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

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ABSTRACT

Majority of the currently available anticancer drugs are designed to have selective toxicity to rapidly dividing cells. Among these agents 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 compound, on cancer and normal cells was evaluated. HESA-A was prepared in normal saline as a stock solution (0.8 mg/ml, pH=7.4), sterilized and further diluted to final concentrations of 0.4, 0.2, 0.1 and 0.05 mg/ml. Cells (MDA-MB-468, Hep-2, Hela as cancer cells; L929 and McCoy as normal cells) were grown in completed RPMI 1640 and seeded in 96 well micro plates at a concentration of $1\text{-}5 \times 10^4$ cells/ml. After incubation for 24 h, different concentrations of HESA-A were added and cells were further incubated for 72 h. Using MTT assay, percent cell survival was determined by ELISA at 540 nm. Doxorubicin was used as a positive control (20 µg /ml). HESA-A (0.4 mg/ml) reduced the number of viable MDA-MB-468 and Hela cells to less than 50%. For Hep-2 cells the IC_{50} was 0.8 mg/ml. In normal cells IC_{50} could not be obtained at any given concentrations. These results suggest that HESA-A in therapeutic doses and in a concentration dependent manner inhibits the growth of cancer cells more selectively than normal cells.

Key Words: Antitumor agents; MTT assay, HESA-A, Natural product, Human cancer cells

INTRODUCTION

After cardiovascular diseases, cancer is the second major cause of death in the western world accounting for 24% of all deaths. In European countries each year over three-quarters of a million people die from cancer (1). Once cancer is diagnosed, a variety of possible treatment options are considered. The choice of treatment depends on the type of cancer and the extent of its progress. Three basic strategies are used in treatment of cancer: surgery, radio-therapy and chemotherapy, which may be employed alone or in combination with the other two methods.

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 all prescription drugs are derived from natural sources. This is more significant with regard to anti-cancer drugs in which more than 80% are plant-derived compounds (1).

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 (2). This leads to a low therapeutic index which results in unacceptable damage to normal organs and consequently put limitation on the dose of the drug that can be administered (3). For example the use of anthracycline antitumor antibiotics especially doxorubicin which have a broad-spectrum of activity, are hampered by their severe dose limiting due to the cumulative cardiotoxicity (4). Several approaches are being considered to handle this problem and to improving the effectiveness and tumor cell specificity of drugs in treatment of cancer. One of these methods involves the use of monoclonal antibodies which are quite expensive and their uses are time consuming.

In this study on the basis of the ethnopharma-cological knowledge effects of a mixture of compounds called HESA-A (patented in Iran) against cancerous and normal cells by MTT assay was investigated.

MATERIALS AND METHODS

Sample preparation for X-ray assay

HESA-A (biologically active compounds with

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marine origin that is patented under Iranian authority) was a gift from Dr. Ahmadi (patent holder). It was grounded to a fine powder and pressed with wax C, to prepare tablets for X-ray assay. A Phillips XRF 2404 X-ray florescent spectrometry (Tarbiat Modarres University) was used for this assay.

Sample preparation for cytotoxicity assay

A portion of HESA-A as a fine powder was dissolved in acidic saline (pH was adjusted to 1.5, using HCl) and shaked for 30 min. The mixture was then filtered and its pH adjusted to 7.4 using NaOH. Using 0.22µ 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 solution the final concentrations of 0.4, 0.2, 0.1 and 0.05 mg/ml were prepared and used in cytotoxicity assay. Doxorubicin hydrochloride (Farmitalia) with a final concentration of 20 µg/ml in PBS was used as positive control.

Chemicals

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

Cell lines and cell culture medium

Three cancerous cell lines, MDA-MB 468 (Human, black, breast adenocarcinoma), Hep-2 (human, Caucasian, larynx carcinoma), Hela (Human, black, cervix carcinoma epitheloid) and two normal cell lines, L929 (Mouse, C34/An, connective tissue) and McCoy (Mouse, fibroblast), which were purchased from Pasture Institute (Tehran, Iran) were used in this study. All cell lines were grown in RPMI 1640 medium supplemented with 10% (V/V) heat-inactivated fetal calf serum (FCS), 1% of L-glutamine (2 mM), 1% of sodium pyruvate (1 mM) and 1% of penicillin/streptomycin (50 IU/ml and 500 µg/ml respectively). The whole media was sterilized by filtering through 0.22µ microbiological filters and kept at 4°C before its use. Cell lines were maintained in a humidified atmosphere of 5% CO_2 – 95% air at 37 °C. Under these conditions doubling time for cancer and normal cell lines were 13-15 and 21-24 h, 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 and results were compared with untreated control (5). In this assay mitochondrial enzyme of viable cells reduces metabolically 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 (5×10^4 cells per ml of media) were seeded in 96 well micro plates and incubated for 24 h (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 h (37 °C, air humidified 5% CO_2). Doxorubicin was used as a positive control and the first column of the micro plate was used as negative control (containing no drug). To evaluate cell survival, each well was incubated with 20 µl of MTT solution (5 mg/ml in phosphate-buffered saline) for 3 h and afterward, 150 µl of the media of each well was gently replaced with DMSO and mixed to dissolve insoluble formazan crystals. All drug concentrations were tested in 8 replicate of on each plate and the average of 8 wells was considered as a single experiment. Then the absorbance was measured spectrophotometrically at 540 nm using an ELISA plate reader. Each experiment was repeated 8 times except for Hep-2 cell line that was repeated 5 times. Surviving fraction or fractional absorbance was calculated by the formula: mean of test sample divided by mean of untreated sample or negative control in which percent cell survival was taken as 100%. Additional controls consisted of media alone with no cells, in the presence or absence of the drug. In this study cytotoxicity was considered whenever percent cell survival was less than 50.

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. X-ray studies demonstrated the presence of some oxides of some compounds 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%). 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 low percentage 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, Fig.1. (n=8). At concentration of 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, Fig.2. (n=8). At concentration of 0.2 mg/ml, cell survival was 53%.

Hep-2 cell line: Aqueous fraction of HESA-A was less toxic towards Hep-2 cells in a dose dependent manner, Fig.3. (n=5). At concentration of 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 was selective. The results indicated that unlike cancer cells, in all tested concentrations, HESA-A was not cytotoxic towards normal cell lines.

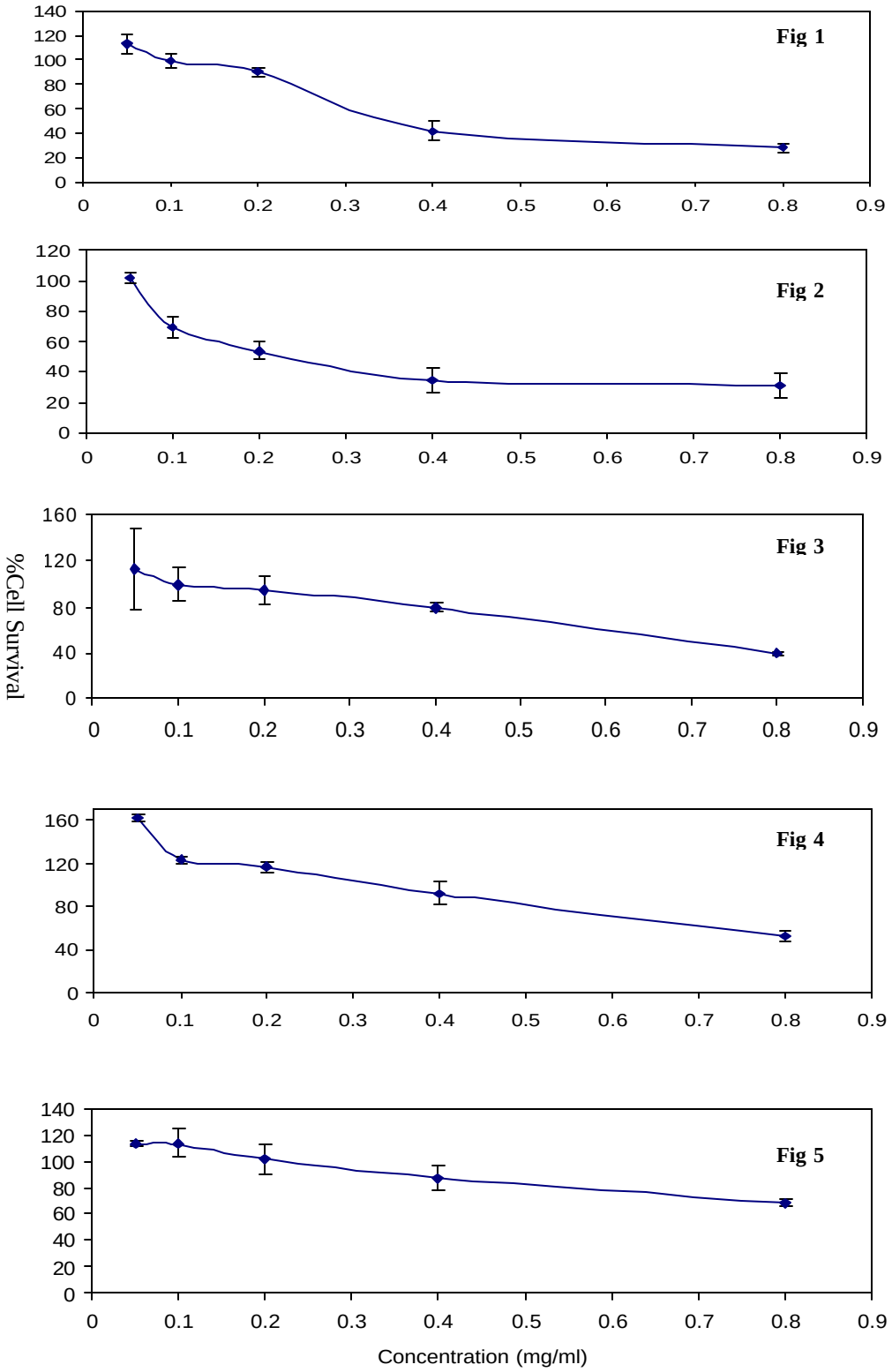
McCoy cells: Aqueous fraction of HESA-A appeared to have low cytotoxicity towards McCoy cells (Fig.4). While at the highest tested concentration (0.8 mg/ml) it reduced cell survival to 53%, in concentrations of 0.2 mg/ml and lower than that, HESA-A acted as a growth inducer in this cell line (n=8).

L929 cells: Aqueous fraction of HESA-A appeared to have low cytotoxicity towards L929 cells (Fig.5) while at highest tested concentration (0.8 mg/ml) it reduced cell survival to 68%, at concentrations equal or lower than 0.2 mg/ml HESA-A acted as a growth inducer in this cell line (n=8).

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. 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 and two normal cells. The aqueous extract exhibited significant growth inhibition of all cells that were tested and at the most tested concentrations, the cell survival was less than 50%. This was the case even though in normal cells the maximum tested concentration never reached to IC_{50} value.

Since the extracted organic fraction of this mixture had no significant effects on cancer cells (unpublished data by this laboratory), 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 and makes them alkaline and, selenium is an effective anti-oxidant nutrient (7) which 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 properties (Wallach in his booklet "a dire warning"). Therefore effects of HESA-A against cancer cells are possibly due to the presence of these trace elements in its composition, which is in agreement with the findings of other investigations. A preliminary report by Patel (8) revealed that tantalum has been used in diagnosis of tumors of the ureter and renal pelvis. Hall et al (9) in their study showed that antitumor activity of mono- and di-methalic transition metal carborane complexes of Ta, Fe, Co, Mo and W was inhibition of human DNA topoisomerase II activity. In another study Auvert and colleagues (10) showed that iridium-192 wiring could be used for the treatment of small malignant bladder tumors. Gallium (11) and thallium-201 (12) have been used in breast carcinoma and neodymium (13) has been employed in treatment of the mobile tongue cancers. A series of complexes containing titanium, as a metal center has been shown to possess a wide spectrum of antitumor properties (14). According to Hiraoka and his colleagues the growth of osteosarcomas in nude mice were inhibited by selenium which showed no toxicity towards normal tissues. This suggested that selenium might offer a novel therapeutic modality for osteosarcoma (15). Selenium may also decreases the risk of occurrence of large size adenomas (16). Trials of Linxian in China revealed that daily supplementation with following nutrient combinations would reduce the cancer mortality: retinol and zinc; riboflavin and niacin; vitamin C and molybdenum; and beta-carotene, alpha-tocopherol, and selenium (17-18). The effects of light rare earth elements on suppression of two cancer cell lines (K562 and PAMC82) were demonstrated by Ji et al (19).



Figures 1.-5 The cytotoxic effect of HESA-A (**fig 1**) at various concentrations on Hela cells (fig1), MDA-MB-468 cells (**fig 2**), Hep-2 cells (**fig 3**), McCoy cells (**fig 4**) and L929 cells (**fig 5**) following 72h continuous exposure. Cells were examined in terms of percent cell survival. Each point represents the mean of 8 determinations ± SD.

In another study (20), Erbium has been used in treatment of common warts and actinic keratoses. In the case of hepatic tumors, Onyx, which is a complex of 28% tantalum, was used in treatment of rabbits which had hepatic cancer (21). Also studies on various cell lines demonstrate the use of vanadium complexes in cancer treatment (22). Jouad and colleagues showed that Ni (II) was toxic to cultured cells and on the basis of the toxicity assays in mice and carcinogenesis assays in rats, nickel complexes can be used as antimitotic agents (23). Since the main composition of HESA-A is calcium salts, Rozen and Holbrook have proposed that addition of calcium supplements to the western-style diets might reduce the risk of colorectal neoplasia (24-25).

Although the exact mechanism of HESA-A action on cancer and normal cells is not known, its effects could be due to the action of important elements or their complexes which are present in this mixture. For example vanadium exerts its antitumor 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 (22). Costello (26) suggests two modes of action for anticancer actions of selenium: first by functioning as an essential nutrient that provides the catalytic centers for a number of selenoenzymes (including some with antioxidant and redox functions); Secondly by serving as a source of selenium metabolites that may prevent carcinogenesis. 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 (27). Ding et al (28) 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).

Our results also revealed that HESA-A is much more effective on cancer cells and in normal cells even at low concentrations, it induces cell growth. More experiments are required to resolve the question that how this mixture can differentiate between cancer and normal cells.

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