Cytotoxicity of Nubein6.8 peptide isolated from the snake venom of *Naja nubiae* on melanoma and ovarian carcinoma cell lines

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Abstract

This study was conducted to examine the cytotoxic effects of Nubein6.8 isolated from the venom of the Egyptian Spitting Cobra *Naja nubiae* on melanoma (A375) and ovarian carcinoma cell lines and to reveal its mode of action. The size of Nubein6.8 (6801.8 Da) and its N-terminal sequence are similar to cytotoxins purified from the venom of other spitting cobras. Nubein6.8 showed a high significant cytotoxic effect on A375 cell line and moderate effect on A2780. A clonogenic assay showed that Nubein6.8 has a significant long-term potency on A375 cell survival when compared to A2780. The molecular intracellular signaling pathways of Nubein6.8 have been investigated using Western blotting analysis, flow cytometry, and microscale protein labeling. This data revealed that Nubein6.8 has DNA damaging effects and the ability to activate apoptosis in both tumor cell lines. Cellular uptake recordings revealed that the labeled-Nubein6.8 was intracellularly present in A375 cells while A2780 displayed resistance against it. SEM examination showed that Nubein6.8 was found to have high accessibility to malignant melanoma cells. The apoptotic effect of Nubein6.8 was confirmed by TEM examination that revealed many evident characteristics for Nubein6.8 apoptotic efficacy on A375 cell sections. Also, TEM reflected many resistant characteristics that faced Nubein6.8 acquisition through ovarian carcinoma cell sections. Accordingly, the snake venom peptide of Nubein6.8 is a promising template for developing potential cytotoxic agents targeting human melanoma and ovarian carcinoma.

Keywords:

Cancer Therapy, Snake venom, Cardiotoxins, Malignant Melanoma, Ovarian Carcinoma, Cytotoxic Potential, Cellular Uptake, Apoptosis

1. Introduction

Cancer is considered to be one of the most common causes of death worldwide. Based on the global cancer statistics of GLOBOCAN, the number of deaths attributed to cancer was around 8.2 million in 2012 (Torre et al., 2015) and has risen to 9.6 million cancer deaths in 2018 (Bray et al., 2018). Various therapeutic strategies including chemotherapy, radiotherapy, and immunotherapy that are being used to treat cancer have numerous side effects. In addition, tumors often acquire resistance against chemotherapeutics (Lai et al., 2012). Thus, the discovery and development of specific/safe anticancer agents is one of the main objectives of pharmaceutical and biotechnological industry. The ability of different natural toxins derived from various venomous animals (such as frogs, scorpions and snakes) to alter the main hallmarks of cancer cells makes them as good templates for developing potential anticancer agents.

According to venomics studies, snake venom is a cocktail of different bioactive molecules which mainly include peptides/proteins (such as disintegrins, cysteine-rich secretory proteins, natriuretic peptides, lectins and three-finger toxins (3-FTxs) and enzymes (such as phospholipase A2, L-amino acid oxidases, serine proteases, hyaluronidases and acetylcholinesterases) (Calvete et al., 2007; Sanhajariya et al., 2018). There are several peptides have been characterized from the venom of elapidae and viperidae snakes targeting various types of cancer cells through certain molecular pathways (such as inducing cell membrane and DNA damage, upregulation proapoptotic signals, inhibition proliferation and metastasis, blocking DNA synthesis and promoting cell cycle arrest)

(Calderon et al., 2014; Liu et al., 2014; Ma et al., 2017). The differential cytotoxicity of these molecules toward tumor cells rather than normal cells makes them potential candidates to be explored for their therapeutic potential (Calderon *et al.*, 2014).

Of these peptides, snake venom disintegrins (as anti-angiogenesis and metastasis agents, see review of Ma et al., 2017) which include salmosin (8.0 kDa, *Agkistrodon halys brevicaudus*), albolabrin (7.5 kDa, *Trimeresurus albolabris*), lebein (7.0 kDa, *Macrovipera lebetina*), saxatilin (7.7 kDa, *Gloydius halys*) and triflavin (7.57 kDa, *Protobothrops flavoviridis*). Cathelicidin-BF (30 amino acids) was purified from the snake venom of *Bungarus fasciatus* and showed in vitro and in vivo activity (as an anti-proliferative, anti-angiogenic and anti-migratory agent) on B16F10 melanoma cell line (Wang et al., 2013). Also, cardiotoxin III (CTX III belongs to 3-FTxs) which purified from the snake venom of *Naja naja atra* induced apoptosis in different cancer cell lines (Colo205 human colorectal cancer, HL-60 human leukemia and MCF-7breast cancer) through mitochondrial and endoplasmic reticulum mediated pathways (Tsai et al., 2006; Chien et al., 2008;; Chiu et al., 2009).

Currently there are no FDA approved anticancer drugs derived from snake venoms, however, three promising candidates (disintegrins), ATN-161, GLPG0187 and vicrostatin which were derived from snake venom are currently being investigated, using in vivo studies and clinical trials, (Phase I, II and preclinical studies) as potent anticancer drugs. Disintegrins have been shown to effect angiogenesis, invasion, and metastasis of various cancer types including malignant glioma, breast and ovarian carcinoma (see review of Ma et al., 2017). Hence, there is a clear rational for investigating snake venoms as potential new effective therapeutics for cancer. Therefore, the main

objectives of this research are: (i) to evaluate the venom derived peptide (Nubein6.8) from the Egyptian Spitting Cobra *Naja nubiae* on different types of cancer cells (human malignant melanoma and ovarian cancer cell lines), and (ii) to determine a mechanism of action of Nubein6.8 using various molecular and imaging approaches.

2. Materials and Methods

2.1. Collection of snakes and venom preparation

About 20 snakes of the Egyptian Spitting Cobra *Naja nubiae* were collected from Aswan Governate, Egypt. The snakes were carefully kept in large wooden boxes at Zoology Department (Suez Canal University, Egypt). The snakes were fed on mice and provided with water every 15 days. The venom was mechanically collected in a 50 mL glass sterilized beaker. The collected venom was freeze-dried and then stored at -20°C until used.

2.2. Separation and proteomic analysis of Nubein6.8

N. nubiae crude venom (50 mg dissolved in 500 µL dH_2O) was fractionated using a Sephadex G-50 column (1.5 x 100 cm; Shimadzu Liquid Chromatograph). The column was equilibrated with 0.1 M ammonium acetate buffer (30 mL/hr) and fractions were collected (5 mL), the column effluent was monitored at 280 nm. The collected venom fractions were lyophilized and stored at -20°C until used. Five fractions were obtained (F1-F5) and F3 (Nubein6.8) was further purified. One mg/mL dH_2O of fraction F3 was applied to a Water Xbridge BEH300-C18 column (4.6 x 50 mm, 3.5 µm particle size). The fractions were eluted using a linear gradient of buffer A (0.05%

TFA in dH_2O) and 60% buffer B (0.05% TFA in acetonitrile) for an hour with a flow rate 1 mL/min. The identification of Nubein6.8 was done by MALDI–TOF/MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometer), LTQ Orbitrap Mass Spectrometer and N-terminal sequencing (first 6 amino acids) by Alta Bioscience Ltd, University of Birmingham, UK.

2.3. Cell Culture

The experimental cell lines: the human tumor cell lines (A375 malignant melanoma, A780 ovarian carcinoma and PANC1 pancreatic carcinoma) and PNT2 normal cell line (model of human differentiated normal epithelia) used in the present study were obtained from the European Collection of Cell Cultures (ECACC), UK. The culture medium used for all cell lines' growing and culturing was BioWhittaker® RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) kanamycin sulfate, 1% (v/v) non-essential amino acid, 1% (v/v) L-glutamine, and 1% (v/v) sodium pyruvate. The cell lines were cultured in cell culture flasks (25 cm^2 and 75 cm^2), 12-well and 96-well plates and then incubated in a CO_2 incubator (37°C, 5% CO_2).

2.4. Cell viability and haemolysis assays

To assess changes in metabolic activity of A375, A780, PANC1 and PNT2 cell lines, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5Diphenyltetrazolium Bromide) viability assay was carried out (n= 3) by seeding cells (75 µL/well) in 96-well plates (1 $x10^4$, 1 $x10^4$, 2 $x10^4$, 6 $x10^3$ cells/well, respectively) for 24 hrs incubation (37°C, 5% CO_2) and adding 25 µL/well of 12 serial concentrations (0.007-14.7 µM) of Nubein6.8 to the incubated cells (100 µL/well). After heating the MTT solution to 37°C until a clear solution was obtained, the assay was performed, protected

from light, after 24 hrs incubation (37°C, 5% CO_2), by adding (10 µL/well) of the MTT solution. After 24 hrs incubation (37°C, 5% CO_2), 200 µL of DMSO (Dimethyl sulfoxide) was added into the culture medium (final volume 300 µL/well). Appropriate control wells were prepared at the same time. The colorimetric signals were measured, by using Absorbance-based SpectraMax®M5 Microplate Reader, at 570 nm then at 630 nm for background absorbance. To obtain normalized absorbance values, the background absorbance was subtracted from the signal absorbance, then divided by MTT activity negative control and multiplied by 100 to calculate % cytotoxicity. Cytotoxicity percentage was identified by measuring the average values of half of the effective concentration (EC_{50}) from plotting % cytotoxicity values obtained from dose-response curves. Using the Tecan plate reader (Infinite M200, Switzerland, at 570 nm), haemolytic activity of Nubein6.8 (0.007-14.7 µM; n=3) was examined on sheep red blood cells (Corzo et al., 2001). Triton X-100 (10%) and bidistilled water were used as positive and negative controls, respectively. Hemolysis percentage was calculated using the following formula: Hemolysis % = [(absorbance of treated sample – absorbance of negative control/ (absorbance of positive control– absorbance of negative control)] x 100.

2.5. Clonogenic assay

Clonogenic assay (Franken *et al.*, 2006) was conducted to determine the long-term potency of the cytotoxic agents on cell survival, growth rate, cell proliferation and therefore metastasis of tumor cell lines under investigation. Cells were cultured (n= 3) into triplicate 25 cm^2 flasks and incubated (37°C, 5% CO_2) until reaching 60-70% confluence. Then, the culture media were replaced with fresh media containing six different concentrations (from 0.01 to 1.8 µM) of Nubein6.8. After 24 hrs incubation (37°C, 5% CO_2), the culture media were aspirated, the cells were washed with 0.5%

HBSS (0.5% $MgCl_2$ and $CaCl_2$-free Hank's Balanced Salt Solution) and detached by incubation (37°C, 5% CO_2) with 0.25% TrypLE^{TM} Express from 1-5 mins according to type of cell lines, and then 2X fresh media were added to counteract the Trypsin action. After counting cells using the hemacytometer, calculated volume containing an appropriate number of viable cells were seeded in triplicate 60 mm Petri dishes (400 cells/dish) for each treatment concentration, and then incubated at 37°C and 5% CO_2. Following 10-14 days of incubation (37°C, 5% CO_2), colony formation was assessed; at least 50 colonies developed in negative control dishes (untreated colonies). Then, the culture media were aspirated; the colonies were washed with HBSS, fixed with methanol for 10 mins, and stained with Giemsa for 10 mins. The stain was carefully aspirated, and the dishes were rinsed with water, then formed colonies were counted manually after dishes drying. Plating efficiency (PE) and survival fraction (SF) were calculated through the following equations: PE= Average no. of control colonies formed / No. of seeded cells. SF= Average no. of colonies formed after treatment / (No. of seeded cells x PE).

2.6. Western blotting analysis

The western blotting analysis was done to evaluate the stimulatory effect of Nubein6.8 on the phosphorylation of the histone variant (H2AX) that produces gamma Histone (γH2AX) or phospho-Histone H2AX (as a specific marker for DNA damage). Cells were seeded (500 µL/well, n= 3) in 12-well plates for 24 hrs incubation (37°C, 5% CO_2), and treated with the 6 different selected concentrations (from 0.01 to 1.8 µM) of Nubein6.8. Additional cells' triplicate wells for negative control cells (untreated) and other triplicate wells for the positive control (treated with Staurosporine as positive DNA damaging control) were plated. After 24 hrs incubation (37°C, 5%

CO_2), the plates placed on ice to stop the reaction, then the culture media were aspirated, the cells were washed with 250 µL cold HBSS, and 150 µL of Laemmli's sample buffer (63 mM Tris-HCl, (pH 6.8), 2 mM Na-4P_2O_7, 5 mM EDTA (ethylenediaminetetraacetic acid), 10% (v/v) glycerol, 2% (w/v) SDS (sodium dodecyl sulfate), 50 mM DTT (dithiothreitol) and 0.007% (w/v) bromophenol blue) was added to each well to get whole cell extracts. Aliquots of cell extract sample volume (20-25 µL) were electrophoresed through SDS-PAGE at a constant voltage of 130 V. Following the electrophoretic transfer of proteins to the nitrocellulose membrane, the protein transfer membrane incubated with specific primary antibody (anti-phospho-histone H2AX/Ser139) overnight in cold room conditions. The blot was then incubated for another 2 hrs with secondary peroxidase-conjugated IgG anti-mouse, directed against the first immunoglobulin. Immunoreactive proteins were detected by incubation in enhanced chemiluminescence (ECL) reagents for 3 mins with gentle agitation. The membrane was then mounted onto an exposure cassette and covered with cling film, then exposed to X-ray film for the required time under dark room conditions and developed using X-OMAT processor (KODAK M35-M X-OMAT). Then, the relative separated bands intensity was measured by ScnImage software. The nitrocellulose membrane was then stripped for detection of loading control protein, by incubated in 15 mL of stripping buffer (0.05M Tris-HCl, 2% SDS, and 0.1M of β-mercaptoethanol), at 60 ^oC for 1 hr with shaking. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as loading control. The blots were undergone the immunological detection protocol as explained previously.

2.7. Cell cycle analysis

Effect of Nubein6.8 on cell cycle progression was evaluated using FACS (Fluorescence-Activated Cell Sorting Analysis)-Flow. Cells were cultured (n= 3) into triplicate 25 cm^2 flasks and incubated

(37°C, 5% CO_2) until reaching 60-70% confluence. Then, the culture media were replaced with fresh media containing the six different selected concentrations (from 0.01 to 1.8 µM) of Nubein6.8. After 24 hrs incubation (37°C, 5% CO_2), the culture media were aspirated, the cells were washed with 0.5% HBSS and detached by incubation (37°C, 5% CO_2) with 0.25% TrypLE™ Express from 1-5 mins according to the cell line, and then 2X fresh media were added to counteract the Trypsin action. Cell suspensions were centrifuged at 1500 rpm for 5 mins, and supernatants were removed, and the cells were fixed in ice-cold 70% (v/v) ethanol. 2 hrs before flow cytometric analysis, the cells were resuspended in FACS tubes with 1 mL of staining solution (propidium iodide (PI) solution (50 µg/mL) and RNase A solution (100 µg/mL) in phosphate buffered saline (PBS). Then, the tubes containing cell suspension were incubated on ice and in the dark for at least 1 hr. Flow cytometric analysis was carried out using a FACScan (Becton Dickinson Systems, Cowley, UK), and data were analyzed using CellQuest Pro software (version 5.1.1; BD Biosciences).

2.8. Cellular uptake assay

Nubein6.8 was labeled with the molecular probe of Alexa Fluor® 488 Microscale Protein Labeling kit according to the manufacturer's instructions. The molecular infiltration and the fluorescence distribution of labeled-fractions throughout the cells allowed monitoring of their localization into the cell membrane, cytoplasm, and nuclear envelope. In this regard, the actin cytoskeleton was stained with Phalloidin, Dylight™ 554 Conjugated red fluorescent stain, and the blue-fluorescent DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) nucleic acid stain was used to stain the nuclei. Cells were seeded (500 µL/well, n= 3) on coverslips in triplicate wells of 12-well plates for

24 hrs incubation (37°C, 5% CO_2), and treated with the six different concentrations (from 0.01 to 1.8 μM) of labeled-Nubein6.8 (500 μL/well). After 24 hrs incubation (37°C, 5% CO_2), the culture media were aspirated from cells grown on coverslips, the cells were washed three times with 250 μL HBSS, 5 mins for each, and fixed by adding of 150 μL of 3.7% formaldehyde to each well for 10 mins. The cell coverslips were rinsed three times in PBS, 5mins for each, and then the cell membranes were permeabilized by immersion in 0.2% triton X-100 in PBS for 5 mins. The triton was aspirated, and the cells were rinsed again three times in PBS, 5 mins for each. The cell coverslips were then ready to undergo the sub-cellular specific staining; DAPI counterstaining alongside phalloidin conjugated protocol. Labeling imaging was carried out using confocal laser scanning microscope, with oil immersion objectives.

2.9. Ultrastructural Microscopy

2.9.1. Scanning Electron Microscopy (SEM)

Cells were cultured (n= 3) into triplicate 25 cm^2 flasks and incubated (37°C, 5% CO_2) until reaching 60-70% confluence. Then, the culture media were replaced with fresh media containing 6 different concentrations (from 0.01 to 1.8 μM) of Nubein6.8 (500 μL/well). Additional cells' triplicate flasks for negative control cells (untreated) were also cultured. After 24 hrs incubation (37°C, 5% CO_2), the culture media were aspirated, the cells were washed with 0.5% HBSS (0.5% $MgCl_2$ and $CaCl_2$-free Hank's Balanced Salt Solution) and detached by incubation (37°C, 5% CO_2) with 0.25% TrypLE™ Express from 1-5 mins according to the cell line, and then 2X fresh media were added to counteract the Trypsin action. Cell suspensions were centrifuged at 5000 rpm for 10 mins; then the cell pellets were washed twice times in 0.1 M sodium phosphate buffer in dH_2O (3.1 g $NaH_2PO_4.H_2O$ and 10.9 g Na_2HPO_4 anhydrous (pH 7.4) with 15 mins intervals, and firstly fixed in 3% glutaraldehyde overnight at 4°C. After first fixation time, pellets wash was repeated

twice in 0.1 M sodium phosphate buffer also with 15 mins intervals, and secondary fixed in 1% osmium tetroxide aqueous for 1 hr, all at room temperature. Then, the pellets were dehydrated with a series of ascending percentages of ethanol (75%, 95%, and twice times 100%) with 15 mins intervals, and then initially placed in 50:50 mixture of 100% ethanol and 100% Hexamethyldisilazane for 30 mins, followed by another 30 mins in 100% Hexamethyldisilazane. Before mounting, the pellets air dried overnight and were mounted on 12.5 mm stubs and attached with Carbon-Sticky Tabs, and then coated in an Edwards S150B sputter coater (with approximately 25 nm of gold). SEM imaging examination was carried out using a Philips XL-20 Scanning Electron Microscope at an accelerating voltage of 20 Kv.

2.9.2. Transmission Electron Microscopy (TEM)

Cells were cultured (n= 3) and treated with the 6 different concentrations (from 0.01 to 1.8 μM) of Nubein6.8 (500 μL/well). The cell pellets were fixed and dehydrated using the same protocol of SEM as explained previously, including negative control cell pellets (untreated). After the 15 mins interval of the dehydration series in 100% ethanol, the pellets were cleared in epoxypropane (EPP), then infiltrated in 50:50 mixture of araldite resin and EPP overnight on a rotor, followed by repeated infiltration twice times in fresh mixture for 8 hrs, before embedding and curing in a 60ºC oven for 48-72 hrs. Ultrathin sections were cut, approximately 85 nm thick, on a Leica UC 6 ultramicrotome onto 200 mesh copper grids. Then, the sections were stained for 30 mins with saturated aqueous Uranyl Acetate, followed by Reynold's Lead Citrate for 5mins. TEM imaging examination was carried out using a FEI Tecnai Transmission Electron Microscope (with an accelerating voltage of 80 Kv) and acquired using a Gatan Orius digital camera.

2.10. Statistical Analysis

GraphPad Prism software (version 4) was used in statistical analysis and illustrations. Data were expressed as mean ± SE. One-way ANOVA and Dunnett's Multiple Comparison test were applied for multiple comparisons. Differences between groups were considered statistically significant with P values less than 0.05.

3. Results

3.1. Characterization of the snake venom peptide Nubein6.8.

Nubein6.8 (Fig. 1A and 1B) was characterized by MALDI-TOF/MS (Fig. 1C) and LC-MS (Supplementary Fig. 1) to reveal that the protein mass fingerprint of Nubein6.8 was the dominant peptide of nearly 6.8 KDa. N-terminal sequencing of Nubein6.8 resulted in a strong clear primary sequence, and the first six amino acids are LKCNQL (Fig. 1D). Sequence alignment of the first 6 N-terminal residues of Nubein6.8 showed a strong similarity with cytotoxins purified from the venom of other spitting cobras (Fig. 1E). The aligned cytotoxins are UniProt:P01469 (Cytotoxin 2, Mozambique spitting cobra *Naja mossambica;* MW 6800 Da), UniProt:P01467 (Cytotoxin 1, Mozambique spitting cobra *Naja mossambica;* MW 6826 Da) and UniProt:P01468 (Cytotoxin 1, Red spitting cobra *Naja pallida;* MW 6827 Da).

3.2. Cytotoxicity of Nubein6.8 on cancer cell lines (A375 human malignant melanoma, A2780 ovarian carcinoma, PANC1 pancreatic carcinoma), PNT2 normal cell line (using MTT viability assay) and sheep RBCs

Cancer cell lines (A375, A2780 and PANC1), normal cell line (PNT2) and sheep RBCs were treated with various concentrations of Nubein6.8 (from 0.007 to 14.7 μM; for 24 hrs) and cytotoxicity was evaluated using MTT viability and haemolytic assays (Fig. 2 and 3). The average EC_{50} values of A375 and A2780 were 0.54 ± 0.04 and 1.249 ± 0.06 μM, respectively. Nubein6.8 showed a slight cytotoxic effect on PANC1 pancreatic carcinoma only in the high concentrations (above 3.7 μM; Supplementary Fig. 2 and 3). The cytotoxic effect of Nubein6.8 on PNT2 normal cell line was also investigated. The data revealed that cytotoxicity of Nubein6.8 on PNT2 (EC_{50} 1.45 ± 0.05 μM) was less than its effect on A375 (EC_{50} 0.54 ± 0.04 μM) and A2780 (EC_{50} 1.249 ± 0.06 μM) cell lines (Fig. 2). Nubein6.8 showed no haemolytic activity on washed sheep RBCs at the highest concentration tested (14.7 μM; Supplementary Fig. 4).

3.3. Antimetastatic potency of Nubein6.8 on human malignant melanoma (A375) and ovarian carcinoma (A2780) using clonogenic assay

Clonogenic assay showed that there was a significant effect ($p<0.01$) of Nubein6.8 on A375 tumor cell survival (1.8 μM, survival fraction (SF value) = 0) and continued until the lowest concentration tested (0.01 μM, SF value =0.73 ± 0.1). Nubein6.8 also significantly ($p<0.01$) decreased the number of colonized ovarian A2780 tumor cells at 1.8 and 0.9 μM (SF values = 0.60 ± 0.04 and 0.70 ±0.06, respectively) when compared with their untreated control cells (Fig. 4 and Supplementary Fig. 5).

3.4. DNA damage induced by Nubein6.8 on human malignant melanoma (A375) and ovarian carcinoma (A2780) using Western Blotting with Anti-phospho-Histone H2AX antibody

The phosphorylation of histone H2AX is considered as a specific marker for DNA damage response and underlies the complex molecular mechanism for DNA repair, after the formation of double-strand breaks (DSBs). Therefore, the evaluation of phospho-Histone H2AX level of the tumor cell lines under investigation was to monitor the ability of Nubein6.8 to induce DSBs which could lead to genomic instability and consequence cell death.. The data in Fig. 5 illustrate the level of phospho-Histone H2AX expression in A375 (Fig. 5A) and A2780 (Fig. 5B) tumor cells treated with Nubein6.8. Nubein6.8 induced a significant increase ($p<0.01$) in phospho-Histone H2AX expression at 0.45 - 0.9 μM in melanoma A375 cells and at 0.9 - 1. 8 μM in ovarian carcinoma A2780 cells when compared with their negative (untreated cells) and positive (Staurosporine) control cells. GAPDH loading control protein assessment revealed that Nubein6.8 positively regulated GAPDH translation in both tumor cells and inhibited GAPDH signaling in A375 cells (0.45 - 1. 8 μM) and A2780 cells at highest concentration tested (1.8 μM).

3.5. Genotoxicity of Nubein6.8 on human malignant melanoma (A375) and ovarian carcinoma (A2780) using FACS-Flow Cytometry

Treatment of both A375 and A2780 cell lines with different concentrations (0.01-1.8 μM) of Nubein6.8 for 24 hrs induced detectable cell cycle arrest when compared with untreated cells (Fig. 6). In treated melanoma A375, the number of cells distributed in the S phase remarkably decreased (2-fold decrease in melanoma) compared with cells in other phases. Also, in the ovarian carcinoma cells, the effective concentrations of nubein6.8 (0.11- 0.9 μM) induced cell cycle blockage at S phase and continued to reach G2-M phase.

3.6. Cellular uptake of labeled-Nubein6.8 by human malignant melanoma (A375) and ovarian carcinoma (A2780)

The distribution of green fluorescence labeled-Nubein6.8 (0.01-1.8 μM) throughout A375 and A2780 tumor cells (Fig. 7) revealed the uptake for this toxin into cellular compartments. The effective concentrations (0.11 - 0.9 μM) of labeled-Nubein6.8 was abled to penetrate through cell membrane and cytoplasm of both cell types, with the peptide accumulating in the nucleoli of cell.

3.7. Ultrastructural alteration induced by Nubein6.8 on human malignant melanoma (A375) and ovarian carcinoma (A2780)

In order to investigate the mechanism of action underlying the cytotoxic effects of Nubein6.8, melanoma (A375) and ovarian carcinoma (A2780) cell lines were incubated with the peptides (0.01-1.8 μM, 24 hrs) for electron microscopic examination (SEM and TEM). SEM micrographs of A375 treated cells (Fig. 8) revealed irregularly shaped cells, rod-shaped cells, cell membrane blebbing, and cellular shrinkage when compared with untreated cells. SEM of A2780 tumor cells (Fig. 8) treated with (EC: 0.11, 0.45, 0.9 μM) Nubein6.8 caused surface concavity, cellular shrinkage, cell membrane irregularity and loss of normal characteristics. TEM studies of A375 tumor cells (Fig. 9) (EC: 0.11, 0.45, 0.9 μM) of Nubein6.8 revealed the loss of most cell organelles, abnormal blebbing cell membrane and peripheral migration of the nuclear chromatin toward the nuclear envelope. TEM examination of A2780 tumor cells (Fig. 9) exposed to (EC: 0.11, 0.45, 0.9 μM) Nubein6.8 revealed an increased number of clear and empty leaking vacuoles, aggregated mitochondria, fragmented nuclei and loose stranded nucleoli.

4. Discussion

Venom research is considered as a potential platform for drug discovery and design (Vonk *et al.*, 2011, Harvey, 2014; Abdel-Rahman et al., 2015; Chen et al., 2018). The novel finding in the present study was the identification of Nubein6.8 (a snake venom peptide isolated from the Egyptian Spitting Cobra *Naja nubiae*) and evaluation its antitumoral efficacy against A375 human malignant melanoma and A2780 human ovarian carcinoma. The molecular mass of Nubein6.8 (6801.8 Da) and the first six amino acids was determined which revealed similarity to peptides (belong to the snake venom 3-FTxs) previously characterized from the venom of other African spitting cobras (Petras *et al.*, 2011). Of these, NAJMO_CTX1/P01467 (6.8 kDa), NAJMO_CTX3/P01470 and NAJMO_CTX4/P01452 were characterized from the snake venom of *Naja mossambica* (Munawar *et al.*, 2014). CTXs are dominate in *Naja* venom and constitute 72.8% of the Black-Necked Spitting Cobra (*Naja nigricollis*) total venom proteins (Petras *et al.*, 2011). Besides, 3-FTxs (consisting of 60–74 amino acids) characterized in elapid venoms represent a superfamily of non-enzymatic polypeptides (Fry *et al.*, 2003).

The possible pharmaceutical applications of natural toxins have been reported for various venomous animals including snakes (Minea *et al.*, 2012). The present investigation studied whether Nubein6.8 was able to decrease the viability and proliferation rate of tumor cells using MTT and clonogenic assays, respectively. The MTT results showed that the growth of A375 and A2780 tumor cells was significantly decreased in a concentration-dependent manner when treated with Nubein6.8. The potential effect of Nubein6.8 on melanoma and ovarian tumor cells proliferation and colony-forming ability was apparent from clonogenic assay. Thus, the clonogenic

assay permits a distinction between cytotoxic (cell death) and cytostatic (decreased growth rate) effects of Nubein6.8. The cytotoxic pattern possessed by Nubein6.8 is similar to cytotoxicity induced by the spitting cobra venoms of *Naja nigricollis* and *Naja mossambica* venoms (Khalid *et al.*, 2015). Uncontrolled division, cell invasion, angiogenesis, metastasis, and evade of apoptosis are significant capabilities of cancer cells and inhibiting these hallmarks may leading to the arrest of tumor growth (Liu et al., 2010; Circu and Aw, 2010).

Interestingly, the cytotoxin of NAJMO_CTX1/P01467 showed inhibitory effect on cellular proteasome (Munawar *et al.*, 2014). The cells are using ubiquitin-proteasome systems for maintaining cellular homeostasis (Wang and Maldonado, 2006). After envenomation by *N. mossambica*, it was supposed that NAJMO_CTX1/P01467 might be involved in the alteration of cellular processes through inhibiting necessary catalytic machinery of the cell (such as the 20S proteasome). Proteasome complex inhibition could be one of the pathways involved in cytotoxin-mediated cell death (Munawar *et al.*, 2014). Thus, Nubein6.8 which is like NAJMO_CTX1/P01467 could be a good template to develop promising anticancer agents via targeting cellular proteasome (Cron *et al.*, 2013).

To reveal the antitumoral mechanisms of Nubein6.8, the pro-apoptotic properties of this peptide (such as DNA damaging and cell cycle arrest) were determined. The activation of γH2AX translation has been detected in melanoma A375 and ovarian A2780 cells treated with Nubein6.8. Nubein6.8 constitutively activated γH2AX signaling by enhancing phosphorylation of the histone variant H2AX. The present study suggested that Nubein6.8 induced DNA double-strand break (DSB) cell death which could involve activation of γH2AX translation. This effect might be related

to the binding of Nubein6.8 with H2AX signal molecules. As a sensitive biomarker of DNA damage, the estimation of cellular γH2AX levels is essential to measure teeny changes in genome stability (Podhorecka et al., 2010). Increased rate of H2AX phosphorylation due to death-related DNA fragmentation leads to rapid apoptosis.

Surprisingly, the blotting analysis revealed that Nubein6.8 inhibited GAPDH signaling in both A375 and A2780 tumor cells. GAPDH (as bioenergetics signature; Ganapathy-Kanniappan and Geschwind, 2013) is a fundamental element of the glycolysis energy system which is actively used in various types of cancer. Birsoy *et al.* (2013) reported that targeting of cancer cells could be accomplished if anticancer agents employ a mechanism specific for cancer such as targeting tumor glycolysis. Anti-glycolytic agents may be used as an efficient strategy of attack in combination therapy. The addition of chemotherapeutic agents with glycolytic inhibitors (mainly targeting GAPDH) has been applied to overcome drug resistance in cancer diseases (Nakano *et al.*, 2011). Also, glycolysis inhibition can transform cancer cells into sensitive forms to immunotherapy (Beneteau *et al.*, 2012). To the best of our knowledge, the effect of Nubein6.8 on γH2AX and GAPDH signals is nearly for the first time interpreted for snake venom toxins.

Further, Nubein6.8 induced cell cycle arrest of both melanoma and ovarian carcinoma cell lines mainly through reduction the number of cells distributed in the S phase leading to mitotic division disruption. The stage of cell cycle arrest induced by Nubein6.8 may explain its molecular mode of action. Malumbres and Barbacid (2009) reported that the arrestment of S-phase can be activated by the suppression of cyclin A and cyclin E via the activation of p21 (through p53 in the presence of DNA damage). Previously, few snake venom peptides/proteins have been characterized with

potential effect on the progression of cell cycle such as Bothropstoxin-I (*Bothrops jararacussu;* Da Silva *et al.,* 2015), BatroxLAAO (*Bothrops atrox;* Alves-Paiva et al., 2011) and ACTX-6 (*Agkistrodon acutus*, Zhang and WU, 2008). More imortantly to mention that the cytotoxicity of nubein6.8 were confirmed by the results of labeled-nubein6.8 cellular uptaking and electron microscopic examinations (SEM and TEM). These imaging appraches supported the capability of nubein6.8 to enter the cell and disrupting the integrity of cell membrane and cellular organelles (such as membrane blebbing, cellular shrinkage, mitochondrial) leading to the cell death of the tumor cells. Taken together, the novel obtained efficacy of Nubein6.8 in reducing cancer cell proliferation, arresting cell cycle, inducing cancer cell DNA double breaks and apoptosis, besides glycolysis inhibition, may making it as a good template to develop effective anticancer agents targeting human melanoma and ovarian carcinoma.

Acknowledgements

Lougin M. Abdel-Ghani acknowledges a Governmental PhD Scholarship (Channel Scheme between Suez Canal University, Egypt and University of Strathclyde, UK) supported by the Egyptian Ministry of Higher Education and Scientific Research to study at the Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK.

Conflict of interest

Lougin M. Abdel Ghani; Tarek R. Rahmy; and Mohamed A. Abdel-Rahman have a patent (Novel selective antitumoral cytotoxicity of natural derived peptide isolated from the Egyptian spitting cobra: no. 1397/2017; Egyptian Patent Office, EGYPT) pending containing some of the information described in this work.

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Figure Captions

Fig.1. Separation of the snake venom *Naja nubiae* and partial identification of the antitumor peptide Nubein6.8. (A) The crude venom of *N. nubiae* (50 mg dissolved in 500 µl dH_2O) was applied on Sephadex G-50 column (1.5 x 100 cm) and equilibrated with 0.1 M Ammonium Acetate buffer. Venom sample was eluted at 30 ml/hr flow rate and 5 ml fractions were collected. Protein concentration was monitored at 280 nm. (*) Five fractions were obtained (F1-F5) and F3 (Nubein6.8) revealed potent antitumor activity on different cell lines. **(B)** F3 was further purified by HPLC which showed a single peak of Nubein6.8 eluated at RT 58 min. **(C)** Molecular mass determination of Nubein6.8 (6801.8 Da) using MALDI/TOF-MS. **(D)** Nubein6.8 n-terminal amino acids sequencing (first 6 residues; LKCNQL) determined by Automated Edman Degradation. **(E)** Sequence alignment of the first 6 N-terminal residues of Nubein6.8 with cytotoxins purified from the venom of other spitting cobras. The aligned cytotoxins are UniProt:P01469 (Cytotoxin 2, Mozambique spitting cobra *Naja mossambica;* MW 6800 Da), UniProt:P01467 (Cytotoxin 1, Mozambique spitting cobra *Naja mossambica;* MW 6826 Da) and UniProt:P01468 (Cytotoxin 1, Red spitting cobra *Naja pallida;* MW 6827 Da). The BLAST analysis shows that the first 6 residues (LKCNQL) are conserved in the aligned toxins.

Fig. 2. Cytotoxicity and EC_{50} determination of Nubein6.8 derived from the snake venom *Naja nubiae* on cancer and normal cell lines. Effect of different concentrations (0.007-14.7 µM) of Nubein6.8 on cell viability of melanoma (A375), human ovarian carcinoma (A2780) and normal cells (PNT2) after 24 hrs incubation (37°C, 5% CO_2) determined by MTT assay. Dose-response

curves revealed that Nubein6.8 showed high selectivity to melanoma A375 (EC_{50} 0.54 ± 0.04 μM) than ovarian A2780 (1.249 ± 0.06 μM) and PNT2 normal cell lines (1.45 ± 0.05 μM). Values represent the mean ± SEM from three independent experiments (n=3 replicates/experiment).

Fig. 3. Morphological alterations of human melanoma (A375) and ovarian carcinoma (A2780) cell lines induced by Nubein6.8. A375 and A2780 tumor cells prepared at optimized seeding density $1x10^4$ cells/ well in 96 well plates then incubated (37°C and 5% CO_2) for 24 hrs. Effective concentrations (EC; 0.54 ± 0.04 and 1.249 ± 0.06 μM for A375 and A2780, respectively) of Nubein6.8 were added and after 24 hrs incubation, the viability of the cells was assessed and images taken from three independent experiments (n=3 replicates/experiment).

Fig. 4. Antimetastatic potency of Nubein6.8 on human malignant melanoma (A375) and ovarian carcinoma (A2780) using clonogenic assay. Clonogenic assay was carried out (n= 3) for Nubein6.8 on A375 tumor cells and A2780 tumor cells in 60 mm petri dishes at optimized density 400 cells/dish. After 9 days incubation (37°C and 5% CO_2), colonies formation was assessed and manually counted, starting with colonies developed in the negative control dishes and images taken. Melanoma (A375): There was an obvious effect for Nubein6.8 on A375 tumor cell survival appeared from the highest concentration (1.8 μM) that have no colonized cells at all, and the formed-colonies average numbers kept too less than the control dishes till the lowest concentration (0.01 μM). Comparison between the long-term effect of Nubein6.8 with control on A375 tumor cell survival revealed a high significant difference. Ovarian carcinoma (A2780): There was a moderate effect of Nubein6.8 on A2780 tumor cell survival which significantly appeared only at the concentrations of 0.9 μM and 1.8 μM, then the formed-colonies average

numbers were quite near to control dishes at the rest of concentrations. Values represent the mean ± SEM. ** Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison test ($p<0.01$).

Fig. 5. DNA damage efficacy of Nubein6.8 on human malignant melanoma (A375) and ovarian carcinoma (A2780) using Western Blotting with Anti-phospho-Histone H2AX antibody. Whole pre-treated cell DNA extracts were prepared, standardized with Amidoblack protein assay, separated by SDS-PAGE, and assessed for phosho-Histone H2AX. (A and B) Western Blotting of Nubein6.8 (0.01-1.8 μM) on the phosho-Histone H2AX expression level of A375 tumor cells and A2780, respectively. Values represent the mean ± SEM. **Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison test ($p<0.01$). GAPDH (Glyceraldehyde-3-phosphate dehydrogenase): Western blotting loading control. Staurosporine: Positive DNA damaging control.

Fig. 6. Genotoxicity of Nubein6.8 on human malignant melanoma (A375) and ovarian carcinoma (A2780) using FACS-Flow Cytometry. Cell cycle representative histogram analysis of A375 and A2780 tumor cells pre-treated with various concentrations (0.01-1.8 μM) of Nubein6.8 (n= 3). Melanoma A375: In a concentration-dependent manner, the number of cells distributed in the S phase remarkably decreased (2-fold decrease in melanoma) compared with cells in other phases. Ovarian cells: Nubein6.8 induced cell cycle blockage at S phase and continued to reach G2-M phase. LC: low concentrations (0.01 and 0.0.3 μM), EC: effective concentrations (0.11- 0.9 μM).

Fig. 7. Cellular uptake of labeled-Nubein6.8 by human malignant melanoma (A375) and ovarian carcinoma (A2780). Cells cultured on coverslips then incubated at 37°C and 5% CO_2 for 24 hrs and then labeled-Nubein6.8 was added (from 0.01 to 1.8 μM). After 24 hrs incubation, the cells were fixed, stained and mounted, then images taken. There was an obvious cellular uptake of labeled-Nubein6.8 (green) by both A375 and **A2780** tumor cells appeared in the range of effective concentrations (EC 0.11 - 0.9 μM). Red indicates actin cellular skeleton, and blue indicates nuclei.

Fig. 8. Scanning Electron Microscopy (SEM) analysis of Nubein6.8 antitumoral efficacy on human malignant melanoma (A375) and ovarian carcinoma (A2780).

SEM of control A375 tumor cells showing intact cell membrane with normal extended microvilli. SEM of A375 tumor cells treated by the effective concentrations (EC: 0.11, 0.45, 0.9 μM) of Nubein6.8 revealing irregularly shaped cells, rod-shaped cells **(R)**, cell membrane blebbing **(B)** and irregularity and discontinuity **(Dc)**. SEM of control A2780 tumor cells showing intact cell membrane with high density of different shaped (plexus-shaped **(P)**) complex receptors and structural proteins. SEM of A2780 tumor cells treated by the effective concentrations (EC: 0.11, 0.45, 0.9 μM) of Nubein6.8 revealing surface concavity, cellular shrinkage, cell membrane irregularity and loss of normal characteristics.

Fig. 9. Transmission Electron Microscopy (TEM) analysis of Nubein6.8 antitumoral efficacy on human malignant melanoma (A375) and and ovarian carcinoma (A2780). TEM of control A375 tumor cells representing intact cell membrane with protruded microvilli, smoothly outlined nuclei **(N)** with normal distributed nuclear chromatin and nucleoli, abundant mitochondria **(M)** and well-preserved cytoplasmic organelles. TEM of A375 tumor cells treated by the effective

concentrations (EC: 0.11, 0.45, 0.9 μM) of Nubein6.8 showing the complete blockade **(arrow heads)** of external compound (which should be Nubein6.8) to the whole cell surface leading to loss of all internal cell compartments differentiation and disappearance of most cell organelles, and in other cells showing obvious abnormal blebbing cell membrane, peripheral migration of the nuclear chromatin toward the nuclear envelope (**NE**). TEM of control A2780 tumor cells representing intact cell membrane with some coiled and rayed gates, nuclei **(N)** of normally distributed chromatin and nucleoli, and some mitochondria **(M)** and lipid vacuoles were seen in the cytoplasm. TEM of A2780 tumor cells treated by the effective concentrations (EC: 0.11, 0.45, 0.9 μM) of Nubein6.8 showing cells with increased number of clear and empty leaking vacuoles **(EV)**, fragmented nuclei **(F)**, loose stranded nucleoli **(Nu)**. Other cells showed nuclear division features as nuclei can be seen of two poled parts and nucleoli **(Nu)** of stranded duplicated nuclear DNA, although of less dense chromatin, besides the aggregated number of mitochondria **(M)**.

Fig. 1.

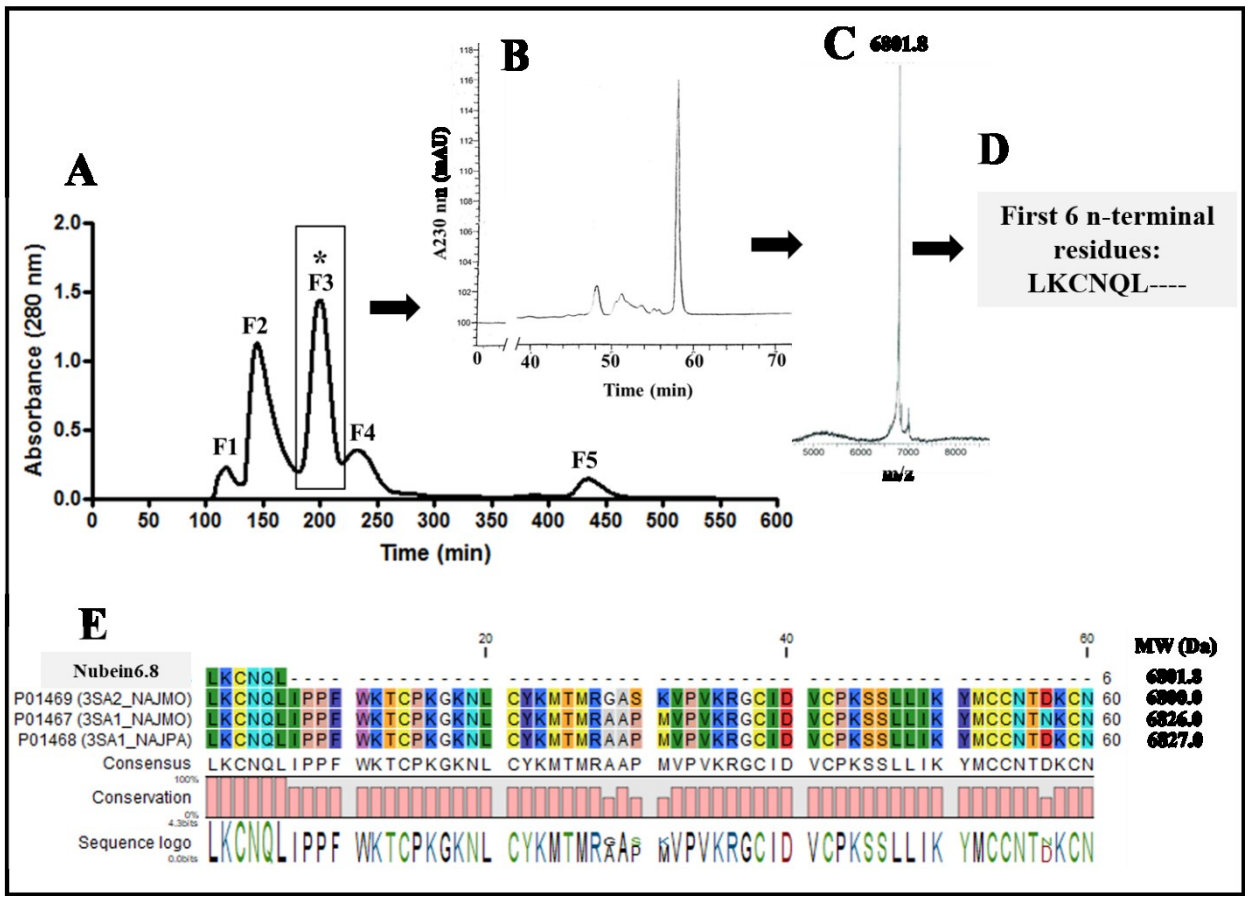

A
B
C
D
E
Absorbance (280 nm)
Time (min)
F1
F2
F3
F4
F5
A230 nm (mAU)
6801.8
m/z
First 6 n-terminal residues:
LKCNQL----
Nubein6.8
P01469 (3SA2_NAJMO)
P01467 (3SA1_NAJMO)
P01468 (3SA1_NAJPA)
Consensus
Conservation
Sequence logo
MW (Da)
6801.8
6800.0
6826.0
6827.0

Fig. 2.

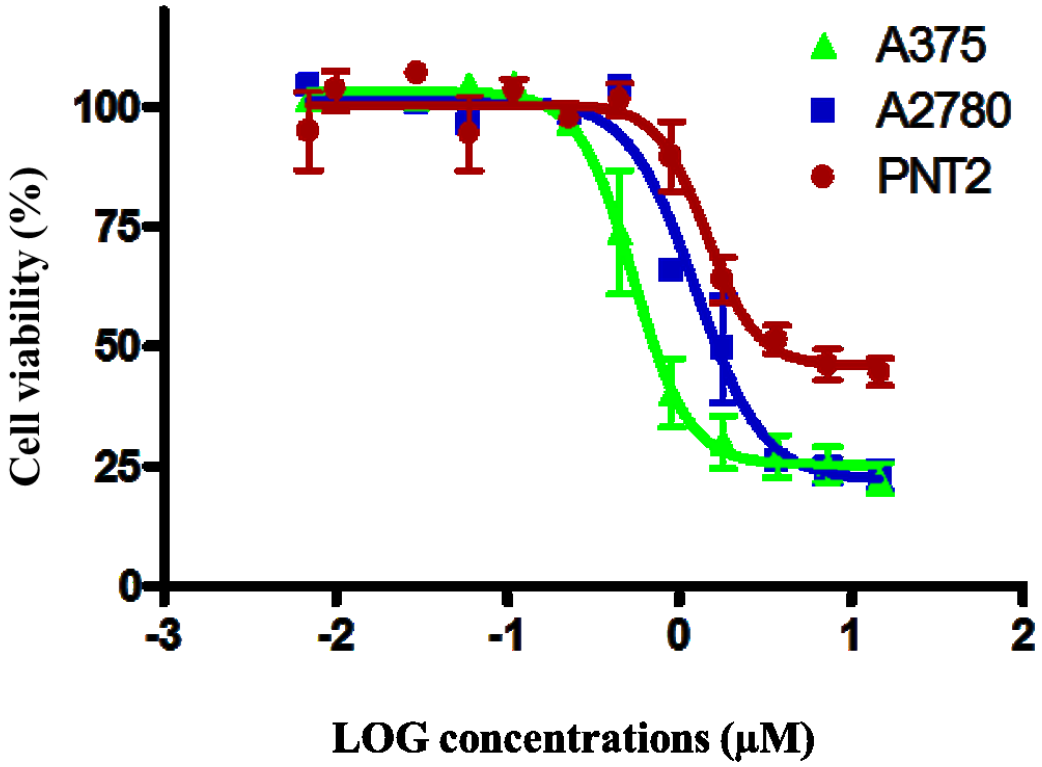

A375
A2780
PNT2
Cell viability (%)
100
75
50
25
0
-3
-2
-1
0
1
2
LOG concentrations (μM)

Fig. 3.

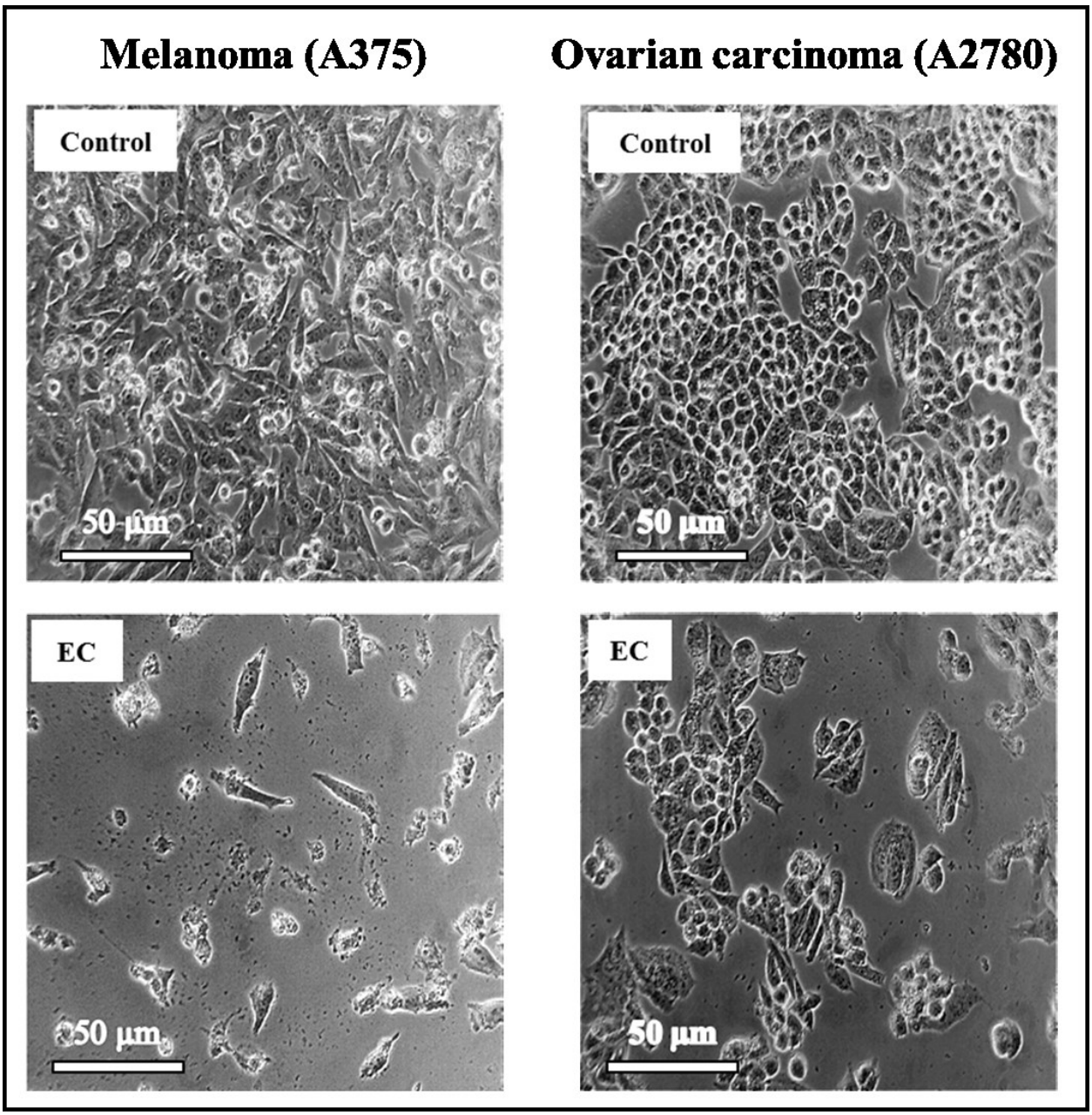

Melanoma (A375)
Ovarian carcinoma (A2780)
Control
50 µm
Control
50 µm
EC
50 µm
EC
50 µm

Fig. 4.

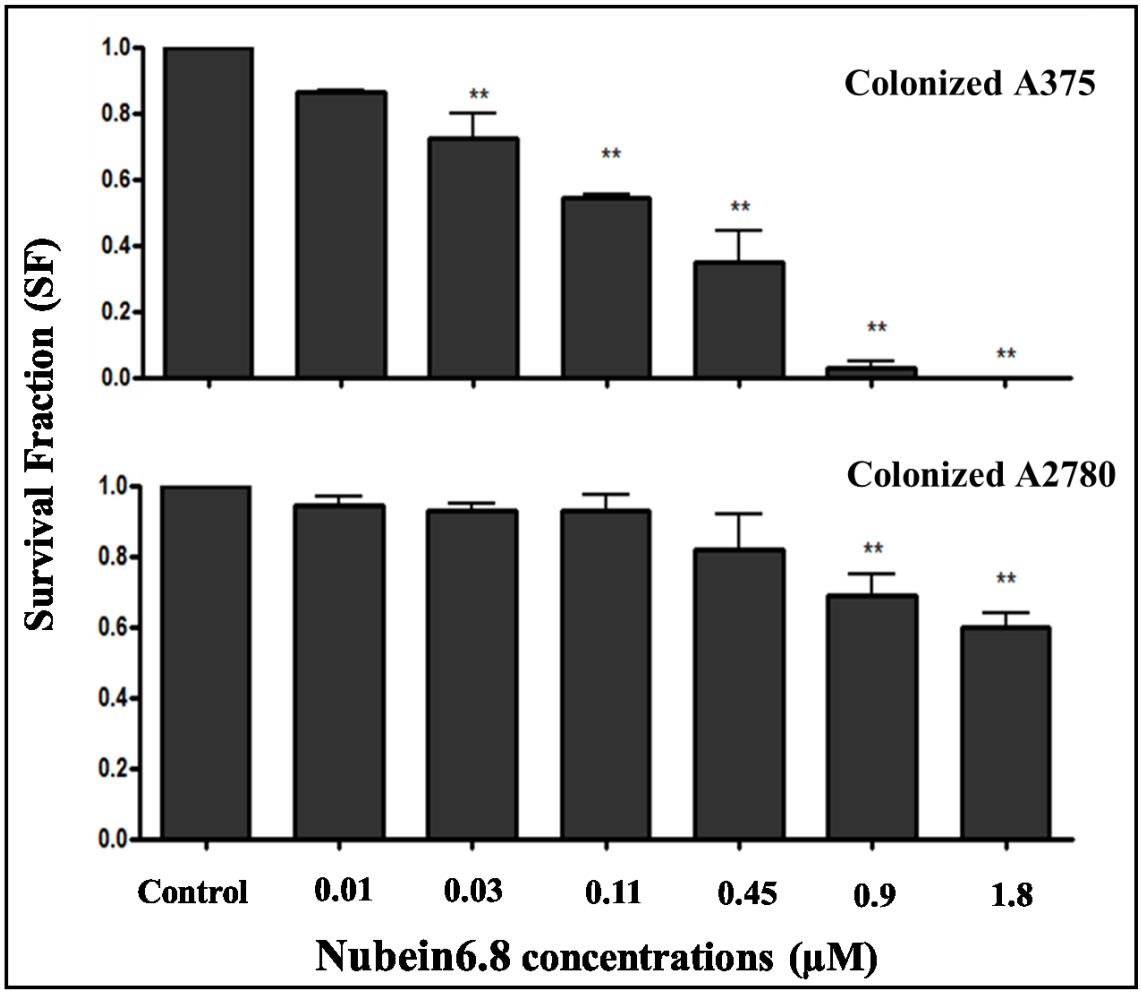

Colonized A375
Colonized A2780
Survival Fraction (SF)
1.0
0.8
0.6
0.4
0.2
0.0
**
Control
0.01
0.03
0.11
0.45
0.9
1.8
Nubein6.8 concentrations (μM)

Fig. 5.

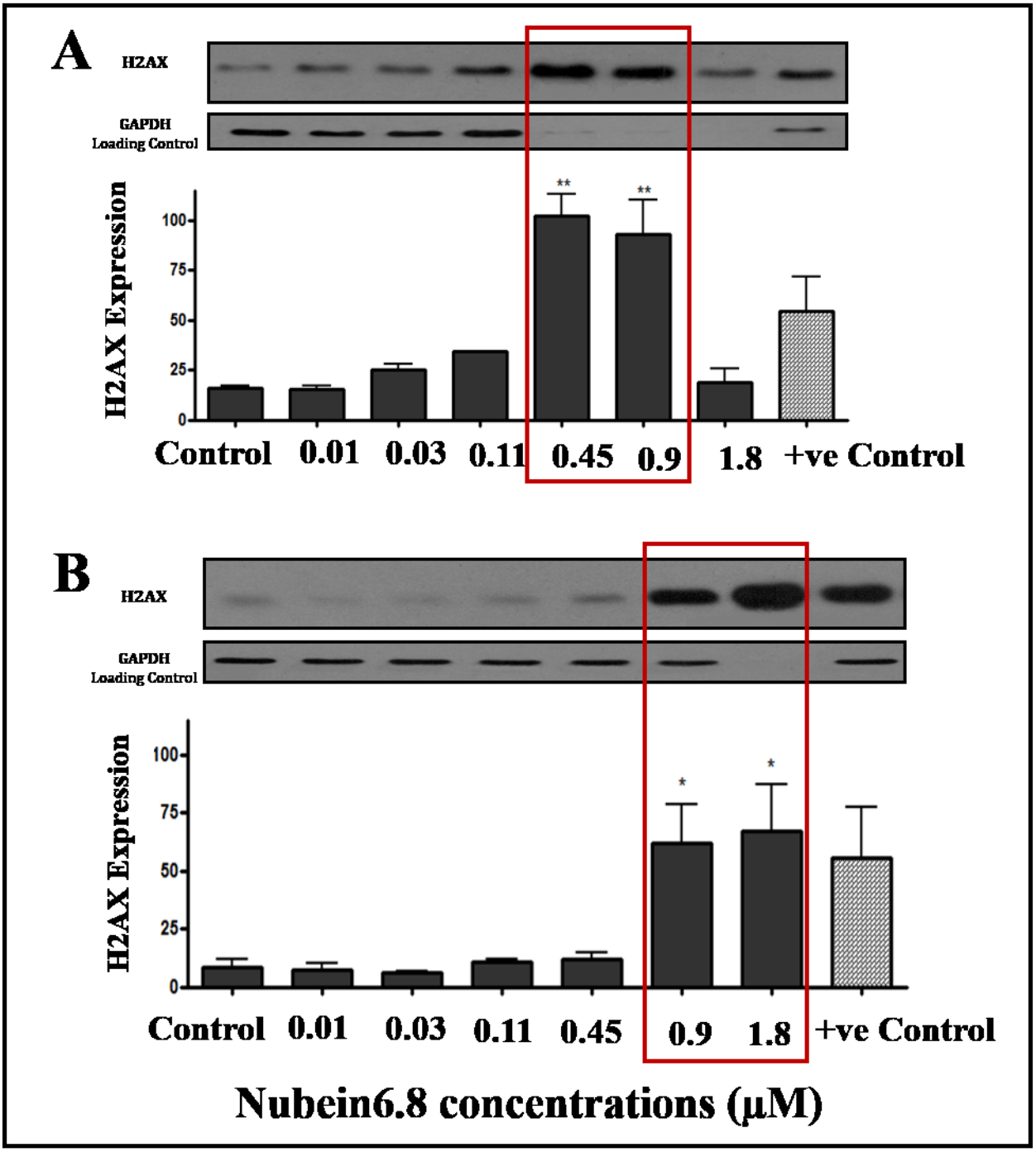

A
H2AX
GAPDH
Loading Control
H2AX Expression
100
75
50
25
0
**
**
Control
0.01
0.03
0.11
0.45
0.9
1.8
+ve Control
B
H2AX
GAPDH
Loading Control
H2AX Expression
100
75
50
25
0
*
*
Control
0.01
0.03
0.11
0.45
0.9
1.8
+ve Control
Nubein6.8 concentrations (μM)

Fig. 6.

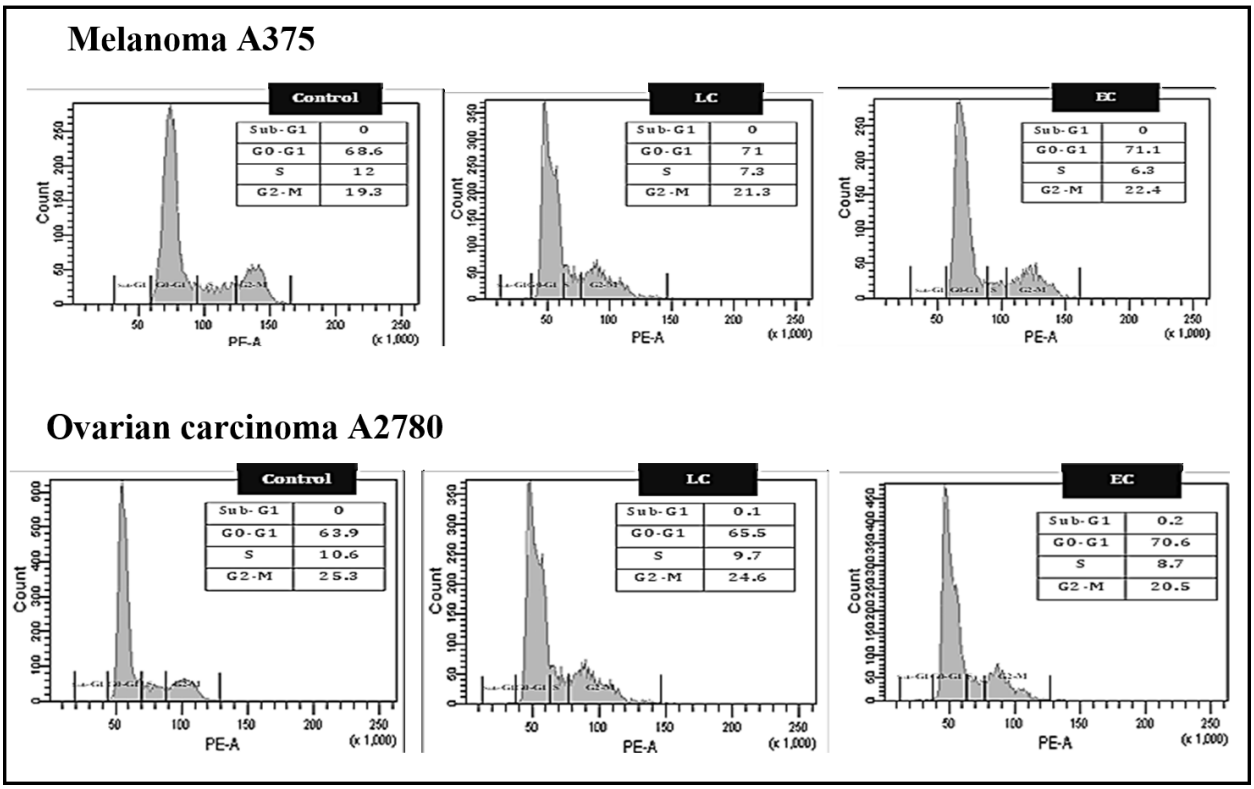

Melanoma A375
Control
Sub-G1 0
G0-G1 68.6
S 12
G2-M 19.3
LC
Sub-G1 0
G0-G1 71
S 7.3
G2-M 21.3
EC
Sub-G1 0
G0-G1 71.1
S 6.3
G2-M 22.4
Ovarian carcinoma A2780
Control
Sub-G1 0
G0-G1 63.9
S 10.6
G2-M 25.3
LC
Sub-G1 0.1
G0-G1 65.5
S 9.7
G2-M 24.6
EC
Sub-G1 0.2
G0-G1 70.6
S 8.7
G2-M 20.5
Count
PE-A
(x 1,000)

Fig. 7.

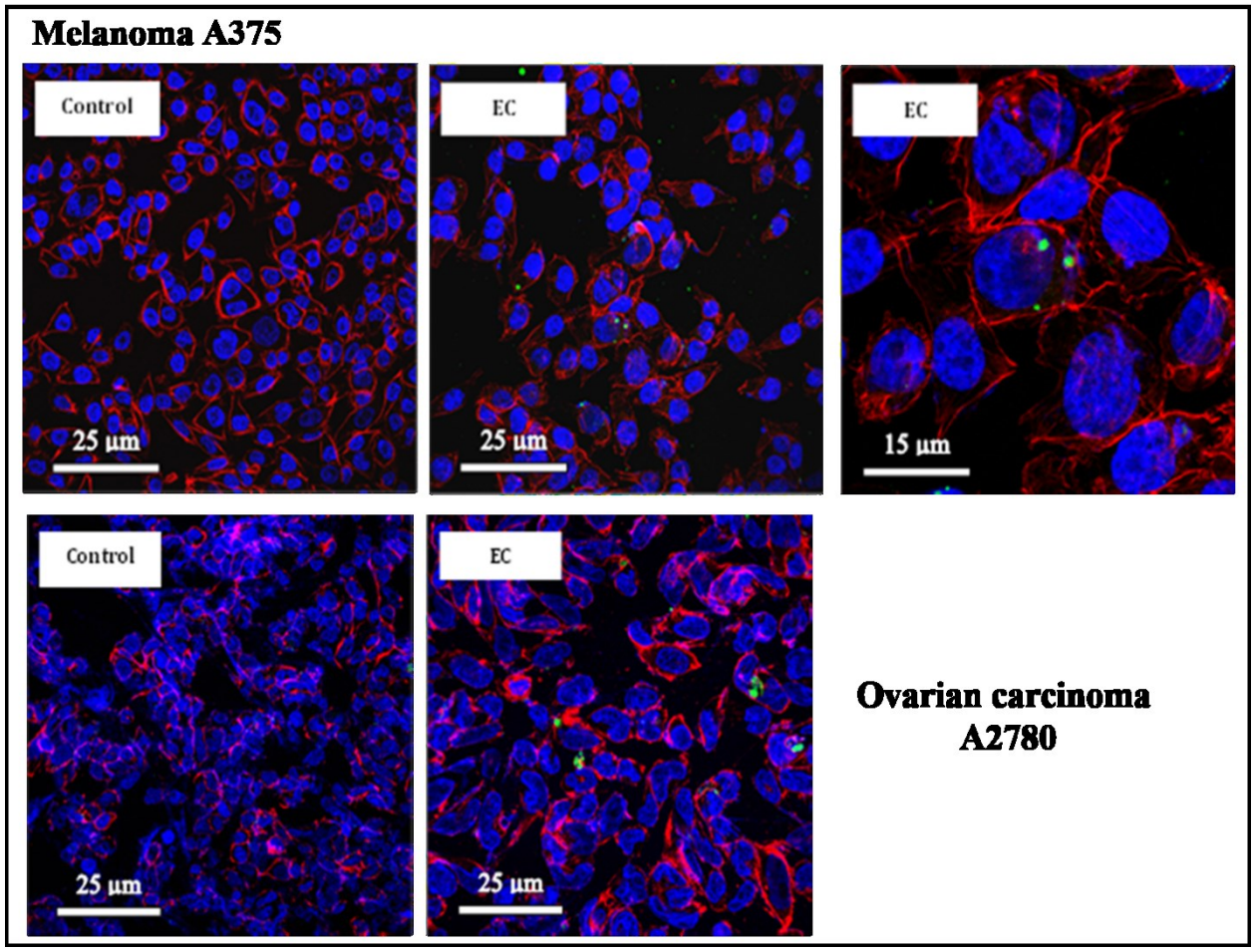

Melanoma A375
Control
25 µm
EC
25 µm
EC
15 µm
Control
25 µm
EC
25 µm
Ovarian carcinoma
A2780

Fig. 8.

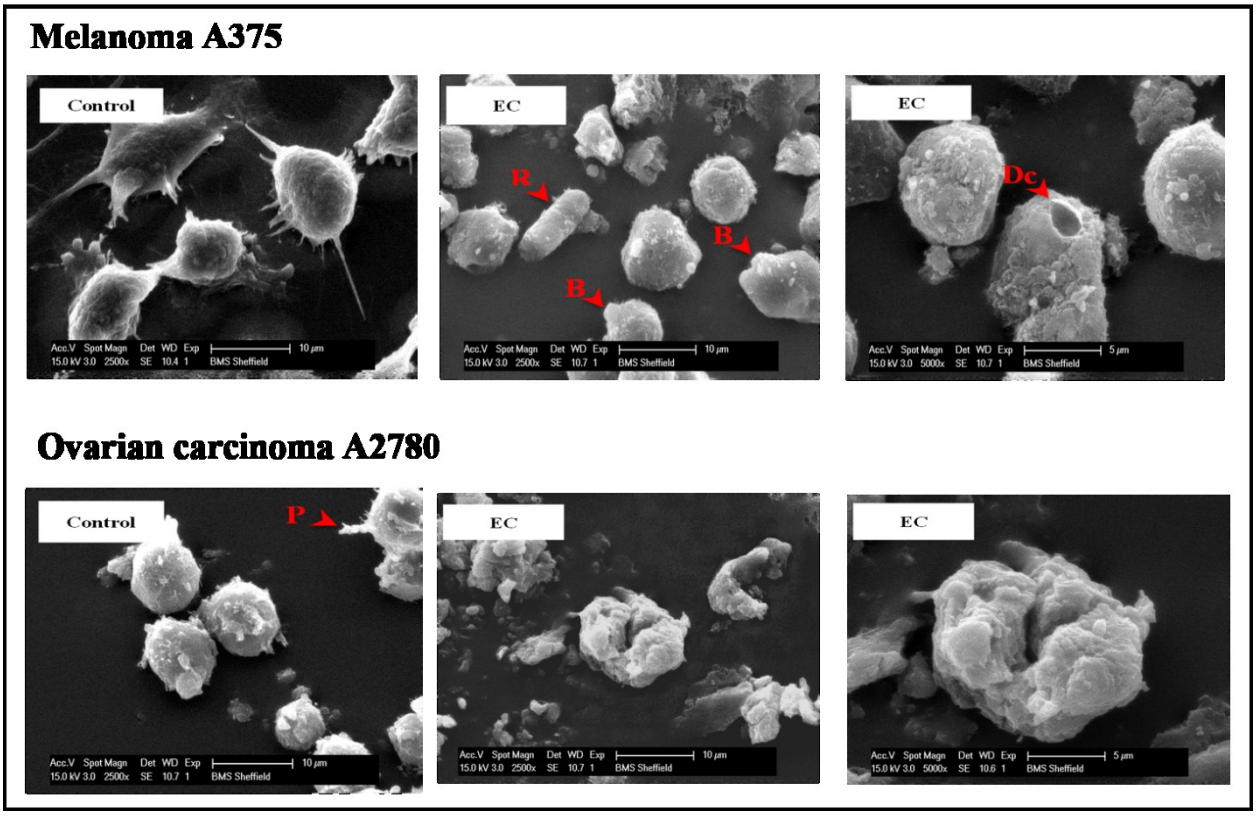
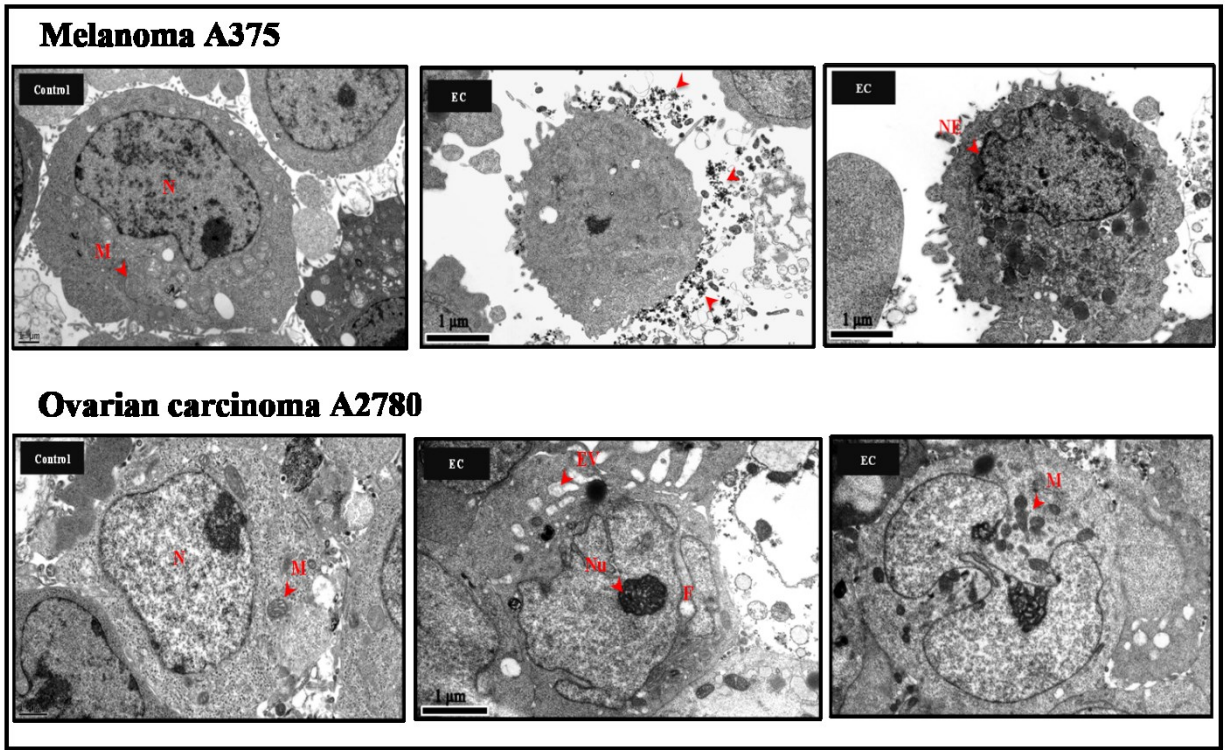
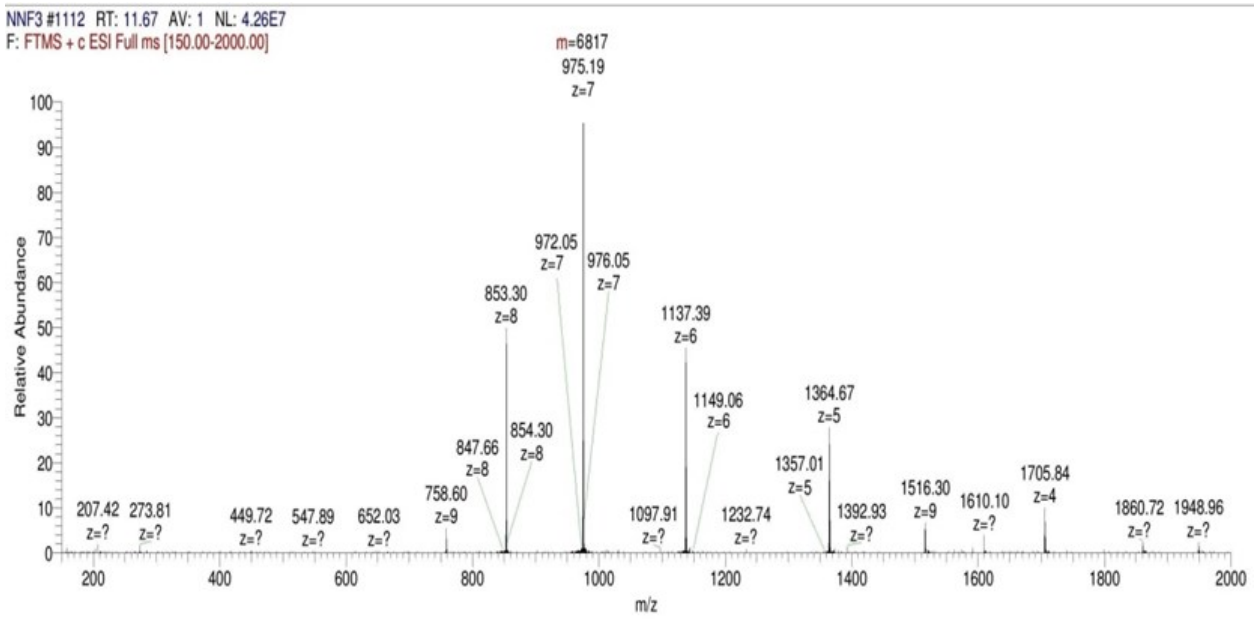
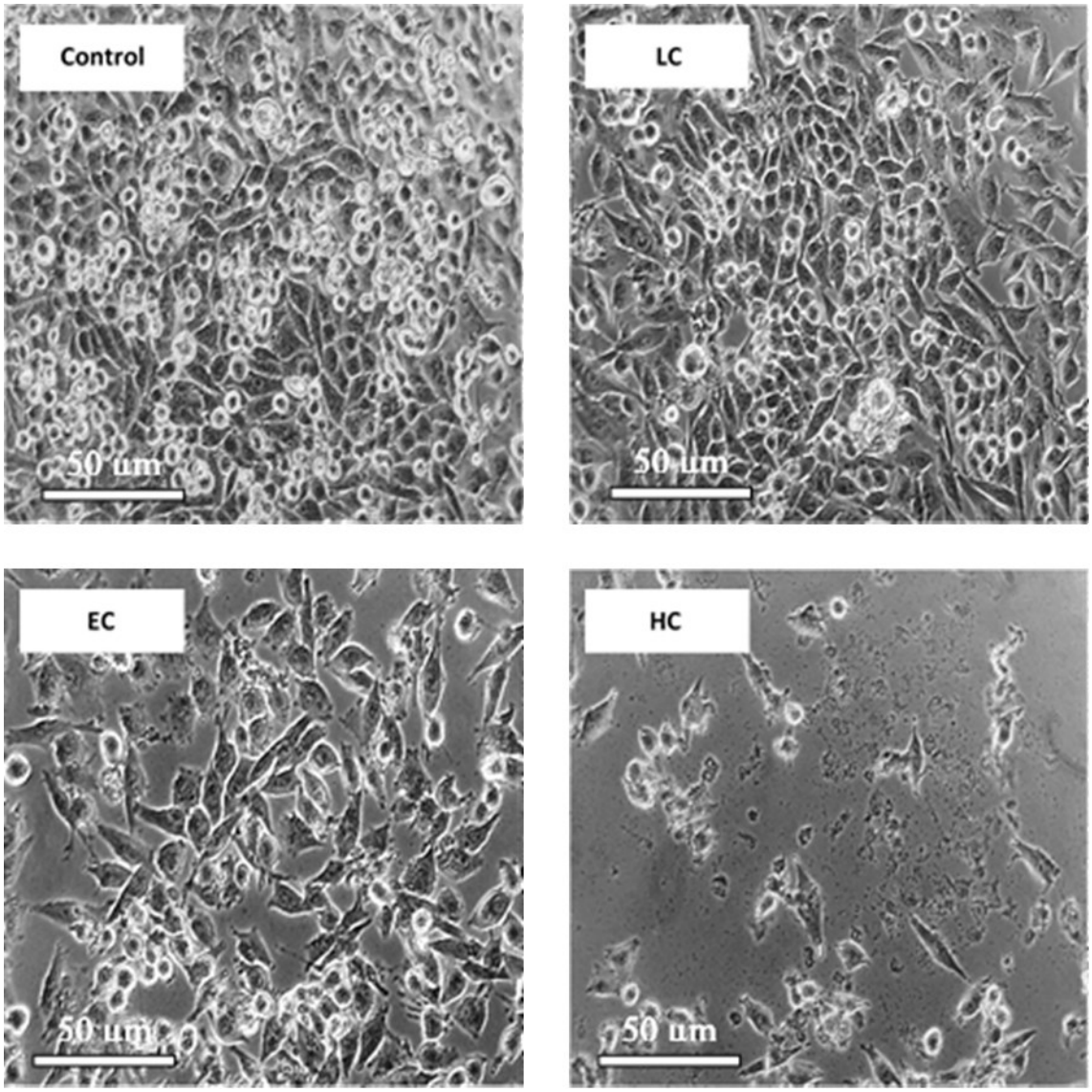

Melanoma A375
Control
EC
R
B
B
EC
Dc
Ovarian carcinoma A2780
Control
P
EC
EC

Fig. 9.

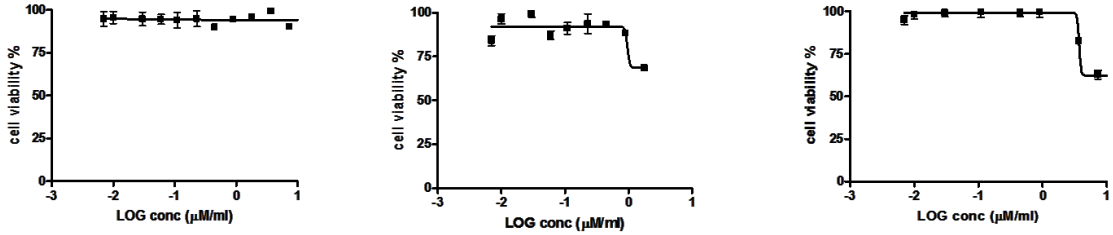

Melanoma A375
Control
N
M
EC
1 µm
EC
NE
1 µm
Ovarian carcinoma A2780
Control
N
M
EC
EV
Nu
F
1 µm
EC
M



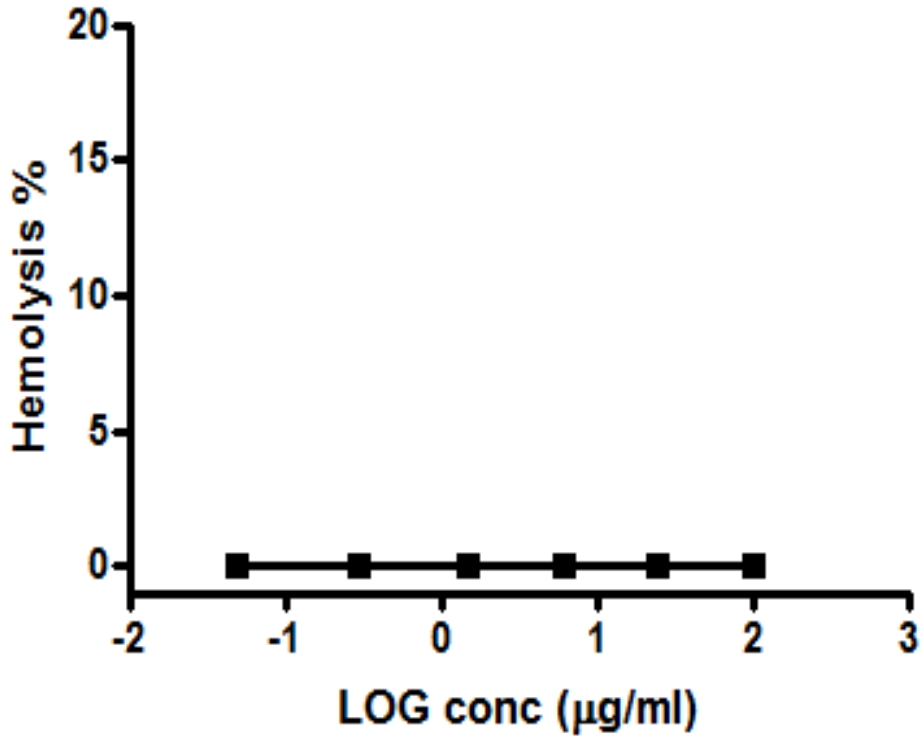
Supplementary Fig. 1. LC-MS of *Naja nubiae* fraction F3 (NNF3) separated by Sephadex G-50 column. The obtained data clearly showed that the fraction F3 contains only one peptide with 6.8 KD (Nubein6.8).



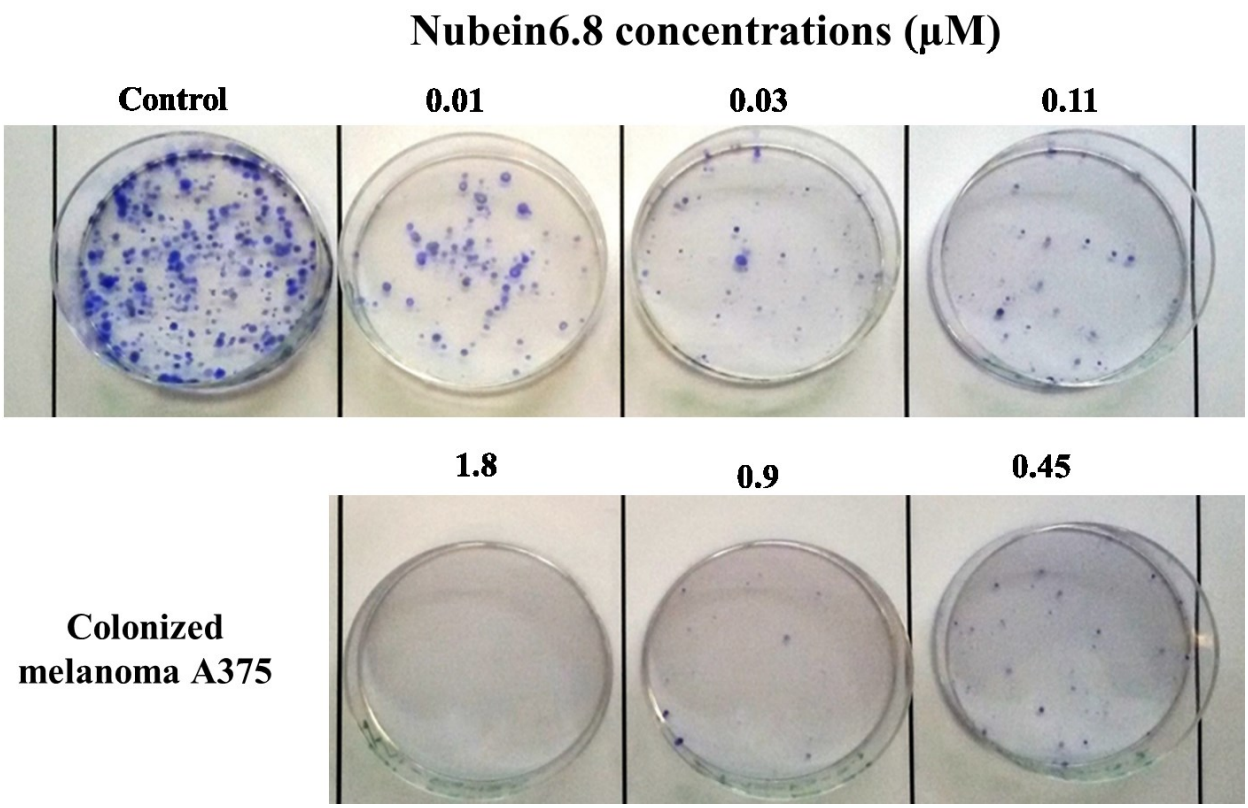
Supplementary Fig. 2. Morphological alterations of PANC1 pancreatic cancer cell line induced by Nubein6.8. PANC1 tumor cells prepared at optimized seeding density 20000 cells/well in 96 well plates then incubated (37°C and 5% CO_2) for 24 hrs. Different concentrations (0.007-14.7 µM) of Nubein6.8 were added and after 24 hrs incubation, the viability of the cells was assessed, and images taken. There was a slight cytotoxic effect of Nubein6.8 on PANC1 tumor cell viability in the high concentrations. HC; high concentrations (above 3.7 µM). EC; effective concentrations (0.11-0.9 µM), LC; low concentrations (0.007-0.06 µM).



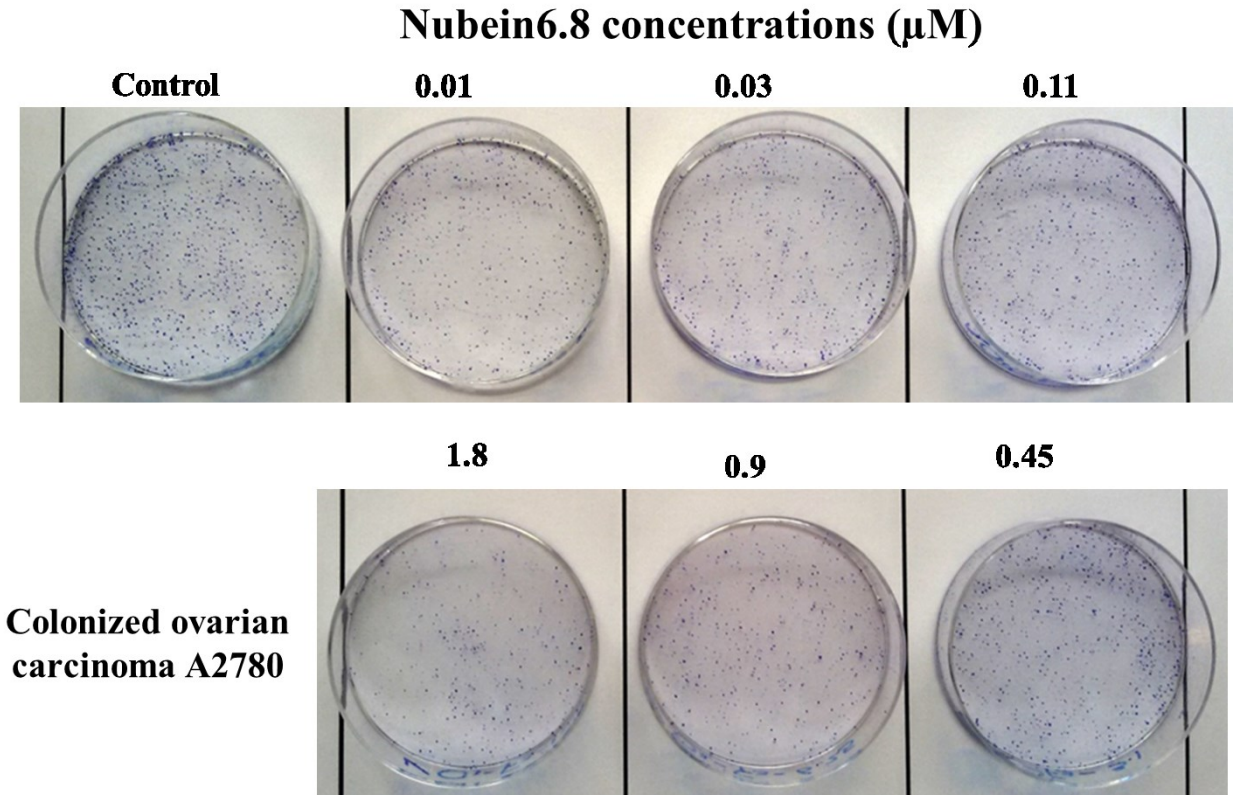
Supplementary Fig. 3. Cytotoxicity of Nubein6.8 derived from the snake venom *Naja nubiae* on PANC1 pancreatic cancer cell line. Dose-response curves and determination of the median effective concentration (EC_{50}) of Nubein6.8 on PANC1 cell line from three independent experiments performed in triplicates. There was no fit EC_{50} and the dose response curves not determined.



Supplementary Fig. 4. Hemolytic effect of Nubein6.8 on washed sheep RBCs. No haemolytic activity was detected of Nubein6.8 on RBCs at the highest concentration tested (14.7 µM).



Supplementary Fig. 5. Anti-metastatic potency of Nubein6.8 on human malignant melanoma (A375) using clonogenic assay. Clonogenic assay was carried out (n= 3) for Nubein6.8 on A375 tumor cells in 60 mm petri dishes at optimized density 400 cells/dish. After 9 days incubation (37°C and 5% CO_2), colonies formation was assessed and manually counted, starting with colonies developed in the negative control dishes and images taken. There was an obvious effect for Nubein6.8 on A375 tumor cell survival appeared from the highest concentration (1.8 µM) that have no colonized cells at all, and the formed-colonies average numbers kept too less than the control dishes till the lowest concentration (0.01 µM).



Supplementary Fig. 6. Anti-metastatic potency of Nubein6.8 on human ovarian carcinoma (A2780) using clonogenic assay. Clonogenic assay was carried out (n= 3) for Nubein6.8 on A2780 tumor cells in 60 mm petri dishes at optimized density 400 cells/dish. After 9 days incubation (37°C and 5% CO_2), colonies formation was assessed and manually counted, starting with colonies developed in the negative control dishes and images taken. There was a moderate effect of Nubein6.8 on A2780 tumor cell survival which significantly ($p<0.01$) appeared only at the concentrations of 0.9 µM and 1.8 µM, then the formed-colonies average numbers were quite near to control dishes at the rest of concentrations.