Research article

Evaluation of the antitumor activity of combretastatin phosphate against hepatic cancer in rats

Esam M. Aboubakr*, Ashraf Taye, Mohamed A. El-Moselhye*****

* Department of Pharmacology and Toxicology, South Valley University
** Department of Pharmacology and Toxicology, Minia University
*** Department of Pharmacology and Toxicology, Minia University

Abstract

Hepatocellular carcinoma (HCC) is a major health problem with a high incidence and mortality all over the world and it is the most frequent primary solid tumor of the liver. HCC aggressiveness and extensive dissemination lead to a poor patient prognosis. Combretastatin phosphate (CA٤-P) is an antitumor agent that acts by tubulin polymerization inhibition, leading to mitosis and microtubule assembly disruption, thereby causing a rapid vascular collapse and tumor necrosis. In this context, the present study was conducted to investigate the antitumor activity of combretastatine against hepatocellular carcinoma both *in vitro* and *in vivo*; in vitro study was conducted by determination their cell cytotoxic effect using MTT assay. In vivo study was conducted using HCC diseased Sprague dewely rats; the antitumor activity was determined by assessment of the number of hepatic nodules and hepatic relative weight. Their regional reactive oxygen species (ROS) generating effect was assessed by means of hepatic tissue malondialdehyde (MDA) and carbonyl content determination, and biochemical evaluation of the antitumor activity was assessed by alpha-feto protein (AFP) determination. **Results:** CA٤-P showed a potent cell cytotoxic effect against HepG٢ cell line, while in vivo study showed a significant decrement in the number of hepatic nodules, hepatic relative weight, and AFP on the other hand a significant increment in the carbonyl content and MDA concentrations were observed. **Conclusion:** CA٤-P possess a potent antitumor activity against hepatic tumors both *in vitro* and *in vivo* which support further research on this recently developed compound.

Key words: Combretastatin, hepatic cancer, anticancer

Introduction

HCC is one of the most vascular solid tumors, in which blood supply plays an important role in its development, progression, and metastasis (Furuse et al., ٢٠٠١). Due to the rapid growth pattern of HCC, tumor cells require a substantial amount of nutrient and oxygen from the circulation. Hence, patent blood vessels are required to provide the nutrient and oxygen for tumor cell survival (Chen et al., ٢٠١٦; Testino et al., ٢٠١٦).

In recent years, therapies targeted specifically at exploiting these tumor vasculature abnormalities have been developed. Vascular disrupting agents (VDAs) cause a rapid and selective shutdown of the tumor vascular by damaging tumor vessel endothelium have now been identified. Treatment with such agents results in the arrest of the blood flow, which in turn acts to starve the tumor of the oxygen and nutrients it needs to survive (Marrelli et al., ٢٠١١).

The tubulin depolymerizing combretastatin A-٤ (CA-٤) emerged as a promising vascular disrupting agent. CA-٤ had been isolated from the Cape Bushwillow tree Combretum caffrum (Borrel et al., ٢٠٠٥). A soluble sodium phosphate salt of CA-٤ (CA٤-P) was later developed, which is readily administered in vivo and rapidly cleaved to CA-٤ by the action of endo-genous non-specific phosphatases (Chaplin & Hill, ٢٠٠٢).

CA٤-P displayed potent activities against a wide range of human cancer cell lines, including multidrug resistant (MDR) cancer cell lines (Gonzalez et al., ٢٠١٢).

Moreover, CA٤-P induces blood flow shutdown in tumors within minutes after administration, resulting in pronounced tumor necrosis, whereas normal tissues are much less affected (Mahal et al., ٢٠١٥).

Therefore, the present study was conducted to investigate the antitumor activity of CA٤-P against hepatocellular carcinoma both in vitro and in vivo with explanation of the underlying mechanism.

Material and methods

Chemicals

Diethylnitrosamine (DENA) and carbon tetra-chloride (CCl٤) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), CA٤-P from Bolise Co. (Shanghai, China). All other chemicals used were of analytical grade.

In vitro study

Anti-tumor activity determination (cell cytotoxicity test)

Cytotoxicity of tested samples was measured against each cell line using the MTT Cell Viability Assay. Based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form a dark blue insoluble formazan crystals which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at ٥٧٠nm (van Meerloo et al., ٢٠١١).

HepG٢ Cells were plated in a flat bottom ٩٦-well microplate and treated with ٢٠ µL of serial concentrations of CA٤P (١٥, ٣٠, ٥٠, ١٠٠ nm) for ٤٨ hr. cytotoxicity test was performed according to method described by (Sun et al., ٢٠١٢; Lin et al., ٢٠١٦)

In vivo study

Animals

Female Sprague-Dawley rats obtained from the animal care unit (Faculty of Agriculture, Minia University, Egypt) with body weights ١٢٠-١٥٠ gm, were allowed ٧ days for acclimatization and fed a standard laboratory diet and water ad libitum. The study protocol was approved by members of "The Research Ethics Committee" as well as by the Pharmacology &Toxicology department, Faculty of Pharmacy, Minia University, Egypt.

Experimental design

After rats acclimatization ٣٠ of them were randomly divided into two main groups. Group ١:١٠ rats untreated; were given ٣ ml/kg saline intraperitoneal (I.P), ٢ weeks later subcutaneously injected with saline (٣ml/Kg/week) for ٦ weeks (normal group). Group ٢:٢٠ rats received single I.P injection of Diethyl nitrosamine (DENA) ٢٠٠ mg/kg body weight dissolved in ٠.٩٪ saline. Two weeks later, animals in group ٢ received S.C injection of carbon tetra-chloride (CCl٤) ٣ml/kg/week for ٦ weeks, as promoter for the carcinogenic effect of DENA. At the end of total ٨ weeks, animals were fasted overnight and animals in group ٢ were subdivided into ٢ groups ١٠ animals for each, treated as following; First group (DENA + CCL٤ group), received i.p injection of saline (٣ml/kg). Second group (CA٤-P group), treated by single dose of ٢٠٠ mg/kg CA٤-P i.p.

Animal left freely moving for ٤ hours, later animals were weighed. At the planned time, animals were sacrificed under diethyl ether anesthesia, blood samples were collected by cardiac puncture then centrifuged at ١٥٠٠g for ٢٠ minutes at ٤°C to collect serum, then kept in plastic vials at −٢٠°C. The abdomens of rats were opened, livers were removed, weighed and the liver surfaces were examined macroscopically for gross visible neoplastic hepatic nodules. Nodules were counted and the approximated spheres of nodules were measured in two perpendicular planes with a digital caliper to the nearest mm to obtain an average diameter of each nodule.

Estimated nodular volume (V) was determined using the formula: $V = ٤/٣ \times r^{٣}$, where r is the half of the average diameter (mm) (Ravenel et al., ٢٠٠٨) and the nodular volume as a percentage of liver volume was determined (relative nodular volume). Rat's hepatic tissues were weighed and suspended in ice-cold saline, rinsed with ice-cold saline, homogenized in Tris–HCl buffer (١٠٠ mM, pH ٧.٤) using Teflon

homogenizer and centrifuged at ١٢,٠٠٠×g for ٣٠ min at ٤°C. The supernatant was pooled and used for the further estimations.

Liver relative weight determination:
Animals liver weights relative to their body weight at the end of experiment were calculated

Hepatic protein carbonyl content determination:
Protein carbonyl content was determined according to method described by (Levine et al., ١٩٩٤; Reznick & Packer, ١٩٩٤), using commercial kit (cayman, USA) and following manufacturer instructions.

Hepatic tissue lipid peroxidation determination: Hepatic tissue lipids were isolated by precipitating them with serum protein using sodium dodecyl sulfate lysis buffer. The level of lipid peroxidation was measured as malondialdehyde (MDA) by reacting with thiobarbituric acid (TBA) in acetic acid solution. The reaction product was assayed by measuring absorption at ٥٣٢ nm (Janero, ١٩٩٠)

Alpha-fetoprotein determination:
This test is based on the sandwich ELISA principle, thus microtiter plate has been pre-coated with a target specific capture antibody. Test was performed using commercial kit (lsbio, USA), following manufacturer instruction.

Results

CA٤-P antitumor activity:
Cells cytotoxicity test using Hep-G٢ cell lines which used to evaluate the antitumor activity of CA٤P revealed that; by using different concentrations of CA٤-P (١٥,٣٠,٦٠ and ١٢٠ nM) resulted in about (٧١.٢٢%, ٥٥.٢٣%, ٣٨٪ and ١٣٪ respectively) cells viability of control.

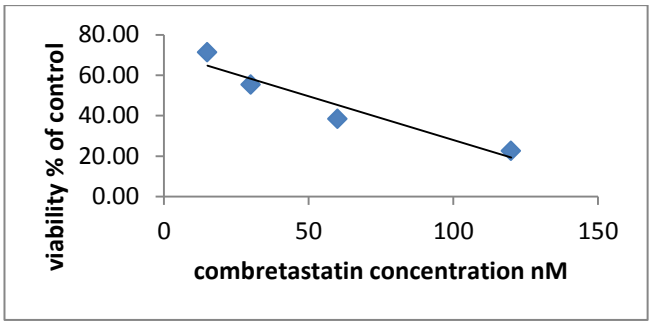

Fig. (١): Antitumor activity of different concentrations of CA٤-P

Effect of combretastatin phosphate on the number of hepatic nodules and the hepatic relative volume.
The number of hepatic nodules and their relative volume were significantly decreased by i.p CA٤-P administration (٥.٩ and ٤.٦٣% respectively) compared to non-treated DENA+CCl٤ group (١٠.٧ and ٧.٤٥% respectively), while normal group did not show any hepatic nodules.

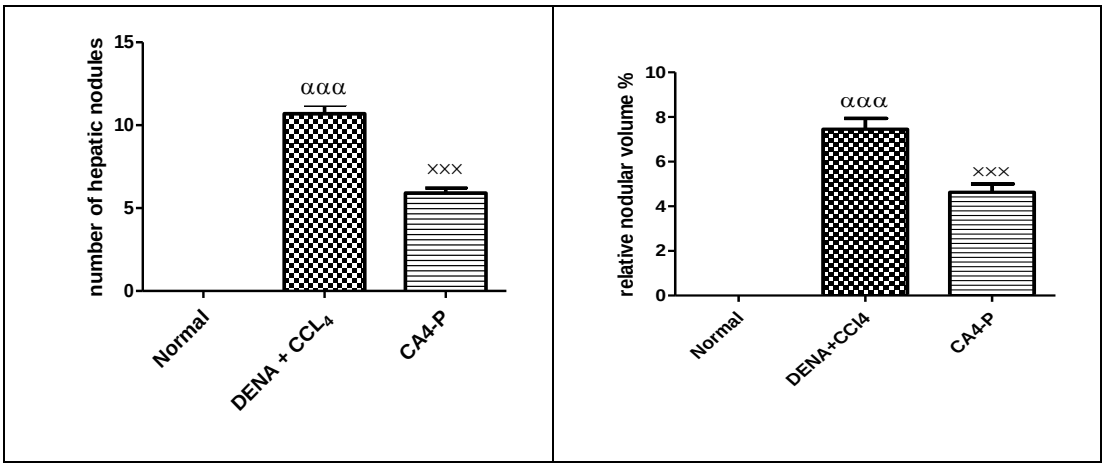

Fig. (٢): Effect of CA٤-P on number of hepatic nodules and nodular relative volume ^{ααα} significantly different from Normal group at p < ٠.٠٠١, , ^{xxx} significantly different from DENA+CCL٤ group at p < ٠.٠٠١.

Effect of CA٤-P on hepatic tissue protein carbonyl content

HCC induction using DENA + CCL٤ increased hepatic tissue content of carbonyl protein from ١.٨٨ nM/mg protein to ٢.١ nM/mg protein, while treatment using CA٤-P markedly increased protein carbonyl content to ٣.٤٥ nM/mg protein which was significantly higher than DENA + CCL٤ non-treated group (p<٠.٠٠١).

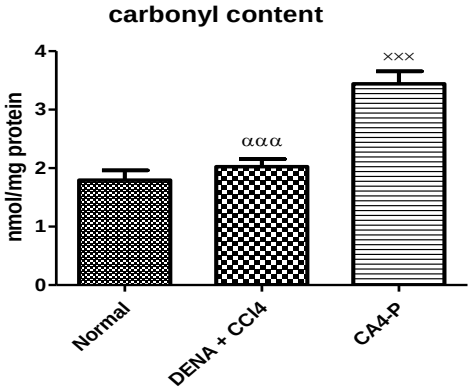

Fig. (٣): Effect of CA٤-P on number of hepatic tissue carbonyl content ^{ααα} significantly different from Normal group at p < ٠.٠٠١, , ^{xxx} significantly different from DENA+CCL٤ group at p < ٠.٠٠١.

Effect of CA٤-P on lipid peroxidation in the hepatic tissue

Normal MDA level inside rats hepatic tissue were determined at ١.٩٧ nM/mg protein, while the i.p administration of CA٤-P caused a noticeable increase in the hepatic levels of MDA (٤.٩٢ nM/mg protein) compare to DENA + CCl٤ non treated group (٣.٢٥).

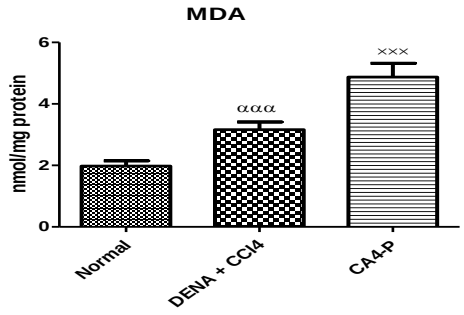

Fig (٤): Effect of CA٤-P on MDA level inside hepatic tissue
^{ααα} significantly different from Normal group at p < ٠.٠٠١, , ^{xxx} significantly different from DENA+CCL٤ group at p < ٠.٠٠١.

Effect of combretastatin on AFP level
The hepatic tissue content of AFP was significantly increased by DENA + CCl٤ treatment (١٤٢.٨ pg /gm protein) compare to normal group (P < ٠.٠١) which was ١٤.٦٢ pg/gm protein. However, a dramatically reduction in the AFP concentrations were observed by treating animals with CA٤-P (١١٠.٨ pg /gm protein).

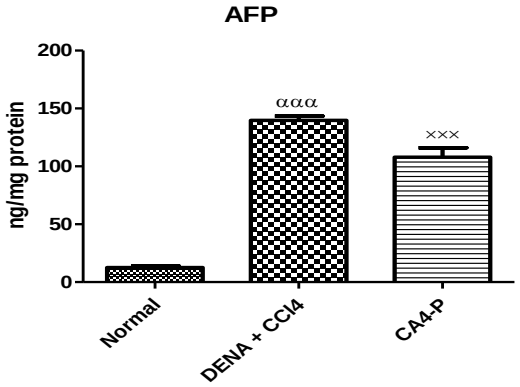

Fig (٢): Effect of CA٤-P on hepatic AFP level
^{ααα} significantly different from Normal group at p < ٠.٠٠١, , ^{xxx} significantly different from DENA+CCL٤ group at p < ٠.٠٠١.

Statistical analysis
All data are expressed as mean SEM. The groups were compared using one-way ANOVA followed by Tukey multiple comparison test. P value of <٠.٠٥ was considered statistically significant.

Discussion
CA٤-P is a potent vascular disrupting agent, acts by binding to tubulin dimers at a distinct region called the colchicine-binding domain (Coderch et al., ٢٠١٢; McNulty et al., ٢٠١٥), disrupt the tubulin-microtubule equilibrium, decreasing the polymer mass. As result of this, an overall destruction of microtubules, causing rapid collapse of existing tumor vessels and indirectly necrosis of the tumor mass (Ji et al., ٢٠١٥).

Accordingly, the present study evaluated the antitumor activity of CA٤-P against hepatocellular carcinoma and the possible underlying mechanisms, which could contribute to this effect.

The present study investigated the possible cytotoxic effect of CA٤-P against hepatocellular carcinoma cell lines to determine its anticancer activity. It was found that there is a significant decrease in cells viability (increased cells toxicity) on a concentration gradient manner, which may attributed to

the severe destabilization of the tubulin cytoskeleton and mitotic spindle that disrupts the cell's ability to successfully cell division leading to cells cytotoxic effect of CA٤-P on tumour cells (Gonzalez et al., ٢٠١٢).

Quantification of hepatic nodules and relative hepatic weight provide a useful tool to study liver cancer and metastases and to assess anticancer drugs in treatment of HCC.

CA٤-P cause endothelial cells shape changes and increased vascular permeability leading to vascular occlusion involving vascular collapse leading to overall tumor tissue collapse in addition to inducing a decrease in tumor perfusion and tumor oxygenation, resulting in an extensive tumor necrosis (Nagaiah & Remick, ٢٠١٠). In accordance with previously founds, the present study on rats HCC showed that DENA+CCl٤ significantly increased hepatic relative weight with a number of macroscopic hepatic nodules. Meanwhile, the i.p administration of CA٤-P significantly decreased both hepatic relative weight and number of hepatic nodules of the treated rat.

ROS generating treatments including ionizing radiation and chemotherapeutic agents are widely used in cancer treatment, based on the rationale that oxidative stresses cause collapse of the antioxidant systems, leading to cell death (Schumann et al., ٢٠١٥). Because molecular products formed from the reaction of free radicals with biomolecules are generally considered more stable than free radicals themselves, most commonly, free radicals have been tracked by measuring stable metabolite concentrations of their oxidation target products, including malondialdehyde (MDA), a by-product of lipid peroxidation, and protein carbonyl (PC) as product of oxidized proteins (Pirinccioglu et al., ٢٠١٠).

In the present study HCC induction significantly increased both PC and MDA inside rat's hepatic tissue which was in consistence with other previous studies (Amin et al., ٢٠١١; Basaran-Kucukgergin et al., ٢٠١٦).

Additionally, studies have demonstrated that, rapid vascular tumor shutdown and tumor ischemia caused by various chemotherapeutic agents, acts by increasing reactive oxygen species (ROS) production inside tumors leading to tumor cells apoptosis (Heinrich et al., ٢٠١٤).

In the present study, CA٤-P treatment significantly increased both hepatic PC and MDA dramatically compare to DENA + CCl٤ group, indicating on a potent oxidative stress was generated inside hepatic tissue as a result of CA٤-P i.p administration which resulted in a tumor necrosis and destruction.

Alpha-fetoprotein (AFP) has been the standard tumor biomarker for HCC (Sauzay et al., ٢٠١٦). In the present study HCC induction using DENA+CCl٤ intensely increased AFP hepatic concentration, while CA٤-P i.p administration significantly decreased AFP level indicating on HCC inhibition.

In conclusion, CA٤-P showed a potent antitumor activity against HCC which could be resulted from tubulin protein inhibition and ROS generation inside hepatic tumors.

References

١. Amin, A., Hamza, A.A., Bajbouj, K., Ashraf, S.S. & Daoud, S. (٢٠١١) Saffron: a potential candidate for a novel anticancer drug against hepatocellular carcinoma. Hepatology, ٥٤, ٨٥٧-٨٦٧.

٢. Basaran-Kucukgergin, C., Bingul, I., Tekkesin, M.S., Olgac, V., Dogru-Abbasoglu, S. & Uysal, M. (٢٠١٦) Effects of carnosine, taurine, and betaine pretreatments on diethylnitrosamine-induced oxidative stress and tissue injury in rat liver. Toxicol Ind Health, ٣٢, ١٤٠٥-١٤١٣.

٣. Borrel, C., Thoret, S., Cachet, X., Guenard, D., Tillequin, F., Koch, M. & Michel, S. (٢٠٠٥) New antitubulin derivatives in the combretastatin A٤ series: synthesis and biological evaluation. Bioorg Med Chem, ١٣, ٣٨٥٣-٣٨٦٤.

٤. Chaplin, D.J. & Hill, S.A. (٢٠٠٢) The development of combretastatin A٤ phosphate as a vascular targeting agent. Int J Radiat Oncol Biol Phys, ٥٤, ١٤٩١-١٤٩٦.

٥. Chen, H., Zhang, J., Cao, G., Liu, P., Xu, H., Wang, X., Zhu, X., Gao, S., Guo, J., Zhu, L. & Zhang, P. (٢٠١٦) Target hepatic artery regional chemotherapy and bevacizumab perfusion in liver metastatic colorectal cancer after failure of first-line or second-line systemic chemotherapy. Anticancer Drugs, ٢٧, ١١٨-١٢٦.

٦. Coderch, C., Morreale, A. & Gago, F. (٢٠١٢) Tubulin-based structure-affinity relationships for antimitotic Vinca alkaloids. Anti-cancer agents in medicinal chemistry, ١٢, ٢١٩-٢٢٥.

٧. Furuse, J., Maru, Y., Mera, K., Sumi, H., Yoshino, M., Yokoyama, Y., Hashimoto, H. & Ejiri, A. (٢٠٠١) Visualization of blood flow in hepatic vessels and hepatocellular carcinoma using B-flow sonography. J Clin Ultrasound, ٢٩, ١-٦.

٨. Gonzalez, M.A., Perez-Guaita, D., Agudelo-Gomez, L.S., Tangarife-Castano, V., Zapata, B. & Betancur-Galvis, L. (٢٠١٢) Synthesis and biological evaluation of combretastatin A-٤ and three combretastatin-based hybrids. Nat Prod Commun, ٧, ١٠٥١-١٠٥٦.

٩. Heinrich, T.A., Tedesco, A.C., Fukuto, J.M. & da Silva, R.S. (٢٠١٤) Production of reactive oxygen and nitrogen species by light irradiation of a nitrosyl phthalocyanine ruthenium complex as a strategy for cancer treatment. Dalton Trans,٤٣,٤٠٢١-٤٠٢٥.

١٠. Janero, D.R. (١٩٩٠) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med, ٩, ٥١٥-٥٤٠.

١١. Ji, Y.T., Liu, Y.N. & Liu, Z.P. (٢٠١٥) Tubulin colchicine binding site inhibitors as vascular disrupting agents in clinical developments. Curr Med Chem, ٢٢, ١٣٤٨-١٣٦٠.

١٢. Levine, R.L., Williams, J.A., Stadtman, E.R. & Shacter, E. (١٩٩٤) Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol, ٢٣٣, ٣٤٦-٣٥٧.

١٣. Lin, C.Y., Chang, T.W., Hsieh, W.H., Hung, M.C., Lin, I.H., Lai, S.C. & Tzeng, Y.J. (٢٠١٦) Simultaneous induction of apoptosis and necroptosis by Tanshinone IIA in human hepatocellular carcinoma HepG٢ cells. Cell Death Discov, ٢, ١٦٠٦٥.

١٤. Mahal, K., Biersack, B., Caysa, H., Schobert, R. & Mueller, T. (٢٠١٥) Combretastatin A-٤ derived imidazoles show cytotoxic, antivascular, and antimetastatic effects based on cytoskeletal reorganisation. Investigational new drugs, ٣٣, ٥٤١-٥٥٤.

١٥. Marrelli, M., Conforti, F., Statti, G.A., Cachet, X., Michel, S., Tillequin, F. & Menichini, F. (٢٠١١) Biological potential and structure-activity relationships of most recently developed vascular disrupting agents: an overview of new derivatives of natural combretastatin a-٤. Curr Med Chem, ١٨, ٣٠٣٥-٣٠٨١.

١٦. McNulty, J., van den Berg, S., Ma, D., Tarade, D., Joshi, S. & Church, J. (٢٠١٥) Antimitotic activity of structurally simplified biaryl analogs of the anticancer agents colchicine and combretastatin A٤. Bioorg Med Chem Lett, ٢٥, ١١٧-١٢١.

١٧. Nagaiah, G. & Remick, S.C. (٢٠١٠) Combretastatin A٤ phosphate: a novel vascular disrupting agent. Future Oncol, ٦, ١٢١٩-١٢٢٨.

١٨. Pirinccioglu, A.G., Gokalp, D., Pirinccioglu, M., Kizil, G. & Kizil, M. (٢٠١٠) Malondialdehyde (MDA) and protein carbonyl (PCO) levels as biomarkers of oxidative stress in subjects with familial hypercholesterolemia. Clin Biochem, ٤٣, ١٢٢٠-١٢٢٤.

١٩. Ravenel, J.G., Leue, W.M., Nietert, P.J., Miller, J.V., Taylor, K.K. & Silvestri, G.A. (٢٠٠٨) Pulmonary nodule volume: effects of reconstruction parameters on automated measurements--a phantom study. Radiology, ٢٤٧, ٤٠٠-٤٠٨.

٢٠. Reznick, A.Z. & Packer, L. (١٩٩٤) Oxidative damage to proteins: spectrophotometric method for carbonyl

assay. Methods Enzymol, ٢٣٣, ٣٥٧-٣٦٣.

٢١. Sauzay, C., Petit, A., Bourgeois, A.M., Barbare, J.C., Chauffert, B., Galmiche, A. & Houessinon, A. (٢٠١٦) Alpha-foetoprotein (AFP): A multi-purpose marker in hepatocellular carcinoma. Clin Chim Acta, ٤٦٣, ٣٩-٤٤.

٢٢. Schumann, C., Taratula, O., Khalimonchuk, O., Palmer, A.L., Cronk, L.M., Jones, C.V., Escalante, C.A. & Taratula, O. (٢٠١٥) ROS-induced nanotherapeutic approach for ovarian cancer treatment based on the combinatorial effect of photodynamic therapy and DJ-١ gene suppression. Nanomedicine, ١١, ١٩٦١-١٩٧٠.

٢٣. Sun, R.B., Xi, Z.G., Yan, J. & Yang, H.L. (٢٠١٢) Cytotoxicity and apoptosis induction in human HepG٢ hepatoma cells by decabromodiphenyl ethane. Biomed Environ Sci, ٢٥, ٤٩٥-٥٠١.

٢٤. Testino, G., Leone, S., Patussi, V., Scafato, E. & Borro, P. (٢٠١٦) Hepatocellular carcinoma: diagnosis and proposal of treatment. Minerva Med, ١٠٧, ٤١٣-٤٢٦.

٢٥. van Meerloo, J., Kaspers, G.J. & Cloos, J. (٢٠١١) Cell sensitivity assays: the MTT assay. Methods Mol Biol, ٧٣١, ٢٣٧-٢٤٥.