REVIEW

The Research Progress of Bufalin in the Treatment of Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is one of the deadliest cancers in the world with a five-year survival rate of less than 20%. Nonetheless, selecting an appropriate therapeutic agent to inhibit the development of hepatoma cells is still a challenge. Bufalin, a component of the traditional Chinese medicine Chansu, has been shown to inhibit the proliferation, invasion and metastasis of HCC through various signaling pathways. In addition, bufalin and sorafenib demonstrate a synergistic effect in cancer therapeutics. This review highlighted on several focal signaling pathways involved in the inhibitory effects of bufalin on HCC and its synergistic mechanisms with sorafenib. The immunotherapy effect of bufalin has also been discussed as a novel property.

Keywords: bufalin, cancer, therapy, signaling pathways, immunotherapy effect

Introduction

Liver cancer is the sixth most commonly diagnosed cancer, and its mortality rate is the third most common among all cancers.[1] Most liver diseases occur in developing countries and most patients with liver cancer are aged between 35 and 65 years, with the number of male patients being higher than that of female patients. These statistics show that hepatocellular carcinoma (HCC) is a life-threatening health problem. The prognosis of liver cancer is very poor such that approximately 5% to 15% of patients are eligible for surgical removal, a procedure suitable only for patients in the early stage of the disease because of diminished hepatic regenerative capacity, typically without cirrhosis. In terms of surgical complexity, right hepatectomy carries a higher risk for postoperative complications than left hepatectomy.[2,3] Consequently, novel therapeutic strategies are essential for improving the clinical management of patients with HCC.

Bufalin, a component of the traditional Chinese medicine Chansu, is extracted from the skin and parotid venom glands of Bufo Bufo gargarizans cantor and has been shown to have antitumor activity.[4] Many studies have reported that bufalin can inhibit proliferation, invasion and metastasis through a variety of mechanisms.

Effect and Mechanism of Action of Bufalin in Inhibiting Hepatocellular Carcinomas

AKT Signaling Pathway

AKT is the key protein of the AKT/GSK3β/β-catenin/E-cadherin signaling pathway in HCC, and its phosphorylation regulates 10 other main regulatory proteins of various signaling pathways. AKT belongs to the serine/threonine protein kinase family,[5] and exists in three isoforms translated from three distinct genes: *AKT1 (PKB alpha), AKT2 (PKB beta),*

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and *AKT 3 (PKB gamma)*.[6] AKT is a crucial regulator that plays a key role in the PI3K/AKT pathway, which regulates several cellular processes including cell survival, cell growth/size, proliferation, transcription, glucose metabolism, synthesis, genome stability, and neovascularization; thus, any disturbance in this pathway has a notable impact on cellular homeostasis.[7] With regard to AKT1, Xu et al[8] showed that AKT1-mediated phosphorylation of mTORC2 is vital for triggering hepatocarcinogenesis in humans and mice, resulting from cellular growth through c-Myc activation. In addition, the increase in the expression of AKT1 was shown to result in an increase in the proliferation and migration of HepG2 cells (HCC cell line).[9] Furthermore, the high expression of AKT2 was related to portal invasion, histopathological differentiation, and the number of tumor nodules.[10] Recent studies on AKT3 have mainly focused on its expression in the testes and brain; however, a few reports have also presented its therapeutic implication in managing HCC.[10]

In the AKT/GSK3β/β-catenin/E-cadherin signaling pathway, AKT promotes the phosphorylation and expression of GSK3β and the nuclear translocation of β-catenin. GSK3β, a multifunctional serine/threonine kinase, regulates various cellular functions including cell survival and cell-fate specification, and participates in numerous signaling pathways, while β-catenin is an essential regulator of cell motility, invasion, and adhesion. The effect on β-catenin inhibits its downstream molecule, E-cadherin, which negatively regulates the expression of MMP-2 and MMP-9. Bufalin was shown to suppress the protein levels of FAK and Rho A, VEGF, MEKK3, MKK7, and uPA, as well as preventing the translocation of nuclear factor kappa B (NF-κB). These processes (signaling pathway and regulation of proteins) are closely related to the proliferation, migration, invasion, and adhesion of hepatoma cells.[11] Qiu et al[4] showed that bufalin could inhibit the AKT signaling pathway by negatively regulating the expression of AKT which is related to reduced levels of NF-κB,[12] to prevent the activities of hepatoma cells.

In addition, Wang et al[13] reported that bufalin inhibited HCC metastasis by negatively regulating the PI3/AKT/mTOR pathway. Through this pathway, HIF-1α expression which can mediate EMT and VEGF involved in the antimetastatic process, is downregulated (Figure 1). Nonetheless, there are many other upstream pathways of HIF-1α besides PI3K/AKT/mTOR; therefore, the suppression of one mere pathway would not be enough to reduce the expression of HIF-1α.

Wnt/β-Catenin Signaling Pathway

The Wnt/β-catenin signaling pathway plays a major role in the occurrence and development of various cancers. It consists of Wnt signaling ligands, viz. Fz receptor proteins and co-receptor LRP5/6, as well as disheveled, axin, β-catenin, APC, casein kinase 1a, and GSK-3β. Among these molecules, β-catenin is an important protein whose accumulation is a sign of the activated state of the Wnt/β-catenin signaling pathway.[14]

In normal mature cells, β-catenin is maintained at a low level because of the lack of Wnt. During embryonic development and tissue regeneration, β-catenin accumulates and passes through the nuclear membrane to conjugate with factors in the nucleus, thereby increasing the downstream target proteins that are related to cell proliferation, apoptosis, matrix dissolution, and angiogenesis, such as cyclin D1, MMP-7, COX-2, c-myc, surviving, and VEGF.[15] Among them, cyclin D1 promotes cell transformation from the G1 phase to the S phase resulting in cell proliferation.[16] MMP-7 not only participates in various extracellular matrix lysis, but also damages diverse tumor suppressor compositions on the cell surface.[17] Consequently, the overexpression of cyclin D1, MMP-7, and COX-2 plays an essential role in the proliferation, invasion, and metastasis of hepatoma cells.[18]

Low E-cadherin expression leads to a significant increase in free β-catenin. In addition, the increased phosphorylation at the GSK-3β ser9 site and reduced expression of E-cadherin are regarded as the main causes of activation of the Wnt/β-catenin pathway in HCC cells.[19]

In a previous study,[14] bufalin decreased the phosphorylation of the GSK-3β Ser9 site and the translocation of β-catenin in HCC cells, then regulated the extranuclear signal transduction and endonuclear target molecules, which subsequently led to lower expression levels of cyclin D1, MMP-7, and COX-2 in HCC cells. In this way, bufalin inhibited the proliferation, invasion, and metastasis of HCC cells.
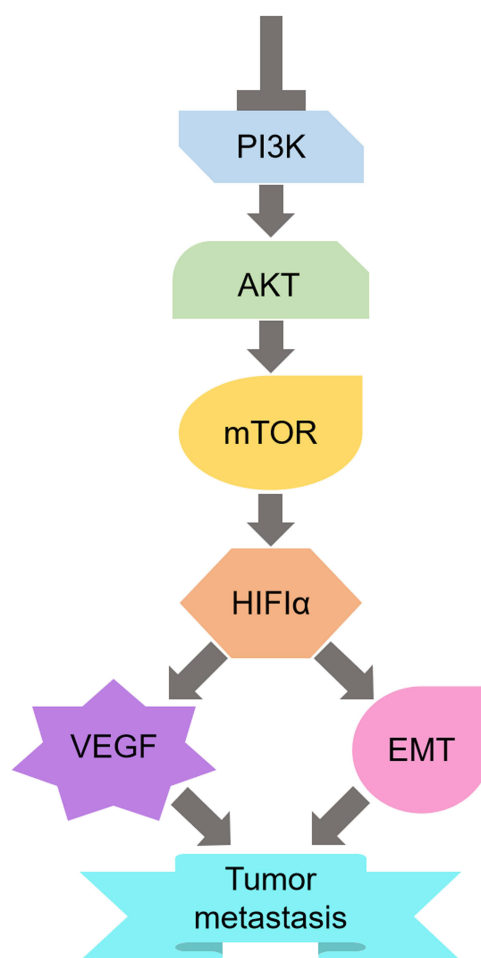
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Figure 1 Schematic of the mechanism by which bufalin inhibits HCC invasion and metastasis.

Hedgehog Signaling Pathway

Hedgehog is a highly conserved intercellular signal transduction system that contains Hedgehog (Hh), Patched-Smoothened, Glioma (Gli), Costal-2, Fuse (Fu), Suppressor of Fuse, and protein kinase A. Among them, Hh, Smo, Gli, and Fu are positive regulatory molecules in the Hh signaling pathway, while the remainder are negative regulators.[20]

Epithelial mesenchymal transition (EMT) is a biological process in which epithelial cells lose polarity through a specific program and obtain the characteristics of mesenchymal cells. Extracellular matrix (ECM) degradation is an important contributor to tumor invasion and metastasis. Bufalin inhibits EMT, ECM degradation, and angiogenesis of hepatoma cells by regulating the expressions of Ptch1, Gli1, and Gli3 proteins in the Hedgehog signaling pathway. In addition, bufalin inhibits the downstream target molecules of MMP-2 and MMP-9 in hepatoma cells, which leads to the degradation of a variety of protein components in the ECM. Similarly, bufalin treatment increases E-cadherin expression and decreases β-catenin and VEGF expression in hepatoma cells by influencing Gli3 protein expression (Figure 2).[21]

Fas- and Mitochondrial-Mediated Apoptotic Pathway

In the intrinsic pathway, Bax and Bcl-2 which belong to the Bcl-2 family of proteins, have crucial effects on initiating the mitochondrial death cascade. Bax, a pro-apoptotic protein, is upregulated by bufalin. Bufalin also inhibits the expression of the anti-apoptotic protein Bcl-2. These changes directly lead to the disruption of ΔΨm and the release of cytochrome c, which activates a caspase cascade consisting of caspase-9 and caspase-3.[22]

In the extrinsic pathway, the Fas pathway has been shown to be highly efficient in inhibiting tumor cells.[22] Bufalin also upregulates the expression of Fas and activates caspase-8 and caspase-10. The major regions of caspase-8 and
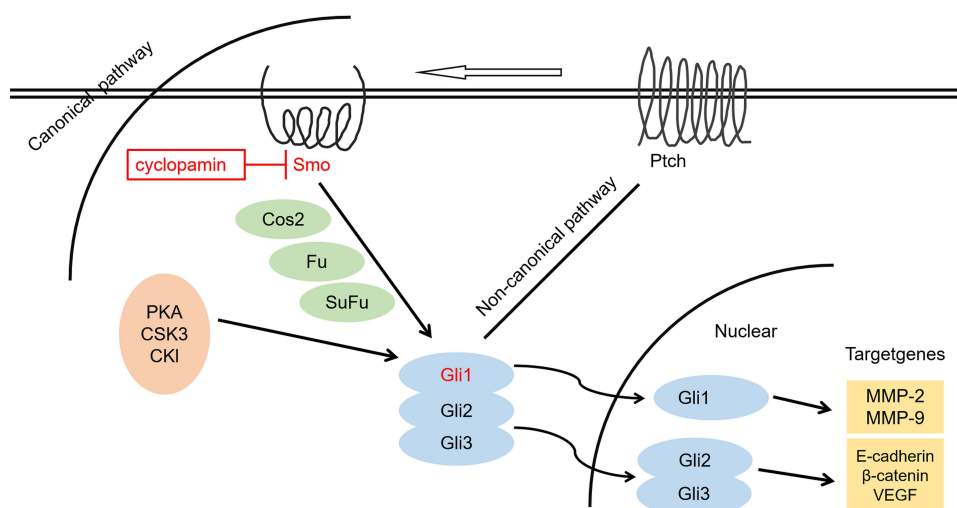
Figure 2 Regulation and control of bufalin on invasive and metastatic molecules of HCC cells via the Hedgehog signaling pathway.

caspase-10 activate caspase-3, while the minor portions cleave Bid to tBid, which also leads to the release of cytochrome c.

Caspase-3 in these caspase cascades induces the cleavage of PRAP together during apoptosis in both intrinsic and extrinsic pathways.[23]

Synergistic Effect of Bufalin and Sorafenib

Wang et al[24] found that the synergistic effect of bufalin and sorafenib is related to the regulation of cells, viz. tumor cells and endothelial cells. The synergistic treatment effects of these molecules regulated HCC cells via the mTOR/VEGF pathway, resulting in the downregulation of VEGF secretion. Consequently, the mTOR/VEGF pathway may be considered a target for strengthening the synergism (Figure 3).

Gao et al[25] demonstrated that bufalin enhanced the anti-cancer effect of sorafenib in PLC/PRF/5 and Hep G-2 cells. The mechanism was postulated to involve sorafenib-mediated inhibition of cell growth by the decreased phosphorylation of ERK, which was positively regulated by bufalin-induced increased phosphorylation of AKT. This proposed effect on AKT by bufalin is contrary to the findings of Zhai et al.[26]
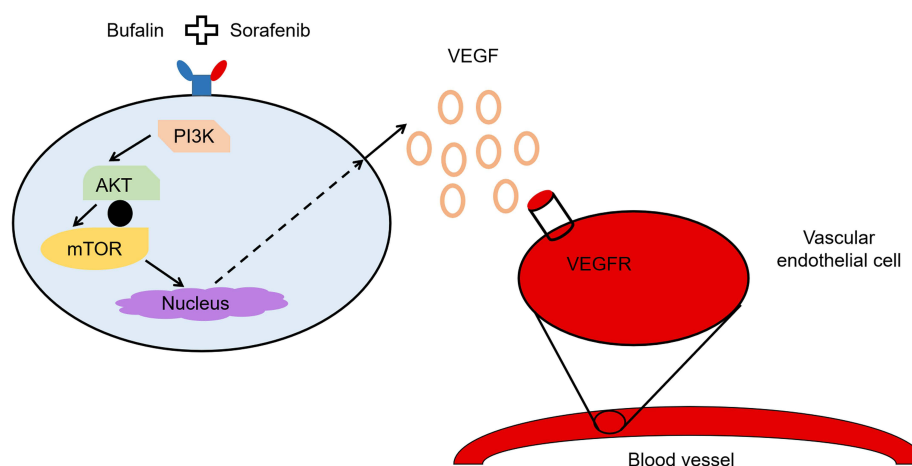

Figure 3 Schematic illustration of the mechanism by which bufalin augments sorafenib-induced inhibition of angiogenesis through the mTOR/VEGF signaling pathway in HCC.

Zhai et al[26] reported that bufalin reversed both inherent and acquired resistance to sorafenib in HCC cells by inhibiting sorafenib-mediated AKT activation. This effect was said to result from bufalin-induced ER stress, which predominantly reduced p-AKT, inhibited cell growth, and promoted apoptosis via the IRE1 pathway. Although the latent mechanism by which bufalin reverses resistance to sorafenib has not been verified, the ER stress pathway participates in crosstalk with various pathways related to cell growth and apoptosis.[26]

From the aforementioned studies, some conclusions seem to be on the contrary, which may result from different perspectives and the advancing technology over the years. However, numerous studies have confirmed that the expression of AKT can be reduced by bufalin, which was consistent with the findings of this review. As previously alluded, bufalin enhances the anti-cancer effect of sorafenib by downregulating the expression of AKT, and AKT is a key protein whose phosphorylation regulates more than 10 signaling proteins to participate in processes such as cell proliferation, apoptosis, migration, and invasion. Molecular signal transduction of cells is a complex process that has multiple paths simultaneously. Different perspectives and experimental conditions may draw different conclusions, which may be associated with simultaneous interferences from other factors. For instance, Gao et al[25] reported an increased synergistic effect of sorafenib from dual administration with bufalin; however, these findings require further study and discussion prior to potential application into clinical settings. Overall, bufalin has been shown to have an inhibitory effect on the occurrence and development of tumors.

Autophagy

Autophagy has received increasing attention in cancer research as it plays a key role in cancer cell survival and death. It is a physiological process that leads to the segregation and degradation of cytoplasmic content through a lysosomal mechanism. Furthermore, it allows cellular components to recycle and supply cellular energy under nutrient starvation, infection, and other stress conditions.[27,28] Miao et al found that bufalin can cause HepG2 autophagy.[29] Their findings concluded that autophagy plays a two-sided role in cancer, viz. in prosurvival and proapoptotic processes, with the detailed mechanisms in cancer cells being dependent on the type of cancer, stage, and microenvironment. In addition, their results showed that autophagy induced by bufalin acted as a proapoptotic mechanism, and bufalin-induced cell death in HCC cancer cells was dependent, at least in part, on the induction of autophagy.

AMP-activated protein kinase (AMPK) is a principal energy-preserving intracellular enzyme that is activated under stress conditions and can induce autophagy through inhibition of the serine/threonine kinase mammalian target of rapamycin (mTOR), a major repressor of autophagy.[30–32] Bufalin induced autophagy in HepG2 cells, which was followed by the inhibition of phosphorylation of both mTOR and its substrate p70S6K. In contrast, bufalin dose-dependently increased AMPK activation. Therefore, the induction of autophagy by bufalin was related to the activation of AMPK and inhibition of the mTOR signaling pathway. Similarly, Tsai et al[33] demonstrated that the AKT/mTOR signaling pathway promotes autophagy in bufalin-treated SK-HEP-1 cells. In addition, Hsu et al[34] have found that hepatoma cells could be effectively killed by bufalin via cell cycle arrest at the G2/M phase due to autophagy and not apoptosis. Sheng et al[35] indicated that bufalin induced autophagy in liver cancer cells by upregulating the protein expression of LC3-II and Beclin-1, and by downregulating that of P62. However, these authors also reported that the therapeutic efficacy of bufalin in HCC can be improved by targeting autophagy-related proteins.

Meanwhile, in another study,[36] the inhibition of autophagy could increase the cytotoxicity of bufalin in HCC cells, resulting from the efficient prevention of apoptosis and cell death mediated by the ER stress response, thus maintaining cell homeostasis and survival.

Immunotherapy Properties of Bufalin

To date, immunotherapy has attracted widespread concern and research. In particular, bufalin was shown to enhance the immune responses in vivo in a leukemia-induced mouse model (BALB/c mice).[37] Shih et al[37] proved that bufalin may increase immune responses not only by promoting the monocyte (CD11b) population as well as T-cell and B-cell proliferation, but also by improving macrophage phagocytosis in mice with leukemia in vivo.

However, not many studies that demonstrate the effect of bufalin in modulating immune responses in HCC in vitro have been reported in literature. A study by Yang et al[38] reported that bufalin inhibited APOBEC3F, CCR9, CCR10,

CXCR4, and pIgR proteins in the intestinal immune network for IgA production. Because the APOBEC3F-induced intestinal immune network of the IgA production signaling pathway plays a key role in tumor progression in HCC, the authors suggested that downregulation of APOBEC3F and inhibiting the activation of the intestinal immune network of the IgA production signaling pathway may be a novel mechanism of the antitumor effect of bufalin. Nevertheless, their experiments were all conducted in vitro, which led to many limitations of the study. Unfortunately, reports on the targeting of other myeloid and lymphoid immune cells for immunotherapy, which proved to be a promising therapeutic strategy for liver cancer influenced by bufalin, were not available in literature.

Toll-like receptor 3 (TLR3) agonists which are polyriboinosinic–polyribocytidylic acid [poly (I:C)] have been reported as potential immunotherapy adjuvants for cancer, with low concentrations having being said to improve the metastatic capacity of TLR3C HCC.[39] In line with this, bufalin has been shown to inhibit the risk of metastasis of poly (I: C).[39] Feng et al[39] showed the potential immunotherapy effect of bufalin. However, further research is needed to explore the mechanism of action of the molecule and the detailed immunotherapy effect in HCC.

Perspective

Traditional Chinese medicines, which have been researched for potential anti-cancer properties for many years have demonstrated low toxicity in normal cancer cells in numerous cases. In particular, bufalin has been recorded as one of the most efficient anti-cancer medicines that has been studied in clinical trials for treatment of various cancers, such as HCC, non-small cell lung cancer, and pancreatic cancer.[40] Fortunately, its effects on proliferation, invasion, and metastasis of HCC cells have been confirmed, and have been summarized in this review. Thus, bufalin has the potential to become a treatment for HCC in clinical settings.

However, the mechanism of its effect on autophagy has not yet attained consensus in the Oncology community. Therefore, autophagy of HCC cells requires further research, and subsequent molecular targeted drug therapy discoveries for autophagy-related proteins may promote the development of bufalin. Importantly, the immunotherapy effect of bufalin has been discovered; however, other myeloid and lymphoid immune cell applications as immunotherapy agents, which are promising therapeutic strategies for liver cancer, were not available in reviewed literature. Therefore, we expect further detailed research on this effect in the coming years.

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Disclosure

The authors report no conflicts of interest in this work.

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