Regorafenib Suppresses Migration of and Induces Cell Cycle Arrest and Apoptosis in MCF-7 Cells

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This study investigated the mechanism underlying the suppression of estrogen receptor-positive MCF-7 cell growth by regorafenib. MCF-7 cells were treated with regorafenib, and the effect of regorafenib on multiple cancer-associated pathways was evaluated. Although regorafenib effectively inhibited the proliferation of MCF-7 cells, it had no effect on the proliferation of the normal breast epithelial cell line MCF10A. Regorafenib suppressed MCF-7 cell migration, probably by regulating the homeostatic expression of matrix metalloproteinases and the tissue inhibitor of MMPs. Furthermore, it upregulated p21 expression, downregulated cyclin B1 and cyclin D1 expresssions, and caused cell cycle arrest. In addition, regorafenib induced apoptosis in MCF-7 cells by reducing Mcl-1 expression and activating caspase signaling. These results demonstrate that regorafenib has the potential to be an effective drug for treating breast cancer.

Keywords: Regorafenib. MCF-7. Apoptosis. Migration. Cell cycle.

INTRODUCTION

Globally, breast cancer is the most common malignancy among women; in fact, approximately 1,300,000 new cases are diagnosed and 450,000 deaths are recorded each year. Breast cancer is a heterogeneous condition and is commonly categorized into three therapeutic types: human epidermal growth factor receptor 2 (HER2)-amplified, estrogen receptor (ER)-positive, and triple-negative types (Palma *et al.*, 2015; Rody, Cornelia, 2017). ER-positive breast cancer is the most commonly observed type, accounting for approximately 80% of all breast cancer cases (Saha Roy, Vadlamudi, 2012). HER2-amplified breast cancer is characterized by an aberration in the DNA copy number of HER2 (Labidi *et al.*, 2016). Triple-negative breast cancer lacks ER, progesterone receptor, and HER2 expressions (Bagaria *et al.*, 2014). Treatment options for breast cancer include radiation therapy, surgery, endocrine (hormone) therapy,

targeted therapy, and chemotherapy (Yamamoto-Ibusuki *et al.*, 2015). For ER-positive breast cancer, endocrine therapy is the standard adjuvant treatment complementary to surgery and has proven to be effective in a majority of patients (Jameera Begam *et al.*, 2017; Santen *et al.*, 2015; Zoghi, Elledge, 2016). However, some patients experience relapse with incurable metastasis, probably because of the development of resistance to endocrine therapy, which underlines the clinical need to develop alternative treatments.

Regorafenib, an orally administered multikinase inhibitor, can block the activity of many protein kinases, including those participating in oncogenesis [rearranged during transcription (RET), BRAF^{V600E}, v-raf murine sarcoma viral oncogene homolog B1 (BRAF), RAF-1, and V-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog (KIT)] and those involved in tumor microenvironment [fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR)] and in the regulation of tumor angiogenesis [TIE-2, vascular endothelial growth factor receptor (VEGFR) 3, VEGFR2, and VEGFR1] (Ettrich, Seufferlein, 2014). Phase I and II clinical trials of regorafenib demonstrated antitumor activity in various types of solid tumors; moreover, phase III trials

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demonstrated the benefits of regorafenib in refractory metastatic gastrointestinal stromal tumor (GIST) and colorectal cancer (CRC) (Sartore-Bianchi *et al.*, 2016; Schvartsman *et al.*, 2016). Moreover, regorafenib has been approved for use in metastatic GIST and CRC in many countries worldwide based on the results of phase III clinical trials (Ettrich, Seufferlein, 2014; Schvartsman *et al.*, 2016). Regorafenib may prove clinically useful in inhibiting breast cancer cell migration and metastasis via PDGFR signaling (Stalker *et al.*, 2014). However, the mechanism underlying the cell autonomous effects of regorafenib, such as cell death in ER-positive breast cancer, is not well understood.

In the present study, the antitumor effects of regorafenib on the ER-positive breast cancer cell line MCF-7 were evaluated and the key pathways crucial for tumor growth, including migration, apoptosis, and cell cycle, were investigated.

MATERIAL AND METHODS

Cell culture and drug treatment

MCF-10A (human breast epithelial cell line) and MCF-7 (human breast cancer cell line) were purchased from the American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were cultured in RPMI-1640 medium supplemented with 3.2-µg/mL insulin, 100-IU/mL penicillin/streptomycin, and 10% fetal bovine serum. MCF10A cells were cultured in Dulbecco's modified Eagle medium/Nutrient Mixture F-12 medium, 5% horse serum, 20-ng/mL epidermal growth factor, 100-ng/mL cholera toxin (Sigma, St. Louis, MO, USA), 10-µg/mL insulin (Sigma), 0.5-mg/mL hydrocortisone (Sigma), and 100-IU/mL penicillin/streptomycin. Regorafenib (Selleckchem, Houston, TX, USA) was diluted with dimethyl sulfoxide. Every procedure used in this study was approved by the Animal Care and Use Committee of the School of Biological and Pharmaceutical Engineering, Wuhan Huaxia University of Technology (Wuhan, Hubei, China), and was conducted in accordance with the guidelines of the National Institutes of Health.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay

The cell lines were seeded in a 96-well plate (1×10^4 cells/well) and incubated with varying concentrations of regorafenib in the growth media for 72 h. The MTS assay was conducted using the MTS assay kit (Promega, Madison, WI, USA) as per the instructions. The Wallac Victor 1420 Multilabel Counter (Perkin Elmer Inc., Waltham, MA, USA) was used for the determination of luminescence. Each assay was duplicated three times. For colony assays, the same number of cells was treated for 48 h in 12-well plates and the attached cells were stained with crystal violet (Sigma).

Transfection

Mcl-1 expression construct was generated by cloning a polymerase chain reaction (PCR)-amplified, full-length human Mcl-1 cDNA fragment into pcDNA3.1/V5-His vector. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Western blotting

According to the procedure previously described by Tong *et al.* (2017b), Western blotting was performed with the antibodies against cleaved caspase-3, cleaved caspase-9 (Cell Signaling Technology Inc.), Bak, Bcl-2, Cyclin B1, Cyclin D1, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax, Mcl-1, and Bcl-Xl (BD Biosciences, San Jose, CA, USA).

Real-time reverse transcriptase PCR

Total RNA was extracted according to the manufacturer's instructions, using the TRIzol RNA Kit (Invitrogen). SuperScript II reverse transcriptase (Invitrogen) and 1 µg of total RNA were used to generate cDNA. PCR was conducted using the Bio-Rad CFX96ä Real-time PCR System and SsoFasrä Probes Supermix (Bio-Rad, Hercules, CA, USA). The final reaction volume was set as 20 µL; the primers were used at the set 35 cycles. Each experiment was conducted in triplicate. TaqMan Gene Expression Real-Time PCR assay was used for the determination of gene expression, results of which were described as the threshold cycle (Ct). The target transcript was quantified using the comparative Ct approach (ΔΔCt) as per the instructions provided. The relative change in gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. To ensure the absence of genomic DNA contamination, controls with no reverse transcription were set up. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control for normalization (Table I).

TABLE I – PCR primers

PCR primers	Forward	Reverse
MMP2	GAGTGCATGAACCAACCAGC	GTGTTCAGGTATTGCATGTGCT
MMP9	CTTTGAGTCCGGTGGACGAT	TCGCCAGTACTTCCCATCCT
TIMP1	GCAATTCCGACCTCGTCATC	TAGACGAACCGGATGTCAGC
TIMP2	CTGCGAGTGCAAGATCACG	TGGTGCCCGTTGATGTTCTT
CDH1	TGAAAAGAGAGTGGAAGTGTCCGAG	GATTAGGGCTGTGTACGTGCTGTTC
CDH2	GGGAAATGGAAACTTGATGGCA	CAGTTGCTAAACTTCACTGAAAGGA
GAPDH	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCCAAATTCGT

Cell cycle analysis

Cells were treated with regorafenib for 48 h. A cell cycle kit was used to evaluate cell cycle distribution according to the instructions. In detail, cells were harvested and then washed twice with phosphate-buffered solution (PBS), followed by fixation with 70% ethanol at 4 ºC for 1 h. Fixed cells were then stained with propidium iodide (PI) solution containing RNase at 4 ºC for 30 min. Becton Dickinson FACScan cytofluorometry (Becton Dickinson, Mansfield, MA, USA) was performed to analyze cells. The ModFIT software (Version 2.01.2) was used to evaluate cell cycle distribution.

Apoptosis assay

Apoptosis was determined by the nuclear staining of cells with Hoechst 33258 (Invitrogen). Annexin V/PI staining was performed using PI and annexin-Alexa 488 (Invitrogen).

Migration detection

The wound healing assay was used to determine cell migration. Briefly, MCF-7 cells were seeded in a 12-well plate (5×10^4 cells/well). A micropipette tip was used to scratch the attached cells to create a wound space after the formation of a monolayer of cells. After PBS washing, cells were then replaced by serum-free RPMI-1640 medium, which contained varying concentrations of regorafenib. Cell migration into the wound was observed using an inverted microscope at 0 and 72 h. The average wound size corresponded to the relative cell migration.

Statistical analysis

Data were presented as mean ± standard deviation. One-way analysis of variance and Student's t-test were conducted using the GraphPad Prism V software. A P value <0.05 was considered to indicate statistically significant difference.

RESULTS

Regorafenib decreased the proliferation of MCF-7 cells

To investigate the anti-proliferation activity of regorafenib in ER-positive MCF-7 cells, the MTS assay was used to assess the viability of MCF-7 cells treated with different doses of regorafenib. Results revealed that the proliferation of MCF-7 cells was inhibited by regorafenib in a dose-dependent manner; however, the proliferation of MCF-10A cells was not decreased (Figure 1A). Observations of regorafenib-treated MCF-7 cells using light microscopy revealed that regorafenib reduced cell viability (Figure 1B). Regorafenib resulted in dose-dependent suppression of growth within 24 h (Figure 1C and 1D). These results demonstrate that the inhibitory effects of regorafenib on proliferation could be specific to tumor cells, suggesting that regorafenib is an ideal drug to treat ER-positive breast cancers.
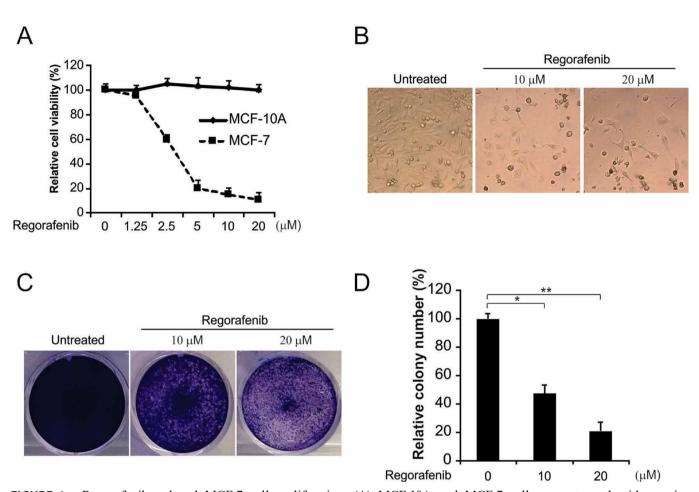
FIGURE 1 – Regorafenib reduced MCF-7 cell proliferation. **(A)** MCF-10A and MCF-7 cells were treated with varying concentrations of regorafenib for 72 h. Cell proliferation was determined using the MTS assay. **(B)** Bright-field images of cell morphology after treatment with 20-μmol/L regorafenib for 24 h. **(C)** MCF-7 cells were treated with 20-**μmol/L regorafenib for 24** h. Adherent cells were stained with crystal violet. **(D)** The quantification of colonies in **C**. Results are described as mean ± standard deviation of three independent experiments performed in triplicate.*$P < 0.05$; **$P < 0.01$.

Regorafenib suppresses MCF-7 cell migration

We used the wound healing assay to evaluate the migration of MCF-7 cells treated by regorafenib. MCF-7 cells treated with 20-μmol/L regorafenib demonstrated significantly reduced migration compared with untreated cells (Figure 2A). Furthermore, we analyzed the effects of regorafenib treatment on the expression of genes involved in cell migration. The expression of genes that promote cell migration [e.g. matrix metalloproteinase (MPP) 2, MPP9, and CDH2] was decreased in regorafenib-treated MCF-7 cells. In contrast, the expression of genes that suppress cell migration [e.g. tissue inhibitor of MMP (TIMP) 1, TIMP2, and CDH1] was increased in regorafenib-treated MCF-7 cells (Figure 2B). Considering all these results, regorafenib suppresses the mobility of MCF-7 cells by regulating migration-related genes.
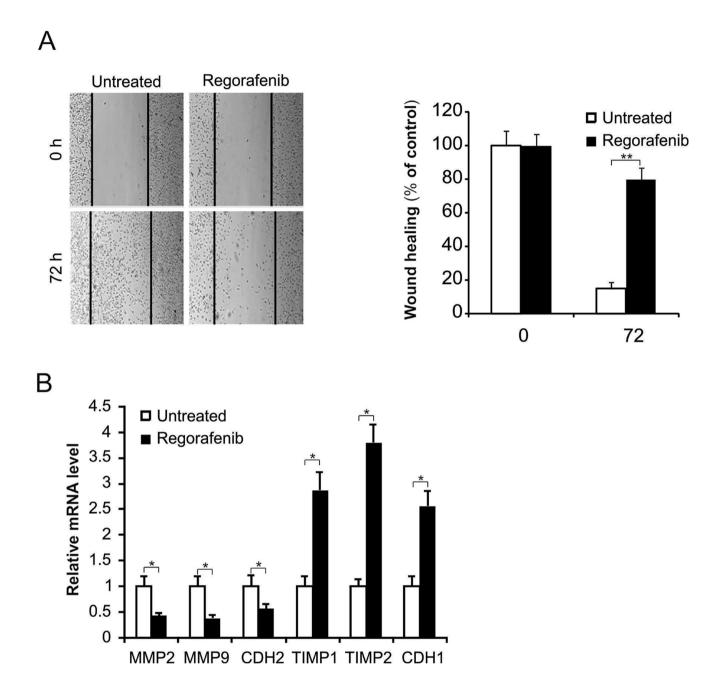
FIGURE 2 – Regorafenib suppresses MCF-7 cell migration. **(A)** MCF-7 cells were treated with 20-µmol/L regorafenib for 72 h, and migratory behavior was analyzed using wound healing assays. **(B)** MCF-7 cells were treated with 20-µmol/L regorafenib for 48 h. The mRNA levels of MMP2, MMP9, CDH1, CDH2, TIMP1, and TIMP2 were analyzed using real-time PCR. Results are described as mean ± standard deviation of three independent experiments performed in triplicate. *$P < 0.05$.

Regorafenib causes MCF-7 cell cycle arrest at the G2/M phase

As regorafenib treatment caused decreased the proliferation of MCF-7 cells, we further investigated whether it inhibited cell proliferation by inducing cell cycle arrest. We observed that the percentage of MCF-7 cells in the G2/M phase significantly increased following regorafenib treatment (Figure 3A). Conversely, the percentage of MCF-7 cells in the G0/G1 phase substantially reduced

following regorafenib treatment (Figure 3A). Furthermore, the effects of regorafenib on proteins that regulated cell cycle progression were investigated. Cyclin-dependent kinase (CDK) 1 and cyclin B1 complex positively regulate G2/M transition; conversely, the G1 phase is shortened by overexpressed cyclin D1 and G1/S transition is blocked by the suppression of cyclin D1 expression (Caldon *et al.*, 2006; Tong *et al.*, 2010). Thus, we assessed the expression of these key molecules in regorafenib-treated MCF-7 cells. Regorafenib treatment decreased the protein levels of cyclin B1 and cyclin D1 but increased that of p21. These results indicate that regorafenib interferes with the proliferation of MCF-7 cells via the induction of cell cycle arrest at different phases (Figure 3B).
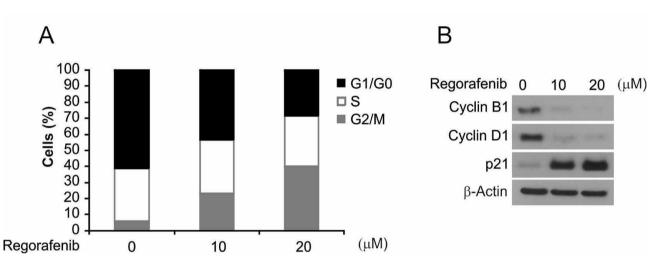

FIGURE 3 – MCF-7 cell cycle arrest is induced by regorafenib at G2/M phase. **(A)** MCF-7 cells were treated with 20-μmol/L regorafenib for 24 h, and flow cytometry was used to analyze cell proportion in the different phases of cell cycle. **(B)** MCF-7 cells were treated with regorafenib at the indicated concentration for 24 h. The expressions of indicated proteins were analyzed using western blotting.

Regorafenib induces apoptosis in MCF-7 cells

As regorafenib showed cytotoxicity in MCF-7 cells, we next examined whether it was related to apoptosis induction. The results of the apoptotic assay revealed that regorafenib treatment at 10 or 20 μmol/L induced apoptosis in MCF-7 cells (Figure 4A). Furthermore, regorafenib treatment increased the proportion of annexin V-positive cells in MCF-7 cells (Figure 4B). The apoptotic response was also found to be attenuated following pretreatment with z-VAD-fmk (pan-caspase inhibitor) (Figure 4C), indicating that the apoptotic response was caspase-dependent. We detected the cleavage of caspase-3 and -9 in regorafenib-treated MCF-7 cells and found that regorafenib promotes caspase-3 and -9 activation in MCF-7 cells (Figure 4D). These findings demonstrate that regorafenib induces caspase-dependent apoptosis in MCF-7 cells.
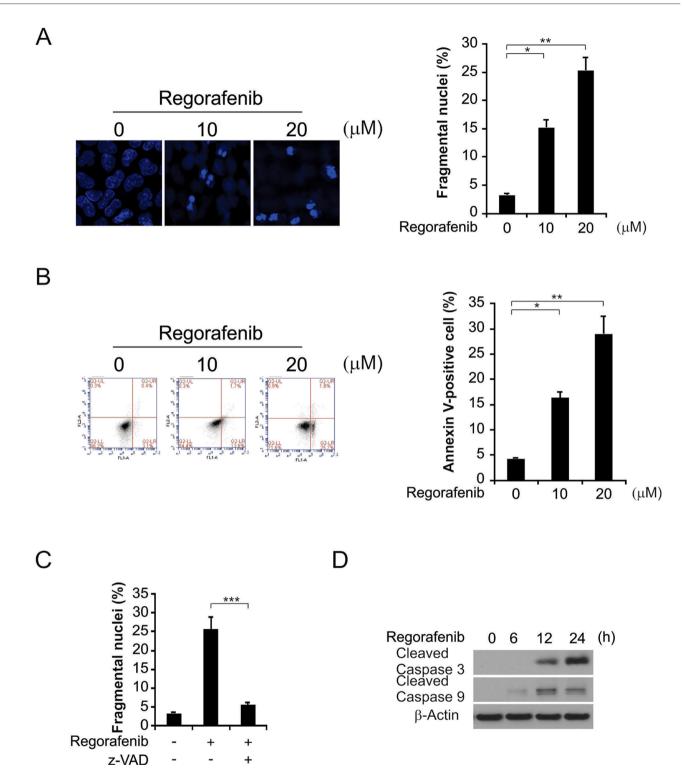
FIGURE 4 – Regorafenib induces apoptosis in MCF-7 cells. (A) MCF-7 cells were treated with regorafenib at indicated concentrations for 48 h. The induction of apoptosis was analyzed using a nuclear fragmentation assay. (B) MCF-7 cells were treated with regorafenib at the indicated concentrations for 48 h. The induction of apoptosis was analyzed using annexin V/PI staining followed by flow cytometry. (C) MCF-7 cells were treated with 20-μmol/L regorafenib with or without z-VAD, and apoptosis was analyzed using a nuclear fragmentation assay. (D) MCF-7 cells were treated with 20-μmol/L regorafenib at the indicated time points. Cleaved caspase-3 and -9 were analyzed using western blotting. The results of (A), (B), and (C) are presented as mean ± standard deviation of three independent experiments performed in triplicate. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Regorafenib reduced Mcl-1 protein level

The potential mechanism underlying regorafenib-induced apoptosis was investigated using western blotting. The levels of proteins in the Bcl-2 family were determined in regorafenib-treated MCF-7 cells. Regorafenib treatment did not affect Bax, Bak, Bcl-2, and Bcl-Xl protein levels but reduced antiapoptotic Mcl-1 protein level (Figure 5A). Furthermore, Mcl-1 overexpression reduced regorafenib-induced caspase-3 and -9 activations in MCF-7 cells (Figure 5B). The above data suggest that regorafenib-induced Mcl-1 downregulation in MCF-7 cells is responsible for the antitumor activity of regorafenib.
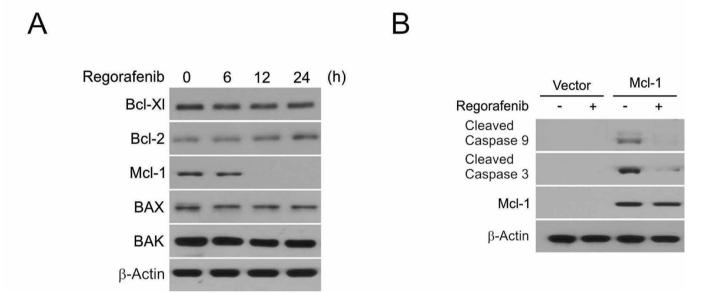

FIGURE 5 – Mcl-1 reduction in regorafenib-treated MCF-7 cells. **(A)** MCF-7 cells were treated with 20-µmol/L regorafenib at the indicated time points. Indicated protein expression was analyzed using western blotting. **(B)** MCF-7 cells transected with Mcl-1 plasmid or vector control were treated with 20-µmol/L regorafenib for 48 h. Cleaved caspase-3 and -9 were analyzed using western blotting.

DISCUSSION

Regorafenib, an oral multikinase inhibitor, can block the activity of several protein kinases that are involved in multiple biological processes of cancer (Ettrich, Seufferlein, 2014). These kinases include: TIE-2 and VEGFR1–VEGFR3, which are involved in tumor angiogenesis; KIT, RET, and BRAF, which are involved in oncogenesis; and RAF-1, FGFR, and PDGFR, which are involved in tumor microenvironment (Ettrich, Seufferlein, 2014; Weng et al., 2015; Yan, Grothey, 2015). In this study, we evaluated the anticancer activity of regorafenib in ER-positive breast cancer cell line MCF-7. We found that regorafenib treatment significantly decreased the viability of MCF-7 cells at different concentrations compared with that of controls. In addition, MCF-10A cell growth was not affected by regorafenib, suggesting that it may not have strong side effects in vivo.

Moreover, we found that MCF-7 cell migration was dramatically suppressed following treatment with regorafenib. Migration is essential for metastasis, which is the primary factor responsible for death among patients with breast cancer (Thakur, Bedogni, 2016; Vilalta et al., 2016). Reportedly, the homeostatic expression of MPP and their inhibitors TIMP is essential for migration and is involved in the metastasis of breast cancer cells (Groblewska et al., 2012; Lawicki et al., 2016). Our results demonstrate that regorafenib downregulate the expression of genes that promote migration and upregulate the expression of genes that suppress migration, which revealed an interesting mechanism of how regorafenib inhibits cell migration; these results are consistent with those of another study (Stalker et al., 2014).

The present study revealed that the viability of MCF-7 cells was suppressed by regorafenib, which enhanced cell cycle arrest at the G2/M phase. CDKs play an important

role in controlling cell proliferation by maintaining cell cycling; moreover, CDK inhibition has been considered as an attractive therapeutic alternative for cancers, such as ER-positive breast cancer (Bendris *et al.*, 2015; Mayer, 2015; Santo *et al.*, 2015). Our results demonstrated that regorafenib significantly suppressed the expression of multiple CDKs, indicating that regorafenib targets multiple CDKs, which may be an effective alternative to completely suppress cell cycling.

More importantly, regorafenib effectively induced apoptosis in MCF-7 cells. It also significantly reduced Mcl-1 expression treated MCF-7 cells (Tong *et al.*, 2017a). Mcl-1 can bind to proapoptotic proteins, such as Bax, and neutralize their proapoptotic functions (Inuzuka *et al.*, 2011; Quinn *et al.*, 2011; Thomas *et al.*, 2010). Therefore, reducing Mcl-1 expression may increase the susceptibility of cells to the stimulation of apoptosis signaling. Mcl-1 has been a target for therapeutic intervention to enhance vulnerability to therapy in ER-positive breast cancer. Further investigations are warranted to elucidate how regorafenib treatment can suppress Mcl-1 expression.

In conclusion, regorafenib may simultaneously inhibit several cancer-associated pathways in ER-positive breast cancer cells, indicating that regorafenib is an effective drug for the treatment of ER-positive breast cancer.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare that there are no conflicts of interest.

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