Bufalin suppresses ovarian cancer cell proliferation via EGFR pathway

Lei Dou[1], Dan Zou[2], Feiran Song[1], Yuxin Jin[3], Yin Li[3], Yi Zhang[1]

[1]Department of Gynecology, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, China;
[2]First Laboratory of Cancer Institute, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, China;
[3]Department of Integration of Chinese and Western Medicine, School of Basic Medical Sciences, Peking University, Beijing 100191, China.

Abstract

Background: Previous studies have shown that bufalin exerts antitumor effects through various mechanisms. This study aimed to determine the antineoplastic mechanism of bufalin, an extract of traditional Chinese medicine toad venom, in ovarian cancer.

Methods: The 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), 5-ethynyl-2′-deoxyuridine (EdU), and colony formation assays were used to investigate the antiproliferative effect of bufalin on the ovarian cancer cell line SK-OV-3. Molecular docking was used to investigate the combination of bufalin and epidermal growth factor receptor (EGFR) protein. Western blotting was performed to detect the expression of EGFR protein and its downstream targets.

Results: Bufalin inhibited the proliferation of SK-OV-3 cells in a dose- and time-dependent manner. Bufalin was confirmed to combine with EGFR protein using molecular docking and downregulate expression of EGFR. Bufalin inhibited phosphorylation of EGFR, protein kinase B (AKT), and extracellular signal-regulated kinase (ERK).

Conclusion: Bufalin suppresses the proliferation of ovarian cancer cells through the EGFR/AKT/ERK signaling pathway.

Keywords: Bufalin; Ovarian cancer; Epidermal growth factor receptor

Introduction

Ovarian cancer ranks seventh among lethal cancers in women.[1] Epithelial ovarian cancer is the main lethal type.[2] Despite the continuous development of cancer therapy, the 5-year survival rate of patients with ovarian cancer has not increased significantly compared with 10 years ago and remains at only 40%.[3,4] Bufalin is an extract of traditional Chinese medicine toad venom, which has digoxin-like immunoactivity, with detoxification, detumescence, and analgesic effects. Previous studies have shown that bufalin [Figure 1] exerts antitumor effects through various mechanisms. For example, bufalin promotes apoptosis of nasopharyngeal carcinoma cells through the mitochondrial ROS/TRAIL pathway[5] and promotes autophagy in gastric cancer cells through the Cbl-b/mammalian target of rapamycin (mTOR)/extracellular signal-regulated kinase (ERK) pathway.[6] Bufalin is also a potent inhibitor of cell growth and migration in ovarian cancer cells through suppression of mTOR activation and hypoxia-inducible factor 1-α induction.[7] In addition, it was demonstrated that bufalin exerts antitumor effects by triggering apoptosis and inducing cell cycle arrest in pancreatic cancer cells. Notably, bufalin can also promote the growth inhibitory effect of gemcitabine in pancreatic cancer cells.[8]

Epidermal growth factor receptor (EGFR) is overexpressed in many solid tumors, including breast, pancreas, head-and-neck, prostate, ovarian, renal, colon, and non-small-cell lung cancers.[9,10] Such overexpression produces strong stimulation of downstream signaling pathways, which induce cell growth, cell differentiation, cell cycle progression, angiogenesis, cell motility, and blocking of apoptosis. The high expression and/or functional activation of EGFR correlates with the pathogenesis and progression of several cancers, which make it an attractive target for both diagnosis and treatment.[11] EGFR expression is also increased in ovarian cancer, and its high expression is related to poor prognosis. EGFR promotes ovarian cancer cell proliferation through the PI3K/AKT/MAPK/STAT pathway. Therefore, inhibition of EGFR signaling is important in suppressing the development of ovarian cancer.[12] Zeng *et al*[13] demonstrated that epidermal growth factor (EGF) secreted by

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000001879

Correspondence to: Dr. Yi Zhang, Department of Gynecology, the First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, China
E-Mail: syzi@163.com
Dr. Yin Li, Department of Integration of Chinese and Western Medicine, School of Basic Medical Sciences, Peking University, Beijing 100191, China
E-Mail: yinli@bjmu.edu.cn

Chinese Medical Journal 2022;135(4)

Received: 29-03-2021; **Online:** 22-12-2021 **Edited by:** Yanjie Yin and Xiuyuan Hao

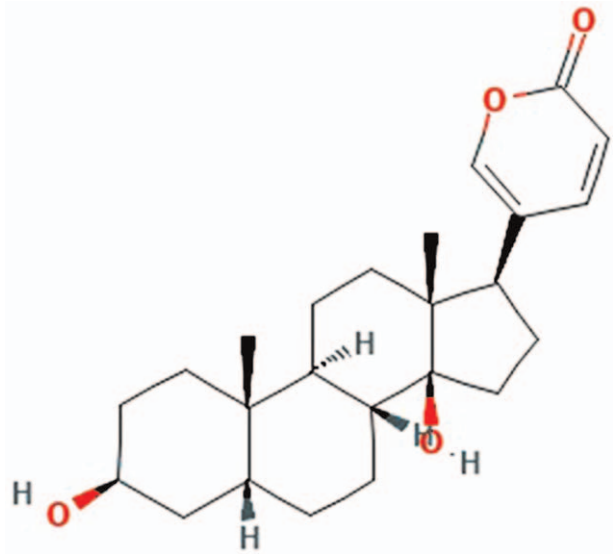

Figure 1: Molecular structure of bufalin (PubChem CID: 9547215).

M2-like tumor-associated macrophages might promote the progression of ovarian cancer by activating the EGFR-ERK signaling pathway.

This study verified the effect of bufalin on EGFR through molecular docking and on ovarian cancer cell proliferation through the EGFR/AKT/ERK pathway *in vitro*.

Methods

Cell lines and culture

Human ovarian cancer SK-OV-3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in a 37°C incubator with 5% carbon dioxide. The cells were cultured in McCoy 5A culture medium (Biological Industries, Israel) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 100 U/mL streptomycin (Biotech, Beijing, China). This research did not involve humans or animals.

Drugs and reagents

Bufalin and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) (thiazolyl blue) were purchased from Sigma (St. Louis, MO, USA). Rapid Wright-Giemsa Staining solution was purchased from Nanjing Jiancheng Institute of Biological Engineering (China). Antibodies to EGFR, phospho-EGFR, AKT, phospho-AKT, ERK, and phospho-ERK were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody to β-actin was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (China).

MTT test

SK-OV-3 cells in the logarithmic growth phase were digested with trypsin and seeded in 96-well plates with 4000 cells/well. After adhesion, different doses of bufalin were applied for 24 or 48 h. Four hours before termination, 20 μL MTT (5 mg/mL) was added to each well. After 10 min incubation with dimethyl sulfoxide, the optical density was read at 570 nm.

EdU assay

As reported,[14,15] cells were treated with 150 μL 5-ethynyl-2′-deoxyuridine (EdU) (Ribobio, Guangzhou, China) for 2 h. After washing with phosphate-buffered saline (PBS) three times, cells were treated with 2 mg/mL glycine and 0.5% TritionX-100 in turn, then stained with 300 μL of 1 × Apollo® reaction cocktail for 30 min followed with 300 μL of Hoechst 33,342 (5 μg/mL) for 30 min. Cells were washed with PBS after each step and then observed under microscopy.

Cloning

SK-OV-3 cells in the logarithmic growth phase were cultured with 0, 100, and 200 nmol/L bufalin for 24 h, then digested with trypsin and seeded in six-well plates with 1200 cells/well for 10 days. Cells were washed twice with PBS, fixed with 700 μL methanol for 15 min, dried and stained with 500 μL Rapid Wright-Giemsa Staining solution reagent one for 1 min, and 1 mL reagent two for 5 to 8 min. The staining solutions were removed, and the cells were washed with distilled water and air-dried for 30 s.

Western blotting

Following treatment, the cells were pelleted by centrifugation for 3 min at 200 × *g*, and homogenized in ice-cold fractionation buffer (50 mmol/L Tris–HCl, pH 7.4, 1 mmol/L ethylenediaminetetraacetic acid, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 10 μg/mL aprotinin, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate and 50 mmol/L sodium fluoride). The cell lysate was incubated on ice for 15 min and then centrifuged at 20,000 × *g* for 30 min at 4°C. The cytosolic fraction was collected and subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The proteins were transferred to a polyvinylidene fluoride membrane. The membrane was incubated successively with 5% bovine serum albumin in Tris-buffered saline with Tween® 20 (TBST) at room temperature for 1 h, with primary antibodies at 4°C for 12 h and then with horseradish-peroxidase-labeled secondary antibody for 1 h. After each incubation, the membranes were washed extensively with TBST, and the immunoreactive bands were detected with enhanced chemiluminescence (ECL)-detecting reagents (Pierce).

Molecular docking

Molecular docking was accomplished with the Schrodinger software (Schrodinger Suites 2018, NY, USA). The structure of bufalin is available at the PubChem website (PubChem CID:9547215). The structure of EGFR protein

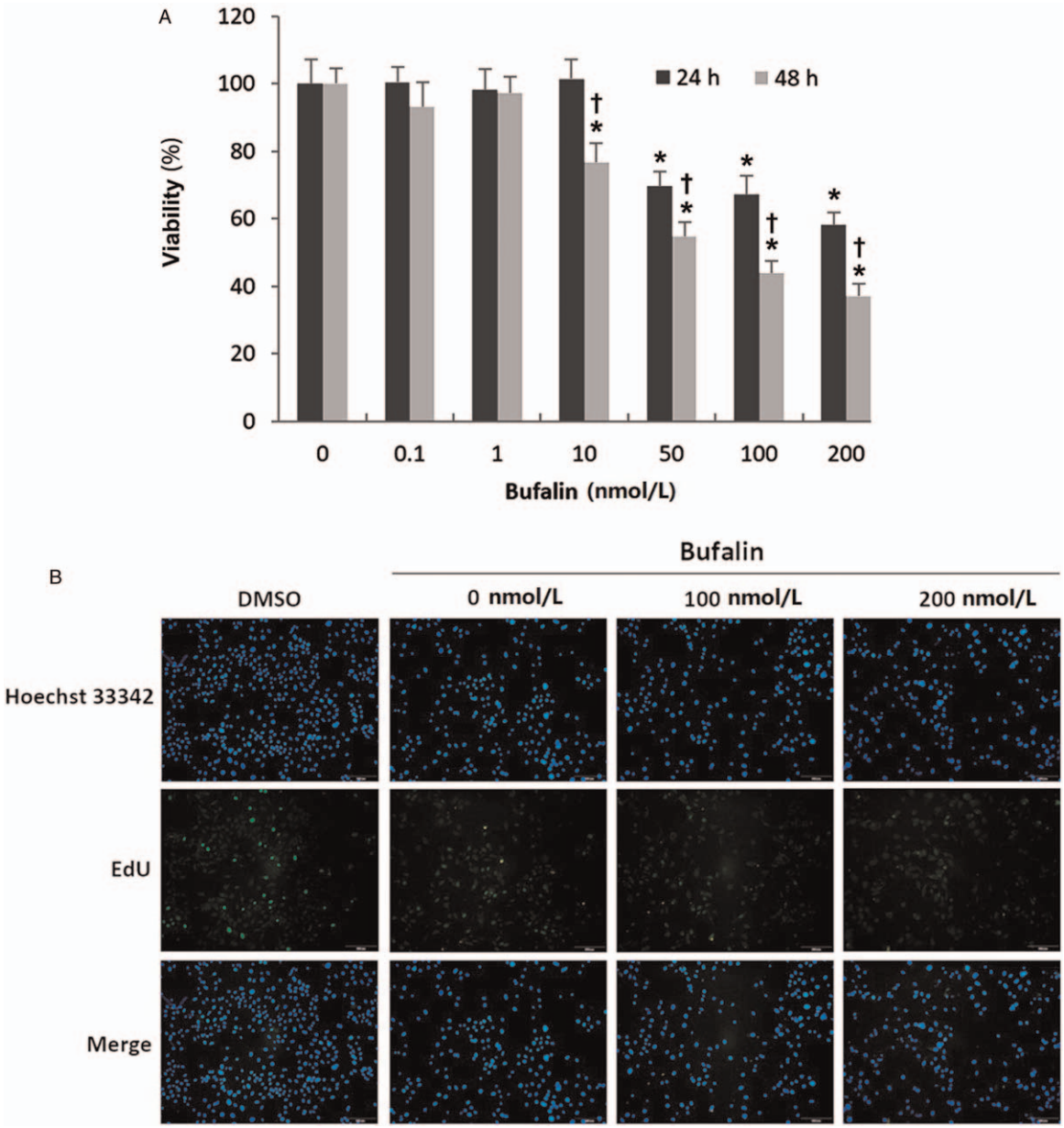

Figure 2: Bufalin inhibited proliferation of SK-OV-3 cells in a dose- and time-dependent manner. (A) MTT assay was used to detect the effect of bufalin on the proliferation of SK-OV-3 cells. $^{*}P < 0.05$ *vs.* control (0 nmol/L group), $^{\dagger}P < 0.05$ *vs.* 24 h. (B) EdU assay was used to detect the effect of bufalin on DNA replication of SK-OV-3 cells (Original magnification ×100). DMSO: Dimethyl sulfoxide; EdU: 5-ethynyl-20-deoxyuridine; MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide.

(PDB:1m17) was downloaded from the Protein Data Bank website (http://www.rcsb.org/). In Schrodinger software, Prepare wizard, Grid generation, Ligand preparation, and Ligand docking were conducted to obtain the docking fraction.

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. All data were expressed as means ± standard error mean. One-way Analysis of Variance (ANOVA) followed by Newman–Keul *post hoc* test was used to compare multiple groups. Unpaired Student *t* test was performed to compare two groups. $P < 0.05$ was considered statistically significant.

Results

Bufalin inhibits ovarian cancer cell proliferation in a dose- and time-dependent manner

As the concentration of bufalin increased, the inhibitory effect on the proliferation of SK-OV-3 cells was enhanced [Figure 2A]. As the duration of treatment increased, the inhibitory effect of bufalin on the proliferation of SK-OV-3 cells was also enhanced [Figure 2A]: 24 h half maximal inhibitory concentration (IC50) = 211.80 nmol/L (95% CI = 136.00–349.80 nmol/L); 48 h IC50 = 74.13 nmol/L (95% CI = 43.35–124.80 nmol/L). The EdU assay demonstrated that as the concentration of bufalin increased, the DNA replication of SK-OV-3 cells decreased significantly [Figure 2B].

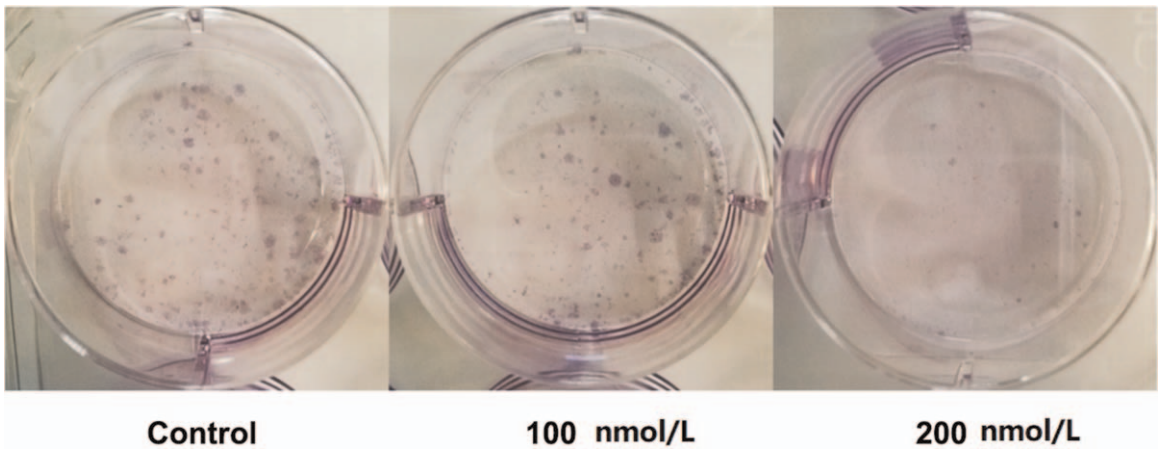

Figure 3: Bufalin inhibits colony formation of SK-OV-3 cells.

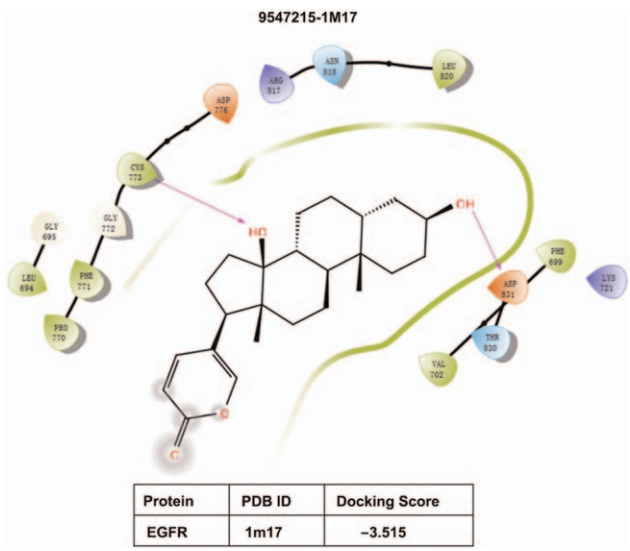

Figure 4: Molecular docking results of bufalin and EGFR protein. EGFR: Epidermal growth factor receptor.

Bufalin inhibits colony formation of ovarian cancer cells

The colony formation assay showed that bufalin reduced the size and number of SK-OV-3 cell colonies. As the concentration of bufalin increased, the inhibitory effect of bufalin on colony formation was enhanced in a concentration-dependent manner [Figure 3].

Bufalin conjugates EGFR protein

Molecular docking using Schrodinger software found that there was a hydrogen bond interaction between bufalin and EGFR protein, and the docking score was −3.515. Bufalin may inhibit EGFR protein [Figure 4].

Bufalin inhibits ovarian cell proliferation by inhibiting the EGFR/AKT/ERK pathway

Western blotting confirmed that bufalin reduced the total protein and phosphorylation levels of EGFR, and the phosphorylation levels of downstream molecules of EGFR, AKT, and ERK were downregulated under 200 nmol/L of bufalin stimulation [Figure 5A and 5B].

Discussion

Ovarian cancer is the most common fatal gynecological tumor.[16] Worldwide, approximately 230,000 women are diagnosed with ovarian cancer each year and 150,000 die.[17] Therefore, there is an urgent need to optimize the treatment of patients with ovarian cancer and improve their prognosis. The present study showed that bufalin inhibited SK-OV-3 ovarian cancer cell proliferation and DNA replication in a dose- and time-dependent manner. A previous study demonstrated that bufalin could inhibit the growth of ovarian cancer cells and induce cell cycle arrest.[18] The mechanism of inhibiting ovarian cancer proliferation is Livin protein inhibition and inhibition of the ITGB2/FAK signaling pathway.[19]

EGFR is a transmembrane glycoprotein consisting of a 170 kDa polypeptide chain. The transmembrane glycoprotein EGFR extracellular domain consists of 622 amino acids and contains two cysteine-rich regions, primarily responsible for ligand binding, and the transmembrane domain structure consists of a single alpha-helix containing 23 residues that constitute the intracellular transmembrane peptide structural domain, consisting of 542 amino acids and containing a 250 amino acid protein tyrosine kinase core, conserved and attached at the C-terminus, containing regulatory tyrosine residues.[20] EGF binds to the extracellular region of EGFR and induces EGFR dimerization, thereby activating EGFR tyrosine kinase activity and autophosphorylation. The tyrosine autophosphorylation sites in activated EGFR interact with downstream signaling proteins, thereby initiating activation of signaling pathways and ultimately promoting cell proliferation and survival.[21,22] In ovarian cancer, amplification and/or high expression of EGFRs have been implicated in the progression and prognosis of the disease.[12] EGFR signaling activation is closely related to ovarian cancer cell proliferation, and in the studies of Kang *et al*[23] and Jiang *et al*,[24] bufalin inhibited EGFR phosphorylation in

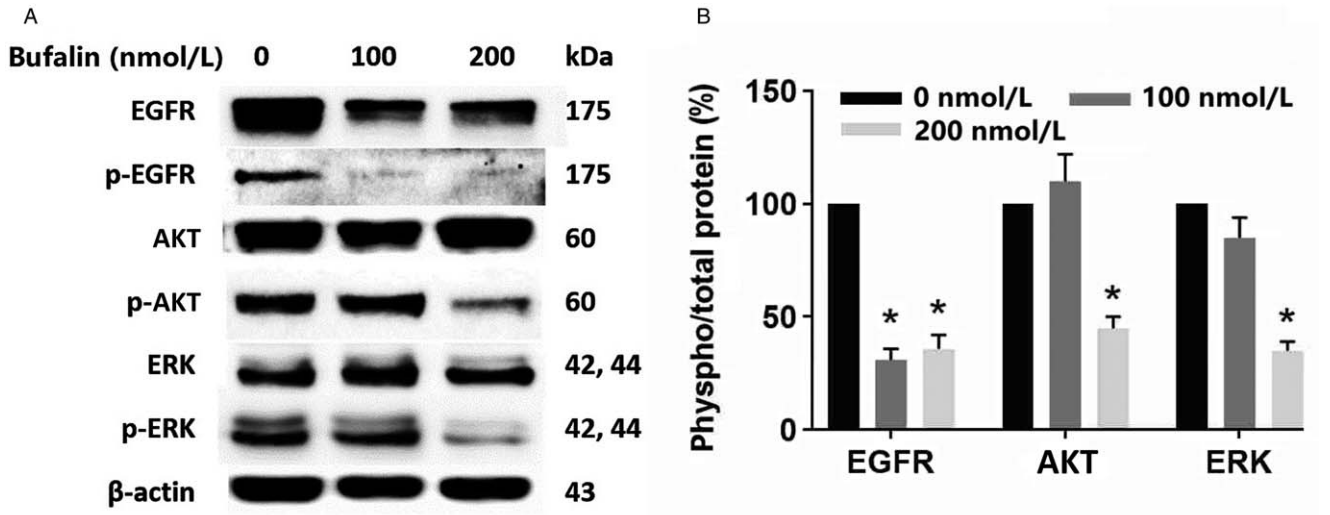

Figure 5: Effect of bufalin inhibition on the EGFR/AKT/ERK signaling pathway. (A) Representative results of western blot. (B) Relative protein signal intensity was quantified and expressed as means ± standard error mean. $^{*}P < 0.05$ *vs.* Control (0 nmol/L group). EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase.

the EGFR mutant lung cancer cell line HCC827 and wild-type cell line A549, respectively. It was also identified that bufalin eliminates resistance to Osimertinib which is an EGFR tyrosine kinase inhibitor, indicating that a combination of Osimertinib and bufalin could be an effective additional treatment to overcome acquired resistance to Osimertinib in non-small cell lung cancer cells.[25] However, there are currently no reports on the inhibition of ovarian cancer cell proliferation by bufalin through the EGFR signaling pathway. We proved that bufalin can inhibit the proliferation of ovarian cancer cells by binding to EGFR through molecular docking, and western blotting confirmed that bufalin can inhibit the proliferation of ovarian cancer cells through the EGFR/AKT/ERK pathway. However, in the studies by Kang *et al*[23] and Jiang *et al*,[24] bufalin only inhibited the phosphorylation of EGFR protein in lung cancer cells without affecting the level of total EGFR protein. In the study by Liu *et al*,[26] bufalin inhibited the expression of transcription factor SOX2, which has been proved to be one of the transcription factors of EGFR gene expression. Therefore, the decrease in EGFR protein may be due to the inhibition of SOX expression. Future studies should examine whether bufalin inhibits activation of the transcription factor SOX2, thereby inhibiting the expression of EGFR protein.

In summary, bufalin inhibits the phosphorylation of AKT and ERK by reducing the total protein and phosphorylation level of EGFR, thus inhibiting the proliferation of ovarian cancer cells, which adds new insight into the mechanism of bufalin in the treatment of ovarian cancer.

Funding

This study was supported by a grant from the Special project for central guidance of local science and technology development of Liaoning province (No. 2019JH6/10400006).

Conflicts of interest

None.

References

1. Morrison J, Thoma C, Goodall RJ, Lyons TJ, Gaitskell K, Wiggans AJ, *et al*. Epidermal growth factor receptor blockers for the treatment of ovarian cancer. Cochrane Database Syst Rev 2018;10:CD007927. doi: 10.1002/14651858.CD007927.pub4.
2. Xu Z, Wang L, Wang WL. Application of lymphangiography in para-aortic lymphadenectomy for ovarian cancer. Chin Med J 2020;134:107–108. doi: 10.1097/CM9.0000000000001087.
3. Allemani C, Matsuda T, Di Carlo V, Harewood R, Matz M, Nikšić M, *et al*. Global surveillance of trends in cancer survival 2000–14 (CONCORD-3): analysis of individual records for 37513025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. Lancet 2018;391:1023–1075. doi: 10.1016/S0140-6736(17)33326-3.
4. Cheng HY, Zeng L, Ye X, Ma RQ, Tang ZJ, Chu HL, *et al*. Age and menopausal status are important factors influencing the serum human epididymis secretory protein 4 level: a prospective cross-sectional study in healthy Chinese people. Chin Med J 2020;133:1285–1291. doi: 10.1097/CM9.0000000000000785.
5. Su EY, Chu YL, Chueh FS, Ma YS, Peng SF, Huang WW, *et al*. Bufalin induces apoptotic cell death in human nasopharyngeal carcinoma cells through mitochondrial ROS and TRAIL pathways. Am J Chin Med 2019;47:237–257. doi: 10.1142/S0192415X19500125.
6. Qi HY, Qu XJ, Liu J, Hou KZ, Fan YB, Che XF, *et al*. Bufalin induces protective autophagy by Cbl-b regulating mTOR and ERK signaling pathways in gastric cancer cells. Cell Biol Int 2019;43:33–43. doi: 10.1002/cbin.11076.
7. Su S, Dou H, Wang Z, Zhang Q. Bufalin inhibits ovarian carcinoma via targeting mTOR/HIF-(pathway. Basic Clin Pharmacol Toxicol 2021;128:224–233. doi: 10.1111/bcpt.13487.
8. Li M, Yu X, Guo H, Sun L, Wang A, Liu Q, *et al*. Bufalin exerts antitumor effects by inducing cell cycle arrest and triggering apoptosis in pancreatic cancer cells. Tumor Biol 2014;35:2461–2471. doi: 10.1007/s13277-013-1326-6.
9. Zhu C, You YH, Nie KK, Ji YX. Icotinib plus osimertinib overcome epidermal growth factor receptor 19del/T790 M/C797S/V834L quadruplet resistance mutation in a patient with non-small cell lung cancer. Chin Med J 2019;132:1115–1116. doi: 10.1097/CM9.0000000000000196.
10. Zhu YC, Xu CW, Zhang QX, Wang WX, Lei L, Zhuang W. Syndecan 4-c-ros oncogene 1 fusion as a mechanism of acquired resistance in epidermal growth factor receptor mutant lung

adenocarcinoma. Chin Med J 2019;132:3015–3017. doi: 10.1097/CM9.0000000000000555.
11. Maennling AE, Tur MK, Niebert M, Klockenbring T, Zeppernick F, Gattenlöhner S, *et al*. Molecular targeting therapy against EGFR family in breast cancer: progress and future potentials. Cancers 2019;11:1826–11826. doi: 10.3390/cancers11121826.
12. Bonello M, Sims AH, Landon SP. Human epidermal growth factor receptor targeted inhibitors for the treatment of ovarian cancer. Cancer Biol Med 2018;15:375–1375. doi: 10.20892/j.issn.2095-3941.2018.0062.
13. Zeng XY, Xie H, Yuan J, Jiang XY, Yong JH, Zeng D, *et al*. M2-like tumor-associated macrophages-secreted EGF promotes epithelial ovarian cancer metastasis via activating EGFR-ERK signaling and suppressing lncRNA LIMT expression. Cancer Biol Ther 2019;20:956–966. doi: 10.1080/15384047.2018.1564567.
14. Liu X, Chen L, Ge J, Yan C, Huang Z, Hu J, *et al*. The ubiquitin-like protein FAT10 stabilizes eEF1A1 expression to promote tumor proliferation in a complex manner. Cancer Res 2016;76:4897–4907. doi: 10.1158/0008-5472.CAN-15-3118.
15. Wang B, Lan T, Xiao H, Chen ZH, Wei C, Chen LF, *et al*. The expression profiles and prognostic values of HSP70s in hepatocellular carcinoma. Cancer Cell Int 2021;21:286. doi: 10.1186/s12935-021-01987-9.
16. Lheureux S, Gourley C, Vergote I, Oza AM. Epithelial ovarian cancer. Lancet 2019;393:1240–1253. doi: 10.1016/S0140-6736(18)32552-2.
17. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359–E386. doi: 10.1002/ijc.29210.
18. Takai N, Ueda T, Nishida M, Nasu K, Narahara H. Bufalin induces growth inhibition, cell cycle arrest and apoptosis in human endometrial and ovarian cancer cells. Int J Mol Med 2008;21:637–643. doi: 10.3892/ijmm.21.5.637.
19. Li H, Hu S, Pang Y, Li M, Chen L, Liu F, *et al*. Bufalin inhibits glycolysis-induced cell growth and proliferation through the suppression of Integrin β2/FAK signaling pathway in ovarian cancer. Am J Cancer Res 2018;8:1288–1296.
20. Coussens L, Yang-Feng T, Liao Y, Chen E, Gray A, McGrath J, *et al*. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 1985;230:1132–1139. doi: 10.1126/science.2999974.
21. Wang Z. ErbB receptors and cancer. Methods Mol Biol (Clifton, NJ) 2017;1652:3–35. doi: 10.1007/978-1-4939-7219-7_1.
22. Shen LS, Jin XY, Wang XM, Tou LZ, Huang J. Advances in endocrine and targeted therapy for hormone-receptor-positive, human epidermal growth factor receptor 2-negative advanced breast cancer. Chin Med J 2020;133:1099–1108. doi: 10.1097/CM9.0000000000000745.
23. Kang XH, Xu ZY, Gong YB, Wang LF, Wang ZQ, Xu L, *et al*. Bufalin reverses HGF-induced resistance to EGFR-TKIs in EGFR mutant lung cancer cells via blockage of Met/PI3k/Akt pathway and induction of apoptosis. Evid Based Complement Altern Med 2013;2013:1–9. doi: 10.1155/2013/243859.
24. Jiang Y, Zhang Y, Luan J, Duan H, Zhang F, Yagasaki K, *et al*. Effects of bufalin on the proliferation of human lung cancer cells and its molecular mechanisms of action. Cytotechnology 2010;62:573–583. doi: 10.1007/s10616-010-9310-0.
25. Cao F, Gong YB, Kang XH, Lu ZH, Wang Y, Zhao KL, *et al*. Degradation of MCL-1 by bufalin reverses acquired resistance to osimertinib in EGFR-mutant lung cancer. Toxicol Appl Pharmacol 2019;379:114662–1114662. doi: 10.1016/j.taap.2019.114662.
26. Liu T, Wu C, Weng G, Zhao Z, He X, Fu C, *et al*. Bufalin inhibits cellular proliferation and cancer stem cell-like phenotypes via upregulation of MiR-203 in glioma. Cell Physiol Biochem 2017;44:671–681. doi: 10.1159/000485279.

How to cite this article: Dou L, Zou D, Song F, Jin Y, Li Y, Zhang Y. Bufalin suppresses ovarian cancer cell proliferation via EGFR pathway. Chin Med J 2022;135:456–461. doi: 10.1097/CM9.0000000000001879