INTRODUCTION

Despite recent decreases in the mortality rate and occurrence of colorectal cancer (CRC), it remains the third most common cancer in the United States. Apoptosis inhibition is a driving force that leads to carcinogenesis and resistance to chemotherapy. Most conventional anti-cancer drugs are designed to kill CRC cells by inducing apoptosis directly or indirectly. Therefore, screening of apoptosis inducers based on a thorough understanding of their mechanisms is needed.

Bufarenogin induces intrinsic apoptosis via Bax and ANT cooperation

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Abstract

Toads have high medicinal value and have been used for medicinal purposes since the Tang Dynasty period (7th-10th Century AD). Bufarenogin, an active anti-tumor constituent of toad venom, shows anti-tumor activity. In this study, we investigated the inhibitory effects of bufarenogin on the growth and metastasis of colorectal cancer (CRC), particularly its effects on mediating intrinsic signaling pathways that initiate apoptosis. An orthotopic CRC model was established in nude mice via surgical orthotopic implantation to investigate tumor growth. Immunohistochemistry, immunofluorescence, and Western blotting assays were performed to evaluate protein expression. The in vitro results revealed the anti-proliferative effect of bufarenogin against CRC cells. Bufarenogin caused cell death via apoptosis, as revealed by Annexin V/7-amino-actinomycin D double staining, which was verified using a pan-caspase inhibitor. Bufarenogin induced B-cell lymphoma 2-associated X protein (Bax)-dependent intrinsic apoptosis, as demonstrated by mitochondrial translocation of Bax and cytoplasm release of HCT116 wild-type cells and cytochrome C (soluble pro-apoptotic factors). Additionally, we showed that adenine-nucleotide translocator interacted with Bax. Bufarenogin induced intrinsic apoptosis through the cooperation of Bax and adenine-nucleotide translocator and inhibited the metastasis and growth of orthotopical CRC cells.

KEYWORDS
ANT, Bax, bufarenogin, intrinsic apoptosis

1 | INTRODUCTION

Despite recent decreases in the mortality rate and occurrence of colorectal cancer (CRC), it remains the third most common cancer...
mechanisms of action is an active and promising area of research for CRC treatment.3

Mitochondrial outer membrane permeabilization (MOMP) is an important event that triggers apoptosis via cytochrome C (cyto C) leakage into the cytosol. There are two distinct mechanisms of MOMP.4 First, the permeability transition pore (PTP) opens into the inner membrane and facilitates the passage of approximately 1.5-kDa molecules and water. Matrix swelling leads to breakage of the outer membrane, resulting in MOMP. The second MOMP mechanism is initiated by members of the B-cell lymphoma 2 (Bcl-2) family of apoptosis-regulating proteins that act directly on the outer mitochondrial membrane (OMM).5 In the first model, the PTP spans both the OMM and inner mitochondrial membrane (IMM). The major PTP components are adenine-nucleotide translocator (ANT) and a voltage-dependent anion channel (VDAC) present in the IMM and OMM, respectively, and a small soluble protein of mitochondria ie cyclophilin D (CypD).5 However, genetic knock-down or inactivation of VDAC or ANT failed to protect cells from mitochondrial permeabilization transition (MPT) induction.5,7 ANT and VDAC are therefore putative partners that regulate MPT and MOMP.5

Bufarenogin is an important bioactive compound in the bufadienolide family with high levels found in toad venom. It has been extensively used in traditional Chinese medicine to treat malignant diseases for hundreds of years. Our previous study showed that arenobufagin, another member of the bufadienolide family, induced intrinsic apoptosis with autophagy in hepatocellular carcinoma cells.8 It also suppressed CRC cell growth and metastasis through Bax-mediated MOMP.9 Additionally, telocinobu-fagin decreased the viability of CRC cells by suppressing the activity of apoptosis inhibitors and upregulating activation of p53 and Bax.10 Ding et al showed that bufarenogin suppressed hepatocellular carcinoma growth via inhibiting receptor tyrosine kinases-regulated signaling,11 while Wilmer H et al showed the importance of the hydroxylation at position C-14 in the bufadienolide skeleton for the inhibitory activity on the Na+/K+-ATPase.12 Here, we evaluated the efficacy of bufarenogin as an apoptosis inducer to trigger MOMP. Furthermore, the anti-CRC capacity of bufarenogin and possible mechanisms were investigated.

2 MATERIALS AND METHODS

2.1 Bufarenogin solution

Tovena lactone compound ‘Bufarenogin,’ with a molecular formula of C_{24}H_{32}O_{6} and molecular weight of 416.5 g/mol, was extracted from toad venom. Bufarenogin was dissolved in normal saline containing 1% dimethyl sulfoxide (DMSO). Bufarenogin (purity >98%, determined via high-performance liquid chromatography) was purchased from Herbest (Baoji, China).

2.2 Cell culture and treatment

Cell lines of human CRC (SW620, HCT116) were purchased from American Type Culture Collection (Manassas, VA). HCT116 Bax^{−/−} was received as a gift from Hong Kong Baptist University. HCT116 and SW620 cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA), whereas HCT116 Bax^{−/−} cells were cultured in McCoy’s 5A medium (Gibco, Grand Island, NY). The media were supplemented with fetal bovine serum (10% (v/v); Invitrogen) and penicillin-streptomycin (1% (v/v); Invitrogen) and maintained in a humidified atmosphere at 37°C in 5% CO2. Additionally, Savelt ™ reagent (Han Heng, Wuhan, China) to kill mycoplasma at a ratio of 1:1000, will be regularly used for three consecutive days and once every half a month. The cells are in good condition.

Small interfering (si)RNA oligos were procured from GenePharma (Shanghai, China). Exponentially growing cells were placed in six-well plates at a density of 0.5 × 10^{5} cells/mL and incubated for 24 hours. siRNA (1 mg) was transfected into the cells using Lipofectamine 2000 (Invitrogen) transfection reagent in reduced serum medium (OPTI-MEM-I; Invitrogen).

2.3 Orthotopic CRC model in BALB/c-nu mice

All procedures involving animals were performed according to the guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication #85–23, revised in 1985) with approval from the Laboratory Animal Care and Use Committee of Southern Medical University. 6-week-old male mice were housed in a sterilized atmosphere for follow-up experiments.

For the orthotopic CRC model, SW620 cells (1 × 10^{7}) were subcutaneously injected into BALB/c-nu (nude) mice. After 10 days, tumor tissue was divided into 1-mm³ pieces. The tumors were introduced into the colon of anesthetized nude mice via surgical orthotopic implantation. We injected penicillin for the first 3 days to prevent its infection and administration on the fourth day. Nude mice were then randomly assigned into different treatment groups and administered bufarenogin (3 or 6 mg/kg/day) via intraperitoneal injection for 21 days. Cisplatin was given at 10 mg/kg/3 days by intraperitoneal injection. Throughout the treatment period, the body weights of the mice were recorded every 5 days. After 21 days, the mice were sacrificed and their tumors were harvested.

2.4 Cell proliferation assays

To evaluate cell proliferation, an MTT assay was carried out according to the manufacturer’s protocol. Briefly, cancer cells (4 × 10^{3}) were placed in 96-well plates (flat-bottomed) and incubated for 24 hours in RPMI medium with 10% fetal bovine serum. The cells were then treated with various concentrations of bufarenogin and further incubated for 24 hours. After incubation, 10 μL of 2.5 mg/mL MTT...
solution (Sigma-Aldrich, St Louis, MO) was added to the cells, following by 4-hours incubation. After removing the medium, formazan crystals were solubilized by adding DMSO (100 µL) and absorbance at 490 nm was measured with a Multiskan Spectrum (Thermo Fisher Scientific, Waltham, MA).

### 2.5 Western blotting

Protein expression was evaluated using 30–60 µg cell lysates by immunoblot analysis in radioimmunoprecipitation assay buffer containing rabbit antibodies for Bax, poly (ADP-ribose) polymerase (PARP), cyto C oxidase subunit IV (Cox IV; 1:1000; Cell Signaling Technology, Danvers, MA), caspase 3, Bcl-2 (1:1000; Proteintech, Rosemont, IL), ANT (1:1000; Abcam, Cambridge, UK), and cyto c (1:500; Abcam), and β-actin (1:500; Santa Cruz Biotechnology, Dallas, TX). Specific protein bands were visualized with an enhanced chemiluminescence (ECL) system (Bio-Rad, Hercules, CA).

### 2.6 Immunofluorescence

Cultured cells were prepared in medium for 24 hours after spreading on a confocal disc. After washing, the cells were fixed with formaldehyde (4%) for 30 minutes, permeabilized for 15 minutes with Triton-100 (0.2%), and incubated with primary antibodies overnight followed by Alexa 488/594-conjugated secondary antibody in the dark for 1 hour. The cells were counterstained with 4′6-diamidino-2-phenylindole and Alexa 488/594-conjugated secondary antibody in the dark for 1 hour. To illustrate the antineoplastic role of bufarenogin in vivo, 1-mm³ tumor pieces were stitched to the colon of BALB/c-nu mice via surgical orthotopic implantation as previously reported9,13 The visual picture can see the changes of body weight clearly in each cell lines (SW620 and HCT116) were used. After treatment with bufarenogin, cell viability was decreased in time- and dose-dependent manners. The SW620 and HCT116 cell viability was decreased by half after 12-hour incubation with 20 µmol/L bufarenogin (Figure 2A). A clonogenic cell survival assay supported that bufarenogin reduced the cell clones compared to in controls. This suggests that bufarenogin is an active antineoplastic agent that inhibits cell proliferation (Figure 2B; Figure S2).

### 2.7 Immunohistochemistry staining

Tumor tissues were fixed with 4% paraformaldehyde followed by embedding in paraffin and sectioning into 5-µm slices. Immunohistochemistry (IHC) staining was conducted according to the manufacturer’s protocol. Endogenous peroxidase activity was blocked with peroxidase blocking solution (0.03% H₂O₂ containing sodium acid) for 5 min. The tissue sections were washed with phosphate-buffered saline, pH 7.2, followed by incubation at −4°C with ANT (1:100; Abcam), Bax (1:50; Abcam), cleaved caspase 3 (1:100; Abcam), and cleaved PARP (1:50; Cell Signaling Technology) antibodies. On the next day, the slides were further incubated with prediluted secondary antibodies (Zhongshan Golden Bridge, Beijing, China) followed by counterstaining with hematoxylin.

### 2.8 Immunoprecipitation

Immunoprecipitation was performed by overnight incubation of the cellular lysate with antibodies followed by 4-hours incubation with protein A/G-Sepharose beads (Santa Cruz Biotechnology). The samples were centrifuged at 2500 g and washed three times with ice-cold lysis buffer. Immunoprecipitated proteins were eluted by denaturing them with Laemmli buffer at 95°C for 5 minutes followed by separation via SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane (Pierce, Rockville, IL) and blocked with 5% nonfat milk. The membranes were incubated with primary secondary antibodies, and proteins were visualized with an ECL detection system (Amersham Biosciences Europe, Amersham, UK).

### 2.9 Statistical analyses

Data are presented as the mean ± SD of three independent experiments. SPSS software (SPSS, Inc, Chicago, IL) was used for statistical analysis. Two-tailed P-values were determined via Student’s t-test. P-values < .05 were considered as statistically significant.
To examine the CRC cell death mechanism of bufarenogin, flow cytometry was performed using annexin V/7-amino-actinomycin D double staining to evaluate the rate of apoptosis and necrosis in DLD-1 and SW620 cells. Bufarenogin induced cell death categorized primarily through apoptosis (Figure 2C; Figure S3). Apoptosis occurs specifically through caspase activation, and an immunoblotting assay revealed the cleavage of caspase by bufarenogin. Furthermore, the presence of cleaved caspase 3 was increased by treatment with 20 μmol/L bufarenogin. Bufarenogin treatment also caused cleavage of the caspase 3 substrate (PARP). In addition, a dose-dependent decrease in Bcl-2 expression was observed (Figure 2D). Bufarenogin-induced apoptosis was significantly inhibited by pretreatment with a broad-spectrum caspase inhibitor (N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk)), indicating that caspase-dependent cell death was influenced by bufarenogin (Figure 2E).

3.3 | Bufarenogin induced Bax-dependent intrinsic apoptosis

We confirmed the role of Bax in bufarenogin-induced apoptosis in HCT116 wild-type (WT) and HCT116 Bax<sup>−/−</sup> cells. After treatment with bufarenogin, apoptosis dose-dependently increased with a decrease in HCT116 WT cell viability as compared to HCT116 Bax<sup>−/−</sup> cells. Cell viability was detected after treatment with bufarenogin by using iMAC2, a mitochondrial apoptosis-induced channels inhibitor. Increased apoptosis and decreased viability associated with bufarenogin were partly reversed by iMAC2 treatment (Figure 3A,B; Figure S4).

To evaluate the mitochondrial membrane potential (ΔΨ<sub>m</sub>), JC-1 is considered as a reliable fluorescent probe. A concentration-dependent red to green shifting in the emission spectrum was observed by flow cytometry, indicating that bufarenogin caused dissipation of ΔΨ<sub>m</sub> in SW620 and HCT116 cells (Figure S5). ΔΨ<sub>m</sub> loss occurs before detectable caspase activation. Hence, HCT116 WT cells showed cleavage of PARP and caspase-3 with decreased Bcl-2 expression, whereas HCT116 Bax<sup>−/−</sup> cells did not. These results indicate that bufarenogin induces Bax-dependent intrinsic apoptosis (Figure 3C).

Bax translocated to the mitochondria in cells treated with bufarenogin, and mitochondrial Bax accumulation in HCT116 WT cells was investigated via mitochondrial cytoplasm separation. As Bax accumulation in the mitochondria triggers MOMP, we analyzed its potential of the cells to secrete cyto c into the cytoplasm. HCT116 WT cells released cyto c into the cytosol (Figure 3E). Immunofluorescent staining provided direct evidence of Bax translocation to the mitochondria (Figure 3D). Based on the Nomenclature Committee on Cell Death, the dissipated Ψ<sub>m</sub> and mitochondrial loss of cyto c demonstrate that bufarenogin-induced cell death occurs through intrinsic apoptosis and highly depends on Bax activation.
FIGURE 2  Bufarenogin induced apoptosis in colorectal cancer (CRC) cells. A, Cell proliferation measured via MTT assay after 12 and 24 h, relative to that in control cells. *P < .01 and **P < .005, one-way ANOVA. Data: means ± SD. B, Clone formation inhibition by bufarenogin in indicated cells. Panels represent the numerous colonies (see Figure S1). Data; means ± SD. ***P < .001, Student’s t-test (two-tailed.) C, Phycoerythrin (PE)-annexin V/7-amino-actinomycin D was measured via flow cytometry in cells treated with bufarenogin (n = 3). Results are shown as the means ± SD (see Figure S2). D, For Western blot analysis, total cell lysates were prepared to investigate the regulatory proteins of apoptosis (n = 3). E, A combination of bufarenogin and z-VAD-fmk (20 μmol/L) was used to treat the cells. MTT assay was used to assess cell viability (n = 6). #P < .05, *P < .01; (one-way ANOVA)
FIGURE 3  Intrinsic apoptosis caused by bufarenogin was Bax-dependent. A, Pretreatment (for 3 h) of HCT116 wild-type (WT) cells with iMAC2 (20 μmol/L) before 24-h incubation with bufarenogin. The viability of HCT116 WT + iMAC2, HCT116 WT, and HCT116 Bax−/− cells was investigated via MTT assay (n = 6 per group). B, flow cytometry-based analysis for annexin V-positive cells. Data; means ± SD, *P < .05 and **P < .01 (one-way ANOVA) (see Figure S3). C, Apoptotic protein expression was detected in two cell lines. D, Immunofluorescence assay was performed to observe the localization of Bax in indicated cells. Magnification: ×1000 for all; scale bar = 10 μm. E, Bax and cyto c expression in cytoplasm and mitochondria of HCT116 WT cells was determined by Western blotting. Mitochondria segregation was assessed by measuring Cox IV. Cytoplasmic segregation was assessed by measuring β-actin.
3.4 | ANT is essential for bufarenogin-induced intrinsic apoptosis

RNA interference was performed to identify the molecular composition of the mitochondrial pore-forming unit that regulates bufarenogin-induced apoptosis. Our results showed that ANT siRNA conferred protection in HCT116 cells treated with bufarenogin. ANT is an abundantly available protein and primarily associated with ADP/ATP exchange across the membrane of mitochondria. Its overexpression facilitates mitochondria-dependent apoptosis.
accompanied by enhanced stimulation of caspase 3 and 9 and the release of cyto c.21

Further evidence showed that ANT siRNA-528 suppressed the apoptosis in HCT116 WT cells but not HCT116 Bax−/− cells following treatment with bufarenogin (Figure 4A). Decreased viability was partially restored by atractyloside, an ANT inhibitor that binds to its intermembrane face22 (Figure 4B). HCT116 cells transfected with ANT siRNA showed PARP cleavage, whereas HCT116 Bax−/− cells did not (Figure 4C). Therefore, we investigated the necessity of ANT for Bax translocation. The merged yellow fluorescence via overlapping of green (mito-tracker) and red (Bax staining) indicated Bax relocation to the mitochondria of SW620 cells induced by bufarenogin, and that ANT inhibited bufarenogin-induced translocation of Bax (Figure 4D). This indicates that ANT plays a central role in bufarenogin-induced apoptosis.

3.5 | Bax interacted with ANT to trigger cell death

A co-immunoprecipitation assay was performed for both HCT116 and SW620 cells to investigate if an association was required between Bax and ANT to induce intrinsic apoptosis. Under apoptotic conditions, the cells exhibited a strong interaction between Bax and ANT (Figure 5A,B). Within the PTP complex (PTPC), the proapoptotic molecule Bax and constitutive mitochondrial protein ANT work together to enhance the membrane permeability of mitochondria to lead to cell death.

**Figure 5** Interaction of Bax with adenine-nucleotide translocator (ANT). A,B, Treatment of HCT116 and SW620 cells with bufarenogin (10 μmol/L) for 2, 4, or 6 h. Immunoprecipitation was conducted by using anti-Bax or -ANT antibody. Co-immunoprecipitation of ANT or Bax was analyzed by Western blotting
3.6 | Bufarenogin inhibited metastasis of orthotopical colorectal carcinoma cells by inducing apoptosis

Bufarenogin suppressed CRC tumor growth in vivo as previously described. Unexpectedly, we found that it also reduced metastatic foci in the liver (Figure 6A,D). We investigated the presence of tumor cells in mouse liver of the model group via hematoxylin staining (Figure S6). Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining indicated that bufarenogin-associated tumor suppression occurred through apoptosis induction in CRC tissues (Figure 6B,C). Similarly, IHC results from orthotopical tumors in mice revealed a significant increase (dose-dependent) in the levels of Bax, ANT, and apoptotic proteins (Figure 6E). These results demonstrate that bufarenogin suppressed the growth and metastasis of CRC cells by inducing apoptosis.

**FIGURE 6** Bufarenogin inhibits metastasis of orthotopical colorectal carcinoma by inducing apoptosis. A,D, Metastatic foci in mouse hepatic tissue. Bar graphs show the percentage of metastasis. *P < .05, **P < .01, one-way ANOVA. B,C, Apoptotic detection was carried out with terminal deoxynucleotidyl transferase dUTP nick-end labeling experiment, and fluorescence was examined by microscopy (magnification: 100×). E, Immunohistochemical staining of Bax, Adenine-nucleotide translocator (ANT), cleaved poly (ADP-ribose) polymerase (PARP), and cleaved caspase 3 expression in tumor tissues. Magnification: 100×; scale bar = 200 μm. F, Schematic mechanism diagram of bufarenogin-induced apoptosis, which requires the interaction between Bax and ANT in colorectal cancer cells.
4 DISCUSSION

Natural products including those derived from animals and plants are a focus of drug development research in screening for targeted cancer therapies. Bufarenogin is a natural compound with anti-tumor efficacy. In this study, bufarenogin showed high efficacy for treating advanced malignant tumors in mice, particularly compared with cisplatin, and weight loss was improved.

Apoptosis is energy-dependent and cautiously planned process resulting from certain morphological changes and biochemical events. Among these morphological and biochemical events, caspase activation plays a key role. Cell death induced by bufarenogin is caspase-dependent apoptosis, as it is sensitive to zVAD. A recombinant phosphatidylserine-binding protein ( Annexin V), which can strongly and selectively attach to phosphatidylserine residues, can be utilized to detect apoptosis. Here, a dose-dependent increase in annexin V was observed. Cell proliferation was also greatly reduced by bufarenogin administration. Several biochemical modifications such as protein cleavage and cross-linking are characteristic features of apoptotic cells. Capsases are overexpressed in numerous cell types in the form of inactive proenzyme; upon activation, they further activate other pro-caspases, leading to protease cascade initiation. Increased apoptosis-related proteins induced by bufarenogin suggest caspase-dependent apoptosis.

The signaling cascades responsible for initiating stimuli for triggering intrinsic apoptosis are highly heterogeneous. However, they all are interlinked mitochondrion-centered control mechanisms. The stimuli are involved in MPT pore opening by creating alterations in the inner membrane of mitochondrion, thus leading to transmembrane potential loss of mitochondria and release of normally sequestered pro-apoptotic proteins from the intermembrane spaces into the cytosol. Release of mitochondrial soluble factor cyto c into the cytoplasm was evident in cells treated with bufarenogin according to mitochondrial cytoplasm separation.

The main PTPC components include VDAC (OMM), ANT (IMM), and CypD (matrix). Although ANT is considered as a non-essential constituent of the PTPC and consequent apoptotic process, our pre-screening experiment showed that ANT silencing only rescued bufarenogin-induced cell death. PTPC is a highly dynamic entity that displays a large degree of context-dependency, and ANT may be a physical and functional interactor functionally associated with PTPC participating in MOMP-dependent apoptosis upon bufarenogin treatment. The regulation and control of mitochondria-based apoptotic events are initiated by members of the Bcl-2 protein family, and translocation of Bax from the cytoplasm to the mitochondria triggers MOMP opening. Cooperation of Bax and ANT within the PTPC results in increased permeability of the mitochondrial membrane, in turn inducing cell death.

Our orthotopical model exhibited that bufarenogin prevents CRC cell growth and metastasis, suggesting that bufarenogin can be used as an anti-CRC agent with low toxicity. Consistently with our in vitro observations, bufarenogin induced apoptosis and Bax activation in vivo. ANT and Bax association was also observed in CRC tissues treated with bufarenogin. This is the first study to show that Bax interacts with ANT in bufarenogin-induced intrinsic apoptosis.

In summary, bufarenogin induced intrinsic apoptosis and significant inhibition in the metastasis and the growth of CRC cells via strong cooperation of Bax and ANT. Bufarenogin-induced apoptosis may be an effective mechanism for achieving the effective killing of tumor cells.

DISCLOSURES
No competing interests are declared by the authors.

AUTHOR CONTRIBUTIONS
QRH and XGS, research design; QRH, CZ, YBZ, and LYW, conducted experiments; XGS, study concepts, manuscript review; QRH, data analysis and interpretation, manuscript editing. All authors read and approved the final manuscript.

ETHICAL STATEMENT
The study was approved by Southern Medical University Experimental Animal Ethics Committee and conducted in accordance with the ethical standards and national guidelines.

DATA AVAILABILITY STATEMENT
All the results and their supporting date of this study are present in the manuscript. Upon a reasonable request, further details can be obtained from the corresponding author.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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