Pre-clinical studies of honey-related synthetic peptides in malignancy control

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Abstract

Background: The challenge with malignancy control approaches, although of all traditional methods of treatment like surgery, chemotherapy, radiotherapy, immunotherapy etc. makes more stress upon the scientific community to look for a solution for this challenge even far from the traditional ways.

Methods: A growth inhibitory assay was performed. Samples of peptide A and peptide F, 5 mg each, were dissolved in 500 μL Dulbecco’s Phosphate-Buffered Saline to prepare the stock solution, which was added into the wells directly. A 1% trifluoroacetic acid (TFA) solution was used as the control. (a) Cells were placed into a 96-well assay plate at a density of 5000 and 10000 cells/well for adherent and suspension cell lines, respectively, with a 50 μL complete cell culture medium. (b) Stock solutions (10 μL) of the compounds were added into the wells. (c) For data analysis, 10 μL 1% TFA solution was used as control. (d) Complete medium of 40 μL was added into the wells to reach a total volume of 100 μL. (e) The plate was incubated at 37°C for 72 hours. (f) CellTiter-Glo assay mix solution (50 μL) was added to each well and mixed gently at room temperature for 10 minutes; subsequently, the luminescence was read using PHERAstar Plus (Molecular Devices).

Results: Apparent inhibitory effects of those peptides were noticed in 17 of 18 tumour cell lines. Similar effects were noted in an in vivo study using the U87MG tumour mouse model.

Conclusion: This study has introduced new synthetic peptides derived from honey proteins, which may help in cancer control through direct apoptotic action not limited to only a certain tumour cell line.

Patent: WO/2014/040605A1, US/2015/0291663A1, and CN105283198A.
1. Background

Bee genome was disclosed and published in 2006 [1], and many hypothetical proteins were postulated. Honey was known as a remedy thousands of years ago as it was mentioned in old books, either religious or others, emphasising its medical and nutritional value [2-6].

Several patents related to honey proteins and peptides were introduced as possible solutions of certain diseases and conditions [7-14].

Royal jelly (RJ) is the most abundant protein found in honey, and some researchers have discussed its inhibitory effects on malignant cells [15].

This study focused on RJ and two hypothetical proteins (XP 001120220 of LOC724386 gene and XP 397512 of LOC408608 gene) for possible anticancer effects [16,17,18]. After a series of experiments, we postulated the following signal peptides in proteins that may present significant anticancer effects [19,20,21]:

1. MAILTYVYVF AVLFIANSMQ A
2. MQLRVLFFFL FVATISYAIA D
3. MTKWLLLVVC LGIACQ

However, these peptides were hydrophobic; many biotechnology companies could manufacture crude products but failed to synthesise highly pure states (>95%) of these peptides to fit our laboratory requirements.

Therefore, we modified the C-terminus of these peptides by adding lysine either after deleting some amino acids in two peptides or without any deletion in the third peptide [22] as follows:

A- MAILTYVYVF AVLFIAKKKK
E- MQLRVLFFFL FVATISKKKKK
F- MTKWLLLVVC LGIACQKKKKK
Because of the increased proportions of ageing populations and habits of smoking and related predisposing factors, the prevalence of cancer is expected to increase, particularly in developing countries. In 2008, approximately 12.7 million cancer cases and 7.6 million cancer deaths were estimated, and 56% of these cases and 64% of the deaths occurred in economically developing countries [23]. Additionally, more than 16 million new cancer cases every year are expected by 2020 [24].

Currently, primary approaches to cancer treatment include surgery, chemotherapy, radiotherapy, and biologic treatment.

The “biologics” option to treat cancer includes the use of proteins, monoclonal antibodies, and peptides. Because of the large size of monoclonal antibodies and protein, its usage was limited to haematological malignancies and suboptimal penetration in other malignancies [25–30]. By contrast, peptides with their small size and high penetration plus easy synthesis and modifications prove their superiority over proteins and monoclonal antibodies in biologic treatment of cancer [31].

Therefore, peptide use in clinical trials has increased from 1.2 per year in the 1970s to 16.8 per year in the 2000s [32].

Peptides have been used in various ways for cancer treatment [33,34,35]; peptides have been used directly as drugs (e.g., as angiogenesis inhibitors) [36], as a target for chemotherapy and radiotherapy [34], hormones [37], and vaccines [38].

Peptides may induce anticancer activity through one or more mechanisms, such as angiogenesis, protein–protein interactions, enzymes, proteins, signal transduction pathways, or gene expression [39–45]. Additionally, peptide antagonists, considering their binding ability to a known receptor, exhibit anticancer activity [46,47]. The peptides assessed in this study induced apoptosis and programmed cell death in tumours [48,49,50].
RGD-(KLAKLAK)2 and NGR-(KLAKLAK)2 are examples of angiogenesis inhibitors, which may help prolong survival in some patients with cancer. By contrast, LyP-1 was limited to certain tumours of lymphatic vessels that inhibit breast cancer cell lines and not melanoma cell lines. Additionally, the inhibition of breast cancer cell lines was found only in vivo in the MDA-MB-435 breast cancer xenografts-mice-animal model [51].

2. Methods

2.1. In Vitro Study

2.1.1. Objectives:

This study evaluated the effect of peptides A, E, F, and AEF on 15 tumour cell lines, namely MG63, U87MG, SH-4, RD, KP1, 5637, 2774, ML-1, Cal-27, Colo-205, 769P.EOL-1, HLE, Calu-3, and MDA-MB-436, using a cell viability assay. [20, 21]

2.1.2. Test and Control Article:

A total of six samples of peptides (1A, 2B, 3C, 4D, 5E, and 6F) in powder form were stored at −20°C. Samples of 1A, 5E, and 6F, 10 mg each, were dissolved in 1 mL Dulbecco’s Phosphate-Buffered Saline (DPBS) to prepare the stock solution, which was directly added into the wells. Trifluoroacetic acid (TFA) solution of 0.1% was used as control.

2.1.3. Test System:

Reagents and materials:

1) Minimum Essential Medium (MEM) Alpha Medium, Cat. # 32561-037, Lot. # 1245518 Gibco

2) RPMI 1640 Medium, Cat. # SH30809.01 B, Lot. # NYA0779 Hyclone

3) Dulbecco’s Modified Eagle Medium (DMEM), Cat. # 11960-044, Lot. # 1267442 Gibco

4) Fetal Bovine Serum (FBS), Cat. # 04-001-1 A, Lot. # 615303 Hyclone

5) Penicillin-Streptomycin, Cat. # SV30010, Lot. # J130071 Thermo
6) 96-well assay plate, Cat. # 3610, Lot. # 27012035 Corning

7) CellTiter, Cat. # G755B, Lot. # 0000047328 Promega Assay Procedures

2.1.4. Assay Procedure:

1) Place the cells in a 96-well assay plate at a density of 5000 and 10000 cells/well for adherent and suspension cell lines, respectively, with 50 μL of complete cell culture medium.

2) Add 10 μL of stock solutions of the compounds to the wells.

3) A 10 μL 0.1% TFA solution was used as control for data analysis.

4) Add 40 μL of complete medium into the wells to reach a total volume of 100 pL.

5) Incubate the plate at 37°C for 72 hours.

6) Add a 50 L CellTiter-Glo assay mix solution to each well and mix gently at room temperature for 10 minutes; subsequently, read the luminescence with PHERAstar Plus (Molecular Devices).

2.2. In Vitro Study:

2.2.1. Objectives:

This study evaluated the effect of peptides A+F (of five concentrations) on four tumour cell lines, namely U87MG, MDA-MB–468, K562, and A375, as proposed for further in vivo studies with cell viability assay and apoptosis assay.

2.2.2. Test and Control Articles:

Growth inhibitory assay was performed with 10 μg, 25 μg, 50 μg, 75 μg, and 100 μg of each peptide.

(A+F) per well: Peptide A and peptide F were stored at −20°C. The samples of peptide A and peptide F, 5 mg each, were dissolved in 500 μL DPBS to prepare the stock solution, which was added to the wells directly. TFA solution of 0.1% was used as control.
2.2.3. Test System:

Reagents and materials:

1) MEM Alpha 11095-080 1179276 Gibco
2) Modified Dulbecco’s Medium 12440-046 841374 Hyclone
3) DMEM 10566-016 1300085 Gibco
4) FBS 10099-141 1227693 Gibco
5) 96-well assay plate 3610 27012035 Corning
6) CellTiter-Glo kit G755B 0000047328 Promega
7) Caspase3/7 G811A 0000014788 Promega

2.2.4. Assay Procedures:

1) Place the cells into a 96-well assay plate at a density of 5000 and 10000 cells/well for adherent and suspension cell lines, respectively, with a 50 μL complete cell culture medium.
2) Add 10 μL stock solutions of the compounds into the wells.
3) A 10 μL 1% TFA solution is used as control for data analysis.
4) Add 40 μL complete medium into the wells to adjust the total volume to 100 μL.
5) Incubate the plate at 37°C for 72 hours.
6) Add 50 μL CellTiter-Glo assay mix solution to each well and mix gently at room temperature for 10 minutes; subsequently, read the luminescence with PHERAstar Plus (Molecular Devices).

2.3. In Vivo Study

The study protocol and the animal use were approved by the IACUC of Genscript.

2.3.1. Dosage and Acute Toxicity:

Before the main experiment, we performed a pilot study to mimic the treatment and
studied mouse tolerance to intravenous (IV) injections of the peptides. We tested IV injections of the mixed peptide A+F in nude mice at different doses: (1) 50 μg/g per peptide, which was a total of 100 μg peptide/g for the mixed peptide; (2) 25 μg/g per peptide, and (3) 12.5 μg/g per peptide. In group (1), both the mice died immediately after injection; in group (2), all three mice tolerated the dose. They showed weakness and reluctance to move after the injection but recovered in a few hours; in group (3), all three mice tolerated the dose well without significant stress. The combination of peptide A+F through IV route showed strong acute toxicity at a relatively high dosage, whereas injection through subcutaneous (SC) route showed tolerate for more than 100 μg/g per peptide, which was a total of 200 μg/g for the mixed peptide. These findings were consistent with the observation in rats with IV injection of peptide F for the PK study.

2.3.2. Efficacy Study of the Peptides A and F in the U67 SC Xenograft Tumour Model:

2.3.2.1. Purpose:

To evaluate the antitumor efficacy of the peptide A and F combination in the human glioblastoma U87 SC xenograft tumour model. [20, 21]

2.3.2.2 Materials:

Cell line U87: The cells were cultured in DME + 10% FBS + 1% Penicillin/Streptomycin (P/S) antibiotics.

Animal species and strain: 20 BALB/c nude mice

Sex, age, and weight: Female, 6-8 weeks, and 20 ± 2 g

Breeder/supplier: SLAC Laboratory Animal Co. Ltd.

Test Facility: In Vivo Pharmacology, Genscript Inc. USA.

Acclimation: 7 days
Room: SPF

Room temperature: 22°C–25°C

Room relative humidity: 40%–70%

Room pressure and air change: 10–20 Pa; 10–15 times/h

Light cycle: 12-hour light cycle 8:00–20:00 with 2-hour dark cycle

Animal housing: 5 mice/cage by treatment group

Food: Free access to food, autoclaved and irradiated, SLAC Laboratory Animal Co. Ltd.

Water: Free access to water from local supply (filtered by Mol animal ultrapure water system and autoclaved)

2.3.2.3 Reagents:

1) DMEM: Invitrogen, Cat No: 11875093

2) FBS: Invitrogen, Cat No: 100999-141

3) P/S antibiotics: Invitrogen, Cat No: 15070-063

4) Trypsin-EDTA: Invitrogen, Cat No: 25200-072

5) P/S antibiotics: Invitrogen, Cat No: 15070-63

6) Matrigel: BD, Cat No: 354234 Order Number: 335369

7) Test article (nasal) name: Peptide A Supplier: Client

Physical description: White powder storage condition: −20°C

8) Test article (nasal) name: Peptide F Supplier: Client

Physical description: White powder storage condition: −20°C

9) Drug formulation: Use normal saline as solvent.

10) Solution storage condition: The peptide solution should be kept at 4°C and used within 48 hours.

2.3.2.4. Assay Procedure:
Cell amplification and implantation: U87 cells were cultured and SC injected into nude mice (right flank). A total number of $2.0 \times 10^6$ tumour cells per mouse were suspended in 0.1 mL of PBS/Matrigel mixture (1:1) and injected (12 tumour-bearing mice were required).

Groups and dosage:

When the tumour size reached a volume of 100–200 mm$^3$, U87 tumour-bearing nude mice were randomly assigned to three groups (four mice/group) and started with dosing immediately:

Group 1 was administered with a vehicle and served as control group.

Group 2 was administrated with peptide A+F (10 g/g of mouse weight with each peptide; IV, biweekly, three injections; then 20 μg/g intratumoral SC injection, every other day, two injections. Therefore, five injections in total).

Group 3 was administrated with peptide A+F (10 μg/g of mouse weight with each peptide, IV, only a single dose at first week; then 20 μg/g intratumoral SC injection, every other day, two injections. Therefore, three injections in total).

The administration period lasted for 12 days.

Peptide injection: Mix the peptide A solution and peptide F solution before each injection and inject.

Physical examination: If abnormal appearance and behaviour or signs of morbidity or mortality were seen through the cage side observation, the veterinarian would be notified and proper physical examination or necropsy would be performed. Frequency: Daily, immediately after cell inoculation and twice a day thereafter starting on first dosing.

Measurement: The tumour volumes and body weight of tumour-bearing mice were measured twice a week, and mice morbidity was recorded immediately before measuring the tumour volume and body weight throughout the whole experiment.

Terminal procedures and necropsy:
Early death/unscheduled necropsy: If a mouse died during the study, the time of death would be estimated as closely as possible and recorded, and the mouse would be necropsied as soon as possible. If the necropsy could not be performed immediately, the mouse would be refrigerated (not frozen) to minimise tissue autolysis and would be necropsied no later than 12 hours following death. If a mouse appeared in poor condition or morbid, the mouse was euthanised after discussing with the client. The mouse might be euthanised (as described later) per the Testing Facility’s policies on humane care of animals. If the tumour burden was larger than 2000 mm$^3$ or the body weight loss was greater than 20% of the baseline body weight, the mice would be euthanised. If the weight dropped significantly, the data would be viewed with a caveat. All unscheduled-necropsy mice would be necropsied immediately, or, if this could not be performed, the mouse would be refrigerated to minimise autolysis and necropsy would be performed no later than 12 hours after death.

Scheduled necropsy: At the end of the experiment, tumour-bearing mice were euthanised with CO$_2$ by the end of the study. The tumour samples were harvested, weighed, and photographed.

Endpoints:

Tumour volume: Tumour volume was measured twice a week in two dimensions using a calliper, and the volume was expressed in mm$^3$ using the formula $V = 1/2 \times a \times b^2$ where $a$ and $b$ were the long and short diameters of the tumour, respectively.

Body weight: The body weight of the tumour-bearing mice was measured and recorded twice a week after administration. If the body weight loss was greater than 20% of the baseline body weight, the dosage would be stopped or decreased.

Tumour weight: The tumour masses were weighed at the end of the experiment after
being harvested. Three tumours, one from each group, were fixed in 10% formalin for paraffin block preparation.

Inhibition rate: Inhibition rate (%) = (average tumour weight of control group − average tumour weight of test group)/average tumour weight of control group × 100%

2.4. In Vivo Study (Pharmacokinetic Study):

The study protocol and all the animal use were approved by the IACUC of Genscript.

2.4.1. Study Purpose:

To evaluate the pharmacokinetic parameters of peptide F in Wistar rats following IV administration.

2.4.2. Materials:

1) Animal species and strain: Wistar rat, three rats
2) Gender and weight: Female, 200–220 g
3) Breeder/supplier: Shanghai SLAC Experimental Animal Center
4) Test Facility: In Vivo Pharmacology Dept., Genscript Inc. Ltd
5) Adaptation: 7 days
6) Room: SPF level
7) Room temperature: 22–25°C
8) Room relative humidity: 40%–70%
9) Light cycle: 12-hour light cycle (8:00–20:00) with 2-hour dark cycle
10) Animal hosting: three rats/cage
11) Food: Free access to food, autoclaved and irradiated, Shanghai SLAC Laboratory Animal Co. Ltd.
12) Water: Free access to water from local supply (filtered by Molanimal Ultrapure Water System and autoclaved).
The study protocol and all the animal use were approved by the IACUC of Genscript.

2.4.3. Reagents and Equipment:

Test Article: Test article (nasal) name: Peptide F Supplier.Client

Physical description: White powder

Storage condition: −20°C

Purity: 95.91%

Molecular weight: 2432.19

Formulation: Use saline as solvent.

2.4.4. Procedure:

a) Peptide F was administrated to the rats through IV route at a single dose of 10 pg/gm.

b) Plasma samples were collected at 0, 5, 15, and 30 minutes and at 1, 2, 4, 6, 8, and 24 hours (total 10 time points) after drug administration for each rat (three rats in all). All the samples were kept in clean tubes and stored frozen at −80°C for further analysis.

c) The compound concentration in plasma samples was analysed using a validated LC-MS/MS method.

2.4.5. LC-MS/MS Analysis:

2.4.5.1. Instrument:

An Agilent Technologies 1260 infinity liquid chromatographic system was used. API4000 mass spectrometer equipped with TurbolonSpray (ESI) Interface (Applied Biosystems, Concord, Ontario, Canada) was used for detection. Analyst 1.5.2 software packages (Applied Biosystems) were used to control the LC-MS/MS system and for data acquisition and processing.

2.4.5.2. Sample Preparation:

An 80 μL aliquot of plasma samples was spiked into a 96-well plate, and 160 μL of
acetonitrile containing internal standard was added for protein precipitation. The vortexes plate and then centrifuged at 4000 rpm for 10 min. Supernatant was transferred into a new 96-well plate and mixed with 1:1 volume of H$_2$O, and the final solution was injected for LC-MS/MS analysis. The whole procedure was operated on ice.

2.4.5.3 Chromatography Condition:

Chromatographic separation was achieved on the Phenomenex Luna 3u NH2 100A (100 × 2.0 mm) column. The column temperature was maintained at ambient temperature (25°C). The flow rate was maintained at 0.2 mL/min, and the following mobile phases were used:

Table 1.

A: Water/Acetonitrile (90:10), 2% Formic acid
B: Acetonitrile

3. Results

3.1. In Vitro Study:

The peptides (A, E, and F and their combination: AEF) were prepared and tested according to the aforementioned protocols. The cell growth inhibition results were shown in the supplementary figures of the final report data. The study demonstrated superior response for peptide F in 14 of 15 cell lines followed by peptide A and lastly peptide E; excellent response was observed when we added peptides F, E, and A in one group.

EOL-1 cell viability assay results are not presented in Figure 1; EOL-1 cells were sensitive to 0.1% TFA control. Approximately 13% cell viability was detected in 1% TFA-treated EOL-1 cells as compared with a medium without TFA-treated EOL-1 cells. Therefore, the data were presented as either normalised by 0.1% TFA control or medium control for EOL-1 cells.
3.2. In Vitro Study:
The peptides (combination A+F) were prepared and tested according to the aforementioned protocols.

3.2.1. Cell Growth Inhibition:
Results are shown in Figures 1 and 2. Apparent tumour cell inhibition for all tested lines increased with increased peptide doses.

3.2.2. Apoptosis Assay:
Apoptosis Effect of Compound A+F on Tumour Cell (TFA as Control).
As shown in Figure 3, high apoptosis was noted with peptides A+F in the U87MG and MDA-MB-468 cell lines, moderate apoptosis was noted in the K562 cell line, and mild apoptosis was noted in the A375 cell line.

3.3. In Vivo Study:

3.3.1 Tumour Growth Curve:
The data showed that the tumour volumes of two treatment groups were reduced compared with the saline group from Day 9 to Day 12 post-treatment but not significantly as shown in Figure 4.

3.3.2 Body Weight Change Curve:
The body weights of the mice who received peptide A+F were not significantly reduced after dosing.

3.3.3 Photo of Tumour Mass and Tumour Weight:
The tumour masses were harvested and weighed.
Tumour weight: Tumour weights of the two treatment groups decreased compared with the saline group without any statistically significant difference.

3.3.4. Inhibition Rate:
The data demonstrated that a combination of peptide A and F moderately inhibited U87 tumours and xenograft growth but not significantly, as shown in Figure 5.

3.4. In Vivo Study (Pharmacokinetic Study):

3.4.1. Data Analysis:

Peak areas of the analyte and internal standard were calculated using Analyst™ 1.5.2 software (Applied Biosystems). Regression analysis was performed in this software, and the standardise on linear curve fit will $1/x$ or $1/x^2$ in the first instance. Acceptance criteria should be $\pm 30\%$ of the target value for standards and quality control (QC). As shown in Fig 6.

A minimum of five calibration points must be used in the quantification. At least three of the four QC standards must be within the $\pm 30\%$ target value. If the sample concentrations are significantly higher than the lowest QC point (100 ng/mL), that particular QC may be disregarded and the run accepted.

At least one of the high QC points must be within the target acceptance. Plasma versus time data were analysed using non-compartmental approaches with the WinNonlin version 5.2 software program.

3.4.2. Pharmacokinetic Parameters:

As shown in Table 2, pharmacokinetic parameters of peptide F after IV administration (10 $\mu$g/g) showed significantly short half-life.

3.4.3. Stability in Plasma and Stability after Sample Preparation: as shown in Table 3 and Table 4.

Peptide F was not stable in plasma at room temperature; therefore, the standard samples must be prepared on ice.

4. Discussion
Flavonoids, phenolics, and many other components in honey are reported for inhibitory effects in cancer colon [52], glioma [53], and melanoma [54] cell lines. Moreover, downregulation of different cellular pathways through tyrosine cyclooxygenase, ornithine decarboxylase, and kinase was reported for this inhibition [52–55]. The results of 3-(4,5-dimethylthiazol–2-y1)-2,5-diphenyl tetrazolium bromide and trypan blue exclusion assays showed a dose- and time-dependence for the inhibitory effects of honey components.[38]. Honey component inhibition of cell growth was due to its perturbation of a cell cycle [53,54]. Moreover, modulation of p53 regulation by honey components was observed [52]. Therefore, honey is a strong antimutagenic agent and presents anticarcinogenic properties. Honey on *Escherichia coli* exposed to radiation exhibits SOS response (SOS is an error-prone repair pathway contributing to mutagenicity) [56]. Additionally, various floral honeys exhibit inhibition of Trp-p-1 mutagenicity [57]. According to an in vivo study of CBA mice for breast carcinoma and fibrosarcoma, RJ administered intraperitoneally and subcutaneously exhibits no effect on the formation of metastases; however, simultaneous IV administration of RJ with tumour cells significantly inhibited metastases [58]. Anti-oestrogenic activities of RJ have been reported, which result in an inhibition of MCF–7 mammary cancer cell proliferation [59,60]. By contrast, if the lipid oestrogenic RJ component is added to MCF–7 cells, the proliferation increases [61]. The protein fraction is of RJP3O, which showed antitumor activities when it was tested in HeLa cells of cervical-uterine carcinoma [62]. Peptides related patents for cancer treatments are growing up and increase nowadays [63–77] As demonstrated in our studies, in vitro efficacy studies presented positive data; however,
studies involving animal model presented only moderate inhibition, although the data were promising for a preliminary human test. These study outcomes present a scope for a debating whether a non-satisfactory effective data in an animal tumour model is sufficient to stop proceeding with the clinical trial before performing a preliminary human test. Considering that several studies have presented positive effective data in animal tumour models while presenting negative outcomes in human models, a reverse possibility may also be contemplated.

The PK study of rats has demonstrated a significantly short half-life of peptide F in plasma. This may partially explain why IV injection of the peptide combination did not present significant tumour inhibition. These findings must be considered for future in vivo studies. Currently, an intratumoral injection, which minimises the loss of peptide during blood transportation, can be an ideal choice. Moreover, administration frequency and dosage might be adjusted accordingly. However, modification of the peptide or formulation to prolong its plasma half-life is necessary.

5. Conclusion

In vitro and in vivo studies have exhibited direct inhibitory effects for the peptides in various types of tumours as an inhibition was observed in 17 of 18 tumour cell lines, namely U87MG, MDA-MB-468, K562,A375, MG63, SH-4, RD, KP1, 5637, 2774, ML-1, Cal-27, Colo-205, 769P, EOL-1, HLE, MDA-MB-436, and Calu-3, and the in U87MG tumour mouse model. Moreover, pharmacokinetics, dosing, safety, and stability of peptide F were investigated, which presented significantly short half-life, instability of peptide at room temperature, and safety in certain doses. Therefore, modification and improvement of peptide F and other peptides was mandatory to yield more potent, stable, and safe peptides using D-form amino acids instead of L-form in both C and N termini [78]. We foresee introduction of new peptides to the scientific community to be tested and modified
for superior long-term malignancy management.

**Abbreviations**

DPBS: Dulbecco’s Phosphate-Buffered Saline. TFA: Trifluoroacetic acid. MEM: Minimum Essential Medium. RJ: Royal jelly. RPMI 1640 medium: Roswell Park Memorial Institute medium. FBS: Fetal Bovine Serum. DMEM: Dulbecco’s Modified Eagle Medium. IV: intravenous. P/S antibiotics: Penicillin/Streptomycin. SC: subcutaneous. T1/2: half-life time. T max: time taken to reach the maximum concentration of a drug. C max: the maximum (or peak) serum concentration of a drug. AUC: the definite integral in a plot of drug concentration in blood plasma vs. time. Vz/F: Volume or volume/kg. CL/F: Volume/time or volume/time/kg

**Declarations**

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Approved by the IACUC of Genscript USA Inc as all in vivo researches were done in Genscript USA Inc. 860 Centennial Ave Piscataway, NJ 08854 United States of America, following the company ethics rules.

**Consent for publication**

Not applicable

**Availability of data and materials**

Data sharing is available by Genscript USA Inc. 860 Centennial Ave Piscataway, NJ 08854 United States of America as said before all experimental studies were done there as a paid service.

**Competing Interest**

Khaled M.E Elawdan as being the founder of Shefaa Research Center & Cancer Control LLC and the owner of patents No:
WO/2014/040605A1, US/2015/0291663A1 & CN105283198A.

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**Authors’ contributions**

Conception and design: K M.E E. Development of methodology: K M.E E. Writing, review, and/or revision of the manuscript: K M.E E, Y K E, E A E & M B S. Study supervision: K M.E E. All authors approved the final manuscript.

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Tables

Due to technical limitations, Tables 1-3 are provided in the Supplementary Files section.

Figures

![Figure 1]

Effect of A and F peptides on tumour cell line proliferation
Figure 2

Doxorubicin inhibition of A375 cell growth occurred as a positive control.
Figure 3

Apoptosis effect of peptides A and F on tumour cells (TFA as control)
Figure 4

Tumour growth curve
Figure 5

Tumour weights of the two groups were decreased compared with the saline group weight.

Figure 6

Typical chromatography of plasma sample (rate 1-5 min, PL, IV, 10 μg/g)
Supplementary Files

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