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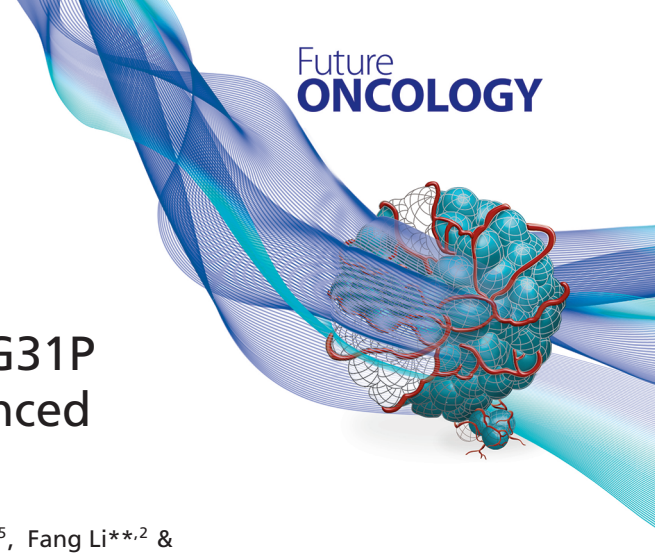

GDC-0941 and CXCL8 (3-72) K11R/G31P combination therapy confers enhanced efficacy against breast cancer

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Aim: Herein is presented the combined effect of PI3K inhibitor (GDC-0941) and CXCR1/2 analogue (G31P) in breast cancer. **Materials & methods:** Breast cancer cell lines and xenograft model were employed to test the efficacy of the combination therapy. **Results:** GDC-0941+G31P treatment substantially inhibited multiplication of all the breast cancer cell lines used in this study (BT474, HCC1954 and 4T1). Even though single therapies caused a meaningful S-phase cell cycle arrest, the inhibition effect was more potent with the combined treatment. Similarly, enhanced apoptosis accompanied GDC-0941+G31P treatment. Furthermore, the migration ability of the breast cancer cell lines were significantly curtailed by the combination therapy compared with the single treatments. **Conclusion:** The findings suggest that combination treatment involving PI3K inhibitor and CXCR1/2 analogue (G31P) could be a potent therapeutic option for breast cancer treatment.

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Keywords: breast cancer • cancer immunology • cell cycle • combination therapy • CXCR1/2 • G31P • GDC-0941 • metastasis • PI3K • proliferation

Mutation in the PI3K encoding gene PIK3CA frequently occurs in many types of tumors including breast cancer. More than 70% of aggressive breast cancers have anomalous alterations in PIK3CA [1], commonly occurring in the helical region or catalytic domain [2]. There are various isoforms of PI3K signaling, and they have substantial degrees of variations in functional and cellular regulation. Mutations in PIK3 gene have immense adverse influence on physiological and cellular activities including metabolism, proliferation, angiogenesis, autophagy, cell cycle progression and apoptosis [3,4], usually via the PI3K/AKT/mTOR pathway [5,6]. Aberrant activation of PIK3CA mutations has been significantly identified in colon, lung, uterus, cervix, head/neck and breast cancers [7–9]. The dominance of PIK3 gene mutations in cancers makes it a potential therapeutic target against cancer development and progression, hence the development and ongoing clinical trials of several PI3K inhibitors [10–12].

One of such well-known inhibitors is GDC-0941. GDC-0941 is a small-molecule inhibitor of pan-class I PI3Ks with a potent inhibition against PI3Kα/δ isoforms, and modest selectivity against p110β and p110γ. Even though clinical trials of PI3K inhibitors have yielded varying degrees of successes, they are commonly rendered inefficacious due to toxicities [13]. Additionally, they are met with the challenge of resistance, both intrinsic and acquired [14]. Recent studies have described several possible resistance mechanisms, including CDK4/6 activation, MYC amplification, enhanced estrogen receptor function, loss of PTEN, activation of PI3K p110β and mTOR complex activation [15–20]. Most of these inhibitors including GDC-0941 seem not to be expectedly efficacious

when used as single agent. This has led to the proposal of combination therapies with PI3K inhibitors to explore potent synergistic permutations for enhanced efficacy. The efficacy of GDC-0941 in combination with hormonal therapy, cytotoxic chemotherapy or cyclin-dependent kinase inhibitors in breast cancers have been reported [21–23].

The CXCL8 chemokine (IL-8), is known as a promoter of tumorigenesis in various types of cancers [24,25]. CXCR1 and CXCR2, two CXCL8 cognate G protein-coupled receptors, are elevated in cancers, and consequently, blockade of these receptors leads to inhibition of CXCL8 production. CXCL8 binding to CXCR1/2 activates cascades of downstream effect leading to its secretion, and activation of diverse pathways including AKT, ERK, STAT3 and transcription factors NF-κB, AP-1, SP-1, Egr1a and HIF-1a [26,27]. A CXCL8 antagonist CXCL8 (3-72) K11R/G31P (G31P), has been shown to be efficacious in breast, liver, lung, ovarian and prostate tumors [24,25].

It has been reported that blockade of PI3K in advanced cancers results in resistance and relapse [26,27]. Additionally, treatment with GDC-0941 increases macrophage infiltration into the tumor microenvironment and elevates the secretion of macrophage associated cytokines and chemokines to promote breast cancer [28]. Signaling of NF-κB, TGFβ and TNFα promote cytokine secretion in the tumor environment, enhancing tumor progression [29]. Also, macrophage associated signaling of NF-κB has been shown to make breast cancer cells resistant to GDC-0941, and this effect was reversed in cotreatment with aspirin [30]. Since G31P has been demonstrated to inhibit NF-κB signaling in macrophages through CXCR1/2 [31,32], we hypothesized that G31P could annul NF-κB signaling to permit the anticancer activity of GDC-0941, particularly in breast cancer. Thus, this study reports that combining PI3K inhibitor (GDC-0941) and G31P as treatment regimen, yields higher efficacy against breast cancer.

Materials & methods

Cell culture & drug preparation

Human HCC1954 (Catalog#: CRL-2338), mouse 4T1 (Catalog#: CRL-2539) and human BT474 (Catalog#: HTB-20) breast cancer cell lines, all from the American Type Culture Collection were used in this study. The detailed characteristics of the cell lines used are available at American Type Culture Collection website. Cell culture media, Dulbecco's Modified Eagle Medium (DMEM) and Fetal bovine serum (FBS) were obtained from Gibco (by Thermo Fisher Scientific, USA) and Biological Industries, USA, respectively, while penicillin-streptomycin solution was purchased from HyClone, USA. GDC-0941 was purchased from BioChemPartner, Shanghai, China, and G31P was synthesized as described previously, and provided by Professor Li Fang.

Cell proliferation assay

Using 12-well culture plates, the cells were seeded at a density of 500 cells per well in 2 ml culture medium (DMEM with 10% FBS, 100 U/ml/100 μg/ml P/S), and incubated for 12 h at 37°C, in 5% CO_2 in humidified atmosphere. The cells were then treated with either GDC-0941 (0.5 μM, dissolved in freshly prepared 0.5% methyl cellulose/Tween 80), G31P 0.03 μM (200 ng/ml, freshly dissolved in 0.9% physiological saline), or both, and both medium and treatment renewed every other day for 10 days. The dosage used in this study was informed by previous studies [27,30]. After the 10th day, the cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min, and stained with 0.2% crystal violet for 10 min. Images were captured with an inverted microscope. Also, the relative cell number was estimated following extraction of the dye with 10% acetic acid and optical density measured at 562 nm wavelengths. In other experiments, we subjected the BT474 and 4T1 breast cancer cells to 3D cultures. Six-well plates coated with 0.6% agar, and overlaid with 0.3% agar were used. Cells were plated at a density of 10^4 cells per well, and incubated for 12 h, followed by treatment. The cells were cultured continuously for 14 days, and medium with treatment was replaced every 3rd day. On the 14th day, images were taken, and colonies enumerated, and presented as ratio to the control.

Cell cycle analysis

Our treatment options were tested for their effect on cell cycle progression in HCC1954 cell lines. At 90–100% confluence, the cells were harvested and divided, subcultured for 24 h. The medium was changed and the cultures treated accordingly for 24 and 48 h, after which the cells were harvested, twice washed with ice-cold PBS and fixed in 75% cold ethanol at 4°C overnight. The cells were washed twice with ice-cold PBS, treated with 50 mg/ml of RNase (Sigma, USA) for 30 min, stained with 20 mg/ml of propidium iodide (Sigma, USA) overnight in darkness and analyzed by flow cytometry (Beckman Coulter, Miami, Inc., FL, USA). Data were analyzed with Multicycle software (Phoenix Flow Systems, San Diego, USA) to determine the cell cycle distributions.

Immunoblotting

We conducted western blot assay to determine the effect of our treatment permutations on HCC1954 and BT474 cells apoptosis by targeting cleaved poly (ADP-ribose) polymerase (c-PARP) expression levels in the various treated groups, and vinculin as reference protein. After 48 h of treatment, the cells were washed with ice-cold PBS, lysed in lysis buffer cocktail containing phosphatase and protease inhibitors. Lysates supernatant were collected by centrifugation at 15000 rpm at 4°C and total protein quantified by bicinchoninic acid (KeyGen Biotech. Inc., Nanjing, China), in accordance with the manufacturer's instruction. 30 μg of protein were separated in 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% skimmed milk in TBST, the membrane was incubated in c-PARP(Catalog#: 5625) and vinculin (Sigma-Aldrich V9131) primary antibodies at 4°C overnight, and subsequently incubated in horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody for 1 h at room temperature. Visualizing of protein was performed by enhanced chemiluminescent detection system (Bio-Rad, USA). The band intensity quantification was performed by Image Lab Software (Bio-Rad Laboratories, Inc., USA).

Cell migration assay

The wound healing assay was used to assess the effect of our treatment on HCC1954 and BT474 cells migration. Cells were cultured in six-well culture dishes till they reached 90% confluence. A scratch was made through the middle of the culture, using a sterile 200 μl pipette tip. After washing to remove unattached cells and initial images of the scratch taken, the cultures were treated for 48 h, and images taken afterward. Wound closure was determined with ImageJ software.

In other experiments, 4T1 cells migration was assessed by transwell migration assay. 4T1 cells were added to the upper chamber, 100 μl at a density of 1×10^5 cells/ml, and the lower chambers filled with 350 μl (20% FBS in DMEM medium) as the attracting agent. After 24 h treatment and incubation, migrated cells were fixed with methanol for 10 min and stained with 0.1% crystal violet. The cells were visualized by an Olympus 1X71 inverted microscope (Olympus Corporation, Tokyo, Japan).

Orthotopic implant of 4T1 cells into mice

The experimental subjects used were 32 female BALB/c mice within the age bracket 6–8 weeks, provided by Dalian Medical University Specific Pathogen Free Animal Center. The mice were housed under sterile conditions with 12 h light 12 h darkness cycle. The mice were provided with sterile water and rodent chow *ad libitum*. After 2 weeks of acclimatization, the mice were inoculated with 4T1 cells (5×10^5 cells, in 200 μl PBS) into both inguinal mammary glands of the mice following anesthesia (200 mg/kg sodium pentobarbital). A week after, 4T1 tumor-bearing mice were collected into a single cage and subsequently randomly grouped into four: vehicle control, GDC-0941(130 mg/kg/day, oral gavage) freshly formulated in 0.5% methyl cellulose/Tween 80, G31P (0.5 mg/kg every other day, by subcutaneous injection) freshly formulated in 0.9% physiological saline and GDC-0941 and G31P combination treatment. Tumor sizes and body weight were measured once every 2 days, and tumor volumes estimated as volume (mm^3) = 0.52 × long axis × (short axis)^2. The mice were euthanized 21 days later under anesthesia (200 mg/kg sodium pentobarbital), and tumors and the lungs harvested for histopathological examination.

Haematoxylin & eosin staining

Fresh lung tissues were fixed in 10% neutral buffer formalin for 24 h and embedded in paraffin. Sections from five different mice from each experimental group were stained with haematoxylin & eosin, and examined microscopically. Lung metastasis index was calculated by the formula: (lung tumor area/lung area) × 100.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Prism Software Inc., San Diego, CA, USA). Comparison between groups was achieved with one-way ANOVA and Tukey's test for multiple comparisons. All values are presented as means ± SEM (Standard Error of Mean), and a $p < 0.05$ was considered significant for differences between groups.

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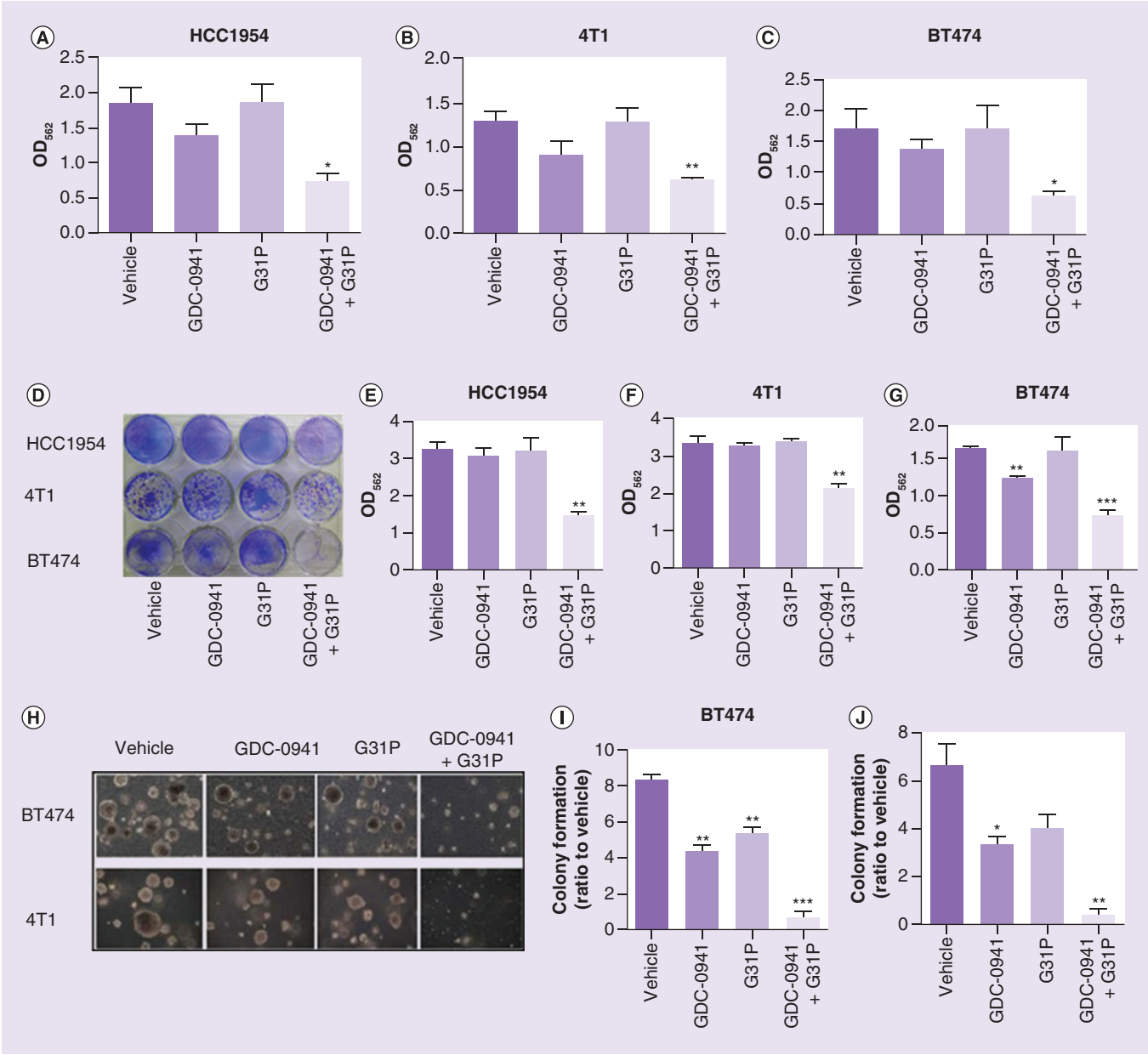

Figure 1. Effect of the treatment options on proliferation of breast cancer cell lines. (A-C) Optical density of cells. **(D-G)** Representative image and quantification of colony formation. **(H-J)** Cell proliferation assay by 3D culture, colony number estimation relative to vehicle. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; all compared with vehicle. Bar represents mean ± SEM, from three independent experiments.

Results

Combined GDC-0941 & G31P treatment synergistically arrests breast cancer cells proliferation *in vitro*

To investigate the treatment effects of GDC-0941 and G31P on cell proliferation, we employed clonogenic assay techniques. The optical density of the cultured cells revealed that only GDC-0941+G31P treatment induced appreciable inhibition of proliferation in all the breast cancer cell lines. No significant proliferation restriction was identified with GDC-0941 treatment, and G31P treatment alone seems indifferent against proliferation when compared with the control treatment (Figure 1A-C). Similarly, the colony forming assay showed that GDC-0941+G31P substantially inhibited multiplication of all the breast cancer cell lines with the greatest inhibition demonstrated in BT474 cell line. Also, BT474 proliferation was meaningfully restricted by GDC-0941 treatment

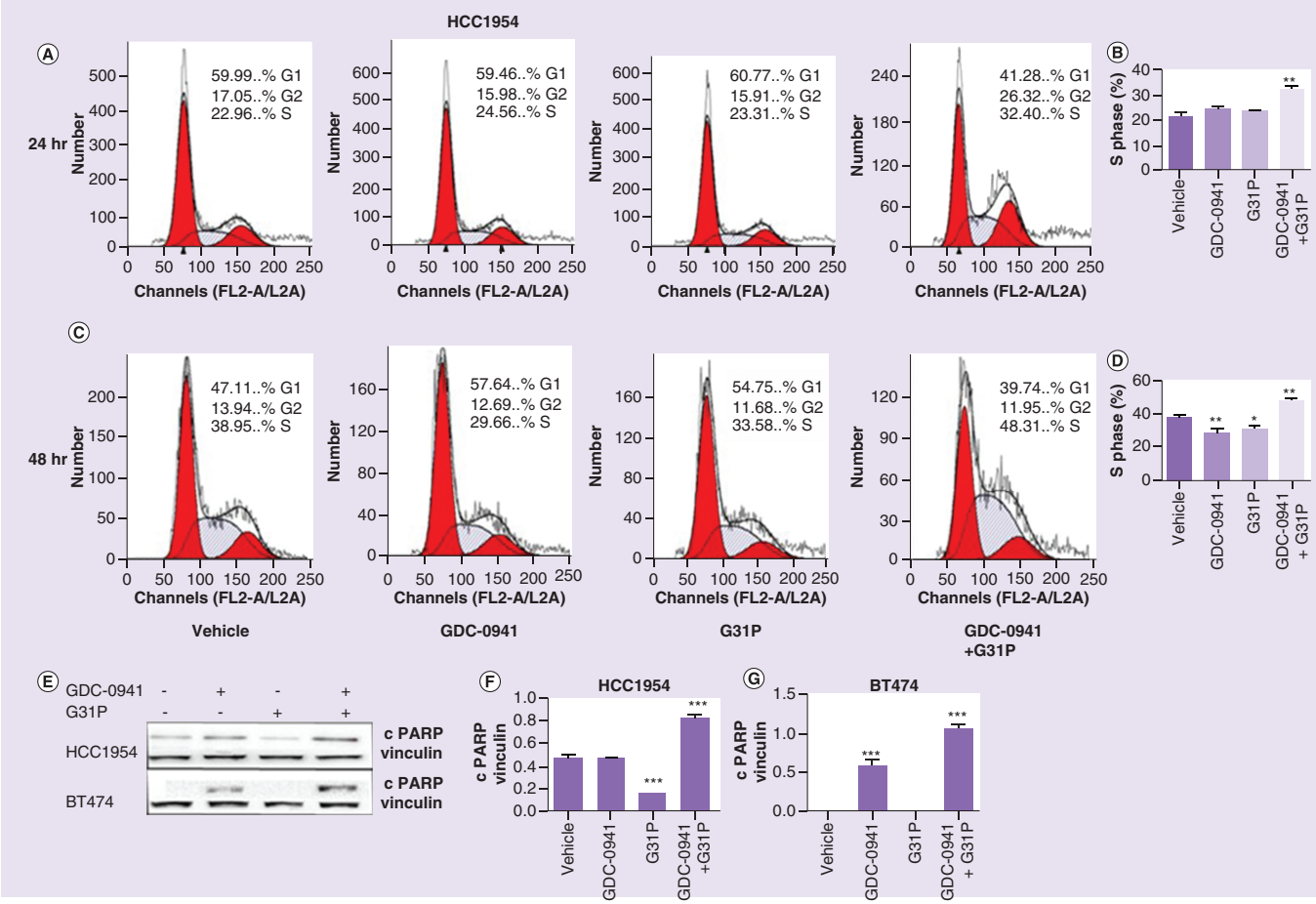

Figure 2. GDC-0941+G31P treatment arrests S-phase of cell cycle and induces apoptosis. (A-D) Representative image of flow cytometry analysis of cell cycle in HCC1954 cells. **(E-G)** c-PARP protein expression in HCC1954 and BT474 cells.
*p < 0.05, **p < 0.01, ***p < 0.001; all compared with vehicle. Bar represents mean ± SEM, from three independent experiments.

alone (Figure 1D-G). Subsequently, two of the cells lines (BT474 and 4T1) were subjected to 3D culture, and surprisingly noted that both GDC-0941 and GDC-0941+G31P treatments offered significant proliferation inhibition, but the greatest growth restriction was demonstrated by GDC-0941+G31P combination (Figure 1H-J). Collectively, these findings suggest that GDC-0941+G31P augment each other to enhance their antiproliferation property, indicating synergistic effect when the two agents are combined in treatment.

Combined GDC-0941 & G31P treatment causes S-phase cell cycle arrest & induces apoptosis in breast cancer cells

The differential proliferation retardation observed informed our decision of investigating the effects of the treatment options on cell cycle progression of HCC1954 cells. The flow cytometry analysis revealed no meaningful effect on cell cycle characteristics after 24 h of treatment with either GDC-094 or G31P alone. However, the combination of the two agents resulted in S-phase cell cycle arrest within 24 h, and this observation was maintained and enhanced after 48 h (Figure 2A-D). In other experiments, HCC1954 and BT474 cell lines were subjected to western blot analysis following treatments to assess the effect of our treatment permutations on apoptosis. The protein expression of c-PARP, which is a hallmark of apoptosis, was measured. In HCC1954 cells, the expression of c-PARP was comparable to the Control in GDC-0941 treatment alone, while G31P treatment demonstrated reduced c-PARP protein expression. However, GDC-0941+G31P treatment resulted in enhanced apoptosis (Figure 2E-G). No detectable c-PARP protein was observed in control and G31P treatment in BT474 cells. Contrarily, apoptosis was induced by GDC-0941 alone and GDC-0941+G31P treatment, with prominent induction in the combined treatment (Figure 2E-G).

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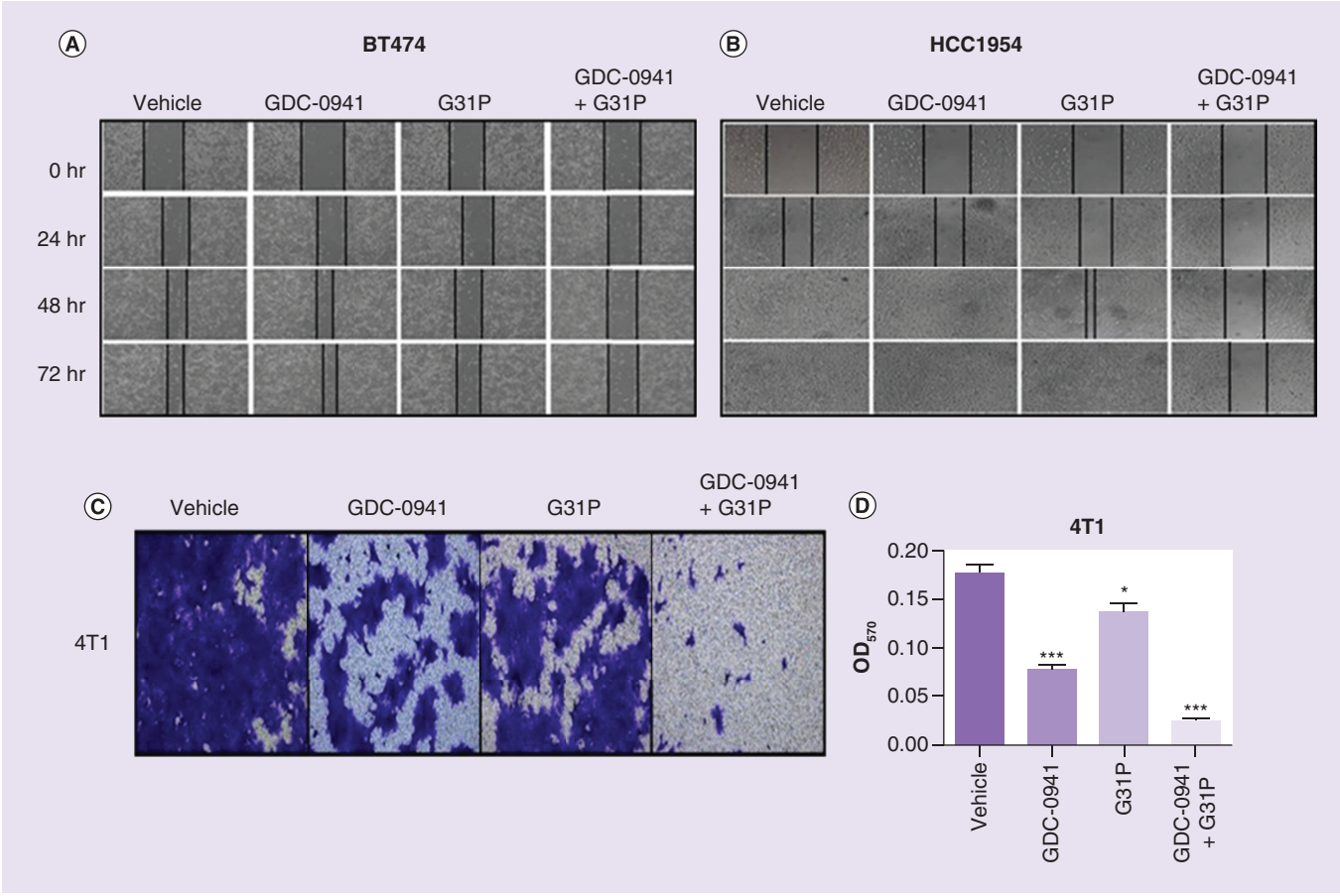

Figure 3. Demonstrations of migration inhibition by the various treatment options. (A, B) Effect of the treatments on BT474 and HCC1954 cells migration, shown by scratch wound assay. **(C, D)** Transwell migration of 4T1 cells after treatment.
*p < 0.05, ***p < 0.001; all compared with vehicle. Bar represents mean ± SEM, from three independent experiments.

GDC-0941+G31P treatment restricts migration & invasion of breast cancer cells

Migration is one of the hallmarks of cancer metastasis, and therapeutic regimens impeding migration correlate with positive treatment outcome. We, therefore, assessed the potency of our treatment combinations on the impedance of migration in the various breast cancer cells. The scratch well assay was performed on BT474 and HCC1954 cells. We noted significantly delayed wound closure by BT474 cells from 0 h of wound creation to after 72 h, however, the wound gap remained significantly larger in GDC-0941+G31P treatment compared with the others. The HCC1953 cell maintained significant wound gap from 0 h of scratch to after 72 h following GDC-0941+G31P treatment. Interestingly, GDC-0941 treatment lost wound gap after 24 h while G31P did same after 48 h in HCC1954 cells. The combined treatment of GDC-0941+G31P kept significant scratch gap even after 72 h. In other experiments, 4T1 cells were subjected to transwell migration assay. Even though G31P treatment showed less migrated cells compared with GDC-0941 treatment after 24 h, the GDC-0941+G31P treatment greatly restricted the cells from migrating (Figure 3).

Combination therapy with GDC-0941 & G31P confers synergistic treatment effects on tumor growth & metastasis

To assess the effect of the treatment permutations *in vivo*, 4T1 cells were selected for orthotopic implant. 4T1 cells implant to the mammary fat pad of mice easily stabilizes, proliferates and metastasizes to various organs. After successful implantation, the mice were randomized into the various experimental groups, and treated accordingly. Throughout the treatment period, there were no significant variations in the body weight of the mice (Figure 4A). The periodic measurement of tumor volumes demonstrated shrinking tumor size by the individual treatments with either GDC-0941 or G31P. However, GDC-0941+G31P intervention resulted in greater depreciation in tumor

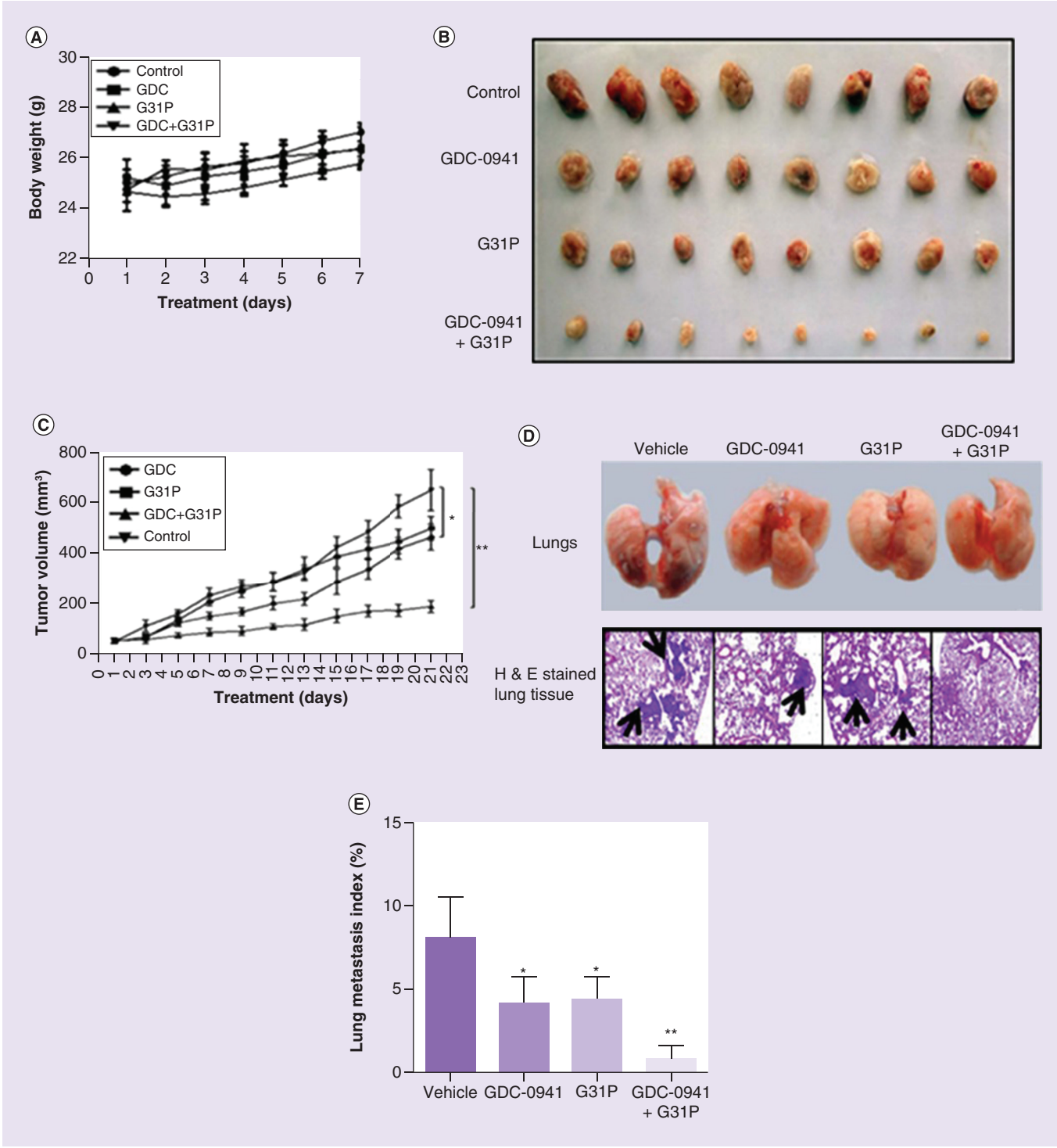

Figure 4. ***In vivo*** **effect of GDC-0941 and/or G31P treatment. (A)** Trend of mice body weight (mean ± SEM, n = 8 mice per group). **(B)** Representation images of tumors at the end of treatment. **(C)** Trends of quantified tumor size (mean ± SEM, n = 8 mice per group). **(D)** Representative lung tissues and H&E stained lung tissues. **(E)** Estimated metastasis.
Results presented as mean ± SEM, n = 10 tissue slides per group, two from each mouse and n = 5 mice per group. *$p < 0.05$, **$p < 0.01$; all compared with vehicle.
H&E: Haematoxylin & eosin.

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volume (Figure 4B). Subsequently, the lungs of the mice were harvested and examined for 4T1 cells metastasis. As indicated in Figure 4C, even though the individual therapeutic agents tangibly impeded metastasis of 4T1 cells, it was noted that GDC-0941+G31P treatment was much significant. These results reveal that either GDC-0941 or G31P treatment exhibits antimetastatic and antigrowth properties against 4T1 cells. However, combination treatment with GDC-0941 and G31P offers a more appreciable arrest of breast cancer metastasis.

Discussion

The immune system plays crucial roles in either cancer progression or regression, and optimum regulation of immune reactions is now seen as pivotal in cancer treatment and potential cure, evident by increasing research into immune-checkpoint blockade in cancer immunotherapy [33,34]. GDC-0941 is a small-molecule inhibitor of pan-class I PI3Ks while G31P is CXCL8 cytokine analogue which interferes CXCL8 production by binding to GPCR CXCR1/2. The inhibitor GDC-0941 functions by inhibiting the PI3K-AKT-mTOR pathway, and leads to diminishing metabolism, proliferation and angiogenic activities, and also influences autophagy, apoptosis and cell cycle activities [6]. G31P, on the other hand, has been reported to interfere with multiple pathways involving the GPCR CXCR1/2, including AKT, ERK1/2, JAK and STAT [35]. The decline in expression of these proteins is associated with the cytokines regulatory potential of G31P.

The main cause of cancer-related death is metastasis, characterized by cancer cells migration, invasion and vascularization. In the present study, we observed remarkable hampering of the breast cancer cells' migration in the combination therapy compared with single treatments with the individual agents. Comparatively, GDC-0941 showed significant migration inhibitory activity than G31P. The role of both agents in limiting cancer cells migration has been reported. G31P restrains the expression of MMP2 and MMP9 during inflammation. These enzymes promote cell migration upon their elevation [32]. Additionally, immune cells infiltration into the tumor microenvironment influences tumor progression or regression. Also, VEGF, a hallmark for tumor vascularization, is limited by G31P, hence obstructing tumor angiogenesis. It has been established recently that macrophages confer resistance to PI3K inhibitor GDC-0941 in breast cancer through the activation of NF-κB signaling [30]. Possibly, the synergism between GDC-0941 and G31P is partly due to the anti-NF-κB signaling effect of G31P.

A positive correlation has been identified between increased copy numbers of CXCL1/2 gene and invasive breast cancer tumors [36]. Additionally, CXCL1/2 is involved in a paracrine loop in the tumor microenvironment and tumor cells to promote chemo-resistance and enhance breast cancer tumor metastasis [36]. In other reports, antagonizing CXCL1 inhibited thrombin-induced endothelial tube formation, and eliminating CXCL1 via shRNA in 4T1 breast cancer cells diminished tumor growth, angiogenesis and metastasis [37]. Malignant MCF10CA1a.c11 breast cancer cells characterized by aggressive metastasis exhibited higher expression of CXCL7 and CXCR2 than their nonmalignant counterparts [38]. Consequently, transfection of nonmalignant MCF10AT cells with CXCL7 made them invasive and was attenuated by antiCXCL7 [38]. G31P attenuates the expression of FGF. Increased in FGFR in epithelial breast cancer cells consequentially arrests the TGFβ/SMAD3 pathways in tumor-associated macrophages, causing elevation of CXCL chemokines, therefore, increasing invasiveness [39]. However, this can be aborted by CXCR2 inhibitor, which G31P does. Furthermore, G31P is able to restrict macrophage infiltration to inflamed colon in an inflammatory bowel disease model, thereby possibly limiting the influence of tumor-associated macrophages in tumor progression.

The production of CXCL1 and CXCL5 by mesenchymal stem cells leads to metastasis of mammary cancer cells, but this can be inhibited by CXCL1/5 and CXCR2 antagonist [40]. In an *in vitro* experiment, CXCR2 silencing by shRNA in metastatic murine mammary tumor cell lines (C166, 4T1) arrests cell invasion, but not proliferation. However, in an *in vivo* study, these CXCR2 down-regulated cells exhibited reduced lung metastasis and enhanced the cytotoxic effects of doxorubicin and paclitaxel [41,42]. Similarly, annulling CXCR1 with either antibody or reparixin was able to deplete breast cancer stem cells in HCC1954, MDA-MB-453 and MDA-MB-231 cell lines, and retarded tumor growth and metastasis in a xenograft model [43,44]. Our findings supported this observation as we noticed comparably enhanced reduction in metastasis by the CXCR1/2 antagonist, G31P.

We have previously demonstrated that a dietary compound, ellagic acid, in combination with GDC-0941 significantly arrests proliferation, migration and invasion of breast cancer cells [45]. Additionally, our report on cisplatin and G31P combination treatment revealed that G31P does not only enhance the efficacy of cisplatin in hepatocellular carcinoma but also mitigates it associated renal damage [46,47]. Appreciating the role of the changes in the tumor microenvironment on cancer progression or otherwise, our laboratory is currently working on ways to

improve the efficacy of established anticancer agents while reducing the associated toxicity via combination therapy with anti-inflammatory and immunoregulatory agents.

Generally, the level of CXCL8 chemokine is significantly elevated in breast cancer patients and positively correlates with the stage of the disease, compared with their healthy counterparts [48]. This indicates that CXCL8 pathway is activated in breast cancers and other forms of cancers. The production of CXCL8 directly or indirectly activates the EGFR/HER2 signaling pathways mediated by sarcoma viral oncogene (SRC), PI3K and MEK in breast cancer stem cells, and intervening with the CXCR1/2 SCH563705 reduced colony formation and enhanced the efficacy of lapatinib (tyrosine kinase inhibitor) [49]. By inference, combination treatment with CXCL8 antagonist via its cognate receptors together with specific kinase inhibitors is efficacious compared with single-agent interventions.

Conclusion

In conclusion, this study has demonstrated that dual targeting of PI3K and the transmembrane receptors CXCR1/2 could be a potent therapeutic option against breast cancer. However, further investigations are required to establish the clinical suitability and applicability of this combination treatment. Additionally, a broader spectrum of combination therapies in various cancers should be explored, with the ultimate aim of significantly minimizing cancer drugs associated toxicity while enhancing efficacy.

Summary points

- GDC-0941+G31P augment each other to enhance their antiproliferation property.
- Combination of the two agents (GDC-0941 and G31P) results in S-phase cell cycle arrest within 24 h, and this is maintained and enhanced after 48 h.
- GDC-0941+G31P treatment greatly restricts the breast cancer cells from migrating compared with the individual treatments.
- GDC-0941+G31P intervention results in a greater depreciation in tumor volume in mice model.
- Though either GDC-0941 or G31P treatment exhibits antimetastatic properties against 4T1 cells, the combination treatment offers a more appreciable arrest of breast cancer metastasis in mice model.
- Optimizing combination therapies could lead to reducing the toxicity associated with some anticancer drugs.

Author contributions

X Li, F Li and F Luo conceived the study. X Li, Y Zhang, W Walana and F Zhao conducted laboratory experiment and data analysis. X Li and W Walana drafted the manuscript. F Li and F Luo supervised the study. All authors read and approved the manuscript.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Availability of data & materials

The data analyzed and presented in manuscript is available from the corresponding author upon reasonable request.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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