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ACT001 reverses resistance of prolactinomas via AMPK-mediated EGR1 and mTOR pathways

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Abstract

Dopamine agonist (DA) is the first choice for the treatment of prolactinomas, and drug resistance is unavoidable during treatment due to the heterogeneity of tumors. The two prolactinoma cell lines (GH3 cells and MMQ cells) were found to have different sensitivity and responding modes to the cabergoline (CAB) and bromocriptine (BRC). In this research, we disclosed the capability of ACT001, a derivative of parthenolide analogs, to activate AMPK by increasing the intracellular reactive oxygen species (ROS) level and AMP/ATP ratio to reverse DA resistance through dual pathways in prolactinoma cells. The results indicated that ACT001 could reverse the CAB resistance in GH3 cells by inhibiting the mTOR signaling pathway, inducing cell death through autophagy, and reverse the BRC resistance in MMQ cells by activating the EGR1 signaling pathway, inducing cell death through apoptosis. Our results suggested that ACT001 is a promising therapeutic compound for treating DA-resistant prolactinomas.

Introduction

Prolactinoma is the most common functional pituitary adenoma, and its mainstay treatment is dopamine agonist (DA) therapy, which usually has favorable efficacy in normalizing the serum prolactin level and shrinking the tumor (Melmed 2020). The most commonly used DAs include cabergoline (CAB) and bromocriptine (BRC). However, approximately 20% of patients respond unsatisfactorily to BRC and the prevalence is 10% for CAB (Maiter 2019).

Endoscopic endonasal transsphenoidal surgery is currently an important adjunct to DA therapy (Vroonen et al. 2019). Despite tremendous technique improvement and efforts to achieve total resection of the tumor, about 34% of the patients suffered from recurrence of hyperprolactinemia after surgery (Primeau et al. 2012).

The underneath molecular mechanisms resulting in DA resistance are complicated and have been reviewed extensively elsewhere (Molitch 2014). Several molecular alterations may involve and even coexist in the same tumor. The reductive expression of long active isoform of the subtype 2 dopamine receptor (D2R) and molecular alterations in the downstream of the D2R might account for the insensitivity in some cases (Caccavelli et al. 1996, Peverelli et al. 2012, Shimazu et al. 2012). The chronic intermittent hypoxia (CIH) may contribute to angiogenesis and play a role in DA resistance in the prolactinomas (Cai et al. 2016). However, the complexity in cellular mechanisms made it difficult to find a potential target to solve this problem.

The prolactinomas have varying levels of susceptibilities to BRC and CAB not only in vivo but
also in vitro. Our previous research indicated that two prolactinoma cell lines responded differently to CAB and BRC via differential mechanisms: GH3 cells were relatively resistant to CAB which mainly induced autophagy via inhibiting the AKT/mTOR pathway, and MMQ cells were relatively resistant to BRC which mainly induced apoptosis via activating the ERK/EGR1 pathway (Tang et al. 2019). Upon the above finding, we hypothesized that the effect of CAB could be enhanced by further inhibiting mTOR to treat CAB-resistant prolactinomas while the effect of BRC can be strengthened via further activating EGR1 to treat BRC-resistant prolactinomas. For the CAB- and BRC-resistant tumor, we can reverse drug resistance through dual pathways.

AMP-activated protein kinase (AMPK) is a key regulator of energy homeostasis, and the interest in the enzyme as a drug target has been stimulated since the physiological regulators of AMPK are proved to be associated with longevity (Lin & Hardie 2018, Steinberg & Carling 2019). It can be activated under conditions of energetic stress to decrease the intracellular AMP/ATP ratio and restore cellular energy balance (Oakhill et al. 2012). What’s more, AMPK, as an upstream target, is capable of regulating both EGR1 and mTOR, which indicates that AMPK could be targeted to solve the problem of DA resistance in prolactinomas.

Intracellular reactive oxygen species (ROS) are by-products of reverse electron transport when energy is produced in mitochondria. The balance of ROS production and elimination is critical for maintaining the homeostasis of the cellular environment and cell survival. Due to mitochondrial dysfunction, oncogene activation and anti-oxidation imbalance, cancer cells generally generate much more ROS than normal cells. A moderate increase in intracellular ROS levels can affect the survival of tumor cells while having no significant impact on normal cells (Panieri & Santoro 2016). The accumulation of ROS in the cell will damage the process of intracellular energy metabolism and increase the ratio of AMP/ATP in the cell and then phosphorylate the α subunit of AMPK to activate it (Filomeni et al. 2015).

As the fumarate of the soluble analog of parthenolide (PTL), ACT001 possesses the ability to increase ROS level in tumor cells by attenuating the function of MnSOD (Xu et al. 2013, Li et al. 2020). In this study, we verified that ACT001 could increase the intracellular ROS level and AMP/ATP ratio to regulate EGR1 and mTOR pathways via activating AMPK and, ultimately, reverse DA resistance by inducing apoptosis and autophagy in DA-resistant prolactinomas.

## Materials and methods

### Materials

Rat prolactinoma cell lines (GH3 cells and MMQ cells) were purchased from National Infrastructure of Cell Line Resource (Beijing, China). ACT001 was supplied by Accendatech Co., Ltd. (Tianjin, China). CAB and BRC were purchased from Abcam. A-769662 (a potent, direct AMPK activator) and compound C (a selective and ATP-competitive AMPK inhibitor) were purchased from MedChemExpress (USA). NAC was obtained from Beyotime Biotechnology (Shanghai, China). Antibodies against AMPKα (ab207442, dilution for Western blot, 1:1000), AMPKα (pThr172) (ab133448, dilution for Western blot, 1:1000; dilution for IHC analysis, 1:100) and EGR1 (ab216964, dilution for Western blot, 1:500) were obtained from Abcam. Antibodies against Raptor (pSer792) (#89146, dilution for Western blot, 1:100; dilution for IHC analysis, 1:100), TSC2 (pSer1387) (#23402, dilution for Western blot, 1:1000), Beclin-1 (pSer14) (#84966, dilution for Western blot, 1:1000; dilution for IHC analysis, 1:100), ULK1 (pSer73) (#37762, dilution for Western blot, 1:1000), ULK1 (pSer75) (#14202, dilution for Western blot, 1:1000), Light chain 3I/II (#12741, dilution for Western blot, 1:1000), BAX (#14796, dilution for Western blot, 1:1000) and Cleaved Caspase-3 (#9664, dilution for Western blot, 1:1000) were obtained from Cell Signaling Technology. Antibody against Bcl-2 (AB1 12, dilution for Western blot, 1:1000) was obtained from Beyotime.

### Cell lines and cell culture

GH3 cells and MMQ cells were cultured in Ham’s F10 medium (Boster, USA) and F12 medium (Boster, USA) containing 15% horse serum, 2.5% fetal bovine serum (Gibco), and 1% penicillin and streptomycin (Gibco) and were maintained at 37°C with 5% CO₂.

### Drug treatment

According to our previous research (Tang et al. 2019), the IC50 values of CAB in GH3 cells and MMQ cells were 84.29 ± 9.16 and 90.34 ± 7.93 μM, respectively, and the IC50 values of BRC in GH3 cells and MMQ cells were 55.61 ± 4.19 μM and 90.34 ± 7.93 μM, respectively, which indicated that GH3 cells were relatively resistant to CAB and MMQ cells were relatively resistant to BRC. In addition, treatment with 50 μM BRC induced more deaths in GH3 cells than in MMQ cells and treatment with 50 μM CAB...
induced more deaths in MMQ cells than in GH3 cells. We also compared the effects of ACT001 on the GH3 and MMQ cells combined with DA at levels of 10, 20 and 40 μM. The results showed that 20 μM was the appropriate treatment concentration (Supplementary Fig. 1A and B, see section on supplementary materials given at the end of this article). The effects of NAC, A-769662, and compound C were tested as single agents to make sure these compounds have no independent effect (Supplementary Fig. 1C and D). The drug concentrations of CAB, BRC, ACT001, NAC, A-769662, compound C for treating cells were 50 μM, 50 μM, 20 μM, 5 mM, 10 μM and 10 μM, respectively.

**Cell Counting Kit (CCK)-8 assay**

Cell viability was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. GH3 cells and MMQ cells were treated with drugs in a 96-well plate for 24, 48, and 72 h and 10 μL CCK-8 reagent was then added. After incubation for 2 h, the optical density was detected by measuring the absorbance at 450 nm by a microplate reader.

**Colony formation assay**

GH3 cells (500 cells/well) were added into a six-well plate. The medium and reagents were changed every 48 h. After 2 weeks, cells were fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet for 20 min. The numbers of colony formation were counted under microscope (100×) by selecting three fields of view randomly.

**Intracellular ROS measurement**

Intracellular ROS levels of GH3 cells and MMQ cells were detected by staining cells with dichlorofluorescin diacetate (DCFH-DA, a cell-permeable probe used to detect intracellular reactive oxygen species) using Reactive Oxygen Species Assay Kit (Beyotime). Briefly speaking, cells were incubated with 10 μM DCFH-DA at 37°C for 30 min and then washed twice with PBS. Laser confocal microscope and fluorescence microplate reader were used to evaluate the intracellular ROS level.

**LC-MS analysis of metabolites**

Metabolites (AMP and ATP) for liquid chromatography coupled to mass spectrometry (LC-MS) were extracted using ice-cold 50% methanol, bead-beaten and then analyzed by LC-MS as described previously (Rabinovitch et al. 2017).

**Western blot analysis**

Cells were collected and lysed using RIPA buffer containing a protease inhibitor cocktail and quantified with a BCA protein assay kit (Beyotime). Cell lysates were resolved by SDS-PAGE, and then equal amounts of proteins were transferred to PVDF membranes (Millipore). After being incubated in blocking buffer, the PVDF membranes were exposed to primary antibodies (described in Materials) at 4°C overnight. A secondary goat anti-rabbit HRP-IgG (Proteintech, Rosemont, IL, USA) was used to detect the primary antibodies. The immunoreactive complexes were visualized using a chemiluminescent substrate, and then the images were acquired on film.

**Cell transfection**

Lentivirus containing an AMPK knockdown sequence (shRNA-AMPK) was generated in GV493. Its target sequence is 5'-GCACGTGCCTACGCTCTTTAA-3' (GeneChem, China). Lentiviral transfection was performed according to the manufacturer's manual. After infection, cells were selected using 5 μg/mL puromycin solution.

**RNA isolation and quantitative real-time PCR**

Total mRNA was extracted from cells using Trizol, and cDNA was synthesized from 100 ng of total RNA as described previously (Rabinovitch et al. 2017). Quantitative real-time PCR (Qrt-PCR) was performed using primers against GAPDH and AMPK. The primer sequences were as follows: GAPDH sense: CTGGAGAAACCTGCCAAGTATG, antisense: GGTGGAAGAATGGGAGTTGCT (138 bp); AMPK sense: CGAAGCCAGAGCAAACCATA, antisense: GGAATCGACACTTGACCAG (294 bp).

**Electron microscopy**

The GH3 cells were fixed with 2% glutaraldehyde for 2 h and then post-fixed in 1% osmium tetroxide for 1 h. Dehydration was induced in increasing concentrations of ethanol followed by dehydration in propylene oxide. When it was incubating in 70% ethanol, the pellet was stained enblock with 1% uranyl acetate. Finally, the pellet was embedded in epon resin. Ultrathin sections were routinely post-stained with uranyl acetate and Reynold's lead citrate. Electron micrographs were acquired using an electron microscope. The numbers of autophagosome were counted in three fields of view randomly of each group.
Quantitative assessment of apoptosis

A quantitative assessment of apoptosis was determined with an annexin V-FITC assay kit (Solarbio Life Sciences, China). After treatment with different drugs for 72 h, MMQ cells were collected and washed twice with cold PBS buffer and suspended in binding buffer. Then, 5 μL of annexin V-FITC was added to a new tube with 100-μL cell suspension and incubated, and 5 μL of PI was added and the sample was incubated in the dark at room temperature for 5 min. Samples were analyzed by flow cytometry according to the manufacturer’s instructions.

Animal model

All animal experiments were approved by the Ethical Committee of the Jinling Hospital. In vivo experiments were performed using 5-week-old female athymic nude mice. GH3 cells suspended in PBS were subcutaneously injected into the right side of the back of each nude mouse. The animals were assigned randomly to five groups after confirming tumor: control group (saline), CAB group, ACT001 group, CAB + ACT001 group, CAB + ACT001 + NAC group (CAB: 5 mg/kg/day; ACT001: 100 mg/kg/day; NAC: 100 mg/kg/day). Tumor burden was monitored every 3 days, and tumor volumes were measured with a Vernier caliper and calculated as (length × width²)/2.

Immunohistochemical staining

After 2 weeks of drug administration, the mice were executed humanely and the tumors were extracted and fixed in 10% formalin and embedded in paraffin for immunohistochemical (IHC) staining. Tumor tissue sections were incubated with primary antibodies (described in Materials) overnight at 4°C, followed by incubating the sections with goat anti-rabbit HRP secondary antibody (1:200 in 1% BSA in TBST) for 1 h at room temperature. The sections were then exposed to DAB substrate (dissolved in Dako substrate buffer) and subjected to a standard dehydration treatment. The staining images were obtained using an Axiovert 200 microscope.

Statistical analysis

The results are shown as the mean ± s.d. with three repeats of each condition per experiment. Comparisons between various conditions were performed using an unpaired t-test. SPSS statistical software version 24 (IBM) was used to analyze the data.

Results

ACT001 induced death of DA-resistant prolactinoma cells via ROS-AMPK pathway

To investigate the effects of ACT001 on prolactinoma cells, we performed CCK-8 assay and clone formation assay on GH3 cells and MMQ cells (Fig. 1A, B and C).

In GH3 cells (relatively resistant to CAB), the results of CCK8 assay (Fig. 1A) showed that the cell viability of the CAB + ACT001 group was significantly lower than that of the CAB group (36.80 ± 6.40% vs 52.07 ± 5.61%, P < 0.05) and ACT001 group (36.80 ± 6.40% vs 84.10 ± 2.95%, P < 0.001). After addition of NAC (an antioxidant), the cell viability of the CAB + ACT001 + NAC group was significantly higher than that of the CAB + ACT001 group (51.10 ± 5.36% vs 36.80 ± 6.40%, P < 0.05) and similar to that of the CAB group (51.10 ± 5.36% vs 52.07 ± 5.61%, P > 0.05).

In MMQ cells (relatively resistant to BRC), the results of CCK8 assay (Fig. 1B) showed that the cell viability of the BRC + ACT001 group was significantly lower than that of the BRC group (52.53 ± 5.05% vs 69.83 ± 1.17%, P < 0.01) and ACT001 group (52.53 ± 5.05% vs 81.80 ± 1.10%, P < 0.01). After addition of NAC, the cell survival rate of the BRC + ACT001 + NAC group was significantly higher than that of the BRC + ACT001 group (75.60 ± 4.55% vs 52.53 ± 5.05%, P < 0.01) and similar to that of the BRC group (75.60 ± 4.55% vs 69.83 ± 1.17%, P > 0.05).

The clone formation experiment cannot be carried out on MMQ cells (suspension growth) and was only performed on GH3 cells. In GH3 cells (Fig. 1C), the clone number of the CAB + ACT001 group was less than that of the CAB group (P < 0.05) and ACT001 group (P < 0.01). After addition of NAC, the clone number of the CAB + ACT001 + NAC group was more than that of the CAB + ACT001 group (P < 0.01) and similar to that of the CAB group (P > 0.05).

The above results showed that NAC can antagonize the anti-tumor effect of ACT001 which indicated that ROS was potentially involved in this process. We further measured the ROS level in different groups.

In GH3 cells (Fig. 1D), the intracellular ROS level in the CAB + ACT001 group was significantly higher than that in the CAB group (P < 0.001) and ACT001 group (P < 0.01). After addition of NAC, the intracellular ROS level in the CAB + ACT001 + NAC group was significantly lower than that in the CAB + ACT001 group (P < 0.001) and was similar to that in the CAB group (P > 0.05).

In MMQ cells (Fig. 1E), the results showed that the intracellular ROS level in the BRC + ACT001 group was significantly higher than that in the BRC group (P < 0.001)
Figure 1

ACT001 induced death of DA-resistant prolactinoma cells via ROS-AMPK pathway. (A) The cell viability of GH3 cells treated with different drugs was detected by CCK-8 assay. (B) The cell viability of MMQ cells treated with different drugs was detected by CCK-8 assay. (C) The proliferation ability of GH3 cells treated with different drugs was detected by clone formation assay. (D) The results of intracellular ROS detection in GH3 cells. (E) The results of intracellular ROS detection in MMQ cells. (F) The intracellular AMP/ATP ratio of GH3 cells was evaluated by LC-MS. (G) The intracellular AMP/ATP ratio of MMQ cells was evaluated by LC-MS. (H) The protein expression of AMPK in GH3 cells was evaluated by Western blot. (I) The protein expression of AMPK in MMQ cells was evaluated by Western blot. CAB: 50 μM; BRC: 50 μM; ACT001: 20 μM; NAC: 5 mM (*P < 0.05, **P < 0.01, ***P < 0.001).
and ACT001 group ($P < 0.01$). After addition of NAC, the intracellular ROS level in the BRC+ACT001+NAC group was significantly lower than that in the BRC+ACT001 group ($P < 0.001$) and similar to that in the BRC group ($P > 0.05$).

In order to verify whether ACT001 can increase the intracellular AMP/ATP ratio by increasing the intracellular ROS level, we further evaluated the intracellular AMP/ATP ratio by liquid chromatography-mass spectrometry (LC-MS).

In GH3 cells (Fig. 1F), the results showed that the intracellular AMP/ATP ratio of the CAB+ACT001 group was significantly higher than that of the CAB group ($P < 0.001$) and ACT001 group ($P < 0.001$). After addition of NAC, the intracellular AMP/ATP ratio of the CAB+ACT001+NAC group was significantly lower than that of the CAB+ACT001 group ($P < 0.001$) and similar to that of the CAB group ($P > 0.05$).

In MMQ cells (Fig. 1G), the results of LC-MS showed that the intracellular AMP/ATP ratio of the CAB+ACT001 group was significantly higher than that of the BRC group ($P < 0.01$) and ACT001 group ($P < 0.05$). After addition of NAC, the intracellular AMP/ATP ratio of the BRC+ACT001+NAC group was significantly lower than that of the BRC+ACT001 group ($P < 0.01$) and similar to that of the BRC group ($P > 0.05$).

We further investigated whether AMPK was activated due to the increased AMP/ATP ratio by measuring the expression of AMPK in GH3 cells and MMQ cells.

In GH3 cells (Fig. 1H), the expression of AMPK (pThr172) in the CAB+ACT001 group was significantly higher than that in the CAB group ($P < 0.01$) and ACT001 group ($P < 0.01$). After addition of NAC, the expression level of AMPK (pThr172) in the CAB+ACT001+NAC group was significantly lower than that in the CAB+ACT001 group ($P < 0.01$) and similar to that in the CAB group ($P > 0.05$).

In MMQ cells (Fig. 1I), the expression of AMPK (pThr172) in the BRC+ACT001 group was significantly higher than that in the BRC group ($P < 0.01$) and ACT001 group ($P < 0.01$). After addition of NAC, the expression level of AMPK (pThr172) in the BRC+ACT001+NAC group was lower than that in the BRC+ACT001 group ($P < 0.01$) and similar to that in the BRC group ($P > 0.05$).

**ACT001 induced autophagic cell death in CAB-resistant GH3 cells by AMPK-mTOR pathway**

In GH3 cells (Fig. 2A), the results of the CCK-8 assay showed that the cell viability of the CAB+ACT001 group was lower than that of the CAB group (35.30 ± 3.33% vs 59.63 ± 1.76%, $P < 0.001$) and ACT001 group (35.30 ± 3.33% vs 84.10 ± 3.90%, $P < 0.001$). After addition of A-769662 (AMPK agonist), the cell viability of the CAB+A-769662 group was similar to that of the CAB+ACT001 group (35.70 ± 3.86% vs 35.30 ± 3.33%, $P > 0.05$). After addition of compound C (AMPK inhibitor), the cell viability of the CAB+ACT001+compound C group was similar to that of the CAB group (57.23 ± 9.86% vs 59.63 ± 1.76%, $P > 0.05$).

The results of cell clone formation experiment (Fig. 2B) showed that the cell clone number in the CAB+ACT001 group was less than that in the CAB group ($P < 0.01$) and ACT001 group ($P < 0.001$). The clone number in the CAB+A-769662 group was similar to that in the CAB+ACT001 group after addition of A-769662 ($P > 0.05$), and the clone number in the CAB+ACT001+compound C group was similar to that in the CAB group after addition of compound C ($P > 0.05$).

We further used AMPK-silenced GH3 cells which were constructed by lentivirus and verified by Western blot and qRT-PCR to confirm the key role of AMPK (Fig. 2C and D). In AMPK-silenced GH3 cells, the inhibitory effect of ACT001 on cell proliferation was greatly weakened in the CAB+ACT001 group (Fig. 2E). The cell survival rate of the CAB+ACT001+A-769662 group was lower than that of the CAB+ACT001 group (40.53 ± 3.56% vs 71.77 ± 3.35%, $P < 0.001$). The cell survival rate of AMPK-silenced GH3 cells which were treated with CAB+ACT001 was higher than that of normal GH3 cells which were treated with CAB+ACT001 (Fig. 2E).

In order to verify whether the activation of AMPK by ACT001 can further inhibit mTOR and induce autophagy, we measured the expression of AMPK (pThr172), mTOR negative regulatory protein Raptor (pSer792) and TSC2 (pSer1387) in each group by Western blot (Fig. 2F). We also measured the expression of Beclin-1 (pSer14), ULK1 (pSer317), ULK1 (pSer757) and autophagy markers LC3 I/II (Fig. 2G). The number of autophagosomes in each group was counted under transmission electron microscope (Fig. 2H).

Western blot results showed that the protein expression levels of AMPK (pThr172), mTOR negative regulatory protein Raptor (pSer792) and TSC2 (pSer1387) in the CAB+ACT001 group were higher than those in the CAB group and ACT001 group. The protein expression levels of Beclin-1 (pSer14) and ULK1 (pSer317) which promote autophagy in the CAB+ACT001 group were higher than those in the CAB group and ACT001 group, and the ratio of LC3 II/LC3 I was also higher. The expression level of ULK1 (pSer757) which inhibits autophagy was significantly lower in the CAB+ACT001 group compared with the CAB group.
Figure 2
ACT001 induced autophagic cell death in CAB-resistant GH3 cells by AMPK-mTOR pathway. (A) The cell viability of GH3 cells treated with different drugs was detected by CCK-8 assay. (B) The proliferation ability of GH3 cells treated with different drugs was detected by clone formation assay. (C and D) AMPK silenced GH3 cells were verified by Western blot. (E) The cell viability of normal GH3 cells and shAMPK GH3 cells treated with different drugs was detected by CCK-8 assay. (F) The protein expression of AMPK (pThr172), mTOR, mTOR negative regulatory protein Raptor (pSer792) and TSC2 (pSer1387) in each group was evaluated by Western blot. (G) The number of autophagosomes in each group was counted under transmission electron microscope. CAB: 50 μM; ACT001: 20 μM; A-769662: 10 μM; compound C: 10 μM ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

and ACT001 group. After addition of AMPK agonist, the protein expression levels in the CAB+A-769662 group were similar to those in the CAB+ACT001 group. After addition of AMPK inhibitor, the protein expression levels in the CAB+ACT001 + compound C group were similar to those in the CAB group.

The results of transmission electron microscope (Fig. 2H) showed that the number of autophagosomes in the CAB+ACT001 group was significantly higher than that in the CAB group ($P<0.001$) and ACT001 group ($P<0.001$). The number of autophagosomes in the CAB+A-769662 group was similar to that in the CAB+ACT001 group ($P>0.05$) after addition of A-769662. After addition of compound C, the number of autophagosomes in the CAB+ACT001 + compound C group was similar to that in the CAB group ($P>0.05$).
ACT001 induced apoptotic in BRC-resistant MMQ cells by AMPK-EGR1 pathway

In MMQ cells (Fig. 3A), the results of CCK8 assay showed that the cell viability of the BRC+ACT001 group was lower than that of the BRC group (49.40 ± 5.46% vs 69.50 ± 3.30%, P < 0.01) and ACT001 group (49.40 ± 5.46% vs 81.00 ± 2.09%, P < 0.01). After addition of A-769662 (AMPK agonist), the cell viability of the BRC+A769662 group was similar to that of the BRC+ACT001 group (49.10 ± 4.52% vs 49.40 ± 5.46%, P > 0.05). After addition of compound C (AMPK inhibitor), the cell viability of the BRC+ACT001+compound C group was similar to that of the BRC group (73.83 ± 3.97% vs 69.50 ± 3.30%, P > 0.05).

AMPK-silenced MMQ cells were also constructed by lentivirus, and the constructed cell lines were verified by Western blot and qRT-PCR (Fig. 3B and C). In AMPK-silenced MMQ cells, the inhibitory effect of ACT001 on cell proliferation was greatly weakened in the BRC+ACT001 group (Fig. 3D). The cell survival rate of the BRC+ACT001+A-769662 group was lower than that of the BRC+ACT001 group (43.13 ± 1.40% vs 68.13 ± 3.47%, P < 0.001). The cell survival rate of AMPK-silenced MMQ cells which were treated with BRC+ACT001 was higher than that of normal MMQ cells which were treated with BRC+ACT001 (68.13 ± 3.47% vs 35.13 ± 2.00%, P < 0.001).

To verify whether ACT001 can activate EGR1 and induce cell apoptosis, we measured the expression levels of AMPK (pThr172) and EGR1 in each group (Fig. 3E), and the expression levels of BAX (promote apoptosis) protein, Bcl-2 (inhibit apoptosis) and apoptosis marker Caspase-3 (Fig. 3F). We also detected the apoptosis of cells in each group by flow cytometry (Fig. 3G).

The protein expression levels of AMPK (pThr172) and EGR1 in the BRC+ACT001 group were significantly higher than those in the BRC group and ACT001 group. The protein expression levels in the BRC+A-769662 group were similar to those in the BRC+ACT001 group after addition of AMPK agonist. After addition of AMPK inhibitor, the protein expression levels in the BRC+ACT001+compound C group were similar to those in the BRC group. The expression level of BAX in the BRC+ACT001 group was higher than that in the BRC group and ACT001 group, while the expression of Bcl-2 was downregulated and Caspase-3 expression was upregulated in the BRC+ACT001 group. The protein expression levels in the BRC+A769662 group were similar to those in the BRC+ACT001 group after addition of A769662, and the protein expression levels in the BRC+ACT001+compound C group were similar to those in the BRC group after addition of compound C.

The results of flow cytometry showed that more cells in the BRC+ACT001 group underwent apoptosis than the BRC group (P < 0.001) and ACT001 group (P < 0.001). The apoptotic level in the BRC+A-769662 group was similar to that in the BRC+ACT001 group (P > 0.05), and the apoptotic level in the BRC+ACT001+compound C group was similar to that in the BRC group (P > 0.05).

Subcutaneous xenograft model in nude mice

Five days after s.c. injection of GH3 cells, the nude mice were randomly divided into five groups: control group, CAB treatment group, ACT001 treatment group, CAB+ACT001 treatment group, and CAB+ACT001+NAC treatment group. The tumor samples were harvested 2 weeks after administration (Fig. 4A).

The results of tumor volumes in nude mice (Fig. 4B) showed that the tumor volume of the CAB+ACT001 group was significantly smaller than that of the CAB group (409.67 ± 87.02 mm³ vs 1392.10 ± 348.16 mm³, P < 0.001) and ACT001 group (409.67 ± 87.02 mm³ vs 1623.00 ± 162.52 mm³, P < 0.001), and the tumor volume of the CAB+ACT001+NAC group was similar to that of the CAB group (409.67 ± 87.02 mm³ vs 1623.00 ± 162.52 mm³, P < 0.001).

The immunohistochemical results of tumor tissue (Fig. 4C) showed that the expression of AMPK (pThr172), Raptor (pSer792) and Beclin (pSer14) in the CAB+ACT001 group was higher than that in the CAB group and ACT001 group, and the expression of AMPK (pThr172), Raptor (pSer792) and Beclin (pSer14) in the CAB+ACT001+NAC group was similar to that in the CAB group.

Discussion

DA is the first choice for the treatment of prolactinomas. However, studies have shown that 20% of prolactinoma patients are resistant to DA, while 10% of patients are resistant to CAB (Maiter 2019).

DA-resistant prolactinomas often need surgical intervention (Vroonen et al. 2019). Total resection of drug-resistant prolactinomas is sometimes difficult because of tumor invasion to the surrounding structures (Wen et al. 2016, Zhu et al. 2021). Therefore, the treatment of DA-resistant prolactinomas is a challenge in clinical practice.

Our previous studies indicated that the resistance of prolactinomas could be reversed by further activating
ACT001 reversed resistance of prolactinomas

Figure 3
ACT001 induced apoptotic cell death in BRC-resistant MMQ cells by AMPK-EGR1 pathway. (A) The cell viability of MMQ cells treated with different drugs was detected by CCK-8 assay. (B and C) AMPK silenced MMQ cells were verified by Western blot and qRT-PCR. (D) The cell viability of normal MMQ cells and shAMPK MMQ cells treated with different drugs was detected by CCK-8 assay. (E) The protein expression of AMPK (pThr172) and EGR1 in each group was evaluated by Western blot. (F) The protein expression of apoptosis-related protein was evaluated by Western blot. (G) The apoptotic rate in each group was detected by flow cytometry. BRC: 50 μM; ACT001: 20 μM; A-769662: 10 μM; compound C: 10 μM (*P < 0.05, **P < 0.01, ***P < 0.001).
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Figure 4
The effect of ACT001 in s.c. xenograft model and the graphic mechanism. (A) Images of xenograft tumors in nude mice. (B) The tumor growth curve on the nude mouse. (C) Immunohistochemical analyses of AMPK (pThr172), Raptor (pSer792) and Beclin (pSer14) in tumor samples of each group. (D) Proposed mechanism for ACT001-mediated reversing resistance in DA resistance prolactinoma cells. CAB: 5 mg/kg/day; ACT001: 100 mg/kg/day; NAC: 100 mg/kg/day.
EGR1 and, at the same time, inhibiting mTOR (Tang et al. 2019). However, due to the lack of cell line which is resistant to both BRC and CAB, we can only study the effects of drugs on CAB-resistant prolactinomas in GH3 cells and BRC-resistant prolactinomas in MMQ cells.

PTL is a natural product which is isolated from medical herb and has a variety of important pharmacological activities. In recent years, it has been found that PTL plays an anticancer role in tumors (such as cholangiocarcinoma, prostate cancer, melanoma, breast cancer, leukemia, etc.) (Lesiak et al. 2010, D’Anneo et al. 2013, Xu et al. 2013). It has also been reported that PTL can increase the level of intracellular ROS by inhibiting the activity of superoxide dismutase in mitochondria (Xu et al. 2013).

However, PTL has been proved to be unstable with low oral bioavailability and poor water solubility (Jin et al. 2007). The researchers converted PTL into micheliolide by structural rearrangement and synthesized ACT001, a new anticancer sesquiterpene lactone, by Michael addition reaction (Zhang et al. 2012). After chemical modification, the stability, bioavailability and water solubility of ACT001 were significantly improved, and ACT001 can penetrate the blood–brain barrier to act on the CNS (Xi et al. 2019). The tissue distribution of ACT001 in Sprague–Dawley rats showed that the plasma’s maximal concentration of ACT001 after the oral administration dose of 100 mg/kg reached 6971.25 ± 1929.43 ng/mL and the concentration of ACT001 in the brain is higher than that in rats’ plasma which reached 12,748.75 ± 4386.25 (Xi et al. 2019).

Toxicity evaluation is crucial to explore the possibility to exploit the drug in clinical practice. The animal study showed that oral administration of DMAMCL (the prodrug of ACT001) at 200 or 300 mg/kg once a day for 21 days did not result in toxicity in the C6 rat tumor model (An et al. 2015). ACT001 has been designated as an orphan drug for glioblastoma by the FDA. It is currently being evaluated in several clinical trials (ACTRN1261600228482, Australian New Zealand Clinical Trials Registry; ChiCTR-OIC-17013604, Chinese Clinical Trial Register). In a phase I dose-escalation study of ACT001 in 13 patients with brain tumors, the treatment (standard 3 + 3 design, dose levels were 100 mg BID, 200 mg BID, 400 mg BID and 600 mg BID) was well tolerated and no dose-limiting toxicities occurred (Licklitter et al. 2021).

Studies showed that ACT001 could also attenuate the activity of superoxide dismutase and increase the level of ROS in cells (Li et al. 2020). Our results also confirmed that ACT001 can increase the level of ROS in tumors.

ROS are by-products of biological reactions and play a dual role in the occurrence and development of tumors. In normal cells, the clearance and production of ROS are balanced, while cancer cells tend to have higher levels of ROS because of their different ways of energy metabolism. The increased level of oxidative stress in cancer cells can be used as a target to have an anti-tumor effect through the regulation of pro-oxidants and antioxidants or redox regulators (Xu et al. 2013). The high level of intracellular ROS will affect the intracellular energy metabolism, which leads to the increase of intracellular AMP content and the decrease of ATP content (Filomeni et al. 2015). Our results showed that ACT001 can increase the ratio of AMP/ATP in tumor cells.

The increase of intracellular AMP/ATP ratio will enhance the activity of AMPK (Filomeni et al. 2015). AMPK is the sensor of intracellular energy metabolism, and its γ subunit can bind to AMP, and then α subunit is phosphorylated, thus activating AMPK signal pathway and maintaining intracellular energy balance (Filomeni et al. 2015). In addition, it has been reported that intracellular ROS can activate AMPK through a pathway independent of the increase of intracellular AMP/ATP ratio (Hart et al. 2015). Our research proved that ACT001 can activate AMPK in tumor cells. For GH3 and MMQ cells, the expression of AMPK (pThr172) in the ACT001 + DA group was significantly higher than that in the DA group and ACT001 group.

Our previous study found that CAB induced autophagic cell death in prolactinoma cells mainly by inhibiting mTOR (Tang et al. 2019), suggesting that further inhibition of mTOR can induce autophagic cell death in CAB-resistant prolactinomas.

mTOR is a nutrition and growth factor receptor complex, located at the intersection of glucose and amino acid metabolism, regulating cell anabolism and autophagy-related cell death (Albert & Hall 2015). Studies have shown that inhibition of mTOR can inhibit the proliferation of pituitary adenoma cells in vitro (Monsalves et al. 2014). Gorvin et al. reported the results of a patient with DA-resistant prolactinoma treated with mTOR inhibitor everolimus. After using everolimus (10 mg/day), the prolactin level decreased by 44%, and the tumor size remained stable 1 year later in this patient (Gorvin et al. 2019).

In cells, mTOR and other proteins such as Raptor constitute mTOR complex 1 (mTORC1) to regulate the downstream pathway. AMPK can phosphorylate Raptor, a regulatory protein, at Ser-792 to prevent mTOR from binding to its downstream molecules, thus inhibiting the function of mTOR. In addition, AMPK can phosphorylate TSC2 at Ser-1387 to indirectly inhibit the function of
mTORC1 (Zhao et al. 2017). Our results showed that ACT001 could phosphorylate and activate AMPK, and the expression levels of Raptor (pSer792) and TSC2 (pSer1387) in CAB+ACT001 group were higher than those in CAB group and ACT001 group.

mTORC1 can phosphorylate ULK1 at Ser-757 and inactivate it to inhibit autophagy (Zhao et al. 2017). AMPK can phosphorylate ULK1 at Ser-317 to initiate Beclin1-mediated autophagy (Shimobayashi & Hall 2014). Our study confirmed that the expression levels of Beclin-1 (pSer14) and ULK1 (pSer317) in CAB+ACT001 group was higher and the expression level of ULK1 (pSer757) was lower compared with CAB group and ACT001 group. The LC3 II/LC3 I ratio was higher and the number of autophagosomes in cells was more in the CAB+ACT001 group compared with the CAB group and ACT001 group, which meant that ACT001 had the ability to induce autophagic cell death and cooperate with CAB to reverse drug resistance.

In our previous published studies, BRC mainly leads to apoptotic death of tumor cells by activating EGR1 (Tang et al. 2019), suggesting that further activation of EGR1 can increase apoptotic cell death in BRC-resistant prolactinomas.

EGR1 can regulate cell proliferation and differentiation (Thiel & Cibelli 2002). The high expression of EGR1 significantly inhibited the proliferation of breast cancer, non-small cell lung cancer and esophageal cancer (Dong et al. 2011, Li et al. 2014). Studies have shown that AMPK can activate EGR1 (Di Biase et al. 2017). In order to verify whether ACT001 can activate EGR1 by activating AMPK and then cooperate with BRC to reverse drug resistance, we detected the expression level of EGR1 and cell survival rate after different drug treatment. The results showed that the expression of EGR1 in BRC+ACT001 group was higher than that in ACT001 and BRC group, and the cell survival rate was significantly lower.

EGR1 is also a pro-apoptotic protein and multiple pathways may be involved in its pro-apoptotic effect (Thiel & Cibelli 2002). In melanoma cells, EGR1 can directly upregulate the transcription of p53 gene, cause p53 overexpression and directly induce apoptosis (Nair et al. 1997). EGR1 can also interact with transcription factor c-Jun and regulate its activity, thus promoting apoptosis (Ham et al. 2000). Our research proved that ACT001 can promote apoptosis by activating EGR1 as reversing the expression level of BAX, Bcl-2, cleaved Caspase-3 and quantitatively assessing apoptosis by flow cytometry.

We also used s.c. xenograft model to further verify whether ACT001 can activate AMPK in vivo and cooperate with DA to reverse drug resistance. Because MMQ cells are suspended cells and can not form tumors in vivo, we only use GH3 cells to construct s.c. xenograft model in nude mice. In vivo, we verified that ACT001 can activate AMPK and promote autophagic death of tumor cells, thus reversing CAB resistance of prolactinomas.

In this article, we verified that ACT001 can increase intracellular ROS level by increasing intracellular AMP/ATP ratio and activate AMPK. For DA-resistant prolactinoma cells, ACT001 can inhibit mTOR and activate EGR1 by phosphorylating AMPK and induce cell death through autophagy and apoptosis in combination with DA.


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