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RESEARCH ARTICLE

Ciprofloxacin and Metformin as Dual Therapeutic Agents: Synergistic Impact on Cervical Cancer Cell line Proliferation: Insight into Cytoplasmic SRC Tyrosine Kinase Targeting

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Abstract

Objective: This study aimed to evaluate the combined effects of ciprofloxacin and metformin on inhibiting cervical cancer proliferation and targeting c-src tyrosine kinase. **Methods:** The anticancer properties and selective toxicity of a ciprofloxacin-metformin mixture were assessed using HeLa cervical cancer and human fibroblast cell lines (HFF) over 24- and 72-hour incubation periods. Concentrations ranged from 0.1 to 1000 µg/ml, with ciprofloxacin and metformin each at 50% of their concentration in the mixture. A combination index value was calculated to evaluate the synergism between the two drugs, and computational molecular docking simulations were performed to analyze their binding affinity with c-Src tyrosine kinase. **Results:** The study found that a mixture selectively inhibited cervical cancer cell proliferation based on concentration and treatment duration, with less impact on HFF cell viability compared to cisplatin. A combination index study showed that ciprofloxacin and metformin had a synergistic effect, demonstrating a favorable selectivity for cancer cells over cisplatin. Molecular docking simulations indicated that both ciprofloxacin (-7.9 kcal/mol) and metformin (-5.1 kcal/mol) interacted with c-Src tyrosine kinase at different sites. **Conclusion:** The study outcomes of the MTT assay, molecular docking, combination index, and selectivity index indicate that the ciprofloxacin-metformin combination presents an effective, safer, and promising option for cervical cancer treatment.

Keywords: Ciprofloxacin-metformin mixture- Cervical cancer- HeLa cell line- Molecular docking- Src tyrosine kinase

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Introduction

Annually, almost 500,000 women get a diagnosis of cervical cancer, resulting in more than 300,000 fatalities. Low- and middle-income countries comprise 90% of cervical cancer cases. In the last 30 years, organized screening programs have shown decreased cervical cancer incidence and mortality in high-income countries by around 50%. The severity of the disease influences diagnostic , therapeutic alternatives and local resources. A radical hysterectomy, chemotherapy, or a combination of both may be necessary [1]. Findings from five randomized clinical investigations indicate [2-7], Invasive cervical cancer patients who meet eligibility criteria should receive cisplatin-based chemoradiotherapy in conjunction with radiation therapy. A recent review of 18 trials conducted across 11 countries indicates that combination chemoradiation enhances prognosis. The research demonstrated a 12% enhancement in overall survival rates [8, 9].

Cervical cancer is often treated with chemotherapy, yet its side effects necessitate the exploration of safer alternatives. In conjunction with this problem, Multiple initiatives have been pursued to discover a more effective and safer alternative treatment for cancer, including the use of drugs that are already employed for the treatment of various diseases other than cancer.

Ciprofloxacin is an antibiotic agent that has shown potential anticancer activities in many investigations; one such study found that ciprofloxacin significantly inhibits

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the growth of transitional cell carcinoma cells [10]. A distinct study indicates that Fluoroquinolone antibiotics induce cell death in breast cancer cells, dependent on the dosage and duration of therapy. Cell death can occur through various mechanisms, including the activation of apoptosis, increased expression of p53, Bax, and Bcl-2 proteins, alterations in cell cycle distribution, DNA fragmentation, disruption of mitochondrial function via the Bax/Bcl-2 pathway, S-phase cell cycle arrest, and inhibition of topoisomerase II. Moreover, studies demonstrate that oligonucleosomal DNA fragmentation is associated with increased p53 expression [11, 12]. Additional studies have implicated that Ciprofloxacin inhibits the proliferation of hepatocellular carcinoma cell lines by inducing DNA breaks and obstructing topoisomerases. When co-administered with cisplatin, it has a synergistic effect [13, 14].

Metformin, a medication often prescribed for diabetes, has shown effectiveness in lowering the risk of various cancers, including pancreatic cancer [15, 16]. The latest study shows that metformin significantly lowers the risk of colon cancer and its mortality rate [17, 18]. Metformin significantly reduces the occurrence of adenomas and polyps in patients undergoing polypectomy [19]. Another study suggests that metformin decreases the risk of developing prostate and liver cancer, as well as lowering mortality rates associated with these cancers [20-24].

Multiple mechanisms have been studied to explore metformin's anticancer effects. Metformin activates AMPK in rat hepatoma cells, reducing the phosphorylation of pS6 [25]. A study conducted in vitro showed that metformin directly inhibited AMP deaminase, resulting in increased AMP levels and subsequent activation of AMPK [26]. A recent study suggests that compounds inhibiting mitochondrial complex I within the respiratory system can increase AMP concentrations, which in turn trigger the activation of AMPK. This activation plays a crucial role in inhibiting mTOR, thereby initiating signaling pathways that promote cellular growth [27, 28]. Metformin may help remove active K-ras from the cellular membrane through a mechanism dependent on protein kinase C (PKC). However, there is no empirical evidence of a direct interaction between metformin and K-ras. Research shows that metformin interacts with and disassembles the PP2A complex in neuronal cells. Furthermore, there is a potential for inhibiting the activity of the PP2A-dependent phosphatase [29].

Numerous recent studies have focused on specific molecular pathways in cancer, particularly the c-src tyrosine kinase, which plays a significant role in cancer incidence due to its involvement in various signalling cascades [30]. Src engages with distinct protein-tyrosine kinase receptors situated within the plasma membrane. This engagement enables a reciprocal transfer of signals, whereby the receptors can modulate the activity of Src, while Src simultaneously influences the receptors' behavior. Of particular significance, Src binds with EGFR (ErbB1) and ErbB2, both of which are crucial protein-tyrosine kinase receptors. Alterations in EGFR are frequently observed in cases of non-small cell lung carcinoma, whereas the overexpression of ErbB2 is associated with breast carcinoma. Additionally, the ErbB family is implicated in various other malignancies, including colorectal, gastric, head and neck, and pancreatic cancers [31, 32]. Furthermore, Src tyrosine kinase interacts with c-MET, also known as the hepatocyte growth factor receptor (HGFR). This receptor, which is a protein-tyrosine kinase, is essential in numerous biological processes, such as embryonic development, wound healing, and cellular functions migration [33, 34]. HGF is synthesized by mesenchymal cells, whereas c-Met is produced by epithelial cells. The dysregulation of c-Met has been associated with various cancers, including those of the bladder, brain, breast, kidney, liver, pancreas, prostate, stomach, and non-small cell lung. Numerous human malignancies exhibit abnormal activation of this pathway, attributed to factors such as overexpression of proteins, mutations, gene amplification, and increased receptor-ligand interactions. Additionally, mutations within the c-MET gene have been detected in the tyrosine kinase domain [35, 36], Juxta membrane domain [37], and extracellular domain [38, 39] of diverse solid tumors, encompassing both hereditary and sporadic human papillary renal carcinomas, lung cancer, ovarian cancer, childhood hepatocellular carcinomas, head and neck squamous cell carcinoma, and gastric cancer [33, 40-42]. The overexpression of c-Met is linked to increased invasion, migration, and metastasis disease [43, 44]. Another type of protein tyrosine kinase receptor that is regulated by c-src tyrosine kinase is Platelet-derived growth factor (PDGF). PDGF signaling plays a role in cellular division, proliferation, migration, survival, and angiogenesis. Platelet-derived growth factor (PDGF) transmits signals through its receptor protein-tyrosine kinases, known as PDGFRα and PDGFRβ. The activity of the PDGFR is linked to several types of cancer, including breast, colorectal, and prostate cancers, as well as gastrointestinal stromal tumors (GISTs), glioblastoma, osteosarcoma, non-small cell lung cancer (NSCLC), and neuroblastoma. Point mutations in the PDGFRα gene are found in approximately 5% of human gastrointestinal stromal tumors (GISTs).

In about 5 to 10% of glioblastoma cases, there is an observed increase in the expression of this gene. The PDGFRα gene amplification has been found in oligodendrogliomas, esophageal squamous cell carcinomas, and sarcomas of the arterial intima. The activation of both PDGFRα and PDGFRβ plays a vital role in enabling cellular invasion and metastasis. Additionally, insulin-like growth factors such as IGF-1 and IGF-2 are involved in various biological processes, including cell division, growth, survival, angiogenesis, wound healing, and embryonic development [45, 46].

Regarding the crucial role of c-src tyrosine kinase in cancer, multiple efforts will be made to target it to establish effective and selective cancer therapies. In this aspect, several agents will develop .as. Dasatinib [47], Saracatinib [48], Bosutinib [49], and KX2-391 [50]. Even though these selective anticancer drugs are available, there are still some limitations in their use, including

financial constraints and serious adverse effects, such as myelosuppression. Cardiovascular complications include QT interval prolongation and arrhythmias, in addition to bleeding complications and Liver toxicity [51-54].

Mixing current drugs that are utilized for non-cancer therapeutic purposes offers a viable approach to developing an effective and safer cancer therapy. Numerous studies explore this subject, with one showing that the amalgamation of amygdalin and esomeprazole successfully eliminates cervical cancer cells. The efficacy of this combination was contingent upon the medicine concentration and the incubation length [55, 56]. The evaluation demonstrated that the combination of ciprofloxacin and laetrile effectively inhibits the proliferation of esophageal cancer cells. [57]. A separate study demonstrated that esomeprazole-amygdaline inhibits the proliferation of the HeLa cancer cell line in a concentration- and time-dependent manner. [58] Numerous studies have investigated the anticancer properties of ciprofloxacin and metformin, but most have focused on each drug individually. The existing literature often fails to comprehensively demonstrate the effectiveness of combining these two agents in targeting c-src tyrosine kinase specifically in cervical cancer. Notably, these identified limitations highlight a significant gap in current research, emphasizing the need for further investigation to clarify the mechanisms and potential therapeutic benefits of this combination approach. To address this gap, the present study aims to systematically examine the inhibitory effects of the ciprofloxacin and metformin combination on the proliferation of cervical cancer cells, while simultaneously assessing their effectiveness in modulating c-src tyrosine kinase activity.

Materials and Methods

Study medications

The Samarra Pharmaceutical Factory supplied the study medications as raw materials. The drugs were diluted with RPMI medium to produce concentrations ranging from 0.1 µg/ml to 1000 µg/ml. For each drug in the mixture, the concentrations of ciprofloxacin and metformin fluctuated between 0.05 and 50 µg/ml, resulting in a final concentration from 0.1 to 1000 µg/ml.

Cell lines

The tissue culture section at ICCMGR produced the HeLa and HFF cell lines from malignant cervical cancer and human fibroblasts, respectively. Cells were cultured in 75 cm² flasks at 37°C with a 5% CO_2 concentration. Ten percent fetal bovine serum (FBS), and to combat bacterial contamination, one hundred units per milliliter of penicillin-streptomycin and other incubation factors were used to grow the cells [59, 60].

Cytotoxicity assay

Ciprofloxacin, metformin, cisplatin, and the combination of ciprofloxacin and metformin were thoroughly evaluated for their effectiveness in inhibiting the proliferation of cervical cancer cells and their cytotoxic effects on human fibroblast cells. The experiments were conducted by culturing these cell lines in 96-well microtiter plates. During the logarithmic growth phase, cancer cell proliferation showed a steady and gradual increase. The treatments' cytotoxic effects were examined over two incubation periods: 24 and 72 hours [61].

Ten percent fetal bovine serum is required to inoculate 10,000 cells in each well. The plates were incubated at 37°C for 24 hours to facilitate cell attachment. Subsequently, serial dilutions of ciprofloxacin, metformin, and cisplatin were prepared in RPMI medium free of calf serum. Dilutions from 0.1 to 1000 µg/ml were created for each compound treatment [57, 62].

After 24 hours of cancer cell proliferation, each treatment concentration was assigned to six wells, with each well receiving 200 µl of RPMI media containing the medication. Control wells received 200 µl of maintenance media, and the exposure durations varied from 24 to 72 hours. After treatment, the plates were securely attached with self-adhesive material and reinserted into the incubator. Subsequently, MTT dye was employed to stain the treated cells.

(ELISA reader) was employed to determine the optical density of the microtiter plate wells at a 550 nm transmission wavelength [63, 64].

The following mathematical equation is utilized to determine the growth inhibition rate [64].

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

IC_{50} values for Ciprofloxacin, metformin, cisplatin, and (Ciprofloxacin- metformin) combination were determined for each incubation duration using GraphPad Prism, version 9.5.0 (2022).

Selective toxicity index

This assay was conducted to examine the selective toxicity of the ciprofloxacin-metformin combination and cisplatin on cancer cells over two incubation periods: 24 hours and 72 hours. The selective cytotoxicity index was computed using the specified formula, following the estimation of the combination's IC_{50} level through cell proliferation curves for both HeLa and HFF cells lines [65]. Cisplatin's selective toxicity was utilized for comparison purposes.

Selective toxicity Index (SI)=(IC_{50} of normal cell lines)/(IC_{50} of cancer cell lines)×100

An SI greater than 1.0 suggests a drug exhibits higher efficacy against tumor cells than its toxicity towards normal cells.

Study of drug combinations

A study was performed to investigate the collective impact of multiple drugs. This evaluation involved generating concentration-effect curves that illustrated the proportion of cells with decreased growth corresponding to drug concentration following 24 and 72 hours of treatment. The drug interactions were analyzed for synergy, additive effects, and antagonism through Compusyn software

(Biosoft, Ferguson, MO, USA), which calculated the combination index and dose reduction index values.

Values of the confidence interval (CI) that are below 1 indicate synergy; values above 1 imply additivity; and those exceeding 1 denote antagonism. The dose reduction index (DRI) assesses the extent to which the concentration of individual components in a mixture can be decreased while still achieving effectiveness similar to that obtained through the independent use of each drug. A DRI score of 1 means that a dose reduction is not favourable. If the DRI exceeds 1, this indicates a beneficial dose reduction, while a DRI below 1 reflects an unfavorable decrease in dosage [66, 67].

Molecular docking

ChemDraw software (Cambridge Soft, USA) was employed to illustrate the chemical structures of ciprofloxacin and metformin, which were then refined with the Chem3D version. Protein Data Bank was referenced to get the molecular structure of Cytoplasmic SRC Tyrosine Kinase (PDB: 1fmk).

Protein structures were optimized and adjusted utilizing AutoDock Tools. The optimal conformation of the ligands was established using AutoDock Tools, subsequently generating a PDBQT file for the ligands.

Following optimization, the structures of each ligand (ciprofloxacin and metformin) and receptor (Cytoplasmic SRC Tyrosine Kinase) were included in AutoDock-Tools. The docking procedure was performed using the same program. The docking energy scores and binding interactions were analyzed using PLIP and BIOVIA Discovery Studio [68, 69].

Ethical approval

This research strictly used in vitro cell line models, avoiding the involvement of human subjects or laboratory animals. All methodologies followed the ethical standards set by the institution for laboratory studies.

Statistical Analysis

The MTT assay results are presented as the mean ± standard deviation (SD) based on six replicates. A one-way analysis of variance (ANOVA) was utilized. The Tukey and LSD tests were employed to compare various groups. The study used statistical software version 20, setting a significance threshold at $p < 0.05$ [70].

The study employed both uppercase and lowercase letters in its data tables to distinguish various statistical groups and significance levels. Means (averages) represented by the same letters indicate no significant difference, while those with different letters signal statistical significance. Uppercase letters are used for comparisons of row means, while lowercase letters are for column means. This method effectively conveys complex statistical results clearly and accessibly, removing the need for lengthy explanations. As a result, readers can quickly identify which groups are similar or different according to the assigned letters.

Results

Cytotoxicity assay

Ciprofloxacin cytotoxicity

The study's findings demonstrated that ciprofloxacin significantly inhibits the proliferation of cervical cancer cells. The inhibition pattern significantly depended on drug concentration, as evidenced by the varying growth inhibition observed at higher and lower concentrations. The inhibition pattern significantly depended on the incubation duration, as evidenced by the decrease in IC_{50} from the 24-hour to the 72-hour incubation period. The results of ciprofloxacin growth inhibition patterns were influenced by both concentration and incubation time: Table 1 and Figure 1.

Metformin cytotoxicity

The study reveals that metformin inhibits the growth of cervical cancer cells. The inhibition effect shows a strong dependence on concentration, highlighted by the varied growth inhibition across all concentrations during each incubation period. Furthermore, the inhibition pattern changes with the duration of incubation, as shown by the differences in IC 50 values between 72-hour and 24-hour incubations: Table 2, Figure 2.

Cisplatin cytotoxicity

The cytotoxic effects of cisplatin on the HeLa cancer cell line indicate that higher drug concentrations and longer incubation times correlate with increased inhibition rates. Growth inhibition varies significantly across concentrations during each incubation period. The results

Table 1. The Impact of Ciprofloxacin on the Survival of HeLa Cancer Cells after 24 and 72 hours

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD [a])		P- value
	24 hr.	72 hr.	
0.1	C 0.00 ± 0.000	C 1.00 ± 1.000	0.158
1	C 0.00 ± 0.000	C 6.00 ± 2.000	0.007*
10	C 4.00 ± 2.000	B 24.00 ± 3.000	0.001*
100	B 21.00 ± 1.000	A 37.00 ± 4.000	0.003*
1000	A 34.00 ± 4.000	A 43.00 ± 3.000	0.036*
[b] LSD value	7.46	10.16	-
IC 50	1486.9 µg/ml	1174.2 µg/ml	-

*, significant at (P<0.05)

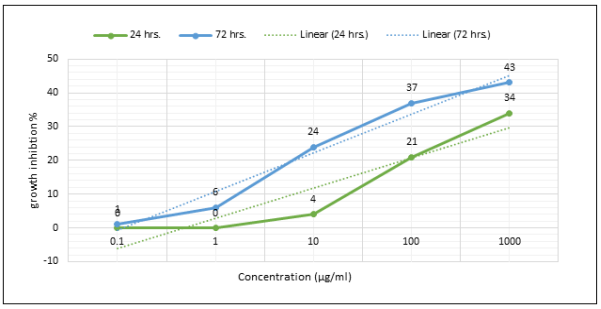

Figure 1. The Influence of Ciprofloxacin on the Viability of HeLa Cancer Cells at 24 and 72 hours

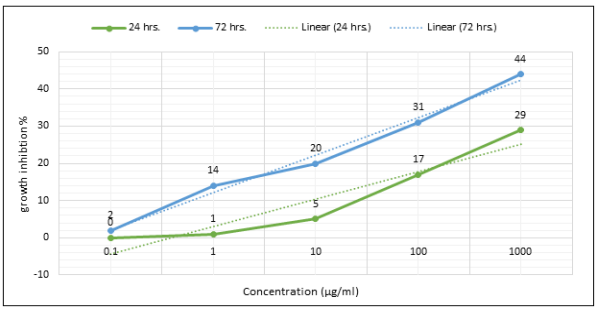

Figure 2. The Influence of Metformin on the Viability of HeLa Cancer Cells at 24 and 72 hours

revealed a notable difference in growth inhibition across various incubation periods for all concentrations, as evidenced by a decrease in the IC_{50} value at 72 hours compared to the 24-hour incubation period.

In contrast, the findings revealed that cisplatin affected the HFF cell line, with growth inhibition patterns depending on concentration and incubation duration. The percentage of growth inhibition is significantly comparable to its cytotoxicity against the HeLa cell line (Table 3, Figure 3).

(ciprofloxacin -metformin) combination cytotoxicity

The study revealed that combining ciprofloxacin with metformin led to a significant reduction in the proliferation of cervical cancer cells. The concentration of the drugs significantly affected the inhibition pattern, as shown by the variations in growth inhibition observed at higher concentrations compared to lower concentrations. The incubation duration significantly influenced the inhibition pattern, as indicated by the marked differences in growth inhibition at all concentrations between the two incubation periods. The decrease in IC_{50} observed during the 72-hour incubation compared to the 24-hour incubation further supports this finding. The pattern of growth inhibition resulting from the ciprofloxacin-metformin combination is affected by concentration and incubation duration (Table 4) (Figure 4).

The combination of ciprofloxacin and metformin demonstrates low cytotoxicity on normal cells (HFF cell line) compared to its impact on cervical cancer cells. The comparison results indicated a significant difference in growth inhibition across all concentrations between the mixture's impact on the HeLa and HFF cell lines (Table 5) (Figure 5).

Additionally, a comparison of the cytotoxic effects of the mixture with ciprofloxacin, metformin, and cisplatin demonstrated that the mixture's cytotoxicity was more significant across all incubation periods. (Table 6, 7) (Figure 6, 7, 13) .

Table 2. Metformin's effect on the Viability of HeLa Cancer Cells after 24 and 72 hours

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD [a])		P- value
	24 hr.	72 hr.	
0.1	C 0.00 ± 0.000	D 2.00 ± 2.000	0.158
1	C 1.00 ± 1.000	C 14.00 ± 1.000	0.0001*
10	BC 5.00 ± 2.000	C 20.00 ± 3.000	0.002*
100	B 17.00 ± 4.000	B 30.33 ± 3.055	0.010*
1000	A 29.00 ± 7.000	A 44.00 ± 2.000	0.023*
[b] LSD value	13.62	8.5	
IC 50	1793.2 µg/ml	1158 µg/ml	

*, significant at (P<0.05)

Table 3. Cisplatin's impact on HeLa and HFF Cell Line Proliferation at 24 and 72 hours.

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD[a])					
	HeLa cell line			HFF cell line		
	24 hr.	72 hr.	P- value	24 hr.	72 hr.	P- value
0.1	D 1.00 ± 1.000	D 4.00 ± 2.000	0.081	C 0.00 ± 0.000	D 7.00 ± 2.000	0.004*
1	CD 3.00 ± 2.000	D 11.00 ± 1.000	0.003*	C 1.00 ± 1.000	D 13.00 ± 3.000	0.003*
10	C 8.00 ± 1.000	C 23.00 ± 3.000	0.001*	C 7.00 ± 1.000	C 37.00 ± 3.000	0.000*
100	B 25.00 ± 3.000	B 44.00 ± 4.000	0.003*	B 29.00 ± 2.000	B 57.00 ± 2.000	0.000*
1000	A 36.00 ± 3.000	A 67.00 ± 1.000	0.0001*	A 47.00 ± 5.000	A 75.00 ± 5.000	0.002*
[b] LSD value	7.98	9.06	-	9.06	11.62	-
IC 50	1420.4 µg/ml	622.1 µg/ml	-	1032 µg/ml	460.5 µg/ml	-

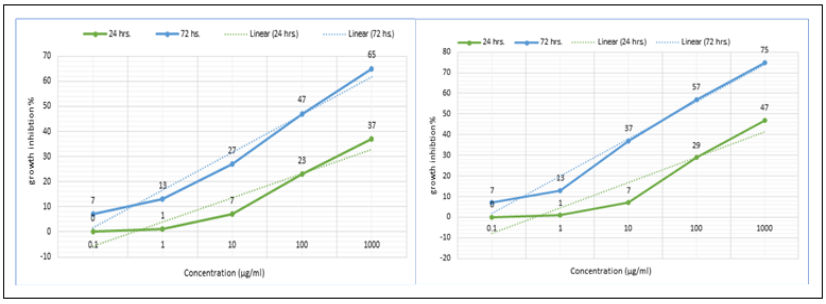

Figure 3. Cisplatin Impact on HeLa (left) and HFF (right) Cell Line Proliferation at 24 and 72 hours

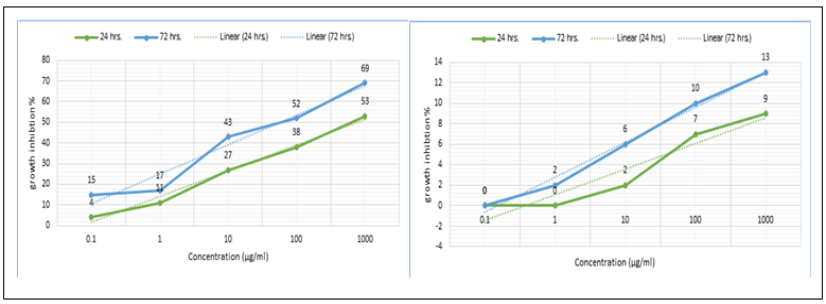

Figure 4. The Influence of Ciprofloxacin-metformin Combination on the Viability of the HeLa cell line (left) and HFF Cell Line (right) at 24 and 72 hours

Studying drug combinations

The study investigating the effects of ciprofloxacin and metformin combinations yielded the following findings. Following incubation periods of 24 and 72 hours, the mixture of ciprofloxacin and metformin at concentrations of 0.1, 1, and 10 µg/ml exhibited very strong synergistic anticancer properties. In contrast, a concentration of 100 µg/ml displayed strong synergy.

A concentration of 1000 µg/ml of the mixture displayed synergism and strong synergism during the 24- and 72-hour incubations, respectively.

The findings of the dose reduction index revealed that the concentrations of the mixture's ingredients necessary to induce cytotoxicity decreased at both 24 and 72 hours of incubation across all concentrations of ciprofloxacin and metformin. This indicates a positive reduction in the effective concentration of the mixture components (Table 8, 9) (Figure 8, 9).

Selective toxicity index

The study findings demonstrated that the selective toxicity index score of the ciprofloxacin-metformin combination was 7.36 and 10 for 24 and 72 hours, respectively. Suggests selectively targeting cervical cancer cells over normal healthy cells, with an increase in the selectivity index corresponding to longer incubation times. In contrast, Cisplatin's selective toxicity index score was 0.72 and 0.74 for 24 and 72 hours, respectively, indicating the lowered selectivity toxicity (Table 10) (Figure 10).

Molecular docking studies

Molecular docking modelling investigated the interaction between ciprofloxacin and metformin with c-Src tyrosine kinase (PDB code: 1fmk). The study utilized AutoDock tools version 1.5.7 and BIOVIA Discovery Studio [71].

The results of our molecular docking studies indicated that the docking score of ciprofloxacin with c-Src tyrosine kinase was (-7.9) kcal/mol. Molecular docking analysis

Table 4. The Influence of Ciprofloxacin-metformin Combination on the Viability of HeLa Cancer Cells at 24 and 72 hours

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD[a])					
	HeLa cell line			HFF cell line		
	24 hr.	72 hr.	P- value	24 hr.	72 hr.	P- value
0.1	C 4.00 ± 2.000	C 15.00 ± 5.000	0.024*	B 0.00 ± 0.000	D 0.00 ± 0.000	N.S
1	C 11.00 ± 3.000	C 17.00 ± 4.000	0.106	B 0.00 ± 0.000	CD 2.00 ± 1.000	0.26
10	B 27.00 ± 4.000	B 43.00 ± 3.000	0.005*	A 2.00 ± 2.000	BDC 6.00 ± 2.000	0.07
100	B 38.00 ± 3.000	B 52.00 ± 2.000	0.003*	A 7.00 ± 2.000	AB 10.00 ± 2.000	0.14
1000	A 53.00 ± 3.000	A 69.00 ± 1.000	0.001*	A 9.00 ± 4.000	A 13.00 ± 3.000	0.238
[b] LSD value	11.16	12.06	-	7.98	6.9	-
IC 50	870.2 µg/ml	487.8 µg/ml	-	6408.7 µg/ml	4881.7 µg/ml	-

*, significant at (P<0.05)

Table 5. Comparison of the Growth Inhibition of Ciprofloxacin-metformin Combination between HeLa and HFF Cell Lines.

Concentration (µg/ml)	Inhibition of cellular proliferation (mean ± SD[a])					
	24 hrs.			72 hrs.		
	Hela	HFF	P- value	Hela	HFF	P- value
0.1	C 4.00 ± 2.000	B 0.00 ± 0.000	0.026*	C 15.00 ± 5.000	D 0.00 ± 0.000	0.007*
1	C 11.00 ± 3.000	B 0.00 ± 0.000	0.003*	C 17.00 ± 4.000	CD 2.00 ± 1.000	0.003*
10	B 27.00 ± 4.000	A 2.00 ± 2.000	0.001*	B 43.00 ± 3.000	BDC 6.00 ± 2.000	0.0001*
100	B 38.00 ± 3.000	A 7.00 ± 2.000	0.0001*	B 52.00 ± 2.000	AB 10.00 ± 2.000	0.0001*
1000	A 53.00 ± 3.000	A 9.00 ± 4.000	0.0001*	A 69.00 ± 1.000	A 13.00 ± 3.000	0.0001*
[b] LSD value	11.16	7.98	-	12.06	6.9	-
IC 50	870.2 µg/ml	6408.7 µg/ml	-	487.8 µg/ml	4881.7 µg/ml	-

*, significant at (P<0.05)

Table 6. A 24-hour Growth Inhibition Comparison of Ciprofloxacin, Metformin, Cisplatin, and a Mix.

Concentration (µg/ml)	Growth inhibition (mean ± SD [a])				[b] LSD value
	Ciprofloxacin	metformin	mix	Cisplatin	
0.1	C 0.00 ± 0.000 [a]	C 0.00 ± 0.000 [a]	C 4.00 ± 2.000 [a]	D 1.00 ± 1.000 [a]	N. S
1	C 0.00 ± 0.000 [b]	C 1.00 ± 1.000 [b]	C 11.00 ± 3.000 [a]	CD 3.00 ± 2.000 [b]	7.04
10	C 4.00 ± 2.000 [b]	BC 5.00 ± 2.000 [b]	B 27.00 ± 4.000 [a]	C 8.00 ± 1.000 [b]	9.42
100	B 21.00 ± 1.000 [b]	B 17.00 ± 4.000 [b]	B 38.00 ± 3.000 [a]	B 25.00 ± 3.000 [b]	11.14
1000	A 34.00 ± 4.000 [b]	A 29.00 ± 7.000 [b]	A 53.00 ± 3.000 [a]	A 36.00 ± 3.000 [ab]	17.16
[b] LSD value	7.46	13.62	11.16	7.98	
IC 50	1486.9 µg/ml	1793.2 µg/ml	870.2 µg/ml	1420.4 µg/ml	

significant at (P<0.05)

was presented.

Two carbon-hydrogen bonds were set up with the ASP A:404 a.a. residue at 3.64 Å distance and LEU A:273 a.a. residues at 3.50 Å distance. Two halogen (fluorine) bonds set up with the MET A:341 a.a residues at 3.21 Å distance and SER A:342 a.a residues at 3.45 Å distance. Three pi-sigma bonds were set up with the LEU A:273 a.a residues at 3.45 Å distance, VAL A:281 a.a residues at 3.74 Å distance, and LEU A:393 a.a residues at 3.56 Å distance. Two pi-alkyl bonds set up with the LEU A:273 a.a residues at 4.98 Å distance and LEU A:303 a.a residues at 5.29 Å distance. Finally, one alkyl bond was set up with VAL A:281 a.a residues at 3.99 Å distances (Figure 10).

Furthermore, molecular docking study data of metformin with c-Src tyrosine kinase revealed a total docking score of (-5.1) kcal/mol. Molecular docking analysis was presented. Six conventional hydrogen-bound sets up with two GLU A: 146, two TYR A: 149, one GLN A: 144, and one GLU A: 147, a.a. residues at 2.86 Å, 2.29 Å, 2.51 Å,2.18 Å,2.42 Å, and 2.34 Å of distance, respectively. One carbon-hydrogen bound with LEU A:89 a.a. residue at 3.43 Å distances (Figure 11).

For comparison purposes, molecular docking study data of bosutinib (c-Src tyrosine kinase inhibitor) revealed a total docking score of (-8.1) kcal/mol. It formed one Conventional hydrogen bond with the LYS A:401 a.a. residues at 2.04 Å. Seven carbon hydrogen bonds with two TYR A: 149, one ARG A: 160, one TYR A: 90, one SER A: 248, PHE A: 150, and LEU A: 248, a.a residues at 3.69 Å, 3.73 Å, 3.35 Å,3.37 Å,3.31 Å, 3.33 Å and 3.65 Å of distance, respectively. One carbon-hydrogen bond with GLY A:135 a.a residues at 2.77 Å of distance. Two pi-Anion bonds with two GLU A:320 a.a residues at 4.05 Å and 3.93 Å of distance. One alkyl bond with MET A:341 a.a residues at 5.28 Å distance. Finally, with three pi-alkyl bonds with ILE A:153, LEU A:161, and

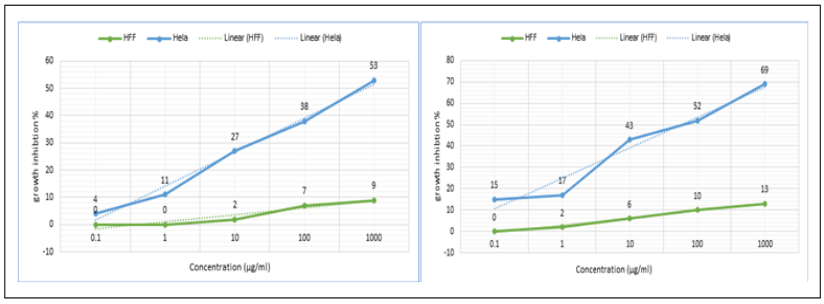

Figure 5. Comparison of the 72-hour Growth Inhibition of Ciprofloxacin-metformin Combination between HeLa and HFF Cell Lines.

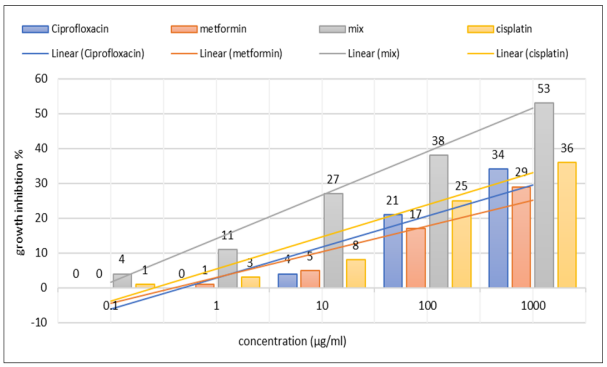

Figure 6. A 24-hour Growth Inhibition Comparison of Ciprofloxacin, Metformin, Cisplatin, and a Mix.

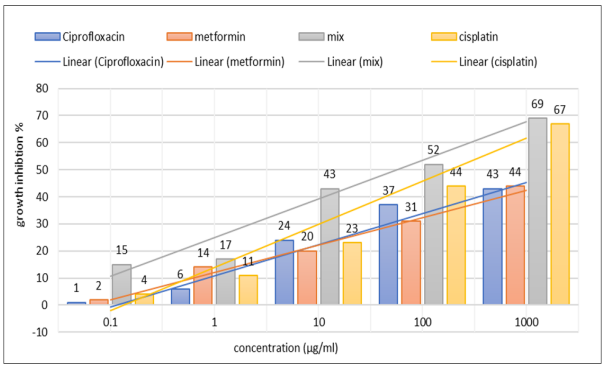

Figure 7. A 72-hour Growth Inhibition Comparison of Ciprofloxacin, Metformin, Cisplatin, and a Mix.

VAL A:399 a.a. residues at 5.11 Å, 5.22 Å, and 5.16 Å of distance, respectively (Figure 12) (Table 11).

The binding site outcomes for both ciprofloxacin and metformin were diverse, indicating that the mixture has a higher docking score than its components due to its complementary targeting ability. These results clarify the mixture's synergistic cytotoxic effect (Figure 13).

Discussion

This study examines the synergistic anticancer effects of the ciprofloxacin-metformin combination on cervical cancer cell survival and investigates its capacity to target cytoplasmic sarcoma tyrosine kinase. The study results revealed that combining ciprofloxacin and metformin inhibits the growth of cervical cancer cells in a manner that depends on both concentration and time, demonstrating effects that are both cell cycle-specific and cell cycle-nonspecific. Additionally, the combination index findings showed that the mixture displayed synergistic activity at all concentrations and incubation periods. The finding of the dosage reduction index indicated a favourable lowering in the effective cytotoxic concentration of the mixture medications, signifying an increase in the safety of the combination and a reduction in the occurrence of side effects.

The docking study results demonstrated the ability of each mixture component to interact with c-Src tyrosine kinase to differing degrees and locations, providing insights into the combination's anticancer mechanism. However, based on the cytotoxicity results on the HFF normal cell line, we proposed a mixture that selectively targets cancer cells, given that both cancerous and healthy cells express c-Src tyrosine kinase. Nonetheless, the cytotoxicity of the mix was predominantly observed in cancer cells relative to normal cells.

Our study's findings of ciprofloxacin cytotoxicity align with previous research; one investigation indicated that ciprofloxacin markedly suppresses the proliferation of transitional cell carcinoma cells [10]. Another study suggests that Fluoroquinolone antibiotics induce cell death in breast cancer cells, contingent upon the treatment dose and duration. Cell death can transpire through multiple mechanisms, such as the initiation of apoptosis, heightened expression of p53, Bax, and Bcl-2 proteins, modifications in cell cycle distribution, DNA fragmentation, impairment of mitochondrial function via the Bax/Bcl-2 pathway, S-phase cell cycle arrest, and suppression of topoisomerase II. Furthermore, research indicates that oligonucleosomal DNA fragmentation correlates with elevated p53 expression [11, 12]. Ciprofloxacin may impede the growth of hepatocellular cancer cell lines by generating DNA breaks and blocking topoisomerases. When administered in conjunction with cisplatin, it has a synergistic impact [13].

Conversely, Metformin, a medication often recommended for diabetes, has been shown to effectively reduce the occurrence of several malignancies, including pancreatic cancer [15, 16]. Recent research has shown that metformin significantly decreases the risk of colon cancer development and the related death rate [17, 18]. Metformin has shown effectiveness in reducing the development of adenomas and polyps in individuals undergoing polypectomy [19]. It reduces the mortality

Table 7. A 72-hour Growth Inhibition Comparison of Ciprofloxacin, Metformin, Cisplatin, and a Mix.

Concentration (µg/ml)	Growth inhibition (mean ± SD a)				b LSD value
	Ciprofloxacin	metformin	mix	cisplatin	
0.1	C 1.00 ± 1.000 [b]	D 2.00 ± 2.000 [b]	C 15.00 ± 5.000 [a]	D 4.00 ± 2.000 [b]	10.98
1	C 6.00 ± 2.000 [b]	C 14.00 ± 1.000 [ab]	C 17.00 ± 4.000 [a]	D 11.00 ± 1.000 [ab]	8.84
10	B 24.00 ± 3.000 [b]	C 20.00 ± 3.000 [b]	B 43.00 ± 3.000 [a]	C 23.00 ± 3.000 [b]	11.3
100	A 37.00 ± 4.000 [bc]	B 30.33 ± 3.055 [c]	B 52.00 ± 2.000 [a]	B 44.00 ± 4.000 [ab]	12.68
1000	A 43.00 ± 3.000 [b]	A 44.00 ± 2.000 [b]	A 69.00 ± 1.000 [a]	A 67.00 ± 1.000 [a]	7.3
[b] LSD value	10.16	8.5	12.06	9.06	-
IC 50	1174.2 µg/ml	1158 µg/ml	487.8 µg/ml	622.1 µg/ml	-

significant at (P<0.05)

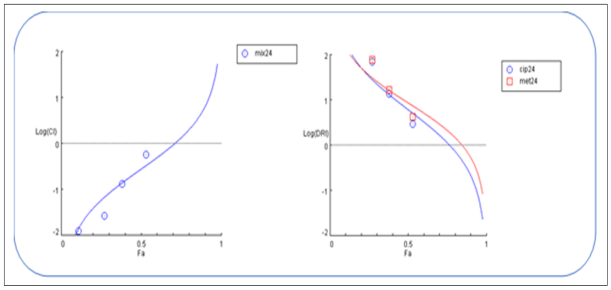

Figure 8. Log Combination Index Plot (left) and Log Dose Reduction Index Plot (right) for the Mixture at 24 hrs. cip; ciprofloxacin, met; metformin.

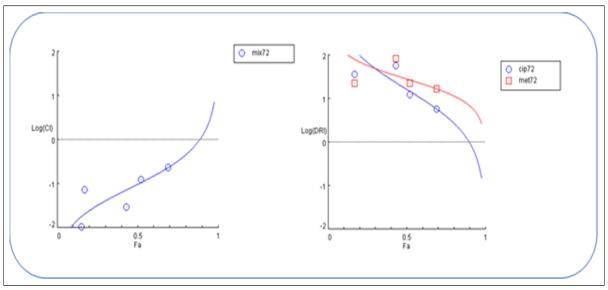

Figure 9. Log Combination Index Plot (left) and Log Dose Reduction Index Plot (right) for the Mixture at 72 hrs. cip; ciprofloxacin, met; metformin.

risk in people with diabetes diagnosed with colon cancer. [20, 72]. Metformin has been shown in another study to reduce the chance of acquiring prostate and liver cancer, as well as the related death rates for these cancers [20-24].

In line with our study concept, numerous studies have been conducted, with one showing that the amalgamation of amygdalin and esomeprazole successfully eliminates cervical cancer cells. The efficacy of this combination was contingent upon the medication concentration and the incubation length [55, 56]. The evaluation demonstrated that the combination of ciprofloxacin and laetrile effectively inhibits the proliferation of esophageal cancer cells [57]. Furthermore, A distinct investigation revealed that the combination of metformin and omeprazole exerts an inhibitory effect on the proliferation of the HeLa cancer cell line in both a concentration- and time-dependent manner, via a mechanism that involves the inhibition of heat shock protein 60 (Hsp60) [73].

Multiple proposed mechanisms have been studied to explore metformin's anticancer properties. The activation of AMPK by metformin in rat hepatoma H4IIE cells results in reduced phosphorylation of pS6 [25]. A particular in vitro study discovered that metformin directly inhibited AMP deaminase, resulting in elevated AMP levels and the following activation of AMPK [26]. Recent studies indicate that agents inhibiting mitochondrial complex I activity within the respiratory system could raise AMP levels and trigger AMPK activation. This, in turn, inhibits mTOR and activates signaling pathways that promote cellular functions and survival [27, 28]. Furthermore, Metformin may remove active K-ras from the cellular membrane via a PKC-dependent method. No empirical evidence exists to suggest a direct interaction between metformin and K-ras. Research indicates that metformin interacts with and disassembles the PP2A complex inside neuronal cells. Furthermore, there is potential to impede the function of PP2A-dependent phosphatase [29].

We performed a molecular docking study to elucidate the novel mechanisms by which the ciprofloxacin metformin combination targets c-Src tyrosine kinase. We focused on this molecular target due to its association with cervical cancer; enhanced phospho-Src expression has been seen in cervical cell lines and clinical cervical cancer tissues, whereas downregulation of phospho-Src results in inhibited cell proliferation [74-76]. Another study discovered that cervical squamous cell carcinoma patients exhibiting phospho-Src expression had a higher likelihood of recurrence. This indicates that phospho-Src may serve

Table 8. Combination Index and Dose Reduction Index Value for the Cytotoxicity of Ciprofloxacin-metformin Mixture at a 24-hour Incubation Period

Concentration µg/ml		Con. ratio	CI value	Combination behaviour	DRI value	
Metformin	Ciprofloxacin	1:01			Ciprofloxacin	Metformin
0.05 µg/ml	0.05 µg/ml		0.00591	Very Strong Synergism	439.545	274.794
0.5 µg/ml	0.5 µg/ml		0.01257	Very Strong Synergism	175.698	145.321
5 µg/ml	5 µg/ml		0.02692	Very Strong Synergism	71.024	77.8946
50 µg/ml	50 µg/ml		0.1332	Strong Synergism	13.5187	16.885
500 µg/ml	500 µg/ml		0.57288	Synergism	2.94011	4.29627

Table 9. Combination Index and Dose Reduction Index Value for the Cytotoxicity of Ciprofloxacin–metformin Mixture at 72 hrs. Incubation Period

Concentration µg/ml		Con. ratio	CI value	Combination behaviour	DRI value	
Metformin	Ciprofloxacin	1:01			Ciprofloxacin	Metformin
0.05 µg/ml	0.05 µg/ml		0.01055	Very Strong Synergism	262.495	148.447
0.5 µg/ml	0.5 µg/ml		0.07236	Very Strong Synergism	36.0144	22.4234
5 µg/ml	5 µg/ml		0.0295	Very Strong Synergism	57.3551	82.871
50 µg/ml	50 µg/ml		0.12517	Strong Synergism	12.3664	22.5712
500 µg/ml	500 µg/ml		0.23566	Strong Synergism	5.70376	16.5729

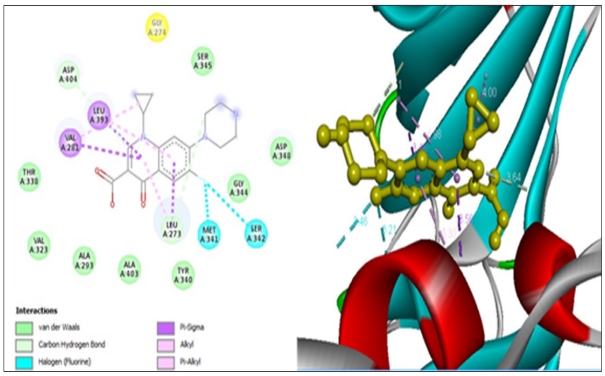

Figure 10. 2D and 3D Structures of the Human c-Src Tyrosine Kinase Binding Site with Ciprofloxacin.

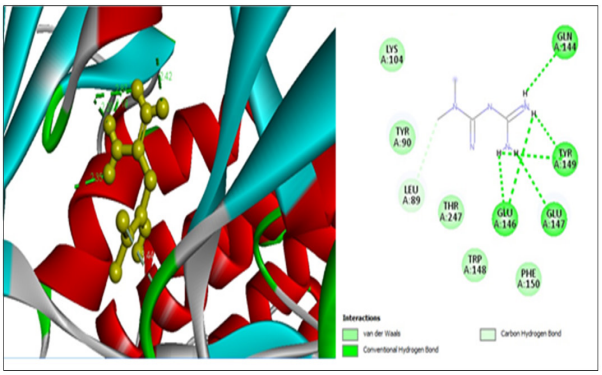

Figure 11. 2D and 3D Structures of the Human c-Src Tyrosine Kinase Binding Site with Metformin.

as a predictor for the development and recurrence of cervical squamous cell carcinoma [77].

The non-receptor protein tyrosine kinase Cancer Src is involved in several signaling pathways [30]. Numerous protein-tyrosine kinase receptors in the plasma membrane interact with Src. These interactions affect Src's activity, which also influences the receptors. EGFR (ErbB1) and ErbB2 are two significant protein-tyrosine kinase receptors involved with Src. Overexpression of ErbB2 is connected to breast cancer, whereas mutations in EGFR are typical in non-small cell lung cancer. Besides glioblastoma, the ErbB family is associated with cancers of the colorectal, gastric, head and neck, and pancreatic regions [31, 32]. The hepatocyte growth factor receptor (HGFR), also known as the c-MET protein-tyrosine kinase receptor, is another tyrosine kinase receptor that is regulated by src tyrosine kinase. C-MET is involved in embryonic development, wound healing, cellular migration, and angiogenesis [33, 34]. Epithelial cells produce c-Met, while mesenchymal cells generate HGF. Various malignancies, such as bladder, brain, breast, kidney, liver, pancreatic, prostate, stomach, and non-small cell lung cancer, exhibit c-Met dysregulation. Many tumors in humans demonstrate abnormal system activation through mechanisms like protein overexpression, mutations, gene amplification, and receptor-ligand upregulation.

Table 10. The Selective Toxicity Index of Ciprofloxacin-metformin Mixture and Cisplatin Across two Incubation Periods

Incubation	SI	
	MIX	Cisplatin
24 hrs.	7.36	0.72
72 hrs.	10	0.74

(An SI greater than 1.0 signifies a drug's enhanced efficacy against tumor cells compared to its toxicity towards normal cells.

Table 11. Comparison of Docking Scores among Ciprofloxacin, Metformin, and Bosutinib

Medications	Docking score
Ciprofloxacin	-7.9
Metformin	-5.1
Bosutinib	-8.1

C-MET mutations have been detected in the tyrosine kinase domain [21], juxtamembrane domain [5], and extracellular domain [16] of numerous solid tumors, which include both hereditary and sporadic human papillary renal carcinomas, lung cancer, ovarian cancer, childhood hepatocellular carcinomas, head and neck squamous cell carcinoma, and gastric cancer [33, 40-42]. Elevated levels of c-Met enhance invasion, migration, and metastasis [9, 43, 44, 78] Src tyrosine kinase plays a role in regulating the activity of platelet-derived growth factor (PDGF), which affects cellular proliferation, migration, survival, and angiogenesis. PDGF binds to its receptor protein-tyrosine kinases (PDGFRα/β). Cancers such as breast, colorectal, prostate, GIST, glioblastoma, osteosarcoma, NSCLC, and neuroblastoma display PDGFR activity. Point mutations in the PDGFRα gene are found in 5% of human gastrointestinal stromal tumors (GISTs). Additionally, 5–10% of glioblastomas show expression of this gene. PDGFRα amplification has also been detected in oligodendrogliomas, esophageal squamous cell carcinoma, and artery intimal sarcomas, with PDGFRα/β activation facilitating cellular invasion and metastasis. Moreover, insulin-like growth factors (IGF-1 and IGF-2) support cellular division, growth, survival, angiogenesis, wound healing, and embryonic development [45, 46].

Our study indicates that the combination index value illustrates a synergistic interaction between ciprofloxacin and metformin. Additionally, our molecular docking analysis shows that ciprofloxacin and metformin work

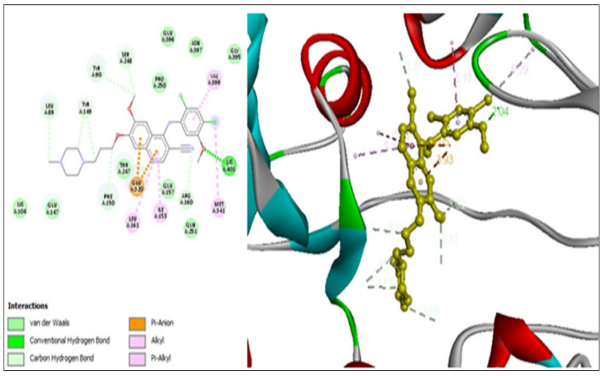

Figure 12. 2D and 3D Structures of the Human c-Src Tyrosine Kinase Binding Site with Bosutinib.

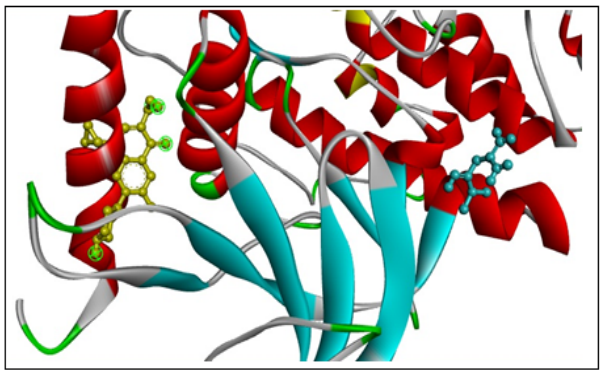

Figure 13. 3D Structure of Binding Sites of Human c-Src Tyrosine Kinase for Ciprofloxacin (yellow) and Metformin (blue).

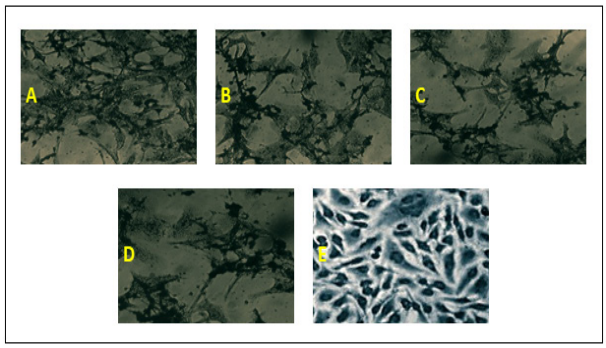

Figure 14. Hela Cancer Cells' histopathological Features. (A) Ciprofloxacin-treated Cancer Cells at 72 hours. (B) metformin-treated Cancer cells at 72 hours. (C) Ciprofloxacin-metformin mix treated Cancer cells at 72 hours. (D) cisplatin-treated Cancer cells at 72 hours. (E) control group.

together by targeting c-src tyrosine kinase at distinct binding sites.

Based on the dose reduction index, combination medications exhibit fewer side effects due to their effective cytotoxic concentration being lower than that of separate components. The combination selectivity index demonstrated superior cytotoxicity against cancer cells compared to cisplatin, suggesting its potential as a cervical cancer treatment.

The limitations of this study primarily revolve around the restricted range of medication concentrations utilized. This choice was made to identify the optimal effective cytotoxic concentration of the ciprofloxacin and metformin combination. Additionally, the lack of in vivo validation represents a significant limitation, which we acknowledge and propose as a critical area for future research (Figure 14).

In conclusion, our study results indicate that using ciprofloxacin along with metformin effectively inhibits the growth of cervical cancer cells. This inhibition occurs through both cell cycle-specific and cell cycle-nonspecific mechanisms. The findings demonstrate that these two medications together produce synergistic cytotoxicity, as measured by the combination index value.

Computational docking simulations demonstrated that ciprofloxacin and metformin interact with c-Src tyrosine kinase. The findings elucidate the synergistic interactions among the combination ingredients, as each drug targets a distinct binding site on c-Src tyrosine kinase, suggesting a complementary mechanism for anticancer activity. Furthermore, the selective toxicity of the ciprofloxacin metformin mixture was greater than that of cisplatin. The dose reduction index study showed that the combined concentration of medications required to elicit significant cytotoxic effects is less than that needed for each medication when administered separately. This finding suggests that the combination possesses enhanced potency compared to the individual drugs, making it a potentially appealing and safer therapeutic option for the treatment of cervical cancer. Despite the well-known pharmacokinetics, drug interactions, and safety profile, Future studies should investigate these factors to optimize dosing regimens and ensure safety, thereby facilitating the translation of preclinical findings to clinical applications in cervical cancer therapy.

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Article composition: Kawakeb N Abdulla, Istikrar M.Hade, Azal hamoody

Critique the essay for significant ideas: Azal Hamoody, Istikrar M.Hade, Youssef Shakuri

Statistical analysis expertise: Azal Hamoody, Kawakeb N Abdulla, Istikrar M.Hade

Ultimate article endorsement and guarantee: Azal Hamoody, Istikrar M.Hade, Youssef Shakuri.

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Conflicts of interest

The authors affirm that there is no conflict of interest.

Proclamation about Generative AI and AI-enhanced technologies in the composition process:

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

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. SD: standard deviation. LSD: Least Significant Difference. DRI: dose reduction index. CI: combination index. Hsp 90: heat shock protein 90. HFF cell line: human fibroblast cell line. PPIs: proton pump inhibitors. c-src tyrosine kinase: cytoplasmic sarcoma tyrosine kinase. SI: selectivity index

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