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## In silico identification and in Vitro assessment of a potential anticancer peptide sequence retrieved from the red sea metagenomics library

Mona Elradi Imam

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***In silico* Identification and *In Vitro* Assessment of a Potential  
Anticancer Peptide Sequence Retrieved from The Red Sea  
Metagenomics Library**

Thesis submitted to the Biotechnology Master's program  
for partial fulfillment of the requirements of a Master's Degree in Science

By:

**Mona Elradi**

Bachelor's degree in Medicine

Under supervision of:

**Dr. Asma Amleh**

Associate Professor, Biology Department

AUC

December 2016

# American University in Cairo

## *In silico* Identification and *In Vitro* Assessment of a Potential Anticancer Peptide Sequence Retrieved from The Red Sea Metagenomics Library

Thesis submitted to the Biotechnology Master's program By:

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December 2016

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Date

## **Dedication**

**With a heart full of love, I dedicate this work**

To the soul of my unforgettable indispensable Mom,

To my beloved Dad,

To my precious siblings, Bassant & Mohamed,

To my lovable husband, Rami

& To my best friend, Shaimaa.

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*In silico* Identification and *In Vitro* Assessment of a Potential Anticancer Peptide Sequence Retrieved from The Red Sea Metagenomics Library

**Abstract**

Cancer is imposing a worldwide health concern with alarming morbidity and mortality rates. Its burden stems from its increasing incidence along with depletion of efficient therapeutic options. Current conventional anticancer therapies; surgery, radiotherapy and chemotherapy are barely effective with deleterious side effects that ruin patients' quality of life. There is a paradigm shift in the interest of pharmaceutical industry towards a new class of peptide based drugs offering more selectivity, easier synthesis, wider safety profile and lower cost of manufacture. Anticancer peptides (ACPs) have gained interest in the last few decades due to their intrinsic properties such as cationicity and small size enabling them to be selective and effective anticancer agents. In our study, we made use of the publicly available databases of ACPs and the Red Sea metagenomics data, generated during AUC/KAUST Red Sea microbiome project. Our experimental design consists of two phases; *in silico* analysis followed by *in vitro* validation of the computational results. *In silico* analysis resulted in a set of peptide hits from our library that share similar composition to ACPs. One hit was submitted for further *in silico* prediction of structure and function. The sequence was then chemically synthesized for subsequent *in vitro* functional assessment through cytotoxicity assay (MTT assay), apoptosis/necrosis detection assay (Annexin/PI assay) and RNA expression analysis of Caspase 3. The cancer cell lines used were U2OS, HepG2, MCF7 and HeLa. The peptide showed evident, yet variable, dose dependent cytotoxicity in all tested cell lines. Membranolysis and Apoptosis could be concluded as possible mechanisms of action from the results of annexin assay and RT-PCR that showed overexpression of Caspase 3 in peptide treated U2OS cells. Our results, despite being consistent and in line with each other, more investigative techniques should be done for confirmation and elucidation of the molecular mechanism of action of our peptide lead.

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## **List of Abbreviations**

aa	amino acid
ACP	Anticancer Peptide
ADME	Absorption, Distribution, Metabolism & Excretion
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
APD	Antimicrobial Peptide Database
BIRC2	Baculoviral IAP repeat-containing protein 2
CAMP	Collection of Antimicrobial Peptides
CsA	Cyclosporin-A
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
HMM	Hidden Markov Model
HNP	Human Neutrophil Peptide
HPLC	High Performance Liquid Chromatography
IARC	International Agency of Research on Cancer
KAUST	King Abdullah University of Science & Technology
LDH	Lactate Dehydrogenase
MDR	Multiple Drug Resistance
PBS	Phosphate Buffered Saline
PDB	Protein Data Bank
PI	Propidium Iodide
PS	Phosphatidyl Serine
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAR	Structure Activity Relationship
SI	Selectivity Index
WHO	World Health Organization

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# **Chapter 1**

## **Literature review & Study objectives**

### **1.1. Literature review:**

#### **1.1.1. Cancer burden:**

There is no debate over the health and economic burdens that cancer yields both at the patient and community levels in developing and developed countries as well. Cancer encompasses a large group of diseases affecting any organ of the body causing uncontrollable cellular proliferation that is commonly metastasizing far from its original tissue and resisting treatment ("What Is Cancer?", 2016).

Cancer incidence is not restricted to any age group, gender or race. Cancer related mortality rate is surpassing cardiac disease related one within the coming few years. According to World Health Organization (WHO) and International Agency of Research on Cancer (IARC), there were 14.1 million new cancer cases, 8.2 million deaths and 32.6 million people living with cancer worldwide in 2012 with expectancy of the annual incidence rate to rise to 22 million cases before 2030 (de Martel et al., 2012).

Current cancer treatments include surgery, chemotherapy, radiotherapy and the recently introduced immunotherapy and biologics. The limited efficiency of current conventional onco-therapeutic options together with emerging resistance of cancer cells to a wide range of chemotherapeutic drugs add up a recent challenge to the burden of cancer (Chen et al., 2014).

Surgery is still the primary treatment approach for most solid malignancies. However, some tumors are inoperable due to anatomical inaccessibility, aggressive tumor nature invading adjacent tissues or risk of injury to a nearby vital unaffected structure. Radiotherapy is restrained in practical use by a certain exposure dose limit beyond which normal tissues are wrecked (Riedl et al., 2011).

The problem with chemotherapy, despite its relative efficacy especially for advanced cases, stems from its uncontrolled bio-distribution and lack of effective selection of malignant cells rather than normal cells. This treatment option is said to transform a deadly cancer into a chronic disease with more survival years, slower clinical deterioration and evident side effects such as bone marrow suppression, alopecia and mucositis which all interfere with patients' quality of life (Riedl et al., 2011).

Moreover, cancer cells developed several multiple drug resistance (MDR) mechanisms that enabled them to tolerate those chemotherapeutic drugs rendering many cancers resistant to therapy (Perez-Tomas, 2006). Given all these drawbacks of the available cancer treatment options in a time where the number of cancer victims is increasing every day, is an alarm for the need of a new alternative stand-alone or adjuvant cancer therapy. Such a new therapy should be of comparable efficacy if not higher and with a wider safety profile.

### **1.1.2. Peptide based drugs:**

The pharmaceutical industry is in continuous search for drug lead molecules that are of high potency, paramount safety and low manufacture cost. During the last few decades, peptides were mined for new lead competitors in the pharmaceutical market. Most drugs entering the market during the 20<sup>th</sup> century were developed in what can be called "chemistry dominated era". Those drugs are almost all small molecule drugs with a molecular weight cutoff of 500 daltons (Newman &Cragg., 2012).

The small size of such drugs grants them adequate oral bioavailability, efficient membrane permeabilization, metabolic stability and low cost of synthesis. On the other hand, this small size renders them non target selective causing side effects. Protein based drugs or biologics e.g. insulin and monoclonal antibodies have been introduced with the advance in molecular biology tools and recombinant protein expression technology in the latter half of the 20<sup>th</sup> century. Their large size (>5000 daltons) renders them finely specific for their targets on the cost of inadequate membrane permeabilization, high cost of synthesis, poor oral bioavailability and metabolic instability, (Craik et al., 2013).

Peptide leads have been the focus of the pharmaceutical R&D process because they simply fill the gap in molecular weight and properties between small molecule drugs and protein biologics. Like biologics, they can bind with high specificity to their molecular targets with few side effects. Their smaller size makes them more membrane penetrable, less immunogenic and more amenable to chemical synthesis with lower cost of manufacture than biologics (Craik et al., 2013, Uhlig et al., 2014).

Furthermore, they display a broad range of targets due to multiple mechanisms of action. They are less toxic than small molecules due to low accumulation in tissues because of their susceptibility to proteolytic degradation. Being discoverable from various natural sources along with their structural and biological diversities offer a rich source for peptide drug lead design and development. Finally, they are amenable to site specific modifications which make them good candidates for tailored drugs with desired therapeutic properties in the era of personalized medicine (Craik et al., 2013).

This paradigm shift in pharmaceutical interest towards peptides is hindered by some technical and financial challenges. Large scale peptide synthesis still needs cheaper methods to be developed. Natural peptides typically possess poor absorption, distribution, metabolism and excretion (ADME) properties. Methods to modify peptides to overcome their low systemic availability and high clearance still need to be optimized. Cyclizations, L-aa substitution with D-aa and modification of N & C termini have shown some desirable effects on peptide stability (Craik et al., 2013, Di, 2015).

Many peptide drugs have been in the market for decades representing 2% of the drug market e.g. Cyclosporin-A (CS-A) (11- aa) as an antifungal and immunosuppressant agent. Many others are still in the preclinical and clinical stages of the drug development pipeline. Others are still under basic research such as the Anticancer peptides (ACPs) (Di , 2015)

Accommodating new chemical and molecular biology tools and skills to efficiently design and optimize peptide based drug leads is becoming increasingly essential. Nonetheless, it mustn't be ignored that developing statistical and *in silico* models for exploitation of the publicly available databases of current knowledge about peptide drugs and for predicting their properties prior to synthesis would greatly enhance the next wave of peptide drug leads (Uhlig et al., 2014).

### **1.1.3. Anticancer peptides (ACPs):**

The attention to peptides as potential anticancer agents was drawn from some studies of antimicrobial peptides (AMPs) showing varying degree of activity against tumor cells (Hoskin & Ramamoorthy, 2008). The interest in understanding AMPs arose in order to develop new alternative resistance-free antimicrobial therapies after the illegitimate use of antibiotics in the last 50 years inducing a worldwide problem of microbial resistance (Reddy, Yedery, & Aranha, 2004).

Antimicrobial peptides constitute one of the weapons of innate immunity in almost all eukaryotes. They are small molecules of low antigenicity. Generally, AMPs are cationic, amphipathic with a high proportion of hydrophobic residues. The ability of these small cationic molecules to disrupt bacterial membranes depends on their biophysical properties such as their amphipathicity, hydrophobicity, size, secondary structure and most importantly the non-specific electrostatic interaction with the anionic lipid rich microbial membrane (Huang et al., 2014).

The rapid non-specific mechanism of membrane permeation and subsequently cell death offers marked potency, selectivity and abrupt changes that the cell machinery can't simultaneously endure to produce a resistant membrane (Chen et al., 2014). AMPs, either natural or synthetic, didn't only show activity against bacterial cells but their activity was also reported against fungal, protozoal, viral and cancer cells (Gaspar, Veiga, & Castanho, 2013).

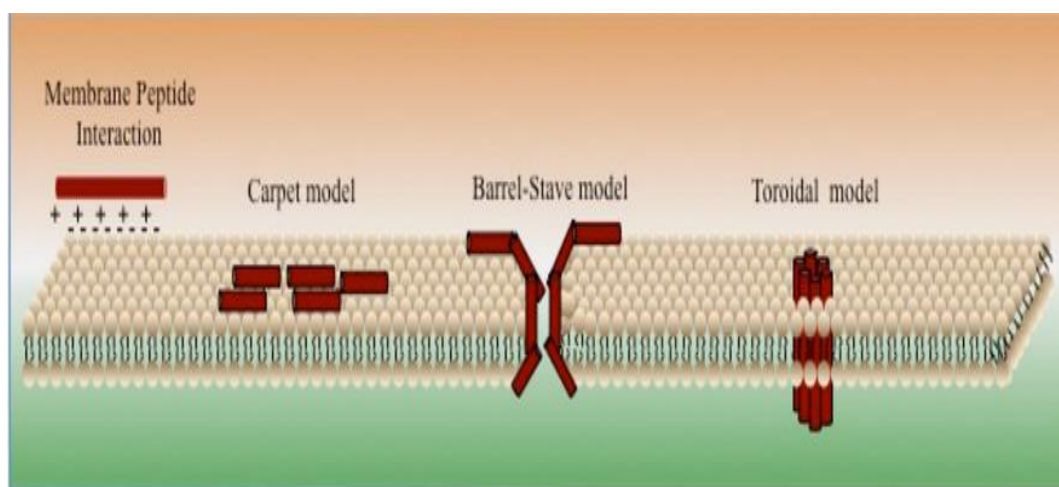
Anticancer peptides (ACPs) exert their cytotoxic activities in a similar way to AMPs. AMPs and ACPs share the same biophysical properties knowing that most ACPs are originally AMPs or synthetic AMP analogs. The increased net surface negative charge is a shared molecular feature of bacterial and tumor cells, relative to healthy normal cells. Hence, ACPs and AMPs can also be said to share the same molecular principle of cytotoxicity and selectivity (Gaspar, Veiga, & Castanho, 2013).

The ability of ACPs to selectively recognize and lyse tumor cells is still researched for the mechanistic details of the cellular death event. Kinetics of cancer cell killing involve membranolytic and non-membranolytic modes of action. However, the non-receptor mediated membrane lysis events still best explain the rapid and selective ACPs induced cytotoxicity (Epand, n.d, 2016).

In 2009, Iwaski et al. provided a proof of the role of phosphatidyl serine (PS) density, a main lipid component of cancer cell membrane, to anticancer activity of ACPs stressing on the proposed concept of electrostatic interaction (Iwaski et al., 2009). Other membrane features that aid ACP cytotoxicity are increased membrane fluidity allowing easy destabilization of the membrane along with its increased surface area due to more numerous microvilli allowing exposure to higher number of ACP molecules (Sok et al., 2002).

There are different modes of action that can explain the mechanism of membrane disruption. It occurs either through pore formation (torroidal and barrel stave model), thinning and membrane dissolution (detergent-like Carpet model), lipid peptide domain formation or membrane depolarization (Fig.1) (Papo & Shai, 2005). Membranolysis involves mitochondrial membrane disruption as well releasing initiators of apoptosis which can explain the late death events with some ACP examples (Constance & Lim., 2012).

From a structural point of view, there is an evident structural diversity in the AMPs and ACPs studied up till now. Certain ACP specific aa and dipeptide composition frequency is observed as well (Tyagi et al., 2013). The conformation of most ACPs fall either into the  $\alpha$ -helical (e.g. Cecropins) or the  $\beta$ -sheet structure (e.g. Defensins). Structure activity relationship (SAR) studies so far have demonstrated that peptide-membrane interaction depends on and affects the 2ry structure acquired by the peptide.



**Figure 1: Various models of membrane permeation by ACPs.** Adapted with permission from/: <http://crdd.osdd.net/raghava/anticp>.

Wang and Zhang found that the  $\alpha$ -helical conformation of MPI-1, a synthetic analog of the AMP Polybia-MPI extracted from *Polybia Paulista* wasp venom, was essential for its membranolytic anticancer activity (Wang et al., 2008, wang et al., 2009b, Zhang et al., 2010). In 2014, Chen et al. designed a series of  $\alpha$ -helical cationic peptides with certain (IHKK) repeats that promoted  $\alpha$ -helical conformation upon interaction with the negatively charged membranes. They found out that the  $\alpha$ -helical conformation with a certain hydrophobic effect is essential for membrane permeabilization. Peptides then got internalized initiated an apoptotic cascade (Chen et al., 2014).

Membrane disruption events are not always ensued with an apoptotic event. Dermaseptin B2, from tree frog *Phyllomedusa bicolor*, was found to induce some necrotic mechanisms following membrane lysis. That was confirmed through increased release of lactate dehydrogenase (LDH), propidium iodide (PI) staining and morphological studies using confocal microscopy (Van Zoggel., et al., 2012). CAMEL peptide, a synthetic hybrid peptide derived from the natural *Hyalophora cecropia* AMP; cecropin and *Apis mellifera* AMP; mellitin, is a necrosis inducing peptide that showed statistically significant *in vivo* tumor regression and relapse delay when combined with an Interleukin-12 carrying plasmid (Smolarczyk et al., 2010).

The direct membrane disrupting effect is not always the trigger for cell death induced by ACPs. LcfinB, isolated from cow's milk, is a known ACP that induces membrane pore formation with subsequent loss of membrane integrity and peptide internalization. LcfinB-induced membrane damage caused necrosis of human neuroblastoma and mouse fibrosarcoma cells (Eliassen et al., 2006). On the other hand, LcfinB induced reactive oxygen species (ROS) generation and subsequent mitochondrial mediated apoptosis in human breast and leukemia cells (Mader, 2005).

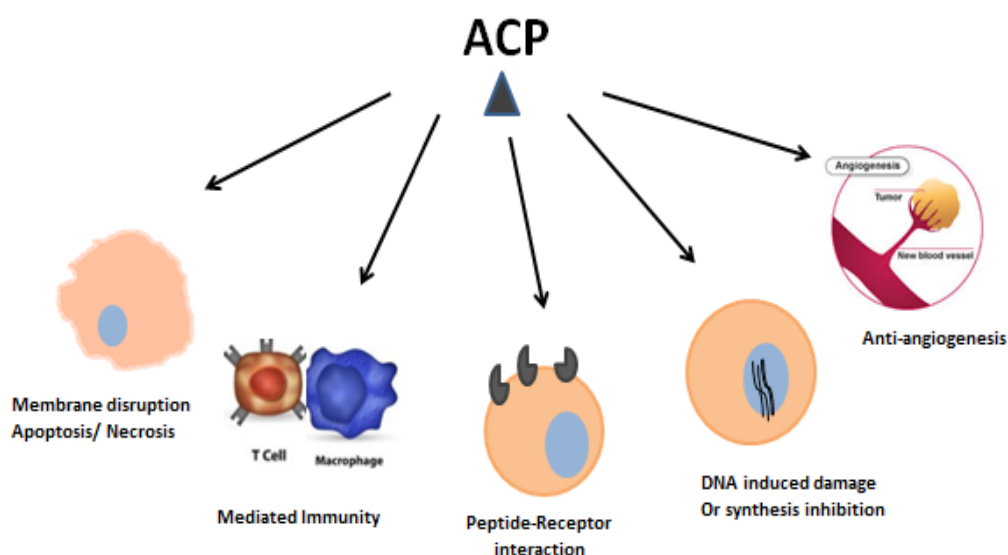
Human neutrophil peptides 1-4 (HNPs 1-4) are a group of defensive peptides (Defensins) released from the neutrophils in response to microbial infections. Their anticancer properties have been investigated for a long time. HNP-1 was found to have dual mechanism of action through damaging cell membrane and causing DNA strand break (Gera and Lichtenstein, 1991). That was recently confirmed as a mechanism of action in prostate cancer cells (Gasper et al., 2015). On the other hand, Wang et al. reported that expressing HNP-1 in breast and colon cancer cells induced



cellular death via recruiting dendritic cells eliciting an immune response (Wang et al, 2009c).

Other non-membranolytic mechanisms of ACPs include inhibition of tumor neovascularization (Koshimaki et al., 2009). Leuschner et al. used a 15 aa segment derived from chorionic gonadotropin as a mechanism for targeting cells expressing receptors for chorionic gonadotropin/Luteinizing hormone (CG/LH) in hormone dependent prostate and breast cancer respectively (Leuschner, 2005). Different mechanisms of ACPs are demonstrated in figure 2.

The different potencies and mechanisms of action adopted by the same peptide in different types of cancers can be explained by the differential expression pattern of negatively charged lipid molecules on the surface of different neoplastic cells which might condition the engagement and conformation of the interacting peptide dictating its preference to kill specific cancer cells. This elucidates the possibility that the mechanism of action depends on the cell type (Eliassen et al., 2006, Gasper et al., 2015).



**Figure 2: Different mechanisms of action of ACPs.** ACPs' activity can be due to a membranolytic mechanism, followed by apoptosis or necrosis, or a non-membranolytic mechanism such as a mediated immune response or DNA synthesis inhibition. Adapted and modified from (Gasper, Veiga, & Castanho, 2013).

The future of peptides as an oncotherapy is still met by some challenges. The concept of peptides being amenable to sequence manipulation during synthesis in order to end up having certain structural conformation and biophysical properties should be dealt with caution to have balanced equilibrium between different characters and maintaining potency and selectivity (Gaspar, Veiga, & Castanho, 2013).

## 1.2. Study Objectives:

This study had two main objectives:

1. Computational scanning of the Red Sea metagenomics library, created during AUC/KAUST Red Sea expeditions in 2008 & 2010, for sequence hits that show composition similarity to known experimentally verified ACPs. Computational structure and function prediction of one hit that shows adequate score and properties and presumably would act as an anticancer peptide (ACP).
2. Validation of the *in silico* results through *in vitro* experiments that would prove or deny the *in silico* result using chemically synthesized peptide hit. Experiments include cytotoxicity assay (MTT assay) against 4 cancer cell lines and one normal cell line, apoptosis and necrosis detection assay (Annexin/PI assay) and RNA expression analysis (RT-PCR) of Caspase 3; a known apoptosis marker.

## Chapter 2

### **Materials & Methods:**

#### **2.1. Computational Analysis:**

##### **2.1.1. Red Sea Metagenomics Library scanning:**

Scanning the Red Sea Metagenomics library, created at AUC after the Red Sea expedition in spring 2010 in collaboration with KAUST, was inherited after Tyagi et al. (Tyagi et al., 2013). Positive and negative examples of ACPs were required. One set of experimentally validated ACPs was retrieved from publicly available AMP databases such as the antimicrobial databases (APD) and (APD2) (Wang, 2004, Wang et al., 2009a), the collection of antimicrobial peptides (CAMP) (Waghu et al., 2014) and the database of Anuran defense peptides (DADP) (Novkovic et al., 2012). Another set of AMPs with no known anticancer activity was recruited, from the same databases, for comparison.

A preliminary investigation of all possible one amino acid (aa) and oligopeptide frequencies was done for the 2 datasets. Certain 1 aa and dipeptide (2 aa) frequencies were naturally observed in each dataset, creating a narrow standard error from their mean, indicating that the reported frequencies are almost consistent within each dataset. Thus, one aa and 2 aa composition were used as input features when comparing the 2 datasets for selecting only those recognizing ACPs (t- test,  $p < 0.05$ , Bonferroni multiple testing correction). A sliding window of an increasing size (minimum 5 aa) was used to generate peptides from metagenomic reads and assemblies. Each generated peptide had the ACPs' specific amino acid and dipeptide frequencies measured and scored accordingly.

A set of 59 peptide sequences with various scores and lengths was generated. Furthermore, the generated peptides were searched for Hidden Markov Models (HMMs) (Eddy, 1998) confirmed for the experimentally validated ACPs. Then, they were fed into an online tool "AntiCP" (Institute of microbial technology, Chandigarh, India) for confirming their prediction as ACPs (<http://crdd.osdd.net/raghava/anticp>) (Tyagi et al., 2013).

Out of the 59 sequences, a homeodomain sequence (PF00046.24) of 30 aa was chosen for synthesis. A short sequence (AAEK), corresponding to 2 Alanines, Glutamate and a Lysine, was added to the 30 aa homeodomain peptide sequence outside the HMM domain as a means to increase its hydrophobicity and charge and hence its penetrability. The new 34 aa sequence (AAEKEFIKYPYPTPLQYQQQLATRLKVEKKLVRRW) was again validated as an ACP using AntiCP webserver provided by (Tyagi et al., 2013) (<http://crdd.osdd.net/raghava/anticp>). The sequence was alone aligned against the downloaded ACP dataset and the non-redundant protein database of PDB and SwissProt using online BLastp (NCBI, NIH) (Altschul et al., 1990).

### **2.1.2. *In silico* prediction of structure & function of the sequence:**

Raghava group provide more online servers that aid the development of peptide drugs especially anticancer ones. The half-life of the peptide sequence in an intestine-like environment could be predicted using "HLP" webserver (<http://www.imtech.res.in/raghava/hlp/>) (Sharma et al., 2014). Then, the sequence was analyzed for toxicity using "Toxinpred" webserver which contains more than 1800 toxic peptides (<http://osddlinux.osdd.net/raghava/toxinpred/>) (Gupta et al., 2014). Finally, aligning the sequence against a database of human apoptotic proteins within cancer context was done using "ApoCanD" webserver (<http://crdd.osdd.net/raghava/apocand/>) (Kumar & Raghava, 2016).

Using some other available online tools, the structure and function of the chosen sequence was predicted. Using I-tasser server, provided by Zhang lab; University of Michigan (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) (Zhang., 2008, Yang & Zhang, 2015), the 3D structure of the sequence could be predicted along with identifying top templates to which the sequence shows high structural identity. Using COACH & COFACTOR, which are other tools provided by the same webserver, the ligand binding site & biological function annotation of the peptide could be predicted respectively.

## **2.2. Peptide synthesis:**

The 34 aa peptide was synthesized using FlexPeptide™ technology (Genscript, USA), purified using High performance liquid chromatography (HPLC). The peptide was of >95% purity and delivered aliquoted in a lyophilized powder form. Sterile filtered phosphate buffered solution (PBS) was used to dissolve the peptide. Using the molecular calculator on Genscript Company's website, these characteristics of the peptide were determined:

Chemical formula: C197H312N52O49

M. Wt: 4192.92 Daltons

Charge: +5 (Cationic)

## **2.3. Cell culture:**

The cell lines used were HepG2, MCF7, HeLa & U2OS. Both MCF7, a human breast adenocarcinoma cell line, and HepG2, a human liver cancer cell line, were purchased from Vacsera, Egypt. HeLa, a human cervical cancer cell line, & U2OS, a human osteosarcoma cell line, were both gifts from Dr. A. kakaroukas (Biology Department, AUC). HepG2 was maintained in RPMI-1640 (Sigma Aldrich, USA) and the other 3 cell lines were maintained in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, USA) supplemented with 10% Fetal bovine serum (FBS) (Gibco, USA) and 5% Penicillin-streptomycin antibiotic (Gibco, USA).

Cells were incubated in a humidified CO<sub>2</sub> incubator (ShellLab, USA) adjusted at 37°C and 5% CO<sub>2</sub>. They were regularly split at 70-80% confluence. Cells were routinely examined using an inverted microscope (Olympus IX70, USA) to detect any morphologic changes or contamination. Viable cell counting was done for all the cells using the Trypan blue staining method via haemocytometer (Hausser Scientific, USA). It was a mandatory step before seeding for the MTT and annexin V/ Propidium iodide (PI) assay.

## 2.4. Cytotoxicity assay (MTT assay)

After counting, cells (HepG2, MCF7, HeLa & U2OS) were seeded in 96-well plates (Greiner Bio-one, Germany) at a density of  $2 \times 10^4$  viable HepG2 cells/ well and  $1 \times 10^4$  viable cells/well for other cell lines. They were left for overnight incubation before peptide treatment. Serial concentrations of the peptide drug (1, 10, 100, 300, 500 ug/ml) were used to treat cells. Triplicates of each condition were run, unless otherwise specified. Fresh media was used to prepare the peptide drug serial dilutions.

In each plate, untreated cells exposed only to fresh media were used as control cells and their absorbance values were used as the 100% viability. Additionally, some cells in each experiment were treated with fresh media along with the solvent, used for preparing the peptide drug (PBS), in an equivalent volume for the highest concentration of the peptide drug in order to check whether any resultant cytotoxicity would be due to the peptide or its solvent.

Cells were left incubated with the peptide for 24 hours before removing the drug and adding MTT. MTT (Serva, 11 Germany) is a yellow chemical tetrazolium compound; 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide. It reacts with the mitochondrial dehydrogenase enzymes of viable cells producing purple precipitate. 20ul MTT (5mg/ml) along with 100ul fresh media were added to each well and incubated for 3-4 hours.

Then, the media with the MTT were removed and the formed crystals in each well were solubilized using 100ul Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). Absorbance was measured using a microplate reader FLUOstar OPTIMA (BMG LabTech, Germany). Cell viability was then determined depending on the absorbance values (at 492nm) of the test cells as a percentage of the control untreated cells (100% viability).

The IC<sub>50</sub> of the peptide (Median inhibitory concentration that kills 50% of the cells) was calculated using Graphpad Prism version 5.0 ([www.graphpad.com](http://www.graphpad.com)) (Haeley, 2005). It was calculated depending on the mean of cellular viability percentages for the tested concentrations after log transformation of data of three independent experiments (unless otherwise specified). Parallel experiments using cisplatin as a standard cytotoxic drug was done for each cell line as a positive control example of cytotoxicity. Serial concentrations of cisplatin (0.5, 1, 2, 4, 8, 16 & 32 ug/ml) were used.

### **2.5. Cancer cell selectivity:**

In order to test for the selectivity of the peptide, its cytotoxic activity was tested on L929 (mouse normal fibroblast cell lines) using MTT assay in the same technique used for the cancer cell lines using the following concentrations (0.01, 0.1, 1, 10 & 100ug/ml). Cisplatin activity against L929 was also assessed using the same concentration range used with the cancerous cell lines.

### **2.6. Annexin V/ Propidium Iodide (PI) assay:**

All the following experiments were done on U2OS cells since it is the only cell line that gave a well-defined statistically significant dose dependent cytotoxicity pattern in three independent experiments. Annexin V/PI assay was performed according to manufacturer's protocol (Thermofischer, USA) and adapted for imaging fluorescent microscopy (Olympus IX70, USA).

Death was induced by incubating  $33 \times 10^4$  U2OS cells in one well of a 6 well plate, after an overnight seeding, with 200ug/ml of the peptide drug for 24 hours. Calculations were optimized according to the seeding density and surface area for a 6 well plate. Equal number of untreated U2OS cells was seeded in another well in the same plate to be used as negative control.

The cells were washed by PBS, trypsinized by quenching and finally centrifuged to get a cell pellet. After trypsinization, 20ul of cell suspension was used for counting using trypan blue to determine viability percentage of the treated and untreated cells. The pellet was then resuspended in a volume of 1X annexin binding buffer that would allow to have a cell count of  $1 \times 10^5$  cells in 15ul cell suspension to be properly deposited onto a glass slide, covered with a cover slip and visualized under the microscope.

For each 100ul cell suspension, 10ul Annexin V conjugate (Alexa flour) and 1 ul of PI working solution was added, incubated at room temperature in the dark for 15 minutes and then visualized under the microscope using the appropriate filters. The fluorophore (Alexa flour AnnexinV conjugate) is a strong green fluorescent dye excited by blue light (488nm) with an emission spectrum similar to FITC (530nm).



Red fluorescent nucleic acid binding PI can be excited using green light (535nm) and emits at 617nm.

## **2.7. Reverse Transcription-PCR (RT-PCR)**

Total RNA was extracted using Trizol (Invitrogen, USA) as per manufacturer's protocol from peptide (IC50; 100.5 ug/ml) treated U2OS cells and control untreated cells. Extraction of RNA was followed by a quantification step in order to use constant concentration throughout the experiments. cDNA synthesis ensued using Revert ID™ First Strand cDNA synthesis kit (Fermentas, USA). PCR experiment for Caspase 3 was done using the following primers F. Primer (GACCATACATGGGAGCAAGT) R. Primer (ATCCGTACCAGAGCGAGA).

## **2.8. Computational assessment of OCT3 expression in studied cell lines:**

Genevestigator, a high performance search engine for gene expression studies was used (Hruz et al., 2008). The tool integrates thousands of manually curated published microarray and RNAseq data for a reference based visualization of gene expression in different biological contexts. OCT3 expression level was assessed in U2OS, HepG2, MCF7 &Hela cell lines. Deficiency of molecular data on OCT6 expression made the analysis of its expression level in different cell lines inapplicable.

## **2.9. Data Analysis**

Statistical analysis was performed using Graphpad Prism version 5.0 ([www.graphpad.com](http://www.graphpad.com)) (Haeley, 2005). Graphical presentations were created using the same program. All results represent the mean  $\pm$  SD of three independent experiments unless otherwise specified. One-way ANOVA was used for the statistical analysis of multiple groups and a single variable. Unpaired t-test was used for pairwise comparison. Results were deemed significant with P-value <0.05.

For RT-PCR experiment, densitometric analysis was done using Image-J (National Institute of Health, USA, <http://www.imagej.nih.gov/ij>). Band intensities was normalized to the intensities of the loading control ( $\beta$ -actin).

## Chapter 3

### **Results:**

#### **3.1. Computational analysis:**

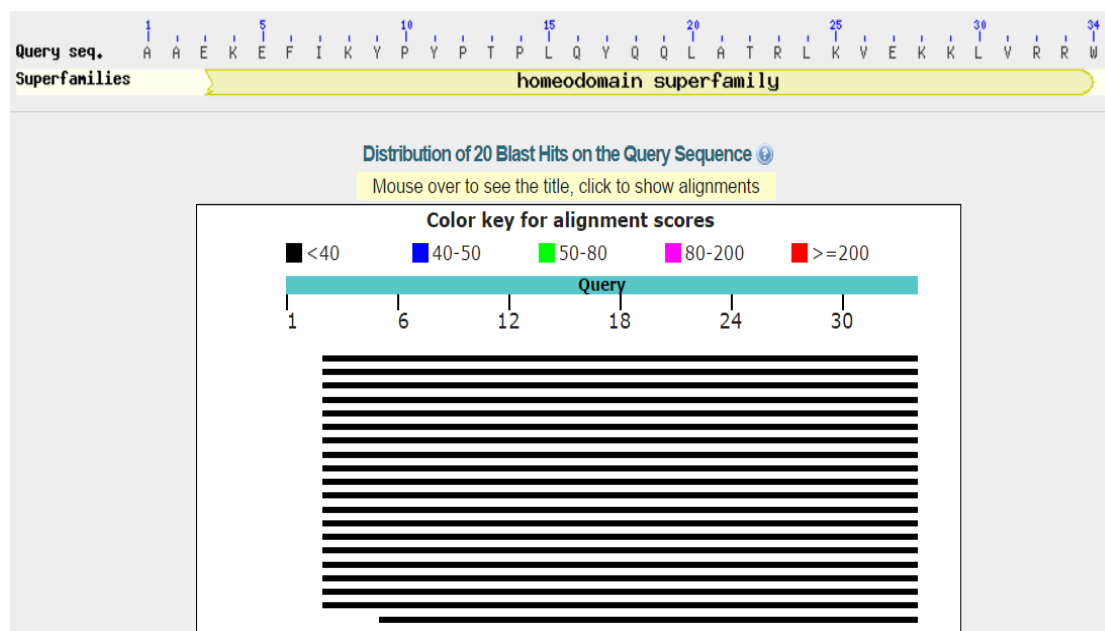
Out of the generated list of 59 peptide sequences with *in silico* predicted anticancer activity, one 30 aa sequence with a homeodomain HMM-ID was chosen for subsequent *in silico* structure analysis, synthesis and *in vitro* functional assessment. The sequence was predicted as an ACP using the "AntiCP" webserver (Tyagi et al., 2013) (Fig.3). The sequence was confirmed to be belonging to homeodomain protein family using Blastp (Fig.4) and HMMER online webtools (<http://hmmer.org/>). Nearly all resulting significant matches were homeodomain proteins of different species. Homeodomain is a well-known family of transcription factors involved in embryogenesis, cell differentiation and carcinogenesis in some instances (Sagan et al., 2013).

When the sequence was alone blasted against the downloaded set of ACPs, it showed significant alignment with one ACP namely, Bacteriocin (Fig.5). When the sequence was analyzed for predicting its half-life in an intestine-like environment, its half-life was predicted to be nearly 1 second with normal stability (Fig.6) and when it was analyzed against a database of toxic peptides, it was predicted to be non-toxic (Fig.7).

The sequence showed significant alignment with multiple apoptotic proteins when fed into the Blastp tool of the apoptotic protein database "ApoCand". The aligned proteins include 17 different apoptotic proteins out of the 82 proteins of the database. The most significant of which are human BIRC2 (Baculoviral IAP repeat-containing protein 2, UniProtKB - Q13490), human tumor necrosis factor receptor superfamily member 1A (UniProtKB - P19438) and different caspases. Such proteins play different roles in the numerous apoptotic pathways some of which are caspase independent (Parthanatos).



**Figure 3: Prediction of the peptide sequence as an ACP.** The sequence was submitted to <http://crdd.osdd.net/raghava/anticp/> and predicted ACP before and after addition of the newly introduced amino acids (AAEK). The sequence exhibits adequate amphipathic and hydrophobic properties of an ACP.



**Figure 4: Blastp Alignment of the peptide sequence against PDB & SwissProt.** The sequence showed significant alignment with hundreds of homeoproteins of different species of which are human POU transcription factors.

Download [Graphics](#)

**Bacteriocins**

Sequence ID: Query\_27336 Length: 26 Number of Matches: 1

Range 1: 8 to 24 [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Method	Identities	Positives	Gaps
17.7 bits(34)	0.10	Compositional matrix adjust.	8/17(47%)	11/17(64%)	0/17(0%)

Query 18 QQLATRLKVEKKLVRRW 34  
 Q AT +K KKL ++W  
 Sbjct 8 QMGATAIKQVKKLFKKW 24

**Figure 5: Blastp alignment of the peptide sequence against a set of experimentally verified ACPs.** It shows significant alignment with one ACP sequence namely Bacteriocin.

## HLP: A webserver for predicting half-life of peptides in intestine like environment

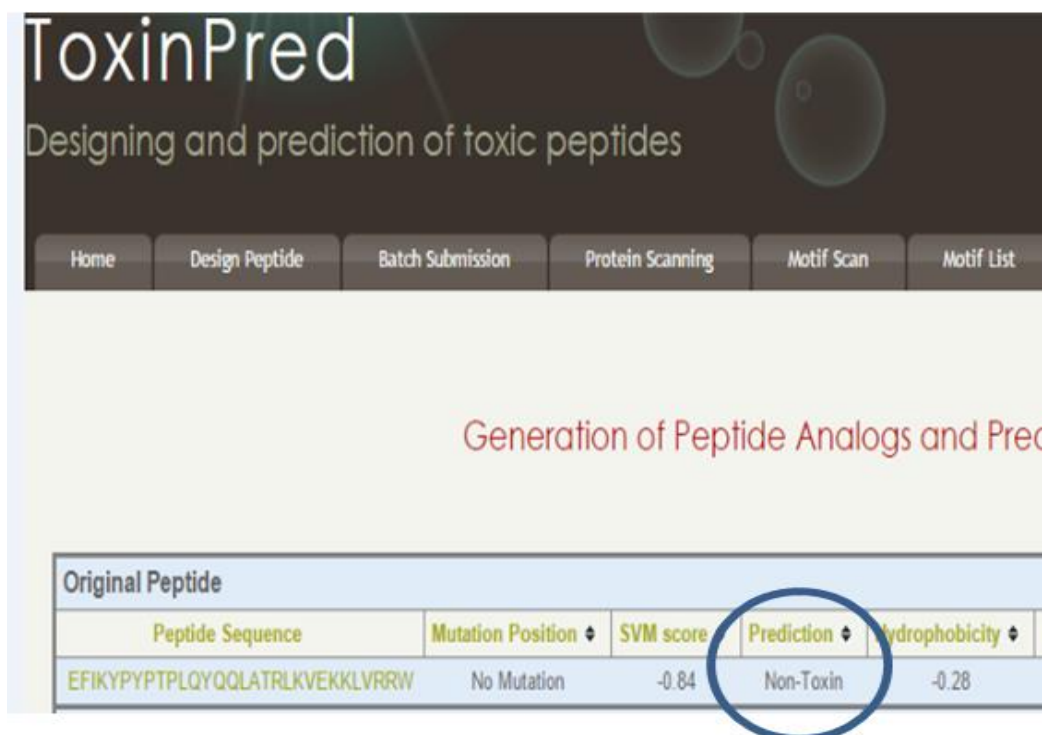
[Home](#) | [Submit: Peptide, Protein, Batch](#) | [Data sets](#) | [Algorithm](#) | [Help](#) | [Links](#) | [Team](#) | [Contact us](#)

[Go Back](#)

### Results Peptide Half-life (10mer)

S.No.	Peptides	Mutation Position	Half-life(sec)	Stability	HPLC parameter	Hydrophobicity (KJ/mol)	pKa	pKb	Residue volume	Molecular weight	Isoelectric point
Original peptide sequence											
0	AAEKEFIKYP	NO	0.945	Normal	1.420	10.400	21.800	93.980	1481.500	1195.510	6.138

**Figure 6: predicted half-life of the peptide .** The tool states a short halflife of the peptide sequence (around 1 second), though with normal stability.



**Figure 7: Predicted peptide toxicity.** Peptide sequence was predicted to be non-toxic.

### 3.2. Peptide structure & function prediction:

Using I-Tasser, the peptide sequence was predicted to be helical (Fig.8) with the predicted 3D model shown in Figure 9. Out of the templates showing highest structure identity to the peptide sequence, 1ocpA (Solution structure of OCT3 POU-homeodomain (POU5F1), PDB hit) (Fig.10.A)& 2XSD (Crystal structure of the dimeric OCT6 (POU3F1), PDB hit)(Fig.10.B) were found to show the highest alignment score with the structure of the peptide sequence under study.

Using COACH, It was found to be nucleic acid binding which goes with the prediction of the sequence as a homeodomain (Fig.11).COFACTOR tool could do biological function annotation based on the anticipated 3D structure of the sequence. Using COFACTOR, the best hit showing highest score was for Antp (Antennapedia) which is a regulatory homeoprotein in Drosophilla that is implicated in developmental

regulatory pathways. Antennapedia is famous for its cell penetrating properties and many cell penetrating peptides (CPPs) make use of its structure to enhance their penetrability (Thoren et al., 2000).

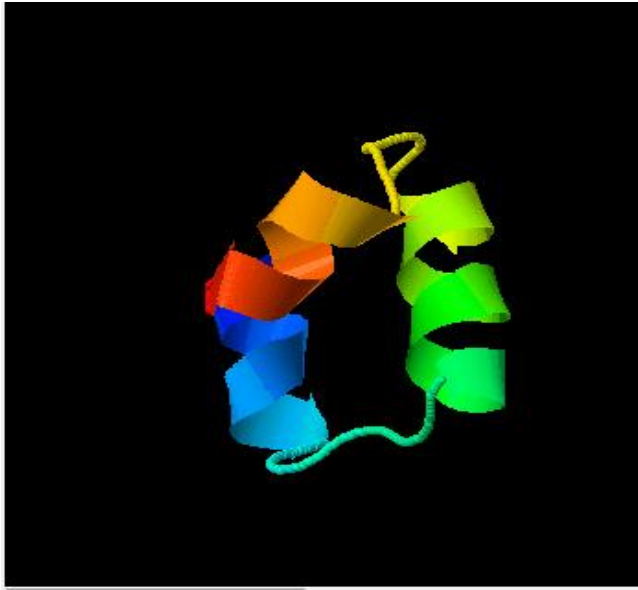
### Submitted sequence in FASTA format

```
>protein
AAEKEFIKYPYPTPLQYQQLATRLKVEKKLVRRW
```

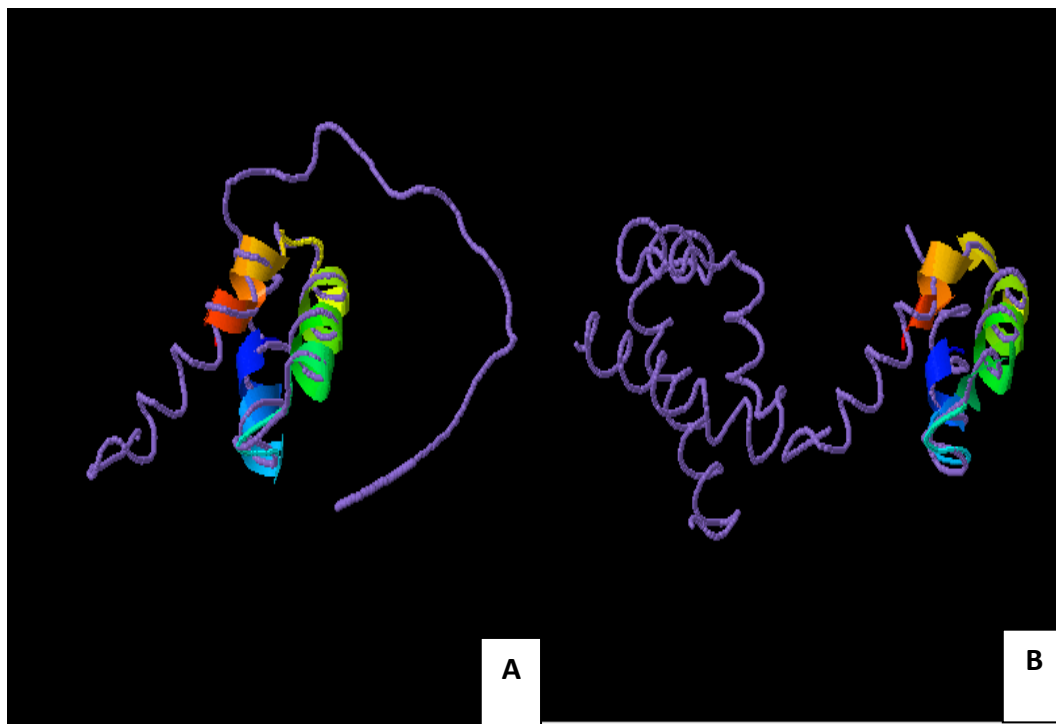
### Predicted secondary structure

	20 
Sequence	AAEKEFIKYPYPTPLQYQQLATRLKVEKKLVRRW
Prediction	CCHHHHHCCCCCCCCHHHHHHHHHHHHHHHHHHHCC
Conf.Score	92120202899994389999999999999997539
	H:Helix; S:Strand; C:Coil

**Figure 8: Predicted peptide 2ry structure.** Using I-Tasser, the 2ry structure of the peptide sequence was found to be  $\alpha$ -helices rich.

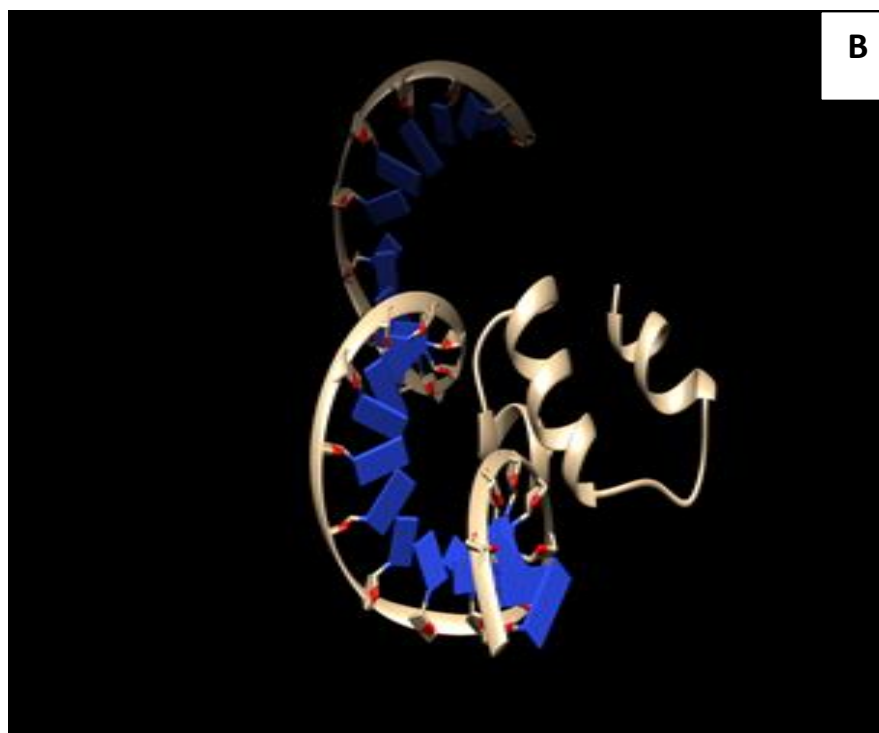


**Figure 9: Predicted 3D model of the peptide sequence using I-Tasser modeler.**  
The image depicts the 3D anticipated helical conformation of the peptide sequence.



**Figure 10: Structural alignment of the predicted model with OCT3 & OCT6.** The TM-align tool of the I-Tasser server aligns the predicted model to all structures in the Protein Databank (PDB) library. (A) Structural alignment of the predicted model of the peptide (colored and helical) to OCT3 structure (violet ribbon). (B) Structural alignment of the predicted model of the peptide (Colored and helical) to OCT6 structure (violet ribbon).

COACH							A
Click to view	Rank	C-score	Cluster size	PDB Hit	Lig Name	Download Complex	Consensus Binding Residues
<input checked="" type="radio"/>	1	0.17	67	<a href="#">1wrkA</a>	TFP	<a href="#">Rep.</a> <a href="#">Mult</a>	14,18,19,22
<input type="radio"/>	2	0.10	39	<a href="#">1hg3C</a>	PO4	<a href="#">Rep.</a> <a href="#">Mult</a>	20,23,24
<input type="radio"/>	3	0.10	41	<a href="#">2vjhA</a>	PEB	<a href="#">Rep.</a> <a href="#">Mult</a>	15,16,19
<input type="radio"/>	4	0.07	27	<a href="#">3v3xD</a>	MTN	<a href="#">Rep.</a> <a href="#">Mult</a>	13,16,17
<input type="radio"/>	5	0.04	16	<a href="#">3v5A</a>	Nuc.Acid	<a href="#">Rep.</a> <a href="#">Mult</a>	16,17,18,28,32
<input type="radio"/>	6	0.03	12	<a href="#">2ve9F</a>	Nuc.Acid	<a href="#">Rep.</a> <a href="#">Mult</a>	21,25,26,27,28,30
<input type="radio"/>	7	0.02	7	<a href="#">4bxcA</a>	Nuc.Acid	<a href="#">Rep.</a> <a href="#">Mult</a>	32
<input type="radio"/>	8	0.02	7	<a href="#">4nahD</a>	2VJ	<a href="#">Rep.</a> <a href="#">Mult</a>	5,6,9
<input type="radio"/>	9	0.01	3	<a href="#">3q5nB</a>	PB2	<a href="#">Rep.</a> <a href="#">Mult</a>	20,21,26,28
<input type="radio"/>	10	0.01	3	<a href="#">1en7B</a>	CA	<a href="#">Rep.</a> <a href="#">Mult</a>	11,16



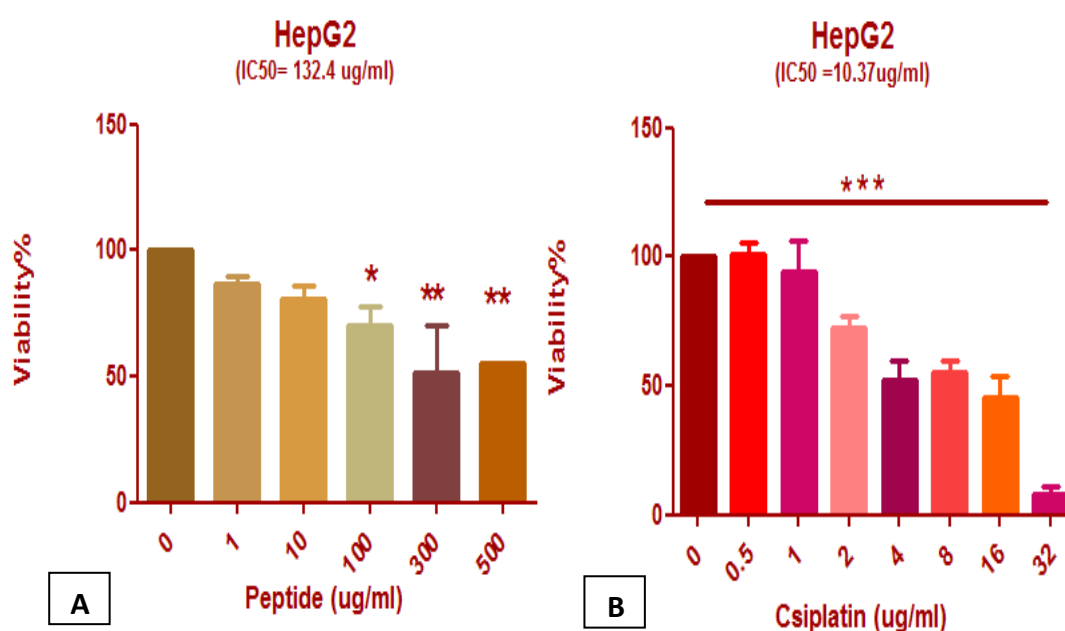
**Figure 11: Ligand binding site prediction using COACH.** (A) COACH tool of I-Tasser server infers the function of the query peptide sequence since it builds its prediction on both sequence and structure features not only a global structural alignment. DNA could be computationally predicted as a binding ligand to the peptide with a good score. (B) The image depicts a model of the peptide binding to DNA which goes with its prediction as a homeodomain. The image is drawn by Chimera 1.11.2rc.



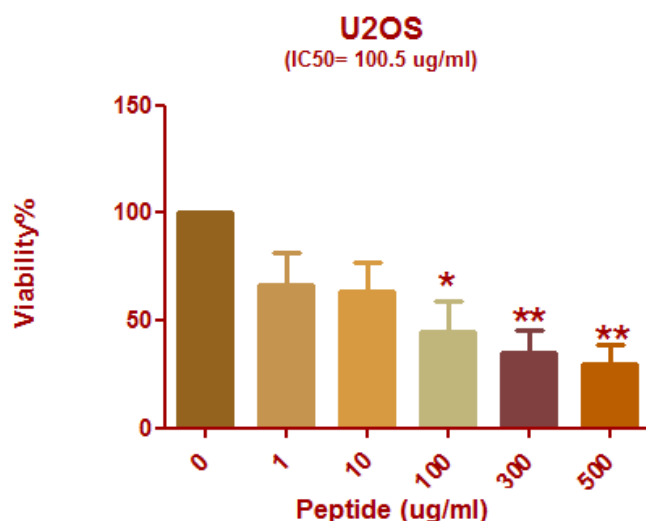
### 3.3. Cytotoxicity:

All tested cell lines showed viability reduction when treated with increasing concentrations of the peptide. HepG2 cells showed dose dependent cytotoxicity in two independent experiments (Fig.12). U2OS cells showed dose dependent cytotoxicity in three independent experiments (Fig.13). Both MCF7 and HeLa (Fig.14) showed dose dependent viability reduction in one experiment with triplicates of each condition.

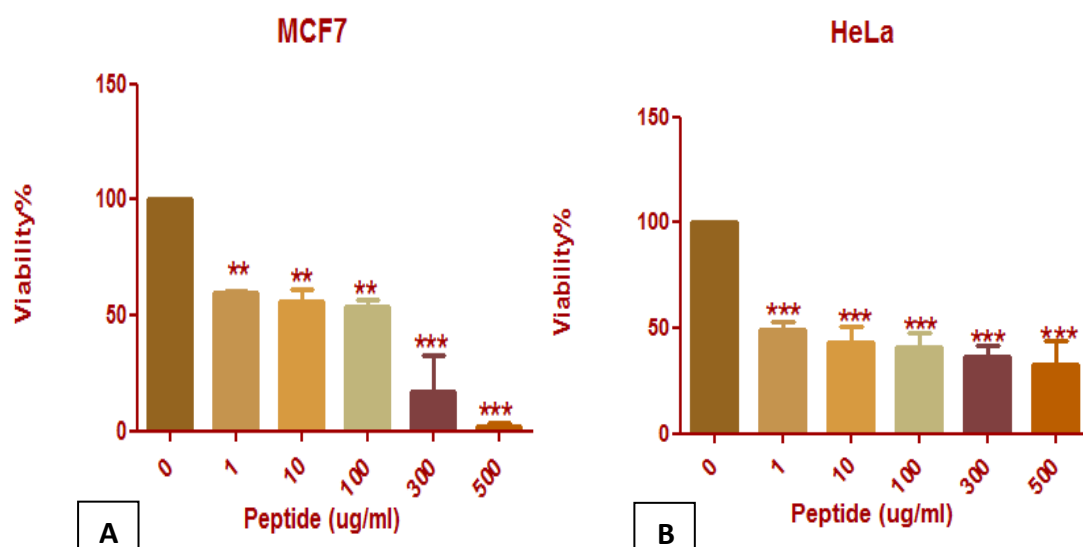
Images of U2OS peptide treated cells (Fig.15) show abnormal cellular morphology together with reduction of number of viable cells with increasing concentration of the peptide drug. Figure 16 shows a combined graph for viability reduction of all cell lines used. Peptide IC<sub>50</sub> value for HepG2 cells was calculated to be 132.4ug/ml. Peptide IC<sub>50</sub> value for U2OS cells was calculated to be 100.5ug/ml. The IC<sub>50</sub> for cisplatin treated HepG2 cells was 10.37ug/ml.



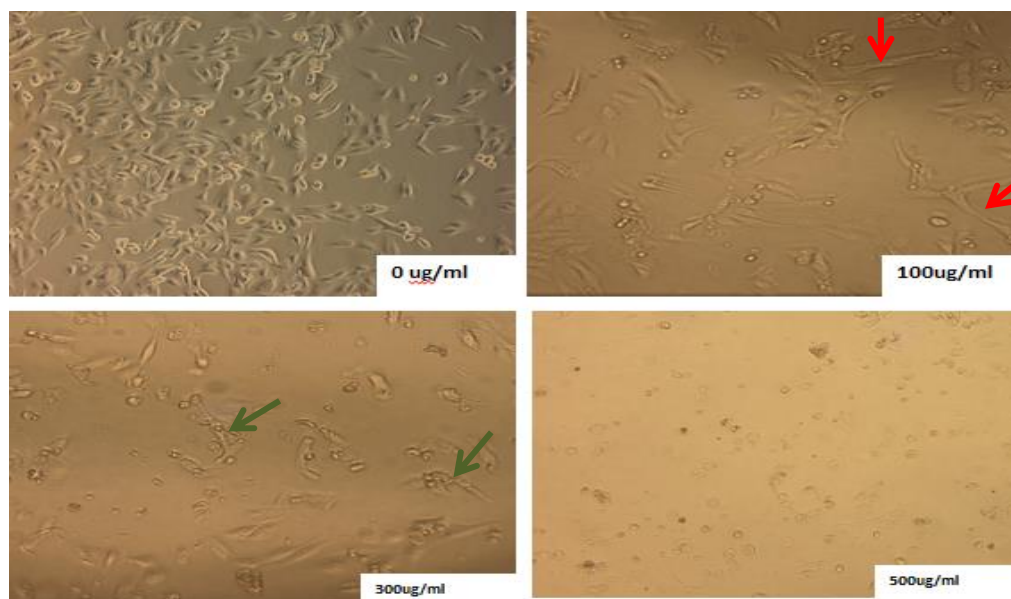
**Figure 12: Dose dependent cytotoxicity of peptide and cisplatin treated HepG2 cells.** Six replicates of each condition were tested. **(A)** One way ANOVA showed a statistical significant difference in viability between control cells (0 ug/ml) and both 300ug/ml and 500ug/ml peptide treated cells at  $p < 0.05$ . **(B)** Higher statistical difference in viability between control cells and (4, 8, 16 & 32 ug/ml) cisplatin treated cells at  $P < 0.05$  is noted.



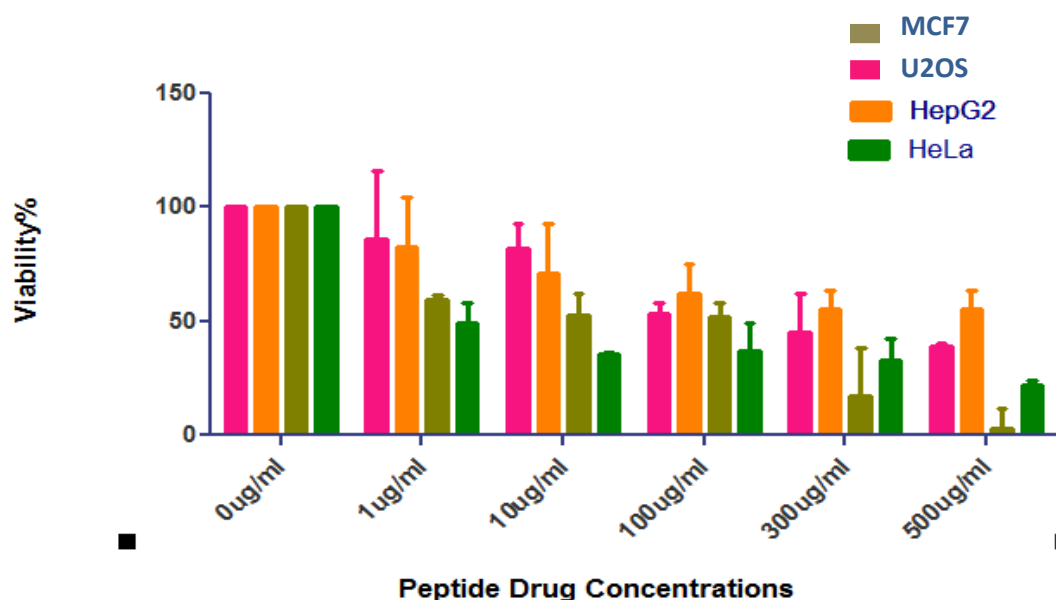
**Figure 13: Dose dependent cytotoxicity of peptide treated U2OS cells.** Eight replicates of each condition were tested. One way ANOVA showed a high statistical significant difference in viability between control cells (0ug/ml) and both 300ug/ml and 500ug/ml peptide treated cells at  $P < 0.05$ .



**Figure 14: Dose dependent cytotoxicity of peptide treated HeLa and MCF7 cells.** Three replicates of each condition were tested. One way ANOVA was used for statistical analysis with  $P < 0.05$ . **(A)** Statistical significant difference in viability between control untreated cells and all concentrations tested. **(B)** Statistical significant difference in viability between control cells and 100ug/ml peptide treated cells and a higher significant difference between control cells and 300ug/ml and 500ug/ml peptide treated cells.



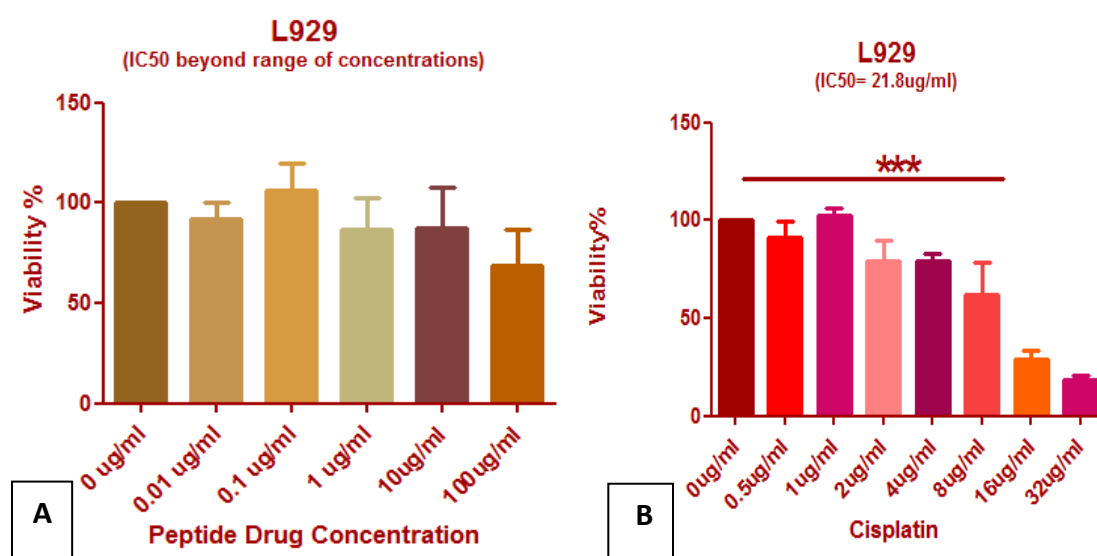
**Figure 15: Morphological changes of U2OS cells upon peptide treatment.** Control untreated cells (0ug/ml) show the normal morphology of U2OS cells being fusiform in shape with intact membrane. At 100ug/ml cells started to be more elongated, sparse with shrunken volume (red arrows). At 300ug/ml, there was a noticeable bizarre appearance of the cells (lost normal architecture) (Green arrows). At 500ug/ml, no normal U-2 OS cells can be noted in the field which is full of round dead cells and cells with shaggy/ragged membranes.



**Figure 16: Variable dose dependent reduction of viability in U2OS, HepG2, MCF7 & HeLa cell lines.** Different sensitivity to peptide treatment of different cell lines is noted with variable dose dependent cytotoxicities.

### 3.4. Cancer cell selectivity

Normal murine fibroblast cell line; L929 showed reduction of viability upon peptide treatment but not in a dose dependent manner. Evident cytotoxicity started at 100ug/ml concentration (Fig.17A). No statistical significant difference was noted among different concentrations and control untreated cells and IC<sub>50</sub> was predicted, using Prism 5, to be above the highest concentration tested. On the other hand, L929 showed dose dependent cytotoxicity when treated with cisplatin with an IC<sub>50</sub> value of 21.8ug/ml (Fig. 17B).

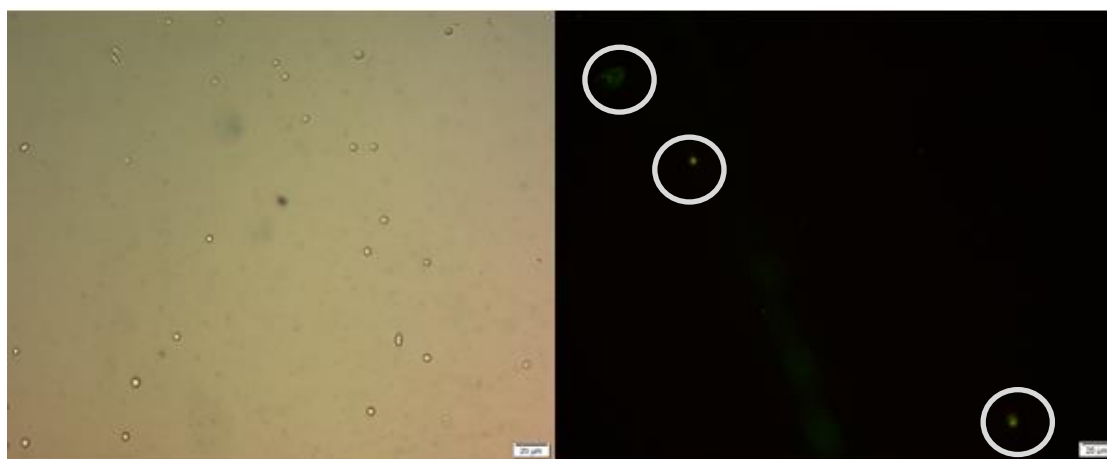


**Figure 17: Effect of peptide and cisplatin on L929 cells.** (A) No pattern of cytotoxicity could be observed with increasing concentrations of the peptide on L929 cells. (B) L929 show statistically significant dose dependent reduction of viability when treated with cisplatin with an IC<sub>50</sub> value of 21.8ug/ml.

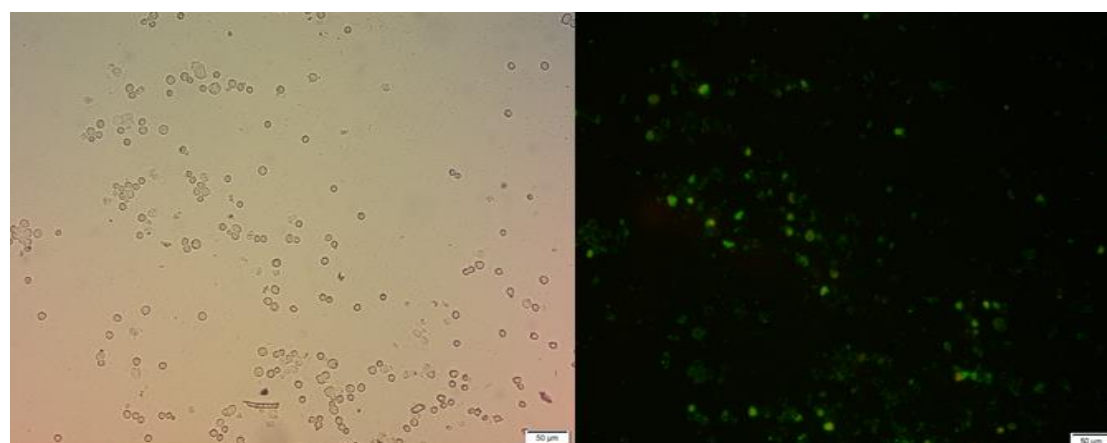
### 3.5. Annexin V/PI assay:

Annexin, a human anticoagulant, has a high affinity to bind Phosphatidyl serine (PS) that gets externalized on the surface of early apoptotic cells. Thus, Alexa fluorophore conjugated Annexin can easily identify apoptotic cells by appearing green when excited by blue light. The red fluorescent DNA binding PI can't penetrate live or early apoptotic cells. It binds tightly to DNA of necrotic and late apoptotic cells staining them with red fluorescence when excited by green light.

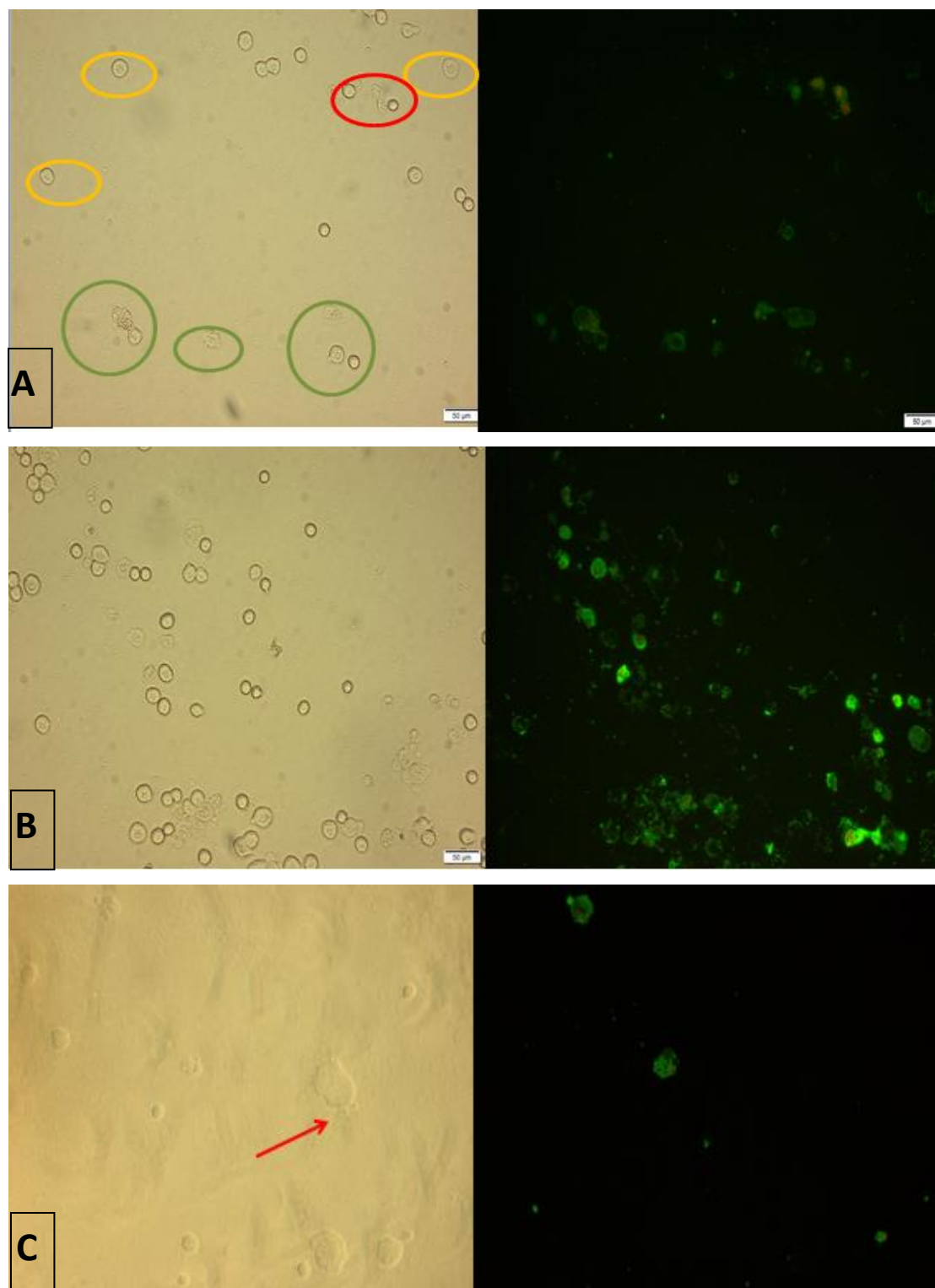
Untreated U2OS cells, showed low number of green, red and green/red cells as well as low fluorescence intensity (Fig. 18). Images of peptide treated U2OS cells shows that most of the cells, appearing in the field, are stained green as a sign of apoptosis. Red fluorescence was not detected alone, but it was always emitted along with green fluorescence indicating necrotic/apoptotic cells (Fig.19). Higher magnification images (20X) show morphological changes of some cells along with illustration of membrane disassembly into apoptotic bodies with no signal detected from completely disrupted cell (Fig. 20).



**Figure18: Annexin V/PI assay of untreated U2OS cells.** The field shows low number of stained dead cells (White circles).



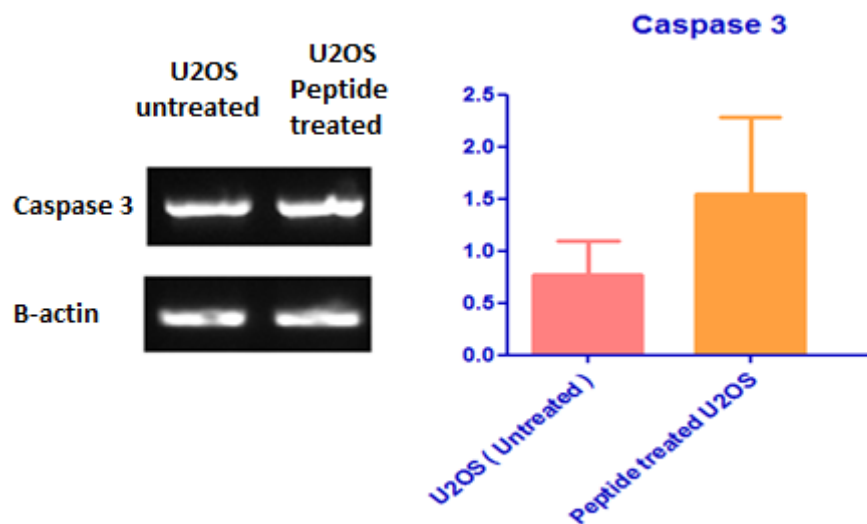
**Figure 19: Annexin V/PI assay of peptide treated U2OS cells (10X magnification).** Field shows dominance of green stained cells indicating apoptotic cells.



**Figure 20: Annexin V/ PI Assay of peptide treated U2OS cells (20X magnification).** (A) Yellow circles highlight viable cells, green circles highlight apoptotic cells and red circles highlight apoptotic/necrotic cells. (B) Almost all of the cells in the field shown are stained green indicating apoptosis. (C) Arrow points to budding from a disrupted cell that is most likely to be apoptotic bodies. It's assumed that the cell is completely disrupted that it didn't take up any dye.

### 3.6. RT- PCR

Caspase 3 shows non-statistically significant higher expression in peptide treated U2OS cells versus control cells (Fig.21).  $\beta$ -actin was used as a housekeeping gene and it shows comparable expression in the 2 samples.

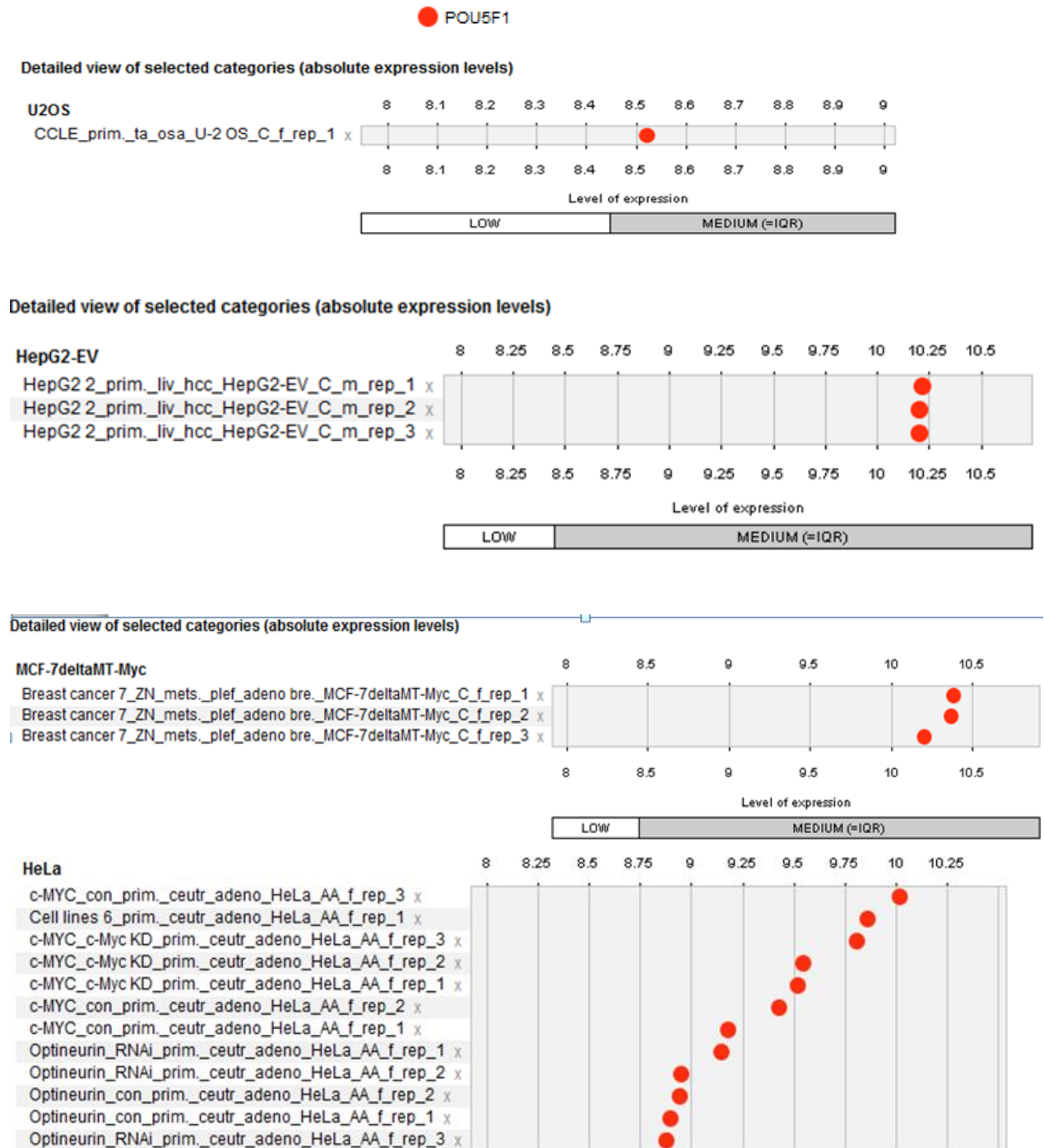


**Figure 21: RNA expression analysis of Caspase 3 in untreated vs peptide treated U2OS cells.** It shows over expression in peptide treated cells

### 3.7. Computational assessment of OCT3 expression in studied cell lines:

Using the cell lines tool of Genevestigator, the expression level of OCT3 (POU5F1) could be visualized in a large collection of cell lines including U2OS, HepG2, MCF7 & HeLa. Its expression level was found to vary among the cell lines under study (Fig.22).





**Figure 22: OCT3 (POU5F1) expression level in U2OS, HepG2, MCF7 & HeLa cell lines.** Computational analysis of OCT3 expression in different cell lines using Genevestigator webtool was done. It demonstrates highest OCT3 expression, among cell lines under study, in MCF7 and lowest in U2OS. HeLa and HeLa derived cell lines show slightly variable OCT3 expression, but still higher than that in U2OS.



## Chapter 4

### Discussion

#### 4.1. Computational Analysis:

The *in silico* prediction of the peptide sequence as a cationic, amphipathic, non-toxic and stable ACP was a strong start of the subsequent structure analysis and functional validation of the potential anticancer activity. Besides its online computational prediction as an ACP (Fig. 3), it showed significant alignment, using Blastp, with bacteriocins (Fig. 5) which encompass a family of bacterial cationic peptide toxins that have lately shown potent cytotoxic and apoptosis inducing effects in many cancer cells (Nguyen & Nguyen, 2016, Kaur & Kaur, 2015).

The peptide structure was predicted to be helical (Fig. 8 & 9) which goes with its prediction as an ACP as the helical conformation is characteristic of many studied ACPs and was confirmed essential for their interaction with the cancer cell membrane (Zhang et al., 2010, Chen et al., 2014).

The half-life of the peptide in an intestine like environment was computationally predicted to be nearly 1 second (Fig. 6) which is quite short and indicates its susceptibility to proteolytic degradation after systemic administration. Its stability should be further assessed using incubation with biological matrices e.g. blood, serum, stimulated gastric or intestinal fluid in order to observe its kinetic profile and degradation products (Di, 2015). However, from another point of view serum instability won't affect its topical or intralesional uses may be for various skin cancers. Predicting the peptide non-toxic (Fig. 7) favors any subsequent planned *in vivo* testing.

Aligning the peptide sequence to a homeodomain superfamily using Blastp (Fig. 4) and modeling its peptide structure against PDB, using I Tasser, and its significant alignment with OCT3 and OCT6 homeodomains (Fig. 10) confirm our *in silico* prediction of it as a homeodomain which gives a solid ground for our *in silico* results. Moreover, COACH predicted its ligand binding site to be DNA (Fig. 11) which is valid for the homeodomain family of transcription factors.

COFACTOR annotated its biological function to Antennapedia (AntP); a regulatory homedomain in *Drosophila* (Fig. 12). AntP is famous now for its use as a fusion cell penetrating peptide (Penetratin) to carry cargo molecules to the interior of the cells (Thoren et al., 2000). Annotating the function of our peptide to Antp suggests it might easily penetrate to the inside of the cells. Following translocation into the cell, it may either initiate an intracellular death cascade or restore the function of downregulated or compete with an upregulated OCT3 or OCT6 according to the carcinogenic events in the studied cancer.

The sequence and structural similarity to OCT3 favors the peptide, if penetrable to the cell membrane, to be acting as a competitor to OCT3 that is found to be upregulated and involved in carcinogenesis of bone, liver, breast and cervix (Fujino et al., 2010, Dong et al., 2012, Wang et al., 2003, Wang et al, 2013a).

#### **4.2. Cytotoxicity**

The peptide exerted a cytotoxic effect against all tested cell lines (HepG2, U2OS, MCF7 and HeLa) in a dose dependent manner (Fig. 12, 13 & 14). The variable sensitivity of the cell lines (Fig.16) to peptide treatment using the same concentrations can be explained as previously discussed in the review chapter by the varied membrane lipid composition of each cell line which dictates peptide preference and affects its conformation upon contact with the membrane (Eliassen et al., 2006, Gasper et al., 2015). Another explanation might be the differential expression of OCT3 in studied cancers, which is predicted from computational analysis of microarray and RNAseq data (Fig. 22), if the proposed mechanism of action would be interference with OCT3 function. Performing Chromatin Immunoprecipitation (ChIP) assay may help prove this proposition.

Under low concentrations (1 & 10 ug/ml), U2OS and HepG2 showed comparable viability reduction (Fig. 16). Starting from 100 ug/ml concentration on, U2OS displayed more viability reduction than HepG2 (Fig. 16). This can be explained according to (Wang et al., 2013b) where it was found that there was a concentration

dependent variation in the anticancer activity of the peptide under study (temporin-1CEa).

They found temporin-1CEa at certain low concentration, with its helical amphipathic conformation, to get bound to the cell membrane with subsequent loss of its integrity. At higher concentrations, it could initiate a caspase dependent apoptotic cascade. They concluded that accumulating concentrations of the peptide on the membrane above a certain threshold induces its damage with subsequent translocation of temporin to the inside of the cells initiating apoptosis and accelerating cell death. This might be the explanation for the increased sensitivity of U2OS to peptide concentrations  $\geq 100 \mu\text{g/ml}$  more than HepG2 as it might have then displayed an additional mechanism of action in U2OS cells and not in HepG2 cells.

HeLa cells (Fig. 14A) show statistically significant viability reduction even at low concentrations that doesn't go much down as peptide concentrations go up. This might be explained by the peptide acting only through an early death inducing mechanism of action e.g. membrane damage and necrosis and so any additive concentrations would accumulate and wouldn't enhance its activity. MCF7 cells (Fig. 14B) show comparable viability reduction at low concentrations (1, 10, 100  $\mu\text{g/ml}$ ) that abruptly goes down at 300 & 500  $\mu\text{g/ml}$  concentrations. This might be due a dual mechanism of action of the peptide in MCF7 cells.

Illustrated in the review chapter two peptide examples (HNP-1 & Lcfin-B) acting through different mechanisms in different cancers. The difference in timing of each mechanism might explain the variability in cytotoxicity results in different cancer contexts. For example, peptides causing membrane disassembly would show faster cytotoxicity than those causing DNA damage or synthesis inhibition. Necrotic peptides show earlier results than those causing apoptosis. Thus, testing cytotoxicities at different incubation times and using a wider range of concentrations might provide a clue for the mechanism of action in each cell line.

An accurate way to assess selectivity is to monitor the IC<sub>50</sub> of the peptide against a normal and a cancerous cell lines and to monitor the IC<sub>50</sub> of a standard chemotherapeutic drug against the same cell lines. Then a selectivity index (SI) would

be calculated for each drug by dividing the IC<sub>50</sub> of the normal cell line by that of the cancerous one. If the index of the experimental drug is higher than that of the standard drug, then it's likely to be a more selective potent drug candidate.

Peptide treated L929 cells (Fig.17A) showed unapparent pattern of cytotoxicity under the range of concentrations tested from which calculation of an IC<sub>50</sub> denoted a wide range that would go higher than the highest concentration tested (100ug/ml). While a cisplatin SI could be calculated (Fig. 12B, 17B) as follows:

$$SI = \frac{\text{IC}_{50} \text{ of cisplatin on L929 cells}}{\text{IC}_{50} \text{ of cisplatin on HepG2 cells}} = \frac{21.8}{10.37} = 2.1$$

Since the IC<sub>50</sub> of peptide treated HepG2 cells = 132ug/ml and apparently the IC<sub>50</sub> for peptide treated L929 cells would go much higher. We suppose its SI would be comparable if not higher to cisplatin.

Mole, in chemistry, is the standard measurement of amount. Thus, converting the gram concentrations applied throughout this study into their molar equivalents would reveal another aspect of the cytotoxic activity of the peptide drug and cisplatin depending on the difference of their molar masses. The molar mass of the peptide drug equals 4192.9042 gm/mol and that of cisplatin equals 300.04 gm/mol.

Using available online molarity to mass converting calculators, we could find that the highest peptide concentration (500ug/ml) tested equals 119.2uM and the highest cisplatin concentration (32ug/ml) tested equals 106.6uM. The peptide IC<sub>50</sub> on HepG2 (132.8ug/ml) equals 31.6uM and the cisplatin IC<sub>50</sub> on HepG2 cells (10.37ug/ml) equals 34.5uM which in terms of molarity is much higher than that of the peptide drug despite being much lower when calculated in grams.

### 4.3. Apoptosis or Necrosis

Morphological changes of peptide treated U2OS cells (Fig.15) show evident shrinkage of cells upon treatment with 100ug/ml but with preserved architecture.

300ug/ml peptide concentration caused more bizarre appearance with shredded membranes of a high proportion of cells. At 500ug/ml concentration, no intact cells could be noted. These changes are consistent with an apoptotic death event in which shrinkage and membrane disintegration are main key events.

Figure 18 shows a few necrotic/apoptotic cells in the field of control untreated U2OS cells compared to figure 19 and figure 20B, where almost all cells are stained green indicating apoptotic cells with very few green/red cells. Higher magnification, 20X, (Fig. 20C) shows a cell ghost with disintegrated budding membrane into apoptotic bodies that did not even give a green signal, may be due to complete damage of its membrane. These results support an apoptotic mode of action.

The morphological changes depicted in figure 15 with absence of necrotic cell swelling, membrane blebbing and cellular rupture diverted our attention from necrosis as a possible mechanism of cell death. However, more electron microscope based morphological studies of apoptosis related nuclear and mitochondrial changes must be done to be more confident of apoptosis occurrence. Mitochondrial transmembrane potential and ATP level should be assessed. Fluorescence conjugated peptide would be a useful approach for investigating where it localizes inside the cell.

#### **4.4. RNA expression analysis**

Increased expression of Caspase 3, an initiator caspase, in peptide treated U2OS cells (Fig.21) supports the apoptotic mode of action as well. However, being non-statistically significant along with the complex molecular nature of apoptosis pathways indicates that more studies to confirm and detect which pathway was involved should be done. DNA laddering and expression of other effector caspases, apoptotic markers e.g. Bcl2, Bax, IAPs and proliferation markers should be done.

Wang et al., (2013b) demonstrated that Temporin-1CEa mediated its effect in a caspase dependent manner at certain concentrations and in a caspase independent manner at other concentrations. Since, in our case RNA expression analysis was done for IC<sub>50</sub> treated U2OS cells, it's recommended to be done for cells at various dose conditions and various incubation times as well.

## Conclusion & Future recommendations

### Conclusion

In summary, we could conclude that we've developed a cationic amphipathic cytotoxic peptide with a potential to be developed into an ACP drug lead. The peptide has an *in silico* sequence and structural similarity to homeoproteins. Its cytotoxicity can be either due to a non-specific selective membranolytic effect on cancer cells being cationic helical and amphipathic, which supports its non-specific electrostatic interaction with cancer cell membranes, or another non-membranolytic mechanism. Moreover, we could conclude that most probably the peptide is adopting a different mechanism of action in each cell line and may be more than one in the same cell line.

The results could support two proposed mechanisms of action other than membranolysis. It might either compete with OCT3 function or be an apoptosis inducing peptide. Sequence and structural similarity to OCT3 homeodomain, being biologically annotated to AntP cell penetrating peptide together with the notion of OCT3 as an upregulated and carcinogenic effector in the studied cancers support the hypothesis that the peptide may be acting through competitive inhibition of OCT3.

The apoptotic mechanism of action is supported by the cytotoxicity results, evident annexin staining and over expression of Capsase 3 in peptide treated cells. No definite pathway could be outlined depending on this data. The *in silico* alignment of the peptide to several apoptotic proteins, implicated in different pathways, support the *in vitro* results. The non-statistical significant overexpression of Caspase 3 doesn't disprove our hypothesis since some apoptosis mechanisms are caspase independent (parthanatos).

## **Future recommendations:**

Developing a peptide drug is challenging but quite rewarding. In order to develop an effective and selective oncolytic peptide, precise data on its structure, biophysical properties and mode of action must be obtained. There is a long avenue of basic research and preclinical optimization to be done before a peptide can be claimed to be an ACP.

Bioinformaticians are doing a great effort to compile data about the newly introduced peptide based drugs in general and ACPs in particular into well-organized publicly available databases. Those web-servers are supported by user friendly tools to manipulate peptide sequence and predict its properties and hence its activity. It would be auxiliary to develop webserver for designing and modeling peptides against 3D models of definite cellular targets (peptide specific docking servers) to optimize peptide based targeted therapy approaches. Moreover, softwares to predict enzymatic cleavage sites of peptides would greatly help directed modification strategies, during peptide synthesis, to optimize peptide stability.

There are upcoming duties for chemists interested in the field of ACPs to develop cheap and efficient methods to modify peptide sequences in a way that would overcome the obstacles of peptide serum instability and rapid plasma clearance. However, these modifications should not disturb the basic required conformation and properties essential for the anticancer activity of the peptide.

The future of the field of ACPs is highly dependent on how successfully growing the basic research errand. Precise structure determination tools such as mass spectrometry and nuclear magnetic resonance should be available for every ACP oriented research. More imaging tools such as scanning electron microscope, transmission electron microscope and confocal microscope should be at hand for accurate determination of the cellular death morphologic events.

Cytotoxicity analysis at different time points and a wider concentration range should be investigated for early and late cellular death modes of actions. The peptide should be tested against a bigger panel of cell lines. Analysis of cytotoxicity using more than one assay such as MTT assay, LDH release assay....etc must be done. Testing the expression of apoptotic and necrotic markers, DNA fragmentation occurrence and other apoptotic studies must be a part of every research aiming to develop an ACP lead.

Finally, more confirmatory studies are essentially needed to confirm our preliminary results. More ACPs oriented studies are needed to exploit this rich source of selective, easy to synthesize and effective weapon to be added to the anticancer arsenal.

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Date: 8-12-2016

Dear Mona,

Mustafa A. Ahmed, hereby authorizes Mona ElRadi Imam to use the data he generated through in silico analysis of the Red Sea Metagenomics library against the publicly available anticancer peptide databases.



Mustafa A. Ahmed

## Permission to use image in my thesis



**Mona El-Radi Imam** <monaradi@aucegypt.edu>

Dec 7 (1 day ago) ☆

to raghava ▾

Dear Dr. Raghava,

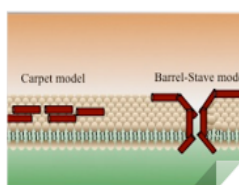
First of all I'd like to thank you deeply for the valuable numerous databases and tools your group developed available for everyone and in a user friendly manner.

My name is Mona Elradi. I'm a biotechnology Master's student in the American University in Cairo (AUC). I'm sending to ask your permission to use the image on the AntiCP webserver, about various models of membrane permeation by ACPs, in the review section of my thesis in which I used and cited many webtools belonging to your group.

Attached is the image.

Best regards and thanks in advance

Sincerely,  
Mona Elradi



**Dr. G.P.S. Raghava**

Dec 7 (1 day ago) ☆

to me ▾

Dear Elradi

Thanks for email, I am happy to permit you to user our image in your review.

With best wishes and regards

Raghava

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