RESEARCH ARTICLE

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Antiproliferative Impact of Linagliptin on the Cervical Cancer Cell Line

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Abstract

Objective: This study aimed to assess linagliptin's inhibitory effects on the proliferation of cervical cancer cell lines and investigate its potential for targeting human heat shock protein 90. **Methods:** Linagliptin's cytotoxicity was assessed on a cervical cancer cell line (Hela cancer cell line) at two different incubation periods, 24 and 72 hours. The molecular docking between linagliptin and the receptor protein human Hsp 90 (PDB code: 5XRE) was performed using the Biovia Discovery Studio and AutoDock tool software. The Discovery Studio visualizer generated three-dimensional (3D) and two-dimensional (2D) interactive images. **Results:** The study's cytotoxicity results demonstrated that linagliptin can inhibit the proliferation of cervical cancer cells. The cytotoxicity exhibited a time-dependent pattern (cell cycle specific). The molecular docking study was conducted to investigate the interaction between linagliptin and human Hsp90. The study identified 11 sites where linagliptin can bind to Hsp90 amino acid residues. The total docking score for this interaction was -10.3 kcal/mol. The most potent binding occurred through conventional hydrogen bonds with the ASP:54 amino acid residues at a distance of 2.93 Å. The docking scores for linagliptin were comparable to those of the reference drug geldanamycin, indicating a strong interaction between linagliptin and Hsp90. **Conclusion:** The study has found that linagliptin successfully reduces the growth of cervical cancer cells with a time-dependent cytotoxic pattern. The potential anticancer mechanism of linagliptin can be inferred by analyzing the docking score and docking pattern between linagliptin and Hsp90, suggesting that linagliptin targets human Hsp 90.

Keywords: linagliptin- cervical cancer- Hela cancer cell line- heat shock protein 90- chemical docking

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Introduction

There were around 529,000 new instances of invasive cervical cancer (ICC), making it the third most prevalent malignancy among women globally. Cervical cancer has significant variation in prevalence throughout various areas of the world, with over 85% of cases concentrated in low-to-medium-resource countries. In these countries, cervical cancer remains the most predominant form of cancer among women [1].

The stage of the disease determines the treatment of cervical cancer at the time of diagnosis and the resources available in the local area. It may entail a radical hysterectomy, chemoradiation, or a combination of both [2]. However, chemotherapy is yet encountered as the one from primary option for cervical cancer treatment, cisplatin, which remains the current standard therapy for advanced, persistent, or recurrent cervical cancer. Topotecan and paclitaxel, combined with cisplatin, have yielded the best response rates; only combining cisplatin and topotecan has enhanced overall survival [3-5]. Although chemotherapy treatment can improve survival, its side effects still pose a major challenge for specific patients, specifically those with chronic disease, old age, and immunocompromised patients. To overcome these hazards that result from chemotherapy side effects, several alternative cancer treatments are under investigation to determine their efficacy and safety compared with traditional cancer chemotherapy; one tactic employed was drug repositioning, which involves finding new uses for pharmaceuticals already on the market. This approach has been demonstrated to be an effective method for developing novel anti-tumor medications [6], An example of a medication with a prospective anticancer effect is aspirin. Aspirin has exhibited the ability to suppress the growth of human gastric cancer cells by inducing apoptosis and causing cell cycle arrest at the G1 phase. In addition, it elevates the expression of caspase-3 and p53 while lowering the expression of cyclin D1, NF-κB, COX-2, and PGE2 [7], The osteosarcoma cell line MG63 successfully grew less rapidly after receiving short-term therapy with therapeutic dosages of paracetamol. This was accomplished by upregulating the expression of antigens (CD80, CD86, and HLA-DR) involved in presenting

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antigens to T lymphocytes and decreasing the synthesis of osteocalcin and phagocyte activity [8], Metformin demonstrated antitumor effects in various types of cancer, such as colorectal, prostate, pancreatic, renal, cervical, endometrial, gastric, lung, breast, and ovarian cancer, By a mechanism that encompasses both direct and indirect methods. Metformin has both direct and indirect effects. The direct effects include actions that are not dependent on AMPK (adenosine monophosphate-activated protein kinase) as well as actions that are dependent on AMPK. The indirect effects of metformin involve lowering glucose levels, reducing hyperinsulinemia, and decreasing the levels of Insulin-like Growth Factor 1 (IGF-1). Metformin boosts the immune system's defense against cancer cells by lowering levels of pro-inflammatory cytokines and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-KB) [9], However, proton pump inhibitor esomeprazole antiacid drugs exhibited a significant antiproliferative impact on cervical cancer cells, with suggested mechanisms including an increase in lysosomal membrane permeability of the cancer cells selectivity [10, 11], Linagliptin, a type 2 antidiabetic medication, has shown the capacity to inhibit cell survival, cell proliferation, and cell migration in Glioblastoma multiforme cells, through its capacity to modulate the levels of phosphorylated NF-kB, proteins involved in cell cycle regulation, and proteins associated with cell adhesion [12], Linagliptin also effectively decreases the viability of Saos-2 cells osteosarcoma cell line by a suggested mechanism involved in induced apoptotic effects [13]. One of the novel targets in cancer cells is heat shock protein; multiple studies have demonstrated that the heightened production of specific Heat Shock Proteins (HSPs) is crucial in regulating the internal balance of a diverse range of cancer types. Multiple studies indicate that the cytoprotection conferred by HSP is partially mediated by suppressing spontaneous and therapy-induced apoptosis. Consequently, overexpression of HSP contributes to tumor advancement and the development of resistance to treatment; aside from inhibiting programmed cell death, the abnormal regulation of Heat Shock Proteins (HSPs) plays crucial roles in the rapid growth, invasiveness, and spread of tumors [14].

Several studies were conducted to investigate the anticancer properties of linagliptin. Still, these studies showed that there was a lack of investigation into the cytotoxicity of linagliptin against cervical cancer cells. Our study aim was to investigate the antiproliferative activity of linagliptin on cervical cancer by studying its ability to target Hsp 90 by employing chemical molecular studies.

Materials and Methods

Linagliptin

Linagliptin, obtained from Samarra Pharmaceutical Factory, was utilized as raw material. A varied range of linagliptin concentrations, from 0.1 µg/ml to 1000 µg/ml, was prepared by diluting it with RPMI media.

Vincristine

Vincristine sulfate 1 mg/ml (Hospira UK/intravenous I.V) was employed at different concentrations which ranged between (1,10,100,1000) µg/ml after diluting with a serum-free medium.

Cell culture

The Hela cancer cell line, derived from malignant cervical carcinoma, was initially developed in the tissue culture unit at (ICCMGR) [15], The cells were cultured in 75 cm2 tissue culture containers under controlled conditions, maintaining a relative humidity of 37°C and 5% CO2. The cells were cultured in RPMI-1640 medium (Sigma Chemicals, England) supplemented with 10% fetal calf serum (FBS) and 100 U/mL penicillin-streptomycin (100 µg/mL streptomycin) [11].

Cytotoxicity study

The cervical cancer cells grown in a 96-well microtiter plate were treated with linagliptin and vincristine. During the logarithmic growth phase, the concentration of cancer cells steadily increased, and the toxicity of the medications being tested was assessed at various incubation durations [16, 17].

Every well comprises 10,000 cells. Seeding involves utilizing a medium containing 10% fetal bovine serum. The plates were incubated for 24 hrs. at 37°C to promote cell attachment. Serial dilutions were performed using RPMI medium without any added serum. Linagliptin and vincristine were diluted in RPMI medium without calf serum to form a series of dilutions ranging from 0.1 to 1000 µg/ml for each component [11, 18].

After being incubated for twenty-four hours, the cancer cells were treated six times in 200 µl increments. Two hundred microliters of maintenance media were injected into each control well, and the exposure times varied between twenty-four and seventy-two hours. The plates were then put back in the incubator after being securely sealed with self-adhesive material. After that, the cells were treated with MTT dye. A microtiter plate ELISA reader was used to measure the optical density of each well at a transmission wavelength of 550 nm [19, 16].

The growth inhibition rate is determined by applying the following mathematical equation [16].

Growth inhibition= (optical density of control wells-optical density of treated wells)/(optical density of control wells)*100%

Molecular docking

The chemical structures of linagliptin were illustrated using ChemDraw software (CambridgeSoft, USA) and subsequently optimized using the Chem3D version. The molecular structure of heat shock protein 90 was obtained from the "Protein Data Bank."

The enzymes and proteins were adjusted using AutoDock Tools to optimize their structures. The ligands' most stable structure was found using AutoDock Tools, and then the PDBQT file of the ligands was created.

After the ligand and protein structures were optimized, they were loaded into AutoDock-Tools, where the docking

procedure was carried out with the aid of the same program. Using PLIP and BIOVIA Discovery Studio, the docking energy scores and binding interactions were comprehensively investigated [20, 21].

Research ethics

There were no human subjects in this study.

Statistical Analysis

The MTT test was conducted with six duplicates, and the results are shown as the mean ± standard deviation (SD) values. The One-way analysis of variance (ANOVA) test was employed. The study analyzed the differences between groups using the Least Significant Difference (LSD) test. The study was conducted using the statistical software version 20 package, and statistical significance was determined at a level of $p < 0.05$ [22].

Results

Study on the cytotoxicity of Hels cancer cell line
1.Linagliptin cytotoxicity

The study revealed that linagliptin exhibits a cytotoxic impact on cervical cancer cells. It demonstrated that the cancer cells' growth suppression depended on the duration of their exposure to linagliptin and the drug's concentration. Growth inhibition varied significantly between the two incubation times for all linagliptin doses. Moreover, substantial differences in the growth inhibition rate were seen across all doses during each incubation period (Table 1, Figure 1.)

Vincristine cytotoxicity

The study found that the growth inhibition pattern of vincristine on cervical cancer cells primarily depended on time. A significant difference in growth inhibition between two incubation periods for all vincristine concentrations supported this. We found that the impact of concentration was less effective than the time factor. Table 2, Figure 2.

Table 1. Shows the Impact of Linagliptin on the Growth Suppression of the Hela Cancer Cell Line at 24- and 72-hour Intervals.

Concentration (µg/ml)	Growth inhibition (mean ± SE a)		P- value
	24 hr.	72 hr.	
0.1	C 4.00 ± 1.155	D 21.00 ± 0.577	0.0001*
1	C6.00 ± 1.732	CD 27.00 ± 1.732	0.001*
10	B 20.67 ± 2.028	CD 34.00 ± 2.309	0.012*
100	A 32.00 ± 1.155	B 53.00 ± 1.732	0.001*
1000	A 38.00 ± 1.732	A 72.00 ± 1.155	0.0001*
b LSD value	10.07	10.16	-
IC_{50}	1418.63 µg/ml	429.42 µg/ml	-

[a], standard error; [b], least significant difference, statistically significant differences are shown by variations in capital letters within the same column, whereas variations in lowercase letters within the same rows also indicate statistically significant differences. *, significant at (P<0.05)

Table 2. The Impact of Vincristine's Ability to Limit the Growth of the Hela Cancer Cell Line after 24 and 72 Hours.

Concentration (µg/ml)	Growth inhibition (mean ± SE [a])		P- value
	24 hr.	72 hr.	
0.1	B10.00 ± 2.887	C 20.00 ± 2.887	0.07
1	B 13.33 ± 4.410	BC 29.00 ± .577	0.009*
10	AB 18.00 ± 1.732	AB 33.00 ± 1.732	0.004*
100	AB 23.00 ± 1.732	AB 38.00 ± 1.732	0.004*
1000	A 33.00 ± 1.732	A 41.00 ± .577	0.012*
[b] LSD value	17.09	10.92	-
IC_{50}	1928.24 µg/ml	1669.26 µg/ml	-

[a], standard error; [b], least significant difference; statistically significant differences are shown by variations in capital letters within the same column, whereas variations in lowercase letters within the same rows also indicate statistically significant differences. *, significant at (P<0.05)

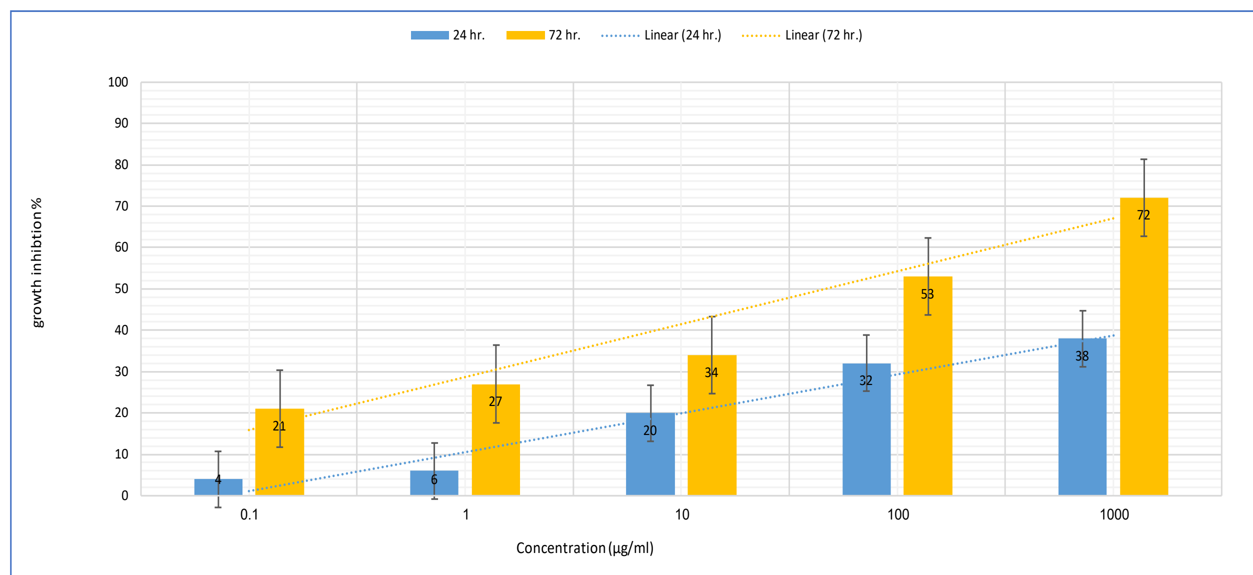

Figure 1. The Impact of Linagliptin on the Growth Suppression of the Hela Cancer Cell Line at 24 and 72-hour Intervals

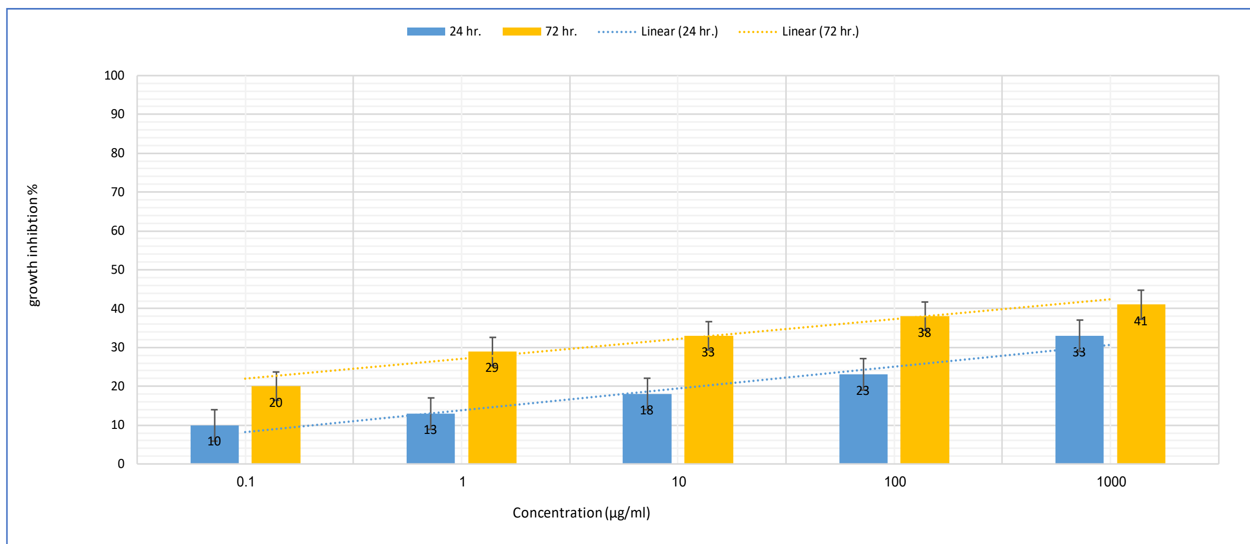

Figure 2. The Impact of Vincristine's Ability to Limit the Growth of the Hela Cancer Cell Line after 24 and 72 hours

Comparison between linagliptin vincristine cytotoxicity

The comparison results between the growth inhibition of linagliptin and vincristine indicate that linagliptin exhibits best growth inhibition. This difference is particularly significant at higher concentrations (100 and 1000) µg/ml and after a 72-hour incubation period. Table (3,4) Figure (3,4)

Table 3. Comparison of Linagliptin and Vincristine Growth Inhibition at 24 hours

Concentration (µg/ml)	Growth inhibition (mean ± SE [a])		P- value
	Linagliptin	Vincristine	
0.1	C 4.00 ± 1.155	B10.00 ± 2.887	0.126
1	C6.00 ± 1.732	B 13.33 ± 4.410	0.056
10	B 20.67 ± 2.028	AB 18.00 ± 1.732	0.374
100	A 32.00 ± 1.155	AB 23.00 ± 1.732	0.012*
1000	A 38.00 ± 1.732	A 33.00 ± 1.732	0.111
[b] LSD value	10.07	17.09	-
IC_{50}	1418.63 µg/ml	1928.24 µg/ml	-

[a], standard error; [b], least significant difference. Statistically significant differences are shown by capital letters within the same column, whereas variations in lowercase letters within the same rows also indicate statistically significant differences. *, significant at (P<0.05)

Table 4. Comparison of Linagliptin and Vincristine Growth Inhibition at 72 hours

Concentration (µg/ml)	Growth inhibition (mean ± SE [a])		P- value
	Linagliptin	Vincristine	
0.1	D 21.00 ± 0.577	C 20.00 ± 2.887	0.751
1	CD 27.00 ± 1.732	BC 29.00 ± .577	0.335
10	CD 34.00 ± 2.309	AB 33.00 ± 1.732	0.746
100	B 53.00 ± 1.732	AB 38.00 ± 1.732	0.004*
1000	A 72.00 ± 1.155	A 41.00 ± .577	0.0001*
[b] LSD value	10.16	10.92	-
IC_{50}	429.42 µg/ml	1669.26 µg/ml	-

[a], standard error; [b], least significant difference. Statistically significant differences are shown by capital letters within the same column, whereas variations in lowercase letters within the same rows also indicate statistically significant differences. *, significant at (P<0.05)

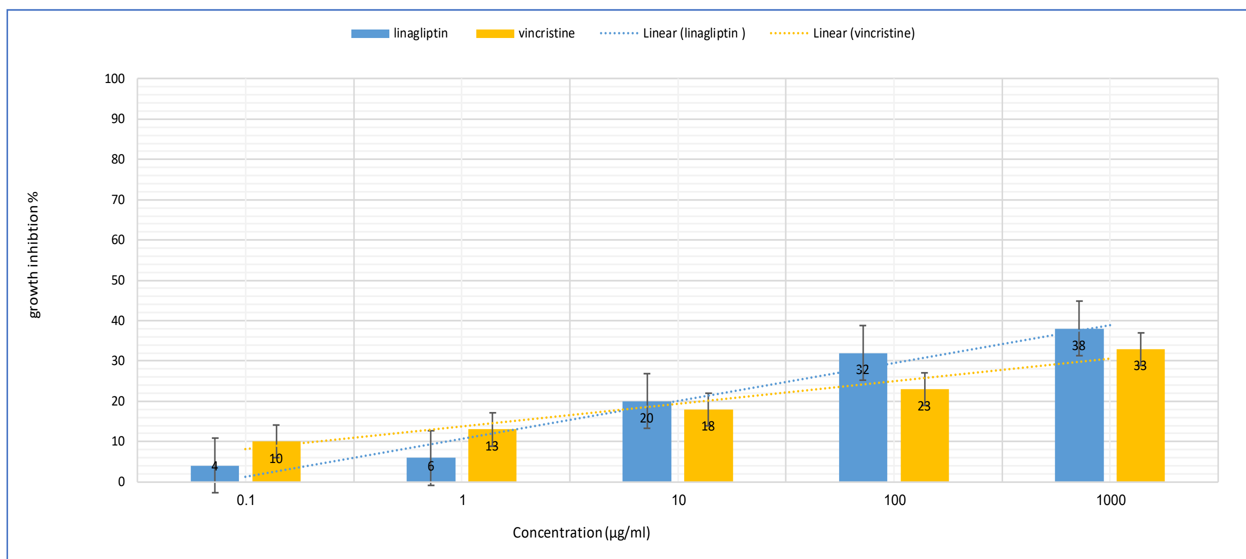

Figure 3. Comparison of Linagliptin and Vincristine Growth Inhibition at 24 hours.

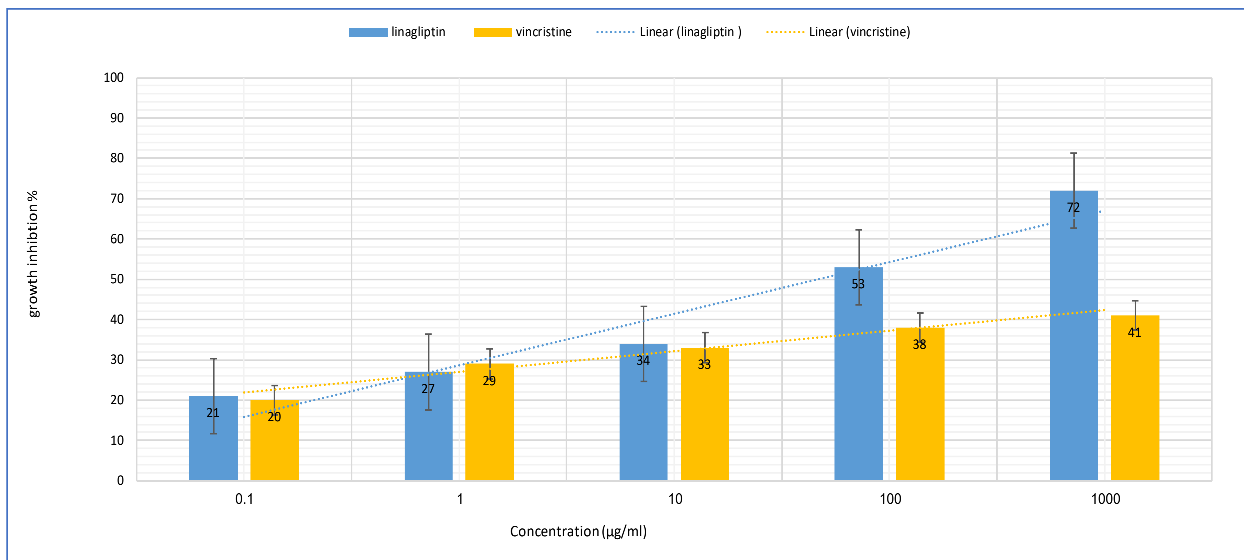

Figure 4. Comparison of Linagliptin and Vincristine Growth Inhibition at 72 hours.

Molecular docking studies

Evaluation of New Drug Compounds for Human Hsp 90 Molecular docking models were constructed using the structures of human Hsp 90 (PDB code: 5XRE) as a basis. The interaction between linagliptin and human Hsp 90 was investigated using molecular docking modeling. The molecular docking analysis was performed using AutoDock tools 1.5.7 and BIOVIA Discovery Studio [21], our study result of Molecular docking studies exhibited the active site for binding linagliptin with Hsp90 was ASP A:54, LEU A:107, ILE A:110, ASN A:51, MET A: 98, PHE A: 138, TRP A:162, VAL A: 150, LYS A:58 and ALA A:55, on another side, with the total docking score was -10.3 kcal/mol. Also, the result of docking showed linagliptin formed Conventional hydrogen bonds with the ASP:54 amino acid residues at 2.93 Å of distance, Pi-alkyl bound with the MET:98, ALA:55, and VAL A:150 amino acid residues at 5.05 Å, 4.65 Å and 4.74 Å of distance subsequently, alkyl bound with the LEU A:107, LYS A:58 and ILE A:110 amino acid residues at 3.94 Å, 4.87 Å and 3.94 Å of distance subsequently, Pi-pi shaped bound with TRP:162 at 5.05 Å of distance, Pi-pi stacked bound with PHE:138 at 3.77 and 4.34 Å of distance (Figure 5)

On the other side, molecular docking study data of geldanamycin (a standard Hsp 90 inhibitor) revealed a total docking score of (-7.9) kcal/mol, formed Conventional hydrogen bonds with the LYS A:54 amino acid residues at 2.03 Å of distance, Pi-alkyl bound with the LEU A:107 and MET A:98 amino acid residues at 5.12 Å and 5.16 Å of distance subsequently, alkyl bound with the LEU A:107 at 5.29 Å of distance, Carbon hydrogen bond with LEU A:107 and ASP A:54 at 3.49 Å and 3.60 Å of distance subsequently (Figure 6). This finding demonstrated the similarities in linagliptin and geldanamycin's capacities to interact with HSP 90 (Figure 7).

Discussion

Our study showed that linagliptin can reduce the growth of cervical cancer cells. This finding is consistent with other studies that have shown that treatment with linagliptin significantly decreased the viability of Saos-2 cells (a human osteosarcoma cell line) and hFOB1.19 cells (a human fetal osteoblastic cell line) at

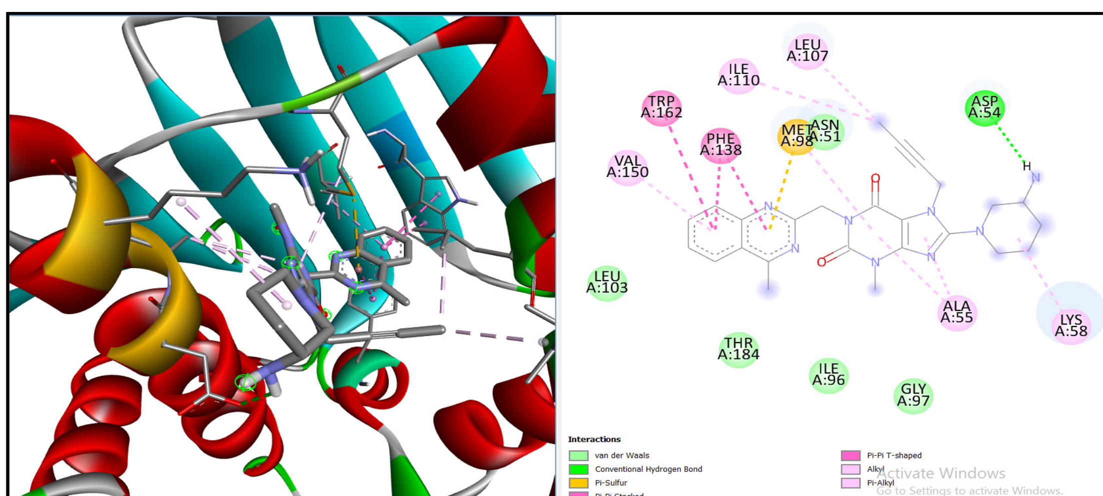

Figure 5. Human Hsp90 Binding Site with Linagliptin

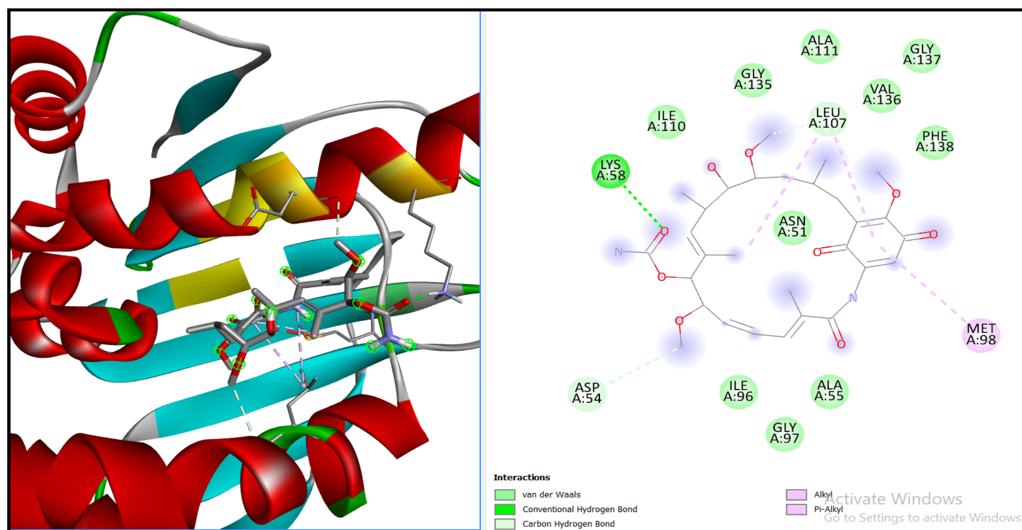

Figure 6. Human Hsp90 Binding Site with Geldanamycin.

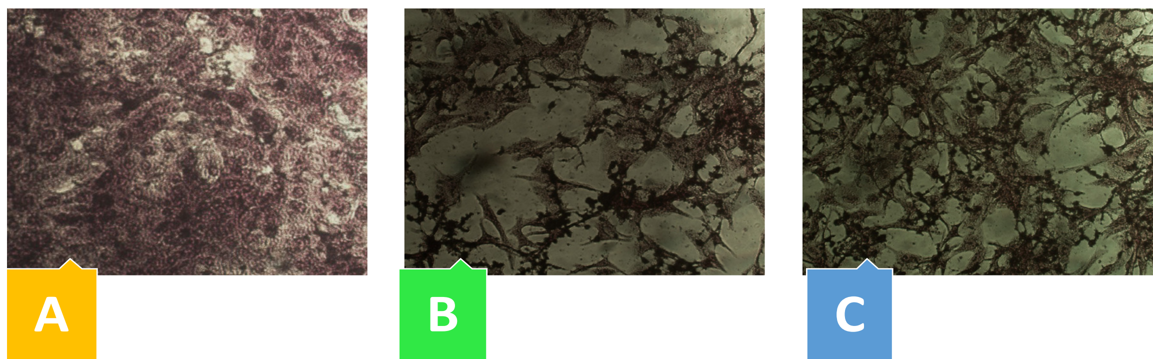

Figure 7. Morphology of Human Cervical Cancer Cells. (B) Cancer cells were subjected to a 1000 µg/ml concentration of linagliptin for 72 hours. (C) Cancer cells were treated with 1000 µg/ml of vincristine for 72 hours. (A) Cervical cancer cells were not treated and served as the control group.

a statistically significant level ($p < 0.001$) [13]. Another study demonstrated that Linagliptin can inhibit cell viability, proliferation, and migration in Glioblastoma cancer cells.[12] Furthermore, linagliptin reflects the capacity to suppress the growth of HCT116 cells, a human colorectal cancer cell line [23], the growth inhibition pattern was mainly dependent on the incubation time, which was greater than its dependency on concentration. Based on this fact, we hypothesized that the mechanism behind the anticancer properties of linagliptin is involved (cell cycle specific). Multiple studies have provided evidence in acceptance of this idea. One study indicated that linagliptin has the potential to cause cell cycle arrest at the G2/M phase when administered in low dosages and at both the G2/M and S phases when administered in large amounts [23]. Another suggested mechanism that supports our findings is the ability of linagliptin to target Aurora kinase B specifically. This kinase is a considerably preserved serine-threonine protein kinase categorized as a constituent of the Aurora family and has a crucial role in regulating mitosis [24], Aurora kinase B is raised in pleomorphic gliomas, malignant mesothelioma, and hematological malignancies. Likewise, this gene is excessively too in colorectal, liver, and breast cancer [25], Linagliptin can target Cyclin Dependent Kinase 1 (CDK1), which plays a critical role in the cell cycle and phosphorylates diverse substrate proteins comprising histones H1, laminin, and Rb. By targeting Aurora kinase B and CDK1, linagliptin can inhibit cell proliferation and inhibit tumor growth.

Linagliptin can induce cell death by inhibiting the phosphorylation of Rb and the production of Bcl 2. Pro-caspase3 [23]. Heat shock protein 90 is identified as a potent and successful pharmacological target for designing novel anticancer drugs, as it plays a critical role in cell growth [26].

The docking scores of Linagliptin ranged from -10.3 to -8.8 kcal/mol. The medication's docking scores were comparable to those of the reference drug geldanamycin, with values ranging from –7.9 to -7.6 kcal/mol. This result exhibited the ability of linagliptin to interact with Hsp90, which is similar to standard Hsp 90 inhibitors (geldanamycin). Furthermore, this result revealed that linagliptin exhibited robust activity, as evidenced by its high docking score and docking pattern, supported by its interaction capacity. Through this targeting, we suggest

that linagliptin minimized cellular energy by interacting with Hsp90 and its associated molecular chaperone. This interaction governs the breakdown of ATP and triggers the start of the Hsp90 molecular chaperone cycle, which supplies energy for cellular processes; Hsp90 and Hsp90 have a role in regulating the folding of proteins in mitochondria. Hsp90 disrupts the folding of mitochondrial proteins during cell mitosis; this mechanism Approved by our cytotoxicity study results that conduction to, the pattern of linagliptin cytotoxicity was manlily cell-cycle specific. Many post-translational changes, such as phosphorylation, acetylation, oxidation, and S-nitrosylation, influence the regulation of Hsp90's chaperone action [27-30]. In addition, Hsp90 plays a key role in conformational maturation and stability of client proteins in transducing proliferative and anti-apoptotic signals. Targeted Hsp 90 causes down-regulation of c-FLIPL; Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) is a significant protein that plays a crucial role in preventing cell death (apoptosis). It also acts as a cytokine and chemotherapy resistance factor, suppressing cell death induced by cytokines and chemotherapy. Additionally, c-FLIP inhibits programmed necroptosis (necrosis) and autophagy. C-FLIP mutations confer resistance to death receptor ligands and chemotherapeutic drugs in cancer cells [31-33], Furthermore, Hsp90 has been shown to control the stability of client proteins, including HER2, CD4, AKT, RAF-1, and Bcr-Abl, which play a vital role in the proliferation of cancer cells. It is crucial to note that HER2 is a highly recognized protein frequently observed in breast cancer cases. Moreover, breast cancer cells exhibit a higher abundance of Hsp90 than normal cells. Increased Hsp90 expression in initial breast cancer has shown a notable association with poorer survival [30].

study tries to explore a novel target of linagliptin to provide a more descriptive mechanism of linagliptin as an anticancer. Still, it must explore more cellular targets and find the best pathway for effectively reaching linagliptin to target cancer cells.

In conclusion, a study demonstrated that linagliptin has a time-dependent cytotoxic pattern, effectively decreasing the proliferation of cervical cancer cells; the putative mechanism of linagliptin's anticancer effect can be inferred from the strong affinity (docking score and docking pattern) between linagliptin and Hsp90, indicating linagliptin's capacity to target Hsp90 specifically.

Author Contribution Statement

All authors contributed equally in this study.

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Conflicts of interest

No evidence indicating a conflict of interest was discovered.

Abbreviations

(ICCMGR): The Iraqi Centre for Cancer and Medical Genetics Research.

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide stain

RPMI: Roswell Park Memorial Institute medium

SAS: Statistical Analysis System

LSD: Least Significant Difference

Å: angstrom

Hsp: heat shock protein

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