Discovery of canine drug toceranib phosphate as a repurposed agent against human hepatocellular carcinoma

Ling Qiao1 | Siyuan Qin2,3 | Ningna Weng4 | Bowen Li2,3 | Maochao Luo2,3 | Zhe Zhang2,3 | Li Zhou2,3 | Dong Wang1 | Canhua Huang1,2,3

1School of Basic Medical Sciences, Chengdu University of Traditional Chinese Medicine, Chengdu, P.R. China
2Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Collaborative Innovation Center for Biotherapy, Chengdu, P.R. China
3West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, P.R. China
4Department of Abdominal Oncology, West China Hospital of Sichuan University, Chengdu, P.R. China

Correspondence Canhua Huang and Dong Wang, School of Basic Medical Sciences, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, P.R. China. Email: hcanhua@hotmail.com and wangdong@cdutcm.edu.cn

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Abstract

Background and Aims: Human hepatocellular carcinoma (HCC) is an aggressive malignancy with poor clinical outcomes. There are limited therapeutic options for those diagnosed with terminal HCC and therefore incorporating novel agents into standard-of-care regimens is urgently needed. In contrast to de novo drug discovery, the strategy of repurposing compounds initially designed to treat animals might yield substantial advantages in terms of efficacy and safety. Given the evidence for the clinical efficacy of toceranib phosphate (TOC) against canine carcinomas, we aimed to investigate its therapeutic effect on human HCC.

Methods: The antitumor effects of TOC were evaluated using human HCC cell lines and cell-line-derived xenograft models. Changes in autophagic response upon TOC exposure were quantified through immunoblotting and immunofluorescence analysis. The role of TOC-triggered autophagy was addressed via pharmacological and genetic inhibition.

Results: We demonstrated TOC exhibited potent antitumor activity against human HCC cells by stimulating apoptosis in vitro and in vivo by a concomitant increase in autophagic flux. Blocking the TOC-triggered autophagy inhibited cellular proliferation and decreased tumour burden, indicating a protective role of autophagy against TOC-mediated HCC cell death. This role played by TOC-induced autophagy was further linked to the inactivation of the Akt/mTOR pathway that could be attributed to the upregulation of Cyr61. Moreover, treatment with sorafenib plus TOC resulted in pronounced synergistic effects on HCC cells.

Conclusion: Our results elucidate a newly identified therapeutic potential of TOC in treating HCC, sparking a growing interest in repurposing such canine drugs for human use.

KEYWORDS

apoptosis, autophagy, hepatocellular carcinoma, toceranib phosphate
1 | INTRODUCTION

Multiple lines of evidence indicate that comparative oncology clinical trials provide biomedical knowledge and test results in the veterinary setting to solve specific obstacles in cancer therapy that cannot be completely addressed by other traditional model species (e.g. mice). In particular, dogs act as a sentinel model for a paradigm shift of human cancer development in terms of aetiology and epidemiology. More specifically, canine cancers appear to precisely recapitulate the histopathology and biology of the corresponding human cancers, highlighting the immense value of comparative and translational studies for therapeutic benefits to both dogs and humans in need. From a therapeutic point of view, existing canine drugs carry considerable important information in terms of efficacy and safety, which supports their incorporation into preclinical trials and, in some ways, enables new antineoplastic drugs to be born with an abbreviated trial-and-error process. Because of this, repurposing of canine drugs may mitigate part of the risk associated with the development of entirely new drugs, providing us with an alternative route to solve clinical dilemmas and achieve the ultimate goal of rapid application in patients with cancers. An encouraging sign is that these fundamental similarities between canine and human carcinomas seem to motivate a concern about agents targeting crucial pathways or molecular drivers shared by cancers in people and dogs. At this point, one of the earliest examples—receptor tyrosine kinase (RTK) inhibitors toceranib (TOC) and sunitinib can be drawn, which might inform the subsequent work with them or other selective RTK inhibitors for veterinary and human use.

Primary hepatocellular carcinoma (HCC) remains one of the leading malignancies globally characterized by high case-fatality rates and rising treatment costs, the incidence of which has reached a peak in recent years despite numerous advances in varied medical, locoregional, and surgical therapies. While these currently emerged as a potent candidate pending further experimental investigation.

As mentioned earlier, dogs as the idealized and anthropomorphic model sharing crucial features with humans including similar responses to the same agent, potentially representing a significantly broader population of cancer patients that could be included in the preclinical drug-development path. For instance, two reagents and even their analogues tailored to target canine cancers (i.e. a non-specific COX inhibitor piroxicam and selective inhibitor of nuclear export (SINE) KPT-330) have already been applied to suppress the growth of lymphoma and urothelial carcinoma in humans, respectively. Implicit in this paradigm is the hypothesis that there may appear to exist more canine drugs harbouring a potential and translational significance to treat a certain form of human tumours. HCC is also prevalent in canines, and such canine HCCs share plenty of similarities with human HCC in clinical characteristics and histological traits, raising the possibility of agents for canine HCC as a neglected “arsenal” that could be used for human HCC. Among the authorized drugs used in dogs, TOC (Palladia®), an aforementioned RTK inhibitor preliminary proved to be effective for dogs with HCC, emerged as a potent candidate pending further experimental investigation. In this regard, another closely related small-molecule inhibitor sunitinib has received FDA approval for treating advanced resistance or adverse reactions. Hence, in order to improve overall survival (OS) of patients with HCC, there exists an imperative medical need for the development of new therapeutic agents as well as optimal multidrug combination strategies. Perhaps ironically, against the backdrop of apparent contradiction between the lengthy process of de novo drug synthesis and urgent requirement of HCC patients, it is necessary for us to find an alternative way of drug management, with a particular focus on drug repurposing.

In light of the above, the study herein aimed to identify the potential therapeutic efficacy of toceranib (TOC) in HCC, to elucidate its mechanism of action, and further explore the possibility of TOC’s application in future clinical studies in patients with HCC.

**Key points**

- There are limited therapeutic options for patients with terminal HCC, highlighting the urgency for identifying novel agents to combat this lethal disease.
- Toceranib phosphate (TOC), a novel agent for treating canine carcinomas, exhibits potent therapeutic efficacy against human HCC.
- Synergetic action can be obtained by combining TOC with an autophagy inhibitor or sorafenib.

**Figure 1** TOC exhibits potent anti-HCC effects by inducing apoptosis in vitro and in vivo. (A) The chemical structure of TOC. (B) MTT assay of HCC cell lines and LO2 incubated with indicated concentrations of TOC for 24 h (left). The IC50 value (μM) of cell lines (right). (C, D) HCC cells were treated with indicated concentrations of TOC. Cell proliferation was measured by colony formation assay (C) and EdU incorporation assay (D). (E) LDH release assay of HCC cells subjected to TOC for 24 h. (F) Cellular morphology of Huh7 and Hep3B cells treated with or without TOC for 24 h. 10 × 10. Scale bar, 100 μm. (G) Immunoblotting analysis of total and cleaved PARP or caspase 3 in Huh7 and Hep3B cells treated with indicated concentrations of TOC for 24 h. (H) MTT assay of Huh7 and Hep3B cells treated with or without TOC (10 μM, 24 h) in the presence or absence of Z-VAD. (I) The images of isolated tumours derived from vehicle or TOC-treated mice bearing Huh7 subcutaneous tumour xenografts (n = 5). (J, K) The volume and weight in (I). (L, M) Immunohistochemical staining of Ki67 and cleaved-caspase3 in tumour tissues. Scale bar, 50 μm. (ns, no significant; *p < 0.05; **p < 0.01; ***p < 0.001). The significant differences were observed between the treatment groups and 0 μM group (C, D and E), or treatment groups and Ctrl group (H, J, K, L and M). Statistic method: unpaired 2-tailed Student’s t-test and two-way ANOVA analysis (J).
TOC elicits autophagy in HCC cells. (A, B) Immunoblotting analysis of LC3, ATG5, and p62 in Huh7 and Hep3B cells treated with indicated concentrations of TOC for 24 h, or 10 μM TOC for indicated times. (C) Autophagic vesicles were detected by transmission electron microscope in Huh7 and Hep3B cells treated with or without 10 μM TOC for 24 h. Scale bar: 2 μm. N, nucleus. Arrows, autophagic vesicles. (D) Immunofluorescence analysis of LC3 in HCC cells treated with or without TOC (10 μM, 24 h) in the presence or absence of 3-MA (10 mM). (E) Immunofluorescence analysis of LC3 in HCC cells treated as in (F). Scale bar: 20 μm. (F) Immunoblotting analysis of LC3 in HCC cells transfected with siScramble, siATG5 for 24 h, followed by treatment with or without TOC (10 μM, 24 h). Scale bar: 20 μm. (*p < 0.05; **p < 0.01; ***p < 0.001).

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human hepatocellular carcinoma cell lines (Huh7, Hep3B, MHCC-97H, PLC/PRF/5, and SK-Hep1) and non-tumour human hepatic cell line (LO2) were all purchased from ATCC or Cell Bank of the institute of Culture Collection of the Chinese Academy of Sciences. Cells were maintained in DMEM supplemented with 1% streptomycin–penicillin (Sigma) and 10% serum (Biowest) at 37°C in a humidified atmosphere with 5% CO2.

2.2 | Reagents

TOC, CQ (Chloroquine) and MG-132 were purchased from Selleck Chemicals. 3-methyladenine (3-MA) and Z-VAD (Z-Val-Ala-Asp (OMe)-FMK) were purchased from MedChem Express. Lipofectamine 3000 reagent was purchased from Invitrogen. The antibodies involved in this study were listed as follows: cleaved caspase-3 (9664 S; Cell Signalling Technology, Danvers, MA, USA), PARP (ab74290; Abcam, Cambridge, MA, USA), cleaved PARP (ab32064; Abcam), LC3 (NB100-2220; Novus, Saint Charles, MO, USA), ATG5 (12994S; Cell Signalling Technology), Akt (4685; Cell Signalling Technology), phosphorylated (p-)Akt (Ser473) (4060; Cell Signalling Technology), p-mTOR (Ser2448) (2971; Cell Signalling Technology), p70S6K (9202; Cell Signalling Technology), p-p70S6K (Ser371) (9208; Cell Signalling Technology), Cyr61 (122190, ZEN BIO), Ki67 (ab66155; Abcam), β-actin (sc-1616; Santa Cruz Biotechnology), and horseradish peroxidase-conjugated anti-rabbit secondary antibody (sc-2004; Santa Cruz Biotechnology), horseradish peroxidase-conjugated anti-mouse secondary antibody (sc-2005; Santa Cruz Biotechnology).

2.3 | Measurement of cell viability

TOC-effects on tumour cell growth were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay and colony formation assay described as before, respectively, for short-term and long-term. Cell proliferation was detected by employing the EdU incorporation assay kit (RiboBio Co., Ltd, C10310). And the operation was completely conducted according to the instruction. Images were collected with a fluorescence microscope.

2.4 | Lactate dehydrogenase release assay

Cell cytotoxicity of TOC was assessed by lactate dehydrogenase release (LDH) assay, using an LDH test kit (Beyotime Biotechnology, Nanjing, China). The experiment was carried out in accordance with the supplier’s instructions.

2.5 | Flow cytometry

Cells were harvested after 24 h of TOC treatment and washed once with PBS, then fixed with 70% medicinal alcohol for 48 h. These fixed cells prepared for cell cycle analysis (Beyotime Biotechnology, Nanjing, China) were washed with PBS, followed by FACS detection. FlowJo software was applied to data analysis.

2.6 | TUNEL assay

Cells were seeded onto glass coverslips in 24-well plates beforehand, subsequently dealing with TOC for 24 h. 30 min fixed in 4% paraformaldehyde later, terminal deoxynucleotidyl transferase-mediated nick-end labelling (TUNEL) staining was conducted with
FIGURE 3  TOC promotes autophagic flux in HCC cells. (A) Immunoblotting analysis of LC3 in Huh7 and Hep3B cells treated with or without TOC (10 μM, 24 h) in the presence or absence of CQ (10 μM). (B) Immunofluorescence analysis of colocalization of LC3 and LAMP2 in HCC cells treated with or without TOC (10 μM, 24 h). (C) Immunofluorescence analysis of HCC cells transfected with RFP-GFP-LC3 and treated with or without TOC (10 μM, 24 h). Cells treated with CQ served as a negative control in (B, C). Scale bar, 20 μm. (D) Acridine orange staining of HCC cells treated as in (B). Scale bar, 20 μm. (*p < 0.05; **p < 0.01; ***p < 0.001).
the Dead End Fluorometric TUNEL system (Promega). Using a fluorescent microscope, the TUNEL-positive cells were detected and photographed in every independent experiment.

2.7 | Transmission electron microscopy

After being treated with or without 10 μM TOC, Huh7 and Hep3B cells were fixed in 3% glutaraldehyde. When the samples were embedded after dehydration, ultrathin sections of ~70 nm were prepared and then double stained with 2% uranyl acetate and Reynolds lead citrate. Autophagic vesicles were analysed by Hitachi HT7800 electron microscopy at 80 kV.

2.8 | Tandem mRFP-GFP-LC3 assay

Tandem mRFP-GFP-LC3 assay was performed to detect autophagy flux. Cells were plated in glass coverslips in 24-well plates and infected with mRFP-GFP-LC3 adenovirus for 24 h. After being treated with or without TOC for another 24 h, cells were fixed in 4% formaldehyde for 2 h, then stained nuclei with DAPI. Image capture was performed using confocal laser scanning microscopy (Zeiss).

2.9 | Acridine orange staining

Acridine orange staining assay was performed to evaluate autophagy as described previously. Cells suspended in PBS were stained with 1 mmol/L acridine orange (Sigma-Aldrich) for 5 min.

2.10 | Immunofluorescence

Cells were plated in glass coverslips in 24-well plates, fixed in 4% formaldehyde for 2 h, and then permeabilized with PBS containing 0.3% Triton X-100 and 5% BSA for 1.5 h. After that, these cells were incubated with antibodies against LC3 and LAMP2 at 4°C overnight and subsequently incubated with Alexa Fluor secondary antibodies for 1.5 h. Nuclei were stained with DAPI (Santa Cruz Biotechnology). Image capture was performed using confocal laser scanning microscopy (Zeiss).

2.11 | RNA interference

The RNA interference assay was conducted using ATG5, Cyr61, and scramble small interfering RNA (siRNA) synthesized by GenePharma (Shanghai, China) following the manufacturer’s instructions, using Lipofectamine 3000 reagent (Thermo Fisher Scientific). The sequences of siRNA included in transfection were human ATG5 siRNA, 5′-GCAACU-CUGGAUGGGAUUGTT-3′; human Cyr61 siRNA, 5′-AACAUC-AGUGCACAUGUAUUG-3′.

2.12 | Western blotting

Cells were harvested after treatment and then lysed in RIPA buffer added with protease and phosphatase inhibitor cocktail (Sigma, p8340). Proteins were isolated using SDS-PAGE and then transferred to a 0.22 μm polyvinylidene difluoride (PVDF) membrane. Blocked the transferred membrane in 5% skimmed milk for 1 h, and the samples were incubated in the suitable primary antibody at 4°C overnight and then incubated with the secondary antibody at room temperature for 1.5 h. Immunoreactive bands were detected with enhanced chemiluminescence reagent, with β-actin serving as an internal control.

2.13 | Cellular thermal shift assay

Cells were harvested after being treated with or without TOC for 12 h and resuspended with PBS. The suspension was divided into six tubes, then heated for 5 min to 54, 57, 60, 64, 67, and 70°C followed by 3 cycles of freeze-thawing with liquid nitrogen and centrifugation at 15000g for 15 min.

2.14 | Animal models

8-weeks-old male nude mice purchased from HFK Bioscience Co., Ltd (Beijing) were employed to establish the subcutaneous model of human HCC cells (Huh7, 1×10⁷ cells/mouse) and were collected and suspended in PBS. Tumour volumes were assessed daily according to the following formula: tumour volume (mm³) = (length×width²)/2. When the tumour volume reached ~100 mm³, the mice were randomly divided into different groups (five mice for each group). In Figure 1, mice were randomized into two groups, 0.1 mL of vehicle (physiologic saline), TOC 5 mg/kg. In Figure 4E, mice were randomized into four groups and received the following treatments, 0.1 mL of vehicle (physiologic saline), TOC 5 mg/kg, CQ 25 mg/kg, or TOC 5 mg+CQ 25 mg/kg. In Figure 7D, mice were randomized into four groups and received the following treatments, 0.1 mL of vehicle (physiologic saline), TOC 5 mg/kg, SOR 15 mg/kg, or TOC 5 mg+SOR 15 mg/kg. Mice in all groups were administered orally once a day. After 14 days of treatment, they were euthanized for analysis. All procedures performed on mice in our study were approved by the Institutional Animal Care and Treatment Committee of Sichuan University.

2.15 | Immunohistochemistry

The tumour samples and major organs from xenograft models were embedded in paraffin and cut into 4-μm-thick sections. All sections were dewaxed, rehydrated, then quenched the endogenous peroxidase activity, and then treated with citrate buffer to remove antigen. After incubation with indicated primary antibodies at 4°C overnight,
sections were stained with diaminobenzidine and re-stained with Mayer haematoxylin. H&E staining was done to the major organs (heart, liver, spleen, lung, and kidney) sections. Imaging was visualized with a DM2500 fluorescence microscope.

### 3.1 | TOC exhibits potent anti-HCC effects by inducing apoptosis in vitro and in vivo

The chemical structure of TOC, a chemotherapeutic agent for canine carcinoma, is shown in Figure 1A. To define the antitumor effects of TOC on human HCC in vitro, we undertook to perform an MTT assay using five different human HCC cell lines (Huh7, Hep3B, MHCC-97H, PLC/PRF/5 and SK-Hep1) and a non-tumorigenic human hepatocyte cell line (LO2). As expected, the cell viability of HCC cell lines (IC_{50} values ranging from 5.90 to 12.35 μM) was repressed greater than that of the LO2 cell line following TOC exposure in a dose- and time-dependent manner (Figures 1B and S1A). Because this repression is most significant in Huh7 and Hep3B cells, these two types of HCC cell lines were selected as representatives for the follow-up exploration. Consistently, TOC treatment also resulted in a lower clonogenic capability of HCC cells compared to that of LO2 cells through 2D (plate)-colony formation assay (Figure 1C). In addition to this tumour-specific growth inhibitory effect exerted by TOC, the reduced proliferative potential, as well as enhanced cytotoxicity, were further observed in HCC cells via EdU incorporation and lactate dehydrogenase (LDH) assays (Figures 1D,E and S1B). Next, it was demonstrated that this impaired proliferative capacity was largely because of TOC-induced cell-cycle arrest in a dose-dependent manner, manifested by a gradually increasing HCC cell arrested in G0/G1 phase (Figure S1C). As for the effective cytotoxicity of TOC against HCC cells, we were curious if its killing effect on Huh7 and Hep3B cells was associated with apoptotic cell death, given the fact that apoptosis is the most common way in which antineoplastic agents work. As predicted, we observed that TOC-treated cells have undergone significant morphological changes, which were manifested by the formation of intracytoplasmic vacuoles and signs of apoptosis (Figure 1F). The expression levels of apoptotic markers, i.e. cleaved caspase 3 and cleaved PARP, were simultaneously upregulated in HCC cells as the increment of TOC dose (Figure 1G), indicating an apoptosis-promoting role played by TOC, as was the case displayed in TUNEL assay (Figure S1D). Moreover, incubating HCC cells with apoptosis inhibitor Z-VAD-FMK and TOC could partially alleviate the growth-inhibitory effect of TOC that was reflected in improved cell viability when compared to the group without Z-VAD-FMK treatment (Figure 1H). To summarize, these results indicated that TOC exhibits a potent anti-HCC effect in vitro via activating apoptosis.

To extend and generalize these findings further, we developed an HCC cell-line (Huh7) derived xenograft mouse model to evaluate the therapeutic effect of TOC against HCC in vivo. Consistently, in comparison with animals in the vehicle group, mice administered with TOC showed a dramatic reduction in tumour size and weight (Figure 1I–K). Corresponding to that, the TOC-treated group compared with vehicle-treated ones exhibited a lower level of Ki67 (a reliable indicator of proliferation) and an increased expression of cleaved caspase 3 in xenograft tumours by immunohistochemical (IHC) staining (Figure 1L,M). Collectively, these results demonstrated that TOC inhibits HCC cell growth both in vitro and in vivo through TOC-mediated apoptosis.

### 3.2 | TOC elicits autophagy in HCC cells

An increasing number of studies have indicated the importance of drug-induced autophagy in cancer therapy.\textsuperscript{15,16} It has been reported that sunitinib, a multityrosine kinase inhibitor that shares structural and functional similarity with TOC, served a controversial role in autophagy regulation within multiple cancers,\textsuperscript{17} which raised the question of whether TOC can also act as an autophagy regulator in HCC cells. Immunoblotting analysis of autophagy markers was performed to investigate whether TOC modulates autophagy in HCC cells, which revealed that Huh7 and Hep3B cells exhibited dramatic autophagic induction in a dose- and time-dependent manner upon TOC treatment, as demonstrated by enhanced expression of LC3-II, ATG5 and reduced the expression of p62 (Figure 2A,B). Furthermore, as evidenced by transmission electron microscopy and immunofluorescence analyses, there was a significant accumulation of autophagic vesicles and LC3 puncta in Huh7 and Hep3B cells after TOC treatment (Figure 2C,D). Consistently, xenograft tumours exhibited increased expression of LC3B following TOC administration (Figure 2E). Notably, this induction manifested by TOC-triggered accumulation of LC3-II could be intensively attenuated...
by pharmacological inhibition of autophagy initiation employing 3-methyladenine (3-MA) (an inhibitor of class III PI3K), or through genetic silencing of ATG5 (a key inducer of autophagosome formation) in HCC cells (Figures 2F–H and S2A). The TOC-induced autophagy in HCC was further verified by the observation that TOC treatment resulted in increased interaction of Beclin1 with Atg14L and the dissociation of Beclin1 with Bcl-2 (Figure S2A). This pro-survival role of TOC-induced autophagy in vitro was further verified by in vivo studies, reflected by the greatly decreased tumour size, growth rate, and weight without obvious weight loss and visceral toxicity following TOC/CQ combination therapy compared to either treatment alone (Figures 4E–G and S4F–G). Consistently, the Ki67 level was much lower in the combinatorial treated tissues detected by IHC staining (Figure 4H). Overall, these data highlighted that TOC-triggered autophagy might show a cytoprotective effect on HCC, elucidating that the anti-HCC potency caused by TOC could be further strengthened when combined with autophagy inhibitors such as CQ.

3.3 | TOC promotes autophagy flux in HCC cells

Considering that TOC-mediated LC3-II accumulation may be attributed to either autophagy initiation or impaired autophagy flux, we next evaluate the autophagy flux upon TOC treatment. As depicted in Figure 3A, the combinatorial treatment of TOC with chloroquine (CQ) (a late-autophagy inhibitor) led to a substantial accumulation of LC3-II compared to a single treatment of TOC or CQ, implying that TOC may prompt autophagic flux in HCC cells. This notion was further validated by the observation indicating significant colocalization of LC3 and LAMP2 in HCC cells treated with TOC rather than CQ, a symbol of the fusion of autophagosomes and lysosomes (Figure 3B), which concert with the results from lysotracker red-staining (Figure S3A). Consistently, using tandem mRFP-GFP-LC3 constructs, there emerged increased autolysosomes (red dots, RFP-GFP) accompanied by reduced autophagosomes (yellow dots, RFP-GFP+) in TOC-treated HCC cells in contrast to that detected in CQ-treated cells (Figure 3C). Moreover, in response to TOC treatment, the induced formation of autophagic vacuoles was monitored by acridine orange (AO) staining (Figure 3D). In summary, these results indicated that TOC boosts the autophagic flux in HCC cells.

3.4 | Inhibition of autophagy enhances the anti-HCC effects of TOC

It is firmly believed that the role of autophagy is context-dependent and may be cytoprotective, cytostatic or cytotoxic to tumour cells. Curious about the function of TOC-activated autophagy in HCC, we therefore assessed the impacts of TOC on HCC growth in combination with a series of inhibitors targeting different autophagic phases. Strikingly, the anti-HCC efficacy of TOC could be significantly exacerbated through both inhibition of initial-stage and late-stage autophagy (3-MA and CQ respectively), or by genetic silencing of ATG5, evidenced by a drastic reduction in cell viability, clonogenic capacity and proliferative potential compared with that of the group treated with TOC alone (Figures 4A–D and S4A–E). This pro-survival role of TOC-induced autophagy in vitro was further verified by in vivo studies, reflected by the greatly decreased tumour size, growth rate, and weight without obvious weight loss and visceral toxicity following TOC/CQ combination therapy compared to either treatment alone (Figures 4E–G and S4F–G). Consistently, the Ki67 level was much lower in the combinatorial treated tissues detected by IHC staining (Figure 4H). Overall, these data highlighted that TOC-triggered autophagy might show a cytoprotective effect on HCC, elucidating that the anti-HCC potency caused by TOC could be further strengthened when combined with autophagy inhibitors such as CQ.

3.5 | TOC induces autophagy by repressing the Akt/mTOR pathway in HCC cells

Cumulative evidence has supported the Akt/mTOR signalling pathway as a major regulator of autophagy. Therefore, we hypothesize that the Akt/mTOR pathway may be responsible for TOC-induced autophagy. In line with our speculation, TOC in a dose- and time-dependent manner decreased the expression of phosphorylated (p-) Akt, mTOR, and p70S6K, and their phosphorylated counterparts in Huh7 and Hep3B cells treated with indicated concentrations of TOC for 24 h (A), or 10 μM TOC for indicated times (B). (C) Immunoblotting analysis of LC3 in HCC cells transfected with empty vector or constitutively active CA-Akt for 24 h, followed by treatment with or without TOC (10 μM, 24 h). (D) Immunofluorescence analysis of LC3 in HCC cells treated as in (D). Scale bar, 20 μm. (E) The MTT assay of HCC cells treated as in (D). (*p < 0.05; **p < 0.01; ***p < 0.001).

3.6 | TOC facilitates autophagy through Cyr61/Akt axis in HCC cells

There have been considerable reports about the contribution made by Cyr61 (a secreted matricellular protein) to Akt inactivation.

FIGURE 5 TOC induces autophagy by suppressing the Akt/mTOR pathway in HCC cells. (A, B) Immunoblotting analysis of Akt, mTOR, p70S6K, and their phosphorylated counterparts in Huh7 and Hep3B cells treated with indicated concentrations of TOC for 24 h (A), or 10 μM TOC for indicated times (B). (C) Immunohistochemical staining of p-Akt in tumour tissues. Scale bar, 50 μm. (D) Immunoblotting analysis of LC3 in HCC cells transfected with empty vector or constitutively active CA-Akt for 24 h, followed by treatment with or without TOC (10 μM, 24 h). (E) Immunofluorescence analysis of LC3 in HCC cells treated as in (D). Scale bar, 20 μm. (F) The MTT assay of HCC cells treated as in (D). (*p < 0.05; **p < 0.01; ***p < 0.001).
To explore whether Cyr61 was involved in TOC-induced Akt inactivation, we conducted a western blot analysis of Cyr61, which revealed the upregulation of Cyr61 in Huh7 and Hep3B cells exposed to TOC (Figure 6A). Similar observations were generated by in vivo models (Figure 6B). Furthermore, the treatment of TOC could engage and stabilize Cyr61 against thermal changes in the cellular thermal shift assay (Figure 6C), and the rate of CYR61-degradation was decreased by MG-132 (Figure S5A). In addition to
the HCC cell growth was repressed by SOR with IC\textsubscript{50} values ranging from 10 to 20\mum in Huh7 and Hep3B cells, while the combinatorial treatment of TOC and SOR showed an additive effect which was reflected in strongly decreased cell viability and impaired clonogenic capability in contrast to the group with a single treatment of TOC or SOR (Figure 7B,C). The synergistic effects of TOC and SOR were further confirmed in vivo by the Huh7 xenograft model, as demonstrated by a slower tumour growth rate and remarkably reduced tumour size and weight in the combinatorial treated group (Figure 7D–F). Furthermore, xenograft tumours with combinatorial treatment exhibited decreased expression of ki67 in comparison with TOC or sorafenib-treated ones (Figure 7G). Taken together, these analyses indicated the potential of TOC applications in both monotherapy and combination pharmacotherapy for HCC.

4 | DISCUSSION

Drug repurposing, known as identifying new indications for approved drugs, offers a shorter approval process for cancer therapy.\textsuperscript{23,24} Several studies have proved that drug repurposing from canine drugs may reveal new targets and pathways for human HCC\textsuperscript{16,25} particularly the small molecule kinase inhibitors.\textsuperscript{26,27} TOC, an orally bioavailable multi-targeted inhibitor blocking RTK, is generally active against several split kinase families, including VEGFR, PDGFR, Kit, and Ret.\textsuperscript{28} For its inhibition of VEGFR and PDGFR known to function in tumour-driven angiogenesis, TOC was initially developed as an antiangiogenic drug, displaying a biological activity in various cancer models.\textsuperscript{4} In addition to its impact on angiogenesis, TOC might also directly inhibit tumour growth via targeting Kit and Ret.\textsuperscript{29} Similar to TOC in terms of structure and function, another RTK inhibitor sunitinib (Sutent) which shares the same targets with TOC, has been reported to exert single-agent activity in HCC, hinting at a potential role of TOC in HCC therapy.\textsuperscript{30} Our present study demonstrated that TOC has a potent anti-cancer effect in HCC both in vitro and in vivo, driven mainly by TOC-stimulated apoptosis in HCC cells. In addition to apoptosis induced by TOC, TOC was demonstrated to activate protective autophagy via the Cyr61/Akt/mTOR axis. Considering this pro-survival role, blocking autophagy using autophagic inhibitors could enhance anti-HCC efficacy mediated by TOC. Our findings may extend the clinical potential of TOC and provide a new paradigm for HCC therapy.
TOC enhances the therapeutic response of HCC cells to sorafenib in vitro and in vivo. (A) MTT assay of Huh7 and Hep3B cells incubated with indicated concentrations of sorafenib for 24 h. (B) MTT assay of HCC cells treated with the indicated concentrations of TOC and sorafenib for 24 h (Every grid showed an absorbance value in 570 nm. The shade of blue represented the relative value of absorbance in 570 nm.). (C) Colony formation assay of HCC cells treated with indicated concentrations of SOR in the presence or absence of TOC (10 μM). (D) The images of isolated tumours derived from vehicle, TOC or sorafenib-treated mice (n = 5). (E) The volume of individual tumours was measured at the indicated time points. (F) The weight of individual tumours in (D). (G) Immunohistochemical staining of Ki67 in tumour tissues. Scale bar, 50 μm. (ns, no significance, *p < 0.05; **p < 0.01; ***p < 0.001).
In general, autophagy, considered as a survival pathway that maintains cell homeostasis, is a mechanism by which cellular materials are delivered to lysosomes for a multi-step degradation process. Growing evidence has proven that autophagy may play context-dependent roles in different tumours or the same tumour at different stages. Furthermore, regulating autophagy has been recognized as a promising therapeutic strategy. Apoptosis, an autonomic regulated physiological process also known as type I programmed cell death (PCD I) characterized by cell membrane blebbing and chromosomal DNA fragmentation, has long been considered a popular target in different cancer treatment strategies. Both autophagy and apoptosis are typically programmed cell death regulated by complicated signal transduction networks, and thus their dysregulation may ultimately contribute to tumour development. Recent studies suggest complex interactions between autophagy and apoptosis with contexts of both physiological and pathological, which have been widely studied mainly for their significance in cancer therapy. In this regard, autophagy usually occurs in advance of apoptosis within the same cell. Meanwhile, whether autophagy activates or suppresses apoptosis depends on specific cell types, stress, or stimuli. In addition to the regulatory role of autophagy in the apoptosis process, there are indeed reports stating that several proteins known to modulate apoptosis might act as inducers of autophagy as well. In the present study, our data demonstrated that HCC cells upon TOC treatment exhibited intense autophagic induction. Importantly, this induction of autophagy by TOC in HCC cells occurred with the activation of apoptosis, and the therapeutic response of HCC cells to TOC could be significantly strengthened via pharmacological inhibition using autophagy inhibitors (3-MA or CQ), or via siRNA-mediated silencing of autophagic machinery components (i.e. ATG5). These results elucidated that the induction of autophagy by TOC displayed a cytoprotective effect against TOC-mediated apoptosis in HCC cells. Stated somewhat differently, the activation of autophagy served as a rapid response mechanism by which the HCC cells could be equipped with a survival advantage in dealing with TOC exposure. Notably, increasing evidence has proved the close correlation between autophagy mechanism and drug resistance. Consistent with our study, there are quite a few cases that some compounds have been used to treat cancers (such as frontline glioma, colon cancer, pancreas cancer) in conjunction with autophagy inhibitor CQ/HCQ in clinical trials. In this regard, TOC-induced autophagy played a cell-protective role in treating HCC, suggesting that the combination of autophagy inhibitors with TOC may provide a better therapeutic response against cancers, especially for apoptosis-resistant diseases. However, given the issue above that autophagy likely affects apoptosis in the opposing directions, the underlying problem of autophagy manipulation is highlighted in clinical practice; that is, blocking autophagy may show no influence on the improvement of response to partner agents, partially owing to the degradation of different pro- or anti-apoptotic regulators by autophagy. The principles behind such combination treatments largely remain unknown as most combinations were introduced based on experimentation among patients. Therefore, the real issue is to evaluate and determine which member possesses a more significant weight in monitoring cellular fate.

The Akt/mTOR signalling pathway is well-recognized in autophagy and apoptosis. Hence, a series of agents targeting the Akt/mTOR-mediated autophagy display considerable anti-tumour effects and promising clinical application prospects in malignancies. Following this, we found TOC decreased phosphorylation levels of Akt and mTOR, which were crucial for the initiation of autophagy in TOC-treated HCC cells. Furthermore, the upstream of mTOR, PI3K/Akt pathway could be inhibited by a secreted matricellular protein Cyr61, a member of the CCN protein family. Past studies have shown that the function of Cyr61 depended on the complex cellular context. Here, we found that Cyr61 was expressed at a low level in HCC cell lines, in accordance with previous reports. Further investigation showed that elevated Cyr61 could inactivate Akt upon TOC exposure, thus giving rise to the initiation of protective autophagy conferring resistance to TOC in HCC cells. Thus, we proposed a novel mechanism of the Cyr61/Akt axis in the context of TOC treatment.

5 | CONCLUSION

In conclusion, our results have demonstrated that the canine drug TOC could be a promising anti-cancer drug for the treatment of human HCC via inducing apoptosis. Intriguingly, TOC could activate protective autophagy in HCC cells with the involvement of the Cyr61/Akt axis, and for this reason the combined use of TOC and autophagy inhibitors could further strengthen its anti-HCC activity. Enlightened by such a mode of action, there is a reason to believe that repurposing canine drugs for human use is not confined to TOC for HCC, but can be extended to other canine drugs for a broad spectrum of human malignancies, and, therefore, further investigation is merited.

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CONFLICT OF INTEREST STATEMENT

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ORCID

Canhua Huang https://orcid.org/0000-0003-2247-7750

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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