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FACILE SYNTHESIS OF THIOPHENE-EMBEDDED BAICALEIN VIA OXIDATIVE CYCLIZATION AND ANTICANCER ACTIVITY AGAINST OVARIAN CANCER CELLS VIA INHIBITING PI3K/MTOR SIGNALING PATHWAY: IN-VITRO AND IN-SILICO STUDY

Zheqi Liu[1#], Lin Zeng[2#], Linshan Wu[3], Daping Song[2], Yanhua Chen[4*]

[1]Department of TCM Gynecology, Hangzhou TCM Hospital Affiliated to Zhejiang Chinese Medical University, Hangzhou, Zhejiang, 310000, China

[2]Department of Pathology, The Third Hospital of Mianyang, Sichuan Mental Health Center, Mianyang Sichuan, 621000, China

[3]Department of General Practice, The General Hospital of Western Theatre Command of Chinese People's Liberation Army, Chengdu, Sichuan,610000, China

[4]Department of Obstetrics, Obstetrics & Gynaecology Hospital of Fudan University, Shanghai, 200090, China

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ABSTRACT. This study illustrated the synthesis of a thiophene-embedded Baicalein (BAC) conjugate through the formation of a chalcone backbone, accomplished by refluxing hydroxyl-acetophenone with 5-nitrothiophene-2-carbaldehyde in the presence of piperidine. The BAC conjugate (5,6,7-trihydroxy-2-(5-nitrothiophen-2-yl)-4H-chromen-4-one) was obtained through the oxidative cyclization of the previously synthesized chalcone using DMSO/I2. The structure of BAC was determined by FTIR, 1H-NMR, 13C-NMR, mass spectrometry, and elemental analysis. The impact of BAC on SKOV-3 ovarian cancer cells was assessed via several pharmacological assays, including viability, apoptosis, migration, invasion, and cell cycle analysis. RT-qPCR analysis was performed to assess the impact of BAC on the mRNA levels of Bcl2, Bax, PI3K, Akt, and mTOR. BAC underwent docking analysis utilizing the 3D crystal structure of PI3K. The results indicate that BAC decreases cell viability, migration, invasion, and induces cell cycle arrest at the G2/M phase in SKOV-3 cells in a concentration-dependent manner. The Annexin FITC assay demonstrated the activation of apoptosis by increasing Bax expression and decreasing Bcl2 expression. The mRNA expression of PI3K, Akt, and mTOR was considerably suppressed relative to the untreated control, potentially through interactions with Trp201, Gln291, Leu657, Arg690, Phe694, and Arg849 of PI3K.

Keyword: Cell-viability, Apoptosis, Migration, Cell-cycle, RT-qPCR, Docking

INTRODUCTION

Ovarian cancer ranks as the eleventh most common type of cancer and is the fifth leading cause of death among women, recognized as one of the most lethal forms of cancer affecting the reproductive system [1]. In 2018, there were around 240,000 new cases of ovarian cancer, making it the sixth most common cancer among women globally. Ovarian cancer often goes unrecognized until it reaches a more advanced stage due to the absence of clear symptoms [2]. Despite the significant attention this cancer has garnered in recent years, the survival rate has not changed substantially due to the difficulties associated with early-stage diagnosis. Furthermore, developing

*Corresponding authors. E-mail: chen20241988@outlook.com
#Co-first authors and contributed equally to this work

a therapeutic strategy that can be universally applied to ovarian cancer is challenging due to the various molecular and genetic abnormalities [3]. The resistance of ovarian cancer cells to standard cancer treatments negatively impacts patient prognoses, complicating the development of innovative therapies [4]. As a result, there is an urgent requirement for innovative pharmaceuticals to address this demand.

Wide experimental studies have demonstrated that phytochemicals such as polyphenols, flavones, and flavonoids exert great potential anti-cancer properties against various types of cancers [5]. Among the flavonoids, Baicalein (BAC), a flavonoid extract (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) derived from the dried root of *Scutellaria baicalensis* Georgi, can inhibit cancer-promoting mechanisms such as metastasis, angiogenesis, and inflammation without harming healthy cells [6]. It showed potent anticancer activity against hepatocellular [7], human breast [8], myeloma [9], T24 bladder cancer cells [10], and prostate cancer via interfering with mitogen-activated protein kinase (MAPK), protein kinase B (Akt), mammalian target of rapamycin (mTOR), MMP-2/-9 (matrix metalloproteinase-2/-9) expression, and caspase-9/-3 activation [11]. However, the pharmacological activity of BAC has been seriously compromised due to its instability and altered pharmacokinetics. Therefore, in the present study, we have elucidated the pharmacological activity of various BAC derivatives and their mechanism of action against ovarian cancer cells.

EXPERIMENTAL

The chemicals utilized in the synthesis were obtained from Sigma Aldrich (USA). FTIR spectra were obtained using a Spectrum 1 spectrophotometer from Perkin Elmer equipped with an attenuated total reflectance (ATR) accessory. Measurements were conducted utilizing thin films fabricated in a heated hydraulic press. The Bruker Avance 400 and Bruker Avance 100 spectrophotometers were utilized for ^{1}H-NMR and ^{13}C-NMR, respectively. The chemical changes were documented in parts per million (ppm), while Hertz (Hz) was employed for the coupling constant. The mass spectrum (MS) was acquired using a Waters ZQ LC/MS, alongside elemental analysis for carbon, hydrogen, and oxygen conducted using a Vario Elemental Analyzer.

*Synthesis of 3-(5-nitrothiophen-2-yl)-1-(2,3,4,6-tetrahydroxyphenyl)prop-2-en-1-one (**1**)*

The synthesis was performed as per the previously reported procedure [12]. A solution containing 0.01 mol of 2,4,6-trihydroxyacetophenone and 0.01 mol of 5-nitrothiophene-2-carbaldehyde was prepared in 50 mL of ethanol. Then, approximately 1 mL of piperidine was added drop by drop to the mixture. The final mixture underwent reflux for a duration of 5 hours. The progress of the reaction was tracked using thin-layer chromatography (TLC) using a mixture of ethanol and acetone in a 1:1 ratio. This resulted in the formation of a raw product in the form of chalcone derivatives, namely compound **1**. The solid obtained was subsequently purified by recrystallization using methanol and then dried to obtain the pure chemical.

*Synthesis of 5,6,7-trihydroxy-2-(5-nitrothiophen-2-yl)-4H-chromen-4-one (**2**)*

The BAC (compound **2**) was synthesized as per the previously reported procedure [12]. A solution was prepared by combining 0.01 mol of the produced compounds 2 with 6 mL of DMSO. Then, 0.01 mol of iodine was added to the solution. The resulting mixture was subjected to reflux for 1 hour on an oil bath. After the completion of the reaction, the mixture was poured into freezing water. The product was then precipitated, filtered, and washed with a 20% solution of sodium thiosulfate. The unrefined product **3** underwent purification using column chromatography utilizing a mixture of hexane and ethyl acetate in a ratio of 8:2 as the eluting solvent.

Yield: 87%; MP: 122-123 °C; MW: 321.26; Rf: 0.68; FT-IR (ν_{max}; cm^{-1} KBr): 3498.24 (OH str), 3086.47 (Ar C-H str), 1718.25 (C=O str), 1526.82 (NO2 str), 1491.58 (C=C of benzene), 1034.69 (C-O str), 686.38 (C-S-C) cm^{-1}; ^{1}H-NMR (400MHz, DMSO, TMS) δ ppm: 9.48 (s, 3H, Ar-OH X3), 8.47 (d, 1H, J = 8.26 Hz, Thiophen-H), 7.98 (d, 1H, J = 8.01 Hz, Thiophen-H), 6.72 (s, 1H, chromen-4-one-H), 6.02 (s, 1H, chromen-4-one-H); ^{13}C-NMR (100 MHz, CDCl3) δ, ppm:182.1, 168.7, 158.5, 155.9, 153.2, 151.8, 144.7, 143.9, 130.4, 128.4, 110.2, 106.9, 95.5; mass: 322.26 (M+1); elemental analysis for $C_{13}H_7NO_7S$: calculated: C, 48.60; H, 2.20; O, 34.86; found: C, 48.63; H, 2.21; O, 34.85.

Cell culture

The human ovarian cancer cells (SKOV-3) were obtained from ATCC, USA. All cell lines have been identified by STR (short tandem repeat) profiling, and cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. (Thermo Fisher Scientific) and penicillin-streptomycin (Gibco) at 37 °C in a 5% CO_2 incubator.

Cellular apoptosis and cell-cycle

Initially, the cells were rinsed twice with chilled PBS. Subsequently, a 100 µL aliquot of a solution containing 105 cells was transferred to a 5 mL culture tube. Subsequently, 5 µL of Annexin V–FITC (BD Biosciences) and 5 µL of propidium iodide (PI) (BD Biosciences) were added to the tube. Subsequently, the solution was gently agitated using a vortex mixer and then allowed to rest for a duration of fifteen minutes at room temperature while being shielded from light. Then, 400 µL of a binding buffer with a concentration of 1x1 was added to each tube. To detect stained cells, we used a FACScan flow cytometer manufactured by BD Biosciences. The data obtained were then processed using the FlowJo V10 software. After being washed twice with cold PBS, the cells were incubated with 70% ethanol at a temperature of 4 degrees Celsius for twelve hours. The cells were stained with PI (BD Biosciences) and analyzed within twenty-four hours. Flow cytometry generated by BD Biosciences was employed to examine the dispersion of the cell cycle.

RT-qPCR analysis

The extraction of total RNA was performed using a Total RNA Isolation Kit (RC101-01, Vazyme) following the instructions provided by the manufacturer. The quantification of total RNA was performed using the NanoDrop 2000 instrument manufactured by Thermo Fisher Scientific. The process of reverse transcription was employed to convert the total RNA into complementary DNA (cDNA) using the HiScript® III All-in-one RT SuperMix (R333-01, Vazyme). The ChamQ SYBR Color qPCR Master Mix (Q411-02, Vazyme) was employed for performing two-step real-time RT-PCR analysis.

Docking analysis

The docking of compound 2 was performed using the CB-Dock default setting as pe the given protocol using the blind docking [13, 14].

Statistical analysis

The GraphPad Prism (version 8.0) program was utilized for statistical analysis. The in vitro trials were replicated at least three times. Variations between the two groups were evaluated using a

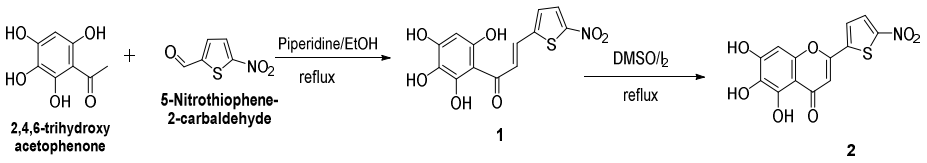
two-tailed Student's t-test. The data is presented as the mean ± SD. The threshold for statistical significance was established at a p-value < 0.05.

RESULTS AND DISCUSSION

Chemistry

The compounds were synthesized using a two-step process following a previously reported method, as shown in Scheme 1. First, we aimed to create the chalcone backbone (**1**) by refluxing hydroxyl-acetophenone with 5-nitrothiophene-2-carbaldehyde in the presence of piperidine. This synthesis of chalcones involves a base-catalyzed Claisen-Schmidt condensation reaction between an aromatic aldehyde and acetophenone. This process begins by dissolving the two starting materials—hydroxyl-acetophenone and an aromatic aldehyde (5-nitrothiophene-2-carbaldehyde)—in a ethanol. A catalytic amount of a base, piperidine, is added to initiate the reaction. The base deprotonates the methyl group (α-hydrogen) of acetophenone, generating a reactive enolate ion. This enolate serves as a nucleophile and attacks the carbonyl carbon of the aromatic aldehyde, forming a β-hydroxyketone intermediate through nucleophilic addition. Following this addition, the reaction mixture is typically refluxed to promote the dehydration of the intermediate. Under these conditions, the hydroxyl group and a neighboring hydrogen atom are eliminated, resulting in the formation of a conjugated α,β-unsaturated ketone, known as chalcone. This conjugation between the carbonyl group and the double bond extends the delocalization of electrons, which imparts characteristic stability and reactivity to the chalcone molecule. In the final step, the target compound, 5,6,7-trihydroxy-2-(5-nitrothiophen-2-yl)-4H-chromen-4-one (**2**), was synthesized through oxidative cyclization of compound **1** using DMSO and iodine (I_2). In this reaction, the chalcone, containing both a hydroxyl group and an α,β-unsaturated ketone, undergoes intramolecular cyclization facilitated by the oxidizing environment created by DMSO/I_2. Initially, the iodine acts as an oxidant, activating the hydroxyl group on the aromatic ring, which promotes the formation of an enolate from the chalcone. This enolate intermediate nucleophilically attacks the nearby carbonyl group within the same molecule, forming a cyclic hemiacetal. Under the influence of DMSO, the reaction proceeds by oxidizing the intermediate to form the desired cyclic structure, chromen-4-one (a flavonoid core). DMSO serves not only as a solvent but also as a mild oxidant, facilitating the elimination of water molecules during the final cyclization step. The result is the formation of a stable chromenone ring system through the closure of the oxygen-containing heterocycle, completing the oxidative cyclization. This mechanism ensures high selectivity and efficiency, yielding the chromen-4-one derivative. The characterization of compound 2, including elemental analysis and melting point measurement, confirmed consistency with previously reported data [12].

The FT-IR spectra provided key insights into the functional groups present in the synthesized compound. A strong absorption band at 3498 cm^{-1} confirmed the presence of an –OH group, indicating hydroxyl functionality. Another band observed at 3086 cm^{-1} corresponded to the stretching vibrations of aromatic C–H bonds, further supporting the aromatic nature of the compound. The characteristic peak at 1718 cm^{-1} validated the presence of a carbonyl (C=O) group attached to the benzene ring. Additionally, a prominent absorption band at 1526 cm^{-1} indicated the presence of a nitro ($–NO_2$) group. The ^1H NMR spectrum offered further structural information. A doublet signal at 8.47 ppm was attributed to protons on the thiophene ring, confirming its incorporation into the molecule. Meanwhile, protons corresponding to the chromen-4-one moiety appeared within the chemical shift range of 6.72–6.02 ppm, aligning well with the expected values for such aromatic environments. Together, the FT-IR and ^1H and ^{13}C NMR, and mass spectra confirmed the structural integrity and successful synthesis of the target compound.



Scheme 1. Synthesis of thiophene-embedded baicalein analog, BAC (compound **2**) (5,6,7-trihydroxy-2-(5-nitrothiophen-2-yl)-4H-chromen-4-one).

Biological activity

Effect on cell viability of SKOV-3 cells

It is usual practice to perform cellular viability and proliferation assays while assessing the effectiveness of potential anti-cancer medications, which may encompass cytostatic and cytotoxic treatments. The purpose of this is to find out how well the treatments work. The quantity of viable cells in a population is called cellular viability and is used to measure how many healthy cells are in a population. As shown in Figure 1, the BAC showed a significant reduction of cell viability as compared to the untreated control. The effect of BAC on the SKOV-3 cells is observed to be concentration- and time-dependent. The most significant activity was reported by the 10 μM treated group 72 h.

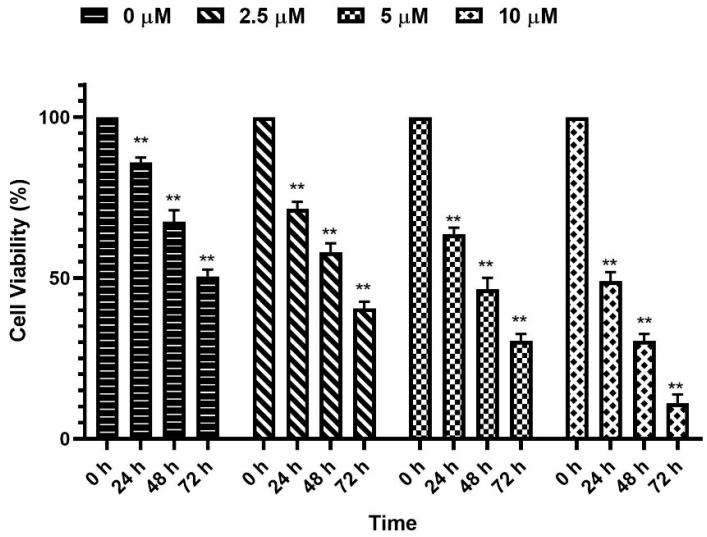


Figure 1. Effect of BAC on cell viability of SKOV-3 cells.

Effect on the apoptosis and cell cycle of SKOV-3 cells

We used flow cytometry with Annexin V-FITC/PI labeling to determine if BAC may induce cell death in SKOV-3 cells. It is visible from the results shown in Figure 2A and B that BAC administration resulted in a significant increase in the rate of apoptosis. In addition, compared to the control group that did not receive any medication, the induction was found to be substantially

stronger at higher BAC concentrations. Within the context of a cell-cycle analysis study (Figure 2C), we found a significantly lower percentage of cells in the S-phase and a significantly higher percentage of cells in the G2/M phase with an increase in BAC concentration. The G0/G1 phase cells, on the other hand, showed minimal change. The activation of apoptosis and the interruption in cell cycle progression during the G2/M phase may be responsible for the significant suppressive effect that BAC has on the proliferation of SKOV-3 cells.

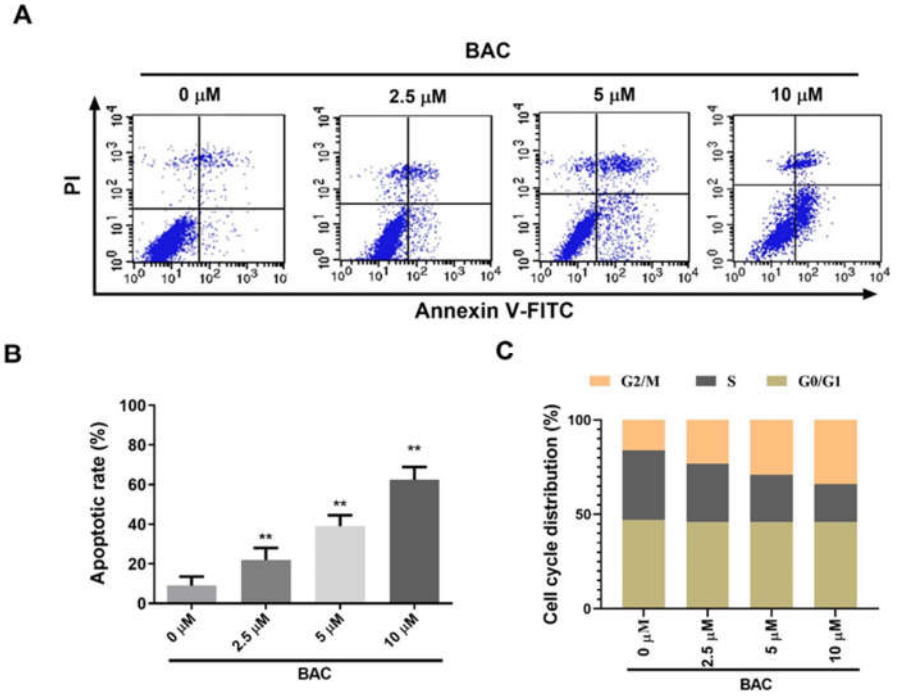


Figure 2. Effect of BAC on the (A and B) apoptosis and (C) cell-cycle analysis of SKOV-3 ovarian cancer cells. **p<0.05 vs. non-treated group. Data are presented as means ± SEM.

Effect on migration and invasion of SKOV-3 cells

The events that trigger tumor cell invasion and metastasis are crucial for assessing cancer prognoses [15], as the progression of malignancies across tissues is a major cause of cancer-related mortality and morbidity [16]. Tumor metastasis involves a multi-step process that leads to the formation of secondary tumors in distant organs. The invasion begins when motile tumor cells penetrate the basement membrane and extracellular matrix; once they enter the lymphatic or vascular circulation through intravasation, they continue their journey. As these metastatic cells traverse the circulatory system, they undergo extravasation by breaching the vascular basement membrane and extracellular matrix. Eventually, the cells adhere to new sites, where they proliferate to form secondary tumors. Each phase of this complex process is characterized by distinct mechanisms involving cell-cell junction markers, the actin cytoskeleton, matrix adhesion, and protease activity [15, 18]. Inhibiting these migration and invasion processes offers a protective pharmacological strategy against cancer. In this context, the present study demonstrates that

compound 2 (BAC) effectively suppresses the migration and invasion of ovarian cancer cells in a concentration-dependent manner, as shown in Figure 3.

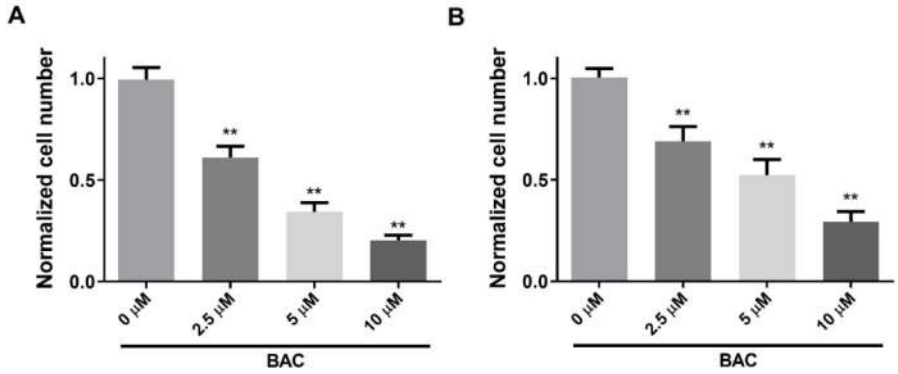


Figure 3. Effect of BAC on the (A) migration and (B) invasion, of SKOV-3 ovarian cancer cells. **p<0.05 vs. non-treated group. Data are presented as means ± SEM.

Effect on protein expression of Bcl2 and Bax using RT-qPCR

Medications used to treat cancer either cause cancer cells to die out naturally or trigger a process known as apoptosis, which is a programmed cell death [19]. Apoptosis is the last line of defense for many cancer treatments, but when cancer cells develop resistance, the treatment may have little to no effect [20]. Several studies have revealed that the form and function of mitochondria play significant roles in the process of cancer cell growth. In addition to their involvement in caspase-dependent apoptosis, mitochondria also have a considerable influence on the Bcl-2 pathway during caspase-independent apoptosis [21]. Alterations in mitochondria are one of the primary routes that regulate caspase-independent apoptosis and proteins belonging to the Bcl family during cell death. The B-cell lymphoma 2 (BCL-2) family of proteins has been the subject of intense research over the past decade due to the important roles they play in regulating cell death, cancer development, and cellular responses to anticancer therapy. These proteins have separate roles in promoting cell death and preventing cell death [22]. Bcl-2 is probably a negative regulator of cell apoptosis due to its function in cell survival and its capacity to inhibit cell apoptosis, a process that can be initiated by various stimuli. Bax is a protein that induces cell death and is located on the outer mitochondrial membrane. Its role in the transfer of apoptotic signals is supported by its ability to migrate to the mitochondria in the initial phases of cell death [23–25]. In earlier studies, Baicalein showed significant induction of apoptosis in a variety of cancers, such as prostate, lung, tongue, liver, bladder, and breast cancer. It showed significant modulation of Bax and Bcl2. In the present study, we have studied the expression of Bcl2 and Bax using RT-qPCR analysis and the results have been presented in Figure. 4. Results suggest that BAC showed a significant increase in mRNA expression of Bax, while the level of Bcl2 was found to be reduced in a concentration-dependent manner. These results suggest that the apoptosis-inducing effect of BAC was due to significant modulation apoptosis.

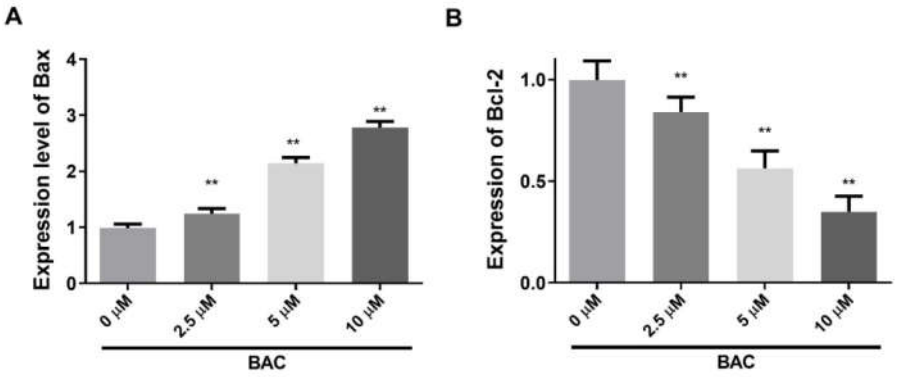


Figure 4. Effect of BAC on the mRNA level of (A) Bax and (B) Bcl2. **p<0.05 vs. non-treated group. Data are presented as means ± SEM.

Effect on PI3K/Akt/mTOR using RT-qPCR

Mammalian cells undergo several processes, including proliferation, differentiation, apoptosis, autophagy, and survival, all of which are influenced by the PI3K/AKT signaling pathway, which is a key regulator of autophagy and plays a substantial role in it [26]. Additionally, it plays a fundamental role in the regulation of autophagy. The activation and inhibition of the PI3K/AKT signaling pathway are responsible for this regulation [27]. This regulation governs the survival of human cancer cells in vitro and the carcinogenicity, invasion, and metastasis of human cancer cells in vivo [28, 29]. It has also been established that this regulation is responsible for the regulation of the signaling pathway. This particular regulator is responsible for determining whether human cancer cells can survive. In human renal carcinoma cells, glioma, human epidermoid carcinoma cells, breast cancer cells, and bladder cancer cells, it has been demonstrated that the modulation of the PI3K/AKT signaling pathway is responsible for the activation of apoptosis and autophagy occurs when baicalein is present [30, 31]. This has been demonstrated by several research that have been conducted. Therefore, in the present study, we use RT-qPCR analysis to study the effect of PI3K/Akt. As shown in Figure 5, it has been found that BAC significantly reduced the mRNA expression of PI3K, Akt, and mTOR. These results suggest that BAC inhibits the progression of ovarian cancer cells via interfering with PI3K/Akt/mTOR signalling pathway.

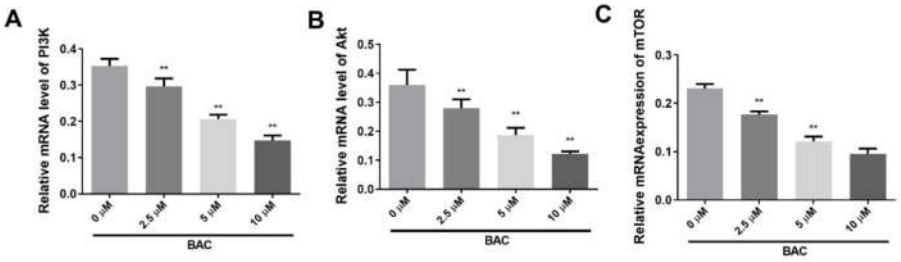


Figure 5. Effect of BAC on the mRNA level of (A) PI3K, (B) Akt, and (C) mTOR using RT-qPCR. **p<0.05 vs. non-treated group. Data are presented as means ± SEM.

Docking analysis with PI3K

An approach to computational drug discovery known as molecular docking models the interaction between a small molecule (drug candidate) and a target protein (usually an enzyme or receptor associated with a disease). This phenomenon can be witnessed during the drug discovery process [32]. Due to the docking technique, the comprehension of molecular interactions at the atomic level has become significantly easier. Consequently, this offers a valuable understanding of the connections between the structure and activity, which are essential for the advancement of medications [33, 34]. This work has a significant influence on various domains, such as expediting the discovery of prospective drug candidates, enhancing the efficacy of lead optimization, and contributing to the advancement of pharmaceuticals that are simultaneously safer as well as efficacious [35–38]. Thus, in the present study, we have demonstrated the docking analysis of BAC into the active site of PI3K. As shown in Figure 6, 7, and 8, and Table 1, BAC showed excellent affinity against PI3K with a Vina score of -8.2. The 2D interaction clearly showed that BAC created various H-bonds and non-bonded interactions (pi-pi, pi-sigma, and others) with Trp201, Gln291, Leu657, Arg690, Phe694, and Arg849 of PI3K. Studies have shown that the interaction of drugs with specific amino acids in the PI3K (Phosphoinositide 3-kinase) protein plays a critical role in modulating enzyme activity and determining the efficacy of inhibitors. Based on the docking results, it has been suggested that BAC created key interaction with Trp201 which contributes to hydrophobic or π-π stacking interactions with aromatic drug moiety, stabilizing the ligand and enhancing its binding within the active site. Gln291 is involved in hydrogen bonding with polar group of BAC, increasing specificity and binding strength. Similarly, Leu657 participates in hydrophobic interactions with BAC that help anchor the drug within the lipid-binding region or regulatory site, further enhancing affinity. The positively charged Arg690 engages BAC in electrostatic or hydrogen bonding interactions, playing a key role in proper ligand orientation and stabilizing inhibitor binding, potentially affecting kinase activity. Moreover, Phe694 provides aromatic interactions through π-π stacking or hydrophobic contacts with BAC, which contribute to correct ligand positioning within the hydrophobic pocket. Finally, Arg849 also forms hydrogen bonds or electrostatic interactions, stabilizing the BAC in the active or allosteric site and possibly altering the enzyme's conformation to block substrate access. Collectively, these interactions ensure high binding affinity, selectivity, and inhibitory potency of BAC, which are essential for effective PI3K inhibition. By stabilizing BAC within the active site, these residues help prevent PI3K from phosphorylating downstream targets in the PI3K/AKT/mTOR pathway, a key signaling cascade implicated in cancer progression and survival. Therefore, targeting these amino acids is crucial for developing potent and selective PI3K inhibitors for cancer therapy.

Table 1. Docked parameter of BAC with the PI3K.

Vina score	Cavity volume ($Å^3$)	Center (x, y, z)	Docking size (x, y, z)	Contact residues
C1	-8.2	6576	32, 0, 15	35, 22, 33

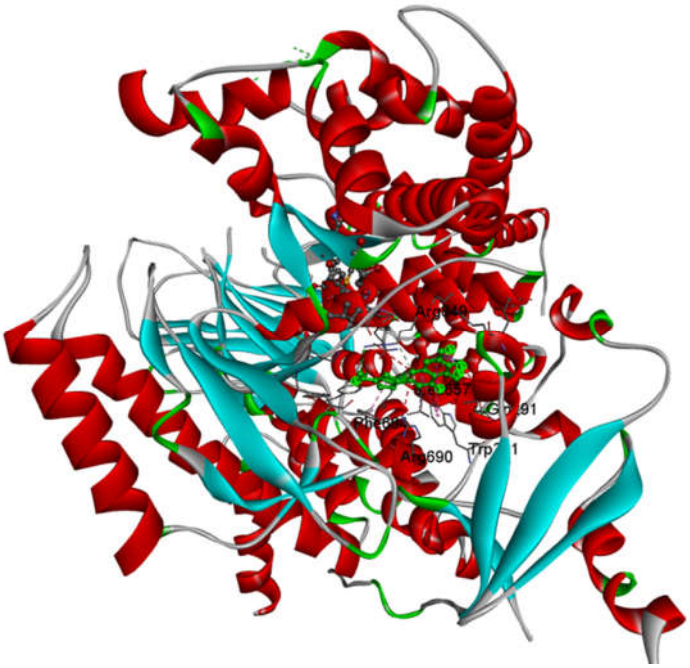


Figure 6. 3D Uncropped docked pose of BAC into the active site of PI3K.

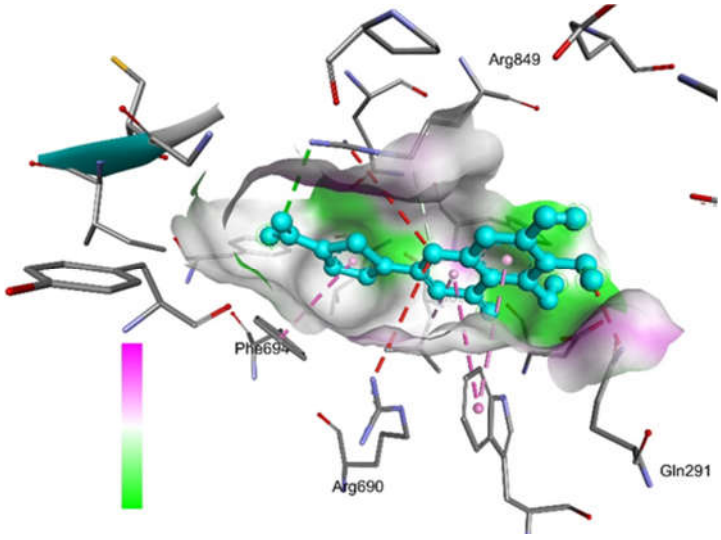


Figure 7. 3D clear-view docked pose of BAC into the active site of PI3K.

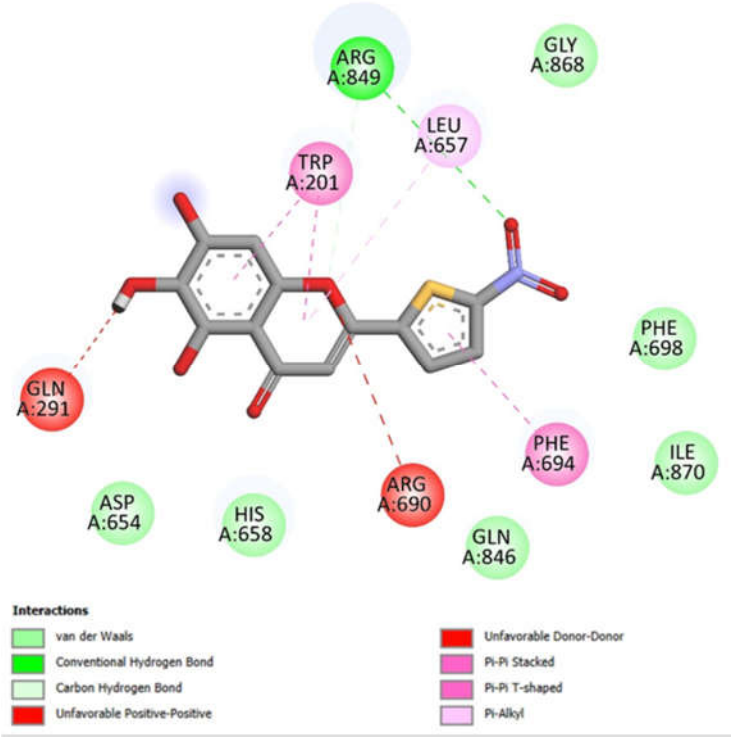


Figure 8. 2D docked pose of BAC into the active site of PI3K.

CONCLUSION

Our study demonstrated the significant anticancer activity of thiophene-embedded Baicalein derivative as a potent anti-cancer agent against ovarian cancer cells. These results showed to be brought by the apoptosis-promoting and inactivation effect of the PI3K/Akt/MTOR signaling pathway of BAC.

Declaration

Ethics approval

The authors declare that the procedures followed were under the regulations of the relevant animal research ethics committee and the study was approved by the Obstetrics & Gynaecology Hospital of Fudan University, China.

Authors' contributions

Z.L. and L.Z. performed the experiments, acquired and analyzed the data. L.W. and D.S. contributed top methodology, software, validation. All authors involved in writing the draft of the article. Y.C. responsible for conceptualization, supervision, resources, writing - review and editing and project administration.

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