = (S-NC)/(PC-NCT) x 100. Data are presented as the means and SD from three independent experiments.

Analysis of $CD4^+$ T cells and Treg. After treatment with 8 and 16 ng/ml of gamabufotalin for 72 and 96 h, respectively, alterations of $CD4^+$ T cells and Treg cells population in mitogen-activated PBMCs were analyzed by staining of cells with specific antibodies using flow cytometry performed on a FACSCalibur™ II (BD Biosciences, Mountain View, CA, USA) according to the method previously described with slight modifications (27,28). Briefly, after treatment for the indicated time periods, ~$1x10^6$ cells were collected and washed once with PBS (pH 7.4) (Gibco®; Thermo Fisher Scientific, Waltham, MA, USA), followed by the addition of 10 μl of monoclonal mouse anti-human CD4 PerCP-Cy5.5-conjugated antibody and 10 μl of monoclonal mouse anti-human CD25 PE-conjugated antibody (BD Biosciences), respectively. To exclude the amount of non-specific binding, 10 μl of PerCP-Cy5.5-conjugated and 10 μl of PE-conjugated mouse IgG1 κ isotype control (BD Biosciences) were used and evaluated as background, respectively. After the incubation for 20 min in the dark at 37˚C, the cells were washed with PBS and resuspended in diluted Foxp3 buffer A (BD Biosciences), followed by the incubation for 10 min in the dark at room temperature. Then, the cells were washed once with PBS and resuspended in 0.2 ml of Foxp3 buffer C composed of 49 parts Foxp3 buffer A and 1 part Foxp3 buffer B (BD Biosciences), and incubated for 30 min in the dark at room temperature. After the incubation, the cells were washed once with PBS and 10 μl of mouse anti-human Foxp3 Alexa Fluor® 488-conjugated antibody or 10 μl of Alexa Fluor® 488-conjugated mouse IgG1 isotype control to re-suspend the pellet was added, and incubation for 30 min in the dark at 37˚C followed. After washing once with PBS, the cells were resuspended in 0.4 ml staining buffer [0.4% (v/v) formaldehyde neutral buffer solution in PBS (pH 7.4); Nacalai Tesque, Inc., Kyoto, Japan] and analyzed by flow cytometry, and then the data were further analyzed using CellQuest software (BD Biosciences). $CD4^+$ T cells in the lymphocyte fraction were gated, and the percentages of $CD4^+$ T and Treg cells in the $CD4^+$ T cells were calculated, respectively.

Statistical analysis. Experiments were independently repeated three times, and the results are presented as the mean ± standard deviation (SD) of three assays. Statistical analysis was conducted using one-way ANOVA followed by the Tukey's or Dunnett's post hoc test methods. A $P<0.05$ was considered to indicate a statistically significant result.

Results

Cytotoxic effects of bufadienolide compounds against the U-87 and SW1990 cells. Gamabufotalin and arenobufagin exhibited dose-dependent cytotoxic effects on both the U-87 and SW1990 cells after treatment with various concentrations of each drug (1.6, 8, 40, 200 and 1,000 ng/ml) for 48 h, and the IC_{50} values of gamabufotalin were 16.8±6.5 and 8.1±1.5 ng/ml in the U-87 and SW1990 cells, respectively (Fig. 2A). Moreover, similar IC_{50} values of arenobufagin were obtained, which were 10.3±3.3 and 9.9±2.2 ng/ml in the U-87 and SW1990 cells, respectively (Fig. 2B). In comparison, a mild cytotoxic effect was observed in both cancer cell lines treated with telocinobufagin, whereas a potent cytotoxic effect was observed in the bufalin-treated cancer cell lines (data not shown). Notably, an ~3- to 5-fold increase in the IC_{50} values of gamabufotalin was obtained in the PBMCs (IC_{50}=44.1±2.4 ng/ml) as compared to both cancer cell lines, although a clear cytotoxic effect was also observed in the PBMCs when the concentration of gamabufotalin increased up to 40 ng/ml (Figs. 2A and 3). Similarly, the IC_{50} values of arenobufagin were 3-4 times higher in the PBMCs (IC_{50}=36.5±15.8 ng/ml) than those in the two cancer cell lines (Figs. 2B and 3). Moreover, the IC_{50} values of telocinobufagin and bufalin were 77.8±13.3 and 16.5±4.7 ng/ml in PBMCs, indicating that the rank order for the cytotoxicity of the four bufadienolide compounds against PBMCs was: bufalin > arenobufagin > gamabufotalin > telocinobufagin (Fig. 3). These results showed that gamabufotalin exerted not only a relatively high cytotoxic effect against cancer cells, but also a relatively low cytotoxic effect on normal PBMCs among the four active bufadienolide compounds. Therefore, the effects of gamabufotalin on the alterations of the $CD4^+$ T and Treg cell populations in mitogen-activated PBMCs were further evaluated.

Gamabufotalin-mediated LDH leakage in both the U-87 and SW1990 cells. The release of LDH provides an accurate measure of the cell membrane integrity and cell viability (24,26). After treatment with various concentrations of gamabufotalin (1.6, 8, 40, 200 and 1,000 ng/ml) for 24 h, LDH leakage analysis was thus performed to examine whether gamabufotalin treatment affects cell membrane integrity. A dose-dependent

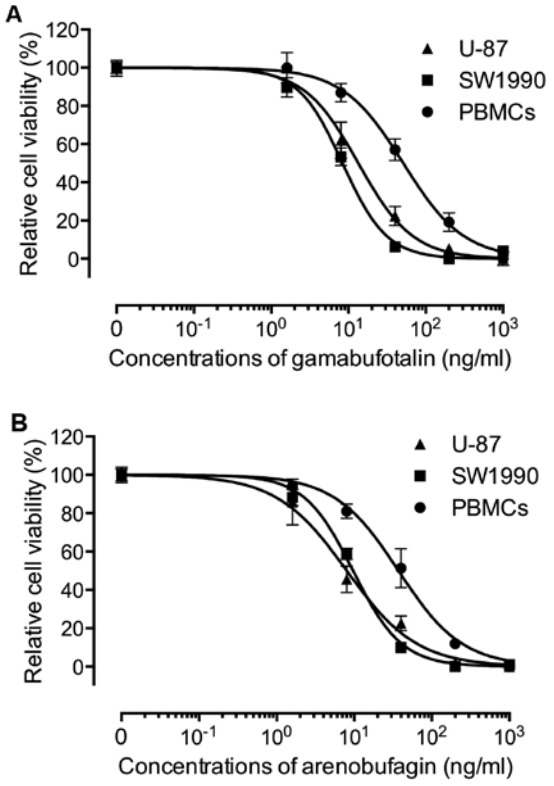

Figure 2. Cytotoxic effects of gamabufotalin and arenobufagin against the U-87 and the SW1990 cells. After treatment with various concentrations (1.6, 8, 40, 200 and 1,000 ng/ml) of (A) gamabufotalin and (B) arenobufagin for 48 h, the viability of U-87, SW1990 and PBMCs was quantified with an XTT assay as described in Materials and methods. Data are presented as the means ± SD from three independent experiments. The IC_{50} value for the PBMCs was significantly greater than that of the U-87 and SW1990 cells (P<0.01). PBMCs, peripheral blood mononuclear cells.

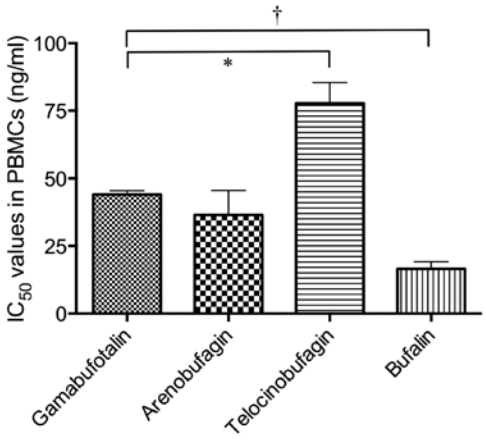

Figure 3. IC_{50} values of active bufadienolide compounds in PBMCs. After treatment with various concentrations (1.6, 8, 40, 200 and 1,000 ng/ml) of each active bufadienolide compound for 48 h, cell viability was quantified with an XTT assay and the IC_{50} values were calculated as described in Materials and methods. Data are presented as the means ± SD from three independent experiments. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post test; *P<0.05 and †P<0.01 were considered to indicate a statistically significant result. PBMCs, peripheral blood mononuclear cells.

LDH leakage was observed in both cancer cell lines (Fig. 4). Furthermore, statistically significant increments in the LDH

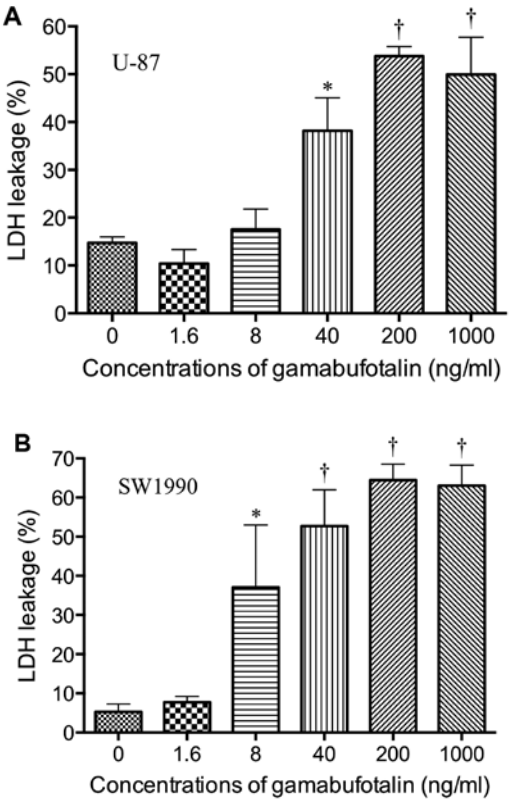

Figure 4. Gamabufotalin-mediated LDH leakage in both the U-87 and SW1990 cells. After treatment with various concentrations (1.6, 8, 40, 200 and 1,000 ng/ml) of gamabufotalin for 24 h, LDH leakage from U-87 (A) and SW1990 (B) was analyzed as described in Materials and methods. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's post hoc test. (B) Significant differences were observed between the control group (0 ng/ml) and the gamabufotalin-treated groups (*P<0.05 and †P<0.01). LDH, lactate dehydrogenase.

leakage were observed at concentrations starting from 40 and 8 ng/ml of gamabufotalin in the U-87 and SW1990 cells, respectively, indicating that the sensitivity of the SW1990 cells to gamabufotalin appeared to be higher than that of the U-87 cells.

Downregulation of the population of Treg cells by gamabufotalin. The populations of $CD4^+$ T and Treg cells in mitogen-activated PBMCs were analyzed by flow cytometry. In this experiment, 8 and 16 ng/ml of gamabufotalin were selected since these two concentrations are very close to its IC_{50} values in both cancer cell lines, and that the concentration of 8 ng/ml was almost non-toxic to PBMCs; the growth inhibition rate by the agent at 16 ng/ml was <20% in PBMCs. Using CellQuest software, $CD4^+$ T cells in the lymphocyte fraction were gated, and the percentages of Treg cells in the $CD4^+$ T cell fraction were further calculated (Fig. 5A). Flow cytometric analysis showed that almost no alteration in the percentages of $CD4^+$ T cells in PBMCs after treatment with either 8 or 16 ng/ml gamabufotalin for 72 and 96 h, respectively, was observed (Fig. 5B and C). Notably, a significant downregulation of the percentages of Treg cells was observed in the $CD4^+$ T cells of PBMCs when treated with either 8 or 16 ng/ml gamabufotalin for 72 h, respectively, and the downregulation continued up to 96 h after treatment with 16 ng/ml gamabufotalin (Fig. 5D and E).

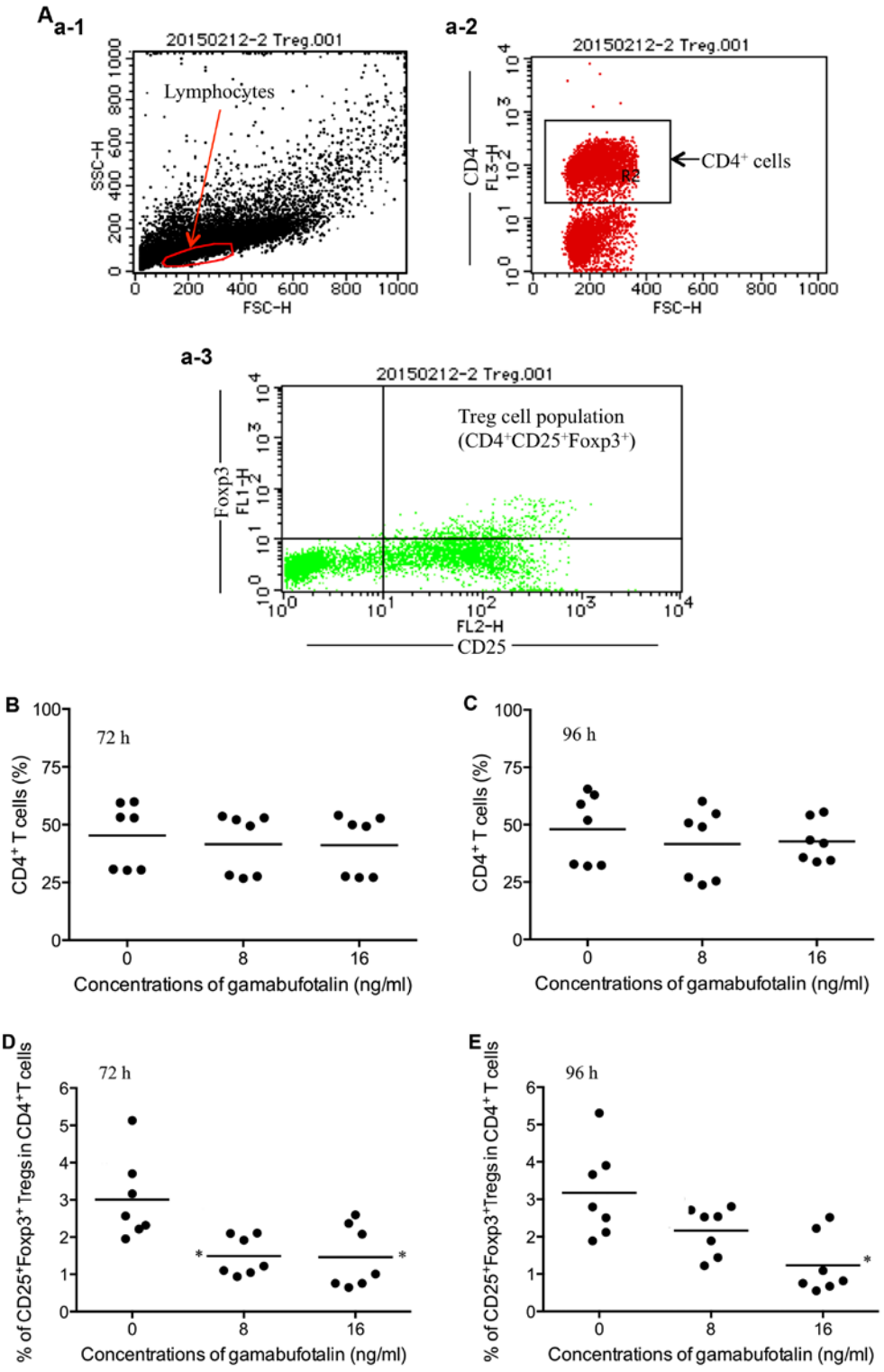

Figure 5. Downregulation of the population of Treg cells by gamabufotalin. After treatment with 8 and 16 ng/ml of gamabufotalin for 72 and 96 h, respectively, alterations of $CD4^+$ T and Treg cell populations in ConA-activated PBMCs were analyzed by a flow cytometer as described in Materials and methods. (A) Lymphocyte fraction of PBMCs (a-1) were gated for $CD4^+$ T cells (a-2), and the percentage of $CD4^+CD25^+Foxp3^+$ T (Treg) cells in the $CD4^+$ cell fraction was calculated (a-3). Percentages of $CD4^+$ T cells [(B and C) represent 72 and 96 h treatment, respectively] and Treg cells [(D and E) represent 72 and 96 h treatment, respectively] in ConA-activated PBMCs treated with 8 and 16 ng/ml of gamabufotalin were calculated, respectively. Data are presented as the means ± SD from three independent experiments. Statistical analysis was conducted using one-way ANOVA followed by Dunnett's post hoc test. $P<0.05$ was considered as statistically significant [$^*P<0.01$ vs. the control (0 ng/ml)].

Discussion

In the present study, we demonstrated that active bufadienolide compounds including gamabufotalin and arenobufagin exhibited dose-dependent cytotoxic effects on the U-87 as well as the SW1990 cells. Notably, among the four active bufadienolide compounds tested in the present study, the IC_{50} values for gamabufotalin and arenobufagin

in the PBMCs were much higher than those in the two different cancer cell lines, whereas similar phenomena were not observed for telocinobufagin and bufalin. In fact, gamabufotalin was recently demonstrated to inhibit the cell growth of non-small cell lung cancer (NSCLC) cell lines such as A549, H1299 and H322, and have only minimal effects on a human normal embryonic lung fibroblast cell line (29). These results thus suggest that bufadienolide compounds such as gamabufotalin and arenobufagin possess selective cytotoxic activity against tumor cells rather than normal cells. In addition, a pilot study aimed to evaluate the efficacy of cinobufacini in patients with advanced cancer demonstrated that no dose-limiting toxicities were observed with the use of cinobufacini at doses up to 8 times higher than the usual therapeutic dose in China, suggesting that cinobufacini has effective anticancer activity with low toxicity and few side-effects (3). Our results, thus, possibly provide supportive evidence for its safety. To the best of our knowledge, this is the first study to show the cytotoxicity of gamabufotalin and arenobufagin against glioblastoma and/or pancreatic cancer cells, although the effects of other bufadienolide compounds with similar structure such as gamabufotalin rhamnoside and arenobufagin diacetate on the U373 glioblastoma cell line has been recently investigated (30).

Growth inhibition and apoptosis induction have been implicated in the antitumor activity of cinobufacini and its main active bufadienolide compounds (30-32). Indeed, bufalin is one of the well-studied active compounds with respect to its *in vitro* activity against a wide spectrum of cancer cell lines including leukemia, breast, prostate, gastric and liver cancer (6), and has been demonstrated to induce apoptosis of these cancer cells via modulation of several signaling pathways such as activation of the intrinsic mitochondrial apoptosis pathway and Fas, downregulation of the PI3K/Akt or MEK/ERK pathway and activation of the MAPK cascade including JNK (6-8). Despite being key active bufadienolide compounds (33,34), the antitumor activity of gamabufotalin and arenobufagin, and their underlying molecular mechanisms have not yet been well determined. Until quite recently, arenobufagin has been demonstrated to induce apoptosis and autophagy through inhibition of the PI3K/Akt/mTOR pathway, and intercalate with DNA leading to G_2 cell cycle arrest in human hepatocellular carcinoma cells (35,36). Moreover, gamabufotalin has recently been demonstrated to induce apoptosis in NSCLC cell lines A549 and H1299, and suppress vascular endothelial growth factor (VEGF)-induced anti-apoptosis of human umbilical vein endothelial cells via the mitochondrial pathway (29,37). In contrast, the present study found no evidence of DNA fragmentation and morphological changes such as nuclear condensation in both the U-87 and the SW1990 cells (data not shown), indicating no involvement of apoptosis induction in these cells following treatment with gamabufotalin and arenobufagin, respectively. Notably, a clear dose-dependent LDH release was observed in both cancer cell lines after treatment with gamabufotalin. It is well known that the release of LDH provides an accurate measure of the cell membrane integrity, and represents a hallmark of necrosis (38). Collectively, our results suggested that gamabufotalin and arenobufagin-induced cell death in both cancer cell lines was predominantly associated with a necrosis-like phenotype. Understandably, further investigation is needed to elucidate details of the mechanism underlying the cell death induction. Furthermore, whether induction of apoptosis or necrosis occurs after treatment with these bufadienolide compounds may be dependent on different cell types.

Since immunosuppression as a result of tumor-induced tolerance and the side-effects of chemotherapeutic drugs is widely recognized in cancer patients (10-14), immune enhancement therapy has become a new attractive field that could be exploited to yield clinical benefit. There is now accumulating evidence that Treg cell population is actively involved in the negative control of a variety of physiological and pathological immune responses including tumor immunity, and its increased number and function are considered to be important for limiting antitumor immune responses and promoting immunological ignorance of cancer cells (10,13,15). Furthermore, increasing evidence supports the existence of elevated numbers of Treg cells in patients with solid tumors as well as hematologic malignancies, and Treg cell depletion has been attempted in tumor therapy (10,13,15,16). In this regard, we demonstrated for the first time that treatment with as little as 8 ng/ml of gamabufotalin, even an almost non-toxic concentration to PBMCs, efficiently downregulated the percentages of Treg cells without impacting the percentages of $CD4^+$ T cells. These results thus suggested that gamabufotalin not only exhibited a cytocidal effect on tumor cells, but also was capable of enhancing antitumor immunity by impeding Treg cell expansion and function. Notably, cinobufagin and telocinobufagin, two important constituents of Chan Su have been demonstrated to significantly stimulate the proliferation of mouse splenocytes, and markedly enhance mouse peritoneal macrophage activation as well as the percentage of $CD4^+CD8^+$ cells (22,23). The two reagents also increased the levels of several Th1 cytokines including interferon-γ and tumor necrosis factor-α, while they decreased the levels of Th2 cytokine interleukin-4, resulting in the increase in the ratio of Th1/Th2 (22,23). Taking these previous results and our observations into account, we thus suggest that gamabufotalin, similar to cinobufagin and telocinobufagin, may also be developed as a novel immunotherapeutic agent to treat cancer such as glioblastoma and/or pancreatic cancer, although there remains urgent need to characterize the function of Treg cells in gamabufotalin-treated PBMCs by evaluating alteration in the levels of Th1/Th2 cytokines, and transforming growth factor-β, known cytokines secreted from Treg cells and also implicated in the induction of Treg cells (39).

As mentioned above, there is a growing concern about immunosuppression in tumor-bearing hosts treated with clinical anticancer drugs (10,11,14). In parallel, we also demonstrated that As_2O_3 and As_2S_2, two different arsenic derivatives with a remarkable efficacy in the treatment of leukemia and myelodysplastic syndrome (40-43), decreased cell viability and proliferation, while they increased the percentages of Treg cells in PBMCs activated by ConA (27,28). Since combination therapy is a frequently used method in the clinical therapy of cancer to improve anticancer effects and reduce toxicities (44), we thus hypothesize that gamabufotalin may serve as a promising candidate, as an adjuvant therapeutic reagent by manipulating Treg cells to enhance the treatment efficacy of arsenic derivatives and lessen their side-effects. The efforts

to evaluate the *in vivo* efficacy of the combination in tumor xenografts in our laboratory are ongoing.

In conclusion, we demonstrated for the first time that gamabufotalin and arenobufagin showed selective cytotoxic effects against tumor cells, but minimal effects on PBMCs, indicating the possibility of using these active compounds for intractable cancers such as glioblastoma and pancreatic cancer. More importantly, we also demonstrated that almost non-toxic gamabufotalin concentrations to PBMCs efficiently downregulated the percentages of Treg cells, suggesting that gamabufotalin may provide therapeutic benefit to patients by not only exhibiting cytocidal effect against tumor cells but also by enhancing antitumor immunity.

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