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ORIGINAL ARTICLE

Network pharmacology-guided elucidation of metformin and irinotecan mechanisms in 2D and 3D cancer cell models

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Abstract

Cancer remains the second leading cause of death globally, following cardiovascular diseases, and continues to represent one of the most critical public health concerns of our time. Beyond its physiological impact, cancer also imposes significant psychological, social, and economic burdens on individuals and societies. Among women, breast and cervical cancers are particularly prevalent. While breast cancer development is associated with genetic predisposition, lifestyle, and socioeconomic factors, cervical cancer is primarily linked to high-risk human papillomavirus (HPV) infections, especially HPV-16 and HPV-18. Despite advances in chemotherapy, commonly used agents such as irinotecan often exhibit toxicity toward healthy cells, induce drug resistance, and face challenges due to the heterogeneous nature of cancer subtypes. These limitations underscore the need for safer, more effective, and targeted therapeutic alternatives. This study aims to explore the potential anticancer effects of metformin, a well-established antidiabetic agent, when used alone or in combination with irinotecan in breast and cervical cancer models. Additionally, it seeks to elucidate the underlying molecular mechanisms governing their cytotoxic and inhibitory effects through experimental and computational analyses. In this study, breast and cervical cancer cell lines were cultured under both two-dimensional (2D) and three-dimensional (3D) in vitro conditions. The cytotoxic effects of metformin and irinotecan, individually and in combination, were assessed through cell viability assays, and IC_{50} values were determined. To evaluate their influence on tumor progression, migration assays were performed in both culture models. Furthermore, network pharmacology and molecular docking analyses were applied to identify shared molecular targets and key biological pathways potentially involved in the observed effects. The results revealed that both metformin and irinotecan exerted significant cytotoxic activity on the tested cancer cell lines. More importantly, their combined administration led to a stronger suppression of cancer cell proliferation, migration, and invasion compared with single-drug treatments. Network pharmacology analysis highlighted several common molecular targets, including *SLC47A1*, *ACHE*, *HRH3*, *EGFR*, *F2*, and *NOS1*, which are associated with critical biological processes such as apoptosis, angiogenesis, cell cycle control, and cellular stress regulation. Overall, this study demonstrates that the combined use of metformin and irinotecan enhances anticancer efficacy and may offer a promising therapeutic strategy for breast and cervical cancers. The integration of in vitro assays with computational approaches provides deeper mechanistic insights into how these drugs exert their effects, thereby supporting the potential repositioning of metformin as an adjuvant agent in cancer treatment.

Keywords Cancer therapy · Metformin · Irinotecan · Cytotoxicity · Breast cancer · Cervical cancer

1 Introduction

Cancer is a major global cause of mortality, marked by the unregulated growth and division of cells. It is well-established that genetic, epigenetic, and environmental factors contribute to this complex process. Cancer cells modify their metabolic pathways to generate energy and biomolecules that support their growth. These metabolic alterations

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open avenues for creating innovative approaches to cancer therapy (Hanahan and Weinberg 2011).

In recent years, there has been growing interest in the potential anticancer properties of antidiabetic medications like metformin. Originally developed as a biguanide derivative for managing type 2 diabetes, metformin enhances insulin sensitivity by suppressing liver gluconeogenesis and promoting glucose uptake in peripheral tissues (Dowling et al. 2012). Epidemiological research has associated metformin usage with a decreased risk of developing cancer and lower mortality linked to cancer in individuals with diabetes (Decensi et al. 2010).

Preclinical and clinical research has revealed that metformin demonstrates both indirect and direct antitumor effects. It has been suggested that the ability of metformin to lower insulin levels enhances its effectiveness in cancers associated with hyperinsulinemia. Additionally, Metformin may suppress tumor growth directly by targeting the AMP-activated protein kinase (AMPK) pathway (Ma et al. 2020). Specifically, in certain malignancies like breast cancer, metformin has been shown to decrease cellular growth and promote programmed cell death (Hadad et al. 2011).

The mechanisms underlying metformin's effects against cancer involve both indirect and direct processes. Epidemiological studies have shown that the use of metformin markedly lowers the risk of cancer occurrence and mortality associated with cancer in individuals with diabetes (Decensi et al. 2010). Metformin suppresses tumor cell growth and alters metabolic processes by activating the AMP-activated protein kinase (AMPK) pathway (Morales and Morris 2015). For instance, it has been suggested that metformin modifies cellular energy metabolism through insulin and insulin-like growth factors, thereby inhibiting cell proliferation and tumor progression (Mallik and Chowdhury 2018). Moreover, metformin has been shown to target cellular energy flux by regulating mitochondrial metabolism and selectively affecting cancer stem cells (Cioce et al. 2020). Additionally, in osteosarcoma models, metformin was found to activate ROS/JNK signaling pathways, causing cell cycle inhibition and triggering programmed cell death (Li et al. 2020).

Metformin also exerts significant effects on the immune system. It modulates the functions of immune cells within the tumor microenvironment, suppressing immunosuppressive mechanisms and supporting antitumor immune cells such as CD8 + T cells (Gupta et al. 2023). Notably, in pancreatic cancer models, metformin has been shown to promote the immune response and exert antitumor effects by activating the STING/IRF3/IFN-β pathways (Ren et al. 2020).

Furthermore, metformin has been suggested to regulate the immune system with the potential to target cancer cells, particularly when combined with immunotherapies (Ma et al. 2020). These results suggest that metformin could function as a versatile anticancer compound by exerting both metabolic and immunological actions.

These mechanisms highlight the promise of metformin as a supportive therapy in cancer treatment. Next-generation clinical studies aim to better understand these effects and evaluate the efficacy of combining metformin with immunotherapy and chemotherapy.

Metformin has shown promising results in preventing tumor growth and recurrence when administered in combination with chemotherapy drugs, particularly in breast cancer and various other cancer types (Hirsch et al. 2009). Although metformin shows potential as an anticancer drug, additional studies are needed to comprehensively understand its modes of action and assess its effectiveness across various cancer types (Bost et al. 2012).

Various studies have shown that metformin is more effective when combined with irinotecan in targeting various cancer cell lines (Taghizadehghalehjoughi et al. 2018; Khader et al. 2021; Bragagnoli et al. 2021). Bragagnoli et al. (2021) carried out a single-arm phase II trial to assess the effectiveness and safety of combining irinotecan with metformin in individuals treatment-resistant colorectal cancer. They reported that the results continued to show positive outcomes. Therefore, the combination of metformin and irinotecan aims to leverage the synergistic benefits stemming from their distinct mechanisms of action. This approach offers advantages such as achieving effective treatment with lower drug doses, thereby reducing side effects and improving treatment response rates. Consequently, the combination strategy has been regarded as a promising option for optimizing both efficacy and tolerability.

A substantial body of work has shown that metformin lowers intracellular ATP levels, thereby activating AMP-activated protein kinase (AMPK). Upon AMPK activation, anabolic programs that drive cell proliferation are suppressed, whereas catabolic pathways are upregulated. The literature further indicates that metformin can mimic the physiological effects of caloric restriction; through AMPK signaling it reduces proliferation and promotes apoptosis (Pierotti et al. 2013; Zhu et al. 2024). In contrast, the chemotherapeutic agent irinotecan, a topoisomerase I inhibitor, induces DNA strand breaks and thereby contributes to cell-cycle arrest. On the premise that metformin's metabolic regulatory actions could complement the DNA-damaging effects of irinotecan to enhance therapeutic efficacy—and that metformin may increase sensitivity to chemotherapeutic agents, including topoisomerase inhibitors, while mitigating resistance and toxicity by reprogramming cellular metabolism—our study aims to examine this potential synergy comprehensively in 2D and 3D in vitro cancer models (Samuel et al. 2020; Elhamipour et al. 2025).

In recent years, studies combining metformin with nanotechnology to enhance its effectiveness in cancer treatment have gained momentum. Particularly, the encapsulation of metformin into nanoparticles has been shown to improve drug delivery to targeted regions and reduce its side effect profile. Taghizadehghalehjoughi et al. (2018) reported promising results of metformin and irinotecan-loaded Poly(lactic-co-glycolic acid) (PLGA) nanoparticles on glioma cell lines, highlighting their high efficacy in suppressing cell proliferation and invasion. In another study, Faramarzi et al. (2019) demonstrated that metformin-loaded PLGA-PEG nanoparticles exhibited greater cytotoxicity in ovarian carcinoma cells compared to free MET, in a time- and dose-dependent manner. These nanoparticle-based approaches not only enhance the bioavailability of drugs but also represent a significant step forward in targeted therapy strategies. Such innovative methods enable a broader understanding of metformin's antineoplastic potential and lay the foundation for the creation of improved treatment strategies.

1.1 Research gaps

Despite the increasing number of studies on metformin, a careful examination of the literature shows that metformin-related cancer research still faces several important methodological limitations. First, there is a noticeable inconsistency between the low plasma concentrations of metformin achieved in patients and the much higher doses commonly required to produce biological effects in vitro. While clinically administered metformin typically reaches only micromolar levels (about 10–40 µM), most cell-based studies rely on millimolar concentrations (1–10 mM) to observe antiproliferative or pro-apoptotic outcomes (Chandel et al. 2016). This discrepancy has long complicated the interpretation of metformin's true anticancer capacity. Second, although many studies use two-dimensional (2D) or three-dimensional (3D) cancer models, they often provide limited quantitative data on migration and invasion, which reduces the ability to compare results across different studies. Third, research exploring the synergistic interactions between metformin and chemotherapeutic agents remains relatively scarce. Consequently, the strength and consistency of potential synergistic effects—particularly between metformin and irinotecan—are still not clearly established.

1.2 Main contributions

In order to address these methodological gaps, the present study focuses on two of the most common cancer types worldwide, cervical and breast cancer, and provides an integrative evaluation of the metformin–irinotecan (MET + IRI) combination. The main contributions of this work can be summarized as follows: (i) a comparative analysis of the phenotypic effects of the MET + IRI combination in 2D and 3D models using HeLa (cervical cancer) and MDA-MB-231 (triple-negative breast cancer) cell lines; (ii) systematic target–pathway mapping through network pharmacology approaches; (iii) derivation of potential binding hypotheses via molecular docking analyses of metformin and irinotecan with cancer-related proteins; and (iv) quantitative assessment of size and viability in 3D spheroids, providing a more physiologically relevant view of drug responses.

1.3 Rest of paper

The rest of this paper is organized as follows: Sect. 2 describes the materials and methods, including the development of 2D and 3D cell models, cytotoxicity, migration, invasion, network pharmacology, and molecular docking analyses. Section 3 presents the results and discussion, providing detailed interpretations of the experimental findings and bioinformatics analyses. Finally, Sect. 4 concludes the study by summarizing the key outcomes and highlighting potential directions for future research.

2 Materials and methods

2.1 Development of cell lines in two-dimensional (2D) culture

Cancer cell lines were cultured in 75 cm² flasks using RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% gentamicin, and 1% penicillin at 37 °C in a 5% carbon dioxide incubator. The cells were passaged using trypsin when they covered 80% of the flask surface. HeLa and MDA-MB-231 cell lines were utilized in the study. Based on the molecular weight of metformin, 0.001 g of metformin was weighed and dissolved in 1 mL of RPMI-1640 medium for the HeLa cell line, while 0.0013 g of metformin was similarly prepared for the MDA-MB-231 cell line. These solutions were used in the experiments.

Metformin was prepared at different starting concentrations across cell lines because HeLa and MDA-MB-231 cells differ in drug sensitivity and metabolic activity. HeLa cells are relatively less sensitive to metformin, whereas MDA-MB-231 cells exhibit greater sensitivity. Accordingly, to achieve comparable ranges of effective exposure in both cell lines, the initial solutions were prepared at different strengths.

2.2 Cytotoxicity assay

An XTT assay was conducted to evaluate the cytotoxic effects of metformin and irinotecan on cancer cells. Cells were seeded into 96-well plates at a density of 5,000 cells per well. One column of the plate was designated as a medium control, to which no cells were added. After a 24–48 h incubation, metformin was introduced to the wells in serial dilutions. Following an additional 48–72 h, the XTT reagent containing formazan dye was added to each well, and the plate was incubated for 2–5 h. Optical densities were then measured using an ELISA reader. Based on the optical density readings, the IC_{50} values of metformin and irinotecan on the cells were determined (Scudiero et al., 1988; Berridge et al., 2005). For irinotecan, the concentration range used in the XTT assay was selected on the basis of half-maximal inhibitory concentrations (IC_{50}) reported in the Genomics of Drug Sensitivity in Cancer database (GDSC, https://www.cancerrxgene.org/, accessed 15 November 2025), namely 66.64 µM for HeLa and 18.05 µM for MDA-MB-231 cells; serial dilutions were prepared from the primary stock solution around these values.

2.3 Development of cell lines in three-dimensional (3D) culturre

Cell lines were propagated as spheroids using the three-dimensional hanging drop method. For this purpose, scaffold-free 96-well cell culture plates were utilized. After preparing the cell suspension, 40–45 µL drops were dispensed into the wells using a pipette, as shown in the protocol. To maintain humidity control, water or agarose gel prepared in a buffer solution was added to the reservoir area of the plates. The agarose gel was boiled in a microwave, cooled to 50 °C, and then placed into the reservoir area. The cells were subsequently cultured at 37 °C in a 5% carbon dioxide (CO_2) incubator (Foty, 2011).

2.4 Migration assay / wound healing assay

This assay was performed using the method summarized by Liang et al. (2007). Six-well plates were used, with three wells designated as controls and three wells treated with metformin and irinotecan. A total of 1.5×10^5 cells were seeded into each well and incubated for 24 h. Images of the wound at the time of creation (0-hour) were captured using a microscope. Subsequently, images were taken every 6 h until the wound closure was complete. The images were analyzed using the ImageJ software.

2.5 Invasion assay

To observe the effects of metformin and irinotecan on the invasive movements of cancer cells, a 24-well plate was prepared with inserts. Each insert was filled with 100 µL of pre-prepared 1:10 Matrigel stock solution. The plates were incubated at 37 °C for 48 h to allow the gel to solidify. Once the desired Matrigel structure was formed, RPMI-1640 medium containing fetal bovine serum (FBS) was added to the wells. Fresh serum-free RPMI-1640 medium was added to the inserts containing the Matrigel, followed by the addition of cancer cells at a concentration of approximately 10^6 cells/mL. Metformin and irinotecan treatments were then applied. The inserts with cancer cells were placed into wells containing serum-containing medium and incubated at 37 °C for 48 h. After incubation, the cells were treated with 10% formaldehyde for 10 min. Subsequently, the cells were stained using Giemsa stain (Shaw, 2005).

2.6 Network pharmacological analysis

In the initial phase of the study, the potential therapeutic targets of metformin and irinotecan were identified. For this purpose, the open-access pharmacological database SwissTargetPrediction (http://www.swisstargetprediction.ch/) was utilized. In parallel, high-scoring genes specific to breast and cervical cancer were retrieved from the GeneCards database (https://www.genecards.org/). The gene sets associated with metformin were then compared with those related to breast and cervical cancer to identify overlapping targets. To achieve this, a Venn diagram was constructed using the Venny 2.1 tool (https://bioinfogp.cnb.csic.es/tools/venny/). Based on the common genes identified, a protein-protein interaction (PPI) network was constructed via the STRING v12.0 platform (https://string-db.org). The resulting network data were exported in .tsv format and imported into Cytoscape v3.10.3 for visualization and topological analysis. Network analysis was then carried out within the Cytoscape environment. Finally, functional enrichment analysis was performed to determine the biological roles of the overlapping genes. These analyses were conducted using the Enrichr (https://maayanlab.cloud/Enrichr/) and Metascape (https://metascape.org) platforms.

2.7 Molecular docking analysis

Molecular docking was performed to evaluate the potential interactions between metformin and irinotecan with their target proteins. The 3D structures of both compounds were retrieved from the PubChem database (CID: Metformin – 4091, Irinotecan – 60838). The three-dimensional crystal

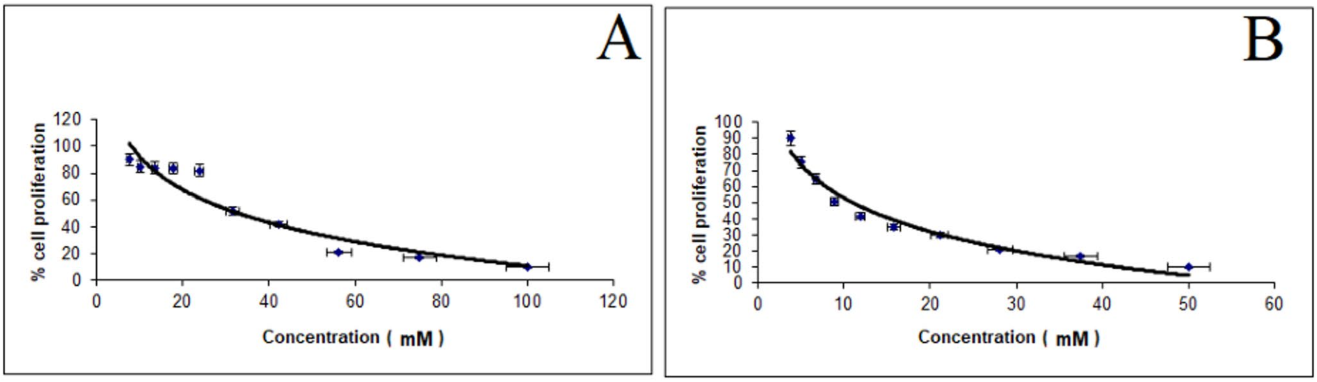


Fig. 1 **A**) Results of the XTT analysis of Metformin (30 mM) in HeLa cell lines **B**) Results of the XTT analysis of Metformin (8 mM) in MDA-MB-231 cell lines

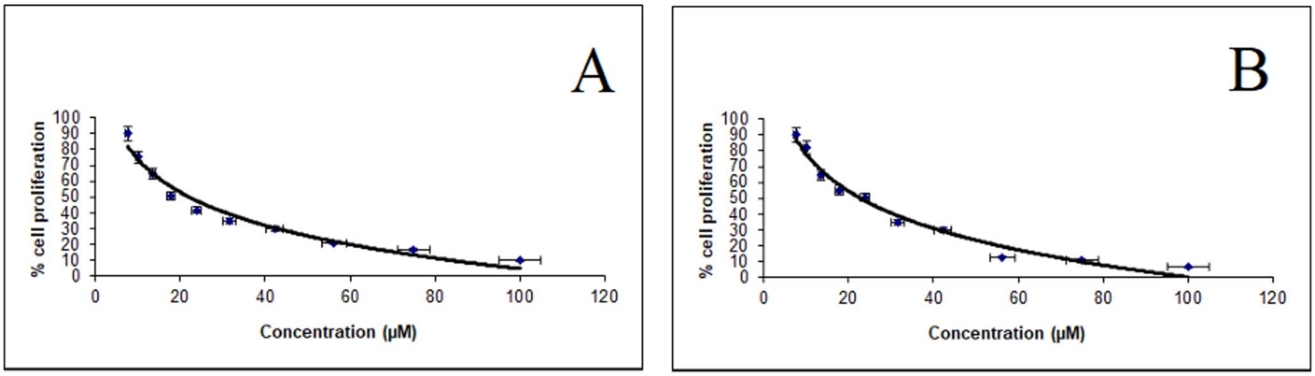


Fig. 2 **A**) Results of the XTT analysis of Irinotecan (83.7 µM) in HeLa cell lines **B**) Results of the XTT analysis of Irinotecan (23 µM) in MDA-MB-231 cell lines

structures of the relevant proteins were obtained from the Protein Data Bank (PDB).

After the preparation of ligands and proteins, the molecular docking procedure was carried out using the SwissDock (http://www.swissdock.ch/) online platform. Default param eters were applied during the docking process, and binding energies (ΔG) were calculated in kcal/mol.

The docking results were ranked according to their binding energies, and complexes with binding energies lower than −5.0 kcal/mol were considered to have strong binding affinities. The molecular interactions and hydrogen bonds of the top-scoring docking complexes, based on binding energy results, were analyzed using the Molegro Molecular Viewer 2019.7.0.0 software.

2.8 Calculations and statistical analysis

The results obtained from the analyses were evaluated using SPSS 22.0 software (SPSS Inc., USA). A p-value less than or equal to 0.05 was considered “statistically significant” ($p \leq 0.05$, p: Significance). The Levene test was used to verify variance homogeneity, while the Shapiro-Wilk test was used to verify data normality. When the normality assumptions were satisfied, parametric tests (one-way ANOVA and post-hoc Tukey) were employed, and non-parametric tests (Kruskal-Wallis and Dunn post-hoc) were.

3 Results and discussion

3.1 Cytotoxicity analysis

In this study, the IC_{50} value of metformin was found to be 30 mM for the HeLa cell line and 8 mM for the MDA-MB-231 cell line (Fig. 1). For irinotecan, the IC_{50} value was determined as 83.7 µM for the HeLa cell line and 23 µM for the MDA-MB-231 cell line (Figs. 2 and 3).

In the study by Kunthur et al. (2011) the impact of metforminin combination with chemotherapeutic drugs like 5-FU, oxaliplatin (O), and irinotecan (I) on colon cancer cells was examined using the MTT assay. The findings revealed that metformin markedly decreased cell viability when paired with single agents. However, no significant change in cell viability was observed when metformin was added to the FU + I or FU + O drug combinations. In conclusion, it was highlighted that metformin possesses antitumor

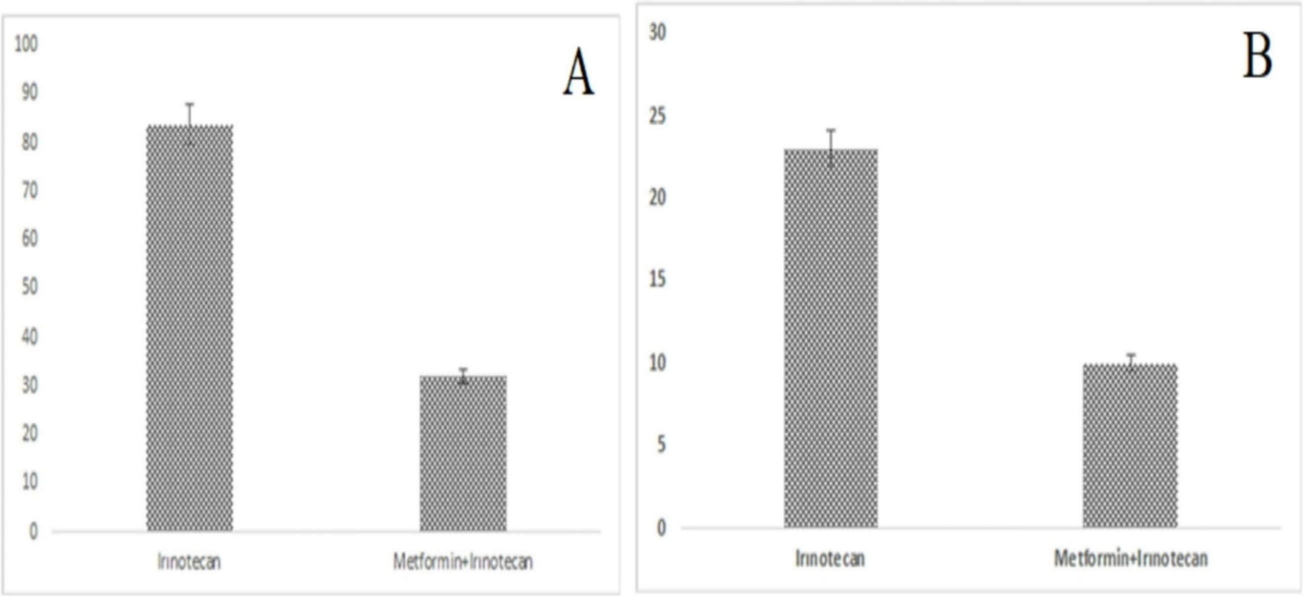


Fig. 3 **A**) HeLa cells after treatment with Metformin and Irinotecan **B**) MDA-MB-231 cells after treatment with Metformin and Irinotecan

activity on its own and boosts the effectiveness of both single-agent and combination chemotherapy when cells are pre-exposed to metformin. In another study, Zordoky et al. (2014) MDA-MB-231 cells were treated with increasing concentrations of metformin under hyperglycemic or normoglycemic conditions. The findings indicated that metformin failed to suppress the growth of MDA-MB-231 cells cultured in a hyperglycemic environment. However, in normoglycemic conditions, metformin significantly suppressed the growth of MDA-MB-231 cells. Furthermore, metformin treatment under normoglycemic conditions triggered notable AMPK activation and emphasized the suppression of various molecular signaling networksdependent on AMPK. These pathways are crucial for regulating protein synthesis and cell proliferation, and such inhibition was not observed under hyperglycemic conditions. Marinello et al. (2016) conducted a study investigating the mechanisms by which metformin acts on MCF-7 and MDA-MB-231 cell lines, with an emphasis on oxidative stress generation, DNA damage, and the activation of transforming growth factor β1 (TGF-β1). Specific concentrations of metformin were administered to both cell lines, and cytotoxic effects, oxidative stress levels, DNA damage, and intracellular metabolic pathways associated with cell proliferation and survival were clinically evaluated following drug exposure. The study findings demonstrated that metformin reduced the metabolic activity of MCF-7 cells in the MTT assay, accompanied by an increase in oxidative stress and DNA damage. However, cell death and a reduction in cell proliferation were observed only at higher concentrations. In MDA-MB-231 cells, decreases in metabolic activity and proliferation were evident only at experimental concentrations and after 24 h of drug exposure, with oxidative stress and DNA damage also being induced under these conditions. These results revealed how metformin functions in both breast cancer cell lines and proposed its potential use as an adjuvant therapy in breast cancer treatment. Tang et al. (2018) conducted a study investigating the combined anticancer effects of carboplatin and metformin on the HeLa cell line. Results from the MTT assay, performed to assess cytotoxicity, demonstrated a significant reduction in cell viability in the HeLa cell line treated with the carboplatin-metformin combination compared to the control group. Furthermore, an increase in apoptotic rates was observed in the group treated with the drug combination compared to the control. These findings revealed that metformin enhances the inhibitory effects of carboplatin on the proliferation of HeLa cells. Additionally, It was determined that metformin enhances the susceptibility of HeLa cells to carboplatin by activating the mitochondria-associated apoptotic signaling pathway.

In a study similar to ours, Khader et al. (2021) treated HCT116 and SW480 colorectal cancer cells with metformin, irinotecan, and their combination. The results demonstrated that metformin enhances the cytotoxic impact of irinotecan on colorectal cancer cells (HCT116 and SW480) when used in combination. This combination reduced cell proliferation by arresting the cell cycle at the G1 and S phases. It was highlighted that this effect is linked to a reduction in cyclin E and CDK2 levels and an increase in p21 levels. In a study conducted by Rizvi et al. (2023), the anticancer effects of Mebendazole, Metformin, and Apricoxib were investigated on MCF-7, HT-29, MDA-MB-231, and HeLa cells. The findings revealed that Metformin demonstrated greater effectiveness at lower doses relative to Mebendazole and Apricoxib in decreasing the survival of MCF-7, HeLa, and MDA-MB-231 cells. The findings indicate that Metformin possesses significant promise as an effective chemotherapeutic adjuvant.

In mechanism-oriented cell culture experiments, metformin is generally applied in the millimolar range. Indeed, doses up to 30 mM have been used to demonstrate effects such as mTOR/c-Myc axis suppression, G0/G1 cell cycle arrest, and increased apoptosis. Furthermore, studies conducted on various cancer cell lines have reported dose-dependent antiproliferative and/or pro-apoptotic responses at concentrations ranging from 32 to 50 mM (Besli et al. 2019; Sarıaydın et al., 2021; Wang et al. 2021). In addition to metformin, irinotecan (CPT-11) was employed in this study as a chemotherapeutic agent, and its in vitro effective concentration range is also consistent with previously reported values in the literature. Irinotecan (CPT-11) is a camptothecin derivative that exhibits cytotoxic activity at tens of micromolar concentrations in cell culture studies, which aligns well with the 23–83.7 µM range used in our experiments. Indeed, literature reports have shown IC_{50} values for irinotecan typically within the 5–60 µM range. For instance, Pavillard et al. (2002) reported IC_{50} values of 15.8 µM in LoVo and 5.17 µM in HT-29 colorectal cancer cell lines, with the formation of DNA–topoisomerase I “cleavable complexes” observed at these concentrations. Similarly, in another study, IC_{50} values of approximately 57.8 µM and 94 µM were reported for CPT-11 in 2D and 3D HeLa cell cultures, respectively (Sarıbaş et al. 2023), further supporting that the concentrations employed in our study are within the biologically relevant and mechanistically active range.

3.2 Development of cell lines in three-dimensional (3D) culture

In 3D cell cultures, cell death was clearly observed following metformin treatment. By the 48th hour, metformin caused significant cell disintegration and dispersion. Similarly, treatment with both irinotecan and metformin in 3D cell cultures resulted in notable cell disintegration and dispersion (Figs. 4, 5, 6, 7 and 8, and 9).

Yilmazer (2018) investigated the effects of metformin in 3D-cultured MCF-7 and U87-MG cells and found that metformin downregulated multidrug resistance genes (ABC transporters), thereby enhancing the anticancer activity of 5-fluorouracil. In another study, Yuan et al. (2018) attempted to investigate the functions and underlying mechanisms of metformin on stem cell properties and epithelial-mesenchymal transition (EMT) in glioma cells. The study revealed that metformin repressed spheroid formation and size in glioma cells and inhibited the expression of CD133, a marker associated with glioma stem cells. Similarly, Zhang and Wang (2019) conducted a study exploring the roles and mechanisms of metformin on stem cell properties and EMT in colorectal cancer cells. The findings demonstrated that metformin reduced spheroid formation capacity and suppressed the expression of stem cell markers in HCT116 cells. Furthermore, metformin was shown to resensitize HCT116 spheroid cells to 5-fluorouracil, overcoming drug resistance.

Bizjak et al. (2019) examined the impact of nutritional components on the activation of AMP-activated protein kinase (AMPK) triggered by metformin. Non-essential amino acids exhibited inhibitory effects in both two-dimensional (2D) and three-dimensional (3D) MDA-MB-231 cell cultures. Glutamine and pyruvate mildly attenuated the effects of metformin in 2D cultures. Additionally, glucose was found to protect tumor cells from metformin-induced disintegration. The findings emphasized the importance of

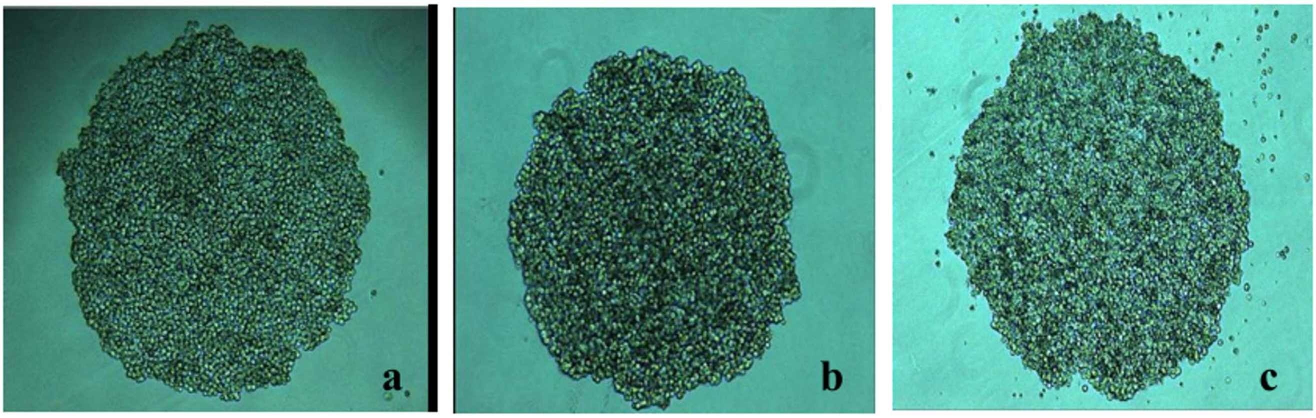


Fig. 4 HeLa cell line control **a**. 0 h **b**. 24th hour **c**. 48th hour

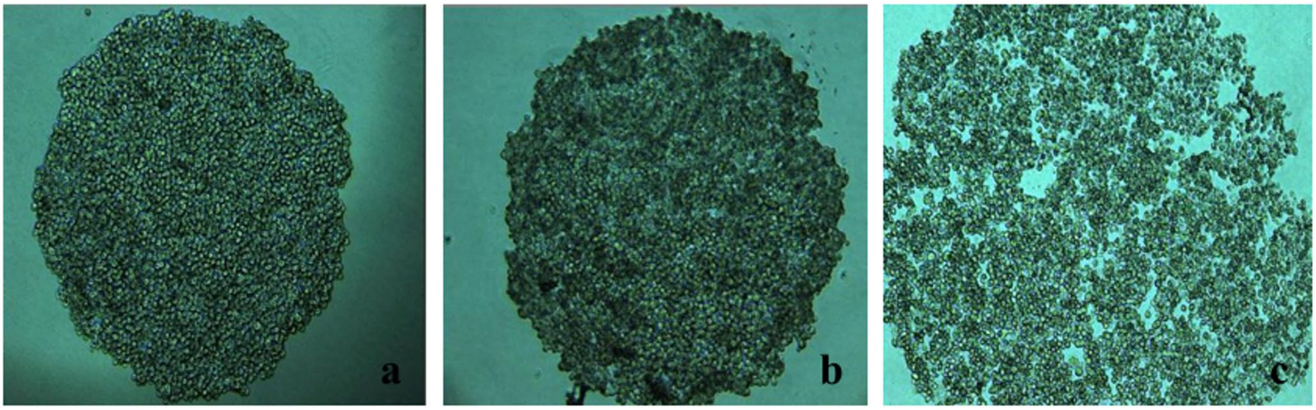


Fig. 5 HeLa cell line after metformin treatment **a**. 0 h **b**. 24th hour **c**. 48th hour

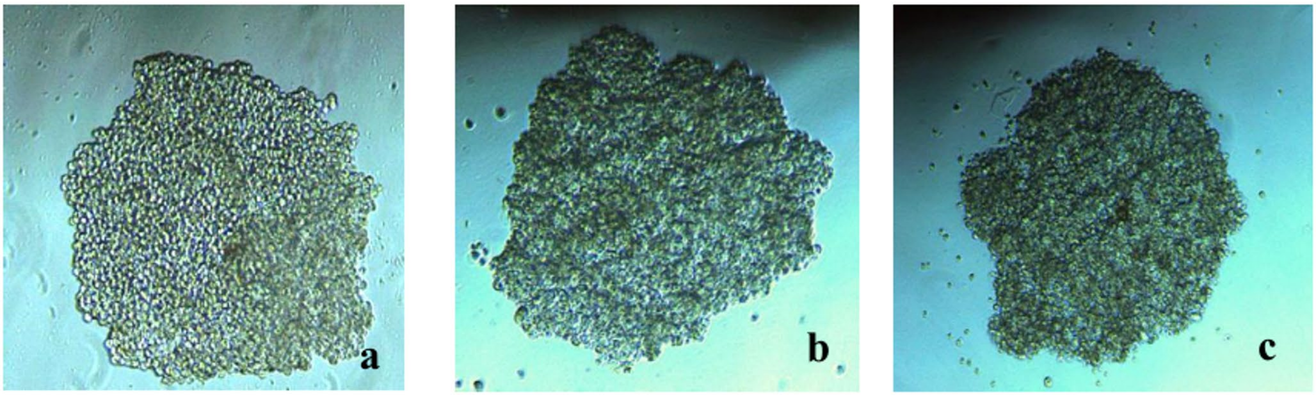


Fig. 6 HeLa cell line after metformin and irinotecan treatment **a**. 0 h **b**. 24th hour **c**. 48th hour

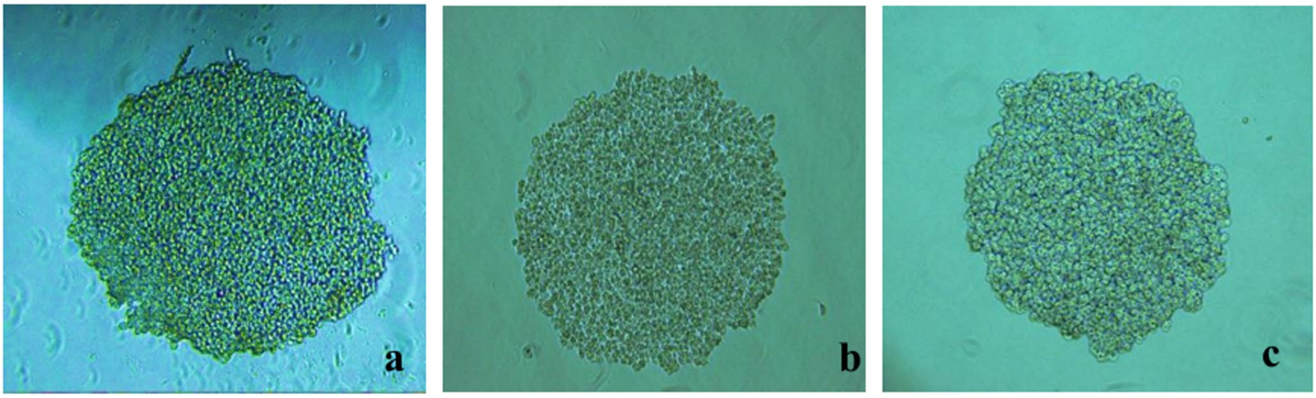


Fig. 7 MDA-MB-231 cell line control **a**. 0 h, **b**. 24th hour, **c**. 48th hour

considering nutritional elements when studying the effects of metformin on 2D and 3D cell cultures. In a study investigating the effects of metformin on germ cell tumors (GCTs), Salatino et al. (2022) demonstrated that metformin inhibited 3D spheroid formation in SEM-1 cells. Furthermore, it reduced the expression of IGFBP1, IGF1R, and MMP-11, thereby inhibiting cell migration and invasion.

In another study, Hahn et al. (2023) investigated the anticancer properties of metformin in a 3D co-culture model for pancreatic ductal adenocarcinoma (PDAC). In the direct 3D co-cultivation of PDAC organoids and primary pancreatic stellate cells (PSCs), metformin was found to reduce the transcription of genes associated with cancer development.

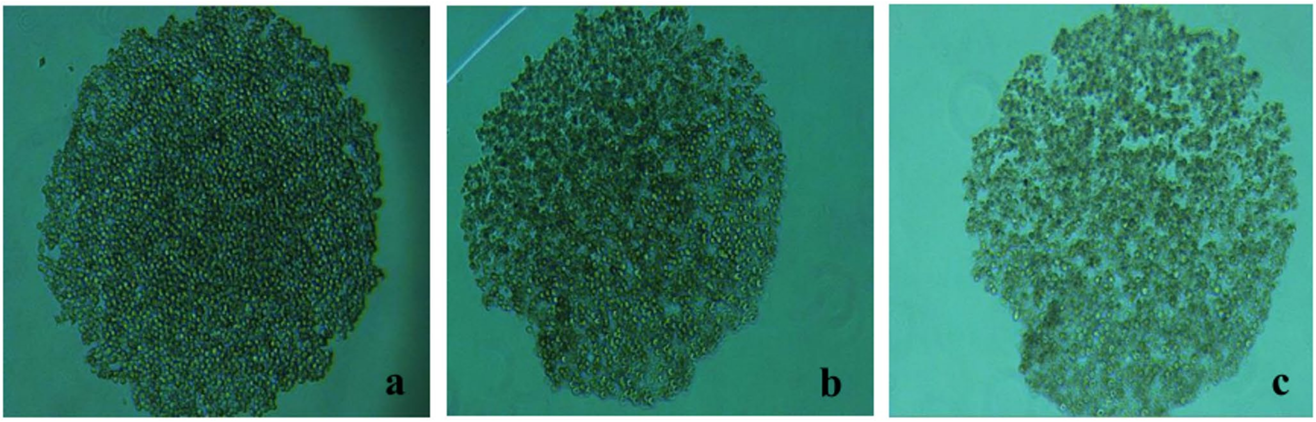


Fig. 8 MDA-MB-231 cell line after metformin treatment **a**. 0 h, **b**. 24th hour, **c**. 48th hour

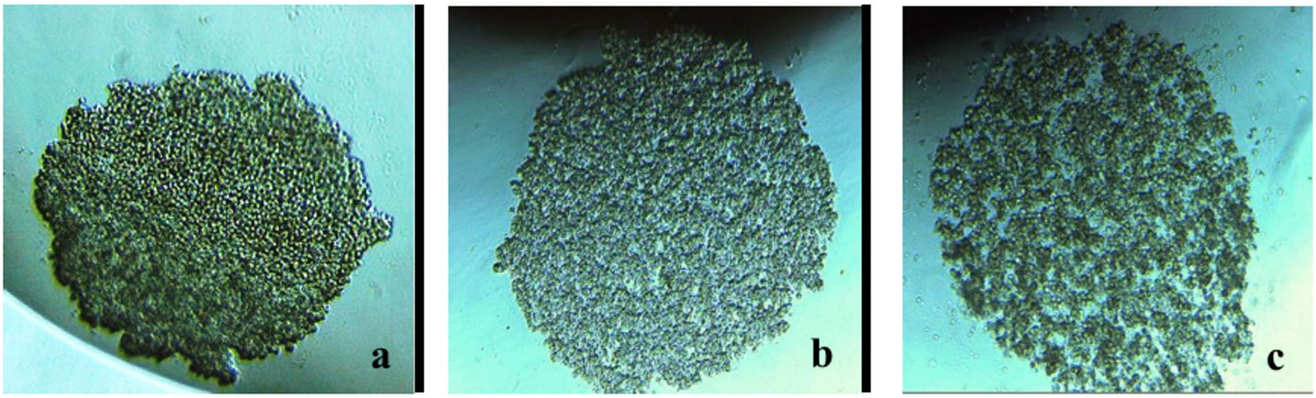


Fig. 9 MDA-MB-231 cell line after metformin and irinotecan treatment **a**. 0 h, **b**. 24th hour, **c**. 48th hour

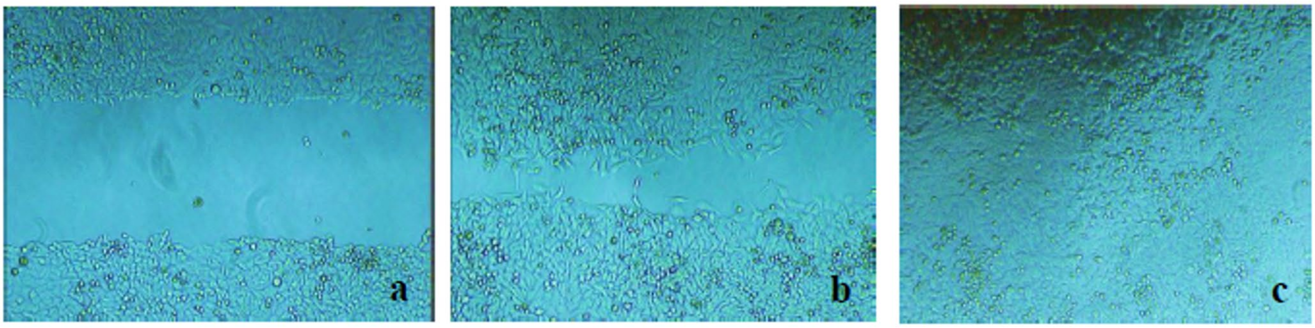


Fig. 10 HeLa cell line control **a**. 0 h, **b**. 24th hour, **c**. 48th hour

3.3 Migration assay / wound healing and invasion assay

The migration and invasion capabilities of cancer cells are among the most critical steps in tumor metastasis. Examining these processes facilitates the creation of efficient strategies for cancer treatment. In this study, the effects of metformin on these fundamental biological processes of cancer cells were evaluated using migration and invasion assays. The findings showed that metformin suppresses the migration of cancer cells (Figs. 10, 11, 12, 13, 14 and 15).

In the study conducted by Cerezo et al. (2013), the effects of metformin on the migration and invasion properties of melanoma cells in the early stages were investigated. Using cell migration assays with Boyden chambers, it was observed that metformin failed to suppress the migration of the 1205Lu and A375 melanoma cell lines after 24 h of treatment. Subsequently, cell invasion was examined using Boyden chambers coated with Matrigel, and it was found

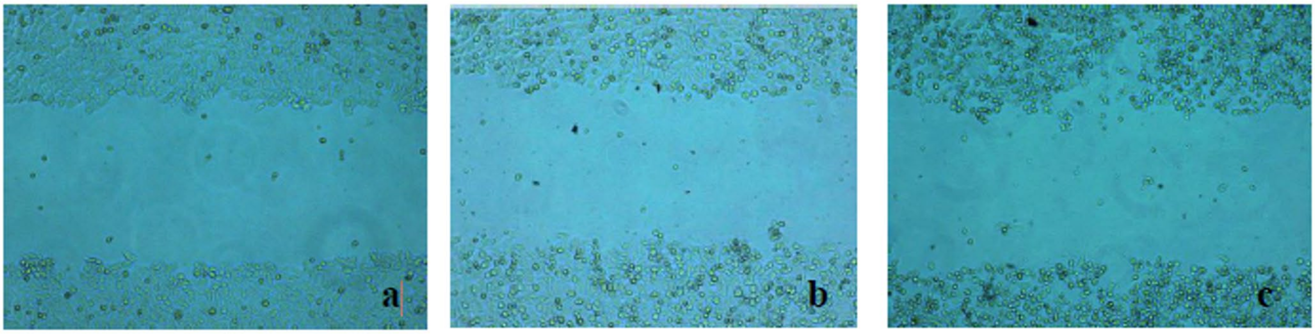


Fig. 11 HeLa cell line after metformin and irinotecan treatment **a**. 0 h, **b**. 24th hour, **c**. 48th hour

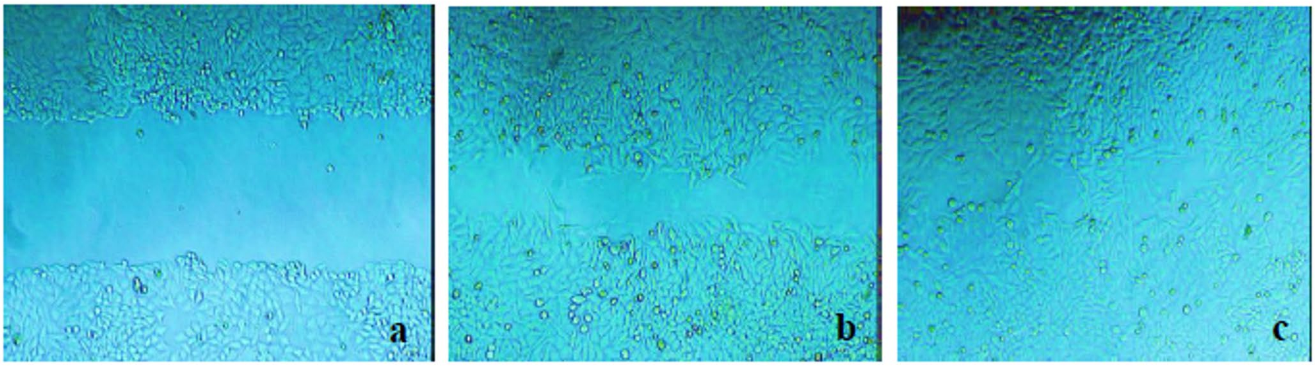


Fig. 12 HeLa cell line after metformin treatment **a**. 0 h, **b**. 24th hour, **c**. 48th hour

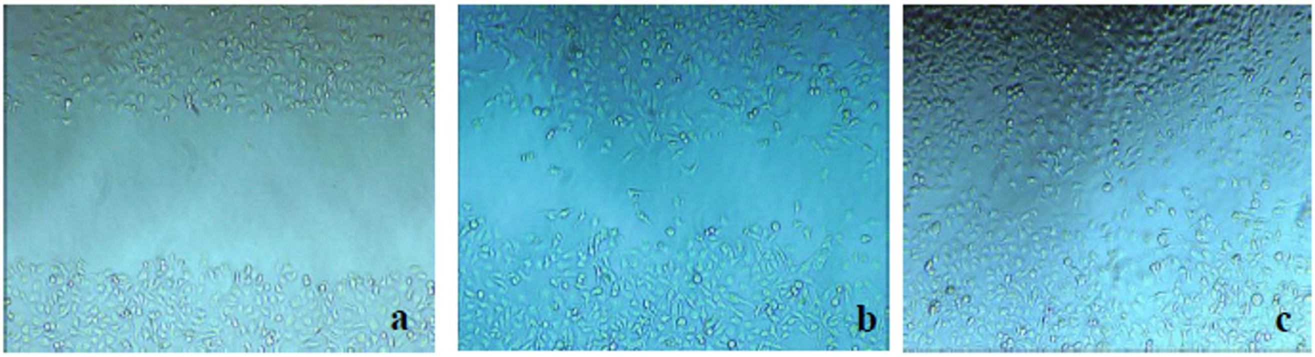


Fig. 13 MDA-MB-231 cell line control **a**. 0 h, **b**. 24th hour, **c**. 48th hour

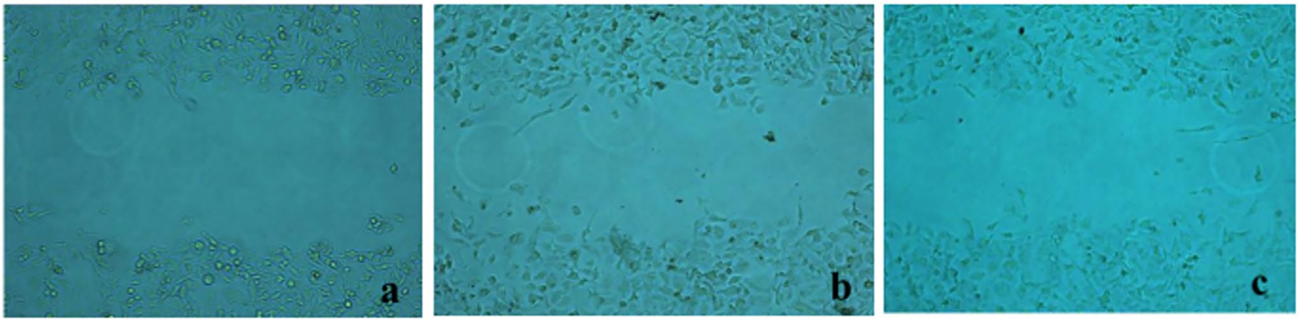


Fig. 14 MDA-MB-231 cell line after metformin treatment **a**. 0 h, **b**. 24th hour, **c**. 48th hour

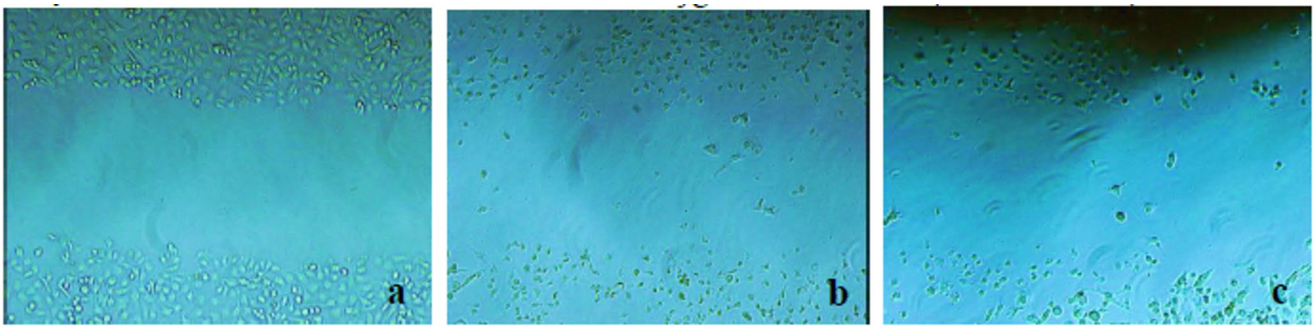


Fig. 15 MDA-MB-231 cell line after metformin and irinotecan treatment **a**. 0 h, **b**. 24th hour, **c**. 48th hour

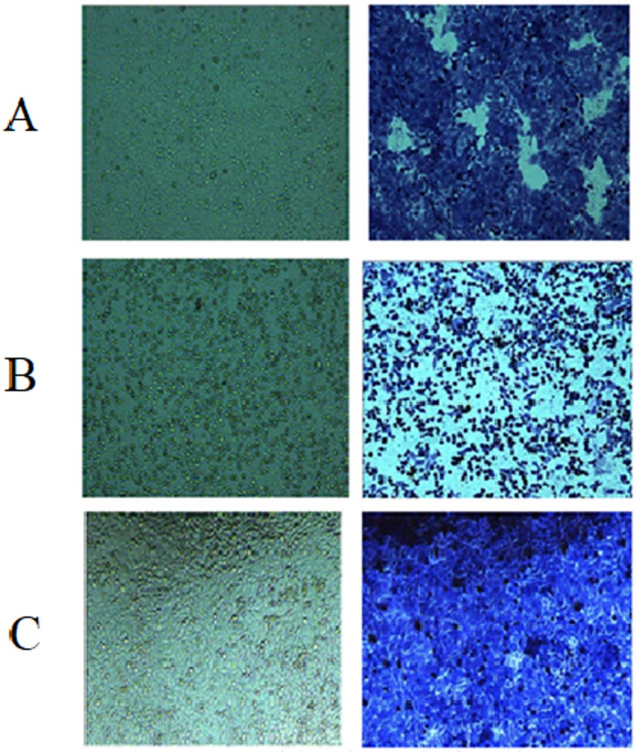


Fig. 16 **A**) HeLa cell line control **B**) HeLa cell line after metformin and irinotecan treatment **C**) HeLa cell line after metformin treatment

that metformin reduced cell invasion in the 1205Lu, A375, and WM9 melanoma cell lines in a dose-dependent manner. These findings indicate that metformin inhibits cell invasion independently of cell migration and antiproliferative effects. In another study by Cheng-jun (2014), which investigated the effects of metformin on the migration and invasion of renal cell carcinoma (RCC) cells, it was determined that metformin suppressed both migration and invasion in 786-O cells following treatment. In the study by Pan et al. (2015), the effect of metformin was investigated using the T24 bladder cancer cell line, which has a remarkably high invasion capacity. Metformin significantly inhibited the migration of T24 cells into the wound area, with the strongest inhibitory effect observed at 24 h in the wound healing assay. Similarly, the migration and invasion of T24 cells were markedly reduced by metformin at 24 h in the Transwell assay. Taken together, these findings indicate that metformin has the potential to inhibit the migratory and invasive abilities of bladder cancer cells in vitro. In the study by Qiang et al. (2019), the effects of metformin on the proliferation and migration of endometrial cancer (EC) Ishikawa cells were investigated. Similarly, the results showed that 10 mM metformin remarkably suppressed the migration and invasion of Ishikawa cells. In the study by Ferretti et al. (2019), the migration and invasion of hepatocellular carcinoma (HCC) cells treated with metformin and subjected to glucose deprivation were analyzed. Metformin was found to be as a strong suppressor of cell migration and invasion, with glucose restriction enhancing these effects.

In another study by Chen et al. (2021), which investigated the synergistic effects of pitavastatin and metformin on pancreatic cancer, it was determined that metformin (30 mM) and pitavastatin (10 μM) notably suppressed cell migration.

The results obtained from this study indicate that metformin not only inhibits the migration of cancer cells but also significantly suppresses their invasion capability. This finding highlights the potential of metformin to target critical processes involved in tumor metastasis, providing promising insights for its use in cancer therapy (Figs. 16 and 17).

In a study conducted by Schexnayder et al. (2018), the effects of low-dose metformin on migration and invasion of MDA-MB-231 cancer cells were examined using Boyden Chamber Flow Cytometry (BCFC). The cells were cultured with or without 100 μM metformin. Cell migration was suppressed by approximately 63% in the absence of the extracellular matrix. Additionally, invasion was suppressed by around 40% in the presence of the extracellular matrix. These results strongly indicate that low-dose metformin plays a significant role in inhibiting critical aspects of breast cancer metastasis, potentially enhancing its suggested therapeutic benefits in breast cancer treatment. In the study by Xia et al. (2018), the anti-neoplastic mechanisms of metformin were investigated by evaluating its effects on the migration and invasion of human cervical cancer cells.

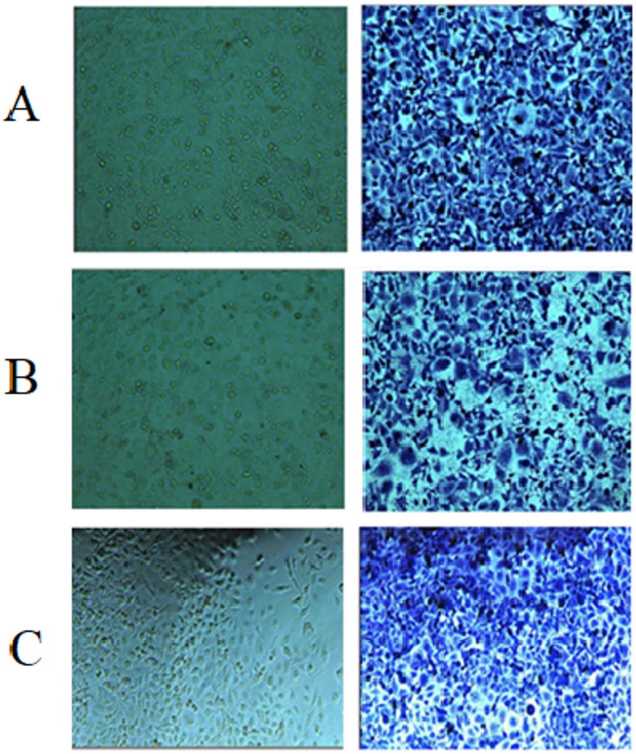


Fig. 17 **A**) MDA-MB-231 cell line control **B**) MDA-MB-231 cell line after metformin and irinotecan treatment **C**) MDA-MB-231 cell line after metformin treatment

The results revealed that metformin is capable of suppressing the migration and invasion of cervical cancer cells.

3.4 Network pharmacology analysis

As a result of the comparison between the targets of metformin and irinotecan and the gene sets specific to breast and cervical cancer, common genes were identified. For this purpose, the metformin targets obtained through SwissTargetPrediction and the high-score gene sets retrieved from the GeneCards database were analyzed using the Venny 2.1 tool, and a Venn diagram was generated. In this study, to evaluate the potential effects of metformin in breast and cervical cancer, the PPI network of 47 common target genes was analyzed via the STRING v12.0 platform. The resulting network consisted of 47 nodes and 79 edges, indicating that each protein interacted with approximately 3.36 other proteins on average. The local clustering coefficient of the network was calculated as 0.605, suggesting that the genes tend to form functionally coherent clusters. While the expected number of interactions for 47 randomly selected genes was only 17, a total of 79 interactions were observed among the analyzed genes. This discrepancy indicates that the network did not arise by chance and reveals the presence of biologically meaningful relationships among the genes. Indeed, the PPI enrichment analysis yielded a p-value of $< 1.0e-16$, indicating high statistical significance (Fig. 18). These results demonstrate that the genes targeted by metformin do not act independently but instead form an interconnected network. These genes are collectively involved in several key biological processes such as cell proliferation, apoptosis, signal transduction, and tumor suppression. This finding suggests that metformin may act as a pleiotropic agent capable of influencing multiple biological pathways.

Venn diagram showing the overlap among metformin, irinotecan, breast cancer, and cervical cancer gene sets. In the comparative analysis, gene sets related to metformin, irinotecan, and those specific to breast and cervical cancers were evaluated to identify overlapping targets. Based on the results of the Venn diagram analysis: Three common genes (EGFR, ESR2, and DHFR) were identified between metformin and breast cancer,

One gene (EGFR) was found to be shared between metformin and cervical cancer. Six common target genes were identified between metformin and irinotecan: SLC47A1, ACHE, HRH3, EGFR, F2, and NOS1. These genes may represent shared molecular mechanisms through which both compounds exert their effects.

To further evaluate the interaction relationships among the common target genes, topological analyses were conducted using Cytoscape v3.10.3. The constructed network represents gene-to-gene interactions through nodes and edges. In the visualization, genes located at the center of the network establish strong connections with surrounding genes, suggesting that these central genes may have greater biological importance and may play regulatory roles (Fig. 19). Based on the network structure, genes such as EGFR, ESR2, TYMS, DHFR, NOS1, and PLAU appeared to be centrally positioned and highly connected. These genes are considered noteworthy candidates due to their involvement in both cancer development and the mechanisms of metformin. This finding indicates that drugs such as metformin and irinotecan may exert their effects not through a single target, but via multiple genes simultaneously. Therefore, these compounds may act as pleiotropic agents, influencing various biological pathways within the cell at once.

In order to further investigate the biological functions and involved pathways of the common genes, gene enrichment analyses were performed. In these analyses, the Enrichr and Metascape platforms were used, and the results were evaluated in terms of both GO and KEGG pathways. According to the results of the GO biological process analysis, most of the common genes were found to be involved in various categories such as: cellular processes, biological regulation, response to stimulus, developmental processes, homeostasis, detoxification, and immune system processes (Fig. 20). According to the KEGG pathway enrichment

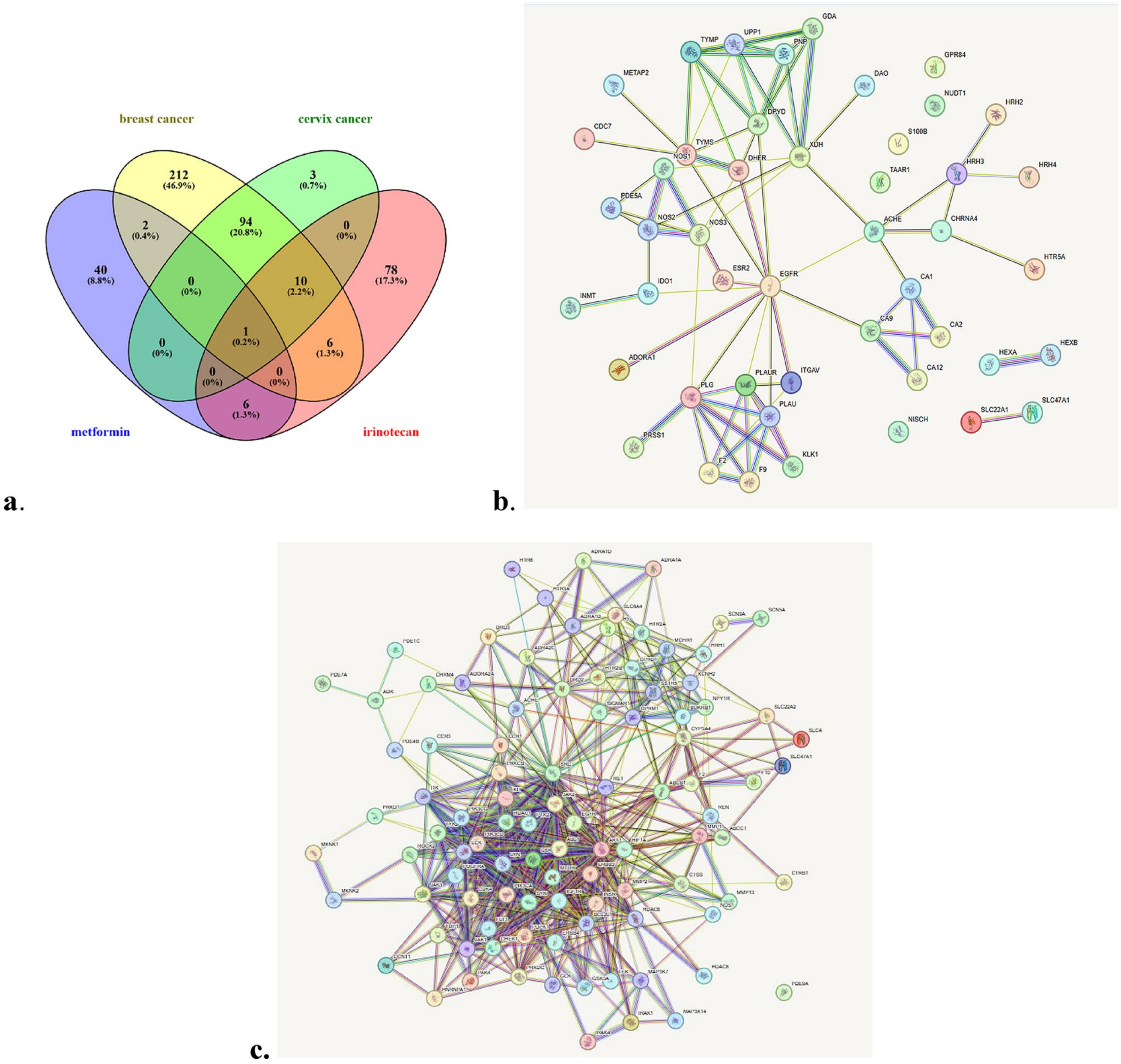


Fig. 18 **(a)** Venn diagram showing the intersection of metformin target genes with breast and cervical cancer-related gene sets. **(b)** PPI network of metformin target genes constructed using the STRING platform. **(c)** PPI network of irinotecan target genes constructed using the STRING platform

analysis, the main pathways in which the common genes are involved include: neuroactive ligand-receptor interaction, nitrogen metabolism, pyrimidine metabolism, complement and coagulation cascades, calcium signaling pathway, drug metabolism, proteoglycans in cancer, and purine metabolism (Fig. 21).

In the study conducted by Shi et al. (2023), a total of 115 key targets of metformin related to Alzheimer's disease (AD) and type 2 diabetes (T2D) were identified. GO analysis revealed that the biological processes were mainly associated with the response to hormones and the regulation of ion transport. KEGG pathway analysis showed that metformin was involved in various biological pathways, primarily including cancer, neurodegeneration, and endocrine resistance. In another study conducted by Liu et al. (2024), KEGG pathway enrichment analysis was performed on 30 core target genes to elucidate the mechanism of action of metformin, and 20 distinct biological pathways were identified. One of the notable findings was the enrichment of apoptosis-related pathways, which was consistent with the results of the previously conducted GO functional annotation. The study also identified the AKT/HIF1A/PDK1

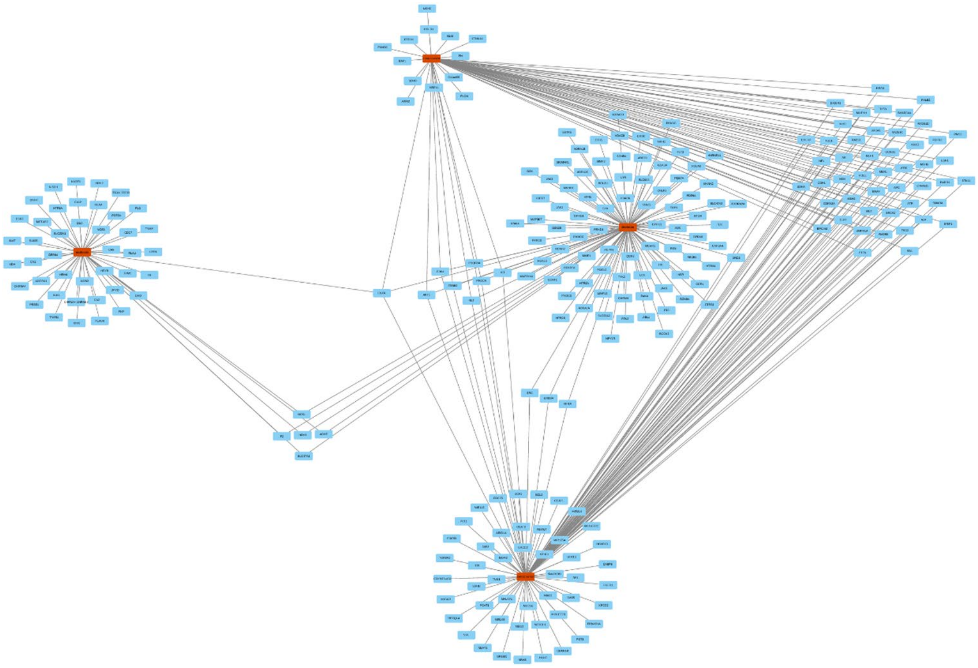


Fig. 19 PPI network visualization created in the Cytoscape environment. The network illustrates the topological relationships among common genes associated with metformin, irinotecan, and breast and cervical cancers

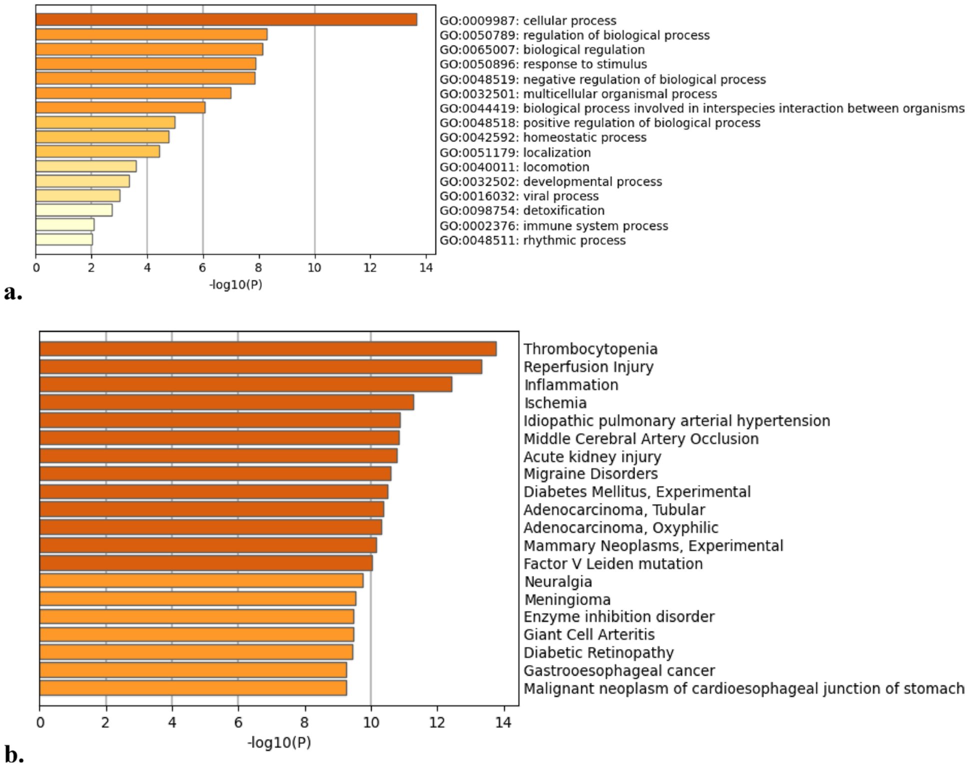
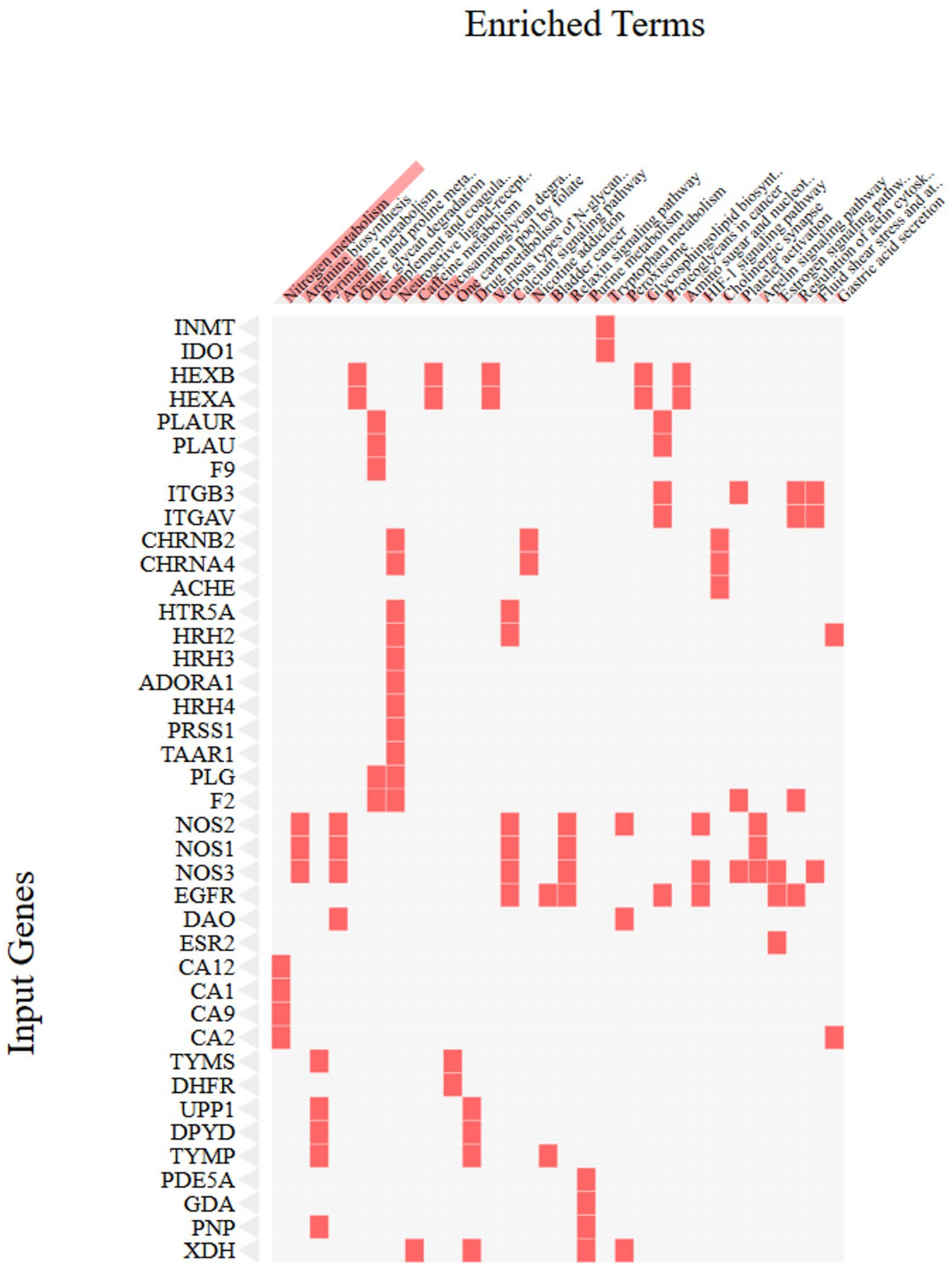


Fig. 20 **(a)** GO biological process enrichment analysis **(b)** The gene-disease association analysis performed using the DisGeNET database

signaling pathway as a potential apoptosis-related pathway influenced by metformin. These findings align with our study, where metformin demonstrated significant interactions with apoptosis-related target proteins, further supporting its potential as an anti-cancer agent.

Figure 21 presents the enriched biological pathways involving the shared genes in the form of a heatmap. The red boxes indicate the involvement of a specific gene in a given pathway. Notably, the genes EGFR, DHFR, and ESR2 are associated with multiple pathways, suggesting that they may play a central role in cancer biology.

Fig. 21 KEGG pathway enrichment analysis



3.5 Molecular docking

All predicted targets were effectively docked with metformin, and the corresponding binding energy results are presented in Table 1. The hydrogen bond locations and interaction visualizations of the best molecular docking results—between AKT-1 and irinotecan, and between KRAS and metformin—are presented in Figs. 22 and 23. Metformin, which shows high binding energy with the KRAS protein, interacts via hydrogen bonding with the Glu-143 residue (1 H-bond), as shown in Fig. 22. Irinotecan, which exhibits high binding affinity with the AKT1 protein, forms three hydrogen bonds with Tyr-152(A), Phe-150(A), and Tyr-215(A) residues (Fig. 23).

The molecular binding energies presented in the table compare the binding potentials of metformin and irinotecan, an established chemotherapeutic agent, to various cancer-related protein targets. As expected, irinotecan demonstrated high binding affinity to several targets (e.g., BAK-BAX: -9.86 kcal/mol, VEGFR1: -9.63 kcal/mol, MMP2: -9.04 kcal/mol). However, metformin also showed notable binding energies with key proteins involved in cancer biology, such as AKT1 (-6.79 kcal/mol), KRAS (-6.86 kcal/mol), and ESR2 (-6.83 kcal/mol). These findings suggest

Table 1 Molecular Docking results of Metformin and Irinotecan with target proteins

Protein	PDB ID	Binding energy (kcal/mol) metformin	Binding energy (kcal/mol) irinotecan
CASPASE 3	3KJF	-6.30	-8.88
CASPASE 8	3KJQ	-6.63	-6.63
TRAIL	1D2Q	-5.89	-8.03
TNF-ALFA	1A8M	-6.04	-7.36
MMP2	7XGJ	-6.71	-9.04
VEGFR1	3HNG	-6.59	-9.63
VEGFA	3QTK	-6.07	-7.28
JAK1	3EYG	-6.03	-7.74
TNFR1	1ICH	-6.07	-7.69
CDK2	8FP0	-6.24	-7.51
BIRC5	2QFA	-6.04	-7.15
AKT1	3MV5	-6.79	-8.96
BCL2	4XLD	-6.40	-6.68
BAK-BAX	8SRX	-6.47	-9.86
BRAF	5VR3	-5.95	-6.80
KRAS	7XKJ	-6.86	-6.60
MAP2K1	8YP4	-6.26	-8.05
FAS	1A1W	-5.92	-6.65
FASL	4MSV	-6.02	-9.00
EGFR	5UGB	-6.52	-6.61
ESR2	1YYE	-6.83	-
DHFR	4KD7	-7.01	-7.03

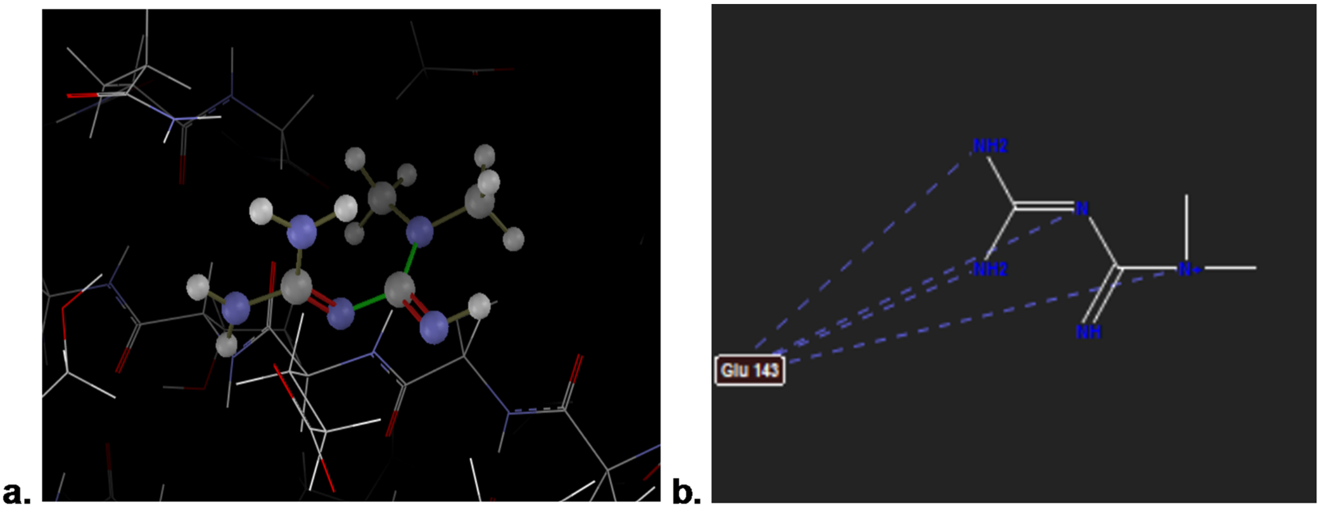


Fig. 22 **(a)** Interaction between KRAS ve metformin and **(b)** Location of hydrogen bonds

that, when compared to a potent chemotherapeutic like irinotecan, metformin's significant interactions with similar targets highlight its potential as an anticancer agent.

In this study, no direct AMPK–mTOR or DNA-damage validation assays were performed. However, network-pharmacology analyses—which encompassed pathways associated with genes such as EGFR, ESR2, DHFR, NOS1, and F2—together with molecular docking results indicate that the metformin–irinotecan combination may concurrently modulate multiple cellular signaling routes. The most biologically plausible explanation is that metformin enhances AMPK activation and thereby suppresses mTOR signaling, which in turn diminishes cellular DNA-repair capacity, allowing irinotecan to exert more potent topoisomerase I (TOP1)–mediated genotoxic effects. Under conditions of energy stress, the cell is less able to repair irinotecan-induced double-strand breaks and consequently undergoes apoptosis. Consistent with this view, prior literature reports that metformin lowers ATP production, induces energetic stress, inhibits the AMPK–mTOR axis, and thereby augments the cytotoxicity of TOP1 inhibitors such as irinotecan in cells with a weakened DNA-damage response (Pommier 2006; Dowling et al. 2012; Ma et al. 2020). This mechanism is also in agreement with our in silico findings.

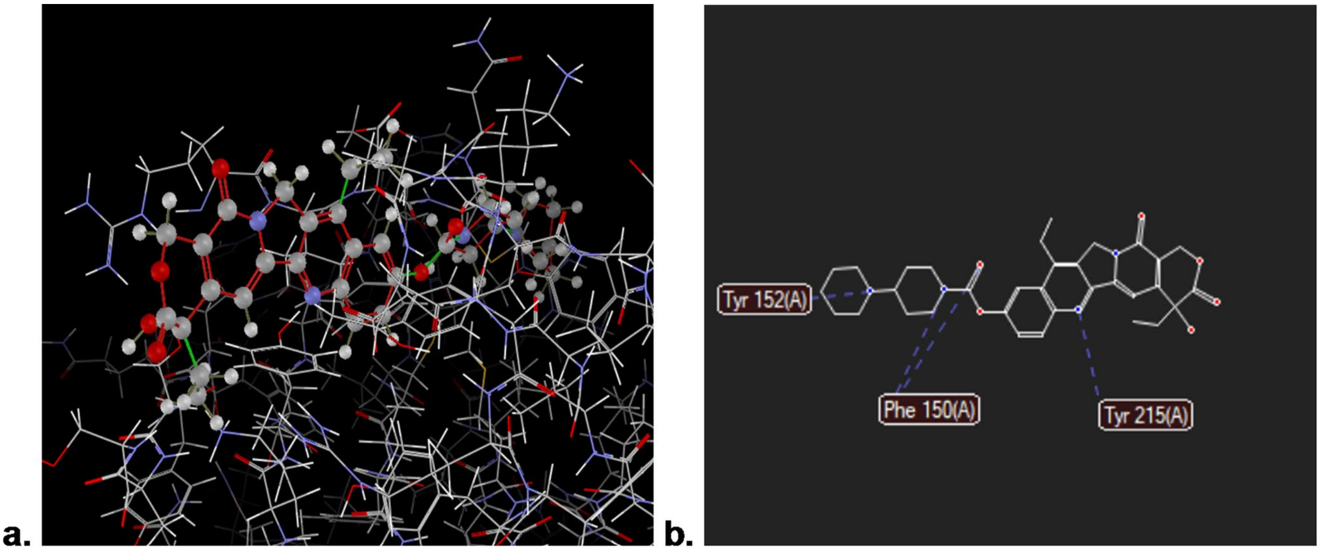


Fig. 23 **(a)** Interaction between AKT-1 and irinotecan and **(b)** Location of hydrogen bonds

Similar results have also been obtained in previous studies. For example, in the study by Shi et al. (2023), the binding energies between metformin and the target proteins ranged from −3 to −6 kcal/mol. Likewise, in the study by Liu et al. (2024), the binding energies of metformin with selected target proteins were found to range from −4.35 to −6.86 kcal/mol. The lower the energy score, the stronger the ligand–receptor binding capacity and the more stable the structure.

3.6 Study limitations and future directions

This study has several limitations that should be taken into account when interpreting the findings. First, all experiments were performed in malignant cell lines (HeLa and MDA-MB-231), and we did not include non-transformed control cell lines to directly assess potential toxicity in normal cells. Nevertheless, published data indicate that metformin generally displays a broad clinical safety profile and does not induce proliferative toxicity in normal cells at therapeutic concentrations, and in vitro work in healthy epithelial cells has reported no major cytotoxic effects at doses comparable to those used here (Erices et al. 2013; Apostu et al. 2023; Mahmoudi et al. 2024). Therefore, while a healthy control cell line could not be included as a comparator, these reports support that the metformin doses used here lie within physiologically tolerable, non-toxic ranges. This approach is consistent with our aim to evaluate the combination therapy's effects through cancer-specific mechanisms. Second, all experiments were conducted in standard RPMI-1640 medium under approximately normoglycemic conditions (glucose ~ 2 g/L, ≈ 11.1 mM). We therefore cannot draw conclusions about how hyperglycemic conditions, which mimic diabetic states, might modulate the response to metformin and the MET + IRI combination. Prior studies have shown that high glucose can attenuate some of the antiproliferative and AMPK-dependent actions of metformin or exert a protective, pro-survival influence in certain settings. Future work will be needed to systematically compare the effects of metformin and MET + IRI under physiological (~ 5–6 mM) versus hyperglycemic (~ 25 mM) glucose concentrations, integrating readouts of AMPK activation, mTORC1 signaling, DNA damage and long-term cell survival (Sinnett-Smith et al. 2013; Villa-Fernández et al. 2024).

4 Conclusion

Cancer remains a major global and national public health challenge, demanding innovative strategies to overcome its multifaceted impact on health, society, and the economy. This study emphasizes the promise of metformin, a commonly prescribed antidiabetic drug, as an effective therapeutic option for cancer therapy, especially in combination with irinotecan. The results demonstrate that metformin not only exerts significant cytotoxic effects on cervical and breast cancer cell lines but also enhances the efficacy of irinotecan in inhibiting cell proliferation, migration, and invasion.

The potential of metformin to target cancer cells while maintaining a favorable safety profile and low cost underscores its potential as an accessible and effective treatment option. Furthermore, the use of both 2D and 3D in vitro models in this study provides robust and clinically relevant insights into the mechanistic actions of these agents. To better understand the underlying mechanisms of these effects, network pharmacology and molecular docking analyses were conducted, revealing that both drugs interact with several common molecular targets involved in key cancer-related biological processes. Most of these targets are associated with pathways such as apoptosis, cell cycle regulation, angiogenesis, and cellular stress responses. The molecular docking results further demonstrated that metformin and irinotecan establish strong and specific bindings with these target proteins.

These findings lay a foundation for further exploration of metformin's role in cancer therapy and underscore the importance of combination therapies in addressing the complexity of cancer biology. By contributing valuable data to the field, this study supports the development of alternative therapeutic approaches that can improve outcomes for patients with cervical and breast cancer.

Authors' contributions Seda Yalçınkaya and Serap Yalçın Azarkan: Conceptualization, Methodology, Data curation, Writing—Original draft preparation, Investigation, Validation, Visualization, Writing.

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Declarations

Conflict of interest There is no conflict of interest.

Ethical approval Approved.

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