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Review

Bufalin for an innovative therapeutic approach against cancer

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ABSTRACT

Bufalin is an endogenous cardiotonic steroid, first discovered in toad venom but also found in the plasma of healthy humans, with anti-tumour activities in different cancer types. The current review is focused on its mechanisms of action and highlights its very large spectrum of effects both in vitro and in vivo. All leads to the conclusion that bufalin mediates its effects by affecting all the hallmarks of cancer and seems restricted to cancer cells avoiding side effects. Bufalin decreases cancer cell proliferation by acting on the cell cycle and inducing different mechanisms of cell death including apoptosis, necroptosis, autophagy and senescence. Bufalin also moderates metastasis formation by blocking migration and invasion as well as angiogenesis and by inducing a phenotype switch towards differentiation and decreasing cancer cell stemness. Regarding its various mechanisms of action in cancer cells, bufalin blocks overactivated signalling pathways and modifies cell metabolism. Moreover, bufalin gained lately a huge interest in the field of drug resistance by both reversing various drug resistance mechanisms and affecting the immune microenvironment. Together, these data support bufalin as a quite promising new anti-cancer drug candidate.

1. Introduction

Bufalin is a cardiotonic steroid composed of a steroid backbone structure bound to a lactone cycle (Fig. 1) [1]. As other cardiotonic steroids such as digoxin and ouabain, its main target is the Na^+/K^+-ATPase pump. Even if it exerts its major and most prominent effects by perturbating the osmotic balance in cells, more recent studies have indicated that non-toxic doses of these molecules affected signal transduction [2]. First focused in the cardiovascular context, the study of cardiotonic steroids has gained interest in other research areas and their inhibitory activity on cancer development has been reported in the literature [3,4]. More specifically, bufalin is a bufadienolide which can be found in many plant or animal species, but their main sources are skin and parotid gland secretions of venomous toads. In plants and animals, bufadienolides are present mainly as a defence mechanism against predators [5]. Bufalin is also a key active ingredient of the traditional Chinese medicine *HuaChansu* [6]. It possesses potential therapeutic effects in various medical conditions such as heart failure, infection or inflammatory diseases [7–9] by harbouring cardiotonic, anti-inflammatory, and diuretic properties, but it also has anti-cancer activities [6,9]. Even if bufadienolides were first and mainly identified in toads, some research highlighted their presence in humans. The presence of endogenous bufadienolides in humans has been known for many years mainly with marinobufagenin (MBG) which is produced in humans mainly during preeclampsia [10]. Their biosynthesis takes place in the adrenal gland and cholesterol is the major substrate [5]. Bufalin-like immunoreactive substances have been observed in human serum for several years [11], but the presence of bufalin in human serum was validated for the first time in 2020 by HPLC-MS/MS [12]. This study reported bufalin concentrations of about 5.7 nM in the serum of healthy individuals and of five times decreased concentrations in the serum of patients with hepatocellular carcinoma, further highlighting its role as a potentially protective molecule against cancer development.

1.1. Clinical trials on HuaChansu

The administration of purified bufalin has never been performed in humans yet but there are studies in the literature reporting the results of clinical trials assessing the effect of *HuaChansu*, a traditional Chinese medicine, whose main active constituent is bufalin, on cancer patients. *HuaChansu* is a sterilized hot water extract of dried toad skin which can be injected.

A pilot study was performed by the Fudan University Cancer Hospital (Shanghai, China) and the M.D. Anderson Cancer Center (Houston,

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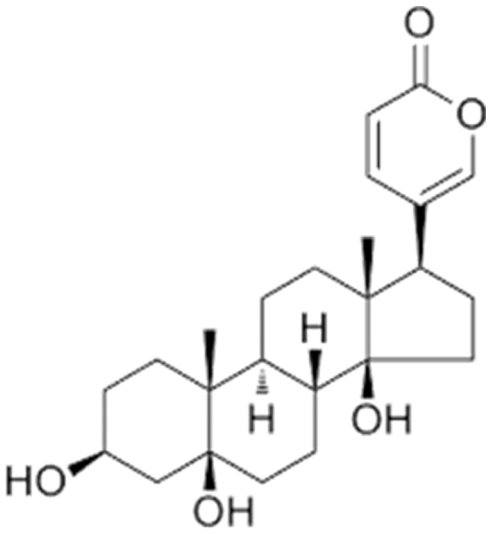

Fig. 1. Structure of bufalin.

Texas, USA) to investigate its adverse effects and its effectiveness against hepatocellular carcinoma, NSCLC and pancreatic cancer. HuaChansu was i.v. administered for 14 days followed by 7 days off. No dose limiting toxicities were calculated as no significant adverse event were observed at doses 8 times higher than what is used in traditional Chinese medicine. Indeed, 73 % of patients had no toxicities greater than grade 1. Moreover, as HuaChansu contains cardiotonic steroids, cardiac function was assessed by electrocardiogram, but no alteration of the cardiac function was observed. The toxicities were haematological (leukopenia and thrombocytopenia), gastrointestinal (loss of appetite, constipation and diarrhea), mucocutaneous (dental ulcers and rashes), and cardiovascular (premature ventricular contraction and hypertension). On the 15 patients enrolled, 6 (40 %) had stable disease (median duration = 6.0 months; range 3.5–11.1 months) and one patient with hepatocellular carcinoma had 20 % regression (duration = 11 months) [13]. These encouraging results highlighted that HuaChansu was well tolerated and capable of disease stabilization, and have led to another phase II clinical trial by the same groups to study the effects of the combination of gemcitabine with HuaChansu in pancreatic cancer (NCT00837239) [14]. This trial compared the effect of gemcitabine plus HuaChansu versus gemcitabine plus placebo in locally advanced or metastatic pancreatic adenocarcinoma. A total of 80 patients were enrolled and no toxicity was observed with the combination. However, no benefit of the combination on progression-free survival (PFS), objective radiographical response rate (ORR) nor time to progression was observed [15]. Three hypotheses are proposed to explain the lack of effectiveness of such combination: 1) a too low dose of HuaChansu, as no toxic effects were observed; 2) a too short exposure to see a positive effect, this short exposure time is in line with the characteristics of advanced pancreatic cancer which evolves very quickly; 3) the resistance of pancreatic cancer to many treatments, as many clinical trials have tested the combination of gemcitabine with other molecules without significative benefit [6,13].

Nevertheless, HuaChansu is approved by the Chinese FDA for use at oncology clinics in China and various clinical studies have recently demonstrated its significant anti-cancer properties in patients [6].

– In hepatocellular carcinoma, a clinical study (379 patients) compared the effect of HuaChansu versus transarterial chemoembolization (TACE) in preventing recurrence in post-resection patients with small tumours. Compared to TACE, HuaChansu prolonged recurrence-free survival and decreased recurrence rate at 1, 2 and 3 years. The adverse events were mild [16]. Another study (60 patients) compared the effect of HuaChansu injection combined

Table 1
Apoptotic mediators affected by bufalin.

Increase	Decrease /Loss	
cleaved caspase 3		Glioma [49,50], glioblastoma [48], osteosarcoma [51], large B cell lymphoma [52], multiple myeloma [39], melanoma [53], lung [35], nasopharyngeal [54], oesophageal [46], hepatocellular [27,32], bladder [38,55], prostate [34], oral [31] and colon cancer in vitro [40,56] but also in vivo [28]
caspase 4		Nasopharyngeal carcinoma [54]
cleaved caspase 7		Bladder carcinoma [55] and colorectal cancer [57]
cleaved caspase 8		Prostate cancer [34], bladder carcinoma [38], melanoma [53], nasopgaryngeal carcinoma [54] and hepatocellular carcinoma [32]
cleaved caspase 9		Glioblastoma [48], osteosarcoma [51], leukemia [58], melanoma [53], nasopharyngeal carcinoma [54], hepatocellular carcinoma [32, 54], prostate [34], colorectal [28, 57], bladder [38,55] and lung cancer [35].
caspase 10		Hepatocellular carcinoma [32]
APAF		Bladder cancer [55], nasopharyngeal carcinoma [54], oral carcinoma [31] and lung cancer [35]
cleaved-Parp		osteosarcoma [51], glioma [50], large B cell lymphoma [52], breast [25], colon [30,57], lung [35], tongue [59], gastric [60], and prostate cancer [47]
Bax/Bcl-2 ratio		Osteosarcoma [51], large B cell lymphoma [52], glioma [50], glioblastoma [48], hepatocellular and nasopharyngeal carcinomas [32, 54,61], colorectal [28,56,57], bladder [38,55], gastric [60] and tongue cancer [59]
Bak		Osteosarcoma [62], leukemia [58], nasopharyngeal carcinoma [54] and colorectal cancer [28]
Bad		Colorectal cancer [40] and oesophageal carcinoma [46]
Bid		Nasopharyngeal [54] and hepatocellular carcinoma [32]
AIF		Osteosarcoma [62], melanoma [53], tongue [59], oral [31] and bladder cancer [55]
cytochrome c release		Leukemia [58], osteosarcoma [51, 62], glioblastoma [48–50], melanoma [53], hepatocellular carcinoma [32], lung cancer [35], nasopharyngeal carcinoma [54], oral carcinoma [31], bladder carcinoma [55] and prostate cancer [34]
endo G release		Osteosarcoma [62], melanoma [53], nasopharyngeal carcinoma [54], tongue carcinoma [59] and lung cancer [35]
intra-cellular Ca^{++} concentration		Melanoma [53] and tongue carcinoma [59]
	mitochondrial membrane potential	Osteosarcoma [51], glioblastoma [48,49], melanoma [27], hepatocellular carcinoma [32], lung cancer [35], tongue carcinoma [59], bladder carcinoma [38] and colon cancer [30]
	Bcl-2	Osteosarcoma [62], leukemia [58], oesophageal [46] and lung cancer [35]
	Bcl-xL	Osteosarcoma [63], nasopharyngeal [54], prostate [24] and colorectal cancer [28,56]

(*continued on next page*)

Table 1 (*continued*)

Increase	Decrease /Loss	
	Mcl-1	Large B cell lymphoma [52], gallbladder carcinoma [44], breast cancer [45] and NSCLC [43]

with stereotactic body gamma knife radiosurgery versus gamma knife alone. The results indicated clear benefits of the combination with improved 1-year survival, overall survival and disease-free progression as well as quality of life with reduced side effects and analgesic use in the combination cohort [17]. Finally, a study using HuaChansu alone (100 patients) reported a lower progression rate, an increased survival rate and improved liver function as well as patient quality of life [18].

- In non-small-cell lung cancer, a metanalysis (32 studies, 2753 patients) investigated the combination of HuaChansu with platinum-based chemotherapy. The results indicated that the combination improved the overall response rate and the 1- and 2-years survival rates as well as the quality of life by alleviating chemotherapy-induced adverse effects (neutropenia, thrombocytopenia, nausea, vomiting, anaemia, liver injury, renal injury, and alopecia) [19]. Another study (64 patients) investigated HuaChansu versus chemotherapy as maintenance therapy after response to chemotherapy. The results reported similar overall survival but improved quality of life and 1-year survival rate [20]. A third study (64 patients on maintenance therapy after a 1st line chemotherapy), more focused on immunity, reported decreased serum concentration of CTLA-4 in patients treated with HuaChansu compared with chemotherapy [21].
- In gastric cancer, a metanalysis (14 studies, 976 patients) investigated the combination of HuaChansu with chemotherapy versus chemotherapy alone and highlighted improved response rate and quality of life with the combination [22].
- In gallbladder carcinoma, a clinical trial (25 patients) assessed the efficacy and safety of gemcitabine-oxaliplatin combined with HuaChansu. The combined treatment was well tolerated with moderate myelosuppression as the main toxicity. In this study, 23 patients were evaluated, 8 (34.8 %) had partial response while 7 (30.4 %) had stable disease [23].

Altogether, these clinical trials reported a benefit to patients from the use of HuaChansu in combination with chemotherapy in many cancers.

1.2. In vitro and animal studies

Nowadays, the vast majority of experiments conducted with bufalin were in vitro studies mainly using cancer cell lines and, in some cases, using primary cultures when specified. In addition, a few in vivo studies were performed and reported below when available.

2. Bufalin induces apoptosis in cancer cells

Bufalin causes DNA fragmentation as observed by TUNEL assay in in vivo models of human prostate, breast, pancreatic, hepatocellular, and colorectal cancer exposed to bufalin [24–28].

2.1. Caspase-induced apoptosis

Bufalin promotes the expression, cleavage, and activity of caspases in various cancers (Table 1). Also, a large scale analysis of genes in lung cancer cells exposed to bufalin indicated that CASP9 gene was upregulated by 5.5 fold [29]. Moreover, the use of pan-caspase inhibitors in colorectal cancer rescued cells from apoptotic death [30]. A similar result was obtained in oral squamous cell carcinoma with the use of caspase 3 or 9 inhibitors [31], as well as in hepatocellular carcinoma exposed to either caspase 3, 8, 9 or 10 inhibitors [32].

Three different mechanisms underly caspase activation by bufalin: 1) in colorectal cancer, caspase 3 activation was linked to Bax and Bak since the knockout of either one prevented bufalin-stimulated increase in cleaved caspase 3 [28]; 2) in lung cancer, bufalin-promoted caspase 3 activation could be prevented by N-acetylcysteine (NAC) thus indicating its stimulation of oxidative stress [33]; 3) in hepatocellular carcinoma, a crucial role of bufalin in inducing Fas-mediated caspase 10-dependent apoptosis pathway was highlighted [32]. Furthermore, in prostate cancer, bufalin increased Fas levels [18], while the use of siRNA to knock down Fas expression led to a restored cell viability. Increased levels of Fas and Fas-ligand were documented also in lung cancer [35]. Of note, in bladder carcinoma, bufalin treatment led to the down-regulation of cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein and X-linked inhibitor of apoptosis protein [36], while an increased expression of APAF, which links cytochrome c to activated caspase 9, has also been reported in many cancers (Table 1).

Bufalin also affects Parp protein cleavage, Parp being a molecular indicator of caspase-mediated cell death. (Table 1). In breast cancer, Parp inhibition prevented bufalin-induced cell death [37]. The cleavage of Parp in bufalin-treated cells is linked to caspases activation. Indeed, the cleaved Parp induction in colon cancer following bufalin exposure was reversed with pan-caspase inhibitors [30]. A study on bladder cancer indicated that the degradation of Parp polymerases was concomitant with the proteolytic activation of caspases 3, 8 and 9 [38]. Parp cleavage was also induced by ROS after bufalin treatment as NAC decreases the cleaved Parp accumulation in colorectal cancer [30]. Finally, a study in multiple myeloma indicated that *PARP1* overexpression partially suppressed bufalin-induced apoptosis [39].

2.2. Bcl-2 protein family-mediated apoptosis

A decreased Bcl-2 expression had been documented in many cancer types (Table 1). In addition, an increased Bax/Bcl-2 ratio has been also observed in many studies (Table 1). Concerning the protein Bax, its expression was upregulated in colorectal cancer and hepatocellular carcinoma [40,41], while it was redirected from the cytosol to the mitochondria in prostate and lung cancers [33,34]. Bax translocation was associated with ROS as NAC inhibited it [33]. In osteosarcoma, a downregulation of TPTC1 (tumour protein translationally-controlled 1), a Bax antagonist, was also observed [42], whereas an increased expression of the pro-apoptotic proteins Bak, Bad and Bid could also be induced by bufalin (Table 1).

Similarly, the expression of the anti-apoptotic protein Bcl-xL as well as Mcl-1 were decreased in many cancers treated with bufalin. In NSCLC, Mcl-1 overexpression moderated the bufalin-induced apoptosis. Moreover, decreased Mcl-1 levels by bufalin are linked to its proteasomal degradation. Indeed, only the Mcl-1 protein level was decreased while the mRNA rate did not change and the use of the proteasome inhibitor MG132 supressed the decrease in Mcl-1 protein level. A Mcl-1 ubiquitination was also observed by immunoprecipitation. This mechanism was linked to the activation of GSK3-β by bufalin as *GSK3* siRNA blocked the Mcl-1 proteasomal degradation [43]. In gallbladder cancer, Mcl-1 decrease was associated to the inhibition of phosphorylated c-Met by bufalin [44], while in breast cancer, it was associated with the inhibition of the transcriptional factor STAT3 [45].

2.3. IAP (inhibitor of apoptosis protein family)-mediated apoptosis

After bufalin treatment, decreases in survivin and livin levels were observed in colorectal cancer [12], and cIAP1 level was decreased in oesophageal carcinoma [46]. In bladder cancer, bufalin caused an increase of Bax/Bcl-2 ratio leading to a downregulation of IAP family members [38]. By contrast, other studies documented increased levels of the AIF in cancers treated with bufalin (Table 1).

2.4. p53-mediated apoptosis

An increased p53 protein level has been observed in prostate and lung cancers treated with bufalin, where it was associated with an increased p21 protein level [24,47]. A study in glioblastoma reported an increased nuclear translocation of p53 following bufalin exposure. This nuclear p53 accumulation was induced by a decrease of the XPO1 exportin expression. These results were highly supported by the fact that exportin inhibitor leptomycin B, a known potent and selective inhibitor of p53 nuclear export, caused the same nuclear retention of p53 as the one obtained by bufalin. Silencing XPO1 led to an increase in nuclear p53 expression and consequently attenuated bufalin effect [48]. Additional studies highlighted the crucial role of p53 in bufalin-induced apoptosis. Indeed, bufalin induced apoptosis in WT p53 colorectal cancer cells and autophagy in mutant p53 cells, while p53 downregulation restored cell viability [34]. Similar results were obtained in prostate cancer [48] where bufalin induced apoptosis in WT and mutant p53 cells but not in p53-null cells [47].

2.5. Death receptor-induced apoptosis

Following bufalin treatment, death receptor proteins and death receptor-related factors were upregulated in bladder cancer [36,38] while DR4 and DR5 were upregulated in nasopharyngeal carcinoma [54]. In breast cancer, bufalin upregulated DR4 and DR5 and this effect was linked to the activation of ERK, JNK and p38-MAPK and the downregulation of Cbl-b [64]. Another study indicated that bufalin promoted the clustering of DR4 and DR5 in aggregated lipid rafts and the use of a cholesterol-sequestering agent reversed such clustering and reduced bufalin-induced apoptosis [65]. In the latter three studies, bufalin also increased TRAIL expression, known to be involved in the induction of apoptosis [54,64,65]. Likewise, in breast cancer, bufalin downregulated Mcl-1 expression and promoted TRAIL-induced apoptosis [45].

2.6. Mitochondria-induced apoptosis

Bufalin modifies mitochondria morphology and biogenesis. A study in glioma using MitoTracker green and Transmission Electron Microscopy reported that bufalin led to a relatively loose mitochondrial network and caused mitochondria to split into many smaller mitochondria which affects their function. [49]. Bufalin also affected the expression of the mitochondrial division/fusion related proteins DRP1 and MFN2. DRP1 was downregulated in the cytoplasm but upregulated in mitochondria, while the opposite observation was reported for MFN2. These data indicate that bufalin can disrupt the mitochondrial division/fusion balance to induce apoptosis [49]. Bufalin also decreased the oxygen consumption rate, an indicator of mitochondrial function, in glioblastoma [48]. Finally, bufalin stimulated the release of cytochrome c from the mitochondria to the cytosol and upregulated the proapoptotic nuclease endonuclease G release by mitochondria and its nuclear translocation [49].

2.7. Stress-induced apoptosis

An increased intracellular Ca^{++} concentration was also observed after bufalin treatment and was associated with the loss of mitochondrial membrane potential (MMP). In osteosarcoma, the loss of MMP was a consequence of the downregulation of protein phosphatase 2 (PPA2) [42] and was associated with a production of mitochondrial ROS in many cancers including melanoma, colon and lung cancer [30,35,53]. In neuroblastoma, this bufalin-induced ROS production led to the disruption of the electron transport chain and NAC inhibited bufalin-induced apoptosis [66]. In lung cancer, the production of ROS by bufalin mediated mitochondrial permeability transition [33]. In glioblastoma, ROS production was associated to bufalin-induced DNA damage. Indeed, an increased fluorescence of phosphorylated H2AX was observed following bufalin treatment and its was attenuated with NAC [48]. A study on pancreatic and oral cancers indicated that bufalin induces apoptosis by downregulating hTERT expression (hTERT protects against mitochondrial damage by binding to mitochondrial DNA and reducing mitochondrial ROS production) related to an increased phosphorylation of JNK and p38-MAPK. Actually, the use of JNK or p38-MAPK inhibitors opposed bufalin-induced hTERT downregulation and hence moderated apoptosis induction by the mitochondrial pathway [67].

Bufalin leads to endoplasmic reticulum (ER)-stress induced apoptosis as indicated by changes in the expression of ER stress markers. In nasopharyngeal carcinoma, bufalin increased GRP78, IRE-1α and IRE-1β (inositol-requiring enzyme 1), ATF-6α, Calpain 1 and GADD153 [54]. In glioma, bufalin exposure led to the upregulation of CHOP (C/EBP homologous protein) and GPR78. The role of ER stress response was confirmed by the attenuated bufalin-induced apoptosis in cells transfected with *siCHOP* RNA. In this context, ER stress was associated with an increased phosphorylation of ERK and eIF2a [50]. In gastric cancer, bufalin led to ER stress via the IRE1-JNK pathway and to a significantly increased expression/phosphorylation of target genes of the ER stress pathway including IRE1, CHOP and p-eIF2a.

Modulation of the anti-apoptotic protein Hsp27 level by bufalin also plays a key role in apoptosis induction. Its expression significantly decreased after bufalin exposure in osteosarcoma and pancreatic cancers [42,68]. A comparative proteomics approach reported that the level of the anti-apoptotic protein Hsp27 remarkably decreased after treatment with bufalin. Bufalin downregulates Hsp27 in vitro and in vivo through proteasomal degradation to induce apoptosis. Ectopic expression of Hsp27 reduced the bufalin-induced apoptosis [42].

2.8. Other mechanisms inducing apoptosis

In glioma, bufalin induces the mitochondrial translocation of Annexin A2 to modulate apoptosis [49]. In prostate cancer, the protein level of AR and its transcriptional target PSA were decreased by bufalin, while the AR coactivators SRC-1 and SRC-3 were suppressed, and these downregulations were not dissociable from apoptosis [47]. In leukemia, Tiam1 was upregulated by bufalin, and this led to the activation of Rac1, PAK and JNK pathway and to apoptosis. Regarding TNF-induced apoptosis, bufalin increased THAP1 in lung cancer [29] and FADD in nasopharyngeal carcinoma [54], suggesting that bufalin could improve the apoptotic effect of TNF.

3. Bufalin induces necroptosis in cancer cells

Necroptosis is another type of cell death distinct from apoptosis that can be induced by DNA damage, oxidative stress, and specific receptors. Necroptosis is mainly mediated by the TNF receptor superfamily. When cancer cells are stimulated to activate TNF receptors, caspase 8 activation is inhibited to block apoptosis. RIP1 and RIP3 are phosphorylated to form a necrotic complex which associates to PGAM5, disintegrates mitochondria and induces necroptosis. Inducing necroptosis could be a way to overcome resistance to apoptosis, for example MDR overexpression in breast cancer [69].

In breast cancer, bufalin upregulated RIP1, p-RIP1, RIP3 and PGAM5 [37,69,70]. The necroptosis inhibitor Nec-1 and the ROS scavenger NAC but not pan-caspases inhibitors decreased bufalin-induced necroptosis indicating that bufalin stimulated ROS production to induce necroptosis [69,70]. In another study, bufalin-induced cell death was prevented by RIP1 inhibitor or *RIP3* shRNA while RIP3 ectopic expression enhanced bufalin-induced cell death [37]. Moreover, it also increased TNFα levels and the phosphorylation of the TNF receptor [70].

In glioblastoma, bufalin induced necroptosis in cells that escaped from apoptosis. Indeed, when caspase-8 was functionally lost, necroptosis occurred through the formation of a necrosome complex containing RIPK1, RIPK2 and MLKL (mixed lineage kinase domain-like

Table 2
Cell cycle and proliferation regulators affected by bufalin.

Increase	Decrease/ Loss	
p27		NSCLC [80], hepatocellular carcinoma [83] and colorectal cancer [28]
p21		Large B cell lymphoma [52], prostate cancer [47] and colorectal cancer [28]
p53 and p-p53		Hepatocellular carcinoma [78], glioma [49], prostate cancer [47] and NSCLC [84]
G2/M arrest		Hepatocellular carcinoma [27,78,85], TNBC [25], gallbladder carcinoma [44], leukemia [58,79], renal cell carcinoma [86], bladder carcinoma [38], osteosarcoma [51], oesophageal carcinoma [87], glioblastoma [88] and multiple myeloma [39] (in vitro) colorectal [28] and prostate cancer [47] (in vivo)
G0-G1 arrest		Oral carcinoma [31], ovarian and endometrial cancers [89], hepatocellular carcinoma [61], bladder [55] and gastric cancer [60], NSCLC [80] and glioma [49]
S phase arrest		Oesophageal carcinoma [87], ovarian and endometrial cancers [89]
	Cyclin A	Hepatocellular carcinoma [27,78] and colorectal cancer [28]
	Cyclin B1	Hepatocellular carcinoma [27,78], renal cell carcinoma [86] and large B cell lymphoma [52]
	Cyclin D1	Bladder carcinoma [31], NSCLC [80] (in vitro) and colorectal cancer (in vivo) [28]
	Cyclin E1	Bladder carcinoma [31], NSCLC[80] (in vitro) and colorectal cancer (in vivo) [28]
	CDK1, p-CDK1	Hepatocellular carcinoma [27,78], renal cell carcinoma [86] and large B cell lymphoma [52]
	CDK2	Bladder carcinoma [55] (in vitro) and colorectal [28] (in vivo)
	CDK4	Bladder carcinoma [55] (in vitro) and colorectal [28] (in vivo)
	CDK6	Hepatocellular carcinoma [83]
	Cdc25a	Hepatocellular carcinoma [27,85]
	Cdc25c, p-Cdc25c	Hepatocellular carcinoma [83,85]

Table 3
Signalling pathway regulators affected by bufalin.

Increase	Decrease/ loss	
PTEN		Colorectal cancer [40]
p-JNK		Hepatocellular carcinoma [78], pancreatic cancer [105], leukemia [98], breast cancer [64], gastric cancer [60], pancreatic and oral carcinomas [67]
p-p38		Hepatocellular carcinoma [78], breast cancer [64], pancreatic and oral carcinomas [67]
	(p-)EGFR	Ovarian carcinoma [106] and hepatocellular carcinoma [83]
	VEGFR1/2	Hepatocellular carcinoma [94,99]
	p-c-Met	Gallbladder cancer [44]
	Axl	NSCLC [107]
	p-PI3K	Hepatocellular carcinoma [94], lung cancer [108] and colorectal cancer [28]
	p-AKT	Ovarian carcinoma [106], renal cell carcinoma[86], hepatocellular carcinoma [27,90,92,94], lung cancer [108,109] and colorectal cancer [28,40]
	p-mTOR	Ovarian carcinoma [93], renal cell carcinoma [86], hepatocellular carcinoma [27] and gastric cancer [72]
	p-ERK	Ovarian carcinoma [106], hepatocellular carcinoma [78, 110], gastric cancer [111], lung cancer [108] and osteosarcoma [112]
	p-p38	Gastric cancer [111] and lung cancer [108]
	(p-)NF-kB	Lung cancer [108], hepatocellular carcinoma [94] and colorectal cancer [81]
	p-AMPK	Hepatocellular carcinoma [75], glioma [50] and oesophageal carcinoma [46]

protein). In this context, increases in TNF, TNFR1 and RIPKK were also observed [71].

4. Bufalin induces autophagy in cancer cells

Bufalin has been reported to induce autophagy but it is highly dependent on the cell characteristics and type. Indeed, in cells harbouring wild type p53, bufalin mainly induces apoptosis while it induces autophagy in mutant p53 cells [30]. Depending on the context, autophagy can be viewed as a protective mechanism to escape apoptosis or as an alternative mechanism to kill the cell. When bufalin-induced autophagy acts as a protective mechanism, it could be validated by using an autophagy inhibitor [49,50,72,73]. For example, in hepatocellular carcinoma, autophagy induction was highlighted by the observation of double-membrane vacuoles by transmission electron microscopy, the detection of acidic vesicular organelles using acridine orange staining, and the cleavage of microtubule-associated protein light chain 3 (LC3). In this context, autophagy inhibitor opposed the effect of bufalin on cell viability but enhanced apoptosis [27]. Bufalin induced autophagy by increasing the conversion of LC3-I to LC3-II thus leading to increased levels of LC3-II. This has been observed in colorectal cancer [74], hepatocellular carcinoma [73,75,76], glioma [50] and gastric cancer [49]. This increase in LC3-II rate was associated to an increased expression of Beclin-1 in colorectal cancer [74] and hepatocellular carcinoma [62–65], and a decreased expression of p62 through proteasomal degradation in hepatocellular carcinoma [76] and gastric cancer [49]. Bufalin-induced autophagy can be triggered by ROS and ER-stress which leads to the activation of the IRE1-JNK pathway. This was observed in gastric [49] and colorectal cancer [74] as well as in hepatocellular carcinoma [76]. Another way to trigger autophagy is through the depletion of ATP and the activation of the AMPK-mTOR pathway. Indeed, bufalin-induced ATP depletion leads to the phosphorylation of AMPK which decreases the phosphorylation of mTOR and its downstream targets 4EBP1 and P70S6K1. The pERK/eIF2α/CHOP pathway also plays a crucial role in this process [50,72,75]. The levels of other autophagy-related proteins such as ATG5 and ATG8 were increased in colorectal cancer [74] and hepatocellular carcinoma [76].

5. Bufalin induces senescence in cancer cells

Studies on multiple myeloma and prostate cancer also highlighted a senescence-like inducing effect of bufalin. It was observed by an increased volume of the cells and by an increase in the number of SA-Gal positive cells [47,77]. In prostate cancer, bufalin induced a selective activation of p53-related senescence. Moreover, bufalin stimulated the expression of genes related to senescence such as *CYR6/CCNI* and its related family member *CTGF/CCN2* as well as the p53 target gene *CDKN1A* (p21).

6. Bufalin modulates cell cycle and proliferation in cancer cells

Bufalin induced a cell cycle arrest in G2/M phase in many cancer types that were associated with a decreased activity of Cdk1 [78]. In leukemia, the effect of bufalin on cell cycle was similar to that of topoisomerase inhibitors with a selective inhibition of the activity of topoisomerase II but not topoisomerase I [79]. Other studies highlighted a G0/G1 phase arrest explained by the decreased expression of cyclin but also of cyclin-dependant kinases (Table 2). Bufalin also modulated the expression and activity of Cdc25a and Cdc25c which have a role in mitotic progression (Table 2). This was often associated with an increased expression of p21 and p27, two universal cyclin inhibitors, p27 controlling cell cycle transition from S to G2 phase [80] (Table 2).

More specific mechanisms implicated in cell cycle and proliferation arrests induced by bufalin are described in the literature. In leukemia, bufalin increased the casein kinase 2 (CK2) activity through its nuclear translocation [79], whereas it inhibited the activity of PKA and PKC [79] or it binds to β-tubulin [58] to decrease proliferation. In colorectal

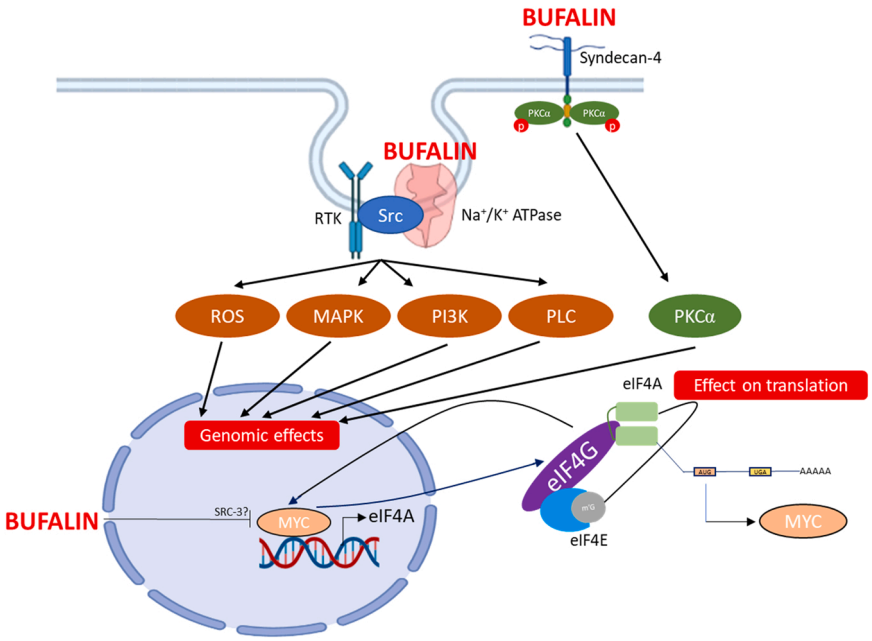

Fig. 2. Direct interactions of bufalin with ATP1A1, SDC-4 and SRC-3 affecting gene transcription and mRNA translation in cancer (*need to be printed in colours*).

cancer, bufalin induced a mitotic arrest through the downregulation of the polo-like kinase 1 (Plk1) [81] or through the inhibition of Aurora A/B activation [82]. Finally, a gene expression study in lung cancer reported the downregulation of CCPG1 (cell cycle progression protein 1) and of CDCA7L (cell division cycle-associated 7-like protein) [29].

7. Bufalin modulates the activation of specific signalling pathways in cancer cells

Bufalin modifies the expression and phosphorylation of some receptors such as EGFR, VEGFR, c-Met or Axl (Table 3).

Bufalin regulates the PI3K/AKT signaling pathway (Table 3) and in oral carcinomas, AKT overexpression is associated with resistance to bufalin [31]. In colorectal cancer and hepatocellular carcinoma, this effect leads to a decrease in mTOR phosphorylation and an increased GSK-3β activity [28,90–92]. In ovarian carcinoma, bufalin decreases kinase S6K phosphorylation downstream of mTOR [93]. In parallel, MAPK pathway was as well modulated by bufalin. Indeed, bufalin decreased MEKK3 and MKK7 in hepatocellular carcinoma [94]. Accordingly, ERK phosphorylation was also decreased in many cancer types following bufalin exposure (Table 3). However, the activity of bufalin on ERK phosphorylation differs among studies and cancer types. Indeed, bufalin generally moderates ERK phosphorylation but, conversely, it can also induce its phosphorylation in some tumour types. For example, in bladder carcinoma, bufalin promoted p-ERK, while ERK inhibitor opposes bufalin inhibitory effect on migration [95]. An increased ERK phosphorylation was also observed in hepatocellular carcinoma cells exposed to bufalin [96] and in breast cancer where it was associated with induction of apoptosis [64]. In leukemia, a persistent activation of MAPK by bufalin was involved in the induction of apoptosis [97]. Of note, the phosphorylation of the p38-MAPK protein was either increased or decreased according to different studies, however, in those where p38 phosphorylation was increased, the use of a p38 inhibitor decreased bufalin-induced apoptosis [64,67].

Bufalin also activates JNK pathway by increasing JNK phosphorylation in many cancer types and nuclear translocation of its target c-Jun in leukemia [98] (Table 3). Moreover, JNK inhibitor decreased bufalin-induced apoptosis [64,67].

The Hedgehog signaling pathway was also inhibited by bufalin. The expression levels of PTCH2 and Gli proteins were downregulated by bufalin in liver cancer [99]. Additionally, NF-*k*B expression, phosphorylation and nuclear translocation were decreased by bufalin (Table 3).

Table 4
Sodium pump is a direct target of bufalin.

Bufalin effect	
ATP1A1↓	• In melanoma: ATP1A1 level positively correlated with bufalin response [115] • In glioblastoma: decreased ATP1A1 expression through proteasomal degradation (in vitro and in vivo) [48,116] and ATP1A1 KO markedly increased the IC50 values of bufalin [48] • High level of ATP1A1 increased cellular levels of glutathione which delayed and reduced apoptosis [117]
ATP1A3↓	• In bladder carcinoma: bufalin supressed expression of ATP1A3. ATP1A3 KO decreased bufalin-induced apoptosis [118] • In hepatocellular carcinoma: ATP1A3 level positively correlated with bufalin response and ATP1A3 KO increased the IC50 value for bufalin [96] • In colorectal cancer: ATP1A3 KO or bufalin decrease COX2 expression in a similar way [28]

Moreover, cell exposure to bufalin promotes the phosphorylation of AMPK leading to the inhibition of its downstream targets mTOR, p70S6K and 4EBP1 in glioma and oesophageal carcinoma [46,50].

Many studies demonstrated that bufalin is a potent inhibitor of the transcriptional coactivators SRC-1 and SRC-3 through proteasomal degradation. This was highlighted in triple-negative breast cancer (TNBC) where SRC-3 is a prognostic marker associated with poor overall survival and progression-free survival [100], but also in other breast cancer subtypes [101,102]. SRC-3 was also emphasized in glioblastoma [103] and in prostate cancer where it is associated with a low level of active androgen receptor (AR) and its downstream target PSA (prostate specific antigen) [47]. A molecular docking model in colorectal cancer indicated that bufalin could directly bind SRC-3 to inhibit its activity [104].

8. Bufalin modulates protein translation

Bufalin affected protein translation by inhibiting mTOR activity, as discussed above, but also acted on the translation of more cancer-specific mRNA related to the eIF4F translation initiation complex. A

Table 5
Effects of bufalin in combination with other treatments.

Effect on	Cancer type	
Macrophages NF-kB/MDR1 ABCB1 transporter Apoptosis Stemness markers	Colorectal cancer	• Enhanced antitumoral effect of oxaliplatin by decreasing M2 macrophages polarization [104] • Increased sensitivity of adriamycin-resistant cells to doxorubicin, mitomycin C, vincristine, and cyclophosphamide in vitro and increased antitumor effects of DOX in vivo. These effects are linked to its activity on the NF-kB/MDR1 pathway [122] • Increased doxorubicin antitumor activity in vitro and in vivo by reducing ABCB1 transporter level [123] • Enhanced cytotoxicity of 5-fluorouracil by a synergistic effect of the combination on apoptosis induction [125] • Chemoprophylactic activity [28] • Synergistic effect of bufalin combined with cisplatin on apoptosis induction and decrease of stemness markers which are associated with resistance to treatment [126]
Cell growth Apoptosis Angiogenesis	Hepatocellular carcinoma	• Increased sensitivity of HBV-associated HCC refractory to sorafenib [127] • Synergistic effect of the bufalin-sorafenib combination on growth inhibition and apoptosis induction [110, 128,129] • Activity on sorafenib-resistant cells [129] • Enhanced anti-angiogenic effect of the bufalin/sorafenib combination [130] • Enhanced the chemosensitivity to 5-FU by inducing apoptosis [61]
Apoptosis	Gastric cancer	• Reversed intrinsic and acquired cisplatin resistance by inducing apoptosis through the AKT pathway [131]
	Ovarian cancer	• Bufalin improves cisplatin responsiveness by decreasing HIF-1a [93]
	Gallbladder carcinoma	• Bufalin enhances chemotherapeutic sensitivity [44]
	Cervical cancer	• Increased the chemotherapeutic efficacy of paclitaxel [132]
	Osteosarcoma	• Apoptosis induction in methotrexate resistant cells [42,63]
	Glioma	• Improved the inhibitory effect of TMZ by activating the mitochondrial apoptotic pathway [133] • Increased sensitivity to radiotherapy by reducing DNA repair function [88]
	Leukemia	• Combination of bufalin with the MEKi PD98059 has a synergistic effect on decreasing proliferation and inducing apoptosis [134]
	Pancreatic cancer	• Enhanced the chemosensitivity to gemcitabine [105] • Increased apoptosis induction by the bufalin-HIFU (High Intensity Focused Ultrasound) combination [135]

recent study in TNBC identified bufalin as a novel inhibitor of eIF4A-mediated translation by acting on c-MYC translational activity to downregulate eIF4A and eIF4G [113] (Fig. 2).

9. Bufalin interacts with the sodium pump

In relation with its steroid structure, bufalin can act on the Na+/K+ ATPase pump to mediate its anticancer effects [49,82]. More specifically, the main targets of bufalin are the isoforms ATP1A1 or ATP1A3, depending on the cancer type (Table 4). However, the effect seems restricted to caveolae to mediate its impact on the Na+/K+ ATPase signalosome [114] (Fig. 2).

Table 6
MicroRNAs involved in bufalin antitumor effect.

Bufalin effect on	
miR-522–3P ↓	Involved in NSCLC development [137]
miR-181a ↑	Involved in apoptosis induction by repressing its target gene Bcl-2 in prostate cancer [138]
miR-298 ↓	Block apoptosis by increasing its target BAX in gastric cancer [139]
miR-497 ↓	Associated with decreased angiogenesis and metastasis in colorectal cancer [140]
miR-203 ↑	Associated with decreased stemness in glioma [141]
miR-148a ↑	Associated with decreased stemness in osteosarcoma [142]

*↑: increase; ↓: decrease.

10. Bufalin acts on cancer cell metabolisms

Bufalin may affect the metabolism of cancer cells by 3 main mechanisms impacting mitochondria, glycolysis and lipid metabolism.

1) ***Mitochondrial metabolism***: Bufalin is associated with disruption of mitochondrial membrane potential and decreased oxygen consumption and ATP production [49,50,88]. The latter leads to AMPK phosphorylation and activation [50]. Moreover, in glioma, using MitoTracker green, bufalin exposure was linked to scattered mitochondria associated with a relatively loose network and a decreased mitochondria fluorescence. In addition, transmission electron microscopy revealed that bufalin can increase mitochondrial number, density and swelling, and reduce its surface area and volume. Thus bufalin causes mitochondrion to split into a number of smaller mitochondria, thereby affecting mitochondrial function [49].
2) ***Glycolysis***: Bufalin moderates glucose uptake and lactate production in ovarian carcinoma in vitro and in vivo and is associated with low levels of glycolysis-related proteins, including GLUT4, LDHB and HK2 [119].
3) *Lipid metabolism*: In hepatocellular carcinoma, bufalin modulates sphingolipid and glycerophospholipid metabolism leading to the disruption of tumour cell membranes but mainly located within the non-necrotic areas [120]. Also, bufalin affects the metabolism of a number of animo acids including phenylalanine, histidine, glutamate, aspartate, methionine, glutamine, isoleucine, proline, serine or carnitine (synthesized in the liver from methionine and lysine). As carnitine plays a crucial role in fatty acid transport within the mitochondrial matrix for β-oxidation, bufalin can compromise energy production [121].

11. Bufalin sensitizes resistant cancer cells to conventional therapies

Combination of bufalin with chemo or targeted therapies have proven beneficial compared to each drug alone (Table 5). Moreover, in the context of cancer with resistance to treatment, bufalin was able to reverse the resistance to certain therapies or to kill resistant cells. These effects were often linked to the inhibition of MDR1 pathway. For example, in colorectal cancer, doxorubicin (DOX) uptake experiment indicated that bufalin significantly increased the intracellular drug concentration [122,123]. In multidrug-resistant hepatocellular carcinoma, bufalin enhanced chemosensitivity by inhibiting drug efflux pump activity via the downregulation of MRP1 [61]. A similar MRP1 downregulation by bufalin was observed in leukemia cells with vincristine-acquired multidrug resistance which then led to an increased intracellular levels of adriamycin [124].

Table 7
Phenotype and stemness associated markers affected by bufalin.

Decrease	
Spheroid formation	TNBC [25], gallbladder cancer [44], osteosarcoma [142,151], glioma [141], pancreatic cancer [150] and colorectal cancer [126]
SOX2	TNBC [25], gallbladder cancer [44], osteosarcoma [142,151], glioma [141] and colorectal cancer [126]
OCT4	TNBC [25], gallbladder cancer [44], osteosarcoma [142,151], glioma [141] and colorectal cancer [126]
CD133	Gallbladder cancer [44], colorectal cancer [122,126] and osteosarcoma [142,151]
CD44	Gallbladder cancer [44] and colorectal cancer [126]

12. Bufalin modulates microRNAs in cancer cells

Bufalin modulates the expression of specific microRNAs responsible for cancer development and has an antitumor effect (Table 6). In prostate cancer, it affected long non-coding RNAs (lncRNA) known to interact with miRNAs. The study reports that bufalin decreased *HOTAIR*, a crucial lncRNA upregulated in bone metastases, and promotes metastases by binding to miR-520b and thus increasing the oncogene FGFR1 and consequently moderating the metastatic potential of prostate cancer [136].

13. Bufalin induces phenotype switching/EMT towards differentiation and reverses cell stemness

In melanoma, bufalin activates tyrosinase and thus stimulates melanogenesis and pigmentation that is associated with a more differentiated/melanocytic phenotype [143]. Many studies also indicate that bufalin is a potent inducer of differentiation in leukemia cell lines and primary cultures accompanied by a loss of cell proliferation and adherence affecting viability and an increased expression of IL-1β. Similarly, the induction of cell differentiation was enhanced by All-trans retinoic acid known to affect cell growth and apoptosis [144–149].

Cancer stem cells (CSC) possess enhanced tumour-forming capabilities and are resistant to current anticancer therapies. In line with its ability to promote cancer cell differentiation, bufalin attenuates stem cell characteristics in many cancer types (Table 7). This was mainly observed through the inhibition of spheroid formation and the decreased expression of stemness-associated proteins SOX2 and OCT4 and stem cell-surface marker proteins CD133 and CD44 (Table 7). In gallbladder cancer, bufalin also attenuates the self-renewal of cancer stem cells [44]. Furthermore, bufalin reduced the expression of other stemness markers, such as ALDH1, TERT, NANOG, Notch and Bim1 in isolated CSC of osteosarcoma primary cultures. Bufalin-treated CSC conferred a lower ability of xenograft formation in mice [142]. In an in vivo pancreatic cancer model, bufalin inhibited tumour growth and prolonged the duration for tumour formation (subcutaneous xenograft), and pre-treatment with bufalin decreased intestinal and lung metastases after IV tail vein administration [150]. In colorectal cancer, both in in vitro and in vivo models, bufalin abrogated cisplatin induced stemness (associated with increased expression of stemness markers such as CD133, CD44, NANOG, OCT4, SOX2, and ABCG2) [126].

14. Bufalin modulates migration and invasion of cancer cells

Bufalin regulates the expression of some common markers involved in epithelial-mesenchymal transition (EMT) in different cancer types (Table 8). In hepatocellular and bladder carcinomas, this was associated with the decrease of the transcription factor ZEB1 [78], the increase of mRNA and protein levels of the tissue inhibitor of metalloproteinase (TIMP) 1 and 2 [95] and the decrease in Gli1 and Gli3 proteins within the Hedgehog (Hh) signaling pathway [99]. While in gastric cancer, bufalin-induced inhibition of migration and invasion was associated

Table 8
Migration and invasion regulators affected by bufalin.

Increase	Decrease/loss	
E-cadherin		Gastric cancer [111,152], pancreatic cancer [26], renal cell carcinoma [86], hepatocellular carcinoma [90,91,99,153], colorectal cancer [57] and gallbladder carcinoma [44]
	Vimentin	Hepatocellular carcinoma [78,153], gastric cancer [111,152] and pancreatic cancer [26]
	β-catenin	Hepatocellular carcinoma [90,91,99] and colorectal cancer [57]
	N-cadherin	Hepatocellular carcinoma [78,153], gastric cancer [111], renal cell carcinoma [86] and colorectal cancer [57]
	Snail	Hepatocellular carcinoma [78,153] and gallbladder carcinoma [44]
	MMP2	Hepatocellular carcinoma [78,90,99], bladder carcinoma [95], osteosarcoma [112], gastric cancer [152] and lung cancer [108]
	MMP7	Hepatocellular carcinoma [91]
	MMP9	Hepatocellular carcinoma [78,90,99], gallbladder carcinoma [44], bladder carcinoma [95], osteosarcoma [112], lung cancer [108] and gastric cancer [152]

with the inhibition of ASCL2 expression. Indeed, *ASCL2 RNAi* mimicked bufalin effects on MMPs, E-cadherin and vimentin [152]. In hepatocellular carcinoma, bufalin moderates the phosphorylation of GSK3-β, which plays a key role in tumour invasion, while the total amount of GSK3-β protein was increased [90,91].

Other more specific mechanisms are also described. In hepatocellular carcinoma, bufalin binds syndecan-4 (SDC4) which is a major endogenous membrane receptor regulating cell cytoskeleton, adhesion, and migration (Fig. 2). Consequently, SDC-4 interaction with its protein substrate DDX23 (Dead-box helicase 12) is increased contributing to genomic instability. A DARTS (Drug Affinity Responsive Target Stability) experiment also indicated that bufalin could stabilize SDC4 leading to an enhanced susceptibility to proteolysis. SDC4-DDX23 KO opposes bufalin inhibition of cell migration [78]. In gastric cancer, bufalin suppressed peritoneal dissemination by reducing the phosphorylation of NOS3 [111]. In bladder carcinoma, bufalin inhibits motility and invasiveness by acting on tight junctions (TJs) [95]. In hepatocellular carcinoma and lung cancer, bufalin opposes TGF-β-induced EMT [153,154] and TGF-β-induced Twist2, zinc finger E-box binding homeobox 2 (ZEB2) as well as the phosphorylation of Smad2 and Smad3 whereas both TGF-β receptor I (TβRI) and TGF-β receptor II (TβRII) were downregulated [154]. In lung cancer, bufalin causes fibronectin overexpression that, in its turn, reinforces bufalin antimigratory activity [155]. In hepatocellular carcinoma, bufalin reduces APOBEC3F which is overexpressed in tumour tissue having roles in cell growth and cell cycle control [156]. Finally, in lung cancer, bufalin downregulates metastasis-related genes, including the small GTPases RhoA, the Rho-associated kinases ROCK1, and the focal adhesion kinase (FAK) [108].

15. Bufalin impairs neo-angiogenesis

Many studies report that bufalin downregulates HIF-1α notably in colorectal cancer [81], renal cell carcinoma [86] and ovarian carcinoma [93]. In hepatocellular carcinoma hypoxia-induced HIF-1α by CoCl2 could be abrogated by bufalin [153] and moderates VEGF expression both in vitro and in vivo in tumour xenografts in mice [99,153]. In colorectal cancer, bufalin suppressed tumour microenvironment-mediated angiogenesis by inhibiting the STAT3 signalling pathway in vascular endothelial cells (HUVEC) [157]. In hepatocellular carcinoma, assays on chick chorioallantoic membrane and rat aortic rings demonstrated that bufalin enhanced anti-angiogenic effect of sorafenib via modulating the AKT/VEGF signaling pathway [78,158]. Moreover, bufalin decreased HUVEC proliferation and migration. Accordingly, as vascular endothelial cells liberate various cytokines to affect angiogenesis, conditioned medium (CM) from bufalin-treated HUVEC significantly inhibited both HUVEC migration and blood vessel formation [78,158].

16. Bufalin modulates immune response

To date, many studies focused on the effect of bufalin on tumour cells and many things are known regarding its cytotoxic activities. However, there are not many studies focusing on the impact of bufalin on the immune system in a context of cancer while a recent study published in May 2022 in the Journal for Immunotherapy of Cancer reported a stronger effect of bufalin on decreasing tumour volume and growth rate in immunocompetent *C57BL/l* mice compared to immunodeficient nude mice. Moreover, in this context, the depletion of T cells with anti-CD4 and anti-CD8 neutralizing antibody or the macrophage deprivation decreased the anti-tumour activity of bufalin in hepatocellular carcinoma (HCC) xenograft models indicating a predominant role of the immune system in bufalin anti-cancer properties [159]. We report in this section the data on the implication of bufalin on macrophages, lymphocytes, and inflammation.

16.1. Macrophages

Bufalin promotes macrophage phagocytosis. Indeed, blood samples from mice treated with bufalin revealed that bufalin treatment led to increased phagocytosis by macrophages isolated from peripheral blood mononuclear cells or from the peritoneal cavity [160]. In colorectal cancer, the anti-inflammatory/pro-tumoral macrophages M2 play a key role in oxaliplatin chemoresistance. Bufalin reverses this chemoresistance by moderating M2 macrophages polarization through inhibition of SCR-3 and its target MIF that is crucial for M2 polarization [104]. In HCC xenograft immunocompetent mice, bufalin increased the recruitment of macrophages to the tumour site and induced their polarization toward an anti-tumour M1 phenotype. This M2 to M1 repolarization is linked to the activation of the NF-κB pathway by bufalin. *Ex vivo*, HCC cells co-cultured with Bone Marrow-Derived Macrophages (BMDMs) differentiated such macrophages into M2 phenotype, while bufalin abrogated this mechanism and increased the M1/M2 ratio. The IL-12/IL-10 ratio was also increased by bufalin (from 0.3 to 7) indicating an increased M1 polarization. Finally, the delivery of these bufalin-primed BMDMs to mice suppressed HCC development [159].

16.2. Lymphocytes

In hepatocellular carcinoma, bufalin exerts its immunomodulatory effects by modifying the balance between stimulatory and inhibitory receptors on the surface of natural killer (NK) cells. Moreover, bufalin promotes MICA expression on the cell membrane and decreases its secretion, which contributes to the induction of cytolytic activity of the NK cells. In hepatocellular carcinoma, low MICA expression in tumours is associated with a poor survival [161]. In leukaemia mouse models, bufalin stimulates NK cell cytotoxic activity as well as B-cell and T-cell proliferation [160]. A study in HCC indicated that bufalin did not directly induce T cell immune response. Indeed, splenic $CD4^+$ and $CD8^+$ T cells did not proliferate faster in the presence of bufalin. However, in bufalin treated mice, M1 polarization by bufalin promoted the migration and accumulation of T cells in the tumour. *Ex vivo*, the treatment of BMDMs by bufalin increases IL-12 and TNF-α and decreased IL-10 and TGF-β and these changes were responsible for T cell proliferation and activation. Moreover, the co-culture of bufalin treated-BMDMs and $CD8^+$ T cells enhanced their killing activity. The delivery of BMDM-primed with bufalin to HCC xenografts mice upregulated the presence T cells in the tumour and provoke their cytotoxic activity [159].

Regarding its impact on immunotherapy, this study on HCC, which is usually not a good candidate for immunotherapy due to its strong immunosuppressive tumour microenvironment, reported that bufalin could increase the efficacy of anti-PD1 antibody in vivo by modifying the microenvironment [159].

16.3. Inflammation

In in vivo colorectal cancer models, bufalin inhibited NF-kB and GSK-3β pathways resulting in suppression of the expression of pro-inflammatory mediators COX-2, TNFα, IL-1β, IL-6, CXCL1–2–5 [28]. Similarly, in a murine model of bone cancer, an anti-inflammatory effect of bufalin was associated with a decrease in cancer-induced bone pain and destruction [162]. Another study confirmed that bufalin exerted its anti-inflammatory and analgesic effects by downregulating iNOS, COX-2, IL-1β, IL-6, and TNFα [163].

17. Toxicity of bufalin on healthy tissues and in vivo models

Bufalin showed no significant toxicity in leucocytes and lymphocytes obtained from healthy donors [58,164] and no apoptosis induction even at 1 μM of bufalin in polymorphonuclear cells [165]. Other studies also showed no damages to normal ovarian and endometrial tissues and cells

Table 9
Bufalin anti-cancer effect in animal models.

Ovarian carcinoma	• Xenograft, decreased tumour size [93] • Xenograft, PET-CT indicated suppressed glucose uptake [119]
Pancreatic cancer	• Xenograft, decreased tumour size, large areas of necrosis and inflammatory exudation and increased number of apoptotic cells [26] • Xenograft, increased antitumor effect of gemcitabine [105] • Xenograft of gemcitabine resistant cells, decreased tumour growth and prolonged duration for tumour formation [150] • Injection in tail vein, fewer intestine and lung metastasis [150]
Hepatocellular carcinoma	• Inhibited HBV-induced tumour development [127] • Orthotopic, decreased tumour volume and increased necrotic areas [167] • Orthotopic, decreased primary tumour size, increased necrotic areas and number of apoptotic cells and decreased lung metastasis number and size [90] • Orthotopic and injection in tail vein, fewer metastatic lesions in lung and liver and fewer mice detected with metastasis [153] • Xenograft, attenuated tumour formation and progression [83] • Orthotopic, decreased tumour size and volume [83]
Gallbladder carcinoma	• Xenograft, decreased tumour volume [44]
Colorectal cancer	• Xenograft, increased antitumor effect of DOX [122] • Orthotopic, decreased tumour growth and prolonged survival [40] • Xenograft, increased effect of cisplatin by reduction of cisplatin-induced stemness markers [126] • Xenograft, decreased tumorigenesis and increased apoptosis [28]
Leukaemia	• Decreased tumorigenesis by enhancing immune responses [160]
Oesophageal squamous cell carcinoma	• Orthotopic, decreased tumour size [46]
Prostate cancer	• Xenograft, decreased tumour growth [47]
Lung cancer	• Xenograft, decreased tumour growth [35] • Xenograft, EGFR mutated, decreased tumour size [168]
Glioblastoma	• Xenograft, suppressed tumour growth [116]
Osteosarcoma	• Xenograft, inhibited MTX-resistant tumour growth [42]
Cervical cancer	• Xenograft, decreased tumour growth and increased paclitaxel activity [132]
Breast cancer	• Xenograft, decreased tumour growth [37]
Gastric cancer	• Xenograft, decreased tumour growth and metastasis [152]

whereas 0.2 μM bufalin eliminated almost all tumour cells sparing healthy cells [89,93,132]. It was also the case with immortalized hepatocytes where up to 1 μM concentration almost eliminated all cancer cells while merely reducing healthy cells by only 20 % [83]. Bufalin also exerts marginal effect on normal gastric mucous epithelium cell line up to 200 nmol/l [60] as well as on human mesangial and breast epithelial cell lines [34]. Finally, bufalin exhibits lower toxicity towards human oesophageal squamous cells compared to cancer cells, supporting its high selectivity [166].

Moreover, the effect of bufalin has been assessed in vivo in many cancer animal models with promising results reporting inhibition of tumour development and metastases (Table 9). None of these studies (Table 9), reported changes in animal weight or specific acute or delayed toxicities. Moreover, a study indicated no morphological changes in the myocardium, brain, liver and kidney tissues [167].

18. Bufalin pharmacokinetics and bioavailability

One of the major concerns for the implementation of bufalin in the clinic is linked to its poor pharmacological properties. Indeed, like other steroids, bufalin harbours a poor water solubility linked to its hydrophobic chemical structure. Moreover, as other cardiotonic steroids, it has a narrow therapeutic window linked to the modification in sodium and potassium balances when it affects the Na^+/K^+-ATPase pump which is ubiquitously expressed in human cells and is crucial for the proper functioning of the heart and the cardiovascular system [169,170].

Table 10
Strategies to improve bufalin bioavailability.

Formulation	Aim
Albumin sub-microspheres loaded with bufalin	Decreased toxicity [176]
Targeting immunomicelles loaded with bufalin	Slow drug release [177]
Immunoliposomes for bufalin and anti-CD40 antibody co-delivery	Improved biodistribution to the tumour and increased antitumor activity [178]
Bufalin-loaded modified abumin-polymer	Efficient tumour-targeted delivery and side effect reduction [179,180]
Acetyl-bufalin	Prodrug increasing half-life of elimination [80]
Bufalin-loaded PEGylated liposomes	Improved solubility, increased time in blood stream and increased half-life [181]
Bufalin-loaded CaP/DPPE-PEG-EGF nanospheres	Improved antitumor effect in vivo in colon cancer and increased release-time [182]
Bufalin-loaded MPEG-PLGA-PLL-CRGD nanoparticles	Prolonged and sustained release of bufalin, able to target the tumour in vivo, increased anticancer effect on colorectal cancer in vivo [183]
Bufalin loaded biotinylated chitosan nanoparticles	Slower bufalin release, increased antitumor effect on breast cancer in vivo [184]
Bufalin-loaded pluronic polyetherimide nanoparticles	Decreased toxicity linked to controlled release and increased antitumor effect on colon cancer in vivo [185]
PEG-based polymeric prodrug of bufalin	Improved water solubility and stability [186]
Octreotide-modified esterase-sensitive tumour-targeting polymeric prodrug of bufalin	Increased cytotoxicity, cellular uptake and apoptotic induction in vitro and improved tumour accumulation in breast cancer in vivo [187]
Folate receptor targeted bufalin/β-cyclodextrin supramolecular inclusion complex	Increased water solubility and increased antitumor efficiency [188]
BU-loaded VES-CSO/TPGS-RGD mixed micelles	Improved stability, sustained release pattern, higher intracellular uptake, and greater cytotoxicity in vitro and reduced side effects in vivo
Platelet-membrane-biomimetic nanoparticles	Increased tumour accumulation leading to more effective tumour growth inhibition [189]
Folic acid-modified MOFs (metal-organic frameworks)	Improved water solubility, stability and intracellular uptake in vitro and improved tumour accumulation and reduced side effects in vivo [190]
BF211, a bufalin derivative	Greater apoptotic induction and lower acute toxicity in multiple myeloma [77] and lung cancer [191]
3-phospho-bufalin, prodrug	Increased water solubility [100]

Some studies also reported a fast distribution in rat and mice (half-life of 0.0693 min in blood after i.v. injected) [80,171]. Bufalin clearance occurs through the hepatic (by CYP3A4 [172–174]) and renal routes with a half-life of 510 min [171].

As bufalin has a poor water solubility and is rapidly distributed and metabolized, many formulation studies are now focusing on developing tools to improve bufalin bioavailability by increasing its water solubility and tumour uptake while decreasing its cardiac toxicity (Table 10). In this context, tumor-vectorized bufalin-grafted magnetic iron oxide nanoparticles could constitute an interesting theranostic strategy combining improved tumour uptake of unmetabolized bufalin and possibility of magnetic resonance imaging [175].

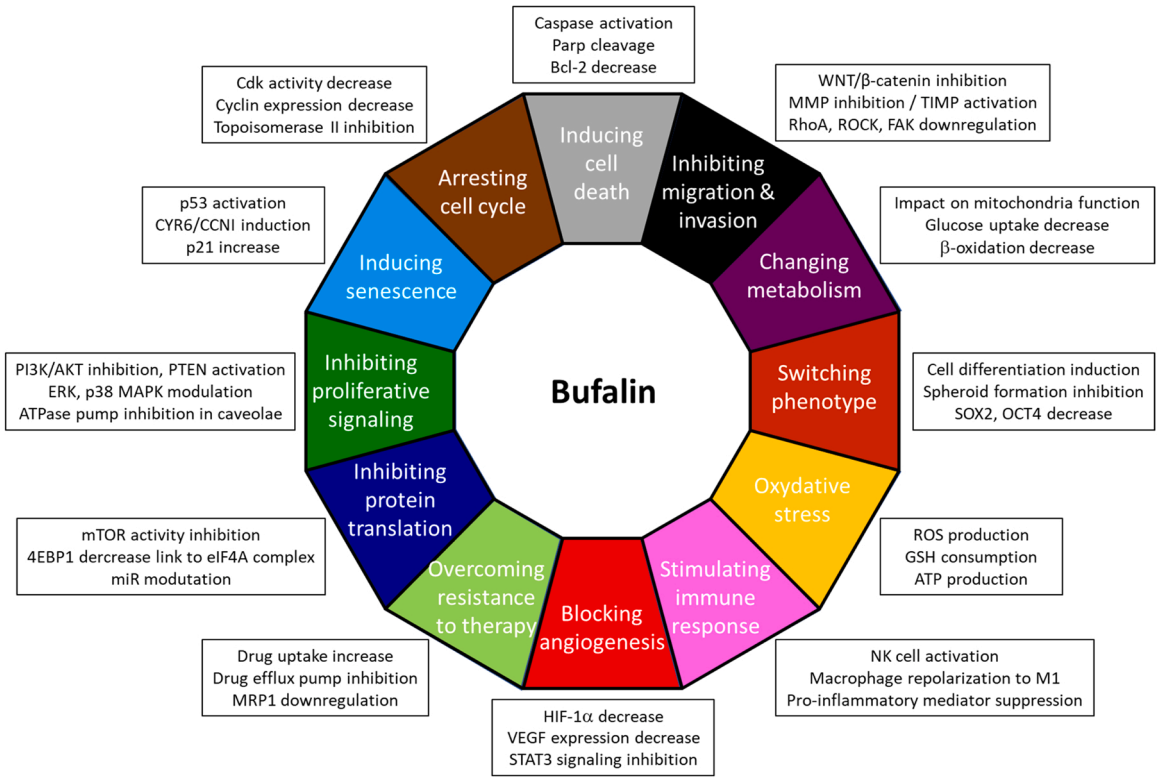

Fig. 3. Hallmarks of bufalin against cancer (*need to be printed in colours*).

19. Discussion and perspectives

Firstly, we will focus on the advantages of bufalin as a potential future cancer treatment.

All the data discussed in the current review indicated that bufalin could act on many hallmarks of cancer which could make it a candidate of choice for the treatment of such diseases. Indeed, actual treatment, such as targeted therapy mainly focus on only one specific oncogenic mechanism which is the main cause of resistance development. As bufalin target many oncogenic pathways, the development of resistance should be delayed or blocked, making combination more effective. Among the mechanisms of action of bufalin, one even more interesting is its ability to reverse stemness which is often associated with resistance and metastasis development.

Its main target is the α-subunit ATP1A1 of the sodium pump which is overexpressed in many cancers. By acting on ATP1A1, bufalin induced the inactivation of the SRC protein kinase and of many downstream signalling pathways, inhibiting cell survival. Another important target of bufalin is the co-activator SRC-3 of transcription factors. Indeed, by inhibiting SRC-3, bufalin is able to affect the transcription of many genes, including genes coding for specific proteins involved in the translation complex toward cancer-associated proteins (Fig. 2). Hence, targeting ATP1A1 and SRC-3, bufalin can impact signalling pathways, gene transcription and translation, all important regulatory processes in cancer.

On the other hand, bufalin is a cardiotonic steroid, as digoxine which has also demonstrated interesting anti-tumour properties [192]. Moreover, epidemiological studies have highlighted lower risks of developing leukemia and renal tumours as well as lower risks of breast cancer recurrence in patient treated with cardiotonic steroids for other heart disorders [102]. But the major problem with these molecules is their potential cardiac toxicity and, as a result, their small therapeutic windows. This problem could be less pronounced for bufalin use since it is already endogenously present in serum of healthy people [12]. Of note, bufalin is detected at a relatively low level (5.7 nM) in healthy humans but this level could be sufficient to fight cancer development as the concentrations used in the literature to decrease tumour development in vitro and in vivo range from 1 to 10 nM. Moreover, bufalin levels are significantly decreased in cancer patients [12]. This major observation shed a completely new perspective suggesting that restoring normal bufalin levels in cancer patients could limit and delay tumour spread.

Then, we will emphasis on the major disadvantage of bufalin and how we could work to improve its use in clinics.

The major drawbacks of bufalin are its potential cardiotoxicity as well as its low solubility and its fast distribution and metabolization observed in in vivo studies. There are a few reasons to hope that bufalin used at low doses should not induce cardiotoxicity as in vivo studies did not observed any morphological changes in the myocardium of mice or rats treated with this compound. Moreover, as explained above, bufalin is endogenously expressed in humans; so if used at physiological doses, it should not induce toxicity. To counteract its low bioavailability, it is crucial to better understand how bufalin is synthesized in the human body and how it is stored, and to try to stimulate its endogenous production. On the other hand, the I.V. administration of bufalin-coupled nanoparticles could be an effective alternative strategy to improve bioavailability of the molecule for cancers.

Regarding the possibility of cancer treatment with bufalin and knowing the current necessity of treatment combinations, it is difficult to imagine a treatment relying only on bufalin today. Nevertheless, bufalin could be used at small doses to improve the efficacy of other conventional chemotherapy or targeted treatments as it already showed synergy with many drugs in different cancer types. In addition, in the context of the development of immunotherapy, bufalin could be a very interesting molecule to switch the immunosuppressive tumour microenvironment into a more immunocompetent one and to improve the response to immunotherapeutic strategies.

20. Conclusions

In the past few years, bufalin has been largely studied in the context

of oncology in many cancer types for its major regulatory role in various tumorigenic pathways. Taken together, all data discussed within the present review support that bufalin exerts its antitumor effect by acting on all the hallmarks of cancer (Fig. 3). Indeed, bufalin acts on tumour growth by inducing cell death, blocking cell cycle, stimulating senescence, or inhibiting proliferative signaling and protein translation. It also moderates metastatic spread by inhibiting cell migration and invasion as well as by blocking angiogenesis. Bufalin also modifies other aspects of cancer cells such as their metabolism, phenotype, or level of oxidative stress. Finally, bufalin could improve the effect of current anticancer therapies by overcoming resistance or/and stimulating immune response. Despite that bufalin could be seen as a molecule of choice to improve cancer treatment, the major problem is linked to a narrow therapeutic window because of a possible cardiotoxicity, that can, however, be easily bypassed by using prodrugs, drug formulations to improve tumour delivery or/and repeated low dose administration.

CRediT authorship contribution statement

Laura Soumoy: Investigation, Writing – original draft, Writing – review & editing, Visualization. **Ghanem Ghanem:** Writing – original draft, Funding acquisition. **Sven Saussez**: Writing – original draft, Funding acquisition. **Fabrice Journe:** Writing – original draft, Writing – review & editing, Supervision, Funding acquisition, Visualization.

Competing interests

The authors have no competing interests.

Data Availability

No data was used for the research described in the article.

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References

[1] A.F.M. Botelho, F. Pierezan, B. Soto-Blanco, M.M. Melo, A review of cardiac glycosides: structure, toxicokinetics, clinical signs, diagnosis and antineoplastic potential, Toxicon Off. J. Int. Soc. Toxinol. 158 (2019) 63–68, https://doi.org/10.1016/j.toxicon.2018.11.429.

[2] J. Bejček, V. Spiwok, E. Kmoníčková, S. Rimpelová, Na+/K+-ATPase revisited: on its mechanism of action, role in cancer, and activity modulation, Molecules 26 (2021) 1905, https://doi.org/10.3390/molecules26071905.

[3] L.-J. Deng, Y. Li, M. Qi, J.-S. Liu, S. Wang, L.-J. Hu, Y.-H. Lei, R.-W. Jiang, W.-M. Chen, Q. Qi, et al., Molecular mechanisms of bufadienolides and their novel strategies for cancer treatment, Eur. J. Pharmacol. 887 (2020), 173379, https://doi.org/10.1016/j.ejphar.2020.173379.

[4] F.-J. Li, J.-H. Hu, X. Ren, C.-M. Zhou, Q. Liu, Y.-Q. Zhang, Toad venom: a comprehensive review of chemical constituents, anticancer activities, and mechanisms, Arch. Pharm. 354 (2021), e2100060, https://doi.org/10.1002/ardp.202100060.

[5] T. Mijatovic, E. Van Quaquebeke, B. Delest, O. Debeir, F. Darro, R. Kiss, Cardiotonic steroids on the road to anti-cancer therapy, Biochim. Biophys. Acta 1776 (2007) 32–57, https://doi.org/10.1016/j.bbcan.2007.06.002.

[6] C.-S. Cheng, J. Wang, J. Chen, K.T. Kuo, J. Tang, H. Gao, L. Chen, Z. Chen, Z. Meng, New therapeutic aspects of steroidal cardiac glycosides: the anticancer properties of huachansu and its main active constituent bufalin, Cancer Cell Int. 19 (2019) 92, https://doi.org/10.1186/s12935-019-0806-1.

[7] Y.N. Utkin, Animal venom studies: current benefits and future developments, World J. Biol. Chem. 6 (2015) 28–33, https://doi.org/10.4331/wjbc.v6.i2.28.

[8] C. Rodriguez, R. Ibáñez, L.A. Rollins-Smith, M. Gutiérrez, A.A. Durant-Archibold, Antimicrobial secretions of toads (Anura, Bufonidae): bioactive extracts and isolated compounds against human pathogens, Antibiot. Basel Switz. 9 (2020), E843, https://doi.org/10.3390/antibiotics9120843.

[9] L.Q. Sousa, K. de; Machado, C. da, S.F. Oliveira, C. de, L. Araújo, S. da, E.D. S. Monção-Filho, A.A. Melo-Cavalcante, C. de, G.M. Vieira-Júnior, P.M. P. Ferreira, Bufadienolides from amphibians: a promising source of anticancer prototypes for radical innovation, apoptosis triggering and Na+/K+-ATPase inhibition, Toxicon Off. J. Int. Soc. Toxinol. 127 (2017) 63–76, https://doi.org/10.1016/j.toxicon.2017.01.004.

[10] V.A. Reznik, V.A. Kashkin, N.I. Agalakova, C.D. Adair, A.Y. Bagrov, Endogenous bufadienolides, fibrosis and preeclampsia, Cardiol. Res. Pract. 2019 (2019) 5019287, https://doi.org/10.1155/2019/5019287.

[11] M. Oda, M. Kurosawa, S. Numazawa, S. Tanaka, T. Akizawa, K. Ito, M. Maeda, T. Yoshida, Determination of bufalin-like immunoreactivity in serum of humans and rats by time-resolved fluoroimmunoassay for using a monoclonal antibody, Life Sci. 68 (2001) 1107–1117, https://doi.org/10.1016/s0024-3205(00)01013-4.

[12] M. Han, G. Yang, Q. Lin, Y. Yang, H. Zhang, Y. Su, Determination of endogenous bufalin in serum of patients with hepatocellular carcinoma based on HPLC-MS/MS, Front. Oncol. 9 (2019) 1572, https://doi.org/10.3389/fonc.2019.01572.

[13] Z. Meng, P. Yang, Y. Shen, W. Bei, Y. Zhang, Y. Ge, R.A. Newman, L. Cohen, L. Liu, B. Thornton, et al., Pilot study of huachansu in patients with hepatocellular carcinoma, non-small cell lung cancer, or pancreatic cancer, Cancer 115 (2009) 5309–5318, https://doi.org/10.1002/cncr.24602.

[14] M.D. Anderson, Cancer center a randomized, placebo-controlled, blinded phase II study of huachansu & gemcitabine in pancreatic cancer, Clin. Gov. (2012).

[15] Z. Meng, C.R. Garrett, Y. Shen, L. Liu, P. Yang, Y. Huo, Q. Zhao, A.R. Spelman, C. S. Ng, D.Z. Chang, et al., Prospective randomised evaluation of traditional chinese medicine combined with chemotherapy: a randomised phase II study of wild toad extract plus gemcitabine in patients with advanced pancreatic adenocarcinomas, Br. J. Cancer 107 (2012) 411–416, https://doi.org/10.1038/bjc.2012.283.

[16] X. Zhai, Z. Chen, B. Li, F. Shen, J. Fan, W. Zhou, Y.-K. Yang, J. Xu, X. Qin, L. Li, et al., Traditional herbal medicine in preventing recurrence after resection of small hepatocellular carcinoma: a multicenter randomized controlled trial, J. Integr. Med. 11 (2013) 90–100, https://doi.org/10.3736/jintegrmed2013021.

[17] C.-Q. Ling, J. Fan, H.-S. Lin, F. Shen, Z.-Y. Xu, L.-Z. Lin, S.-K. Qin, W.-P. Zhou, X.-F. Zhai, B. Li, et al., Clinical practice guidelines for the treatment of primary liver cancer with integrative Traditional Chinese and Western Medicine, J. Integr. Med. 16 (2018) 236–248, https://doi.org/10.1016/j.joim.2018.05.002.

[18] Z. Chen, X.-F. Zhai, Y.-H. Su, X.-Y. Wan, J. Li, J.-M. Xie, B. Gao, Clinical observation of cinobufacini injection used to treat moderate and advanced primary liver cancer, Zhong Xi Yi Jie He Xue Bao 1 (2003) 184–186, https://doi.org/10.3736/jcim20030311.

[19] X. Tan, X. Liang, J. Xi, S. Guo, M. Meng, X. Chen, Y. Li, Clinical efficacy and safety of Huachansu Injection Combination with platinum-based chemotherapy for advanced non-small cell lung cancer, Medicine 100 (2021), e27161, https://doi.org/10.1097/MD.0000000000027161.

[20] Y. Jiang, L.-S. Liu, L.-P. Shen, Z.-F. Han, H. Jian, J.-X. Liu, L. Xu, H.-G. Li, J.-H. Tian, Z.-J. Mao, Traditional Chinese Medicine treatment as maintenance therapy in advanced non-small-cell lung cancer: a randomized controlled trial, Complement. Ther. Med. 24 (2016) 55–62, https://doi.org/10.1016/j.ctim.2015.12.006.

[21] Y. Jiang, L.B. Wu, L.P. Shen, P. Zhang, W.J. Jiang, J.H. Tian, L.S. Liu, Effect of traditional Chinese medicine treatment as maintenance therapy on regulating the serum concentration of sCTLA-4 in patients with advanced non-small-cell lung cancer and its relationship with prognosis, Zhonghua Zhong Liu Za Zhi 38 (2016) 757–762, https://doi.org/10.3760/cma.j.issn.0253-3766.2016.10.009.

[22] J. Wu, D. Zhang, M. Ni, J. Xue, K. Wang, X. Duan, S. Liu, Effectiveness of huachansu injection combined with chemotherapy for treatment of gastric cancer in China: a systematic review and meta-analysis, J. Tradit. Chin. Med. Chung Tsa Chih Ying Wen Pan 40 (2020) 749–757, https://doi.org/10.19852/j.cnki.jtcm.2020.05.004.

[23] T.-J. Qin, X.-H. Zhao, J. Yun, L.-X. Zhang, Z.-P. Ruan, B.-R. Pan, Efficacy and safety of gemcitabine-oxaliplatin combined with huachansu in patients with advanced gallbladder carcinoma, World J. Gastroenterol. 14 (2008) 5210–5216, https://doi.org/10.3748/wjg.14.5210.

[24] R. Gu, Q. Zhang, Effects of low-dose bufalin combined with hydroxycamptothecin on human castration-resistant prostate cancer xenografts in nude mice, Exp. Ther. Med. 22 (2021) 1015, https://doi.org/10.3892/etm.2021.10447.

[25] F. Chen, L. Zhu, J. Hu, S. Jiang, H. Liu, J. Zheng, J. Wang, F. Wang, Z. Li, Bufalin attenuates triple-negative breast cancer cell stemness by inhibiting the expression of SOX2/OCT4, Oncol. Lett. 20 (2020) 171, https://doi.org/10.3892/ol.2020.12028.

[26] X. Liu, Y. Zhou, J. Peng, B. Xie, Q. Shou, J. Wang, Silencing C-Myc enhances the antitumor activity of bufalin by suppressing the HIF-1α/SDF-1/CXCR4 pathway in pancreatic cancer cells, Front. Pharmacol. 11 (2020) 495, https://doi.org/10.3389/fphar.2020.00495.

[27] S.-C. Tsai, J.-S. Yang, S.-F. Peng, C.-C. Lu, J.-H. Chiang, J.-G. Chung, M.-W. Lin, J.-K. Lin, S. Amagaya, C. Wai-Shan Chung, et al., Bufalin increases sensitivity to AKT/MTOR-induced autophagic cell death in SK-HEP-1 human hepatocellular carcinoma cells, Int. J. Oncol. 41 (2012) 1431–1442, https://doi.org/10.3892/ijo.2012.1579.

[28] X. Sun, T.T.H. Ng, K.W.Y. Sham, L. Zhang, M.T.V. Chan, W.K.K. Wu, C.H. K. Cheng, Bufalin, a Traditional Chinese Medicine Compound, prevents tumor formation in two murine models of colorectal cancer, Cancer Prev. Res. Phila. Pa 12 (2019) 653–666, https://doi.org/10.1158/1940-6207.CAPR-19-0134.

[29] S.-H. Wu, Y.-T. Hsiao, J.-C. Chen, J.-H. Lin, S.-C. Hsu, T.-C. Hsia, S.-T. Yang, W.-H. Hsu, J.-G. Chung, Bufalin alters gene expressions associated DNA damage, cell cycle, and apoptosis in human lung cancer NCI-H460 cells in vitro, Mol. Basel Switz. 19 (2014) 6047–6057, https://doi.org/10.3390/molecules19056047.

[30] D. Wu, W.-Y. Zhou, X.-T. Lin, L. Fang, C.-M. Xie, Bufalin induces apoptosis via mitochondrial ROS-mediated caspase-3 activation in HCT-116 and SW620 human

colon cancer cells, Drug Chem. Toxicol. 42 (2019) 444–450, https://doi.org/10.1080/01480545.2018.1512611.
[31] S.-C. Tsai, C.-C. Lu, C.-Y. Lee, Y.-C. Lin, J.-G. Chung, S.-C. Kuo, S. Amagaya, F.-N. Chen, M.Y. Chen, S.-F. Chan, et al., AKT serine/threonine protein kinase modulates bufalin-triggered intrinsic pathway of apoptosis in CAL 27 human oral cancer cells, Int. J. Oncol. 41 (2012) 1683–1692, https://doi.org/10.3892/ijo.2012.1605.
[32] F. Qi, Y. Inagaki, B. Gao, X. Cui, H. Xu, N. Kokudo, A. Li, W. Tang, Bufalin and cinobufagin induce apoptosis of human hepatocellular carcinoma cells via fas- and mitochondria-mediated pathways, Cancer Sci. 102 (2011) 951–958, https://doi.org/10.1111/j.1349-7006.2011.01900.x.
[33] L. Sun, T. Chen, X. Wang, Y. Chen, X. Wei, Bufalin induces reactive oxygen species dependent bax translocation and apoptosis in ASTC-a-1 cells, Evid. Based Complement. Altern. Med. ECAM 2011 (2011), 249090, https://doi.org/10.1093/ecam/nep082.
[34] C.-H. Yu, S.-F. Kan, H.-F. Pu, E. Jea Chien, P.S. Wang, Apoptotic signaling in bufalin- and cinobufagin-treated androgen-dependent and -independent human prostate cancer cells, Cancer Sci. 99 (2008) 2467–2476, https://doi.org/10.1111/j.1349-7006.2008.00966.x.
[35] S.-H. Wu, D.-T. Bau, Y.-T. Hsiao, K.-W. Lu, T.-C. Hsia, J.-C. Lien, Y.-C. Ko, W.-H. Hsu, S.-T. Yang, Y.-P. Huang, et al., Bufalin induces apoptosis in vitro and has antitumor activity against human lung cancer xenografts in vivo, Environ. Toxicol. 32 (2017) 1305–1317, https://doi.org/10.1002/tox.22325.
[36] K.-H. Kang, M.H. Han, J.-W. Jeong, C. Park, S.-H. Lee, H.W. Lee, S.H. Hong, Y. H. Choi, S.H. Hong, Bufalin sensitizes human bladder carcinoma cells to TRAIL-mediated apoptosis, Oncol. Lett. 14 (2017) 853–859, https://doi.org/10.3892/ol.2017.6223.
[37] Y. Li, X. Tian, X. Liu, P. Gong, Bufalin inhibits human breast cancer tumorigenesis by inducing cell death through the ROS-mediated RIP1/RIP3/PARP-1 pathways, Carcinogenesis 39 (2018) 700–707, https://doi.org/10.1093/carcin/bgy039.
[38] S.-H. Hong, Y.H. Choi, Bufalin induces apoptosis through activation of both the intrinsic and extrinsic pathways in human bladder cancer cells, Oncol. Rep. 27 (2012) 114–120, https://doi.org/10.3892/or.2011.1451.
[39] H. Huang, Y. Cao, W. Wei, W. Liu, S.-Y. Lu, Y.-B. Chen, Y. Wang, H. Yan, Y.-L. Wu, Targeting poly (ADP-Ribose) polymerase partially contributes to bufalin-induced cell death in multiple myeloma cells, PLoS One 8 (2013), e66130, https://doi.org/10.1371/journal.pone.0066130.
[40] J. Wang, C. Chen, S. Wang, Y. Zhang, P. Yin, Z. Gao, J. Xu, D. Feng, Q. Zuo, R. Zhao, et al., Bufalin inhibits HCT116 colon cancer cells and its orthotopic xenograft tumor in mice model through genes related to apoptotic and PTEN/AKT pathways, Gastroenterol. Res. Pract. 2015 (2015), 457193, https://doi.org/10.1155/2015/457193.
[41] W. Gu, K. Han, Y. Su, X. Huang, C. Ling, Inhibition action of bufalin on human transplanted hepatocellular tumor and its effects on expressions of Bcl-2 and Bax proteins in nude mice, Zhong Xi Yi Jie He Xue Bao 5 (2007) 155–159, https://doi.org/10.3736/jcim20070211.
[42] X. Xie, J. Yin, L. Wen, Z. Gao, C. Zou, J. Wang, G. Huang, Q. Tang, C. Colombo, W. He, et al., Critical role of heat shock protein 27 in bufalin-induced apoptosis in human osteosarcomas: a proteomic-based research, PLoS One 7 (2012), e47375, https://doi.org/10.1371/journal.pone.0047375.
[43] X.-H. Kang, J.-H. Zhang, Q.-Q. Zhang, Y.-H. Cui, Y. Wang, W.-Z. Kou, Z.-H. Miao, P. Lu, L.-F. Wang, Z.-Y. Xu, et al., Degradation of Mcl-1 through GSK-3β activation regulates apoptosis induced by bufalin in non-small cell lung cancer H1975 cells, Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol. 41 (2017) 2067–2076, https://doi.org/10.1159/000475438.
[44] L. Qian, H. Su, G. Wang, B. Li, G. Shen, Q. Gao, Anti-tumor activity of bufalin by inhibiting c-MET mediated MEK/ERK and PI3K/AKT signaling pathways in gallbladder cancer, J. Cancer 11 (2020) 3114–3123, https://doi.org/10.7150/jca.38393.
[45] Y. Dong, S. Yin, J. Li, C. Jiang, M. Ye, H. Hu, Bufadienolide compounds sensitize human breast cancer cells to TRAIL-induced apoptosis via inhibition of STAT3/Mcl-1 pathway, Apoptosis Int. J. Program. Cell Death 16 (2011) 394–403, https://doi.org/10.1007/s10495-011-0573-5.
[46] Y. Liu, X. Wang, Y. Jia, Y. Liu, Effects of bufalin on the MTOR/P70S6K pathway and apoptosis in esophageal squamous cell carcinoma in nude mice, Int. J. Mol. Med. 40 (2017) 357–366, https://doi.org/10.3892/ijmm.2017.3039.
[47] Y. Zhang, Y. Dong, M.W. Melkus, S. Yin, S.-N. Tang, P. Jiang, K. Pramanik, W. Wu, S. Kim, M. Ye, et al., Role of P53-senescence induction in suppression of LNCaP prostate cancer growth by cardiotonic compound bufalin, Mol. Cancer Ther. 17 (2018) 2341–2352, https://doi.org/10.1158/1535-7163.MCT-17-1296.
[48] Y.-L. Lan, Y.-J. Zou, J.-C. Lou, J.-S. Xing, X. Wang, S. Zou, B.-B. Ma, Y. Ding, B. Zhang, The sodium pump A1 subunit regulates bufalin sensitivity of human glioblastoma cells through the P53 signaling pathway, Cell Biol. Toxicol. 35 (2019) 521–539, https://doi.org/10.1007/s10565-019-09462-y.
[49] Y. Li, Y. Zhang, X. Wang, Q. Yang, X. Zhou, J. Wu, X. Yang, Y. Zhao, R. Lin, Y. Xie, et al., Bufalin induces mitochondrial dysfunction and promotes apoptosis of glioma cells by regulating annexin A2 and DRP1 protein expression, Cancer Cell Int. 21 (2021) 424, https://doi.org/10.1186/s12935-021-02137-x.
[50] S. Shen, Y. Zhang, Z. Wang, R. Liu, X. Gong, Bufalin induces the interplay between apoptosis and autophagy in glioma cells through endoplasmic reticulum stress, Int. J. Biol. Sci. 10 (2014) 212–224, https://doi.org/10.7150/ijbs.8056.
[51] Y. Chen, M. Li, Z. Li, P. Gao, X. Zhou, J. Zhang, Bufalin induces apoptosis in the U-2OS human osteosarcoma cell line via triggering the mitochondrial pathway, Mol. Med. Rep. 13 (2016) 817–822, https://doi.org/10.3892/mmr.2015.4583.
[52] J. Song, D. Zou, X. Zhao, Y. Chen, F. Lv, S. Wang, D. Sui, Q. Han, C. Yang, X. Wang, et al., Bufalin inhibits human diffuse large B-Cell lymphoma tumorigenesis by inducing cell death through the Ca2+/NFATC1/CMYC pathway, Carcinogenesis 42 (2021) 303–314, https://doi.org/10.1093/carcin/bgaa108.
[53] Y.-P. Hsiao, C.-S. Yu, C.-C. Yu, J.-S. Yang, J.-H. Chiang, C.-C. Lu, H.-Y. Huang, N.-Y. Tang, J.-H. Yang, A.-C. Huang, et al., Triggering apoptotic death of human malignant melanoma A375.S2 cells by bufalin: involvement of caspase cascade-dependent and independent mitochondrial signaling pathways, Evid. Based Complement. Altern. Med. ECAM 2012 (591241) (2012), https://doi.org/10.1155/2012/591241.
[54] E.-Y. Su, Y.-L. Chu, F.-S. Chueh, Y.-S. Ma, S.-F. Peng, W.-W. Huang, C.-L. Liao, A.-C. Huang, J.-G. Chung, Bufalin induces apoptotic cell death in human nasopharyngeal carcinoma cells through mitochondrial ROS and TRAIL pathways, Am. J. Chin. Med. 47 (2019) 237–257, https://doi.org/10.1142/S0192415×19500125.
[55] W.-W. Huang, J.-S. Yang, S.-J. Pai, P.-P. Wu, S.-J. Chang, F.-S. Chueh, M.-J. Fan, S.-M. Chiou, H.-M. Kuo, C.-C. Yeh, et al., Bufalin Induces G0/G1 phase arrest through inhibiting the levels of cyclin D, Cyclin E, CDK2 and CDK4, and triggers apoptosis via mitochondrial signaling pathway in T24 human bladder cancer cells, Mutat. Res. 732 (2012) 26–33, https://doi.org/10.1016/j.mrfmmm.2011.09.010.
[56] Z. Zhu, E. Li, Y. Liu, Y. Gao, H. Sun, G. Ma, Z. Wang, X. Liu, Q. Wang, X. Qu, et al., Inhibition of Jak-STAT3 pathway enhances bufalin-induced apoptosis in colon cancer SW620 cells, World J. Surg. Oncol. 10 (2012) 228, https://doi.org/10.1186/1477-7819-10-228.
[57] N. Zhang, Y. Xie, Y. Tai, Y. Gao, W. Guo, W. Yu, J. Li, X. Feng, J. Hao, Y. Gao, et al., Bufalin inhibits HTERT expression and colorectal cancer cell growth by targeting CPSF4, Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharm. 40 (2016) 1559–1569, https://doi.org/10.1159/000453206.
[58] S. Abdelfatah, X. Lu, G. Schmeda-Hirschmann, T. Efferth, Cytotoxicity and antimitotic activity of Rhinella schneideri and Rhinella marina venoms, J. Ethnopharmacol. 242 (2019), 112049, https://doi.org/10.1016/j.jep.2019.112049.
[59] H.-Y. Chou, F.-S. Chueh, Y.-S. Ma, R.S.-C. Wu, C.-L. Liao, Y.-L. Chu, M.-J. Fan, W.-W. Huang, J.-G. Chung, Bufalin induced apoptosis in SCC-4 human tongue cancer cells by decreasing Bcl-2 and increasing bax expression via the mitochondria-dependent pathway, Mol. Med. Rep. 16 (2017) 7959–7966, https://doi.org/10.3892/mmr.2017.7651.
[60] H. Zhao, Q. Li, J. Pang, H. Jin, H. Li, X. Yang, Blocking autophagy enhances the pro-apoptotic effect of bufalin on human gastric cancer cells through endoplasmic reticulum stress, Biol. Open 6 (2017) 1416–1422, https://doi.org/10.1242/bio.026344.
[61] W. Gu, L. Liu, F.-F. Fang, F. Huang, B.-B. Cheng, B. Li, Reversal effect of bufalin on multidrug resistance in human hepatocellular carcinoma BEL-7402/5-FU cells, Oncol. Rep. 31 (2014) 216–222, https://doi.org/10.3892/or.2013.2817.
[62] C.-H. Lee, Y.-L. Shih, M.-H. Lee, M.-K. Au, Y.-L. Chen, H.-F. Lu, J.-G. Chung, Bufalin induces apoptosis of human osteosarcoma U-2 OS cells through endoplasmic reticulum stress, caspase- and mitochondria-dependent signaling pathways, Mol. Basel Switz. 22 (2017), E437, https://doi.org/10.3390/molecules22030437.
[63] J.-Q. Yin, J.-N. Shen, W.-W. Su, J. Wang, G. Huang, S. Jin, Q.-C. Guo, C.-Y. Zou, H.-M. Li, F.-B. Li, Bufalin induces apoptosis in human osteosarcoma U-2OS and U-2OS methotrexate300-resistant cell lines, Acta Pharmacol. Sin. 28 (2007) 712–720, https://doi.org/10.1111/j.1745-7254.2007.00559.x.
[64] S. Yan, X. Qu, C. Xu, Z. Zhu, L. Zhang, L. Xu, N. Song, Y. Teng, Y. Liu, Down-regulation of Cbl-b by bufalin results in up-regulation of DR4/DR5 and sensitization of TRAIL-induced apoptosis in breast cancer cells, J. Cancer Res. Clin. Oncol. 138 (2012) 1279–1289, https://doi.org/10.1007/s00432-012-1204-4.
[65] S. Yan, X. Qu, L. Xu, X. Che, Y. Ma, L. Zhang, Y. Teng, H. Zou, Y. Liu, Bufalin enhances TRAIL-induced apoptosis by redistributing death receptors in lipid rafts in breast cancer cells, Anticancer Drugs 25 (2014) 683–689, https://doi.org/10.1097/CAD.0000000000000095.
[66] L. Pan, L. Nie, S. Yao, A. Bi, Y. Ye, Y. Wu, Z. Tan, Z. Wu, Bufalin exerts antitumor effects in neuroblastoma via the induction of reactive oxygen species-mediated apoptosis by targeting the electron transport Chain, Int. J. Mol. Med. 46 (2020) 2137–2149, https://doi.org/10.3892/ijmm.2020.4745.
[67] X. Tian, S. Dai, J. Sun, S. Jiang, C. Sui, F. Meng, Y. Li, L. Fu, T. Jiang, Y. Wang, et al., Bufalin induces mitochondria-dependent apoptosis in pancreatic and oral cancer cells by downregulating HTERT expression via activation of the JNK/P38 pathway, Evid. Based Complement. Altern. Med. ECAM 2015 (2015), 546210, https://doi.org/10.1155/2015/546210.
[68] X.-B. Xie, L.-L. Wen, J.-Q. Yin, H.-Y. Liao, C.-Y. Zou, B. Wang, G. Huang, J.-N. Shen, Proteomics research of bufalin-induced apoptosis in osteosarcoma cell lines, Zhongguo Zhong Yao Za Zhi Zhongguo Zhongyao Zazhi China J. Chin. Mater. Med. 39 (2014) 2739–2743.
[69] Y. Li, P. Gong, C. Kong, X. Tian, Bufalin engages in RIP1-dependent and ROS-dependent programmed necroptosis in breast cancer cells by targeting the RIP1/RIP3/PGAM5 pathway, Anticancer Drugs 30 (2019), e0770, https://doi.org/10.1097/CAD.0000000000000770.
[70] X.-D. Liu, C.-Y. Song, C.-C. Kong, X. Tian, Bufalin induces programmed necroptosis in triple-negative breast cancer drug-resistant cell lines through RIP1/ROS-mediated pathway, Chin. J. Integr. Med. (2021), https://doi.org/10.1007/s11655-021-3458-7.
[71] H.R. LingHu, H. Luo, L. Gang, Bufalin induces glioma cell death by apoptosis or necroptosis, OncoTargets Ther. 13 (2020) 4767–4778, https://doi.org/10.2147/OTT.S242567.

[72] H.-Y. Qi, X.-J. Qu, J. Liu, K.-Z. Hou, Y.-B. Fan, X.-F. Che, Y.-P. Liu, Bufalin induces protective autophagy by Cbl-b Regulating MTOR and ERK signaling pathways in gastric cancer cells, Cell Biol. Int. 43 (2019) 33–43, https://doi.org/10.1002/cbin.11076.
[73] X. Sheng, P. Zhu, J. Qin, Q. Li, The biological role of autophagy in regulating and controlling the proliferation of liver cancer cells induced by bufalin, Oncol. Rep. 39 (2018) 2931–2941, https://doi.org/10.3892/or.2018.6365.
[74] C.-M. Xie, W.Y. Chan, S. Yu, J. Zhao, C.H.K. Cheng, Bufalin induces autophagy-mediated cell death in human colon cancer cells through reactive oxygen species generation and JNK activation, Free Radic. Biol. Med. 51 (2011) 1365–1375, https://doi.org/10.1016/j.freeradbiomed.2011.06.016.
[75] Q. Miao, L.-L. Bi, X. Li, S. Miao, J. Zhang, S. Zhang, Q. Yang, Y.-H. Xie, J. Zhang, S.-W. Wang, Anticancer effects of bufalin on human hepatocellular carcinoma HepG2 cells: roles of apoptosis and autophagy, Int. J. Mol. Sci. 14 (2013) 1370–1382, https://doi.org/10.3390/ijms14011370.
[76] F. Hu, J. Han, B. Zhai, X. Ming, L. Zhuang, Y. Liu, S. Pan, T. Liu, Blocking autophagy enhances the apoptosis effect of bufalin on human hepatocellular carcinoma cells through endoplasmic reticulum stress and JNK activation, Apoptosis Int. J. Program. Cell Death 19 (2014) 210–223, https://doi.org/10.1007/s10495-013-0914-7.
[77] X.-Y. Wu, F. Tian, M.-H. Su, M. Wu, Y. Huang, L.-H. Hu, L. Jin, X.-J. Zhu, BF211, a derivative of bufalin, enhances the cytocidal effects in multiple myeloma cells by inhibiting the IL-6/JAK2/STAT3 pathway, Int. Immunopharmacol. 64 (2018) 24–32, https://doi.org/10.1016/j.intimp.2018.08.016.
[78] H. Yang, Y. Liu, M.-M. Zhao, Q. Guo, X.-K. Zheng, D. Liu, K.-W. Zeng, P.-F. Tu, Therapeutic potential of targeting membrane-spanning proteoglycan SDC4 in hepatocellular carcinoma, Cell Death Dis. 12 (2021) 492, https://doi.org/10.1038/s41419-021-03780-y.
[79] Y. Jing, M. Watabe, S. Hashimoto, S. Nakajo, K. Nakaya, Cell cycle arrest and protein kinase modulating effect of bufalin on human leukemia ML1 cells, Anticancer Res. 14 (1994) 1193–1198.
[80] L. Yang, F. Zhou, Y. Zhuang, Y. Liu, L. Xu, H. Zhao, Y. Xiang, X. Dai, Z. Liu, X. Huang, et al., Acetyl-bufalin shows potent efficacy against non-small-cell lung cancer by targeting the CDK9/STAT3 signalling pathway, Br. J. Cancer 124 (2021) 645–657, https://doi.org/10.1038/s41416-020-01135-6.
[81] C.-M. Xie, X.-Y. Liu, S. Yu, C.H.K. Cheng, Cardiac glycosides block cancer growth through HIF-1α- and NF-KB-mediated Plk1, Carcinogenesis 34 (2013) 1870–1880, https://doi.org/10.1093/carcin/bgt136.
[82] C.-M. Xie, X.-T. Lin, D. Wu, Y. Tan, C.H.K. Cheng, J. Zhang, Cardiac glycoside bufalin blocks cancer cell growth by inhibition of Aurora A and Aurora B activation via PI3K-Akt pathway, Oncotarget 9 (2018) 13783–13795, https://doi.org/10.18632/oncotarget.24475.
[83] Z. Yu, H. Feng, X. Sun, Y. Zhuo, M. Li, Z. Zhou, L. Huang, Y. Jiang, X. Zhu, X. Zhang, et al., Bufalin suppresses hepatocarcinogenesis by targeting β-catenin/TCF signaling via cell cycle-related kinase, Sci. Rep. 8 (2018) 3891, https://doi.org/10.1038/s41598-018-22113-2.
[84] S.-H. Wu, T.-Y. Wu, Y.-T. Hsiao, J.-H. Lin, S.-C. Hsu, T.-C. Hsia, S.-T. Yang, W.-H. Hsu, J.-G. Chung, Bufalin induces cell death in human lung cancer cells through disruption of DNA damage response pathways, Am. J. Chin. Med. 42 (2014) 729–742, https://doi.org/10.1142/S0192415×14500475.
[85] C.-M. Hsu, Y. Tsai, L. Wan, F.-J. Tsai, Bufalin Induces G2/M phase arrest and triggers autophagy via the TNF, JNK, BECN-1 and ATG8 pathway in human hepatoma cells, Int. J. Oncol. 43 (2013) 338–348, https://doi.org/10.3892/ijo.2013.1942.
[86] J. Xie, W. Lin, L. Huang, N. Xu, A. Xu, B. Chen, M. Watanabe, C. Liu, P. Huang, Bufalin suppresses the proliferation and metastasis of renal cell carcinoma by inhibiting the PI3K/Akt/MTOR signaling pathway, Oncol. Lett. 16 (2018) 3867–3873, https://doi.org/10.3892/ol.2018.9111.
[87] X. Tian, Y. Luo, Y. Yan, C. Sui, F. Meng, Y. Liu, Effect of bufalin on cellular proliferation and apoptosis in human esophageal squamous carcinoma EC9706 cells, Zhongguo Yi Xue Ke Xue Yuan Xue Bao 34 (2012) 556–562, https://doi.org/10.3881/j.issn.1000-503X.2012.06.004.
[88] X. Zhang, Q. Huang, X. Wang, Y. Xu, R. Xu, M. Han, B. Huang, A. Chen, C. Qiu, T. Sun, et al., Bufalin enhances radiosensitivity of glioblastoma by suppressing mitochondrial function and DNA damage repair, Biomed. Pharmacother. Biomed. Pharmacother. 94 (2017) 627–635, https://doi.org/10.1016/j.biopha.2017.07.136.
[89] N. Takai, T. Ueda, M. Nishida, K. Nasu, H. Narahara, Bufalin induces growth inhibition, cell cycle arrest and apoptosis in human endometrial and ovarian cancer cells, Int. J. Mol. Med. 21 (2008) 637–643.
[90] Z.-J. Zhang, Y.-K. Yang, W.-Z. Wu, Bufalin attenuates the stage and metastatic potential of hepatocellular carcinoma in nude mice, J. Transl. Med. 12 (2014) 57, https://doi.org/10.1186/1479-5876-12-57.
[91] J.Q. Gai, X. Sheng, J.M. Qin, K. Sun, W. Zhao, L. Ni, The effect and mechanism of bufalin on regulating hepatocellular carcinoma cell invasion and metastasis via Wnt/β-catenin signaling pathway, Int. J. Oncol. 48 (2016) 338–348, https://doi.org/10.3892/ijo.2015.3250.
[92] D.-Z. Qiu, Z.-J. Zhang, W.-Z. Wu, Y.-K. Yang, Bufalin, a component in chansu, inhibits proliferation and invasion of hepatocellular carcinoma cells, BMC Complement. Altern. Med. 13 (2013) 185, https://doi.org/10.1186/1472-6882-13-185.
[93] S. Su, H. Dou, Z. Wang, Q. Zhang, Bufalin inhibits ovarian carcinoma via targeting MTOR/HIF-α pathway, Basic Clin. Pharmacol. Toxicol. 128 (2021) 224–233, https://doi.org/10.1111/bcpt.13487.
[94] Y.-Y. Chen, H.-F. Lu, S.-C. Hsu, C.-L. Kuo, S.-J. Chang, J.-J. Lin, P.-P. Wu, J.-Y. Liu, C.-H. Lee, J.-G. Chung, et al., Bufalin inhibits migration and invasion in human hepatocellular carcinoma SK-Hep1 cells through the inhibitions of NF-KB and matrix metalloproteinase-2/-9-signaling pathways, Environ. Toxicol. 30 (2015) 74–82, https://doi.org/10.1002/tox.21896.
[95] S.H. Hong, G.-Y. Kim, Y.-C. Chang, S.-K. Moon, W.-J. Kim, Y.H. Choi, Bufalin prevents the migration and invasion of T24 bladder carcinoma cells through the inactivation of matrix metalloproteinases and modulation of tight junctions, Int. J. Oncol. 42 (2013) 277–286, https://doi.org/10.3892/ijo.2012.1683.
[96] H. Li, P. Wang, Y. Gao, X. Zhu, L. Liu, L. Cohen, Z. Meng, P. Yang, Na+/K+-ATPase A3 mediates sensitivity of hepatocellular carcinoma cells to bufalin, Oncol. Rep. 25 (2011) 825–830, https://doi.org/10.3892/or.2010.1120.
[97] M. Watabe, Y. Masuda, S. Nakajo, T. Yoshida, Y. Kuroiwa, K. Nakaya, The cooperative interaction of two different signaling pathways in response to bufalin induces apoptosis in human leukemia U937 cells, J. Biol. Chem. 271 (1996) 14067–14072, https://doi.org/10.1074/jbc.271.24.14067.
[98] M. Watabe, K. Ito, Y. Masuda, S. Nakajo, K. Nakaya, Activation of AP-1 is required for bufalin-induced apoptosis in human leukemia U937 cells, Oncogene 16 (1998) 779–787, https://doi.org/10.1038/sj.onc.1201592.
[99] X. Sheng, X. Sun, K. Sun, H. Sui, J. Qin, Q. Li, Inhibitory effect of bufalin combined with hedgehog signaling pathway inhibitors on proliferation and invasion and metastasis of liver cancer cells, Int. J. Oncol. 49 (2016) 1513–1524, https://doi.org/10.3892/ijo.2016.3667.
[100] X. Song, C. Zhang, M. Zhao, H. Chen, X. Liu, J. Chen, D.M. Lonard, L. Qin, J. Xu, X. Wang, et al., Steroid receptor coactivator-3 (SRC-3/AIB1) as a novel therapeutic target in triple negative breast cancer and its inhibition with a phospho-bufalin prodrug, PLoS One 10 (2015), e0140011, https://doi.org/10.1371/journal.pone.0140011.
[101] Z. Zou, X. Luo, P. Nie, B. Wu, T. Zhang, Y. Wei, W. Wang, G. Geng, J. Jiang, Y. Mi, Inhibition of SRC-3 enhances sensitivity of human cancer cells to histone deacetylase inhibitors, Biochem. Biophys. Res. Commun. 478 (2016) 227–233, https://doi.org/10.1016/j.bbrc.2016.07.063.
[102] Y. Wang, D.M. Lonard, Y. Yu, D.-C. Chow, T.G. Palzkill, J. Wang, R. Qi, A. J. Matzuk, X. Song, F. Madoux, et al., Bufalin is a potent small-molecule inhibitor of the steroid receptor coactivators SRC-3 and SRC-1, Cancer Res. 74 (2014) 1506–1517, https://doi.org/10.1158/0008-5472.CAN-13-2939.
[103] M. Gong, X. Wang, L. Mu, Y. Wang, J. Pan, X. Yuan, H. Zhou, J. Xing, R. Wang, J. Sun, et al., Steroid receptor coactivator-1 enhances the stemness of glioblastoma by activating long noncoding RNA XIST/MiR-152/KLF4 pathway, Cancer Sci. 112 (2021) 604–618, https://doi.org/10.1111/cas.14685.
[104] J. Chen, H. Wang, L. Jia, J. He, Y. Li, H. Liu, R. Wu, Y. Qiu, Y. Zhan, Z. Yuan, et al., Bufalin targets the SRC-3/MIF pathway in chemoresistant cells to regulate M2 macrophage polarization in colorectal cancer, Cancer Lett. 513 (2021) 63–74, https://doi.org/10.1016/j.canlet.2021.05.008.
[105] Y. Chen, Q. Guo, B. Zhang, M. Kang, Q. Xie, Y. Wu, Bufalin enhances the antitumor effect of gemcitabine in pancreatic cancer, Oncol. Lett. 4 (2012) 792–798, https://doi.org/10.3892/ol.2012.783.
[106] L. Dou, D. Zou, F. Song, Y. Jin, Y. Li, Y. Zhang, Bufalin suppresses ovarian cancer cell proliferation via EGFR pathway, Chin. Med. J. (2021), https://doi.org/10.1097/CM9.0000000000001879.
[107] N.-Y. Kim, Y.-A. Suh, S. Kim, C. Lee, Bufalin down-regulates axl expression to inhibit cell proliferation and induce apoptosis in non-small-cell lung cancer cells, Biosci. Rep. 40 (2020), https://doi.org/10.1042/BSR20193959.
[108] S.-H. Wu, Y.-T. Hsiao, C.-L. Kuo, F.-S. Yu, S.-C. Hsu, P.-P. Wu, J.-C. Chen, T.-C. Hsia, H.-C. Liu, W.-H. Hsu, et al., Bufalin inhibits NCI-H460 human lung cancer cell metastasis in vitro by inhibiting MAPKs, MMPs, and NF-KB pathways, Am. J. Chin. Med. 43 (2015) 1247–1264, https://doi.org/10.1142/S0192415×15500718.
[109] Z. Zhu, H. Sun, G. Ma, Z. Wang, E. Li, Y. Liu, Y. Liu, Bufalin induces lung cancer cell apoptosis via the inhibition of PI3K/Akt pathway, Int. J. Mol. Sci. 13 (2012) 2025–2035, https://doi.org/10.3390/ijms13022025.
[110] Y. Gao, H.-X. Li, L.-T. Xu, P. Wang, L.-Y. Xu, L. Cohen, P.-Y. Yang, K. Gu, Z.-Q. Meng, Bufalin enhances the anti-proliferative effect of sorafenib on human hepatocellular carcinoma cells through downregulation of ERK, Mol. Biol. Rep. 39 (2012) 1683–1689, https://doi.org/10.1007/s11033-011-0908-x.
[111] D. Zou, J. Song, M. Deng, Y. Ma, C. Yang, J. Liu, S. Wang, Z. Wen, Y. Tang, X. Qu, et al., Bufalin inhibits peritoneal dissemination of gastric cancer through endothelial nitric oxide synthase-mitogen-activated protein kinases signaling pathway, FASEB J. . Publ. Fed. Am. Soc. Exp. Biol. 35 (2021), e21601, https://doi.org/10.1096/fj.202002780R.
[112] F.-S. Chueh, Y.-Y. Chen, A.-C. Huang, H.-C. Ho, C.-L. Liao, J.-S. Yang, C.-L. Kuo, J.-G. Chung, Bufalin-inhibited migration and invasion in human osteosarcoma U-2 OS cells is carried out by suppression of the matrix metalloproteinase-2, ERK, and JNK signaling pathways, Environ. Toxicol. 29 (2014) 21–29, https://doi.org/10.1002/tox.20769.
[113] C.M. Howard, M. Estrada, D. Terrero, A.K. Tiwari, D. Raman, Identification of cardiac glycosides as novel inhibitors of EIF4A1-mediated translation in triple-negative breast cancer cells, Cancers 12 (2020), E2169, https://doi.org/10.3390/cancers12082169.
[114] M. Liu, L.-X. Feng, P. Sun, W. Liu, T. Mi, M. Lei, W. Wu, B. Jiang, M. Yang, L. Hu, et al., Knockdown of *Apolipoprotein E* enhanced sensitivity of Hep3B cells to cardiac steroids via regulating Na^+/K^+-ATPase signalosome, Mol. Cancer Ther. 15 (2016) 2955–2965, https://doi.org/10.1158/1535-7163.MCT-15-0961.
[115] L. Soumoy, M. Wells, A. Najem, M. Krayem, G. Ghanem, S. Hambye, S. Saussez, B. Blankert, F. Journe, Toad venom antiproliferative activities on metastatic melanoma: bio-guided fractionation and screening of the compounds of two different venoms, Biology 9 (2020), E218, https://doi.org/10.3390/biology9080218.

[116] Y.-L. Lan, X. Wang, J.-C. Lou, J.-S. Xing, Z.-L. Yu, H. Wang, S. Zou, X. Ma, B. Zhang, Bufalin inhibits glioblastoma growth by promoting proteasomal degradation of the Na+/K+-ATPase A1 subunit, Biomed. Pharmacother. Biomed. Pharmacother. 103 (2018) 204–215, https://doi.org/10.1016/j.biopha.2018.04.030.
[117] Y. Lin, D.H. Ho, R.A. Newman, Human tumor cell sensitivity to oleandrin is dependent on relative expression of Na+, K+ -ATPase subunitst, J. Exp. Ther. Oncol. 8 (2010) 271–286.
[118] H. Huang, W. Zhang, Bufalin induced apoptosis of bladder carcinoma cells through the inactivation of Na+K+-ATPase, Oncol. Lett. 16 (2018) 3826–3832, https://doi.org/10.3892/ol.2018.9142.
[119] H. Li, S. Hu, Y. Pang, M. Li, L. Chen, F. Liu, M. Liu, Z. Wang, X. Cheng, Bufalin inhibits glycolysis-induced cell growth and proliferation through the suppression of integrin B2/FAK signaling pathway in ovarian cancer, Am. J. Cancer Res. 8 (2018) 1288–1296.
[120] J. Zhang, Y. Hong, P. Xie, Y. Chen, L. Jiang, Z. Yang, G. Cao, Z. Chen, X. Liu, Y. Chen, et al., Spatial lipidomics reveals anticancer mechanisms of bufalin in combination with cinobufagin in tumor-bearing mice, Front. Pharmacol. 11 (2021), 593815, https://doi.org/10.3389/fphar.2020.593815.
[121] J. Zhang, Y. Hong, L. Jiang, X. Yi, Y. Chen, L. Liu, Z. Chen, Y. Wu, Z. Cai, Global metabolomic and lipidomic analysis reveal the synergistic effect of bufalin in combination with cinobufagin against HepG2 cells, J. Proteome Res. 19 (2020) 873–883, https://doi.org/10.1021/acs.jproteome.9b00681.
[122] Y. Zhan, Y. Qiu, H. Wang, Z. Wang, J. Xu, G. Fan, J. Xu, W. Li, Y. Cao, V.-M. Le, et al., Bufalin reverses multidrug resistance by regulating stemness through the CD133/Nuclear factor-KB/MDR1 pathway in colorectal cancer, Cancer Sci. 111 (2020) 1619–1630, https://doi.org/10.1111/cas.14345.
[123] Z.-T. Yuan, X.-J. Shi, Y.-X. Yuan, Y.-Y. Qiu, Y. Zou, C. Liu, H. Yu, X. He, K. Xu, P.-H. Yin, Bufalin reverses ABCB1-mediated drug resistance in colorectal cancer, Oncotarget 8 (2017) 48012–48026, https://doi.org/10.18632/oncotarget.18225.
[124] X. Zhai, J. Lu, Y. Wang, F. Fang, B. Li, W. Gu, Reversal effect of bufalin on multidrug resistance in K562/VCR vincristine-resistant leukemia cell line, J. Tradit. Chin. Med. Chung Tsa Chih Ying Wen Pan 34 (2014) 678–683, https://doi.org/10.1016/s0254-6272(15)30082-0.
[125] X.-Y. Dai, B.-F. Zhou, Y.-Y. Xie, J. Lou, K.-Q. Li, Bufalin and 5-fluorouracil synergistically induce apoptosis in colorectal cancer cells, Oncol. Lett. 15 (2018) 8019–8026, https://doi.org/10.3892/ol.2018.8332.
[126] J. Sun, K. Xu, Y. Qiu, H. Gao, J. Xu, Q. Tang, P. Yin, Bufalin reverses acquired drug resistance by inhibiting stemness in colorectal cancer cells, Oncol. Rep. 38 (2017) 1420–1430, https://doi.org/10.3892/or.2017.5826.
[127] Z. Yu, H. Feng, Y. Zhuo, M. Li, X. Zhu, L. Huang, X. Zhang, Z. Zhou, C. Zheng, Y. Jiang, et al., Bufalin inhibits hepatitis B virus-associated hepatocellular carcinoma development through androgen receptor dephosphorylation and cell cycle-related kinase degradation, Cell. Oncol. Dordr. 43 (2020) 1129–1145, https://doi.org/10.1007/s13402-020-00546-0.
[128] H. Wang, C. Zhang, H. Chi, Z. Meng, Synergistic anticancer effects of bufalin and sorafenib by regulating apoptosis associated proteins, Mol. Med. Rep. 17 (2018) 8101–8110, https://doi.org/10.3892/mmr.2018.8927.
[129] B. Zhai, F. Hu, H. Yan, D. Zhao, X. Jin, T. Fang, S. Pan, X. Sun, L. Xu, Bufalin reverses resistance to sorafenib by inhibiting Akt activation in hepatocellular carcinoma: the role of endoplasmic reticulum stress, PLoS One 10 (2015), e0138485, https://doi.org/10.1371/journal.pone.0138485.
[130] H. Wang, C. Zhang, H. Chi, Z. Meng, Synergistic anti-hepatoma effect of bufalin combined with sorafenib via mediating the tumor vascular microenvironment by targeting MTOR/VEGF signaling, Int. J. Oncol. 52 (2018) 2051–2060, https://doi.org/10.3892/ijo.2018.4351.
[131] H. Zhao, D. Zhao, H. Jin, H. Li, X. Yang, L. Zhuang, T. Liu, Bufalin reverses intrinsic and acquired drug resistance to cisplatin through the AKT signaling pathway in gastric cancer cells, Mol. Med. Rep. 14 (2016) 1817–1822, https://doi.org/10.3892/mmr.2016.5426.
[132] F. Liu, D. Tong, H. Li, M. Liu, J. Li, Z. Wang, X. Cheng, Bufalin enhances antitumor effect of paclitaxel on cervical tumorigenesis via inhibiting the integrin A2/B5/FAK signaling pathway, Oncotarget 7 (2016) 8896–8907, https://doi.org/10.18632/oncotarget.6840.
[133] J. Liu, Y. Zhang, S. Sun, G. Zhang, K. Jiang, P. Sun, Y. Zhang, B. Yao, R. Sui, Y. Chen, et al., Bufalin induces apoptosis and improves the sensitivity of human glioma stem-like cells to temozolamide, Oncol. Res. 27 (2019) 475–486, https://doi.org/10.3727/096504018×15270916676926.
[134] Z. Zhu, E. Li, Y. Liu, Y. Gao, H. Sun, Y. Wang, Z. Wang, X. Liu, Q. Wang, Y. Liu, Bufalin induces the apoptosis of acute promyelocytic leukemia cells via the downregulation of survivin expression, Acta Haematol. 128 (2012) 144–150, https://doi.org/10.1159/000339424.
[135] Z. Ning, Z. Zhu, H. Wang, C. Zhang, L. Xu, L. Zhuang, X. Yan, D. Wang, P. Wang, Z. Meng, High-intensity focused ultrasound enhances the effect of bufalin by inducing apoptosis in pancreatic cancer cells, OncoTargets Ther. 12 (2019) 1161–1170, https://doi.org/10.2147/OTT.S185953.
[136] J.-J. Zhang, X.-H. Zhou, Y. Zhou, Y.-G. Wang, B.-Z. Qian, A.-N. He, Z. Shen, H.-Y. Hu, Y. Yao, Bufalin suppresses the migration and invasion of prostate cancer cells through HOTAIR, the Sponge of MiR-520b, Acta Pharmacol. Sin. 40 (2019) 1228–1236, https://doi.org/10.1038/s41401-019-0234-8.
[137] J. Jin, Z. Yao, H. Qin, K. Wang, X. Xin, Bufalin inhibits the malignant development of non-small cell lung cancer by mediating the Circ_0046264/MiR-522-3p axis, Biotechnol. Lett. 43 (2021) 1229–1240, https://doi.org/10.1007/s10529-021-03081-6.
[138] X. Zhai, F. Fang, Q. Liu, Y. Meng, Y. Guo, Z. Chen, MiR-181a contributes to bufalin-induced apoptosis in PC-3 prostate cancer cells, BMC Complement. Altern. Med. 13 (2013) 325, https://doi.org/10.1186/1472-6882-13-325.
[139] H. Zhao, D. Zhao, G. Tan, Y. Liu, L. Zhuang, T. Liu, Bufalin promotes apoptosis of gastric cancer by down-regulation of MiR-298 targeting bax, Int. J. Clin. Exp. Med. 8 (2015) 3420–3428.
[140] Y. Qiu, Q. Hu, Q. Tang, W. Feng, S. Hu, B. Liang, W. Peng, P. Yin, MicroRNA-497 and bufalin act synergistically to inhibit colorectal cancer metastasis, Tumour Biol. J. Int. Soc. Oncodev. Biol. Med. 35 (2014) 2599–2606, https://doi.org/10.1007/s13277-013-1342-6.
[141] T. Liu, C. Wu, G. Weng, Z. Zhao, X. He, C. Fu, Z. Sui, S.-X. Huang, Bufalin Inhibits Cellular Proliferation and Cancer Stem Cell-Like Phenotypes via Upregulation of MiR-203 in Glioma, Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol. 44 (2017) 671–681, https://doi.org/10.1159/000485279.
[142] Y. Chang, Y. Zhao, W. Gu, Y. Cao, S. Wang, J. Pang, Y. Shi, Bufalin inhibits the differentiation and proliferation of cancer stem cells derived from primary osteosarcoma cells through Mir-148a, Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol. 36 (2015) 1186–1196, https://doi.org/10.1159/000430289.
[143] L. Zhang, T. Yoshida, Y. Kuroiwa, Stimulation of melanin synthesis of B16-F10 mouse melanoma cells by bufalin, Life Sci. 51 (1992) 17–24, https://doi.org/10.1016/0024-3205(92)90213-9.
[144] L. Zhang, K. Nakaya, T. Yoshida, Y. Kuroiwa, Induction by Bufalin of differentiation of human leukemia cells HL60, U937, and ML1 toward macrophage/monocyte-like cells and its potent synergistic effect on the differentiation of human leukemia cells in combination with other inducers, Cancer Res. 52 (1992) 4634–4641.
[145] L.S. Zhang, K. Nakaya, T. Yoshida, Y. Kuroiwa, Bufalin as a potent inducer of differentiation of human myeloid leukemia cells, Biochem. Biophys. Res. Commun. 178 (1991) 686–693, https://doi.org/10.1016/0006-291x(91)90162-z.
[146] K. Yamada, K. Hino, S. Tomoyasu, Y. Honma, N. Tsuruoka, Enhancement by Bufalin of retinoic acid-induced differentiation of acute promyelocytic leukemia cells in primary culture, Leuk. Res. 22 (1998) 589–595, https://doi.org/10.1016/s0145-2126(98)00044-7.
[147] S. Numazawa, N. Inoue, H. Nakura, T. Sugiyama, E. Fujino, M. Shinoki, T. Yoshida, Y. Kuroiwa, A cardiotonic steroid bufalin-induced differentiation of THP-1 Cells. Involvement of Na+, K(+)-ATPase inhibition in the early changes in proto-oncogene expression, Biochem. Pharmacol. 52 (1996) 321–329, https://doi.org/10.1016/0006-2952(96)00210-9.
[148] E. Szabo, J. Francis, M.J. Birrer, Alterations in differentiation and apoptosis induced by bufalin in CJun overexpressing U-937 cells, Int. J. Oncol. 12 (1998) 403–409, https://doi.org/10.3892/ijo.12.2.403.
[149] Y. Amano, Y. Cho, M. Matsunawa, K. Komiyama, M. Makishima, Increased nuclear expression and transactivation of vitamin D receptor by the cardiotonic steroid bufalin in human myeloid leukemia cells, J. Steroid Biochem. Mol. Biol. 114 (2009) 144–151, https://doi.org/10.1016/j.jsbmb.2009.01.022.
[150] H. Wang, Z. Ning, Y. Li, X. Zhu, Z. Meng, Bufalin suppresses cancer stem-like cells in gemcitabine-resistant pancreatic cancer cells via hedgehog signaling, Mol. Med. Rep. 14 (2016) 1907–1914, https://doi.org/10.3892/mmr.2016.5471.
[151] Y. Chang, Y. Zhao, H. Zhan, X. Wei, T. Liu, B. Zheng, Bufalin inhibits the differentiation and proliferation of human osteosarcoma cell line HMG63-derived cancer stem cells, Tumor Biol. 35 (2014) 1075–1082, https://doi.org/10.1007/s13277-013-1143-y.
[152] J. Wang, H. Cai, Y. Xia, S. Wang, L. Xing, C. Chen, Y. Zhang, J. Xu, P. Yin, Y. Jiang, et al., Bufalin inhibits gastric cancer invasion and metastasis by down-regulating Wnt/ASCL2 expression, Oncotarget 9 (2018) 23320–23333, https://doi.org/10.18632/oncotarget.24157.
[153] H. Wang, C. Zhang, L. Xu, K. Zang, Z. Ning, F. Jiang, H. Chi, X. Zhu, Z. Meng, Bufalin suppresses hepatocellular carcinoma invasion and metastasis by targeting HIF-1α via the PI3K/AKT/MTOR pathway, Oncotarget 7 (2016) 20193–20208, https://doi.org/10.18632/oncotarget.7935.
[154] L. Zhao, S. Liu, X. Che, K. Hou, Y. Ma, C. Li, T. Wen, Y. Fan, X. Hu, Y. Liu, et al., Bufalin inhibits TGF-β-induced epithelial-to-mesenchymal transition and migration in human lung cancer A549 cells by downregulating TGF-β receptors, Int. J. Mol. Med. 36 (2015) 645–652, https://doi.org/10.3892/ijmm.2015.2268.
[155] D.-M. Zhang, L.-X. Feng, M. Liu, W.-H. Jin, J. Luo, A.-Y. Nie, Y. Zhou, Y. Li, W.-Y. Wu, B.-H. Jiang, et al., Possible target-related proteins and signal network of bufalin in A549 cells suggested by both ITRAQ-based and label-free proteomic analysis, Proteomics 16 (2016) 935–945, https://doi.org/10.1002/pmic.201500418.
[156] Z. Yang, Y. Tao, X. Xu, F. Cai, Y. Yu, L. Ma, Bufalin inhibits cell proliferation and migration of hepatocellular carcinoma cells via APOBEC3F induced intestinal immune network for IgA production signaling pathway, Biochem. Biophys. Res. Commun. 503 (2018) 2124–2131, https://doi.org/10.1016/j.bbrc.2018.07.169.
[157] K. Fang, Y. Zhan, R. Zhu, Y. Wang, C. Wu, M. Sun, Y. Qiu, Z. Yuan, X. Liang, P. Yin, et al., Bufalin suppresses tumour microenvironment-mediated angiogenesis by inhibiting the STAT3 signalling pathway, J. Transl. Med. 19 (2021) 383, https://doi.org/10.1186/s12967-021-03058-z.
[158] H. Wang, C. Zhang, Z. Ning, L. Xu, X. Zhu, Z. Meng, Bufalin enhances anti-angiogenic effect of sorafenib via AKT/VEGF signaling, Int. J. Oncol. 48 (2016) 1229–1241, https://doi.org/10.3892/ijo.2016.3326.
[159] Z. Yu, Y. Li, Y. Li, J. Zhang, M. Li, L. Ji, Y. Tang, Y. Zheng, J. Sheng, Q. Han, et al., Bufalin stimulates antitumor immune response by driving tumor-infiltrating macrophage toward M1 phenotype in hepatocellular carcinoma, J. Immunother. Cancer 10 (2022), e004297, https://doi.org/10.1136/jitc-2021-004297.

[160] Y.-L. Shih, J.-S. Chou, Y.-L. Chen, S.-C. Hsueh, H.-Y. Chung, M.-H. Lee, C.-P. Chen, M.-Z. Lee, H.-T. Hou, H.-F. Lu, et al., Bufalin enhances immune responses in leukemic mice through enhancing phagocytosis of macrophage in vivo, Vivo Athens Greece 32 (2018) 1129–1136, https://doi.org/10.21873/invivo.11355.
[161] R. Fu, F. Yu, W. Wu, J. Liu, J. Li, F. Guo, L. Xu, F. Wang, X. Cui, Bufalin enhances the killing efficacy of NK Cells against hepatocellular carcinoma by inhibiting MICA shedding, Int. Immunopharmacol. 101 (2021), 108195, https://doi.org/10.1016/j.intimp.2021.108195.
[162] D. Ji, Z. Liang, G. Liu, G. Zhao, J. Fang, Bufalin attenuates cancer-induced pain and bone destruction in a model of bone cancer, Naunyn. Schmiede Arch. Pharmacol. 390 (2017) 1211–1219, https://doi.org/10.1007/s00210-017-1419-7.
[163] L. Wen, Y. Huang, X. Xie, W. Huang, J. Yin, W. Lin, Q. Jia, W. Zeng, Anti-inflammatory and antinociceptive activities of bufalin in rodents, Mediat. Inflamm. 2014 (2014), 171839, https://doi.org/10.1155/2014/171839.
[164] E. Fujii, Y. Inada, M. Kakoki, N. Nishimura, S. Endo, S. Fujiwara, N. Wada, Y. Kawano, Y. Okuno, T. Sugimoto, et al., Bufalin induces DNA damage response under hypoxic condition in myeloma cells, Oncol. Lett. 15 (2018) 6443–6449, https://doi.org/10.3892/ol.2018.8091.
[165] Y. Jing, H. Ohizumi, N. Kawazoe, S. Hashimoto, Y. Masuda, S. Nakajo, T. Yoshida, Y. Kuroiwa, K. Nakaya, Selective inhibitory effect of bufalin on growth of human tumor cells in vitro: association with the induction of apoptosis in leukemia HL-60 cells, Jpn. J. Cancer Res. Gann 85 (1994) 645–651, https://doi.org/10.1111/j.1349-7006.1994.tb02408.x.
[166] S. Lin, J. Lv, P. Peng, C. Cai, J. Deng, H. Deng, X. Li, X. Tang, Bufadienolides induce P53-mediated apoptosis in esophageal squamous cell carcinoma cells in vitro and in vivo, Oncol. Lett. 15 (2018) 1566–1572, https://doi.org/10.3892/ol.2017.7457.
[167] K.-Q. Han, G. Huang, W. Gu, Y.-H. Su, X.-Q. Huang, C.-Q. Ling, Anti-tumor activities and apoptosis-regulated mechanisms of bufalin on the orthotopic transplantation tumor model of human hepatocellular carcinoma in nude mice, World J. Gastroenterol. WJG 13 (2007) 3374–3379, https://doi.org/10.3748/wjg.v13.i24.3374.
[168] F. Cao, Y.-B. Gong, X.-H. Kang, Z.-H. Lu, Y. Wang, K.-L. Zhao, Z.-H. Miao, M.-J. Liao, Z.-Y. Xu, Degradation of MCL-1 by bufalin reverses acquired resistance to osimertinib in EGFR-mutant lung cancer, Toxicol. Appl. Pharmacol. 379 (2019), 114662, https://doi.org/10.1016/j.taap.2019.114662.
[169] M. Strauss, W. Smith, O.V. Fedorova, A.E. Schutte, The Na+K+-ATPase inhibitor marinobufagenin and early cardiovascular risk in humans: a review of recent evidence, Curr. Hypertens. Rep. 21 (2019) 38, https://doi.org/10.1007/s11906-019-0942-y.
[170] H. Shao, B. Li, H. Li, L. Gao, C. Zhang, H. Sheng, L. Zhu, Novel strategies for solubility and bioavailability enhancement of bufadienolides, Molecules 27 (2021) 51, https://doi.org/10.3390/molecules27010051.
[171] Z. Yang, J. Liu, Q. Huang, Z. Zhang, J. Zhang, Y. Pan, Y. Yang, D. Cheng, Radiosynthesis and pharmacokinetics of [18F]Fluoroethyl Bufalin in hepatocellular carcinoma-bearing mice, OncoTargets Ther. 10 (2017) 329–338, https://doi.org/10.2147/OTT.S110281.
[172] B. Jiang, F. Cai, S. Gao, L. Meng, F. Liang, X. Dai, W. Xu, W. Chen, Induction of cytochrome P450 3A by shexiang baoxin pill and its main components, Chem. Biol. Interact. 195 (2012) 105–113, https://doi.org/10.1016/j.cbi.2011.12.001.
[173] X.-L. Xin, P.-P. Dong, X.-H. Sun, S. Deng, N. Zhang, C. Wang, X.-K. Huo, Y. Li, R. Lan, L. Chen, et al., Identification of the hydroxylated derivatives of bufalin: phase I metabolites in rats, J. Asian Nat. Prod. Res. 18 (2016) 239–247, https://doi.org/10.1080/10286020.2015.1071358.
[174] H. Li, W. Xu, X. Zhang, W. Zhang, L. Hu, Bufalin inhibits CYP3A4 activity in vitro and in vivo, Acta Pharmacol. Sin. 30 (2009) 646–652, https://doi.org/10.1038/aps.2009.42.
[175] T. Vangijzegem, D. Stanicki, S. Laurent, Magnetic iron oxide nanoparticles for drug delivery: applications and characteristics, Expert Opin. Drug Deliv. 16 (2019) 69–78, https://doi.org/10.1080/17425247.2019.1554647.
[176] Y. Xu, Y. Liu, Q. Liu, S. Lu, X. Chen, W. Xu, F. Shi, Co-delivery of bufalin and nintedanib via albumin sub-microspheres for synergistic cancer therapy, J. Control. Release Off. J. Control. Release Soc. 338 (2021) 705–718, https://doi.org/10.1016/j.jconrel.2021.08.049.
[177] H. Gou, R.-C. Huang, F.-L. Zhang, Y.-H. Su, Design of dual targeting immunomicelles loaded with bufalin and study of their anti-tumor effect on liver cancer, J. Integr. Med. 19 (2021) 408–417, https://doi.org/10.1016/j.joim.2021.05.001.
[178] Y. Li, J. Yuan, Q. Yang, W. Cao, X. Zhou, Y. Xie, H. Tu, Y. Zhang, S. Wang, Immunoliposome co-delivery of bufalin and anti-CD40 antibody adjuvant induces synergetic therapeutic efficacy against melanoma, Int. J. Nanomed. 9 (2014) 5683–5700, https://doi.org/10.2147/IJN.S73651.
[179] Y. Xu, L. Tang, P. Chen, M. Chen, M. Zheng, F. Shi, Y. Wang, Tumor-TArgeted Delivery of Bufalin-loaded Modified Albumin-polymer Hybrid for Enhanced Antitumor Therapy and Attenuated Hemolysis Toxicity and Cardiotoxicity, AAPS PharmSciTech 22 (2021) 137, https://doi.org/10.1208/s12249-021-02000-2.
[180] H. Hu, Q. Qi, Z. Dong, X. Yu, Y. Mo, J. Luo, Y. Wang, S. Du, Y. Lu, Albumin coated trimethyl chitosan-based targeting delivery platform for photothermal/chemo-synergistic cancer therapy, Carbohydr. Polym. 241 (2020), 116335, https://doi.org/10.1016/j.carbpol.2020.116335.
[181] J. Yuan, C. Zeng, W. Cao, X. Zhou, Y. Pan, Y. Xie, Y. Zhang, Q. Yang, S. Wang, Bufalin-loaded PEGylated liposomes: antitumor efficacy, acute toxicity, and tissue distribution, Nanoscale Res. Lett. 14 (2019) 223, https://doi.org/10.1186/s11671-019-3057-0.
[182] J. Xu, Y. Sun, Z. Yuan, Y. Bao, R. Li, C. Liu, Y. Qiu, K. Xu, X. Shi, H. Yu, et al., Bufalin-loaded CaP/DPPE-PEG-EGF nanospheres: preparation, cellular uptake, distribution, and anti-tumor effects, J. Biomed. Nanotechnol. 15 (2019) 329–339, https://doi.org/10.1166/jbn.2019.2681.
[183] P. Yin, Y. Wang, Y. Qiu, L. Hou, X. Liu, J. Qin, Y. Duan, P. Liu, M. Qiu, Q. Li, Bufalin-loaded MPEG-PLGA-PLL-CRGD nanoparticles: preparation, cellular uptake, tissue distribution, and anticancer activity, Int. J. Nanomed. 7 (2012) 3961–3969, https://doi.org/10.2147/IJN.S32063.
[184] X. Tian, H. Yin, S. Zhang, Y. Luo, K. Xu, P. Ma, C. Sui, F. Meng, Y. Liu, Y. Jiang, et al., Bufalin loaded biotinylated chitosan nanoparticles: an efficient drug delivery system for targeted chemotherapy against breast carcinoma, Eur. J. Pharm. Biopharm. Off. J. Arb. Pharm. Verfahr. EV 87 (2014) 445–453, https://doi.org/10.1016/j.ejpb.2014.05.010.
[185] Q. Hu, B. Liang, Y. Sun, X.-L. Guo, Y.-J. Bao, D.-H. Xie, M. Zhou, Y.-R. Duan, P.-H. Yin, Z.-H. Peng, Preparation of bufalin-loaded pluronic polyetherimide nanoparticles, cellular uptake, distribution, and effect on colorectal cancer, Int. J. Nanomed. 9 (2014) 4035–4041, https://doi.org/10.2147/IJN.S64708.
[186] T. Liu, X. Yuan, T. Jia, C. Liu, Z. Ni, Z. Qin, Y. Yuan, Polymeric prodrug of bufalin for increasing solubility and stability: synthesis and anticancer study in vitro and in vivo, Int. J. Pharm. 506 (2016) 382–393, https://doi.org/10.1016/j.ijpharm.2016.04.041.
[187] T. Liu, T. Jia, X. Yuan, C. Liu, J. Sun, Z. Ni, J. Xu, X. Wang, Y. Yuan, Development of octreotide-conjugated polymeric prodrug of bufalin for targeted delivery to somatostatin receptor 2 overexpressing breast cancer in vitro and in vivo, Int. J. Nanomed. 11 (2016) 2235–2250, https://doi.org/10.2147/IJN.S100404.
[188] A. Zou, X. Zhao, U.A. Handge, V.M. Garamus, R. Willumeit-Römer, P. Yin, Folate receptor targeted bufalin/β-cyclodextrin supramolecular inclusion complex for enhanced solubility and anti-tumor efficiency of bufalin, Mater. Sci. Eng. C Mater. Biol. Appl. 78 (2017) 609–618, https://doi.org/10.1016/j.msec.2017.04.094.
[189] H. Wang, J. Wu, G.R. Williams, Q. Fan, S. Niu, J. Wu, X. Xie, L.-M. Zhu, Platelet-membrane-biomimetic nanoparticles for targeted antitumor drug delivery, J. Nanobiotechnol. 17 (2019) 60, https://doi.org/10.1186/s12951-019-0494-y.
[190] H. Zeng, C. Xia, B. Zhao, M. Zhu, H. Zhang, D. Zhang, X. Rui, H. Li, Y. Yuan, Folic acid–functionalized metal-organic framework nanoparticles as drug carriers improved bufalin antitumor activity against breast cancer, Front. Pharmacol. 12 (2022), 747992, https://doi.org/10.3389/fphar.2021.747992.
[191] M. Liu, L.-X. Feng, P. Sun, W. Liu, W.-Y. Wu, B.-H. Jiang, M. Yang, L.-H. Hu, D.-A. Guo, X. Liu, A novel bufalin derivative exhibited stronger apoptosis-inducing effect than bufalin in A549 lung cancer cells and lower acute toxicity in mice, PLoS One 11 (2016), e0159789, https://doi.org/10.1371/journal.pone.0159789.
[192] J. Ren, X. Gao, X. Guo, N. Wang, X. Wang, Research progress in pharmacological activities and applications of cardiotonic steroids, Front. Pharmacol. 13 (2022), 902459, https://doi.org/10.3389/fphar.2022.902459.