

# Novel Imidazo[2,1-b]oxazole Derivatives Inhibit Epithelial Cell Transformation and Triple Negative Breast Cancer Tumorigenesis

POSHAN YUGAL BHATTARAI<sup>1\*</sup>, CHANG-HYUN OH<sup>2\*</sup>, GARAM KIM<sup>1</sup>,  
MIN SOO KIM<sup>1</sup>, BONG SANG LEE<sup>3</sup> and HONG SEOK CHOI<sup>1</sup>

<sup>1</sup>College of Pharmacy, Chosun University, Gwangju, Republic of Korea;

<sup>2</sup>Department Centre for Biomaterials, Korea Institute of Science and Technology, Seoul, Republic of Korea;

<sup>3</sup>CTCScience Inc., Hwaseong, Republic of Korea

**Abstract.** *Background/Aim:* Triple negative breast cancer (TNBC) is an aggressive type of breast cancer with limited targets for chemotherapy. This study evaluated the inhibitory effects of novel imidazo[2,1-b]oxazole-based rapidly accelerated fibrosarcoma (RAF) inhibitors, KIST0215-1 and KIST0215-2, on epithelial cell transformation and TNBC tumorigenesis. *Materials and Methods:* Immunoblotting, BrdU incorporation assay, reporter gene assay, and soft agar assay analyses were performed. *In vivo* effects were studied using the BALB/c mouse xenograft model. *Results:* KIST0215-1 and KIST0215-2 inhibited the RAFs-MEK1/2-ERK1/2 signalling pathway induced by EGF in MDA-MB-231 cells, which inhibited c-fos transcriptional activity and activator protein-1 transactivation activity. KIST0215-1 and KIST0215-2 also prevented neoplastic transformation of JB6 C141 mouse epidermal cells induced by EGF and consistently suppressed the growth of tumours formed by 4T1 cells in BALB/c mice. *Conclusion:* Inhibition of RAF kinases using KIST0215-1 and KIST0215-2 is a promising chemotherapeutic strategy to treat TNBC.

Breast cancer is the leading cause of cancer-related deaths in women worldwide (1). Clinically, breast cancer is characterised based on the expression levels of the oestrogen receptor, progesterone receptor (PgR), and human epidermal growth factor-2 (HER2) receptor (2). These receptors are not expressed

in triple negative breast cancer (TNBC) patients, and therefore, do not respond to hormone targeted therapies, such as tamoxifen, and to specific antibodies, such as trastuzumab, which are effective in other types of breast cancers (1, 3, 4). Currently, TNBC is treated with adjuvant chemotherapies, such as platinum compounds, taxanes, and anthracycline derivatives, combined with surgery and radiotherapy (5). However, high drug toxicity and relapse rates limit the therapeutic usefulness of these compounds (5). Numerous efforts have been made to develop targeted chemotherapies for TNBC. Several compounds with novel targets such as PARP, VEGF/VEGFR, EGFR, PI3K, HDAC or mTOR are potential chemotherapy candidates in TNBC (5-7). However, these compounds have not been as clinically successful as anticipated. Thus, alternative molecular targets have to be identified to develop truly effective targeted chemotherapies.

Several cancer driver genes are either mutated or amplified in TNBC, making it a heterogeneous disease (1, 4). Mitogen-activated protein kinase (MAPK) signalling pathway up-regulation is frequently seen in TNBC (8-10). The Cancer Genome Atlas dataset analysis has revealed that approximately 80% of basal-like TNBC have some degree of genomic amplification or activation of major components of the MAPK signalling pathway (11). MAPK signalling is a cascade of protein kinases, regulating cell proliferation, survival, and apoptosis in response to growth factors such as EGF, IGF-1, or PDGF (10, 12). Activation of the RAS oncogene is the initial step in the activation of the MAPK pathway (13). Oncogenic RAS recruits RAF kinases, a family of serine/threonine-specific protein kinases composed of A-RAF, B-RAF and C-RAF, to the cell membrane and phosphorylates serine or threonine residues (13). RAF kinases then activate MEK1/2 by phosphorylating multiple serine residues. MEK1/2 subsequently phosphorylates ERK1/2, leading to their activation (13). MEK1/2 and ERK1/2 are dual-specificity kinases that modulate the

\*These Authors contributed equally to this study.

*Correspondence to:* Hong Seok Choi, College of Pharmacy, Chosun University, 309 Pilmun-daero, Dong-gu, Gwangju 501-759, Republic of Korea. Tel: +82 622306379, Fax: +82 622225414, e-mail: chs@chosun.ac.kr

**Key Words:** Imidazo[2,1-b]oxazole derivatives, RAF inhibitors, TNBC, chemotherapy.

expression of several cancer driver genes *via* the phosphorylation and activation of transcription factors such as c-Fos, c-Jun, c-Myc, and activator protein-1 (AP-1) (13). Sustained activation of the MAPK pathway leads to the enhanced proliferation of TNBC cancer cells.

Many cancer cell types harbour mutations in the *RAS* and *RAF* genes, whose proteins are constitutively active (10). Activating mutations in the *RAS* oncogene are most predominant in pancreatic adenocarcinoma (90%), colorectal (50%), thyroid (50%), and lung cancers (30%) (10). Activating mutations in *B-RAF* oncogenes are mostly found in melanoma (70%), thyroid (40-70%), and colorectal carcinomas (18%) (10). The high frequency of activating *RAS* and *RAF* oncogene mutations in cancer has led to the development of small molecule inhibitors for these proteins (10, 13, 14). However, *RAS* inhibitors are not feasible for various reasons including high affinity of *RAS* for GTP (10). *RAF* inhibitors including vemurafenib, sorafenib, and dabrafenib have been clinically approved for treating melanoma, non-small-cell lung cancer, renal cell carcinoma, and thyroid cancer (15).

MAPK pathway up-regulation is frequently observed in TNBC due to wild-type *B-RAF* and *C-RAF* overexpression. The Cancer Genome Atlas database analysis revealed that the *B-RAF* gene is amplified in approximately 31% of TNBC specimens, with activating *B-RAF* gene mutations in only 1-2% of cases (11). Therefore, *RAF* kinase inhibitors are suggested as potential candidates for the chemotherapy of TNBC patients (16). However, clinically approved *RAF* kinase inhibitors have failed to yield clinical benefits for TNBC patients. *RAF* kinase inhibitors, such as vemurafenib and dabrafenib, are effective on tumours containing the *B-RAF*-V600E mutation but are not indicated for TNBC patients, since the V600E mutation is usually absent in TNBC (17). Sorafenib, which inhibits multiple kinases, including *C-RAF*, has modest activity against TNBC when combined with capecitabine or paclitaxel (18, 19). However, sorafenib failed to demonstrate a similar clinical benefit against TNBC during a phase III clinical trial (20). Sorafenib belongs to the class of ATP-competitive inhibitors, which are relatively poor inhibitors of wild-type *B-RAF* kinase (21). Indeed, ATP-competitive *RAF* inhibitors might lead to the paradoxical activation of the MAPK pathway in *B-RAF* wild-type cancer cells, including TNBC, by promoting *B-RAF* dimerization (22). Pan-*RAF* inhibitors, which inhibit all three forms of *RAF* kinases, may avoid the paradoxical activation of MAPK pathway (23). Furthermore, pan-*RAF* inhibitors have an enhanced therapeutic response in mutant cancer cells lacking *B-RAF*-V600E, such as TNBC cells (23). Therefore, to explore the therapeutic efficacy of *RAF* inhibitors against TNBC, it is necessary to develop novel pan-*RAF* kinase inhibitors or oncogenic *B-RAF* and *C-RAF* kinase inhibitors.

Here, we developed two novel imidazo[2,1-b] oxazole derivatives, KIST0215-1 and KIST0215-2, as dual inhibitors of *B-RAF* and *C-RAF* kinases and show that inhibition of *RAF* kinases using these compounds reduces MDA-MB-231 cell proliferation. KIST0215-1 and KIST0215-2 reduced the MEK1/2 and ERK1/2 phosphorylation induced by EGF. Furthermore, these novel compounds also induced apoptosis and cell cycle arrest in MDA-MB-231 cells. Our study findings support the therapeutic potential of dual *RAF* inhibitors in TNBC treatment.

## Materials and Methods

**Cell lines and establishment of stable cell lines.** MDA-MB-231 and 4T1 breast cancer cells were grown in Eagle's minimal essential medium and Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum, respectively. All cell lines were cultured and maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Reagents and antibodies.** Antibodies against MEK1/2 (1:1,000), ERK1/2 (1:1,000), c-Fos (1:1,000), total caspase 3 (1:1,000), phospho-MEKs (1:1,000), phospho-ERKs (1:1,000), phospho-c-Fos (1:1,000), and cleaved caspase 3 (1:1,000) were acquired from Cell Signalling Technology Inc. (Danvers, MA, USA). The antibody against PARP (1:5,000) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse IgG-HRP and anti-rabbit IgG-HRP were purchased from Invitrogen (Carlsbad, CA, USA).

**Mammalian expression vectors.** The *c-fos-luc* promoter (pFos WT-GL3) was provided by Dr. Ron Prywes (Columbia University, New York, NY). The AP-1 luciferase reporter plasmid (pGL4.44[luc2P/AP1 RE/Hygro] vector; Cat. No., E4111) was purchased from Promega (Madison, WI, USA).

**Immunoblot assay.** The cells were disrupted in RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.25% sodium deoxycholate, 1 mM EDTA, 1% NP40, 1 mM NaF, 0.2 mM phenylmethyl sulfonyl fluoride, 0.1 mM sodium orthovanadate, and a protease inhibitor cocktail (Roche Life Science, Indianapolis, IN, USA). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA) blocked in 5% skim milk and probed with the indicated antibodies. The immunoblots were visualised using a SuperSignal West Femto chemiluminescence substrate (Thermo Fisher Scientific, Waltham, MA, USA) and detected using the LAS 4000-mini biomolecular imaging system (FUJIFILM, Tokyo, Japan).

**In vitro kinase assay.** Kinase assay was performed as described previously (24). Briefly, the Reaction Biology Corp. Kinase HotSpotSM service was used for the kinase assay using 1 µM concentration of ATP.

**Cell proliferation assay (BrdU incorporation).** Cell proliferation was assessed using a cell proliferation ELISA kit (Roche Life Sciences, Penzberg, Germany) according to the manufacturer's instructions. Briefly, MDA-MB-231 cells were seeded (5,000 cells per well) in 96-well plates in 100 µl of medium supplemented with 10% FBS. After

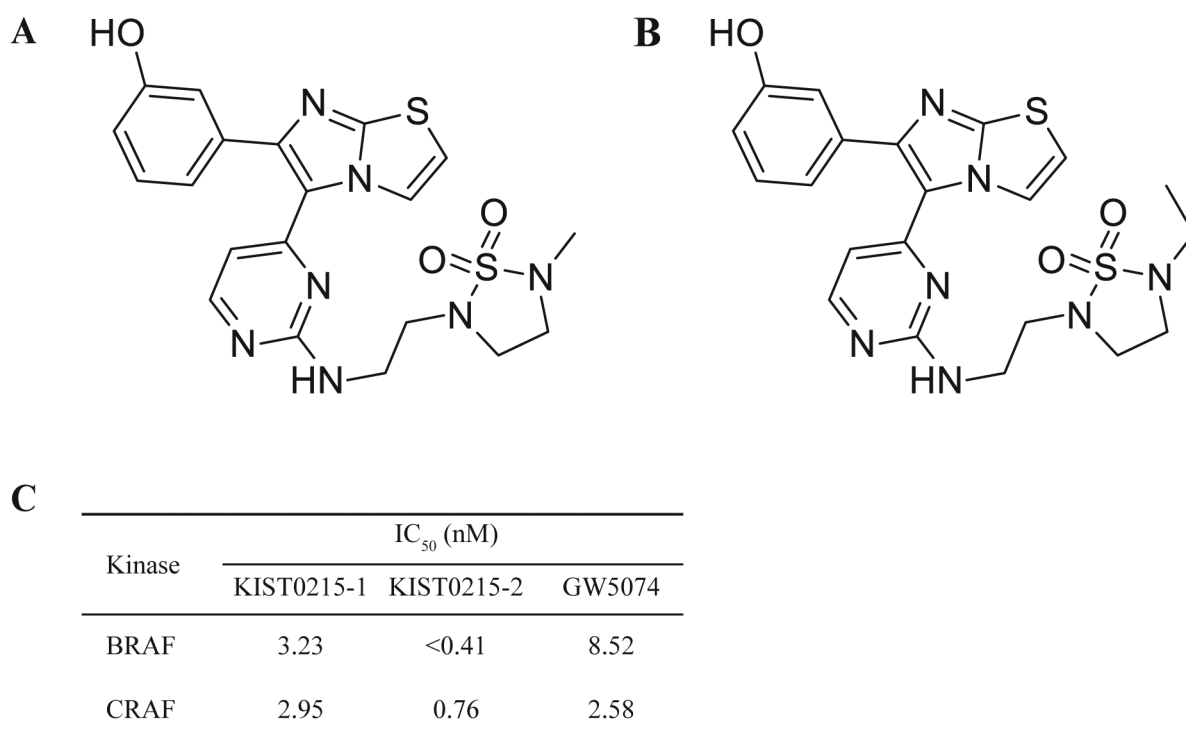


Figure 1. Chemical structure of novel imidazo[2,1-*b*]oxazole derivatives. (A) Structure of 2-(2-((4-(6-(3-hydroxyphenyl)imidazo[2,1-*b*]oxazole-5-yl)pyrimidine-2-yl)amino)ethyl)-5-methyl-1,2,5-thiazolidine 1,1-dioxide, KIST0215-1. (B) Structure of 2-ethyl-5-(2-((4-(6-(3-hydroxyphenyl)imidazo[2,1-*b*]oxazole-5-yl)pyrimidine-2-yl)amino)ethyl)-1,2,5-thiazolidine 1,1-dioxide, KIST0215-2. (C) IC<sub>50</sub> values of KIST0215-1 and KIST0215-2 against RAF kinases.

24 h, the cells were treated with KIST0215-1 and KIST0215-2 for 48 h, labelled with 10  $\mu$ L/well BrdU-labelling solution, and incubated for an additional 4 h at 37°C in 5% CO<sub>2</sub>. The medium containing the BrdU-labelling reagent was aspirated, and FixDenat solution was added into each well. The plate was incubated at room temperature (RT) for 30 min and the solution was removed. Anti-BrdU-POD-working solution was then added to each well and incubated for a further 90 min at RT. The cells were washed three times with washing solution, followed by addition of 100  $\mu$ L of substrate solution to each well and incubation for another 30 min. Cell proliferation was estimated by measuring the absorbance at 370 nm.

**Reporter gene assay.** The reporter gene assay for firefly luciferase activity was performed using lysates from AP-1 or c-Fos transfected JB6 Cl41 cells. In addition, the reporter gene vector pRL-TK-luciferase plasmid (Promega) was co-transfected into each cell line, and the Renilla luciferase activity generated by this vector was used to normalise the results for transfection efficiency. Cell lysates were prepared by washing the transfected JB6 Cl41 cells once with phosphate-buffered saline (PBS). After removing the PBS completely, passive lysis buffer (Promega) was added, and the cells were incubated at RT for 1 h with gentle shaking. The supernatant fraction was used to measure firefly and Renilla luciferase activities. Cell lysates (50  $\mu$ L each) were mixed with 50  $\mu$ L of luciferase assay II reagent (Promega), and firefly luciferase catalysed light emission was measured using a Glomax luminometer (Promega). Subsequently, 50  $\mu$ L of Renilla luciferase substrate was added, and the luminescence produced was measured.

**Anchorage-independent cellular transformation assay.** The effect of KIST0215-1 and KIST0215-2 on transformation was investigated in JB6 Cl41 cells. Briefly, 8×10<sup>3</sup> cells were exposed to the indicated doses of KIST0215-1 or KIST0215-2 in 1 ml of 0.3% Eagle's basal medium agar containing 10% FBS, 2 mM L-glutamine, and 25  $\mu$ g/ml gentamicin. The cultures were maintained at 37°C in a 5% CO<sub>2</sub> incubator for 14-20 days. Cell colonies were scored using an Axiovert 200 M fluorescence microscope and Axio Vision software (Carl Zeiss Inc., Thornwood, NY, USA).

**Detection of apoptosis.** The induction of apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining and detected with an *in situ* Cell Death detection kit (Roche Life Sciences). Briefly, 3×10<sup>5</sup> cells were cultured for 24 h in 6-well plates. The cells were then exposed to indicated concentrations of KIST0215-1 and KIST0215-2 for 24 h. The treated cells were washed with PBS and fixed with Cytfix/Cytoperm reagent (BD Biosciences, San Diego, CA, USA) at 4°C for 20 min. Cells were stained with 50  $\mu$ L TUNEL solution at 37°C for 1 h, washed twice with PBS, and fixed. DNA fragmentation was detected using the Axiovert 200 M fluorescence microscope.

**Tumorigenicity assay in mice.** Six-week-old female BALB/c mice (18-20 g) were obtained from Samtako Co. (Osan, Republic of Korea). The mice were acclimatised for 1 week and maintained in a clean room at the College of Pharmacy, Chosun University. Animal study protocols were approved by the Animal Care

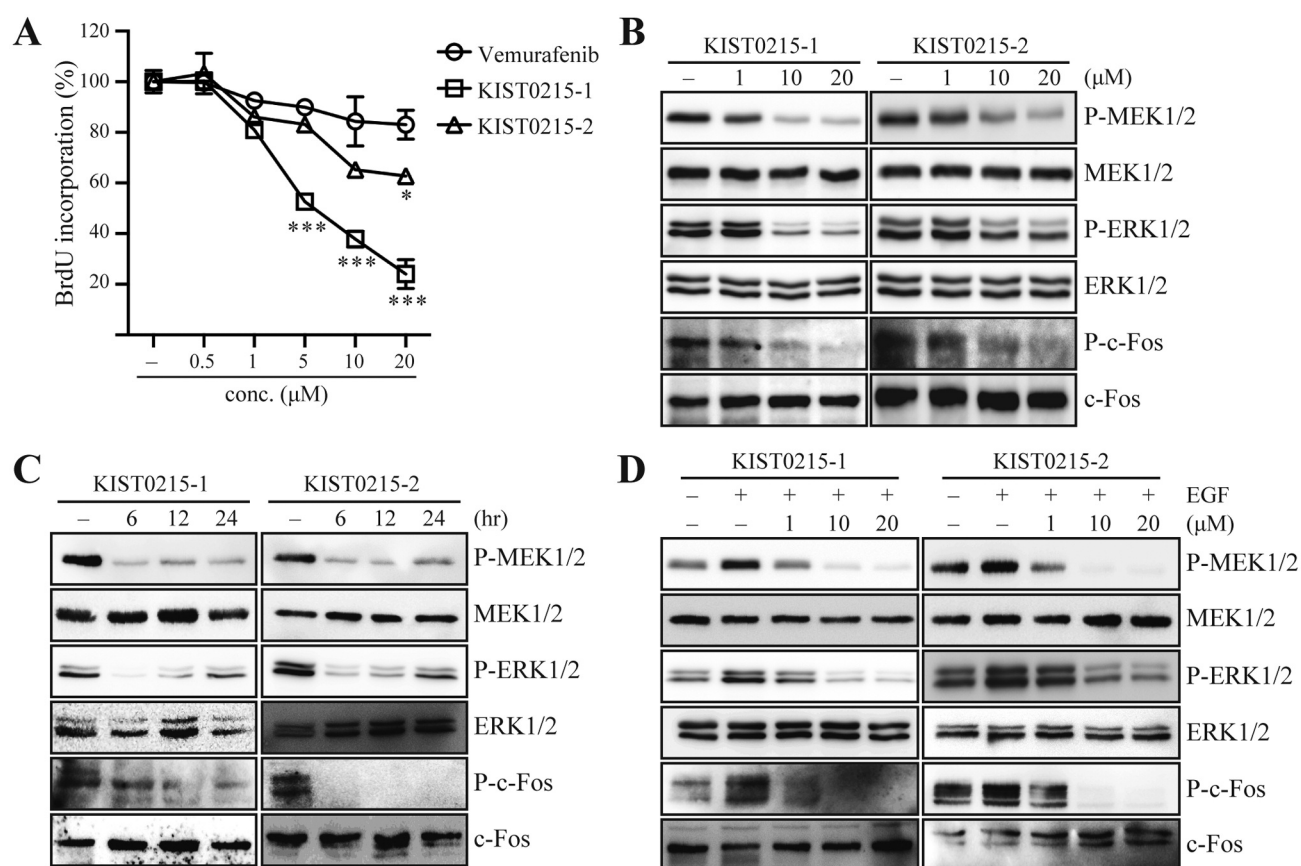


Figure 2. Effects of KIST0215-1 and KIST0215-2 on cell proliferation and signalling cascades of MEK1/2-ERK1/2-c-Fos in MDA-MB-231 cells. (A) Cells were treated with different concentrations of KIST0215-1 and KIST0215-2 for 48 h, and cell proliferation was estimated using the BrdU incorporation assay. Columns correspond to triplicate measurements of three experiments. The bars denote standard deviation (S.D). \* $p < 0.05$ , \*\*\* $p < 0.001$ , compared with control cells. (B and C) Cells were treated with the indicated doses of KIST0215-1 and KIST0215-2 for 24 h (B) or 20 μM KIST0215-1 and KIST0215-2 for the indicated times (C) and harvested. Levels of phosphorylated and total proteins related with MEK1/2, ERK1/2, and c-Fos in whole cell lysates were determined by immunoblotting analysis using specific antibodies against the corresponding proteins. (D) Cells were exposed to 10 ng/ml EGF with/without treatment with KIST0215-1 and KIST0215-2 at the indicated concentrations for 24 h and harvested. The phosphorylated and total proteins levels related with MEK1/2, ERK1/2, and c-Fos in whole cell lysates were determined by immunoblotting analysis using specific antibodies against the corresponding proteins.

Committee of Chosun University. Mice were randomly divided into two or four groups of 20 animals each, and 4T1 cells were trypsinised, washed, resuspended with PBS, and adjusted to a concentration of  $1 \times 10^6$  cells/100 μl in PBS. Cells were injected into the mammary gland of the mice together with 50 μM KIST0215-1 and/or KIST0215-2 and allowed to form tumours. The tumour volume was calculated using the formula: tumour volume =  $0.5 \times [(large\ diameter) \times (short\ diameter)^2]$ .

**Cell cycle assay.** MDA-MB-231 cells were seeded ( $3.0 \times 10^5$  cells per plate) in 60 mm dishes in 3 ml of Modified Eagle's medium supplemented with 10% FBS and incubated at 37°C in 5.0% CO<sub>2</sub>. After 24 h, cells were treated with 20 μM of KIST0215-1 and KIST0215-2 and incubated for 48 h. Cells were harvested and fixed with 500 μl of 70% cold ethanol at -20°C and analysed using the Muse Cell Analyser (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

**Statistical analysis.** Statistical calculations were carried out using Prism 4 for Macintosh (GraphPad Software Inc., La Jolla, CA, USA). Results are expressed as the mean ± standard error of triplicate measurements of three independent experiments. For multiple comparison analyses, a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used. Differences were considered significant when the calculated  $p$ -value was  $< 0.05$ .

## Results

**KIST0215-1 and KIST0215-2 inhibits cell proliferation and MAPK signalling in MDA-MB-231 cells.** An inhibition assay directed at B-Raf and C-Raf kinases was performed using the novel imidazo[2,1-b]oxazole compounds, KIST0215-1 and KIST0215-2, respectively (Figure 1A and B). Both compounds displayed inhibitory concentration (IC<sub>50</sub>) in

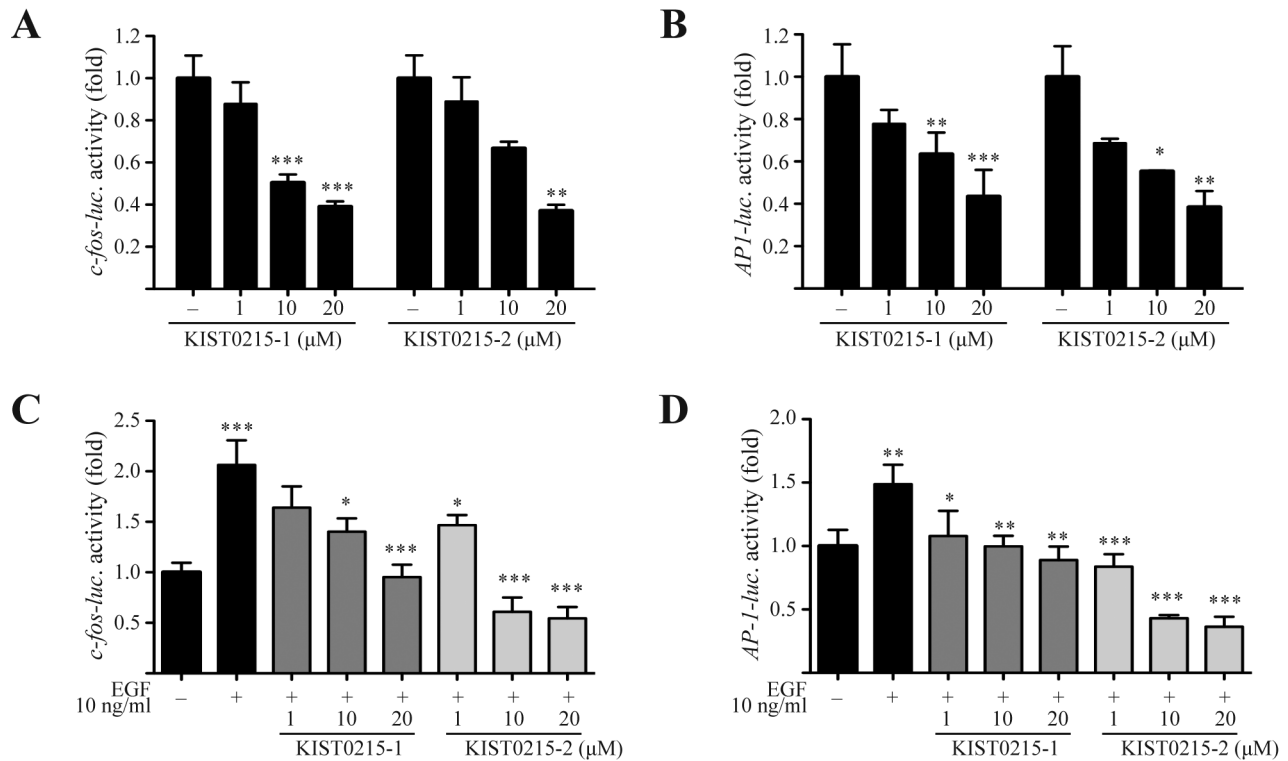


Figure 3. Effects of KIST0215-1 and KIST0215-2 on c-fos and AP-1 promoter activities in MDA-MB-231 cells. (A and B) Cells were transfected with a plasmid mixture containing c-fos-luc (A) and AP-1-luc promoter gene (B) with the pRL-TK vector. Twenty-four hours after transfection, cells were treated with the indicated concentrations of KIST0215-1 and KIST0215-2 for 24 h and the reporter gene assay was performed. (C and D) Cells were transfected with a plasmid mixture containing c-fos-luc (C) and AP-1-luc promoter gene (D) with the pRL-TK vector. Twenty-four hours after transfection, cells were serum-starved for 12 h, and then treated with 10 ng/ml EGF in the presence or absence of the indicated doses of KIST0215-1 and KIST0215-2 for 24 h. In all the promoter assays, the firefly luciferase activity was determined in cell lysates and normalised against Renilla luciferase activity. The luciferase activities are expressed relative to control cells. Columns, mean of triplicate measurements of two experiments. The bars denote S.D. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared to control cells.

nanomolar ranges against B-Raf and C-Raf kinases (Figure 1C). Therefore, KIST0215-1 and KIST0215-2 were characterised as B-Raf and C-Raf inhibitors. Next, we examined whether these compounds can reduce the MDA-MB-231 cell proliferation using the BrdU incorporation assay. They significantly reduced cell proliferation in a dose-dependent manner (Figure 2A). To investigate whether KIST0215-1 and KIST0215-2 can inhibit MEK1/2 and ERK1/2 phosphorylation, MDA-MB-231 cells were treated with various doses of KIST0215-1 and KIST0215-2 for various times. The levels of phosphorylated MEK1/2 and ERK1/2 was detected by immunoblotting. KIST0215-1 and KIST0215-2 inhibited MEK1/2, ERK1/2, and c-Fos phosphorylation in a dose- and time-dependent manner (Figure 2B and 2C). To examine the effects of KIST0215-1 and KIST0215-2 on MEK1/2 and ERK1/2 phosphorylation induced by EGF, MDA-MB-231 cells were exposed to EGF in the absence or presence of various concentrations of KIST0215-1 and KIST0215-2. Both compounds inhibited the

EGF-induced phosphorylation of MEK1/2, ERK1/2, and c-Fos (Figure 2D). Hence, these data suggested that KIST0215-1 and KIST0215-2 inhibit the Rafs-MEK1/2-ERK1/2 signalling pathway, and thereby inhibit MDA-MB-231 cell proliferation.

*KIST0215-1 and KIST0215-2 inhibit EGF-induced c-Fos and AP-1 promoter activity.* The AP-1 transcription factor is important in TNBC tumorigenesis (25). AP-1 is a heterodimer composed of Jun and Fos proteins, whose activity is regulated by the MAPK pathway in response to growth factors including EGF. To ascertain whether KIST0215-1 and KIST0215-2 can suppress c-Fos and AP-1 transcriptional activity in MDA-MB-231 cells, reporter plasmids carrying the luciferase gene under the control of c-Fos promoter or AP-1 response elements were used. The c-Fos and AP-1 activities were inhibited by KIST0215-1 and KIST0215-2 in a dose-dependent manner (Figure 3A and B). These compounds also suppressed the EGF-induced c-Fos

and AP-1 promoter activities in a dose-dependent manner (Figure 3C and D). These results indicated that KIST0215-1 and KIST0215-2 can inhibit the activities of c-Fos and AP-1 transcription factors induced by the RAF-MEK1/2-ERK1/2 pathway in MDA-MB-231 cells.

*KIST0215-1 and KIST0215-2 induces apoptosis and cell cycle arrest in MDA-MB-231 cells.* Next, we examined whether KIST0215-1 and KIST0215-2 can regulate apoptosis. We determined the levels of cleaved caspase 3 and cleaved-PARP using immunoblotting. KIST0215-1 and KIST0215-2 induced the cleavage of caspase 3 and PARP in a dose-dependent manner (Figure 4A). Furthermore, these compounds induced cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase (Figure 4B). Consistent with previous results, TUNEL staining revealed that KIST0215-1 and KIST0215-2 increased DNA fragmentation compared to control (Figure 4C). Taken together, these results suggest that KIST0215-1 and KIST0215-2 can induce apoptosis and cell cycle arrest in MDA-MB-231 cells.

*KIST0215-1 and KIST0215-2 suppress transformation and tumorigenesis of epithelial cells.* AP-1 transcription factor activation by various tumour promoters is highly important in the neoplastic transformation of the JB6 C141 cell line. Given that KIST0215-1 and KIST0215-2 reduced the activity of AP-1 in MDA-MB-231 cells, we examined whether these compounds can inhibit the transformation of JB6 C141 cells. Firstly, we examined the effects of the novel compounds on JB6 C141 cell proliferation induced by EGF. Both compounds inhibited EGF-induced cell proliferation (Figure 5A). Secondly, to examine the effect of KIST0215-1 and KIST0215-2 on cell transformation induced by EGF, JB6 C141 cells were treated with EGF in the absence or presence of KIST0215-1 and KIST0215-2 in a soft agar matrix and incubated for 14 days. KIST0215-1 and KIST0215-2 significantly reduced EGF-induced colony formation by JB6 C141 cells (Figure 5B and 5C). Finally, we studied the anti-tumorigenic effects of these compounds *in vivo*. The results showed that KIST0215-1 and KIST0215-2 significantly reduced the size and weight of tumours formed by 4T1 cells in BALB/c mice (Figure 5D).

## Discussion

TNBC accounts for 15% of all the breast cancers diagnosed worldwide (26). The pathophysiological features of TNBC are often more aggressive compared to those of other hormone receptor-positive breast cancers, and are represented by high relapse rates and increased metastatic potential (26). TNBC chemotherapy remains challenging since it does not express a distinct therapeutic target (2, 4). The current approach to treat TNBC is focused on the discovery of novel therapeutic targets (5). Pre-clinical trials involving AKT inhibitors that include patasertib have shown

promising results against TNBC (27). However, AKT inhibitors have been effective only in combination with the monoclonal antibodies atezolizumab and paclitaxel (27). Similarly, VEGF inhibitors, such as bevacizumab, have failed to improve overall survival in TNBC patients (28). Recently, the United States Food and Drug Administration (USFDA) approved the PARP inhibitor talazoparib for a small subset of TNBC patients having germline mutations in BRCA1/2 (29). The results from these clinical trials clearly suggest that therapeutic targets are still required in TNBC.

Emerging evidence suggests that the MAPK pathway is frequently activated in TNBC that is often accompanied by RAF kinase up-regulation (9, 16). MAPK pathway up-regulation leads to enhanced cell proliferation and survival of TNBC cells. Targeted inhibition of RAF kinases has thus been suggested as a potential chemotherapy option for TNBC patients. Sorafenib inhibits multiple kinases including C-RAF, and has shown promising results against TNBC (5). In a multinational double-blind, randomised phase IIb study evaluating the efficacy and safety of sorafenib in combination with capecitabine (SOLTI-0701 trial), sorafenib significantly improved the median progression-free survival of metastatic breast cancer patients (30). However, the confirmatory phase III, randomised, double-blind RESILIENCE trial comparing sorafenib with capecitabine failed to show similar results (20). Previous studies have shown that all ATP-competitive RAF inhibitors – including sorafenib, dabrafenib and vemurafenib – are poor inhibitors of wild-type B-RAF that paradoxically activate MAPK pathway in B-RAF wild-type cells, including TNBC cells (21, 31). Therefore, significant efforts have been made towards the development of pan-RAF inhibitors for chemotherapy with little attention being paid towards the use of pan-RAF inhibitors in TNBC treatment. We developed two novel imidazo[2,1-b]oxazole compounds, KIST0215-1 and KIST0215-2, as dual inhibitors of B-RAF and C-RAF, which decreased MEK1/2 and ERK1/2 phosphorylation induced by EGF and consequently reduced the EGF-induced proliferation of MDA-MB-231 cells.

Oxazole compounds have significant anti-microbial, anti-viral, and anti-proliferative effects (24). Several oxazole derivatives including linezolid, oxacillin, and sulfisoxazole that have pronounced bioactivity, low toxicity, and excellent pharmacokinetic profiles have been used clinically for a long time (24). A dihydroimidazo[2,1-b]oxazole derivative, delamanid, was recently approved by the USFDA for multi-drug resistant tuberculosis treatment (32). However, the anti-cancer properties of these derivatives are poorly understood. A recent study reported that imidazo[2,1-b]oxazole-based compounds can inhibit RAF kinases, thereby reducing the viability of colon cancer and melanoma cell lines (24). Here, we developed a series of imidazo[2,1-b]oxazole compounds and found that KIST0215-1 and KIST0215-2 are potent

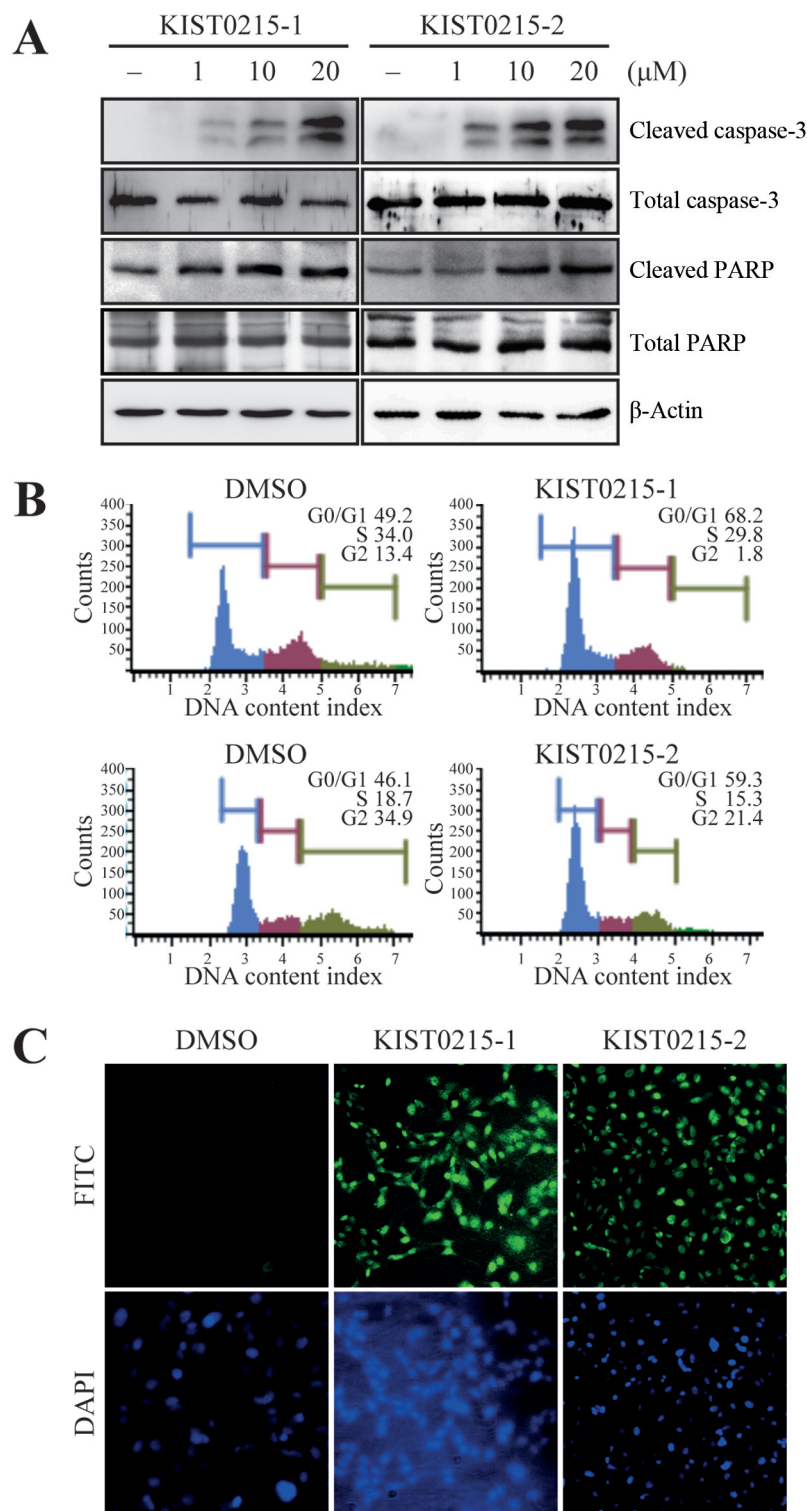


Figure 4. Effects of KIST0215-1 and KIST0215-2 on apoptotic signalling pathway in MDA-MB-231 cells. (A) Cells were treated with the indicated doses of KIST0215-1 and KIST0215-2 for 24 h and harvested. The levels of cleaved and total proteins of caspase 3 and PARP in whole cell lysates were determined by immunoblotting analysis using specific antibodies against the corresponding proteins, respectively. (B) Cells were treated or not treated with 20  $\mu$ M KIST0215-1 and KIST0215-2 for 24 h. The percentage of cells in the G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases was calculated using a Muse Cell Analyser. (C) Cells were treated or not treated with 20  $\mu$ M of KIST0215-1 and KIST0215-2 for 24 h. Cells were fixed and stained with TUNEL solution and DNA fragmentation induced by both compounds was measured.

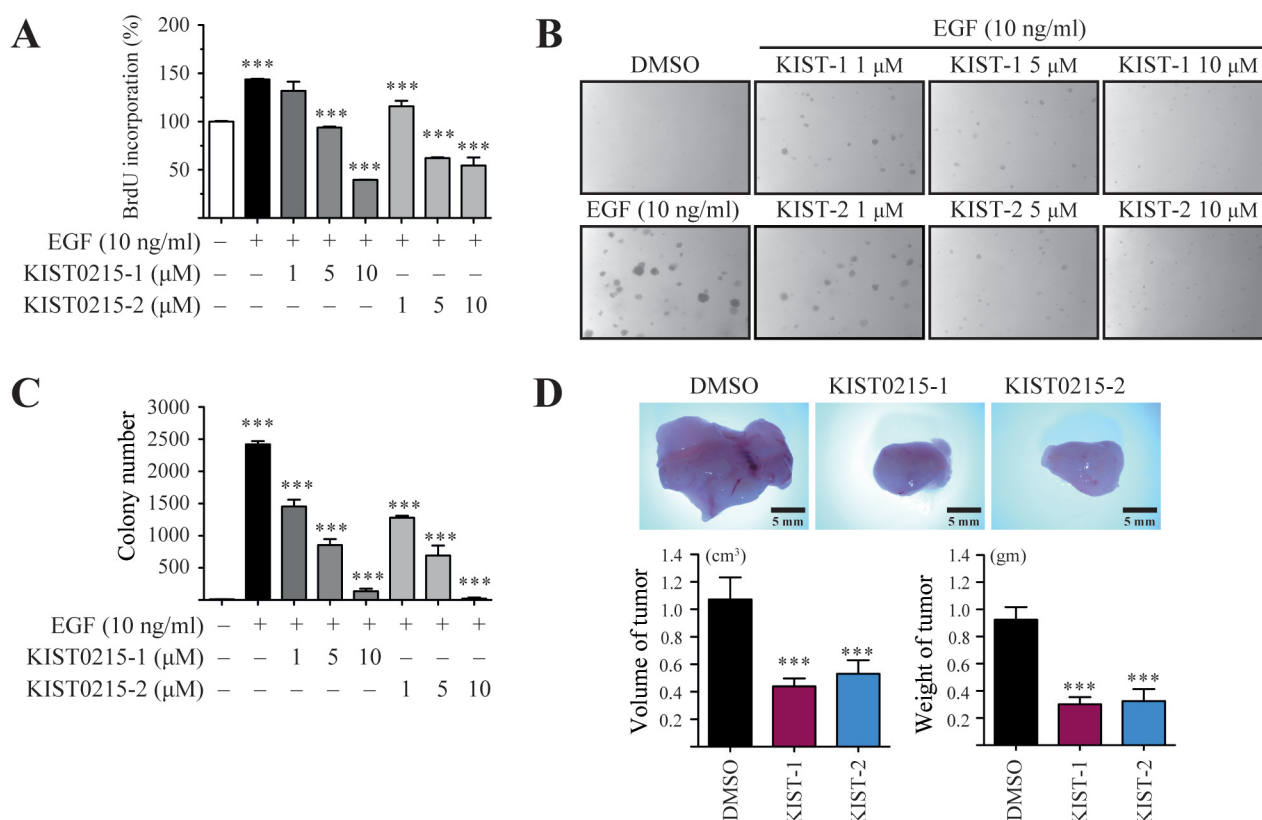


Figure 5. *In vitro* and *in vivo* effects of KIST0215-1 and KIST0215-2 on neoplastic cell transformation and tumorigenicity. (A) Cells were treated with different concentrations of KIST0215-1 and KIST0215-2 in the absence or presence of 10 ng/ml EGF for 72 h. Cell proliferation was estimated using the BrdU incorporation assay. Columns correspond to triplicate measurements of three experiments. The bars denote S.D. \*\*\* $p < 0.001$ , compared with control cells. (B and C) Cells were exposed to 10 ng/ml EGF with/without treatment with KIST0215-1 and KIST0215-2 in soft agar. The colonies were counted under a microscope using the Image-Pro Plus software. Representative colonies from three separate experiments are shown in (B). The colonies were counted under a microscope with the aid of the Image-Pro Plus software. (C) Columns correspond to triplicate samples. The bars denote S.D. \*\*\* $p < 0.001$ , compared with cells treated only with dimethyl sulfoxide (DMSO). (D) 4T1 cells were treated with 50 μM KIST0215-1 and KIST0215-2. The cells were injected into the mammary glands of BALB/c mice ( $n=30$ ) and allowed to grow until tumours formed (14 days). Representative pictures of tumours (top) and tumour volumes and weights (bottom) are shown. Columns correspond to triplicate samples. The bars denote S.E. \*\*\* $p < 0.001$ , compared to the only-DMSO treated group.

inhibitors of B-RAF and C-RAF kinases. We further clarified the anti-cancer properties of these compounds against MDA-MB-231 cells. These novel inhibitors of RAF kinases reduced MEK1/2 and ERK1/2 phosphorylation induced by EGF and consequently reduced c-Fos promoter and AP-1 transactivation activities and prevented the neoplastic transformation of JB6 C141 cells induced by EGF, demonstrating the anti-cancer properties of KIST0215-1 and KIST0215-2 as dual RAF inhibitors.

The AP-1 transcription factor plays an important role in epithelial cell transformation and TNBC tumorigenesis (33, 34). AP-1 composed of a variety of members including c-Fos, c-Jun, activation transcription factor is involved in mediating many biological processes such as proliferation, differentiation and cell death (35). MAPK signalling pathway regulates the

function of AP-1 transcription factor (34). Phosphorylation of c-Fos and c-Jun transcription factors by ERKs allows them to dimerise to form a functional AP-1 transcription factor, which modulates the expression of several cancer driver genes including DNA methyltransferase 1, EGFR, cyclin D1, and Bcl2 by binding with TRE/AP-1 response elements found in the promoter region (35, 36). AP-1 family members are significantly up-regulated in TNBC cells and promote the progression of cancer induced by proinflammatory cytokines (33). Furthermore, AP-1 mediated transcription promoted the invasiveness of TNBC cells *in vivo* in a zebrafish tumour xenograft model (25). Presently, KIST0215-1 and KIST0215-2 reduced the transcriptional activity of AP-1 to prevent epithelial cell transformation and also reduced TNBC cell proliferation.

In summary, the inhibition of RAF kinases using two novel imidazo[2,1-b]oxazole derivatives inhibits epithelial cell transformation and reduces MDA-MB-231 cell tumorigenicity. Furthermore, these novel compounds reduce the growth of tumours formed by 4T1 cells in BALB/c mice. RAF inhibitors are often used in combination with MEK1/2 inhibitors or AKT inhibitors to improve their efficacy and prevent acquired resistance. Therefore, further studies are required to evaluate the efficacy of KIST0215-1 and KIST0215-2 in combination with other compounds, such as MEK inhibitors or AKT inhibitors, to achieve maximum clinical benefit for TNBC patients. Our study not only reports dual inhibitors of RAF kinases but also illustrates the therapeutic potential of dual RAF inhibitors in the chemotherapy of TNBC.

### Conflicts of Interest

The Authors declare no conflicts of interest.

### Authors' Contributions

P.Y.B., G.K., M.S.K. and C.H.O. designed and performed experiments, analysed and interpreted data, and wrote the manuscript. H.S.C. conceived the study, designed experiments, interpreted data, and wrote the manuscript. All Authors reviewed the manuscript.

### Acknowledgements

This work was supported by research funds from Chosun University (2015).

### References

- 1 Aysola K, Desai A, Welch C, Xu J, Qin Y, Reddy V, Matthews R, Owens C, Okoli J, Beech DJ, Piyathilake CJ, Reddy SP and Rao VN: Triple negative breast cancer - an overview. *Hereditary Genet* 2013(Suppl 2): 001, 2013. PMID: 25285241. DOI: 10.4172/2161-1041.S2-001
- 2 Waks AG and Winer EP: Breast cancer treatment: A review. *JAMA* 321(3): 288-300, 2019. PMID: 30667505. DOI: 10.1001/jama.2018.19323
- 3 Damaskos C, Garmpi A, Nikolettos K, Vavourakis M, Diamantis E, Patsouras A, Farmaki P, Nonni A, Dimitroulis D, Mantas D, Antoniou Ea, Nikolettos N, Kontzoglou K And Garmpis N: Triple-negative breast cancer: The progress of targeted therapies and future tendencies. *Anticancer Res* 39(10): 5285-5296, 2019. PMID: 31570423. DOI: 10.21873/anticancer.13722
- 4 Stockmans G, Deraedt K, Wildiers H, Moerman P and Paridaens R: Triple-negative breast cancer. *Curr Opin Oncol* 20(6): 614-620, 2008. PMID: 10934195. DOI: 10.1097/CCO.0b013e328312efba
- 5 Hwang SY, Park S and Kwon Y: Recent therapeutic trends and promising targets in triple negative breast cancer. *Pharmacol Ther* 199: 30-57, 2019. PMID: 30825473. DOI: 10.1016/j.pharmthera.2019.02.006
- 6 Garmpis N, Damaskos C, Garmpi A, Kalampokas E, Kalampokas T, Spartalis E, Daskalopoulou A, Valsami S, Kontos M, Nonni A, Kontzoglou K, Perrea D, Nikiteas N And Dimitroulis D: Histone deacetylases as new therapeutic targets in triple-negative breast cancer: Progress and promises. *Cancer Genomics Proteomics* 14(5): 299-313, 2017. PMID: 28870998. DOI: 10.21873/cgp.20041
- 7 Pohl SG, Brook N, Agostino M, Arfuso F, Kumar AP and Dharmarajan A: Wnt signaling in triple-negative breast cancer. *Oncogenesis* 6(4): e310, 2017. PMID: 28368389. DOI: 10.1038/oncsis.2017.14
- 8 Bartholomeusz C, Gonzalez-Angulo AM, Liu P, Hayashi N, Lluch A, Ferrer-Lozano J and Hortobagyi GN: High ERK protein expression levels correlate with shorter survival in triple-negative breast cancer patients. *Oncologist* 17(6): 766-774, 2012. PMID: 22584435. DOI: 10.1634/theoncologist.2011-0377
- 9 Huang J, Luo Q, Xiao Y, Li H, Kong L and Ren G: The implication from RAS/RAF/ERK signaling pathway increased activation in epirubicin treated triple negative breast cancer. *Oncotarget* 8(64): 108249-108260, 2017. PMID: 29296238. DOI: 10.18632/oncotarget.22604
- 10 Santarpia L, Lippman SM and El-Naggar AK: Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. *Expert Opin Ther Targets* 16(1): 103-119, 2012. PMID: 22239440. DOI: 10.1517/14728222.2011.645805
- 11 Johnson GL, Stuhlmiller TJ, Angus SP, Zawistowski JS and Graves LM: Molecular pathways: adaptive kinome reprogramming in response to targeted inhibition of the BRAF-MEK-ERK pathway in cancer. *Clin Cancer Res* 20(10): 2516-2522, 2014. PMID: 24664307. DOI: 10.1158/1078-0432.CCR-13-1081
- 12 De Luca A, Maiello MR, D'Alessio A, Pergameno M and Normanno N: The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches. *Expert Opin Ther Targets* 16 Suppl 2(sup2): S17-27, 2012. PMID: 22443084. DOI: 10.1517/14728222.2011.639361
- 13 Molina JR and Adjei AA: The Ras/Raf/MAPK pathway. *J Thorac Oncol* 1(1): 7-9, 2006. PMID: 17409820. DOI: 10.1016/S1556-0864(15)31506-9
- 14 Chen RE and Thorner J: Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1773(8): 1311-1340, 2007. PMID: 17604854. DOI: 10.1016/j.bbamer.2007.05.003
- 15 Khazak V, Astsaturon I, Serebriiskii IG and Golemis EA: Selective Raf inhibition in cancer therapy. *Expert Opin Ther Targets* 11(12): 1587-1609, 2007. PMID: 18020980. DOI: 10.1517/14728222.11.12.1587
- 16 Giltneane JM and Balko JM: Rationale for targeting the Ras/MAPK pathway in triple-negative breast cancer. *Discov Med* 17(95): 275-283, 2014. PMID: 24882719.
- 17 Sharma A, Shah SR, Illum H and Dowell J: Vemurafenib: targeted inhibition of mutated BRAF for treatment of advanced melanoma and its potential in other malignancies. *Drugs* 72(17): 2207-2222, 2012. PMID: 23116250. DOI: 10.2165/11640870-000000000-00000
- 18 Baselga J, Costa F, Gomez H, Hudis CA, Rapoport B, Roche H, Schwartzberg LS, Petrenciuc O, Shan M and Gradishar WJ: A phase 3 trial comparing capecitabine in combination with Sorafenib or placebo for treatment of locally advanced or metastatic HER2-Negative breast Cancer (the RESILIENCE

- study): study protocol for a randomized controlled trial. *Trials* 14(1): 228, 2013. PMID: 23876062. DOI: 10.1186/1745-6215-14-228
- 19 Loibl S, Rokitta D, Conrad B, Harbeck N, Wullner M, Warm M, Schwedler K, Gerber B, Schrader I, Eidtmann H, Mehta K, Fuhr U and von Minckwitz G: Sorafenib in the treatment of early breast cancer: Results of the neoadjuvant phase II study - SOFIA. *Breast Care (Basel)* 9(3): 169-174, 2014. PMID: 25177258. DOI: 10.1159/000363430
- 20 Baselga J, Zamagni C, Gomez P, Bermejo B, Nagai SE, Melichar B, Chan A, Mangel L, Bergh J, Costa F, Gomez HL, Gradishar WJ, Hudis CA, Rapoport BL, Roche H, Maeda P, Huang L, Meinhardt G, Zhang J and Schwartzberg LS: RESILIENCE: Phase III randomized, double-blind trial comparing sorafenib with capecitabine *versus* placebo with capecitabine in locally advanced or metastatic HER2-negative breast cancer. *Clin Breast Cancer* 17(8): 585-594 e584, 2017. PMID: 28830796. DOI: 10.1016/j.clbc.2017.05.006
- 21 Holderfield M, Deuker MM, McCormick F and McMahon M: Targeting RAF kinases for cancer therapy: BRAF-mutated melanoma and beyond. *Nat Rev Cancer* 14(7): 455-467, 2014. PMID: 24957944. DOI: 10.1038/nrc3760
- 22 Poulikakos PI, Zhang C, Bollag G, Shokat KM and Rosen N: RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* 464(7287): 427-430, 2010. PMID: 20179705. DOI: 10.1038/nature08902
- 23 Molnar E, Rittler D, Baranyi M, Grusch M, Berger W, Dome B, Tovar J, Aigner C, Timar J, Garay T and Hegedus B: Pan-RAF and MEK vertical inhibition enhances therapeutic response in non-V600 BRAF mutant cells. *BMC Cancer* 18(1): 542, 2018. PMID: 29739364. DOI: 10.1186/s12885-018-4455-x
- 24 Abdel-Maksoud MS, Ammar UM, El-Gamal MI, Gamal El-Din MM, Mersal KI, Ali EMH, Yoo KH, Lee KT and Oh CH: Design, synthesis, and anticancer activity of imidazo[2,1-b]oxazole-based RAF kinase inhibitors. *Bioorg Chem* 93: 103349, 2019. PMID: 31627060. DOI: 10.1016/j.bioorg.2019.103349
- 25 Zhao C, Qiao Y, Jonsson P, Wang J, Xu L, Rouhi P, Sinha I, Cao Y, Williams C and Dahlman-Wright K: Genome-wide profiling of AP-1-regulated transcription provides insights into the invasiveness of triple-negative breast cancer. *Cancer Res* 74(14): 3983-3994, 2014. PMID: 24830720. DOI: 10.1158/0008-5472.CAN-13-3396
- 26 Goncalves H, Jr., Guerra MR, Duarte Cintra JR, Fayer VA, Brum IV and Bustamante Teixeira MT: Survival study of triple-negative and non-triple-negative breast cancer in a Brazilian cohort. *Clin Med Insights Oncol* 12: 1179554918790563, 2018. PMID: 30083066. DOI: 10.1177/1179554918790563
- 27 Dent R, Im SA, Espie M, Blau S, Tan AR and Isakoff SJ: Overall survival (OS) update of the double-blind placebo (PBO)-controlled randomized phase 2 LOTUS trial of first-line ipatasertib (IPAT) plus paclitaxel (PAC) for locally advanced/metastatic triple-negative breast cancer (mTNBC). *J Clin Oncol* 36(15): 1008-1008, 2018. DOI: 10.1200/JCO.2018.36.15\_suppl.1008
- 28 Ribatti D, Nico B, Ruggieri S, Tamma R, Simone G and Mangia A: Angiogenesis and antiangiogenesis in triple-negative breast cancer. *Transl Oncol* 9(5): 453-457, 2016. PMID: 27751350. DOI: 10.1016/j.tranon.2016.07.002
- 29 Beniey M, Haque T and Hassan S: Translating the role of PARP inhibitors in triple-negative breast cancer. *Oncoscience* 6(1-2): 287-288, 2019. PMID: 30800714. DOI: 10.18632/oncoscience.474
- 30 Baselga J, Roché H, Costa F, Getúlio Martins Segalla J, Pincowski H, Ma Ciruelos E, Cabral Filho S, Gómez P and Van Eyll B: SOLTI-0701: A multinational double-blind, randomized phase 2b study evaluating the efficacy and safety of sorafenib compared to placebo when administered in combination with capecitabine in patients with locally advanced or metastatic breast cancer (BC). *Cancer Res* 69(24 Suppl): 45-45, 2009. DOI: 10.1158/0008-5472.Sabcs-09-45
- 31 Durrant DE and Morrison DK: Targeting the Raf kinases in human cancer: the Raf dimer dilemma. *Br J Cancer* 118(1): 3-8, 2018. PMID: 29235562. DOI: 10.1038/bjc.2017.399
- 32 Xavier AS and Lakshmanan M: Delamanid: A new armor in combating drug-resistant tuberculosis. *J Pharmacol Pharmacother* 5(3): 222-224, 2014. PMID: 25210407. DOI: 10.4103/0976-500X.136121
- 33 Qiao Y, He H, Jonsson P, Sinha I, Zhao C and Dahlman-Wright K: AP-1 is a key regulator of proinflammatory cytokine TNFalpha-mediated triple-negative breast cancer progression. *J Biol Chem* 291(10): 5068-5079, 2016. PMID: 26792858. DOI: 10.1074/jbc.M115.702571
- 34 Eferl R and Wagner EF: AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* 3(11): 859-868, 2003. PMID: 14668816. DOI: 10.1038/nrc1209
- 35 Hess J, Angel P and Schorpp-Kistner M: AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci* 117(Pt 25): 5965-5973, 2004. PMID: 15564374. DOI: 10.1242/jcs.01589
- 36 Sharma SC and Richards JS: Regulation of AP1 (Jun/Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation. *J Biol Chem* 275(43): 33718-33728, 2000. PMID: 10934195. DOI: 10.1074/jbc.M003555200

Received May 15, 2020

Revised June 25, 2020

Accepted June 30, 2020