Pituitary Tumor Suppression by Combination of Cabergoline and Chloroquine

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Context: The dopamine agonist cabergoline (CAB) has been used widely in the treatment of prolactinomas and other types of pituitary adenomas, but its clinical use is hampered by intolerance in some patients with prolactinoma and lack of effectiveness in other pituitary tumor types. Chloroquine (CQ) is an old drug widely used to treat malaria. Recent studies, including our own, have revealed that CAB and CQ are involved in induction of autophagy and activation of autophagic cell death.

Objective: To test whether CAB and CQ can function cooperatively to suppress growth of pituitary adenomas as well as other cancers.

Results: *In vitro* studies using the rat pituitary tumor cell lines MMQ and GH3, human pituitary tumor cell primary cultures, and several human cancer cell lines showed that CQ enhanced suppression of cell proliferation by CAB. These results were confirmed in *in vivo* xenograft models in nude mice and estrogen-induced rat prolactinomas. To understand the mechanism of combined CAB and CQ action, we established a low-CAB-dose condition in which CAB was able to induce autophagy but failed to suppress cell growth. Addition of CQ to low-dose CAB blocked normal autophagic cycles and induced apoptosis, evidenced by the further accumulation of p62/caspase-8/LC3-II.

Conclusion: The data suggest that combined use of CAB and CQ may increase clinical effectiveness in treatment of human pituitary adenomas, as well as other cancers, making it an attractive option in tumor and cancer therapies. (***J Clin Endocrinol Metab*** **102: 3692–3703, 2017)**

Cabergoline (CAB) is a dopamine agonist widely used clinically for the treatment of pituitary adenomas and Parkinson disease (1, 2). It is the first choice for the treatment of prolactinomas because it effectively reduces prolactin (PRL) secretion and shrinks tumors in most patients (2, 3). Recently, its clinical use has been expanded to the treatment of other types of pituitary tumors, including acromegalic and adrenocorticotropic hormone–secreting adenomas, as well as nonfunctioning pituitary adenomas (4–6). Although CAB is well tolerated in general, severe adverse events as well as tumor resistance occur in some patients. Despite its clinical uses for decades, the detailed molecular mechanisms of CAB action are not fully understood, especially pertaining to its tumor-suppressive effect. We have previously reported that CAB is capable of inducing autophagy through inhibition of the mammalian target of rapamycin pathway. Furthermore, CAB caused autolysosome acidification, resulted in impaired lysosomal content degradation, and thus blocked autophagic flux and induced cell death (7).

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Abbreviations: CAB, cabergoline; CQ, chloroquine; GFP, green fluorescent protein; LC3, light chain 3; MRI, magnetic resonance imaging; PARP, poly-adenosine diphosphate ribose polymerase; PBS, phosphate-buffered saline; PRL, prolactin; RNAi, RNA interference.

Chloroquine (CQ) has been used to treat malaria since World War II (8) and has also been used to treat some autoimmune diseases (9, 10). Recent studies have found that CQ can also induce growth arrest and apoptosis in several cancer cells and sensitize cancer cells to chemotherapy (11–17). Several clinical trials using CQ as an adjuvant therapy in combination with traditional chemotherapies and radiotherapy have shown some promising results (12, 18–20). A well-studied mechanism for the antiproliferative function of CQ is the inhibition of autophagy.

These recent findings prompted us to explore the combined use of CAB and CQ on tumor suppression. We report the *in vitro* antiproliferation and *in vivo* tumor suppression in pituitary tumors and other cancer cells by CAB and CQ and investigate the tumor-suppressive mechanisms of autophagy and apoptosis.

Materials and Methods

Cell culture, pituitary tumor tissues, and other reagents

Rat pituitary cell lines GH3 and MMQ cells were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle medium and F12 medium, respectively (Gibco, Life Technologies, Grand Island, NY), supplemented with 2.5% fetal bovine serum (Gibco) and 15% horse serum (Gibco). All other human cancer cell lines (U87, SW480, SCG7901, SHG66, hepG2) were gifts from Dr. Liang-Fu Zhou (Department of Neurosurgery, Huashan Hospital, Shanghai, China) and cultured in DMEM supplemented with 10% fetal bovine serum. All cells were cultured in an atmosphere of 5% CO_2: 95% air at 37°C.

Primary human pituitary tumor cells were obtained from patients who underwent surgery for pituitary tumors between October 2014 and May 2016 at the Department of Neurosurgery, Ruijin Hospital of Jiaotong University, Shanghai, China. The adenoma tissues from surgery were enzymatically and mechanically dispersed (21), and the tumor cells obtained were carefully washed by repeated centrifugation and were cultured in DMEM with 10% fetal bovine serum and 100U/mL penicillin/streptomycin (Gibco). Cells were seeded onto six-well plates at a density of 200,000 cells per well and were cultured in the previously-described cell culture medium for 48 hours before treatment.

Different cell lines as well as 15 human pituitary tumor primary cell cultures (growth hormone–secreting adenoma, n = 3; prolactin-secreting adenomas, n = 4; clinically nonfunctioning pituitary adenomas, n = 8) were analyzed for cell viability in response to CAB and CQ treatment.

The Ethics Committee of Shanghai Jiaotong University School of Medicine approved this study. All patients whose tumor tissues were used in this study provided written informed consent.

Chemical compounds used in this study include CAB (2664, Tocris Bioscience, Bristol, United Kingdom), Z-IETD-FMK (ab141382, Abcam, Cambridge, MA), dimethyl sulfoxide (D2650, Sigma-Aldrich, St. Louis, MO), and CQ (C6628, Sigma-Aldrich).

Cell viability and cell death assays

A total of 1×10^4 cells were plated in 96-well tissue culture plates and treated with different doses of each drug for certain time as indicated. Cell viability was assayed by using the MTS-based CellTiter 96AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. Upon addition of MTS solution, the plate was incubated at 37°C for 4 hours, and the absorbance was read at 490 nm with a plate reader (TECAN, Männedorf, Switzerland). Data from at least three independent experiments (at least twice for primary cell cultures) were averaged. To measure apoptotic cell population, cells were stained with annexin V-PE as described by the manufacturer's protocol (BD Biosciences, San Jose, CA) and assayed by fluorescence-activated cell sorting (Beckman Coulter, Brea, CA).

Transfection and RNA interference

MMQ and GH3 cells were stably transfected with green fluorescent protein (GFP)–LC3 lentivirus from GenePharma (Shanghai) to generate GFP-LC3-expressing cells. Briefly, cells were seeded in six-well plates and transfected with 20 and 100 multiplicity of infection of GFP-LC3 lentivirus using Polybrene (2 μg/mL; Sigma-Aldrich). Cells with GFP fluorescence were selected by MoFlo XDP Cell Sorter (Beckman Coulter) and cultured in complete cell culture medium containing G418 at 200 mg/mL for future experiments. RNA interference was performed as previously described (22). The small interfering RNAs against rat p62 and caspase-8 were purchased from GenePharma (Shanghai, China), with the following sequences: for sip62: 5′ GUGAUGAGGAACUGACAAUTT 3 and 5′ AUUGUCAGUUCCUCAUCACTT 3′; for siCaspase-8: 5′GCUCUGAGUAAGACCUUUATT3′ and 5′UAAAGGUCUUACUCAGAGCTT3′.

Caspase activity assays

Caspase-8 or caspase-3/7 activity was measured by the Caspase-Glo 8 or Caspase-Glo 3/7 Assay (Promega) that uses a luminogenic caspase-8 (LETD) or caspase-3/7 (DEVD) substrate. Measurements were performed by using a luminescence reader (TECAN), according to the recommended protocol by Promega.

Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting

Cells were lysed by radioimmunoprecipitation assay buffer (Beyotime Biotechnology, Beijing, China), and total protein concentration was measured by using a bicinchoninic acid protein assay kit (Tiangen Biotech, Beijing, China). Extracted proteins were separated in sodium dodecyl sulfate– polyacrylamide gel electrophoresis and analyzed by Western blot. Antibodies used in Western blot analyses included anti- glyceraldehyde 3-phosphate dehydrogenase (ab181602, Abcam, Cambridge, MA), anti-LC3 (L7543, Sigma-Aldrich), anti-p62 (ab56416, Abcam), anti–caspase-8 (*p*-18, ab25901, Abcam), anti–total caspase-8 (catalog #4790, Cell Signaling Technology, Danvers, MA), and poly-adenosine diphosphate ribose polymerase (PARP; catalog #9532, Cell Signaling Technology). All antibodies were used according to manufacturers' instructions. Each Western blot was repeated at least three times. Intensities of protein bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence and microscopy

For colocalization of caspase-8, p62, and LC3, GH3 cells cultured on Laboratory-Tek-II Chamber slides (Sigma-Aldrich) were washed three times in phosphate-buffered saline (PBS), fixed for 20 minutes in 4% paraformaldehyde at room temperature, washed with PBS, and blocked with blocking buffer (5% bovine serum in PBS) for 1 hour at room temperature. Primary antibodies were diluted in blocking buffer with 0.1% saponin and incubated with cells at 4°C overnight. After three washes with PBS, cells were incubated with secondary antibodies conjugated with Alexa 594, Alexa 488 (Invitrogen, Carlsbad, CA), or Alexa 647 (Cell Signaling) for 1 hour at room temperature. Then the nuclei were stained with 0.1 mg/mL 4′, 6-diamidino-2-phenylindole (D9542, Sigma-Aldrich) for 15 minutes at room temperature. Staining was visualized and photographed by using an LSM710 laser scanning confocal microscope with a ×63 oil immersion lens (Carl Zeiss, Oberkochen, Germany).

Xenograft animals and rat prolactinoma model

Female athymic nude mice (nu/nu) were purchased from Shanghai Slack Laboratory Animal Co., Ltd. (SLAC, Shanghai, China), and kept under specific pathogen-free conditions. One million GH3 or SCG7901 gastric cancer cells were mixed with PBS and injected subcutaneously into the flanks of 5-week-old athymic nude mice. The animals were randomly assigned to four groups with six animals in each group: control group, CAB group, CQ group, and CAB plus CQ group. For drug administration, the animals were injected intraperitoneally with 100 μL vehicle control (0.1% dimethyl sulfoxide in 0.9% saline), CAB (0.5 mg/kg), CQ (50 mg/kg) alone, or CAB plus CQ (the same dose as single-drug treatment) for 14 days. Tumor ellipsoid volume was estimated as previously described (23). The weight of mice and tumor dimensions were measured every other day after drug injection. When the tumor sizes reached approximately 50 mm^3, all mice were euthanized and photographed and tumors were collected and used for Western blotting. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Ethics Committee of Shanghai Jiaotong University School of Medicine approved the care and use of athymic nude mice.

For the rat prolactinoma model, twenty 4-week-old female F344 rats were purchased from Vital River Laboratories (Beijing, China) and were fed *ad libitum* in a controlled 12-hour light/12-hour dark environment. The local animal care and use committee approved all experimental protocols. The prolactinomas were induced by 17β-estradiol for 6 weeks, as described by Cao *et al.* (24). After validation by magnetic resonance imaging (MRI) for pituitary tumor formation, the rats were divided into the CQ group (50 mg/kg, daily intragastric administration, n = 5), CAB group (0.5 mg/kg, intragastric administration once every 3 days, n = 5), CAB plus CQ group (daily CQ 50 mg/kg plus CAB 0.5 mg/kg once every 3 days, n = 5), or control group (vehicle, n = 5). Tumor sizes were monitored by using the high-field Bruker ClinScan 7.0-Tesla magnetic resonance scanner for small animals equipped with a surface coil (Bruker, Ettlingen, Germany) before animals were euthanized after the 14-day treatment. Tumors were weighed and stored for further analyses. The tumor volumes were calculated as follows: anteroposterior diameter × transverse diameter × transverse diameter × $\pi/6$.

Statistics

Statistical significance in experiments was assessed by using GraphPad Prism, version 5 (GraphPad Software, La Jolla, CA). Student unpaired, two-tailed *t* test with a 95% confidence interval was used to analyze data involving direct comparison of an experimental group with a control group. Reported *P* values were adjusted to account for multiple comparisons. A *P* value <0.05 was considered to indicate statistical significance.

Results

Cooperation of CAB and CQ in cell death and tumor suppression

We have previously shown that CAB induces autophagy but blocks autophagic flux, leading to cell death (7). It has been reported that CQ is an inhibitor of autophagy, which augments the effectiveness of chemotherapy and radiotherapy in cancers (13, 25). Treatment of the rat pituitary tumor cell line MMQ with CAB at 50 μM for 24 and 48 hours decreased cell viability by 46.2% and 55.6%, respectively ($P < 0.001$); combined treatment with CAB at 50 μM and CQ at 20 μM for 24 and 48 hours further reduced cell viability, by 81.0% and 89.7%, respectively ($P < 0.001$) [Fig. 1(A)]. Meanwhile, treatment of MMQ cells with CQ alone at 20 μM for 24 and 48 hours had no major effect on cell viability [Fig. 1(A)]. Similarly, CAB treatment (at 100 μM) of another rat pituitary tumor cell line GH3 for 24 and 48 hours resulted in a decrease of cell viability by 39.1% and 51.6%, respectively ($P < 0.01$); combined treatment of GH3 cells with CAB at 100 μM and CQ at 20 μM for 24 and 48 hours resulted in a decrease of cell viability by 61.3% and 80%, respectively ($P < 0.05$) [Fig. 1(B)].

Next, we examined the effect of CAB and CQ on the cell viability of primary human pituitary tumor cell cultures. As shown in Supplemental Table 1, combined CAB (at 50 μM for prolactinomas and 100 μM for other types of pituitary adenomas) and CQ (at 20 μM) treatment of the primary pituitary tumor cell culture resulted in cell viability reduction for 13 of 15 tumor samples tested. It is interesting that in our experiment, 7 of 8 clinically nonfunctioning pituitary adenomas also showed responsiveness to combined CAB and CQ treatment, as it is generally believed that this type of pituitary adenoma is not responsive to CAB.

Interestingly, the observation that CQ enhances CAB-mediated suppression of cell viability is not restricted to pituitary tumor cells. Similar effects were also observed in several different human malignant cancer cell lines, such as glioblastoma U87 and U66, gastric cancer SCG7901, liver cancer HepG2, and colon cancer SW480 (Supplemental Table 1). For example, treatment of human gastric cancer SCG7901 with CAB at 100 μM for 24 and 48 hours resulted in a slight reduction of cell viability by

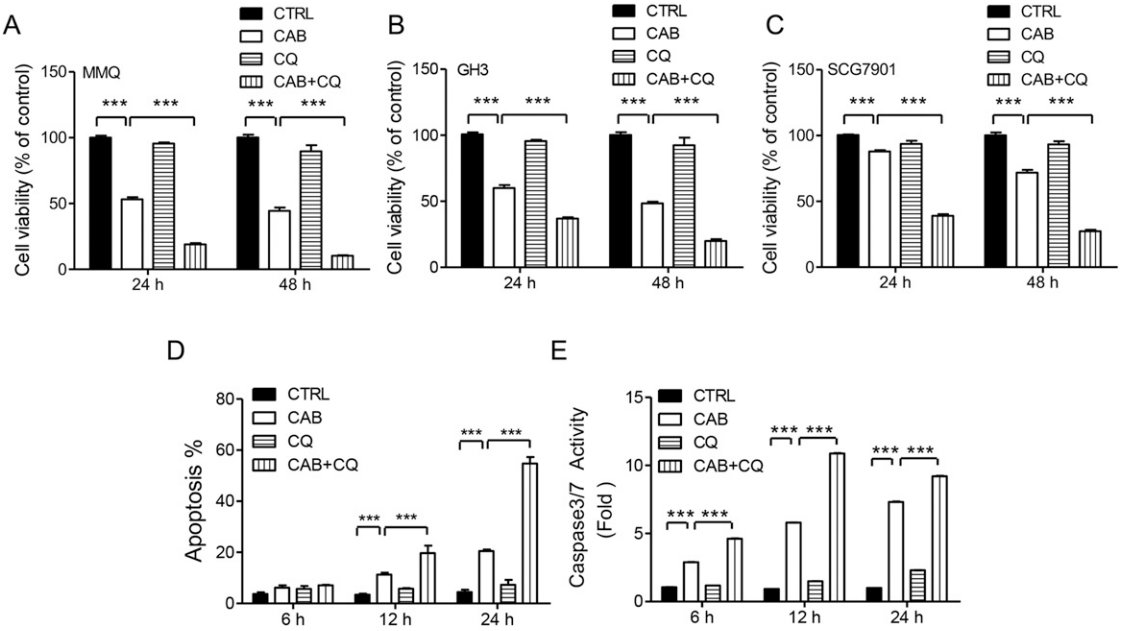

Figure 1. Inhibition of tumor growth treated with CAB and CQ *in vitro*. Combination CAB and CQ treatment of (A) MMQ, (B) GH3, and (C) gastric cancer SCG7901 resulted in decreased cell viability compared with CAB treatment alone. (D) Percentage of apoptotic cell population in MMQ cells with different treatments as indicated, measured by phosphatidylserine (PS) exposure and monitored by annexin V-PE binding. (E) Caspase-3/7 activation in MMQ cells with different treatments as indicated, evaluated by a luminescent activity assay kit. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Scale bar, standard error of the mean. CTRL, control.

11.7% and 28.3%, respectively ($P < 0.05$); when CAB was combined with CQ at 20 μM, the cell viability was reduced by 59.8% and 72.7% ($P < 0.01$) [Fig. 1(C)]. Therefore, it seems that suppression of tumor cell growth by CAB and CQ is a general phenomenon.

We then examined whether the addition of CQ led to further increase in apoptosis using fluorescence-activated cell sorting. As shown in Fig. 1(D), at 12 and 24 hours, the apoptotic cell population in MMQ cells treated with CAB (at 50 μM) was 16.1% and 22.3% ($P < 0.05$), respectively. When CAB and CQ (at 20 μM) were combined, the apoptotic cell population increased to 20.3% at 12 hours and 58.3% at 24 hours ($P < 0.01$). Furthermore, combined treatment led to an increase in caspase 3/7 activity, from 2.8-, 5.8-, and 7.3-fold to 4.5-, 11-, and 10-fold at 6, 12, and 24 hours, respectively, compared with the untreated controls ($P < 0.001$) [Fig. 1(E)]. Similar results were obtained from GH3 and SCG7901 cells (Supplemental Fig. 1). These data demonstrate that CQ is able to enhance CAB-mediated apoptosis.

We next examined the effect of CAB and CQ combination on *in vivo* tumor growth. First, GH3 cells were injected into nude mice to allow *in vivo* tumor formation and growth. In the treatment groups, CAB (0.5 mg/kg, once in 2 days) and/or CQ (50 mg/kg, once daily) was administrated starting on day 9 of GH3 cell injection. On day 24, a clear difference in tumor sizes was observed between the CAB/CQ combined group and the control or single-drug groups [Fig. 2(A)]. On day 27 of GH3 cell injection, the mice were euthanized and tumors were removed and weighed. A clear decrease in tumor size for tumors from the CAB/CQ combined group was found compared with the size of tumors from the control group (80.0 ± 32.86 mg *vs*. 243.33 ± 126.12 mg; $P < 0.05$) [Fig. 2(B) and 2(C); Supplemental Table 2), although CAB or CQ alone had no effect on tumor growth at the dose used here. Similar experiments were performed with human gastric cancer SCG7901 cells. After SCG7901 cells were injected into nude mice, CAB (0. 5 mg/kg, once in 2 days) and/or CQ (50 mg/kg, once daily) was administrated starting on day 4 of cell injection. On day 8, a clear difference in tumor sizes was observed between the CAB/CQ combined group and the control group [Supplemental Fig. 2(A)]. On day 14 of cell injection, the mice were euthanized and tumors were removed and weighed. A clear decrease in tumor size for tumors from the CAB/CQ combined group was found compared with that for tumors from the untreated control group (167.50 ± 44.55 mg *vs*. 535.0 ± 68.56 mg; $P < 0.05$) [Supplemental Fig. 2(B) and 2(C); Supplemental Table 2].

We also generated a rat prolactinoma model by estrogen induction. After the rats were treated with 17β-estradiol for 6 weeks, pituitary tumors *in situ* were confirmed by MRI [Fig. 2(D)]. From this moment on, 17β-estradiol was withdrawn and CAB and/or CQ

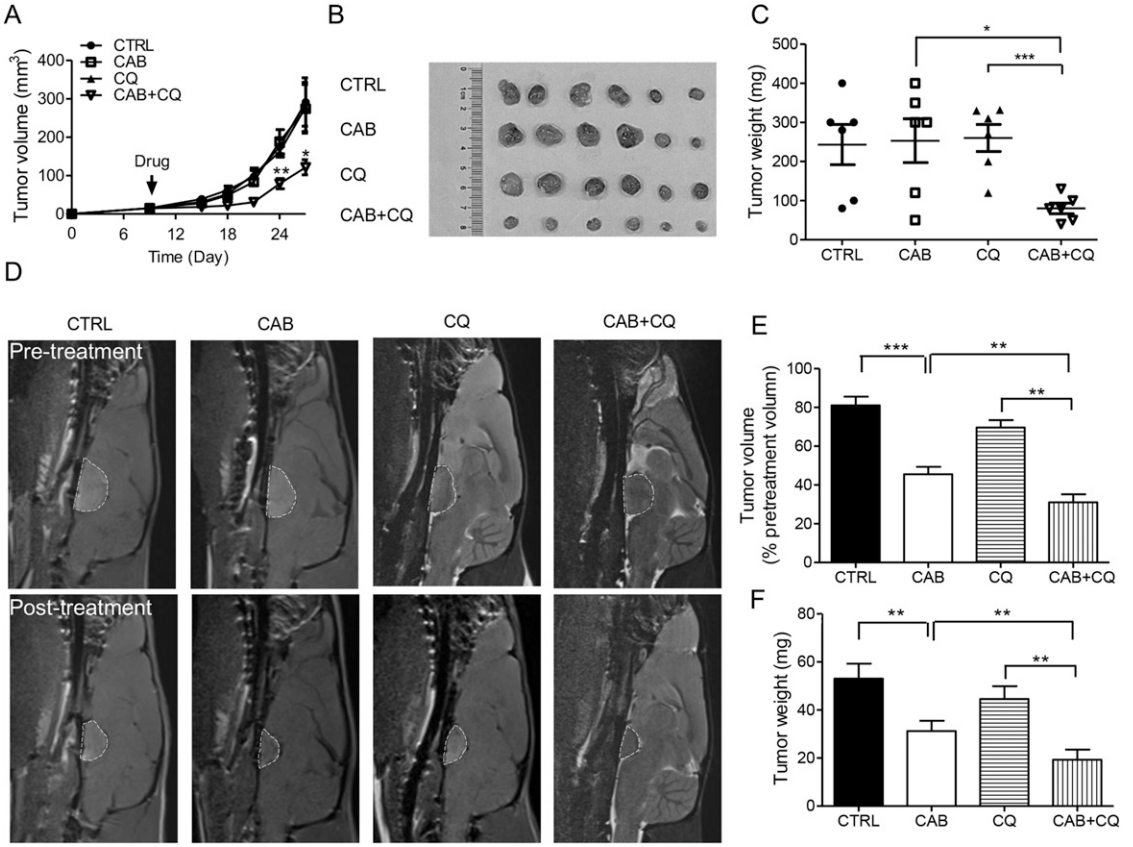

Figure 2. *In vivo* inhibition of tumor growth by CAB and CQ treatment. (A) Tumor volume growth curves of nude mice in different treatment groups. (B) Tumors obtained from mice injected with GH3 cells and treated with control (CTRL) vehicle, CAB, CQ, and CAB plus CQ at day 27 of drug administration. (C) Tumor weights from mice injected with GH3 cells and treated with control vehicle, CAB, CQ, and CAB plus CQ at day 27 of drug administration. (D) Representative MRI scan of tumor sizes of rat estrogen–induced prolactinomas in different treatment groups. (E) Tumor volume and (F) weight decreased significantly with CAB plus CQ treatment (n = 5). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Scale bar, standard error of the mean.

administration was started. After 14 days of CAB and/or CQ treatment, MRI revealed that although tumors shrank slightly after estrogen withdrawal in the control group (19.0% ± 4.5%), CAB treatment resulted in shrinkage of tumor volume by approximately 55% compared to the size before treatment. Furthermore, tumors in the CAB plus CQ group further shrank by 69% (average tumor size: CAB plus CQ, 31.0% ± 4.2% *vs.* CAB alone: 45.5% ± 3.9% compared with pretreated tumors; $P < 0.01$) [Fig. 2(E)]. The mean tumor weight was reduced from 53.0 ± 6.3 mg in the control group to 31.2 ± 4.3 mg in the CAB group and 19.3 ± 4.2 mg in the CAB plus CQ group [Fig. 2(F)]. These results showed the enhancement of the antitumor effect of CAB by CQ on prolactinomas *in situ*.

Taken together, our data clearly demonstrate that CQ is able to enhance the tumor suppressive function of CAB *in vivo* and *in vitro*.

CQ enhances CAB-mediated accumulation of autophagic protein p62

We recently reported that CAB was able to induce autophagy, resulting in cellular accumulation of p62, an autophagy cargo protein (7). Western blotting revealed that in MMQ, GH3, and SCG7901 cell lines, as well as primary cultured cells from a human pituitary acromegalic adenoma, CAB treated resulted in accumulations of p62, along with the conversion of microtubule-associated protein light chain 3 (LC3)-I to lipidated LC3-II, a classic hallmark of autophagy activation; these accumulations were further enhanced by the combination of CAB and CQ [Fig. 3(A–D); see Supplemental Fig. 3 for bar graph presentations], indicating an initiation of autophagy. Similarly, Western blotting also revealed that in tumors obtained from the aforementioned *in vivo* studies with GH3 and SCG7901 cells, there was a clear increase in p62 and LC3-II in tumors from the CAB/CQ combined group when compared with that in tumors from the CAB-alone group [Fig. 3(E) and Supplemental Fig. 2(D)], paralleling the effects observed in the *in vivo* experiments. In MMQ cells, immunofluorescence staining and confocal microscopy revealed that cellular p62 protein level increased upon CAB treatment [Fig. 3(F)], and this increase was further enhanced by CQ [Fig. 3(G)]. Taken together, CQ is able to enhance CAB-induced accumulation of autophagy-related proteins.

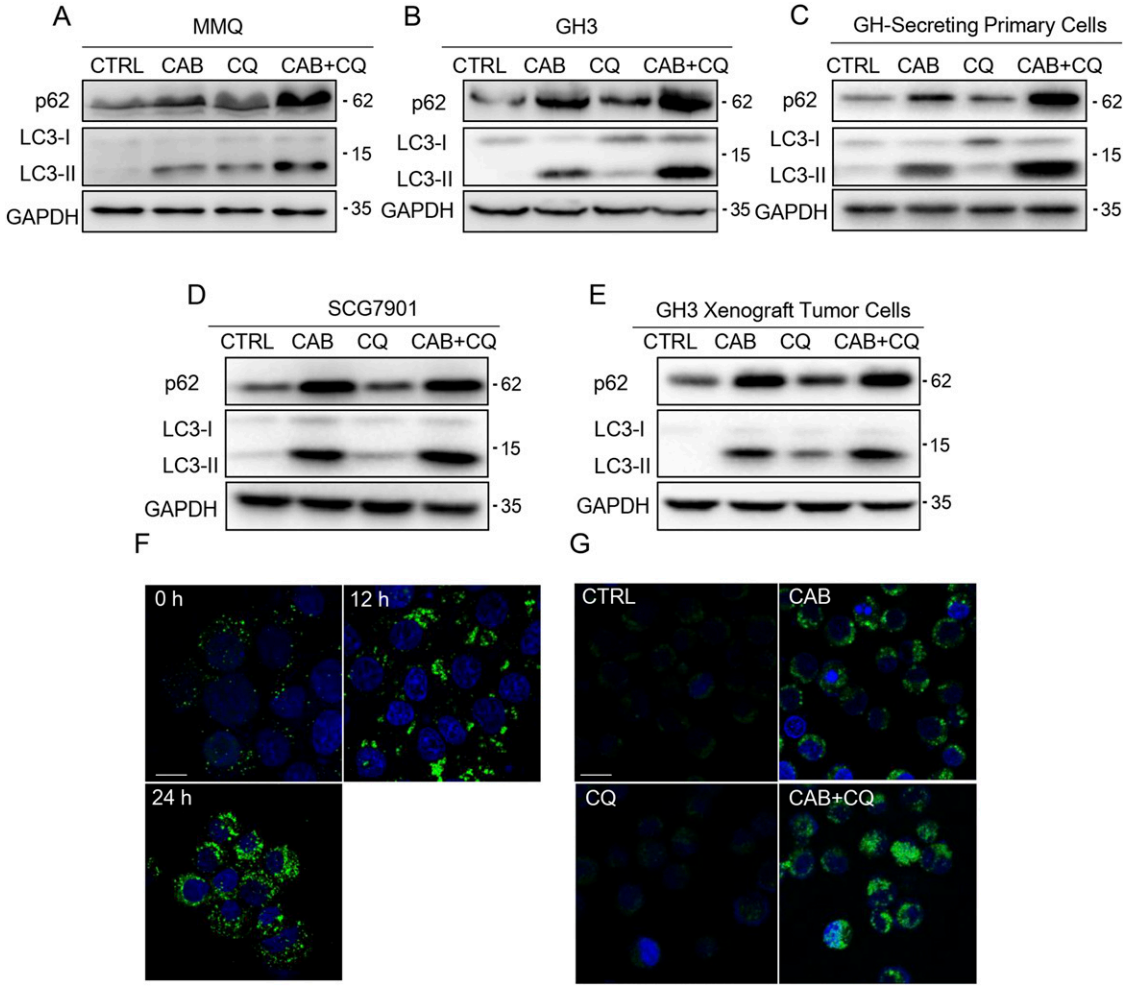

Figure 3. CQ enhances CAB-mediated p62 protein accumulation. (A–E) Western blotting showed that in (A) MMQ, (B) GH3, (C) primary cultured human GH–secreting tumor cells, (D) gastric cancer SCG7901, and (E) tumors from GH3-injected nude mice, CAB plus CQ treatment resulted in increase of autophagy-related proteins p62 and LC3-II. (F) In MMQ cells treated with CAB, p62 proteins were induced, as shown by immunostaining at 12 and 24 hours. (G) Enhanced p62 protein expression was observed in MMQ cells treated with CAB plus CQ for 24 hours, compared with p62 proteins in MMQ cells treated with CAB alone. Scale bar: 5 μm. CTRL, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

CAB and CQ combination enhanced disruption of autophagy

Next we explored the mechanism for tumor suppression by CAB and CQ. We hypothesized that CAB and CQ work concordantly to block the normal cycles of autophagy, thus causing cellular crisis and leading to cell death. To confirm this hypothesis, we first determined the CAB concentration at which it could induce autophagy but had no effect on tumor cell growth. As shown in Fig. 4(A) and 4(C), CAB treatment of MMQ cells at 10 μM or GH3 cells at 50 μM alone did not inhibit cell viability; however, when CQ at 20 μM was added to the treatment, a significant decrease in cell viability was observed at 48 and 72 hours.

To understand the impact of CAB and CQ combination on autophagy, Western blotting was used to monitor the expression of autophagic proteins p62 and LC3-II. As shown in Fig. 4(B), when MMQ cells were treated with CAB at 10 μM alone, induction of p62 and LC3-II was observed at 24 hours, indicating an initiation of autophagy. However, p62 and LC3-II levels were decreased at 48 hours and returned nearly to baseline level at 72 hours, indicating that a normal autophagy process was completed without cell damage. However, when CAB at 10 μM and CQ at 20 μM were combined, not only were the p62 and LC3-II levels at 24 hours higher than that of CAB-alone treatment, they were still increased at 48 hours and 72 hours, without clearance. This result was further confirmed by immunofluorescence staining and confocal microscopy: CAB induced p62 and LC3-II at 24 hours, but these proteins were cleared at 48 and 72 hours. However, when CAB and CQ were combined, p62 and LC3-II were still visible at 72 hours, indicating the blockage of the normal autophagic cycles [Fig. 4(E)]. Similar results were obtained in GH3 cells by Western blotting [Fig. 4(D)]. These data indicated that combined CAB and CQ treatment of MMQ and GH3 cells resulted in induction of autophagy

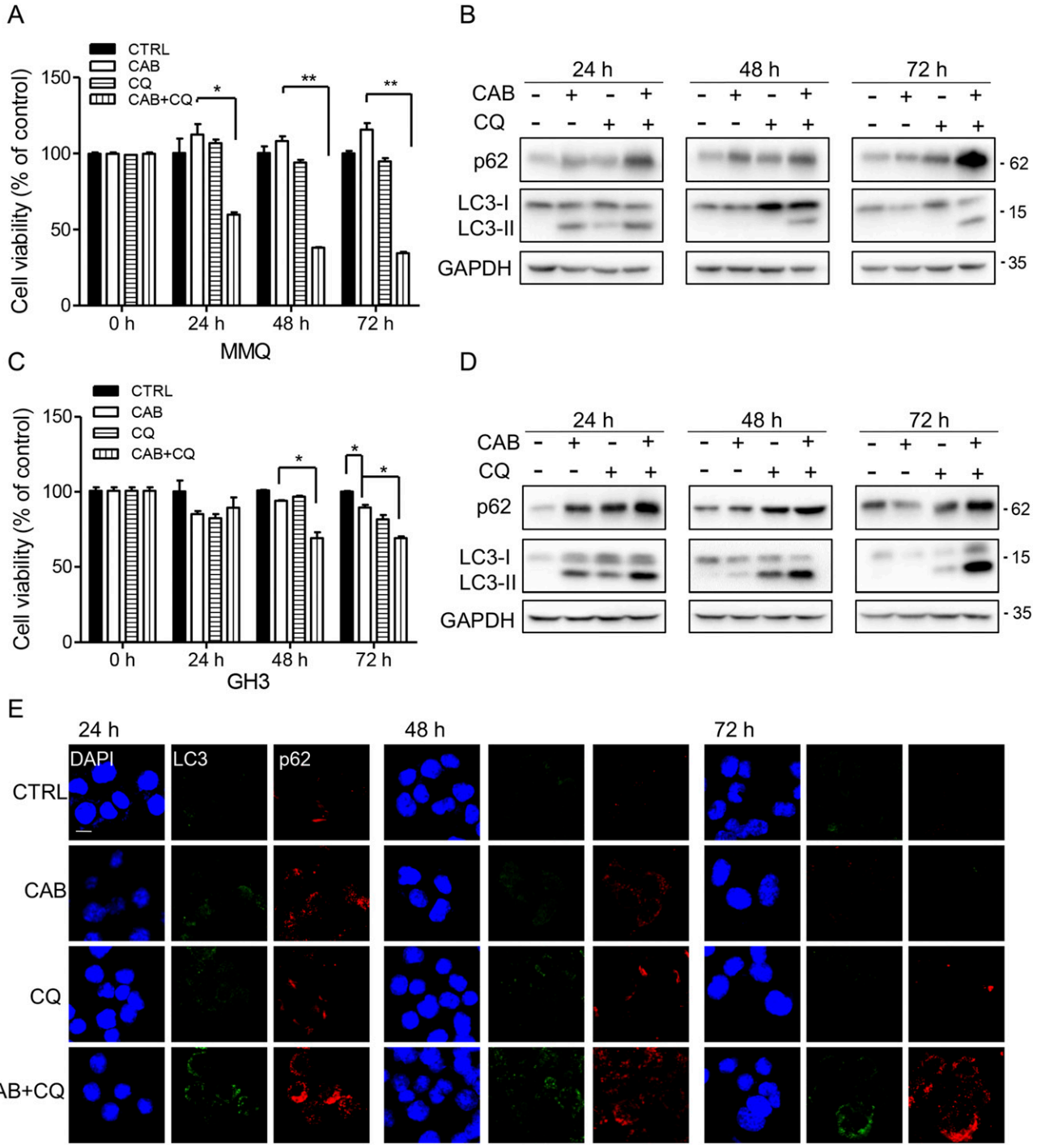

Figure 4. CAB and CQ combination enhanced disruption of autophagy. (A) Low-dose treatment of MMQ cells with 10 μM of CAB or 20 μM of CQ did not lead to major change in cell viability. However, when CAB at 10 μM and CQ at 20 μM were combined, cell viability decreased in a time-dependent manner. (B) In MMQ cells, Western blotting showed that CAB treatment at 10 μM resulted in a slight increase in autophagy-related protein p62 at 24 and 48 hours; however, this increase was eliminated at 72 hours. Similarly, another autophagy-related protein, LC3-II, was induced by CAB treatment at 10 μM at 24 hours but disappeared after 48 hours. In contrast, when CAB at 10 μM and CQ at 20 μM were combined, p62 levels steadily increased and LC3-II levels were sustained over time. (C) Low-dose treatment of GH3 cells with 50 μM of CAB or 20 μM of CQ does not lead to major change in cell viability. However, when CAB at 50 μM and CQ at 20 μM were combined, cell viability was decreased after 48 hours. (D) In GH3 cells, Western blotting showed that CAB treatment at 50 μM resulted in a slight increase in autophagy-related protein p62 at 24 and 48 hours; however, this increase was eliminated at 72 hours. Similarly, LC3-II was induced by CAB treatment at 50 μM at 24 hours but disappeared after 48 hours. In contrast, when CAB at 50 μM and CQ at 20 μM were combined, the p62 level was steadily increased and the LC3-II level was sustained over the time points. (E) Immunostaining revealed that in GH3 cells, CAB treatment at 50 μM resulted in an increase of p62 protein at 24 hours but was cleared at 72 hours, indicating a completion of autophagy. In contrast, steady expression of p62 and LC3-II was observed when CAB at 50 μM and CQ at 20 μM were combined. *$P < 0.05$; **$P < 0.01$. Scale bar: 5 μm. CTRL, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

but blockage of autophagic flux, leading to cellular crisis.

Apoptosis induced by CAB and CQ

Because we observed a marked increase in the autophagy cargo protein p62 upon the combined CAB and CQ treatment, we next explored the involvement of p62 in CAB-mediated apoptosis, as shown in Fig. 1(D) and 1(E). It has been reported that accumulation of p62 promotes caspase-8 self-aggregation and activation and results in apoptosis (26, 27). Following the strategy used for Fig. 4, GH3 cells were treated with a low dose of CAB (50 μM), with or without CQ (20 μM), and the activities of apoptotic enzymes caspase 3/7 and caspase 8 were measured. Treatment of CAB at 50 μM or CQ at 20 μM alone at 24 and 48 hours had no effect on these caspase activities in GH3 cells, although a slight increase was observed at 72 hours. In contrast, when CAB and CQ were combined, a significant increase of caspase 3/7 and caspase 8 activities was observed at 48 hours, and the activities further increased at 72 hours [Fig. 5(A)]. Similar results were obtained from MMQ cells [Fig. 5(B)]. Immunofluorescence staining and confocal microscopy revealed that combined CAB and CQ treatment resulted in an increase and colocalization of p62 and LC3-II in GH3 cells at 24 hours, indicating the formation of autophagosomes and autolysosomes. This is followed by the appearance of caspase 8 at 48 hours. The colocalization of caspase 8 and the p62/LC3-II complex suggested cleavage of organelles in the cells and activation of apoptosis [Fig. 5(C) for GH3 cells and Fig. 5(D) for MMQ cells]. Consistent with this observation, combined CAB and CQ treatment in MMQ cells resulted in cleavage of PARP, a signature of cell death [Fig. 5(E)]. This was confirmed by a dramatic decrease of living cells with combined CAB and CQ treatment [Fig. 5(F)]. Taken together, our data indicate that CQ could enhance the functions of CAB, achieving a tumor-killing effect at a lower dose of CAB at which CAB alone is ineffective.

Involvement of caspase 8 in CAB-induced cell death

After observing the increase of caspase 8 and its association with p62 in cells treated with CAB and CQ, we explored whether caspase 8 was indeed involved in cell death induced by CAB. To avoid treating cells with too many reagents, we used a higher concentration of CAB (at 100 μM) on MMQ cells for this mechanistic study. As shown in Fig. 6(A), treatment of MMQ cells with 100 μM of CAB increased caspase 8 activity and protein expression in a time-dependent manner. Treatment of MMQ cells with a caspase 8–specific inhibitor, Z-IETD-FMK, partially relieved growth suppression by CAB, increasing cell viability from 31.0% to 55.2% compared with the control [Fig. 6(B)]. Similarly, knockdown of caspase 8 by RNA interference (RNAi) in CAB-treated MMQ cells also increased cell viability from 37.1% to 53.5% [Fig. 6(C)]. Correspondingly, Z-IETD-FMK treatment or caspase 8 knockdown by RNAi also led to a decrease of PARP cleavage [Fig. 6(D)], indicating the decrease of cell death. Taken together, these data confirmed the involvement of caspase 8 in CAB-induced cell death.

We have shown the colocalization of caspase 8 and p62/LC3-II complex during CAB and CQ-induced cell death. To confirm the importance of p62 in activation of cell death, RNAi was used to knockdown p62 in MMQ cells. As shown in Fig. 6(E) and 6(F), treatment of CAB at 100 μM increased caspase 3/7 activity by 9-fold [Fig. 6(E)] and caspase 8 activity by 2.7 fold [Fig. 6(F)]. Knockdown of p62 decreased CAB-induced caspase 3/7 and caspase 8 activity to 4.4-fold and 1.6-fold, respectively; whereas Z-IETD-FMK treatment decreased CAB-induced caspase 3/7 and caspase 8 activity to 0.1-fold and 0.3-fold, respectively. Further reduction in caspase 3/7 and caspase 8 activity was observed when p62 knockdown and Z-IETD-FMK treatment were combined. These data demonstrate the importance of p62 in CAB-induced cell death.

Discussion

In this study, we have shown that in multiple cancer cells as well as different human pituitary adenoma primary cultures, CQ was able to enhance the functions of CAB in initiation of autophagy and induction of apoptosis *in vitro*. Furthermore, combination treatment of CAB and CQ increased tumor suppression *in vivo* in two animal models. The addition of CQ allowed CAB to exert its growth suppressive and apoptotic functions at a lower dose.

For pituitary tumor treatment, CAB is primarily used in the treatment of prolactinomas. However, CAB has also been used occasionally to treat other pituitary adenomas that are nonresponsive to traditional therapeutic approaches (4–6). Using primary cultures of different pituitary tumor types, we observed *in vitro* growth suppression in most pituitary tumor samples. Furthermore, growth suppression by CAB was also observed in different human cancer cells. Previous studies have revealed that dopamine receptors are expressed in cells of many cancers, such as breast, lung, gastric, ovarian, and liver cancer, as well as esophageal squamous cell carcinoma and different neuroendocrine tumors, including pituitary tumors (28–33), which is also confirmed by our own studies (34) (Supplemental Fig. 4). These data suggest that CAB may have a much broader use in cancer treatment and that its antitumor effect can be further enhanced in combination with CQ.

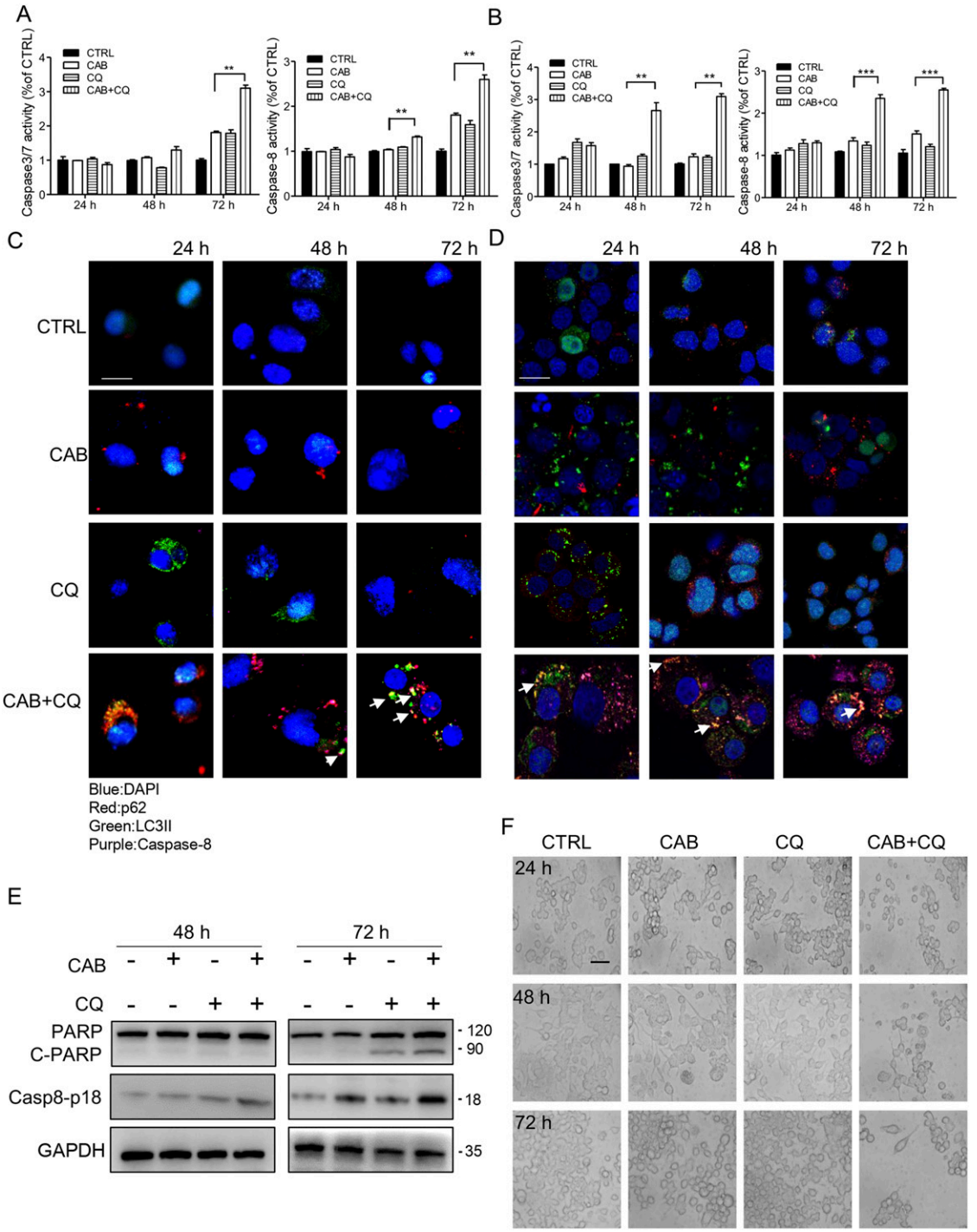

Figure 5. Apoptosis induced by CAB and CQ. (A) Low-dose treatment of 50 μM of CAB combined with 20 μM of CQ increased caspase 3/7 (left panel) and caspase 8 (right panel) activity in GH3 cells. (B) Low-dose treatment of 10 μM of CAB combined with 20 μM of CQ increased caspase 3/7 (left panel) and caspase 8 (right panel) activity in MMQ cells. (C) Confocal microscopy revealed that in GH3 cells, low-dose treatment of 50 μM of CAB combined with 20 μM of CQ induced p62 and LC3-II at 24 hours and caspase 8 at 48 hours, with colocalization of p62, LC3-II, and caspase 8 at 72 hours. (D) Confocal microscopy revealed that in MMQ cells, low-dose treatment of 10 μM of CAB combined with 20 μM of CQ induced p62, LC3-II, and caspase 8 expression at 24 hours and colocalization at 48 hours. (E) Western blotting showed that in MMQ cells, low-dose treatment of 10 μM of CAB combined with 20 μM of CQ resulted in cleavage of PARP and caspase 8, indicating cell death. (F) Decreased viable MMQ cells observed in low-dose treatment of 10 μM of CAB combined with 20 μM of CQ. $^{**}P < 0.01$; $^{***}P < 0.001$. Scale bar: 5 μm. C-PARP, cleaved poly-adenosine diphosphate ribose polymerase; CTRL, control; DAPI, 4′,6-diamidino-2-phenylindole.

Recently, we reported that CAB induced autophagy by inhibition of mammalian target of rapamycin pathway (7). CAB treatment resulted in the formation of autophagosomes; however, CAB also blocked autophagic flux, thus blocking the normal autophagy process from completion, leading to autolysosome accumulation

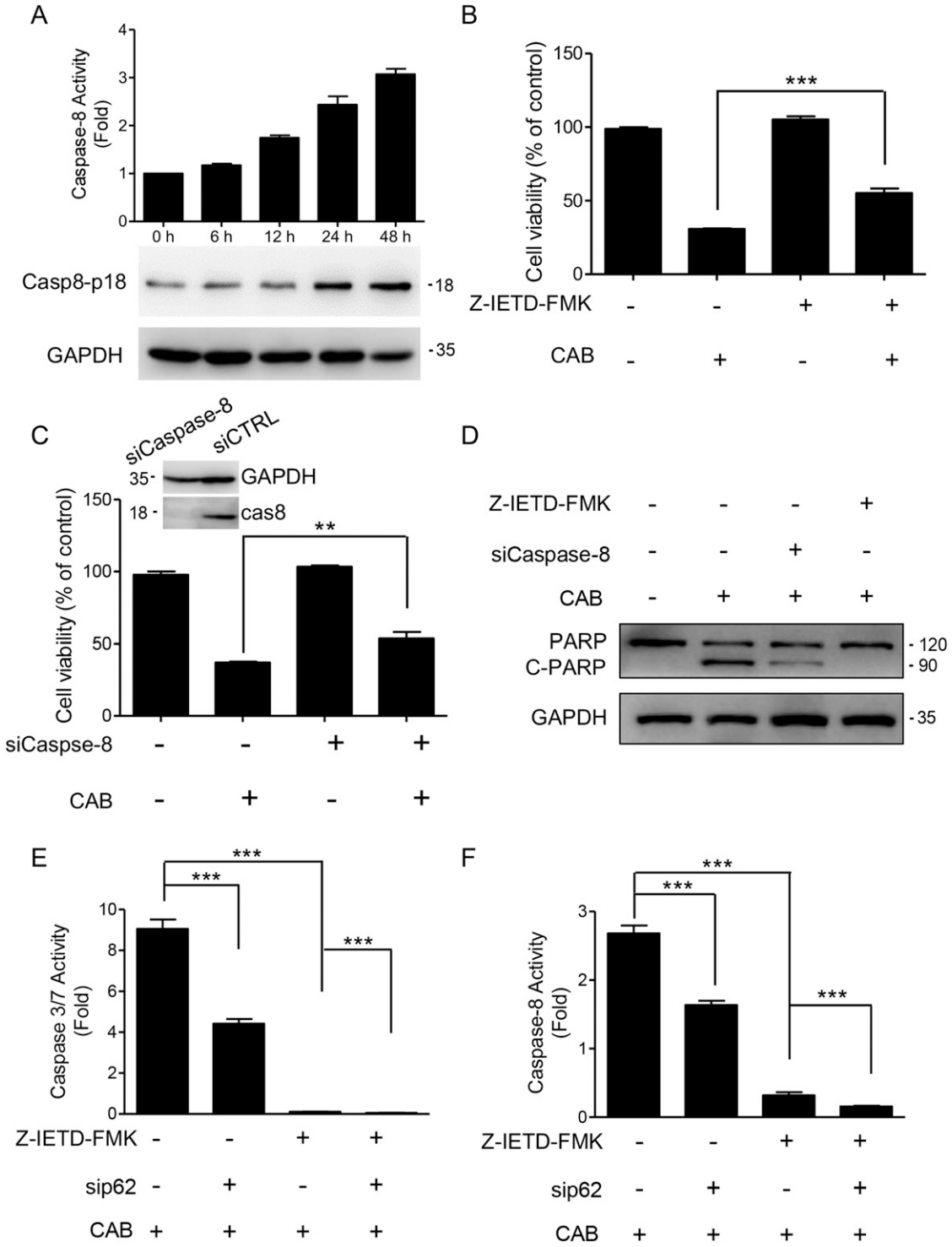

Figure 6. Involvement of caspase 8 in CAB-induced cell death. (A) MMQ cells treated with 100 μM CAB and analyzed by Western blot or caspase-8 activity assays. Combination of CAB and (B) caspase-8 inhibitor Z-IETD-FMK or (C) capase-8 small interfering RNA treatment of MMQ resulted in increase in cell viability compared with CAB treatment alone, and (D) the same cell lysates with similar protein concentrations were analyzed by Western blot. Treatment with 100 μM of CAB combined with caspase-8 inhibitor Z-IETD-FMK or p62 small interfering RNA decreased caspase-3/7 (E) and caspase-8 (F) activity in MMQ cells. Scale bar: standard error of the mean. C-PARP, cleaved poly-adenosine diphosphate ribose polymerase; DAPI, 4′,6-diamidino-2-phenylindole; siCTRL, small interfering control.

within the cells, thereby causing autophagic cell death (7). Here we showed that in MMQ and GH3 cells, a low dose of CAB induced the initiation of autophagy (featured by induction of autophagy-related protein, such as p62 and LC3-II) without blockage of autophagic flux; in this circumstance, no growth suppression or apoptosis was observed. In contrast, when the low dose of CAB was combined with CQ, p62 and LC3-II accumulated without

clearance, demonstrating a block in autophagic flux. This caused a cellular crisis leading to activation of apoptotic enzymes, such as caspase 3/7 and caspase 8 (26, 35, 36). Indeed, our data showed that in cells treated with low-dose CAB and CQ, p62 and LC3-II started to accumulate after 24 hours, followed by activation of caspases after 48 hours and cell death, whereas no caspase activation or cell death was observed in cells treated with low-dose CAB alone. CQ is a known inhibitor of autophagy by increasing pH in acidic cellular compartments, such as lysosomes, thus inhibiting lysosomal enzyme activities. Under the condition used in this study, CQ alone could not cause the complete disruption of autophagy; however, the combination of CQ and CAB could amplify the functions of CAB and thus lead to cell death. These data suggest that high doses of CAB and/or CQ may achieve a better clinical outcome in patients who are resistant to the treatment at the regular dose, as long as the patients are tolerant to the drugs. Indeed, it has been reported that in a patient with a giant prolactinoma, a clear dose–response effect between cabergoline dose and PRL level was observed up to a total weekly dose of 9 mg CAB (37). Another study including 150 patients showed that high doses of CAB (up to 12 mg/wk) normalized PRL level in all patients except one (38).

Although CAB is well tolerated in general, still some patients cannot tolerate it at the doses for clinical use. In addition, many tumors are nonresponsive to CAB used at clinical doses. Our study has revealed that when CAB was combined with CQ, it can function to suppress cell growth at a lower dose. Thus, adding CQ to the treatment plan may provide a solution to both problems mentioned previously: Clinical efficacy may be achieved at a lower dose of CAB, and tumors may become responsive because of increased CAB efficacy. Because CQ is a well-tolerated drugs with mild side effects, combined CAB and CQ therapy is likely to be safe and effective (39).

Several clinical trials have been or are currently being conducted for the use of CQ as an adjuvant cancer therapy in patients with different types of cancers (40). Most of these studies have shown that CQ is generally safe and well tolerated, with promising results (11, 12, 14, 18, 19). For example, in a randomized, double-blind, placebo-controlled trial among patients with glioblastoma multiforme, those receiving CQ had a significantly longer survival time than the control group (33 months vs 11 months; $P = 0.0002$) (14). Our study here clearly demonstrates the benefit of combining CAB and CQ in tumor suppression and provides a rationale for conducting pilot clinical studies of this combination therapy for human pituitary tumors and other cancers.

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