CYTOTOXICITY AND ANTITUMOR PROPERTIES OF A MARINE COMPOUND ON CANCER CELLS (HESA-A)

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SUMMARY: The lack of specificity for tumor cells, which is associated with conventional cancer chemotherapy, is the main cause of failure of a new anticancer agent. Therefore, majority of the currently available anticancer drugs are designed to have selective toxicity to rapidly dividing cells. Among these agents and the focus of many studies are compounds obtained from natural products with high therapeutic index. In this study the cytotoxicity of HESA-A (a marine biological compound) was evaluated on cancer and normal cells.

HESA-A was dissolved in normal saline (pH 1.5), shacked for 30 minutes and filtered. Prior to its use, this stock solution (0.8 mg/ml, pH 7.4) was sterilized using 0.22 μ microbiological filters and diluted to final concentrations of 0.4, 0.2, 0.1 and 0.05 mg/ml. 180 μl of cells (MDA-MB-468, HepII, Hela as cancer cells; L929 and McCoy as normal cells) were grown in completed RPMI1640 and seeded in 96 well micro plates at a concentration of 1-5 x 10^4 cells/ml. After their incubation for 24 hours, 20 μl of different concentrations of HESA-A was added and cells were further incubated for 72 hours. Using MTT assay, percent cell survival was determined by ELISA at 540 nm. Doxorubicin was used as a positive control.

HESA-A (0.1 mg/ml) reduced the number of viable MDA-MB-468 cells to less than 50%. For Hela and HepII cells the $IC_{50's}$ were 0.2 and 0.4 mg/ml, respectively. In normal cells IC_{50} was not obtained at any given concentration. Therefore these results suggest that HESA-A selectively and in a concentration dependent manner inhibits the growth of cancer cells.

Key Words: Antitumor agents, MTT assay, HESA-A, cancer cells.

INTRODUCTION

After circulatory system diseases, cancer is the second major cause of death in the Western world accounting for 24% of all deaths occurred. In European countries each year over three-quarters of a million people die from cancer (4).

Once cancer is diagnosed, a variety of possible treatment options is considered. The choice of treatment is dependent on the type of cancer and the extent of its progress. Three basic strategies are used in the treatment of cancer: surgery, radiotherapy and chemotherapy. Each may be used alone or in combination with the other/s.

Systemic chemotherapy is mainly used for the treatment of the metastatic forms of neoplastic disease. Chemotherapeutic agents are classified into two major groups: synthetic and natural products. Despite enormous progress in the field of organic chemistry, currently 25% of

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all prescription drugs are derived from natural sources. This is more significant in regards to anti-cancer drugs in which more than 80% are plant-derived compounds.

The toxicity associated with the conventional cancer chemotherapy arises primarily from the lack of specificity for tumor cells. Majority of the currently available anticancer drugs are designed to have selective toxicity towards rapidly dividing cells (26). This would lead to a low therapeutic index, which results in unacceptable damage to normal organs, thus putting limitation on the dose of the drug that can be administered (10). For example anthracycline anti-tumor antibiotics, especially doxorubicin with its broad-spectrum of activity, are hampered by their severe dose limiting, cumulative cardiotoxicity (5). Several approaches are being considered to handle this problem and thus improving the effectiveness and tumor cell specificity of cancer treatment drugs. Many such methods involve the use of monoclonal antibodies, which are quite expensive and time consuming.

Since the aim of cancer chemotherapy is selective toxicity towards tumor cells, our research focuses on natural products, which induce damage to tumor cells without affecting the normal ones.

Based on the ethno-pharmacological knowledge and screening of natural resources against cancer cells, a mixture of compounds called HESA-A (patented in Iran) has been produced which is used in the present study to test against cancerous and normal cells utilizing MTT assay.

MATERIALS AND METHODS

Sample preparation

HESA-A (biologically active compounds with marine origin that is patented under Iranian authority) was a gift from Dr. Ahmadi (patent holder). It was grounded to a fine powder and was dissolved and shacked for 30 min in acidic saline (pH was adjusted to 1.5, using HCl). The mixture was then filtered and its pH adjusted to 7.4 using NaOH. Using 0.22 μ microbiological filters, this solution was sterilized and kept frozen as a stock solution prior to its use. Concentration of HESA-A in this solution was 0.8 mg/ml. From this the final concentrations of 0.4, 0.2, 0.1 and 0.05 mg/ml were prepared and used as cytotoxic agents.

Chemicals

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Fluka, via a local dealer. All other cell biology chemicals were purchased from Life Technologies unless otherwise stated.

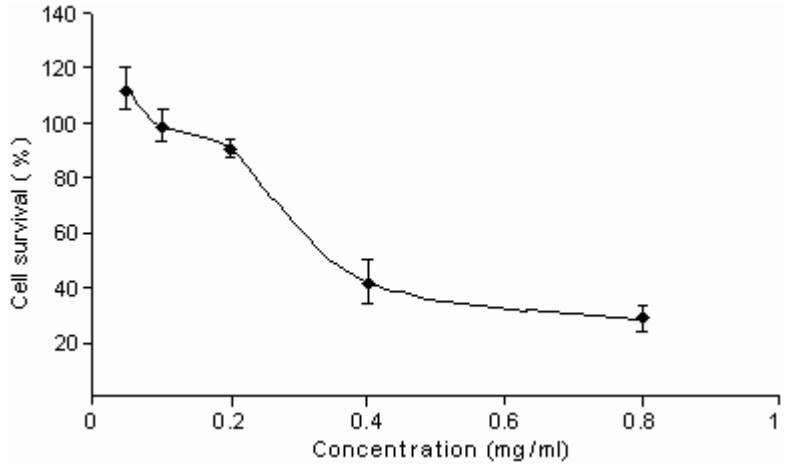
Cell lines and cell culture medium

Three cancerous cell lines, MDA-MB468 (Human, black, breast, adenocarcinoma), HepII (human, Caucasian, larynx, carcinoma), Hela (human, black, cervix, carcinoma, epithelioid) and two normal cell lines, L929 (Mouse, C34/An, connective tissue) and McCoy (Mouse, fibroblast) were used in this study (purchased from Pasteure Institute of Iran, Tehran). All cell lines were grown in RPMI1640 medium supplemented with 50 ml heat-inactivated fetal calf serum (FCS), 5 ml of L-glutamine (2 mM), 5 ml of sodium pyruvate (1 mM) and 5 ml of penicillin/streptomycin (50 IU/ml and 500 μg/ml respectively). Completed media was sterilized by filtering through 0.22 μ microbiological filters and kept at 4°C before use. Cell lines were maintained in a humidified atmosphere of 5% CO_2 - 95% air at 37°C. Under these conditions the doubling time for cancer and normal cell lines were 13-15 and 21-24 hours, respectively.

In-vitro cytotoxicity assay

The cytotoxic effects of HESA-A against tumor and normal cell lines were determined by a rapid colorimetric assay using MTT. The results were compared with untreated control (22). In this assay, mitochondrial enzyme activity of viable cells would metabolically reduce the soluble MTT into an insoluble colored formazan product which in turn can be dissolved in DMSO and measured spectrophotometrically (6). Briefly, 180 μl of cells (5 x 10^4 cells per ml of media) were seeded in 96 well micro plates and incubated for 24 hours (37°C, air humidified 5% CO_2). Then 20 μl of various concentrations of HESA-A were added and the micro plates were further incubated for 72 hours (37°C, air humidified 5% CO_2). Doxorubicin was used as a positive control. The first column of the micro plate was used as negative control (containing no drug or doxorubicin). To evaluate cell survival, each well incubated with 20 μl of MTT solution (5 mg/ml in phosphate buffer solution) for 3 hours. Afterwards, gently 150 ml of the media on each well was replaced with DMSO and pipetted up and down to dissolve any formed formazan crystals. Then the absorbance of each well was measured at 540 nm using an ELISA plate reader. Each concentration of HESA-A was assayed in 8 wells. These experiments were repeated for 6 times. Standard curves (absorbance against number of cells) for each cell lines were constructed and used for the calculation of percent cell survival. In negative control, percent cell survival was taken as 100%.

Figure 1: Dose response curve for Hela cells following 96 hours continuous exposure to HESA-A, n=8.



Statistical Analysis

SIGMASTAT™ (Jandel Software, San Raphael, CA) was used for statistical analysis. Analysis of variance followed by Dunkan test was used to see the differences amongst various groups. Significance was assumed at 5%.

RESULTS

General composition of HESA-A (X-ray results)

HESA-A has a marine origin (collected from sea clay) and is a mixture of many inorganic salts or complexes. We do not have the exact composition of this mixture since it is patented in Iran, but the X-ray studies identified its general composition. These studies demonstrated the presence of oxides of some elements such as CaO (43.787%), P_2O_5 (6.163%), Na_2O (3.689%), MgO (2.897%), SO_3 (2.193%), K_2O (1.988%), SiO_2 (1.09%), Fe_2O_3 (0.375%), Al_2O_3 (0.354%). Low percent of other elements such as Br, Sr, Ti, Mn, Ni, As, Ag, Cu, Zn, W, Tm, Lu, Tl, Er, V, Cs, Ba, Cd, Te and so forth were found in salt or complex forms in HESA-A mixture.

The effect of HESA-A on cancer cells

Hela cell line: Aqueous fraction of HESA-A appeared to be toxic towards Hela cells in a dose dependent manner, n=8, (Figure 1). At 0.4 mg/ml cell survival was 42%.

MDA-MB 468 cell line: Aqueous fraction of HESA-A appeared to be toxic towards MDA-MB-468 cells in a dose dependent manner, n=8, (Figure 2). At 0.2 mg/ml cell survival was 53%.

HepII cell line: Aqueous fraction of HESA-A appeared to be toxic towards HepII cells in a dose dependent manner, n=5, (Figure 3). At 0.8 mg/ml cell survival was 39%.

Effect of HESA-A on normal cells

In addition to its effects on cancer cells, HESA-A was used against two normal cell lines, McCoy and L929, in order to determine whether its effect were selective. The results indicate that unlike cancer cells, HESA-A was not cytotoxic towards normal cell lines.

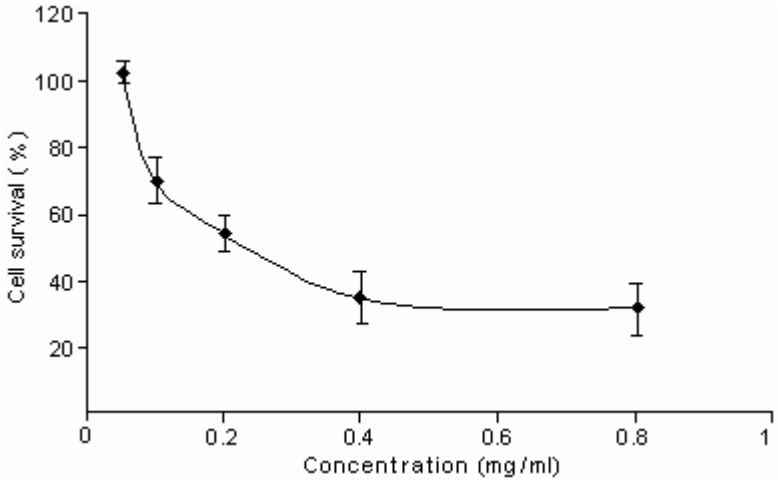
McCoy cells: Aqueous fraction of HESA-A appeared to be non-cytotoxic towards McCoy cells (Figure 4). The maximum tested concentration reduces cell survival to 53%; however, in concentrations of 0.2 mg/ml and lower HESA-A acted as a growth inducer.

L929 cells: Aqueous fraction of HESA-A appeared to be non-cytotoxic towards L929 cells (Figure 5). The maximum tested concentration reduced cell survival to 68%; however, in concentrations of 0.2 mg/ml and lower HESA-A acted as a growth inducer.

DISCUSSION

This *in-vitro* study was undertaken to demonstrate the effects of HESA-A aqueous extract on different classes of human cancer and normal cells.

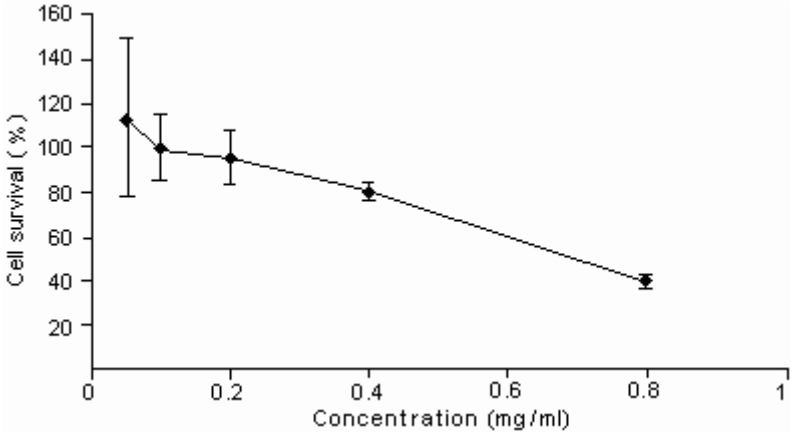
Figure 2: Dose response curve for MDA-MB 468 cells following 96 hours continuous exposure to HESA-A, n=8.



The purpose of the study was to determine whether this compound has a selective cytotoxic effect against cancer cells. MTT based cytotoxic assay was carried out using three cancer cells and two normal ones. The aqueous extract exhibited significant growth inhibition of all cells tested and in most of the tested concentrations the cell survival was less than 50%. This was the case even though in normal cells the maximum tested concentration never reached LD50.

Since the extracted organic fraction of this mixture had no significant effects on cancer cells, it could be suggested that the effect of this mixture is due to the presence of inorganic trace elements and/or their different complexes. This mixture contains elements that are essential for body survival and their deficiency may cause some problems such as cancer. For example cesium is a cancer aid element that enters cancer cells making them alkaline. Germanium deficiency can cause cancer; molybdenum is an essential part of several methalloenzymes; selenium is an effective antioxidant nutrient (3) and its deficiency increases the risk of cancer; strontium is an essential trace element; thulium, lanthanum, neodymium, samarium, europium and yttrium enhance growth of normal cells. Vanadium and gallium have anti-cancer property (Wallach in his booklet "a dire warning"). Therefore the effects of HESA-A against cancer cells are possibly due to the presence of these trace elements in its composition. This is in agreement with the findings of other researchers. A preliminary report by Patel (24) revealed that tantalum has been used in diagnosing tumors of the ureter and renal pelvis. Hall *et al.* (14) in their study showed that anti-tumor activity of mono- and dimethalic transition metal carborane complexes of Ta, Fe, Co, Mo and W act by inhibition of human DNA topoisomerase II activity. In another study Auvert and colleagues (1) showed that iridium-192 wiring could be used for the treatment of small malignant bladder tumors. Gallium (9) and thallium-201 (23) in breast carcinoma and neodymium (20) in the treatment of the mobile tongue cancer have been used. A series of complexes containing titanium, as a metal center has been shown to possess a wide spectrum of anti-tumor properties (21). According to Hiraoka and his colleagues the growth of osteosarcomas in nude mice were inhibited by selenium without toxicity towards normal tissues. This suggested that selenium might offer a novel therapeutic modality for osteosarcoma (15). Selenium may also decrease the risk of occurrence of large size adenomas (13). The Linxian trials in China revealed that the daily supplementation with the following nutrient combinations would reduce the cancer mortality: retinol and zinc; riboflavin and niacin; vitamin C and molybdenum; and β-carotene, α-tocopherol, and selenium (2, 27). The effects of light rare earth elements on sup-

Figure 3: Dose response curve for HepII cells following 96 hours continuous exposure to HESA-A, n=5.



pression of two cancer cell lines (K562 and PAMC82) were demonstrated by Ji *et al.* (17). Erbium in another study (28) has been used in the treatment of common warts and actinic keratoses. In the case of hepatic tumors, Onyx, which is a complex of 28% tantalum, was used in the treatment of rabbits having hepatic cancer (19). Also studies on various cell lines demonstrate the use of vanadium complexes in cancer treatment (12). Jouad and colleagues showed that Ni (II) was toxic to cultured cells although based on the toxicity assays in mice and carcinogenesis assays in rats nickel complexes can be used as antimitotic agents (18). Majority of HESA-A is composed of calcium salts. In this regard Rozen and Holbrook mentioned that the addition of calcium supplements to the western-style diets might reduce the risk of colorectal neoplasia (16, 25).

Although the exact mechanism of action of HESA-A on cancer and normal cells is not known, its effects could be due to the action of important elements or their complexes present in this mixture. For example vanadium exerts its anti-tumor effects through inhibition of cellular tyrosine phosphatases and/or activation of tyrosine phosphorylases which in either case result in the activation of signal transduction pathways leading to apoptosis and/or activation of tumor suppressor genes (12). Costello (7) suggests two modes of action for anticancer actions of selenium: first by functioning as an essential

Figure 4: Dose response curve for McCoy cells following 96 hours continuous exposure to HESA-A, n =10.

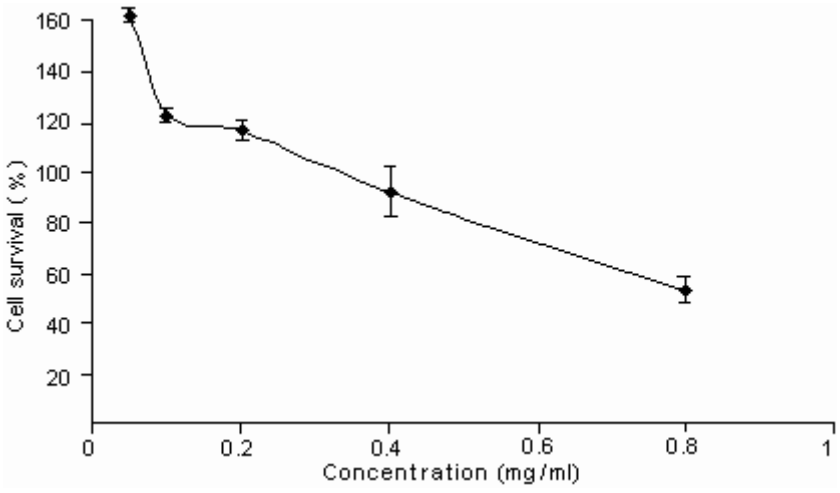
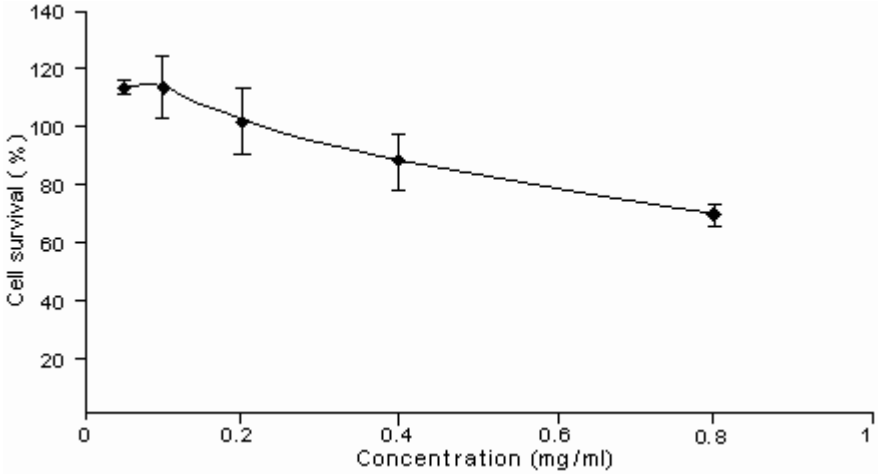


Figure 5: Dose response curve for L929 cells following 96 hours continuous exposure to HESA-A, n = 8.



nutrient that provides the catalytic centers for a number of selenoenzymes (including some with antioxidant and redox functions); second mode of action of selenium is by serving as a source of selenium metabolites that affects carcinogenesis in other ways. Other well-documented results suggest that carcinogenic metal such as arsenic (III), chromium (VI) and vanadium (V) can cause cell death through DNA damage, protein modifications or lipid peroxidation (8). Ding *et al.* (11) showed that metals such as arsenic, beryllium, chromium, nickel and vanadium might enhance generation of reactive oxygen species (which are implicated in the pathogenesis of cancer). However some recent findings in our laboratories showed that HESA-A in a concentration range of 0.1-0.9 mg/ml acts as an antioxidant agent and could scavenge free radicals (unpublished data).

Recent studies from a mini clinical trial done on 5 end-stage volunteer patients revealed that HESA-A had a good antitumor effect in every patient without any adverse effects (unpublished data). Our results also revealed that HESA-A is much more effective on cancer cells. In normal cells even in low concentrations it induces cell growth. About the question of how this mixture can differentiate between cancer and normal cells, we do not have the complete answer yet and experiments are needed to explore this and other important questions.

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