


Evaluation of Active Hexose Correlated Compound (AHCC) in Combination With Anticancer Hormones in Orthotopic Breast Cancer Models

Integrative Cancer Therapies
2017, Vol. 16(3) 300–307
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DOI: 10.1177/1534735417704948
journals.sagepub.com/home/ict


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Abstract

Objective. To determine the impact on antitumor activity when active hexose correlated compound (AHCC) in combination with anticancer hormonal agents in orthotopic mouse models of human estrogen receptor positive breast cancer and evaluate impact of AHCC on aromatase activity. **Methods.** The study consisted of 7 treatment arms (n=10) conducted in 2 breast cancer mouse models: MCF-7 and ZR-75. Treatment groups included untreated, vehicle, AHCC 50 mg/kg, AHCC 50 mg/kg + tamoxifen 10 mg/kg, tamoxifen 10 mg/kg, AHCC 50 mg/kg + letrozole 10 µg/mouse, or letrozole 10 µg/mouse. All treatments were administered daily by oral gavage for 12 weeks. Tumors were measured 3 times a week. In vitro estrone and 17β-estradiol enzyme immunoassay was used to evaluate aromatase activity. **Results.** There was no difference in the activity with the combination of AHCC + tamoxifen compared with tamoxifen ($P = 0.29$). In the ZR-75 model (catechol-O-methyltransferase [COMT] wild-type), there was no difference in activity with the letrozole + AHCC compared with letrozole. However, in the MCF-7 model (COMT variant), AHCC + letrozole resulted in a decrease in activity compared with letrozole ($P < 0.01$). Immunoassay data suggested that AHCC is a potential inducer of aromatase activity. In both tumor models, there was cytotoxicity observed with AHCC compared with untreated ($P < 0.02$). **Conclusion.** AHCC did not change the activity of tamoxifen. AHCC may have some interaction with letrozole in patients with COMT variant genotype. AHCC had cytotoxicity that warrents additional studies to evaluate its potential role for consolidation/prevention of breast cancer.

Keywords

AHCC, tamoxifen, letrozole, breast cancer, drug interactions

Submitted February 19, 2016; revised February 8, 2017; accepted February 19, 2017

Introduction

Breast cancer is the most frequently diagnosed form of cancer and the second leading cause of death in Western women, with 240 190 new cases diagnosed in 2015.¹ Death and most of the complications associated with breast cancer are a result of metastasis developing in distant organs, including bone, lung, liver, and brain.^{2,3} More than half of new breast cancer cases occur in women older than 65 years.⁴ Fortunately, numerous clinical trials have demonstrated the beneficial role of anticancer hormone agents for prevention of recurrence in patients with hormone-responsive tumors.⁵⁻⁷ Hence, it is critical not to interfere with the activity of these agents in breast cancer patients who have already achieved initial clinical complete response.

Active hexose correlated compound (AHCC; Amino Up Chemical Co, Ltd, Sapporo, Japan) is a fermented extract prepared from mycelia of a *Basidiomycete* mushroom (*Lentinula edodes*) that has been proposed to have numerous health benefits mediated by both immunomodulatory and

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antitumor effects.⁸⁻¹¹ The primary active component is acylated α -glucan and contains <0.2% β -glucans, which have a molecular weight of 10 000 to 500 000, with the lower molecular weight α -glucans having much better oral absorption.

Over the years, there have been numerous clinical studies on AHCC that have demonstrated benefit in decreasing the side effects associated with anticancer chemotherapy.^{12,13} A previous study evaluating the phase I metabolism found that AHCC was a substrate as well as inducer of the CYP450 2D6 pathway.¹⁴ Otherwise, the overall data suggested that AHCC would not interact with the other CYP450 pathways and would be generally safe to administer with most other chemotherapy agents that are not metabolized via the CYP450 2D6 pathway.¹⁴ Tamoxifen, however, is a substrate of CYP450 2D6 pathway and has growing literature demonstrating the potential for altered activity caused by polymorphism in the CYP2D6 pathway.^{15,16} The phase II metabolism pathway demonstrates that AHCC did not have potential for drug interactions with substrates of glutathione-S-transferase, catechol-O-methyltransferase (COMT), or uridine diphosphate-glucuronosyltransferase (UGT) 2B17 pathways but may have potential interactions with quinone oxidoreductase, UGT1A3, or UGT 1A6.¹⁷ Although AHCC induction of the COMT pathway was less than that of the control inducer (rifampin), a difference was observed between low and higher concentrations of AHCC, suggesting some potential for interaction with the COMT pathway, which is the primary pathway for estradiol metabolism. COMT plays a critical role in the detoxification of reactive catechol estrogen intermediates. The conversion of 17β -estradiol via CYP450-mediated metabolism results in 4 primary estrogen metabolites. First 2-hydroxyestrone (2-OHE₁), which is considered the “good estrogen” not associated with carcinogenesis, undergoes methylation to 2-methoxyestrone. The 2 catechol estrogens (2-hydroxycatechol estrogen [2-OHE₂] and 4-hydroxycatechol estrogen [4-OHE₂]) have been widely associated with the formation of estrogen-mediated cancers as well as 16-hydroxycatechol estrogen (16-OHE₁), which undergoes reduction to estriol, which is known to increase the risk of breast cancer.¹⁸ 2-OHE₂ is formed by metabolism of 17β -estradiol by CYP450 1A1, 1A2, and 3A isoenzymes.¹⁹ CYP450 1B1 is primarily responsible for the formation of 4-OHE₂ and is constitutively expressed in the breasts, ovaries, adrenal glands, and uterus as well as in several other tissues, linking its formation with a variety of cancers in women, such as breast cancer.¹⁸

The expression of the COMT variant genotype is associated with alteration in detoxification of both 2-OHE₂ and 4-OHE₂ free radicals. Hence, there were some concerns as to whether there could be a potential for a drug-herbal interaction. Letrozole is a substrate of the CYP450 3A4 pathway and, therefore, was thought to have less potential for drug-herbal interaction; however, the role of estrogen metabolism via aromatase pathways also had to be considered. The

aromatase enzyme is responsible for the peripheral conversion of hormones, specifically androstenedione to estrone and testosterone to estradiol.

The primary objective of this study was to determine the activity of AHCC in combination with commonly used anticancer hormonal agents, tamoxifen or letrozole, in an orthotopic mouse model of human estrogen receptor positive (ER+) breast cancer, one a COMT variant (MCF-7) and the other a COMT wild type (ZR-75). In addition, the secondary objective was to conduct *in vitro* studies to evaluate the impact of AHCC on aromatase activity.

Materials and Methods

Chemicals and Reagents

AHCC was generously provided by Amino Up Chemical Co, Ltd, Sapporo, Japan. Estradiol pellets (0.72 mg) were purchased from Innovative Research of America (Sarasota, FL). Fetal bovine serum (FBS) and trypsin-EDTA were purchased from GIBCO Invitrogen Co (Carlsbad, CA); 3-(4,5-dimethylthiazole)-2, 5-diphenyl tetrazolium bromide (MTT), dimethyl sulphoxide, insulin, and antibiotics were purchased from Sigma-Aldrich Co (St Louis, MO). Estrone and 17β -estradiol enzyme immunoassay kits were purchased from Arbor Assays (Ann Arbor, MI).

Cell Culture

Human breast cancer cell lines MCF-7 (ER+/COMT-variant) and ZR-75 (ER+/COMT-wild type) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The MCF-7 breast cancer cell line was propagated in a medium consisting of Eagles Minimum Essential Media (EMEM) with 2 mM L-glutamine and Earl's Buffered Saline Solution (BSS) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% FBS, 1.0 mM sodium pyruvate, and 4 mg/mL insulin solution. The ZR-75 breast cancer cell line was propagated in a medium consisting of RPMI 1640 with 10% FBS and 1% antibiotics. Human ovarian granulosa-like tumor cell line (KGN) was obtained from Cell Bank, RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). The KGN cell line was propagated in a medium consisting of DMEM: F12 with 10% FBS and 1% antibiotics. These cells were grown in 75-cm² culture flasks in 5% CO₂ in air at 37°C to 90% confluence. Cell lines used for this study were maintained for less than 15 passages in order to prevent major changes in cell line characteristics.

Human Breast Cancer Orthotopic Mouse Models

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee prior to initiating any

animal work. For each mouse study, 70 female nude mice, 6 to 8 weeks old, were obtained from Charles River Laboratories (Wilmington, MA). All the mice weighed about 22 to 26 g, and they were maintained 5 per cage in a specific pathogen-free barrier room, at temperatures of $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and $45^{\circ}\text{C} \pm 3^{\circ}\text{C}$ at room humidity. They had free access to autoclaved food and reverse osmosis autoclaved water. The experimental procedures and handling of the mice were in strict accordance with the guidelines for the care and use of laboratory animals.

The 2 most widely used hormone breast cancer cell lines, MCF-7 and ZR-75, were used in this study. These cell lines require estrogenic supplementation for tumorigenesis in nude mice; therefore, slow-release estrogen pellets (0.75 mg) were implanted subcutaneously in each mouse on the day of cell injection. To implant the estradiol pellets, mice were anesthetized with 5% isoflurane, using an automated anesthesia machine, which was continued throughout the procedure using 2.5% isoflurane introduced via a nose cone. Both MCF-7 and ZR-75 express the CYP19A1/aromatase pathway associated with estrogen metabolism.^{20,21}

MCF-7 cells (5×10^6) and ZR-75 (5×10^6) were dispersed in medium with 25% matrigel and were injected into female athymic (*nu/nu*) mice subcutaneously. Each mouse grew 1 tumor on the dorsal surface. Tumor measurements were obtained 3 times per week with electronic calipers (Mitutoyo, Utsunomiya, Japan). Mice were monitored daily for signs/symptoms of morbidity, including but not limited to lethargy, weight loss, anorexia, or hunching. Mice were killed humanely via CO_2 inhalation followed by cervical dislocation, when the tumor diameter was greater than 12 mm^2 and if a 10% or greater decrease in body weight was found during the study period. At the end of the study, all the remaining mice were killed. When killed, total tumor burden was determined by macroscopic dissection. Immediately after the mice were killed, tumors were surgically removed and stored at -80°C for further use.

Treatment

The mice were divided into 7 groups with 10 mice in each group. There were 7 arms in this study: no treatment control arm ($n = 10$); a vehicle control arm ($n = 10$); an AHCC treatment arm ($n = 10$), which received an oral dose of 50 mg/kg in 0.2-mL gastric gavage once daily for 12 weeks; the tamoxifen-alone treatment arm ($n = 10$), which received an oral dose of 10 mg/kg in 0.2-mL gastric gavage; the AHCC + tamoxifen treatment arm ($n = 10$), which received an oral dose of AHCC of 50 mg/kg in 0.2-mL gastric gavage and tamoxifen 10 mg/kg in 0.2-mL gastric gavage once daily for 12 weeks; the letrozole-alone treatment arm ($n = 10$), which received an oral dose of 10 $\mu\text{g}/\text{mouse}$ in 0.2-mL gastric gavage once daily for 12 weeks; and the AHCC + letrozole treatment arm ($n = 10$), which received an oral dose of

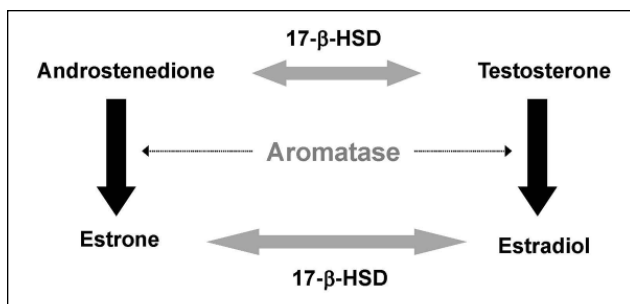


Figure 1. Summary diagram of aromatase activity in hormone metabolism.

AHCC of 50 mg/kg in 0.2-mL gastric gavage and letrozole 10 $\mu\text{g}/\text{mouse}$ in 0.2-mL gastric gavage once daily for 12 weeks. The vehicle control arm received an oral dose of 70:30 autoclaved water:polysorbate 80 mixture (0.2 mL, gastric gavage) per day for 12 weeks. All treatments started after a palpable tumor of at least 5 mm^2 was achieved and continued until completion of the study (12 weeks). All doses were selected based on mouse equivalent to human doses approved by the Food and Drug Administration or recommended by the manufacturer (Amino Up Chemical, Sapporo, Japan).

In Vitro Estrone and 17 β -Estradiol Enzyme Immunoassay

As per the study by Ohno et al,¹⁸ the KGN cell line, which has relatively high aromatase activity, was used for in vitro estrone and 17 β -estradiol enzyme immunoassays. Because the KGN cell line lacks 17 α -hydroxylase, it secretes little or no androstenedione, 17 β -estradiol, or estrone, making it a suitable experimental tool for aromatase assay (Figure 1). As per this study, briefly, 50 000 KGN cells/mL were added to each well and were grown at 37°C in DMEM F12 medium for 2 days. After 2 days, cells were treated with 3 clinically relevant concentrations of AHCC alone (low, 0.42 mg/mL; medium, 0.85 mg/mL; and high, 1.28 mg/mL), 1 clinically relevant concentration of letrozole alone (0.35 $\mu\text{g}/\text{mL}$), and AHCC plus letrozole for 24 hours. After 24 hours, 10 μL of 0.1 μM androstenedione and testosterone were added to respective plates as substrates for estrone and 17 β -estradiol, respectively, and cells were further incubated for 24 hours. Control wells had only media and no drugs or substrates. After the completion of treatment of cells, estrone and 17 β -estradiol enzyme immunoassays were performed according to the manufacturer's protocols. The concentration (pg/mL) of estrone and 17 β -estradiol produced in each well was measured and was compared with action of letrozole, which served as a positive inhibitor control with 100% aromatase inhibition activity. Each experiment was conducted in triplicate, and standard curves for estrone and

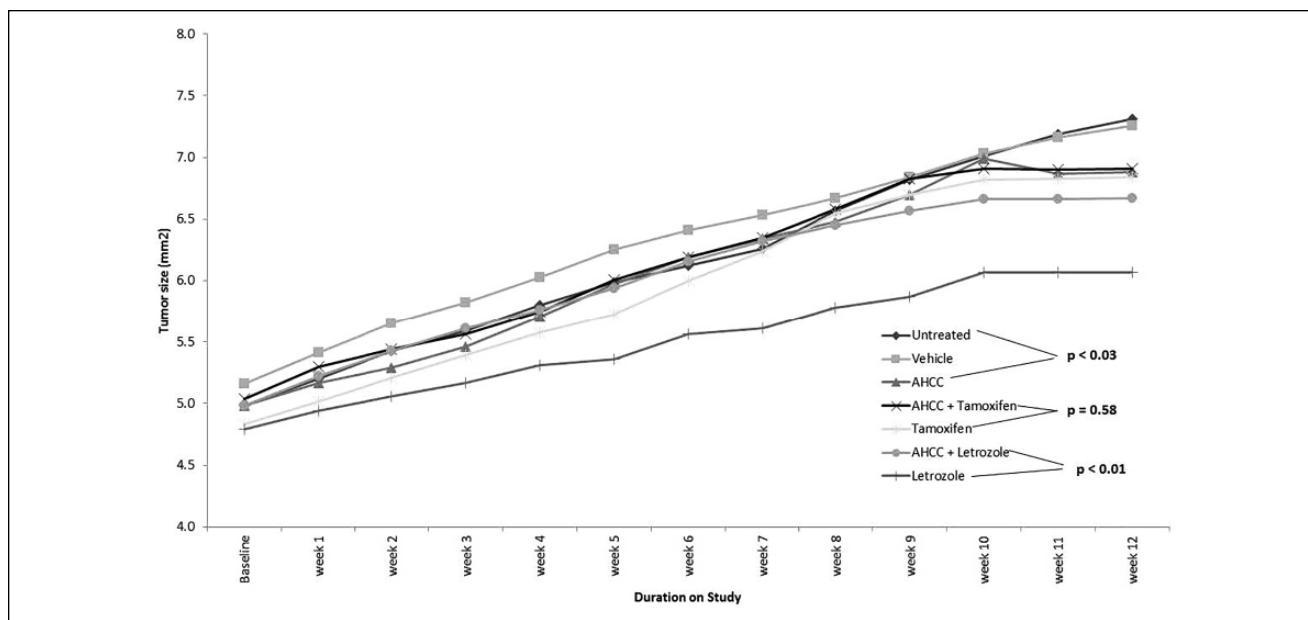


Figure 2. Effect of active hexose correlated compound (AHCC) on aromatase activity of letrozole: the KGN cell line, which has a relatively high aromatase activity, was used for in vitro estrone and 17β -estradiol enzyme immunoassays.¹⁸ Because the KGN cell line lacks 17α -hydroxylase, it secretes little or no androstenedione, 17β -estradiol, or estrone, making it a suitable experimental tool for aromatase assay. 17β -Estradiol and estrone were measured in picogram per milliliter concentrations and reported as the percentage change in concentrations in the presence of AHCC compared with the known aromatase inhibitor, letrozole. Data demonstrate that AHCC alone is a potential inducer of aromatase and potentially enhanced the inhibition activity of letrozole in converting androstenedione to estrone.

17β -estradiol were constructed. Figure 2 represents the percentage change in concentrations in the presence of AHCC compared with the known aromatase inhibitor, letrozole.

Statistical and Data Analysis

Each study treatment arm, AHCC alone, tamoxifen alone, letrozole alone, AHCC + tamoxifen or AHCC + letrozole included 10 mice (50 total) in which tumor growth inhibition/reduction after treatment was independently compared with the control group (no treatment/vehicle alone) with 10 mice. A total of 70 mice were included in the study. Power calculations were completed to determine that a sample size of 10 mice would be required to detect a 20% difference in response rate. An independent-sample Student's *t*-test was used to evaluate the differences in tumor size/growth over the duration of the study. For these experiments, α is set at 5% and β is set at 20% (power = 80%).

Results

MCF-7 (ER+, COMT Variant Type) Orthotopic Human Breast Cancer Mouse Model

In the MCF-7 orthotopic model, there was a significant 22.6% decrease in tumor growth with AHCC alone

compared with untreated or vehicle alone ($P < .03$) and a 10.8% decrease in tumor growth with the addition of a AHCC supplement to tamoxifen treatment compared with tamoxifen alone ($P = .58$). However, the combination of AHCC supplement to letrozole treatment resulted in a significant 27% increase in tumor growth compared with letrozole alone ($P < .01$; Figure 3).

ZR-75 (ER+, COMT Wild Type) Orthotopic Human Breast Cancer Mouse Model

In the ZR-75 orthotopic model, there was a 26.7% decrease in tumor growth with AHCC alone compared with untreated or vehicle alone ($P > .01$), and there was an 18.3% increase in the tumor growth with the addition of AHCC supplement to tamoxifen treatment compared with tamoxifen alone ($P > .07$). There was also a 29.3% decrease in tumor growth between the letrozole alone and letrozole + AHCC arms ($P > .01$; Figure 4).

In Vitro Estrone and 17β -Estradiol Enzyme Immunoassay

The effect of AHCC on aromatase activity of letrozole was evaluated by a previously validated in vitro model of estrone and 17β -estradiol enzyme immunoassays using the

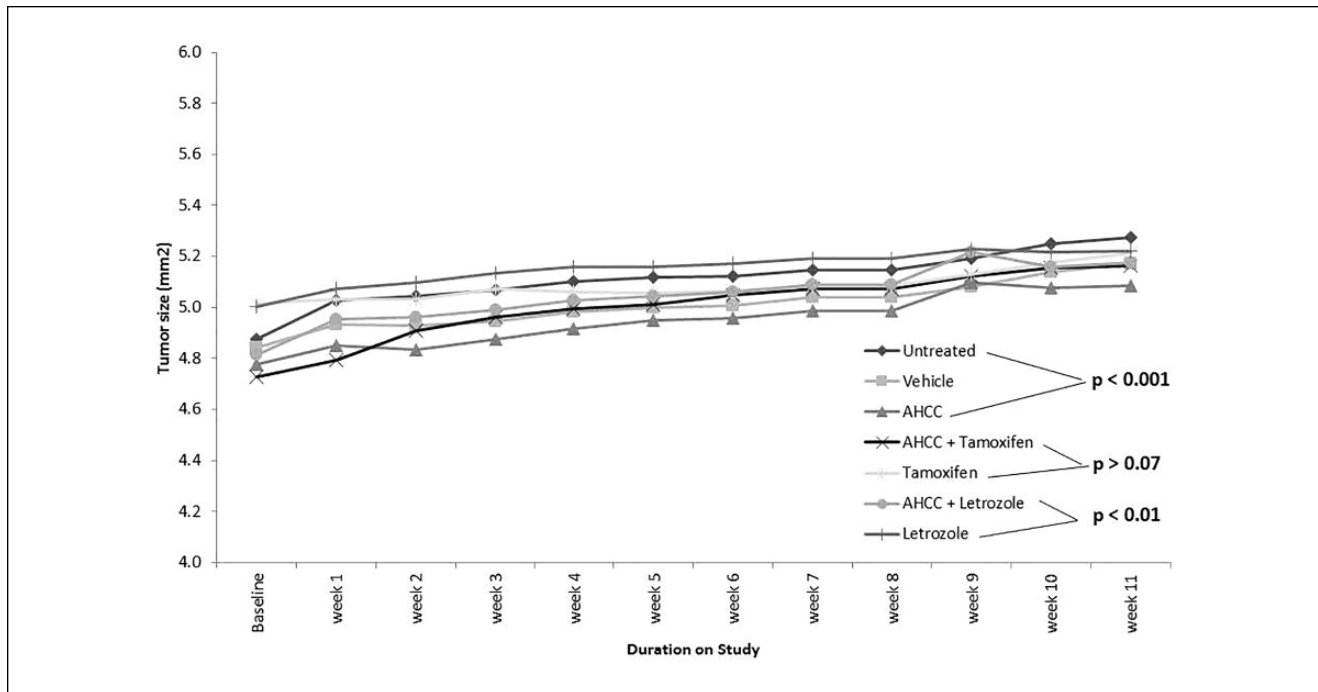


Figure 3. Efficacy of AHCC and a combination of AHCC with anticancer hormonal agents in an MCF-7 (ER+, COMT variant) orthotopic breast cancer mouse model after 12 weeks of treatment. Abbreviations: AHCC, active hexose correlated compound; ER+, estrogen receptor positive; COMT, catechol-*O*-methyltransferase.

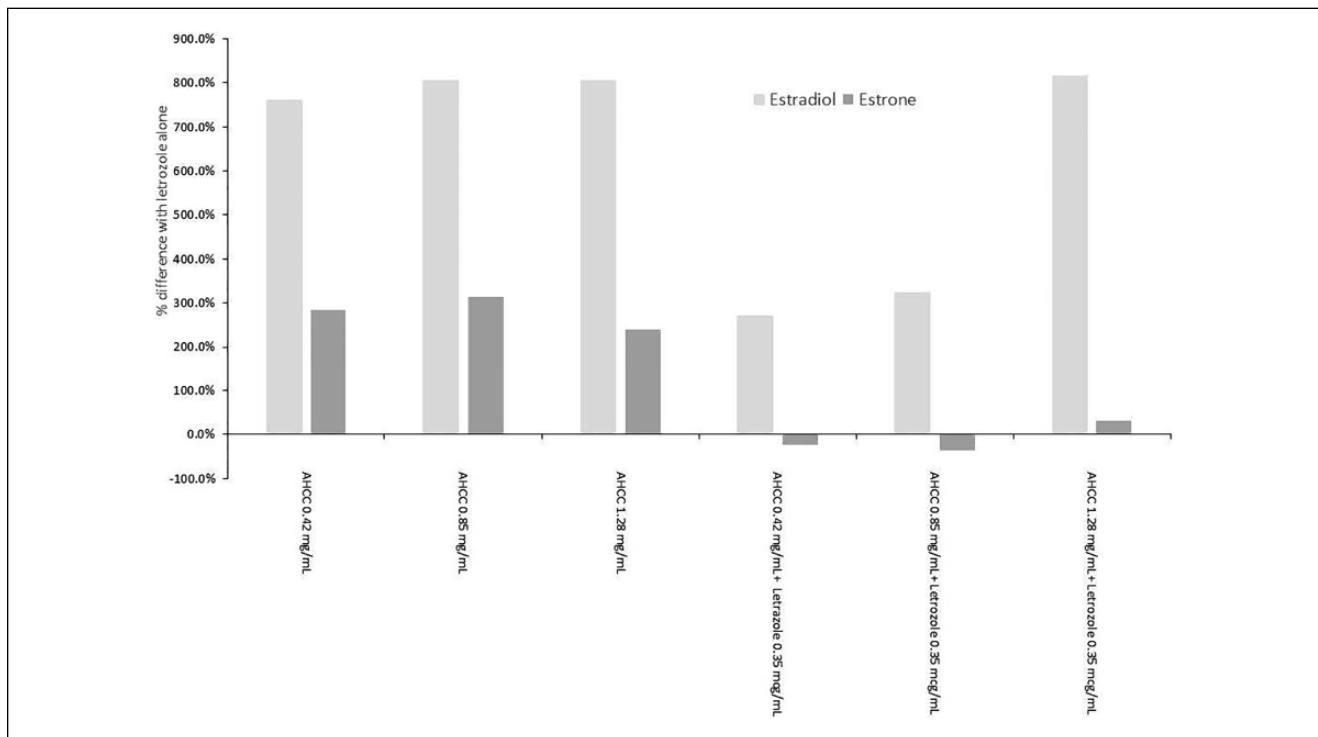


Figure 4. Efficacy of AHCC and a combination of AHCC with anticancer hormonal agents in a ZR-75 (ER+, COMT wild-type) orthotopic breast cancer mouse model after 12 weeks of treatment. Note that the y-axis starts at 4 to allow better display of data. Abbreviations: AHCC, active hexose correlated compound; ER+, estrogen receptor positive; COMT, catechol-*O*-methyltransferase.

KGN cell line.¹⁸ The data demonstrated that AHCC alone induced the conversion of testosterone by 7.6- to 8.0-fold and conversion of androstenedione to estrone by 2.4- to 3.1-fold. In combination with letrozole, a known aromatase inhibitor, there was only a 2.7- and 3.2-fold increase at the 2 lower concentrations of AHCC—0.42 and 0.85 mg/mL—but still an 8.1-fold increase at 1.28 mg/mL of AHCC in the conversion of testosterone to estradiol. However, the AHCC 0.42 mg/mL and AHCC 0.85 mg/mL concentrations slightly improved the aromatase inhibition of letrozole conversion of androstenedione to estrone, with only a slight increase at the supratherapeutic concentration of AHCC 1.28 mg/mL. The aromatase activity of AHCC alone and in combination with letrozole is summarized in Figure 2.

Discussion

Although tamoxifen is a substrate of the CYP450 2D6 pathway and AHCC was previously reported as a potential inducer of the CYP2D6 pathway, this study demonstrated that AHCC did not interfere with activity of tamoxifen in the orthotopic breast cancer mouse models. Potential growth inhibitory activity was also observed with AHCC alone. Tamoxifen is a potent selective estrogen receptor modulator that inhibits tumor growth by modulation/blocking of the estrogen receptor. AHCC is not known to have any impact or binding affinity to the estrogen receptor site; therefore, it was not surprising that the combination of AHCC with tamoxifen did not significantly affect tumor growth compared with tamoxifen alone. Letrozole is potent aromatase inhibitor with activity in both the MCF-7 and ZR-75 mouse models in this study. Although AHCC did not demonstrate interaction with the aromatase inhibitor letrozole in the COMT wild-type model (ZR-75), there was significant decrease in letrozole activity when combined with AHCC in the COMT variant model (MCF-7). In a follow-up study, the aromatase assay data demonstrated that AHCC is a potential inducer of aromatase enzyme activity when given alone, yet it did demonstrate potential slight improvement in inhibiting conversion of androstenedione to estrone at the 2 lower AHCC concentrations but not at the higher AHCC concentration. Additional studies to evaluate its potential effect are warranted.

The COMT pathway is the primary metabolic pathway for detoxification of catechol estrogens, 4-OHE₂ and 2-OHE₂, via methylation to form 4-methoxy catechol estrogen (4-MeO-E₂) and 2-methoxy catechol estrogen (2-MeO-E₂), which could be oncoprotective in addition to its role in the prevention of quinone formation. The COMT pathway does not have an impact on 16-OHE₂ levels. There are emerging data that there is a relationship between risk of estrogen-mediated cancers and inhibition of the COMT detoxification pathway, but this is also more dependent on

the COMT wild-type (108Val/Val) versus variant (108Met/Met) genotype expression. This variant polymorphism is associated with 3- to 4-fold less COMT metabolic activity, which could result in a significant decrease in catechol estrogen detoxification, ultimately leading to an increase in oncogenic activity. In the US population, 25% of the Caucasian population is homozygous for the variant polymorphism in the COMT gene and potentially may have an increased risk for the development of estrogen-mediated cancers.²¹ In general, women have a 20% to 30% lower COMT activity in comparison to men, and with potential for the presence of COMT polymorphism, this risk could be even greater.²² In this animal study, there was a difference observed between the COMT wild type (ZR-75) and COMT variant (MCF-7). In the COMT wild-type model (ZR-75), there was a significant decrease in tumor growth with the combination of letrozole +AHCC compared with letrozole alone. However, in the COMT variant model (MCF-7), there was a significant decrease in letrozole aromatase inhibitor efficacy when given in combination with AHCC compared with letrozole alone, which may have been a result of AHCC induction of aromatase activity in the presence of COMT variant that decreases detoxification of oncogenic catechol estrogens.

Other mushroom extracts, such as the white button mushroom, have demonstrated some potential aromatase inhibition.^{23,24} The study by Shuiian et al²³ identified the stuffing mushroom as the most potent inhibitor of aromatase activity; however, the shiitake, white button mushroom, portobello, crimini, and baby button mushrooms also showed significant inhibitory effects. The potential aromatase inhibitory effects suggest that mushroom extracts may have a role in the management of hormone-mediated cancers such as breast cancer.²⁴ Several studies have reported a variety of therapeutic effects, including antioxidant and anticancer activity, prevention of the onset of diabetes, prevention of liver injury, and improvement of immune response.⁸⁻¹⁰ AHCC is predominantly used for its purported ability to stimulate the immune system and as an adjuvant therapy to reduce the adverse side effects of chemotherapy.¹¹ AHCC is also well tolerated with minimal to no side effects reported in clinical trials.^{25,26} In this current study, the potential growth inhibition of AHCC in breast cancer was observed, which might be attributed to multiple mechanisms that need closer evaluation in future studies. Previously Hunter et al²⁷ demonstrated AHCC induction of apoptosis via the bcl-2 pathway, which may have contributed to the antitumor activity observed in the breast cancer mouse models in this study.²⁸ However, the applicability of data is limited because it was evaluated only in the 2 selected ER+ breast cancer orthotopic mouse models. The activity of AHCC in ER-, especially triple-negative breast cancer, is unknown. Confirmation in other breast cancer cell line mouse models is necessary as well as close

evaluation of the impact that COMT variant hybrid versus COMT wild-type expression has on the aromatase inhibition pathway.

Conclusion

Based on the data from the 2 orthotopic human breast cancer mouse models in this study, it appears that AHCC can be coadministered with tamoxifen without affecting antitumor activity. AHCC may have some interaction with letrozole in tumors with COMT variant genotype, which is the primary pathway for detoxification of oncogenic catechol estrogen metabolites. However, additional studies would need to be conducted in humans to determine the clinical significance, if any, of this potential interaction. AHCC alone demonstrated potential decreased tumor growth compared with no treatment. Additional studies to evaluate the potential role of AHCC in consolidation/prevention are warranted.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was generously supported by an unrestricted research grant from the Amino Up Chemical Company, Ltd, Sapporo, Japan.

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