Effect of organogermanium complexes on hepatocellular carcinoma cell-line

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ABSTRACT

Organogermanium complexes are used as dietary supplement. Studies revealed that organogermanium compounds exhibited analgesic, anti-inflammatory,antioxidant, immune-modulating, antiviral and anticancer activity. Ascorbic acid is an essential vitamin for human body it is well known with its antioxidant activity. High doses of ascorbic acid showed anticancer activity in different cancer cells. In this study, the effect of organogermanium compounds was tested on HEPG2 cell line. In addition, a combination of selected complexes with high dose of ascorbic acid was done. The results showed a strong antitumor activity of the newly tested compounds. A great down regulation was observed on proliferation marker Ki67 and anti-apoptotic marker BCl2 and up regulation of apoptotic marker caspase3. The combination with ascorbic acid increased the activity of some compounds acting in a synergetic way. However, other compounds showed no difference upon combination.

Key words: *Hepatocellular carcinoma,Organogermanium complexes, Ascorbic acid, Apoptosis.*

INTRODUCTION

Cancer is one of the leading causes of mortality worldwide with an estimation of 14 million new cases in 2012.The number of new cases is expected to increase by 70% over the next 2 decades (World Health Organisation, 2018). In Egypt, the five most common cancers in both sexes combined were breast cancer (17.0%), liver cancer (16%), bladder cancer (8.2%), non-hodgkin lymphoma (5.2%), and central nervous system cancer (5%). While according to mortality rate the liver cancer represented the most common cause of cancer death with (23%) of the total death (I.A.R.C., 2018). Hepatocellular carcinoma incidence and mortality rate increase worldwide in a fast rate, it is more frequent in males than females. In addition, it is more frequent in Africa and Asia than in Europe. The major risk factors for this cancer comprise infection with hepatitis B virus, chronic hepatitis C virus, alcoholic liver disease and non-alcoholic fatty liver disease (Castelli *et al.,* 2017). Organogermanium complexes are powerful antioxidant that showed great effect to protect against cancer. They reduce cancer growth and inhibit metastasis through modifying the immune response. Researchers observed that germanium has limited to no interference with normal healthy cells (Yang *et al*., 2009). Carboxyethylgermaniumsesquioxide Ge-132) is one of the most common water-soluble organic germanium compounds. It is known for broad spectrum biological activities including anti-oxidative, anti-tumor, anti-

inflammatory, cardiovascular activities with low toxicity (Kim *et al*., 2017). Bis-methioninogermanate and bis-glutathionogermanate are considered as interferon inducer that enhance theimmunologic function against cancer. It was found that both compounds increased the levels of serum IL-12 and IFN via mechanisms, which act as neurochemical messengers to the immune system. This permits the communication and execution of the immune system strategies that might provide sufficient immune responses to cancer diseases (Badawi *et al*., 2015). Vitamin C or ascorbic acid is a water-soluble vitamin. Reports have suggested that high-dose vitamin C has anticancer effects (Uetaki *et al*., 2015). Recent study found that high-dose vitamin C enhanced the anticancer activities of Methotrexate on breast cancer cells, including MCF-7 cells and MDA-MB-231 cells (Wu *et al*., 2017). In this study the antitumor activity of organogermanium complexes (having the general formula [GeCl2 (amino acids, peptides, nucleosides or fatty amines) $_4$]$^{2+}$ Cl2) was evaluated *in vitro* against HEPG2 cell line.Selected complexes were combined with ascorbic acid. In the present investigation the effect of these complexes alone and combined with ascorbic acid was investigated on different biomarker such as Ki67, BCl2 and caspase 3.

MATERIALS AND METHODS

Organogermanium complexes which are amino acids complexes: G (cysteine), G (lysine), G (betaine), G (arginine) and G (seleno-methionine), fatty amines complexes: G (lauryl amine) and G (adamantly amine), peptide complexes: G (carnosine) and G (gly-hist) and nucleosides complexes:G (cytosine), G (adenosine) and G(cytidine) were obtained from the Egyptian Petroleum Research Institute. Hepatocellular carcinoma cell line HEPG2 used in this study was obtained frozen in liquid nitrogen (-180 ºC) from the American Type Culture Collection (ATCC; Washington, DC, USA). The tumor cell line was maintained by serial sub-culturing at the National Cancer Institute (NCI), Cairo, Egypt. These cells were maintained in a humidified atmosphere containing 5% CO2 at 37˚C and cultured as "monolayer culture" in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were used when 70% confluence was reached. The adherent cell line was harvested with 0.025% trypsin and viability was determined using trypan blue.

Determination of potential cytotoxicity of organogermanium complexes using Sulphorhodamine-B (SRB)

(Skehan *et al*., 1990) Cells were seeded in 96-wellmicro titer plates at a concentration of 3 $\times 10^3$ cells/well in fresh medium and left to attach to the plates for 24 hours. Then cells were treated with different concentrations of germanium complexes (0, 1, 2.5, 5 and 10ug/ml) in triplicate for each dose and incubated for 48h the negative control was treated with saline as it is the complexes solvent. Doxorubicin was used as positive control. After 48h cells were fixed using trichloroacetic acid, washed and stained with SRB. Excess stain was washed with acetic acid and attached stain was recovered with tris base solution. Optical density (O.D) was measured at 570 nm with an ELISA micro plate Reader and the mean values for each drug concentration were calculated.

Sample preparation

Only four complexes with the less IC50 values were chosen for molecular analysis and combination with ascorbic acid. Cells were plated in T75 flasks supplemented with

medium. After 24 h the medium was replaced with fresh one containing the tested complexes as follow: ascorbic acid 50ug/ml, G (lauryl amine) 2.5ug/ml, G (arginine) 3.2ug/ml, G (adenosine) 2.1ug/ml and G (gly-histidine) 3ug/ml.Then combination was done as follow: Ascorbic acid + G (lauryl amine), Ascorbic acid + G (arginine), Ascorbic acid + G (adenosine) and Ascorbic acid + G (gly-histidine). For negative controls the medium was replaced with fresh medium containing saline.For positive controls, the medium was replaced with fresh medium containing doxorubicin 8ug/ml and doxorubicin + Ascorbic acid. After 24 h, the cells were collected for molecular tests.

Cell lysis

Supernatant was collected and cells were washed with phosphate buffer saline. Cells were harvested by scraping and gentle centrifugation and PBS was aspirated leaving an intact pellet. Every 5 X 10^6 cells were resuspended in 1 ml lysis Buffer and incubated for 60 minutes at room temperature with gentle shaking. The extracts were then transferred to new tubes and centrifuged to obtain a clear lysate.

Molecular markers

Human caspase3 instant ELISA and human Bcl2 platinum ELISA were purchased from eBioscience (An Affymetrix Company, USA). Human antigen KI67 ELISA kit was purchased from Eiaab Company, china. The lysate was treated according to the kits manufacturers' instructions, a yellow color is developed which is proportional to the amount of Caspase-3, Bcl2 or Ki67 bound in each kit. The intensity of the color is measured at 450 nm.

Statistics

The percentage of cell survival was calculated as follows:Survival fraction = O.D. (treated cells)/ O.D. (control cells). The I.C.50 values (the concentrations of the complex required to produce 50% inhibition of cell growth) were calculated using dose response curve-fitting models (GraphPad Prism software, version 5). IBM SPSS 21 (IBM, USA) was used for statistical analysis. The statistical significance of results in all of the experiments was determined by one way ANOVA. $P< 0.05$ was deemed for statistically significance.

RESULTS

Inhibition concentration 50%

Organogermanium complexes produced a concentration dependent decrease in cell viability of HEPG2 compared to the non-treated control. Doxorubicin was used as positive control to evaluate the effect of the newly synthesized complexes. The 50% inhibition concentration value (I.C.50) was as follow:Amino acids complexes: G (cysteine) 4.6 ug/ml,G (lysine)5.05ug/ml, G (betaine) 4.8ug/ml, G (arginine) 3.2ug/ml and G (seleno-methionine) 3.5ug/ml.Fatty acids complexes: G (lauryl amine) 2.5ug/ml and G (1-adamantyl amine) 4ug/ml.Peptide complexes: G (carnosine) 6ug/ml and G (gly-hist) 3ug/ml.Nucleosides complexes**:**G (cytosine) 5.4ug/ml, G(adenosine) 2.1ug/ml and G (cytidine) 5.06ug/ml. This was significantly less than Doxorubicin with IC50 value 8ug/ml (Fig. 1). G (adenosine) 2.1ug/ml, G (lauryl amine) 2.5ug/m, G(gly-hist) 3ug/ml and G (arginine) 3.2ug/ml as complexes with the less IC50 value were chosen to test their effect on the expression of cell proliferation and apoptotic marker Ki67, BCl2 and caspase3.

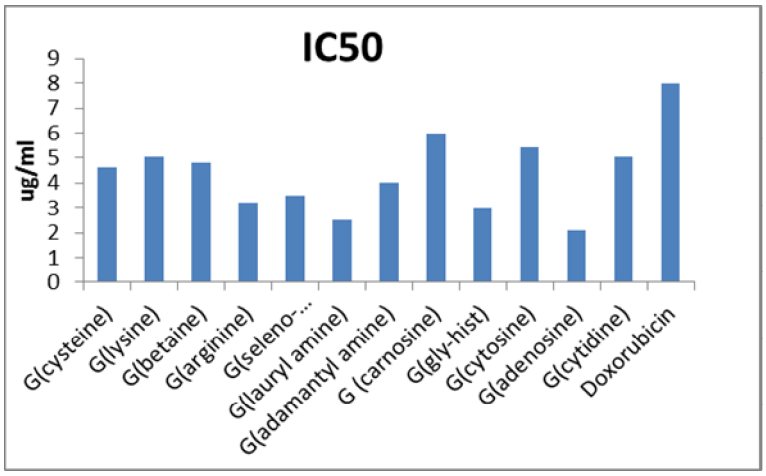

Fig.(1): IC50 of different organogermanium complexes on HEPG2 cell line after 48 hours.

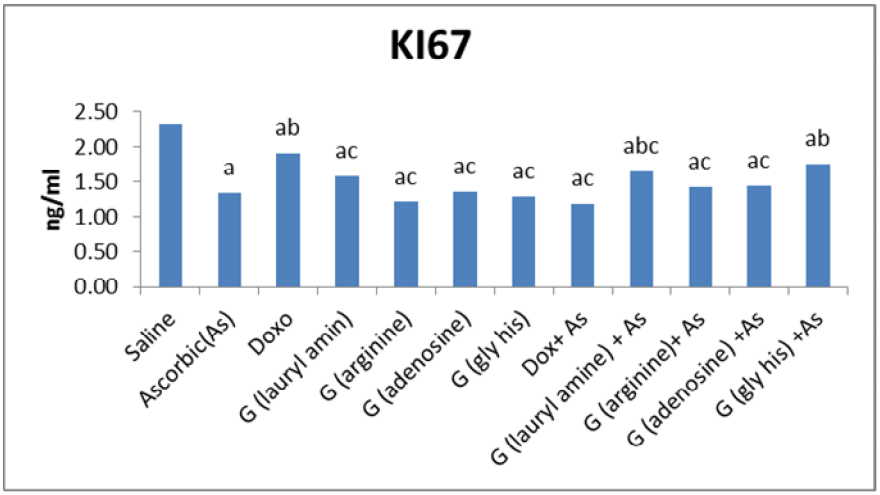

Fig. (2): Effect of 48 hours treatment with selected organogermanium complexes and ascorbic acid alone and their combination on expression of KI67 in HEPG2 cell line.Statistical significance of results was analyzed using one way ANOVA. a Significantly different from negative control (saline), b from ascorbic acid and c from positive control doxorubicin (P <0.05).

Ki67 expression

Ki67 a cellular proliferation marker was clearly inhibited in HEPG2 cell line upon treatment with organogermanium complexes alone, high dose of ascorbic acid alone and their combination together when compared to negative control (Fig. 2). In addition, it was the case when compared to doxorubicin, which indicate that the new complexes have greater effect on Ki67 inhibition than doxorubicin. With G (arginine) and G (gly-his) giving the maximum inhibition among complexes. However, when compared to ascorbic acid there was no significant change in the inhibition rate between all the complexes and ascorbic acid, which indicate that they almost

have the same effect. This was applicable to all the complexes and their combination with ascorbic acid except for G (lauryl amine) and G (gly-his) combination with ascorbic acid, which showed over expression of Ki67 than each of these compound alone.

Bcl2 expression

Bcl2 is known for its function as anti-apoptotic protein. All the compounds and their combinations exhibited significant inhibition on BCl2 expression in HEPG2 cells compared to negative control, ascorbic acid and doxorubicin (Fig. 3). The combination of G (lauryl amine)+As achieved the maximum inhibition rate among all the complexes and their combinations followed by G(lauryl amine) alone. All the compounds showed a remarkable greater inhibition than doxorubicin.

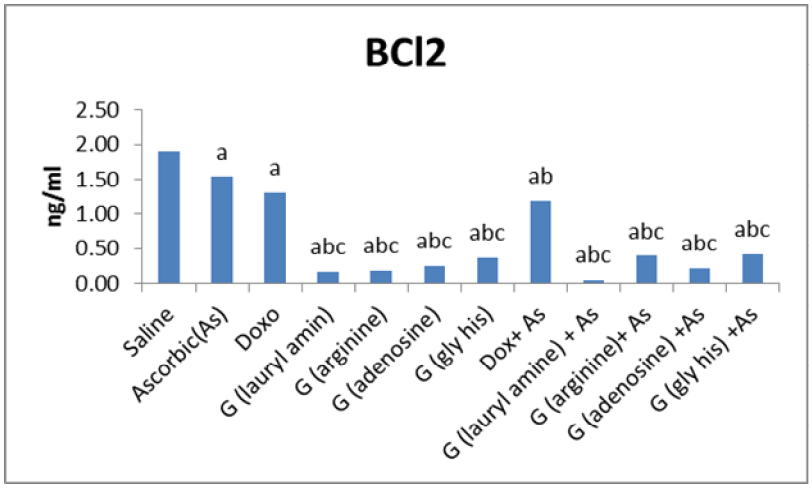

Fig.(3): Effect of 48 hours treatment with selected organogermanium complexes and ascorbic acid alone and their combination on expression of KI67 in HEPG2 cell line. Statistical significance of results was analyzed using one way ANOVA. a Significantly different from negative control (saline), b from ascorbic acid and c from positive control doxorubicin (P <0.05).

Caspase3 expression

Caspase3 expression was greatly affected by the treatment of different complexes and their combination on HEPG2 cells. As an apoptotic marker its expression increased significantly compared to the negative control (Fig. 4). The combination of G (adenosine) with ascorbic acid showed the highest expression of caspase3 among all other complexes and their combinations followed by the effect of G (gly-his) alone. All the organogermanium complexes and their combinations did not give a significant change in caspase3 expression when compared to ascorbic acid or doxorubicin. This indicates that all the compounds have similar effect to ascorbic acid and doxorubicin on caspase3 expression.

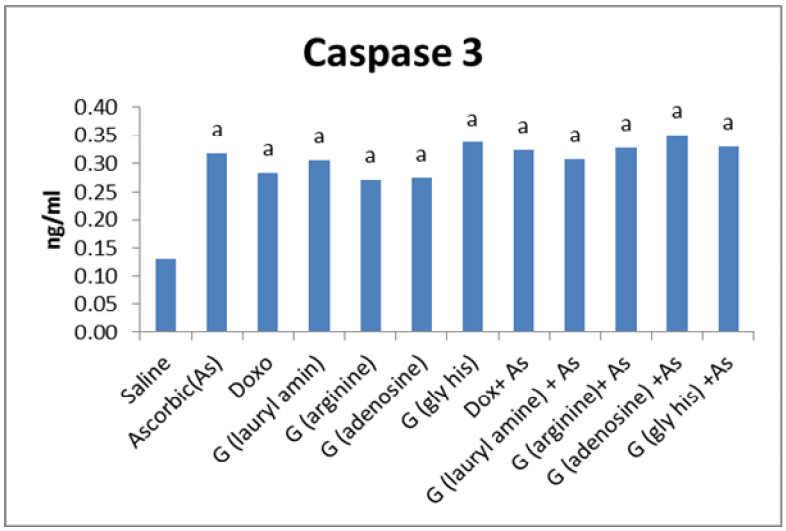

Fig. (4): Effect of 48 hours treatment with selected organogermanium complexes and ascorbic acid alone and their combination on expression of Caspase3 in HEPG2 cell line.Statistical significance of results was analyzed using one way ANOVA. a Significantly different from negative control (saline), b from ascorbic acid and c from positive control doxorubicin (P <0.05).

DISCUSSION

Liver cancer is one of the main causes of cancer-related death in the world. It is the second most common cause of cancer mortality rate (Castelli *et al*., 2017). Organogermanium compounds have been used as dietary supplement and their therapeutic attributes include fighting cancer and improving the immune system (Cheong, 2009). Studies revealed that high-dose vitamin C is more cytotoxic to cancer than it is to normal cells. vitamin C induces death of various types of cancer cells including mesothelioma, pancreatic, and leukemia cells (Uetaki *et al.*, 2015) doxorubicin is one of the most commonly used antitumor drugs which acts on proliferating cells to trigger apoptosis. The use of doxorubicin in cancer therapy has been limited by its serious side effects especially cardiotoxicityand the development of cellular resistance (Xiao *et al.,* 2015).

In this study, 12 newly synthesized organogermanium compounds were tested in vitro on HEPG2 cell line. The inhibitory concentration 50% value of all the complexes was less than doxorubicin, which is conventionally used for hepatocellular carcinoma treatment.This indicates a higher cytotoxicity than doxorubicin on HEPG2 cell line. Organogermanium as a constituent of manymedicinal plants such as ginseng root, ginger and garlic is a powerful antioxidant with no interference to normal healthy cells (Singh *et al.*, 2012). So when compared to doxorubicin the new complexes can exhibit a higher cytotoxicity on tumor cells with lower side effects on normal cells. Among the amino acids Organogermanium complexes group G (arginine) exhibited the best IC50 value. Argininеis involved in a number of biosynthetic pathways that significantly influence carcinogenesis. its supplementation augmented specific and nonspecific anti-tumor mechanisms,retarded tumor growth, and

prolonged survival in some animal tumor models (Lind, 2004). Arginine significantly enhanced defense mechanisms in patients with breast cancer and significantly reduced the incidence of colorectal cancer due to a nonspecific stimulation of immune system (Cao *et al.*, 2016). Our results show that G (arginine) alone act on inhibiting ki67 expression giving the maximum inhibition among all the other complexes. It also acts on inhibiting BCl2 expression and over expressing caspase3. The combination of G (arginine) with ascorbic acid enhance its effect on caspase3 expression in HEPG2 cell line which act on activating the apoptotic pathway. In nucleosides complexes group adenosine showed the best IC50 value. Adenosine, which is an endogenous purine nucleoside composed of an adenine attached to a ribose sugar molecule is well known for its role in activating apoptosis, through intrinsic and extrinsic pathways in different cancer cells (Tsuchiya and Nishizaki, 2015). In this experiment G (adenosine) had a great effect in up regulation of caspase3 and down regulation of both BCl2 and Ki67 with greater effect on BCl2. The combination of G adenosine with ascorbic acid ameliorate the effect of the compounds on caspase3 and BCl2 expression when compared to the compound alone. Peptides, which are short linear chains of amino acids, have high activity, specificity and minimal drug-drug interaction. Peptides do not accumulate in specific organs like liver or kidney which encourage their usage in cancer treatment (Marqus *et al.*, 2017). G (gly-his) complex showed the best IC50 value in its group. It also exhibited remarkable effect on HEPG2 cell line by activating apoptosis through down regulation of BCl2 and ki67 and up regulation of caspase3. However the combination of G (gly-his) with ascorbic acid did not increase its activity. G(lauryl amine) a fatty acid complex showed the highest cytotoxicity to HEPG2 cell line in it group with the highest effect on BCl2 expression compared to all other complexes. G(lauryl amine) effect was magnified when combined with ascorbic acid giving the maximum inhibition to BCl2 expression among all the compounds, which directly affect the cell proliferation and steered the cell toward apoptosis.

CONCLUSION

Newly synthesized organogermanium compounds showed high antitumor activity on hepatocellular carcinoma cell line. The treatment of HEPG2 cell line showed down regulation of proliferation marker Ki67 and anti apoptotic protein BCl2 and up regulation of apoptotic marker caspas3. The combination of some compounds with ascorbic acid increased their antitumor activity while others remained unaffected. Organogermanium complexes represent a promising cancer treatment with lower side effect than conventional treatment.

Author Disclosure Statement

The authors have no conflicts of interest to disclose.

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الملخص العربي

تأثير متراكبات الجرمانيوم العضوي على سلالة خلوية من سرطان الكبد

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تستخدم متراكبات الجرمانيوم العضوي كمكملات غذائية. اظهرت الدراسات علي متراكبات الجرمانيوم العضوي نشاطها كمسكن للالام ، مضاد للالتهابات ، مضادات الأكسدة ، محفز لجهاز المناعة ، مضاد للفيروسات ومضاد للسرطان. حمض الأسكوربيك هو فيتامين أساسي لجسم الإنسان ، وهو معروف جيداً بنشاطه المضاد للأكسدة. أظهرت الجرعات العالية لحمض الأسكوربيك نشاط مضاد للسرطان في الخلايا السرطانية المختلفة. في هذه الدراسة تم اختبار تأثير متراكبات الجرمانيوم العضوي على سلالة خلوية من سرطان الكبد و تم إجراء توليفة من المتراكبات المختارة مع جرعة عالية من حامض الاسكوربيك. أظهرت النتائج نشاط مضاد للأورام قوي للمركبات المختبرة حديثا. كما لوحظ تراجع كبير بمعدل دلالات الانتشار الخلوى التى تشمل انزيمات BCl2 و ki67 و زيادة بمعدل دلالات الموت المبرمج الكاسبر .التوليفة مع حمض الأسكوربيك زاد من نشاط بعض المركبات التي تعمل بطريقة متضافرة.ومع ذلك لم تظهر بعض المركبات فرق عند التوليفة.