A research update on the anticancer effects of bufalin and its derivatives (Review)

YU-LONG LAN[1-3*], JIA-CHENG LOU[1,2*], XUE-WEN JIANG[1,2*], XUN WANG[1,2], JIN-SHAN XING[1,2], SHAO LI[3] and BO ZHANG[1,2]

[1]Department of Neurosurgery, The Second Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116023; [2]Department of Neurosurgery, Shenzhen People's Hospital, Shenzhen, Guangdong 518020; [3]Liaoning Provincial Key Laboratory of Cerebral Diseases, Department of Physiology, Dalian Medical University, Dalian, Liaoning 116044, P.R. China

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Abstract. Bufalin (BF) is a cardiotonic steroid that has recently been found to have substantial anticancer activity; however, more efforts should be directed toward clarifying the detailed molecular mechanisms underlying this activity. BF could exert its anticancer effect by inducing apoptosis in various human cancer cells and thus triggering autophagic cancer cell death. The anti-inflammatory activities of BF are potentially important for its anticancer functions. Notably, some promising synthetic BF derivatives, including poly (ethylene glycol)-based polymeric prodrug of BF and BF211, have shown potent anticancer activity. Additionally, clinical trials regarding the use of BF-related agents in patients have supported the positive effect of BF as an anticancer treatment. Currently, large-scale randomized, double-blind, placebo or positive drug parallel controlled studies are required to confirm the anticancer potential of BF in various cancer types in the clinical setting. The present review will evaluate the potential mechanisms mediated by BF in intracellular signaling events in cancer cells and various promising BF derivatives that may have greater anticancer activity, thereby clarifying BF-mediated anticancer effects. The experimental and clinical results reviewed strongly emphasize the importance of this topic in future investigations.

Correspondence to: Dr Bo Zhang, Department of Neurosurgery, The Second Affiliated Hospital of Dalian Medical University, 467 Zhong Shan Road, Dalian, Liaoning 116023, P.R. China
E-mail: zhangbodl@126.com

Dr Shao Li, Liaoning Provincial Key Laboratory of Cerebral Diseases, Department of Physiology, Dalian Medical University, 9 Western Section, Lvshun South Street, Dalian, Liaoning 116044, P.R. China
E-mail: lishao89@dmu.edu.cn

*Contributed equally

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Contents

1. Introduction

Bufalin (BF) is a cardiotonic steroid isolated from the Chinese toad venom, Chansu, a galenical preparation of the dried white venom of Chinese *Bufo gargarizans* (Asiatic toad) (1,2), with a molecular formula of $C_{24}H_{34}0_4$ and a relative molecular weight of 386.5 g/mol. As an active compound extracted from a Chinese traditional medicine, BF exerts various biological effects, including pain relief, myocardial contraction stimulation, blood pressure stimulation, anti-inflammatory and antineoplastic activities (3-5). Since 2010, BF has received increased attention due to its anticancer effects on a wide range of cancer types (e.g., lung, liver, prostate, gastric, colon and gastric cancer) (6-12). This compound could mediate cell cycle arrest, cell growth inhibition, apoptosis and the expression of genes associated with the malignant phenotype in human cancer cells (13). In addition, BF can be used safely for an extended period without marked side effects (12,13). Furthermore, transformed cells could be more susceptible to the effects of BF than normal cells (13). All of these factors have prompted the present review to analyze the potential of BF in anticancer treatments. However, the precise molecular mechanisms by which BF induces tumor suppression remain unclear. Microarray analysis revealed possible target-related proteins and genes of BF in cancer cells (11). A proteomic-based study was performed by Xie *et al* (11), and following BF treatment,

24 differentially expressed proteins were identified using a comparative proteomics approach. The study found that the downregulation of heat shock protein 27 (Hsp27) could serve a critical role in BF-induced apoptosis in osteosarcoma cells. Subsequently, Zhang *et al* (14) explored target-related proteins using two quantitative proteomic methods (isobaric tags for relative and absolute quantitation-based and label-free proteomic analysis) in lung cancer. These two proteomic methods were complementary, and suggested that oxidative stress and regulation of relevant gene expression were significantly involved in the effects of BF, while the fibronectin-associated pathway was found to be important. Bioinformatics analysis revealed that the fibronectin-associated pathway is the most distinct pathway in the signal network of BF, and BF-induced protein expression changes, including decreased expression of fibronectin, increased expression of paxillin, calpain 2 and cell division control protein 42 homolog, , have been further confirmed in the fibronectin-associated pathway using immunoblotting (14). In addition, the genetic mechanisms underlying BF-induced DNA damage and apoptosis in lung cancer cells have been further elucidated (15). Wu *et al* (15) demonstrated that numerous genes associated with cell cycle regulation, apoptosis and DNA repair are significantly altered following BF treatment. Analysis of these gene alterations by Wu *et al* (15) and Zhang *et al* (14) could aid the elucidation of the mechanism underlying the cytotoxicity of BF at the genetic level and potentially offer various biomarkers for the treatment and diagnosis of lung cancer. Certainly, further studies are required to improve the understanding of how BF suppresses cancerous cells and does not affect normal cells. Furthermore, combinations of BF with cytotoxic agents, differentiation-inducing agents and even gene therapy may represent potential novel therapeutic strategies for cancer treatment. The present review aims to evaluate the anticancer properties of BF from the perspective of emerging treatment options for cancer patients.

2. BF could exert antitumor activity by inducing the apoptosis of various human cancer cells

Inducing apoptosis in target cells could be a key mechanism for the majority of anticancer therapies. BF is a cardiotonic steroid that has the potential to induce cancer cell apoptosis (12). Cell apoptosis was induced in human non-small cell lung cancer A549 cells following treatment with BF, while suppression of cell proliferation occurred in a time- and dose-dependent manner, and induced cell cycle arrest at the G_1 phase was found (6). Li *et al* (7) identified that BF exerts antitumor effects by triggering apoptosis and inducing cell cycle arrest in pancreatic cancer cells. Notably, in pancreatic cancer cells, BF could also promote the growth inhibition effect of gemcitabine (7). For the first time, Jiang *et al* (16) indicated that BF could be a potential therapy for treating gallbladder cancer. The study demonstrated that BF induces cell cycle arrest and apoptosis in gallbladder carcinoma cells. Treatment of human bladder carcinoma T24 cells with BF had a significant growth inhibition effect ($P<0.05$), compared with vehicle treatment. This effect is likely attributable to the prominent arrest of cancer cells in the G_2/M phase of the cell cycle and the apoptosis stimulated by BF (17), as evidenced by formation of apoptotic bodies, chromatin condensation and cell accumulation in the sub-G_1 phase. In breast cancer, BF greatly sensitized estrogen receptor (ER) α-positive MCF-7 and ERα-negative MDA-MB-231 human breast cancer cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (18), compared with vehicle treatment. Notably, BF increases TRAIL-induced apoptosis from 2.0±0.5 to 30.1±1.2% in MCF-7 cells, and from 6.9±1.8 to 41.5±1.4% in MDA-MB-231 cells, which indicated that MCF-7 cells are more sensitive to BF than MDA-MB-231 cells. The enhanced apoptotic effects of the TRAIL/BF combination were also associated with the augmentation of caspase activation (18). In leukemia, BF could exert strong differentiation-inducing activity in three human leukemia-derived cell lines (myeloblastic ML1, human promyelocytic HL60 and monoblastic U937) at a concentration of 10 nM. However, treatment of human leukemia K562 cells with other cardiotonic steroids, including digitoxigenin, cinobufagin and ouabain, at the same concentration only had a weak or no effect on these cells (19,20). These findings indicate that BF may have potential in human myelogenous leukemia differentiation therapy (19-21). Notably, Jing *et al* (22) demonstrated that in normal polymorphonuclear and mononuclear cells, apoptotic cell death was not induced by BF, indicating that the anticancer effects of BF may be cell-type specific. In gastric cancer, BF could inhibit the proliferation of gastric cancer MGC803 cells by inducing apoptosis, and the phosphatidylinositol 3-kinase/protein kinase B (Akt) pathway may serve a key role in this process (23). The antiproliferative and apoptosis-inducing mechanisms of BF in prostate cancer cells have also been investigated (24,25). In hepatocellular carcinoma, BF exhibited significant anticancer effects on the orthotopic transplantation tumor model of human hepatocellular carcinoma in nude mice and could promote the apoptosis of transplanted tumor cells with no marked toxicity (26). In our previous study investigating glioma (27), it was demonstrated that BF inhibits glioma growth by promoting proteasomal degradation of the sodium/potassium-adenosine 5'-triphosphatase α-1 subunit (ATP1A1), and ATP1A1 deficiency could inhibit the proliferation of glioma via promotion of apoptosis. Notably, BF was revealed to penetrate the blood-brain barrier (BBB) (27). Rats were intraperitoneally injected with BF and cerebrospinal fluid (CSF) was collected from the cerebello-medullary cistern by penetrating the foramen magnum. The content of BF in the CSF was detected using liquid chromatography/mass spectrometry assays to prove that BF could traverse the BBB (27). Therefore, we hypothesize that BF can enter the brain via the BBB and thus exert a more powerful effect on tumors in the central nervous system.

3. BF could exert anticancer effects by triggering autophagic cancer cell death

Apoptosis, necrosis and autophagic cell death are the three major morphological processes responsible for cell death (28,29). Autophagy is an important cellular catabolic process that maintains homeostasis by degrading dysfunctional cellular organelles and excessive proteins in living cells (30). The cytoplasms and double smooth membranes (phagophores) of numerous types of organelles, including the endoplasmic reticulum, peroxisomes and mitochondria, can form autophagosomes. The autophagosome then fuses with the lysosome to form autophagolysosomes, finally leading to

degradation of the captured proteins or organelles by lysosomal enzymes (31-33). Promoting autophagic cell death is an important strategy for the chemotherapeutic treatment of cancer (34-36). Xie *et al* (10) demonstrated that BF could induce autophagic cell death via c-Jun N-terminal kinase activation and the generation of reactive oxygen species in human colon cancer HT29 cells. Tsai *et al* (37) also found that in SK-HEP-1 cells, BF induced autophagic cell death and cell cycle arrest via the Akt/mechanistic target of rapamycin signaling pathway. These findings indicate that BF may be a possible treatment of human hepatocellular carcinoma. Notably, Shen *et al* (38) explored the potential of BF in glioma for the first time. The study found that preventing autophagy promoted apoptosis and increased the induction of ER stress-associated proteins, indicating that autophagy could exert a cytoprotective effect on cell death and ER stress induced by BF. This result indicates that BF could inhibit glioma cell growth and induce interplay between autophagy and apoptosis via ER stress, and more importantly, provides a molecular basis for developing BF into a drug candidate for glioma treatment. The amount of research on the association between autophagy and cancer has increased rapidly. Cancer cells may utilize autophagy to enhance their survival in the hostile tumor microenvironment with an altered metabolism, indicating that suppression of autophagy is required from therapeutic cancer treatment strategies (39). Indeed, the interaction between autophagy and apoptosis, which depends on the use of chemotherapeutic drugs, as well as the type of cancer cell, could significantly impact the fate of human cancer cells.

4. BF could exert anticancer effects via its anti-inflammatory activity

Chansu has been used in the treatment of inflammatory diseases in China for thousands of years (40); however, little is known about the anti-inflammatory mechanisms of BF. Nuclear factor-κB (NF-κB) is a central regulator of the inflammatory process and could regulate a group of proinflammatory mediators, including inducible nitric oxide synthase, cyclooxygenase-2, interleukin (IL)-1β, TNF and IL-6 (41). In 2011, Ye *et al* (42) suggested that BF suppresses the nuclear translocation of NF-κB in response to TNF in human kidney tissue cells. BF was also shown to modulate NF-κB activity in human osteosarcomas in a previous study (11). NF-κB signaling could be a potential therapeutic target of BF for the pathogenesis of inflammation and cancer. Wen *et al* (43) demonstrated that compared with vehicle treatment, BF exerts a strong anti-inflammatory effect on carrageenan-induced paw edema in rats, which is a commonly used model for the investigation of inflammation. The study demonstrated that BF significantly ($P<0.05$) suppressed the activation of NF-κB *in vivo* by maintaining IκBα levels and inhibiting the nuclear translocation of NF-κB p65. Furthermore, the downstream NF-κB proinflammatory mediators, cyclooxygenase-2, inducible nitric oxide synthase, TNF-α, IL-6 and IL-1β were also suppressed. Therefore, BF could possess strong *in vivo* anti-inflammatory activity, thus serving a role in reducing NF-κB activation and the inhibition of downstream proinflammatory mediators. Inflammatory pathways have been targeted in attempts to treat cancer (44-46), and the association between cancer and inflammation has returned to the forefront of clinical oncology. These findings support BF as a potential novel therapeutic agent for alleviating inflammation, and more importantly, for treating various cancer types.

5. Promising BF derivatives may have even greater anticancer activity

While research into BF has revealed its potential in the treatment of different human cancer types, further investigation is required prior to its use as a cancer treatment. BF has a narrow therapeutic window, extremely low water solubility, unsatisfactory bioavailability and severe adverse effects, including high cardiac toxicity, which limits its clinical applications (47,48). Since the purification and total synthesis of BF, various methods have been used to improve its activity and expand its therapeutic potential in different biological systems by modifying its structure (49,50). More importantly, efforts should be directed towards identifying additional BF derivatives that exert stronger anticancer effects and have a lower toxicity level compared with the natural compound to promote the development of novel anticancer agents from cardiac steroids, including BF.

Notably, Liu *et al* (51) synthesized a novel polymeric prodrug of BF, poly (ethylene glycol)-based polymeric prodrug of BF (PEGS-BF). The water solubility and stability of PEGS-BF were improved without loss of its anticancer activity compared with BF. In addition, *in vitro* and *in vivo* experiments showed that PEGS-BF exerted anticancer effects comparable to those of unbound BF. The reported improved stability, water solubility and controlled drug release features of this polymeric prodrug provide strong arguments for its use in potential clinical applications.

BF211 is a BF derivative with a stronger cytotoxic activity than BF in cancer cells (52,53). In nude immunodeficient Balb/c-nu-nu mice inoculated with A549 cells, BF211 also exhibited significantly ($P<0.05$) stronger suppressive effects on tumor growth compared with BF (53). Notably, as the acute toxicity of BF211 was lower compared with that of BF [lethal dose 50 (LD_{50}) value of BF211 was 14.75 mg/kg for male mice and 18.21 mg/kg for female mice, whereas LD_{50} value of BF in mice was ~2.2 mg/kg], BF211 could be used at higher concentrations for cancer treatment, indicating that BF211 could have a wider therapeutic window. In summary, studies by Liu *et al* (53) demonstrated that BF211 is a potential novel anticancer compound with a relatively lower toxicity compared with BF as aforementioned (BF211 LD_{50} value could be >6 times higher than BF LD_{50} value), and this may be due to its specific binding characteristics for sodium-potassium adenosine triphosphatase. Furthermore, Sun *et al* (54) attempted to identify target-related proteins of BF211 in A549 cells to further elucidate the mechanism underlying its anticancer effects. Their findings suggested that BF211 may impact multiple cellular functions, including translation, transcription and protein synthesis. In addition, a previous study (54) also explored the effect of BF211 on regulating proteasome activities and revealed only moderate inhibitory effects; thus, there is currently no direct evidence that the anticancer effects of BF211 are mediated by proteasome inhibition and it is possible that the contribution of proteasome inhibition to the cytotoxicity of BF211 is minimal.

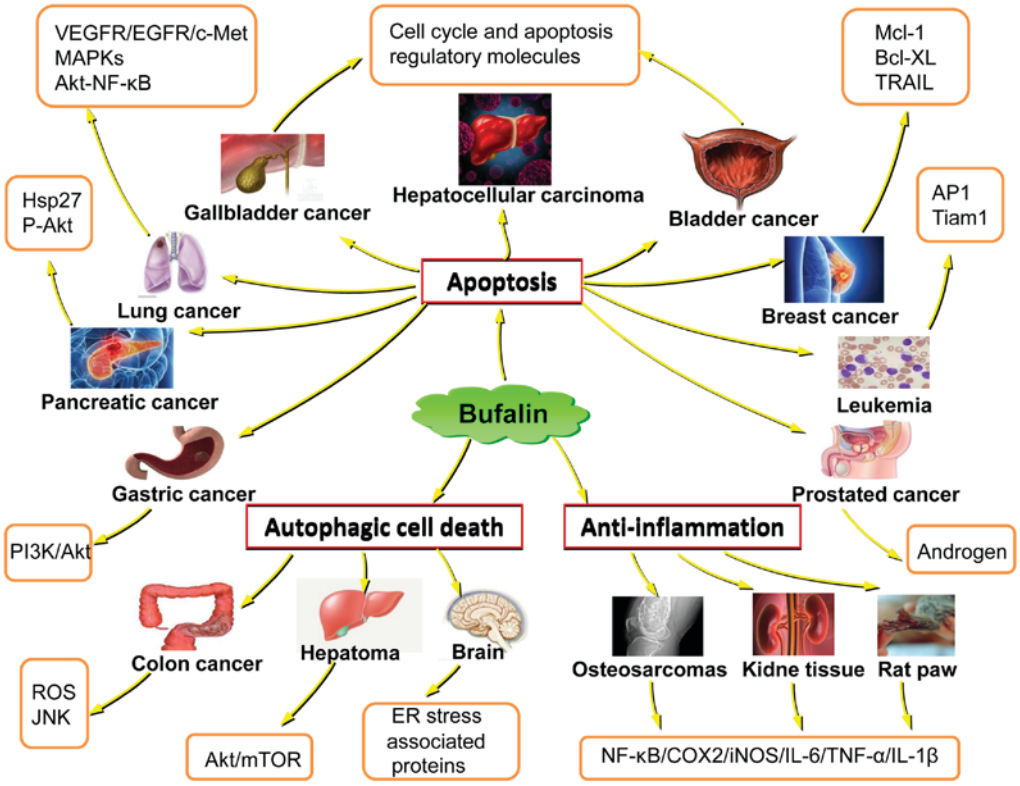

Figure 1. Schematic diagram displaying the anticancer properties of bufalin, from the perspective of the emerging treatment targets for various cancer types. Hsp27, heat shock protein 27; P-Akt, phosphorylated protein kinase B; VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor, cMet, mesenchymal-epithelial transition factor; MAPKs, mitogen-activated protein kinases; NF-κB, necrosis factor-κ B; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; JNK, c-Jun N-terminal kinases; mTOR, mechanistic target or rapamycin; ER, endoplasmic reticulum; COX2, cyclo-oxygenase 2; iNOS, inducible nitric oxide synthase; IL, interleukin; TNF-α, tumor necrosis factor-α; AP1, activating protein 1; Tiam1, T-lymphoma invasion and metastasis-inducing protein; Mcl-1, induced myeloid leukemia cell differentiation protein; Bcl-XL, B-cell lymphoma-extra large; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

6. Clinical trials regarding the use of BF in patients

Chansu, the galenical preparation of the dried white venom of Chinese *Bufo gargarizans*, has been widely used for the treatment of cancer at oncology clinics in China (55). Huachansu, one of the main biologically active components of BF, is a Chinese medicine derived from dried toad venom from the skin glands of *Bufo bufo gargarizans* Cantor or *Bufo melanotictus* Schneider (55). Huachansu is manufactured by Anhui Jinchan Biochemistry Company Ltd., in Huaibei, China (Chinese FDA no. ISO9002) and includes 2 primary biologically active chemical components: Indole alkaloids (bufotenidine, bufotenine, serotonin and cinobufotenine) and steroidal cardiac glycosides (>28 have been identified, including BF, cinobufagin, resibufogenin, marinobufagin, cinobufotalin and bufotalin) (55). A previous study confirmed that BF, cinobufagin and resibufogenin are the 3 major cardiac glycosides, which the anticancer activity of Huachansu can be attributed to (56). A pilot study investigating the use of Huachansu in patients with advanced cancer was performed by Meng *et al* (57), using a phase I trial design. Huachansu was intravenously administered for 2 weeks followed by 1 week off, for each cycle. Without significant adverse events or progressive disease, treatment continued beyond two cycles. A total of 15 patients (11 with hepatocellular cancer, 2 with pancreatic cancer and 2 with non-small cell lung cancer) were included in the trial. Overall 6 patients (40%) had stable disease (median duration, 6.0 months; range, 3.5-11.1 months). In addition, 1 patient with hepatocellular cancer had a 20% reduction in tumor mass (duration of disease stability in response to Huachansu alone, 11 months). The plasma BF concentration reached maximal levels at the end of the 2-h infusion and was proportional to the amount of drug administered. Notably, a dose-dependent increase in BF levels was observed in all the patients, with no evidence of drug accumulation in the plasma, which may have been due to the short half-life of the drug. Although there was no correlation between the plasma BF level and the anticancer effect of Huachansu, the small number of patients studied precluded drawing an association between the drug dose and response. One limitation of the study by Meng *et al* (57) was the absence of a control group. Significantly, Huachansu has been found to be well tolerated, even at doses eight times those normally administered (normally administered doses: Level l, 10 ml/m^2; level 2, 20 ml/m^2; level 3, 40 ml/m^2; level 4, 60 ml/m^2; and level 5, 90 ml/m^2) in China, and can lead to disease stabilization in certain patients according to the results of a phase I clinical trial (57).

7. Conclusion

In summary, the potential roles of BF in various cancer types have been increasingly recognized, but the specific mechanisms have not been fully clarified (Fig. 1). The present review demonstrates that BF has marked antitumor activity. BF could exert an antitumor effect by inducing apoptosis and triggering autophagic cell death in various human cancer cells.

The anti-inflammatory activities of BF are also important for its antitumor function. Notably, an increasing number of BF derivatives have shown potent anticancer activity, including PEGS-BF and BF211. Clinical trials regarding the use of BF in cancer patients have supported the positive effect of BF on cancer treatment.

An increasing number of clinical studies will focus on investigating the use of BF in cancer treatment in the near future. Research has revealed that BF has potential as a potent, novel and effective anticancer therapy for cancer patients. However, more BF derivatives with stronger anticancer effects and lower toxicity levels should be identified to promote the development of additional novel anticancer agents. Furthermore, large-scale randomized studies are required to investigate the efficacy of BF in various cancer types. This review provides critical information for the design of larger and more focused clinical studies that are necessary to systematically and definitively evaluate the role of BF in cancer treatments.

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Availability data and materials

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Author contributions

YL, JL, XJ, XW, JX, SL and BZ contributed in the design and writing of the review. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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