RESEARCH ARTICLE

Anti-Proliferative Effects of Hesa-A on Human Cancer Cells with Different Metastatic Potential

Rana Jahanban-Esfahlan^{1,2}, Mozghan Abasi¹, Hakimeh Moghaddas Sani^{1,3}, Mehran Mesgari Abbasi^{2,4}, Abolfazl Akbarzadeh^{3,5*}

Abstract

Background: During the past few years, Hesa-A, a herbal-marine mixture, has been used to treat cancer as an alternative medicine in Iran. Based on a series of studies, it is speculated that Hesa-A possesses special cytotoxic effects on invasive tumors. To test this hypothesis, we investigated the selective anticancer effects of Hesa-A on several cancer cell lines with different metastatic potential. **Materials and Methods:** Hesa-A was prepared in normal saline as a stock solution of 10 mg/ml and further diluted to final concentrations of 100 µg/ml, 200 µg/ml, 300 µg/ml and 400 µg/ml. MTT-based cytotoxicity assays were performed with A549 (lung non small cancer), MCF-7 (breast adenocarcinoma), SKOV3 (ovarian cancer), and PC-3 (prostate adenocarcinoma) cells. **Results:** All treated cancer cells showed significant ($P < 0.01$) or very significant ($P < 0.0001$) differences in comparison to negative control at almost all of the tested doses (100-400 µg/ml). At the lower dose (100 µg/ml), Hesa-A reduced cell viability to 66%, 45.3%, 35.5%, 33.2% in SKOV3, A549, PC-3 and MCF-7 cells, respectively. Moreover, at the highest dose (400 µg/ml), Hesa-A resulted in 88.5%, 86.6%, 84.9% and 79.3% growth inhibition in A549, MCF-7, PC-3 and SKOV3 cells, respectively. **Conclusions:** Hesa-A exert potent cytotoxic effects on different human cancer cells, especially those with a high metastatic potential.

Keywords: Hesa-A - metastatic cancer cells - anti tumoral effects

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Introduction

In unaltered form, natural chemotherapeutic agents are acknowledged as effective remedies to combat different types of human disease including cancer with less concern about the possible side effects on healthy cells (Nasiri et al., 2013; Valiyari et al., 2013; Abbasi et al., 2014b). Hesa-A is an Iranian new immunomodulating medication with natural biological compounds, which has been patented by Ahmadi et al, 2002 in Iran for its biological properties. Hesa-A is a mixture of herbal-marine substances and includes *Penaeus latisculatus* (king prawn), *Apiumgraveolens* and *Carumcarvi*. It is composed of 50% inorganic substance, 45% organic substance (aminoanthraquinone) and 5% water. The inorganic component consists of calcium carbonate, potassium, magnesium phosphate and sulfate and sodium and elements such as zinc, aluminum, potassium, cobalt, chrome, bromine, iron, and strontium at high concentrations (Ahmadi et al., 2010a).

Ahmadi et al in 2005, tested the effects of 50 mg/kg/day of Hesa-A on twenty four end staged breast cancer patients with retina choroid metastases. Their findings

indicated that 92% of the patients who received Hesa-A at a dosage of 50 mg/kg/day orally lived with notably improving quality of life through the six months of the study. These patients suffered fewer complications and survived longer (Ahmadi et al., 2005).

In the second study by Ahmadi et al in 2009, authors investigated therapeutic effects of Hesa-A in fifty consecutive patients with end-stage colon cancer and liver metastasis. Patients received Hesa-A 50 mg/kg/d orally in 2 to 3 divided doses for 6 months. The authors concluded that Hesa-A is an effective and safe anticancer drug, in treatment of selected patients with less side effects (Ahmadi et al., 2009).

In another clinical trial study by Ahmadi et al in 2010, thirty consecutive patients (18 men, 12 women) with end-stage cancers and liver metastasis were studied. Patients received Hesa-A 50 mg/kg/d orally in 2 to 3 divided doses for 3 months. Result showed that a total of 90.4% of the patients who remained in the study were alive for 12 weeks. No significant hepatic or hematologic adverse effect was seen during the study (Ahmadi et al., 2010b).

In 2013, Mehdipour et al evaluated the effect of two systemic doses of Hesa-A on prevention of induced tongue

¹Department of Medical Biotechnology, ²Student Research Committee, ³Department of Genetics, Biotechnology Research Center, Research & Development Complex, ⁴Drug Applied Research Center, ⁵Department of Medical Nanotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran *For correspondence: akbarzadehab@tbzmed.ac.ir

neoplasm in rats. Their results indicated that Hesa-A has dose-dependent inhibitory effects on the development of neoplasms of the tongue (Mehdipour *et al.*, 2013)

At the other hand, our unpublished data indicate that oral doses of Hesa-A improve clinical outcome of tongue carcinoma in rat. Tongue carcinoma is the most malignant neoplasm of oral cavity that mostly represents an unfavorable fatal prognosis (Abbasi *et al.*, 2014a; 2014c).

It is speculated that aggressive tumors with high propensity for invasion may benefit more from cytotoxic effect of Hesa-A, in order to test this hypothesis we evaluated the effects of Hesa-A on a series of human cancer cells with a different metastatic potential.

Materials and Methods

Hesa-A preparation

A portion of Hesa-A as a fine powder was dissolved in acidic saline (pH was adjusted to 1.5, using HCl) and shaken for 30 min. The mixture was then filtered and its pH adjusted to 7.4 using NaOH. Using 0.22 µm 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 10 mg/ml). From stock solution the final concentrations of 100, 200, 300 and 400 µg/ml were prepared and used in the cytotoxicity assay.

Cell Culture

MCF-7 (human breast adenocarcinoma), SKOV3 (human ovarian carcinoma), A549 (lung non small cancer) and PC-3 (human prostate adenocarcinoma) cells were obtained from the Pasteur Institute (Tehran-Iran). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (10 U/ml), streptomycin (10 µg/ml) and 0.2 mM sodium pyruvate. Cultures were incubated in the presence of 5% CO₂ at 37°C and 100% relative humidified atmosphere.

Determination of Cell Survival by the MTT Reduction Assay

Cancer cells were seeded in 96-well microplates at a density of 10×10³ cells/well and grown for 24 h at 37°C in 5% CO₂ prior to the addition of test samples. Cells were treated with various concentrations of samples (100-400 µg/ml) dissolved in Dulbecco's phosphate buffered saline (PBS). After 72 h of incubation, cell viability was determined using the colorimetric MTT assay. MTT solution at 5 mg/ml was dissolved in 1 ml of PBS and 200 µl of it was added to each of the 96 wells and were incubated at 37°C for 2 hours. Afterwards, the solution in each well were removed and were replaced with 100 µl of DMSO in each well. Then cell survival (%) was measured as reduction of MTT into formazan at 550 nm. Toxol with a final concentration of 20 µg/ml was used as positive control. Untreated cells (at 0 µg/ml) were chosen as the negative control. Controls and samples were assayed in triplicate for each concentration and replicated three times. The percentage of cytotoxicity and cell viability were calculated using following equation:

$$\% \text{ Growth Inhibition} = (1 - \text{OD extract treated}) / \text{OD negative control} \times 100.$$

$$\% \text{ Cell viability} = 100 - \% \text{ Growth inhibition}$$

Statistical analysis

All the data represented in this study are mean ± SEM of three identical experiments made in three replicate. Statistical significance was determined by analysis of variance, followed by LSD test and p-value ≤ 0.01 was considered significant. All analyses were conducted using the SPSS 16.

Results

The cell proliferation inhibition was registered at 4 different doses and after a 72h exposure (Table 1, Figure 1 and Figure 2).

SKOV3 cell line: Except dose 100 µg/ml, all tested doses showed significant differences compared to negative control. IC₅₀ for this cancer cells obtained at 200 µg/ml doses in which Hesa-A caused to 51.28% growth inhibition of ovarian cancer cells. At the highest concentration, Hesa-A caused to maximum growth inhibition of 79.3%. In this cell lines, 20 µg/ml Toxol (positive control) caused to 93.76% growth inhibition of SKOV3 cancer cells.

MCF-7 cancer cells: Significant (P<0.01) or very significant (P<0.0001) differences were found in comparison to negative control at all tested doses (100-400

Table 1. % Growth inhibition of Hesa-A extract on different human cancer cells

Doses (µg/ml)	% Growth Inhibition (72 h)			
	SKOV3	MCF-7	PC-3	A549 cells
400	79.3±5.2	86.57±7.2	84.85±7.7	88.45±7.1
300	67.85±3.0	77.85±3.6	75.57±3.8	75.0±3.3
200	51.28±4.4	71.28±2.4	70.25±8.1	64.28±4.9
100	34.0±6.1	66.85±8.3	64.47±5.5	54.71±4.5

Figure 1. Hesa-A Inhibit Different Human Cancer Cell Proliferation *in vitro*. *p<0.01; **p<0.001, compared to the negative control

Figure 2. Cytotoxic Effect of Hesa-A on human SKOV3 (a), MCF-7 (b), PC-3(c) and A549 (d) cell lines after 72h exposure

$\mu\text{g/ml}$), reaching values to 33.15%, even the lowest dose ($100\mu\text{g/ml}$) after 72 h of treatments. Hesa-A caused to 50% growth inhibition obtained at doses $<100\mu\text{g/ml}$ (66.85%) and the highest dose ($400\mu\text{g/ml}$) caused to 86.57% growth inhibition of breast cancer cells, value comparable to that of $20\mu\text{g/ml}$ Toxol which caused to 95.1% growth inhibition of MCF-7 breast adenocarcinoma cells.

PC-3 cancer cells: Hesa-A caused the lowest cell viability of 15.15% at a concentration of $400\mu\text{g/ml}$ after 72 h of treatment. Toxol reduced the cell viability to 6.8% in PC-3 cancer cells (fig1). All tested doses showed significant differences compared to negative controls. Even the lowest dose ($100\mu\text{g/ml}$) caused to $>50\%$ growth inhibition (66.47%) in this cell line (Table 1). Figure 2 shows the potent cytotoxic properties of Hesa-A on PC-3 cells ($400\mu\text{g/ml}$) after a 72h exposure.

A549 cell line: All tested doses showed significant differences compared to negative controls. At the highest doses, cell viability reduced to 11.55%, value close to $20\mu\text{g/ml}$ Toxol which caused to 10.25% cell survival of A549 cells. IC50 for this cancer cells obtained at $100\mu\text{g/ml}$ doses in which Hesa-A caused to 54.71% growth inhibition of lung cancer cells. At the highest concentration, it caused to maximum growth inhibition close to 88.45% (Table 1). Figure 2 shows the potent cytotoxic properties of Hesa-A on A549 cells ($400\mu\text{g/ml}$) after a 72h exposure.

Discussion

Cancer still continuous to be world wide killer

(Jahanban Esfahlan et al., 2011). Global cancer burden rises to 14.1 million new cases in 2012 and marked increase in breast cancers must be addressed. Furthermore, according to the 2014 cancer statistics in United States, Among men, cancers of the prostate, lung and bronchus, and colorectum will account for about 50% of all newly diagnosed cancers ,as prostate cancer alone will account for 27% (233,000) of incident cases in men. The 3 most commonly diagnosed types of cancer among women in 2014 will be breast, lung and bronchus, and colorectum, accounting for one-half of all cases in women. Breast cancer alone is expected to account for 29% (232,670) of all new cancers among women. Cancers of the lung and bronchus, prostate, and colorectum in men and cancers of the lung and bronchus, breast, and colorectum in women continue to be the most common causes of cancer death. These 4 cancers account for almost half of the total cancer deaths among men and women, with more than one-quarter of all cancer deaths due to lung cancer. An urgent need in cancer control today is to develop effective and affordable approaches to the early detection, diagnosis, and treatment of cancer (Siegel et al., 2014).

In this regard, Hesa-A is an Iranian patented natural product with herbal/marine origin which is showed to increase the survival rate of end staged patients with metastasis. There are several *in vitro* studies that evaluated the cytotoxic effects of this extract on different cancer cells.

In a recent study, Muhammadnejad et al assessed selective growth inhibitory effects of Hesa-A on breast (MCF-7), prostate (PC-3), colon (HCT-116) and glioblastoma multiforme (U-87MG) neoplastic cell lines . They indicated that Hesa-A at the highest concentration ($100\mu\text{g/mL}$) significantly inhibited the growth of HCT-116 cell line (40.13% growth inhibition) and no satisfactory results obtained by the other tested cell lines(Muhammadnejad et al., 2014).

Aliabadei et al evaluated the cytotoxicity of HESA-A on MDA-MB-468 (breast cancer), Hep-2 (human liver carcinoma cell line), Hela as cancer cells (Cervical cancer). L929 and McCoy used as normal cells. Different concentrations of 0.4, 0.2, 0.1 and 0.05 mg/ml of Hesa-A was used in MTT assay. 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 IC50 was 0.8 mg/ml . In normal cells IC50 could not be obtained at any given concentrations. The authors suggested that Hesa-A in therapeutic doses and in a concentration dependent manner inhibits the growth of cancer cells more selectively than normal cells (Sadighi-Aliabadi and Ahmadi, 2003).

Roudkenar, et al concluded that cytotoxic effects of Hesa-A on Chinese hamster ovary (CHO) and human embryonic kidney (HEK293T) could be attributed to its potent anti-oxidant and anti radical activity on these cells (Roudkenar et al., 2012).

It is speculated that Hesa-A is more effective against aggressive and end staged cancers as well as end staged breast cancer, metastatic colon cancer and also aggressive forms of oral neoplasms as confirmed by our group. The results of our study confirms that Hesa-A exert potent cytotoxic effects on different human cancer cells including

breast, ovarian, prostate and lung cancer even at the lower doses (100 µg/ml). The most promising results obtained with lung non small cell cancer cells which is a rapid growth tumor with a high propensity for invasion and metastasis and shows a fatal prognosis (Shindo-Okada et al., 2002). Similar results obtained with MCF-7 hormone responsive breast cancer cell line. It is shown that MCF-7 cells form tumors when injected into athymic nude mice. These tumors are able to metastasize to lungs, liver and spleen. MCF-7 cells secrete into the culture media collagenases able to lyse types I and IV collagens (Shafie and Liotta, 1980). In our study, doses 400 µg/ml of Hesa-A caused to more than 85% growth inhibition of aggressive lung and breast cancer cells. Hesa-A also showed substantial anti-proliferative effects on PC-3 prostate adenocarcinoma cells. It is well documented that PC-3 cells have high metastatic potential to bone compared to other prostate cell lines such as DU145 cells which have a moderate metastatic potential and to LNCaP cells which have low metastatic potential (Sanchez-Sweatman et al., 1998).

At the other hand, we tested the effects of Hesa-A on ovarian cancer cells which is a less aggressive tumor compared to lung, prostate and breast cancer cells. Our result indicated that 400 µg/ml doses of Hesa-A caused to 79.3% growth inhibition of ovarian cancer cells. The highest IC₅₀ of 200 µg/ml required to result in 50% growth inhibition of SKOV3 cells whilst in the other tumor cells doses ≤100 µg/ml of Hesa-A suffice to reduce cell viability to less than 50% and values close and comparable to that of Taxol as positive control.

In conclusion, the results of this study suggest that Hesa-A possess potent cytotoxic effects on the most human common cancers even at the lower doses and yet it is more effective against tumors with high propensity for invasion and metastasis as well as lung non small cancer cells, breast adenocarcinoma and prostate adenocarcinoma, however the mechanism of action need to be addressed.

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