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In Vitro Anticancer Effects of a Novel Immunostimulant: Keyhole Limpet Hemocyanin

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Background. **Keyhole limpet hemocyanin (KLH) is a recently described immune stimulant and hapten carrier derived from a circulating glycoprotein of the marine mollusk *Megathura crenulata*. It has been reported to be a potent form of intravesical immunotherapy for the treatment of transitional cell carcinoma of the bladder and has been used in a variety of genitourinary tumors. We hypothesized that KLH would be effective against other cancer cells *in vitro*.**

Methods. **Multiple cancer cell lines were tested, including estrogen-dependent breast (MCF-7), estrogen-independent breast (ZR75-1), pancreas (PANC-1, MIA-PaCa), and prostate (DU145). Serial twofold dilutions of KLH were prepared in sterile 96-well plates. Dose–response curves were performed beginning with a concentration of 100 μg of KLH/well and ending at a concentration of 0.8 ng/well. Cells were added at concentrations of 5×10^4 cells per well. Cell viability was evaluated at 24 and 72 h by MTT assay at an absorbance of 570 nm.**

Results. **Significant ($P < 0.05$) cancer cell growth inhibition was observed in four of the five cell lines tested at both time treatment intervals. The breast cancer line ZR75-1 exhibited a mean growth inhibition of 43 ± 1.1% (range 37 to 59%) at 72 h, whereas treated MCF-7 cells had an average of 39 ± 9.1% growth inhibition (range 35 to 44%) at these same concentrations. Treated PANC-1 cells had a mean growth inhibition of 19 ± 0.8% (range 4 to 46%) at 72 h. The DU145 prostate cancer cell line averaged a 6 ± 1.3% growth inhibition (range −19 to 55%) over the concentrations tested.**

Conclusions. **The direct growth inhibition of multiple tumor cell-lines exhibited by KLH is significant and warrants further *in vitro* mechanistic studies and *in vivo* experiments. Investigation into the efficacy and mechanism of response could directly lead to more effective treatment regimens for patients suffering from these diseases.**

Key Words: **novel cancer therapy; keyhole limpet hemocyanin; breast, pancreas, and prostate tumor cell lines.**

INTRODUCTION

Keyhole limpet hemocyanin (KLH) is a high-molecular-weight, copper-containing protein found in the hemolymph of the sea mollusk *Megathura crenulata* [1]. Its primary biological role is the uptake, transport, and release of oxygen during respiration. For over 40 years, researchers have been discovering that the KLH polymer has many other attributes, including the ability to enhance the host's immune response by interacting with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes [2]. It is a nonspecific immune stimulant that induces both a cell-mediated and a humoral response in both animals and man. Clinically, KLH is used specifically as a carrier for vaccines and antigens and as adjuvant treatment in regimens such as antimicrobial therapy.

KLH acts as a nonspecific immune stimulant when used as a conjugate vaccine. Cancer vaccines can stimulate antibody- and cell-mediated immune responses against tumor-associated antigens, namely cell surface carbohydrates and peptides found on melanomas, sarcomas, and cancers of the breast, prostate [3], and ovary. Researchers are finding, however, that breaking tolerance to these tumor antigens is best achieved using vaccines containing antigens chemically conjugated to KLH [3]. For example, sialyl-Tn, a carbohydrate associated with the MUCI mucin on a number of human cancer cells, is currently being tested in pa-

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tients with distant metastatic breast cancer [4]. Vaccination strategies using KLH, however, are directed against a single tumor antigen, and the search for new experimental modalities is emerging. Antitumor immune responses *in vitro* are currently being explored using pancreatic tumor cell lysate supplemented with the immunogenic protein KLH [5]. Results show that T cells specific for pancreatic carcinoma cells can be generated *in vitro* by lysate-pulsed dendritic cells and that the T cell response is enhanced by KLH.

KLH has been used as a form of therapy for patients with superficial bladder cancer for years in both the United States and Europe. In 1974, Olsson *et al.* immunized patients with 5 mg of KLH and observed a marked reduction in the recurrence of superficial bladder cancer [6]. Using a MBT-2 murine model of transitional cell carcinoma, Lamm *et al.* reported reduced tumor growth and prolonged survival in mice [7]. KLH has been tested against mitomycin C chemotherapy in patients and was found to be superior in preventing bladder tumor recurrence with no adverse local or systemic side effects [8, 9]. More recently, multicenter clinical trials have confirmed the efficacy of KLH given intravesically for 6 weeks to patients with various stages of bladder cancer [10]. Based on these studies, KLH is regarded as a safe and highly effective immunotherapy for superficial bladder cancer. KLH may also have considerable possibilities for the treatment of other carcinomas, in particular the epithelial-derived adenocarcinomas.

We hypothesized that KLH might have beneficial effects in other cancers. To assess the response of cancer cells to KLH *in vitro,* we tested human breast (MCF-7, estrogen dependent, and ZR75-1, estrogen independent), pancreas (PANC-1, MIA-PaCa), and prostate (DU145) cell lines.

MATERIALS AND METHODS

Keyhole limpet hemocyanin. KLH was supplied as a lyophilized powder from Biosyn Corp. (Carlsbad, CA). KLH was resuspended to the desired assay concentrations in tissue culture medium for each cell line. The highest KLH concentration tested was 100 μg/well. Serial two fold dilutions were carried out in 96-well microtiter plates to the final, lowest concentration of 800 pg/well.

Cell culture and reagents. All cell lines were purchased from American Type Tissue Collection (Manassas, VA). The breast cancer lines tested were the ZR75-1 and the MCF7. The MCF7 cell line has maintained its ability to respond to estrogens in culture. Cells were maintained as monolayers in DMEM, 10% FBS, and 1% antibiotics. The ZR75-1 line was derived from the tumor of a 63-year-old white female and can be propagated *in vivo* in nude mice. Cells were cultured in RPMI medium supplemented with sodium pyruvate, 10% FBS, and 1% antibiotics. The pancreas cancer cell lines tested were the MIA-PaCa and the PANC-1 cell lines. The MIA-PaCa-2 cell line was isolated from the tumor tissue of the pancreas from a 65-year-old white male. Cells were incubated in DMEM supplemented with 10% FBS, 2.5% horse serum, and 1% antibiotics. The PANC-1 line was originally derived from the pancreas of a 56-year-old white male. Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. The prostate cell line tested was DU145, which originated from a brain metastasis of a patient with prostate cancer. Cells were cultured in RPMI medium with Hepes (25 mM) and L-glutamine (2 mM), 10% FBS, and 1% antibiotics. All cultures were maintained at 37°C in humidified atmosphere containing 5% CO_2. Cells were harvested at 70–80% confluence and washed twice with filter-sterilized phosphate-buffered saline (0.15 M K_2HPO_4, 0.15 M Na_2HPO_4, 0.85% NaCl, pH 7.2) to remove dead cells. Live cells were detached by using 0.25% trypsin in 0.1% EDTA (Sigma Chemical, St. Louis, MO), centrifuged at 1000 rpm for 8 min, and resuspended in growth medium in all experiments. Viability (>80%) of cultures was confirmed with trypan blue exclusion. Cells were added at concentrations of 5×10^4 cells per well for 24 and 72 h of incubation.

MTT assay. The MTT colorimetric assay [11] was employed to detect tumor cell viability after both 24 and 72 h of incubation in the presence of KLH. MTT, a tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue; Sigma] was added to the each well in the assay in a volume of 20 μl at a concentration of 7.5 mg/ml MTT. Plates were incubated in the presence of MTT dye for 4 h. Mitochondrial dehydrogenase activity reduces the yellow MTT dye to a purple formazan, which is then solubilized with acidified isopropanol, and absorbance was read at 570 nm on an ELISA plate reader.

Statistical analysis. Determination of statistical significance was performed by analysis of variance. *Post hoc* comparison of individual concentration means with the control was completed using the Tukey–Kramer multiple comparison test. All data are reported as means ± SE.

RESULTS

Breast Cancer Cell Lines

The breast cancer line, ZR75-1, exhibited a mean growth inhibition of 18 ± 1.4% (range 9 to 32%) at 24 h as shown in Fig. 1A. All KLH concentrations exhibited significant antiproliferative effects compared to the positive control ($P < 0.001$). At 72 h, ZR75-1 exhibited a mean growth inhibition of 43 ± 1.1% (range 37 to 59%). All 72-h KLH concentrations also exhibited significant antiproliferative effects compared to controls ($P < 0.001$). The growth inhibitory effect of KLH observed in the ZR75-1 breast cell line was significantly increased at 72 h compared to 24 h incubation ($P < 0.001$).

The MCF-7 cells exhibited an average of 19 ± 4.4% growth inhibition at 24 h (range 12 to 29%) as shown in Fig. 1B. All the KLH concentrations tested exhibited significant antiproliferative effects ($P < 0.001$) compared to controls, with the exception of 400 ng, 3.2 ng, and 800 pg. At 72 h, the MCF-7 cells showed 39 ± 9.1% growth inhibition (range 35 to 44%) at the concentrations tested. All 72-h concentrations exhibited significant antiproliferative effects compared to controls ($P < 0.001$). As was observed with the ZR75-1 cell line, the antiproliferative response in the MCF-7 cells was also time dependent. The anti-proliferative response at 72 h was significantly increased compared to 24 h after KLH administration ($P < 0.001$).

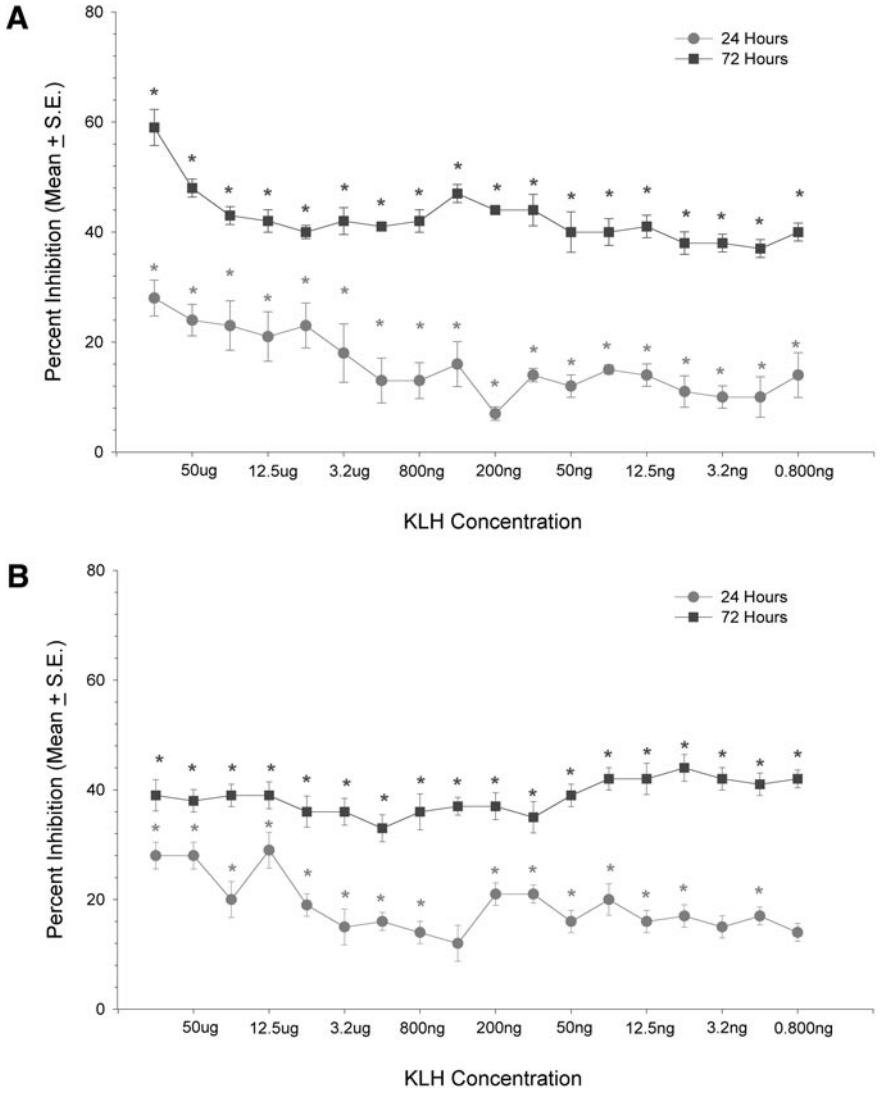

FIG. 1. The MTT assay of breast cancer cell lines incubated with various concentrations of KLH for 24 and 72 h. Graph A represents the percent age inhibition from control for the ZR75-1 (estrogen-independent) cell line: Graph-B represents the results for the MCF-7 cell line. Data are expressed as means ± SE. *$P < 0.001$.

Pancreas Cancer Cell Lines

PANC-1 cells treated with KLH exhibited a mean growth inhibition of 11 ± 1.2% (range −1 to 32%) at 24 h as shown in Fig. 2A. concentrations of 100 and 50 μg KLH were the only concentrations to significantly inhibit cell growth compared to the control ($P < 0.001$). Treated PANC-1 cells had a mean growth inhibition of 19 ± 0.8% (range 4 to 46%) at 72 h. All 72-h concentrations exhibited significant antiproliferative effects compared to the control ($P < 0.001$), with few exceptions. There was no increase in growth inhibition after 24 h incubation compared with 72 h incubation with KLH.

MIA-PaCa cells treated with KLH exhibited little or no cell inhibition; cell proliferation averaged −43 ± 10% (range −62 to 4%) at 24 h (Fig. 2B). MIA-PaCa cells treated with KLH exhibited no significant antiproliferative effects compared to the control. In contrast, significant ($P < 0.05$) proliferation was observed in the cells treated with 12.5-ng, 6.3-ng, 3.2-ng, and 400-pg concentrations of KLH. At 24 h, KLH had little or no antiproliferative effects on the MIA-PaCa cells at 72 h incubation, mean response was −41 ± 9.7% (range −68 to 34%). Compared to control, 100 μg was the only dose to provide a significant antiproliferative response ($P < 0.001$). Lower concentrations of KLH, beginning with 12.5 μg, showed significant cell growth compared to the control ($P < 0.01$). In the PANC-1 cells, there was not a time-dependent increase in growth inhibition with KLH.

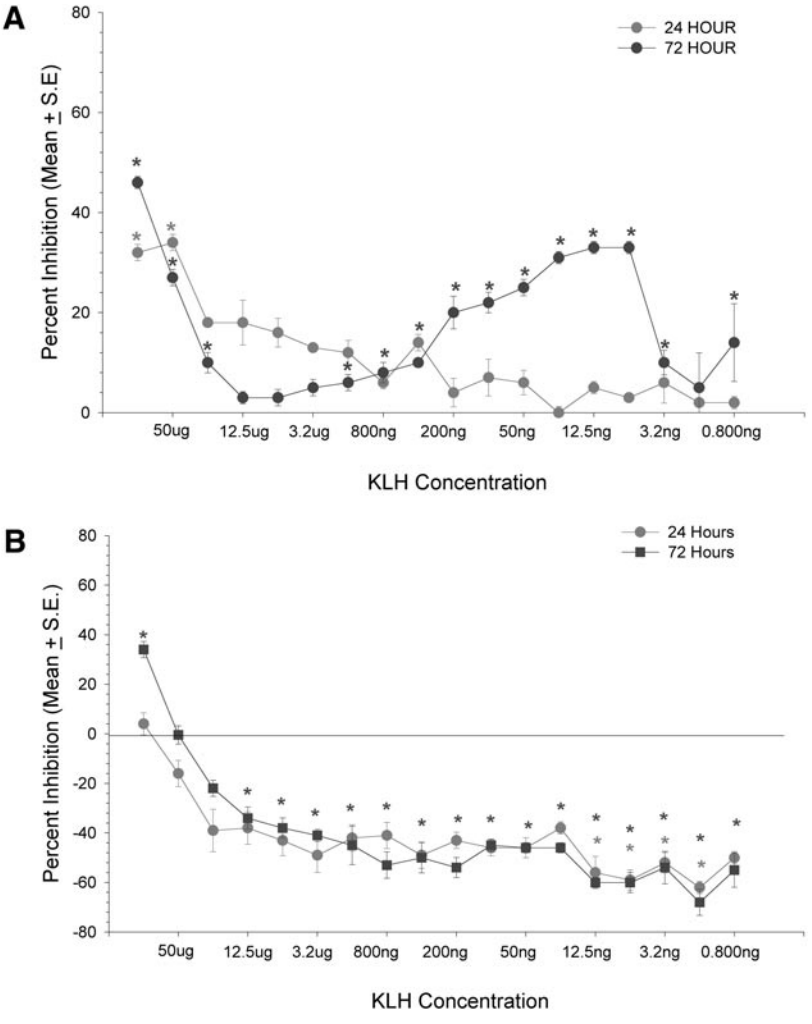

FIG. 2. The MTT assay of pancreatic cancer cell lines incubated with various concentrations of KLH for 24 and 72 h. Graph A represents the percentage inhibition from control for the PANC-1 cell line. Graph B represents the results for the MIA-PACa cell line. Data are expressed as means ± SE. *$P < 0.001$.

Prostate Cancer Cell Line

The DU145 prostate cancer cell line averaged 15 ± 3.5% growth inhibition (range −0.7 to 37%) over the concentrations tested at 24 h incubation. KLH concentrations of 100, 50, 25.0, 12.5, and 6.3 μg exhibited significant ($P < 0.05$) antiproliferative effects compared with controls, as shown in Fig. 3. After 72 h incubation with KLH, growth inhibition averaged 6 ± 1.3% (range −19 to 55%); KLH concentrations of 100, 50, 25.0, and 12.5 μg exhibited significant ($P < 0.05$) inhibition of cell growth. Time effects were not observed in the DU145 prostate cells within treatments.

DISCUSSION

The search for new cytotoxic agents and novel targeted therapies for adjuvant treatment of early breast cancer continues to evolve. The "gold standard" drug combination of cyclophosphamide, methotrexate, and fluorouracil (CMF) was considered the mainstay treatment for early stage breast cancer for nearly 25 years [12]. Adjuvant chemotherapy now includes an armamentarium of anthracyclines, including doxorubicin and epirubicin [13]. The anthracycline-containing regimens have provided women with small, albeit significant, benefits in relapse-free and overall survival compared with standard CMF as adjuvant chemotherapy [14]. Ongoing research is focusing on combining anthracyclines with other novel agents in an effort to continue to improve outcomes following adjuvant therapy. We continue to explore alternative treatments that resist growth regulation by the breast tumor. Studies from this laboratory show that Peptide YY (PYY), a gut regulatory peptide, and vitamin E inhibit *in vitro* growth of breast cancer cells with variable hormone receptor status [15]. Anthracycline-containing polychemotherapy regimens, however,

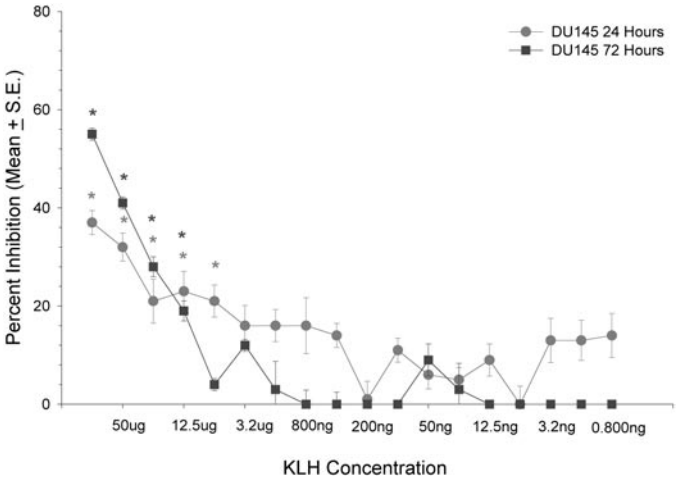

FIG. 3. The MTT assay of DU145 prostate cancer cell line incubated with various concentrations of KLH for 24 and 72 h. Data are expressed as means ± SE. *$P < 0.001$.

have yet to include the use of keyhole limpet hemocyanin as an alternative to cytotoxic agents such as taxanes, trastuzumab, or biphosphonate treatments. Multicenter clinical trials have already confirmed the efficacy of KLH given intravesically for 6 weeks to patients with various stages of bladder cancer [10] with no adverse effects. To determine if KLH would be an effective cytotoxic agent against other cancer cell types, we treated breast cancer cells with increasing concentrations of the drug and evaluated mitochondrial respiration. The results of this study, although preliminary, show that KLH inhibits the growth of two breast cancer cell lines *in vitro* by an average of 30%. Furthermore, KLH was less effective in inhibiting cell proliferation with the estrogen-dependent MCF-7 breast cells compared to the estrogen-independent cells. The differential response of the two breast cell lines to KLH may be related to whether the tumors are estrogen receptor positive or negative. Overall, the cell growth inhibition by KLH observed in this study equaled or surpassed response rates to drug therapy reported in documented clinical trials [13]. *In vitro* studies are ongoing to determine if anthracycline–KLH combinations are effective in inhibiting growth or inducing differentiation or apoptosis of breast cancer cells.

Pancreatic cancer is considered one of the malignancies most resistant to therapy and prognosis of the disease has not been improved markedly. Although major advances have occurred in the standard surgical treatment of the disease, the efficacy of radiotherapy and chemotherapy remains a challenge. Local radiotherapy may provide pain relief, but gastrointestinal toxicity is significant [16]. The standard chemotherapeutic agent was 5-fluorouracil (5-FU); however, it has been replaced by gemcitabine, the most widely used agent against the disease. Pilot phase II studies combining gemcitabine with 5-FU, irinotecan, docetaxel, or cisplatin show improved outcomes [17]. Current research is targeted at combining gemcitabine with other novel agents, including angiogenesis inhibitors, matrix metalloproteinase inhibitors, antisense compounds, inhibitors of cell signaling such as epidermal growth factor, vascular endothelial growth factor, and inhibitors of oncogene activation [16]. We have recently described the growth inhibitory properties of PYY, a regulatory gut peptide, upon human pancreatic ductal adenocarcinomas both *in vitro* and *in vivo* [18–20] by an unknown mechanism. We continue to investigate novel biologic agents that can be combined with traditional therapies such a chemotherapy or radiotherapy for the treatment of pancreatic cancers. Here we show that *in vitro* administration of the potent marine immunoactivator KLH to human pancreatic adenocarcinoma cells reduces their growth by approximately 20%. While growth inhibition of the pancreatic cancer cells was very modest compared with the breast cells, the use of KLH as an agent for cellular immunotherapy shows potential. The antiproliferative effects of KLH warrant further investigation into the mechanisms of carcinogenesis and the progression of pancreatic cancer.

Genitourinary malignancies account for an estimated annual morbidity of 225,000 patients per year in the United States and the need for new systemic agents that can arrest tumor growth and prevent metastasis is paramount. Localized prostate cancer is potentially curable with localized therapies (radical prostatectomy or irradiation therapy); however, there are no curative therapies for metastatic prostate cancer. Gemcitabine alone or in combination with other agents has begun to play a major role in the treatment of testicular cancer and to a lesser extent for refractory prostate cancer because of its activity and low toxicity [21]. Conventional chemotherapy and radiotherapy, however, offer no more than palliation. Intermittent androgen ablation and combining initial hormonal therapy with chemotherapeutic agents may be more promising, but androgen independence and bone metastasis remain a challenge. Phase II evaluation of docetaxel and oral estramustine phosphate for patients with androgen-independent prostate carcinoma shows improvement in the therapeutic index [22] with fewer complications from toxicity. Even newer on the horizon is the use of corrective gene therapy for the treatment of prostate cancer. In animals studies, gene therapy has been aimed at correcting normal patterns of tumor suppressor gene (p53, Rb, p21, and p16) expression or negating the effects of mutated tumor-promoting oncogenes (ras, myc, erbB2, and bcl-2) [23]. However, this approach suffers from the lack of efficient gene delivery by local and systemic routes. Immunomodulatory therapy may be a reasonable alternative to the above ther-

apies as it generates an effective local immune response that will translate to systemic antitumor activity in prostate cancer. Here we demonstrate, for the first time, a 15% reduction in growth of DU145 prostate adenocarcinoma cells in response to the protein KLH. We have reason to believe that this may be an immune response to KLH. In preliminary studies with other cancer cells, we have observed changes in immunostimulatory cytokines (interleukin-2 and -12) with KLH administration (unpublished data).

In summary, we have shown for the first time significant growth inhibition in breast, pancreatic, and prostate cancer cells in response to treatment with hemocyanin from the giant keyhole limpet marine organism. Promising results from the use of KLH in inhibiting growth of cancer cells have led to the initiation of *in vitro* studies to determine if KLH is best suited to use alone or in combination with conventional therapy.

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