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Bioactive Immunomodulatory Compounds: A Novel Combinatorial Strategy for Integrated Medicine in Oncology? BAIC Exposure in Cancer Cells

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Abstract

The Standardized Cultured Extract of Lentinula edodes Mycelia (also known as Active Hexose Correlated Compound, AHCC) and *Wasabia japonica* (Wasabi) are natural nutritional supplements known for their immunomodulatory and anticancer potential. The aim of this study was to evaluate the combinatorial effect of the bioactive immunomodulatory compound (BAIC), obtained by combining Wasabi and AHCC, on human breast (MCF-7) and pancreatic (Panc02) adenocarcinoma cell lines. Data obtained revealed that BAIC determines a striking decline in cancer cell growth at minimal concentrations compared with the use of Wasabi and AHCC as single agents. A significant increase in the G_0/G_1 subpopulation together with a marked augmentation in the percentage of apoptotic cells was demonstrated by flow cytometry, together with a significant upregulation in the expression of genes associated to the apoptotic cascade in both cell lines. The inhibitory role BAIC plays in mammospheres formation from MCF-7-derived cancer stem cells was shown with a marked reduction in size and number. Interestingly, when BAIC was exposed to monocytic cells, no cytotoxic effects were observed. A monocytes-to-macrophages differentiation was rather observed with the concomitant acquisition of an anti-inflammatory phenotype. Taken together, our findings suggest that BAIC could be used as a potential integration of standard chemotherapy treatments because of the improved inhibitory activity on cancer cell proliferation and reduced potential adverse effects.

Keywords

integrative medicine, Wasabi, *Wasabia japonica*, cell cycle, immunomodulation, gene regulation, apoptosis, cancer cells, glucans, paracrine signals, Active Hexose Correlated Compound

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Introduction

The first studies reporting the efficacy of natural compounds in integrative medicine date back to the past 2 decades. However, during recent years, the use of nutritional supplements to integrate standard medical treatments has considerably increased. A plethora of natural products are currently being screened for their potential as adjuvant therapeutics for the control of the side effects of particularly aggressive approaches, including but not limited to chemotherapy. To date, complementary therapies relying on the use of natural compounds have demonstrated efficacy in the treatment of serious diseases, such as cancers and inflammatory diseases, including rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis, and inflammatory brain diseases.[1-3] Our journey in integrative medicine began with the enzyme-fermented extract of the mushrooms *Lentinula edodes*, the Standardized Cultured Extract of Lentinula edodes Mycelia. Our journey in integrative medicine began

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with the Standardized Cultured Extract of Lentinula edodes Mycelia, widely known as Active Hexose Correlated Compound (AHCC). Its most active component, α-glucan, plays multiple roles in cancer (including breast,[4] ovaries,[5] and pancreas[6]), host protection during viral[7] or bacterial[8,9] infections, chronic diseases (ie, diabetes[10]), and cardiovascular pathologies. In this context, it is worth mentioning the anticancer effect of α-glucans, which has been reported in patients with squamous cell lung carcinoma, as well as their role in reducing the adverse effects observed in patients with advanced cancer during chemotherapy.[4,6] Moreover, several groups around the world are currently exploiting the beneficial properties that α-glucan exerts on human health in combination with other bioactive molecules, respectively, with CpG-oligodeoxynucleotide and tamoxifen showing they modulate oxidative stress and immune responses in cancer patients and anticancer hormonal agent.[11,12] Furthermore, the effectiveness of AHCC supplementation has been mainly ascribed to its role in interfacing with the immune system. Specifically, AHCC stimulates the immune system by modulating the response against pathogens[13] and is capable of enhancing it via multiple mechanisms, including through the augmentation of macrophage and natural killer cell proliferation.[13] In particular, AHCC is known to induce a high production of various cytokines by macrophages and T lymphocytes, such as interferon-γ (IFN-γ), interleukin (IL)-8, IL-1β, and tumor necrosis factor (TNF-α, IL-2, and IL-12).[14] Whereas *Wasabia japonica* (reported here as Wasabi) is an aromatic component of the Japanese typical pungent spice used as a condiment in Asia to prepare traditional foods like sashimi and sushi. The active component of Wasabi is methylsulfinyl hexyl isothiocyanate (6-MITC), which is also known for its apoptotic effects on cancer cells,[15] anti-inflammatory potential,[16] and detoxifying properties.[17] In the inflammatory process, macrophages play a central role in the activation of the metabolic pathways responsible for the release of inflammatory enzymes, cytokines, chemokines, and other inflammatory factors. Overexpression of these inflammatory factors by macrophages has been attributed to the pathophysiology of many inflammatory diseases. It was observed that Wasabi retains the capability to suppress the expression of cyclooxygenases (*Cox-2*) and prostaglandins (*Pge2*) in human U937 monocytic cells[16] and macrophages.[18,19] Furthermore, 6-MITC has been shown to selectively suppress breast cancer and melanoma cell progression as reported by Nomura et al.[20] According to Watanabe et al, the apoptotic properties and the consequent anticancer effect associated to Wasabi is mediated by the presence of another compound found in Wasabi, allyl isothiocyanate (6-HITC), which has been found to also induce detoxification through the activation of enzymes such as glutathione S-transferases.[21] Based on this evidence, the aim of this study was to demonstrate a combinatorial effect between AHCC and Wasabi (depicted in this study as bioactive immunomodulatory compound [BAIC]) in contrasting the growth of 2 human adenocarcinoma cell lines, the breast adenocarcinoma (MCF-7) and pancreas adenocarcinoma (Panc02) cells. To this end, we first defined the minimal dose of AHCC and Wasabi able to reduce cell viability in MCF-7 compared with their use as single agents (AHCC or Wasabi). Subsequently, we verified if the observed effect was due to a cell cycle arrest or to the induction of apoptotic cascade within the cells following the treatment. In parallel, preliminary insights of the role the combination of AHCC and Wasabi plays in modulating the size and growth of mammospheres produced by MCF-7-derived cancer stem cells (CSC) were also obtained. Finally, we asked whether the combination of AHCC and Wasabi at the concentrations found to be effective in reducing cancer cell progression could concomitantly lead to side effects. To answer this question, the minimal concentration able to induce a detrimental role on MCF-7 and Panc02 cells was administered to monocytic cells (ThP-1 cell line) to evaluate viability and phenotype, as compared with the treatment with their single counterparts.

Materials and Methods

AHCC and Wasabia japonica

AHCC used in this study was provided by Amino Up Chemical Co, Ltd (Sapporo, Japan). A lyophilized extract titrated and standardized by the rhizome of *Wasabia japonica* was purchased from Pharmagen BG-Sofia (Bulgaria), its official suppliers.

Cell Culture

Cells used in this study include human breast adenocarcinoma (MCF-7), human pancreas adenocarcinoma (Panc02), and human leukemia monocytic (ThP-1) cell lines (from ATCC). Cancer cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (Corning) supplemented with 10% fetal bovine serum (Corning), 1% L-glutamine, and 2% antimitotic/antibiotic. ThP1 cells were cultured in RPMI-1640 media (Gibco) supplemented with 10% fetal bovine serum (Corning), 1% L-glutamine, and 2% antimitotic/antibiotic. Cells were maintained at 37°C in a humid atmosphere with 5% CO_2.

Experimental Design

For the treatments, a stock solution of the single components was prepared in Dulbecco's phosphate-buffered saline (Sigma), incubated for 72 hours, filtered using a 0.45-μm filter, and stored at 4°C. MCF-7 and Panc02 cells were treated with different concentrations of Wasabi and AHCC (ranging from 7.5 to 500 μg/mL) for 24 and 48 hours, in combination or as single components. At the end of each time point, a cell viability assay was used to determine the

minimal concentration able to induce a significant reduction. Once defined through the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, the optimal combination was used to perform further analyses and assess the effect on cell cycle and apoptosis. The cytotoxic effect as well as the immunomodulatory potential of the Wasabi and AHCC combination have been also investigated on ThP-1 cells after 48-hour treatment as reported below.

Evaluation of Cancer Cells Viability

The effect on cell viability of Wasabi and AHCC, as single compounds or in combination (BAIC), was determined on MCF-7 and Panc02 following 24- and 48-hour treatment using MTT. The colorimetric MTT assay allowed identifying the minimum doses of BAIC able to reduce cell viability. Briefly, cells were seeded at the density of 10 000 cells/well into 96-well flat-bottomed plates to allow them to cover the whole surface of the dish. Cells were then treated with different concentrations of Wasabi and AHCC (range = 7.5-500 μg/mL) and analyzed following the manufacturer's indications (Vybrant MTT Cell Proliferation Assay Kit, Life Technologies). Absorbance was measured at 570 nm using a microplate reader (Biotech), and data were analyzed by using the software Gen05.

Cell Cycle Assessment

The effect of BAIC on cell cycle distribution was examined using flow cytometry. In brief, MCF-7 and Panc02 were seeded at a density of $1 \times 10^4/cm^2$ on 6-well plates and treated with the optimal combination of BAIC (7.5 μg/mL for Wasabi and 10 μg/mL for AHCC) or with Wasabi (7.5 μg/mL) and AHCC (10 μg/mL) for 48 hours. Following the treatment cells were collected, centrifuged at room temperature at 500 × *g* for 5 minutes, and incubated overnight with cold 70% ethanol. Cells were then resuspended in phosphate-buffered saline containing propidium iodide (40 μg/mL) and RNase (100 μg/mL). Flow cytometry data were acquired using a Guava Millipore cytometer. At least 20 000 cells/sample were run. The percentage of cells in sub G0, G1, S, and G2/M was established using FlowJo software.

Evaluation of Apoptosis

To analyze the possible apoptotic effect induced on MCF-7 and Panc02 by BAIC, the Annexin V-FITC Apoptosis Detection Kit I (BioLegend) was used. Briefly, cells were treated with Wasabi and AHCC in combination (7.5 μg/mL for Wasabi and 10 μg/mL for AHCC) or as single agents (7.5 μg/mL and 10 μg/mL for Wasabi and AHCC, respectively) for 48 hours, collected, and washed in a binding buffer solution. Cells were then incubated in the staining solution containing propidium iodide and Annexin V-FITC for 15 minutes at room temperature and in the dark. Flow cytometry data were acquired using a Guava Millipore cytometer. The percentage of normal, early apoptotic, apoptotic, and necrotic cells was established using FlowJo software by comparing experimental cells to control groups (untreated cells).

Molecular Analysis

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed to evaluate the expression of specific pro-apoptotic markers on cancer cells and markers associated to inflammation on monocytes as shown in Table 1. In all cases, total RNA was isolated using TRI-reagent (Invitrogen). DNAse (Sigma) treatment followed the reaction. RNA concentration and purity were measured using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). The cDNA was synthesized from 500 ng of total RNA using the PrimeScript RT Master Mix (TAKARA), and quantitative PCR was run in the StepOnePlus Real-time PCR system (Applied Biosystems) using commercially available master mix (PowerUp SYBER Green Master Mix; Applied Biosystems).

Monocytic Cells Viability and Differentiation

To evaluate the side effects of the doses of BAIC defined as the most efficient in inhibiting cancer cells proliferation, monocytic cells (ThP1) were seeded at the density of $2 \times 10^5/cm^2$ on 6-well plates and treated accordingly. After 48 hours of incubation, cells were harvested, and the percentage of viable cells was evaluated by trypan blue exclusion dye using a Burker chamber.

Nonadherent Mammosphere Formation Assay and Treatment

Cancer stem cells from MCF-7 cells (ProMab) were cultured at a density of 1×10^3 cells/mL and grown in a 6-well ultralow attachment plate with Premium Cancer Stem Cell Media (ProMab) to enable the cells to grow and form spheres. A further 1 mL of fresh medium was added to each well every other day. Incubation of the primary culture MCF-7-derived CSC with BAIC was conducted under mammosphere-forming conditions for 5 days when the size of the formed mammospheres was compared with those found in the control group (CTRL, untreated cells). A BX51 microscope (Nikon) was used to acquire images that have been captured by using the open source Spot Advanced software and analyzed by ImageJ.

Statistical Analysis

Statistical analysis was performed using GraphPad Instat 3.00 (GraphPad Software). Three replicates for each

Table 1. Transcripts and Sequence of Each Primer Used in qPCR to Investigate the Apoptosis and the Inflammation[a].

Gene	Sequences (5′ → 3′)	T_m (°C)	Product Size (bp)
Pro-apoptotic markers			
BCL-2 associated X protein (*BAX*)	S: TCCCCCCGAGAGGTCTTTT A: CGGCCCCAGTTGAAGTTG	57	68
Apoptotic peptidase activating factor-1 (*Apaf-1*)	S: TGGCTGCTCTGCCTTCT A: CCATGGGTAGCAGCTCCTTC	57	142
p53	S: CCCCTCCTGGCCCCTGTCATCTTC A: GCAGCGCCTCACAACCTCCGTCAT	62	265
Immunomodulatory markers			
Interleukin 1β (*Il-1β*)	S: TGCTCTGGGATTCTCTTCAGC A: CTGGAAGGAGCACTTCATCTG	56	164
Transforming growth factor-β (*Tgf-β*)	S: ATGGTGGAAACCCACAACG A: GGAATTGTTGCTGTATTTCTGG	56	171
Cyclooxygenase-2 (*Cox-2*)	S: TGAGTTATGTGTTGACATCCAG A: TCATTTGAATCAGGAAGCTGC	56	197
Glyceraldehyde-3-phosphatase dehydrogenase (*Gapdh*)	S: TCCACTGGCGTCTTCACC A: GGCAGAGATGATGACCCTTT	57	78

Abbreviations: qPCR, quantitative polymerase chain reaction; S, sense primer; A, antisense primer.
[a]For each gene, oligonucleotide sequence (5′ → 3′), melting temperature (T_m), and the length of the product are also reported.

experiment (quantitative PCR, flow cytometry analyses, and cell counts) were performed, and the results are reported as mean ± standard deviation (SD). One-way analysis of variance for multiple comparisons by the Student-Newman-Keuls multiple comparison test was used to assess differences between groups. Differences were considered statistically significant for P values <.05. For quantitative PCR data, nonparametric tests were used.

Results

BAIC Inhibits, at Defined Concentrations, Cancer Cell Growth

Before starting the experiments, we aimed at determining the minimal concentration of Wasabi and AHCC able to drastically reduce the percentage of viable cancer cells at 48 hours. To this the MCF-7 cell line was chosen and the MTT assay was applied to determine the effect of the combination of AHCC and Wasabi at different concentrations, as reported in Supplementary Figure 1A (available online). The percentage of active cells after the treatment with different combinations are shown in Supplementary Figure 1B (available online).

Results of this part of the study indicated no significant differences among the treatments although significant differences ($P < .01$) were found compared with the control cells (CTRL). Among the possible combinations, we identified in the concentrations 7.5 and 10 μg/mL for Wasabi and AHCC, respectively, the efficient cocktail to inhibit the growth of MCF-7 cells. Figure 1A shows a statistically significant ($P < .01$) effect of BAIC on MCF-7 cells compared with AHCC (10 μg/mL) and Wasabi (7.5 μg/mL) provided as single agents, with a marked inhibition in cell proliferation at 48 hours (values assessed 48%). The percentage of active Panc02 cells registered following the exposure to Wasabi and AHCC demonstrated a slight increase compared with control at 24 hours. Values assessed around 109% and 110%, respectively. In contrast, a slight reduction was observed at 48 hours. At 48 hours, the reduction of proliferative cells was assessed around 45% for BAIC, 43% for Wasabi, and 64% for AHCC (Figure 1B).

BAIC Determines an Arrest in Cell Cycle Progression

Cell cycle represents the most fundamental and important processes in eukaryotic cells. Cell cycle analysis was performed to determine the influence of BAIC on cell cycle phase distribution (G_0, G_1, S, G_2, and M) in MCF-7 and Panc02 cells at 48 hours. Our results indicated that the combination of Wasabi and AHCC halted the cell cycle progression in the G_0/G_1 phase ($P < .05$) in both cell types (Figure 2). They also suggest that such inhibition occurs in a crucial phase of the cycle, being the transition from G1 to S responsible for controlling cell proliferation.[22] Figure 2A shows a greater G_0/G_1 phase arrest in MCF-7 cells, which accounts for 55.7% following the treatment with BAIC ($P < .05$). MCF-7 cells treated with single Wasabi and AHCC revealed similar G_0/G_1 phase distribution (40.8% and 42.3%), which was comparable to control cells (40%). An increase in the percentage of cells in the G_0/G_1 phase (51%) was also registered when Panc02 were treated with AHCC compared with control cells (43%; Figure 2B). Intermediate results for the

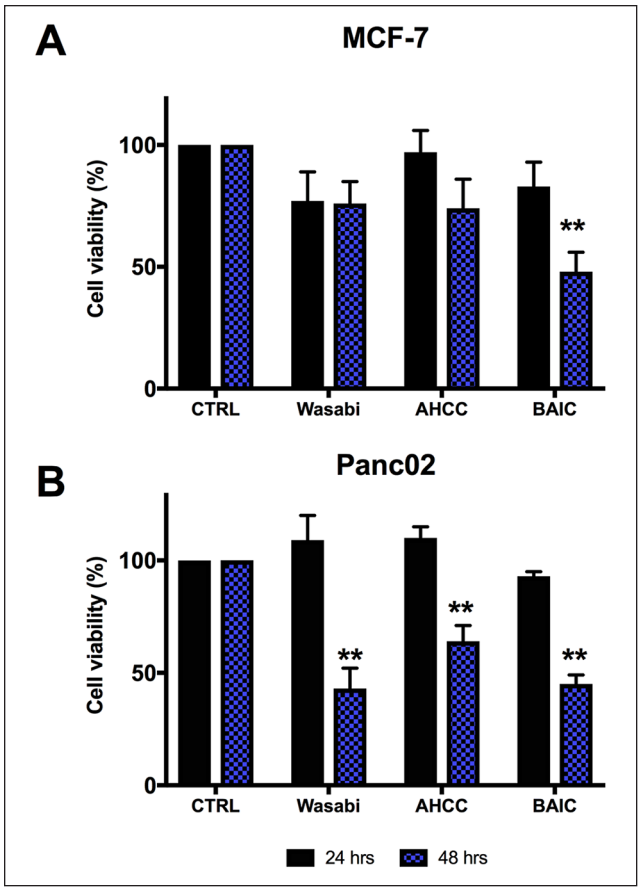

Figure 1. MTT assay demonstrating the effect of bioactive immunomodulatory compound (BAIC; 7.5 and 10 µg/mL, respectively) on the viability of MCF-7 (A) and Panc02 (B) after 24 and 48 hours of treatment. Data obtained exposing both cell lines to active hexose correlated compound (AHCC) and Wasabi used as single agents are also reported for comparison. Data are normalized to control cells (CTRL) and reported as mean ± standard deviation (n = 3). **Highly significant ($P < .01$) and *significant ($P < .05$) compared with CTRL.

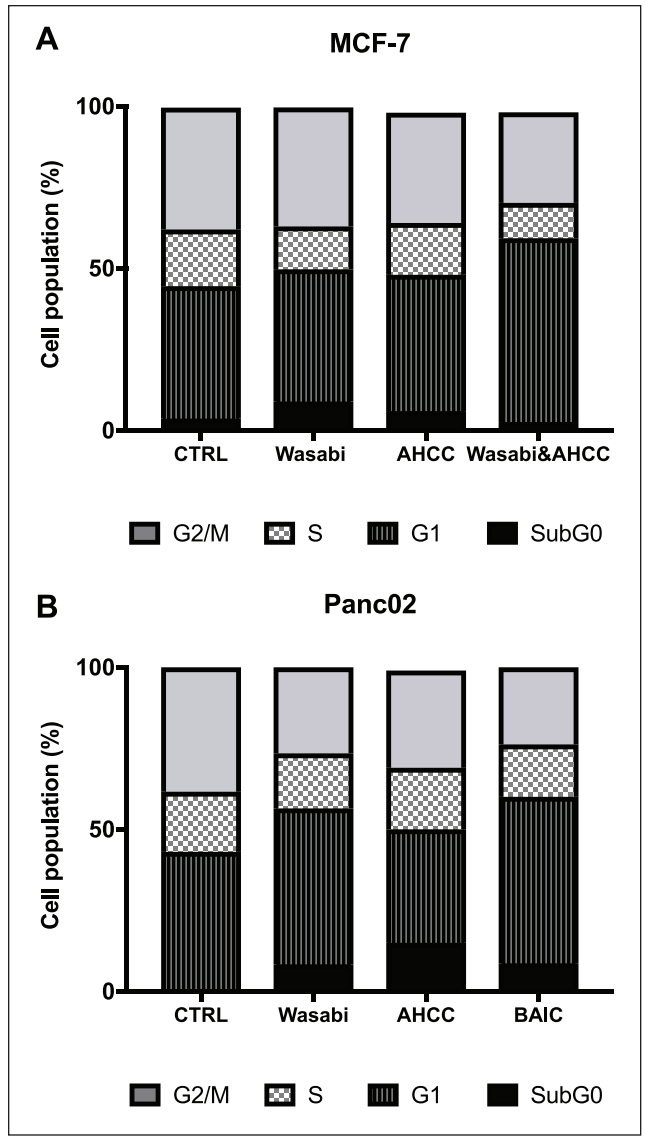

Figure 2. Cell cycle analysis performed on MCF-7 (A) and Panc02 cells (B) following the treatment with bioactive immunomodulatory compound (BAIC) for 48 hours. Data obtained from untreated cells (CTRL) and cells treated with the single compounds (7.5 µg/mL Wasabi and 10 µg/mL active hexose correlated compound [AHCC]) are also reported for comparison. Data are reported as average of the percentage of cells distributed in the subG0, G1, S, and G2/M phase ± SD (n = 3). **$P < .01$, highly significant differences compared with CTRL.

percentage of cells in G_0/G_1 phase were found as the consequence of the single treatment with Wasabi, with values assessed around 48%. On the contrary, a marked reduction (30%) in the percentage of cells in G_0/G_1 was found as the result of AHCC exposure, although the percentage of resting cells ($subG_0$) was greater than the other experimental groups.

BAIC Induces Apoptosis in Cancer Cells

Annexin V assays and gene expression evaluation were performed to confirm data obtained from the cell cycle and proliferation assays. Although all treatment groups showed significant increases in apoptosis compared with control groups ($P < .05$), flow cytometry assays showed marked changes in MCF-7 and Panc02 cell profiles after treatment with BAIC at 48 hours, compared with Wasabi (7.5 µg/mL) and AHCC (10 µg/mL) used as single agents (Figure 3A and B). Apoptotic rates ranged around 17% for Wasabi- and AHCC-treated Panc02 cells and increased to 25% when cells were treated with BAIC, registering a 5-fold increase compared with untreated cells (5% apoptotic cells).

These rates were found even greater in MCF-7 where 50% of apoptotic cells were detected, whereas the percentage was decreased to 30% and 36% following the treatment

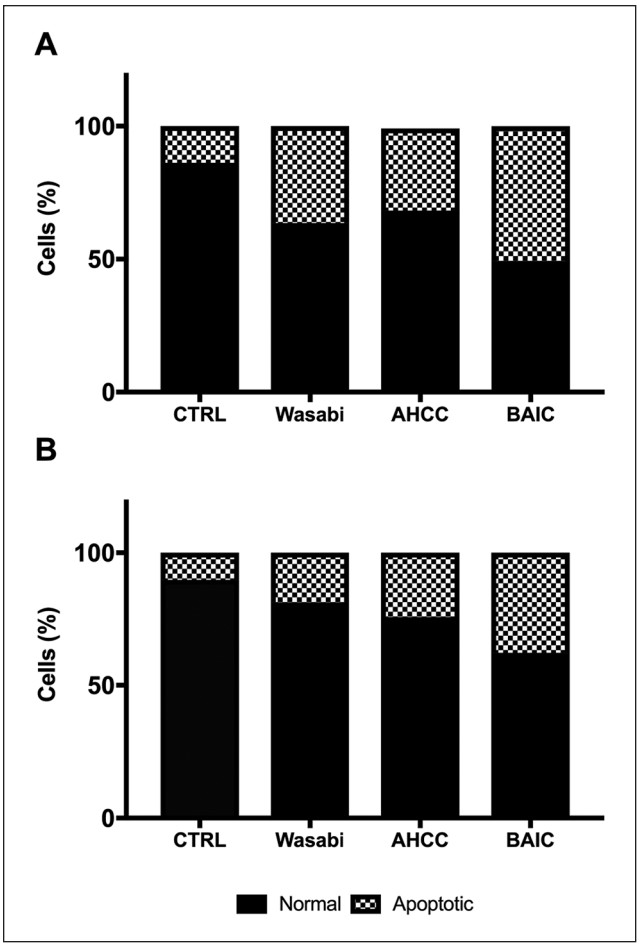

Figure 3. Representative flow cytometric analysis of MCF-7 (A) and Panc02 (B) showing normal and apoptotic cells after 48 hours treatment with Wasabi and active hexose correlated compound (AHCC) in combination (bioactive immunomodulatory compound [BAIC]), as single agents (Wasabi at 7.5 µg/mL and AHCC 10 µg/mL), or in standard conditions (CTRL). Data represent the means ± standard deviations (n = 3). *Significant and **highly significant differences compared with CTRL at $P < .05$ and $P < .01$, respectively. Solid bars depict normal cells, and patterned bars depict apoptotic cells.

with AHCC and Wasabi, respectively. The apoptotic cascades are tightly regulated by a variety of factors; among these factors, the Bcl-2 protein family plays central roles in apoptotic events.[23] We further investigated the expression of effectors of the apoptotic mechanisms, such as *Bax-2* and *Apaf-1*, both at 24 and 48 hours (Figure 4).

Following the treatment with BAIC, a statistically significant increase compared with control in the expression of *Bax-2* was found in MCF-7 (8.32 ± 0.41) but not in Panc02 cells (1.14 ± 0.34) at 48 hours. When MCF-7 cells treated with Wasabi and AHCC as single agents a slight increase in the expression levels of *Bax-2* was found at the same time point (48 hours), with values assessed around 1.28 ± 0.04 for Wasabi and 1.5 ± 0.16 for AHCC. A 2-fold increase in *Apaf-1* expression was found at mRNA level in MCF-7 cells treated with BAIC (2.08 ± 0.25) compared with the control group and to the other experimental groups at 48 hours. On the contrary, significant differences compared with control cells were found in the expression of *Apaf-1* when Panc02 cells were exposed to the combination of Wasabi and AHCC (3.68 ± 0.04) at 48 hours. A 2-fold increase in the expression of *Apaf-1* was observed following the treatment with AHCC at 24 hours (1.98 ± 0.14). Furthermore, the expression of P53 included as a stress-responsive transcription factor and potent tumor suppressor was investigated (Figure 5). A slight increase in the expression of *p53* was observed when MCF-7 cells were treated with BAIC and AHCC (1.3 ± 0.7 and 1.6 ± 0.3-fold, respectively) compared with control cells, showing an advantage in the use of BAIC compared with Wasabi. In Panc02 cells, a greater upregulation was found following the treatment with values assessed around 2.3 ± 0.1-fold for BAIC, 2.1 ± 0.1-fold for AHCC, and 1.9 ± 0.1-fold for Wasabi, compared with control.

Effect of BAIC on Monocytic Cells

To analyze the role BAIC has on the immune system, a proof of concept study was performed. Following the exposure to BAIC, monocytic cells have been tested for viability and the expression of inflammatory-associated markers. As reported in Figure 6, the combination of Wasabi and AHCC at low concentrations is capable of supporting the proliferation of monocytic cells. No cytotoxic effects were found in any of the experimental groups considered, where viability was comparable to control (Figure 6A). A 2-fold increase in the number of adherent cells was found on the surface of the dish where monocytes were grown in presence of BAIC compared with cells grown in standard conditions (CTRL; Figure 6B).

Similar or reduced numbers were found when cells were exposed to Wasabi and AHCC at the same concentrations, respectively. Molecular analysis performed on ThP-1 treated with BAIC for the evaluation of inflammatory genes demonstrated the immunomodulatory potential of the mixture compared with the single components (Figure 7). In particular, the expression of all the tested genes was found dramatically increased as a consequence of the treatment with Wasabi at 24 hours, with values of around 682 (±59)-fold for *Tfg-β*, 8060 (±53)-fold for *Cox-2*, and 3167(±16.7)-fold for *Il-1β* compared with control cells, respectively. Monocytes treated with BAIC increased the expression levels of 4 ± 0.27-fold, 185 ± 27-fold, and 90 ± 38-fold, respectively. An average of 2-fold increase with regard to control was observed in cells treated with AHCC at the same time point. An interesting downregulation was found at 48 hours,

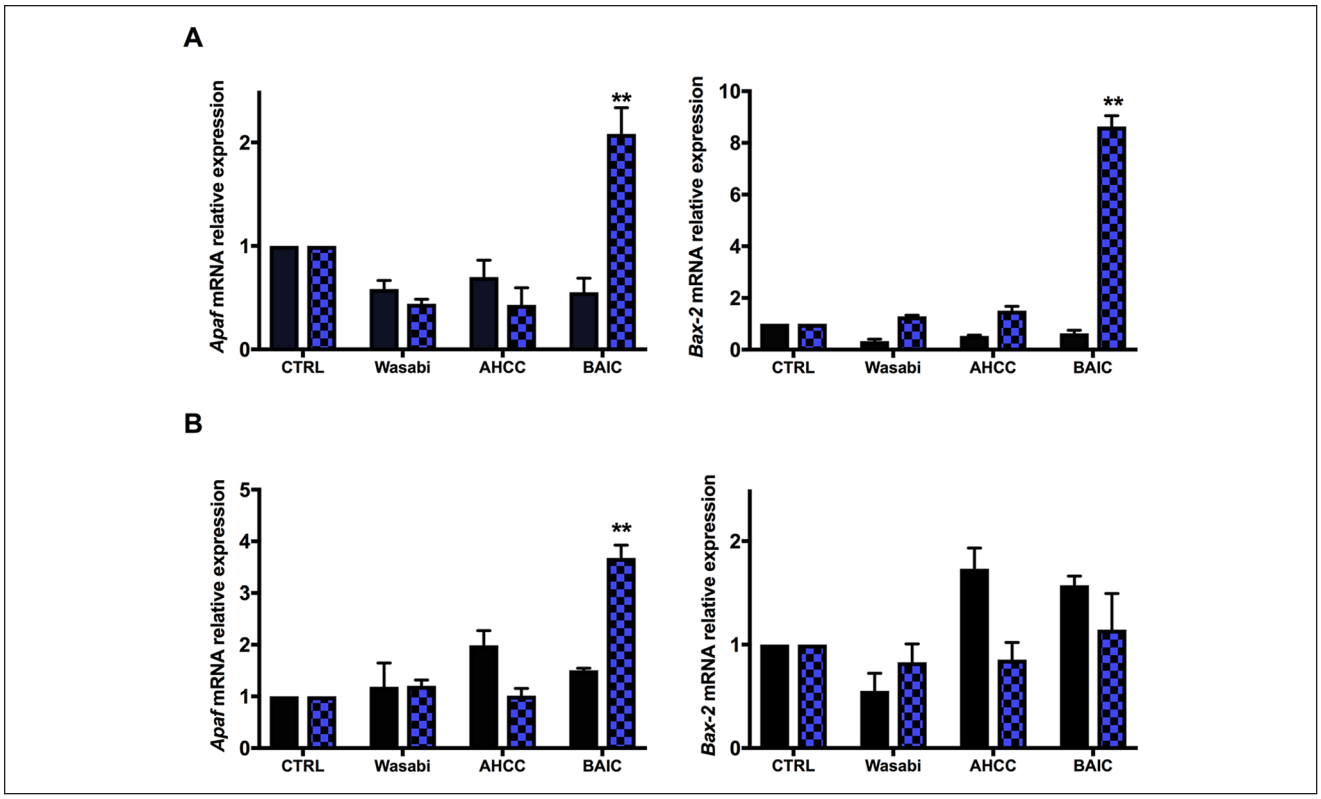

Figure 4. Quantitative polymerase chain reaction for the expression of pro-apoptotic genes (*Apaf-1* and *Bax-2*) on MCF-7 (A) and Panc02 (B) cell lines following the treatment with bioactive immunomodulatory compound (BAIC), 7.5 µg/mL Wasabi (Wasabi), and 10 µg/mL active hexose correlated compound (AHCC). Data are represented as fold-change compared with the expression levels found in untreated cells (CTRL, baseline). *Significant and **highly significant differences compared with CTRL at $P < .05$ and $P < .01$, respectively.

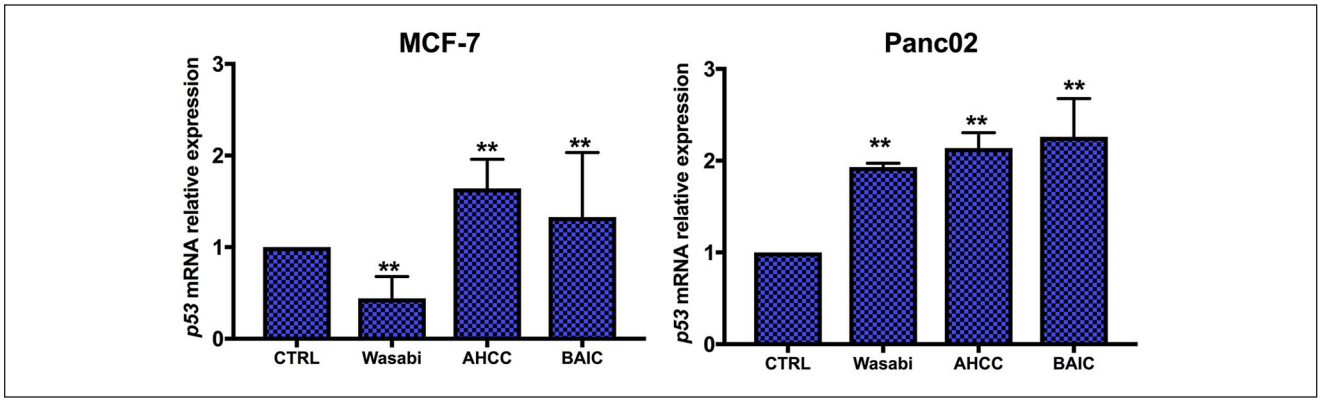

Figure 5. Quantitative polymerase chain reaction for the expression of the oncosuppressor p53 following the treatment of MCF-7 and Panc02 cells with bioactive immunomodulatory compound (BAIC), 7.5 µg/mL Wasabi (Wasabi), and 10 µg/mL active hexose correlated compound (AHCC), at 48 hours. Data are represented as fold-change compared with the expression levels found in untreated monocytic cells (CTRL) (n = 3; **$P < .01$).

when the expression levels of *Il-1β* and *Cox-2* were significantly decreased following the treatment with BAIC, thus becoming more comparable to or even lower than untreated cells.

BAIC Inhibits Mammosphere Growth

The assessment of mammosphere growth following the treatment with a bioactive compound is crucial to define its

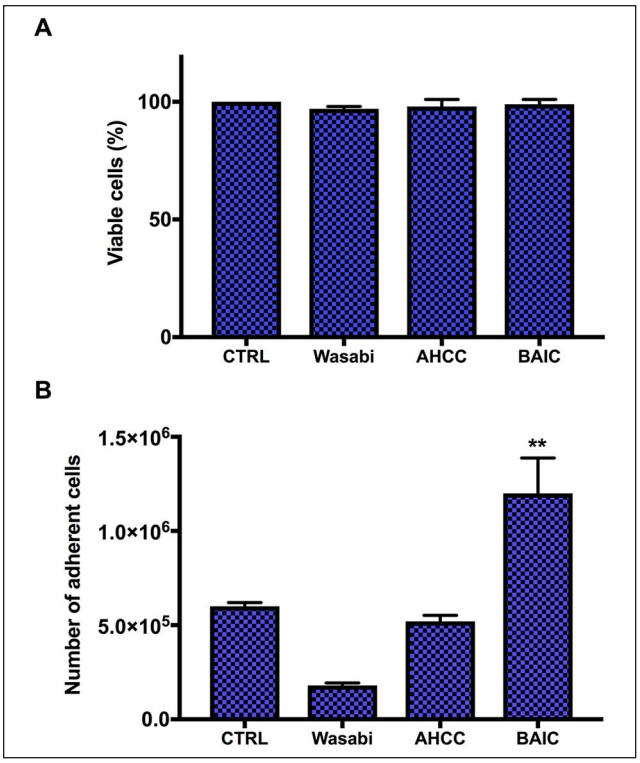

Figure 6. Graphs showing the effect of the combination BAIC on monocytic cells (ThP1 cell line). Cell viability following the treatment with bioactive immunomodulatory compound (BAIC) at 48 hours, compared with cells grown in standard conditions (CTRL), or treated with 7.5 µg/mL Wasabi (Wasabi) and 10 µg/mL active hexose correlated compound (AHCC) (A). The number of adherent cells is also reported following the treatment with BAIC (B).

potential role in inhibiting tumor initiative and progression. Formed mammospheres from MCF-7-derived CSC were visualized with a BX51 microscope (Nikon). Mammosphere size was determined following the exposure to BAIC (Figure 8A) and compared with those obtained treating cells with standard media (CTRL, Figure 8B). Results clearly demonstrated a 2-fold decrease in size, which was induced by the treatment with AHCC and Wasabi compared with CTRL. Values assessed around 40.61 ± 9.10 and 24.26 ± 5.1, respectively (Figure 8C).

Discussion

In this study, we aimed at evaluating the combinatorial effect between 2 natural compounds, AHCC and Wasabi, for their possible use as a nutritional supplement for integrative medicine. We first tested different concentrations of AHCC and Wasabi (ranging from 7.5 to 500 µg/mL) and evaluated their effectiveness in reducing viability in 2 adenocarcinoma cell lines (MCF-7 and Panc02). Once the minimal concentration

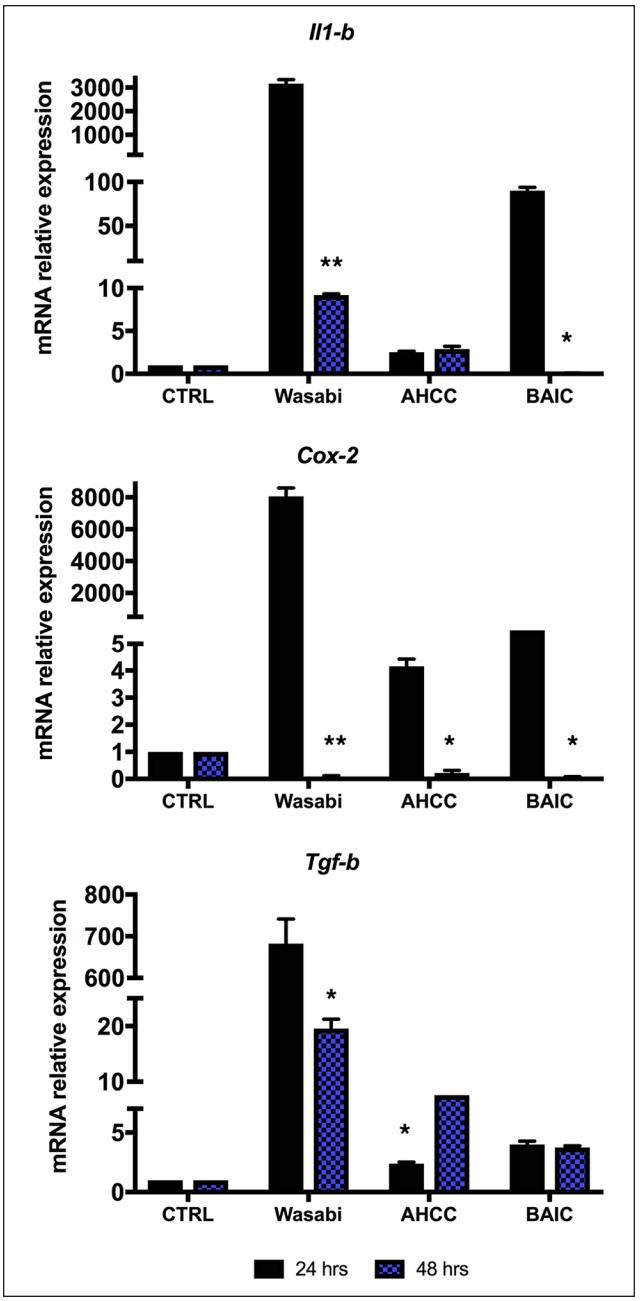

Figure 7. Quantitative polymerase chain reaction for the expression of immunomodulatory genes (*Tgf-β, Il-1β*, and *Cox-2*) following the treatment of ThP1 cells with bioactive immunomodulatory compound (BAIC), 7.5 µg/mL Wasabi (Wasabi), and 10 µg/mL active hexose correlated compound (AHCC), at 24 and 48 hours. Data are represented as fold-change compared with the expression levels found in untreated monocytic cells (CTRL) (n = 3; $^{**}P < .01$).

capable of inducing more than a 50% decrease in cell proliferation following 48 hours exposure was defined, we further tested whether the observed effect relied on the combination of AHCC and Wasabi, thus estimating whether by providing cells with the

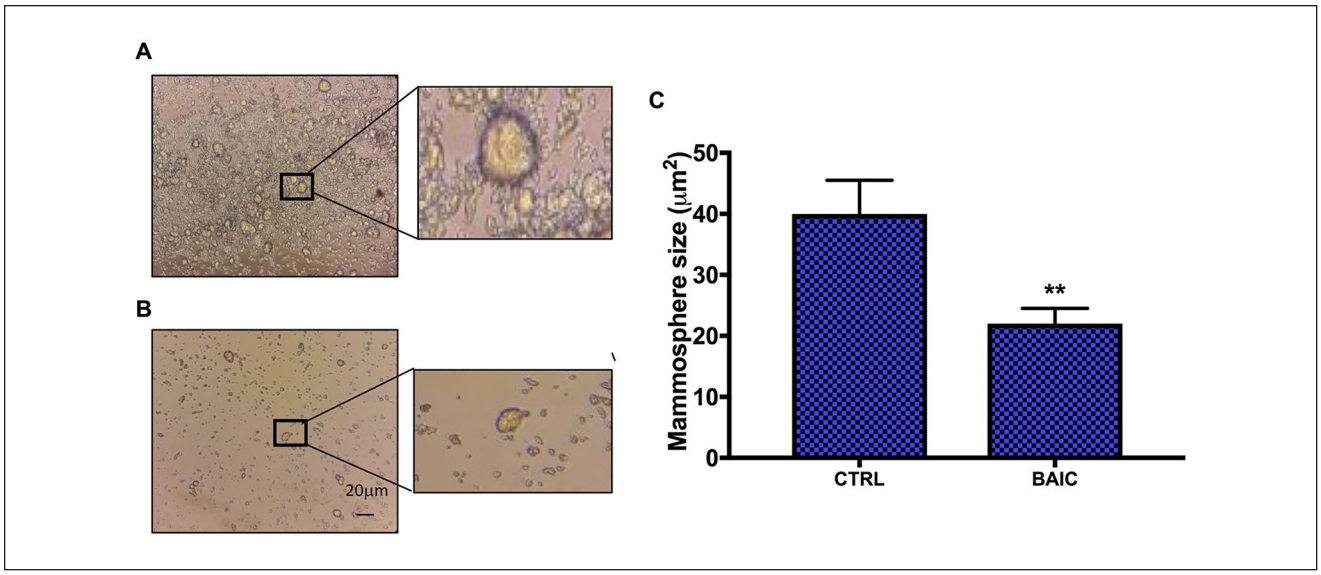

Figure 8. The potential inhibitory effect of bioactive immunomodulatory compound (BAIC) on mammospheres formation. Preliminary results show mammospheres containing MCF-7 cancer stem cells on day 5 after being cultured in the presence of BAIC (7.5 μg/mL Wasabi and 10 μg/ml active hexose correlated compound [AHCC]) (A) or in standard media (CTRL, B). Following the treatment, a statistically significant ($P < .01$) reduction in number and size of primary mammospheres is observed compared with control cells (CTRL) (C). Data are represented as means ± standard deviations (n = 3).

single compounds, it would be possible to achieve the same result. Interestingly, data obtained showed a marked reduction in viability when the compounds were combined even at very low concentrations (7.5 μg/mL for Wasabi and 10 μg/mL for AHCC) compared with their single counterparts. Results were assessed around 52% and 55% for MCF-7 and Panc02, respectively. Literature reports that a greater reduction (of about 64.9%) in MCF-7 proliferation can be achieved by the sole use of Wasabi only if a greater concentration (250 μg/mL) of the crude extract was applied.[24] Contrarily, we only observed a slight decrease (23%) in the percentage of proliferative MCF-7 cells following the treatment with Wasabi as single agent. However, by combining Wasabi with AHCC, we demonstrated that only a 33.3-fold less Wasabi is required to obtain a marked reduction in MCF-7 proliferation. When the pancreatic Panc02 cell line was tested, we showed that also the single agents (Wasabi and AHCC) were able to induce a significant reduction in cell proliferation (57% and 36%, respectively), with Wasabi revealing a reduction that was found similar to BAIC (55%). Furthermore, according to the existing literature, no cytotoxic activity of AHCC used as single agent was observed at 24 hours on MCF-7 and Panc02[12] (data not shown). By providing cells with 10 μg/mL AHCC, we found a 20% reduction at 48 hours in MCF-7. It is worth mentioning that previous evidence coming from other research groups highlights no cytotoxic effect that could be ascribed to AHCC even at clinically relevant concentrations.[25] The above findings demonstrated that BAIC has an antiproliferative impact on the adenocarcinoma cell lines tested (MCF-7 and Panc02) with differences that are probably associated to the nature of the cells used. According to our study, Chen et al also reported a great reduction in human pancreatic cancer cells viability following the exposure to the natural Wasabi compound, the 6-MITC, and its chemical derivatives.[26] Based on the evidence provided by other studies on the role of natural compounds on cancer cells,[27,28] the antiproliferative effects could be the result of the induction of apoptosis, and/or cell cycle arrest. The cell cycle analysis we performed demonstrated an increase in the percentage of cells in G_0/G_1 phases when MCF-7 and Panc02 cells were treated with BAIC at 48 hours. Similar results were observed following the treatment with other natural compounds with anticancer effect,[29,30] including curcumin,[30] casticin,[23] arctigenin,[31] and α-mangostin.[32] Although in line with these previous experiments, our data are in conflict with those obtained by other groups on the role of Wasabi on cell cycle distribution, which demonstrated a dose-dependent marked arrest in the G_2/M phase by providing colon cancer cells with *Wasabia japonica* extracts.[26,33] We hypothesize that discrepancies between ours and previously reported data may be the result of the lower concentrations used in the study. However, according to our studies, groups focusing on the effect of the active component of Wasabi, the 6-MITC, on other human cell lines (ie, acute promyelocytic leukemia, HL-60) demonstrated an increased percentage in cells at the G_0/G_1 phase following the treatment with 0.8 μg/mL Wasabi.[34] On the other hand, no significant differences have been noticed in cell cycle distribution following the treatment with AHCC, which is consistent with its role as coadjutant of antitumorigenic therapies than as single agent.[25,35,36]

Since the mode of action of natural compounds inducing the cell cycle arrest has been closely related to the activation of the apoptotic cascade,[37] we investigated whether the reduction of viable cells following the treatment with BAIC could also be ascribed to apoptosis in both cell lines. Following the treatment, a statistically significant increase in the percentage of apoptotic Panc02 and MCF-7 cells was demonstrated (up to 24% for apoptotic cells for Panc02 and 50% for MCF-7). A general increase was also observed following the treatment with Wasabi and AHCC as single agents compared with control cells. In both cases, the results are in line with those obtained from the MTT assay. To confirm our observations, we proceeded with analyzing the expression of 2 factors related to the apoptotic cascades, the pro-apoptotic member of the *Bcl-2* family Bax-2[38] and the effector molecule responsible for the activation of apoptosis through the caspases pathway, *Apaf-1*.[39] Our data indicated that an increase in the mRNA levels of *Bax-2* was induced in MCF-7 cells by the BAIC treatment and was found enhanced compared with the single counterparts (Wasabi and AHCC). A relative upregulation in the expression of *Apaf-1* was also observed although at lower levels with regard to those displayed by Panc02. In fact, we hypothesize that BAIC activates different apoptotic mechanisms in the 2 cell lines tested, with MCF-7 undergoing a marked trigger of the Bcl-2-dependent cascade[40] and Panc02 eliciting the family of cysteine proteases, the caspases.[41] Whether this activation is p53 dependent is still under consideration in our laboratory as data obtained show differences between the cell lines and support the existing literature on the discrepancy in the apoptotic pathways elicited by cancer cells following the exposure to Wasabi[42] or AHCC.[43]

It has been widely established that a small population of tumorigenic cells (CSC) exists in several human cancers. CSC are able to self-renew and to perpetually proliferate to initiate and develop cancer.[44] As such, their fundamental involvement in tumor progression has highlighted the urgency to develop therapeutic strategies capable of targeting CSC for cancer treatment or prevention.[45,46] With this in mind, we also wanted to test the combinatorial effectiveness of BAIC on the activity of MCF-7-derived CSC. The preliminary data obtained emphasize the potential role of the combination of AHCC and Wasabi in suppressing breast CSC progression and were consistent with previously published studies showing antitumorigenic agents with marked potential in inhibiting mammospheres development.[40] Specifically, our study demonstrates a 2-fold decrease in mammosphere size and number when mammospheres produced by MCF-7-enriched CSC were treated with BAIC for 5 days. Fani et al suggested that the reduction in mammosphere size is correlated to the decrease in cancer progression.[40] While no reports exist on the effect of Wasabi on CSC-derived mammospheres, a recently published article highlights the role of AHCC extracts to inhibit mammosphere growth in 3 cell lines.[47]

Finally, when BAIC were provided to monocytes to understand whether such combination could concomitantly exert negative effects on immune cells, no cytotoxic effects were detected. Cell viability was found to be comparable to control groups in all experimental conditions. Interestingly, we also observed a hypothetical activation of the monocytic cells used following the treatment, as demonstrated by the adhesive properties they acquired.[48] Based on these observations and on previously published evidence on the role ThP-1-derived macrophages play in modulating the apoptotic response to cancer cells,[49] we moved forward and aimed at shedding light on the phenotype of such macrophages-like cells. We then evaluated the expression levels of inflammatory genes suggesting the immunomodulatory potential of the BAIC mixture compared with the single components. Our data demonstrated an upregulation of the cyclooxygenase-2 (*Cox-2*) soon after the treatment with a significant reduction compared with control cells at 48 hours. This gene has not been generally associated to normal cells or tissues, nor to resting cells, although its results are highly expressed following cell exposure to inflammatory cytokines, growth factors, and molecules able to induce carcinogenesis.[50] We also analyzed the expression of *IL1-β* following the exposure to BAIC and found a statistically significant increase following the exposure to Wasabi at 24 hours, which appeared to be reduced over time, with expression levels that were comparable to control. This result is particularly interesting as it has been reported that macrophage-derived *Il-1β* also stimulates the growth of cancer cells,[51] and a decrease in its expression could lead to the inhibition of cancer progression. According to previous studies showing a marked increase in *Il-1β* in in vivo setting following the treatment with AHCC,[48] we only observed an average of 2.5-fold upregulation in its expression at 24 and 48 hours. Furthermore, there was no increase in the expression levels of the third marker analyzed, transforming growth factor-*β* (*Tgf-β*), which has been suggested to determine an augmentation of the angiogenic properties of tumor and support its progression.[52,53] In our experiments, an average 4-fold increase was found at 24 and 48 hours in the BAIC group, which was reduced compared with the single use of AHCC and Wasabi.

Taken together, data obtained herein not only indicate a combinatorial effect of BAIC but also highlight the immunomodulatory role of the combination as no cytotoxic effect to immune system was observed while beneficial effects were rather found. Further studies will be required to elucidate the mechanisms targeted by such combination of natural compounds. Particular emphasis will be given to the NF-κb, Wnt, and Notch self-renewal pathways, which have been demonstrated to be activated

by other bioactive natural products such as curcumin[54,55] in tumor cells and CSC, as well as to the apoptotic cascades activated by the BAIC. Our preliminary studies on the immunomodulatory role of BAIC opens the possibility to further investigate paracrine signals between cancers cells and the immune system. Our study provides evidence on the combinatorial action that occurs when 2 natural compounds with demonstrated anti-inflammatory and anticancer effects are combined and suggests such combination as novel adjuvant therapy to support chemotherapy while controlling side effects. Further investigations are required to highlight the role BAIC play in modulating the immune system in case of inflammation as compared with physiological conditions.

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Supplemental Material

Supplemental material for this article is available online.

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