

# Advancement in research of anti-cancer effects of toad venom (ChanSu) and perspectives

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## ABSTRACT

Toad venom, called as ChanSu in China, is a widely used traditional Chinese medicine (TCM) whose active components are mainly bufadienolides. ChanSu could exhibit cardiotoxic, anti-microbial, anti-inflammatory and, most importantly, anti-cancer effects. In the present review, reports about the *in vitro*, *in vivo* and clinical anti-cancer effects of ChanSu or its representative component, bufalin, were summarized. And, reported anti-cancer mechanisms of cardenolides, structure analogues of bufadienolides, were also introduced. Based on the results got from research of ChanSu/bufalin and the results from cardenolides, possible signal network related to the anti-cancer effects of ChanSu/bufalin was predicted. Furthermore, future potential use of ChanSu in anti-cancer therapy was discussed.

**Key words:** ChanSu, Anti-cancer, Bufadienolides, Bufalin, Cardenolides

**Abbreviations:** TCM, traditional Chinese medicine; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; JNK, c-Jun N-terminal protein kinase; PAK, p21-activated kinase; CK2, casein kinase 2; PARP, poly ADP-ribose polymerase; COX-2, cyclooxygenase-2; VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; NF- $\kappa$ B, nuclear factor- $\kappa$ B; ERK, extracellular signal-regulated kinase; ROS, reactive oxygen species; Hsp27, heat shock protein 27; TNF, tumor necrosis factor; BECN, Beclin; ATG8, autophagy related 8; Bid, BH3 interacting domain death agonist; AMPK, adenosine monophosphate kinase; mTOR, mammalian target of rapamycin; 4EBP1, eukaryotic translation initiation factor 4E binding protein 1; p70S6K1, ribosomal protein S6 kinase, 70kDa, polypeptide 1; ER, endoplasmic reticulum; AKT, protein kinase B; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; TJs, tight junctions; GSK3 $\beta$ , glycogen synthase kinase 3 beta; FAK, focal adhesion kinase; Rho A, ras homolog gene family, member A; MEKK3, MAPK/ERK kinase 3; MKK7, mitogen-activated protein kinase kinase 7; uPA, urokinase plasminogen activator; ER- $\alpha$ , estrogen receptor- $\alpha$ ; TRAIL, TNF-related apoptosis-inducing ligand; STAT3, signal transducer and activator of transcription 3; Mcl-1, myeloid cell leukemia sequence 1; BNPs, bufalin-loaded mPEG-PLGA-PLL-cRGD nanoparticles; Bax, B-cell lymphoma 2-associated X protein; TACE, transcatheter arterial chemoembolization; THM, traditional herbal medicine; ORR, objective response rate; PI3K, phosphoinositide-3-kinase; Bu-BCS-NPs, bufalin encapsulating nanoparticles.

## INTRODUCTION

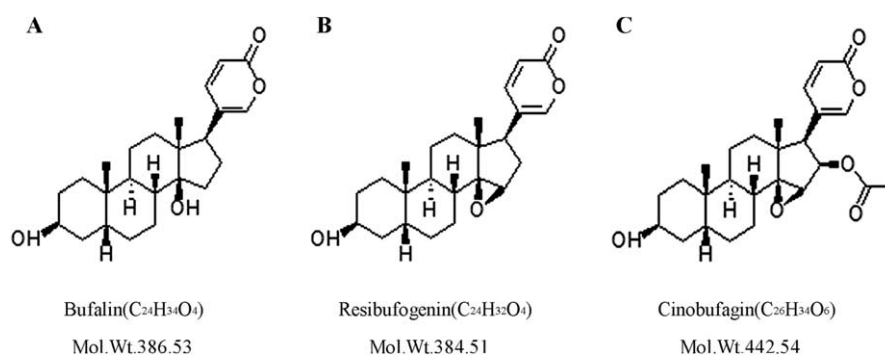
The dried secretion from the skin and parotid venom glands of *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider is a kind of TCM named as ChanSu. The earliest record of ChanSu appeared in Tang Dynasty (618-907)<sup>[1]</sup>. Since then, it had been widely used as a cardiotoxic, anodyne, anti-microbial, anti-inflammatory and anti-neoplastic agent<sup>[2]</sup>. ChanSu could be used either as a single agent or in combination with other TCMs in formulas. Cinobufacini, also known as Huachansu, is the water-soluble extract of the dried skin of *Bufo bufo gargarizans* Cantor and has been successfully used in clinic to treat patients with various cancers in China<sup>[3-5]</sup>. Representative TCM formulas containing ChanSu are Shexiang Baoxin Pill for treatment of cardiovascular diseases<sup>[6]</sup>, Mei-Hua-Dian-She-Wan and Liu-Shen-Wan for treatment of inflammatory diseases<sup>[7]</sup>, and etc. Recently, anti-cancer effects of Liu-Shen-Wan was also reported<sup>[8]</sup>. Up to now, lots of studies about the anti-cancer effects of ChanSu or its

components such as bufalin has been reported<sup>[9-17]</sup>. Notably, in the past two decades, study of anti-cancer effects of cardiac glycosides, including both bufadienolides and cardenolides, was one of the hot topics in the anti-cancer drug research area and some important reviews had been published<sup>[18-23]</sup>. Therefore, it is time to summarize what we have known about the anti-cancer effects of ChanSu and discuss future perspectives concerning the use of ChanSu in anti-cancer therapy.

## CHEMICAL CONSTITUENTS OF ChanSu

ChanSu contains mainly bufadienolides, indoleamines, peptides, amino acids, fatty acids, polysaccharides, and sterols<sup>[24]</sup>. Bufadienolides were reported to be the major active constituents of ChanSu<sup>[25, 26]</sup>. So far, more than 100 bufadienolides with a unique steroidal skeleton has been identified<sup>[27-29]</sup>. Bufalin, resibufogenin, and cinobufagin (as shown in Figure 1) were reported to be the three major bufadienolides in ChanSu

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**Figure 1.** The major components of bufadienolides. (A) Bufalin. (B) Resibufogenin. (C) Cinobufagin.

and they weighed as high as 5% of the dry weight in the crude drug<sup>[23, 27, 30, 31]</sup>. Generally, bufalin was used as a representative component of ChanSu to study the mechanism of ChanSu. In the present review, both studies using crude extract of ChanSu and studies using bufalin are included and discussed to clarify the anti-cancer mechanisms of ChanSu/bufalin.

## ANTI-CANCER ACTIVITIES AND POSSIBLE MECHANISMS OF ChanSu/BUFALIN

### 1. In vitro anti-cancer effects

#### 1.1. Inhibition of proliferation of cancer cells

Numerous experimental studies have indicated that ChanSu and its representative component bufalin inhibited proliferation in a dose-dependent and time-dependent manner in human cancer cells including hepatocellular carcinoma cells<sup>[32–39]</sup>, lung cancer cells<sup>[40–43]</sup>, prostate cancer cells<sup>[31, 44]</sup>, gastric cancer cells<sup>[45]</sup>, leukemia cells<sup>[46–53]</sup>, breast cancer cells<sup>[54]</sup>, colon cancer cells<sup>[55, 56]</sup>, osteosarcoma cells<sup>[16, 17, 57–59]</sup>, bladder carcinoma cells<sup>[60]</sup>, pancreatic cancer cells<sup>[61]</sup>, multiple myeloma cells<sup>[62]</sup>, gallbladder cancer cells<sup>[63]</sup>, malignant melanoma cells<sup>[64]</sup>, skin squamous cell carcinoma cells<sup>[65]</sup>, endometrial cancer cells and ovarian cancer cells<sup>[66]</sup>. Detailed

information about the cell lines in which anti-proliferative effects of ChanSu/bufalin has been reported is shown in Table 1.

#### 1.2. Induction of cell cycle arrest in cancer cells

Treatment of ChanSu/bufalin could induce cell cycle arrest of cancer cells. While, the kinds of cell cycle arrest induced by ChanSu/bufalin might depend on the specific types of treated cancer cells. For example, G2/M phase arrest was induced by ChanSu/bufalin in bladder carcinoma T24 cells<sup>[60]</sup>, pancreatic cancer cell lines (PANC-1 and CFPAC-1)<sup>[61]</sup>, multiple myeloma cells (NCI-H929, U266, RPMI8226 and MM.1S)<sup>[62]</sup>, gastric cancer MGC803 cells<sup>[45]</sup> and hepatoma cells (HepG2 and SK-HEP-1)<sup>[33, 37]</sup>. While, S phase arrest was induced by ChanSu/bufalin in gallbladder cancer cells (GBC-SD and SGC996)<sup>[63]</sup>. G0/G1 phase arrest was induced by ChanSu/bufalin in hepatocellular carcinoma cells with multi-drug resistance (BEL-7402/5-FU)<sup>[15]</sup>, lung carcinoma A549 cells<sup>[41]</sup> and endometrial cancer and ovarian cancer cells<sup>[66]</sup>.

#### 1.3. Induction of apoptosis in cancer cells

Apoptosis induced by ChanSu/bufalin has been well studied. The earliest report about bufalin-induced apoptosis was

**Table 1.** Human cancer cell lines in which the anti-proliferation effects of ChanSu/bufalin had been reported

Type of cancer	Name of cell lines	Refs
Hepatocellular Carcinoma	Bel-7402, HepG2, Huh7, HCCLM3, HA22T SK-Hep-1, PLC/PRF/5, SMMC7721	32–39
Lung cancer	A549, ASTC-a-1, NCI-H460	40–43
Prostate cancer	LNCaP, DU145, PC3	31, 44
Gastric cancer	MGC803	45
Leukemia	K562, HL60, U937, ML1, Jurkat, NB4	46–53
Breast cancer	MCF-7, MDAMB-231, MCF-10A	54
Colon cancer	HT-29, Caco-2, SW620	55, 56
Osteosarcoma	U-2OS, MG-63, SAOS2, IOR/OS9	16, 17, 57–59
Bladder carcinoma	T24	60
Pancreatic cancer	PANC-1, CFPAC-1	61
Multiple myeloma	NCI-H929, U266, RPMI8226, MM.1S	62
Gallbladder cancer	GBC-SD, SGC996	63
Melanoma	A375.S2	64
Skin squamous cell Carcinoma	SSCC-1	65
Endometrial cancer	HHUA, HEC-1	66
Ovarian cancer	SK-OV-3, omc-3	66

published in 1994 by research group of Nakaya K<sup>[50]</sup>. Characteristics of apoptotic cells, such as condensed and fragmented nuclei, fragmented DNA smaller than that of the G1 phase, DNA ladder in agarose gel electrophoretic analysis, were observed in HL-60, ML1, and U937 leukemia cells treated with bufalin at 100 nM and above<sup>[50]</sup>. Research group of Nakaya K. then conducted a series of study investigating the expression of apoptosis-related genes and activation of signal transduction pathways in apoptosis by bufalin<sup>[47–49, 67–75]</sup>. In their studies, they reported that Ras, Raf-1, Mitogen Activated Protein Kinase (MAPK) kinase and MAPK were sequentially activated in U937 cells treated with bufalin<sup>[71]</sup>. And, the effect of overexpression of Bcl-2 on the MAPK cascade in bufalin-induced apoptotic process in U937 cells was investigated. Results indicated that Bcl-2 acted downstream of MAPK kinase-1 but upstream of MAPK and the transcriptional activity of activator protein-1 (AP-1) might be down-regulated through the inhibition of MAPK activity by Bcl-2<sup>[69]</sup>. They found that the activation of AP-1 via a MAPK cascade including c-Jun N-terminal protein kinase (JNK) was required for the induction of apoptosis by bufalin<sup>[48]</sup>. They also identified another apoptosis-related gene, which was homologous to a human gene for Tiam1, induced by bufalin. Tiam1 might play a critical role in bufalin-induced apoptosis through the activation of the Rac-1-PAK-JNK signaling pathway<sup>[49]</sup>. Tiam1 was confirmed as a downstream mediator of bufalin-induced apoptosis in the cervical tumor HeLa cells<sup>[72]</sup>. Besides, topoisomerase II was also identified as a target plays an important role in bufalin-induced apoptosis. In HL-60 cells, the amount and activity of topoisomerase II decreased markedly after the start of bufalin-treatment and the decrease of topoisomerase II preceded the fragmentation of DNA, a typical feature of apoptosis<sup>[74]</sup>. In U937 cells, the activity of topoisomerase II reached a maximum after 3 h and then decreased markedly after treatment with bufalin for 9 h. The amount of the complex of casein kinase 2 (CK2) and topoisomerase II  $\alpha$  increased just after the start of treatment with bufalin and reached a maximum at 6 h. It suggested that the bufalin signal was transmitted to the nucleus by the translocation of CK2, which formed a complex with topoisomerase II  $\alpha$  and modulated the activity of this enzyme, leading to the induction of apoptosis<sup>[75]</sup>.

Other researchers also conducted study of possible mechanisms of apoptosis induced by ChanSu/bufalin. In A549 cells, bufalin induced apoptosis by affecting the protein expressions of Bcl-2/Bax, cytochrome c, caspase-3, poly ADP-ribose polymerase (PARP), p53, p21WAF1, cyclinD1, and COX-2. And, bufalin decreased the protein expressions and/or phosphorylation of VEGFR-1, VEGFR-2, EGFR, c-Met, Akt, NF- $\kappa$ B, p44/42 MAPK (ERK1/2) and p38 MAPK in A549 cells. The results suggested that bufalin might exert its effects on cells via VEGFR1/VEGFR2/EGFR/c-Met–Akt/p44/42/p38–NF- $\kappa$ B signaling pathways<sup>[41]</sup>. In ASTC-a-1 cells, Sun *et al.* found that bufalin induced apoptosis via ROS-dependent mitochondrial death pathway<sup>[42]</sup>. In HepG2 cells, bufalin induced apoptosis via both Fas- and mitochondria-mediated pathways<sup>[76]</sup>. Activation of both intrinsic and extrinsic apoptotic pathway by bufalin

was also found in T24 cancer cells<sup>[60]</sup> as well as in human malignant melanoma A375.S2 cells<sup>[64]</sup>.

New technologies such as proteomics, genomics and computational docking analysis shed new lights on understanding of the mechanism of apoptosis induced by ChanSu/bufalin. For example, possible target-related proteins of bufalin were searched by comparing the protein expression profiles of cells with or without bufalin treatment with proteomic methods<sup>[59, 77, 78]</sup>. Interestingly, an anti-apoptotic protein heat shock protein 27 (Hsp27), was found to be possible target-related protein of bufalin in both study of HeLa cells<sup>[78]</sup> and U2OS cells<sup>[77]</sup>. Down-regulation of Hsp27 expression was induced by bufalin treatment in both cultured U2OS cells and *in vivo* osteosarcoma xenografts. And, Hsp27 over-expression protected against bufalin-induced apoptosis and reversed the alterations of its partner signaling molecules (decrease in p-Akt, nuclear NF- $\kappa$ B p65, and co-immunoprecipitated cytochrome c/Hsp27) induced by bufalin<sup>[77]</sup>. The important role of Hsp27 was also observed in PANC-1 and CFPAC-1 cells<sup>[79]</sup>. Genomic analysis was also conducted by researchers to search possible target-related genes of bufalin<sup>[43, 80, 81]</sup>. In HL-60 cells treated with bufalin, 21, 272, 461 or 659 genes, which related functionally to cell proliferation, apoptosis, differentiation, were found to be altered in their expression level with  $\geq 2$  fold after 6, 12, 24 and 48 h treatment, respectively<sup>[81]</sup>. In NCI-H460 cells treated with bufalin for 24 h, 6 genes were over 20-fold up-regulated and 21 genes were over 10-fold up-regulated. And, 11 genes were over 10-fold down-regulated and 42 genes were over 6-fold down-regulated. Among those affected genes, 165 genes were associated with apoptosis while 107 genes were associated with DNA damage and repair<sup>[43]</sup>. In K562 cells treated with bufalin, 2185 were up-regulated and 2111 genes were down-regulated compared with control. The most up-regulated genes were associated with transcription regulation and the most down-regulated genes were associated with the non-coding RNA metabolic processes and DNA repair<sup>[80]</sup>. By using computational docking analysis, bufalin was suggested to interact directly with PARP1 which was a highly conserved DNA binding protein involved in maintaining the genomic stability, repairing the DNA damage, and regulating transcriptional processes<sup>[62]</sup>.

#### 1.4. Induction of autophagy in cancer cells

Compared with the well-documented apoptosis-inducing activity of ChanSu/bufalin, autophagy induced by ChanSu/bufalin was reported only recently<sup>[14, 34, 36, 37, 55, 82]</sup>. Researchers reported that, in some kinds of cancer cells, bufalin-induced cell death was autophagy but not apoptosis. For example, Xie *et al.* reported that bufalin did not cause caspase-dependent cell death in HT-29 and Caco-2 cells but activated an autophagy pathway, which linked to the generation of ROS. ROS antioxidants (N-acetylcysteine and vitamin C), the JNK-specific inhibitor SP600125, and JNK2 siRNA could attenuate bufalin-induced autophagy<sup>[55]</sup>. Hsu *et al.* reported that, in Huh7, Hep3B and HA22T cells, bufalin inhibited the proliferation and regulated the cell death program in a dose- and time-dependent manner without typical features of apoptosis. Bufalin synergized with the JNK pathway to

induce autophagy and was closely associated with the up-regulation of TNF, BECN-1, MAPK and ATG8, together with the down-regulation of Bcl-2 and Bid<sup>[36]</sup>. While, other researchers reported that both apoptosis and autophagy were induced by bufalin in cancer cells though whether autophagy was pro-survival or pro-apoptotic had no unified answers. Shen *et al.* reported that, in U87MG cells, the mechanism of bufalin-induced autophagy might be associated with ATP depletion involved an increase in the active form of AMPK, decreased phosphorylation levels of mTOR and its downstream targets 4EBP1 and p70S6K1. Blockage of autophagy increased expression of endoplasmic reticulum (ER) stress associated proteins and the ratio of apoptosis, indicating that autophagy played a cytoprotective role in bufalin induced ER stress and cell death<sup>[14]</sup>. Hu *et al.* reported that, in Huh7 and HepG2 cells, bufalin also induced both apoptosis and autophagy. The pro-survival role of bufalin-induced autophagy was suggested when the autophagy pathway was blocked with specific chemical inhibitors<sup>[34]</sup>. Tsai *et al.* reported that bufalin might trigger autophagic cell death and cell cycle arrest through the Akt/mTOR signaling pathway in SK-HEP-1 cells. And, inhibition of autophagy by 3-methyladenine or bafilomycin A1 enhanced the effect of bufalin on apoptosis<sup>[37]</sup>. On the contrary, Miao *et al.* reported that, in HepG2 cell, the mechanism of action of bufalin-induced autophagy was at least partly AMPK-mTOR dependent. The results that blockage of autophagy by selective inhibitor 3-MA decreased apoptotic ratio in bufalin-treated HepG2 cells suggested a proapoptotic role of bufalin-induced autophagy<sup>[82]</sup>.

### Anti-migration and anti-invasion effects on cancer cells

Besides of cytotoxicity, ChanSu/bufalin also could exhibit anti-migration and anti-invasion effects<sup>[16, 35, 83–85]</sup>. Bufalin inhibited the cell migration and invasion of U-2OS cells *in vitro* through blocking MAPK signaling and resulting in the inhibition of matrix metalloproteinase (MMP)-2<sup>[16]</sup>. In A549 cells, the aqueous extract of ChanSu induced marked inhibition of cell motility and invasiveness by inhibiting the activities of MMP-2 and MMP-9 and up-regulating the mRNA expression of tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2<sup>[40]</sup>. Through its inhibition of the Akt/GSK3 $\beta$ / $\beta$ -catenin/E-cadherin signaling pathway, bufalin inhibited migration, invasion and adhesion in HCCLM3 and HepG2 cells<sup>[35]</sup>. Anti-migration effects of bufalin were also observed in the HCT116 cells and HUVECs<sup>[83]</sup>. Within the concentration range that was not cytotoxic, bufalin markedly inhibited the cell motility and invasiveness of T24 cells. The inhibitory effects of bufalin on cell migration and invasion of T24 cells might be related to modulating the activity of tight junctions (TJs) and MMPs, possibly in association with the activation of ERK<sup>[84]</sup>. In SK-Hep1 cells, bufalin markedly inhibited activity, mRNA expression and protein levels of MMP-2 and -9, suppressed protein levels of focal adhesion kinase (FAK) and ras homolog gene family, member A (Rho A), VEGF, MAPK/ERK kinase 3 (MEKK3),

mitogen-activated protein kinase kinase 7 (MKK7), and urokinase plasminogen activator (uPA), and diminished NF- $\kappa$ B translocation. These results suggested that bufalin might act as an anti-invasive agent by inhibiting MMP-2 and -9 and involving PI3K/Akt and NF- $\kappa$ B pathways<sup>[85]</sup>.

### 1.5. Enhancing the cytotoxicity of other anti-cancer reagents

ChanSu/bufalin could be used as an enhancer of other anti-cancer chemotherapeutic drugs. Bufalin greatly sensitize both estrogen receptor- $\alpha$  (ER- $\alpha$ )-positive MCF-7 and ER- $\alpha$ -negative MDA-MB-231 cells to TRAIL-induced apoptosis via suppression of STAT3/Mcl-1 pathway<sup>[54]</sup>. And, bufalin also enhanced the anti-cancer effect of gemcitabine in pancreatic cancer<sup>[86]</sup> as well as the anti-proliferative effect of sorafenib on human hepatocellular carcinoma cells<sup>[38]</sup>.

## 2. In vivo anti-cancer effects in nude mice models

The anti-cancer effects of ChanSu/bufalin had been observed in *in vivo* study using nude mice transplanted with human cancer cells<sup>[63, 83, 87–91]</sup>. Intraperitoneal injection of bufalin at 0.1-0.4 mg/kg significantly inhibited the growth of gallbladder carcinoma (GBC-SD) xenografts in athymic nude mice<sup>[63]</sup>. Intravenous administration of bufalin at 1 mg/kg inhibited colorectal cancer metastasis and improved quality of survival of nude mice inoculated with HCT-116 cells<sup>[83]</sup>. In the study of Zhang *et al.*, intraperitoneal injection of bufalin at 1.5 mg/kg decreased the sizes and qualities of orthotopic transplanted HCCLM3-R tumors as well as pulmonary metastasis. Bufalin treatment also caused changes in expression of Akt/GSK3 $\beta$ / $\beta$ -catenin/E-cadherin signaling pathway-related proteins in tumor tissues<sup>[87]</sup>. In studies using MCF-7 tumor models in nude mice, both the remarkable therapeutic effects of bufalin and bufalin encapsulating nanoparticles were observed<sup>[88]</sup>. Bufalin-loaded pluronic polyetherimide nanoparticles also exhibited anti-metastatic effects on HCT116 colon cancer-bearing mice<sup>[89]</sup>. The targeting efficacy and anti-cancer effects of bufalin-loaded mPEG-PLGA-PLL-cRGD nanoparticles (BNPs), were observed in SW620 colon cancer-bearing mice<sup>[90]</sup>. Intraperitoneal injection of bufalin at 0.5-1.5 mg/kg exhibited significant anti-tumor activities in the orthotopic transplantation tumor model of human hepatocellular carcinoma (Bel-7402) in nude mice and was able to induce apoptosis of transplanted tumor cells mainly via up-regulating the expression of apoptosis-regulated gene Bax<sup>[91]</sup>.

### Clinical anti-cancer effects of ChanSu preparations

As a well-known anti-cancer TCM, ChanSu could be used by TCM doctors in their TCM prescriptions for anti-cancer therapy. While, it is difficult to collect clinical data of these prescriptions containing ChanSu. In the present review, we focused on summarizing the published results about clinical anti-cancer use of Cinobufacini, a water-soluble extract of the dried skin of *Bufo bufo gargarizans* Cantor<sup>[5, 92–100]</sup>. In treating moderate and advanced primary liver cancer, Cinobufacini injection was found to not only inhibited the

proliferation of cancer, but also protected liver function, improved quality of life and prolonged survival time<sup>[101]</sup>. Combined use of Cinobufacini with gemcitabine-oxaliplatin in patients with advanced gallbladder carcinoma was found to be well tolerated, effective, thus improving the quality of life of patients<sup>[92]</sup>. In 2009, Meng *et al.* conducted a pilot study of Cinobufacini using a phase 1 trial design. Totally 15 patients (hepatocellular cancer, n = 11; nonsmall cell lung cancer, n = 2; pancreatic cancer, n = 2) were enrolled in the trial. The results indicated that no dose-limiting toxicities was observed with the use of Cinobufacini at doses up to 8 x higher than typically used in China. Six patients had prolonged stable disease or minor tumor shrinkage<sup>[5]</sup>. In 2012, Meng *et al.* performed a randomized, single-blinded, phase II clinical study of Cinobufacini plus gemcitabine versus placebo plus gemcitabine in patients with locally advanced and/or metastatic pancreatic adenocarcinomas (ClinicalTrials.gov Identifier NCT00837239). While, the results indicated that Cinobufacini, when combined with gemcitabine, did not improve the outcome of patients<sup>[94]</sup>. Xie *et al.* conducted a meta-analysis to evaluate the efficacy and safety of Cinobufacini combined with chemotherapy for advanced gastric cancer based on literatures. Analysis results of fifteen eligible randomized controlled trials showed that, compared with chemotherapy control group, Cinobufacini combined with chemotherapy provide benefits for advanced gastric cancer on improving the response rate, increasing Karnofsky score, reducing leucocytopenia and major side effects such as gastrointestinal side effects caused by chemotherapy<sup>[95]</sup>. Yang *et al.* analyzed all randomized clinical trials of Chinese herbs for advanced or late gastric cancer to assess the effectiveness of Chinese medicinal herbs in the short-term remission of advanced or late gastric cancer. The pooled results from the four injected TCMs, Cinobufacini, Aidi, Fufangkushen, and Shenqifuzheng showed statistically significant differences for the improvement of leukopenia. Limited, weak evidence showed that Cinobufacini, Aidi, and Fufangkushen were of benefit for adverse events in the digestive system caused by chemotherapy. While, these TCMs did not improve the rate of short-term remissions<sup>[96]</sup>. In 2012, research group of Ling CQ reported a case-control trial in which a total of 120 patients in Changhai hospital were enrolled from December 2001 to December 2006. Among the patients, 60 patients were treated with Jiedu granules plus Cinobufacini injection to prevent tumor recurrence after operation (CM group) while the other 60 patients were treated with transcatheter arterial chemoembolization (TACE group) after surgical resection of hepatocellular carcinoma. The results showed that the survival rate of CM group was significantly higher than that of TACE group and the survival time of CM group was significantly longer than that of TACE group.<sup>[93]</sup> In 2013, research group of Ling CQ reported a multicenter, open-label, randomized, controlled study, which was undertaken in five centers of China. A total of 379 patients were enrolled and 188 patients were assigned to the traditional herbal medicine (THM) group and received Cinobufacini injection and Jiedu Granule, while the

other 191 patients were assigned to the TACE group and received one single course of TACE. The results suggested that, in comparison with TACE therapy, the THM regimen was associated with diminished risk of recurrence of small-sized hepatocellular carcinoma after resection, with comparable adverse events<sup>[97]</sup>. Sun *et al.* reported a case concerning treatment of a 63-year-old man in advanced lung cancer with a large amount of pericardial effusion. Pericardium puncture and drainage combined with instillation of Cinobufacini injection in the pericardial cavity to treat pericardial effusion were utilized. After treatment with Cinobufacini injection for two weeks, the cardiac tamponade symptoms such as difficult breathing, chest distress, and palpitations were significantly relieved and the patient's quality of life was effectively improved with KPS scores increased. Furthermore, the levels of tumor marker CA-125 in the pericardial effusion decreased and pericardium B ultrasound showed that the quantity of pericardial effusion reduced significantly after Cinobufacini treatment<sup>[98]</sup>. Wang *et al.* searched 9 electronic databases and 6 gray literature databases in April 20, 2013 to compare clinical efficacy and safety among Chinese herb injections for gastric cancer. A total of 541 records were searched and 38 RCTs met the inclusion criteria (2,761 participants), involving 10 Chinese herb injections. The results of network meta-analysis showed that compared with FOLFOX alone, combinations with Kanglaite, Astragalus polysaccharides, Cinobufacini, or Yadanziyoure injections could further strengthen pooled objective response rate (ORR), improve the quality of life, reduce nausea and vomiting, and reduce the incidence of leukopenia (III-IV)<sup>[99]</sup>. In 2014, Wu *et al.* reported a meta-analysis to evaluate the clinical efficacy of Cinobufacini combined with TACE in the treatment of advanced hepatocellular carcinoma. Nine studies including a total of 659 subjects (333 in Cinobufacini plus TACE and 326 in TACE only) were finally included in this meta-analysis and the results suggested that, cinobufacini combined with TACE could significantly increase the objective response rate and 2-year survival rate compared with TACE only<sup>[100]</sup>.

## ANTI-CANCER MECHANISMS OF CARDENOLIDES, STRUCTURE ANALOGUES OF BUFADIENOLIDES

The family of cardiac glycosides contains two categories, cardenolides and bufadienolides. The difference between cardenolides and bufadienolides is the nature of the lactone ring at position 17 of cardiac glycosides, i.e. cardenolides with an unsaturated butyrolactone ring and the bufadienolides with an  $\alpha$ -pyrone ring<sup>[102]</sup>. With similar chemical structures, cardenolides and bufadienolides are supposed to have similar biological effects and mechanisms. Therefore, research results got from study of cardenolides are presented in the present review to provide some hints for clarifying the mechanism of ChanSu/bufalin. Cardenolides such as digoxin, digitoxin, ouabain and oleandrin have long been used as positive inotropic agents in the treatment of congestive heart failure in Western countries.

Interestingly, in the use of cardenolides, epidemiological data suggested that, more benign histologic characteristics and lower proliferative capacity were observed in tumors from women on digitalis treatment compared with their counterparts not taking cardiac glycosides<sup>[103]</sup>, women on digitalis also had a lower recurrence rate<sup>[104]</sup>, and their death rate from breast carcinoma was significantly lower compared with that of their counterparts in a 20-year follow up study<sup>[105]</sup>. These results induced the research interests of developing cardenolides into new anti-cancer agents and clarifying the mechanisms of anti-cancer effects of cardenolides in the last two decades. New cardenolides were evaluated in clinical trials for anti-cancer therapy. For example, the Phase I clinical trials of PBI-05204 (Nerium oleander extract) (NCT00554268) had finished and the results showed that PBI-05204 was well tolerated in heavily pretreated patients with advanced solid tumors<sup>[106]</sup>. A Phase I study of the combination of carboplatin, docetaxel, and increasing doses of sublingual Anvirzel (Nerium oleander extract) in advance non-small cell lung cancer is being conducted (NCT01562301). The Phase I clinical trial of a modified cardenolide UNBS1450 was conducted in Europe (Belgium and The Netherlands)<sup>[18, 19]</sup>.

Up to now, Na<sup>+</sup>/K<sup>+</sup>-ATPase on plasma membrane is still the only well-accepted direct target of cardiac glycosides. Na<sup>+</sup>/K<sup>+</sup>-ATPase is the first P-type ion translocating ATPase ever identified and the significance of this class of proteins is highlighted by the 1997 Nobel Prize in Chemistry awarded to Jens C. Skou for the discovery of Na<sup>+</sup>/K<sup>+</sup>-ATPase in 1957<sup>[107]</sup>. By using the energy from ATP hydrolysis, Na<sup>+</sup>/K<sup>+</sup>-ATPase exports three Na<sup>+</sup> ions from the cell and imported two K<sup>+</sup> ions into the cell against an electrochemical gradient. It is composed of catalytic  $\alpha$  subunits, regulatory  $\beta$  subunits and tissue-specific regulatory subunits belonging to the FXFD proteins family. So far, four different  $\alpha$  isoforms ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 4$ ) and three different  $\beta$ -isoforms ( $\beta 1$ ,  $\beta 2$  and  $\beta 3$ ) have been identified in mammalian cells<sup>[108, 109]</sup>. Na<sup>+</sup>/K<sup>+</sup>-ATPase has an evolutionarily conserved cardiac glycosides binding site and cardiac glycosides inhibit the sodium pump by interacting with an extra-cellular surface binding "groove" composed of multiple functional groups in the  $\alpha$  subunits and to a lesser extent the  $\beta$  subunits<sup>[19, 102]</sup>. Since Na<sup>+</sup>/K<sup>+</sup>-ATPase is a very difficult protein to crystallize, only in 2011 the first crystal structure of Na<sup>+</sup>/K<sup>+</sup>-ATPase was reported<sup>[107]</sup> and then the structure characteristics of binding between Na<sup>+</sup>/K<sup>+</sup>-ATPase and ouabain were studied<sup>[110–113]</sup>.

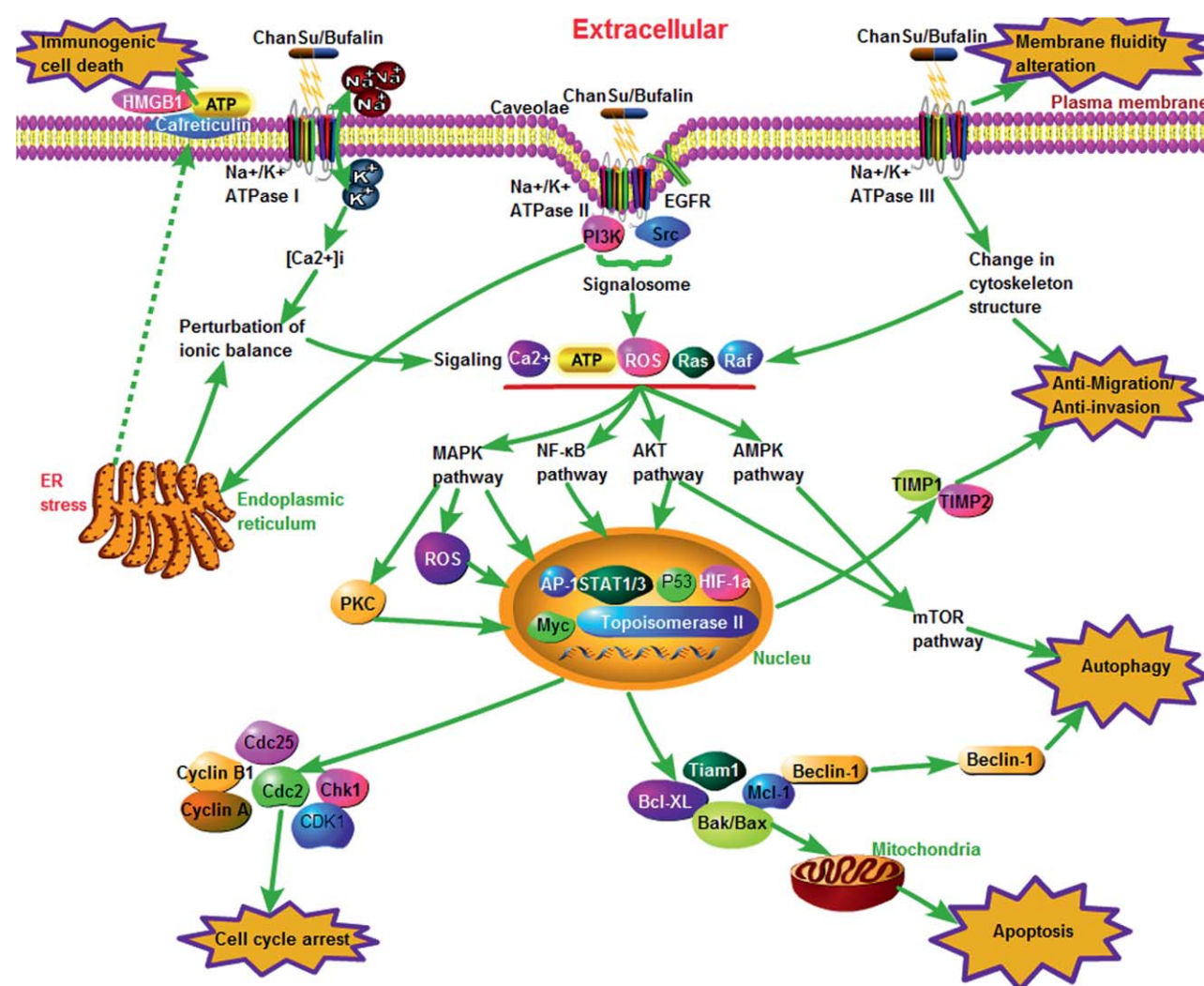
It has been found that, besides of acting as Na<sup>+</sup>/K<sup>+</sup> pump, Na<sup>+</sup>/K<sup>+</sup>-ATPase also has other functions such as acting as a signal transducer, playing roles in the formation and maintenance of adhesion complexes, induction of epithelial cell tight junctions (TJs) and polarity, as well as cell motility and actin dynamics<sup>[19]</sup>. The concept of "Na<sup>+</sup>/K<sup>+</sup>-ATPase signalosome" appeared at about 2000<sup>[114–116]</sup>. The existence of two pools of Na<sup>+</sup>/K<sup>+</sup>-ATPase within the plasma membrane was proposed: one being the classical pool of the enzyme acting as an energy transducing ion pump and the other being the signal transducing pool of the enzyme that was restricted to the caveolae, forming the so-called "Na<sup>+</sup>/K<sup>+</sup>-ATPase signalosome"<sup>[117]</sup>. The identification of a mutant  $\alpha 1$  Na<sup>+</sup>/K<sup>+</sup>-ATPase

that had normal pumping function but was defective in signal transduction suggested the role of  $\alpha$  subunit in the Na<sup>+</sup>/K<sup>+</sup>-ATPase signalosome<sup>[118]</sup>. The Na<sup>+</sup>/K<sup>+</sup>-ATPase signalosome has been found to play an important role in the anti-cancer effects of cardiac glycosides. During the last decade, multiple experiments implicated that, when cardiac glycosides bind to the Na<sup>+</sup>/K<sup>+</sup>-ATPase, Na<sup>+</sup>/K<sup>+</sup>-ATPase signalosome provided normal cells with survival signals but death signals in cancer cells<sup>[102]</sup>. More importantly, increased expression and activities of Na<sup>+</sup>/K<sup>+</sup>-ATPase in cancer cells resulted in increased susceptibility of cancer cells to cardiac glycosides which supported the potential use of cardiac glycosides in anti-cancer therapy<sup>[119]</sup>. On the other hand, Na<sup>+</sup>/K<sup>+</sup>-ATPase, possibly mainly through its  $\beta$  subunits, were actively involved in cell adhesion, cell motility, epithelial integrity, assembly of TJs and etc<sup>[120–125]</sup>. Accordingly, by interacting with Na<sup>+</sup>/K<sup>+</sup>-ATPase, cardenolides such as ouabain could affect cell adhesion, TJs, and invasion of cells<sup>[120, 126–129]</sup>. For example, ouabain decreased cell attachment to fibronectin<sup>[126]</sup> and relaxed cell attachment<sup>[120, 127]</sup>. Ouabain also promoted cell-cell communication by means of gap junctions by specifically enhancing the expression of connexin 32<sup>[128]</sup>. At concentrations that neither inhibit K<sup>+</sup> pumping nor disturb the K<sup>+</sup> balance of the cell, ouabain modulated the degree of sealing of the TJ as measured by transepithelial electrical resistance and the flux of neutral 3 kDa dextran, accompanied by changes in the levels and distribution patterns of claudins 1, 2, and 4<sup>[129]</sup>.

Interestingly, there are also some new findings about the anti-cancer mechanism of cardenolides which might need to be confirmed by more studies. Menger *et al.* reported that, cardenolides might induce specific types of cell death such as immunogenic cell death<sup>[130–133]</sup>. Immunogenic cell death involves changes in the composition of the cell surface as well as a release of soluble mediators, which finally induce an immune response against dead-cell antigens<sup>[134]</sup>. By binding to Na<sup>+</sup>/K<sup>+</sup>-ATPase, digoxin/digitoxin might induce ER stress which resulted in immunogenic cell death featuring the exposure of calreticulin at the cell surface, the secretion of ATP as well as the release of the nuclear protein HMGB1 into the extracellular space<sup>[133]</sup>.

## POSSIBLE SIGNAL NETWORK RELATED TO ANTI-CANCER EFFECTS OF ChanSu/BUFALIN

Like cardenolides, bufadienolides could directly bind to the Na<sup>+</sup>/K<sup>+</sup>-ATPase on plasma membrane and act as Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitors<sup>[135, 136]</sup>. The binding to Na<sup>+</sup>/K<sup>+</sup>-ATPase was the basis of the regulative effects of ChanSu/bufalin on cardiovascular system such as cardiotoxic, renal sodium excretion and blood pressure stimulating effects<sup>[137–139]</sup>. The mechanism of the anti-inflammatory effects<sup>[140, 141]</sup>, immunoregulatory effects<sup>[142–145]</sup>, and anti-cancer effects<sup>[146]</sup> of ChanSu/bufalin have not been fully clarified. While, though the possibility that ChanSu/bufalin might have other direct targets which play roles in its anti-cancer effects could not be excluded, the important role of Na<sup>+</sup>/K<sup>+</sup>-ATPase in the



**Figure 2.** Possible signal network related to the anti-cancer effects of ChanSu/bufalin.

anti-cancer effects of ChanSu/bufalin is supported by most of the available research results. In the present review, based on both the research results of anti-cancer effects of ChanSu/bufalin and the reported anti-cancer mechanisms of cardenolides, possible signal network related to the anti-cancer effects of ChanSu/bufalin is predicted (Figure 2).

As shown in Figure 2, three  $\text{Na}^+/\text{K}^+$ -ATPases (I, II and III) on the plasma membrane indicate the three types of functions of  $\text{Na}^+/\text{K}^+$ -ATPase, respectively. Firstly, ChanSu/bufalin, by inhibiting the ion pump function of  $\text{Na}^+/\text{K}^+$ -ATPase ( $\text{Na}^+/\text{K}^+$ -ATPase I), could induce perturbation of ionic balance. Secondly, ChanSu/bufalin, by affecting the signal transducer function of  $\text{Na}^+/\text{K}^+$ -ATPase in caveolae ( $\text{Na}^+/\text{K}^+$ -ATPase II), could induce change in the activation states of signalosome components such as Src, EGFR, and PI3K. Thirdly, ChanSu/bufalin, by disturbing the membrane structure formation function of  $\text{Na}^+/\text{K}^+$ -ATPase ( $\text{Na}^+/\text{K}^+$ -ATPase III), could induce perturbation of membrane fluidity and also change in intracellular cytoskeleton structure. The signaling produced from these three effects of ChanSu/bufalin would then affected pathways including MAPK

pathway, NF- $\kappa$ B pathway, AKT pathway and AMPK pathway. By changing expression of genes such as Cyclin A, Cdc25 and etc., cell cycle arrest would be induced. By changing expression of genes such as Bax, Tiam 1, and etc., apoptosis would be induced. Autophagy could also be induced based on change in mTOR pathway as well as expression of genes such as Beclin-1. And, change in expression of genes such as TIMP-1, TIMP-2 and direct influence on cytoskeleton structure might both contribute to the anti-migration and anti-invasion effects of ChanSu/bufalin. Possible mechanisms of ChanSu which have not been reported such as the induction of immunogenic cell death through ER stress are shown in dashed line. And, to further decipher the mechanisms of anti-cancer effects of ChanSu/bufalin, more experimental data would be needed.

## PERSPECTIVES

Chansu has been successfully used in anti-cancer therapy for a long time in China. The discovery and research of the anti-cancer effects of cardenolides further support the role of

ChanSu in anti-cancer therapy. To develop new anti-cancer agents based on the anti-cancer effects of ChanSu, efforts have been made to either synthesize chemical derivatives as drug candidates or to design tumor-targeted delivery of ChanSu/bufalin. Lots of derivatives of bufalin were synthesized and their anti-cancer effects were evaluated. Some promising compounds were suggested<sup>[147–149]</sup>. On the other hand, tumor-targeted drug delivery systems of bufalin were designed. For example, Tan *et al.* designed a tumor-targeted drug delivery system of bufalin based on enhanced permeability and retention effect by using biotinylated chitosan which resulted in bufalin encapsulating nanoparticles (Bu-BCS-NPs) with mean hydrodynamic size of 171.6 nm. The remarkable therapeutic effect of Bu-BCS-NPs were confirmed in *in vivo* studies using MCF-7 tumor models in nude mice<sup>[88]</sup>. Both kinds of study would contribute to the future potential use of ChanSu in anti-cancer therapy. Hopefully, derivatives of bufalin with stronger anti-cancer activity delivered by tumor-targeted delivery system might be developed in the near future. While, further clarification of their anti-cancer molecular mechanisms is necessary for the development of new anti-cancer agents from ChanSu as well as more rational use of ChanSu.

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