Anti-metastatic Potential of Natural Triterpenoid Cucurbitacin B Against Cholangiocarcinoma Cells by Targeting Src Protein

Putthaporn Kaewmeesri, MS1, Piman Pocasap, PhD1, Veerapol Kukongviriyapan, PhD1,2, Auemduan Prawan, PhD1,2, Sarinya Kongpetch, PhD1,2, and Laddawan Senggunprai, PhD1,2

Abstract

Owing to the crucial role of Src in cancer metastasis, interruption of Src and its signaling has been considered a promising strategy for cancer metastasis treatment. Cucurbitacin B, a dietary triterpenoid, has been shown to possess anti-proliferative and apoptosis-inducing activities in cholangiocarcinoma (CCA) cells via suppressing the activation of FAK which is a main downstream Src effector. We hypothesized that cucurbitacin B might act as a Src suppressant which conferring anti-metastasis effect against CCA cells. To investigate this, the role of Src in regulating metastasis behavior of CCA cells and the effect of cucurbitacin B on Src-mediated metastatic phenotype of these cells were determined. The results showed that activation ofSrc significantly enhanced the migratory and invasive abilities of CCA cells. Molecular analysis revealed that Src-facilitated metastasis behavior of CCA cells occurred by modifying expression of a wide range of metastasis-related genes in the cells. Consistent with gene expression results, activation of Src significantly induced the protein expression of 2 important metastasis-associated molecules, MMP-9 and VEGF. Cucurbitacin B markedly suppressed activation of Src and its key effector, FAK. As a consequence, the alteration of expression profiles of metastasis-associated genes induced by Src activator in CCA cells was diminished by cucurbitacin B treatment. The compound also down-regulated Src-induced expression of MMP-9 and VEGF proteins in the cells. Moreover, molecular docking analysis revealed that cucurbitacin B could interact with Src kinase domain and possibly restrain the kinase from being activated by hindering the ATP binding. In conclusion, cucurbitacin B exhibited anti-metastatic property in CCA cells via negatively influencing Src and Src-related oncogenic signaling. This compound may therefore be a potential therapeutic drug for further development as an anti-Src agent for treatment of metastatic CCA.

Keywords
cucurbitacin B, cholangiocarcinoma, metastasis, Src, FAK

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Introduction

Cancer is one of the major causes of death worldwide with the burden growing in almost every country. Based on World Health Organization data, approximately 10 million deaths were mentioned globally in 2020. Cholangiocarcinoma (CCA) or bile duct cancer is an invasive malignancy arising from the biliary duct epithelium, and has a complicated etiology. According to data available through 2020, the overall incidence of CCA has increased worldwide in recent decades, accounting for 0.3 to 6 per 100,000 population per year.1 The highest prevalence of CCA has been reported in Southeast Asian countries, especially in Thailand. Chronic inflammation by liver fluke Opisthorchis viverrini infection might be an important risk factor for the development of CCA in this region.2 Primary sclerosing cholangitis and

1Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
2Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen, Thailand

Corresponding Author:
Laddawan Senggunprai, Department of Pharmacology, Faculty of Medicine, Cholangiocarcinoma Research Institute, Khon Kaen University, 123 Mitraphab Road, Khon Kaen 40002, Thailand.
Email: laddas@kku.ac.th
chronic viral infection represent other risk factors of this cancer. Most CCA patients are diagnosed at an advanced stage where curative surgery is not applicable. At present, treatment of unresectable CCA remains challenging for clinicians because response to conventional chemotherapeutic agents is poor. Moreover, dysregulation of intracellular signaling networks in CCA cells leads to an aggressive metastatic feature which significantly affects the prognosis of the disease. Previous studies have shown that metastasis contributed to the failure of cancer treatment and was a significant clinical predictor of mortality among CCA patients. Therefore, metastasis-associated signaling pathways and molecules are potential targets for the establishment of new therapeutic approaches for metastatic CCA therapies.

It is well established that the metastatic potential of cancers relies on the ability of malignant cells to migrate and invade. Activation of several regulatory proteins is required for driving the machinery of cell motility and invasion. Among them, Src protein is recognized as one of the prominent regulators of the metastasis process. Src is an intracellular non-receptor tyrosine kinase encoded by a proto-oncogene c-Src. Src has been strongly implicated as one of the prominent regulators of the metastasis process. Src is an intracellular non-receptor tyrosine kinase encoded by a proto-oncogene c-Src. Src has been strongly implicated as one of the prominent regulators of the metastasis process. For regulation of metastasis process, Src cooperatively works with focal adhesion kinase (FAK) by acting as an important kinase involved in the catalytic activation of FAK. Downstream signaling cascades, which are associated with metastatic features including mobility, invasion, epithelial-mesenchymal transition and angiogenesis, are regulated by the FAK-Src complex. It has been reported that over-activation of Src has been found in surgical tissues of CCA patients with metastatic potential. Similarly, elevated expression and sustained activation of Src have been observed in several other cancers such as breast cancer, non-small cell lung cancer, and colorectal cancer and these phenomena have contributed to metastasis and angiogenesis of the cancer cells. Furthermore, overexpression and increased activity of Src has also been demonstrated to be associated with shorter survival of cancer patients. Accordingly, agents capable of compromising the function of Src may be a promising therapeutic drug to inhibit oncogenic signaling for treatment of cancers, especially those exhibiting a metastasis phenomenon.

For several decades, bioactive constituents of herbal plant origin have been proposed as valuable resources for novel therapeutic agents. The information about medicinal plants and bioactive phytochemicals that have been identified as potential candidates for anti-cancer drug development as well as their molecular mechanism of actions has been intensively reviewed. Cucurbitacin B (Figure 1) is a naturally occurring triterpenoid obtained from plants in cucurbitaceous species. Several plants in these species are commonly consumed vegetables and used in traditional Chinese medicine. Previous studies reported that cucurbitacin B possessed beneficial medical values according to its numerous pharmacological activities such as anti-diabetic and anti-inflammatory. The compound also elicits anti-cancer activity in various cancer cell types. Our recent studies have found that cucurbitacin B triggered apoptosis programed cell death and suppressed growth of CCA cells through inhibition of FAK. In a preliminary study, we found the anti-metastasis potential of the compound in CCA cells. In the present study, an in-depth analysis of the anti-metastasis activities of cucurbitacin B against CCA cells was performed. Given that Src is an important upstream signaling molecule to FAK by functioning as a critical kinase for FAK phosphorylation. In this study, the role of Src in the CCA metastasis process and the action of cucurbitacin B as modulator of Src in CCA cells were investigated.

Materials and Methods

Materials

Cucurbitacin B was purchased from Sigma Chemical (St. Louis, MO). Src activator was from Santa Cruz Biotechnology, Inc. (California, USA). The antibodies against phospho-Src and phospho-FAK were obtained from Cell Signaling Technology (Dancer, MA, USA). The primary antibody against Src was from Affinity Biosciences LTD. (Victoria, Australia). The antibodies against β-actin, matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), and the secondary horseradish peroxidase (HRP)-linked antibodies were purchased from Santa Cruz Biotechnology, Inc. (California, USA). Proteinase and phosphatase inhibitors were obtained from Thermo Scientific (Rockford, IL, USA).

Cell Lines and Cell Culture

The human CCA cell lines, KKU-100, KKU-213 and KKU-452 (kindly provided by the Cholangiocarcinoma Research
Institute, Khon Kaen University) were cultivated in Ham’s F12 media (Gibco-BRL Life Technologies, Grand Island, New York) containing 10% fetal calf serum (HiMedia Laboratories, Mumbai, India) supplemented with 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, pH 7.3), sodium bicarbonate, 100 μg/mL gentamicin and 100 U/mL penicillin at 37°C in a humidified incubator with 5% CO₂ and 95% air.

**Protein Preparation and Western Blotting Analysis**

Whole cell lysates of CCA cells treated with various concentrations of test compounds for indicated time points were prepared using RIPA lysis buffer (Thermo Scientific, USA) containing proteinase and phosphatase inhibitors according to the manufacturer’s instructions. Protein concentration of samples was quantified using Bradford Reagent (Bio-Rad, Hercules, CA, USA). For Western blot analysis, the 30 μg of protein samples were subjected to 10% SDS-PAGE electrophoresis and then transferred on to a polyvinylidene difluoride (PVDF) membrane. The membrane was subsequently incubated with appropriate dilution of primary antibodies targeting specific proteins at 4°C overnight, followed by incubation with HRP-conjugated secondary antibody at room temperature for 2 hours. The target immune complexes were detected using an enhanced chemiluminescence detection system (Luminata™ Forte Western HRP Substrate, Merck Millipore, Watford, UK) and visualized by ChemiDoc™ MP Imaging system.

**Cytotoxicity Assay**

The effects of test compounds on the viability of KKU-213 CCA cells were assessed using sulforhodamine B colorimetric assay. The CCA cells were seeded onto a sterile culture plate and allowed to adhere overnight. The old media was replaced with new serum free medium containing various concentrations of test compounds, and the cultured cells were additionally incubated for 24 hours. Thereafter, the cells were fixed with 10% trichloroacetic acid for 1 hour at 4°C and subsequently stained with 0.4% sulforhodamine B in 1% acetic acid for 30 minutes. Unbound dye was discarded by rinsing with 1% acetic acid, and the protein-bound dye was then dissolved in 10 mM Tris solution. The color intensity was quantified at 540 nm using a microplate spectrophotometer.

**Cell Invasion and Migration Assays**

The Transwell Boyden chamber (Corning, Lowell, MA) was used for determination of invasive and migratory ability of CCA cells. For the invasion assay, an 8 μm pore size of polycarbonate membrane Transwell inserts was coated with 0.3 mg/mL of matrigel (BD Biosciences, Bedford, MA). Thereafter, the KKU-213 cells treated with either vehicle or different concentrations of test compounds in serum-free medium were seeded on to the upper section of the chamber and incubated for 24 hours. The lower chamber of the apparatus was added with a medium supplement with 10% fetal calf serum as a chemoattractant. After complete incubation, the invasive cells were fixed with methanol and stained with crystal violet. The cells were then photographed and quantified. For the migration assay, the Transwell Boyden chamber on polycarbonate membrane filters without matrigel was used and the migration ability of KKU-213 cells was determined as described in the invasion assay.

**Analysis of the Expression of Metastasis-associated Genes Using PCR Array**

The Human Tumor Metastasis RT² Profiler PCR Array; PAHS-028ZA (Qiagen, Hilden, Germany) was used to quantify the expression of 84 genes involved in the metastasis process, following the manufacturer’s instructions. Briefly, after treatment of KKU-213 cells with test compounds for designated time points, total RNA was collected and cDNA was synthesized using RT² First Strand Kit (Qiagen). The PCR reaction was performed using RT² SYRB Green qPCR Mastermix (Qiagen) under the following conditions: one cycle of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute in a QuantStudio™ 6 Flex Real-Time PCR System (Applied Bio-systems, Foster, CA, USA). The detection limit of gene expression (CT cutoff) was set at 35 cycles and the data of expression of genes that were below the detection limit were not included for analysis. The quality control was conducted using the manufacturer’s controls for reverse transcription process, positive PCR and genomic DNA contamination. The relative expression of each gene was determined by the 2−ΔΔCp equation as specified in the manufacturer’s manual.

**Determination of MMP-9 Level**

The KKU-213 cells were exposed to various concentrations of test compounds for 24 hours. After that, the treated cells were detached using trypsin solution, washed with PBS and the number of cells adjusted to be 1 × 10⁶ cells for each reaction. Subsequently, the CCA cells were lysed using an ultrasonic cell disruptor. The lysis cell suspension was centrifuged to sediment the cellular debris and the clear supernatants were then collected. The amount of MMP-9 in the supernatants was quantified using an ELISA kit (Elabscience, Houston, Texas) following the manufacturer’s instructions.
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Molecular Docking Analysis

The 3D structures of ligand were obtained from PubChem database. Ligand energy minimization was performed to acquire the lowest energy before docking process using PyRx software (Pyrx-Python Prescription 0.8). The 3D crystal structure of human Src protein was obtained from Protein Data Bank and was refined by removing water, solvent, and ligand using Pymol software (V2.4.1) (Pymol Molecular Graphics System, Schrödinger) prior to being imported to PyRx. Molecular docking analysis was performed using PyRx according to a previous report.23 The complexes were then visually analyzed by Pymol and 2D interaction was examined using Discovery Studio Visualizer (v21.1.0.20298) (BIOVIA, San Diego, CA, USA).

Statistical Analysis

The data were analyzed using one-way analysis of variance with post hoc Student-Newman-Keuls (SNK) test. Results were considered to be statistically significant with a P-value less than .05. The statistical analyses were conducted using GraphPad Prism 8.0 software.

Results

Src Activator Stimulated Phosphorylation of Src and FAK in CCA KKU-213 Cells

The basal level of p-Src was initially determined in various CCA cells including KKU-100, KKU-213 and KKU-452. The results showed that KKU-100 and KKU-452 had high levels of p-Src protein expression, whereas KKU-213 had a very low level of p-Src (Figure 2A). Therefore, KKU-213 cells were employed for further experiments to examine the role of Src in the CCA metastasis process. The KKU-213 cells were treated with specific Src activator at 3 µM. Under these conditions, the cell viability was virtually unaffected (data not shown). The results showed that after treatment with Src activator, phosphorylation of Src in KKU-213 cells
was markedly increased (Figure 2B). As expected, the phosphorylation of FAK was also significantly increased after exposure to Src activator (Figure 2B).

**Cucurbitacin B was a Src Suppressant**

To investigate whether cucurbitacin B acts as a Src suppressant in CCA cells, the effect of the compound on Src activator-stimulated Src phosphorylation in KKU-213 was determined. As shown in Figure 2B, cucurbitacin B diminished Src activator-stimulated Src phosphorylation in KKU-213 cells in a concentration-dependent manner. The phosphorylation of FAK in KKU-213 cells mediated by Src activator was also significantly decreased after cucurbitacin B treatment. Moreover, cucurbitacin B was also able to suppress the constitutive expression level of p-Src in KKU-100 and KKU-452 cells (Figure 2C and D). These results suggested that the compound was a Src suppressant and that its effect was not restricted to a special cell type.

**Cucurbitacin B Suppressed Src Activator-mediated Metastatic Behaviors of KKU-213 Cells**

To confirm the role of Src in the metastasis process of CCA cells, KKU-213 cells were treated with Src activator at 3 μM for 24 hours. Thereafter, the migratory and invasive abilities of the cells were determined. The results showed that Src activator significantly increased migration and invasion of KKU-213 cells (Figure 3A and B). Treatment with cucurbitacin B suppressed Src activator-mediated migration and invasion of KKU-213 cells in a dose-dependent fashion (Figure 3A and B). It is noted that at these concentrations of cucurbitacin B, the KKU-213 cell viability was only minimally affected as confirmed in separate experiments by which IC$_{50}$ value of cytotoxicity effect of the compound on the cells is 360.90 ± 3.10 nM. These data indicated that the anti-metastasis activity of cucurbitacin B was not from its cytotoxicity effect on the tumor cells.

**Cucurbitacin B Interfered with Src Activator-regulated Expression of Metastasis-associated Genes**

To investigate whether activation of Src alters the expression profile of genes involved in the metastasis process in KKU-213 cells, and whether cucurbitacin B can restore this condition, RT$^2$ Profiler™ PCR Array Human Tumor Metastasis was employed. All PCR array experiments showed the appropriate performance and passed the quality control checks. Of the 84 genes examined, the expression of 29 genes was below the limit of detection and these data...
Cucurbitacin B Diminished Src Activator-induced Expression of Metastasis-related Proteins

It is known that matrix metalloproteinase-9 (MMP-9) is one of the critical enzymes participating in the degradation of extracellular matrix constituents for enabling movement and invasion of metastatic malignant cells.24 We therefore evaluated the action of Src activator on the expression of MMP-9 in KKU-213 cells. The results showed that MMP-9 expression was significantly up-regulated after Src activator treatment, and further that curcurbitacin B could significantly alleviate Src activator-stimulated MMP-9 expression (Figure 4B). Determination of MMP-9 protein levels was also carried out to quantify the amount of expression by ELISA assays (Figure 4C). Additionally, exposure to Src activator also up-regulated the expression of VEGF protein in KKU-213 cells (Figure 4B). As expected, the Src activator-induced expression of VEGF was diminished by curcurbitacin B treatment (Figure 4B).

Cucurbitacin B Interacted with Src in Molecular Docking Analysis

To further verify whether Src is a molecular target of curcurbitacin B for conferring its anti-cancer activities against CCA cells, an analysis of molecular interplay between these 2 molecules was performed. The molecular docking was applied to examine the possible interaction. Src is a non-receptor tyrosine protein kinase that consists of 3 domains including SH3, SH2 (both acting as regulatory domains), and SH1 (acting as a kinase domain). The SH1 plays a pivotal role in catalytic activity, and the phosphorylation state of the domain (Tyr:416) contributes to its active form.25 Therefore, the kinase (SH1) was selected in the docking study since it corresponds to our finding that curcurbitacin B affects the phosphorylation of Src. The results showed that curcurbitacin B bound with the Src kinase domain with a binding affinity of −8.0 Kcal/mol via both hydrogen and hydrophobic bonds. The binding residues and relevant interaction are shown in Figure 5A and B. The sequence analyses of crystal structure, comparing between the Src kinase and its ligands, indicated that curcurbitacin B bound with Src at the pocket site overlapping with a known Src inhibitor, bosutinib (Figure 5B and C).

Discussion

Metastasis remains a major obstacle for treatment of various cancers including CCA. Understanding the underlying mechanisms mediating the metastasis process of CCA cells is crucial for the development of effective anti-metastatic agents to improve the outcome of CCA therapy. In the present study, it was demonstrated that Src plays a crucial role in regulating the metastasis phenotype of CCA cells. Cucurbitacin B, a dietary triterpenoid, altered Src oncogenic signaling and suppressed the migratory and invasive abilities of CCA cells. This compound may therefore serve as a complementary approach for inhibiting CCA metastasis.

Migration and invasion abilities are particularly important properties of metastatic malignant cells.26 Growing evidence indicates that Src, a proto-oncogene, acts as an essential regulator in mediating signal transduction to regulate cell differentiation, proliferation, growth, migration, and invasion.7,9 These features make Src a potential target for anti-cancer drug development. Previous studies elucidated the network of Src interacting with Src effector proteins to transduce the intracellular signaling pathways. FAK is a downstream Src effector that plays a major role in the regulation of the metastasis process.27 Apparently, activation of Src in CCA cells by a specific Src activator provoked the phosphorylation of FAK. It has been reported that increased activity of Src has contributed to metastasis and angiogenesis of numerous cancer cell types such as ovarian cancer, lung cancer, colon cancer, and breast cancer.11-13 Additionally, a clinical association between elevated activity of Src kinase and shorter overall survival in colorectal cancer patients presenting a metastatic stage was also demonstrated.11 In accordance with previous reports, in this study it was found that activation of Src significantly enhanced the migratory and invasive abilities of CCA cells. Src over-expression and activation have impact on several downstream transcriptional events.7 Further in depth investigation for the molecular mechanism underlying Src-facilitated metastasis phenotype of CCA cells revealed that Src activation modified expression of a wide range of metastasis-related genes in the cells. The influence of Src on expression levels of 2 important metastasis-associated proteins, MMP-9 and VEGF, in CCA cells was also explored in the present study. MMP-9 is a pivotal enzyme participating in matrix proteolysis for facilitating metastatic tumor cell motility and invasiveness.24 VEGF is responsible for angiogenesis and vascular permeability.28 Consistent with PCR array results, activation of Src markedly induced the expression of MMP-9 as well as VEGF proteins in CCA cells. Quantification of MMP-9 concentration was also employed to ascertain Src-mediated MMP-9 production in CCA cells. These data suggested that Src is a critical mediator in the regulation of metastasis of CCA cells. Thus, Src is
Figure 4. Effect of cucurbitacin B on Src activator-mediated metastasis-associated genes and protein expressions. (A) PCR array analysis of metastasis-related gene expression. KKU-213 cells were exposed to vehicle, Src activator alone (3 μM) or combination of Src activator and cucurbitacin B (5 nM) for 12 hours. Thereafter, the level of mRNA expression was quantified using qRT-PCR with the Human Tumor Metastasis RT² Profiler PCR Array. Heat map illustrating increased or decreased gene expression in the treatment group compared with that in the control group. Representative gene expression profiles from 2 reproducible independent experiments are shown. (B) KKU-213 cells were treated with vehicle, Src activator alone (3 μM) or combination of Src activator and cucurbitacin B (5 nM) for 24 hours. Whole cell lysates were then prepared for Western blot analysis. Representative figures and quantification graphs are illustrated. Data are expressed as mean ± SEM of 3 independent experiments. (C) Quantitative determination of MMP-9 protein level in cell lysates was carried out using commercial ELISA kit. Results are mean ± SEM averaged of 4 determinations from one experiment. CuB, cucurbitacin B. *P < .05 versus control group. #P < .05 versus Src activator alone.
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An appealing molecular target for development of new therapeutic agents for treatment of CCA metastasis.

A large number of diverse bioactive constituents found in natural plant are valuable resources for discovery and development of new anti-neoplastic agents. Some phytochemicals exhibit anti-cancer activities through suppression of Src and its related signaling cascades in cancer cells. Thymoquinone inhibits phosphorylation of Src and other proteins which eventually suppresses metastatic ability of renal carcinoma cells. Silibinin exerts anti-metastasis efficacy in prostate cancer cells via reduction of Src phosphorylation. Denbinobin shows anti-metastasis activities against breast cancer cells via inhibiting of the Src-mediated signaling pathways. In the present study, the results demonstrated that cucurbitacin B significantly suppressed Src activator-mediated phosphorylation of Src in CCA KKU-213 cells. As a consequence, FAK phosphorylation mediated by Src activation was also inhibited. Moreover, the compound was also able to potently inhibit constitutive high expression level of p-Src in other CCA cell lines, KKU-100, and KKU-452. These findings indicated that cucurbitacin B exhibits an anti-Src effect in diverse type of CCA cells, and this offers a high potential therapeutic opportunity. The impact of cucurbitacin B on Src-mediated metastasis activities of CCA cells was then evaluated. As expected, 2 important metastasis behaviors of cancer cells, namely migration and invasion induced by Src activation, were significantly suppressed after cucurbitacin B exposure. Detailed analyses revealed that the alteration of expression profiles of metastasis-associated genes induced by Src activator in CCA cells was diminished by cucurbitacin B treatment. Accordingly, cucurbitacin B down-regulated the expression of MMP-9 and VEGF proteins induced by Src activation. The data suggested that cucurbitacin B possessed its anti-metastatic property in CCA cells via negatively impacting on Src and Src-related pathways. Furthermore, molecular docking analysis indicated that cucurbitacin B could bind to Src in the overlapping area with chemotherapeutic agent bosutinib ( Src inhibitor). This drug binds to the Src SH-1 domain, resulting in interference with ATP binding and phosphorylation of Src at Tyr:416, consequently preventing Src from taking an active form. Since cucurbitacin B shares binding site with bosutinib, there is the possibility that cucurbitacin B hinders ATP binding to Src and therefore restrains the kinase from changing to its phosphorylated active form. This finding is consistent with the observed p-Src reduction found in our experiment. Nonetheless, the definitive binding study such as X-ray crystallography between cucurbitacin B and Src should be performed to confirm the statement. In addition, the other molecular targets and signaling pathways might also contribute to the anti-metastasis action of cucurbitacin B in CCA, and this question requires further study.

Figure 5. Cucurbitacin B interaction with Src. (A) Amino acid residues at cucurbitacin B binding pocket (5 Å away from cucurbitacin B molecule), H-bonds are displayed in blue dotted line. Inset displays H-bond residues and distance. (B) 2D interaction diagram of cucurbitacin B at binding pocket. Blue residues represent H-bonding and green residues represent hydrophobic interaction. Amino acid residues with red circle represent overlapping binding site of cucurbitacin B with bosutinib ( Src inhibitor). (C) Overlay structure of cucurbitacin B (cyan) and bosutinib (magenta) at binding pocket in 3D. Limon surface displayed the overlapping area of amino acid residues occupied by both cucurbitacin B and bosutinib. PDB ID: 4MXO. Cucurbitacin B, PubChem ID: 5281316.
In conclusion, cucurbitacin B suppresses the metastasis properties of CCA cells by disrupting the activation of Src and its oncogenic signaling. Additional pharmacological studies and development of this compound for use as an anti-Src agent for metastatic CCA treatment are challenges for future research.

Author Contributions
PK participated in performing experiments, analyzing the results and writing the manuscript. PP performed molecular docking analysis and wrote the manuscript. VK reviewed the manuscript and providing critical comments. AP and SA collaborated in analyzing the data. LS participated in designing the study, planning the experiments, analyzing the results and writing the manuscript. All authors read and approved the final manuscript.

Data Availability Statement
The datasets deriving from this study are available from the corresponding author on reasonable request.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD
Laddawan Senggunprai
https://orcid.org/0000-0001-5509-8664

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