The synergistic anticancer effects of apatinib combined with PD-L1 inhibitor on breast cancer

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Research Article

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Abstract

Purpose: Apatinib have potential anti-angiogenic and anti-tumor activities in breast cancer. PD-L1 inhibitor also has a good effect on breast cancer . In this study we aimed to discuss the synergistic effect of apatinib combined with PD-L1 inhibitor and explore its mechanisms in breast cancer.

Method: We investigated the synergistic effects of apatinib and PD-L1 inhibitor on breast cancer in vitro and in vivo. MTT assay, Wound-healing, and transwell invasion assays were used to determine the effects of apatinib and PD-L1 inhibitor on cell proliferation, migration, and invasion of MCF-7 and MDA-MB-231 cells in vitro. Western blotting was performed to assess the expression of Erk, p-Erk, NF-κB and slug. In vivo, xenograft model were established to test the effect of single drug or the combined therapy in the nude mice.

Results: The results indicated that apatinib and PD-L1 inhibitor significantly inhibit proliferation, migration and invasion of MCF-7 and MDA-MB-231 cells. The combined treatment showed synergistic anticancer activities. Moreover, apatinib and PD-L1 inhibitor significantly downregulated the expression of p-Erk, NF-κB and slug in breast cancer cells.

Conclusion: Apatinib and PD-L1 inhibitor show synergistic effects both in vitro and in vivo,which may provide a more effective treatment option for breast cancer patients.

1. Introduction

Breast cancer (BC) is the malignancy tumor with the highest incidence in women in the world[1]. There are a variety of treatment methods for breast cancer, including chemotherapy, endocrine therapy and targeted therapy. Apatinib is a novel tyrosine kinase inhibitor that selectively binds and inhibit vascular endothelial growth factor receptor 2 (VEGFR-2). Apatinib can suppress tumor development by blocking VEGF/VEGFR2[2]. Untill now, anecdotal evidence suggests that apatinib has a good efficacy in many tumors including breast cancer[3–5], indicating that apatinib has a strong inhibitory effect on breast cancer cells. However, targeted drugs are not a panacea. Therefore, there have been efforts to develop better therapeutic schedule with better targeted therapeutic effects and fewer side-effects.

PD-L1 was discovered and used as a ligand of PD-1,which is highly upregulated on tumor cells, intratumoral macrophages and dendritic cells. In the presence of T cells,tumor cells can suppress the immune function of T cells through the PD-1/PD-L1 signaling pathway, so as to evade immune surveillance and achieve immune escape[6]. In the absence of T cells, PD-L1 inhibitors can also play a corresponding anti-tumor effect. Tumor cells can also be identified and killed by T cell-independent pathways such as ADCC and CDC. In recent years, a variety of PD-1/PD-L1 antibodies have been approved for the clinical application of tumor therapy, whether single drug or combined chemotherapy[7]. However, previous studies have shown that PD-L1 blocking alone agent causing tumor occurrence and

adverse clinical consequences[8, 9]. The efficacy of monotherapy PD-1/PD-L1 blockade was only 20–40% in most cancer types[10–12]. Immunotherapy is once again at the forefront of cancer research.

Since both drugs are approved for clinical use, our studies have a very strong clinical releva. In this study, We explored that the anti-tumor effects of PD-L1 inhibitor or apatinib alone and the combination therapy were assessed in vitro and vivo,and explore the mechanism of synergistic effect,which may help to make the best use of apatinib and PD-L1 inhibitor combination therapy for appropriate patients in cancer treatment. Treatment for breast cancer has been revolutionized with the interaction of immunotherapy and targeted therapy.

2. Materials & Methods

2.1 Cell culture and compounds

Human ATC cell lines MDA-MB-231 and MCF-7 were purchased from the China Center for Type Culture Collection (CCATCC, China). The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. The cells were cultured at 37℃ in a humidified incubator with 5% CO2. All cultures were mycoplasma-free.

Apatinib was obtained from Hengrui Medicine Co. Ltd. (Jiangsu,China), dissolved in DMSO and diluted with PBS medium to the desired concentration with a final DMSO concentration of 0.1% for in vitro studies. PD-L1 inhibitor(SHR-1316) was obtained from Shengdia Biomedical Co,Ltd. (Suzhou,China), dissolved in glycerinum in vitro and in vivo studies.

2.2 Histology and immunohistochemical staining

PD-L1 expression was analyzed in a broad panel of MCF-7 and MDA-MB-231 cells by immunohistochemistry. PD-L1 primary antibody was used for overnight incubation. The immunostaining score was estimated based on the positive cell and the staining intensity.

2.3 Cell viability assay

Cell viability was assayed by the MTT assays. MCF-7 and MDA-MB-231 cells were seeded in 96-well plates at a density of 3000–5000 cells/well and were treated for 48 h with DMSO, apatinib, PD-L1 inhibitor or both drugs in combination. The MTT solution was added and then cultured for another 4 h, and the medium was subsequently removed. Next, 150 μl of DMSO was added to dissolve the formed formazan crystals. The absorbance of each well was measured at 570 nm by a microplate reader (Bio-Tek, Norcross, GA, U.S.A.). The mean IC50 values were calculated by SPSS. Combination index (CI) values were calculated using CompuSyn (ComboSyn Inc.).

2.4 Scratch-wound healing assay and Transwell invasion assay

Cell migration ability was measured by scratch-wound healing assay. Briefly, We plated 1×10^6 cells/well in 6-well plates and cultured overnight until the cells reached 90% confluence. A straight scratch was created by a sterile pipette tip at 0 h and 24h. The cells that migrated into the denuded zone of each dish were quantitated in a field of view using ImageJ software (NIH, Bethesda, MA, USA.).

Cell invasion assays were performed using 24-well plates and 100 μl of cell suspension (0.5×10^6 cells/ml) containing apatinib and PD-L1 inhibitor. For the invasion assay, the upper chamber was coated with Matrigel. There was serum-free medium in upper chamber while medium contains 20% FBS in lower chamber. The cells were cultured in an incubator at 37˚C with 5% CO2 for 24 h. The migrant cells were fixed with 4% paraformalde-hyde, stained with 0.1% crystal violet and counted in five random fields at 100× magnification.

2.6 Flow cytometry analysis of cellcycle &apoptosis

For cell cycle arrest assay, The MCF-7 and MDA-MB-231 cells were seeded in six-well plate at a density of 0.5×10^6 cells per well overnight. After treatment for 24 h, the treated cells were harvested, washed with PBS, and fixed with precooled 70% ethanol in the dark at -20°C for 1 h. Then, the fixed cells were washed with PBS and applied in the RNase I treatment at 37°C for 30 min. Finally, the cells were stained with PI staining at 4°C for additional 30 min and then measured through BD FACS caliber.

Regarding the cell cycle apoptosis, cells were treated as previously described. Cells treated with Annexin V-FITC/PI apoptosis detection kit based on the manufacturer's protocol. Finally, the cells were detected by BD FACS caliber, using the BD CellQuest Pro software for analysis.

2.7 Western blotting

After treatment, MCF-7 and MDA-MB-231 cells were harvested and lysed, and prepared for cytosolic and nuclear protein extraction using a cytoplasmic and nuclear protein extraction kit (Cowin Bio, Beijing, China) according to the manufacturer's instructions. The membrane was incubated with primary antibody at 4°C overnight. Subsequently, secondary antibodies were incubated at 37°C for 1h. Finally, the membranes were washed with TBST and detected in Tanon 2500 chemiluminescence imaging system (Tanon, Shanghai, China). The protein levels were quantitated by densitometric analysis using ImageJ software (NIH, Bethesda, MA, USA.). Erk, p- Erk, NF-κB and Slug was purchased from Cell Signaling Technology (Boston, MA, USA).

2.8 In vivo animal studies

Female nude mice (4–6 weeks) were raised in a specific pathogen-free (SPF) animal facility (Temperature, 20–26°C; humidity, 40–60%; 12/12-h light/dark cycle; free access to food and water). MDA-MB-231 cells were subcutaneously injected with 0.2ml suspension (1×10^6 cells) and inoculated into the abdominal cavity of female BALB/C nude mice. After injection, the nude mice were observed without reaction, and the mice were routinely fed for observation. When the tumor volume reached nearly $60mm^3$, mice were randomly assigned into 4 groups. Each group of mice (n = 6) was treated via daily oral gavage

with vehicle apatinib (50mg/kg/d), intravenous injection PD-L1 inhibitor (10 mg/kg) on days 3,6 and 10, or a combination of both drugs. The weight and tumor size were measured twice a week. Tumor volume was calculated as V= (length × width^2)/2. All procedures involving animals were approved and monitored by the animal care and use committee of Henan University of Science and Technology, and were performed in accordance with the institutional guidelines.

2.6 Statistical analysis

All experiments were repeated independently at least three times. Statistical analysis was performed using IBM SPSS 25.0 software (SPSS, Chicago, IL, USA). All the data were presented as mean value ± SD, and statistically significant differences between different experimental groups and control group were examined using one-way analysis of variance (ANOVA), $P<0.05$ was considered as statistical significant difference. In the figures, symbols were used as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3. Results

3.1 PD-L1 inhibitors can inhibit the growth of breast cancer cells in vitro. Apatinib and PD-L1 inhibitors have a synergistic inhibitory effect on breast cancer cells

As shown in Fig. 1, the results showed that both apatinib and PD-L1 inhibitor could inhibit the cell viability of the two types of cells in a dose-dependent manner(Fig. 1a,g). PD-L1 inhibitors can inhibit the growth of breast cancer cells in vitro,and the ability of inhibited cancer cells of apatinib is more than PD-L1 inhibitor. Also ,we can see that the apatinib sequential PD-L1 inhibitor group had a stronger anti-tumor effect than the two other combination therapies(Fig. 1b,h). Therefore, we used apatinib sequential PD-L1 inhibitor regimen for subsequent animal studies. Next, we investigated the combined effects of the two drugs at different concentrations(Figure 1d-f,j-l),with significant anticancer effects observed at higher concentrations. By detecting the combination index of the two drugs, we found that apatinib combined with PD-L1 had a synergistic effect. CI values<1(Fig. 1c,l).

3.2 The combination of apatinib and PD-L1 inhibitor can inhibit the metastasis and invasion of breast cancer cells to the greatest extent.

The effect of apatinib and PD-L1 inhibitor on the migration of MCF-7 and MDA-MB-231 cell was analyzed by wound-healing assays. In MCF-7 cells (Fig. 2a), the migration rate of MCF-7 cells significantly decreased with the increase of the concentration of apatinib and PD-L1 inhibitor ($p<0.05$) compare with the control group. The migration rate of PD-L1 inhibitor group was slower than apatinib group. The combination group had less mobility and better drug response. We got similar results in MCA-MB-231 cells (Fig. 2b).

Similar conclusions were found in the transwell invasion assay(Fig. 2c,d). Except for the low group of PD-L1 inhibitor, the remaining groups were statistically significant in MCF-7 cells ($p < 0.05$, Fig. 2c). However, in MDA-MB-231 cells, only the high concentration group and the combination group had statistical

significance($p < 0.05$,Fig. 2d). In summary, The combination of apatinib and PD-L1 inhibitor can synergistically inhibit the metastasis and invasion of breast cancer cells.

3.3 Apatinib and PD-L1 inhibitor induced apoptosis but did not affect cell cycle

The distribution of cell cycle phase in MCF-7 and MDA-MB-231 cells treated with apatinib and PD-L1 inhibitor(Fig. 3a ,b). Compared with the control group, neither Apatinib nor PD-L1 inhibitor affected the cell cycle in MCF-7 and MDA-MB-231 cells.

To further investigate the apoptosis on MCF-7 and MDA-MB-231 cells under the effect of apatinib and PD-L1 inhibitor, the apoptosis rate was conducted by Annexin V-FITC/PI double staining followed (Fig. 3c,d). Apoptosis rate increased in drug treatment group compared to the control group in MCF-7 cells(Fig. 3c) and MDA-MB-231(Fig. 3d). However, PD-L1 inhibitors can promote apoptosis in a concentration-dependent manner. The combination group had better apoptotic effect than single group. In MCF-7 cells, apoptosis mainly occurred in the early stage of cells, and the combination of drugs significantly increased the rate of apoptosis in the early stage. However, there are relatively many late apoptotic cells of MDA-MB-231 cells. Apoptosis rate can be inhibited by apatinib and PD-L1 inhibitors, and the combination of the two drugs can achieve the maximum inhibition degree. Compared to the control, apatinib and PD-L1 inhibitor promotes apoptosis and induces cellular stress in MCF-7 and MDA-MB-231 cells.

3.4 The combined action of apatinib and PD-L1 inhibitor can inhibit the phosphorylation of ERK, reduce the protein synthesis of NF-KB and reduce the expression of slug.

We further elucidated the molecular mechanism for the synergistic anti-tumor efficacy of apatinib and PD-L1 inhibitor in breast cancer cells. The results showed that p-Erk, NF-κB and Slug activity were tremendously decreased by apatinib and PD-L1 inhibitor treatment after 48 h incubation (Fig. 4a,b). The effect of single drug on the reduction of ERK and NF-KB was not obvious through statistical analysis (Fig. 4c,d). Some monotherapy groups were not statistically significant($P>0.05$), but they were statistically significant after combination therapy($P<0.05$). Neither apatinib or PD-L1 inhibitor effected the expression of total Erk. These results could explain the superior synergistic effects of apatinib and PD-L1 inhibitor. Apatinib and PD-L1 inhibitor decreased invision of breast cancer cells by the singling pathway of p-Erk/NF-κB/slug.

At the same time, we examined the survival analysis of the three factors in Kaplan-Meier Plotter(Fig. 4e). The results showed that p-Erk,NF-κB and Slug were all significant in the breast cancer($p<0.05$). Inhibition of the expression of these markers could inhibit the occurrence and development of breast cancer to a certain extent.

3.5 The synergistic effects of apatinib and PD-L1 inhibitor effectively inhibited tumour growth in mice

The tumor model was established to further evaluate the inhibitory effect of the two drugs on tumor growth either individually or synergistically. Experimental mice were treated on the planned schedules:

apatinib once a day, PD-L1 inhibitor started on day 3(Figure 5a). In vivo antitumor efficacy studies, BALB/C nude mice were treated with placebo, apatinib, PD-L1 inhibitor or apatinib combined with PD-L1 inhibitor (Fig. 5b). The growth rate of cancer cells in control group is very fast and unbounded,with the average of tumors reaching sizes 874mm^3 at day 21 (Fig. 5d).Compared to control group, apatinib and PD-L1 inhibitor alone displayed mild inhibition effects on tumor growth. Then on days 21, tumour sizes and weight in Joint group significantly decreased compared with the single drug group (Fig. 5c, d). The combined treatment group had the best effect on the tumor compared with the monotherapy group. (Fig. 5b,c). Futhermore,the results indicated that apatinib was more effective than PD-L1 inhibitor about the ability of inhibiting tumor growth ($p<0.05$, Fig. 5c,d). There was no significant difference in the weight of mice in each group. (Fig. 5e). All the treatment caused a significant inhibition in tumor growth compared with the control group ($p<0.05$). Collectively, apatinib in combintion with PD-L1 inhibitor may result in tumor regression or stasis in breast cancer.

Discussion

It is well known that breast cancer is the most common cause of death in women[13]. There are various treatment therapeutic regimen,including targeted therapy and immunotherapy. Apatinib has a good antitumor effect in breast cancer[14]. Correspondingly, cancer cell will death when researcher block the PD-1/PD-L1 by activating tumor cell-intrinsic signaling[15]. However, The effective rate of single drug is only about 20%[16]. There have clinical studies shown that the combination of immunotherapy and targeted therapy has a good effect on tumor treatment. Zhang yu[17] suggested that apatinib combined with PD-L1 blocking therapy could synergistically enhance the anti-tumor immune response in GC. And in liver cancer,the control rate of advanced liver cancer is 93.3% through the combination of the two drugs[18]. This combination can extend the benefit of PD-L1 inhibitor treatment to a larger population of breast cancer.

In this study, we demonstrate a critical role of PD-L1 inhibitor and apatinib in conferring breast cancer with a variety level of studies. We find PD-L1 expressed in both MCF-7 and MDA-MB-231, while PD-L1 expressed stronger in MDA-MB-231 than in MCF-7 (Supplementary 1). Then, we showed that the targeted drug apatinib synergized with PD-L1 inhibitor against breast cancer in vitro and in vivo. MTT assay showed that PD-L1 inhibitor significantly inhibited the cell viality of the breast cancer MCF-7 and MDA-MB-231 cell line in a concentration-dependent manner. The same conclusion is true for apatinib. At the same time, a large number of studies have proved that apatinib[19]and PD-L1 inhibitor[20]can inhibit the occurrence and development of MCF-7 and MDA-MB-231 cells.we observed increased antitumor efficacy when apatinib was combined with anti–PD-L1 in the in vitro. Samely,Sha Zhao[21]think that apatinib can potentiates antitumor effect of PD-L1 blockade in cancer. In the synergistic experiment, the combination of the two drugs plays a synergistic effect in the treatment of cancer. Moreover, We find that apatinib sequential PD-L1 inhibitor have better effect than the other two combination mode. There are studies that apatinib combined with PD-L1 blockade synergistically enhances antitumor immune responses[22, 23]. In the invasion and metastasis experiment, Our study showed that apatinib and PD-L1 inhibitor significantly

inhibited cell migration and invasion in the breast cancer cells. We observed different therapeutic effects with different concentrations of the drug. When the drug concentration increases, the invasion and metastasis ability of cells decreases. Compared with the single drug group, the number of cells invading the lower chamber in the combined group decreased by about twice. In Haige Zhang ‘s study,apatinib can suppress proliferation and invasion of the cancer cells via angiomotin inhibition[24]. Samely, a Phase 1B JAVELIN solid tumor study achieve expectations with acceptable side effects in metastatic breast cancer[25]. The effects of apatinib and PD-L1 inhibitor mainly occurred in the early stage of apoptosis on MCF-7 cells. While the result of drugs on MDA-MB-231 cells mainly focused on the late stage of apoptosis. We suggest that apatinib may induce apoptosis in cells by PI3K/AKT or MAPK/ERK signaling pathways. Then, we find that apatinib can also triggers apoptotic cell death via influencing PD-L1 signaling in cancer[26]. In a certain dose range, with the increase of concentration, the depressor effect of the drug is stronger. And again, we found that apatinib Combined with PD-L1 inhibitor synergistically enhances antitumor immune responses in other malignant tumor[23, 24].

To gain insight into the potential molecular mechanisms and signaling pathways involved in the synergistic anticancer effects in breast cancer cell line, we performed the western blotting to evaluate molecular changes upon mono or combination therapy. Apatinib and PD-L1 inhibitor through downregulated p-Erk and reduced the protein level of NF-κB in the breast cancer cells, and then downregulated its regulatory target Slug, therepy inhibit cell invasion and metastasis. The inhibition of p-Erk, NF-κB and Slug at lower concentrations was not obvious. In parallel, the inhibition of signaling pathways was most significant when combined drug therapy was used. Consistent with the result of Zhou[27] et al. Our results suggest that PD-L1 inhibitors and apatinib can inhibit Erk expression in breast cancer in a concentration-dependent manner. Recent evidenced have shown that the mechanisms of PD-L1 inhibitor were related with a variety of signaling pathways and specific molecular mechanism[28, 29]. The antitumor effects of apatinib reportedly have been reported at downstream of the VEGFR2 signaling pathway via PI3K/AKT/mTOR or MAPK/ERK[30]. Mechanistically, PD-L1 prominently activated epithelial mesenchymal transition (EMT) process through the MEK/Erk signaling pathway[31]. In this study, PD-L1 inherent in cancer cells can also promote the transduction of intracellular signaling pathway Ras/p-Erk/slug[31]. More deeply, apatinib can weaken the expression of PD-L1 by inhibiting the ERK signaling pathway. However, Inhibition of activation of ERK was a key point for suppression of NF-κB/p65 modulated metastatic mechanism in Jin's study[32]. The inhibition of NF-κB/p65 can take the edge off the slug and reverts the EMT expression pattern[33]. The Slug may be involved in the resistance of epithelial cells to apoptosis and promote tumor invasion through some mechanisms[34],and is regulating the invasion and growth of breast cancer[35]. Consistent with prior study, PD-L1 overexpression or knockout affects the proliferation and migration of cancer cells through Erk signaling pathway[36]. Meanwhile, MAPK/ERK signaling pathway can affect the expression of PD-L1[37, 38]. Therefore, these findings indicate that combination of apatinib and PD-L1 inhibitor can inhibit cell proliferation, reduce migration and invasion ,increase apoptisis possibly by attenuating the expression of p-Erk/NF-κB/Slug in breast cancer cells.

To confirm this synergy on tumor cells, we also test the effects of apatinib and PD-L1 inhibitor in vivo. The results indicated that the anticancer effect of the combinatorial treatment was higher than any other single drug, which was consistent with in vitro. In addition, nude mices were treated with the combinatorial treatment did not show worse body weights than the patients in the groups treated with apatinib or PD-L1 inhibitor alone. It indicates that the drug toxicity is within the acceptable range. Hence, our data show that a combination of apatinib and PD-L1 inhibitor led to enhanced antitumor activity. The studies from Schmittnaegel[39] evidenced that antiangiogenic agents could improve anti–PD-L1 therapy in various tumor models. The Combination of antiangiogenic and anti–PD-L1 therapy can stimulate Body tumor immunity in a previous study[40]. There were studies that demonstrated the effect of apatinib in combination with PD-L1 mAb from another perspective[41]. There are a closing correlation between targeted therapy and immunotherapy in cancer[42]. Similarly, apatinib enhances anti-tumor efficacy when combined with immunotherapy in preclinical studies[40, 43]. Apatinib not only inhibits angiogenesis, but also affects PD-L1, thereby mediating antitumor immunity. Nevertheless, We need to conduct more experiments to confirm the clinical efficacy of apatinib combined with PD-L1 inhibitor on breast cancer.

In summary, this work reveal the antitumor activity of apatinib combined with PD-L1 inhibitor on breast cancer in vitro and in vivo. The study supposed that the interaction of apatinib and PD-L1 inhibitor induced the activation of p-Erk/NF-κB/Slug cascade, thus promoting Breast cancer cell migration/invasion (Fig. 6). It provides partial theoretical basis for the combination of targeted therapy and immunotherapy for breast cancer. However, there are many challenges remain to be overcome before the potential of The combination therapy can be realized.

Declarations

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Author contributions

Xinshuai Wang conceived and designed the experiments. Danyang Han, Hongxia Hu, Jing Li performed the experiments. Zhiwei Liu, Xinyang Li, Ziming Wang analyzed the data. Danyang Han and Xinshuai Wang wrote the paper. All authors reviewed the manuscript.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Disclosure

The authors report no conflicts of interest in this work.

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Figures

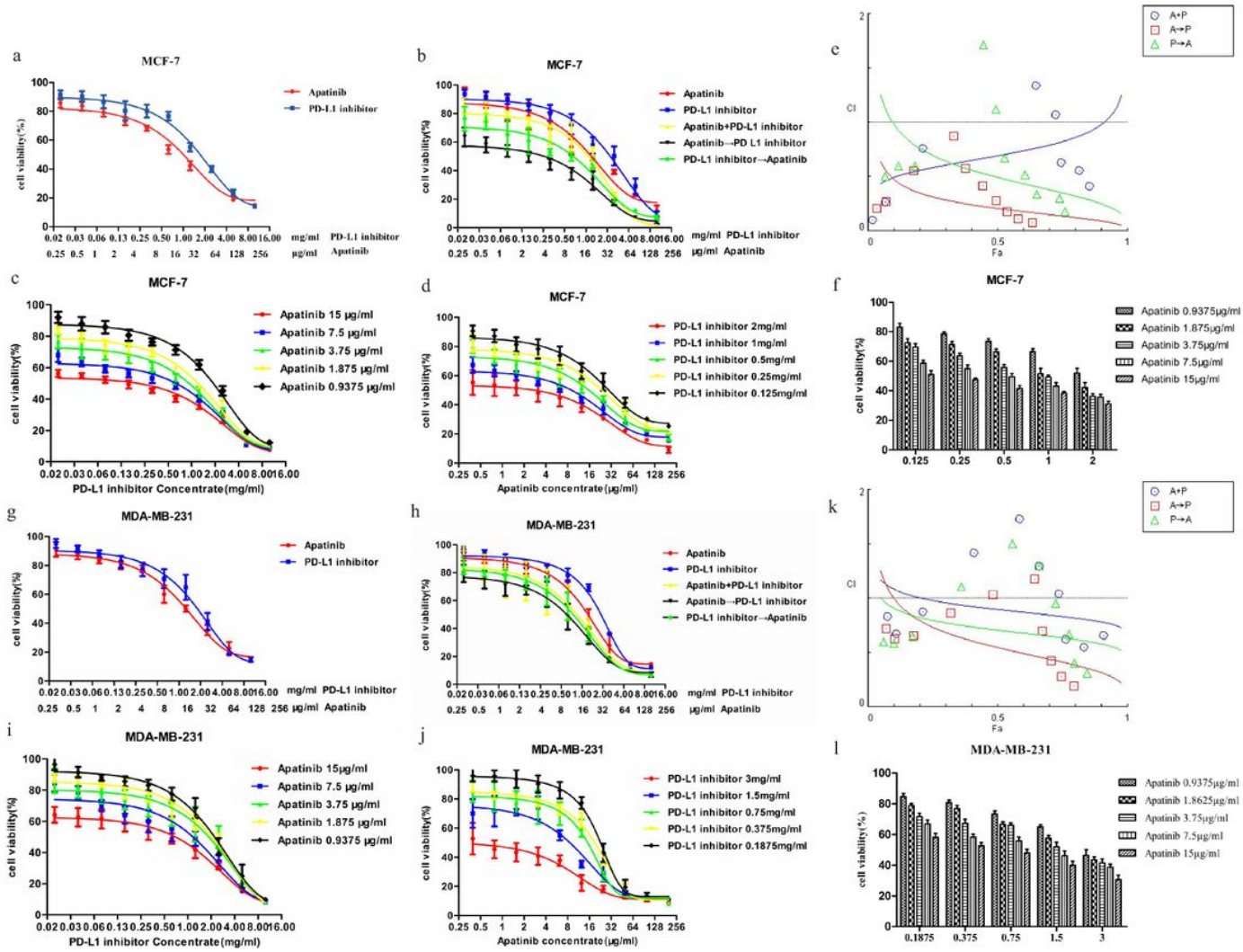

Figure 1

Apatinib and PD-L1 can synergistically inhibit breast cancer cell growth

(a,g) Proliferation activity of MCF-7 or MDA-MB-231 cells was determined by the MTT assay after incubation for 48h with different concentrations of apatinib or PD-L1 inhibitor. (b,e) MCF-7 or MDA-MB-231 cells were treated with apatinib or PD-L1 inhibitor alone or in combination or in sequences (apatinib first for 6 h followed by PD-L1 inhibitor or PD-L1 inhibitor first for 6 h followed by apatinib). (c,f) The combination index (CI) affected plot was calculated by Compusyn and depicted the combination effects. Synergy is defined as CI values < 1.0; antagonism as CI values > 1.0; and additivity as CI values=1.0. (d,e,j,k) Proliferation activity of MCF-7 cells was determined after incubation for 48h with different concentrations of PD-L1 inhibitor combination with apatinib. (f,l) Quantifying j and d.

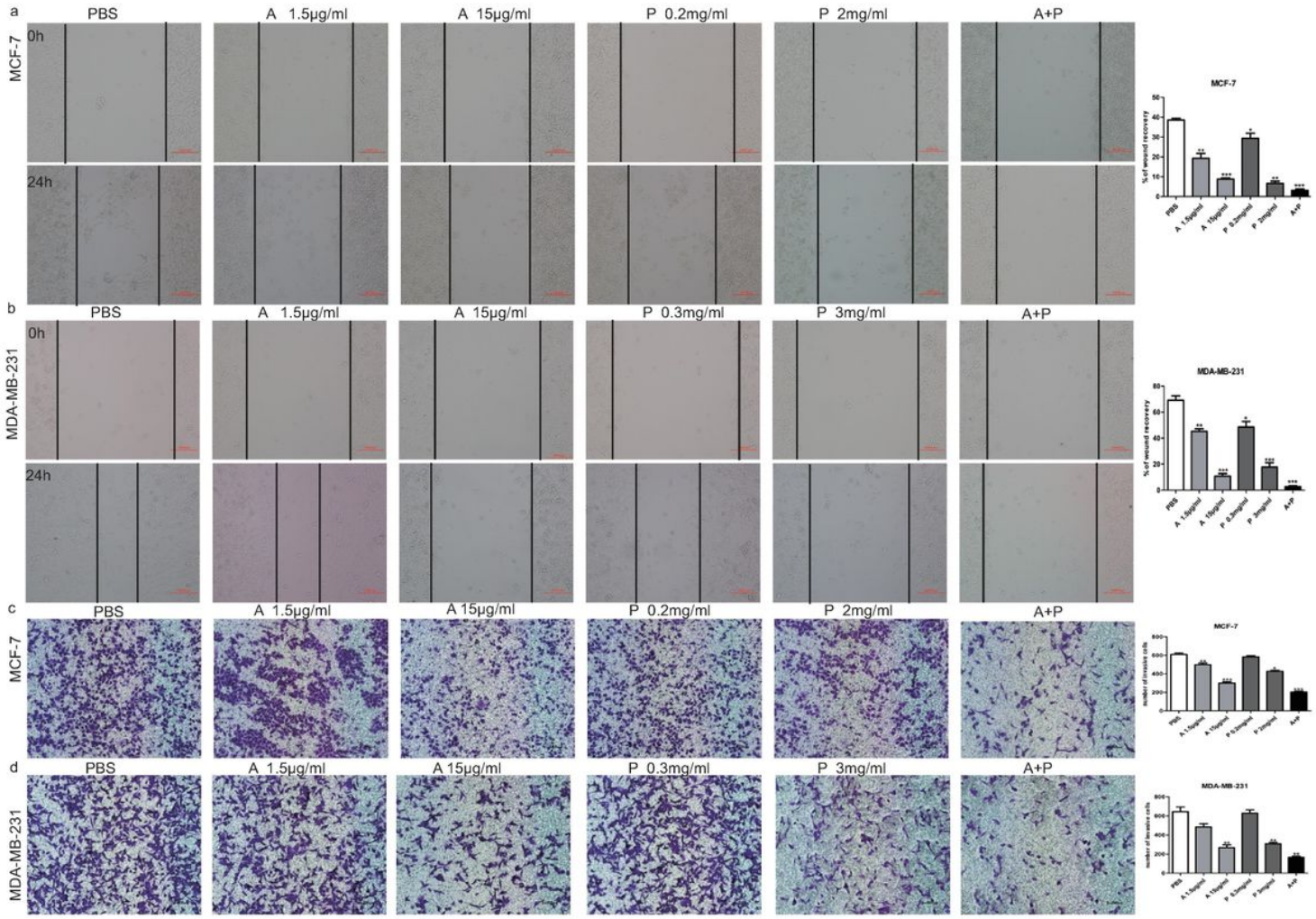

Figure 2

Effects of apatinib and PD-L1 inhibitor on migration and invision of cancer cells.

Wound healing assay assessed the effect of apatinib and PD-L1 inhibitor on cell migration ability . (a) The migration of MCF cells changed with drug treatment . (b) The migration of MDA-MB-231 cells changed with drug treatment .

Transwell invasion assays assessed the effect of apatinib and PD-L1 inhibitor on cell invasion ability. (c) MCF-7 cells were treated with PBS, different concentrations of apatinib (1.5, 15μg/ml) and PD-L1 inhibitor (0.2, 2mg/ml) for 24 h. (d) MDA-MB-231 cells were treated with PBS, different concentrations of apatinib (1.5, 15μg/ml) and PD-L1 inhibitor (0.3, 3mg/ml) for 24 h. Data represent the mean ± S.D. of three independent experiments. *p□0.05 , **p□0.01 and ***p□0.001 compared with the control

A:Apatinib; P:PD-L1 inhibitor; A+P:Apatinib+PD-L1 inhibitor

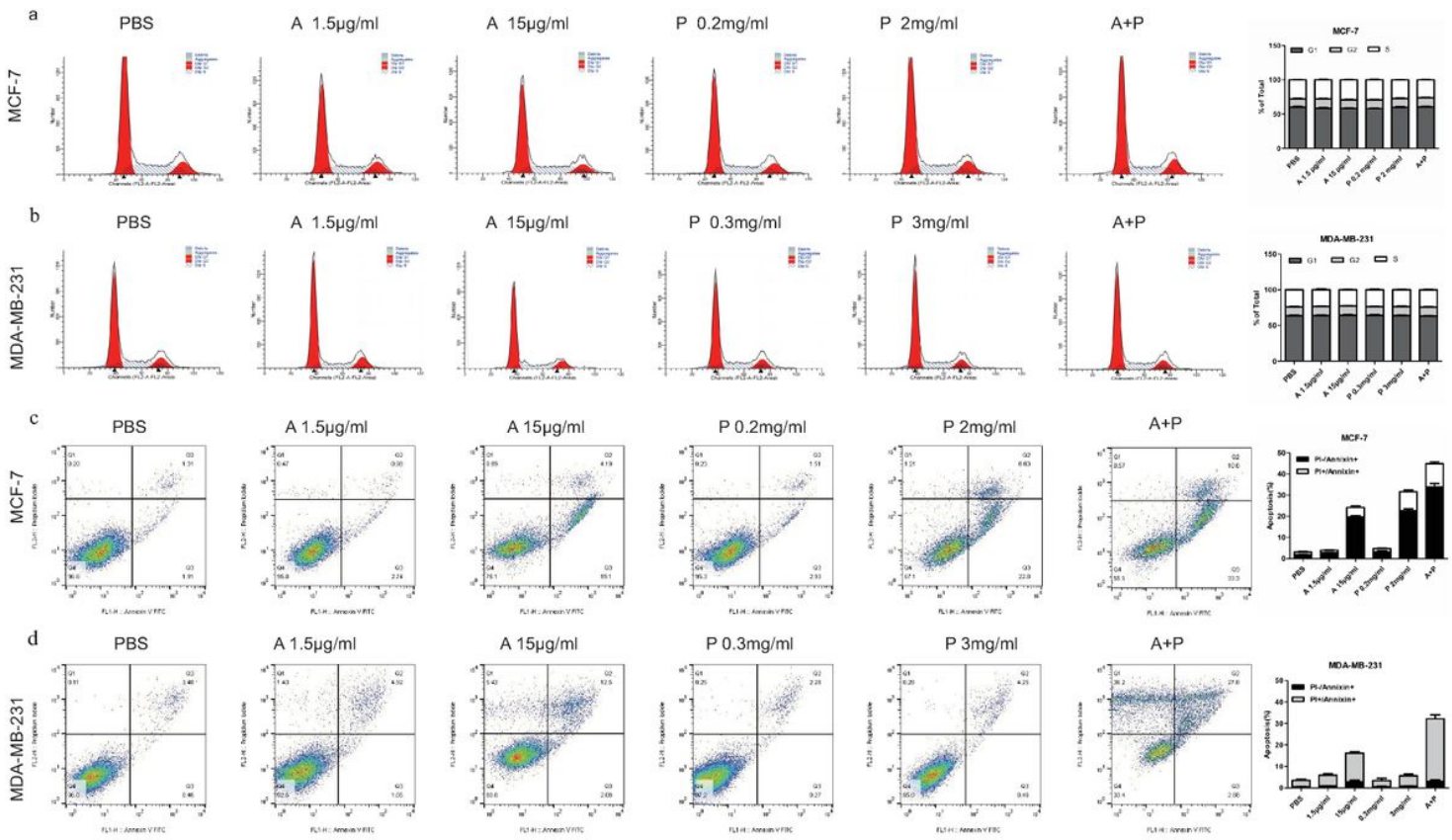

Figure 3

Effects of Apatinib and PD-L1 inhibitor on the cell cycle and apotosis.

(a) Cell cycle analysis through PI staining and following flow cytometry for MCF7 or MDA-MB-231 cells after incubated with PBS, different concentrations of apatinib(1.5, 15μg/ml) and PD-L1 inhibitor (0.2, 2μg/ml) . (b)Cell cycle analysis for MDA-MB-231 cells after incubated with PBS, different concentrations of apatinib(1.5, 15μg/ml) andPD-L1 inhibitor (0.3, 3μg/ml) for 24 h. The histograms were the representative results.

(c)Cell apoptosis was detected through Annexin V-FITC/PI double staining and following flow cytometry for MCF-7 or MDA-MB-231 cells after incubated with PBS, different concentrations of apatinib(1.5, 15μg/ml) and PD-L1 inhibitor (0.2, 2μg/ml) or). (d)Cell apoptosis was detected for MDA-MB-231 cells . The histograms were the representative results,

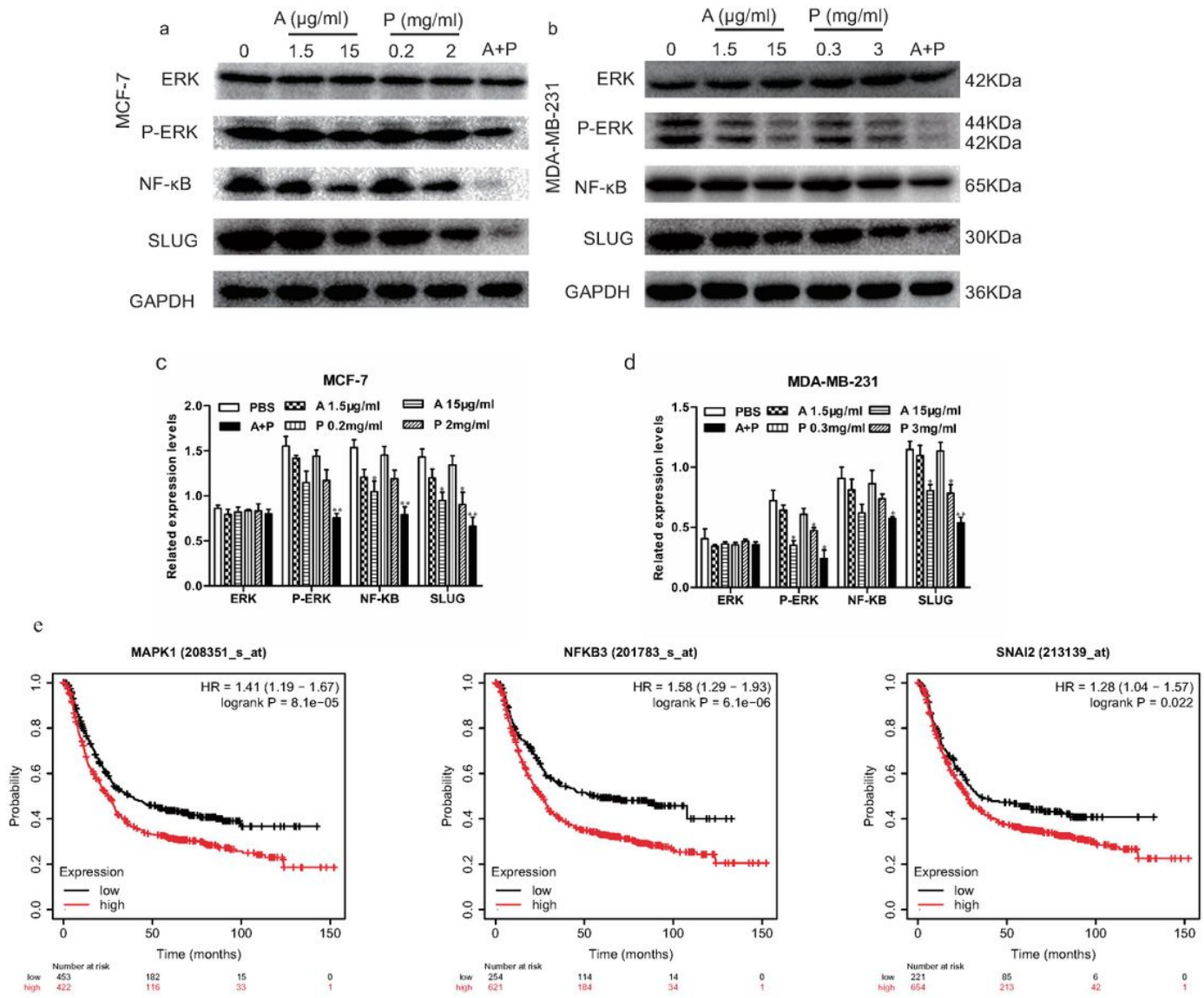

Figure 4

Molecular mechanism in MCF-7 or MDA-MB-231 cells after treatment of PBS, Apatinib, PD-L1 inhibitor or a combined treatment.

(a,b) The results of Western blot for Erk, p- Erk, Nf-κB and Slug in the nuclear fractions and cytosolic extracts, respectively in MCF-7(a) and MDA-MB-231(b). (c,d) Quantitative analysis of the Western blotting results. *p□0.05 , **p□0.01 and ***p□0.001 compared with the control.□e□Survival analysis of three factors was tested in Kaplan-Meier Plotter

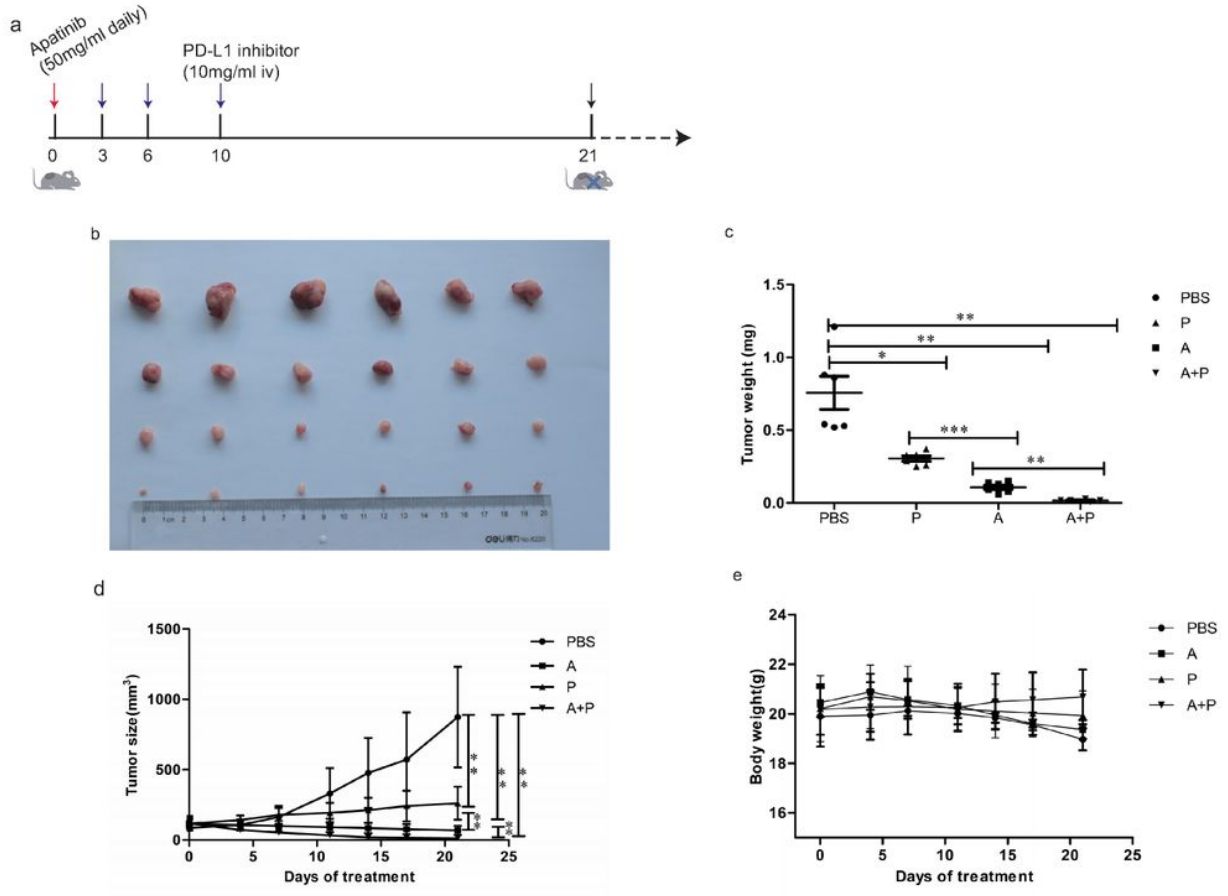

Figure 5

In vivo anticancer effect of apatinib and PD-L1 inhibitor in breast cancer xenograft models.

(a) Chart describing the treatment schedule of xenograft model. The red arrow indicates taking apatinib. The blue arrow indicates the injection of PD-L1 monoclonal antibody. (b) Randomly grouped nude mice were treated with PBS, Apatinib,PD-L1 inhibitor, or a combination treatment (A+P) for 21 days. Photos of the excised tumors obtained on day 21 after treatment. (c) Weight of tumor after the day 21. (d) Tumor growth ratio curve and body weight changes every three days after the onset of treatment. (e) The mice weight changes every three days after the onset of treatment.

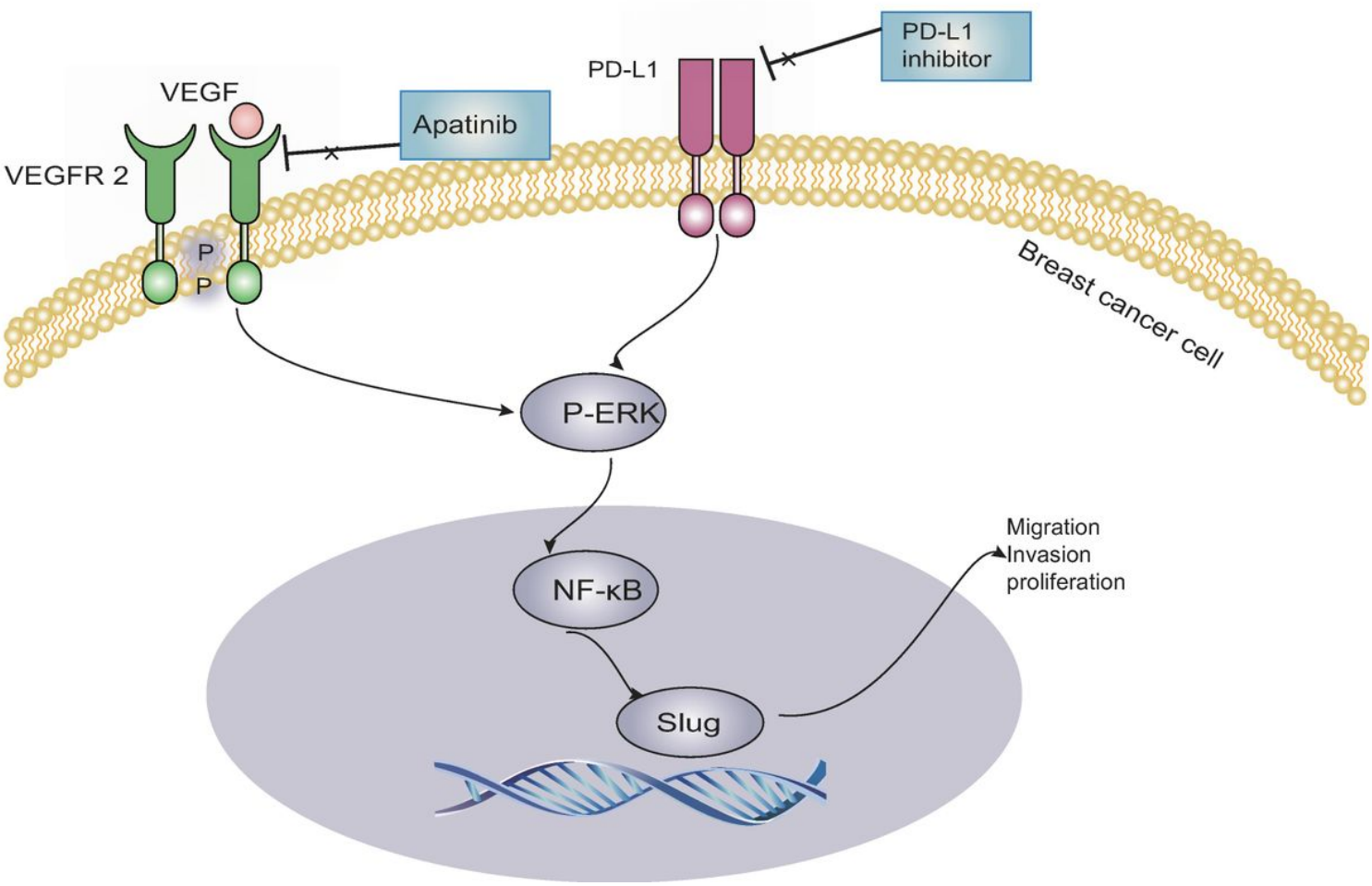

Figure 6

A proposed model for the apatinib and PD-L1 inhibitor-mediated inhibitory effects on proliferation, migration and invasion of breast cancer.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary1.pdf