RESEARCH ARTICLE

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Antiproliferative Potential of Linagliptin-Rivaroxaban Mixture in Cervical Cancer: Mechanistic Insights into Targeting Mutant MAPK, RAS kinase Signal Protein

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Abstract

Background: Repositioning existing marketed drugs represents a viable strategy for identifying new anticancer agents. This study employed an approach that combined these drugs and examined their molecular mechanisms of action against cancer. **Objective:** This study evaluated the anticancer properties of the linagliptin-rivaroxaban mixture and its molecular anticancer mechanism by screening its ability to target the mutant MAPK-RAS kinase signal proteins. **Methods:** Following 24 and 72 hours of incubation, HeLa and human-derived adipose tissue (NHF) cell lines were utilized to investigate the anticancer and safety properties of a linagliptin-rivaroxaban mixture and cisplatin at concentrations between 0.1 and 1,000 µg/ml. A combination and selectivity index study assessed the potential synergistic effects between mixture ingredients and selective toxicity. Computational molecular docking simulations were employed to investigate the binding affinity of linagliptin and rivaroxaban to various mutant kinase signal proteins within the MAPK-RAS kinase pathway. **Results:** The study results indicated that the combination of linagliptin and rivaroxaban significantly suppressed the growth of cervical cancer cells compared to the inhibitory effects of cisplatin, linagliptin, and rivaroxaban individually. Furthermore, the mixture cytotoxicity on the NHF cell line was significantly lower than that of cisplatin. The interaction between linagliptin and rivaroxaban exhibited synergistic cytotoxicity, as evidenced by the combination index score. The mixture exhibited selective cytotoxicity against cancer cells, suggesting a favorable toxicity index score. The outcomes of the molecular docking pilot study of diverse mutant MAPK-RAS kinase signal proteins with the mixture's ingredients are indicated. Linagliptin and rivaroxaban's best interactions were with mutant P38 MAPK and RAS kinase signal proteins, with docking scores of -7.9 kcal/mol and -8.7 kcal/mol, respectively. **Conclusion:** Regarding the study findings and the established pharmacokinetic and safety profiles of mixture drugs. The linagliptin-rivaroxaban mixture offers an attractive and safer alternative for cervical cancer treatment.

Keywords: Linagliptin- rivaroxaban- HeLa cell line- P38 MAPK kinase- RAS kinase- molecular docking

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Introduction

Cervical cancer is a major global health issue, especially in low- and middle-income countries (LMICs), where it ranks as the fourth most prevalent cancer among women. [1]. Persistent infection with high-risk human papillomavirus (HPV) types, particularly HPV-16 and HPV-18, is the main etiological factor responsible for around 70% of cases [2, 3]. Despite the existence of effective screening programs and HPV vaccination, disparities in access to these preventive measures result in elevated incidence and mortality rates in low- and middle-income countries compared to high-income nations [4, 3]. Early detection via Pap smears and HPV testing has markedly decreased cervical cancer rates in areas with strong healthcare systems. However, achieving global equity in prevention and treatment constitutes a significant public health challenge [5, 6, 3, 7]. Chemoradiotherapy is the primary treatment modality for cervical cancer. Chemotherapy for cervical cancer, typically utilizing cisplatin-based regimens, is constrained by notable drawbacks, such as systemic toxicity, drug resistance, and unintended effects on healthy tissues [8]. The side effects, including nephrotoxicity, myelosuppression, and gastrointestinal distress, frequently diminish patients' quality of life and restrict treatment efficacy [9].

The negative consequences linked to chemotherapy highlight the need for safer alternatives. Numerous trials have been conducted to identify an effective treatment for cervical cancer by utilizing a combination of medications

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that were originally developed for different therapeutic purposes rather than for cancer[10-15]. Along with this concept, Linagliptin and rivaroxaban are two examples of drugs that may possess anticancer properties. The selection criteria for these drugs were grounded in extensive pharmacokinetic studies and safety profiles, alongside demonstrated anticancer efficacy, as evidenced by multiple previous studies.

Linagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor commonly utilized in managing type 2 diabetes, has demonstrated potential anticancer properties in preclinical studies. It exerts antitumor effects by inhibiting cancer cell proliferation and inducing apoptosis by modulating key signaling pathways, such as PI3K/AKT and ERK, essential for tumor survival and growth [16]. Linagliptin has been shown to inhibit angiogenesis, an essential mechanism in tumor progression, through the downregulation of vascular endothelial growth factor (VEGF) expression [17]. In addition to its direct impact on cancer cells, linagliptin may improve immune-mediated tumor surveillance by increasing the activity of natural killer cells and cytotoxic T lymphocytes, thus enhancing antitumor immunity [18].

In contrast, Rivaroxaban, an oral anticoagulant that acts as a direct factor Xa inhibitor, has recently attracted interest for its potential anticancer properties and primary application in thromboembolic disorders. Preclinical studies indicate that rivaroxaban may impede cancer progression by targeting mechanisms like tumor-associated thrombosis, which is associated with tumor growth and metastasis [19]. Additionally, Rivaroxaban has been demonstrated to decrease the expression of pro-inflammatory and pro-angiogenic factors, such as tissue factor (TF) and vascular endothelial growth factor (VEGF), thus restricting tumor angiogenesis and metastasis [20]. Recent evidence suggests that rivaroxaban may directly influence cancer cell survival by inducing apoptosis and inhibiting proliferation via the modulation of signaling pathways, including PI3K/AKT [21]. Additionally, its anticoagulant properties may lower the risk of venous thromboembolism associated with cancer, a prevalent complication among cancer patients [22].

Along with the hypothesis that suggested that understanding the MAPK-RAS pathway is critical for designing successful cancer therapies, the current study focuses on the role of the mixture in targeting this crucial pathway in cancer.

The MAPK-RAS kinase signaling system regulates cell proliferation, differentiation, survival, and disruption, which are characteristic of many malignancies. RAS proteins, including KRAS, NRAS, and HRAS, function as molecular switches that activate the MAPK cascade, which includes RAF, MEK, and ERK kinases, to convey growth signals from cell surface receptors to the nucleus [23] . Mutations in RAS or upstream receptors, including EGFR, are commonly found in pancreatic, colorectal, and lung cancers. These mutations result in the constitutive activation of the MAPK pathway and subsequent uncontrolled cell proliferation [24].This hyperactivation facilitates tumorigenesis by enhancing cell cycle progression, inhibiting apoptosis, and promoting angiogenesis and metastasis [25]. Additionally, the MAPK pathway interacts with other oncogenic signaling networks, including PI3K/AKT, enhancing its pro-tumor effects [26]. Another critical member of the MAPK-RAS kinase pathway is p38 MAPK. This stress-responsive kinase plays a role in cancer by supporting tumor survival and metastasis in others [27-29].

Related to the crucial role of MAPK-RAS kinase signal protein in cancer, Multiple trials were performed to identify medications that can target these protein kinases, such as Vemurafenib [30], Dabrafenib [31], Trametinib [32] , Cobimetinib [33], Sotorasib [34], Adagrasib [35], Erlotinib [36], Gefitinib [37] and Ralimetinib [38].

Incorporating existing marketed drugs for cancer therapy presents a promising strategy for developing effective cancer treatments. Several studies have been conducted on this topic, one of which has shown that the amygdalin—esomeprazole mixture effectively kills cervical cancer cells via a pattern of inhibition contingent upon the concentration of the medication and the incubation period [10, 11]. Another recent study demonstrated that the laetrile-vinblastine mixture significantly inhibited the proliferation of esophageal cancer, indicating a synergistic interaction between the components [39, 40]. A separate study indicates that the combination of ciprofloxacin and laetrile effectively inhibits the proliferation of esophageal cancer cells. [12]while another exhibited the ability of the linagliptin-metformin Combination to inhibit the Growth of the HeLa cancer Cell Line synergistically [14].

Despite numerous studies on this issue, they have not demonstrated the anticancer effects of the linagliptin-rivaroxaban combination and its capacity to target mutant MAPK-RAS kinase signaling proteins. This study was conducted to address this topic.

This study explored the linagliptin-rivaroxaban mixture's anticancer properties and its molecular anticancer mechanism by screening its ability to target the mutant MAPK-RAS kinase signal proteins.

Materials and Methods

Study medications

Linagliptin and rivaroxaban were sourced from Samarra Pharmaceutical Factory as raw materials. They were diluted in MEM medium to create a concentration ranging from 0.1 to 1,000 µg/ml for each linagliptin and rivaroxaban. The mixture's concentration ranged from 0.05 to 50 µg/ml for each medication, resulting in an overall concentration of 0.1 to 1,000 µg/ml.

Cytotoxicity Assay

This cytotoxicity assay evaluates the anticancer properties of linagliptin, rivaroxaban, cisplatin, and the linagliptin-rivaroxaban combination on HeLa cancer cell lines. Furthermore, mixture cytotoxicity on the NHF cell line was detected to identify the mixture's safety and determine whether any harmful impact of a product may arise from the mixture's drug-pharmaceutical interaction.

We intend to ascertain the drugs' cytotoxic profile by evaluating cell viability in response to concentrations ranging from 0.1 to 100 µg/ml. This will aid in determining if these medications induce cell death, essential for

assessing anticancer efficacy and safety properties.

Cell Lines Used

Hela cell line: The cell line that originates from cervical cancer cells [41, 42].

NHF cell line: The cell line that originates from Normal human-derived adipose tissue [43].

Cell culture conditions

The cell lines were grown in MEM medium (US Biological, USA) as a growth-bolstering supply with 10% (v/v) fetal bovine serum (FBS) (Capricorn-Scientific, Germany) and fortified with 100 IU of penicillin and 100 µg of streptomycin (Capricorn-Scientific, Germany) to avert bacterial contamination. They were incubated in a humidified atmosphere at 37°C. Exponentially proliferating cells were utilized for experimentation [44].

MTT cytotoxicity assay

This colourimetric assay relies on viable cells' capacity to reduce yellow MTT to purple formazan crystals via mitochondrial dehydrogenases. In the MTT assay, cells are typically cultured in a 96-well plate and exposed to varying concentrations of the test compound. Following an incubation period, MTT is introduced to each well and incubated further. Viable cells convert MTT into formazan, which can be solubilized, and its concentration is quantified by measuring the absorbance at a specific wavelength using a spectrophotometer.

The quantity of formazan generated is directly related to the count of viable cells. Following treatment with the test substance, a reduction in formazan formation, and thus a drop in absorbance, signifies cytotoxicity. The half-maximal inhibitory concentration (IC_{50}), the concentration of the test drugs that diminishes cell viability by 50%, can be derived from the dose-response curve [45].

Cells were inoculated at a density of 10,000 cells in a 96-well microplate and incubated at 37°C for 72 hours until monolayer confluence was attained. Cytotoxicity was assessed using the MTT test. The cells were subjected to various concentrations (0.1, 1, 10, 100, and 1000 µg/ml) throughout six wells for each concentration of rivaroxaban, linagliptin, cisplatin, and the mixture. Several untreated wells were left without any treatment, representing a negative control. After 24 and 72 hours of treatment, 28 µL of MTT dye solution (2 mg/ml) was applied to each well. The incubation persisted for three hours. 100 µl of DMSO was administered to each well and incubated for 15 minutes. The optical density was assessed at 570 nm utilizing a microplate reader. The percentage of cytotoxicity was determined using the below equation.

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

OD control is the mean optical density of untreated wells, and OD Sample is the optical density of treated wells [46].

Selective toxicity index

This assay assessed the selective toxicity of the linagliptin-rivaroxaban combination and cisplatin against cancer cells at each incubation period (24 and 72 hours). The selective cytotoxicity index was calculated using the following mathematical equation after determining the IC_{50} levels for the mixture and cisplatin using cell growth curves for both HeLa and NHF cell lines [47].

Selective toxicity Index (SI)=(IC_{50} of normal cell lines)/(IC_{50} of cancer cell lines)

An SI score over 1.0 indicates a drug's enhanced efficacy in targeting tumor cells relative to its toxicity towards normal cells.

Molecular docking

The chemical structures of linagliptin and rivaroxaban were precisely constructed with the ChemDraw program (Cambridge Soft, USA) and enhanced using Chem3D. Based on the outcomes of a pilot study exploring the chemical docking of linagliptin and rivaroxaban with the mutant RAS-MAPK kinase signalling proteins, based on the outcomes of the pilot study, the mutant p38 MAPK kinase and mutant RAS kinase signalling proteins were chosen. The molecular structures of mutant p38 MAPK (PDB: 5o8u) and mutant RAS (PDB: 7l0f) were obtained from the Protein Data Bank.

Protein structures were optimized and modified using AutoDock Tools. AutoDock Tools identified the optimal configuration of the ligands and generated a PDBQT file for them. Following optimization, the structures of the ligands (rivaroxaban and linagliptin) and the human mutant p38 MAPK and RAS kinase proteins were entered into AutoDock-Tools. The docking technique was then performed using the same application. The docking energy scores and binding interactions were evaluated utilizing BIOVIA Discovery Studio, UCSF Chimera, and AutoDock Vina [48, 49].

Drug combinations pattern assessment

Compusyn, a computational simulator, was utilized to determine the combination index (CI) and dose reduction index (DRI) scores. The evaluation of the CI score sought to assess the likelihood of synergistic, additive, or antagonistic interactions among the mixture's components. Concentration-effect curves can demonstrate the percentage of cells displaying reduced growth concerning drug concentration, assessed after 24 and 72 hours of treatment. CI values below 1 suggest synergistic impact, equal to 1 denote additivity, and beyond 1 reflect antagonism.

The DRI score estimation quantifies the extent to which the concentration of each drug in a mixture can be lowered while preserving its cytotoxic efficacy. A DRI exceeding 1 signifies a favorable concentration reduction, while a DRI below 1 denotes an unfavorable concentration reduction.

The combination and dose reduction index values were calculated using Compusyn software (Biosoft, Ferguson, MO, USA) [50, 51].

Ethical approval

This investigation does not include human or animal subjects within its scope.

Statistical Analysis

The cytotoxicity assay outcomes are expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was utilized to determine the variation among research groups. The paired t-test and LSD tests were employed to analyze the differences among separate groups. The study used SPSS version 20 for statistical analysis, setting the significance level at $p < 0.05$ [52].

Results

Cytotoxic study

Initially, we assessed the cytotoxicity of rivaroxaban and linagliptin singularly before evaluating the cytotoxic effects of their combination. This initial evaluation sought to elucidate the mechanisms of cytotoxicity and examine the interactions among the mixture components, specifically assessing whether these interactions demonstrate synergistic, antagonistic, or additive effects.

Linagliptin Cytotoxicity

The finding of Linagliptin cytotoxicity revealed its ability the reduce cervical cancer multiplication in a concentration- and time-dependent style, lessening the IC_{50} from 24 to 72 hours. Supported time-dependent cytotoxicity, Table 1.

Rivaroxaban cytotoxicity

The assessment of cytotoxicity for rivaroxaban revealed antiproliferative properties that varied with changes in incubation duration. With the fluctuation in the growth inhibition across the concentrations Table 2.

Cisplatin cytotoxicity

Cisplatin was selected as a positive control for comparative purposes. The cytotoxicity study indicated that the cytotoxic effects of cisplatin on each cell line were dependent on concentration and duration of exposure. Table 3.

(Linagliptin -Rivaroxaban) mixture cytotoxicity

The study results indicated that the combination of linagliptin and rivaroxaban inhibited the growth of human cervical cancer, with the mode of inhibition primarily influenced by the concentration of the mixture. Moreover, the mixture's cytotoxicity on the NHF cell line was less pronounced than on the cancer cell line, indicating a favourable safety profile and selective toxicity towards cancer cells, Tables (4 and 5).

The comparison of cytotoxicity between the mixture and its ingredients and cisplatin revealed that the mixture exhibited more significant cytotoxicity at higher concentrations. Indicating a synergistic cytotoxic impact between the mixture ingredients. And as the best alternative to traditional chemotherapy, Supplementary Tables (1 and 2).

Table 1. The Influence of Linagliptin on the Survival of Cervical Cancer Cells at 24 and 72 Hours

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD [a])		P- value
	24 hr.	72 hr.	
0.1	D 1.00 ± 1.000	C 12.00 ± 2.000	0.001*
1	CD 8.00 ± 3.000	BC 23.00 ± 3.000	0.004*
10	BC 17.00 ± 2.000	AB 33.00 ± 4.000	0.003*
100	AB 20.00 ± 5.000	A 37.00 ± 1.000	0.004*
1000	A 30.33 ± 3.055	A 44.00 ± 4.000	0.009*
[b] LSD value	11.32	11.04	-
IC_{50}	1861.5 µg/ml	1252.6 µg/ml	-

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

Table 2. The Influence of Rivaroxaban on the Survival of Cervical Cancer Cells at 24 and 72 Hours

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD a)		P- value
	24 hr.	72 hr.	
0.1	D 5.00 ± 3.000	C 17.00 ± 1.000	0.003*
1	CD 9.00 ± 2.000	BC 22.00 ± 2.000	0.001*
10	BC 18.00 ± 3.000	B 32.00 ± 2.000	0.003*
100	A 29.00 ± 1.000	AB 44.00 ± 4.000	0.003*
1000	AB 26.00 ± 4.000	A 39.00 ± 5.000	0.007*
[b] LSD value	10.16	11.5	-
IC_{50}	2024.8 µg/ml	1239.7 µg/ml	-

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

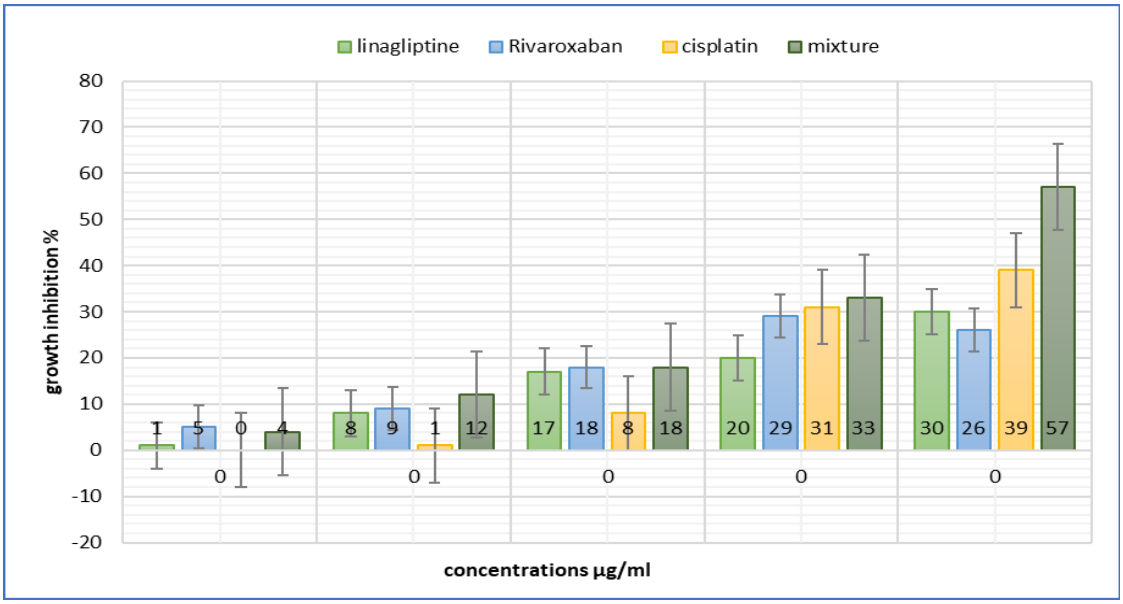

Figure 1. A 24-hour Growth Inhibition Comparison of Linagliptin, Esomeprazole, Cisplatin, and a Mixture

Table 3. The Influence of Cisplatin on the Survival of Hela and NHF Cell Lines at 24 and 72 Hours

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD[a])					
	Hela cell line			NHF cell line		
	24 hr.	72 hr.	P- value	24 hr.	72 hr.	P- value
0.1	D 0.00 ± 0.000	D 4.00 ± 2.000	0.026*	C 3.00 ± 2.000	C 10.00 ± 5.000	0.088
1	D 1.00 ± 1.000	CD 10.00 ± 2.000	0.002*	C 7.00 ± 2.000	C 21.00 ± 1.000	0.000*
10	C 8.00 ± 3.000	C 17.00 ± 1.000	0.012	C 14.00 ± 2.000	B 36.00 ± 3.000	0.000*
100	B 31.00 ± 1.000	B 36.00 ± 2.000	0.018	B 33.00 ± 3.000	A 50.00 ± 5.000	0.007*
1000	A 39.00 ± 1.000	A 52.00 ± 3.000	0.001*	A 47.00 ± 6.000	A 63.00 ± 3.000	0.014
[b] LSD value	5.64	7.28	-	12.28	13.52	-
IC_{50}	1280.8 µg/ml	904.7 µg/ml	-	1037.9 µg/ml	598.4 µg/ml	-

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

Selective toxicity index assessment

The SI score for the linagliptin–rivaroxaban mixture was 5.63 and 5.1 at 24 and 72 hours, respectively, indicating that the mixture possessed a higher selectivity in targeting cancer cells than its impact on healthy cells, in contrast, the SI score for cisplatin was 0.81 and 0.66 at 24 and 72 hours, exhibiting less selectivity in targeting cancer cells than its impact on healthy cells.

Molecular docking studies

A computational molecular docking simulation was utilized to explore the affinity of the mixture ingredients (linagliptin and rivaroxaban) for binding with mutant Ras/(MAPK) signal protein kinase. The finding demonstrated

Table 4. The Influence of Mixture on the Survival of HeLa and NHF Cell Lines at 24 and 72 Hours

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD[a])					
	Hela cell line			NHF cell line		
	24 hr.	72 hr.	P- value	24 hr.	72 hr.	P- value
0.1	D 4.00 ± 3.000	D 10.00 ± 2.000	0.045*	D 0.00 ± 0.000	C 0.00 ± 0.000	N.S
1	CD 12.00 ± 3.000	CD 18.00 ± 3.000	0.045*	CD 2.00 ± 1.000	BC 6.00 ± 1.000	0.008*
10	C 18.00 ± 4.000	BC 27.00 ± 4.000	0.051	BC 7.00 ± 2.000	AB 14.00 ± 4.000	0.053
100	B 33.00 ± 3.000	B 36.00 ± 2.000	0.223	AB 11.00 ± 1.000	A 18.00 ± 5.000	0.076
1000	A 57.00 ± 2.000	A 70.00 ± 3.000	0.003*	A 14.00 ± 3.000	A 22.00 ± 2.000	0.018*
[b] LSD value	10.54	10.14	-	6.3	11.04	-
IC_{50}	805.3 µg/ml	579 µg/ml	-	4541.7 µg/ml	2956.1 µg/ml	-

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

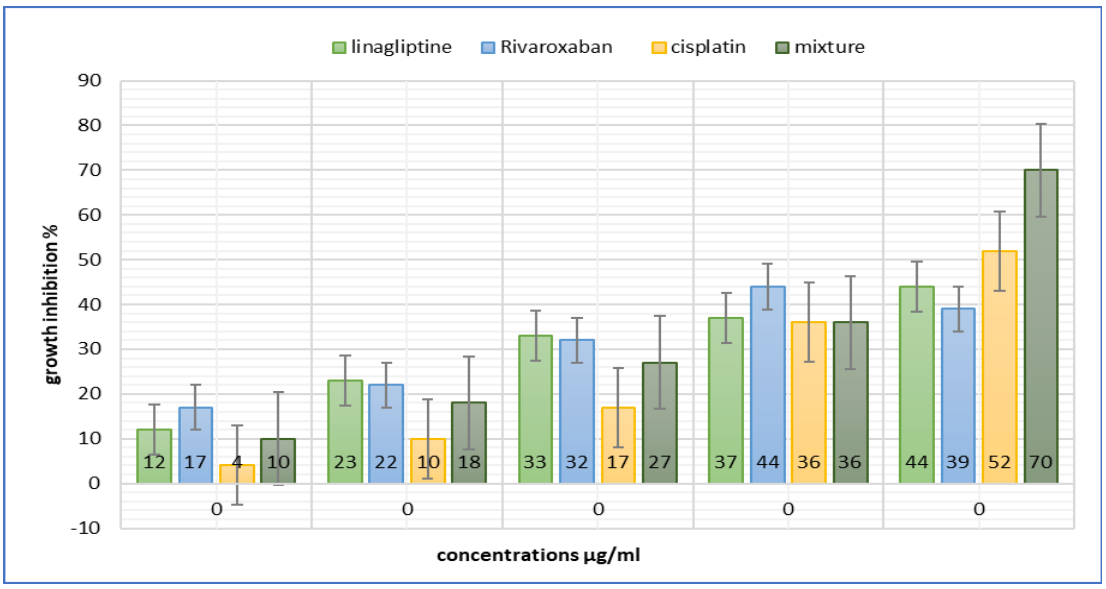

Figure 2. A 72-Hour Growth Inhibition Comparison of Linagliptin, Esomeprazole, Cisplatin, and a Mixture

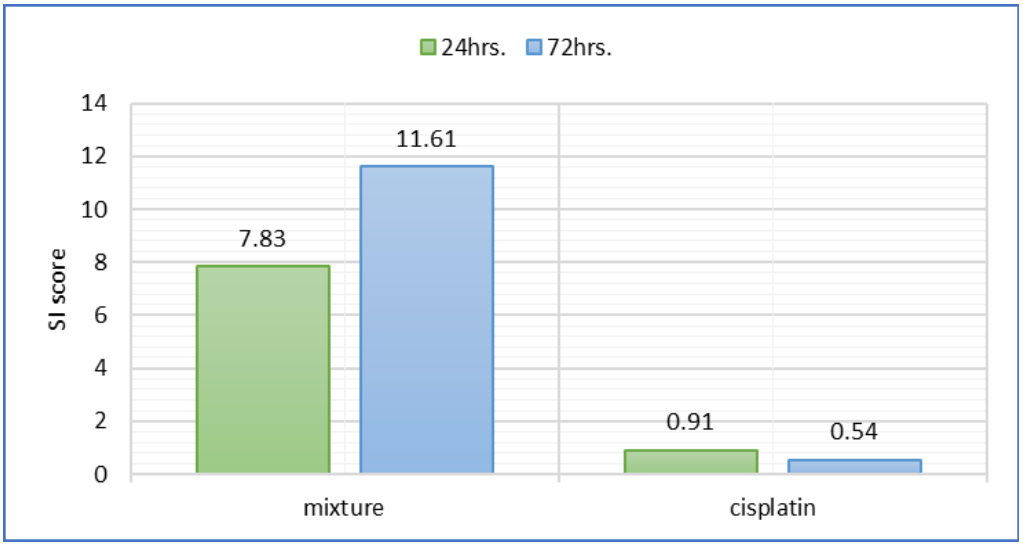

Figure 3. Comparison of Mixture with Cisplatin SI over 24 and 72 hours. (An SI greater than 1.0 indicates a drug's increased effectiveness against tumor cells relative to its toxicity towards normal cells).

that the best interaction of linagliptin was identified with mutant p38 MAPK (PDB code: 5o8u), yielding a docking score equal to (-7.9) kcal/mol. Meanwhile, rivaroxaban showed a higher affinity for interaction with Ras signal

Table 5. Comparing the Impacts of the Mixture on the Growth of the Hela and NHF Cell Lines at the 24-and 72-hour Marks

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD[a])					
	24 hr.			72 hr.		
	Hela cell line	NHF cell line	P- value	Hela cell line	NHF cell line	P- value
0.1	D 4.00 ± 3.000	D 0.00 ± 0.000	0.082	D 10.00 ± 2.000	C 0.00 ± 0.000	0.001*
1	CD 12.00 ± 3.000	CD 2.00 ± 1.000	0001*	CD 18.00 ± 3.000	BC 6.00 ± 1.000	0.003*
10	C 18.00 ± 4.000	BC 7.00 ± 2.000	0.013*	BC 27.00 ± 4.000	AB 14.00 ± 4.000	0.016*
100	B 33.00 ± 3.000	AB 11.00 ± 1.000	0.000*	B 36.00 ± 2.000	A 18.00 ± 5.000	0.004*
1000	A 57.00 ± 2.000	A 14.00 ± 3.000	0.000*	A 70.00 ± 3.000	A 22.00 ± 2.000	0.000*
[b] LSD value	10.54	6.3	-	10.14	11.04	-
IC_{50}	805.3 µg/ml	4541.7 µg/ml	-	579 µg/ml	2956.1 µg/ml	-

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

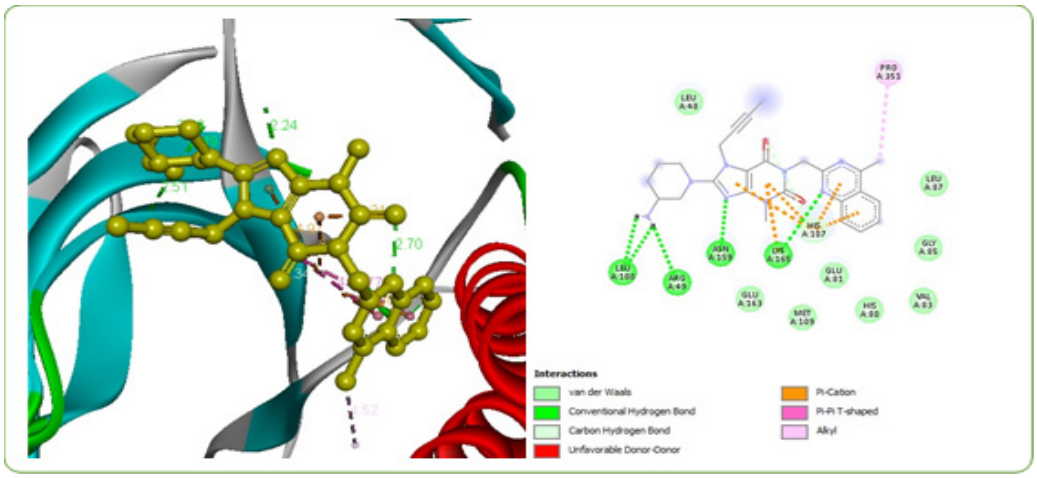

Figure 4. 2D and 2D Structure for Human Mutant *MAPK* Binding Site with Linagliptin

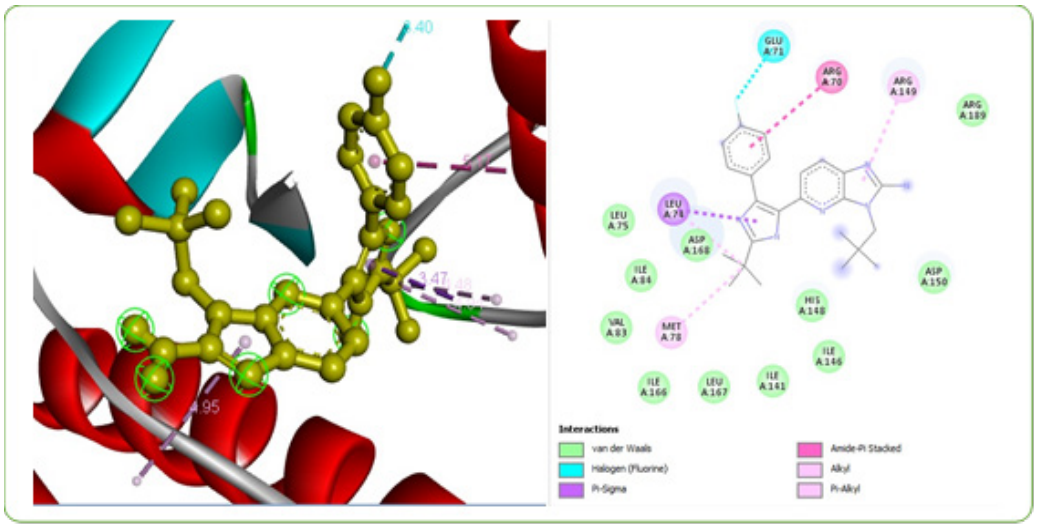

Figure 5. 2D and 2D Structure for Human Mutant *MAPK* Binding Site with Ralimetinib

protein kinase (PDB code: 7l0f), yielding a docking score equal to (-8.7) kcal/mol. The study utilized AutoDock tools version 1.5.7, BIOVIA Discovery Studio UCSF Chimera, and AutoDock Vina [53].

Analysis of molecular docking for the interaction of linagliptin with mutant p38 MAPK was conducted. Five "conventional hydrogen bonds" with the ASN A:159, LYS A:165, ARG A:49, and two LEU A:108 amino acid residues at 2.24 Å, 2.70 Å, 2.51 Å, 2.48 Å, 2.48 Å of distance formed subsequently. One "carbon-hydrogen bond" constricts with HIS A:107 amino acid residues at 3.33 Å of distance. Two "pi-cation bonds" formed with the HIS A:107 amino acid residues at a 4.96 Å and 4.44 Å distance, respectively. Three "pi-cation; pi-donor hydrogen bonds" with LYS A:165 and two HIS A:107 amino acid residues at 2.21 Å, 2.39 Å, and 2.81 Å of distance. Subsequently. Two "pi-pi-T-shaped bonds" with two HIS A:107 amino acid residues at a 4.41 Å and 4.77 Å distance, respectively. Finally, one "alkyl bond" constricts with PRO A:351 amino acid residues at 4.51 Å of distance, Figure 1.

For comparative purposes, a molecular docking study assessed the interaction between Ralimetinib, a " p38 MAPK inhibitor." [54], and mutant p38 MAPK, resulting in a docking score of -8.7 kcal/mol for binding and presenting. One "halogen (fluorine) bond" constricts with GLU A:71 amino acid residues at 3.39 Å of distance. One "pi-sigma bond" constricts with LEU A:74 amino acid residues at 3.46 Å of distance. One "amid-pi stacked bond" constricts with ARG A:70 amino acid residues at a 5.17 Å distance. Two "alkyl bond" constraints with LEU A:74 and MET A:78 amino acid residues at 4.47 Å and 4.94 Å of distance, respectively. Finally, one "pi-alkyl bond" constricts with ARG A:194 amino acid residues at a 4.95 Å distance, Figure 2.

Regarding the other mixture ingredient, "rivaroxaban," Analysis of molecular docking for interaction with mutant RAS yielded one "conventional hydrogen bond" constricted with ASN A:116 amino acid residues at 2.30 Å of distance. Three "carbon-hydrogen bonds" constrict with two GLY A:15, SER A:17, and GLU A:31 amino acid residues at 3.47 Å, 3.52 Å, and 3.32 Å distances, respectively. One "pi-sigma bond" constricted with VAL A:29 amino acid residues at 3.59 Å of distance. One "pi-sulfur bond" constricted with PHE A:28 amino acid residues at 5.56 Å of distance. One "pi-pi-T-shaped

bond" with two PHE A:28 amino acid residues at a 5.07 Å distance. Two "alkyl bonds" with LEU A:120 and LYS A:147 amino acid residues at a 4.67 Å and 4.98 Å distance, respectively. Finally, three "pi-alkyl bound" with ALA A:18, LYS A:117, and LYS A:147 amino acid residues at 5.32 Å, 4.42 Å, and 4.69 Å distance, respectively. Figure 3.

A molecular docking study assessed the interaction between Sotorasib, a RAS inhibitor, for comparative purposes. [55] And mutant RAS, resulting in a docking score of (-6.2) kcal/mol for binding and presenting. Two "conventional hydrogen bonds" constricted with GLY A:60 and GLY A:62 amino acid residues at 2.43 Å, 2.84 Å of distance. One "carbon-hydrogen bond" constricts with GLY A:63 at 3.26 Å of distance. Three "halogen (fluorine) bonds" constrict with two GLU A:62 and GLU A:63 amino acid residues at 3.09 Å, 3.69 Å of distance. And finally, one "pi-alkyl bond" constricts with ALA A:59 amino acid residues at 4.83 Å of distance, Figure 4.

To explain the effectiveness of the mixture's ability to target mutant p38 MAPK and RAS kinase signal proteins, their docking scores were compared with those of standard medications that target MAPK and RAS kinase signal proteins. The comparison showed that the docking score of linagliptin was relatively close to that of Ralimetinib (a standard mutant p38 MAPK inhibitor). In contrast, the docking score of rivaroxaban was higher than that of Sotorasib (a standard RAS kinase inhibitor), Supplementary Table 3, Figure 5 and Supplementary Figures 1, 2.

Identifying the Maximizing Efficacy

The study findings suggested a possible synergistic impact among the mixture components, resulting in the following consequences. Following a 24-hour incubation, the interaction of linagliptin and rivaroxaban demonstrated a Very Strong Synergism at 0.1, 1, 100, and 1,000 µg/ml concentrations. While 10 µg/ml concentrations demonstrate a Strong Synergism Supplementary Table 4 and Supplementary Figure 3.

After 72-hour incubation, the pattern of the linagliptin-rivaroxaban mixture showed a Strong Antagonism, Very Strong Antagonism, Moderate Synergism, Synergism, and Very Strong Synergism at 0.1,1, 10,100, and 1,000 µg/ml concentrations. Subsequently, Supplementary Table 5 and Supplementary Figure 4. The dose reduction index outcomes demonstrated that the concentrations of the drugs in the mixture necessary to induce cytotoxicity were lower than those required when used separately (Supplementary Figure 5). The decline was observed after 24 hours of incubation, except for the lower rivaroxaban concentration. At 72 hours of incubation, a decline was observed at 5.50 and 500 µg/ml concentrations for both rivaroxaban and linagliptin. Supplementary Tables 3 and 4; Figures 3 and 4).

Discussion

Reevaluating existing marketed drugs for cancer therapy represents an effort to identify effective anticancer alternatives. Along with this aspect, the present study focused on exploring the anticancer properties of a mixture of the antidiabetic "linagliptin " with the anticoagulant "rivaroxaban." The selection of these medications is motivated by findings from various previous studies suggesting each one possesses anti-cancer properties. Furthermore, each medication was thoroughly examined for its pharmacokinetics and safety profile.

The results of the MTT cytotoxicity assay indicated that the linagliptin-rivaroxaban combination exhibited an inhibitory effect on cervical cancer proliferation while demonstrating reduced cytotoxicity towards normal cell lines. The combination showed enhanced anticancer efficacy compared to cisplatin cytotoxicity and the mixture of medications. The combination index score indicates that the two drugs act synergistically. Furthermore, from a safety standpoint and regarding the dosage reduction index, the results suggest that the mixture presents a lower likelihood of unwanted effects than its components. The mixture's safety is also corroborated by its advantageous selectivity index score, signifying that it exhibits selective toxicity towards cancer cells relative to healthy cells.

The mixture's anticancer properties can be elucidated via two avenues: first, by examining the proposed anticancer mechanisms of each constituent in several previous studies, and second, by the novel anticancer mechanism proposed by the current molecular docking study.

Several studies have been conducted regarding linagliptin's anticancer properties. Linagliptin showed the ability to minimize the proliferation of human bone cancer cell lines [57]. And inhibits the survival, proliferation, and invasion of Glioblastoma cancer cells via a pattern that primarily depends on incubation periods, suggesting that linagliptin's anticancer impact correlates with its effects on specific cell cycle stages. [58]Furthermore, Linagliptin could activate cell cycle arrest at low doses in the G2/M phase, while at high doses, it arrested both the G2/M and S phases [58]. Another suggested mechanism regarding linagliptin's anticancer properties is supposed to be its ability to interact highly with Cyclin-Dependent Kinase 1 (CDK1), an essential protein in cell cycle control. many substrate proteins were phosphorylated by CDK1, including histones H1, laminin, and Rbis. On the other hand, Linagliptin exhibited a significant antiproliferative impact via selective targeting of Aurora kinase B and CDK1, leading to a decline in the phosphorylation of Rb and a lowering in the production of Bcl-2 2 [58]. Aurora kinase B is an essential kinase for cell division regulation [59].

In contrast, Multiple studies were conducted to evaluate the anticancer properties of rivaroxaban. One study indicated that rivaroxaban could reduce cancer growth invasion in fibrosarcoma mice via A proposed mechanism that enhances immunity against cancer, specifically through increased infiltration of dendritic and cytotoxic T-cells [60]. In another study, Rivaroxaban demonstrates a synergistic effect with immunotherapy, resulting in a greater inhibition of cancer growth compared to the impact of immunotherapy alone. Rivaroxaban activated CD103 + F4/80 - CCR7 + dendritic cells and GrB + cytotoxic CD8+ T cells specifically within the tumor microenvironment [61, 62]. A separate study indicates that rivaroxaban

exhibits an antiangiogenic effect in a dose-dependent manner, with a score of 0.7 observed at a concentration of 10−4 μmol/l [63]. In contrast, A separate study indicated that while the direct oral anticoagulants rivaroxaban and dabigatran effectively inhibited coagulation, they did not impede cancer orthotopic growth and metastasis [64].

In contrast to linagliptin's cytotoxicity pattern, which exhibited a linear dose-response curve, rivaroxaban's curve was non-linear. This biphasic response can be explained through the hormesis and dose-response relationships. At low doses, specific agents may stimulate protective cellular mechanisms, such as DNA repair or antioxidant responses, inhibiting cancer development. Conversely, high doses can overwhelm these mechanisms, causing DNA damage, genomic instability, and carcinogenesis [65].

Furthermore, in addition to the anticancer mechanisms elucidated earlier, the current study examines novel anticancer mechanisms for each linagliptin and rivaroxaban, as demonstrated by the findings of the molecular docking study, suggesting their affinity to target p38 MAPK, RAS kinase signal protein, particularly the mutant types.

The RAS-MAPK signalling pathway is a key regulator of cellular processes, including proliferation, differentiation, survival, and apoptosis. Dysregulation of this pathway is a characteristic feature of cancer [66].

p38 MAPKs regulate stress responses, inflammation, and cell survival, playing roles in cancer by promoting progression, metastasis, and therapy resistance by modulating EMT, the tumor microenvironment, and survival pathways [67, 68]. RAS proteins (KRAS, NRAS, HRAS) are small GTPases that function as molecular switches. Gain-of-function mutations in RAS genes occur in roughly 30% of all human cancers, including pancreatic, colorectal, and lung cancers [69]. Mutations result in the constitutive activation of the MAPK cascade, comprising RAF, MEK, and ERK kinases, thereby promoting unregulated cell growth and tumor progression [69]. For instance, BRAF mutations, especially V600E, are common in melanoma and lead to the hyperactivation of downstream signalling pathways [70]. Targeting components of the RAS-MAPK pathway, including BRAF and MEK inhibitors, has demonstrated potential in treating several cancer types, such as melanoma and non-small cell lung cancer. However, mutation incidence may develop, and Resistance mechanisms diminish the efficacy of these medications [71]. For this reason, the present study focuses on the ability of drugs in the mixture to target the mutant form of MAPK and RAS kinase signal proteins. Combinatorial therapies and novel inhibitors targeting upstream regulators or downstream effectors are being explored to overcome resistance and improve outcomes [72]. Comprehending the complex dynamics of RAS-MAPK signaling is essential for formulating successful cancer therapies and personalized treatment approaches [73].

The significant function of MAPK and RAS kinase signaling proteins in cancer designates them as potential targets for effective cancer therapies. As a result of Targeting, p38 MAPKs show potential in overcoming resistance and inhibiting metastasis. Several trials were performed to identify p38 MAPK inhibitors as doramapimod [74], Talmapimod [75] , Ralimetinib [38] , Neflamapimod [76] Along with this line, several attempts were made to identify an agent, such as Sotorasib, that can target the RAS kinase protein. [34], Adagrasib [35] and GDC-6036 [77]. Despite identifying multiple p38 MAPK and RAS kinase inhibitors, challenges persist in their application, including Toxicity and Side Effects, the emergence of resistance, Limited Efficacy in Certain Cancers, Narrow Spectrum of Activity, and High Cost. [78-81]

The findings from the molecular docking study suggest that the linagliptin-rivaroxaban combination acts through a dual mechanism, particularly by targeting cancer cells via the mutant form of p38 MAPK and RAS kinase signaling protein. This mechanism clarifies the synergistic interaction among the mixture's components. The study has limitations, including laboratory validation concerning the molecular study, due to several factors, such as financial obstacles.

In conclusion, this study aimed to identify an effective and safe anticancer option by focusing on repurposing a marking agent (linagliptin and rivaroxaban) for cancer treatment. MTT assay findings indicate that the linagliptin-rivaroxaban mixture significantly inhibits the growth of cervical cancer compared to cisplatin, linagliptin, and rivaroxaban cytotoxicity. Linagliptin and rivaroxaban act synergistically in the mixture, as indicated by the combination index score, particularly after 24 hours of incubation and at high concentrations following 72 hours of incubation.

From a safety perspective, the concentrations of the drugs in the mixture that induce cytotoxicity were low compared to when used solely, suggesting that the mixture possesses a low propensity for adverse effects. The mixture exhibits a favorable selectivity index, indicating it's selectively targeting cancer cells relative to healthy cells. Moreover, the study explores a novel anticancer mechanism of the mixture, representing a dual targeting of two essential kinase signaling proteins in the MAPK-RAS pathway by the mixture. Via p38 MAPK targeting by linagliptin and RAS kinase targeting by rivaroxaban, with molecular docking scores of -7.9 and -8.7 kcal/mol, respectively. This mechanism clarifies the synesthetic effects resulting from a complementary anticancer mechanism.

Regarding all these findings. Linagliptin-rivaroxaban mixture presents an effective and safer alternative for cervical cancer treatment, particularly concerning their established pharmacokinetic and safety profiles. a further study regarding the in vivo study and clinical study was recommended.

Abbreviations

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

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

MEM: Minimum Essential Medium
SAS: Statistical Analysis System
LSD: Least Significant Difference
DRI: dose reduction index
CI: combination index
Hsp 60: heat shock protein 60
NHF cell line: human-derived adipose tissue cell line
PPIs: proton pump inhibitors
HSB: heat shock protein
MAPK: mitogen-activated protein kinase

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Declaration of Generative AI and AI-assisted technologies in the writing process

The authors clarify that this work does not employ generative AI or AI-assisted technologies.

Conflicts of interest

The authors affirm that there are no conflicts of interest to disclose.

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