RESEARCH ARTICLE

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# Low Dose Berberine Suppresses Cholangiocarcinoma Cell Progression as a Multi-Kinase Inhibitor

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## Abstract

**Background:** Berberine (BBR), a natural isoquinoline alkaloid, possesses diverse pharmacological properties and anti-cancer effects that have been demonstrated in many *in vitro* and *in vivo* studies. In this study, the inhibitory effects and molecular mechanism of low dose BBR on EMT-induced cell migration, and invasion capability of cholangiocarcinoma (CCA) cell lines were demonstrated. **Methods:** The commercially available BBR chloride powder with purity ≥ 95% was used in this study. Effects of BBR on cell growth of two human CCA cell lines, KKU-213A and KKU-213B were measured using MTT assay. The progressive phenotypes-cell adhesion, migration, and invasion were evaluated using cell adhesion, wound healing, and Boyden chamber assays. Molecular docking analysis was performed to assess the possible binding mode of BBR against EGFR, Erk, STAT3 and Akt. The effects of BBR on the activations of EGF/EGFR and its downstream effectors were demonstrated using Western blotting. **Results:** BBR inhibited growth of CCA cells in a dose dependent manner. At sub-cytotoxic dose, BBR significantly inhibited cell adhesion, migration, invasion and decreased expression of vimentin, slug, and VEGFA of both CCA cell lines. Molecular docking suggested the simultaneous inhibitory activity of BBR on EGFR, Erk, STAT3 and Akt. The Western blot analyses revealed that upon the EGF/EGFR activation, BBR considerably attenuated the activations of EGFR, Erk, STAT3 and Akt. **Conclusion:** Low dose of BBR suppresses EMT and thus aggressiveness of CCA cells, in part by its multi-kinase inhibitor property on EGFR and its downstream pathways. BBR might be beneficial for therapy of human CCA.

**Keywords:** Benzylisoquinoline alkaloid- berberine- bile duct cancer- epithelial-mesenchymal transition- EGFR- VEGFA

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## Introduction

In the past few decades, the incidence and the mortality rate of cholangiocarcinoma (CCA), a highly lethal malignancy of the hepatobiliary system, have been increasing (Brindley et al., 2021). Prognosis of CCA patients is dismal and the treatment option is also limited because most patients present with unresectable or metastatic tumors. Moreover, CCA is highly resistant to the conventional chemotherapeutic drugs such as 5-fluorouracil, gemcitabine and cisplatin. A detailed information regarding epidemiology, pathophysiological mechanisms, diagnosis, and management of CCA was discussed in a recent review (Brindley et al., 2021). Taken together, CCA remains a highly devastating disease and there is a critical need to identify new efficacious therapeutic strategies to improve patients' outcome.

Medicinal plants represent an invaluable source of chemicals for novel drug discovery, especially for cancers and infectious diseases. The potential therapeutic effects of these medicinal plants have been characterized by high safety, availability, accessibility, and low cost (Atanasov et al., 2021). Numerous studies have demonstrated a wide range of pharmacological activities of berberine (BBR), an isoquinoline alkaloid (Figure 1a), extracted from roots, rhizomes, and stem bark of various medicinal plants, e.g., Ranunculaceae, Rutaceae, and Berberidaceae families (Neag et al., 2018). BBR possesses diverse/numerous effects against bacteria, chronic inflammation, hyperlipidemia, diabetes, and coronary artery disease (Cicero and Baggioni, 2016; Ai et al., 2021). In addition, numerous evidences support that BBR and its derivatives

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could be a promising anti-cancer agent (Zhang et al., 2019; Rauf et al., 2021). As evident in various cancers, BBR could inhibit cell growth, cell invasion and metastasis; induced cell apoptosis and autophagic cell death; and reduced cancer stemness (Zhang et al., 2019). Moreover, BBR can act as a radiosensitizer and a chemosensitizer of many cancer types (Devarajan et al., 2021).

Despite several investigations on the actions of BBR in various cancers, little is known about the effects of BBR on CCA progression. Few reports indicated that BBR inhibited CCA cell growth, and induced G1 cell cycle arrest and apoptosis (Puthdee et al., 2017) by inducing pro-apoptotic protein Bax and decreasing anti-apoptotic proteins Bcl-2 and Bcl-xL (He et al., 2012). In the present study, the effects of sub-cytotoxic dose BBR on CCA progressive phenotypes—cell adhesion, migration and invasion were investigated. Molecular docking approach was applied to identify the possible direct targets of BBR responsible for its anti-cancer activity and the actual involvements of those predicted molecules in the anti-cancer pathways of BBR was validated using a Western blotting assay.

## Materials and Methods

*Chemicals*

The study protocol was approved by the Khon Kaen University Ethics Committee for Human Research (HE641574). BBR chloride powder (purity ≥ 95%) was purchased (Sigma-Aldrich, St. Louis, MO, U.S.A.) and prepared as described previously (Puthdee et al., 2017).

*Cell lines and cell cultures*

Two human CCA cell lines, KKU-213A and KKU-213B, established from a Thai CCA patient with *Opisthorchis viverrini* infection (Sripa et al., 2020), were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. The cell lines were tested for mycoplasma free, and the short tandem repeat (STR) analysis was used for authentication of the currently used cell lines. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Calsbad, CA) supplemented with 25 mM glucose, 10% fetal bovine serum (FBS, Gibco; Paisley, Scotland, UK) and 1% antibiotic-antimycotic mixture (Gibco), at 37 °C in a humidified atmosphere of 5% $CO_2$. The protocol of the study was approved by the Khon Kaen University Ethics Committee for Human Research (HE641574).

*Cell viability assay*

CCA cells were plated at $5x10^3$ cells/well in a 96-well plate, overnight. BBR treatment was performed by incubating cells in a medium containing various concentrations of BBR (3.125, 6.25, 12.5, 25, and 50 µM) for 24 h. Control cells were incubated in the medium without BBR. Viable cells were determined using MTT assay. Briefly, a final concentration of 0.5 mg/mL MTT solution (Sigma-Aldrich) was added to each well and incubated at 37°C for 2 h. The formazan crystal formed was solubilized with 100 µL dimethyl sulfoxide and the absorbance at 540 nm was recorded using a microplate reader.

*Wound healing and cell adhesion assay*

The wound healing and cell adhesion assay were performed in the medium with or without 3.125 µM BBR as defined earlier with minor modification (Indramanee et al., 2019). CCA cells ($4x10^4$ cells) were allowed to adhere to a Matrigel (BD Biosciences, Bedford, MA) pre-coated well for 3 h at 37°C, 5% $CO_2$. The adhered cells were stained and counted under a microscope at 100x magnification.

*Cell migration and invasion assay*

The migration and invasion abilities of CCA cells were determined using Boyden chamber assay (8 µm pore size Transwell® inserts, Corning Inc.) according to the previous study (Indramanee et al., 2019).

*Expression of EMT markers and EGFR signaling pathway proteins*

To examine the effect of BBR on EMT marker expression, CCA cells ($2x10^5$ cells/well of a 6-well plate) cultured in a complete medium overnight were incubated in the medium containing the indicated concentration of BBR for further 24 h. Cells cultured in the medium without BBR was used as controls. For the effects of BBR on epidermal growth factor receptor (EGFR) signaling pathway, cells were cultured in the complete medium containing 12 µM BBR for 12 h and then in the medium containing 12 µM BBR and 100 ng/mL EGF (PeproTech, Rocky Hill, NJ) for further 12 h. Cells cultured in the complete medium for 12 h and then in the medium with 100 ng/mL EGF for further 12 h were used as a control.

*SDS-PAGE and Western blotting*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as previously described (Detarya et al., 2021). In brief, cell lysates (20 µg protein/well) were electrophoresed in a 10% SDS-PAGE and transferred onto a PVDF membrane. Blots were probed with the specific antibodies. Signals were developed with the ECL™ Prime reagent and then captured and analyzed on Image Quant™ Imager (GE Healthcare). The source of the primary antibodies: anti-slug (C19G7; 9585S), vimentin (D21H3; 5741S), EGFR (D38B1; 4267S), pEGFR (Y1068, 2234S), signal transducer and activator of transcription 3 (STAT3; D3Z2G; 12640S), pSTAT3 (S727; 9134S), extracellular signal-regulated kinase (Erk1/2, 9102S), and pErk1/2 (P202/Y204; 9101S), were as mentioned by Detarya et al (Detarya et al., 2021). In addition, anti-β-catenin (D10A8; 8480P), anti-claudin-1 (B5H1D, 13255S), anti-E-cadherin (24E10; 3195S), anti- protein kinase B (Akt, 9272S) and anti-pAkt (S473, 9271S), from Cell signaling, anti-VEGF (Santa Cruz Biotechnology, C-1, SC-7269) and anti-GAPDH (EMD Millipore, MAB374) were used.

*Molecular docking analysis*

Crystal structures of human EGFR (PDB ID: 1M17), Erk1 (2ZOQ), STAT3 (1BG1), and Akt (4GV1) were

obtained from Protein Data Bank. SWISS-MODEL server was used to complete missing residues of the protein structures. The protonation state of all ionizable amino acids of the studied targets was characterized at pH 7.0 using H++ server, and the structure of BBR ligand was constructed using GaussView 5 program. Molecular docking simulation of BBR against EGFR, Erk, STAT3, and Akt proteins was performed using Autodock Vina software (Trott and Olson, 2010). Lamarckian genetic algorithm was employed to predict bound conformations and free energies of association (Fuhrmann et al., 2010).

*Statistical analysis*

Statistical analyses were conducted using SPSS statistics 24.0 software (IBM Corp., Armonk, NY, USA). All assays were performed as three independent experiments with triplicate assays each. Data presented are mean ± SD. Statistical comparison between groups were done using the Student's t test. $P < 0.05$ was considered as statistical significance.

## Results

*BBR inhibited CCA cell viability*

Compared with the untreated controls, BBR-treatment for 24 h suppressed the viability of both CCA cell lines in a dose dependent manner (Figure 1b). Compared to the vehicle control cells, cell viability of CCA cell lines were decreased in the presence of BBR; at low dose of BBR (3.125 µM); KKU-213A was 90% ± 4.7% and KKU-213B was 81.5 ± 6%. To avoid the effect of BBR on cell viability, BBR at 3.125 µM that suppressed cell viability less than 20% of the controls was chosen for the subsequent experiments to investigate the effects of BBR on the progressive phenotypes.

*Low dose BBR suppressed progressive phenotype expression of CCA cells*

BBR-treated and untreated control cells were allowed to adhere to the Matrigel pre-coated culture plate. The results showed that BBR significantly suppressed cell adhesion, 25.4 % ± 14.4% in KKU-213A and 23.6% ± 3.3%, in KKU-213B compared with those of the untreated controls (Figure 2a).

The effect of low dose BBR (3.125 µM in culture) on cell motility was examined using three methods, the wound healing assay, cell migration assay and cell invasion assay. As shown in Figure 2b, BBR treatment caused significant delay of wound closure of KKU-213A cells. The wound closure of the control cells vs. BBR treated cells was 44.3 ± 3.58% vs. 24.7 ± 2.30% ($P < 0.05$) at 8 h, and 94.4% ± 2.26% vs. 69.7% ± 5.29% ($P < 0.05$) at 16 h. Similar results were observed for KKU-213B at 12 h, (93.0% ± 0.70% of control cells vs. 73.0% ± 2.20% of the BBR treated cells $P < 0.05$).

Cell migration and invasion assessed using Boyden chamber assay revealed that cell migration potential of both CCA cell lines were inhibited by BBR treatment ($P < 0.05$). The number of migrated cells were reduced to 40.4% ± 10.7% in KKU-213A and 32.5% ± 16.8% in KKU-213B as compared with their respective untreated control cells (Figure 2c). Similarly, BBR treatment caused considerable decrease of cell invasion capability of CCA cells; 57.8% ± 15.7% in KKU-213A and 24.9% ± 3.8% in KKU-213B compared with untreated control cells ($P < 0.05$; Figure 2d).

*BBR suppressed expression of key proteins related to tumor progression*

To investigate the mechanisms underlying BBR-induced suppression of the observed phenotypes adhesion, wound healing, migration and invasion of CCA cells, semi-quantitative Western blotting analysis was performed for the expression of various epithelial mesenchymal transition (EMT) protein markers. The results were summarized in Figures 3a and 3b. BBR treatment for 24 h resulted in a decrease of protein expression of two key mesenchymal markers, slug and vimentin in both CCA cell lines ($P < 0.01$). Conversely, BBR treatment enhanced the expression of claudin-1, an epithelial phenotype marker ($P < 0.05$), but not β-catenin and E-cadherin. Moreover, the expression of vascular endothelial growth factor A (VEGFA), an angiogenesis and metastasis mediator, was strongly suppressed by

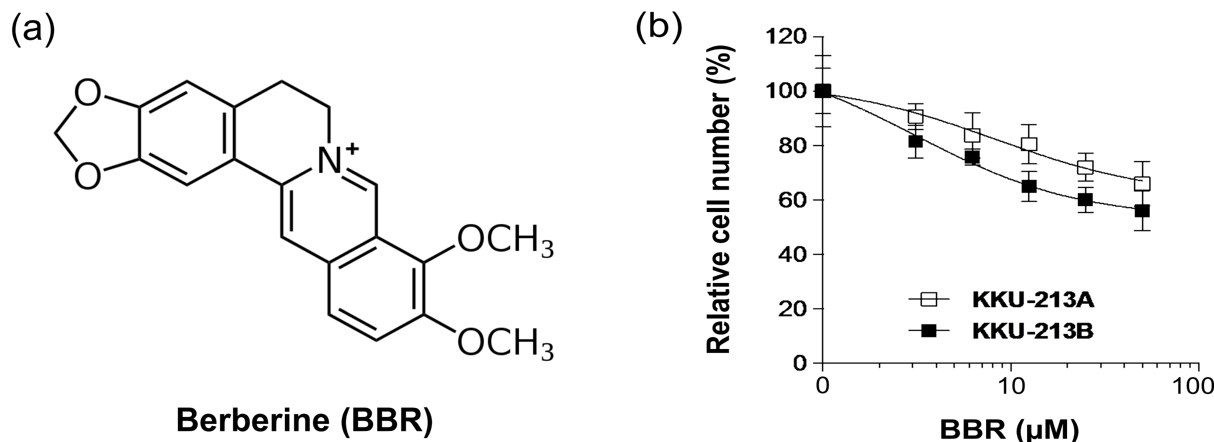


Figure 1. Structure and the Growth Inhibitory Effect of BBR on CCA Cell Lines. (a) Chemical structure of BBR. (b) Cell viability of KKU-213A and KKU-213B treated with various concentrations of BBR or medium alone for 24 h. Data are mean ± SD from three independent experiments, triplicate measurements each.

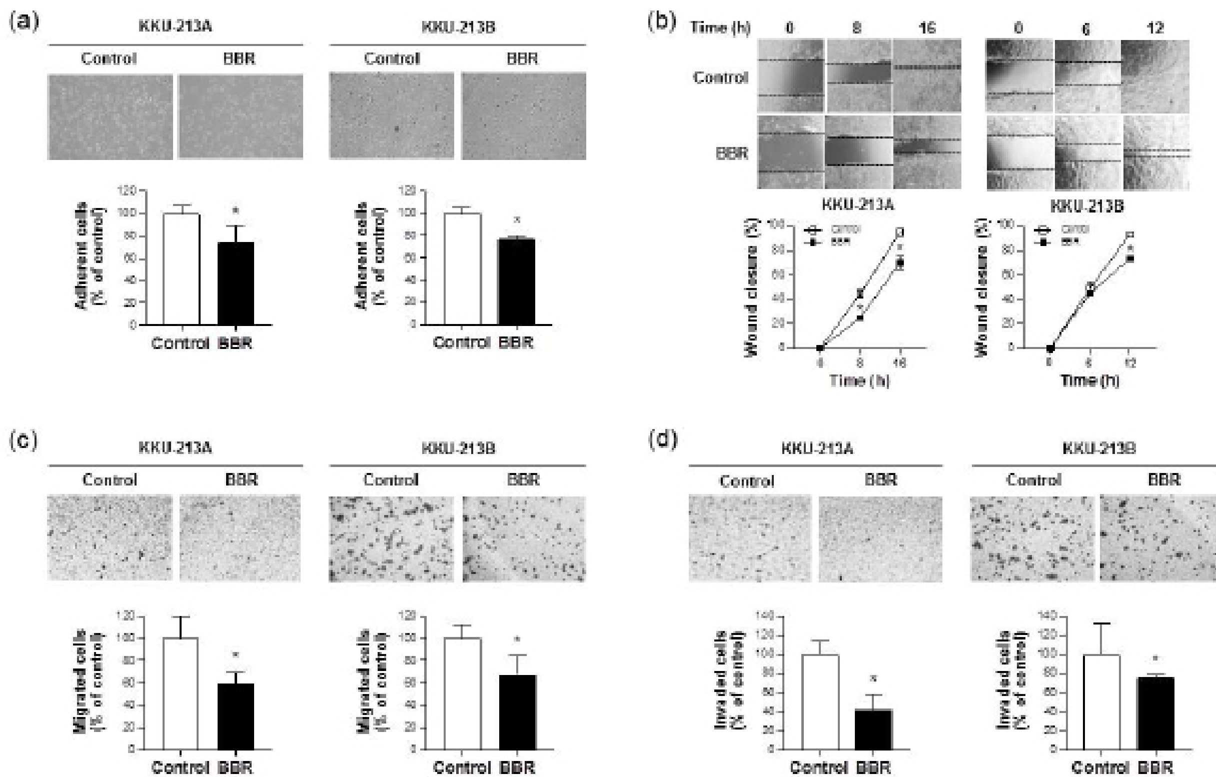


Figure 2. BBR Suppressed Progression of CCA cells. KKU-213A and KKU-213B cells were treated with BBR at the specified doses and time as described in each method. (a) Cell adhesion, (b) Wound healing, (c) Cell migration and (d) cell invasion. CCA cells in medium alone were used as controls. The data represent mean ± SD from three independent experiments by giving those of controls as 100%. *P < 0.05.

BBR treatment.

Molecular docking suggested BBR as a multi-kinase inhibitor of EGFR and its downstream signaling effectors. As epidermal growth factor receptor (EGFR)-mediated activation of Erk, STAT3, and PI3K/Akt signaling are necessary for EMT regulation, whether BBR acts on these key active proteins was determined in silico using a molecular docking analysis. As shown in Figure 4a, the molecular docking simulation revealed that BBR could bind to the ATP-binding pocket of EGFR (-8.8 kcal/mol), Erk1 (-8.5 kcal/mol), and Akt (-9.0 kcal/mol), indicating the action of BBR as the ATP competitive inhibitor of

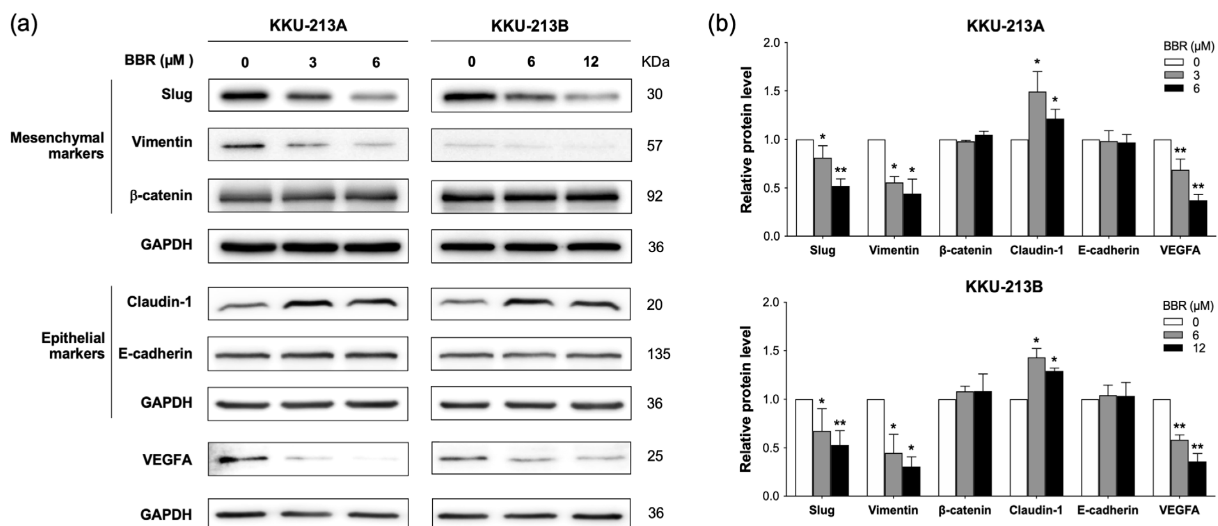


Figure 3. Western Blot Analysis of EMT Marker Proteins and VEGFA. CCA cells were treated with indicated concentrations of BBR for 24 h before subjecting to SDS-PAGE and Western blotting. (a) Western blot, (b) Quantitative analysis of band intensities by giving the value of the untreated control as 1. Expression of GAPDH protein was used as a loading control. Data are mean ± SD from three independent experiments. *P < 0.05, **P < 0.01.

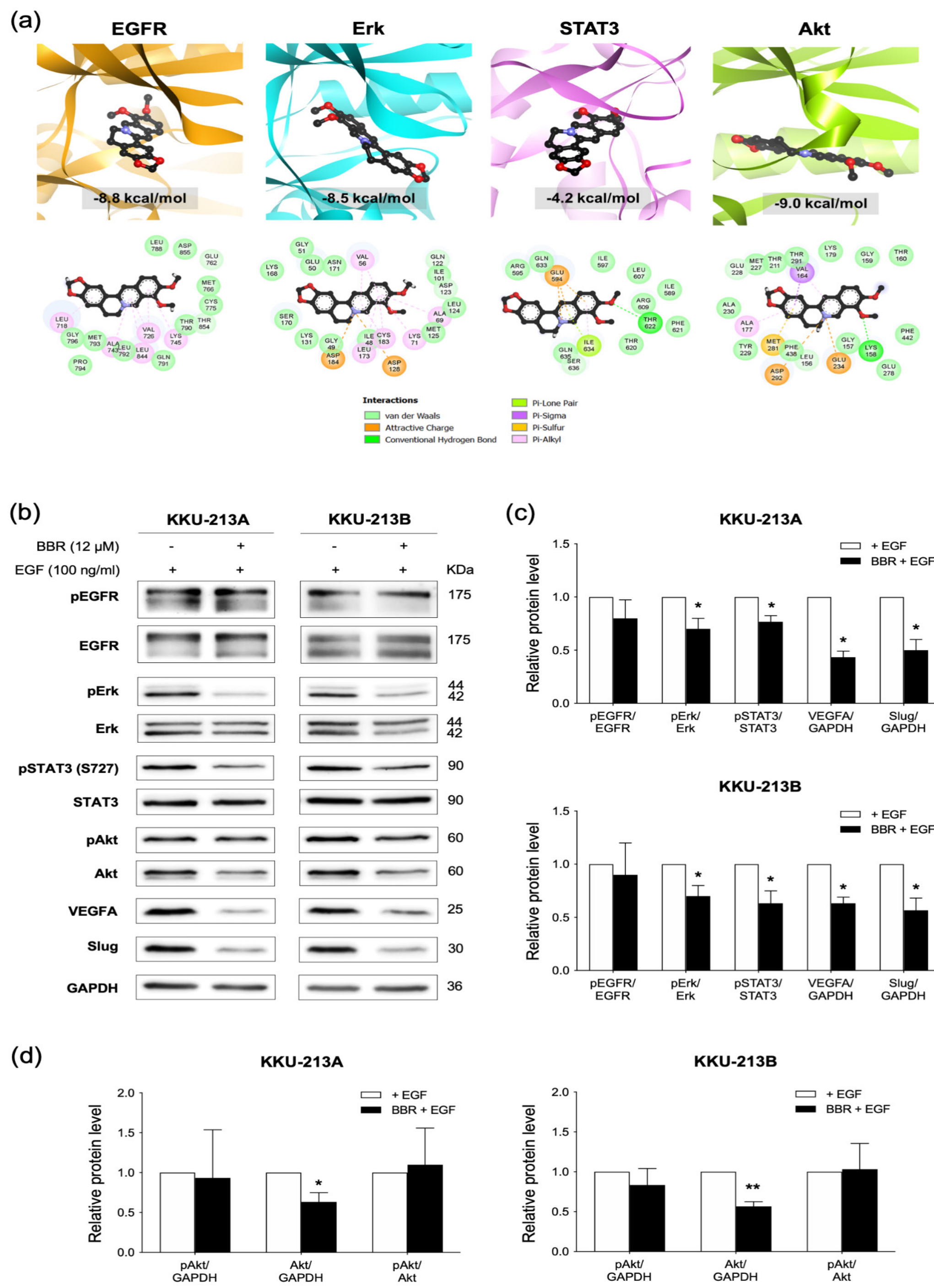


Figure 4. Effect of BBR Treatment on the EGF/EGFR Activation. (a) Molecular docking simulates the binding of BBR to the ATP-binding pocket of EGFR, Erk1 and Akt, and to the SH2 domain of STAT3, suggesting the inhibition of BBR on the functions of these molecules. (b) The Western blotting demonstrates the effect of BBR on EGFR, its downstream effectors and the target proteins. (c) The quantitative analyses of the phosphorylated form and total form of EGFR, VEGFA and Slug; and (d) the phosphorylated form and total form of pAkt and Akt. The data are presented by giving those of the EGF treated cells as 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

EGFR, Erk and Akt. For STAT3, BBR could bind to the SH2 domain of STAT3 (-4.2 kcal/mol) and inhibit the binding of STAT3 to its target proteins.

Although van der Waals interactions is assumed to be the main stabilizing forces for BBR binding against all studied targets, the positively charged nitrogen atom of BBR can form electrostatic interactions with the negative charge of (i) Asp128 and Asp184 of Erk1, (ii) Glu594 of STAT3, and (iii) Glu234 and Asp292 of Akt. As the structure of BBR contains three aromatic rings, it can form several additional pi interactions (i.e., pi-lone pair, pi-sigma, pi-sulfur, and pi-alkyl) with neighboring residues. These data agreed well with the previous report emphasizing the multi-kinase inhibitory activity of BBR on these signal molecules (Jabbarzadeh Kaboli et al., 2019).

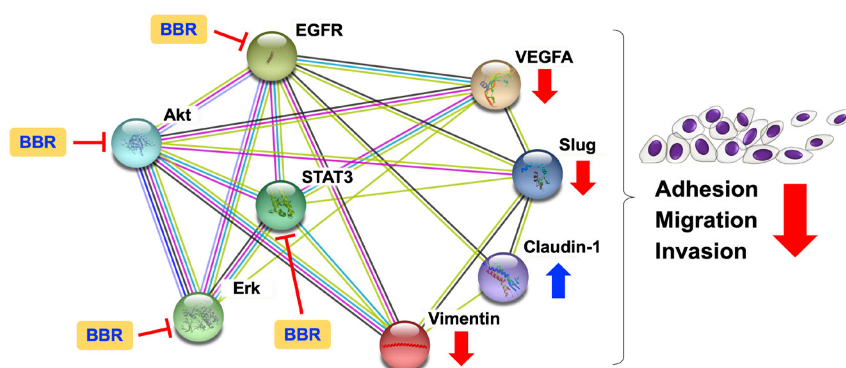


Figure 5. A Schematic Summarizes the Multi-Kinase Inhibiting Effect of BBR on the Progression of CCA. The interactions of EGFR, its downstream effector proteins and the protein targets-VEGFA, slug, claudin 1 and vimentin were predicted using STRING analysis (https://www.string-db.org). Colored lines represent the types of evidence for associations: light blue, curated database; purple, experimental; green, neighborhood evidence; red, gene fusion; blue, co-occurrence; yellow, text mining; black, co-expression; sky blue, protein homology.

*BBR simultaneously inhibited EGFR and its downstream signaling*

To demonstrate the multi-kinase inhibitor of BBR as suggested from the molecular docking data, the effects of BBR on the activation of EGF/EGFR and downstream signaling was examined. CCA cells were cultured in the medium containing 12 µM of BBR for 24 h, with the presence of 100 ng/mL EGF in the last 12 h. Cells cultured in the medium without BBR for 24 h, with the presence of 100 ng/mL EGF for the last 12 h were used as controls. Compared with the controls, semi-quantitative Western blot analysis (Figures 4b and 4c) revealed that BBR treatment caused significant decrease of the activation of the downstream signaling of EGFR-Erk and STAT3, and the expression of VEGFA and slug in both KKU-213A and KKU-213B cells. The ratios of pErk/Erk and pSTAT3/STAT3, but not pAkt/Akt, were markedly decreased upon BBR treatment. Expression level of Akt, however, was significantly decreased under the BBR treatment (Figure 4d). For EGFR, the pEGFR/EGFR ratio tended to decrease without statistical significance in BBR treated cells. In addition, EGF induced EGFR degradation was apparently observed in the EGF treatment, indicated by the presence of the lower molecular weight EGFR band (Sigismund et al., 2008). All those changes caused by BBR treatment were consistently observed in both CCA cell lines. Taken all these together, the results indicated the multi-target action of BBR on EGFR and its downstream signaling pathways, resulting in the suppression of EMT and CCA progression (Figure 5).

## Discussion

BBR, a secondary metabolite of plants, has a definite potential as a drug in a wide spectrum of clinical applications and is currently used widely in both basic research and clinical trials (Rauf et al., 2021). BBR-mediated G1 growth arrest was shown in two CCA cell lines; KKU-213A and KKU-213B by suppressing expression of cyclin D1 and cyclin E1 (Puthdee et al., 2017). The present results demonstrated further that the sub-growth inhibitory concentration of BBR could significantly inhibit cell adhesion, motility, and invasion of CCA cells, *in vitro*. BBR exhibited these effects through the suppression of VEGFA and slug expression by simultaneously targeting the activation of EGF/EGFR and its downstream signaling pathways—Erk, STAT3 and Akt. These findings provide a molecular understanding of the anticancer actions of BBR as the therapeutic drug for human CCA.

Cell adhesion is essential for cell growth, survival, and communication with neighboring cells. In the current study, low dose of BBR could significantly inhibit CCA cell adhesion to Matrigel. This observation may be resulted from the interference of BBR on the expression of cell adhesion molecules or extracellular matrix (ECM) proteins. In fact, BBR treatment suppressed the expression of cell surface proteins, VCAM-1 and ICAM-1 (Huang et al., 2013; Jeong et al., 2018), which were enhanced by VEGF through Nuclear Factor Kappa B (NF-κB) activation (Kim et al., 2001). In CCA cells, BBR exhibited a strong inhibition on the expression VEGFA as shown in the current study, and NF-κB pathway as shown in the previous report (Puthdee et al., 2017), BBR possibly inhibit CCA cell adhesion through VCAM-1 and ICAM-1 via suppression of NF-κB and VEGFA. This postulation, however, needs further validation.

EMT is a critical mechanism that regulates cellular development, wound healing, and cancer cell metastasis. Cancer cells possess EMT to convert immotile epithelial cells to a highly invasive mesenchymal cells (Kalluri and Weinberg, 2009) for metastatic dissemination. In several cancers, such as malignant melanoma, osteosarcoma and cervical cancer, BBR could either suppress or reverse EMT through various pathways (Zhang et al., 2019). In this study, BBR treatment showed significant inhibitory effects on the expression of slug and vimentin, the mesenchymal markers, and enhanced the expression of claudin-1, an epithelial marker (Figure 3). Vimentin, slug, and VEGFA play important roles in the EMT process and may be responsible for the decrease of CCA cell migration and invasion observed upon BBR treatment. The significant role of BBR as a modulator of EMT pathway

of CCA as observed in the present study is consistent with the previous reports in cervical cancer and malignant melanoma (Chu et al., 2014; Kou et al., 2016). The impact of VEGF on EMT was also reported in many studies. VEGF triggered EMT through an autocrine loop during malignant transformation (Gonzalez-Moreno et al., 2010), and reciprocally, its expression was enhanced upon EMT phenotype switching (Fantozzi et al., 2014).

EGFR, the ErbB family of receptor tyrosine kinases (RTKs), is frequently mutated and/or overexpressed in different types of human cancers, including CCA (Claperon et al., 2014). Activation of EGFR signaling regulates migration and invasion of malignant cells through EMT processes (Al Moustafa et al., 2012; Claperon et al., 2014). Blocking of EGFR and consequent effector pathways such as RAS/MAPK, PI3k/Akt, and JAK/STAT, by tyrosine kinase inhibitors or monoclonal antibody, inhibit cellular migration and invasion, suggesting EGFR as an essential target for controlling cancer progression (Al Moustafa et al., 2012). The multi-kinase inhibiting effects of BBR on EGFR and its effector pathways was predicted by molecular docking approach (Figure 4) and demonstrated using the EGF activation in the presence of BBR. BBR treatment significantly diminished the actions of EGF, and its downstream effector pathways Erk, STAT3, and Akt, which consequently suppressed the EMT markers. Unlike Erk and STAT3, the decrease of total Akt protein was consistently noted under BBR treatment. This observation is supported by the previous report of Tak and colleagues (Tak et al., 2019) in that BBR down-regulated Akt expression at both mRNA and protein levels. The mechanism was further shown to be the effect of BBR on the post-translational stability of Akt protein. The half-life of Akt protein was reduced 80% upon BBR treatment.

The multi-target and multi-kinase inhibiting property of BBR is more beneficial than other anti-cancer agents. Based on the current findings and evidence, the use of BBR as an anticancer agent may provide several clinical advantages. Firstly, BBR acts as the multi-kinase inhibitor that inhibits several kinases signaling simultaneously. Secondly, BBR inhibits several cancer-related proteins, e.g., VEGF, HIF-1α, HSP90AB1, neuraminidase, and protein tyrosine phosphatase 1B, etc. Thirdly, BBR shows selective action on cancer cells than the normal cells (Qi et al., 2014; Wang et al., 2016). In CCA, the growth inhibitory effect of BBR in the primary human intrahepatic cholangiocyte cell line and the immortalized human cholangiocyte cell line, MMNK-1, was 20-30 times lesser than those of CCA cell lines (He et al., 2012; Puthdee et al., 2017). These properties impact the efficacy of low dose BBR. The use of BBR in combination with currently used chemotherapeutic drugs may improve the efficacy and decrease the usage dose and side effects of those drugs, especially for the patients with a low tolerance to the side effects. The benefits of prolonged low dose therapy, known as low-dose metronomic chemotherapy, has been demonstrated in several clinical trials with clinical safety and without any significance reduction in the anti-cancer effects (Lien et al., 2013). Nevertheless, the efficacy of BBR on the anti-progression of CCA needs to be further investigated in the animal model.

In conclusion, the findings of the current study emphasize the significant effects of low dose BBR on inhibiting CCA progression. BBR inhibits EMT-induced migration and invasion of CCA through suppression of EGF/EGFR and its downstream effector activations namely Erk, STAT3 and Akt which mediated the EMT inducersVEGF and slug. Owing to its multi-kinase inhibitory property, BBR may be served as a potential therapeutic/adjuvant agent for the treatment of advanced CCA and overcoming the EGFR inhibitor-resistant tumor.

## Author Contribution Statement

KV, SO, SW: Conceived, designed, and interpreted the data; PM, KV, SO, SW: contributed data or analysis tools; SOb, MD, PB, PS, PM Performed the experiments and analysis; SOb, MD, KV, SW: wrote the paper; SOb, KV, SO: funding supports; All Authors read and approved the final version of the article.

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*Conflicts of Interest*

The authors declared that there is no conflict of interest regarding the publication of this article.

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