Evaluation of active hexose correlated compound (AHCC) in combination with pegylated liposomal doxorubicin for treatment of ovarian cancer

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Summary: The objective was to define the mechanism of the growth inhibition of active hexose correlated compound (AHCC) alone and evaluate its activity in combination with pegylated liposomal doxorubicin (PLD). Scientific Methods: In vitro growth inhibition assays were completed with AHCC alone and in combination with PLD in panel of human cancer cell lines and findings confirmed in vivo in an ovarian cancer xenograft mouse model. AHCC mechanism of action was evaluated with immunoblotting and flow cytometry studies. Major Findings: The in vitro growth inhibition assays demonstrated additive activity when AHCC is co-administered with PLD. The combination of AHCC with PLD demonstrated a 64.1% reduction in tumor growth compared to the untreated group (p value = 0.03) and a 31.2% improvement in tumor response with combination regimen compared to PLD alone. No difference in toxicity was observed in the control or treatment groups. An increased expression of Bcl-2 was observed and induction of apoptosis confirmed in presence of AHCC. Conclusions: There is potential improvement in PLD activity when co-administered with AHCC and decrease side effects of PLD. A clinical study to evaluate the combination of AHCC plus PLD in the treatment of ovarian cancer is being pursued.

Industrial Relevance: This study presents an example of the successful integration of a well-known herbal supplement, AHCC, with traditional western medicine cytotoxic agent, pegylated liposomal doxorubicin, for the treatment of recurrent ovarian cancer. In addition to providing evidence of the efficacy of AHCC, the mechanism of improved activity was also investigated. Using a traditional approach this study provides pre-clinical data to support the benefits previously observed and reported in the clinical setting and supports future endeavors to integrate AHCC into standard of care to be given with chemotherapy. These finding are particularly beneficial in the treatment of recurrent cancer when maintaining a good quality of life during chemotherapy is a priority and allows patients to have a natural, nutritional approach to preventing and managing chemotherapy adverse effects.

Keywords: AHCC, Doxil, ovarian, cancer, drug resistant

Introduction

Ovarian cancer is the fifth leading cause of cancer among women and the number one gynecologic fatality with an estimated 21,880 women will be newly diagnosed and 13,850 deaths will occur in 2011.(American Cancer Society, 2011) Ovarian cancer is difficult to diagnose early and patients often initially present with advanced disease with a poor prognosis.(Barnes & Grizzel 2002) The current standard of care for first line treatment includes surgery and cytotoxic chemotherapy with 70 % of patients achieving an initial clinical complete response.(Partiridge & Barnes 1999) However, over 50% of patients will have recurrence within the first two years from completion on first line treatment which is often associated with taxane/platinum-resistant disease with a poor prognosis.(Partiridge & Barnes 1999, Martin 2002; Barnhill & Kurman 1995) The response rates with second-line agents such as gemcitabine, topotecan, or pegylated liposomal doxorubicin have not been impressive ranging from 15 to 30% in platinum resistant ovarian cancer. (Martin 2002; Barnhill & Kurman 1995).
Pegylated liposomal doxorubicin (PLD) (Doxil®, Ortho Biotech Products, L.P., Bridgewater, NJ) which was formulated in an attempt to further reduce cardiac toxicities associated with doxorubicin but still has significant dermatologic and hematologic toxicity. PLD has demonstrated clinical activity in the treatment of recurrent platinum resistant ovarian cancer patients with response rates of 17-26% with a median progression free survival of 5-6 months. (Thigpen & Aghajanian 2005, Muggia & Hainsworth 1997) While PLD has clinical activity the toxicities are often challenging for this patient population who is typically of advanced age and may have other co morbid conditions. (Thigpen & Aghajanian 2005)

Active Hexose Correlated Compound (AHCC, Amino Up Chemical Co., Ltd., Sapporo, Japan), is mixture of polysaccharides, amino acids, lipids and minerals extracted from the culture the basidiomycete mushroom Lentinula edodes (shiitake) that has been proposed to have many health benefits including both immunomodulatory and anti-tumor effects. (Miura & Kitadta 2010, Kidd 2000) In animal studies AHCC has demonstrated benefit to decrease the side effects associated with anticancer chemotherapy and to have a role in treatment and prevention of cancer. (Hirose & Sato 2007, Shigama & Nakaya 2009, Gao & Zhang 2006) The functions of particular interest in the oncology arena are AHCC’s immunomodulating and potential restorative effects on natural killer (NK) cells, macrophages and cytokines after anti-cancer chemotherapy as well as the potential to integrate AHCC with cytotoxic regimens for the treatment of cancer. (Gao & Zhang 2006, Sun & Wakame 1997)

Since there has been limited success with traditional cytotoxic chemotherapy agents in the persistent and recurrent ovarian cancer, alternative approaches need to be considered, including the integration of natural supplements with potential to improve growth inhibitory activity and adverse effects. This preclinical study was designed to evaluate the in vitro growth inhibition and confirm the in vivo activity of active hexose correlated compound (AHCC) alone and when given in combination with pegylated liposomal doxorubicin (PLD).

Materials and Methods

Supplies: All human cancer cell lines- ES-2, SKOV3, MES-SA, MCF-7 and HeLa were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and the SKOV3-IP1 ovarian cancer cell line was generously provided by Dr. Isaiah J. Fidler at The University of Texas M.D. Anderson Cancer Center (UTMDACC). All cell lines were maintained for less than fifteen passages. Pegylated liposomal doxorubicin (PLD) (Doxil®, Ortho Biotech Products, L.P., Bridgewater, NJ) and doxorubicin HCL (Bedford Laboratories®, Bedford, OH) was purchased from UTMDACC Division of Pharmacy (Houston, Texas). The active hexose correlated compound was generously provided by Amino Up Chemical Company, Ltd (Sapporo, Japan). Fetal bovine serum (FBS) and trypsin-EDTA were purchased from Gibco Invitrogen Co (Carlsband, CA). (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO), and tris base were purchased from Sigma-Aldrich Co (St. Louis, MO).

Chemicals and reagents: The BCA protein estimation kit was obtained from Pierce (Rockford, IL). All primary antibodies, including Bcl-2, Parp-1/2, and Caspase-3 and Goat and Mouse anti-rabbit IgG purified AB were obtained from Calbiochem-Novabiochem Company (San Diego, CA). The buffer solutions including: 40% Acrylamide/Bis Solution, 19:1, N,N,N’,N”- Tetra-methylene diamine (TEMED), 10% SDS, 10x Tris/Glycine/SDS buffer, 10x Trs/Glycine buffer, 10x Tris-Buffered Saline (TBS), Tween 20, Blotting Grade Blocker- Non fat dry milk, Immun-Blot PVDF Membrane for protein blotting (0.2 µM) were all purchased from Bio-Rad Laboratories (Hercules, CA). Finally, the ECL plus Western Blotting Detection Reagents were obtained from Amersham Biosciences Co (Piscataway, NJ).

Standard Solutions: In the in vitro studies a 100 mg/mL stock solution of AHCC was prepared by dissolving 1000mg of AHCC in 10 mL sterile distilled water. Doxorubicin was used in the in vitro studies was prepared by dissolving doxorubicin HCL 90 mg in 3 mL of sterile water for injection to achieve final concentration of 5 mg/mL. All further dilutions were prepared using respective cell culture media for each cell line. The MTT stock solution was prepared by dissolving 54 mg of MTT in phosphate buffered saline (PBS) to achieve a final concentration of 0.3 mg/mL.

For the animal studies the AHCC oral suspension was prepared once every seven days with 150 mg of AHCC powder of polysorbate-80 which achieved a final concentration of 5 mg/mL and stored in the 4°C. The intravenous doses of PLD were prepared by drawing up diluting 2 mg/mL solution with dextrose 5% water to achieve a final concentration of 0.25 mg/0.15 mL.

Cell Culture: The human uterine sarcoma cell line (MES-SA) was propagated in medium consisting of McCoy’s 5A medium with 10% FBS. The MCF-7 human breast cancer cell line was propagated in a media consisting of Eagles minimum essential medium (EMEM), 0.01 mg/mL bovine insulin and 10% FBS. The ES-2 clear cell ovarian carcinoma cell line and both the SKOV3-IP1 and SKOV3 adenocarcinoma ovarian cancer cell lines were both propagated with media consisting of McCoy’s5a medium with 2mM L-glutamine and 10% FBS. Finally, the
HeLa adenocarcinoma cervical cancer cell line was propagated in a media consisting of EMEM with 2 mM L-glutamine and Earl’s 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.

**Growth Inhibition Assay:** Growth inhibition assays were conducted as previously described. (Smith & Brown 2004). Briefly, cells were plated at 2,500-5,000 cells per well in 96-well and incubated at 37°C for 24-hours. Each of the cancer cell lines were treated with AHCC or doxorubicin at concentrations ranging from 1 x 10^{-6} µg/mL to 1000 µg/mL. Control wells had either no drug, media alone or were blank wells (no cells, drug or media). After a 72-hour incubation period, 25 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and 96-well plates incubated for two hours. Plates were then centrifuged and the supernatant was removed and then 50 µL of DMSO was added to each well and absorbance measured at 563 nm by FL600 Dual-Band plate reader from BioTek Instruments, Inc. (Winooski, VT).

The inhibitory concentration to achieve 20% and 50% cell death (IC_{20} and IC_{50}) for each drug and each cell line were calculated. The IC_{20} concentration was selected and use in combination AHCC + doxorubicin studies to ensure that enough viable cells were available completing for immunoblotting and flow cytometry experiments. All experiments were done in quadruplicate.

**SKOV_{3}-IP1 Xenograft Mouse Model:** The animal protocol was approved by the institutional animal care and utilization committee (IACUC) prior to initiating any animal work. All mice were handled according to the Guide for the Care and Use of Laboratory Animals. Although in vitro activity in the MTT assays also demonstrated synergistic activity in the MCF-7 cell line, only the SKOV_{3}-IP1 cell line was selected to evaluate the combination of AHCC with PLD in this animal study because the FDA approved indication of PLD is for the treatment of recurrent ovarian cancer with long term toxicities being the major treatment limitation hence the priority for translation to human correlative studies. On day -10, SKOV_{3}-IP1 cells (9 x 10^6) were injected intraperitoneal (IP) into each athymic mice for all treatment groups. Ten days after inoculation, mice were randomized to receive 0.25 mL of oral vehicle by gastric gavage once daily, single 0.15 mL dose of intravenous (IV) vehicle (0.9% sodium chloride solution), AHCC (50 mg/kg) via gastric gavage once daily, a single dose PLD (10 mg/kg) IV, or the combination of AHCC (50 mg/kg) via gastric gavage once daily and a single dose PLD (10 mg/kg) IV.

To assess IP tumor growth in the study the body weight, abdominal circumference, and accumulation of ascites were utilized as surrogates to reflect tumor burden were quantified three times a week. Abdominal circumference measurements were obtained at the body’s widest point throughout the experimental period while the animal was held in a prone position. In addition, mice were monitored daily for evidence of signs/symptoms of moribundity (weight loss, anorexia, hunching, listlessness, extensive enlarge abdominal cavity, lethargy, etc.). Ascites was removed and the volume of ascites measured as appropriate to relieve abdominal bloating up to three times then animals were sacrificed. At the end of the experiment, all remaining mice were euthanized via CO_{2} inhalation followed by cervical dislocation as per the institutional protocol. Post-mortem, macroscopic evaluation was completed to evaluate extent of tumor burden and all tumors were harvested, weighed and then stored for evaluation of molecular markers.

**Immunoblotting:** Evaluation of apoptosis included assessment of three commonly observed up-regulated proteins in ovarian cancer pathogenesis were evaluated including Bcl-2, Parp-1/2, and caspase-3. Samples were obtained from tumors in each of the respective treatment arms of the animal study and protein extracts were prepared by lysing cells on ice in 300-400 µL of NP40 lysis buffer. Pierce Micro BCA Protein Assay Kit (Pierce: Rockford, IL) was used to determine the protein concentration. For each series of protein determinations, a standard curve was constructed with known concentrations of bovine serum albumin (BSA). For direct immunoblotting, 50 µg protein were run on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes and probed with the appropriate antibodies using manufacturer’s protocol (Calbiochem- Novabiochem Co. San Diego, CA).

**Flow analysis of cell cycle:** Flow cytometry was performed as confirmation of apoptosis observed in the immunoblotting experiments. Following the 24 hour in vitro treatment of SKOV_{3} cells with IC_{20} concentration of AHCC alone, IC_{20} concentration of PLD alone, or combination of IC_{20} concentration of AHCC and PLD, all cells were pooled and washed in ice-cold PBS then fixed in ice-cold 1% paraformaldehyde (PFA) in PBS for 30 minutes on ice. Next, cells were washed again with ice-cold PBS then were fixed in 70% ethanol for overnight. After overnight incubation, the cells washed in ice-cold PBS then stained with propidium iodide (PI) in presence of RNase at 37° C for fifteen minutes. Cell cycle distribution was analyzed on 10,000 cells for each experimental condition. Data analysis was performed using Flow Cytometry and Cellular Imaging Core Facility of UTMDACC. Quantitative data from cell cycle analysis were graphed to illustrate the accumulation of percentage of cells in various phases of cell cycle.
Data Analysis: In the growth inhibition assay the interaction index to determine antagonism, additive or synergistic activity as described by Tallarida and colleagues was determined with following equation: \( \gamma = \frac{a}{A} + \frac{b}{B} \). Whereas \( A = \) dose of drug A alone that gives the specified effect; \( B = \) dose of drug B alone that gives specified effect; \( a = \) dose of drug A used in combination to achieve specified effect; and \( b = \) dose of drug B used in combination to achieve specified effect. (Tallarida 1996)  

The interaction index measures drug combination as follows: \( \gamma \) value equal to one it is additive; \( \gamma \) value that is less than one is synergistic and \( \gamma \) value greater than one is antagonistic. (Tallarida 1996)  

Descriptive statistics were utilized to summarize in vitro study results. Data was analyzed using a paired two tailed Student’s t-test for comparison between the treatment groups. Differences between groups were considered statistically significant at \( P < 0.05 \).  

In the animal study, each control, single chemotherapy treatment arm included ten mice in which tumor growth was measured and independently compared. A total of 60 mice were utilized for completion of the study. Power calculations were completed to determine that a sample size of ten mice is required to detect a 10% increase in inhibition of tumor growth of each of the respective study arms. An independent sample t-test was used in the data analysis to evaluate differences in tumor growth inhibition. For these experiments the \( \alpha \) was set at 5% and the \( \beta \) was set at 20% (power = 80%). The Holm’s methods were employed for evaluating differences between the multiple treatment agents and combination regimens. (Aickin & Gensler 1996)  

Results  

MTT studies employed determined the concentration to achieve IC\(_{20}\) for doxorubicin was 0.54 ng/mL, 0.1 ng/mL, 0.1 ng/mL, 0.005 ng/mL, and 0.004 ng/mL in the ES-2, SKOV\(_3\), MES-SA, MCF-7 and HeLa cancer cell lines, respectively. AHCC did not demonstrate any cytotoxic activity as a single agent at clinically relevant concentrations and however IC\(_{20}\) was achieved at concentrations of 0.478 mg/mL, 0.42 mg/mL, 0.823 mg/mL, 0.77 mg/mL, and 0.75 mg/mL in the ES-2, SKOV\(_3\), MES-SA, MCF-7 and HeLa cancer cell lines, respectively. The interaction index was determined for each cell line determined that AHCC plus doxorubicin was at least additive and potentially synergistic in the panel of human cancer cell lines. (Table 1)  

<table>
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<tr>
<th></th>
<th>ES-2</th>
<th>MES-SA</th>
<th>MCF-7</th>
<th>HeLa</th>
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This table represents the interaction index (\( \gamma = \frac{a}{A} + \frac{b}{B} \)) measure of drug synergism between PLD in plus AHCC in a panel of selected human cancer cell lines. \( a = \) dose of AHCC alone that achieved specified effect (IC\(_{20}\)); \( A = \) dose of AHCC used in combination to achieve same effect (IC\(_{20}\)); \( b = \) dose of PLD alone that achieved specified effect (IC\(_{20}\)); \( B = \) dose of PLD used in combination to achieve same effect (IC\(_{20}\)). \( \gamma = 1 \) it is additive; \( \gamma < 1 \) is synergistic and \( \gamma > 1 \) is antagonistic.
These *in vitro* findings were confirmed with *in vivo* ovarian cancer xenograft mouse model study to determine if the combination of AHCC plus PLD would have improved efficacy compared to PLD alone. The combination of AHCC plus PLD reduced tumor growth by 15.2% and 33.9% more than PLD or AHCC alone, respectively. The percent tumor reduction observed in the PLD plus AHCC and PLD group alone were reduced by 64.1% and 48.9%, respectively, compared to the untreated group. (Figure 1)

![Figure 1](image-url)  
*Figure 1 Mean percent reduction in tumor burden after completion of 28 day treatment cycle*

In a mouse xenograft model of SKOV3ip1 60 mice were randomly divided into six treatment groups (N=10) and received AHCC once daily alone, PLD once alone, or combination of AHCC and PLD, vehicle, or were untreated controls. The mean percent reduction was determined by calculating difference from untreated control with each respective treatment group. Data was analyzed using a paired two tailed Student’s t-test for comparison between the study groups. Differences between groups were considered statistically significant at P < 0.05.

Postmortem macroscopic evaluation of all mice was performed to confirm all results of tumor location and adjacent organ involvement. Tumors were present on the surface of the peritoneum cavity, uterus, liver, lung, intestines, and mesentery in all mice except the AHCC plus PLD treatment group the tumor was restricted to peritoneum cavity in 9 of 10 mice. The mean total tumor weight was reduced by 29.8% in the AHCC plus PLD treatment group compared to the PLD alone group. (Figure 2).

The safety and tolerability of AHCC was also evaluated during the study. There were no significant differences in the baseline total body weight nor any differences in the mean total body weight distribution throughout the treatment period between any of the treatment groups. No toxicity or morbidity observed during the study associated with the AHCC or PLD alone or in combination.
In a nude mouse xenograft model of SKOV3/JP1, 60 mice were randomly divided into six treatment groups (N=10) and received AHCC once daily alone, PLD, once alone, or combination of AHCC and PLD, vehicle, or were untreated controls. At postmortem examination, tumors were found on the surface of the peritoneum, uterus, liver, lung, intestines, and mesentery in all treatment and control groups. Data was analyzed using a paired two-tailed Student’s t-test for comparison between the study groups. Differences between groups were considered statistically significant at P < 0.05.

Following the completion of the in vivo ovarian cancer xenograft mouse study demonstrating AHCC plus PLD demonstrated improved efficacy compared to PLD alone, molecular studies were embarked to define the mechanism of additive cytotoxicity activity of this combination. Down regulation of unphosphorylated Bcl-2 was observed only in the AHCC treatment group and AHCC plus PLD which was suggestive of increase in apoptosis via the Bax-Bcl-2 pathway. (Figure 3)

**Figure 2** Summary of the mean tumor weight after completion of 28 day treatment cycle

Blot represent the protein expression analysis on six tumor samples collected at completion of the mouse study. 1- protein standard (+); 2- AHCC (A) + pegylated liposomal doxorubicin (D); 3- pegylated liposomal doxorubicin (D); 4- AHCC alone (A); 5- PO vehicle control; 6- IV vehicle control; 7- untreated

**Figure 3** AHCC down regulation of unphosphorylated Bcl-2 in tumor samples
Apoptosis was confirmed by flow cytometry studies that illustrated with a larger relative proportion of cells in the M1 phase of the cell cycle in the AHCC alone and combination AHCC plus PLD tumors compared to the control groups. (Figure 4)

**Figure 4** Confirmation of increase cytotoxicity observed with AHCC plus PLD

Flow cytometry experiments were performed on *in vitro* samples collected from SKOV3 treated with AHCC, pegylated liposomal doxorubicin, and combination of both at concentrations at the IC\textsubscript{20}. PI staining was utilized to quantify DNA content in samples.

**Discussion**

The integration of nutritional supplements and herbal products with traditional cytotoxic chemotherapy regimens has become increasingly of more interest in clinical practice based on patient preferences. (Navo & Phan 2004) However, there is growing evidence that herbal products, nutritional supplements and functional foods are not completely benign when combining such agents with other medications. Although a time consuming process, each combination should be evaluated for potential drug interactions specifically if antagonistic, additive or synergistic. When increase activity is observed *in vitro*, then appropriate *in vivo* studies should be conducted to confirm findings and ultimately studies should be conducted to define the mechanism of the improved cytotoxicity activity.

This study systematically evaluated the potential activity of AHCC alone and in combination with PLD. First the *in vitro* data demonstrated potential additive or synergistic activity, the SKOV3-IP1 xenograft which then was confirmed in the combination of AHCC plus PLD limited the spread of the tumor growth to the peritoneal cavity as well as reduced tumor volume close to one third compared to PLD alone. The combination regimen was well tolerated as well. The potential mechanism of improved activity with the addition of AHCC to PLD was also evaluated to determine AHCC has role in induction of apoptosis via the Bcl-2/BAX pathway achieved with doses of AHCC of 50 mg/kg in mice which is approximate equivalent to the recommended supplement dose of 3 g/day for humans.

There has been clinical benefit in patients with hepatocellular carcinoma, gastric cancer or colon cancer with use of single agent AHCC. (Matsui & Uhara 2002, Kawaguchi 2009) In the clinical study by Matsui and colleagues of 269 hepatocellular cancer patients, 113 received AHCC (3g/day) orally following curative hepatocellular surgery while the other 157 patients were just observed, and the AHCC treatment group had a significantly longer
progression free survival (HR, 0.639; CI 95%, 0.429-0.952; P = 0.0277) and overall survival (HR, 0.421; 95% CI, 0.253-0.701; P = 0.0009) compared to the observation group. (Matsui & Uhara 2002)

There is a growing body of literature that has demonstrated the immunomodulatory activity of AHCC. (Miura & Kitadte 2010, Gao & Zhang 2006, Matsui & Uhara 2002, Uno & Kosuna 2000) Since an athymic mouse has no immune function, the immunomodulatory pathways could be eliminated as potential mechanisms of additive cytotoxicity activity observed in this study. Multiple potential pathways for mechanisms of cytotoxicity were investigated with only the positive finding suggesting induction of apoptosis via down regulation of unphosphorylated Bcl-2 was observed in this study. This is one mechanism but is unlikely the only mechanism that has contributed to the anti-tumor activity of AHCC that has been demonstrated in this study as well as multiple clinical studies. (Miura & Kitadte 2010, Gao & Zhang 2006, Matsui & Uhara 2002, Uno & Kosuna 2000) This study only evaluated the non-immunomodulatory activity of AHCC in the xenograft mouse model. There is a potential for additional activity to be observed with the combination of AHCC to PLD in humans with functional immune systems both improved antitumor activity as well as decreasing side effects similar to what has been observed in AHCC single agent studies. A confirmatory clinical study is warranted to further evaluate the activity of PLD when given in combination with AHCC in recurrent ovarian cancer patients.

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References