Human Growth Hormone Fragment 176–191 Peptide Enhances the Toxicity of Doxorubicin-Loaded Chitosan Nanoparticles Against MCF-7 Breast Cancer Cells

Mahmoud M Habibullah1,2, Syam Mohan3,4, Nabeel Kashan Syed5, Hafiz A Makeen6,7, Qazi Mohammad Sajid Jamal6, Hani Alothalid6,7, Farkad Bantun8, Alaa Alhazmi1,2, Ali Hakamy2,9, Yahia A Kaabi1, Ghalia Samlan10, Mothashim Lohan11, Neelaveni Thangavel12, Mohamed Ahmed Al-Kasim5

1Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Jazan University, Jazan, Saudi Arabia; 2SMIRES for Consultation in Specialized Medical Laboratories, Jazan University, Jazan, Saudi Arabia; 3Substance Abuse and Toxicology Research Center, Jazan University, Jazan, Saudi Arabia; 4School of Health Science, University of Petroleum and Energy Studies, Dehradun, Uttarakhand, India; 5Pharmacy Practice Research Unit, Department of Clinical Pharmacy, Faculty of Pharmacy, Jazan University, Jazan, Saudi Arabia; 6Department of Health Informatics, College of Public Health and Health Informatics, Qassim University, Al Bukayriyah, Saudi Arabia; 7Department of Basic Sciences, Faculty of Applied Medical Sciences, Al-Baha University, Al-Baha, Saudi Arabia; 8Department of Microbiology, Faculty of Medicine, Umm Al-Qura University, Makkah, Saudi Arabia; 9Respiratory Therapy Department, Faculty of Applied Medical Sciences, Jazan University, Jazan, Saudi Arabia; 10Department of Food Science and Nutrition, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia; 11Emergency Medical Services Department, Faculty of Applied Medical Sciences, Jazan University, Jazan, Saudi Arabia; 12Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Jazan University, Jazan, Saudi Arabia

Correspondence: Mahmoud M Habibullah, Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Jazan University, Al Maarefah Road, Jazan, Saudi Arabia, Tel +966 55644205, Email mhhabibullah@jazanu.edu.sa

Introduction: Numerous drugs with potent toxicity against cancer cells are available for treating malignancies, but therapeutic efficacies are limited due to their inefficient tumor targeting and deleterious effects on non-cancerous tissue. Therefore, two improvements are mandatory for improved chemotherapy: 1) novel delivery techniques that can target cancer cells to deliver anticancer drugs and 2) methods to specifically enhance drug efficacy within tumors. The loading of inert drug carriers with anticancer agents and peptides which are able to bind (target) tumor-related proteins to enhance tumor drug accumulation and local cytotoxicity is a most promising approach.

Objective: To evaluate the anticancer efficacy of Chitosan nanoparticles loaded with human growth hormone hGH fragment 176–191 peptide plus the clinical chemotherapeutic doxorubicin in comparison with Chitosan loaded with doxorubicin alone.

Methods: Two sets of in silico experiments were performed using molecular docking simulations to determine the influence of hGH fragment 176–191 peptide on the anticancer efficacy of doxorubicin: 1) the binding affinities of hGH fragment 176–191 peptide to the breast cancer receptors, 2) the effects of hGH fragment 176–191 peptide binding on doxorubicin binding to these same receptors. Further, the influence of hGH fragment 176–191 peptide on the anticancer efficacy of doxorubicin was validated using viability assay in Human MCF-7 breast cancer cells.

Results: In silico analysis suggested that addition of the hGH fragment to doxorubicin-loaded Chitosan nanoparticles can enhance doxorubicin binding to multiple breast cancer protein targets, while photon correlation spectroscopy revealed that the synthesized dual-loaded Chitosan nanoparticles possess clinically favorable particle size, polydispersity index, as well as zeta potential.

Conclusion: These dual-loaded Chitosan nanoparticles demonstrated greater anti-proliferative activity against a breast cancer cell line (MCF-7) than doxorubicin-loaded Chitosan. This dual-loading strategy may enhance the anticancer potency of doxorubicin and reduce the clinical side effects associated with non-target tissue exposure.

Keywords: anticancer potency, nanoparticles, cytotoxicity, docking analysis

Introduction

Nearly around 19.3 million new cases of cancer along with 10 million cancer-related deaths have been reported by the GLOBOCAN figures for 2020.1 Moreover, it is also being predicted that cancer will soon eclipse cardiovascular diseases
as the leading cause of death.² According to these estimates, female breast cancer has overtaken lung cancer (11.7% vs 11.4% of all cases) as being the most commonly diagnosed cancer worldwide.¹

Many drugs as well as drug combinations with potent toxicity against cancerous cells have been developed for chemotherapy. However, these drugs can also damage non-cancerous cells, so a major challenge in cancer treatment is to deliver optimal amounts of drug precisely to the tumor sites without any exposure to the normal cells.³ In addition, bio-transformation, acquired drug resistance, and altered drug clearance can further diminish the efficacy of conventional chemotherapeutic drugs.³

To mitigate these problems, many novel drug delivery techniques have been developed for tumor-targeted chemotherapy.⁴–⁸ These include drug-loaded nanoparticles that which are able to bind with high affinity to cancer-associated proteins.⁶,⁸,⁹ For instance, targeting of a drug delivery vehicle such as a nanoparticle complexed with chemotherapeutic agent could be enhanced by co-loading the vehicle with peptide ligand to a receptor over-expressed on the tumor.¹⁰ Recent reports have also mentioned magnetic nanoparticles loaded with luteinizing hormone-releasing hormone (LHRH) to be able to aid in breast cancer diagnosis as well as enhancing its drug delivery.¹¹

Some other potential carriers against breast cancer include those studied by Azandaryani et al,¹² who studied the efficiency of Letrozole containing folate-conjugated polymer-lipid hybrid nanoparticles in the treatment of breast cancer. It was observed that the entrapment as well as therapeutic efficiency of letrozole within the amphiphilic carrier were significantly increased by the use of lipid nanoparticles along with surface modification, respectively.¹²

Alqaraghuli et al¹³ evaluated the effectiveness of the delivery of Epirubicin (Epr) through encapsulation with horse spleen apoferritin (HsAFr) cavity. Additionally, in this study, the surface of HsAFr-encapsulated Epr was also modified with folic acid (FA) for enhanced as well as optimal targeting of the breast cancer cells (MCF-7). It was also noticed in the drug release study that the HsAFr provided controlled drug release from Epr loaded with HsAFr, carried out at 37°C and at a pH of 7.4. The HsAFr–Epr–FA complex was tested on a human breast cancer cell line and the results demonstrated highly elevated toxicity against the MCF-7 cell line as compared to the free drug. It was also observed that 2 µM of free Epr, Epr-loaded HsAFr and HsAFr–Epr–FA demonstrated decreased cell viability.¹³

Human growth hormone (hGH or somatotropin), is a 191-amino acid peptide hormone that is secreted by the anterior pituitary gland that stimulates longitudinal bone growth and tissue expansion.¹⁴ Receptors for hGH are widely expressed throughout the body, especially in the liver. In addition to tissue growth promotion, hGH modulates body fat composition as well as carbohydrate and protein metabolism.¹⁵ In adipose tissue, hGH induces breakdown of triglycerides by stimulating hormone-sensitive lipase and also inhibits glucose uptake which is essential for adipocyte differentiation.¹⁶ Moreover, hGH is an autocrine oncogenic factor that promotes the proliferation of breast cancer stem cells.¹⁷ Therefore, hGH-related peptides may be extremely beneficial in drug targeting and possibly for disrupting hGH-dependent breast cancer progression as well.

Human growth hormone is composed of 4 major α-helices which are arranged in an up-up-down-down topology required for receptor interaction along with three mini helices.¹⁸ A C-terminal hGH fragment 176–191 with a tyrosine to phenylalanine substitution at the last position has been reported to enhance lipid breakdown and fat utilization in mice.¹⁹–²¹ And hence, in the present study we examined if hGH fragment 176–191 peptide can facilitate the anticancer efficacy of doxorubicin-loaded nanoparticles.

Chitosan nanoparticles (CN NPs) are particularly efficient delivery vehicles for both peptides as well as for chemotherapeutic drugs that which are used in breast cancer treatment due to their good biocompatibility, susceptibility to enzymatic hydrolysis, and for their high molecular carrying capacity.¹¹ The topo isomerase inhibitor doxorubicin (Adriamycin) is frequently used alone or in combination for the eradication of solid tumors, but a major limitation of this agent is that it also induces non-cancer cell death, thereby resulting in numerous side effects including cardiovascular toxicity. Doxorubicin toxicity to healthy cells can be reduced by targeted drug delivery as well as by reducing the administered dose. Therefore, in the present study, we examined if the addition of hGH fragment 176–191 peptide to doxorubicin-loaded CN NPs can enhance in silico target binding as well as in vitro toxicity against breast cancer cells. Furthermore, we also examined if these dual-loaded CN NPs have physicochemical properties favorable for clinical application.
Materials and Methods
Part 1: In silico Analysis
Two sets of experiments were performed in silico to determine (1) the binding affinities of hGH fragment 176–191 peptide to the breast cancer receptors, (2) the effects of hGH fragment 176–191 peptide binding on doxorubicin binding to these same receptors, (3) to evaluate the influence of hGH fragment 176–191 peptide on the anticancer efficacy of Doxorubicin.

Modeling of Doxorubicin Structure
The two-dimensional structure of doxorubicin (Figure 1) was downloaded (DB00997) in .mol format from Drug Bank database ([https://go.drugbank.com/drugs/DB00997](https://go.drugbank.com/drugs/DB00997)) and converted to Protein Data Bank (PDB) file format (.pdb) for 3D modeling using PEP-FOLD3.5 and docking analysis using Autodock 4.2 tools. The doxorubicin model was also subjected to the CHARMM energy minimization protocol in Discovery Studio Visualizer 2021 [BIOVIA, 2021].

Modeling of hGH Fragment 176–191 3D Structure
The hGH fragment 176–191 peptide sequence (YLRIVQCRSVEGSCGF) was submitted to the PEP-FOLD3.5 webserver. It uses the Hidden Markov Model sub-optimal conformation sampling approach for predicting small peptide 3D structures. After model generation using PEP-FOLD3.5, the 3D structure was further refined and assessed for quality using the MolProbity tool of the Swiss Model server. Finally, the suitability of the peptide structure for docking was analyzed using the Ramachandran plot function in Discovery Studio Visualizer.

Modeling of Target Protein Structures
The 3D structure files of the breast cancer biomarkers estrogen receptor (PDB:1QKU), progesterone receptor (PDB:2OVH), human epidermal growth factor receptor (PDB:3GOP), mind bomb (Mib) protein (PDB:4TSE), and Ki-67 (PDB:5J28) were then obtained from Protein Data Bank ([Figure 2; Table 1](#)). Published structures were subsequently edited in order to remove non-standard residues by making use of the Discovery Studio Visualizer 2021. For energy minimization, the UCSF Chimera program ([www.cgl.ucsf.edu>chimera](http://www.cgl.ucsf.edu>chimera)) with the following conjugate gradient minimization settings was used: removal of steric collision with steepest descent steps set to 1000, steepest descent size to 0.02 Å, conjugate gradient steps set to 1000, as well as the conjugate gradient step size to 0.02 Å. Energy minimized structures were saved as PDB files for docking simulations.

Predicting Peptide–Receptor (Breast Cancer Biomarker) Interactions
Receptor–peptide complexes were modeled by the PatchDock online docking server ([https://bioinfo3d.cs.tau.ac.il/](https://bioinfo3d.cs.tau.ac.il/)), which makes use of a geometry-based molecular docking algorithm as a scoring function. After docking analysis, complexes were additionally refined by using the FireDock online server.

![Figure 1 Two-dimensional (2D) structure of doxorubicin.](image-url)
Molecular Docking Analysis

Docking of doxorubicin with tumor biomarkers (receptors) was simulated using Autodock Version 4.2. Autodock searches for the best conformation of receptor and doxorubicin were on the basis of its binding energy. Water molecules were subsequently removed from the 3D files of receptor molecules prior to docking and then later on hydrogen atoms were added to all the target proteins. Gasteiger charges were also added to the receptors. A 60×60×60 Å grid box was also set for covering the entire surface of the receptor with a default grid point spacing of 0.375 Å. The Lamarckian genetic algorithm (LGA) was then used for making receptor-drug flexible docking calculations. The LGA parameters were set as follows: population size (ga_pop_size) = A, energy evaluations (ga_num_generation) = B, mutation rate = C, crossover rate = D, step size = E, and number of LGA runs = 10. Molecular docking simulations of doxorubicin to peptide-bound receptors were also conducted using a 75×75×75 Å grid and default parameters.

**Figure 2** Crystal 3D structure of (A) estrogen receptor (PDB:1QKU), (B) progesterone receptor (PDB: 2OVH), (C) human epidermal growth factor receptor (PDB:3GOP), and (D) mind bomb (Mib) protein (PDB:4TSE). All 3D graphics were obtained from RCSB-PDB.

### Table 1 Structural Information on Selected Breast Cancer Biomarkers

<table>
<thead>
<tr>
<th>Receptor Name</th>
<th>UniProt ID</th>
<th>PDB ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen receptor (ER)</td>
<td>P03372 (ESR1_HUMAN)</td>
<td>1QKU</td>
<td>[40]</td>
</tr>
<tr>
<td>Progesterone receptor (PR)</td>
<td>P06401 (PRGR_HUMAN)</td>
<td>2OVH</td>
<td>[41]</td>
</tr>
<tr>
<td>Human epidermal growth factor receptor (HER2)</td>
<td>P00533 (EGFR_HUMAN)</td>
<td>3GOP</td>
<td>[42]</td>
</tr>
<tr>
<td>Mind bomb (Mib) protein</td>
<td>Q86YT6 (MIB1_HUMAN)</td>
<td>4TSE</td>
<td>[43]</td>
</tr>
</tbody>
</table>
**Part - II: Nanoparticle Preparation**

Doxorubicin, low molecular weight (LMW) Chitosan (95% deacetylated) and glacial acetic acid were purchased from Sigma-Aldrich (Merck group, U.S.A.) while gum Arabica (Gum Acacia) was purchased locally from Gizan, Jazan Province. Other pharmaceuticals along with analytical grade materials were used in the present study as received.

**Preparation of Dual hGH Fragment 176–191 Peptide- and Doxorubicin-Loaded Chitosan Nanoparticles (Ch-hGH-DOX)**

Chitosan nanoparticles were synthesized using the ionic gelation method of Avadi et al., with slight modification. Negatively charged gum Arabica was dissolved in water at normal room temperature under constant stirring, while positively charged LMW Chitosan was dissolved in 0.1% aqueous acetic acid also at normal room temperature, and under constant stirring. The pH of both solutions was subsequently adjusted to 5.5 with the addition of 0.5 M sodium hydroxide. The indicated concentrations of doxorubicin and hGH 176–191 fragment were added to the Chitosan solution. Nanoparticles were then prepared by making use of dropwise addition of gum Arabica to LMW Chitosan solution under constant magnetic stirring (200–300 rpm) at normal room temperature for three hours. Composition of the optimal dual-loaded nanoparticles is presented below in **Table 2**.

**Measurement of Particle Size, Polydispersity Index, as Well as the Zeta Potential**

Photon correlation spectroscopy was used for measuring the mean particle size (PS), polydispersity index (PDI), as well as the zeta potential (ZP) (Malvern, Nano ZS90, UK). All formulations were dissolved in double-distilled water (Millipore) prior to the analysis. To avoid multi-scattering effects, the appropriate particle concentration was obtained for each dilution. All measurements were made in triplicate.

**Part - III: Effects of Ch-hGH-DOX on Breast Cancer Cell Viability**

Human MCF-7 breast cancer cells were procured for the present study from American Type Culture Collection (ATCC, Manassas, VI, USA) and were grown in RPMI-1640 medium (pH 7.4). It was further supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 g/mL streptomycin. For both propagation as well as the treatment, cells were maintained in an incubator (Heraeus, GmbH, Germany) at 37°C; humidity (90%) along with CO₂ (5%).

**Cell Viability Assay**

The cytotoxicity profiles of the indicated formulations were analyzed by making use of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) microculture tetrazolium viability assay. Cells were then seeded in multi-well plates and later incubated for 48 hours with the indicated concentration of Ch-hGH-DOX, Ch-DOX, or DOX (0–2.5 µg/mL). Each concentration was tested in triplicate, with each plate also including untreated controls along with a blank cell-free control. Subsequent to the incubation, MTT (5 mg/mL) was then added to each of the wells and the plates were further incubated for another 4 hours. The medium was then removed and DMSO (100 µL/well) was added for solubilizing the formazan crystals formed from MTT by viable cells. The optical density (OD) at 490 nm was recorded as an estimate of remaining viable cell number by making use of a microtiter plate reader (BioTek Instruments).

**Table 2** Composition of Dual-Loaded Chitosan Nanoparticles

<table>
<thead>
<tr>
<th>Factor</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Gum Arabica</td>
<td>10 mg/mL</td>
</tr>
<tr>
<td>hGH (176–191) fragment</td>
<td>50 nm/mL</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1 mg/mL</td>
</tr>
</tbody>
</table>
Growth inhibition (%) was calculated from the OD according to the following formula:

\[
\text{Growth inhibition} = \frac{\text{OD control} - \text{OD treated}}{\text{OD control}} \times 100
\]

The cytotoxicity of the sample on cancer cells was expressed as the IC50 (the concentration that reduces the OD by 50% relative to untreated cells).

**Statistical Analysis**

Statistical Package for the Social Sciences (SPSS-10 Inc., Chicago, IL., USA) version 23 was used for data analysis. All data are presented as mean ± standard deviation (SD). One-way ANOVA was used for comparing treatment group means. \( p<0.05 \) was considered statistically significant.

**Results and Discussion**

**In silico Analysis**

The estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Mib1/Ki-67 proliferation index are firmly established biomarkers used for the diagnosis, prognosis, and treatment guidance of primary, recurrent, and metastatic breast cancer patients. Additionally, these biomarkers are established therapeutic targets for breast cancer as well.

**The 3D Structure of hGH Fragment 176–191 Peptide**

In total, 63.64% of peptide residues were in the favorable zone for docking simulation according to the Ramachandran plot, while only 9.09% were in Ramachandran Outlier and 7.69% in Rotamer Outlier regions (Figure 3A).\(^{33}\) In addition, the MolProbity score was 2.00, and no C-Beta deviations, bad angles, or bonds were found\(^{34}\) (Figure 3B).\(^{35}\) Thus, the peptide 3D structure proved suitable for docking analysis.

The docking simulations in our study depicted that the hGH fragment 176–191 peptide binds with high affinity to Ki-67, MiB protein, and the estrogen receptor, but not with the progesterone receptor or HER2 (Table 3). The peptide also influenced simulated doxorubicin binding to these breast cancer receptors (Table 4). For example, the binding energy score for doxorubicin to the progesterone receptor was \(-10.09\) kcal/mol in the absence of hGH fragment 176–191 peptide, but was \(-11.31\) kcal/mol after peptide binding. Furthermore, peptide binding reduced the doxorubicin inhibition constant for the progesterone receptor from 139.01 to 14.41 nM. In addition to the progesterone receptor, hGH fragment 176–191 peptide binding also augmented doxorubicin binding to HER2 and MiB protein, but slightly reduced binding to the estrogen receptor and Ki-67.

There is compelling evidence indicating that progesterone receptors (PRs) playing a hierarchical role in breast cancer growth and therefore they might potentially be useful in improving the success of endocrine treatments.\(^{36}\) Likewise, Ki-67 and MiB proteins promoting the proliferation in breast cancer can be the choice of targeting breast cancer treatment (Soliman and Yussif, 2016). Increased affinity of doxorubicin to the progesterone receptor, Ki-67 and MiB protein hence proves worthiness of the hGH fragment 176–191 peptide in targeting breast cancer cells with increased efficacy.

In silico work presents with a good predictive model of what may actually happen in the cellular environment. Therefore, the efficacy of the hGH fragment 176–191 peptide was further evaluated in in-vitro condition.

**Nanoparticle Preparation**

Ch-hGH-DOX was successfully prepared by the ionic gelation technique developed by Avadi et al to produce Chitosan nanoparticles from a mixture of Chitosan and gum Arabica.\(^{31}\) The electrostatic interactions occurring between the positively charged Chitosan as well as the negatively charged Arabica gum also facilitate the incorporation of other charged compounds such as peptides. The formula for the synthesis of Ch-hGH-DOX (Table 3) was optimized on the basis of PS, uniformity of dispersion, and ZP.
PS, Polydispersity Index, and ZP

These Ch-hGH-DOX nanoparticles were first examined for PdI value, ZP, and PS distribution using a Malvern Zetasizer. Mean PS (90.25 nm), PdI (0.197), and ZP (31.7 mV) were all considered acceptable for subsequent testing in biological preparations (Table 5; Figure 4A and B).

In the present study, we found the particle size to be less than 100 nm and hence we can suggest that these nanoparticles can be considered suitable for the effective delivery of the chemotherapeutic agents. According to Danaei et al 2018,37 nanocarriers having a particle size lesser than 150nm (or 200 nm) are considered to be extremely beneficial and effective for the treatment of cancers as they passively target the tumor cells by means of improved permeability as well as retention.37 The polydispersity index (PdI) of Ch-hGH-DOX nanoparticles was found to be less than 0.2 this indicates that our formulation not only has a monodispersity conduct but it also has a much lesser tendency
With regard to the Zeta potential it was observed in the present study that the Ch-hGH-DOX nanoparticles showed high positive values. With a ZP greater than 30 mV it can be said that Ch-hGH-DOX nanoparticles have a tendency of stabilizing colloidal preparations and thus preventing particle aggregation.

With the aforementioned findings, we can conclude that the Ch-hGH-DOX nanoparticles prepared by the present method have shown to possess characteristics that are favorable for cellular uptake as well as having colloidal stability, including suitable diameter, surface charge along with a low polydispersity index that which is indicative of a reasonably homogeneous size distribution.

**Table 3** Docking Results of the hGH Fragment 176–191 Peptide Model with Breast Cancer Receptor Models After Refinement Using the FireDock Server

<table>
<thead>
<tr>
<th>Receptor Names</th>
<th>PDB IDs</th>
<th>Global Energy</th>
<th>Attractive VdW</th>
<th>Repulsive VdW</th>
<th>ACE</th>
<th>HB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen receptor (ER)</td>
<td>1QKU</td>
<td>−48.29</td>
<td>−30.94</td>
<td>22.87</td>
<td>−11.63</td>
<td>−3.04</td>
</tr>
<tr>
<td>Progesterone receptor (PR)</td>
<td>2OVH</td>
<td>−21.78</td>
<td>−23.16</td>
<td>10.44</td>
<td>−0.43</td>
<td>−1.03</td>
</tr>
<tr>
<td>Human epidermal growth factor receptor</td>
<td>3GOP</td>
<td>−31.65</td>
<td>−17.47</td>
<td>2.92</td>
<td>−4.21</td>
<td>−2.26</td>
</tr>
<tr>
<td>(HER2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mind bomb (Mib) protein</td>
<td>4TSE</td>
<td>−51.27</td>
<td>−25.50</td>
<td>18.61</td>
<td>−11.15</td>
<td>−6.86</td>
</tr>
<tr>
<td>Ki-67</td>
<td>5J28</td>
<td>−56.62</td>
<td>−25.30</td>
<td>11.74</td>
<td>−17.16</td>
<td>−2.50</td>
</tr>
</tbody>
</table>

**Notes:** Global Energy - The binding energy of the solution. Attractive and Repulsive VdW - The contribution made by the van der Waals forces to the global binding energy. ACE - The contribution made by the atomic contact energy (ACE) to the global binding energy. HB - The contribution made by the hydrogen bonds to the global binding energy.

**Table 4** Docking Results for Doxorubicin with Unbound Breast Cancer Receptors and hGH Fragment 176–191 Peptide-Bound Breast Cancer Receptors

<table>
<thead>
<tr>
<th>Breast Cancer Biomarkers Receptor</th>
<th>Receptor vs Doxorubicin</th>
<th>Receptor + Peptide vs Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final Intermolecular Energy (Kcal/mol)</td>
<td>Inhibition Constant (Ki)</td>
</tr>
<tr>
<td>Estrogen receptor (PDB:1QKU)</td>
<td>−2.77</td>
<td>NA</td>
</tr>
<tr>
<td>Progesterone receptor (PDB:2OVH)</td>
<td>−10.09</td>
<td>139.01 mM</td>
</tr>
<tr>
<td>Human epidermal growth factor receptor (PDB:3GOP)</td>
<td>−6.55</td>
<td>39.10 mM</td>
</tr>
<tr>
<td>Mind bomb (Mib) Protein (PDB:4TSE)</td>
<td>−5.76</td>
<td>158.20 mM</td>
</tr>
<tr>
<td>Ki-67 (PDB:5J28)</td>
<td>−7.62</td>
<td>2.81 mM</td>
</tr>
</tbody>
</table>

**Table 5** Physiochemical Characterization of Ch-hGH-DOX by Photon Correlation Spectroscopy

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Size (Z-Average) (d.nm) (n = 3) ±SD</th>
<th>PdI (n = 3) ±SD</th>
<th>Zeta Potential (mV) (n = 3) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch-hGH-DOX nanoparticles</td>
<td>90.25 ± 0.94</td>
<td>0.197 ± 0.01</td>
<td>31.7 ± 2.36</td>
</tr>
</tbody>
</table>

Towards aggregation. With regard to the Zeta potential it was observed in the present study that the Ch-hGH-DOX nanoparticles showed high positive values. With a ZP greater than 30 mV it can be said that Ch-hGH-DOX nanoparticles have a tendency of stabilizing colloidal preparations and thus preventing particle aggregation.

With the aforementioned findings, we can conclude that the Ch-hGH-DOX nanoparticles prepared by the present method have shown to possess characteristics that are favorable for cellular uptake as well as having colloidal stability, including suitable diameter, surface charge along with a low polydispersity index that which is indicative of a reasonably homogeneous size distribution.
Anticancer Efficacy of Ch-hGH-DOX Nanoparticles

Finally, we compared the cytotoxic effects of these Ch-hGH-DOX nanoparticles to Ch-DOX nanoparticles. MCF-7 breast cancer cells when treated with varied concentrations of Ch-hGH-DOX nanoparticles for 48 hours yielded an IC50 value.

Figure 4 (A) Size distribution of Ch-hGH-DOX nanoparticles (size = 91.33 d.nm) (n = 1), (B) Zeta potential distribution of Ch-hGH-DOX nanoparticles (ZP = 32.4 mV) (n = 1).

Anticancer Efficacy of Ch-hGH-DOX Nanoparticles

Finally, we compared the cytotoxic effects of these Ch-hGH-DOX nanoparticles to Ch-DOX nanoparticles. MCF-7 breast cancer cells when treated with varied concentrations of Ch-hGH-DOX nanoparticles for 48 hours yielded an IC50 value.
lower than those for doxorubicin alone and doxorubicin-loaded Chitosan nanoparticles (1.5 µg/mL vs 1.85 µg/mL as well as 1.7 µg/mL, receptively) (Figure 5). In contrast, neither unloaded Chitosan nanoparticles nor peptide-loaded Chitosan nanoparticles demonstrated substantial cytotoxicity (Figure 6). Furthermore, within the intermediate dose range (0.15 to 1.25 mg/mL), the proportion of viable MCF-7 cells remaining was significantly lower after 48 hours of Ch-hGH-DOX treatment than after Ch-DOX or DOX treatment (Figure 5). Therefore, hGH fragment 176–191 peptide appears to substantially enhance the toxicity of doxorubicin-loaded Chitosan nanoparticles, possibly by enhancing doxorubicin binding to breast cancer target proteins (Table 4).

Some of the potential limitations of the study are its inability to presently conduct any morphological studies, entrapment efficiency, as well as in-vitro release study.

**Conclusion**

Chitosan nanoparticles loaded with both doxorubicin and hGH fragment 176–192 peptide demonstrate both favorable physicochemical properties and enhanced cytotoxic efficacy against breast cancer cells. Molecular docking simulations
suggest that the enhanced cytotoxicity stems from greater binding of doxorubicin to peptide-bound target proteins compared to unbound targets. Moreover, the inclusion of hGH fragment 176–192 peptide may promote accumulation of doxorubicin-loaded Chitosan nanoparticles in breast tumors while reducing non-target tissue exposure, thereby enhancing therapeutic efficacy and reducing side effects.

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The author N.K.S., would like to dedicate this manuscript to his beloved and most dear father Mr. Alhaj Syed Maqbool who recently passed away. M.A.K would also like to dedicate this manuscript to his beloved father Dr Ahmed Al-kasim who recently passed away. M.A.K would also like to dedicate this manuscript to Prof. Mamdouh and Mr. Mohammed Baity, our respected colleagues who passed away very recently.

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Disclosure
The authors report no conflicts of interest in this work.

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