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Research Article

Overcoming Hormone Resistance in Breast Cancer Cell Lines: The Impact of Combined Treatment with Sorafenib and Palbociclib on Cell Survival and Proliferation Pathways

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Abstract

Objectives: The primary objective of this study was to investigate the potential synergy between Sorafenib, a multi-kinase inhibitor, and Palbociclib, a cyclin-dependent kinase 4/6 (CDK4/6) inhibitor, in the treatment of hormone receptor-positive breast cancer. Specifically, we aimed to determine whether the combination of these two drugs could enhance cell death in breast cancer cell lines.

Methods: Cell Culture: Hormone receptor-positive breast cancer cell lines expressing estrogen receptors (ER-positive) and/or progesterone receptors (PR-positive) were selected for the study.

Drug Treatment: Cells were treated with Sorafenib, Palbociclib, or a combination of both drugs.

Cell Viability Assays: Cell viability and proliferation were assessed using MTT and BrdU assays, respectively.

Immunoblotting: Protein expression and phosphorylation levels of key signaling molecules were analyzed to investigate the intracellular pathways affected by drug treatments.

Statistical Analysis: Statistical comparisons were made between single-drug and combination-drug treatments to evaluate their effects on cell viability and proliferation.

Results: Our study revealed the following key findings:

Hormone receptor-positive breast cancer cells were chosen for this study due to their dependence on estrogen and progesterone for growth and division.

Sorafenib, a multi-kinase inhibitor, effectively targeted multiple signaling pathways involved in cell proliferation, angiogenesis, and apoptosis, including Raf, VEGFR, PDGFR, and FLT3.

Palbociclib, a CDK4/6 inhibitor, arrested cancer cells in the G1 phase of the cell cycle, preventing their progression into the S phase and subsequent proliferation.

Contrary to expectations, the combination of Sorafenib and Palbociclib in hormone receptor-positive breast cancer cell lines did not result in enhanced cell death. Instead, it exhibited a proliferative effect.

These unexpected results highlight the complexity of intracellular pathways and the potential for cross-talk between signaling pathways when drugs are combined.

Conclusion: In conclusion, our study emphasizes the intricate and multifaceted nature of intracellular pathways in hormone receptor-positive breast cancer. The unanticipated proliferative effect of the Sorafenib and Palbociclib combination underscores the importance of considering all possible mechanisms of action when designing drug combinations for cancer treatment. This study serves as a valuable reminder that therapies should not solely depend on the modulation of a single pathway but rather take into account the intricate web of interactions within the cellular environment. Further research is warranted to elucidate the underlying molecular mechanisms responsible for the observed outcomes and to guide the development of more effective treatment strategies for hormone receptor-positive breast cancer.

Keywords: Breast cancer, Cyclin-Dependent Kinase, mitogen-activated protein kinase

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Breast cancer (BC) is the most common cancer type in women with a rising incidence in the last 4 decades. According to a recent data covering 2010 – 2019, breast cancer rate increased by 0.5% annually.[1] Breast cancer survival varies significantly by stage at diagnosis. The 5-year relative survival for stage-I patients has been reported as >99% while this ratio was 29% for stage IV.[2]

The subtypes of breast cancer are classified according to the expression of hormone receptors, estrogen receptor (ER) and/or progesterone receptor (PR) (~70-75% of cases), and overexpression of the human epidermal growth factor receptor-2 (HER-2). Tumors that lack expression of such receptors are often referred to as triple-negative breast cancers (TNBCs).[3] Patients HR+/HER-2– subtype have most favorable survival rate (92.5% at 4th year), followed by HR–/HER2+ (82.7%). Among these types, triple negative breast cancer has the worst survival (77.0%).[4]

Targeting estrogen has made hormone receptor-positive breast cancer a manageable disease. Despite the wide variety of endocrine therapies, acquired or de novo resistance to this drugs remains a major challenge.[5] Autonomic cell cycle is known as most important mechanism of this resistance. Selective cyclin-dependent kinase inhibitors, which were tested in order to inhibit this feature that causes uncontrolled proliferation, denoted great success first in preclinical and then in clinical studies.[6-8]

In addition to the success of CDK 4/6 (Cyclin-Dependent Kinases) inhibitors in HR+ breast cancer, most of the patients develop primary or secondary resistance.[9] A wide variety of factors have been implicated in treatment resistance or failure and one of the main reasons is mitogen-activated protein kinase (MAPK) activation.[10] The MAPK pathway is responsible for controlling the function and expression of D-type cyclins and CDK4/6 need to bind to D-type cyclins to work properly.[11]

In previous literature it was reported that MAPK activation was a reason of CDK4/6 resistance and thus implicates the MAPK signaling pathway as potential drug target for tumors that can escape the inhibition of the CDK4/6 pathway.[12-14]

Since cross-activation of kinase-related signaling pathways is one of the main cause of drug resistance and tumor progression in breast cancer, the effects of various multi-kinase inhibitors on breast cancer have been investigated.[15, 16] In this sense, the most studied drug is the multi-kinase inhibitor sorafenib.[17, 18] In studies so far, Sorafenib has shown anti-tumor activity in mammarian cancer cells. This drug improved survival of patients who had diagnosed with breast cancer by inhibiting cancer cells metastatic, invasive and angiogenic properties.[17-19]

While both Sorafenib and Palbociclib have shown activity against breast cancer, it is possible that the combination could have additive or synergistic effects on inhibiting cell survival and proliferation pathways. The rationale for exploring the combination of palbociclib and sorafenib in breast cancer stems from previous research conducted in HCC. In hepatocellular carcinoma, the combination of palbociclib and regorafenib has been shown to exhibit synergistic effects, leading to enhanced antitumor activity.[20] This efficacy was associated with a significant down-regulation of CDK4/6-Rb-myc and mTORC1/p70S6K signaling pathways. Furthermore, regorafenib was found to suppress the palbociclib-induced expression of cyclin D1, thereby contributing to the cytotoxic effects of the combination therapy.

In addition to inhibiting cell viability and proliferation, the combination of palbociclib and regorafenib also demonstrated effects on glucose uptake. However, the impact of these treatments on glucose uptake varied depending on the specific cell model and the availability of oxygen (normoxia or hypoxia). The combination treatment was found to impair glucose uptake and utilization, which was accompanied by a down-regulation of various proteins involved in glucose metabolism, such as HIF-1α, HIF-2α, GLUT-1, and MCT4. Additionally, the activity and expression of glycolytic enzymes, including HK2, PFKP, aldolase A, and PKM2, were also reduced.[20]

The shared molecular pathways targeted by both drugs, such as the MAPK pathway, have been implicated in hepatocellular carcinoma, suggesting potential relevance in breast cancer as well. Therefore, the simultaneous targeting of the MAPK pathway by Sorafenib and Palbociclib could have a complementary effect and potentially enhance their overall anti-cancer activity in breast cancer cells.

Within the scope of this research, we aimed to elucidate the effects of sorafenib and palbociclib combination on pathways with an important role in cell survival and proliferation, such as AKT, ERK1/2 (extracellular signal-regulated kinase), Caspase-3 in hormon positive (MCF-7) and hormon negative (MDA-MB-231) cell lines.

Methods

No patient data or animal models were used in this study. The materials used for the study were only cell lines and this research was conducted in accordance with the principles of the Declaration of Helsinki.

Cell Culture

Hormone positive (MCF-7) and hormone negative (MDA-MB-231) breast cancer cell lines were prepared from the American Type Culture Collection (ATCC, Rockville, Md., USA). These cells cultured in a humidified incubator with

Eagle's Minimum Essential Medium (Capricorn, Cat No: MEM-A) containing 10% fetal bovine serum (FBS11-A), 2 mM L-Glutamine (Capricorn, Cat No:,GLN-B), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Capricorn, Cat No: PS-B) at 37°C with 5% CO_2. The medium was changed every two days and cells were passaged every 5 days.

Cell Viability Assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis was used to measure cell viability. Two thousand five hundred breast cancer cell lines were planted into 96-well plates. Fresh medium was added to the cells' medium after 24 hours. Cells were exposed to sorafenib and palbociclib at concentrations of 0.25, 0.5, 1, 2, 4, 8, and 16 M for 24, 48, and 72 hours. 15 ml of MTT solution with a concentration of 5 mg/ml were added to each well after the treatment period and incubated for 4 hours at 37 °C. The 100 l of DMSO was then added to media which contain MTT. At a wavelength of 570 nm, the microplate reader read the absorbance readings (Figs. 1, 2).

The following formula was used to assess cell viability: (mean optic density of samples (samples-blank)/(mean optic density of controls (controls-blank)) = viability (%) x100

Combinational Treatment

At doses between IC10 and IC25, sorafenib and palbociclib were used to treat breast cancer cell lines. Sorafenib (8 μM) was combine treated with palbociclib at 4 μM and 8 μM doses in MFC-7 cells. Also, sorafenib (4μM) was combine treated with palbociclib at 2 μM and 4 μM doses in MDA-MD-231 cells. MTT assay was used to find changes in cell viability (Fig. 3).

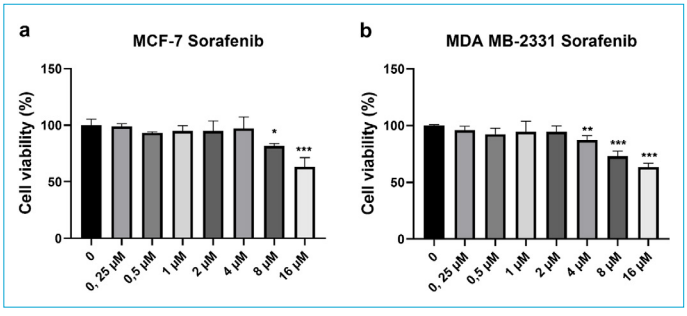

Figure 1. Effects of Sorafenib and Palbociclib on cell viability in MCF-7 cell lines.

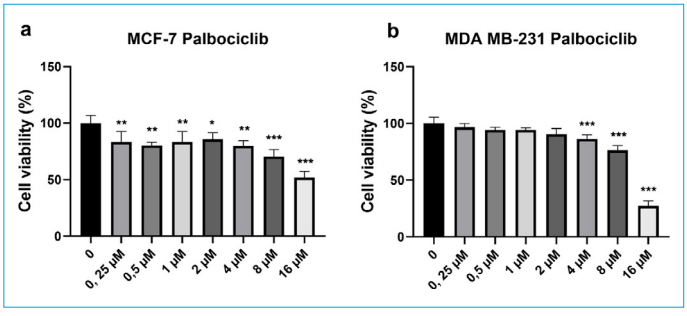

Figure 2. Effects of Sorafenib and Palbociclib on cell viability in MDA MB-231 cell lines.

Western Blot

Following the manufacturer's instructions, a BCA protein concentration determination test was used to determine the presence of proteins. For protein electrophoresis, 10% SDS polyacrylamide gels were produced, and 50 g of protein was injected into each well. Until the protein bands were visible, the samples were run. Proteins were transferred to PVDF membrane after the procedure had run. PVDF membranes were blocked for an hour at room temperature in a blocking solution made up of 5% BSA, Tris-buffered saline, and 0.1% Tween20. ERK1/2 (Cell Signaling, Cat. No. CS-4696), phospho-AKT (Ser473) (Cell Signaling, Cat. No: CS - 4060), AKT (Cell Signaling, Cat. No: CS - 2920), Caspase 3 (Cell Signaling, Cat. No: CS - 9662), and LC3 primer antibodies were treated with blocked membranes (Cell Signaling, Cat. No: CS -12741) and Calnexin (Santa Cruz, sc-23954) in 3%BSA at a 1:1000 concentration. Overnight,

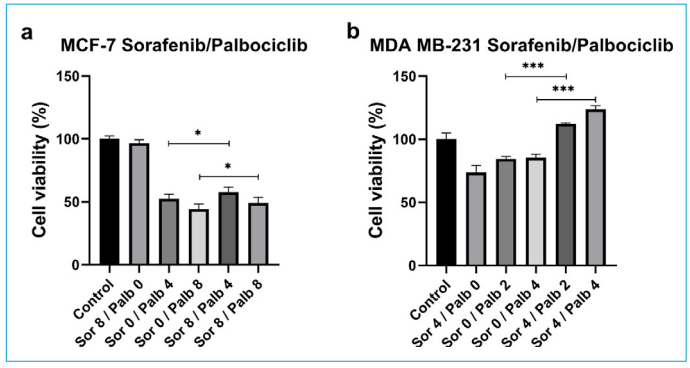

Figure 3. Effects of Sorafenib and Palbociclib combinations on cell viability.

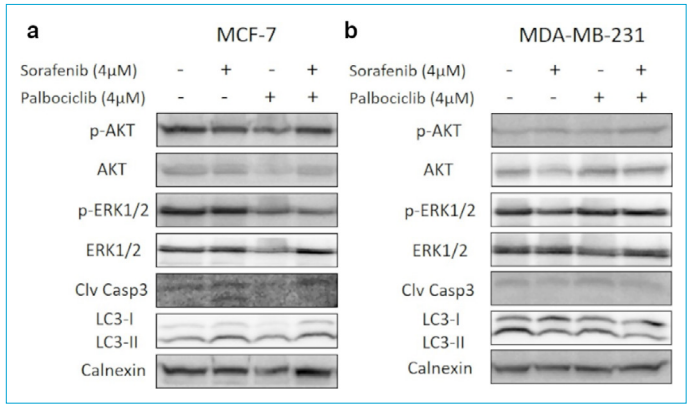

Figure 4. Determination of the effects of Sorafenib and Palbociclib combinations on AKT, ERK1/2 and apoptosis pathways.

the antibodies were incubated at +4 °C. Following incubation, the membranes were incubated for 2 hours at room temperature with secondary antibodies (Thermo, Cat. No. 23430, Thermo, Cat. No. 23460). Membranes were incubated in the dark with ECL (Enhanced chemiluminescence) solution for 5 minutes and protein expression levels were determined by imaging the membranes on a Kodak gel logic imaging device (Fig. 4).

Results

It was observed that a significant decrease was initiated in cell viability in MCF-7 cell lines starting from 8μM sorafenib dose. Palbociclib, caused a significant decrease in cell viability starting from the lowest dose of 0.25 μM. As a result, it was determined that both sorafenib and Palbociclib decreased cell viability in MCF-7 cell lines in a dose-compatibel manner.

Decreased cell viability was observed starting from the 4μM dose of sorafenib in MDA MB-231 cell lines. Palbociclib caused a significant decrease in cell viability from 4 μM dose. As a result, both Sorafenib and Palbociclib decreased cell viability in a dose-dependent manner in MDA MB-231 cell lines.

When the combination of 8 μM sorafenib + 4μM Palbociclib and 4μM Palbociclib alone were compared in MCF-7 cell lines, it was detected that 4μM Palbociclib alone reduced cell viability more than the combined application. However, both 2 and 4μM Palbociclib combinations caused an increase in cell viability in MDA MB-231 cell lines compared to their application alone.

No significant expression changes were detected in the level of phosphorylated AKT in both cell lines. ERK1/2 phosphorylation has decreased at the administered doses of Palbociclib compared to the doses administered with sorafenib in MCF-7 cell lines.

Additionally, no difference has been observed in ERK1/2 phosphorylation levels in MDA MB-231 cell lines. In MCF-7 cell lines, administration of sorafenib alone induced truncated caspase-3 expression, while Palbociclib alone reduced reverse truncated caspase-3 expression. The combination of sorafenib + Palbociclib, truncated caspase-3 expression and accordingly apoptosis decreased compared to the sorafenib administration alone. Decreased level of caspase-3 expression in MDA MB 231 cell lines was seen with combination regimen too.

Discussion

In this study, the effects of the combination of Palbociclib and Sorafenib on cell survival and proliferation pathways in human breast cancer cell lines were shown in detail.

The inhibition of the cell cycle by CDK 4/6 inhibitors results in phosphorylation of RB, which represses the transcription of multiple genes essential for cell cycle progression.[21] These drugs are very effective at inhibiting the growth of HR+ tumor cells and after these properties were proven by clinical studies and then started to be used widely in the clinic.[22]

Palbociclib is among best known cdk4/6 inhibitors and has significant clinical effectiveness in combination with aromatase inhibitors or fulvestrant for the treatment of metastatic BC (HR+ HER2-).[23] In our study Palbociclib dose-appropriately reduced cell viability in MCF-7 cell lines.

Drug resistance is the most important obstacle to treatment success. Unfortunately, in this type of cancer, resistance can develop against cdk 4/6 inhibitors as well as hormonal agents.[24]

Hormone resistance in breast cancer can arise through various mechanisms, including alterations in hormone receptor expression or function, activation of alternative signaling pathways, genetic mutations, and epigenetic modifications.[25] These changes can lead to the growth and survival of cancer cells independent of hormonal stimulation. Studies have identified several mechanisms of hormone resistance, including mutations or loss of ER expression, altered co-regulatory protein expression, and activation of downstream signaling pathways such as the PI3K/AKT/mTOR pathway.[25,26] In various experimental contexts, it has been observed that CDK4/6 inhibition can stimulate the activity of AKT or MTOR, two other proteins involved in cell signaling. Specifically, when CDK4/6 is blocked, there is a RB-dependent activation of AKT, facilitated by a protein complex called mTORC2. Conversely, it has also been demonstrated that CDK4/6 inhibition can cause changes in cellular metabolism that promote the activity of mTORC1.[27] There is evidence of crosstalk between CDK4/6 and MAPK signaling pathways. CDK4/6 activity can regulate the MAPK pathway by modulating the expression or activity of key components involved in MAPK signaling. Conversely, MAPK pathway activation can influence the activity of CDK4/6 complexes.[28]

Biopsy samples taken from tumors which were resistant to CDK 4/6 inhibitors indicated enrichment of functional hyperactivating changes in multiple genes: FGFR genes, RAS genes, ERBB2, PTEN, and AKT1.[29]

Preclinical and clinical studies have explored the use of combination therapies targeting both CDK4/6 and MAPK or PI3K/AKT/mTOR pathways to overcome resistance. For example, combining CDK4/6 inhibitors with MAPK pathway inhibitors (e.g., MEK inhibitors) has shown synergistic effects in preclinical models and may improve treatment

outcomes in patients with resistant breast cancer.[30]

One study by Vora et al. in 2014 demonstrated that combining CDK4/6 inhibitors with PI3K inhibitors had promising results in reversing resistance to endocrine therapy.[31] Another study by Michaloglou et al. in 2018 explored the combination of CDK4/6 inhibitors with mTORC1/2 inhibitors. mTORC1 and mTORC2 are two distinct complexes of the mTOR pathway.[32] The study suggested that combining CDK4/6 inhibitors with mTORC1/2 inhibitors could reverse resistance to endocrine therapy. By targeting both CDK4/6 and mTOR pathways simultaneously, the combination therapy exhibited a potential to overcome resistance mechanisms that develop during endocrine therapy. Todd W. Miller et all. in their research showed that directly inhibiting PI3K and mTOR resulted in the maximal inhibition of hormone-independent cell growth and the induction of apoptosis. However, the statement also indicates that inhibiting signaling kinases upstream (IGF-IR/InsR/ErbBs) and downstream (mTOR) of PI3K had only partial inhibitory effects.[33]

Due to the limited efficacy of these combinations, more potent kinase inhibitors that inhibit many downstream pathways needed to be tested with combined therapies. As a multikinase inhibitor, sorafenib shows antiproliferative and antitumoral effects by causing inhibition of both the down-regulation of the RAF/MEK/ERK pathway and angiogenesis.[34,35] Chinese investigators showed that Sorafenib significantly downregulated mTOR protein levels.[36] It was also shown that sorafenib inhibits cell proliferation in concentration-dependent manner.[37] A study published in 2014 examined the effects of sorafenib on tamoxifen-resistant hormone receptor-positive breast cancer cells. The researchers found that sorafenib inhibited cell growth and induced apoptosis in tamoxifen-resistant cells, suggesting its potential in overcoming resistance to endocrine therapy.[38]

In our research, sorafenib inhibited cell viability in both cell lines only at high doses which was consistent with previous study results.

Subsequently, researchers began to investigate the effectiveness of combining these multikinase inhibitors with other anticancer drugs.[20,39,40] Claudine Isaacs et al., demonstrated that combination of sorafenib and anastrozole produced an encouraging clinical benefit, suggesting that sorafenib may be able to restore sensitivity to hormone therapy.[41] The phase II study investigated the addition of sorafenib to endocrine therapy in patients with hormone receptor-positive breast cancer. The study included a total of 11 patients, with 7 cases receiving tamoxifen and one case each receiving anastrozole, letrozole, exemestane, and fulvestrant in combination with sorafenib. The addition of sorafenib to endocrine therapy was generally well-tolerated, with manageable toxicity, with most patients developing stable disease.[42]

The rationale behind combining CDK 4/6 inhibitors with sorafenib or regorafenib is based on the potential synergistic effects of targeting multiple pathways involved in tumor growth.[20,43] Sorafenib and regorafenib inhibit the RAF/MEK/ERK signaling pathway, while CDK 4/6 inhibitors disrupt the cell cycle progression. Combining these agents may lead to increased cell cycle arrest and apoptosis (programmed cell death) in cancer cells. Regorafenib showed increase cell cytotoxicity, inhibition of migration and invasion in hepatocellular carcinoma (HCC) cell lines, when combined with Cdk 4/6 inhibitors, more efficaciously than individual treatments.[20]

TNBC is known as the most aggressive type among breast cancer types.There is a little chance of targeted treatment for this breast cancer type. Therefore, there is an unmet need to identify molecular pathways that could be then therapeutically targeted. Disregulation of cell proliferation due to the activation of intracellular and intercellular signaling pathways is a common feature of all human cancers, and the maintenance of this signals is also an important marker for TNBC.[44]

The results of previous studies on a roll of CDK4/6 inhibitors in TNBC cell lines presented contradictory outcomes. Some preclinical studies demonstrated sensitivity of TNBC cell lines to the CDK4/6 inhibitors. Yajing Huang and collegues reported that 1/4 of TNBC patients showed amplification or deletion of CDKN2A. Hence, 23% of the patients showed significant mutations in the RB gene. All these mutations contribute to the overactivation of the CDK4/6 pathway and uncontrolled cell proliferation, so this type of tumors would likely benefit from CDK 4/6 inhibitors.[45] Another study showed that palbociclib significantly inhibited cell growth in RB-sufficient cell lines, but did not inhibit cell cycle in RB-negative cell lines.[46] In our study, it was observed that Palbociclib lines significantly affected cell viability in MDA MB-231 cell lines at higher doses (especially at 16mM doses).

But despite their in vivo and xenograft activity, in clinical trials they showed lowest clinical benefit rate. It is known that MAPK/ERK signaling pathway plays a vital role in the progression of TNBC too.[47,48] Activation of MAPK-kinase and phosphoinositide 3-kinase (PI3K)/Akt pathways, leads to tumor invasion, proliferation, metastasis and angiogenesis in TNBC.[48,49] Activation of these pathways causes resistance to CDK 4/6 inhibitors also in this type of breast cancer.[50] On the other hand, it has been shown in previous studies that sorafenib may be effective in triple negative

breast cancer. In various studies, sorafenib was found to induce cell death in hormone negative (MDA-MB-231 cells) cell lines.[34] Sorafenib also showed meaningful efficacy in triple negative breast cancer during clinical use.[51]

In our own research, we demonstrated the antiproliferative efficacy of using these drugs separately. We thought that we could benefit from the synergistic activity in combination for inhibition of both the cell cycle and intracellular pathways. The results of the study were not as we expected and we found both an increase in cell viability and a decrease in apoptosis.

In different studies it was shown that a significant proportion of patients develop endocrine therapy resistance due to activation of various signaling pathways, including dysregulation of (PI3K)/Akt/ (mTOR) signaling. Approximately, 35-40% of HR+/HER2− breast cancer cases showed hyperactivation of this pathway.[52]

In previous literature it was reported that Sorafenib inhibited Raf/MAPK signaling but activated PI3K/Akt pathway and this led to drug resistance.[53] The increase in cell viability with the addition of Palbociclib to sorafenib in MDA MB 231 cell lines and the addition of sorafenib to Palbociclib in MCF-7 series might be due to the activation of the PIK3 pathway. This has also to decreased caspase-3 activity. Thus, this combination caused suppression of apoptosis in tumor cells and increased viability rather than tumor inhibition.

In this study, we demonstrated that the simultaneous combination of the CDK4/6 inhibitor Palbociclib with multikinase inhibitor sorafenib could not induce enhanced anti-tumor effects in hormone receptor positive and triple negative cell lines although they have such effectiveness in separation.

Conclusion

Regarding the results of this study, one can say that intracellular pathways are more complex than they are thought. The drug combinations should not depend on a single pathway rather than considering all possible involved mechanisms of action.

Disclosures

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Ethics Committee Approval: The study was approved by the Local Ethics Committee.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – E.A.; Design – E.K; Supervision – E.G; Materials – E.A; Data collection/proccesing – E.A; Analysis and/or interpretation – E.K; Literature search – E.A; Writing – E.A; Critical review – E.G.

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