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RESEARCH PAPER

Synergistic anti-tumour effects of tetrandrine and chloroquine combination therapy in human cancer: a potential antagonistic role for p21

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BACKGROUND AND PURPOSE

Tetrandrine, a bisbenzylisoquinoline alkaloid isolated from the Chinese medicinal herb *Stephaniae tetrandrae*, has a long history in Chinese clinical applications to treat diverse diseases. Tetrandrine induced apoptosis or, at low concentrations, autophagy of human hepatocellular carcinoma cells. Here we have tested the effects of inhibitors of autophagy such as chloroquine, on the response to low concentrations of tetrandrine in cancer cells.

EXPERIMENTAL APPROACH

Cultures of several cancer cell lines, including Huh7, U251, HCT116 and A549 cells, were exposed to tetrandrine, chloroquine or a combination of these compounds. Cell viability and content of reactive oxygen species (ROS) were measured and synergy assessed by calculation of the combination index. Western blot and RT-PCR assays were also used along with fluorescence microscopy and histochemical techniques.

KEY RESULTS

Combinations of tetrandrine and chloroquine were more cytotoxic than the same concentrations used separately and these effects showed synergy. Such effects involved increased ROS generation and were dependent on caspase-3 but independent of Akt activity. Blockade of tetrandrine-induced autophagy with 3-methyladenine or bafilomycin-A1 induced apoptosis in cancer cells. Lack of p21 protein ($p21^{-/-}$ HCT116 cells) increased sensitivity to the apoptotic effects of the combination of tetrandrine and chloroquine. In a tumour xenograft model in mice, combined treatment with tetrandrine and chloroquine induced ROS accumulation and cell apoptosis, and decreased tumour growth.

CONCLUSIONS AND IMPLICATIONS

The combinations of tetrandrine and chloroquine exhibited synergistic anti-tumour activity, *in vitro* and *in vivo*. Our results suggest a novel therapeutic strategy for tumour treatment.

Abbreviations

3-MA, 3-methyladenine; ATG7, autophagy-related gene 7; BAF, bafilomycin-A1; CI, combination index; LC3, microtubule-associated protein 1 light chain 3; MDA, lipid peroxidation product; NAC, *N*-acetyl-L-cysteine; PI, propidium iodide; ROS, reactive oxygen species

Tables of Links

TARGETS
Enzymes
Akt
Caspase 3
Cathepsin D
ERK
MAP kinases
p38 kinase
PI3K

LIGANDS
Chloroquine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guideto PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Cancer is a major public health concern for people all over the world, and it leads to approximately 12.7 million cases and 7.6 million deaths every year (Jemal *et al.*, 2011). Although complete tumour removal via surgery could cure cancer, there are many restrictive conditions for choosing surgery, such as small and non-metastatic carcinomas (Forde *et al.*, 2014; Minuti *et al.*, 2014). To date, systemic chemotherapy is still a standardized treatment. The common mechanism of targeted chemotherapies is to induce cancer cells to undergo programmed cell death (apoptosis and autophagy) or sensitize them to established cytotoxic agents or radiation therapy (Khan *et al.*, 2014). Because many tumour cells are resistant to single-agent chemotherapeutic drugs and have poor clinical outcomes, targeting multiple signalling pathways with synergistic chemotherapy drugs could potentially increase treatment efficiency in many types of cancer (Burdach, 2014). The development of novel drugs and effective combination chemotherapy pattern with minimal side effects is an urgent necessity to reduce cancer mortality (Cheng *et al.*, 2013; Lai *et al.*, 2013).

Tetrandrine is a key compound found in the traditional Chinese medicinal herb *Stephania tetrandra* S. Moore. It has been widely used for thousands of years in China to treat rheumatoid arthritis, sepsis, endotoxin-induced uveitis, silicosis and hypertension (Ho *et al.*, 2004). We recently demonstrated that relatively high concentrations of tetrandrine induce apoptosis and low doses trigger autophagy of liver cancer cells, suggesting that tetrandrine exhibits strong anti-tumour effects and has the potential to be a cancer chemotherapeutic agent (Liu *et al.*, 2011; Gong *et al.*, 2012a). The induction of apoptosis is a common mechanism underlying the killing of cancer cells by many chemotherapeutic agents. However, autophagy is a cellular response to stress in which organelles, cytoplasm and proteins are degraded and cellular components are recycled to ensure cell survival during starvation (Hurley and Schulman, 2014). This process has recently received considerable attention in the oncogenesis field for its role in the response to anti-cancer therapies (Ma *et al.*, 2013; Munz, 2013). Inhibition of autophagy may disrupt this compensatory process, resulting in the accumulation of metabolic stress products and induction of cell death (Zhu *et al.*, 2013; Zhao *et al.*, 2014b). Inhibition of autophagy or targeting of autophagy pathway inducers may be useful in the development of anti-cancer agents as treatments (Lamoureux *et al.*, 2013; Altman *et al.*, 2014).

The 4-aminoquinoline drug chloroquine is a lysosomotropic agent that prevents endosomal acidification and was widely used to treat or prevent malaria and other diseases (Salas *et al.*, 2013). Chloroquine has been used in recent years as an autophagy inhibitor because it raises the lysosomal pH, leading to the inhibition of autophagosome–lysosome fusion and lysosomal protein degradation (Egger *et al.*, 2013; Kimura *et al.*, 2013). In basic and clinical research, chloroquine is often used in combination with chemotherapeutic drugs and radiation to enhance cancer treatment efficacy (Maycotte *et al.*, 2012; Lamoureux *et al.*, 2013). However, research that has been reported to date has not led to incorporation of chloroquine in widely used cancer treatment protocols (Vlahopoulos *et al.*, 2014). Thus a refinement of the combination of chloroquine with other drugs is needed to improve clinical effects.

Our previous findings showed that low concentrations of tetrandrine induced autophagy without causing cell death (Gong *et al.*, 2012a). Tetrandrine treatment results in cellular stress, including mitochondrial dysfunction resulting in intracellular reactive oxygen species (ROS) production. ROS accumulation activates the ERK/MAP kinase pathway, which contributed to tetrandrine-induced autophagy (Liu *et al.*, 2011; Gong *et al.*, 2012a). Although autophagy is a survival mechanism, it has also been suggested that autophagy could be cytotoxic when cells suffer treatment-induced stress. Excessive cell damage will lead to autophagic cell death (Fang *et al.*, 2013; Li *et al.*, 2014b). In the present study, we have investigated, in cancer cells the effects of tetrandrine-induced stress in the presence of chloroquine, which prevents autophagy by blocking autophagosome fusion and degradation. To test our hypothesis, we treated cancer cells with a combination of tetrandrine and chloroquine and measured cell viability. Our results demonstrate that blocking tetrandrine-induced cell autophagy by chloroquine causes

the cell to undergo caspase-dependent apoptosis *in vitro* and *in vivo*. Our data suggest that intracellular ROS accumulation and p21 expression play a role in regulating the cellular apoptosis induced by the combination therapy. Thus, tetrandrine and chloroquine combination therapy has a synergistic anti-tumour activity, representing a promising effective therapeutic strategy against tumours.

Methods

Cell lines and cell culture

The human hepatoma cell lines (Huh7 and FHCC98), human glioma cell lines (U87 MG and U251), human lung cancer cell line (Calu-1), human cervical adenocarcinoma Hela cells, immortalized non-malignant human normal human hepatic cells (L02) and mammary epithelial cells (HBL100) were obtained from the cell culture collection of Wuhan University (Wuhan University, China). These cells were all cultured in DMEM. Human colon cancer cells (HCT116 and HCT116 $p21^{-/-}$) cultured in McCoy's 5A medium and the human lung cancer cell line (A549) cultured in RPMI1640 medium were kindly provided by Dr Xiaodong Zhang (College of Life Sciences, Wuhan University, China). All cell culture media were supplemented with 10% FBS, 100 $U \cdot mL^{-1}$ penicillin and 100 $U \cdot mL^{-1}$ streptomycin. Cell culture dishes and plates were obtained from Wuxi NEST Biotechnology Co., Ltd. (Wuxi, Jiangsu, China).

Combination index

The effect of agents was defined as combination index (CI), a synergistic effect as CI < 1, an additive effect as CI = 1 and an antagonistic effect as CI > 1 (Tazzari *et al.*, 2008; McCormack *et al.*, 2012). CI values were calculated from dose–response curves to test for synergistic effects. The computer program CompuSyn for drug combinations and for general dose–effect analysis was used and the method of Chou and Talalay was utilized for formal synergy analyses (Chou, 2006). Synergy was defined based upon the terminology of Chou.

Cell clone formation assay

Tumour cells were plated in six-well plates at approximately 2000 cells per well and exposed to drugs for 24 h once the cells were adherent. The medium was then replaced with fresh media, and the tumour cells were allowed to grow for approximately 2 weeks at 37°C, 5% CO_2 before staining with crystal violet (Sigma-Aldrich).

Cell viability assay

Tetrandrine was dissolved in DMSO and chloroquine was dissolved in PBS before use. Cell viability was measured by Trypan blue dye staining assay. To determine whether the combined effects were synergistic, the cells were treated with the indicated doses of tetrandrine and chloroquine for 48 or 72 h, and the CI was determined using the method of Chou and Talalay, using the software package Calcusyn (Biosoft, Cambridge, UK) (Chou, 2010).

Western blot analysis

After each treatment, as indicated in the figure legends, proteins were collected and their expression was assessed by Western blot as previously described (Zhan *et al.*, 2012).

Acridine orange staining

Cells were incubated with or without 5 μM tetrandrine, for the indicated time after they were seeded on sterile coverslips overnight. Cells were then incubated with 1 $\mu g \cdot mL^{-1}$ acridine orange dye in serum-free medium for 20 min. The acridine orange dye was removed, and fluorescence micrographs were obtained by an inverted fluorescence microscope. The cytoplasm and nucleus of the stained cells fluoresced bright green, and the acidic autophagic vacuoles fluoresced bright orange-red.

Detection of intracellular ROS

ROS levels were measured using a flow cytometer (Beckman-Coulter, Miami, FL, USA). Following treatment, cells were harvested and washed with PBS and then suspended in serum-free DMEM or RPMI1640 containing 1 μM DCFH-DA at 37°C, 5% CO_2 for 20 min. Cells were washed with PBS, and flow cytometry analysis was performed.

Plasmids and transfections

An empty vector pHAGE.puro plasmid was kindly provided by Dr Zan Huang (College of Life Sciences, Wuhan University, China), and pHAGE.puro-p21 plasmid was obtained from our laboratory (Li *et al.*, 2014a). The empty vector p-USE and p-USE-CA-Akt plasmids were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Cells were seeded in 24-well plates, and plasmids were transfected for 48 h with FuGENE HD transfection reagent according to the manufacturer's protocol (Roche, Indianapolis, IN, USA).

Tumour xenograft

The animal care and experimental protocols were approved by the Experimental Animal Center of Wuhan University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 56 animals were used in the experiments described here.

Athymic nude mice (4- to 5-week-old male BALB/c) were provided by the Disease Prevention Center of Hubei Province (Wuhan, Hubei, China). A549 cells (approximately 6×10^6), HCT116 and HCT116 $p21^{-/-}$ cells (approximately 5×10^6) were implanted subcutaneously into the right flank of each mouse. Once the tumour volume of A549 cells reached approximately 50 mm^3, tumour-bearing mice were randomized into four experimental groups. Each group of mice was given vehicle (0.5% methylcellulose), chloroquine (50 $mg \cdot kg^{-1}$), tetrandrine (25 $mg \cdot kg^{-1}$) or chloroquine with tetrandrine, three times a week for 30 days via gavage. For HCT116 and HCT116 $p21^{-/-}$ cells, tumour-bearing mice were randomized into two test groups and were administered vehicle (0.5% methylcellulose) or tetrandrine (25 $mg \cdot kg^{-1}$) plus chloroquine (50 $mg \cdot kg^{-1}$) by gavage every other day for 24 days. Mouse weights and tumour volumes were measured and recorded at the same time. Tumour volumes were estimated by direct measurement with calipers and calculated by the formula: $\pi/6 \times (\text{length}) \times (\text{width})^2$.

In vitro *apoptosis assay*

Treated cells were washed with PBS, resuspended in binding buffer and stained with Annexin V-FITC and propidium iodide

(PI) for 15 min according to the protocol kit from Bipec Bioreagent (Cambridge, MA, USA). Annexin V-FITC and PI fluorescence was estimated with a flow cytometer (Beckman-Coulter). For TUNEL assays, tumour tissue samples were prepared and treated according to the TUNEL kit instructions (Roche, Indianapolis, IN, USA), and tumour sections were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

MDA assay

The tissue samples from the tumours were prepared according to the MDA assay kit protocol (Beyotimes, Nantong, China). MDA levels were detected using Multi-Mode Microplate Readers (SpectramMax M5) at 532 nm.

Data analysis

A Student's *t*-test was used for statistical analysis, and all experiments were performed three times, and the accepted level of significance was $P < 0.05$. The data from the flow cytometer (Beckman) were analysed by Flow-Jo software (Tree Star Software, San Carlos, CA, USA).

Materials

Tetrandrine was provided by Shanghai Ronghe Medical, Inc. (Shanghai, China). Z-VAD-FMK was purchased from R&D Systems (Minneapolis, MN, USA). DCFH-DA was obtained from Invitrogen (Carlsbad, CA, USA). The antibody against microtubule-associated protein 1 light chain 3 (LC3) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and the antibodies against Bax, p53 and cathepsin D were acquired from Proteintech Group Inc. (Chicago, IL, USA). The antibody against GAPDH was obtained from Beyotime (Shanghai, China). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), and all other chemicals were provided by Sigma-Aldrich.

Results

Tetrandrine and chloroquine combination therapy showed synergistic anti-tumour activity

To determine the effect of tetrandrine and chloroquine combination therapy on cancer cells, we first evaluated their effects on the survival of the lung cancer cell line A549 and liver cancer line Huh7. As shown in Figure 1A, tetrandrine or chloroquine given alone had a concentration-dependent effect on cell survival. The viabilities of A549 and Huh7 cells were about 80% when they were treated with 5 μM tetrandrine or 20 μM chloroquine alone. However, after treatment with 5 μM tetrandrine plus 20 μM chloroquine for 72 or 48 h, cell viabilities of A549 and Huh7 cells significantly decreased. Similar results were obtained in other cancer cell lines such as Hela, Calu1, FHCC98, U251 and U87 cells (Figure 1B), which suggests that the combination effect of tetrandrine and chloroquine was common to many cancer cell lines, regardless of tissue source. We examined cell viability after treating the cells with various concentrations of tetrandrine and chloroquine, and we found that 5 μM tetrandrine and 20 μM chloroquine had a clearly synergistic anti-tumour activity (Figure 1C and Supporting Information Table S1). The colony formation assay also confirmed that this combined treatment dramatically suppressed long-term cell survival of cancer cells (Figure 1D). However, immortalized non-malignant human normal human hepatic cells (L02) were less sensitive to this combined treatment (Figure 1E). Taken together, our data suggest that combination treatment of tetrandrine and chloroquine has a significant synergistic therapeutic effect on cancer cells but not on normal cells.

Combination therapy induces caspase-dependent apoptosis independent of Akt activity

In general, anti-cancer drugs kill malignant cells via programmed cell death, including apoptosis, autophagic death or non-programmed cell death (necrosis). Therefore, we determined the mode of cell death induced by the combination therapy. As shown in Figure 2A, Annexin V-FITC/PI staining indicated that tetrandrine and chloroquine combination treatment induced apoptosis in A549, Huh7 and Hela cells. Western blot analysis indicated that the tetrandrine and chloroquine combination activated the apoptotic-related proteins PARP and caspases in cancer cells including A549, Huh7, Hela, U251, Calu1 and FHCC98 (Figure 2B and Supporting Information Fig. S1). To further examine the role of caspases in the combination-induced apoptosis, cells were pretreated with the pan-caspase inhibitor Z-VAD-FMK and then treated with combination therapy. The data show that Z-VAD-FMK significantly blocked cell death in A549 and Huh7 cells induced by the tetrandrine/chloroquine combination (Figure 2C).

Our previous reports showed that tetrandrine induced apoptosis of hepatocellular carcinoma (HCC) cells through the inhibition of Akt activity. Here, we found that the tetrandrine/chloroquine combination dramatically decreased total Akt and phospho-Akt protein levels. However, ectopic Akt overexpression did not rescue cell viability after combination treatment (Supporting Information Fig. S2). Therefore, these data demonstrate that the tetrandrine/chloroquine combination therapy-induced cell apoptosis was mediated by caspase activation, but not Akt activity.

Intracellular ROS is involved in tetrandrine/chloroquine combined therapy-induced apoptosis

Chemotherapeutic drugs have multiple effects on tumour cells, including ROS production, which has been shown to be a critical event in anti-cancer drug-induced cell death (Gong and Li, 2011; Gong *et al.*, 2012b; Lee *et al.*, 2013b). Therefore, we examined whether tetrandrine/chloroquine combination therapy-induced apoptosis was associated with intracellular ROS activation. We measured intracellular ROS production after treatment with chloroquine, tetrandrine or the combination in A549 and Huh7 cells. We found that the combination treatment resulted in significantly higher intracellular ROS levels compared with the single-agent treatments (Figure 3A). The free radical scavenger *N*-acetyl-L-cysteine (NAC) markedly inhibited combination treatment-induced ROS generation, which confirms that the tetrandrine and chloroquine combination results in intracellular ROS accumulation (Figure 3B). To examine whether the enhanced ROS

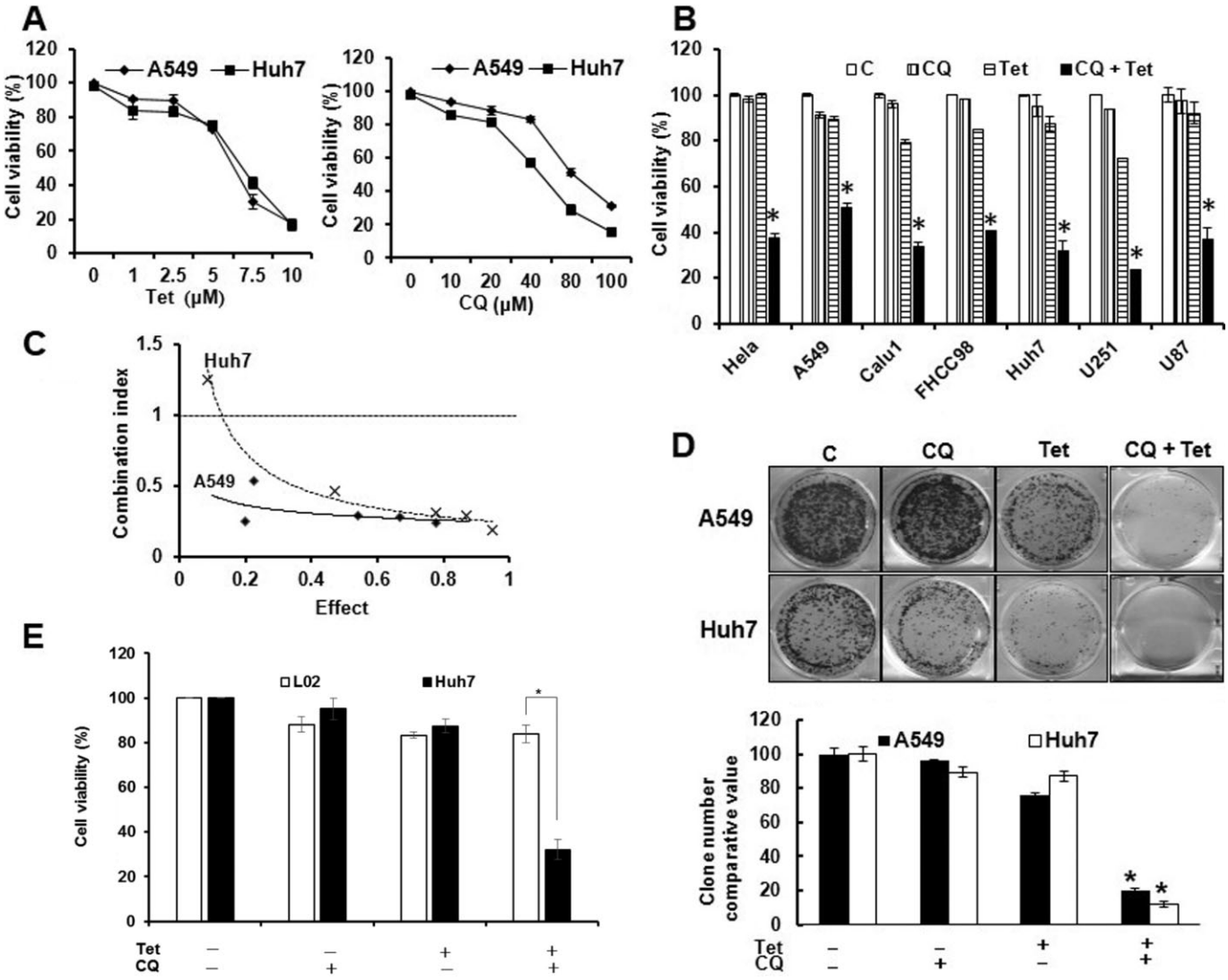

Figure 1

Tetrandrine (Tet) and chloroquine (CQ) combination treatment has a synergistic anti-tumour activity in various cancer cells. (A) A549 cells were treated with serial concentrations of tetrandrine or chloroquine for 72 h, and Huh7 cells were treated with the same concentrations for 48 h. Tumour cell viability was determined by cell viability assay. Data represent means from at least three independent experiments ± SD. (B) Cells were treated with tetrandrine (5 μM), chloroquine (20 μM) or both for 72 h, except Huh7 and U251 were treated for 48 h. *$P < 0.05$, significantly different from control (no treatment). Values represent mean ± SD ($N = 3$). (C) A549 and Huh7 cells were treated with different concentrations of tetrandrine (1–10 μM) plus chloroquine (4–40 μM) at a fixed ratio of 1:4 or with single agents for the indicated time, and the combination index (CI) was calculated. (D) A549 and Huh7 cells were stained with Crystal violet after 14 days of treatment for the colony formation assay. The number of colonies is shown, and the inset shows a representative figure. (E) Immortalized non-malignant normal human hepatic cells (L02) and cancer cells (Huh7) were treated with tetrandrine (5 μM), chloroquine (20 μM) or both for 72 h.

generation plays a role in combination therapy-induced apoptosis, we determined A549 and Huh7 cell viability after pretreatment with NAC for 1 h, followed by combination treatment. The results indicate that NAC treatment prevented the decrease in A549 and Huh7 cell viability induced by the combination treatment.(Figure 3C). Consistent with our observed cell survival data, Western blot analysis of PARP and caspases also indicated that NAC treatment reduced combination treatment-induced cell apoptosis (Figure 3D). Thus, these results suggest that intracellular ROS activation is an essential event in the induction of cell apoptosis by the tetrandrine/chloroquine combination treatment.

Inhibition of tetrandrine-induced cell autophagy causes cell apoptosis

We have previously demonstrated that tetrandrine is a potent autophagy agonist and induces cell autophagy but not death at low concentrations in HCC cells (Gong *et al.*, 2012a). In the present study, we found that tetrandrine also induces cell autophagy in several other cancer cell lines. As shown in Figure 4A and B, tetrandrine significantly increased the LC3-II level and the number of acidic autophagolysosome vacuoles. Chloroquine is an established autophagy inhibitor in the autophagic degradation process

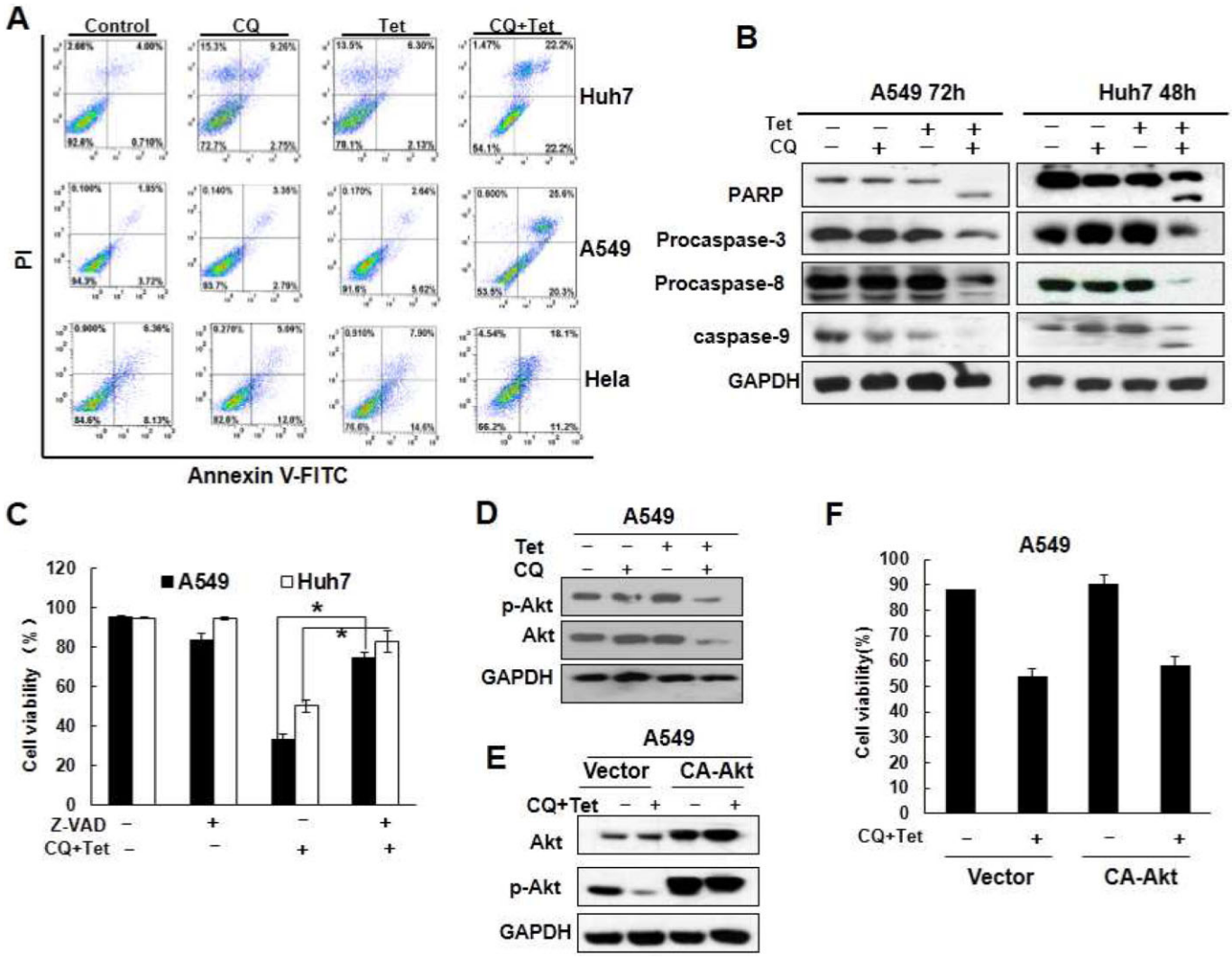

Figure 2

The synergistic effect of tetrandrine (Tet) and chloroquine (CQ) combination induces caspase-dependent apoptosis independent of Akt activity. (A) FACS analysis was performed for detecting apoptosis after tetrandrine (5 μM), chloroquine (20 μM) or combination treatment of Huh7, A549 and Hela cells for the indicated times. (B) PRAP, caspase-9, procaspase-3, procaspase-8 and GAPDH protein expression levels were determined by Western blot. (C) Viability of Huh7 and A549 cells after co-treatment of tetrandrine (5 μM) and chloroquine (20 μM) in the absence or presence of 50 μM Z-Vad-fmk for 48 h (Huh7) or 72 h (A549); *$P < 0.05$, significantly different as indicated. Values represent the mean ± SD ($N = 3$).

via disruption of lysosomal function, leading to lipid modification of LC3-I into LC3-II. As shown in Figure 4C, we further confirmed that chloroquine inhibits tetrandrine-induced cell autophagy. Combined with the results of Figure 1, we hypothesized that the inhibition of autophagy progress would lead to cell death under tetrandrine stress. To further confirm this hypothesis, we treated cells with two other widely used autophagy inhibitors, 3-methyladenine (3-MA) and bafilomycin-A1 (BAF), and combined them with tetrandrine treatment of various cancer cells, including A549, Huh7, Hela and U251 cells. As expected, tetrandrine clearly induced cancer cell death in combination with the autophagy inhibitors 3-MA and BAF (Figure 4D and E and Supporting Information Fig. S3). Therefore, the above data suggested that blocking tetrandrine-induced cell autophagy triggers apoptosis.

p21 expression is involved in apoptosis induced by combination treatment

p21 $^{CIP1/WAF1}$, a potent inhibitor of cyclin-dependent kinase (CDK), has been identified as a key element in chemotherapeutic agent- and DNA-damaging agent-induced apoptosis (Stivala *et al.*, 2012; Lee *et al.*, 2013a). We previously reported that p21 is a critical regulator of combination treatment-induced apoptosis (Li *et al.*, 2014a). Thus, we next examined whether p21 expression was related to the sensitivity of cancer cells to apoptosis induced by tetrandrine/chloroquine

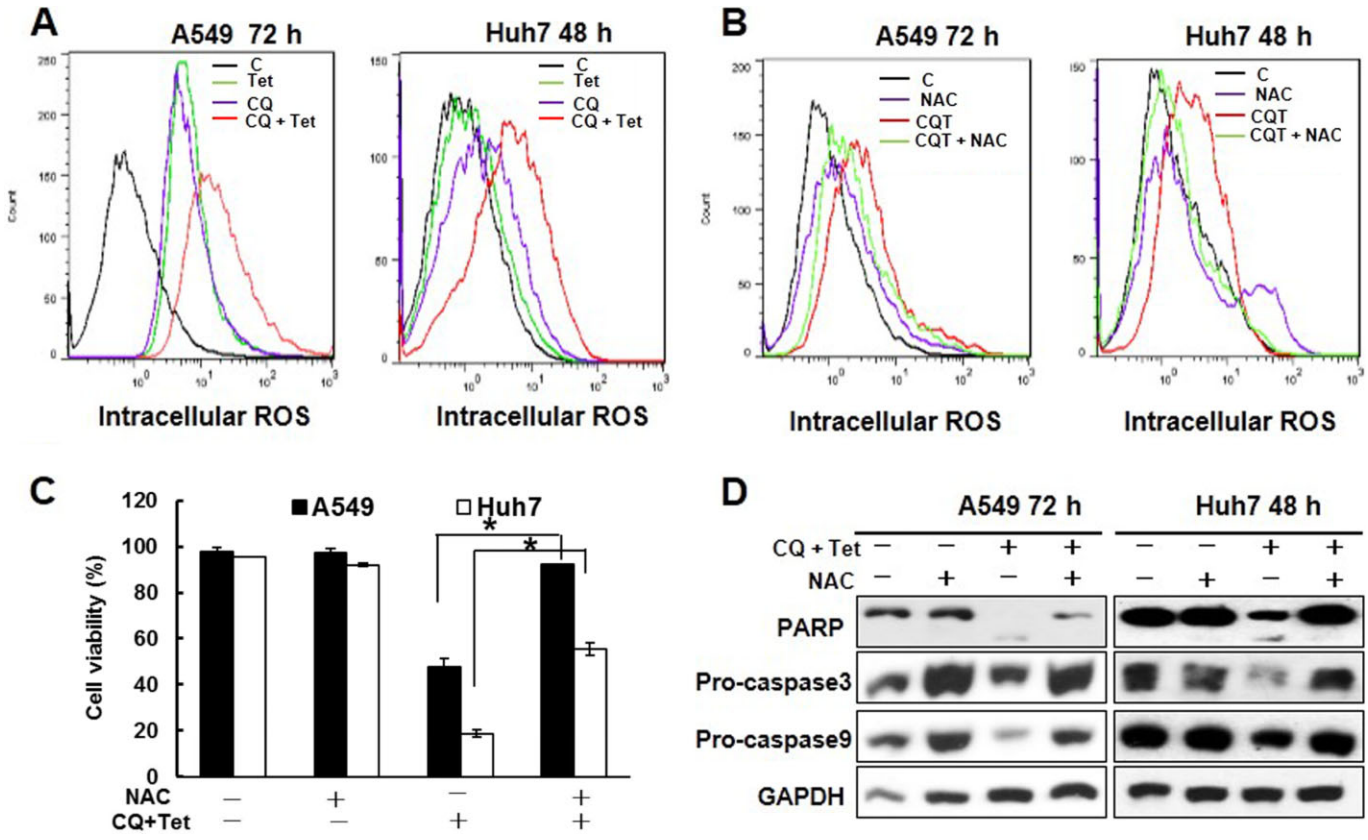

Figure 3

Intracellular reactive oxygen species (ROS) are involved in tetrandrine (Tet) and chloroquine (CQ)-induced apoptosis. (A) Huh7 cells were treated with tetrandrine (5 μM), chloroquine (20 μM) or both for 48 h, and A549 cells were treated for 72 h, then subjected to DCFDA, an indicator for analysis of intracellular peroxide, fluorescence analysis to evaluate intracellular ROS levels. (B) Cells pretreated with 15 mM NAC for 1 h were incubated with both tetrandrine (5 μM) and chloroquine (20 μM) for the indicated time, and DCFDA fluorescence intensity was detected by flow cytometry. (C) After co-treatment with tetrandrine and chloroquine, cellular viability was determined in the presence of 15 mM NAC for 1 h; $^*P < 0.05$. Values represent the mean ± SD ($N = 3$). (D) Western blot analysis was performed in cells pretreated with NAC and co-treatment with 5 μM tetrandrine and 20 μM chloroquine.

combination. We treated the wild-type and $p21^{-/-}$ HCT116 cell lines with tetrandrine and chloroquine for 72 h. Cell viability results indicated that the $p21^{-/-}$ HCT116 cell lines were markedly more sensitive to combination treatment compared with wild-type HCT116 cells and these data were consistent with the results from Western blot analysis of PARP and caspases (Figure 5A and B). In contrast, p53 or Bax knockout did not affect the sensitivity to combination drug therapy (Supporting Information Fig. S4). Moreover, the combination treatment dramatically decreased p21 protein levels (Figure 5C). To further confirm these observations, we transiently transfected p21 plasmid into Huh7 cells, which expressed relatively low endogenous p21 levels, and then analysed their sensitivity to combination treatment. The results showed that ectopic expression of p21 inhibited combination treatment-induced apoptosis in Huh7 cells (Figure 5D). These results suggest that p21 expression plays an important role in apoptosis induced by the tetrandrine and chloroquine combination treatment.

Because ROS generation and p21 repression are required for cell sensitivity to combination treatment, we next examined whether there existed regulatory relationships between them. Western blot analysis of p21 in the presence of an ROS scavenger indicated that NAC restored p21 levels when cells were treated with tetrandrine and chloroquine (Figure 5E). Meanwhile, ectopic expression of p21 also decreased combination therapy-induced ROS levels in Huh7 cells (Figure 5F). These results suggested that intracellular ROS and p21 regulate each other to control the effect of combined chemotherapy in our treatment model.

Combination therapy has synergistic therapeutic effects in an in vivo *xenograft model*

To assess the synergistic therapeutic effects of tetrandrine/chloroquine combination treatment *in vivo*, 28 athymic nude mice with established A549 tumour xenografts were randomly divided into four groups and treated with vehicle, tetrandrine (25 mg·kg^{-1}), chloroquine (50 mg·kg^{-1}) or both compounds (tetrandrine 25 mg·kg^{-1} plus chloroquine 50 mg·kg^{-1}) three times per week for 30 days. The results demonstrated that combination treatment strongly inhibited tumour growth and tumour weight compared with monotherapy (Figure 6A). Consistent with the *in vitro* results, combined treatment also

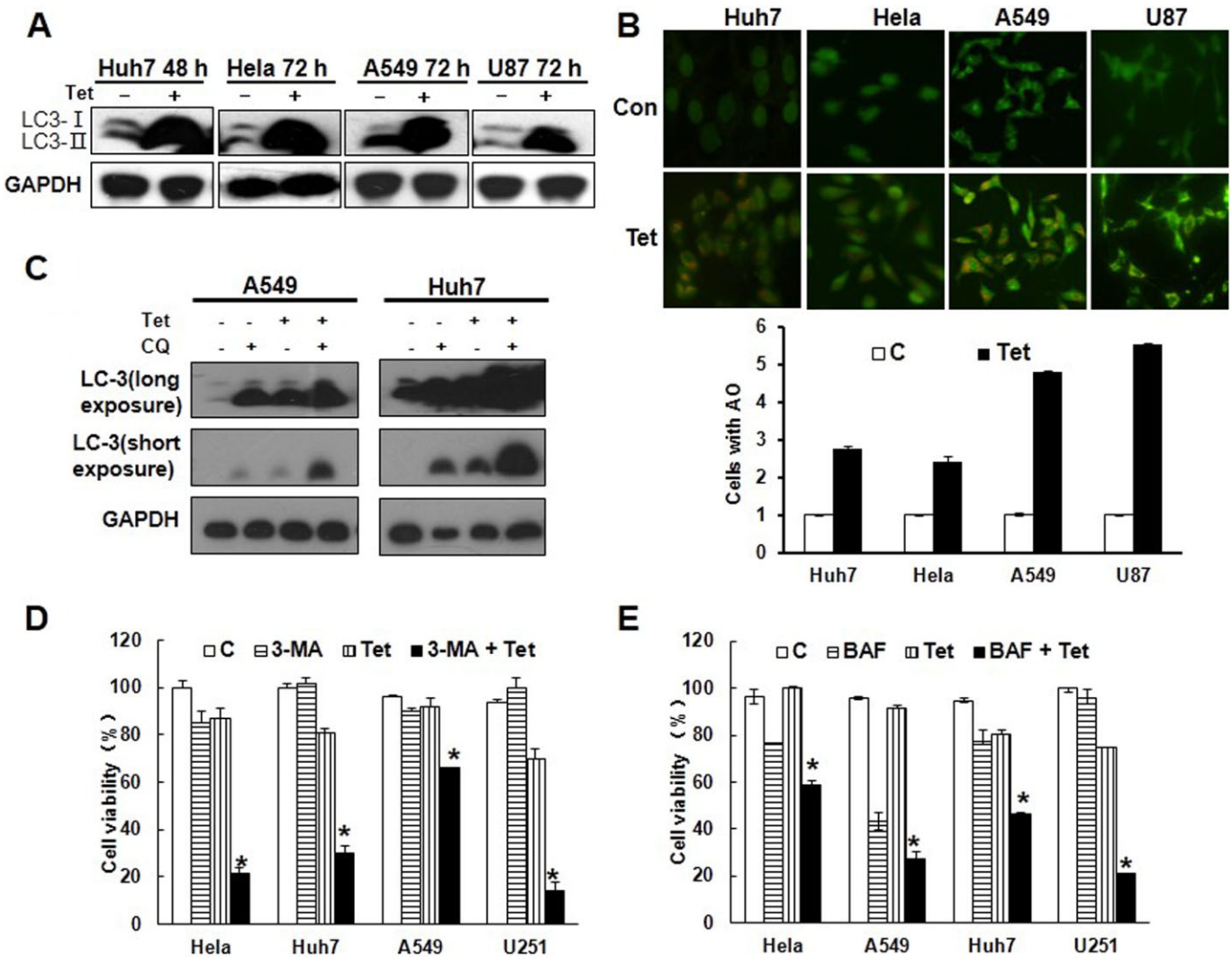

Figure 4

Inhibition of tetrandrine(Tet)-induced autophagy is linked to apoptotic cell death. (A) Huh7, A549, Hela and U87 cells were treated with tetrandrine (5 μM), and LC3 levels were determined by Western blot assay. GAPDH was used as a loading control. (B) Tetrandrine (5 μM) induced the accumulation of autophagic vacuoles in Huh7, A549, Hela and U87 cells. Cells were stained with Acridine Orange for fluorescence detection. (C) Western blot analysis of LC3 in A549 and Huh7 cells after tetrandrine (5 μM), chloroquine (CQ;20 μM) or combination treatment for the indicated time. GAPDH was used as a loading control. (D, E) Cell viability of Hela, Huh7, A549 and U251 cells following treatments of 5 μM tetrandrine and 5 mM 3-methyladenine (3-MA) or 50 nM bafilomycin (BAF) as individual or combination treatments for 72 h. Values represent the mean ± SD (N = 3).

induced a significant increase in the number of apoptotic cells in the tumour tissue (Figure 6B) and the lipid peroxidation product MDA, which is a measure of ROS-mediated injury *in vivo* (Figure 6C). To examine the effect of p21 protein levels on sensitivity to combination treatment *in vivo*, nude mice bearing either HCT116 wild-type or HCT116 $p21^{-/-}$ cell xenograft tumours were treated with the combination of tetrandrine and chloroquine via gavage every other day for 24 days. The results showed that the HCT116 $p21^{-/-}$ cell xenograft tumours were sensitive to combination treatment, but HCT116 wild-type cells were resistant, which is consistent with the *in vitro* results (Figure 6D and E). TUNEL assay of tissue further confirmed that HCT116 $p21^{-/-}$ cells underwent apoptosis after combination treatment (Figure 6F). In addition, the combined treatment was well tolerated by all mice, as the animals did not display additional weight loss or other signs of acute trauma compared to the monotherapy groups (Supporting Information Fig. S5). Therefore, these data suggest that tetrandrine and chloroquine exert significant synergistic anti-cancer effects *in vivo* by inducing cell apoptosis. Moreover, p21 protein expression also plays a key role in combination treatment *in vivo*.

Discussion

The traditional Chinese medicine tetrandrine displays good potential anti-cancer efficacy because it can induce cell G1

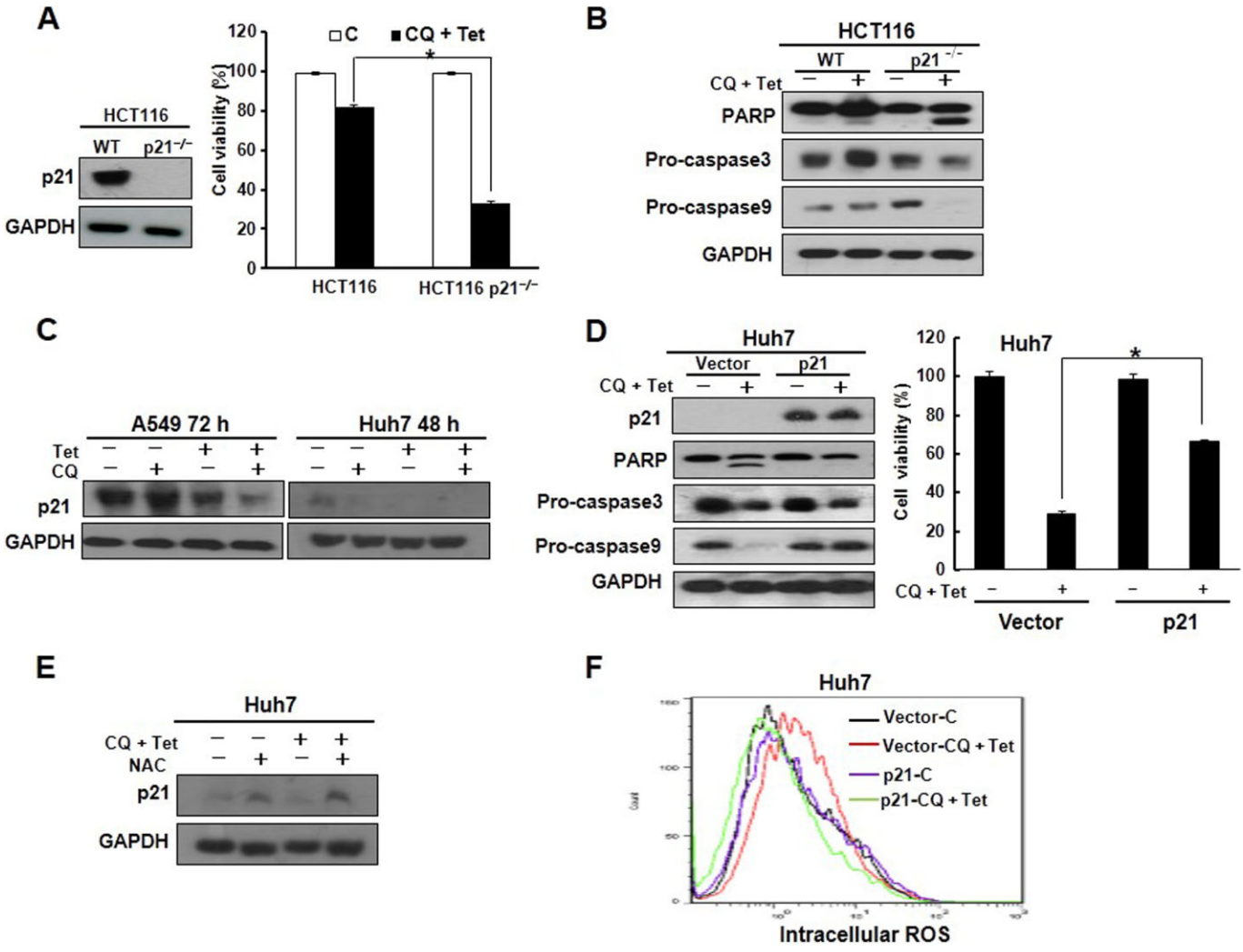

Figure 5

p21 suppresses tetrandrine(Tet) and chloroquine (CQ) combination treatment-induced apoptosis. (A) Cellular viability was calculated after HCT116 wild-type and HCT116 p21$^{-/-}$ cells were treated with tetrandrine (5 μM) plus chloroquine (20 μM) for 72 h. *P < 0.05, significantly different as indicated. (B) PARP and caspase antibody levels were determined by Western blot analysis. (C) p21 protein levels were determined in A549 and Huh7 cells after incubation with tetrandrine (5 μM) and chloroquine (20 μM) singly or together for the indicated time. (D) Huh7 cells were transfected with p21 overexpression plasmid or its vehicle, and cells were treated with tetrandrine (5 μM) and chloroquine (20 μM) for 48 h. Cellular viability, and p21 and apoptosis-associated protein levels were determined. *P < 0.05, significantly different as indicated. (E) Huh7 cells were pretreated with 15 mM NAC for 1 h and then incubated with tetrandrine (5 μM) and chloroquine (20 μM) for 48 h. Western blot analysis of p21 and caspase-9 protein levels was performed. (F) Huh7 cells transfected with pHAGE/pHAGE-p21 plasmids for 48 h were treated with tetrandrine (5 μM) and chloroquine (20 μM) for 48 h and then intracellular ROS was detected by flow cytometry.

blockade, induce apoptosis, impair lysosomal function and inhibit signalling pathways in many cancer cells (Li *et al.*, 2012; Qin *et al.*, 2013; Qiu *et al.*, 2014). Although Huang have reported the combination of tetrandrine and chloroquine briefly in a previous paper (Huang *et al.*, 2013), in this paper, chloroquine did not markedly affect tetrandrine-iduced cell death at high concentrations, which differs from our data where tetrandrine effects were very clear at low concentrations. We think that the results are different due to the differences in assay conditions, including cancer cell source, treatment concentrations and time courses. In our work, we consider that tetrandrine (5 μM) induced cancer cell death when the autophagy induced by tetrandrine was suppressed by the inhibitor of autophagy, chloroquine (20 μM). Our data showed that tetrandrine (5 μM) alone induced autophagy and did not markedly affect cell viability in cancer cells later shown to be sensitive to the combination of tetrandrine and chloroquine. However, a concentration of tetrandrine (25 μM) did increase cell death (to about 50%) and did induce autophagy in human oral cancer SAS cells (Huang *et al.*, 2013). It is possible that chloroquine did not markedly affect the cell death at high concentrations of tetrandrine in human oral cancer SAS cells, in contrast to our present results, because of the different cancer cells used and the different treatments.

Autophagy is a double-edged sword in tumour oncogenesis and anti-cancer therapies, and the status of autophagy as

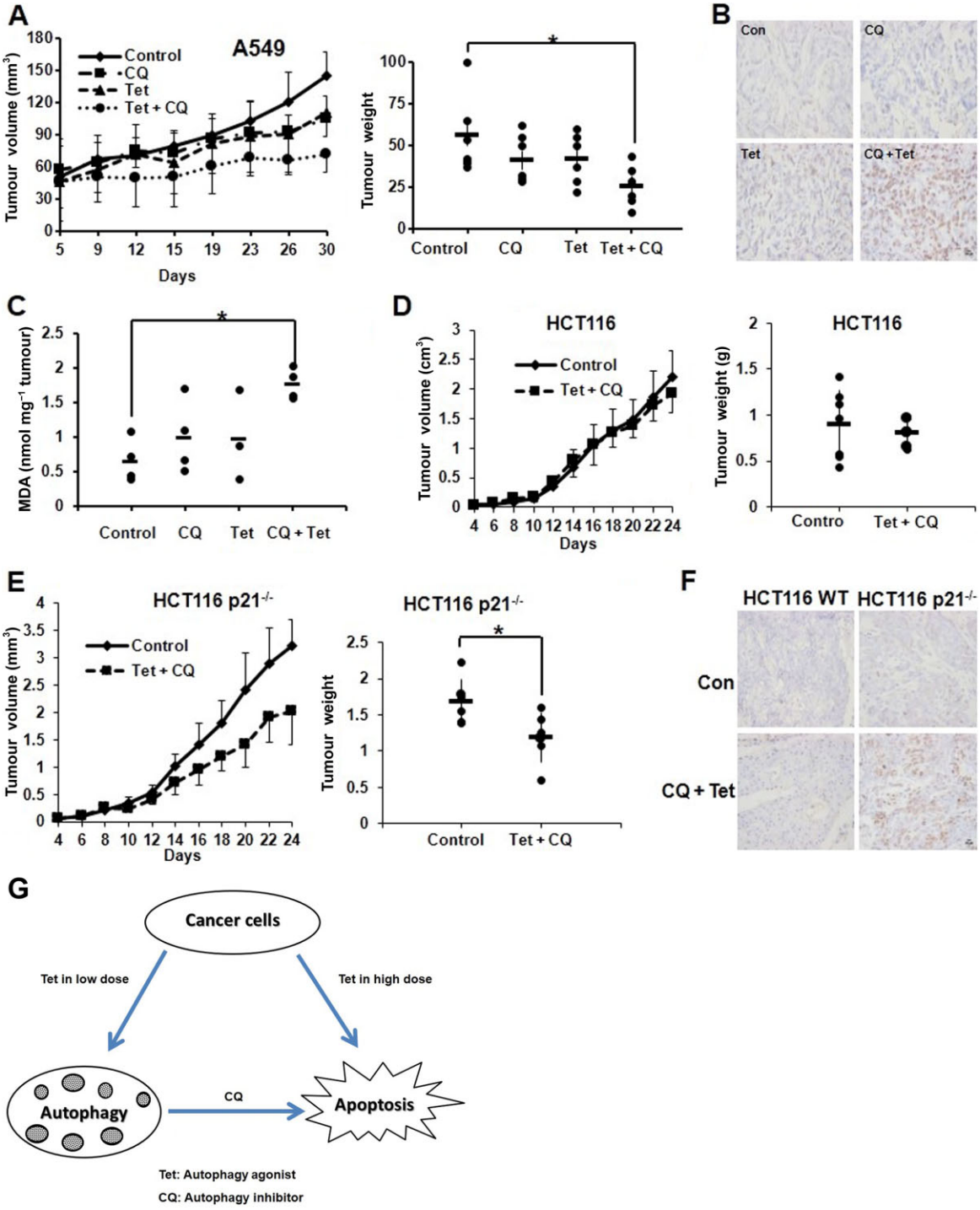

Figure 6

Tetrandrine(Tet) and chloroquine (CQ) combination synergistically represses tumour growth in an *in vivo* xenograft model and is dependent upon p21 expression. Male nude mice bearing A549 tumours were randomly distributed into four groups ($n = 7$) and treated with control, tetrandrine (25 mg·kg^{-1}) and chloroquine (50 mg·kg^{-1}) individually or in combination. Male nude mice bearing HCT116 or HCT116 p21$^{-/-}$ tumours were randomly distributed into two groups ($n = 7$) and treated with control, tetrandrine (25 mg·kg^{-1}) and chloroquine (50 mg·kg^{-1}) combination. (A) Tumour sizes were measured with calipers three times per week for 30 days, and the tumour weight was measured after 30 days of treatment. The data are presented as a scatter plot; the bars represent the SD. *$P < 0.05$, significantly different as indicated. (B)TUNEL assay of A549 tumour tissues was performed as described in the Methods section. (C) MDA levels of tumour tissue proteins exacted from A549 xenograft tumours. *$P < 0.05$, significantly different as indicated. (D, E) Tumour volumes and tumour weights in HCT116 and HCT116 p21$^{-/-}$ cell xenografts. Mean tumour volume in each experimental group and tumour weight are presented as a scatter plot; *$P < 0.05$, significantly different from control group. (F) TUNEL assay analysis of HCT116 and HCT116 p21$^{-/-}$ tumour tissues. (G) Putative model for combined treatment strategy.

a crucial target for cancer treatments has recently received considerable attention (Ma *et al.*, 2013). We previously reported that tetrandrine induced autophagy through regulation of autophagy-related gene 7 (ATG7) expression and ROS/extracellular signal-regulated kinase in human hepatocellular carcinoma (Gong *et al.*, 2012a; Wang *et al.*, 2015). As a mechanism for cell survival, autophagy triggers cells to degrade unnecessary proteins and organelles to obtain a source of macromolecular precursors to prevent the cells from dying when they are under adverse stress, such as nutrient starvation or exposure to chemotherapeutic agents. Of course, ongoing stress will result in cell death (apoptosis and autophagic death) (Sharma *et al.*, 2014). Several reports have shown that the inhibition of autophagy, either at early or late stages of the process, may lead cancer cells to apoptose for many reasons (Lamoureux *et al.*, 2013; Zhu *et al.*, 2013; Li *et al.*, 2014b; Zhao *et al.*, 2014b). In the present study, we found that inhibition of tetrandrine-induced autophagic flux by the autophagy inhibitor chloroquine caused cancer cells to undergo caspase-dependent apoptosis. Thus, our results demonstrate that the combination of tetrandrine and chloroquine has a synergistic anti-tumour activity.

Autophagy is a dynamic process, consisting of initiation, nucleation, elongation and maturation. 3-MA, chloroquine and BAF are well-established autophagy inhibitors that block autophagic flux at different stages. 3-MA inhibits early autophagy sequestration events by suppressing PI3K activity and preventing the formation of autophagic vacuoles, which is essential for autophagy initiation (Li *et al.*, 2009; Wu *et al.*, 2013). Chloroquine effectively blocks later autophagy events by inhibiting lysosomal proteases and autophagosome–lysosomal fusion (Harhaji-Trajkovic *et al.*, 2012; Lamoureux *et al.*, 2013). BAF prevents autophagic vacuole maturation by blocking fusion of autophagosomes with lysosomes (Pivtoraiko *et al.*, 2010; Juhasz, 2012). Here, our data indicate that, in addition to chloroquine, the other two types of autophagy inhibitors, 3-MA and BAF, enhanced anti-tumour activity in combination with tetrandrine. Even when autophagy was blocked upstream of autophagosome formation by 3-MA, tetrandrine-induced stress could still lead to cell death. It is there possible that any inhibition of tetrandrine-induced autophagy will trigger cell apoptosis. If this hypothesis is correct, the combination of cell autophagy agonists with inhibitors may be a useful cancer treatment. However, the exact mechanism and the relationship between autophagy and apoptosis in our model need to be further explored.

Systemic toxicity side effects and acquired drug resistance caused by single-drug therapy have significantly limited the clinical applications of some established chemotherapy agents (Burdach, 2014). The combination of two or several chemotherapeutic agents will potentially improve treatment efficacy and enhance tumour response and patient survival, as well as develop novel therapeutic applications (Fang *et al.*, 2014; Morfouace *et al.*, 2014; Wang *et al.*, 2014). With a long history of clinical application in China to treat various diseases, tetrandrine is considered to be a safe agent (Wang *et al.*, 2012). Similarly, chloroquine has been used for the treatment of diverse diseases, including malaria, rheumatoid arthritis, lupus erythematosus and amoebic hepatitis, and it has relatively low toxicity and well-understood pharmacological properties (Meier *et al.*, 2013; Zhao *et al.*, 2014a). Chloroquine has also been shown to enhance tumour cytotoxicity and promote tumour cell death when used in combination with other chemotherapeutic agents and radiation in clinical trials (Egger *et al.*, 2013; Kimura *et al.*, 2013). We have previously reported valid combination therapy strategies for cancer treatments (Wan *et al.*, 2013). Here, we have demonstrated that the particular combination of tetrandrine and chloroquine had considerable synergistic anti-tumour activity *in vitro* and in an *in vivo* xenograft model. Mechanistically, this combination treatment triggered intracellular ROS accumulation and subsequently activated the caspase signalling cascade, leading to cancer cell apoptosis. Moreover, p21, the CDK inhibitor belonging to the Cip/Kip family, has now been reported to play additional and key roles in other important pathways, including oncogenic properties, apoptosis and DNA repair (Cheng *et al.*, 2010; Stivala *et al.*, 2012; Lee *et al.*, 2013a; Liang *et al.*, 2014). Overexpression of p21(Cip1) maintains cells longer in the G1 phase and attenuates shikonin-induced apoptosis. Immunohistochemistry and *in vitro* binding assays showed that the cytoplasmic p21(Cip1) actually binds to and inhibits the activity of ASK1, regulating the cell cycle progression at G1 (Ahn *et al.*, 2013). Moreover, recent studies demonstrate that degradation of cytoplasmic p21 also by binding to apoptotic proteins such as caspases or phosphorylated by Lats2, activation of caspase-3 and caspase-9, thus promotes apoptosis (Abbas and Dutta 2009; Suzuki *et al.*, 2013). We believe it is possible that caspase or autophagy-related proteins may interact with p21,-to mediate degradation p21 protein, which leads to apoptosis. $p21^{-/-}$ HCT116 cell lines were more sensitive to the combination treatment compared with wild-type HCT116 cells in an *in vivo* xenograft tumour, which is consistent with observations that p21 exerts an anti-apoptotic function as a tumour suppressor protein.

Overall, our present results reveal that the two agents already widely used in clinical treatments, tetrandrine and chloroquine, have a synergistic activity against tumour cells in both *in vitro* cell culture experiments and a xenograft model *in vivo*. These findings indicate that a tetrandrine and chloroquine combination treatment may be a potential therapeutic regimen for tumour patients.

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Liufeng Mei, Yicheng Chen, Zhimeng Wang, Jian Wang and Jiali Wan performed the research and analysed the data. Wenhua Li, Chunrong Yu and Xin Liu designed the research study. Wenhua Li wrote the manuscript and contributed essential reagents or tools.

Conflict of interest

The authors declare no conflict of interest.

References

Abbas T, Dutta A (2009). p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer 9: 400–414.

Ahn J, Won M, Choi JH, Kim YS, Jung CR, Im DS *et al.* (2013). Reactive oxygen species-mediated activation of the Akt/ASK1/p38 signaling cascade and p21(Cip1) downregulation are required for shikonin-induced apoptosis. Apoptosis 18: 870–881.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Altman JK, Szilard A, Goussetis DJ, Sassano A, Colamonici M, Gounaris E *et al.* (2014). Autophagy is a survival mechanism of acute myelogenous leukemia precursors during dual mTORC2/mTORC1 targeting. Clin Cancer Res 20: 2400–2409.

Burdach S (2014). Molecular precision chemotherapy: overcoming resistance to targeted therapies? Clin Cancer Res 20: 1064–1066.

Cheng PH, Lian S, Zhao R, Rao XM, McMasters KM, Zhou HS (2013). Combination of autophagy inducer rapamycin and oncolytic adenovirus improves antitumor effect in cancer cells. Virol J 10: 293.

Cheng X, Xia W, Yang JY, Hsu JL, Chou CK, Sun HL *et al.* (2010). Activation of p21(CIP1/WAF1) in mammary epithelium accelerates mammary tumorigenesis and promotes lung metastasis. Biochem Biophys Res Commun 403: 103–107.

Chou TC (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58: 621–681.

Chou TC (2010). Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70: 440–446.

Egger ME, Huang JS, Yin W, McMasters KM, McNally LR (2013). Inhibition of autophagy with chloroquine is effective in melanoma. J Surg Res 184: 274–281.

Fang K, Li HF, Hsieh CH, Li DY, Song DC, Cheng WT *et al.* (2013). Differential autophagic cell death under stress with ectopic cytoplasmic and mitochondrial-specific PPP2R2B in human neuroblastoma cells. Apoptosis 18: 627–638.

Fang XJ, Jiang H, Zhu YQ, Zhang LY, Fan QH, Tian Y (2014). Doxorubicin induces drug resistance and expression of the novel CD44st via NF-κB in human breast cancer MCF-7 cells. Oncol Rep 31: 2735–2742.

Forde PM, Brahmer JR, Kelly RJ (2014). New strategies in lung cancer: epigenetic therapy for non-small cell lung cancer. Clin Cancer Res 20: 2244–2248.

Gong K, Li W (2011). Shikonin, a Chinese plant-derived naphthoquinone, induces apoptosis in hepatocellular carcinoma cells through reactive oxygen species: a potential new treatment for hepatocellular carcinoma. Free Radic Biol Med 51: 2259–2271.

Gong K, Chen C, Zhan Y, Chen Y, Huang Z, Li W (2012a). Autophagy-related gene 7 (ATG7) and reactive oxygen species/extracellular signal-regulated kinase regulate tetrandrine-induced autophagy in human hepatocellular carcinoma. J Biol Chem 287: 35576–35588.

Gong K, Xie J, Yi H, Li W (2012b). CS055 (Chidamide/HBI-8000), a novel histone deacetylase inhibitor, induces G1 arrest, ROS-dependent apoptosis and differentiation in human leukaemia cells. Biochem J 443: 735–746.

Harhaji-Trajkovic L, Arsikin K, Kravic-Stevovic T, Petricevic S, Tovilovic G, Pantovic A *et al.* (2012). Chloroquine-mediated lysosomal dysfunction enhances the anticancer effect of nutrient deprivation. Pharm Res 29: 2249–2263.

Ho LJ, Juan TY, Chao P, Wu WL, Chang DM, Chang SY *et al.* (2004). Plant alkaloid tetrandrine downregulates IkappaBalpha kinases-IkappaBalpha-NF-kappaB signaling pathway in human peripheral blood T cell. Br J Pharmacol 143: 919–927.

Huang AC, Lien JC, Lin MW, Yang JS, Wu PP, Chang SJ *et al.* (2013). Tetrandrine induces cell death in SAS human oral cancer cells through caspase activation-dependent apoptosis and LC3-I and LC3-II activation-dependent autophagy. Int J Oncol 43: 485–494.

Hurley JH, Schulman BA (2014). Atomistic autophagy: the structures of cellular self-digestion. Cell 157: 300–311.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011). Global cancer statistics. CA Cancer J Clin 61: 69–90.

Juhasz G (2012). Interpretation of bafilomycin, pH neutralizing or protease inhibitor treatments in autophagic flux experiments: novel considerations. Autophagy 8: 1875–1876.

Khan KH, Blanco-Codesido M, Molife LR (2014). Cancer therapeutics: targeting the apoptotic pathway. Crit Rev Oncol/Hematol 90: 200–219.

Kimura T, Takabatake Y, Takahashi A, Isaka Y (2013). Chloroquine in cancer therapy: a double-edged sword of autophagy. Cancer Res 73: 3–7.

Lai CH, Park KS, Lee DH, Alberobello AT, Raffeld M, Pierobon M *et al.* (2013). HSP-90 inhibitor ganetespib is synergistic with doxorubicin in small cell lung cancer. Oncogene 33: 4867–4876.

Lamoureux F, Thomas C, Crafter C, Kumano M, Zhang F, Davies BR *et al.* (2013). Blocked autophagy using lysosomotropic agents sensitizes resistant prostate tumor cells to the novel Akt inhibitor AZD5363. Clin Cancer Res 19: 833–844.

Lee MS, Seo J, Choi DY, Lee EW, Ko A, Ha NC *et al.* (2013a). Stabilization of p21 (Cip1/WAF1) following Tip60-dependent acetylation is required for p21-mediated DNA damage response. Cell Death Differ 20: 620–629.

Lee WL, Huang JY, Shyur LF (2013b). Phytoagents for cancer management: regulation of nucleic acid oxidation, ROS, and related mechanisms. Oxid Med Cell Longev 2013: 925804.

Li J, Hou N, Faried A, Tsutsumi S, Takeuchi T, Kuwano H (2009). Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. Ann Surg Oncol 16: 761–771.

Li J, Chen Y, Wan J, Liu X, Yu C, Li W (2014a). ABT-263 enhances sorafenib-induced apoptosis associated with Akt activity and the expression of Bax and p21 in human cancer cells. Br J Pharmacol 171: 3182–3195.

Li X, Lu X, Xu H, Zhu Z, Yin H, Qian X *et al.* (2012). Paclitaxel/tetrandrine coloaded nanoparticles effectively promote the apoptosis of gastric cancer cells based on 'oxidation therapy. Mol Pharm 9: 222–229.

Li YC, He SM, He ZX, Li M, Yang Y, Pang JX *et al*. (2014b). Plumbagin induces apoptotic and autophagic cell death through inhibition of the PI3K/Akt/mTOR pathway in human non-small cell lung cancer cells. Cancer Lett 344: 239–259.

Liang YY, Zheng LS, Wu YZ, Peng LX, Cao Y, Cao X *et al*. (2014). RASSF6 promotes p21(Cip1/Waf1)-dependent cell cycle arrest and apoptosis through activation of the JNK/SAPK pathway in clear cell renal cell carcinoma. Cell Cycle 13: 1440–1449.

Liu C, Gong K, Mao X, Li W (2011). Tetrandrine induces apoptosis by activating reactive oxygen species and repressing Akt activity in human hepatocellular carcinoma. Int J Cancer 129: 1519–1531.

Ma Y, Galluzzi L, Zitvogel L, Kroemer G (2013). Autophagy and cellular immune responses. Immunity 39: 211–227.

Maycotte P, Aryal S, Cummings CT, Thorburn J, Morgan MJ, Thorburn A (2012). Chloroquine sensitizes breast cancer cells to chemotherapy independent of autophagy. Autophagy 8: 200–212.

McCormack E, Haaland I, Venas G, Forthun RB, Huseby S, Gausdal G *et al*. (2012). Synergistic induction of p53 mediated apoptosis by valproic acid and nutlin-3 in acute myeloid leukemia. Leukemia 26: 910–917.

Meier FM, Frerix M, Hermann W, Muller-Ladner U (2013). Current immunotherapy in rheumatoid arthritis. Immunotherapy 5: 955–974.

Minuti G, D'Incecco A, Landi L, Cappuzzo F (2014). Protein kinase inhibitors to treat non-small-cell lung cancer. Expert Opin Pharmacother 15: 1203–1213.

Morfouace M, Shelat A, Jacus M, Freeman BB 3rd, Turner D, Robinson S *et al*. (2014). Pemetrexed and gemcitabine as combination therapy for the treatment of Group3 medulloblastoma. Cancer Cell 25: 516–529.

Munz C (2013). Autophagy in cellular transformation, survival and communication with the tumor microenvironment. Semin Cancer Biol 23: 299–300.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP; *et al*. NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl. Acids Res 42 (Database Issue): D1098–D1106.

Pivtoraiko VN, Harrington AJ, Mader BJ, Luker AM, Caldwell GA, Caldwell KA *et al*. (2010). Low-dose bafilomycin attenuates neuronal cell death associated with autophagy-lysosome pathway dysfunction. J Neurochem 114: 1193–1204.

Qin R, Shen H, Cao Y, Fang Y, Li H, Chen Q *et al*. (2013). Tetrandrine induces mitochondria-mediated apoptosis in human gastric cancer BGC-823 cells. PLoS ONE 8: e76486.

Qiu W, Su M, Xie F, Ai J, Ren Y, Zhang J *et al*. (2014). Tetrandrine blocks autophagic flux and induces apoptosis via energetic impairment in cancer cells. Cell Death Dis 5: e1123.

Salas PF, Herrmann C, Cawthray JF, Nimphius C, Kenkel A, Chen J *et al*. (2013). Structural characteristics of chloroquine-bridged ferrocenophane analogues of ferroquine may obviate malaria drug-resistance mechanisms. J Med Chem 56: 1596–1613.

Sharma K, Le N, Alotaibi M, Gewirtz DA (2014). Cytotoxic autophagy in cancer therapy. Int J Mol Sci 15: 10034–10051.

Stivala LA, Cazzalini O, Prosperi E (2012). The cyclin-dependent kinase inhibitor p21CDKN1A as a target of anti-cancer drugs. Curr Cancer Drug Targets 12: 85–96.

Suzuki H, Yabuta N, Okada N, Torigata K, Aylon Y, Oren M *et al*. (2013). Lats2 phosphorylates p21/CDKN1A after UV irradiation and regulates apoptosis. J Cell Sci 126: 4358–4368.

Tazzari PL, Tabellini G, Ricci F, Papa V, Bortul R, Chiarini F *et al*. (2008). Synergistic proapoptotic activity of recombinant TRAIL plus the Akt inhibitor Perifosine in acute myelogenous leukemia cells. Cancer Res 68: 9394–9403.

Vlahopoulos S, Critselis E, Voutsas IF, Perez SA, Moschovi M, Baxevanis CN *et al*. (2014). New use for old drugs? Prospective targets of chloroquines in cancer therapy. Curr Drug Targets 15: 843–851.

Wan J, Liu T, Mei L, Li J, Gong K, Yu C *et al*. (2013). Synergistic antitumour activity of sorafenib in combination with tetrandrine is mediated by reactive oxygen species (ROS)/Akt signaling. Br J Cancer 109: 342–350.

Wang H, Liu T, Li L, Wang Q, Yu C, Liu X *et al*. (2015). Tetrandrine is a potent cell autophagy agonist via activated intracellular reactive oxygen species. Cell Biosci 5: 4.

Wang LH, Li Y, Yang SN, Wang FY, Hou Y, Cui W *et al*. (2014). Gambogic acid synergistically potentiates cisplatin-induced apoptosis in non-small-cell lung cancer through suppressing NF-κB and MAPK/HO-1 signalling. Br J Cancer 110: 341–352.

Wang TH, Wan JY, Gong X, Li HZ, Cheng Y (2012). Tetrandrine enhances cytotoxicity of cisplatin in human drug-resistant esophageal squamous carcinoma cells by inhibition of multidrug resistance-associated protein 1. Oncol Rep 28: 1681–1686.

Wu Y, Wang X, Guo H, Zhang B, Zhang XB, Shi ZJ *et al*. (2013). Synthesis and screening of 3-MA derivatives for autophagy inhibitors. Autophagy 9: 595–603.

Zhan Y, Gong K, Chen C, Wang H, Li W (2012). P38 MAP kinase functions as a switch in MS-275-induced reactive oxygen species-dependent autophagy and apoptosis in human colon cancer cells. Free Radic Biol Med 53: 532–543.

Zhao Q, Tensfeldt TG, Chandra R, Mould DR (2014a). Population pharmacokinetics of azithromycin and chloroquine in healthy adults and paediatric malaria subjects following oral administration of fixed-dose azithromycin and chloroquine combination tablets. Malar J 13: 36.

Zhao X, Gao S, Ren H, Huang H, Ji W, Hao J (2014b). Inhibition of autophagy strengthens celastrol-induced apoptosis in human pancreatic cancer *in vitro* and *in vivo* models. Curr Mol Med 14: 555–563.

Zhu S, Cao L, Yu Y, Yang L, Yang M, Liu K *et al*. (2013). Inhibiting autophagy potentiates the anticancer activity of IFN1@/IFNalpha in chronic myeloid leukemia cells. Autophagy 9: 317–327.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 The protein expression of PARP in Hela, U251, FHCC98 and Calu 1 cell lines after treatments of tetrandrine (5 μM), chloroquine (20 μM) or their combination for 48 h or 72 h.

Figure S2 (A) Western blot analysis of p-Akt and Akt after A549 cells were treated with tetrandrine (5 μM), chloroquine (20 μM) or both for 72 h. (B and C) A549 cells transfected with constitutively active Akt (CA-Akt), or vector plasmid (pUSE) were incubated with tetrandrine (5 μM) and chloroquine (20 μM) for 72 h. Western blot analysis and cell viability are shown.

Figure S3 The protein expression of LC3 in Huh7, following treatments of 5 μM tetrandrine and 5 mM 3-methyladenine (or 50 nM bafilomycin) as individual or combination treatments for 72 h.

Figure S4 Cellular viability of HCT116 wild-type, HCT116 $p53^{-/-}$ and HCT116 $Bax^{-/-}$ cells following treatment with tetrandrine (5 μM) and chloroquine (20 μM) for 72 h.

Figure S5 (A) Weight change (mean) in mice bearing A549 cell xenograft tumours during treatment with vehicle, tetrandrine (25 $mg{\cdot}kg^{-1}$), chloroquine (50 $mg{\cdot}kg^{-1}$), or both compounds for 30 days. (B and C) Weight change (mean) in mice bearing either HCT116 wild type or HCT116 $p21^{-/-}$ cell xenograft tumors when treated with combination of tetrandrine 25 $mg{\cdot}kg^{-1}$ and chloroquine 50 $mg{\cdot}kg^{-1}$ every other day for 24 days, the data are presented as a scatter plot; the bars represent the SD. $P < 0.05$.

Table S1 CI values of tetrandrine at concentrations in combination with chloroquine in U87 and U251 cell lines. CI < 0.9 represents synergism.